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Leaf proteome alterations in the context of physiological and morphological responses to drought and heat stress in barley (*Hordeum vulgare* L.)

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Abstract

The objective of this study was to identify barley leaf proteins differentially regulated in response to drought and heat and the combined stresses in context of the morphological and physiological changes that also occur. The Syrian landrace Arta and the Australian cultivar Keel were subjected to drought, high temperature, or a combination of both treatments starting at heading. Changes in the leaf proteome were identified using differential gel electrophoresis and mass spectrometry. The drought treatment caused strong reductions of biomass and yield, while photosynthetic performance and the proteome were not significantly changed. In contrast, the heat treatment and the combination of heat and drought reduced photosynthetic performance and caused changes of the leaf proteome. The proteomic analysis identified 99 protein spots differentially regulated in response to heat treatment, 14 of which were regulated in a genotype-specific manner. Differentially regulated proteins predominantly had functions in photosynthesis, but also in detoxification, energy metabolism, and protein biosynthesis. The analysis indicated that *de novo* protein biosynthesis, protein quality control mediated by chaperones and proteases, and the use of alternative energy resources, i.e. glycolysis, play important roles in adaptation to heat stress. In addition, genetic variation identified

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in the proteome, in plant growth and photosynthetic performance in response to drought and heat represent stress adaption mechanisms to be exploited in future crop breeding efforts.

Introduction

Drought and heat are among the main abiotic stresses dramatically limiting crop growth and productivity worldwide (Wang *et al.*, 2003). In the field, the co-occurrence of several abiotic stresses, rather than an individual stress condition is most damaging to crop production (Mittler, 2006). For example, the combined effects of heat and drought on yield are more detrimental than the effects of each stress alone, as seen in sorghum (*Sorghum bicolor* L.; Craufurd *et al.*, 2008), wheat (Prasad *et al.*, 2011), and barley (Savin and Nicolas, 1996). However, most studies to date have addressed the effects of single stresses on plant performance (Ugarte *et al.*, 2007, Harb *et al.*, 2010), and little is known about the molecular mechanisms underlying the acclimation of plants to a combination of different stresses (Mittler, 2006). Recent studies have revealed that the response of plants to a combination of different abiotic stresses is unique and cannot be directly extrapolated from the response of plants to each of the different stresses applied individually (Rizhsky *et al.*, 2004; Ahmed *et al.*, 2013). Breeding of stress-tolerant crops is the most efficient strategy to maintain yield in stress-prone marginal land. It is thus important to identify genetic resources with high stress tolerance and to understand the mechanisms contributing to adaptation to stresses typically co-occurring in the field such as heat and drought.

Barley (*Hordeum vulgare* L.) is worldwide among the most widely cultivated crops in marginal environments and is often the most common crop in the driest rain-fed farming areas as it is well adapted to abiotic stresses (Baum *et al.*, 2007). Barley has thus been selected or bred for specific adaptation to abiotic stresses in geographically distinct areas of the world. This adaptation of genetically diverse germplasm to similar environmental conditions over a wide geographical range can be exploited for breeding and germplasm exchange. For example, barley germplasm bred by the International Center for Agricultural Research in the Dry Areas, Syria (ICARDA) for the marginal environments of West Asia and Northern Africa (WANA) showed good adaptation to the dry Southern Australian environments (Coventry *et al.*, 2004) and vice versa (Shakhathreh *et al.*, 2001). This germplasm is also interesting in order to study the genetic basis of adaptation to stress in genetically diverse genotypes. Stress adaptation in barley has been attributed to the plasticity of morphological traits such as biomass production, plant growth, tiller number, and peduncle extrusion (von Korff *et al.*, 2008; Shakhathreh *et al.*, 2010). Growth-related responses to stress are typical of stress avoidance strategies which allow the plant to maintain a homeostasis despite changes in the environments. Barley has also been characterized for genetic variation in physiological parameters such as relative water content and chlorophyll fluorescence parameters under stress (Oukarroum *et al.*, 2007; Ahmed *et al.*, 2013). These differences are indicative of differences in stress-tolerance mechanisms which allow the plant to maintain cellular activities under stress (Bartels and Sunkar, 2005). Studies on molecular changes in response to stress in

barley have primarily relied on quantification of mRNA changes under stress (Talamè *et al.*, 2007; Guo *et al.*, 2009 von Korff *et al.*, 2009). These studies showed that a large proportion of the transcriptome responded to drought including genes implicated in stress signalling and stress response. However, while mRNA transcript levels can dictate protein abundance, the differential expression of the two macromolecules is not always well correlated (Stylianou *et al.*, 2008; Baerenfaller *et al.*, 2012). Proteomic approaches provide information missing in DNA or mRNA analysis methods in that they focus on the actively translated portion of the genome. Stress resistance is conferred by the proteins, which function in stress signalling, transcription regulation, cellular detoxification, protection of macromolecules, and a panoply of other processes. In recent years, methods for the analysis of the proteome have advanced considerably, and together with emerging sequence information in model crops like barley (The International Barley Sequencing Consortium, 2012), the plant proteome has become increasingly useful for understanding gene function and networks in response to environmental stimuli. Conventional gel-based proteomics has proved useful in barley research to quantify changes in protein abundance in grains during development (Finnie *et al.*, 2006), in roots in response to salt stress (Witzel *et al.*, 2009) and in shoots in response to heat stress (Süle *et al.*, 2004). Proteomic research using fluorescent labels and two-dimensional difference gel electrophoresis (2D-DIGE) has been successfully applied in barley for the identification of proteins associated with malting quality (March *et al.*, 2012) and in *Arabidopsis* (Shi *et al.*, 2011) and wheat (Gao *et al.*, 2011) to identify proteins responsive to salt. By quantifying changes in protein abundance, one can gain insight into the biochemical processes that underlie the plant's morphological and physiological acclimations to abiotic stress.

