Genetic and Proteomic Basis of Abiotic Stress Responses in Barley (*Hordeum vulgare*)

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Abstract

Drought and heat stress are the two leading abiotic stresses that limit crop productivity. Understanding the range of responses that a model crop like barley can exhibit in different environments to avoid or tolerate stress will be crucial in unraveling the basis of abiotic stress resistance. The objectives of the present study were to identify i) morphological and physiological traits associated with abiotic stress resistance, ii) genetic loci linked to agronomic performance traits under drought, and iii) proteins differentially regulated in response to heat and drought stress.

A barley recombinant inbred line population derived from a cross between the Syrian landrace Arta and the Australian cultivar Keel was grown in a greenhouse under well watered conditions and subjected to drought treatment that began at anthesis and persisted until maturity. Using genotyping data from over 700 genetic markers, a multi-environmental quantitative trait loci (QTL) analysis of morphological and physiological traits was performed. For the proteomic analysis, Arta and Keel were grown in a growth chamber under well watered conditions and subjected to drought, high temperature, or a combination of both treatments starting at anthesis. The leaf proteome of Arta and Keel were visualized and changes in protein spot abundance due to the stress treatments were quantified using difference gel electrophoresis (DIGE). Mass spectrometry was used to identify protein spots excised from the gels.

The drought treatment was characterized by morphological plasticity and stability on the physiological and proteomic level. In contrast, the heat treatment caused perturbations on the physiological and proteomic level which were more prominent than the morphological responses that occurred. The QTL analysis revealed nineteen loci for traits associated with agronomic and physiological performance under drought. The proteomic analysis identified 99 protein spots differentially regulated in response to the heat treatment, 14 of which were regulated in a genotype specific manner. Differentially regulated proteins with potential roles in the observed morphological and physiological changes under heat stress included photosynthetic proteins Rusbisco activase B and chlorophyll a-b binding proteins in addition to the glycolytic enzymes fructose-bisphosphate aldolase and glyceraldehyde-3-phosphate dehydrogenase. Of the stress responsive traits, significant differences in plant height, spike fertility, and photosynthetic performance were detected between Arta and Keel. Altogether, the detection of genetic variation in traits responsive to abiotic stress and in protein abundance represent unique stress adaption mechanisms which can be exploited in future crop breeding efforts.

Zusammenfassung

Trockenheit und Hitze sind die zwei führenden abiotischen Stressfaktoren, die sich limitierend auf den Ernteertrag auswirken. Ein Verständnis davon welche umfangreichen Reaktionen eine Modelpflanze wie Gerste – wenn unterschiedlichen Umweltbedingungen ausgesetzt – aufweisen kann, um Stressfaktoren zu umgehen, beziehungsweise zu tolerieren, wäre entscheidend bei der Aufklärung abiotischer tressresistenz. Die Zielsetzung der vorliegenden Arbeit war die Identifizierung von i) morphologische und physiologische Reaktionen auf Stress durch Hitze und Trockenheit, ii) genetischen Loci, welche mit agronomischen Merkmalen bezüglich der Ertragsleistung unter trockenen Bedingungen, verbunden sind und iii) Proteinen, die als Reaktion auf Hitze und Trockenheit unterschiedlich reguliert werden.

Eine durch rekombinante Zucht erzeugte Gerstensorte, die eine Kreuzung aus der syrischen Landgerste Arta und der australischen Sorte Keel ist, wurde in einem Gewächshaus unter guten Bewässerungsbedingungen gewachsen, und anschließend einer Trockenheitsbehandlung, beginnend mit der Anthesis und die bis zur Reife anhielt, unterzogen. Unter Verwendung von genotypischen Daten von über 700 genetischen Markern wurde eine Multi-umweltbedingte-Merkmal-Loci Analyse (engl.: multi-environmental quantitative trait loci (QTL)) morphologischer und physiologischer Merkmale durchgeführt. Für die proteomische Analyse wurden die parentalen Linien Arta und Keel in einem Gewächshaus unter guten Bewässerungsbedingungen gewachsen und mit der Anthesis startend folgenden Bedingungen ausgesetzt: Trockenheit, erhöhten Temperaturen, oder einer Kombination dieser beiden Bedingungen. Das Blattproteom von Arta und Keel wurde visualisiert, und mittles 2D-Gelelektrophorese (engl.: difference gel electrophoresis (DIGE)) wurde eine Änderung in der Menge an Proteinflecken (protein spots) aufgrund der Stressbehandlungen quantifiziert. Für die Identifizierung der aus den Gelen herausgeschnittenen Proteinflecken wurde Massenspektronomie genutzt.

Die Trockenheitsbehandlung war durch morphologische Plastizität und einer Beständigkeit auf physiologischer und proteomischer Ebene charakterisiert. Im Gegensatz dazu hatte eine Hitzebehandlung einen markanteren Störeinfluss auf die Pflanzenphysiologie und Proteinanreicherung als auf die Morphologie. Die QTL Analysen ließen 19 Loci erkennen für Merkmale, die mit einer agronomischen und physiologischen Leistung in Zusammenhang stehen. Durch die Proteomanalyse der parentalen Linien ließen sich 99 Proteine (Proteinflecken) identifizieren, die als Reaktion auf die Hitzebehandlung unterschiedlich reguliert wurden, 14 davon wurden in Abhängigkeit des Genotyps unterschiedlich reguliert. Der Großteil dieser unterschiedlich regulierten Proteine spielt eine Rolle im Stoffwechsel und der Photosynthese. Unterschiedlich regulierte Proteine mit möglichen Funktionen

verantwortlich für die beobachteten morphologischen und physiologischen Veränderungen als Reaktion auf Hitzebehandlung, schließen photosynthetische Proteine Rusbisco Aktivase B, Chlorophyll a-b bindende Proteine, als auch Glykolytische Enzyme wie Fructosebiphosphataldolase und Glyceraldehyd-3-Phosphatdehydrogenase ein. Einige Merkmale, die Stressabhängig zu sein scheinen, wie z.B. Pflanzengröße, Fertilität der Ähren und die photosynthetische Leistung, haben sich zwischen den zwei Genotypen unter Stressbehandlung deutlich unterschieden. Zusammenfassend erlaubt eine Erkennung der genetischen Variationen dieser stressabhängigen Merkmale sowie der Häufigkeit an Proteinen, dass diese Eigenschaften für spätere Züchtungen ausgenutzt werden.

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Chapter One - QTL Analysis of Agronomic Traits in Barley Under Water Limited Conditions

Introduction

As the world population grows and weather patterns become more unpredictable, the limitation of water available for agriculture will have an increasingly larger impact on the world's food supply [1]. To combat this problem is necessary to develop crops that are more resistance to drought. Drought resistance is the ability of a plant to maintain yield despite limitations in available water. The ability to maintain yield is achieved by plant responses to drought on the morphological, physiological and molecular level. The occurrence of drought is made complex by variations in its severity, duration, and timing [2]. Thus, the responses to drought are accordingly complex.

To date, studies concerned with the molecular basis of the complex responses to drought have been primarily performed in Arabidopsis (*Arabidopsis thaliana*). Such work has partially uncovered responses to drought and the signaling pathways that invoke them. Unfortunately, the majority of research in Arabidopsis has been limited to severe drought stress being applied in the early stages of plant development [3], which only simulates a portion of the stress that crops experience in the field. Additionally, Arabidopsis is not considered a drought tolerant species [4] and may lack the response mechanisms to drought that tolerant species employ. However, barley (*Hordeum vulgare*) is a genetically diverse species adapted to marginal, drought prone, agricultural areas as well as temperate, favorable environments. In comparison to the work in Arabidopsis, our understanding of the genetic basis of drought resistance in barley is lagging behind.

Barley is a drought resistant model crop with established genomic resources that make it suitable for studies concerned with the genetic basis of drought tolerance. Quantitative trait loci (QTL) analysis has been used successfully in Arabidopsis [5] and rice [6] to locate genes participating in the drought response. QTL analysis has also been made possible in barley by the development of segregating populations and recombination maps. A QTL analysis of traits responsive to water limited conditions during anthesis will allow the genetic basis of drought resistance to be further unraveled.

Barley is a model crop with worldwide agricultural importance

Barley originated in the steep ecoclines of the Fertile Crescent where it was under a variety of selection pressures for thousands of years. Since its origin, wild barley (*Hordeum spontaneum)* has been domesticated into numerous landraces due to selection for agronomic traits and has given rise to various

elite cultivars as a result of modern breeding programs. Barley is the fourth most important cereal crop, after maize, rice and wheat in terms of world production. In the year 2010, 123.4 million tons of barley were harvested worldwide from an area totaling 47.8 million hectares [7]. Top world producers of barley in 2010 were Russia (17.8 million tons), Germany (11.8 million tons), and Canada (11.6 million tons) [7]. The majority (~66%) of barley produced worldwide is used for animal feed and a small portion (2%) is used in the production of food stuffs for human consumption while the rest (~32%) is used for malt in the production of fermented beverages [8]. Additional to the agricultural importance of barley is the value of barley as a genetic model for other crops.

The value of barley as a model crop also lies in the genomic tools and research that have been established for it. The barley genome is diploid with seven pairs of chromosomes designated 1H to 7H with an estimated size of 5.1 Gbp [9]. Attempts to sequence the entire genome of barley have been initiated [10][11] and a gene-based marker map of the barley genome has been completed [12]. Comparison of barley gene sequences and gene order to rice, sorghum and *Brachypodium* revealed high collinearity between the four genomes [13] and the comparison will aid in future efforts to assemble the complete genome of barley and more complex genomes such as wheat. In addition to the genomic tools available, microarrays for barley gene expression have been established based on a library consisting of 350,000 high-quality ESTs [14]. Reverse genetics approaches to discovering gene function are also possible in barley thanks to the development of Targeting Induced Local Lesions in Genomes (TILLING) populations [15]. Despite the size and complexity of the barley genome, the above resources combined with the genetic diversity of barley make this species an ideal model for use in abiotic stress research.

Molecular responses to drought

Drought resistance mechanisms can be divided into escape, avoidance and tolerance. Drought escape is the completion of the life cycle before water deficits occur. An example of drought escape would be the completion of flowering and grain filling before the onset of seasonal drought. Drought avoidance is the ability of a plant to maintain a high water status despite experiencing water limited conditions. Drought avoidance mechanisms include: stomatal closure, inhibition of shoot growth, promotion of root growth, and the accumulation of osmolytes [16]. Drought tolerance is the ability to maintain cellular activity despite a decrease in water status. Drought tolerance mechanisms include the increased production of free radical scavengers, protein protecting molecular chaperones, and proteases [4].

One of the most immediate drought avoidance responses to water limitation is the closure of the stomata to reduce transpiration and avoid dehydration. Stomata closure is mediated by the hormone abscisic acid (ABA) in a pathway recently characterized thanks to the discovery of the PYR/PRL family of

ABA receptors. In brief, binding of ABA to the PYR/PRL receptor allows the receptor/ligand complex to inhibit the phosphatase PP2C which would otherwise dephosphorylate and inhibit the kinase SnRK2.6 [17]. Thus, in the presence of ABA, SnRK2.6 is free to phosphorylate and activate the anion channel protein SLAC1 found in guard cells. Once active, SLAC1 regulates the efflux of anions into the apoplast of guard cells resulting in membrane depolarization and subsequent stomata closure [18]. Drought avoidance can also occur through a reduction in the growth rate of leaves and stems as the plant acclimates to water limited conditions. A reduction in the leaf cell number and cell size has been observed in Arabidopsis plants under osmotic stress [19][20]. However, despite the inhibition of above ground growth, roots can continue to elongate despite inhibition of shoot growth [21] in an attempt to access more soil water.

Maintaining a water potential more negative than that of the surrounding soil is crucial for the continued uptake of water into the plant. The accumulation of compatible solutes under drought helps maintain the positive water flow into the plant by reducing the water potential of the cell. Compatible solutes increase the osmolarity in the cell without disturbing ionic interactions and include amino acids (e.g. proline), quaternary amines (e.g. glycine and betaine) and polyols (e.g. mannitol and sorbitol) [22]. In addition to playing a role in drought avoidance, compatible solutes can assist in drought tolerance by acting as free radical scavengers [23] and chemical chaperones [24].

When attempts to avoid drought fail, the plant is left to tolerate the effects of the stress. Two of the major sources of damage during drought stress come from reactive oxygen species and misfolded proteins. Reactive oxygen species (ROS) such as super oxide anion, hydroxyl and singlet oxygen, are primarily formed due to the improper flow of energy during light harvesting [25][26] or due to oxidation of photorespiration products during the light-independent reactions of photosynthesis [27]. Drought increases the production of ROS mainly through the limitation of available carbon dioxide as a final electron acceptor [28][29]. Once formed, ROS may oxidize and damage components crucial for cellular function such as proteins, membrane lipids, and nucleotides [30]. Prevention of damage by ROS is accomplished by the production of enzymatic and non-enzymatic antioxidants which can scavenge ROS by converting them into less reactive forms. Scavenging is performed in part through the action of the enzymes superoxide dismutase, ascorbate peroxidase, glutathione reductase and catalase [22] as well as by the above mentioned compatible solutes. Despite an increase in ROS scavenging drought stress can still result in the damage of proteins which can cause them to denature and aggregate.

Misfolding of proteins during drought can occur due to low cellular water content in addition to damage by ROS. Low cellular water content can lead a to a smaller cytoplasmic volume which results in increased interactions between proteins and a of lack water molecules to form hydration shells around

proteins necessary to maintain proper folding and stability [31]. While some compatible solutes can temporally replace water in providing a hydration shell for proteins [32] the heat shock family of proteins can also act as molecular chaperones for proteins. The heat shock family includes members that are inducible by drought stress [33], and convey drought tolerance by preventing the denaturation of proteins, refolding misfolded proteins, or preventing the aggregation and subsequent accumulation of misfolded proteins [34]. Non-functional proteins that cannot be refolded must be degraded via proteolysis. Proteolysis is increased in response to drought stress [35][36] and is thought to promote drought tolerance by removing damaged proteins and mobilizing nitrogen [37].

Together, small molecules such as compatible solutes and macromolecules such as enzymes comprise a large portion of the drought avoidance and drought tolerance responses known to date. Drought avoidance is concerned with maximizing water uptake and minimizing water loss so as to maintain high plant water status. Drought tolerance is concerned with minimizing cellular damage incurred by ROS and protein denaturation.

Quantitative trait loci for drought resistance

Quantitative trait loci (QTL) analysis has proven to be a valuable method in discovering the genetic basis of quantitative traits. Quantitative traits, or continuous traits, are those that cannot be placed into discrete classes. The genes underlying quantitative traits have been successfully mapped and subsequently identified using QTL analysis in a diverse set of organisms including: rat [38], mouse [39], cow [40], tomato [41], Arabidopsis [5] and rice [6]. The requirements for performing a QTL analysis are: species that can be inbred to produce a segregating population, genetic markers distributed across each chromosome to generate a recombination map, and a trait with quantitative variation that can be phenotyped. QTL analysis is a statistical test performed for a genetic marker which determines if significant differences exist between the phenotype exhibited by progeny carrying one parental allele and progeny carrying another parental allele. The test is repeated for every marker in the recombination map and when a significant difference is found the QTL is considered to be linked to that marker. Since its inception, QTL analysis has evolved to include more sophisticated techniques such as simple interval mapping [42], composite interval mapping [43], and permutation testing [44] which have increased the resolution and reliability in detecting QTL.

QTL analysis has been used to identity chromosomal regions involved in drought stress responses in barley since 1997 by Teulat et al. [45]. Since then, at least eleven studies using barley grown under water limited conditions in the greenhouse and field have been published which used only three mapping populations: Tadmor x Er/Apm [45][46][47][48][49][50][51][52], Arta x *Hordeum spontaneum*

41-1 [53][54], and Steptoe x Morex [55]. From these studies, over 200 significant QTL for a variety of drought resistance related traits have been discovered. These traits, among others, include: relative water content, chlorophyll fluorescence parameters, grain yield, days to maturity, plant height, and kernel weight. These QTL are useful in mapping genes responsible for conveying drought resistance and can be applied in marker assisted breeding programs [56]. Of the QTL studies enumerated above, some have demonstrated the specificity of some QTL to one environment, for example, in a growth chamber experiment using the Tadmor x Er/Apm population QTL for relative water content and leaf number were detected under drought conditions but not well watered conditions [45].

Development dependent responses to drought

Responses to drought are dependent on when the stress occurs (i.e. which developmental stage the barley plant is in) [57], how severe the stress is [58], and how long it persists [59]. Of the eleven barley drought resistance QTL studies mentioned above, six were conducted in the field under low rainfall conditions and five were conducted in the controlled environment of a greenhouse or growth chamber. In rainfed field studies, where the rainfall is not easily controlled, the timing, severity and duration of the drought differs between replications. Therefore, the average recorded phenotypes in rainfed studies are the integration of the varied responses to the different types of drought stress experienced by the population over the experimental replications. While QTL studies under rainfed field conditions provide valuable insight to the genetic basis of barley performance in the field, they provide less information on developmental stage specific responses to drought. In controlled environment studies, the timing of the stress event can be chosen and coinciding factors such as light intensity and humidity can be controlled. In four of the five QTL studies conducted in controlled environments, the stress was applied at the four-leaf stage of development i.e. during vegetative growth. The remaining experiment, which was only concerned with chlorophyll fluorescence parameters, applied drought stress starting post-flowering. A review of the literature revealed an absence of QTL studies in barley that characterized the morphological and physiological responses to drought applied during flowering.

The objective of this study was to identify marker-trait linkages in a barley RIL population, derived from the Syrian landrace Arta and the Australian cultivar Keel, under moderate drought applied during anthesis using quantitative trait loci analysis. Additionally, this study aimed to understand which morphological and physiological traits respond to drought stress and how they correlate with yield performance under well watered and drought conditions. These objectives were realized by measuring

grain yield and yield related traits at maturity in addition to morphological and physiological responses that occur during drought stress.

Materials and methods

Experimental overview

This chapter is concerned with the genetic basis of physiological and morphological changes in barley that occur in response to long term drought stress applied at anthesis. Control plants were well watered and maintained at a soil water content (SWC) of 50% while the SWC of drought treated plants was maintained at 15%. These treatments were applied to barley genotypes Arta and Keel and to a core population of 56 recombinant inbred lines (RILs) from a cross of the parental lines Arta and Keel. Physiological traits were measured in parental lines one day and three days after the target SWC of drought treated plants was reached. Morphological measurements were made during grain filling and agronomic measurements were made after plant maturity. A list of all traits considered and the abbreviations used in this text can be found in **Table 1**. The variance in each trait measured in Arta, Keel and the RIL population was attributed to effects of genotype, the treatment, or the interaction of two, by using a two way ANOVA. Phenotyping in the RIL population under each treatment was used to calculate genetic correlations between grain yield and the physiological, morphological and agronomic traits. By utilizing the recombination map available for the RIL population, Quantitative trait locus (QTL) analysis was performed using the collected phenotypic measurements to locate genetic loci containing potential drought resistance genes.

Plant material

Two *Hordeum vulgare* genotypes, Arta and Keel, were grown in well watered and water limited conditions in the greenhouse to evaluate their performance. Arta is a pure line selection from a Syrian landrace adapted to the driest areas in Syria. Keel is a feed-quality cultivar from Southern Australia with good performance under dry conditions in Australia. A recombinant inbred line population (RIL) generated at International Center for Agricultural Research in the Dry Areas (ICARDA) consisting of 188 F8 plants from the cross Arta x Keel (Arke) was available for genetic analyses. A subset of 54 individuals from the Arke population was selected to represent the maximal diversity in the population by using Core Hunter version 1.0b (CIMMYT)[60] set to optimize the modified Rogers distance.

Table 1. Traits measured experimentally including the abbreviation used, units used and the procedure by which the measurements were taken.

Abbr.	Trait	Units	Procedure
GY	Grain yield	g	Total weight of kernels
BM	Total biomass	g	Weight of all above ground plant biomass
HI	Harvest index	g/g	Ratio of grain yield over total biomass
PH	Plant height	cm	Distance from top of soil to top of primary spike
Pedex	Peduncle extrusion	cm	Distance from peduncle to bottom of primary spike
SN	Spike number		Number of spikes over half of grains filled
AS	Number of aborted spikes		Number of pikes with over half of all grains unfilled
GS	Grains per spike		Number of grains divided by number of spikes
TKW	Thousand kernal weight	g	Extrapolated weight of 1000 kernals
DM	Days to maturity	days	Days in LD until kernels were mature
WU	Total water used per pot	L	Water given to plant from LD to maturity
WUE	Water use efficency of grain yield	g/L	Ratio of grain yield over water used
RWC_1	Relative water content at day 1	%	Ratio of leaf fresh weight over fully turgid weight
RWC_3	Relative water content at at day 3	%	Ratio of leaf fresh weight over fully turgid weight
Fv/Fm_1	Maximum PSII quantum yield at day 1	arb. unit	Chl flouresence of dark adapted leaf before the flag leaf
Fv/Fm_3	Maximum PSII quantum yield at day 3	arb. unit	Chl flouresence of dark adapted leaf before the flag leaf
PI_1	PSII performance index at day 1	arb. unit	Chl flouresence of dark adapted leaf before the flag leaf
PI_3	PSII performance index at day 3	arb. unit	Chl flouresence of dark adapted leaf before the flag leaf

Drought treatment

The response to drought stress was tested under controlled conditions in the greenhouse by withholding water at the generative stage (Zadoks scale 49-53) [61]. Plants were sown in 96 well trays, vernalized at 4°C for 5 weeks with an 8 h light/16 h dark short day (SD) photoperiod and then potted in four liter pots containing 1.8 kg of soil with three plants in each pot. The field capacity of 1.8 kg soil was calculated as the difference in weight between fully hydrated soil and dried soil. The soil was allowed to fully hydrate by flooding the pot with water and allowing any excess water to drain over 24 h while the pot was covered. Soil was dried by heating at 70°C for seven days. The soil water content (SWC) of potted plants was adjusted to 50% of the field capacity (FC) based on trial experiments concerned with finding the moisture content for optimum growth (data not shown). The pots were arranged in two random blocks in a climatically controlled SD greenhouse for 10 days before being moved to a climatically controlled greenhouse with a 16 hr/8 hr long day (LD) photoperiod for the remainder of the experiment. In the mornings, evenings and during overcast days, supplemental lighting was used to ensure that the light levels were maintained at no less than 200 µmol photons m^{-2} s^{-1}. Humidity of the greenhouse was maintained between 50% and 60%.

For the water stress treatment, the water content of the soil was reduced to 15% FC by withholding water in a controlled manner; all pots were weighed every day and then watered to match the weight of

the heaviest pot. Following this gravimetric method, the reduction of the water content of the soil was equal across all pots in the stress treatment. The SWC of 15% FC for the stressed plants and 50% FC for the control plants was maintained until maturity of the plants. The amount of water given to each four liter pot was recorded and used to determine the water use efficiency. To control for the added weight of the growing plants the volumetric water content of random pots were periodically checked using a TDR 100 soil moisture meter (FieldScout) fitted with 12 cm probe rods and set to standard soil mode. If the projected SWC was lower than the measured water content than additional water would be added to correct for the increased plant mass.

Physiological measurements

Samples were taken from control and drought treated plants one and three days after the target FC of 15% had been reached in drought stressed pots. The leaf directly under the flag leaf was used for both physiological measurements. Fast chlorophyll fluorescence induction kinetics was measured on every plant per pot using the Handy PEA chlorophyll fluorimeter (Hansatech Instruments). Fluorescence was induced using a 3000 µmol photons m^{-2} s^{-1} flash of light persisting for 1 s on leaves dark adapted with leaf clips for a minimum of 20 min. Care was taken to place the leaf clips so as to avoid taking measurements that included the midrib. Chlorophyll fluorescence induction curves were analyzed using the PEA plus software version 1.02 (Hansatech Instruments) and the maximum quantum efficiency (Fv/Fm) and the Performance Index (PI) were calculated. The relative water content of the leaf (RWC) was calculated from one ~3 cm leaf cutting per plant and calculated according to $\frac{fresh\ weight - dry\ weight}{turgid\ weight - dry\ weight} \times 100$ [62]. Freshly cut leaves were immediately weighed to determine the fresh weight. The turgid weight was determined after submerging the leaf cuttings in distilled water in closed tubes and storing them overnight at 4 °C in the dark. Dry weight of the leaf cuttings was determined after drying the cuttings at 70 °C for 48 hours.

Morphological and agronomic measurements

Plants were considered to head when the spikelet had visibly emerged from the ear in the main tiller and was used as a developmental marker to apply the drought stress. Peduncle extrusion (Pedex) and plant height (PH) were recorded post-anthesis by measuring the distance from the flag leaf collar to the bottom of the main tiller and the distance from the soil surface to the top of the spike, respectively. In cases where the spike remained booted in the leaf sheath, negative Pedex values were recorded. The number of grains on each primary spike (GS) was counted as well as the number of spikes per plant (SN). The days until maturity (DM) was recorded for each pot from the time of transfer to LD until the majority

of the grains could no longer be dented by fingernail. Mature plants were harvested and the following measurements were taken per pot: above ground biomass dry weight (BM), total grain weight (GY), and 1000 kernel weight (TKW). Harvest index (HI) was calculated as the ratio of the total grain weight over the biomass above ground dry weight. The water used by the plants in each pot (WU) was recorded starting after the plants were potted in four liter pots. To control for the water lost due to evaporation alone, four additional pots were included in the experiment which contained 1.8 kg of soil but no plants and were watered to maintain SWC of either 50% or 15% FC. Water use efficiency (WUE) was calculated as the ratio of the grain yield over the water given to each pot.

Statistical analysis

Statistical analyses were conducted using SAS software version 9.1.3 for Windows (SAS Institute Inc, copyright 2003) using data from the parental barley genotypes and from the RIL population. The command PROC MEANS was used to determine the mean, minimum and maximum values for Arta, Keel and the RIL population. A two way analysis of variation (ANOVA) was performed for each trait in Arta, Keel, and the RIL population with a general linear model using the PROC GLM command with the model: $Y_{ij} = G_i + E_k + G_i \times E_{k,}$ where G_i is the fixed effect of the genotype, E_k is the fixed effect of the treatment, and $G_i \times E_k$ is the interaction of the two effects. Pearson correlation coefficients between all traits recorded in the RIL dataset under control conditions and stress conditions were generated separately using the PROC CORR command.

Linkage mapping

Arta, Keel and 188 RILs were genotyped with six gene specific PCR markers, 103 microsatellite (SSR) markers and 623 Diversity array technology (DArT) markers. DArT genotyping was carried out by Triticarte Pty. Ltd. (Australia). Markers with a segregation distortion higher than 20% in the 188 RILs were excluded from linkage map construction. As a first step SSR markers were assigned to barley chromosomes based on a previously published barley consensus map Alsop et al. [63] . Linkage groups of SSR-, DArT- and PCR-markers were defined using a LOD threshold of 3.0 using the mapping software JoinMap 3.0 (Kyazma B.V.). Genetic distances between markers were calculated using the Haldane mapping function of the software package. For the seven barley chromosomes (1H-7H) 661 markers were assigned to 11 linkage groups with a total map size of 1147.9 cM and an average marker distance of 1.7 cM. Insufficient linkage was found between markers on chromosomes 1H, 2H and 7H, therefore they were assigned to the separate linkage groups 1Ha/1Hb, 2Ha/2Hb and 7Ha/7Hb/7Hc, respectively. Linkage groups of chromosomes 1H, 2H and 7H were combined into a single linkage group per

chromosome with an arbitrary genetic distance of 20 cM added in between them. Minor changes in marker positions and marker order in the range of 1-10 cM were detected when compared to the consensus map of Alsop et al. [63].

Quantitative trait loci analysis

The QTL analysis was conducted with MultiQTL version 2.5 [64]. The population type selected was "RIL selfing" and the multiple environment option was used to calculate significant effects across control and drought treatments. Simple interval mapping using the Haldane function was employed for each trait. A permutation test consisting of 1001 iterations was used to detect significant QTL ($p < 0.05$).

Results

Robustness of barley physiology despite drought

Physiological measurements in drought experiments are important for understanding the extent of stress experienced by the plant due to the treatment. Such measurements allow morphological changes in the treated plants to be put into context of how the plant is responding on the cellular level and can facilitate the comparison of results between different experimental setups. Two physiological measurements are considered here: the leaf relative water content and the chlorophyll fluorescence induction curve. Leaf relative water content (RWC) is a measurement of the how much water is present in the leaf relative to the maximum amount of water the leaf can contain and is an indicator of the overall water status of the plant. After one day of drought treatment, the mean RWC dropped significantly in the RIL population from 87.3% in control conditions to 81.2% in drought conditions (**Table 2**). However, no significant differences in the mean RWC of stressed Arta and Keel plants were detected as compared to controls. After three days of drought treatment, significant differences in mean RWC between conditions were seen in Arta and Keel with respective values of 96.5% and 95.9% in control conditions and 80.2% and 76.0% in drought conditions. Conversely, the mean RWC of the RIL population was not statistically different between conditions after three days of treatment.

The chlorophyll fluorescence induction curve is obtained by measuring the rise in chlorophyll fluorescence in photosystem II (PSII) from a dark adapted state where the reaction centers are fully oxidized to a light saturated state where the reaction centers are fully reduced. One of the parameters calculated from the induction curve is the maximum quantum efficiency of photosystem II which is the ratio of the difference in fluorescence between the fully reduced state (Fo) and the fully oxidized state (Fm) over Fm, this ratio $\frac{Fm-Fo}{Fm}$ is also known as Fv/Fm. The maximum quantum efficiency of

photosystem II (Fv/Fm) describes how often the primary plastoquinone acceptor (Q_a) is reduced per photon absorbed and is an indicator of the status of the light harvesting complex of PSII. The other, more comprehensive parameter considered was the performance index (PI), which incorporates three independent aspects of photosynthesis; the force of the light reactions, the force of the dark reactions and the efficiency of light trapping by the light harvesting complex.

Table 2. The mean**,** minimum (Min) and maximum (Max) values of the traits measured for Arta, Keel and the Arke RIL population either under control or drought conditions. Means that are not significantly ($p<0.05$) different share the same letter subscript.

	Control Arta			Control Keel			Control Arke RILs			Drought Arta			Drought Keel			Drought Arke RILs		
Trait	**Mean**	**Min**	**Max**	**Mean**	**Min**	**Max**	**Mean**	**Min**	**Max**	**Mean**	**Min**	**Max**	**Mean**	**Min**	**Max**	**Mean**	**Min**	**Max**
GY	7.6 a	6.0	8.2	5.3 cd	3.2	6.7	5.4 c	2.4	9.8	3.7 bd	2.8	4.4	3.0 b	2.6	3.8	3.3 b	1.9	4.7
BM	14.2 a	11.9	15.5	10.0 cd	6.4	12.4	10.8 c	5.7	18.6	7.3 bd	5.7	8.5	6.0 b	5.0	7.5	6.9 b	4.2	9.6
HI	0.54 a	0.50	0.64	0.52 a	0.42	0.57	0.49 a	0.41	0.58	0.50 a	0.48	0.52	0.50 a	0.48	0.53	0.48 a	0.34	0.71
PH	54.4 a	50.3	57.8	50.7 ac	48.6	53.5	53.1 a	42.3	63.5	44.8 bc	39.4	49.2	42.8 b	39.7	45.6	42.8 b	31.4	55.0
Pedex	1.5 a	-1.5	15.8	-4.6 bc	-5.8	-2.5	-4.0 c	-9.1	4.8	-6.8 bc	-8.1	-4.6	-5.7 bc	-10.3	0.9	-6.3 b	-11.2	3.3
SN	8.9 a	5.0	10.3	7.5 ab	4.0	10.0	5.5 b	3.3	36.5	3.7 bc	3.0	4.0	3.5 bc	3.0	4.0	3.6 c	1.3	5.0
GS	14.1 ab	11.7	16.3	12.7 a	10.7	14.7	13.6 a	10.0	17.7	15.4 b	13.7	17.0	13.5 ab	12.3	14.3	12.8 a	10.0	16.7
TKW	55.0 b	50.8	58.1	54.0 ab	49.2	62.0	49.1 a	32.2	60.8	51.6 ab	48.3	53.9	53.8 ab	49.2	59.4	50.5 ab	41.7	60.2
DH	19.5 a	18.0	20.0	14.5 bc	13.0	20.0	16.5 ab	13.0	23.0	19.1 a	18.0	21.0	13.4 c	13.0	14.0	16.8 ab	13.0	22.0
DM	68.0 a	63.0	70.0	69.8 a	68.0	70.0	68.1 a	56.0	70.0	60.8 b	60.0	62.0	61.8 b	60.0	62.0	63.4 b	57.0	70.0
WU	12.88 a	11.54	13.59	9.58 c	7.34	11.48	10.50 c	6.75	14.39	7.22 b	6.44	7.90	6.08 b	5.20	6.57	6.31 b	4.32	8.35
WUE	1.8 b	1.5	2.1	1.6 ab	1.2	1.9	1.5 a	1.0	2.1	1.5 ab	1.3	1.7	1.5 ab	1.3	1.7	1.6 ab	1.1	1.9
RWC_1	87.2 abc	63.4	100.0	87.3 ac	62.0	100.0	93.1 a	78.8	100.0	81.6 bc	71.9	100.0	84.2 abc	64.7	100.0	81.2 b	69.5	95.6
RWC_3	96.5 a	78.3	100.0	95.9 a	64.7	100.0	89.4 b	75.2	100.0	80.2 bc	62.5	95.8	76.0 c	44.4	100.0	88.4 b	64.4	100.0
Fv/Fm_1	0.832 a	0.823	0.839	0.833 a	0.831	0.838	0.840 a	0.821	0.850	0.832 a	0.816	0.841	0.837 a	0.832	0.839	0.838 a	0.806	0.851
Fv/Fm_3	0.844 ab	0.841	0.847	0.842 ab	0.840	0.844	0.843 b	0.829	0.852	0.845 ab	0.844	0.846	0.844 ab	0.843	0.844	0.840 a	0.833	0.850
PI_1	3.4 a	2.9	4.0	4.1 a	3.9	4.431	3.9 a	1.7	4.9	3.1 a	2.6	3.6	4.1 a	3.8	4.4	3.8 a	1.863	4.768
PI_3	3.8 ab	3.5	4.2	4.3 ab	4.3	4.3	4.1 b	2.7	5.1	3.2 ab	3.1	3.3	4.2 ab	4.0	4.4	3.6 a	2.5	4.5

GY grain yield, BM biomass, HI harvest index, PH plant height, Pedex peduncle extrusion, SN spike number, GS grains per main spike, TKW 1000 kernel weight, DH days to heading, DM days to maturity, WU water use, WUE water use efficiency, RWC _1/3 leaf relative water content at 1 or 3 days after treatment start, Fv/Fm_1/3 at 1 or 3 days after treatment start, PI_1/3 performance index 1 or 3 days after treatment start.

The mean Fv/Fm of all plants was similar one day after the target SWC of 15% was reached, with values ranging from 0.832 to 0.840 (**Table 2**). Three days after the target SWC was reached, there was a significant difference in the mean Fv/Fm in the RIL population between control (0.843) and in drought (0.840) conditions. The mean Fv/Fm between treatments was not different for Arta and Keel after three days of treatment. The mean PI after one day of stress treatment was not different between Arta, Keel and the RIL population in either control and drought conditions with mean values ranging from 3.1 to 4.1 (Table 2). After three days at 15% SWC, mean PI values between treatments were not different for Arta and Keel while the RIL population showed a significant decrease in the mean PI from 4.1 in control conditions to 3.6 in drought stressed conditions. The PI values in the RIL population after three days at 15% SWC was normally distributed under control and drought conditions (**Figure 1**). It is notable that

under control conditions two RILs had PI values two standard deviations higher than either parental line which was indicative of transgressive segregation for this trait.

Altogether, these results show that the RWC, Fv/Fm and the PI of the plants were relatively unaffected by the drought treatment as seen in the slight reduction in the water status of the plants and in the intermittent decrease in the photosynthetic performance as compared to control.

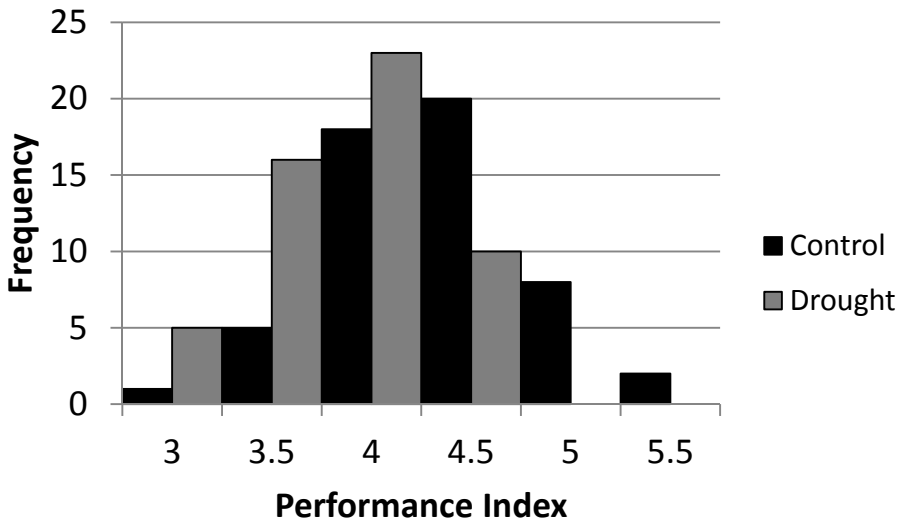

Figure 1. Distribution of the mean performance index three days after the SWC reached 15% in the RIL population under the two watering schemes. Under control conditions the mean performance index and standard deviation was 3.84±0.54 for Arta and 4.3±0.02 for Keel. Under drought conditions, the mean performance index and standard deviation was 3.22±0.12 for Arta and 4.21±0.30 for Keel.

Reduction in grain yield and yield components due to drought stress

When evaluating the performance of a crop plant the most important trait is yield. Grain yield is a complex trait which is dependent on yield component traits such as the ones measured here: number of spikes per plant, the number of grains per spike and the thousand kernel weight. The mean grain yield of Arta under control conditions (7.6 g) was significantly higher than that of Keel (5.3 g) and the RILs (5.4 g) (**Table 2**). However, the mean yield under drought stress conditions of Arta (3.7 g), Keel (3.0 g), and the RIL population (3.3 g) were not different from each other. Comparing yields between treatments revealed significant decreases in yield for Arta, Keel and the RIL population under drought conditions as compared to control conditions. By dividing the grain yield achieved under drought conditions by the yield achieved under control conditions one can obtain the yield tolerance index of each group (**Figure 3**). The highest yield stability was seen in the RIL population (0.60), the second highest in Keel (0.57), with Arta having the lowest index (0.48). The yield of the RIL population was normally distributed under

control conditions and under drought conditions (**Figure 2**). Four RILs had grain yield two standard deviations higher than either parent under control conditions and eight RILs were higher under drought conditions, which is indicative of transgressive segregation for yield in the RIL population.

To better understand how drought affects yield, the performance of individual yield components were also measured under control and drought conditions. There are also genetic components to yield component traits, for example, some genotypes may produce more grains by producing more tillers while other genotypes may favor having more grains per spike. Because the two genotypes used in this study are adapted to different drought prone areas it is possible that have adapted different strategies to maximize yield. The number of spikes developed under water limited conditions can indicate how well the plant has continued to grow despite a perceived limitation in available water. There was a significant decrease in the mean spike number for Arta, Keel and the RILs in drought conditions (3.7 and 3.5, and 3.6, respectively) as compared to control conditions (8.9, 7.5, and 5.5, respectively) (**Table 2**). The mean spike number under control conditions was not different between Arta and Keel but was significantly different between Arta and the RILs. Under drought conditions, the mean spike number was similar between Arta, Keel and the RILs. The mean number of grains per primary spike under control conditions for Arta (14.1), Keel (12.7) or the RILs (13.6) were not different from the number present in plants grown under drought conditions, respectively (15.4, 13.5, and 12.8). The number of grains per primary spike between conditions was similar for Arta, Keel and the RILs with mean values ranging from 12.7 to 14.1 under control conditions and from 12.8 to 15.4 under drought conditions (**Table 2**). The third yield component trait considered was the thousand kernel weight. Under control conditions, Arta had a higher mean thousand kernel weight than the RILs with respective values of 55.0 g and 49.1 g; the kernel weight of Keel was intermediate between the two (54.0 g)(**Table 2**). The mean thousand kernel weight was not different between Arta, Keel and the RILs under drought nor was it different between conditions for Arta, Keel and the RILs.

In summary, grain yield in Arta, Keel and the RILs was reduced due to the drought treatment and this appears to be due to a reduction in the spikes that developed during the drought treatment. It appeared that the reduction in grain yield between conditions was neither due to differences in the number of grains per spike nor due to reductions in the overall weight of individual grains. Arta produced more grain yield than Keel under control conditions but not under drought conditions. In addition to yield component traits, morphological and developmental traits classically associated with yield in an agricultural setting were considered. Many of these agronomic traits, such as plant height, peduncle extrusion, and days to maturity were seen here to be positively and significantly correlated to grain yield under control and drought conditions (**Table 4**).

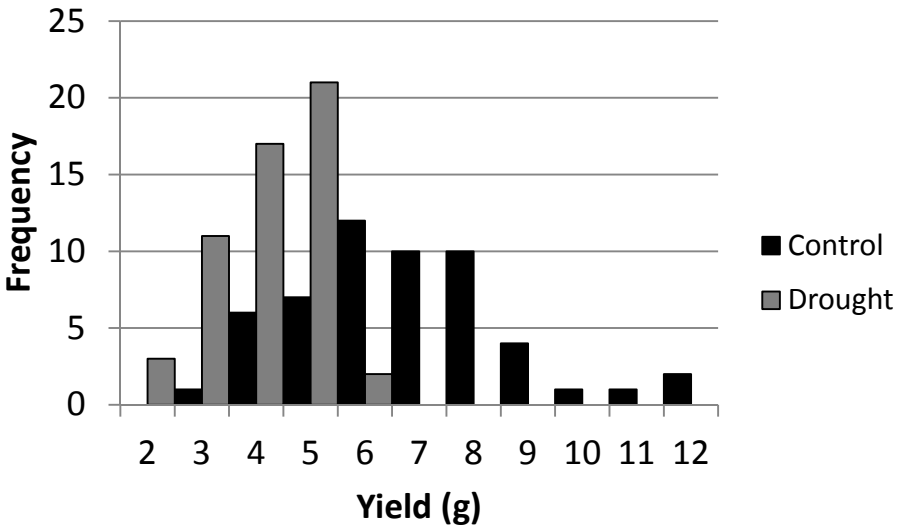

Figure 2. Distribution of the mean grain yield weight per RIL under the two watering schemes. Under control conditions the mean grain yield per plant and standard deviation was 7.59±0.71 for Arta and 5.27±1.46 for Keel. Under drought conditions, the mean grain yield per plant and standard deviation was 3.66±0.49 for Arta and 3.03±0.04 for Keel.

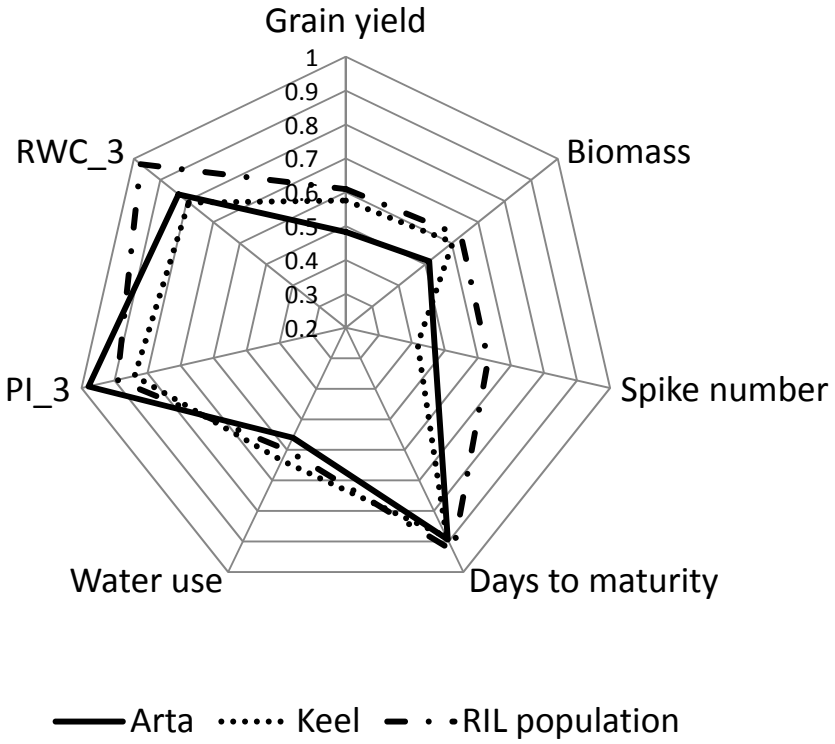

Figure 3. Tolerance index of: grain yield , biomass , number of spikes per plant , days until maturity , water use (WU), the performance index (PI_3), and the relative water content (RWC_3) of Arta, Keel and the average of the RIL population. Units of each axis are the fraction of the trait value maintained under stress conditions as compared to control.

Evaluation of agronomic traits under drought stress

Aside from grain yield and its components, additional agronomic traits pertaining to growth, water use and development were considered. Specifically, the traits measured were: biomass, harvest index, days to maturity, peduncle extrusion, water use, and water use efficiency. The above ground biomass of Arta under control conditions was significantly higher than that of Keel and the RILs with mean per pot weights of 14.2 g, 10.0 g and 10.8 g, respectively (**Table 2**). Under drought conditions there was no significant difference in the biomass between Arta, Keel and the RILs. Due to the drought treatment, the mean biomass of Arta (7.3 g), Keel (6.0 g) and the RILs (6.9 g) were significantly lower than their control counterparts. The tolerance index of biomass was the highest in the RILs and lowest in Arta (**Figure 3**). Harvest index, the ratio of grain yield over the total above ground biomass, is a measure of how the photosynthate has been allocated in the plant. The harvest index was neither different between Arta, Keel and the RILs under either condition nor between conditions for Arta Keel, and the RILs. Mean values of the harvest index ranged from 0.48 to 0.54 (**Table 2**). The time taken for barley grains to mature dictates how long the plant has to fill its grains as well as how long the plant must be kept in the field before harvest. Arta, Keel and the RILs matured faster under drought conditions (61 d, 62 d and 63 d, respectively) than under control conditions (68 d, 70 d and 68 d, respectively)(**Table 2**). The time taken for plant maturity was neither significantly different between Arta, Keel and the RILs under control conditions nor under drought conditions. The mean plant height under control conditions in Arta (44.8 cm), Keel (42.8 cm) and the RILs (42.8 cm) was significantly diminished due the drought treatment (54.4 cm, 50.7 cm and 53.1 cm respectively). No significant differences in plant height were detected between Arta, Keel and the RILs under either condition (**Table 2**). The peduncle extrusion, or Pedex, is a measurement of the distance between the flag leaf collar and the spike bottom and can be negative when the spike remains booted in the leaf sheath. Arta had a positive mean Pedex under control conditions (1.5 cm) which was significantly higher than the Pedex of Keel and the RILs (-4.6 cm and -4.0 cm) (**Table 2**). Due to the drought conditions the mean Pedex of Arta and the RILs (-6.8 cm and -6.3 cm, respectively) was significantly lower than in control conditions.

Both control and drought treatments consisted of maintaining soil water content constant by replenishing lost water. This allowed for the total amount of water given to each pot to be recorded. The mean water use per pot in well watered conditions was significantly higher for Arta (12.9 l) than for Keel (9.6 l) or the RILs (10.5 l) (**Table 2**). The water use under drought conditions was not different between Arta (7.2 l), Keel (6.0 l) and the RILs (6.3 l) and was significantly lower than the water used under control conditions. The water use data was combined with the yield data to calculate the water use efficiency

(WUE); the ratio of yield over the water lost from the pot. The WUE of Arta (1.8) was significantly higher than for the RILs (1.5) under control conditions with Keel (1.6) being intermediate between the two. No significant differences in the WUE were detected between conditions for Arta, Keel or the RILs (**Table 2**).

In summary, the significant differences that appeared between Arta, Keel and the RILs occurred under control conditions. Under control conditions, Arta had more biomass, had peduncles extruded further from the leaf sheath and used more water than Keel or the RIL population. Due to the drought treatment Arta, Keel, and RILs plants had less biomass, were shorter, matured faster, and used less water. Additionally, Pedex values were lower in drought treated Arta and RILs as compared to control treated plants.

Phenotypic variation of traits due to genotype and drought conditions

The variation in each trait due to effects of genotype, watering treatment and the interaction between the two factors was calculated for Arta, Keel, and RILs plants (**Table 3**) using a two factor AVOVA. Such an analysis allows the influence of the genotype alone and the treatment alone to be estimated on each trait. A list of the traits analyzed with the ANOVA and the abbreviations of the traits used in this test are found in **Table 1**. The factor condition explained a significant proportion of the variance for the highest number of traits, nine in total. The factor genotype and the interaction between genotype and condition explained a significant proportion of the variance for seven traits each. The treatment conditions had a significant effect on the expression of the following traits: water use (35%), Days to maturity (32%), RWC_2, (32%), biomass (31%), grain yield (30%), plant height (22%), Spike number (16%), Pedex (15%), RWC_1 (7%) and water use efficiency (3%). A significant effect of the genotype was detected for the traits: thousand kernel weight (10%), grains per spike (9%), PI_1 (8%), grain yield (6%), Pedex (5%), biomass (5%), water use (4%), and spike number (4%). The interaction between genotype and condition explained a significant amount of variation for the traits: RWC_2 (26%), Pedex (8%), water use efficiency (6%), grains per spike (5%), RWC_1 (3%), grain yield (2%), days to maturity (2%), and biomass (2%). The traits harvest index, Fv/Fm_1, Fv/Fm_3, and PI_3 did not have a significant proportion of the variation explained by the ANOVA model.

The factor condition explained a significant amount of variation for more traits than genotype. For traits explained both by genotype and condition: grain yield, biomass, Pedex, spike number, and water use efficiency; a larger proportion of the overall variation was explained by the factor condition than the factor genotype. The trait water use was the only trait where the interaction effect explained more of the variance (6%) than genotype (3%) or condition alone (3%).

Table 3. Results of two-way analysis of variance for the traits measured in Arta, Keel and the RIL population under control and drought conditions. For each trait, the F-value, coefficient of determination and significance probability is given for the two main effects (genotype and condition) and the interaction between the two. Morphological traits appear in the top table, physiological traits appear in the bottom table.

	GY			BM			HI			PH			Pedex			SN			GS			TKW			DM		
Genotype	8.5	0.03	***	7.8	0.05	***	3.7	0.00	n/s	1.5	0.01	n/s	5.0	0.05	**	3.1	0.04	*	6.9	0.09	**	7.3	0.10	**	1.5	0.01	n/s
Condition	87.3	0.18	***	93.7	0.31	***	2.9	0.00	n/s	66.0	0.22	***	28.1	0.15	***	27.5	0.16	***	1.2	0.01	n/s	0.3	0.00	n/s	82.8	0.32	***
G*C	4.0	0.02	*	3.5	0.02	*	0.3	0.00	n/s	0.5	0.00	n/s	8.0	0.08	***	2.8	0.03	n/s	4.2	0.05	*	1.6	0.02	n/s	3.1	0.02	*

	WU			WUE			RWC_1			RWC_3			Fv/Fm_1			Fv/Fm_3			PI_1			PI_3		
Genotype	11.9	0.05	n/s	1.8	0.03	***	0.2	0.00	n/s	1.7	0.02	n/s	2.9	0.00	n/s	0.9	0.00	n/s	4.8	0.08	**	2.6	0.04	n/s
Condition	170.1	0.35	***	4.3	0.03	*	14.9	0.07	***	68.2	0.32	***	0.0	0.00	n/s	0.0	0.00	n/s	0.3	0.00	n/s	3.3	0.02	n/s
G*C	2.6	0.01	n/s	4.0	0.06	*	3.4	0.03	*	27.7	0.26	**	0.3	0.00	n/s	0.7	0.00	n/s	0.1	0.00	n/s	0.4	0.01	n/s

GY grain yield, BM biomass, HI harvest index, PH plant height, Pedex peduncle extrusion, SN spike number, GS grains per main spike, TKW 1000 kernel weight, DM days to maturity, WU water use, WUE water use efficiency, RWC 1/3 leaf relative water content at 1 or 3 days after treatment start, Fv/Fmc_1/3 at 1 or 3 days after treatment start, PI_1/3 performance index 1 or 3 days after treatment start. * p<0.05, ** p<0.01, *** p<0.001, n/s non-significant

Genetic correlations of traits to yield under control and drought conditions

By looking at significant correlation indices between traits in a segregating population, traits that are influenced by the same gene or the same set of genes can be detected. To find genetic correlations to yield in the RIL population, Pearson correlation coefficients were calculated for traits measured under control conditions separately from traits under drought conditions. The results showed that under both conditions grain yield was significantly correlated to plant height, biomass, Pedex, spike number, grains per spike, thousand kernel weight and days to maturity (**Table 4**). Under control conditions, all of the significant correlations to yield were positive with coefficients ranging from 0.95 for biomass to 0.26 for days to maturity. Under drought conditions, the correlations to yield were positive with the exception of Pedex and days to maturity which had coefficients of -0.28 and -0.31, respectively. No significant correlation was found between RWC_3 and grain yield or between PI_3 and grain yield under either condition.

Significant correlations between traits other than grain yield were also detected under each of the two conditions. Pedex was positively correlated with plant height (0.39), biomass (0.37) spike number (0.24) and thousand kernel weight (0.25) under control conditions but not under stress conditions. Thousand kernel weight was positively correlated with biomass (0.69) grains per spike (0.29), days to maturity (0.41), and RWC_3 (0.25) under control conditions and was negatively correlated with days to maturity (-0.25) under drought conditions. Under drought conditions, days to maturity was positively correlated to RWC_3 (0.28) but not under control conditions. Spike number was correlated with biomass (0.48) plant height (0.24) and grain per spike (0.39) under drought conditions while under

control conditions spike number was negatively correlated to PI_3 (-0.36). Additionally, under drought conditions, PI_3 was correlated to plant height (0.29), but this correlation was not found under control conditions.

Differences in the correlation coefficients between conditions were observed for some traits. The number of spikes had a higher correlation to grain yield under drought conditions (0.54) than under control conditions (0.21). The opposite was true for the correlation between thousand kernel weight and grain yield, the correlation was higher under control conditions (0.73) than under drought conditions (0.33). The difference in the correlation index between days to maturity and grain yield between conditions was the most pronounced; under control conditions the correlation was positive (0.26) while under drought conditions the correlation was negative (-0.31). Of the morphological traits considered all were significantly correlated to grain yield, however, neither of the analyzed physiological traits were correlated to grain yield.

While genetic correlations can indicate which traits are influenced by the same genes, the analysis cannot reveal where the influential genes are located. In an attempt to position genes underlying the traits measured under control and drought conditions a quantitative trait loci analysis was performed.

Table 4. Pearson correlation coefficients for selected traits measured in the Arke RIL population under well watered and water limited conditions. Values above and below the diagonal are correlations between the traits under control or stressed conditions, respectively.

	GY	BM	PH	Pedex	SN	GS	TKW	DM	RWC_3	PI_3
GY		0.95	0.34	0.37	0.21	0.44	0.73	0.26	0.13	-0.01
BM	0.80		0.40	0.37	0.16	0.41	0.69	0.23	0.05	-0.06
PH	0.37	0.46		0.39	-0.01	0.09	0.24	0.09	-0.20	0.13
Pedex	-0.28	-0.19	0.17		0.24	-0.04	0.25	0.16	0.05	0.13
SN	0.54	0.48	0.24	-0.21		0.06	0.12	0.06	0.13	-0.33
GS	0.39	0.25	0.06	-0.03	0.39		0.29	0.00	-0.07	-0.16
TKW	0.33	0.21	-0.01	-0.18	0.16	0.19		0.41	0.25	-0.02
DM	-0.31	-0.17	-0.13	0.07	-0.18	-0.23	-0.25		0.20	-0.02
RWC_3	0.03	0.14	0.02	-0.09	0.09	-0.19	-0.03	0.28		0.12
PI_3	-0.08	-0.08	0.29	0.08	-0.10	-0.11	-0.36	0.01	-0.22	

Significant correlations (P<0.05) are underlined. GY grain yield, BM biomass, PH plant height, Pedex peduncle extrusion, GS grains per main spike, SN spike number, TKW thousand kernel weight, DM days to maturity, RWC_3 leaf relative water content 3 days after treatment start, PI performance index 3 days after treatment start.