The objectives of this study were to study the molecular basis of abiotic stress responses in two drought-adapted, genetically diverse genotypes, the Syrian landrace selection Arta and the Australian barley cultivar Keel. The specific aims were to: (i) characterize the physiological and morphological responses to drought and heat stress; and (ii) identify leaf proteins differentially regulated in response to drought and heat stress using a proteomics approach based on DIGE and mass spectrometry. The changes in protein abundance are discussed in the context of the physiological and morphological trait plasticity that occurs due to the abiotic stresses.

Materials and methods

Plant material

Two cultivated barley two-row genotypes, Arta and Keel, were grown in a growth chamber to evaluate their performance. Arta is a pure line selection from a Syrian landrace adapted to the driest areas in Syria and is winter barley. These genotypes were selected because they are genetically diverse but adapted to similar drought-prone environments, thus allowing the study of the diversity of molecular and phenotypic changes in response to drought and heat.

Drought and heat treatments

Response to the single or combined effects of drought and heat treatments applied at the generative stage was tested in a duplicated growth chamber experiment. Plants were sown in 96-well trays, stratified at 4 °C for 4 d, and then grown in 8/16 light/dark short-day (SD) conditions for 24 d. Plants were transferred to 16/8 light/dark long-day (LD) conditions for 2 d to acclimate before being potted in 4 l pots containing 1.8kg soil with three plants in each pot. Plants remained in LD for the remainder of the experiment. The SD followed by LD treatment was applied to synchronize flowering. The relative humidity of the chambers was set to 50%, the light intensity was 350 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and the temperature was set to 21/17 °C light/dark. The field capacity (FC) was calculated as the difference in weight between fully hydrated soil and dried soil (Colman, 1947). The soil water content (SWC) of potted plants was adjusted to 50% of the FC. For the drought treatment, the SWC was reduced to 15% FC by controlled withholding of water; pots were weighed daily and watered to match the weight of the heaviest pot. The reduction of the SWC was equal across all drought-treated pots. The SWC of 15% FC for the stressed plants and 50% FC for the control plants was maintained until plant maturity. To control for the added weight of the growing plants, the volumetric water content of random pots was checked weekly using a TDR 100 soil moisture meter (FieldScout) fitted with 12 cm probe rods. When the SWC reached 15% FC in the drought-treated plants, the heat treatment was applied to a subset of the well-watered and drought-treated plants by moving the pots at ZT 3 to an identical chamber set to 21 °C and gradually raising the temperature to 36 °C over 4h. Heat-treated plants remained at 36/32 °C light/dark for 1 week, at which point the temperature was decreased to 21 °C over 4h. Detailed information on phenotyping can be found in Table 1 and in the [Supplementary Materials and Methods](#) (available at [JXB](#) online).

Table 1.

Summary of traits measured in the genotypes Arta and Keel grown under control, drought, heat, and combination treatments

Trait Abbreviation Unit Grain yield GY g Total biomass BM g Harvest index HI g g⁻¹ Plant height PH cm Peduncle extrusion Pedex cm Spike number SN – No. of aborted spikes AS – Grains per spike GS – Thousand kernel weight TKW g Days to maturity DM days Total water used per pot WU l Water use efficiency of grain yield WUE g l⁻¹ Leaf temperature on day 1 LT_1 °C Leaf temperature on day 3 LT_3 °C Leaf temperature on day 7 LT_7 °C Relative water content on day 1 RWC_1 % Relative water content on day 3 RWC_3 % Relative water content on day 7 RWC_7 % Maximum PSII quantum yield at day 1 Fv/Fm_1 Arbitrary Maximum PSII quantum yield at day 3 Fv/Fm_3 Arbitrary Maximum PSII quantum yield at day 7 Fv/Fm_7 Arbitrary PSII performance index at day 1 PI_1 Arbitrary PSII performance index at day 3 PI_3 Arbitrary PSII performance index at day 7 PI_7 Arbitrary

Two-dimensional gel electrophoresis

Leaf proteins from three biological replicates were extracted using a trichloric acetic acid/acetone precipitation modified from Cascardo *et al.* (2001) and fluorescently labelled as detailed in [Supplementary Table S1](#) and [Supplementary Materials and Methods](#). The protein sample was

diluted to a total volume of 340 µl in rehydration buffer consisting of 7M urea, 2M thiourea, 2% CHAPS, 20mM DTT, and 0.5% biolyte 3-10 ampholytes (Bio-Rad) and applied to 18 cm immobiline strips pH 3-10 NL (GE Healthcare Life Sciences). Strips for DIGE gels contained a total of 150 µg protein while strips for gels to be post-stained with PageBlue (Fermentas life sciences) contained 500 µg protein. Focusing was accomplished at 20 °C using a Protean isoelectric focusing cell (Bio-Rad) with the following conditions: 14h passive rehydration, 250V for 15min, ramping to 2000V for 1h 45min, and ramping to 10kV for 3h, before maintaining the voltage at 10kV for a total of 30kV/h. The resulting strips were equilibrated in 2D equilibrium buffer (0.1M Tris, 6M urea, 30% glycerol, 2% SDS) containing 2% DTT for 15min and then with 2D equilibrium buffer containing 2.5% iodoacetamide (Sigma) for 15min. Equilibrated strips were placed on 1 mm thick 12% SDS-PAGE gels sized 26×20cm and covered with 0.5% agarose (Bio-Budget). The second dimension was separated using 12 mA/gel for 12h with the EttanDaltSix electrophoresis system (GE Healthcare Life Sciences).