QTL for agronomic traits

QTL simple interval mapping was employed for each trait measured in the RIL population using a multi-environmental model for control and drought conditions. A total of 19 significant QTL were discovered

for the traits grain yield , harvest index, plant height, grain per spike, thousand kernel weight, days to heading, water use, RWC_1 RWC_3, PI_1 and PI_3 (**Table 5**). The single QTL for grain yield located on chromosome 2H explained 15.9% of the variation under control conditions and 22.4% of the variation under drought conditions. Based on the calculated substitution effect, the Arta allele at this QTL increased grain yield by 1.28 g under control conditions and 3.20 g under drought conditions. On chromosome 2H, a QTL for harvest index was detected where the Arta allele had opposing effects on the harvest index between conditions; under control conditions the harvest index was decreased by 0.03 due to the Arta allele and increased by 0.04 under drought conditions. The mean harvest index under drought conditions of RILs carrying the Arta allele at this locus was significantly higher than RILs carrying the Keel allele, but under control conditions, the mean harvest index was similar between RILs carrying the two different alleles (**Figure 4**).

Table 5. Simple interval mapping of QTL detected for various traits using a multi-environmental model for control (50% SWC) and drought (15% SWC) conditions. For the peak LOD of each QTL the chromosome (Chr.) and position in centimorgans is given as well as the marker closest to the peak. The substitution effect (Sub.Eff.) expressed as the change in the value of each trait due to the contribution of an allele from Arta as compared to Keel under control or drought conditions. The percent variance (PEV) explained by each QTL under control or drought conditions is given.

					Control		Drought	
Trait	**Chr.**	**Position**	**Locus**	**LOD**	**Sub.Eff.**	**PEV**	**Sub.Eff.**	**PEV**
GY	2H	68.7	bPb_1569	2.97	1.28	15.90	3.20	22.40
HI	2H	15.3	bPb_9220	2.65	-0.03	9.40	0.04	24.20
PH	2H	5.4	bPb_2880	2.76	-3.43	15.80	-3.80	16.60
PH	5H	164.9	bPb_6367	4.21	-3.80	20.00	-5.00	26.10
GS	1H	37.5	bPb_9423	2.97	-0.71	9.80	-1.94	35.50
TKW	1H	55.8	bPb_840	3.02	-2.79	15.60	-6.40	27.00
TKW	2H	22.8	bPb_1098	3.08	1.92	8.90	6.47	25.50
TKW	2H	81.5	bPb_7160	3.12	2.15	20.00	4.80	22.70
TKW	3H	104.6	Hvm60	3.21	-1.49	14.60	-1.11	22.40
TKW	5H	85.7	bPb_46127	4.36	-2.46	18.40	-5.69	26.10
WU	2H	22.9	bPb_4523	2.70	1.85	8.40	1.86	25.20
WU	2H	71.3	bPb_0994	3.63	0.69	19.90	1.43	23.10
RWC_1	2H	65.0	bPb_6313	4.41	-5.60	25.60	4.44	21.40
RWC_3	1H	49.2	Bmag718	3.82	-9.90	38.00	-0.67	4.00
RWC_3	4H	70.4	bPb_0561	4.24	7.66	29.50	3.41	7.80
PI_1	1H	5.6	bPb_9280	2.41	0.48	18.30	0.32	9.50
PI_1	4H	95.2	bPb_1329	2.87	-0.42	18.00	-0.17	15.70
PI_3	1H	8.4	bPb_0690	2.42	0.26	11.50	0.37	16.30
PI_3	4H	114.8	bPb_6627	3.49	-0.39	24.30	-0.27	12.90

GY grain yield, HI harvest index, PH plant height, GS grains per main spike, TKW thousand kernel weight ,WU water use, RWC_1/3 relative water content 1 or 3 days after treatment start, PI performance Index 1 or 3 days after treatment start.

Two QTL for plant height were detected, one on chromosome 2H and the other on chromosome 5H. The Arta allele at the QTL on chromosomes 2H and 5H decreased the plant height under control conditions by 3.4 cm and 3.8 cm, respectively, and under drought conditions by 3.8 cm and 5.0 cm, respectively. A single QTL for grains per spike was detected on chromosome 1Ha where the Arta allele decreased the number of grains per spike by -0.71 under control conditions and by -1.94 under drought conditions. Five QTL for thousand kernel weight were detected on chromosomes 1H, 2H, 3H and 5H. At the QTL on 1H, 3H and 5H, the Arta allele caused a decrease in the kernel weight under control conditions by 2.79 g, 1.49 g, and 2.46 g and a decrease under drought conditions by 6.40 g, 1.11 g, and 5.69 g, respectively. The allele effect on kernel weight at the QTL on chromosome 1H was significant between RILs carrying the two alleles under both conditions tested (**Figure 5**). At the other two QTL for thousand kernel weight on chromosome 2 at 22 cM and 80 cM, the Arta allele was responsible for an increase in the kernel weight under control conditions of 1.92 g and 2.15 g, respectively, and an increase under drought conditions of 6.47 g and 4.80 g, respectively.

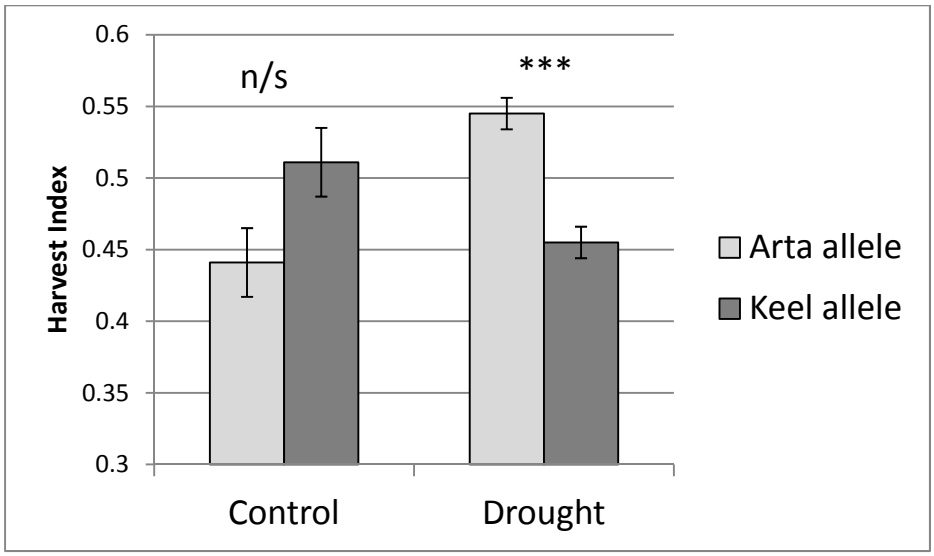

Figure 4. Allele effect of a QTL for harvest index on chromosome 2H where the Arta allele had opposing effects under control and drought conditions. Bars represent average harvest index of RILs that possess the Arta or Keel allele at the peak marker for the QTL. Average values for both control and drought conditions are shown. Error bars are standard deviation. *** $p<0.001$, n/s non-significant.

Two QTL for water use were found; one on chromosome 2H at 22 cM, where the Arta allele increased water use by 0.18 l under control conditions and by 1.85 l under drought conditions. At the other QTL on chromosome 2H at 71 cM the Arta allele increased water use by 0.68 l under control conditions and 1.43 l under drought conditions. A QTL for RWC_1 was found on chromosome 2H, where the Arta allele resulted in a decrease in the leaf water content under control conditions by 5.6% and an increase under drought conditions by 4.4%. Two other QTL for RWC_3 were found on chromosomes 1H

and 4H; the Arta allele was responsible for a decrease in the water content by 9.9% under control conditions and a decrease under drought conditions by 0.67% at the QTL on 1H. At the QTL on 4H the Arta allele was responsible for an increase in the relative water content by 7.6% under control conditions and 3.4% under drought conditions. Two QTL were discovered for the trait PI_1, one on chromosome 1H and one on chromosome 4H. Two QTL for PI_3 were also discovered on chromosomes 1H and 4H which overlapped with the QTL found for PI_1. At the QTL on chromosome 1H for PI_1 and PI_3, the Arta allele resulted in an increase in the performance index by 0.48 and 0.26 under control conditions and an increase under drought conditions by 0.32 and 0.37, respectively. At the QTL on chromosome 4H for PI_1 and PI_3 the Arta allele resulted in an decrease in the performance index by 0.42 and 0.17 under control conditions and an increase under drought conditions by 0.39 and 0.27, respectively.

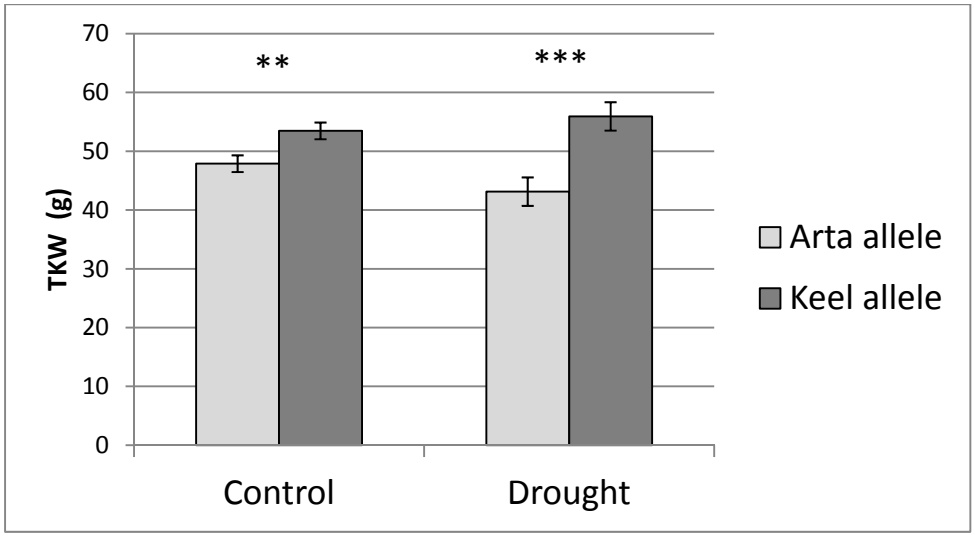

Figure 5. Allele effect of a QTL for thousand kernel weight on chromosome 1H where the Arta allele had similar effects in control and drought conditions. Bars represent average thousand kernel weight of RILs that possess the Arta or Keel allele at the peak marker for the QTL. Average values for both control and drought conditions are shown. Error bars are standard deviation. ** $p<0.01$, *** $p<0.001$.

The percent of variance explained by each of the QTL ranged from 8.4% to 38% under control conditions and from 4% to 35.5% under drought conditions. For many of the QTL, the difference in percent explained variance between control and drought conditions was less than 10%. The QTL that differed by more than 10% between conditions were those for: harvest index (2H), grains per spike (1H), thousand kernel weight (on 1H and 2H), water use (on 2H), RWC_3 (on 1H and 4H) and PI_3 (on 4H). Of these QTL, the ones for morphological traits had more variation explained by the QTL under drought conditions while the QTL for physiological traits had more of the variation explained under control conditions.

In summary, QTL were detected for all of the classes of traits considered: physiological, yield morphological and agronomic. The majority of QTL resulted in the Arta allele affecting traits in the same direction under both conditions except for two QTL, for harvest index and RWC, resulted in the Arta allele having opposing effects between the two conditions. Based on the calculated substitution effect, both parent genotypes contributed beneficial alleles to the RIL population for agronomic traits. Several of the QTL were clustered on neighboring loci, for example, the QTL for harvest index, plant height, thousand kernel weight, and water use on chromosome 2Ha and the QTL for grain yield, thousand kernel weight, water use and RWC_1 on chromosome 2Hb.

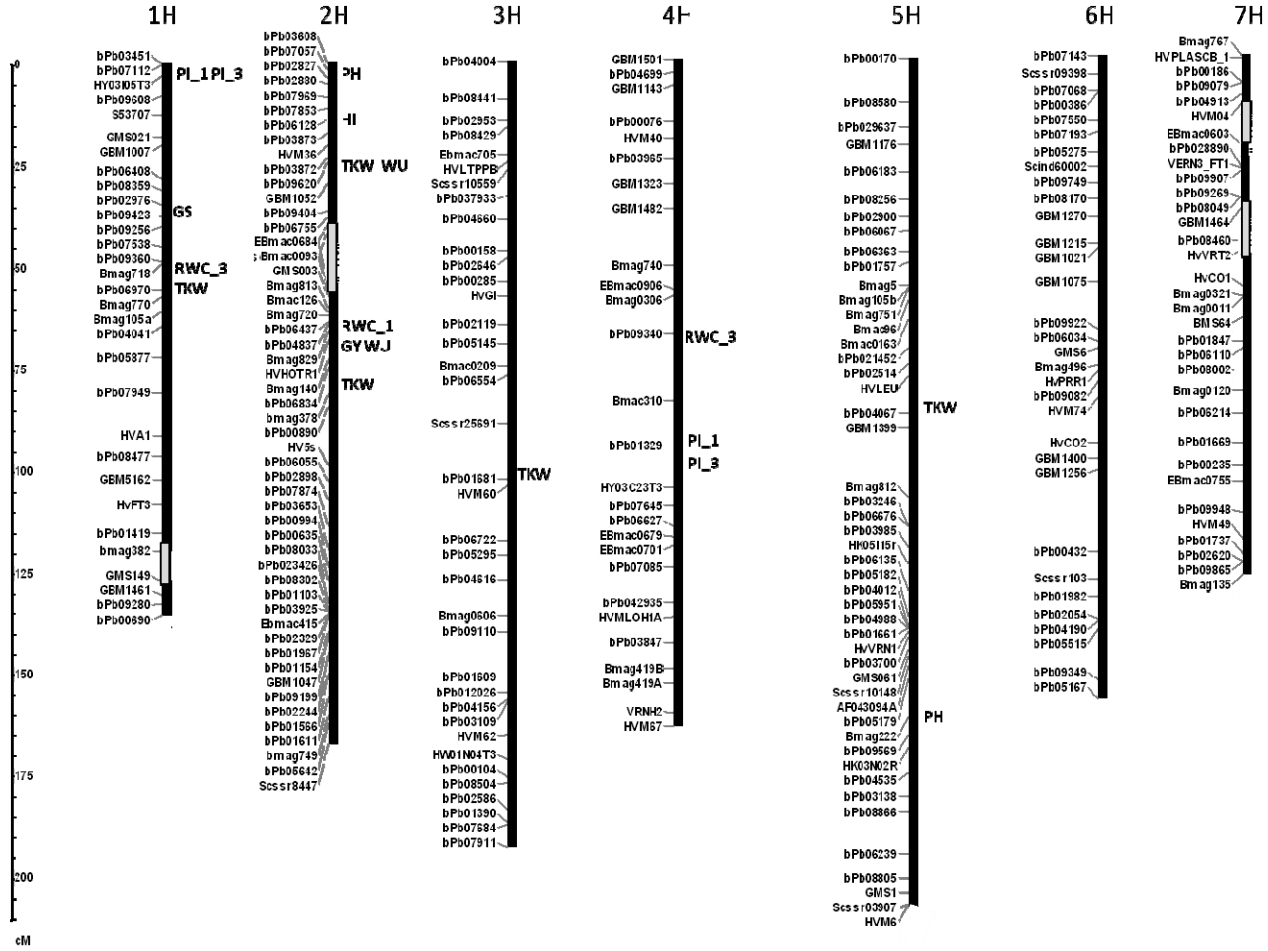

Figure 6. A genetic linkage map of the Arke RIL population with approximate positions of multi-environmental QTL detected. The two environments considered were control (50% soil water content) and drought (15% soil water content) conditions. Positions of the QTL shown are based on the peak marker with the highest LOD score. A detailed list of the QTL is given in **Table 5.** GY grain yield, HI harvest index, PH plant height, GS grains per main spike, TKW thousand kernel weight ,WU water use, RWC_1/3 relative water content 1 or 3 days after treatment start, PI performance Index 1 or 3 days after treatment start. Different linkage groups belonging to the same chromosome are separated by white boxes.

Discussion

The genetic basis of morphological trait plasticity and physiological responses to drought was considered in a segregating population developed by crossing the barley genotypes Arta and Keel. Resilience to drought was exhibited by both genotypes in their ability to maintain leaf water status and to photosynthesize when challenged with drought that started at anthesis and lasted until maturity. However, the drought treatment had pronounced effects on the morphology of the plants as seen in the reduction of grain yield and spike number. Under control conditions, differences in performance were exhibited between genotypes, for example, Arta had ~30% more grain yield and biomass than Keel. As the genotypes Arta and Keel are adapted to two different drought prone ecogeographic areas it is hypothesized that they have evolved unique mechanisms in resisting drought. A multi-environmental QTL analysis revealed 19 loci for agronomic performance under control and drought conditions with beneficial alleles being contributed by both Arta and Keel. Several of the QTL in this study clustered together, suggesting that the traits are controlled by the same gene or closely linked genes. QTL discovered in this study also co-localize with QTL found in other segregating populations grown under water limited conditions thus strengthening the possibility of locating candidate genes that convey drought resistance at those locations.

Reduction of growth and yield in the absence of physiological stress

The leaf water status of the plants remained relatively unaffected by the drought treatment; the lowest mean relative water content recorded was 76% (**Table 2**). This suggests that the plants were able to prevent their water loss despite limitation of available water. Water loss is primarily prevented by stomata closure as mediated by abscisic acid [65] but is accompanied by a decrease in carbon dioxide uptake necessary for carbon fixation [66]. A lack of available carbon dioxide for carbon fixation can limit the light-independent steps of photosynthesis [67] and subsequent sugar accumulation necessary for optimal plant growth. However, photosynthesis was not detected to be perturbed by the drought treatment in the present study despite reductions in plant growth as observed in the morphological data. The inhibition of growth despite no detectable inhibition of photosynthesis suggests that growth was not down-regulated by a limitation of photosynthate availability.

Drought treated plants had reduced biomass, plant height, grain yield and spike number compared to control plants which could have been caused by limitations in photosynthate availability. However, the chlorophyll fluorescence data did not show significant signs of inhibition of photosynthesis in the maximum quantum efficiency or in the photosynthetic performance, which is sensitive to changes in light-independent reactions [68] expected under limited carbon dioxide levels. The reduction of leaf

growth in *Arabidopsis thaliana* beyond the limitation of available photosynthate has been observed for osmotic stress [19] as well as drought stress [69]. The phenomenon of reducing growth above photosynthate limitations caused by drought stress can be an advantage during periods of extreme stress as it might prevent plant death. However, under mild stress, such as the treatment used here, the reduction in growth can be viewed as an unnecessary loss in yield [70].

In cereals, inhibition of growth can affect the number of tillers produced as well as the rate of leaf expansion [71]. In the present study, the inhibition of tillering under drought was observed in the reduction of the final spike number (**Table 2**). The importance of tillering was evident in this study as a significant positive correlation of spike number to grain yield was observed under both control and drought conditions (**Table 4**). Other barley studies have shown the reduction of tillering due to drought [72][57][73], but the molecular basis of drought induced inhibition of tillering is not fully understood at present. However, the inhibition of tillering during anthesis is well studied. The repression of tillering that occurs during anthesis is thought to be due to a combination of auxin signaling and resource competition between the apical buds, which form new tillers, and the stem apex [74]. It is possible that drought induced and anthesis induced inhibition of tillering share similar pathways and the mechanism is worthy of future investigation. Minimizing the yield loss by drought induced growth inhibition in future crop breeding programs will take an understanding of what traits are involved, which genotypes are able to maintain growth despite a perceived limitation, and which genes are taking part in this process.

Genotype specific environmental responses

As Arta and Keel have been selected to grow in different agricultural settings it was hypothesized that they would acclimate to the different environmental conditions by exhibiting different phenotypes. Significant differences in morphology were detected between Arta and Keel under control conditions. When well watered, Arta had significantly higher biomass, grain yield, and plant height than Keel. Additionally, Arta had a mean peduncle extrusion that was positive while Keel had a negative peduncle extrusion. Due to the drought treatment, all of the above traits were significantly reduced in both genotypes. As seen in the tolerance index (**Figure 3**) of the grain yield and biomass, these traits were reduced to a greater extent under drought in Arta than in Keel as compared to control conditions. Arta was thus more prone to drought induced growth inhibition than Keel. Genotype specific growth inhibition under drought has been observed before in barley and wheat [75]. Specifically, leaf expansion rates of Seeva, an elite Israeli cultivar, were reduced more under drought stress than in wild barley [76].

The peduncle, as the last internode to elongate [77], is a measure of late developmental stem elongation and plant growth. The peduncle extrusion in Arta decreased from a mean of 1.5 cm in control

conditions to a mean of -6.8 cm under drought conditions. This decrease in peduncle extrusion was larger than the changes in peduncle extrusion observed in Keel, which were -4.6 cm under control conditions and -5.7 cm under drought conditions. The significant reduction in peduncle extrusion due to the drought treatment in Arta but not is Keel is a further indication of increased growth inhibition due to drought in Arta.

The ANOVA of the traits measured in the parental lines and RIL population under control and drought conditions (**Table 3**) revealed interacting effects between the genotype and treatment on grain yield, biomass and peduncle extrusion. Interacting effects between genotype and treatment for the grain yield, biomass and peduncle extrusion suggests that these traits are reacting to the drought treatment in a genotype specific manner and that unique alleles between the two genotypes differentially regulate these traits under drought stress [78]. Interestingly, significant genetic correlations between grain yield, biomass, plant height and peduncle extrusion were revealed under control conditions as well as drought conditions (**Table 4)**. The correlations between grain yield, biomass, plant height and peduncle extrusion suggests that pleiotropic genes control these traits [79]. However, the correlation between peduncle extrusion and grain yield was negative under drought suggesting that genes participating in peduncle growth were responsible for decreased grain yield.

In summary, the genotypes exhibited morphological differences under control conditions and differences in growth inhibition under drought conditions which have a genetic basis. A possible influence of the same genes on different traits was seen in the clustering of the QTL discovered in this analysis.

Clustering of QTL

In the 19 QTL detected under control and drought treatments (**Table 5**), Arta provided the beneficial allele under drought for 10 QTL demonstrating that both parents had alleles to contribute to drought resistance. Contribution of beneficial alleles from both parents was also evident in the distribution of grain yield and photosynthetic performance index in the RIL population (**Figure 2** and **Figure 1**) where several RILs outperformed either parent under control or drought conditions. Such transgressive segregation of grain yield can be due to complementary action of additive alleles inherited from the parental lines [80]. Several QTL for different traits were found in genetic proximity to each other and were considered QTL clusters. Clusters of QTL detected in this study were observed on chromosomes 1H and 2H (**Table 5**) (**Figure 6**). QTL that are clustered together can be due to pleiotropy or linkage [81]. The cluster on chromosome 1H contained QTL for: thousand kernel weight, grains per spike, and for leaf relative water content. The Keel allele at this locus was responsible for increased kernel weight, more

grains per spike and higher leaf water content under control and drought conditions. Additionally, a significant positive genetic correlation between relative water content and thousand kernel weight was detected between these two traits, which further supports that these two traits are controlled by the same locus [79]. The cluster on 2H distal from the centromere contained a QTL for thousand kernel weight and a QTL for water use. At this locus, the Arta allele was responsible for increased kernel weight and increased water use under both conditions. The cluster on 2H proximal to the centromere contained four QTL for: grain yield, water use, relative water content, and thousand kernel weight. The Arta allele at this locus was responsible for increased kernel weight, water use, and thousand kernel weight under both control and drought conditions. However, the Arta allele at this same locus was also responsible for an increase in leaf relative water content under drought but a decrease in relative water content under control conditions. The opposing effect of the Arta allele on relative water content in the same cluster where the Arta allele had main coinciding effects makes it likely that the QTL for relative water content is caused by a different gene than the QTL for grain yield, water use and thousand kernel weight. In addition to QTL clustering between traits, QTL discovered in this study were seen to overlap with QTL for the same traits discovered in other segregating barley populations grown under water limited conditions.

The multi-environmental QTL analysis used in MultiQTL considers the phenotypes under control and drought conditions simultaneously to determine the significance of each locus [64]. While this approach increases the statistical power to detect significant loci, drought environment specific QTL cannot be identified based on the LOD alone. However, the effect of a QTL under each environment is indicated by percent of trait variance explained by the QTL in that environment. The QTL cluster on 2H for grain yield, water use, relative water content, and kernel weight all had high percent explained variances, ranging from ~15% to ~25%, in both control and drought environments which suggested that the QTL effect was present under both environments. However, for other QTL the majority of the QTL effect is limited to one environment. For example, the percent explained variance for thousand kernel weight at 22 cM on chromosome 2H was under drought conditions (25.5% was over twice that explained under control conditions (8.9%). Similar differences in the explained variance between the drought and control were also observed for grains per spike in chromosome 1H (35.5% and 9.8%, respectively) and water use on chromosome 2H at 22 cM (25.2% and 8.4%, respectively).

Confirmed and novel QTL

The QTL discovered here were compared to the QTL discovered in other studies concerned with drought resistance which used populations from the barley crosses: Tadmor x Er/Apm [50][47], Steptoe x Morex

[55] and Arta x Keel [82]. Tadmor is a black-seeded landrace common to Syria and Er/Apm is a cultivar bred for high yields under favorable conditions [47]. Steptoe is a cultivar used for animal feed considered sensitive to drought and Morex is a malting cultivar considered to be drought resistant [55]. In one QTL study, the Tadmor x Er/Apm population was grown in a growth chamber where physiological traits, like relative water content, were considered [50]. In the other study involving the cross Tadmor x Er/Apm, the population was grown in drought prone fields in Syria where agronomic traits like grain yield and kernel weight were considered [47]. The Steptoe x Morex population was grown in drought prone fields in Iran where agronomic traits were considered. The Arta x Keel (Arke) population was grown over four seasons in drought prone fields in two regions of Syria where agronomic traits were considered [82].

Comparison of QTL found in the present study to published QTL can be considered as a confirmation of the QTL method in general [83]. On chromosome 1H distal to the centromere, the QTL found in the present study for leaf relative water content, with Keel contributing the beneficial allele, was also discovered in the Tadmor x Er/Apm cross with Tadmor providing the allele for higher water content (**Figure 6**). On chromosome 1H proximal to the centromere, QTL were discovered in the population Steptoe x Morex for grains per spike and for thousand kernel weight, which coincided with QTL for the same traits in the present study, where Keel contributed the beneficial allele for both traits. At this locus on 1H, Steptoe provided the positive allele for increased seed number per spike and Morex provided the allele for increased kernel weight. On chromosome 2H, the QTL for harvest index at 15 cM in the present study with Arta providing the beneficial allele under drought treatment, was also found in the field grown Arke population. Additionally on chromosome 2H, the QTL in the present study for grain yield at 68 cM and for kernel weight at 81 cM, where Arta contributed the beneficial alleles, were also found in the Arke population grown in the field. The QTL on 3H for kernel weight, where Keel contributed the beneficial allele, was also discovered in the Tadmor x Er/Apm population with the beneficial allele being contributed from Tadmor. The QTL for kernel weight discovered on 5H with Keel providing the positive allele was also found in the cross Tadmor x Er/Apm where Er/Apm contributed the positive allele and in the population Stepoe x Morex where Morex contributed the positive allele.