Protein identification

Protein gels were imaged and spots were detected as detailed in [Supplementary Materials and Methods](#). Spots from 2D gels were excised using the Proteineer sp II and tryptically digested and spotted on MTP 600/384 AnchorChip plates using the Proteineer dp system (Bruker Daltonics). Aliquots of the digests were automatically spotted on MTP 600/384 AnchorChip plates for subsequent mass spectrometric analysis according to the thin-layer protocol of Gobom *et al.* (2001) using the Proteineer dp robot. Peptide mass fingerprints were obtained using the Ultraflex III MALDI ToF/ToF mass spectrometer (Bruker Daltonics). The resulting spectra were processed into peak files with the flexAnalysis version 2.4 software (Bruker Daltonics) by means of the sophisticated numerical annotation procedure algorithm. Peak data were imported into the ProteinScape database system version 3.0 (Bruker), which initiated Mascot version 2.3 (Matrix Science) searches against the UniProt knowledgebase (<http://www.uniprot.org/>) for *H. vulgare* (release 2011_06) and the DFCI Barley Gene Index (<http://compbio.dfci.harvard.edu/tgi/>) version 12 genome database.

Data analysis

Analysis of variance (ANOVA) and correlations were performed using SAS version 9.1 (SAS Institute) and are described in details in [Supplementary Materials and Methods](#). The regulation factor, the log2-transformed fold change in the normalized spot intensity, was calculated between 36 °C and 21 °C treated plants across genotypes and drought treatments as well as between Arta and Keel across both stress treatments. Gene ontology terms for identified proteins were retrieved from AgBase (McCarthy *et al.*, 2006). Singular enrichment analysis was performed with the AgriGO toolkit (Du *et al.*, 2010) as outlined in [Supplementary Materials and Methods](#).

Results

Comparison of effects of drought, heat, and combination treatments on plant performance

Morphological changes caused by the treatments are depicted in the representative photographs of control and stress-treated Arta and Keel plants taken the end of the heat treatment, 9 days after heading initiated (Fig. 1). Drought caused strong reductions in plant height as compared to control in Arta, but not in Keel. In addition, the drought, heat, and combination treatments caused noticeably stronger senescence of lower leaves in Arta than in Keel.

Fig. 1.

Representative Arta and Keel plants after 7 days of control (21 °C) or high (36 °C) temperature and control (50%) or drought (15%) soil water content. Each 4 l pot contains three plants.

Overall, the drought treatment had a stronger effect on morphological traits than on physiological traits. For example, the water regime explained 26% of the variation in grain yield (GY), 57% of the variation for biomass (BM), and 79% of the variation for spike number (SN), while it explained only up to 18% of the variation in relative water content (RWC) and less than 1% of variation in photosynthetic performance index (PI) (Supplementary Table S2). In contrast, the heat treatment had significantly stronger effects on physiological traits than the drought treatment; it explained up to 54% of the variation in water use efficiency (WUE), 34% of the variation in RWC, and 74% of the variation in PI. The heat treatment did not have strong effects on plant growth; it only explained 8% of the variation in BM and 2% for plant height (PH). The strongest phenotypic changes were observed under the combination treatment (Table 2). The combination treatment caused the strongest reduction in GY. GY under the combination treatment was significantly lower in Keel than GY under heat or drought alone, while significant reductions in GY caused by drought and heat were not significantly different from each other (Table 2). While the reductions in GY under drought and heat were similar, drought and heat had different effects on yield component traits. Whereas the drought and combination treatments caused significant reductions in BM and PH, heat did not significantly affect these traits. In addition, the spike number (SN) was significantly reduced by drought and combination treatments as compared to controls, but not significantly affected by heat in both genotypes. In contrast, heat and combination treatments significantly increased the number of aborted spikes (AS) and decreased thousand kernel weight (TKW) in Arta and Keel, while drought did not have significant effects on AS and TKW as compared to control conditions. The harvest index (HI) was significantly reduced under drought, heat, and combination treatments as compared to controls, while the reductions in HI were also significantly greater under heat and combination treatments as compared to the drought treatment in both genotypes. The number of grains per spike (GS) and peduncle extrusion (Pedex) were reduced under drought, heat, and combination treatments,

but the differences were not significant. The heat treatment had thus strong effects on generative traits; it explained 32% of the variation of AS, and 72% of variation in TKW. In contrast, drought showed the strongest effects on vegetative traits such as vegetative biomass and plant height.

Trait means, minimums, and maximums for Arta and Keel under control or drought conditions at either 21°C or 36°C

Trait			Control 21°C Arta			Control 21°C Keel			Drought 36°C Arta			Drought 36°C Keel			Mean		
Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean
Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min
11.3	6.4 ^b	4.3	8.2	6.0 ^{bd}	3.1	8.5	7.7 ^b	6.0	9.0	3.2 ^{cd}	0.8	6.0	2.9 ^c	1.5	4.8	BM	20.7 ^a
16.2	24.3	20.2 ^a	9.5	26.1	18.7 ^{ab}	15.3	23.3	18.6 ^{ab}	13.3	23.3	13.6 ^c	11.0	17.3	15.1 ^{bc}	12.8	18.3	12.3 ^{cd}
9.6	16.0	9.8 ^d	5.1	13.2	HI	0.56 ^a	0.40	0.73	0.57 ^a	0.44	0.79	0.29 ^b	0.14	0.52	0.35 ^{bd}	0.27	0.45
0.44 ^{cd}	0.23	0.62	0.51 ^{ac}	0.45	0.58	0.25 ^b	0.08	0.47	0.30 ^b	0.22	0.43	PH	49.6 ^{ab}	44.0	53.8	52.5 ^a	
46.5	56.4	44.9 ^{bd}	36.9	54.2	50.8 ^a	43.4	59.8	38.9 ^c	35.2	42.2	49.9 ^{ab}	45.7	54.0	42.8 ^{cd}	37.7	48.7	45.3 ^{bd}
39.7	51.7	Pedex	-3.7 ^a	-4.6	-2.6	-5.1 ^{ab}	-9.7	-1.9	-3.7 ^a	-8.8	2.9	-5.9 ^{ab}	-9.0	-2.9	-6.1 ^{ab}	-9.1	-2.5
-5.8 ^{ab}	-11.3	-3.4	-5.5 ^{ab}	-7.8	-1.2	-6.8 ^b	-8.7	-4.5	SN	22.6 ^{ac}	16.0	30.0	22.2 ^{ac}	10.7	33.0	25.9 ^a	16.6
35.3	26.7 ^a	20	36.3	14.9 ^b	13	16.7	17.6 ^{bc}	12.7	25.0	18.5 ^{bc}	11.7	25.7	14.9 ^b	5.7	21.3	AS	
3.5 ^{ac}	0.3	10.7	0.4 ^a	0.0	2.7	11.1 ^b	4.0	22.0	6.1 ^{cd}	0.3	16.0	3.2 ^{ac}	1.7	5.3	2.1 ^a	0.0	
8.0	8.8 ^{bd}	4.7	13.7	3.3 ^{ac}	0.7	7.7	GS	12.2 ^a	10.9	14.2	12.1 ^a	9.3	17.2	10.7 ^a	6.8	15.1	
10.4 ^a	8.2	14.7	11.2 ^a	5.7	13.6	11.3 ^a	9.6	13.0	10.8 ^a	6.2	14.4	10.0 ^a	7.8	12.6	TKW	50.2 ^a	
40.6	63.8	44.1 ^b	33.1	55.0	35.5 ^c	32.1	41.2	30.4 ^{cd}	27.0	33.9	46.0 ^{ab}	36.7	51.3	44.5 ^{ab}	41.2	48.4	
29.9 ^{cd}	24.3	37.1	26.3 ^d	23.4	29.6	DM	90 ^a	68	107	88 ^a	63	109	97 ^a	82	105	93 ^a	68
107	90 ^a	74	105	88 ^a	68	98	96 ^a	88	102	90 ^a	63	100	WU	7.54 ^a	5.89	9.27	
6.70 ^a	4.71	7.96	7.65 ^a	6.27	9.94	6.65 ^a	4.81	8.06	3.45 ^b	1.63	4.26	3.42 ^b	2.91	3.95	3.61 ^b	1.66	
5.60	3.21 ^b	2.62	3.98	WUE	1.6 ^{ad}	0.9	2.1	1.7 ^{ab}	0.8	2.7	0.7 ^c	0.4	1.4	1.0 ^{cd}	0.6	1.3	
1.8 ^{ab}	1.3	2.8	2.3 ^b	1.8	3.1	0.9 ^c	0.2	1.8	0.9 ^c	0.4	1.7	LT_1	20.3 ^a	17.1	24.5	21.1 ^{ab}	18.7
25.0	35.0 ^c	32.8	36.2	33.2 ^d	31.8	35.3	22.8 ^e	19.5	25.4	22.0 ^{be}	19.5	25.1	36.5 ^{cf}	33.8	39.0	37.2 ^f	35.7
38.4	LT_3	21.7 ^a	18.1	24.4	21.9 ^a	20.0	24.3	37.0 ^b	35.7	39.3	35.9 ^b	32.9	37.9	22.5 ^a	19.1	25.8	
22.6 ^a	19.7	25.3	37.1 ^b	35.5	38.6	37.0 ^b	35.5	38.9									

Under drought, GY was positively correlated to BM (0.70) and PH (0.56), while under heat and combination treatments GY correlated with BM (0.57 and 0.46, respectively), but not with PH ([Supplementary Table S3](#)). Under heat alone, GY correlated negatively with AS (-0.54) and positively with TKW (0.58). Different yield components were thus correlated to GY under the different treatments.

WUE, RWC, maximum quantum efficiency of photosystem II (Fv/Fm), PI, and leaf temperature (LT) were significantly affected by heat and combination treatments, but not by drought (Table 2). WUE dropped from 1.6 and 1.7 under control to 0.7 and 1.0 under heat and 0.9 and 0.9 under combination treatments in Arta and Keel, respectively. In addition, at 3 and 7 days after start of the treatment, Arta and Keel plants had significantly lower RWC due to the combination treatment (Table 2). The integrated chlorophyll fluorescence measurement PI was significantly reduced under heat and combination treatments in both genotypes at all analysed time points. In contrast, Fv/Fm was only significantly reduced in Keel under heat and combination treatments at all three time points analysed. LT (as opposed to the ambient temperature) was significantly increased by drought 1 day after treatment start in Arta plants (22.8 °C) compared to well-watered controls (20.3 °C), but no significant differences in LT were detected between drought-treated and well-watered Keel plants (Table 2). LT was significantly higher in Arta and Keel plants grown under heat than plants grown under control temperatures, with mean values ranging from 33.2 to 37.0 °C and from 20.3 to 23.0 °C, respectively. After 7 days of heat treatment, both Arta and Keel plants experienced higher LT when additionally treated with drought (36.4 and 37.2 °C, respectively) as compared to the heat treatment alone (33.2 and 34.6 °C, respectively).

The experiment revealed the strongest genetic variation for the traits PH (24%), AS (17%), and Fv/Fm (15%) ([Supplementary Table S2](#)). Arta showed a significantly stronger reduction in PH under drought than Keel. In addition, heat resulted in a significantly higher amount of AS in Arta than in Keel (Table 2). Finally, in Keel, the combination treatment resulted in a significantly lower Fv/Fm and PI as compared to the heat treatment alone and as compared to the Fv/Fm and PI under the combination treatment in Arta. The two genotypes thus showed different physiological responses to the heat and combination treatments.