In total, 8 of the 19 QTL revealed in this study were also found in other barley QTL analysis. The appearance of QTL for the same traits in different studies, despite differences in growing conditions, demonstrated that some loci for morphological and physiological traits are common between different mapping populations. QTL confirmed by the comparison of QTL studies using other mapping populations, highlights loci that should be given priority for identification of the causal genes in future studies [84]. The remaining, unconfirmed QTL in this study are considered novel. The ability to discover previously

undiscovered QTL exemplifies the utility of using original mapping populations, growing conditions, and applications of stress as performed in the present study.

As sequencing, assembly and annotation for the barley genome progresses, more tools are becoming available to assist in narrowing QTL intervals and pinpointing candidate genes [13]. One such tool, comparative genetics as used by Hazen et al. [85], will allow concordant QTL from barley, Brachypodium, rice and sorghum to be aligned and the region of overlap to be interrogated for candidate genes. The QTL presented here will facilitate this process by confirming loci previously found from other segregating populations and adding novel loci that are possibly unique to the Arta x Keel population. Until now, QTL for grain yield and growth related traits in barley grown under water limited conditions are based on only few segregating populations: The addition of Arta x Keel QTL into this dataset expands the possibly of applying a combined cross analysis to narrow QTL regions or dissect significant QTL under drought conditions [86].

Conclusion

The search for traits associated with yield performance under mild drought stress applied during anthesis revealed the importance of tillering regulation in drought resistance. Drought treatments applied early in developmental growth would have missed this association and this result highlights the importance of applying treatments at varying stages of barley development in future experiments. The robustness of barley physiology under drought is likely due to some the same molecular responses found to be active in Arabidopsis and rice like production of compatible solutes and ROS scavengers. However, the data here suggests that the maintained homeostasis of plant physiology under drought, as seen in the stability of the leaf water status and photosynthetic performance, can also be due to plasticity in morphological traits.

QTL for traits positively correlated to grain yield under drought such as plant height, grains per spike, and thousand kernel weight were discovered and potentially contain genes responsible for conveying drought resistance. Of particular interest were QTL which explained a greater percent of trait variance under drought than under control conditions, for example the QTL for grains per spike on chromosome 1H, as they may contain genes that act exclusively under stress. Of the 19 QTL discovered in the present study, 8 were confirmed to exist in other QTL studies that used different mapping populations or different applications of drought treatment. Such confirmed QTL exemplify the universality of some drought stress mechanisms which are present in multiple genotypes or during several stages of development. Conversely, the ability to detect previously undiscovered QTL exemplifies

the uniqueness of the present study and alludes to the presence of numerous more genetic loci to be found.

Chapter Two - Leaf Proteome Analysis of Barley Subjected to Drought and Heat Stress

Introduction

High temperature negatively impacts crop production worldwide [87][88]. Models of climate change predict that the global mean temperature will rise by ~2°C by 2100 [89] suggest that heat stress will occur more frequently and more severely in the future. Preventing the reduction of crop yields due to heat stress will require the development of crops with enhanced heat resistance. However, continued development of heat resistant crops will require further investigation of the mechanisms that convey heat tolerance. Heat resistance, as defined by Wahid et al. [90], is the "ability of plants to grow and produce economic yield under high temperatures". The ability to maintain yield is achieved by plant responses to heat on the morphological, physiological and molecular level. Plant responses to heat are dependent on the severity, duration, and timing of the stress [91] as well as its coincidence with other abiotic stresses such as drought.

Heat and drought stress commonly occur simultaneously in agricultural settings [92]. Extensive studies have been performed to elucidate plant reactions to heat [90][93][94] and drought [95][96][97], but relatively few studies have been concerned with the combined effects of heat and drought stress on plants [98], especially during the reproductive stage of development [99]. The combined effects of heat and drought on yield are more detrimental than the effect of each stress alone, as seen in sorghum [100], maize [101] and barley [72]. Physiological responses to heat and drought can be antagonistic to each other when applied in combination [102]. For example, opening of stomata to increase transpirational cooling observed under heat stress was inhibited when a combination of heat and drought was applied to tobacco (*Nicotiana tabacum*), resulting in higher leaf temperatures than heat treatment alone [92]. Additionally, accumulation of the osmoprotectant proline which normally occurs in Arabidopsis under drought stress, was not detected in plants subjected to combined heat and drought stress [103]. The majority of work related to the molecular basis of drought and heat resistance in plants has been limited to the transcriptome.

Microarray analysis has allowed the identification of hundreds of stress inducible genes with putative roles in conveying abiotic stress tolerance [104]. Included in this identification are genes that encode regulatory proteins such as transcription factors and kinases as well as functional proteins such as proteases and enzymes for osomolyte biosynthesis [105]. Transcriptional analysis of responses to

combined heat and drought stress in Arabidopsis revealed that several of the genes inducible by drought were also inducible by heat stress, which suggests that the molecular responses to the two stresses overlap [103]. However, the same study also showed that the combination of both stresses regulated transcripts by that were not detected to be altered by either stress independently. Changes in the barley transcriptome under drought [106][107] and combination drought and heat [108] have provided insight as to which gene networks, like osmolyte metabolism are altered under stress in barley. Despite the popularity and success of transcriptomic analysis the technique is limited in the information it can provide about molecular responses.

Microarrays are typically designed to quantify mRNA transcripts [109] which no not typically possess enzymatic or structural functions. Instead, the vast majority of mRNAs contain the sequence information necessary to synthesize a protein by ribosomes. The synthesized protein, once properly folded, transported and chemically modified can then fulfill its enzymatic or structural role. While protein abundance is at least partially dependent on mRNA transcript levels, the abundance of the two macromolecules are generally not well correlated to each other [110][111]. Therefore, inferring protein abundance from transcript abundance can be problematic. However, a proteomic approach to stress responses should more accurately reflect changes in the cellular state than profiling the expression of mRNAs [112].

Molecular responses to abiotic stress

Heat resistance mechanisms, like drought resistance mechanisms, can be divided into heat avoidance and heat tolerance. Heat avoidance is the ability of the plant to maintain low internal temperatures. Mechanisms of heat avoidance include stomata opening to increase leaf cooling via transpiration, changes in leaf orientation, and reflection of solar radiation [113]. Heat tolerance is the ability of the plant to maintain cellular activity despite high internal temperatures. Heat tolerance mechanisms share similarities to drought tolerance mechanisms. For example, heat tolerance mechanisms include increased production of free radical scavengers, protein protecting molecular chaperones, and proteases. For an overview of the molecular responses to drought, the reader is referred to the introduction in chapter one.

Regulation of stomatal conductance by plant water status and carbon dioxide availability is well characterized in the literature but the regulation by temperature is not [114]. In brief, stomata close under water limited conditions in an abscisic acid dependant pathway. While the molecular pathway for heat induced stomata opening has not been elucidated, the pathway is known to be independent of the carbon dioxide status of the plant but dependant on the water status of the plant [115]. Changes in leaf

orientation can reduce the amount of incident solar radiation that the leaf experiences and helps to keep the leaf cooler. The presence of epicuticular wax can also reduce incident solar radiation that the leaf experiences by reflecting the light before it is absorbed by the leaf [116].

Disruption of photosynthesis is a symptom of both heat and drought stress. At the onset of drought stress, disruption of photosynthesis occurs in part due to the limitation of carbon dioxide as the final electron acceptor[28][29]. In contrast, the disruption of photosynthesis during heat stress is due to the decreased stability of Rubisco activase which results in decreased Rubisco activity [117]. In addition, photosynthesis can be disrupted by the heat induced dissociation of the oxygen evolving complex from the photosystem II reaction center [118]. While the cause of the disruption of photosynthesis between the two abiotic stresses is different the outcome is the same; increased production of reactive oxygen species (ROS) and oxidative stress. An overview of ROS scavenging mechanisms can be found in the introduction of chapter one.

High temperatures, like oxidative damage, can cause proteins to unfold and lose their enzymatic or structural properties. Heat denaturation occurs when the thermal energy exceeds the binding energy of the hydrogen bonds that hold the secondary structure of the protein together [119]. Prevention of denaturation is achieved by molecular chaperones which can assist proteins to fold into a functional state, provide stability to properly folded proteins, and prevent aggregation of denatured proteins [34]. Molecular chaperone functions are provided by members of the heat shock proteins [120] family which contains member that are readily induced upon heat stress [94].

Quantitative proteomics

Quantification of protein abundance is possible using modern proteomic approaches which incorporate two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separation of proteins with mass spectrometric identification. A typical proteomic workflow begins with a protein extract that is electrophoretically separated by isoelectric point in one dimension followed by separation in another dimension according to the molecular weight. The proteins resolved in two dimensions can then be visualized and the spot intensities compared between genotypes or treatments using image analysis software. Protein spots of interest can be excised from the gel, enzymatically digested with trypsin and analyzed by mass spectrometry. Identification of proteins with mass spectrometry can be achieved using peptide mass fingerprinting, which compares the acquired peptide masses to theoretical digests of proteins annotated in public databases. Additionally, proteins can be identified using tandem mass spectrometry, which determines the amino acid sequence of digested peptides to be used in homology-based database query.

An extension of conventional 2D gel electrophoresis (2D-PAGE) is 2D difference gel electrophoresis (DIGE) which allows the concurrent separation and quantification of a mixture of protein samples in the same gel [121]. This is achieved by labeling each protein extract with a different fluorescent dye (Cy5, Cy3, or Cy2) prior to mixing and running the extracts together on the same 2D gel. Fluorescence imaging at wavelengths specific for each dye is then used to visualize the protein spot pattern of each sample separately and allows quantification of similar protein spots between the two samples. Advantages of DIGE include reduced gel to gel variation, sensitivity of ~0.1 ng, and a dynamic range higher than 3.5 orders of magnitude [122]. In comparison, coomassie blue based stains used for protein visualization in 2D gels only offer a sensitivity of approximately 15 ng and dynamic range of 1 [123]. Additionally, DIGE allows for the use of an internal standard, consisting of a pool of all samples to be compared in an experiment, to be included in each gel analyzed. Normalization of each sample to the internal standard can then be performed and each protein spot can be measured as a ratio to its corresponding spot present in the internal standard [124]. By incorporating an internal standard, experimental variation can be separated from the more interesting biological variation [124].

Conventional 2D-PAGE has proved useful in barley research to quantify changes in protein abundance in seeds [125–129], roots [130] and shoots [131] in response to salt stress, heat stress, or during development. Barley proteomic research using DIGE has not, to date, been published but the technique has been successfully applied in Arabidopsis [132], grape [133], and wheat [134]. These studies were able to quantity the regulation and identify proteins responsive to salt stress in Arabidopsis and wheat as well as proteins active in the withering process in grape. By quantifying changes in protein abundance one can gain insight into the biochemical processes that underlie the morphological and physiological acclimations that occur when a plant is challenged with abiotic stress.

The objectives of this study were to i) characterize the physiological and morphological responses to drought and heat stress and ii) identify barley leaf proteins differentially regulated in response to drought stress and heat stress using a proteomics approach based on DIGE and mass spectrometry. The changes in protein abundance were placed into context of the physiological and morphological trait plasticity that also occurred due to the abiotic stresses. The barley genotypes Arta and Keel were included in the analysis to allow stress specific and genotypic specific responses to be considered.

Materials and Methods

Experimental overview

This chapter is primarily concerned with the changes in the barley leaf proteome in response to the effects of drought treatment and heat treatment applied separately as well as simultaneously. Control plants were well watered by maintaining a soil water content (SWC) of 50% and were kept at a control temperature of 21°C. Drought treated plants were given reduced water starting at anthesis by maintaining a SWC of 15% and were grown at 21°C. Heat treated plants were well watered like controls, but were grown at a high temperature of 36°C for seven days starting at anthesis. Combination treated plants were subjected to both drought and heat treatments simultaneously. The aim of this study is to discover genetic differences in response to drought and heat as revealed by two genetically distinct barley genotypes, Arta and Keel. Physiological traits were measured one, three and seven days after the application of the heat treatments started. Morphological measurements were made either during grain filling or after plant maturity. A list of all traits considered and the abbreviations used in this text can be found in

. The variance in each trait was attributed to effects of genotype, soil water content (SWC), temperature or the interaction of all three, by use of an ANOVA. Phenotypic correlations between grain yield and the other traits were calculated for heat treated and non-heat treated plants separately. A barley leaf proteome map was generated using 2D-PAGE and by identifying coomassie-stained protein spots with a combination of peptide mass fingerprinting and tandem mass spectrometry. Individual spots in the barley leaf proteome were quantified utilizing difference in gel electrophoresis for each genotype under each treatment using samples taken three days after the application of the heat treatments began. The eight proteomes were compared and significant effects on protein spot intensity by the genotype and each treatment were calculated using ANOVA.

Plant material

See chapter 1 materials and methods for description of the genotypes Arta and Keel.

Drought and heat treatments

The response of Arta and Keel to the single or combined effects of drought and heat treatments at the generative stage under controlled conditions in growth chambers was tested. The chamber experiment was conducted independently in duplicate. Plants were sown in 96 well trays, stratified at 4°C for 4 d, transferred to 8 h light /16 h dark short day (SD) for 24 d and then grown in 16 h/8 h long day (LD)

conditions for 2 d to acclimate before being potted in four liter pots containing 1.8 kg of soil with three plants in each pot. The chamber was set to 21°C lights on/17°C lights off with a humidity of 50%. The plants remained in LD for the remainder of the experiment. The field capacity of 1.8 kg soil was calculated as the difference in weight between fully hydrated soil and dried soil [135]. The soil was allowed to fully hydrate by flooding the pot with water and allowing any excess water to drain over 24 h while the pot was covered. Soil was dried by heating at 70°C for 7 d. The SWC of potted plants was adjusted to 50% of the field capacity (FC). The relative humidity of the chambers were set to 50%, the light intensity was 350 µmol photons m^{-2} s^{-1} and the temperature was set to 21°C when the light was on and 17°C when lights were off. The drought treatment was applied at anthesis as described in the section drought stress in chapter 1. The heat treatment was applied to a subset of the well watered and drought treated plants when the SWC reached 15% in the drought treated plants by moving the plants to an identical chamber set to 21°C and then gradually raising the temperature to 36°C over 4 h. During the heat treatment, the temperature was set to 32°C when the lights were off. Heat treated plants remained at 36°C lights on/32°C lights off for one week at which point the temperature was decreased to 21°C over 4 h. Photographs of Arta and Keel plants from under each treatment were taken with a EOS 450D digital camera (Cannon) once the heat treatment had ended.

Physiological measurements

The second leaf down from the top of the spike was used for all physiological measurements and was harvested for later protein extraction. Samples were taken one, three and seven days after the target FC of 15% had been reached in drought treated pots. Sampling on each day began at ZT 3 and was completed ZT 6 to minimize possible effects from the circadian clock on the measurements. For each pot, one leaf was harvested from each of the three plants and the three leaves were immediately flash frozen together in liquid nitrogen and stored at -80 °C for subsequent protein extraction. Each pool of three leaves was considered as one biological replicate. Fast chlorophyll fluorescence induction kinetics was measured on every plant per pot using the Handy PEA chlorophyll fluorimeter (Hansatech Instruments). Fluorescence was induced using a 3000 µmol photons m^{-2} s^{-1} flash of actinic light persisting for 1 s on leaves dark adapted with leaf clips for a minimum of 20 min. The induction curves were analyzed using the PEA plus software version 1.02 (Hansatech Instruments) and the maximum quantum efficiency (Fv/Fm) and the Performance Index (PI) were calculated. The relative water content of the leaf (RWC) was calculated from one ~3 cm leaf cutting per plant and calculated according to the equation: $\frac{fresh\ weight - dry\ weight}{turgid\ weight - dry\ weight} \times 100$ [136]. Freshly cut leaves were immediately weighed to determine the fresh weight. The turgid weight was determined after submerging the leaf cuttings in

distilled water and storing them for overnight at 4 °C in the dark. Dry weight of the leaf cuttings was determined after drying the cuttings at 70 °C for 48 hours. Leaf temperature was measured using an Optris LS LT portable infrared thermometer (Optris) set to close focus mode and with the emissivity set 0.99. Temperature measurements were taken prior to sampling and done on the middle portion of blade on the leaf below the flag leaf.

Table 6. Summary of traits measured in the genotypes Arta and Keel grown under control, drought, heat and combination treatments. The abbreviation used, the unit used and the procedure by which each measurement was made is given.

Abbr.	Trait	Units	Procedure
GY	Grain yield	g	Total weight of kernels
BM	Total biomass	g	Weight of all above ground plant biomass
HI	Harvest index	g/g	Ratio of grain yield over total biomass
PH	Plant height	cm	Distance from top of soil to top of primary spike
Pedex	Peduncle extrusion	cm	Distance from peduncle to bottom of primary spike
SN	Spike number		Number of spikes over half of grains filled
AS	Number of aborted spikes		Number of pikes with over half of all grains unfilled
GS	Grains per spike		Number of grains divided by number of spikes
TKW	Thousand kernal weight	g	Extrapolated weight of 1000 kernals
DM	Days to maturity	days	Days in LD until kernels were mature
WU	Total water used per pot	L	Water given to plant from LD to maturity
WUE	Water use efficency of grain yield	g/L	Ratio of grain yield over water used
LT_1	Leaf temperature at day 1	°C	Temperature of the leaf before the flag leaf
LT_3	Leaf temperature at day 3	°C	Temperature of the leaf before the flag leaf
LT_7	Leaf temperature at day 7	°C	Temperature of the leaf before the flag leaf
RWC_1	Relative water content at day 1	%	Ratio of leaf fresh weight over fully turgid weight
RWC_3	Relative water content at at day 3	%	Ratio of leaf fresh weight over fully turgid weight
RWC_7	Relative water content at at day 7	%	Ratio of leaf fresh weight over fully turgid weight
Fv/Fm_1	Maximum PSII quantum yield at day 1	arb. unit	Chl flouresence of dark adapted leaf before the flag leaf
Fv/Fm_3	Maximum PSII quantum yield at day 3	arb. unit	Chl flouresence of dark adapted leaf before the flag leaf
Fv/Fm_7	Maximum PSII quantum yield at day 7	arb. unit	Chl flouresence of dark adapted leaf before the flag leaf
PI_1	PSII performance indexat day 1	arb. unit	Chl flouresence of dark adapted leaf before the flag leaf
PI_3	PSII performance indexat day 3	arb. unit	Chl flouresence of dark adapted leaf before the flag leaf
PI_7	PSII performance indexat day 7	arb. unit	Chl flouresence of dark adapted leaf before the flag leaf

Morphological and agronomic measurements

Heading was considered to have initiated when the awns had visibly emerged from the ear in over half of the main tillers present in each pot. Heading had initiated in both genotypes within two days of each other. Peduncle extrusion (Pedex) and plant height (PH) were recorded post-anthesis on the primary spike by measuring the distance from the flag leaf collar to the bottom of the spike and the distance

from the soil surface to the top of the spike, respectively. The days until maturity (DM) were counted from the time of transfer to LD until the majority of the grains on all spikes could no longer be dented by fingernail. After maturity, each plant was harvested separately. First, all above ground biomass was cut from the soil and then weighed for the biomass (BM) measurement. Then spikes were separated into non-aborted spikes and aborted spikes. A spike was considered aborted if more than 50% of the florets on the spike were empty and had not produced a seed. Spikes of the three plants from a single pot were threshed together. The kernels were counted and weighed using a Marvin-universal seed analyzer (GTA Sensorik GmbH) connected to a digital scale. The included software was used to extrapolate the thousand kernel weight (TKW). The number of grains per spike was calculated as $\frac{spike\ number}{grain\ number}$. Harvest index (HI) was calculated per pot as $\frac{grain\ yield}{biomass}$. The water used by the plants in each pot (WU) was recorded starting after the plants were potted in four liter pots. Water use efficiency (WUE) was calculated for each pot with the ratio $\frac{grain\ yield}{water\ use}$.

Protein extraction

All chemical reagents were purchased from Carl Roth GmbH unless stated otherwise. Frozen leaf samples in tubes containing two ball bearings were set in a liquid nitrogen cooled metal block and powdered using the GenoGrinder 2000 (SPEX Sample Prep) set at 1250 strokes per min for 1 min. Approximately 100 mg of powdered sample was placed in 1.5 ml of 10% trichloric acetic acid (TCA) in acetone containing 20 mM dithiothreitol (DTT) and sonicated in a Biorupter UCD-200 (Diagenode) set on medium intensity for seven cycles (30 s on/1 min off). Samples were then incubated at -20 °C for at least 1 h, centrifuged 5 min at 12,000 x g at 4 °C and the resulting supernatant was removed. The pellet was washed with 1.5 ml of acetone containing 20 mM DTT and then incubated at -20 °C for 30 min. The washing step was repeated for a total of three times. After washing, the dried pellet was suspended overnight at RT in rehydration buffer containing 7 M urea, 2 M thiourea, 2% CHAPS, 20 mM DTT and 0.5% biolyte 3-10 ampholytes (Bio-Rad). Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad).

Fluorescent labeling of proteins

Protein samples from the first replication of the chamber experiment were used for the proteomic analysis. Protein extracts were minimally labeled with fluorescent Cy2, Cy3, or Cy5 N-hydroxysuccinimide (NHS) esters (Lumiprobe,LLC). Protein solutions were diluted to 5 µg/µl with labeling buffer (30 mM Tris, 7 M urea, 2 M thiourea, 4% CHAPS, pH of 8.5) and 1 µl of working solution containing 400 picomoles of

fluorescent ester was added to 50 µg of protein. The protein and dye solution was briefly vortexed and centrifuged before keeping on ice in the dark for 30 min. To quench any remaining unreacted esters 1 µl of 10 mM lysine was added to the reaction and the tube was vortexed and centrifuged before keeping on ice in the dark for 10 min. The labeled protein solution was then subsequently used for 2D- difference gel electrophoresis (2D-DIGE). Cy2 was exclusively used for labeling of pooled internal standards consisting of an equal mixture of all protein samples used in a given experiment; dyes Cy3 and Cy5 were used on individual samples as seen in **Table 7**; sample names in the two right columns represent biological replicates.

Table 7. Dye swap setup of the Differential Gel Electrophoresis (DIGE) experiment using Cy2, Cy3 and Cy5. The experiment consisted of two genotypes and four treatments for a total of eight groups. Three biological replicates were used for each group for a total of 24 protein samples in the experiment. The internal standard contained an equal amount of each biological replicate.

Gel	Cy2	Cy3	Cy5
1	Internal standard	Drought 36°C Keel	Control 36°C Keel
2	Internal standard	Control 21°C Keel	Drought 21°C Keel
3	Internal standard	Control 36°C Arta	Drought 36°C Arta
4	Internal standard	Drought 21°C Arta	Control 21°C Arta
5	Internal standard	Drought 21°C Keel	Drought 36°C Keel
6	Internal standard	Control 36°C Keel	Control 21°C Keel
7	Internal standard	Drought 36°C Arta	Drought 21°C Arta
8	Internal standard	Control 21°C Arta	Control 36°C Arta
9	Internal standard	Drought 36°C Keel	Drought 36°C Arta
10	Internal standard	Control 36°C Arta	Control 36°C Keel
11	Internal standard	Drought 21°C Arta	Drought 21°C Keel
12	Internal standard	Control 21°C Keel	Control 21°C Arta

Two dimensional gel electrophoresis

Protein sample diluted in rehydration buffer to a total volume of 340 µl was applied to 18 cm immobiline strips pH 3-10 NL (GE Healthcare Life Sciences) and allowed to rehydrate at room temperature for 16 hr. Strips for 2D-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 of protein. Focusing was accomplished at 20°C using a Protean isoelectric focusing cell (Bio-Rad) with the following conditions: 14 h passive rehydration, 250 V for 15 min, ramping to 2000 V for 1hr 45 min, ramping to 10000 V for 3 hr before maintaining the voltage at 10000 V for a total of 30000 volt/hours. The resulting strips were equilibrated in 2D equilibrium buffer (0.1 M Tris, 6 M urea, 30% glycerol, 2% SDS) containing 2% DTT for 15 min and then with 2D equilibrium buffer containing 2.5% iodoacetamide (Sigma) for 15 min. Equilibrated strips were placed on 1 mm thick 12% SDS-PAGE gels sized 26 × 20 cm and covered with 0.5% agarose (Bio-budget).

The second dimension was separated using 12 mA/gel for 12 h with the Ettan DaltSix electrophoresis system (GE Healthcare life sciences).

Colloidal Coomassie Brilliant Blue staining

Prior to staining, gels were fixed in an aqueous solution containing 25% isopropyl alcohol and 10% acetic acid for 1 h at RT with shaking. Gels were then rinsed twice with distilled water over a period of 1 h and then stained over night with PageBlue solution (Fermentas Life Sciences). Destaining was performed using a minimum of eight rinses with warm (40°C) distilled water over a 4 h period.

Gel image analysis

Two dimensional gels stained with PageBlue solution were digitally imaged at a resolution of 100 µm using a daylight scanner integrated within the Proteineer spII spotting robot (Bruker Daltronics). Two dimensional gels containing protein labeled with Cy2, Cy3 and Cy5 NHS esters were imaged at a 100 µm resolution with the Typhoon FLA 9000 (GE Healthcare Life Science) using long pass filters for either 520 nm 580 nm or 670 nm. Photomultiplier tube gain voltage was adjusted for each Cy dye for each experiment so that pixel saturation only occurred in the largest spot on the gel, known to contain Rubisco. Typical gain settings were 700 V, 950 V, and 850 V for Cy2, Cy3, and Cy5, respectively. Images were imported into Delta 2D version 3.2 (Decodon) and were assigned the appropriate gel, channel and sample name. The option 'Use Internal Standard' was enabled in the project properties. Imported images were grouped according to genotype and condition, and analyzed following the standard workflow of the program. Gel images were warped using in-gel standard warping strategy included in the program. All direct gel warping were manually checked for proper alignment of gels and spots incorrectly matched were re-matched. A master fusion image was created from the union of all gel images with the options to remove background and to rescale the amplitude of each gel before fusing. Spot detection was done on the resulting fusion image with the following setting: local background region set to 50 pixels, spot size set to 20 pixels, and the sensitivity set to 50%. Manual curation was used to remove background incorrectly designated as spots and to add spots missed by the detection algorithm. Spots were assigned an arbitrary identification number by the software. The normalized spot volume of all spots in each gel image was automatically calculated by the software and exported for further analysis in SAS (Statistical Analysis System, SAS Institute Inc.).

Protein identification

Spots from 2D gels were excised using the Proteineer sp II (Bruker Daltonics) and tryptically digested with DP Chemical 96 kits (Bruker Daltonics) using the Proteineer dp (Bruker Daltonics). Aliquots of the

digests were automatically prepared on MTP 600/384 AnchorChip plates (Bruker Daltonics) using the Proteineer dp digest and sample preparation robot (all from Bruker Daltonics). Peptide mass fingerprints (PMFs) were obtained using the Ultraflex III MALDI ToF/ToF mass spectrometer (Bruker Daltonics). The resulting spectra were processed into peak files with the FlexAnalysis ver 2.4 software (Bruker Daltonics) by means of the sophisticated numerical annotation procedure (SNAP) algorithm. Peak data were imported into the ProteinScape database system version 3.0 (Protagen/Bruker), which initiated Mascot version 2.3 (Matrix Science) searches against the UniProt Knowledgebase for *Hordeum vulgare* (release 2011_06) [137] and the DFCI Barley Gene Index (HvGI) version 12 genome database [138]. Mascot PMF search parameters were: mass tolerance of 50 ppm, one allowed missed cleavage, and oxidation (of Met) and carbamidomethylation (of Cys) were allowed as variable modifications. In the event that post calibration using trypsin failed, the search was repeated with a mass tolerance of 100 ppm. MS/MS spectra were collected based on peak intensity and isolation from other peaks observed in the PMF. The resulting peptide fragmentation fingerprints (PFFs) was used in Mascot searches of the above two databases with a mass tolerance of 0.4 Da for both parent and fragment masses.