Characterization and quantification of the barley leaf proteome

Spot detection on Coomassie-stained gels (Fig. 2) revealed a total of 525 distinct spots that were submitted for identification by peptide mass finger printing and subsequent peptide fragmentation analysis. Mascot search against the Uniprot database for *H. vulgare* or the DFCI Barley Gene Index database revealed the identity of 296 proteins from a total of 525 spots. The 296 identified spots were matched to a total of 145 unique accessions. Spots identified with the same protein accession were considered to be isoforms. Sixty-two of the proteins identified in the barley leaf proteome had isoforms present.

Fig. 2.

Representative Coomassie-stained 12% SDS-PAGE containing 400 µg total leaf protein. Numbered arrows indicate spots that were identified by MS and significantly regulated between conditions or between genotypes ([Supplementary Table S4](#)).

Using a singular enrichment analysis with gene ontology terms and the UniProt database, over half of the identified proteins were present in intracellular compartments (59%) and the cytoplasm (52%), as well as in organelles (51%) and were significantly enriched compared to the background (22, 9, and 15%, respectively; [Supplementary Fig. S1](#)). The largest fold enrichment was for the term 'plastid', which was present in 45% of leaf proteins and in less than 2% of background proteins.

To quantify differences in protein accumulation due to heat and drought treatments, the barley leaf proteome of samples harvested three days after treatment start was visualized using DIGE, as seen in a representative image in [Supplementary Fig. S2](#). Samples from this time point were chosen based on the RWC and PI measurements; 3 days after treatment start was the first time point where the combination treatment resulted in significant changes in RWC and PI in both genotypes compared to controls. Spot detection on a composite fluorescent image comprising all superimposed gel images resolved 1005 distinct spots. Based on DIGE, this study identified 305 spots significantly differentially regulated by the heat treatment, 473 spots different between genotypes, and 35 spots different due to the interaction between temperature and genotype. However, no spots were found to be significantly regulated by the drought treatment. Of the 305 spots found to be differentially regulated by heat, 99 were identified via mass spectrometry (32 downregulated, 67 upregulated). Of the 473 spots found to be differentially regulated between genotypes, 125 were identified (90 downregulated in Keel, 35 downregulated in Arta). Additionally, mass spectrometry helped identify 14 out of the 35 proteins controlled by a significant interaction effect of the genotype and heat treatment ([Supplementary Figs. S3 and S4](#)).

The biological functions of differentially regulated proteins included roles in metabolism, photosynthesis, transport, response to abiotic stimulus, and response to stress (Table 3 and [Supplementary Table S4](#)). The proteomic analysis revealed the upregulation of structural components of the photosystem, i.e. the chlorophyll a-b binding protein of LHCII type III (Lhcb3) (spot 870), chloroplast oxygen-evolving enhancer protein 1 (PsbO) (spots 846, 847, and 851) and oxygen-evolving enhancer protein 2 (PsbP) (spots 97 and 221). While the overall trend of genotype-specific protein regulation was to be downregulation in Keel, photosynthesis-associated proteins had the opposite trend; 14 out of the 20 photosynthetic proteins were detected to be upregulated in Keel. In addition, proteins involved in detoxification, energy metabolism, and protein biosynthesis were differentially regulated by heat and between genotypes. These involved, for example, the upregulation of glycolytic proteins under heat, i.e. fructose-bisphosphate aldolase (spot 788) and cytosolic glyceraldehyde-3-phosphate dehydrogenase (spots 327 and 330). In addition, chaperones (spots 564 and 939), proteases (spots 586

and 527), elongation factors (spots 533 and 723), and initiation factor 4A (spot 703 and 710) were upregulated under heat. A large number of proteins differentially regulated between genotypes had functions in photorespiration and these were primarily upregulated in Arta as compared to Keel irrespective of the treatment. The proteomic data thus revealed a large number of proteins differentially regulated between genotypes and between heat and control conditions and the majority of these proteins had functions in photosynthesis, energy metabolism, and detoxification.

Table 3.

Proteins discussed in the text which were differentially regulated by temperature (T), genotype (G), or an interaction effect (G×T)

Proteins quantified by DIGE, identified via mass spectrometry and grouped according to their biological function. Spot number (No.) in Fig. 2 is given in addition to the Uniprot protein name and accession number. Predicted molecular weight (MW), isoelectric point (pI), Mascot score (Score), and percentage sequence coverage (SC%) are based on Mascot searches. The regulation factors, the log₂ fold change in protein expression, are given for plants grown at 36 °C over plants grown at 21 °C (36/21) across both genotypes, for Keel plants over Arta plants (K/A) across all treatments, for heat-treated Keel plants over heat-treated Arta plants (K36/A36) and for control Keel plants over control Arta plants (K21/A21). Regulation factors corresponding to significant ($P < 0.05$) changes in expression are underlined. The complete list of differentially regulated proteins can be found in [Supplementary Table S3](#).

No.	Protein name	UniRef100	MW	pI	Score	SC%	SE	36/21	K/A	K36/A36	K21/A21
327	Carbohydrate metabolic process Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	P26517	122.3	7.0	154.6	2.4	T	<u>1.29</u>	1.02	-1.18	1.30
	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	P26517	48.3	7.0	49.0	2.6	T/G/G×T	<u>1.68</u>	<u>-1.97</u>	<u>-2.36</u>	<u>-1.49</u>
788	Photosynthesis Fructose- biphosphatealdolase	F2ELD1	41.9	7.5	304.6	10.8	T	<u>1.22</u>	1.09	1.07	1.10
97	Photosynthesis Oxygen-evolving enhancer protein 2, chloroplastic	Q00434	96.0	6.9	44.9	0.9	T/G	<u>1.46</u>	<u>2.14</u>	2.10	2.21
221	Oxygen-evolving enhancer protein 2, chloroplastic	Q00434	96.0	6.9	91.5	7.0	T/G	<u>1.29</u>	<u>1.86</u>	1.84	1.88
846	Predicted protein	F2CRK1	34.4	5.6	306.6	11.6	T/G	<u>1.29</u>	<u>1.80</u>	1.82	1.78
847	Chloroplast oxygen-evolving enhancer protein 1	A5JV93	68.7	7.0	198.6	3.7	T/G	<u>1.55</u>	<u>2.53</u>	2.52	2.55
851	Predicted protein	F2CRK1	34.4	5.6	199.0	11.6	T/G	<u>1.63</u>	<u>2.39</u>	2.28	2.58
870	Chlorophyll a-b binding protein of LHCII type III, chloroplastic	P27523	91.6	7.0	108.6	1.2	T/G	<u>1.75</u>	<u>1.98</u>	2.05	1.86
586	Response to abiotic stimulus ATP-dependent zinc metalloprotease	FTSH1	80.0	6.9	74.2	9.2	T	<u>2.69</u>	<u>-1.29</u>	-1.37	-1.12
527	ATP-dependent Clp protease ATP-binding subunit clpA homologue	CD4B	173.3	6.9	124.0	7.9	T/G	<u>-2.07</u>	<u>-1.76</u>	-2.35	-1.55
564	Chloroplast heat shock protein 70	A4ZYQ0	134.0	7.0	170.2	1.8	T	<u>1.85</u>	<u>-1.10</u>	-1.21	1.08
939	Heat-shock protein	Q43638	103.9	7.0	122.8	2.2	T/G	<u>2.90</u>	<u>-1.43</u>	-1.54	-1.16
533	Translation Elongation factor EF-G	Q9SI75	61.3	6.9	64.2	16.0	T/G/G×T	<u>2.41</u>	<u>-1.45</u>	<u>-1.52</u>	<u>-1.29</u>
703	Eukaryotic initiation factor 4A	P41378	143.9	6.9	121.0	4.8	T/G/G×T	<u>1.73</u>	<u>-1.65</u>	<u>-1.96</u>	<u>-1.24</u>
710	Eukaryotic initiation factor 4A	P41378	68.3	7.0	74.4	2.0	G	<u>-1.11</u>	<u>-1.56</u>	<u>-1.86</u>	<u>-1.34</u>
723	Elongation factor Tu	Q8W2C3	144.5	6.9	298.2	2.4	G	<u>-1.08</u>	<u>-1.34</u>	<u>-1.40</u>	<u>-1.28</u>

Discussion

Drought affected plant growth but not photosynthesis

The reductions in grain yield due to the drought and heat treatments, while of similar magnitude, were due to different changes in yield component traits. Drought had major effects on plant growth, notably biomass and spike number, while the heat stress primarily affected the generative organs of the plant, the number of aborted spikes, and kernel weight. In addition, while drought did not significantly impact photosynthetic efficiency, photosynthesis was significantly compromised under heat and under the combination treatment. Consequently, drought affected plant growth but not photosynthesis. The strong reduction in growth and simultaneous maintenance of photosynthesis under drought as seen in the present study confirm recent studies in *Arabidopsis*, which demonstrated that mild drought primarily affected plant growth, but had minor effects on the photosynthesis rate (Muller *et al.*, 2011; Skirycz *et al.*, 2011; Verelst *et al.*, 2012). The results are thus consistent with the hypothesis that plants reduce their growth as a primary adaptation response to stress rather than as a secondary consequence of resource limitations (Muller *et al.*, 2011). In particular, under drought, biomass was positively correlated with yield. Thus, under non-lethal stress, limiting growth reduction might provide a strategy to increase productivity under stress. In the present study, drought also reduced the number of spikes presumably through reducing tiller number. The reduction in spike number due to the inhibition of tillering is a known reaction to drought (El Soda *et al.*, 2010), but its molecular basis is not established. However, drought-induced inhibition of tillering may share similar pathways to the inflorescence-induced inhibition of tillering. The repression of tillering that occurs during anthesis is thought to be due to a combination of auxin signalling and resource competition between the apical buds, which form new tillers, and the stem apex (Jewiss, 1972).

Analysis of leaf proteomes under drought did not reveal significant changes of protein abundance as compared to control conditions. This is in contrast to the large number of transcripts differentially regulated in barley plants subjected to drought at the generative stage as seen by Guo *et al.* (2009). Homeostasis of the proteome could imply that the plants had acclimated to the drought stress (Harb *et al.*, 2010). Acclimation to drought on the physiological level in the present study was evident in the maintenance of photosynthesis and water status under drought. Differences in stress symptoms might also be due to differences in the severity and duration of the stress application as compared to other studies (Talamè *et al.*, 2007). However, the present data may also indicate a higher stability of the proteome as compared to the transcriptome under drought stress. Interestingly, a recent study has shown that while 1222 transcripts were differentially regulated in *Arabidopsis* subjected to mild drought, 34 out of 2081 proteins were significantly changed with an average regulation factor of 1.5 (Baerenfaller *et al.*, 2012). This suggests a higher stability of the proteome as compared to the transcriptome in response to environmental perturbation. These data suggest that barley adapted to non-lethal drought by avoidance mechanisms, in particular through the reduction of growth. The resistance strategy ensured homeostasis of

the cell which was reflected in the maintenance of photosynthesis and stability of the proteome under drought.