Annotation of identified proteins with gene ontology terms

Gene ontology (GO) terms were retrieved for identified proteins based on their Uniprot IDs. Three datasets were submitted for comparison with all *Hordeum vulgare* UniProt entries, namely all proteins identified in the proteome map, the subsets that were differentially regulated by the temperature treatment, and another subset that was differentially regulated between genotypes. Uniprot IDs were submitted to the online tool GORetriever, which is part of the public resource AgBase [139]. The resulting GO Summary file, containing all annotated GO terms for each of the submitted protein IDs, was then downloaded and submitted to another AgBase online tool, GOSlim viewer. GoSlim viewer was used to summarize GO terms from the database from using the plant GO slim set which contains a high level subset of GO terms pertinent to plants. Manual annotation of differentially regulated proteins in **Table 11** with biological functions was based on the results from the GOSlim view. Proteins with two or more biological functions were assigned one function based on the following hierarchy where specific functions were given higher priority than broad functions: response to stress, response to abiotic stimulus, protein metabolic process, lipid metabolic process, nucleobase-containing compound metabolic process, catabolic process, cellular homeostasis, carbohydrate metabolic process, translation, transport, photosynthesis, metabolic process.

Singular Enrichment Analysis

To find GO terms present significantly more often in the leaf proteome map as compared to the background of all *Hordeum vulgare* UniProt entries , the Singular Enrichment Analysis (SEA) tool in the agriGO toolkit [140] was used. Go terms exported from GORetreiver for proteins that were indentified in the proteome map and for all *Hordeum vulgare* UniProt entries were submitted to the SEA tool as the customized annotation and the customized reference, respectively. Under advanced options the statistical test method chosen was Fisher, the multi-test method was Hochberg False Discovery Rate (FDR), the significance level was 0.05, and the gene ontology type chosen was Plant GO slim.

Statistical analysis

Statistical analyses were conducted using SAS software version 9.1.3 for Windows (SAS Institute Inc, copyright 2003) using trait measurements of Arta and Keel plants. The command PROC MEANS was used to determine the mean, standard error, minimum and maximum values of traits. A four way analysis of variation (ANOVA) of morphological and physiological traits was calculated with a general linear model using the PROC GLM command with the model: $Y_{ijkl} = G_i + S_j + T_k + R_l + G_i * S_k + G_i * T_k + T_k * S_k + G_i * T_k * S_k + \underline{e}ijklm$, where G_i is the fixed effect of the genotype, S_j is the fixed effect of the soil water content, T_k is the fixed effect of the temperature treatment and R_l is the random effect of the experimental replication. The interaction of effects are denoted with a *, i.e. the interaction of genotype and temperature is written as $G_i * T_k$. Significant ($p < 0.05$) differences between genotypes under each of the four treatments were calculated within the PROC GLM command using the LSMEANS statement for the effects $G_i * T_k * S_k$. Within the LSMEANS statement the option PDIFF was used with the Tukey adjustment for multiple comparisons enabled. Spearman correlation coefficients between all traits recorded in Arta and Keel plants under control temperatures of 21°C and heat stress temperatures of 36°C were generated separately using the PROC CORR command.

To determine significant ($p < 0.05$) single and interacting effects of the genotype, soil water content, and temperature on each of the spot intensities from the DIGE experiment a three way ANOVA was used using the PROC GLM command with the model: $Y_{ijkl} = G_i + S_j + T_k + G_i * S_k + G_i * T_k + T_k * S_k + G_i * T_k * S_k + \underline{e}ijklm$. To correct for the multiple testing that occurred due to the high number of protein spots considered in the ANOVA, p-values generated for each effect and interacting effects were adjusted using the PROC MULTTEST command with the FDR option [141].

Results

Single and combined effects of drought and heat treatments on physiological traits over time

In order to assess the level of stress experienced by the plants due to each of the treatments, physiological measurements for leaf relative water content (RWC), leaf temperature (LT), maximum quantum efficiency (Fv/Fm) and performance index (PI) were taken one, three, and seven days after treatment start.

Relative water content

The leaf RWC is relevant for both heat and drought stress as it is an indicator of how well the plant maintained its water status despite high temperatures or limitations in available water. After one day of treatment, the mean relative water content of Arta plants under combination treatment (74.2%) was significantly lower than that under control conditions (90.3%) or drought treatment (87.1%) (**Figure 7A**, **Figure 7B** and (**Table 9**). Arta plants treated with either heat or drought were not significantly different from controls. No significant differences in the mean relative water content were detected between Keel plants under control (83.2%), high temperature (78.3%), drought (85.1%) or combination treatment (79.2%) conditions after one day of treatment. There was no significant difference in the RWC between Arta and Keel due to any of the four treatments after one day of treatment.

After three days of treatment, the RWC of heat treated (76.2%) and combination treated (67.3%) Arta plants were significantly lower than those of control plants (88.5%). However, no difference in the RWC was detected between drought treated Arta plants (79.8%) and Arta controls (88.5%). Keel plants under combination treatment (72.9%) had a significantly lower RWC than drought treated (78.3%) and control (87.5%) Keel plants. At this time point, no differences between Keel control plants (87.5%), heat treated (76.2%), or drought treated (78.3%) were detected. There was no significant difference in the RWC between Arta and Keel due to any of the four treatments after three days of treatment.

After seven days of treatment, the RWC of heat treated (70.4%) and combination treated (60.8%) Arta plants remained significantly lower than controls (82.9%). The RWC of Arta drought treated plants (76.7%) were not different from controls. At this time point, the mean RWC of combination treated Keel plants (59.4%) was significantly lower than controls (79.4%). No differences in the RWC were detected between drought treated (75.6%), heat treated (74.4%) and control Keel plants (79.4%). No differences between drought treated Arta plants and control plants were detected at this time point.

In summary, the leaf RWC was affected most by the combination treatments. At all three time points, Arta plants had significantly lower RWC due to the combination treatment when compared to controls. The combination treatment was also responsible for a significant decrease in the leaf RWC of Keel plants three and seven days after treatment. The heat treatment affected the leaf RWC but to a lesser extent than the combination treatment. Significant decreases in the RWC due to the heat treatment, as compared to control plants, were detected only in Arta plants three and seven days after treatment start. Despite the observation that Arta and Keel plants sometimes reacted differently to the stress treatments compared to their respective controls, no significant differences in the RWC between the two genotypes were detected due to any of the treatments.

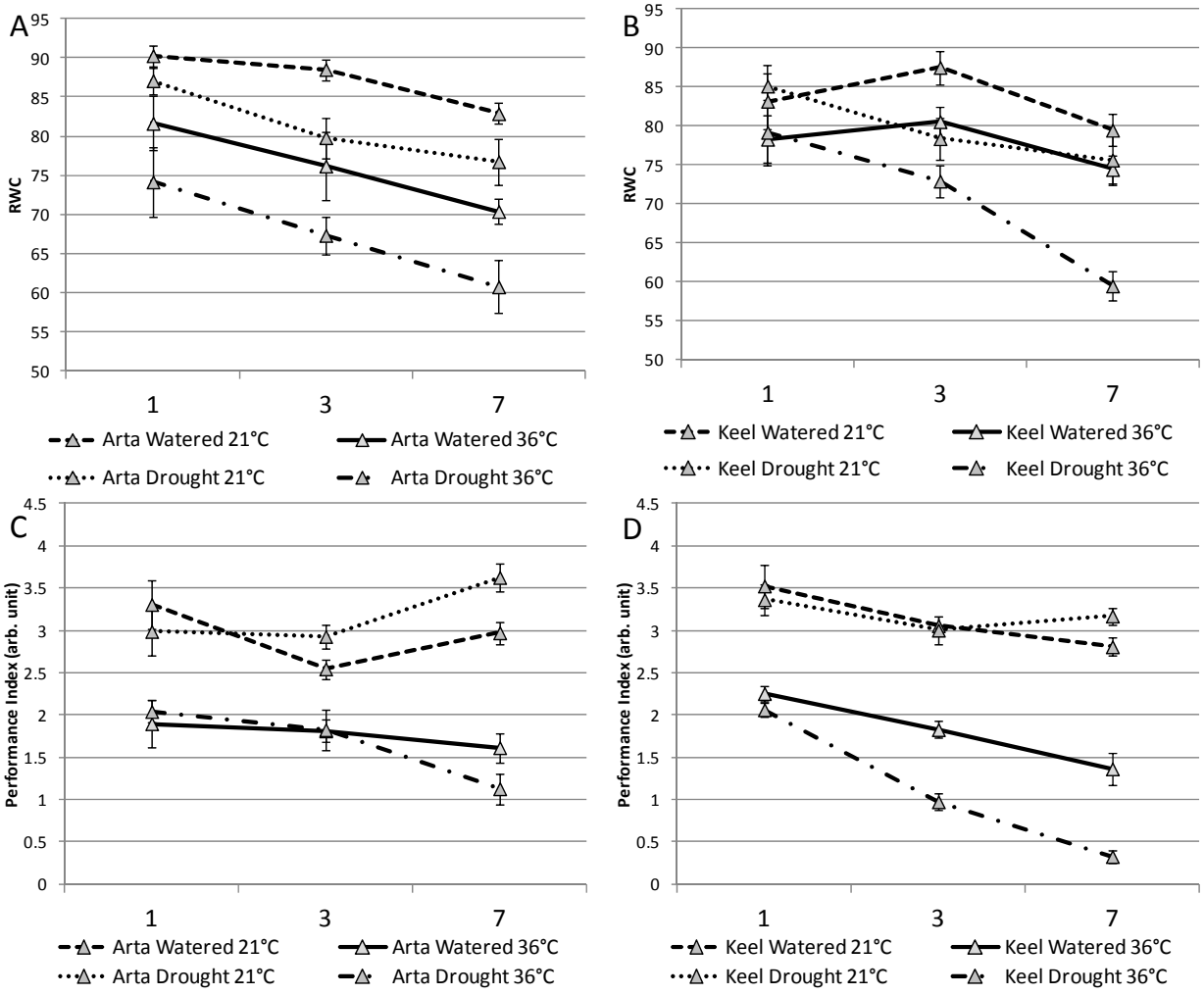

Figure 7. The single and combined effects of drought and heat on plant leaf water status and photosynthesis over seven days of treatment. After three days of treatment, differences in the leaf RWC in Arta (A) and in Keel (B) due treatments became evident. As early as one day after treatment start, differences in the Performance Index in Arta (C) and Keel (D) became evident. Error bars are SEM, n=10

Leaf temperature

Leaf temperature is an indicator of how well the plant can cool itself (e.g. via transpiration) and gives an accurate measurement of the temperature being experienced by the plant as opposed to the ambient temperature. After one day of treatment, the mean leaf temperature of Arta plants under drought (22.8°C) was significantly higher than control Arta plants (20.3°C) (Table 9). The mean leaf temperature of heat treated (35.0°C) and combination treated (36.5°C) Arta plants were not different from each other but both were higher than the leaf temperature of control (20.3°C) or drought treated (22.8°C) Arta plants. The leaf temperature of combination treated Keel plants (37.2°C) was significantly higher than heat treated (33.2°C) Keel plants. No difference in leaf temperature was found between Keel control plants (21.1°C) and Keel drought treated plants (22.0°C) but both temperatures were significantly lower than heat and combination treated Keel plants. Leaf temperatures were not different between Arta and Keel plants under control conditions, drought treatment, or combination treatment but Keel plants subjected to heat treatment (33.2°C) were significantly cooler than Arta plants (35.0°C) subjected to the heat treatment. However, at the other two time points no differences were detected between Arta and Keel plants due to control, drought, heat or combination treatments

After three days of treatment, there was neither a difference in the leaf temperature between Arta heat treated (37.0°C) and Arta combination treated (37.1°C) plants nor between Arta control (21.7°C) and Arta drought treated (22.5°C) plants (**Table 9**). However, heat treated and combination treated plants showed significantly higher leaf temperature than control or drought treated plants. The same was true for the leaf temperature of Keel plants after three days of treatment; Keel heat treated (35.9 °C) and combination treated (37.0 °C) plants were not different from each other but both were higher than control (21.9°C) or drought treated (22.6°C) Keel plants. There were no differences in the leaf temperature between drought treated and control Keel plants.

After seven days of treatment, there were significant differences in the leaf temperature between heat treated plants and combination treated plants for both Arta plants and Keel plants. Arta and Keel heat treated plants had mean leaf temperatures (33.2°C and 34.6°C, respectively) significantly lower than combination treated Arta and Keel plants (36.4°C and 37.2°C, respectively) (**Table 9**). In turn, the heat treated Arta and Keel plants had a leaf temperature higher than either Arta and Keel control plants (23.0°C and 21.4°C, respectively) or drought treated plants (22.3°C and 21.8°C, respectively). There were neither differences in the leaf temperature between control and drought treated Arta plants nor between control and drought treated Keel plants.

In summary, significant differences in the leaf temperature were detected at all three time points between plants grown at 21°C and plants grown at 36°C with mean values ranging from 20.3°C to 20.0°C and from 33.2°C to 37.2°C, respectively. At high ambient temperature, the drought treatment resulted in higher leaf temperatures than the control treatment; these differences were significant for Keel plants one day after the treatment was started and for Arta and Keel plants seven days after the treatment was started. At control temperatures, the drought treatment did not result in a significant increase in the leaf temperature with the exception of Arta plants at the first time point.

Maximum quantum efficiency of photosystem II

The maximum quantum efficiency (Fv/Fm) of photosystem II is a measure of how well the photosystem can trap the energy of absorbed light into the electron transport chain. Fv/Fm values for unstressed leaf samples typically range from 0.800 to 0.840. One day after treatment start, no differences in the Fv/Fm were detected between Arta control plants (0.836) and Arta drought treated plants (0.835) but both values were higher than the Fv/Fm of heat treated (0.795) and combination treated (0.785) Arta plants (**Table 9**). The Fv/Fm of heat treated and combination treated Arta plants were not different from each other. The Fv/Fm of Keel plants followed the trend of Arta plants. In Keel plants there was no difference between control (0.835) and drought treated (0.826) plants and both values were higher than heat treated (0.784) and combination treated (0.775) plants. There was no difference in the Fv/Fm of heat treated and combination treated Keel plants at this time point. The Fv/Fm of Arta and Keel plants were not different from each other due to any of the treatments.

Three days after start of the treatment, the Fv/Fm of control Arta plants (0.820) were not different from drought treated Arta plants (0.829) but both were higher than heat treated (0.789) and combination treated (0.791) Arta plants. The Fv/Fm was not different between heat treated and combination treated Arta plants. In Keel plants, the Fv/Fm of control plants (0.816) were not different from drought treated plants (0.811) but both values were significantly higher than heat treated (0.770) Keel plants which were in turn higher than combination treated (0.732) plants. The mean Fv/Fm of combination treated Arta plants (0.791) was higher than combination treated Keel plants (0.732) but was not different between the two genotypes due to any of the other treatments.

Seven days after the treatment start, the Fv/Fm of control Arta plants (0.829) were neither different from drought treated (0.834), heat treated (0.782), nor combination treated (0.761) plants. However, the mean Fv/Fm of drought treated plants was significantly higher than combination treated plants. In Keel plants, the mean Fv/Fm of control plants (0.803) was not different from drought treated plants (0.815) but both values were significantly higher than heated treat plants (0.728) which was in

turn significantly higher than the Fv/Fm of combination treated plants (0.564). Due to the combination treatment, the Fv/Fm of Arta plants (0.761) was significantly higher than Keel plants (0.564) but Fv/Fm ratios were not different between Arta and Keel due to any of the other treatments.

In summary, the drought treatment did not result in a decrease in Fv/Fm compared to controls in either genotype at any of the three time points. However, the heat treatment resulted in significantly lower Fv/Fm compared to controls in Keel plants at all three time points and in Arta at the first two time points. Significant differences in the Fv/Fm between Arta and Keel combination treated plants were present three and seven days after treatment start but there were no differences between the two genotypes due to the other treatments.

Performance Index of photosystem II

The overall functionality of photosystem II was estimated using the integrated chlorophyll fluorescence measurement performance index (PI). Differences in the PI between control and high temperature treatments were apparent after one day of treatment. In Arta plants, the mean PI of control plants (3.3) was not different than drought treated plants (3.0) but both PIs were significantly higher than the PI of heat treated (1.9) or combination treated (2.0) Arta plants (**Figure 7C** and **Table 9**). The same trend was true for Keel plants at this time point, the PI of control plants (3.5) were not different from drought treated plants (3.4) but both were significantly higher than heat treated (2.3) or combination treated (2.1) Keel plants. No differences in the PI were detected between Arta and Keel plants due to any of the treatments.

After three days of treatment, the PI of control Arta plants (2.5) were still not different than drought treated Arta plants (2.9) but both values remained significantly higher than heat treated (1.8) and combination treated (1.8) Arta plants. At this time point, the PI of heat treated and combination treated Arta and combination treated plants were not different. In Keel plants, there were again no differences in the PI of control (3.1) and drought treated (3.0) plants but both values were significantly higher than the PI of heat treated (1.8) and combination treated Keel plants (1.0). The difference in PI between heat treated plants and combination treated Keel plants was significant. Due to the combination stress the PI of Keel plants (1.0) was significantly lower than Arta plants (1.8) but there were no differences in the PI due to the other treatments at this time point.

On the seventh day after the start of treatment the PI of drought treated Arta plants (3.6) were significantly higher than controls (3.0). The PI of control Arta plants at this time point were significantly higher than heat treated (1.6) and combination treated (1.1) Arta plants. The PI of heat treated and combination treated Arta plants were not different from each other. In Keel plants, however, the PI of

control plants (2.8) were not different from drought treated plants (3.2) but both values were higher than the PI of heat treated (1.4) Keel plants which were, in turn, higher than the PI of combination treated Keel plants (0.3). At this time point, the PI of Arta plants were higher than the PI of Keel plants due to the combination stress, but not due to any of the other treatments.

At each of the three time points, the PI was significantly reduced in both genotypes due to the heat treatment but not due to the drought treatment. In Keel plants, the PI was significantly lower due to the combination stress than due to the heat stress at the last two time points. At the same time points, the PI of Keel plants was lower than Arta plants due to the combination stress. However, the PI of Arta and Keel plants were not significantly different from each other due to control conditions, drought stress, or heat stress.

Of the four physiological traits considered, RWC, LT, Fv/Fm and PI, all were significantly affected by the combination stress treatment in both genotypes within seven days of treatment. The heat treatment also caused changes in all four traits in at least one genotype within the seven days of treatment. Conversely, the drought treatment did not have a significant effect on any physiological traits in either genotype at any of the three time points. Arta and Keel plants often had similar physiological responses to the stress treatments; no differences in the RWC or LT between Arta and Keel due to any of the treatments were detected. However, significant differences in Fv/Fm and PI between Arta and Keel due to the combination stress were detected three and seven days after treatment start.

Single and combined effects of drought and heat stress on morphological traits

Grain yield and yield components

The effects of drought, heat and combination stress on yield and the yield component traits: spike number, grains per spike and thousand kernel weight were analyzed. Mean grain yield harvested from control conditions in Arta (11.8 g) was significantly higher than the yield harvested from Arta plants subjected to drought (6.0 g), heat (5.6 g), or combination stress (3.2 g) (**Figure 8** and **Table 9**). There was no detected difference in grain yield between Arta plants under drought, heat or combination treatments. For Keel plants, the mean grain yield harvested from control conditions (11.7 g) was also significantly higher than the yield harvested from plants subjected to drought (7.7 g), heat (6.4 g), or combination treatments (2.9 g). The grain yield achieved by Keel under drought (7.7 g) was not different than the yield from heat treatment (6.4 g) but both yields were significantly higher than that from combination treated (2.9 g) plants. The grain yield was not significantly different between Arta and Keel under each of the four conditions.

The first yield component trait to be considered was the number of spikes per plant. Plant spikes were divided into spikes that had developed normally and those that had more than half of the florets aborted. The vast majority of aborted spikes were fully aborted i.e. no grains were present on the spike. Counting the number of aborted spikes was done to assess the amount of yield lost to floret infertility. The total number of non-aborted, fertile spikes present in Arta control plants (19.1) was not significantly different from the number of non-aborted spikes present in heat treated Arta plants (14.8) but it was higher than the number in drought treated (11.6) and combination treated (9.7) Arta plants. The number of spikes in Arta drought, heat, and combination treated plants were not significantly different from each other. In Keel plants, the number of spikes from control treated plants (21.8) was not different than the number of spikes from heat treated plants (20.7) but it was significantly higher than the number of spikes from drought (15.6) and combination (11.6) treated Keel plants. The number of spikes between heat, drought and combination treated Keel plants were not different. No significant differences in the number of spikes between Arta and Keel plants were present due to the control, drought, heat or combination treatments.

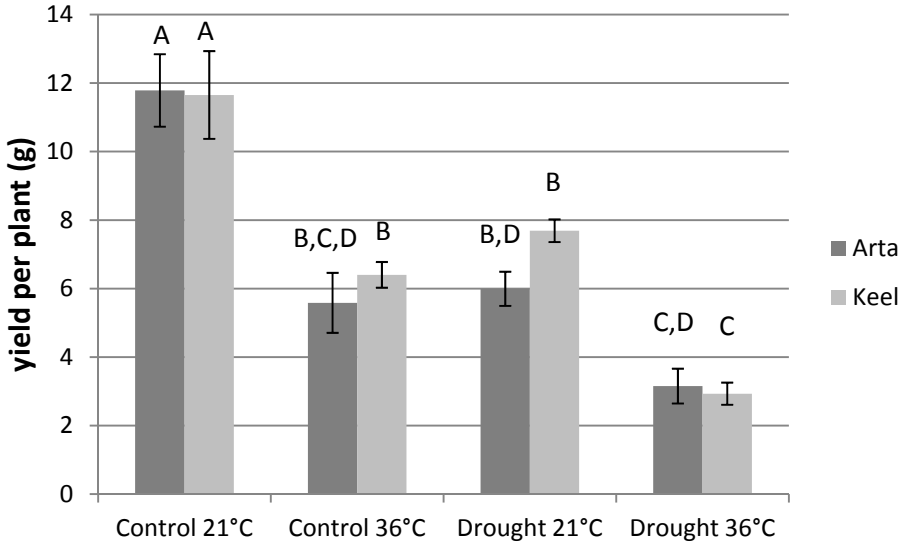

Figure 8. Grain yield of Arta and Keel under control or drought conditions at either 21°C or 36°C. Letters designate statistically similar groups ($p<0.05$). The two genotypes had similar yields to each other under low temperature, control conditions. The effect of the heat treatment was similar to the effect of the drought treatment in terms of grain yield in both genotypes. The combined effect of the heat and drought treatment reduced yield in Keel more than either treatment alone. Bars are SEM, n=10.

Arta spikes were aborted significantly more due to heat treatment (11.1) and combination treatment (8.8) treatment than due to control (3.5) or drought (3.2) treatments. No difference in the number of aborted spikes was detected between heat and combination treatments or between control and drought treated Arta plants. In Keel plants, significantly more spikes were lost due to the heat (6.1)

treatment than were lost due to the control (0.4) or drought treatments (2.1). No difference in the number of aborted spikes was detected between heat and combination (3.3) treatments or between control and drought treated Keel plants. The number of aborted spikes between Arta and Keel under control conditions and under drought conditions was not different from each other. However, heat treated Arta plants (11.1) had significantly more spikes aborted than Keel heat treated plants (6.1). More spikes were also aborted in combination treated Arta plants (8.8) than were aborted in combination treated Keel plants (3.3).

Together, the number of spikes per plant and the number of grains per spike dictates the number of grains produced in a plant. No differences in the mean number of grains per spike were detected in Arta plants between control (12.2), heat (10.7), drought (11.2), and combination (10.8) treatments. Additionally, no differences in the grains per spike were detected in Keel plants between control (12.1), heat (10.4), drought (11.3), and combination (10.0) treatments. The number of grains per spikes was not different between Arta and Keel due to any of the treatments.

The thousand kernel weight is a measurement of kernel size and is the third yield component trait considered here. The number of grains per plant and the mean weight of individual kernels together dictate grain yield. The mean thousand kernel weight of Arta control plants (50.2 g) was not different from the kernel weight of drought treated Arta plants (46.0 g) but both weights were significantly higher than the thousand kernel weight of heat treated (35.5 g) and combination treated (29.9 g) Arta plants (**Table 9**). No difference in thousand kernel weight was detected between heat treated Arta plants and combination treated Arta plants. In Keel plants, the thousand kernel weight of control plants (44.1 g) were also similar to drought treated plants (44.5 g) and both weights were significantly higher than heat treated (30.4 g) and combination treated (26.3 g) Keel plants. The thousand kernel weight of heat treated and combination treated Keel plants were not different from each other. Under control conditions, the thousand kernel weight of Arta plants (50.2 g) was significantly higher than Keel plants (44.1) but the thousand kernel weight between Arta and Keel plants were not different due to any of the other treatments.

In summary, the drought, heat and combination treatments all resulted in significant losses in grain yield compared to control conditions in both genotypes. The losses due to the drought treatment were not significantly different from the losses due to heat treatment. The loss in grain yield due to combination treatment was significantly higher than the losses due to the drought and heats treatments in Keel plants but not in Arta plants. The number of spikes per plant affected by the drought treatment and combination treatment but not by the heat treatment as compared to controls in both genotypes. A significant number of spikes were aborted in both genotypes due to the heat treatment but not due to

the drought treatment as compared to control conditions. Neither drought, heat, nor combination treatment had an effect on the number of grains per spike in either genotype as compared to control conditions. The thousand kernel weight in heat and combination treated plants was significantly decreased in both genotypes compared to their respective controls. Comparing values between genotypes revealed significantly more aborted spikes in Arta plants than Keel plants due to the heat and combination treatments. Additionally, under control conditions only, Arta plant had a significantly higher thousand kernel weight than Keel plants.

Agronomic traits

Morphological changes caused by drought and heat treatments are depicted in the representative photographs of Arta and Keel plants taken after seven days of heat treatment of control and drought treated plants (**Figure 9**). Under control conditions, Arta plants had more and broader leaves than Keel plants. Under control temperatures, drought treated Arta plants were noticeably smaller than the control Arta plants and showed signs of chlorosis while drought treated Keel plants did not show obvious phenotypic differences to Keel plants grown under control conditions. At high temperatures, both control Arta plants and drought treated Arta plants exhibited considerable wilting and chlorosis. In comparison, Keel plants at high temperatures also showed signs of wilting and leaves were lighter green than under control temperatures. Additionally, heat treated Keel plants were more prone to lodging than Keel plants grown at control temperatures.

The above ground biomass is the total amount of plant material that can be recovered from the field and has value as fodder for livestock. The mean above ground biomass of control Arta plants (20.7 g) was not different from the biomass of heat treated (18.7 g) plants but both weights were significantly higher than drought treated Arta plants (13.6 g) and combination treated Arta plants (12.3 g) (**Table 9**). No significant difference between drought treated Arta plants and combination treated Arta plants was detected. In Keel plants, there was no difference between the mean biomass of control plants (20.2 g) and heat treated plants (18.6 g) but the biomass of drought treated plants (15.1 g) and combination treated (9.8 g) were both significantly lower than control plants. Additionally, the biomass of combination treated Keel plants was significantly lower than drought treated Keel plants. No differences in the biomass due to any of the treatments were detected between Arta and Keel plants.

The biomass measurements and grain yield measurements were used to calculate the harvest index; the proportion of the above ground biomass invested into grains. The harvest index of control Arta plants (0.56) was significantly higher than drought treated (0.44), heat treated (0.29), and combination treated (0.25) Arta plants (**Table 9**). No differences in the harvest index were detected

between heat treated and combination treated Arta plants but both values were significantly lower than the harvest index of drought treated plants. In Keel, the mean harvest index of control plants (0.57) was not different from drought plants (0.51) but both values were significantly higher than the harvest index of heat treated (0.35) and combination treated plants (0.30). No significant differences in the harvest index were detected between heat treated and combination treated Keel plants. No differences in the harvest index due to any of the treatments were detected between Arta and Keel plants.

The time taken for a barley plant to mature is important because it dictates how long the plant must remain in the field before harvest. There were neither differences in the days to maturity between treatments for either genotype nor differences between Arta and Keel due to any of the treatments. The mean number of days to maturity ranged from 88 to 97 days after transfer of the plants to LD conditions (**Table 9**).

The plant height of control Arta plants (49.6 cm) were not different from heat treated Arta plants (44.9 cm). However, control Arta plants were significantly taller than drought treated (38.9 cm) and combination treated (42.8 cm) Arta plants (**Table 9**). Combination treated Arta plants were not different from drought or heat treated Arta plants in terms of plant height. The mean plant height of Keel control plants (52.5 cm) was neither different from drought treated (49.9 cm) nor heat treated (50.8 cm) Keel plants but control plants were significantly taller than combination treated Keel plants (45.3 cm). Heat treated Keel plants (50.8 cm) were significantly taller than heat treated Arta plants (44.9 cm) and drought treated Keel plants (49.9 cm) were also significantly taller than drought treated Arta plants (38.9 cm).

The peduncle extrusion, or Pedex, is a measurement of the distance between the flag leaf collar and the spike bottom and can be negative when the spike remains booted in the leaf sheath. The amount of Pedex is partially dependent on the development of the peduncle, the last node to elongate. There were neither significant differences in the mean Pedex between treatments for either genotype nor differences between Arta and Keel due to any of the treatments. Mean Pedex values ranged from -6.8 cm to -3.7 cm.

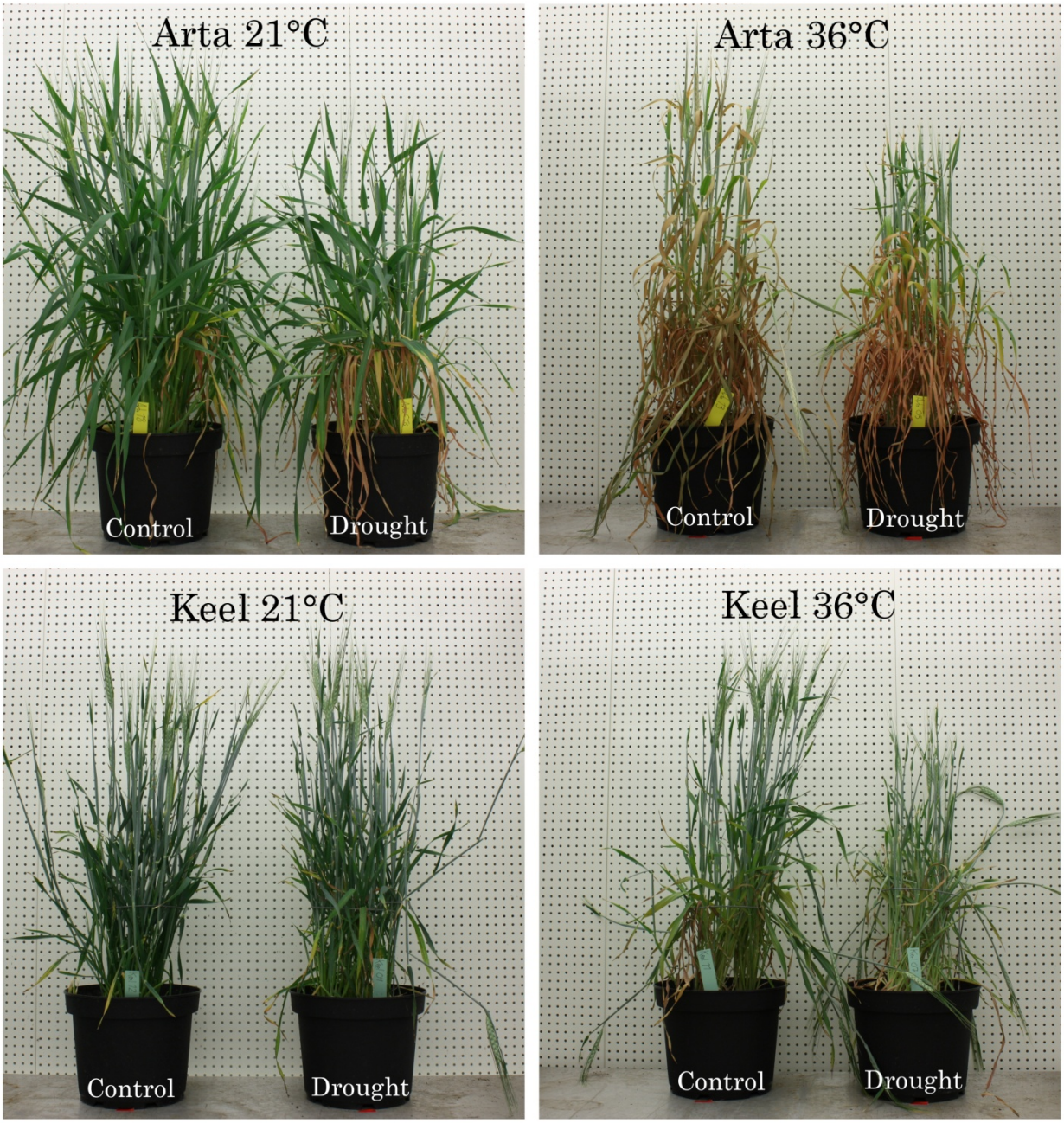

Figure 9. Representative Arta and Keel plants after seven days of control (21°C) or high (36°C) temperature and with the soil water content at control (50% SWC) or drought (15% SWC) levels. Each four liter pot contains three plants.

The water used by the potted plants was recorded starting when the plants were moved into LD conditions and stopped when the plants were mature. The water used by Arta was significantly lower in drought and combination treated plants (3.45 l and 3.61 l, respectively) than in control and heat treated plants (7.54 l and 7.65 l, respectively). This difference was also exhibited in Keel plants where drought and combination treated plants (3.42 l and 3.21 l, respectively) used less water than control and heat

treated plants (6.70 l and 6.65 l). No difference in the water use due to any of the treatments was detected between Arta and Keel plants.

Water use efficiency, the amount of yield per water used, was not different between Arta control plants (1.6 g/l) and Arta drought treated plants (1.8 g/l) but both values were significantly higher than the mean water use efficiency of heat treated (0.7 g/l) and combination treated (0.9 g/l) Arta plants. The water use efficiency between heat treated and combination treated Arta plants were not different from each other. For Keel plants, the water use efficiency of control plants (1.7 g/l) was not different than drought treated plants (2.3 g/l) but both were significantly higher than the water use efficiency of heat treated (1.0 g/l) and combination treated (0.9 g/l) plants. The water use efficiency between heat treated and combination treated Keel plants were not different from each other. No differences in the water use efficiency due to any of the treatments were detected between Arta and Keel plants.

In summary, the agronomic traits had different responses to the control, drought, heat and combination treatments. Compared to controls, plant height, biomass and water use were all significantly decreased due to drought and combination treatment but not due to heat treatments. Conversely, harvest index and water use efficiency were both significantly lowered due to the heat and combination treatments but not due to the drought treatment. Pedex and days to maturity were not different from controls due to drought, heat, or combination treatments.

Phenotypic variation of traits due to main and interacting effects of the genotype and treatments

The variation of traits due to the fixed effect of genotype, soil water content (SWC), ambient temperature, the interaction between the three fixed effects, all possible pair wise combinations of the fixed effects and the random effect of the experimental replication, were considered for Arta and Keel plants by use of ANOVA. The effect of temperature (heat treatment) explained a significant proportion of the variation for 21 traits, soil water content (drought treatment) explained 17 traits, and genotype had a significant effect on 13 traits (**Table 10**). A list of traits considered in the analysis and the abbreviations used in this text are found in (**Table 6**). The random effect of the experimental replication explained a significant proportion of the variance for 14 traits. Each of the pair wise interactions of the fixed effects explained a significant proportion of variation for several traits; eight traits were affected by the interaction of SWC and temperature, five traits by the interaction of genotype and temperature and four traits by interaction of genotype and SWC. The interaction of all three main effects explained a significant proportion of the variation for three traits.

The effect of the temperature explained a significant amount of the variation for more traits than genotype or SWC. Traits affected by temperature included: grain yield, biomass, harvest index, plant height, spike number, aborted spikes, thousand kernel weight, days to maturity, water use efficiency, leaf temperature at all three time points, RWC at all three time points, Fv/Fm at all three time points and PI at all three time points. Of the temperature responsive traits, the greatest amount of variance explained was for leaf temperature at the three time points with percent explained variances ranging from 85.6% to 95.6%. The least amount of significant variance was explained for plant height (3.8%).

The effect of the soil water content explained a significant amount of the variation for the traits: grain yield, biomass, harvest index, plant height, Pedex, spike number, grains per spike, thousand kernel weight, water use, water use efficiency, leaf temperature at all three time points, RWC_3, RWC_7, Fv/Fm_1 and Fv/Fm_7. Of these traits, the greatest amount of variance explained by the soil water content was for water use (76.9%) and biomass (56.7%); the least amount of significant variance was explained for leaf temperature three days after the onset of the heat stress (LT_3) (0.2%).

The effect of the genotype explained a significant amount of the variation for the traits: harvest index, plant height, Pedex, spike number, aborted spikes, thousand kernel, water use, water use efficiency, days to maturity, water use, water use efficiency, Fv/Fm at all three time points, and PI_7. Of these traits, the greatest amount of variance explained by the genotype was for plant height (23.5%) and the least amount for the traits water use (1.8%) and Fv/Fm_7.

The random effect of the experimental replication explained a significant proportion of the variance for the traits: days to maturity (63.4%), RWC_1 (35.6%), harvest index (15.5%), aborted spikes (6.5%), number of spikes (10.3%), grain yield (8.1%), RWC_7 (8.0%), water use (7.6%), LT_7 (7.1%), Fv/Fm_7 (3.0%), Fv/Fm_7 (3.0%), LT_1 (2.5%) and LT_3 (1.2%).

By looking at the interaction between effects, synergistic changes in traits can be identified. Of the four interaction effects tested, the interaction effect between soil water content and temperature explained a significant proportion of variation for the highest number of traits: PI_7 (7.2%), Fv/Fm_7 (6.7%), PI_3 (3.7%), RWC_7 (2.9%), aborted spikes (3.3%), Fv/Fm_3 (1.7%), grain yield (1.5%), LT_7 (1.1%) and LT_1 (0.1%). The interaction effect between genotype and temperature explained a significant proportion of variation for the traits: Fv/Fm_7 (6.9%), PI_3 (5.3%), Fv/Fm_3 (3.7%) aborted spikes (3.1%) and LT_7 (0.5%). The interaction effect between genotype and soil water content explained a significant proportion of variation for the traits: PI_3 (4.5%), Fv/Fm_3 (3.3%), Fv/Fm_7 (3.1%), and PI_7 (0.7%). The interaction effect of genotype, soil water content, and temperature explained a significant amount of variation in the traits plant height (6.3%), Fv/Fm_7 (3.7%) and LT_1 (0.5%).

Overall, the temperature treatment had a significant effect on more traits than the drought treatment. The temperature treatment had the strongest effect on physiological traits, followed by the drought treatment and then the genotype. Of the fourteen traits that temperature and the soil water content both had significant effects on, more variation was explained by the effect of the temperature for ten of the traits. Of the eleven traits that the temperature and genotype both had significant effects on, more variation was explained by the effect of the temperature for nine of the traits. Of the eight traits that the genotype and the soil water content both had significant effects on, more variation was explained by genotype for four of the traits.

Phenotypic correlation of traits to grain yield under control and high temperature treatments

Spearman's rank correlations for traits measured in Arta and Keel were calculated separately for plants grown at a control temperature of 21°C and a high temperature of 36°C (**Table 8**). Heat and control temperature treated plants were chosen for the correlation analysis because the heat treatment explained more of the variance in the traits than the drought treatment (**Table 10**). For simplicity, not all traits were included in the analysis; traits that are measured based on grain yield (e.g. harvest index) were omitted and physiological traits measured three days after the start of the treatment were chosen as representatives for all three time points.

The traits biomass, total spike number, thousand kernel weight, water use, and RWC_3 were significantly and positively correlated to grain yield under both temperature treatments. Plant height, Pedex, and grains per spike were positively correlated to grain yield only when grown at 21°C. Under control temperatures, biomass was positively correlated to: plant height, Pedex, spike number, thousand kernel weight, water use and RWC_3. Under high temperature treatment, biomass was also positively correlated to Pedex, spike number, thousand kernel weight, water use and RWC_3 but not plant height.

Plant height positively correlated to Pedex, spike number, and water use under control temperatures but not the heat treatment. However, plant height was negatively correlated to LT_3 under the heat treatments. Pedex was positively correlated to water use and thousand kernel weight but negatively correlated to PI_3 under control temperatures. The spike number was positively correlated to water use under control and high temperatures and to RWC_3 under control temperature. The number of grains per spike was correlated to thousand kernel weight under control temperature but not under high temperature. Thousand kernel weight was positively correlated to water use under control and high temperature treatments. Under the heat treatment, thousand kernel weight was positively correlated to PI_3 and positively correlated to RWC_3 under control temperature. In the presence of control and high

temperature water use was correlated to RWC_3. Leaf temperature was negatively correlated to RWC_3 and PI_3 under high temperature.

In summary, more positive correlations were found between traits in plants grown at 21°C than in plants grown at 36°C. Additionally, when correlations were found between the same traits under both temperature treatments the coefficients were often higher under control temperature. However, some traits were correlated exclusively in heat treated plants, these correlations were between: thousand kernel weight and Pedex, biomass and PI_3, PI_3 and thousand kernel weight, LT_3 and RWC_3, and LT_3 and PI_3.

Table 8. Spearman's rank correlation coefficients for phenotypic traits measured in both genotypes under control (21°C) or high (36°C) temperatures. Values above the diagonal are from control temperature conditions while values below the diagonal are from high temperature conditions. Significant ($p<0.05$) coefficients are underlined.

	GY	BM	PH	Pedex	SN	GS	TKW	WU	LT_3	RWC_3	PI_3
GY		**0.88**	**0.46**	**0.33**	**0.86**	**0.35**	**0.45**	**0.68**	-0.02	**0.53**	-0.23
BM	**0.76**		**0.43**	**0.32**	**0.79**	0.31	**0.38**	**0.65**	-0.26	**0.56**	-0.23
PH	0.10	0.27		**0.43**	**0.40**	0.16	-0.06	**0.35**	-0.15	0.10	-0.08
Pedex	0.18	**0.32**	0.28		0.17	0.23	0.15	**0.43**	0.02	0.07	**-0.45**
SN	**0.88**	**0.65**	0.13	-0.02		-0.10	0.12	**0.58**	-0.01	**0.50**	-0.14
GS	0.20	-0.05	-0.01	0.21	-0.16		**0.51**	0.27	-0.12	0.12	-0.06
TKW	**0.38**	**0.61**	-0.02	**0.37**	0.10	-0.01		**0.35**	0.14	**0.35**	-0.14
WU	**0.67**	**0.78**	0.07	0.25	**0.53**	0.12	**0.44**		0.15	**0.52**	-0.31
LT_3	-0.04	-0.16	**-0.33**	-0.04	-0.02	0.13	-0.12	-0.17		-0.25	-0.21
RWC_3	**0.33**	**0.37**	0.19	0.24	0.29	-0.03	0.27	**0.37**	**-0.35**		0.11
PI_3	0.20	**0.48**	0.17	0.22	0.09	-0.09	**0.54**	0.31	**-0.37**	0.30	

GY grain yield, BM biomass, PH plant height, Pedex peduncle extrusion, SN spike number, GS grains per main spike, TKW thousand kernel weight, WU water use, LT_3 leaf temperature 3 days after treatment start, RWC_3 leaf relative water content 3 days after treatment start, PI_3 performance index 3 days after treatment start.

Mapping and characterization of the barley leaf proteome

For the creation of a barley leaf proteome map, protein spots were picked from 2D-gels, tryptically digested, and subsequently identified via peptide mass fingerprinting and tandem mass spectrometry. Automated protein spot picking was limited to spots detectable by Coomassie staining as silver staining was not compatible with the trypsin digestion. Spot detection on stained gels (**Figure 10**) revealed a total of 525 distinct spots that were submitted for identification by peptide mass finger printing and subsequent peptide fragmentation analysis. Mascot searches against the Uniprot database for *Hordeum vulgare* or the DFCI Barley Gene Index database allowed a total of 296 proteins from the 525 spots detected to be identified. The 296 identified spots were matched to a total of 145 databases accessions.

Spots identified with the same protein accession were considered to be isoforms of each other. Sixty-two of the proteins identified in the barley leaf proteome had isoforms present.

To better understand the bias of the barley leaf proteins identified in this manner, the cell component gene ontology terms for all proteins were compared to a background consisting of all terms present for barley proteins in the UniProt database using a singular enrichment analysis (SEA). Based on the gene ontology terms, over half of the identified proteins were present in intracellular compartments (59%), the cytoplasm (52%), as well as in organelles (51%) and were significantly enriched compared to the background (22%, 9% and 15% respectively) (**Figure 11**). The largest enrichment was for the term plastid, which was present in 45% of leaf proteins and in less than 2% of background proteins. Additional cellular component terms enriched in the barley leaf proteome as compared to the background were: membrane (31% vs. 14%), envelope (16% vs. 1%), macromolecular complex (15% vs. 7%), extracellular region (12% vs. 2%) and mitochondrion (5% vs. 1%).

In summary, the majority of protein spots were able to be identified via mass spectrometry and Mascot searches using two barley databases containing transcript and protein data. Based on the presence of spots being matched to the same protein accession, isoforms were detected for several of the proteins. A comparison of the peptides identified in the barley leaf proteome to all proteins in the barley Uniprot database revealed an enrichment of intracellular, cytoplasmic and organellular proteins as well as proteins in other cellular compartments.

Quantification of differentially expressed proteins

In order to quantify differences in protein accumulation due to heat and drought treatments, the barley leaf proteome was visualized using difference gel electrophoresis (DIGE). Leaf samples harvested three days after treatment start were used for DIGE. Samples from this time point were chosen based on the RWC and PI measurements (**Figure 7**); three days after treatment start was the first time point where the combination treatment resulted in significant drops in RWC in both genotypes compared to controls as well as the first time point where significant differences in the PI were detected between Arta and Keel due to the combination treatment. Separation of proteins fluorescently labeled with CyDyes was achieved in the first dimension using immobilized pH gradient gels with a pH range of 3-10 followed by a separation in the second dimension using 12% SDS-PAGE. Spot detection on a composite fluorescent image consisting out of all gel images allowed 1005 distinct spots to be resolved. A representative fluorescent image of a gel used in the analysis is seen in **Figure 12.**

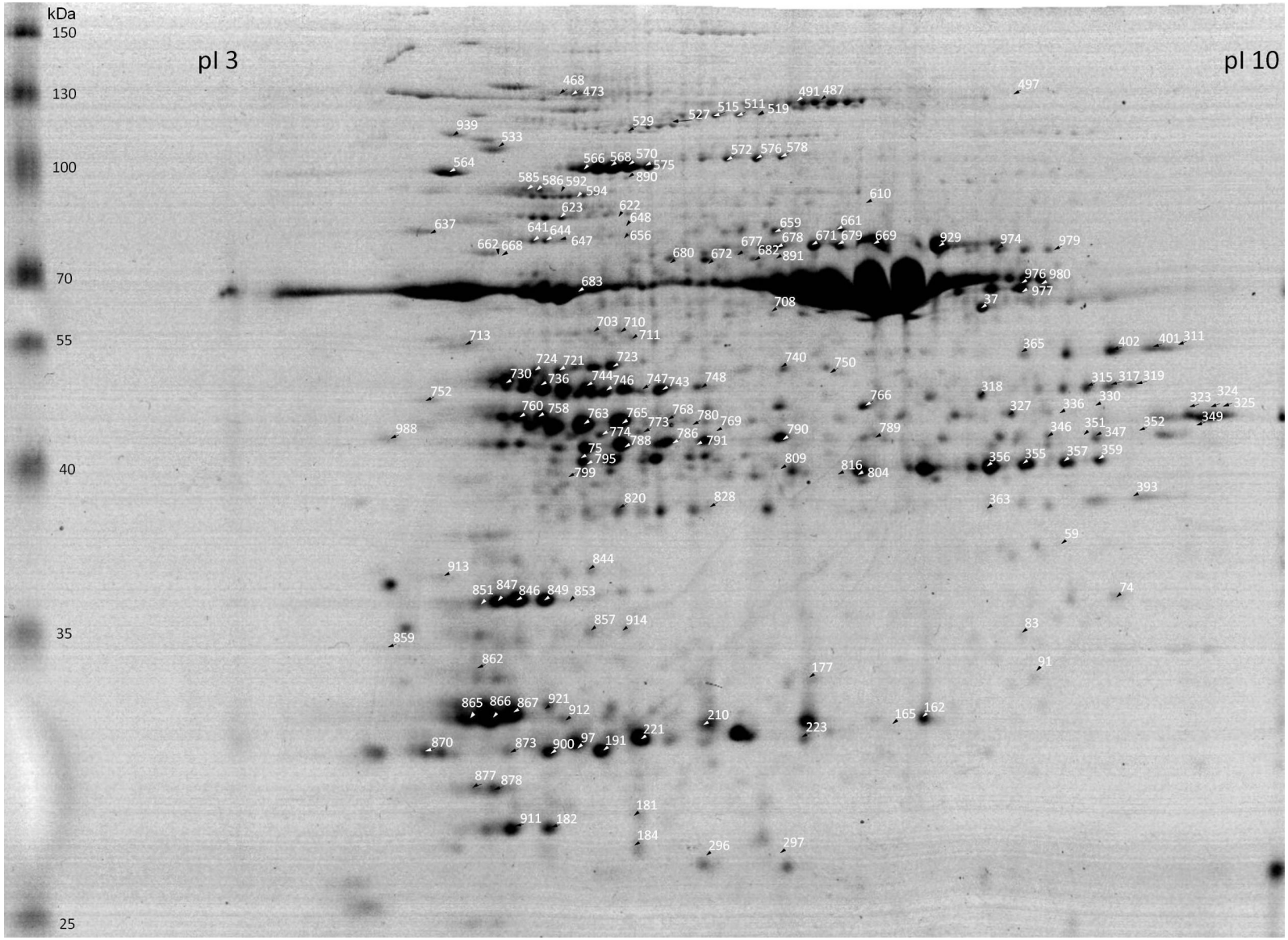

Figure 10. Representative coomassie stained 12% SDS-PAGE containing 400 µg total leaf protein. Numbered arrows represent spots that were identified by MS and significantly regulated between conditions or between genotypes as listed in **Table 11**.

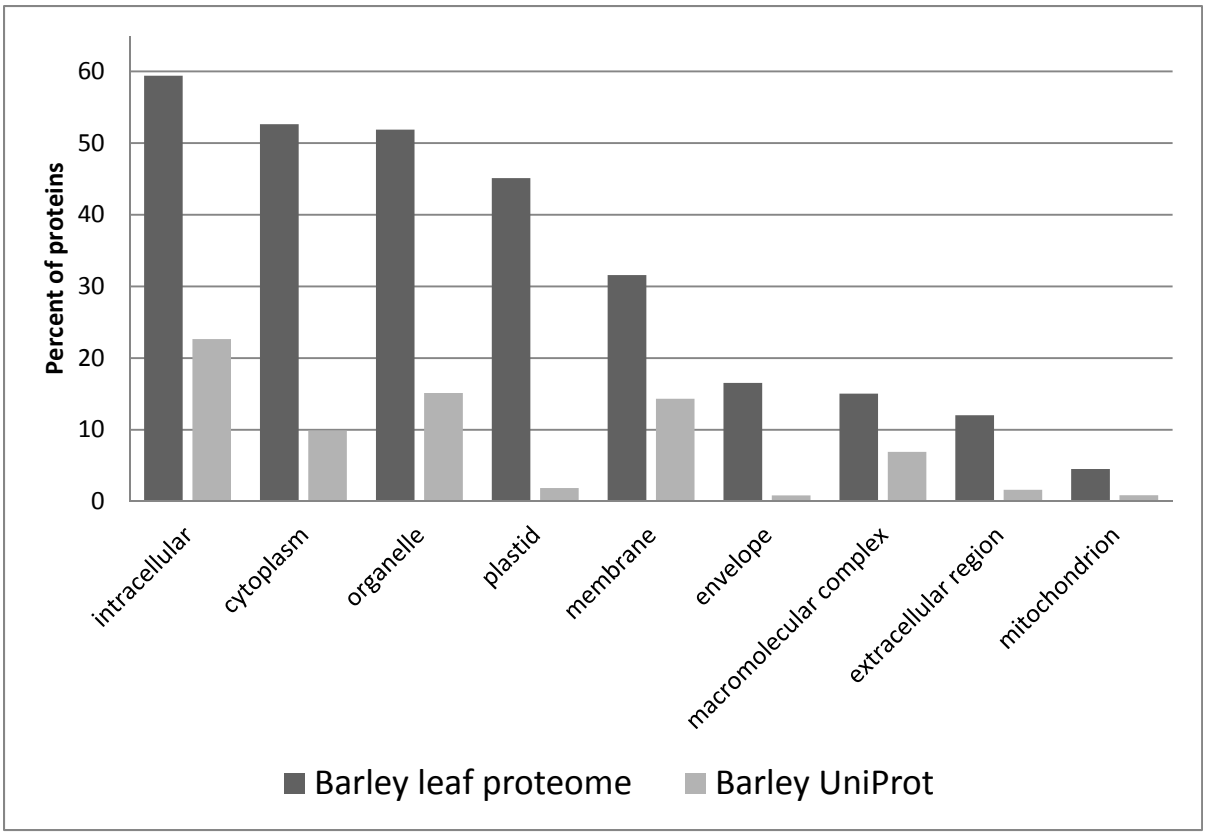

Figure 11. Singular enrichment analysis of cellular component gene ontology terms present in the barley leaf proteome as identified by MS compared to all terms present in Uniprot entries for *Hordeum vulgare*.

Based on the results from the three way ANOVA and subsequent correction for the false discovery rate, 305 spots were found to be significantly differentially regulated by the heat treatment, 473 spots were significantly different between genotypes and 35 spots were significantly different due to the interaction between temperature and genotype. However, no spots were found to be significantly regulated by the drought treatment after correction for the false discovery rate. Of the 473 spots differentially regulated between Arta and Keel, 196 of these were among the 305 temperature responsive spots.

Of the 305 spots found to be differentially regulated by temperature 99 were identified via mass spectrometry, 32 of which were down-regulated and 67 of which were up-regulated. Of the 473 spots found to be differentially regulated between genotypes 125 were identified, 90 of which were down regulated in Keel and 35 of which were down regulated in Arta. Of the proteins controlled by a significant interaction effect of the genotype and heat treatment, fourteen were identified, thirteen of which were down regulated under heat treatment in Keel and ten of which were also down regulated under control temperature in Keel. All differentially regulated proteins were placed into categories based on their gene ontology (GO) biological function.