Heat affected photosynthesis rates

The heat treatment caused significant changes in reproductive organs and affected TKW and spike abortion rates, while the vegetative traits biomass and plant height remained relatively unaffected. At the same time, heat caused a significant reduction of RWC and photosynthetic efficiency. The RWC was likely reduced by an increase in transpiration as seen in the reduced leaf temperature under heat as compared to the ambient temperature. As ambient temperature increases, plants attempt to cool themselves by opening stomata and increasing transpiration (Schulze *et al.*, 1973). However, the RWC under heat was not significantly different from the RWC under drought conditions, while photosynthesis rates were significantly different between these two stresses. This suggested that photosynthesis was significantly reduced under heat not because of suboptimal water content in the cells but rather by a direct detrimental effect of heat on the photosynthetic apparatus. These findings are in line with previous observations that the optimum temperature for photosynthesis in barley is 20 °C and photosynthesis rates are decreased by more than 50% at 35 °C (Todd, 1982). The detrimental effect of heat on the photosynthetic apparatus was also indicated by the proteome data, which demonstrated an increase in the turnover of photosynthesis-related proteins under heat.

A reduction in Fv/Fm and PI in the leaves and senescence of the lower leaves (Fig. 1) indicated that the ability to photosynthesize was permanently decreased under heat. In addition, the PI was significantly reduced under heat which suggests that both the light-dependent and light-independent mechanisms, e.g. carbon fixation, were damaged or inhibited due to high temperature. Thus, the heat treatment had a detectable effect on photoinhibition and the availability of carbon dioxide was probably limited by stomata closure. Evidence of a reduction of the carbohydrate pool in heat-treated plants was also provided by the reduction in kernel weight and by the upregulation of glycolysis enzymes observed in the proteomic data.

Heat also reduced total grain yield by increasing the abortion rate of spikes. Floret fertility and grain setting, as measured by the number of aborted spikes, was reduced under the heat treatment. Reproductive growth is known to be more sensitive to heat stress than vegetative growth in barley. In particular, anthers are prone to growth inhibition and lose the ability to produce pollen when heat stressed (Oshino *et al.*, 2007). Heat thus caused yield reductions primarily by affecting photosynthesis, carbon fixation, and floret fertility. Strong effects of the short-term heat treatment on physiology indicated that the homeostasis of the cells was disturbed.

As in stress-prone environments drought and heat often co-occur, this study compared the combined effects of drought and heat with that of the single stresses on plant performance. The changes in plant performance were greater under the combination treatment than under heat or drought alone (Table 2). The strongest effect of the combination treatment was observed for PI suggesting that photosynthesis was most sensitive to the simultaneous application of drought and heat,

presumably because leaf cooling through transpiration was severely reduced by the limited water supply. Reduced transpiration and leaf cooling in the combination treatment was also suggested by the difference in leaf temperature between the heat (33–34 °C) and the combination treatment (36–37 °C) (Table 2). In addition, grain yield was significantly more reduced by the combination treatment than by the single stresses, presumably because drought and heat affected unique yield component traits such as spike number and spike fertility, which resulted in an overall reduced yield in the combination treatment.

Proteomic basis of morphological plasticity and physiological responses to heat stress

The proteome analysis identified significant differences in protein abundance only under heat, and not under drought. This reflects the strong effects of heat on plant physiology in contrast to the physiological homeostasis seen under drought. Under heat, a large number, 99 out of 296 detected proteins were differentially regulated, and these had predominantly functions in photosynthesis, detoxification, energy metabolism, and protein biosynthesis (Table 3 and [Supplementary Table S4](#)). In many cases, several spots were detected per protein. These may represent close homologues, which could not be resolved based on the mass spectrometric data, but may also be isoforms due to differential post-translational modifications (Röhrig *et al.*, 2006). The high frequency of differentially regulated proteins with functions in photosynthesis may be explained by the enrichment of plastid proteins in the present leaf proteome analysis ([Supplementary Fig. S2](#)). The proteomic analysis revealed structural components of the light-harvesting complex (Lhcb3) and the oxygen-evolving complexes (PsbO and PsbP) as being significantly upregulated under the heat treatment. It is tempting to speculate that the upregulation of Lhcb3, PsbO, and PsbP might be due to the *de novo* synthesis of peptides that are en route to replace them and the lag in degradation of damaged proteins removed from the photosystem. The upregulation of Lhcb3, PsbO, and PsbP proteins was therefore likely due to their increased replacement after damage by heat as indicated by the chlorophyll fluorescence data. The reduction of the PI due to the heat treatment was indicative of inhibition of the light-independent reactions of photosynthesis. Rubisco, the enzyme catalysing the rate-limiting step of carbon fixation, is inhibited by side-products of photorespiration which stabilize the active site of Rubisco in a closed conformation (Pearce and Andrews, 2003). Interestingly, Rubisco activase B showed the highest upregulation under heat, while Rubisco activase A was downregulated under heat (Table 3). Rubisco activase frees the catalytic site of Rubisco from inhibitory sugar phosphates by forcing the active site into an open conformation (Portis *et al.*, 2007). Rubisco activases have been characterized on the genomic level in barley (Rundle and Zielinski, 1991), wheat (Law and Crafts-Brandner, 2001), rice (*Oryza sativa* L.) (To *et al.*, 1999), cotton (*Gossypium hirsutum* L.) (Salvucci *et al.*, 2003), maize (*Zea mays* L.) (Ayala-Ochoa *et al.*, 2004), and *Arabidopsis* (Werneke *et al.*, 1989). Rubisco activase A with two different splice variants is present in all mentioned species and is known to be heat inactivated starting at 35 °C (Crafts-Brandner *et al.*, 1997). In contrast, Rubisco activase B has only been detected in barley, wheat, maize, and cotton but not in rice or *Arabidopsis*. It has already been

shown that Rubisco activase B is induced by heat on the transcript level in wheat (Wang *et al.*, 2011) and on the protein level in cotton (Law *et al.*, 2001). However, the thermostability of Rubisco activase B is currently untested. The upregulation of Rubisco activase B under heat treatment suggested a specific role for Rubisco activase B in maintaining the activity of Rubisco under high temperature conditions, possibly by being more thermostable than Rubisco activase A.