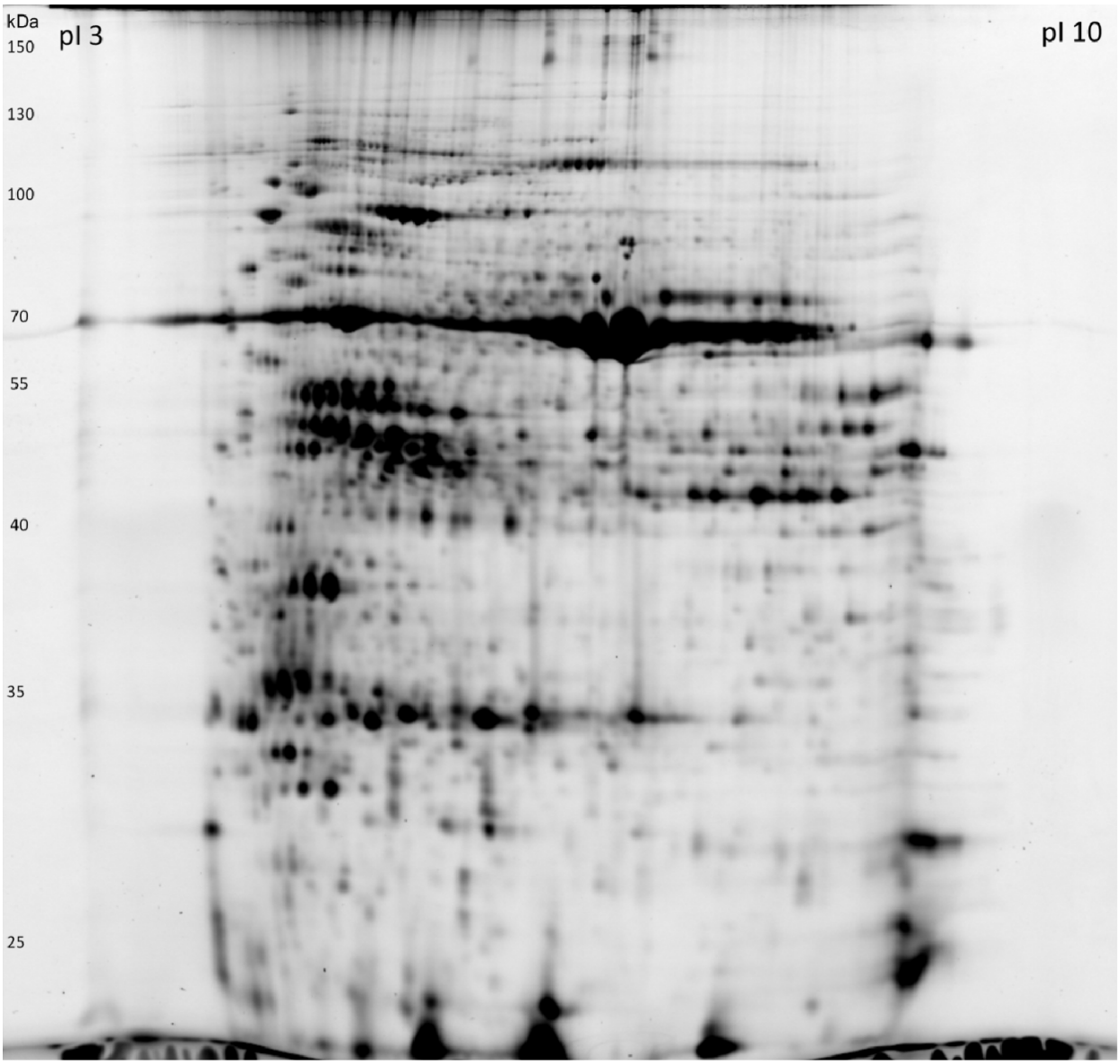

Figure 12. Representative fluorescent image of a 12% SDS-PAGE DIGE gel containing 50 µg leaf total protein labeled with Cy3. First dimension separation was achieved using pH 3-10 IPG strips

Differential regulation of proteins in response to heat treatments

Both Arta and Keel plants were affected by heat treatments on the physiological and morphological level as seen in the phenotyping results. To gain an overview of which processes were affected by the heat treatment on the protein level, the differentially regulated proteins were assigned, when possible, to groups based on their biological functions. The biological functions of heat responsive proteins, as described by plant GO slim terms, were diverse. The functions included roles in metabolic processes, photosynthesis, transport, response to abiotic stimulus, and response to stress (**Table 11**). The percent of proteins in each functional group can be seen in **Figure 13A**; groups smaller than 3% were placed

together into the category 'other'. The most frequent protein function was in the broad category of metabolism; 17% of heat responsive proteins were assigned to this function. Other heat responsive proteins were assigned to more specific metabolic functions; 7% in carbohydrate metabolism, 6% in protein metabolism, 5% in nucleobase-containing compound metabolism and 3% in lipid metabolism. The total percentage of proteins assigned to any metabolic function was 38%.

The regulation factor (RF), the log2-transformed fold change in the normalized spot intensity, was calculated for plants grown at 36°C over plants grown at 21°C, regardless of the drought treatment or genotype. Of the 17% of heat-responsive proteins with functions in general metabolism the majority (12 out of 16) were up-regulated due to the heat treatment with RFs ranging from 1.15 to 1.65; the down-regulated proteins had RFs from -1.22 to -1.37.

Carbohydrates play integral roles in energy storage and structural components. Carbohydrate metabolism proteins represented 7% of the heat-responsive proteins and were almost equally up or down-regulated due to the heat treatment; four were up-regulated with RFs ranging from 1.22 to 1.68 and three were down-regulated with RFs ranging from -1.17 to -1.47. Protein metabolism includes processes involving specific proteins and protein modification. The protein metabolic proteins that responded to the heat treatment were primarily up-regulated with five spots with RFs ranging from 1.99 to 3.14 and one spot with a RF of -1.43. Nucleobase-containing compound metabolic processes are those that involve nucleosides or nucleic acids such as DNA or RNA. Of the five nucleobase-containing compound metabolic protein spots, three were up-regulated due to the heat treatment with RFs ranging from 1.22 to 1.68 and two were down-regulated with RFs of -1.15 and -1.34. The protein spots with functions in lipid metabolism were all down-regulated due to the heat treatment with RFs ranging from -2.21 to -2.72.

The sixteen heat responsive proteins with roles in photosynthesis were all up-regulated with RFs of 1.21 to 1.82 except one, which was down-regulated by a factor of -1.37. A total of fifteen proteins with functions in transport were differentially regulated due to the heat treatment. Twelve of the fifteen transport proteins were up-regulated due to the heat treatments with RFs ranging from 1.09 to 1.81, while the other three were down-regulated with RFs ranging from -1.19 to -1.30.

The functional group 'response to abiotic stimulus' included any protein known to change plant activity due to abiotic signals. Therefore, it should be noted that this group could contain proteins that also match other GO biological function categories. This group represented 13% of the heat responsive proteins. In contrast to the other groups, the majority of proteins in this group were down-regulated due to the heat treatment. The eight down-regulated proteins had RFs ranging from -1.21 to -1.88 while the RFs of the five up-regulated proteins ranged from 1.68 to 6.65. The intensity of spot 773 in each of the

24 images used for the analysis can be seen in **Figure 14**. Spot 773 had an RF of 4.62 one of the highest of all heat-responsive proteins. Under control temperature spot 773 was virtually undetectable by the human eye regardless of the drought treatment but the spot intensity significantly increased due to heat treatment and intensified due to combined treatment as compared to heat treatment alone.

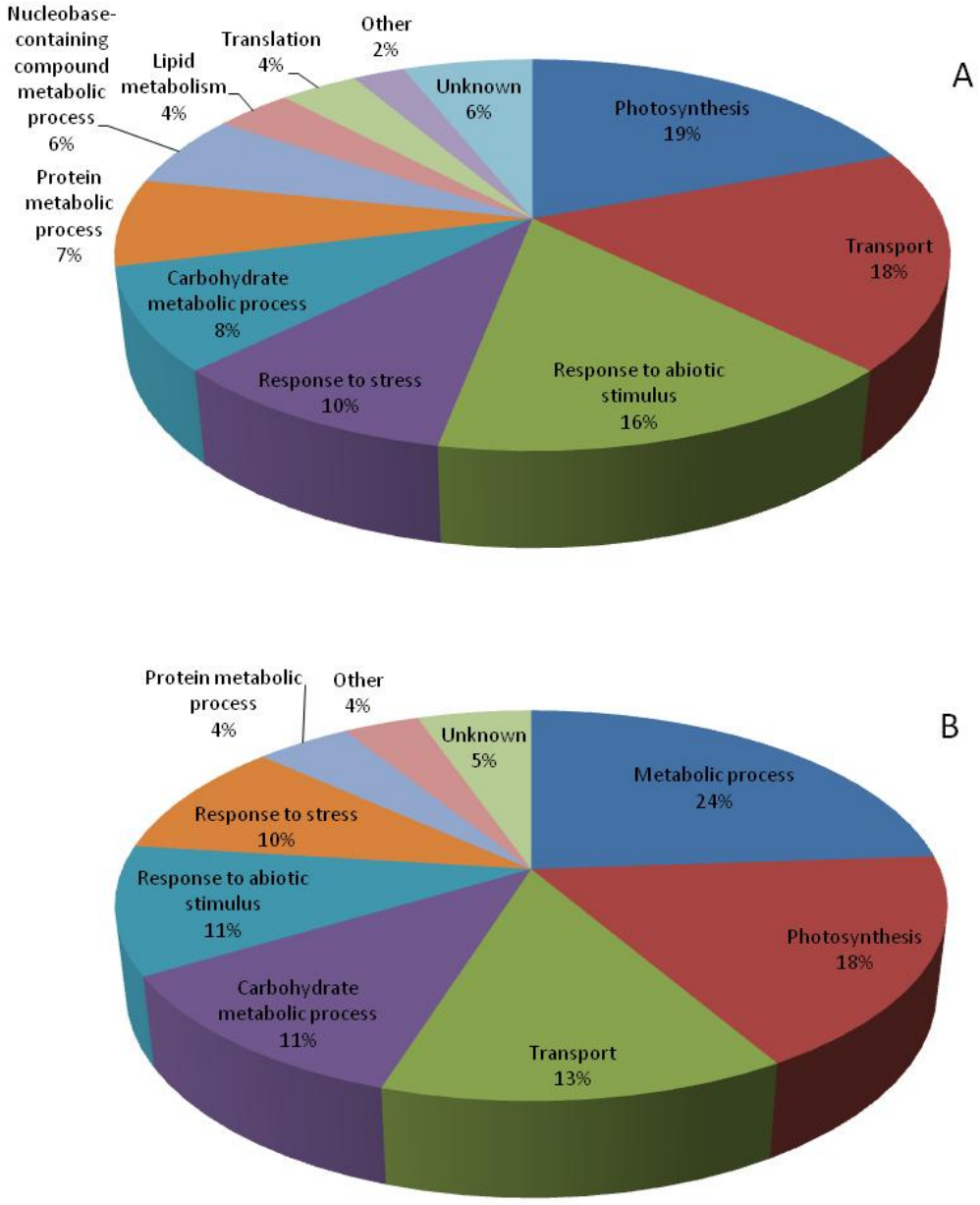

Figure 13. Distribution of biological functions of identified proteins that were differentially regulated due to A, the heat treatment or due to B, the genotype. Functional annotation was based on plant gene ontology slim terms.

Eight proteins with annotated biological functions in stress response were significantly induced by the heat treatment, the majority of which were up-regulated. The six up-regulated proteins had RFs

ranging from 1.44 to 2.90 and the two down-regulated proteins had RFs of -1.28 and -2.07. Three proteins with annotated functions in translation were found to be heat responsive with RFs of -1.39, 1.73 and 2.41. The heat responsive proteins placed into the group 'other' contained one up-regulated catabolic protein (RF of 1.33) and a down regulated protein with functions in carbon utilization.

In summary, the general trend of heat responsive proteins was to be up-regulated due to the heat treatment. The heat responsive proteins were placed into ten categories based on their biological GO functions: general metabolism, carbohydrate metabolism, protein metabolism, nucleobase metabolism, lipid metabolism, photosynthesis, transport, response to stimulus, response to stress and translation. Proteins in the categories carbohydrate metabolism and nucleobase-containing compound metabolism did not follow the general trend as proteins in these groups were near equally up and down-regulated due to the heat treatment. The groups lipid metabolism and response to abiotic stimulus did not follow the general trend either; proteins in the two groups were predominantly down regulated due to the heat stress.

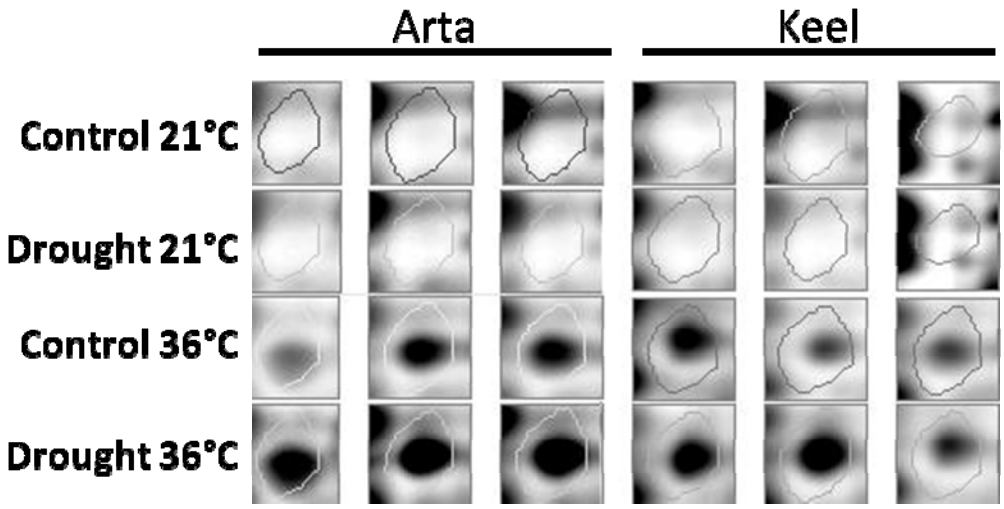

Figure 14. Intensity of spot 773, identified as Rubisco activase B, in each of the DIGE fluorescent images. Outlines represent the area considered by the quantification software for calculating the normalized spot intensity.

Differential protein expression due to genotype

Protein spots that had a significant proportion of their normalized intensity explained by the genotype were considered to be genotype dependent proteins. Of the 125 identified genotype dependent proteins, 24% had a GO biological function relating to general metabolic processes. Additional genotype dependent proteins had more specific metabolic functions in carbohydrate metabolism (11%) and protein metabolism (4%) (**Figure 13B**). In total, 39% of the genotype dependent proteins had functions pertaining to metabolism. The other biological function groups represented in the genotype dependent

data set were photosynthesis (18%), transport (13%), response to stimulus (11%), and response to stress (10%).

The regulation factor (RF) was calculated for Keel plants over Arta plants, regardless of the treatment. The majority (19 out of 27) of the proteins with broad functions in metabolism were down-regulated in Keel as compared to Arta with RFs ranging from -1.23 to -2.39. The eight down-regulated proteins had RFs ranging from 1.26 to 2.04.

Similar to proteins assigned to general metabolism, the majority (11 out of 13) of proteins with functions in carbohydrate metabolism were down-regulated in Keel. The down-regulated proteins had RFs ranging from -1.15 to -2.45 and the two up-regulated proteins had RFs of 1.37 and 1.50. The five proteins with functions in protein metabolic processes were all down-regulated in Keel and had RFs ranging from -1.35 to -3.10.

The majority of the proteins differentially expressed between genotypes with functions in photosynthesis were up-regulated in Keel; 14 were up-regulated and 6 were down-regulated. The down regulated proteins had RFs ranging from -1.11 to -1.57. The up-regulated proteins had RFs ranging from 1.22 to 2.53.

Most proteins with functions in transport were down-regulated in Keel (11 out of 15). The down-regulated proteins had RFs ranging from -1.19 to -3.88. The up-regulated proteins in Keel had RFs ranging from 1.35 to 1.51.

Ten out of twelve proteins with functions in abiotic stress response were down-regulated in Keel. The down-regulated proteins had RFs ranging from -1.12 to -3.23 and the two up-regulated proteins had RFs of 1.41 and 1.56.

All but one of the eleven differently regulated proteins with functions in stress response were down-regulated in Keel. The down regulated proteins had RFs ranging from -1.17 to -1.76 and the one up-regulated protein had an RF of 1.85. The remainder of proteins differentially regulated between genotypes had biological functions in translation, cellular homeostasis, carbon utilization or had no known biological function assigned to them and were primarily down-regulated in Keel.

The overall trend in the genotype dependent proteins was that they were down-regulated in Keel plants as opposed to Arta plants. This trend was seen for proteins with biological roles in general metabolism, carbohydrate metabolism, protein metabolism, transport, abiotic stimulus response, and stress response. However, for proteins with roles in photosynthesis the trend was reversed and the majority of proteins were up-regulated in Keel.

Interacting effects of genotype and heat on protein abundance

The distribution of biological functions of proteins differentially regulated due to the heat treatment and due to genotype was similar to one another. In both datasets, the three groups represented the most were metabolism, photosynthesis and transport. This similarity is partially due to the fact that 65 of the identified protein spots detected as being differentially regulated between heat treatments were also detected as being differentially regulated between genotypes. However, only some of these spots were regulated due to interaction effects between the heat treatment and the genotype.

Fourteen spots with a significant proportion of the variance in their intensity explained by the interaction between genotype and temperature were identified (**Table 13**). Of the fourteen spots with genotype by temperature interacting effects, three had biological functions in general metabolism, two in carbohydrate metabolism, one in protein metabolism, one in nucleobase-containing metabolism, one in responding to abiotic stimulus, two in translation, and three in transport. The remaining spot did not have a known biological function. These proteins were placed into two groups based on their expression profiles under control conditions. The first group contained proteins that were not differentially regulated between genotypes under control conditions (**Figure 15**). The second group contained proteins that were differentially regulated between genotypes under control conditions (**Figure 16**).

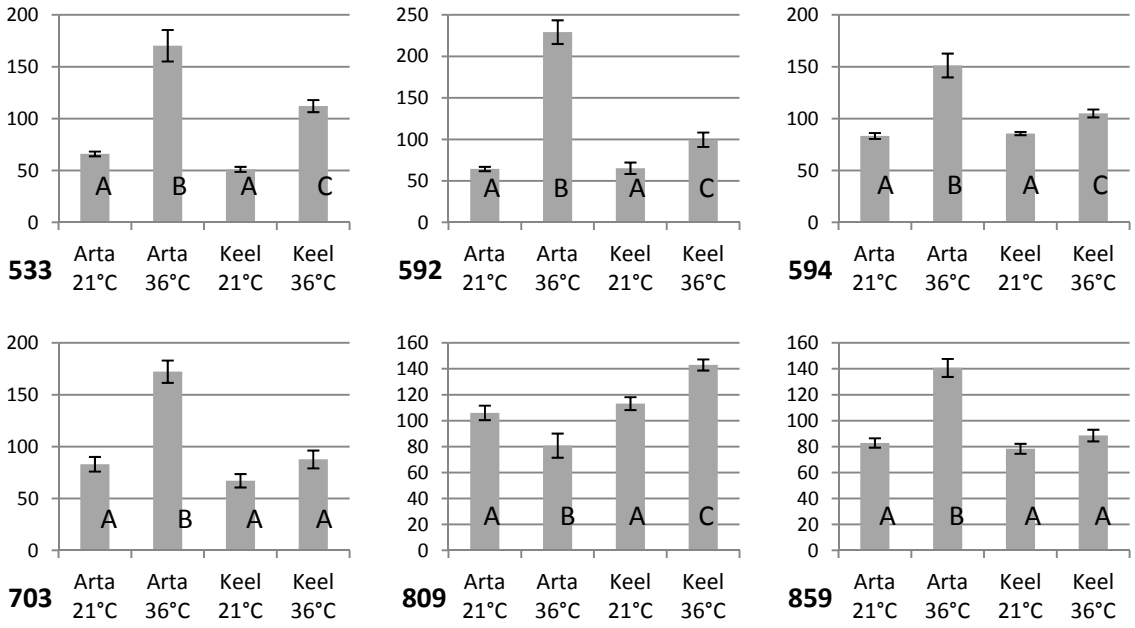

Figure 15. Spot intensities of proteins with genotype by temperature interacting effects where the spot intensity is not significantly different between genotypes under control conditions. The corresponding protein spot number is written in bold. Letter subscripts delineate significantly ($p < 0.05$) different mean spot intensities. n=6, error bars are SEM.

The first group contained six protein spots with interaction effects: 533, 592, 594, 703, 809 and 859 which were not differentially regulated between genotypes under control conditions (**Figure 15**). The protein spots 533, 592 and 594 were up-regulated by the heat treatment in both genotypes but significantly more in Arta than in Keel. The spots 703 and 859 were only up-regulated under heat treatment in Arta. The remaining protein spot, 809 was differentially regulated in both genotypes under the heat treatment as compared to controls but in different directions; the intensity at 36°C was lower in Arta and higher in Keel.

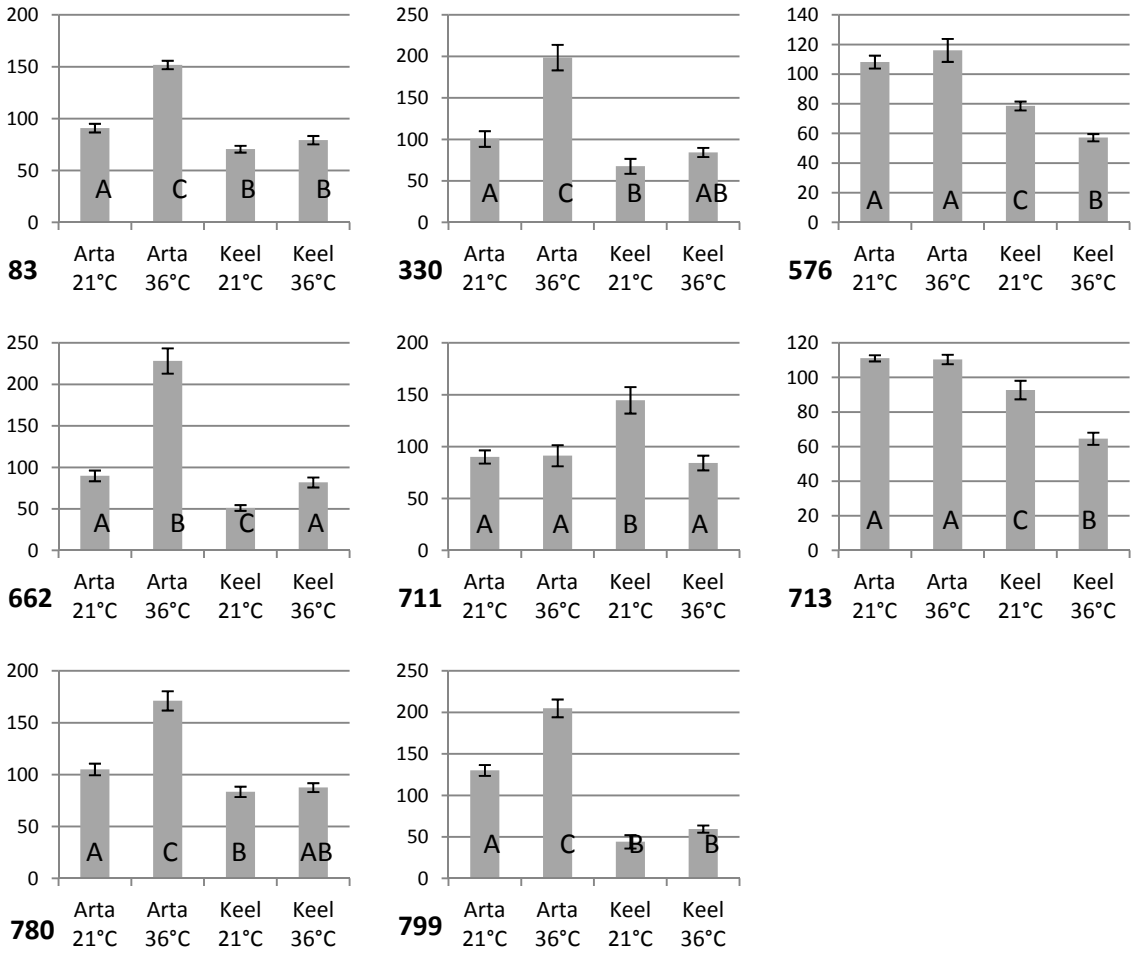

Figure 16. Spot intensities of proteins with genotype by temperature interacting effects where the spot intensity is significantly different between genotypes under control conditions. The corresponding protein spot number is written in bold. Letter subscripts delineate significantly ($p < 0.05$) different mean spot intensities. n=6, error bars are SEM.

The second group contained eight protein spots with interaction effects: 83, 330, 576, 622, 711, 713, 780, and 799 which were significantly different between genotypes under control temperatures (**Figure 16**). The protein spots 576 and 713 were in higher abundance in Arta than in Keel but were not responsive to the heat treatment in Arta. However, the proteins spots 576 and 713 were down-regulated

in Keel due to the heat treatment. In spots 83, 330, 780 and 799, Arta control samples were significantly higher than Keel control samples and were detected to be significantly up-regulated under the heat treatment in Arta only. The protein spot 662 was up-regulated by the heat treatment in both genotypes but significantly more in Arta than in Keel and the spot 703 was up-regulated under heat treatment in Arta only.

In summary, 14 proteins were identified which reacted differently to the temperature treatments in Arta and Keel. Such interactions included proteins that were responsive to the heat treatment in only one genotype or were responsive in both genotypes but to different extents or in different directions. Additionally, seven heat responsive proteins were seen to be differentially regulated between genotypes at control temperatures.

Discussion

The changes of physiological, morphological and molecular traits due to drought and heat treatments in two barley genotypes were considered in this study. This study showed that the morphological traits were more plastic in their response to the drought treatment than the heat treatment. In contrast, the physiological traits were affected more by the heat treatment than the drought treatment. Some of the detected physiological and morphological responses were unique to one genotype such as the higher abortion rate of spikes in Arta due to the heat treatment. On the molecular level, changes in protein abundance in the leaf proteome were detected due to the heat treatment but not due to the drought treatment. Differential expression of proteins between genotypes was also detected. The majority of heat responsive proteins had functions in metabolism and photosynthesis. The same was found to be true for the proteins differentially regulated between genotypes. In addition, proteins were differentially regulated due to interacting effects between the genotype and the environment. The majority of proteins regulated due to interacting effects had functions in metabolism. Characterizing multiple levels of plant performance under heat and drought stress will advance our understanding of how different physiological, morphological and molecular responses interact to maintain cellular homeostasis and continue plant growth.

Morphological traits were more plastic under drought than under high temperature

The drought treatment had a stronger effect on the majority of morphological traits as compared to the heat treatment. The morphology of barley was therefore more plastic when challenged with limited water conditions than with high temperature. The morphological traits that exhibited more plasticity

under drought than high temperature were: spike number, grains per spike, biomass, and plant height (**Table 10**). For example, the spike number in Arta was reduced by 39% under drought and by 22% under high temperature as compared to plants under control conditions. Similarly, the biomass was reduced by 34% under drought and by 10% under high temperature in Arta. Not all morphological traits were affected more by the drought treatment than the heat treatment. The traits thousand kernel weight, harvest index and aborted spikes exhibited more plasticity in response to high temperature than to drought. For example, the harvest index ranged from 0.29 to 0.35 under the heat treatment and ranged from 0.44 to 0.51 under the drought treatment (**Table 9**).

In contrast to the present study where grain number was significantly reduced by drought, a study in barley by Savin and Nicolas [72] reported that grain number was not significantly reduced by heat or drought in a simultaneous comparison of the two stresses. In the study by Savin and Nicolas [72], grain weight was reduced by ~5% due to the heat treatment and by ~20% due to the drought treatment which is not in agreement with the present study where the kernel weight was decreased by ~30% due to the heat treatment and up to 8% by the drought treatment. The different effects of heat and drought on grain number and weight between the two studies may be caused by differences in when and how the stress treatments were applied. Thus far, the combined effects of heat and drought on the number of grains per spike, biomass, and plant height have not been reported in barley.

In the study by Savin and Nicolas [72], the stress treatments began 15 days after anthesis as opposed to at anthesis as in the present study. As tillering is suppressed later in barley development [142], the application of drought 15 days after anthesis could have been too late to have an effect on tillering and thus grain number as seen in the present study. The drought treatment by Savin and Nicolas was applied by withholding all water to the plants for 10 days as opposed to maintaining lower soil water content as in the present study. The water withholding used by Savin and Nicolas resulted in a minimum leaf relative water content of 40% as opposed to 75% in the present study which implies that their drought treatment was more severe and could explain the stronger impact of the drought treatment on the kernel weight than in the present study. Savin and Nicolas treated the plants at 40°C for 6 h and 15°C for 18 h a day for 10 days while the present study heat stressed plants at 36°C for 16 h and 32°C for 8 h a day for 7 days. Despite the heat treatment of Savin and Nicolas being more intense by 4°C and lasting 3 days longer, the plants in the current study experienced higher daily mean temperatures of 34.6°C as opposed to 21.5°C in the other study. As shown in a later study by Savin et al. [143], grain weight decreases with increasing average temperature, which could explain the difference in kernel weight due to the average heat treatment between the two studies. From the comparison of these two studies, it is

apparent that the timing, intensity and duration of heat and drought treatments can dictate what morphological changes occur, especially in yield component traits.

The heat and drought treatments both resulted in equal reductions in the grain yield. However, yield component traits were affected by the two treatments differently i.e. the drought and heat treatments caused differences in how grain yield was achieved. Significantly fewer spikes were recorded in plants treated with drought but not with high temperature as compared to their respective controls. Conversely, neither the grains per spike nor the fertile spike number were significantly reduced by the heat treatment. Instead, the number of aborted spikes was increased and the thousand kernel weight decreased in heat treated plants.