Interestingly, the proteomic data showed that several glycolytic proteins were upregulated under the heat treatment. The prime functions of glycolysis are to generate carbon skeletons, reductants, and ATP, which can confer a bioenergetic advantage that can extend the survival time of plant cells that have become ATP-depleted due to environmental stresses. Increased levels of GAPDH transcripts have been observed under environmental stress conditions, such as dehydration in *Craterostigma plantagineum* (Velasco *et al.*, 1994), during heat shock in *Arabidopsis* plants (Yang *et al.*, 1993), and during anaerobic stress in maize (Chang *et al.*, 2000). Thus, there is supporting evidence for the activation of ATP-generating pathways under different stresses, presumably to cope with a higher demand for ATP to maintain homeostasis under stress conditions. A higher energy demand was also suggested by the upregulation of ATP synthase subunit alpha in the mitochondria and plastids upon heat stress (Table 3). For example, higher transcript levels of ATP synthase were observed in rice upon salt and osmotic stress (Zhang *et al.*, 2006). A higher energy was probably required for a higher rate of protein degradation and biosynthesis as suggested by the upregulation of chaperones, proteases, elongation factors, and initiation factor 4A. Analysis of the proteome of heat-stressed barley plants thus suggested thermolability and a more rapid protein turnover of photosynthesis-related proteins, while drought did not affect cellular homeostasis.

Barley genotypes Arta and Keel responded uniquely to heat and drought treatments

The identification of genetic differences in stress responses is an important basis for improving plant performance under stress. The differences in responses to drought and heat, in particular in growth, spike fertility, and chlorophyll fluorescence seen between Arta and Keel, suggested that the two genotypes have unique mechanisms for coping with environmental stresses. Arta was characterized by a stronger growth reduction under drought than Keel. Under heat, Arta showed a higher spike abortion rate as compared to Keel. Finally, under the combination treatment Arta had a higher Fv/Fm than Keel. Genetic variation in the efficiency of photosynthesis under stress has already been detected in diverse barley genotypes (Oukarroum *et al.*, 2007; Ahmed *et al.*, 2013). Interestingly, Keel tended to show higher yield under drought and heat alone, while Arta tended to show a higher yield under the combination treatment. Differences in growth and yield under the different stress regimes suggested that Keel is better adapted to mild stress as it maintains growth and yield. In contrast, under more severe stress as represented by the combination treatment Arta performed better presumably through a stronger morphological adaptation and maintenance of photosynthesis.

The genetic difference was supported by the high number of proteins differentially regulated between the genotypes. Interestingly, a large number of these differential proteins had roles in photorespiration, and these were primarily downregulated in Keel. Especially under stress conditions that lead to reduced rates of photosynthetic CO₂ assimilation, photorespiration serves as energy sink preventing the over-reduction of the photosynthetic electron transport chain and photoinhibition. Differences in the efficiency of photorespiration between Arta and Keel may explain the difference in photosynthetic performance under the combination stress. This study also observed 14 protein spots regulated by interaction effects between genotype and environment which are thus potential outputs of unique adaptations to heat stress that have evolved between Arta and Keel ([Supplementary Figs. S3 and S4](#)). Arta and Keel are parents of a recombinant inbred population, which shows transgressive segregation for agronomic performance under drought in Mediterranean environments (J. Rollins, B. Drosse M. A. Mulki, S. Grando, M. Baum, M. Singh, S. Ceccarelli, M. von Korff, in revision) unpublished results). Identification of the unique stress adaptation strategies in Arta and Keel will allow further dissection of the genetic basis of this transgressive performance in the offspring.

Conclusion

The data presented in this study suggested that barley has adapted to non-lethal drought by avoidance mechanisms, such as the reduction of growth which allowed the plants to maintain a cellular homeostasis as seen in the stability of photosynthesis and of the proteome under drought. In contrast, heat affected RWC, photosynthesis, and floret fertility and caused a rapid turnover of photosynthesis-related proteins. Based on the protein changes observed, it is proposed that important heat-tolerance mechanisms include protein quality control and *de novo* synthesis, which cause a higher energy demand as seen in the upregulation of ATP generating pathways. Reduced CO₂ availability due to stomatal closure under heat was counter-balanced by the activation of Rubisco by Rubisco activase B. Inhibition of the light-independent reactions of photosynthesis likely caused the production of radical oxygen species as seen in the increase of scavenging and detoxifying enzymes. Genetic variation in stress responses between Arta and Keel, in particular in growth, spike fertility, and photosynthesis can be exploited in future crop breeding efforts.

Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Materials and Methods

[Supplementary Table S1](#). Dye swap setup of the DIGE experiment using Cy2, Cy3, and Cy5.

[Supplementary Table S2](#). Summary of the four-way analysis of variance for the traits measured in Arta and Keel genotypes under the two soil water content treatments at 21 or 36 °C.

[Supplementary Table S3](#). Spearman's rank correlation coefficients for phenotypic traits measured in both genotypes under control, drought conditions, under heat, and combination conditions.

[Supplementary Table S4](#). All proteins differentially regulated due to temperature, genotype, or an interaction effect of the two as quantified by DIGE and identified via mass spectrometry

[Supplementary Fig. S1](#). Singular enrichment analysis of cellular component gene ontology terms present in the barley leaf proteome as identified by mass spectrometry compared to all terms present in Uniprot entries for *Hordeum vulgare*.

[Supplementary Fig. S2](#). Representative fluorescent image of a 2D-DIGE gel containing leaf total protein labelled with Cy3.

[Supplementary Fig. S3](#). Spot intensities of proteins with genotype by temperature interaction effects where the spot intensity is not significantly different between genotypes under control conditions.

[Supplementary Fig. S4](#). Spot intensities of proteins with genotype by temperature interaction effects.

Supplementary Data

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