Reduction in spike number due to the inhibition of tillering is a known reaction to drought [73] [74] but the molecular basis of the inhibition 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 signaling and resource competition between the apical buds, which form new tillers, and the stem apex [74].

The reduction of kernel weight due to pre-anthesis heat stress was observed in barley, wheat, and triticale [93] and due to post-anthesis heat stress in wheat [143]. The effect of heat on the thousand kernel weight is particularly interesting because the heat treatment had ended before grain filling had started, suggesting that the treatment had persisting effects on the plant. A higher kernel weight confers greater fitness to progeny by increasing seedling early growth vigor [144] and growth potential [145]. Grain filling is primarily dependant on two factors, the capacity to produce photosynthate during the grain filling stage of development [146] and the accumulation of reserves before the grain filling begins [147] [148]. In the present study, grain filling was likely reduced due to damage of the photosynthetic apparatus and due to decreased stem reserves. Senescence of the lower leaves (**Figure 9**) indicated that the ability to photosynthesize was permanently decreased [149]**.** Additionally, the ability of the higher leaves to photosynthesize, as based on the chlorophyll fluorescence measurements, was likely permanently damaged, as discussed in the next section.

The stem reserves can contribute as much as 70% of the carbon content to the seed during grain filling [150] in barley. Under favorable conditions in wheat, stem reserves are mobilized after anthesis when grain filling is at the maximum rate [151], but mobilization can take place during anthesis if photosynthesis is limited [152]. The reduced photosynthetic capacity caused by the heat treatment, in the present study, could have resulted in premature mobilization of stem reserves leaving less energy stored to mobilize later for grain filling. Evidence of the lowered carbohydrate status of the heat treated

plants was observed in the proteomic data by the up regulation of glycolysis enzymes, which is discussed in more detail in a following section.

The number of fertile spikes and spikes with more than half of the florets aborted (hereafter called aborted spikes) were counted separately. Spikes aborted due to the heat treatment were more numerous than the spikes aborted due to the drought treatment; for example, an average of 11.1 spikes was aborted under the heat treatment but an average of 3.2 was aborted under the drought treatment in Arta plants. 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 [153]. Floret sterility directly affects fitness by reducing the number of grains that a plant is able to produce.

The duration of heat and drought stress and the rate that they are applied can determine the effect stress has on the plant. The drought treatment was applied gradually over two days as compared to the heat treatment which was applied over a span of four hours. Additionally, the drought treatment was maintained from heading until maturity while the heat treatment lasted for a week. Barley plants thus had more time to acclimate to the drought treatment than the heat treatment. Furthermore, the drought treatment persisted through several developmental stages of the plants life (e.g. anthesis, grain filling and maturity) while the heat treatment was applied only during anthesis. The dependence of the response to drought on the abruptness of its application is best exemplified in the work by Talamè et al. [107] which compared the effects of "shock" and gradually increasing drought stress on transcript profiles in barley. The comparison of the two treatments revealed a greater number of differentially regulated transcripts, especially transcripts related to photosynthesis and metabolism, due to the shock treatment as compared to the gradually stressed plants. Talamè et al. also reported that the "shock" treatment caused a lower relative water content (~40%) as compared to gradually increasing drought stress (~ 80%) which suggested that gradually stressed plants were better able to maintain their water status than drought shocked plants. Law and Crafts-Brandner [154] demonstrated in wheat and cotton that the plant's response depends on the severity and rate of application of the stress. They reported that photosynthesis was inhibited more when the temperature was rapidly increased and that photosynthetic acclimation was possible when the temperature was gradually increased. The rate of application of the heat treatment in the present study is perhaps best seen in the number of spikes aborted, the plants had not the time to acclimate to the temperature difference on the physiological level and thus the consequences were drastic, loss of spike fertility that is otherwise crucial to

reproduction [155]. In comparison, the gradual application of the drought treatment used in this study resulted in significantly less spikes being aborted compared the heat treatment.

The duration of the stress treatments can be as important as the rate at which they are applied. The finding that biomass and plant height were not significantly reduced due to the heat treatment indicated that the plants resumed growth rates similar to control plants after the treatment ended. In comparison, the significant decreases in biomass and plant height due to the drought treatment indicated that growth was continuously inhibited. The sustained reduction of leaf growth has been previously reported in Arabidopsis treated with long term mild drought stress [19]. The continuation of growth but the loss of yield during the heat treatment was seen in the reduction of the harvest index which ranged from 0.56 to 0.57 under control conditions and ranged from 0.29 to 0.35 under the heat treatment in Arta and Keel; approximately 45% less grain yield was generated per biomass produced. Additional evidence that growth continued after the heat treatment is the differential effect of heat and drought treatments on the peduncle extrusion. The peduncle extrusion was seen to be significantly affected by the drought treatment but not the heat treatment. As the last node to elongate during development [77], the peduncle length is linked to late developmental growth. Since peduncle extrusion was not affected by the heat treatment, growth continued in those plants while the drought treated plants had a reduction in the peduncle extrusion which signified a reduction in overall growth. The importance of the peduncle extrusion in the present study was seen in its positive correlation to yield, biomass and height under control temperature (**Table 8**).

In summary, barley morphology is plastic in response to both drought stress and heat stress. Some trait plasticity was exclusive to one treatment, such as the abortion of spikes due to heat stress or the reduction of peduncle extrusion under drought stress. In general, barley morphology was more plastic in its response to drought than to heat. Plasticity can be considered to be adaptive, i.e. to confer fitness to the plant, or to be non-adaptive i.e. to be due to limitations in resources [156]. Characterizing phenotypic plasticity as adaptive or non-adaptive can aid in understanding the value of the trait in environmental acclimation and the evolutionary origin of the plasticity [157].

In this experimental setup, the most accurate indicator of fitness is the grain yield, which is a product of the number and weight of the seeds produced. The reduction in biomass, plant height, and spike number due to drought can be considered adaptive plasticity as the decrease in plant growth is minimizing water use to ensure seed maturation [5]. If reduction in biomass, plant height, and spike number under drought were due solely to limitations in photoassimilate availability then similar reductions in the kernel weight would be expected [158], which was not detected in the present study. Additionally, the adaptive plasticity of plant height and spike number was supported by their significant

positive correlation to grain yield under drought in the RIL population as described in chapter 1. Plasticity in floret fertility is a non-adaptive trait as is appears to be due to the inability to maintain anther growth [153] and results in a loss of fitness though the reduction of seed number. The reduction in thousand kernel weight due to heat can be considered non-adaptive as the reduction was likely due to limitations in photosynthate due to the reduced photosynthetic capacity of senesced lower leaves and impaired photosystem of higher leaves. The traits that had the greatest plasticity under drought: spike number, biomass, and plant height were considered to exhibit adaptive plasticity. The traits with the greatest plasticity under the heat treatment, thousand kernel weight and number of aborted spikes were both considered here to exhibit non-adaptive plasticity.

The application of drought and heat treatments were chosen to simulate how crops experience the two stresses in the field. Gradual drought starting late in the growing season and persisting until harvest are common in marginal environments where barley is cultivated under low input conditions. Additionally, heat waves often cause a rapid rise in temperature that persists for several days [159]. In the present study, the separate application of heat and drought stress as they frequently occur in the field resulted in similar reductions of grain yield. When the two stresses occurred in combination they had an interacting effect on grain yield which resulted in further losses in yield. Considering the shorter duration of the heat treatment, high temperature stress was more detrimental per unit time to the plant than the drought stress. Detrimental effects caused by the heat treatment were in part due to the rate at which it was applied; presumably too fast for the plant to acclimate. Conversely, the gradually applied drought treatment was more amenable to acclimation and detrimental effects from drought were attributed to the duration it persisted.

Physiological traits were negatively impacted by heat stress but not drought stress

The heat treatment explained a larger proportion of the phenotypic variance for each physiological trait than the drought treatment did (**Table 10**). Under the heat treatment, both genotypes had higher leaf temperatures, lower relative leaf water content, reduced maximum quantum efficiency, and reduced overall photosynthetic performance. In contrast, the drought treatment only significantly affected the leaf temperature one day after the start of the treatment and the maximum quantum efficiency three days after the start of the treatment (**Table 9**). Such changes in physiology suggest that the heat treated plants experienced a higher level of stress than the drought treated plants, at least for the duration of the heat treatment. While the effects of heat and drought treatments on plant physiology depend

heavily on their duration and rate of application, it is also well established that photosynthesis is the major physiological process impacted by high temperatures [160].

As ambient temperature increases, plants attempt to cool themselves by opening stomata and increasing transpiration [161]. Even if soil water is not limited, the cooling effect of transpiration is dependent on the surrounding humidity as well as the maximal stomatal conductance [162]. In this study, drought treated plants but not heat treated plants maintained leaf temperatures close to those measured under control temperatures. However, the heat treated plants maintained average leaf temperatures below the ambient temperature of 36°C. Under the heat treatment, the leaf temperature was negatively correlated with the leaf relative water content (**Table 8**) which suggested that the increase in transpiration had an effect on the water status of the plant.

The leaf relative water content of heat treated plants was lower than that of drought treated plants. For example, in Arta plants seven days after the start of the treatment, the leaf relative water content was 70.4% at 37°C and was 74.4% at 21°C. This suggests that leaf cooling and avoidance of extreme temperatures had a higher priority than maintaining the water status. This priority is best explained by the previous observation that the optimum temperature for photosynthesis in barley is 20°C and photosynthesis rates are decreased by more than 50% at 35°C [163]. In contrast, the photosynthetic metabolism of drought treated plants is typically not compromised until the relative water content decreases below 75% [164]. The results showed that under the heat treatment, increased transpiration was important to lower the leaf temperature, even at the expense of reduced water status. However, if a plant is subjected to high temperatures and limited water, the balance between leaf temperature and water status shifts; as was seen here in plants subjected to the combination treatment. Combination treated plants, in this study, often had leaf temperatures ~2°C higher and leaf relative water content ~8% lower than heat treated plants. Work by Rizhsky et al. 2002 [92] demonstrated that tobacco plants subjected to a combination of drought stress and heat shock had stomata that remained closed and that the prevention of transpiration resulted in leaf temperatures 2°C to 3°C warmer than plants treated with heat shock alone. Thus, it appeared that as the water status drops to levels that could inhibit photosynthesis, cooling via transpiration occurs less and leaf temperatures rise to ambient temperatures. The leaf temperature of heat stressed plants was negatively correlated to the performance index which suggested (**Table 8**) that the inability of the plants to sufficiently dissipate heat had noticeable effects on the ability of the plants to photosynthesize.

The maximal quantum efficiency of photosystem II was significantly reduced in heat treated plants but not in drought treated plants, as compared to controls. A reduction in the maximal quantum efficiency suggests permanent damage to the light harvesting complex, oxygen evolving complex, or the

reaction center of photosystem II that results in less energy from absorbed photons being trapped by the reaction center under light saturating conditions [165]. Based on the results here, the light harvesting complex was damaged due to the heat treatment but was unaffected due to the drought treatment. However, trapping of light energy under saturating light conditions as measured by the maximum quantum efficiency represents only one aspect of photosynthesis. The photosynthetic performance index integrates three independent aspects of the photosystem: the proportion of active reaction centers, the probability of trapping the energy of absorbed photons in the reaction center, and the efficiency of transferring trapped energy beyond the primary electron acceptor Qa. Therefore, the performance index is sensitive to changes in the light-independent reactions in addition to changes in the light-dependant reactions. As one of the primary responses to drought is the closure of stomata and the subsequent reduction of carbon dioxide available for light-independent reactions, the performance index is generally considered more suitable for detecting changes in photosynthesis due to drought treatment than the maximal quantum efficiency [166]. While there was an overall trend of the performance index being decreased under the drought treatment as compared to controls, it was not significantly lower. Thus, the drought treatment did not have a detectable effect of photoinhibition and the availability of carbon dioxide was not limited by stomata closure during the time points tested. In contrast, the heat treatment resulted in a significant decrease in the performance index in both genotypes at all three time points which suggests that both the light-dependant and light-independent mechanisms, e.g. carbon fixation, were damaged or inhibited due to high temperature.

The sensitivity of the photosystem to heat stress as compared to drought stress has been previously reported. Three of the physiological traits considered here: performance index, maximum quantum efficiency and relative water content were also measured in a growth chamber drought experiment by Oukarroum et al. [167] where barley plants were withheld water for one week (considered moderate stress) or for two weeks (considered severe stress) starting two weeks after sowing. Based on the reported relative water content (ranging from 67% to 84% in the ten genotypes considered), the moderate stress treatment was most comparable to the drought treatment used here. In their work, neither the performance index nor the maximum quantum efficiency was significantly affected by the moderate drought treatment, which is in agreement with our results. The effect of one and seven days of high temperature stress (40°C) on the performance index of barley seedlings was studied by Kalaji et al. [168]. Despite differences in the barley genotypes and the developmental stage of the plants used in the two experiments, Kalaji et al. 2011 also reported reductions of 40% in the performance index under high temperature conditions as in the present study.

In summary, the heat treatment resulted in leaf temperatures higher than in plants under control conditions. Cooling of the plant to temperatures 1°C to 3°C below the ambient temperature of 36°C was partially achieved, presumably through an increase in transpiration which resulted in a decreased water content of the plant compared to controls or drought treated plants. However, the capacity of the leaves to cool themselves and maintain water status was diminished by the combination treatment. Based on the chlorophyll fluorescence, both light dependant and light-independent reactions of PSII were adversely affected by the heat treatment which implied inhibition or damage of the light harvesting complex and molecular machinery further down the electron transport chain from the primary electron acceptor Qa. In comparison to the heat treatment, the drought treatment did not cause significant changes of physiological traits. The absence of physiological changes due to drought suggests that the plants acclimated to the drought stress primarily through morphological plasticity. The morphological plasticity and physiological responses were sometimes divergent between the two genotypes and potentially represent unique stress adaption mechanisms.

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

Differences in morphological and physiological traits and how these traits changed under stress between Arta and Keel suggest that the two genotypes have unique mechanisms for coping with environmental stresses. Arta and Keel are each adapted to different environments that experience abiotic stresses at varying times and to varying degrees. Environmental pressures on populations such as barley landraces and cultivars have not only shaped which traits the different populations can exhibit under optimal growth conditions [169] but also the range of trait plasticity that they can exhibit in sub-optimal conditions [170]. Morphological traits with the most variance explained by the effect of the genotype were: number of spikes aborted, peduncle extrusion, and plant height (**Table 10**). The physiological trait with the most variance explained by the effect of the genotype was the maximum quantum efficiency.

The major morphological difference between Arta and Keel was the number of spikes aborted due to the heat treatment. The difference in the two genotypes to maintain spike fertility could be due to genotypic differences in the sex organs where Keel plants are hardier under higher temperatures. Difference in floret mortality and grain setting between different barley genotypes has been previously reported between two-row and six-row barley [73] but not between winter and spring barley. Another explanation is that the florets have the same temperature hardiness but Keel was able to better protect the florets from stress. A possible adaptation to protect spikes is keeping them booted within the leaf

sheath. Under all conditions Keel plants had a more negative peduncle extrusion than Arta which means that more of the spike was covered by the leaf sheath. A spike covered in this manner receives less solar irradiance which means the floral organs receive less infrared radiation as well as less photosynthetically active radiation. This is especially important during periods of high temperature as the photosystem is more susceptible to light damage [171]. Despite the popularity of measuring peduncle extrusion in agronomic studies [172][47][173][174], it has yet to be tested if the leaf sheath has a protective effect on the florets under heat stress by shielding them from light damage.

Plant height was the trait influenced the most by genotypic effects; over 23% of trait variance was explained by the effect of the genotype in the ANOVA. Under all treatments the trend was for Keel plants to be taller than Arta plants. Under drought conditions, Arta was significantly shorter than under control conditions while Keel was not. This suggested that Arta is more plastic in its stem elongation than Keel. Plasticity in plant height has been documented before in Arta plants grown in drought prone field conditions by Baum et al. [53]. A reduction in plant height can mean less biomass is generated, as seen in the positive correlation between plant height and biomass under control temperatures in this study (**Table 8**). Biomass reduction is a possible drought avoidance mechanism to water limited conditions by generating less of a transpirational surface to lose water from [175] and by minimizing resource use. A negative correlation between plant height and grain yield in barley grown under water limited field conditions has been established in another barley study [47] which implies that inhibition of growth can be advantageous in periods of drought. However, in the present study no significant correlation between plant height and grain yield under the drought treatment was detected (data not shown). In addition to the morphological traits, a difference in photosynthetic performance between the two genotypes was detected.

Genetic variation in maximum quantum efficiency of photosystem II (PSII) was identified across all treatments. The maximum quantum efficiency as measured by chlorophyll fluorescence measures how often the energy from an absorbed photon of light is transferred from the light harvesting complex to the primary electron carrier of PSII, plastoquinone Qa. Under all conditions and at all time points Arta consistently had higher mean maximum quantum efficiency than Keel. Differences in the maximum quantum efficiency between genotypes were significant three and seven days after the start of the treatment under combination stress. Genetic variation in the maximum quantum efficiency has been detected between wheat genotypes [54] and other barley genotypes [176] which supports the suggestion that there is a genetic component to maintaining the efficiency of photosynthesis. On the molecular level, changes in the maximum quantum efficiency can be due to dissociation of the light harvesting complex from the reaction center, damage to the reaction center, changes in the state of the

oxygen evolving complex or to changes in the size of the plastoquinone pool. Changes in several of the proteins involved in the above processes were detected in this study using a proteomic analysis and will be discussed in the next section.

In summary, Arta exhibited more morphological plasticity due to the drought treatment than Keel, while Keel responded more on the physiological level than Arta to the heat treatment. Arta exhibited non-adaptive plasticity in floret fertility, in response to the heat treatment used here, as seen in the increased number of spikes with the majority of florets aborted. The peduncle extrusion of Keel was consistently shorter than Arta and the resulting booted spikes were a possible explanation for the increased floret fertility under the heat stress in Keel as compared to Arta. Plant height was seen to be an adaptive plastic trait in Arta which suggested that growth is more affected by drought in Arta than in Keel. The maximum quantum efficiency of Keel was more sensitive to the heat, drought and especially the combination treatment than Arta. The identification of natural genetic variation in traits relevant for agronomic performance under stress can be utilized in breeding programs. In an attempt to understand the molecular basis of the morphological plasticity and physiological responses observed above, differences in protein abundance between treatments and were quantified in the leaf proteome of Arta and Keel.

Proteomic basis of morphological plasticity and physiological responses to heat stress

The proteomic analysis revealed differences in the barley leaf proteome dependant on the genotype and on the heat treatment but not due to the drought treatment. The heat treatment resulted in the differential expression of 99 proteins (**Table 11**), the majority of which were up-regulated due to the heat treatment. Difference in the genotypes resulted in 123 proteins being differentially regulated with the majority of proteins being down-regulated in Keel as compared to Arta (**Table 12**). The majority of the proteins differentially regulated due to effects of the heat treatment or the genotype had functions in metabolism, photosynthesis and transport (**Figure 13**). However, it should be noted that the distribution of proteins with functions in photosynthesis in the analysis can be due to the tendency for the leaf proteome to be enriched in plastid proteins (**Figure 11**). Following is a discussion of the differently regulated proteins which have potential roles in the morphological plasticity and physiological responses to heat stress observed in this study. Additionally, 14 proteins were detected to be responsive to the heat treatment in a genotype dependant manner (**Table 13**). Proteins regulated by interacting effects between the temperature and genotype are candidates for further study as they are potentially under unique genetic regulation between Arta and Keel in response to stress.

No significant differences in protein abundance were detected under the drought treatment. This suggested that the plants had acclimated to the drought stress before sampling. A model of drought response in Arabidopsis was proposed by Harb et al. [97] in which responses were divided into an early, intermediate, and late stages. The early responses were characterized by perception of the perturbation of homeostasis and subsequent stress signaling. The following intermediate stage was seen as the preparatory stage for acclimation to drought and was characterized by changes in cell well consistency. The late stage of drought acclimation proposed by Harb et al. was characterized by the establishment of a new cellular homeostasis and reduced growth to save energy. Acclimation on the physiological level in the present study was evident in the maintenance of the photosynthesis performance and water status under drought. It appears that the growth of the plants was inhibited in response to the drought until a new homeostatic balance between water uptake and water loss could be maintained. Evidence in the inhibition of growth in response to drought was seen in the plant morphology where plant height, biomass, and spike number were all significantly reduced. The molecular changes responsible for the growth inhibition probably occurred in the days preceding the time point used for the proteome analysis. Future proteome analyses of drought should consider time points earlier than three days after the soil water content of the pots had reached the target of 15%, the so-called early or intermediate stages [97] of drought acclimation. The heat treatment, unlike the drought treatment, resulted in perturbations in plant physiology and significant changes in protein accumulation in the leaf proteome.

One of the most prominent effects of the heat treatment on morphology was the reduction of thousand kernel weight. One proposed reason for the reduction in the kernel weight was the reduction of stem reserves due to early remobilization during the heat stress to compensate for the reduced photosynthetic ability. The proteomic data supports this hypothesis in that several glycolytic proteins: fructose-bisphosphate aldolase (spot 788), [177] glyceraldehyde-3-phosphate dehydrogenase (spots 327and 330) [178] and triosephosphate isomerase (spot 912) [178] were up regulated under the heat treatment. An increase in glycolysis and a reduction in photosynthetic efficiency as seen in the chlorophyll fluorescence measurements signify a reduction in the pool of carbohydrates available for the storage as reserves in the stem [179].

The chlorophyll fluorescence data showed the reduction of the maximum quantum efficiency due to the heat treatment. The reduction in the maximum quantum efficiency can be due to damage in the light harvesting complex or in the oxygen evolving complex of PSII [180]. The proteomic analysis revealed one structural component of the light harvesting complex, the chlorophyll a-b binding protein of LHCII type III (Lhcb3) in spot 870 as being significantly up-regulated under heat treatment with a regulation factor of 1.75, as compared to controls. A structural component of the oxygen evolving

complex, chloroplast oxygen-evolving enhancer protein 1 (PsbO) was identified as being up-regulated in spots 846, 847 and 851. An additional structural component of the oxygen evolving complex, oxygen-evolving enhancer protein 2 (PsbP) was detected to be up-regulated under the heat treatment in spots 97 and 221. These proteins are known sites for damage under stress [181][182]. The apparent up-regulation of Lhcb3, PsbO and PsbP in this study is proposed to 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. An example of a repair cycle in photosystem II is the maintenance of the D1 subunit [183][184]. The proposed repair cycle of the D1 subunit involves disassembly of the photosystem complex followed by a synchronized replacement and removal of the D1 subunit before the complex is reassembled. Because the subunit is not removed and subsequently degraded until the replacement peptide is poised to be inserted into the complex the amount of protein in the cell is increased for the duration of the repair cycle. Subunits that are repaired more often should therefore appear in higher abundance in the proteome than subunit repaired less often. The up-regulation of Lhcb3, PsbO and PsbP proteins, which are known components of complexes indicated by the chlorophyll fluorescence parameters be damaged by the heat treatment, is therefore likely due to their increased repair.

The chlorophyll fluorescence data revealed the reduction of the performance index due to the heat treatment which is indicative of inhibition of the light-independent reactions of photosynthesis. The protein Rubisco activase B was identified in two spots, 773 and 774, which were both up-regulated under the heat treatment with regulation factors (RFs) of 4.62 and 6.65, respectively. Additionally, Rubisco activase A was indentified in five spots, 721, 724, 763, 765, and 768 that were down-regulated under the heat treatment with RFs ranging from -1.21 to -1.88. The rate limiting step of carbon fixation in the light-independent reactions is Rubisco. Photorespiration, the oxygenation of ribulose-1,5-bisphosphate by Rubisco, occurs more frequently than photosynthesis at elevated temperatures [185]. Rubisco is inhibited by side-products of photorespiration which stabilize the active site in a closed conformation [186]. Rubisco activase frees the catalytic site of Rusbisco from inhibitory sugar phosphates by forcing the active site into a open conformation [187]. Rubisco activases have been characterized on the genomic level in barley [188], wheat [189], rice [190], cotton [191], maize [192] and *A. thaliana* [193]. In these species, three activase polypeptides are known to exist. Two of the activase polypeptides are present in all of the above species and are alternate splice variants of the gene RcaA. The two RcaA splice variants encode the α-isoform and β-isoform of Rubisco activase A. The two isoforms of activase A are known to be heat inactivated starting at 35°C and have different thermostabilities [194]. The other polypeptide, Rubisco activase B, is encoded by the gene RbaB present in barley, wheat, maize and cotton

but is not known to exist in rice and *A. thaliana*. The thermostability of Rubisco activase B is currently untested or unpublished.

The up-regulation of Rubisco activase B and down-regulation of Rubisco activase A under heat treatment suggests 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. In previous studies, the isoforms of Rubisco activase A were neither seen to be differentially regulated by heat on the transcript level in *A. thaliana* [195] nor on the protein level in cotton [196]. Such results are in conflict with the present study in which both isoforms of activase A were down regulated under heat stress. However, Rubisco activase B is known to be induced by heat on the transcript level in wheat [197] and on the protein level cotton [196] which was in agreement with the present study.

The protein spots identified as Rubisco activase B, 773 and 774, resolved differently based on the first dimension, the isoelectric point, but not based on the second dimension, the molecular weight (**Figure 10**). This suggested that two isoforms of Rubisco activase B are present in the barley leaf proteome which differ in a post translational modification that noticeably alters the isoelectric point of the protein but not the mass. One such modification known to shift the isoelectric point of modified proteins with a minimal change in mass is phosphorylation [198]. Such modifications, if confirmed, would be a novel finding for Rubisco activase B. Multiple sub-isoforms of the alternative spliced isoforms of activase A were detected in the present study. Three isoforms of each of the two splice variants of activase A were previously detected in cotton by Law et al. [196]. While the study in cotton also showed the presence of sub-isoforms of activase A, they were not detected to be down-regulated under high temperature treatment. However, the experimental setup used by Law et al. [196] was not designed to be quantitative as in the present study. Altogether, the differential accumulations of the several isoforms of Rubisco activase are candidates to explain the decrease in the performance index and represent an alternative mode of maintaining the activity of Rubisco under high temperatures than what is known to exist in *A. thaliana*.

The differential regulation of 125 proteins between genotypes suggested that Arta and Keel achieve homeostasis through different molecular mechanisms. A comparison of proteins regulated between genotypes and proteins regulated between temperature treatments revealed an overlap of 65 proteins in both datasets which suggested that constitutive differences in Arta and Keel protein levels determine how they respond to heat. For example, a member of the heat shock protein family indentified in spot 939 was down-regulated in Keel compared to Arta with an RF of -1.54 and was up-regulated under high temperature compared to control temperature with a RF of 2.90. As heat shock proteins have known roles as molecular chaperones and assisting protein re-folding (reviewed by Hurang

and Xu [199]), higher constitutive levels of this protein could prime tolerance against heat stress in Arta. Of the 65 protein spots that were detected to have constitutive differences between genotypes and to be heat responsive, some were also detected as having interacting effects between genotype and environment i.e. the proteins responded to the heat treatment in a genotype specific manner.

The 14 protein spots regulated by interacting effects between genotype and environment are of particular interest as they are potential outputs of unique adaptations to heat stress that have evolved between Arta and Keel. To better understand how the heat responsive proteins were regulated between the two genotypes, the 14 proteins were grouped based on their abundance under control temperatures. The first group, represented in **Figure 15**, consists of proteins that had similar expression between genotypes under control temperatures and the second group, represented in **Figure 16**, consists of proteins that had significantly different expression between genotypes under control conditions. If the proteins in the second group indeed convey heat tolerance, their constitutive expression may prime the plant to respond to heat by maintaining high expression even under control temperatures. However, constitutive expression of tolerance genes is known to reduce fitness under non-stress conditions [200]. Therefore, proteins from the first group, which appear to be induced in both genotypes only when needed, are considered for discussion below.

One example of a protein in the first group that may convey an advantageous adaptation to heat stress is elongation factor G. Elongation factor G (spot 533) was significantly up-regulated in response to the heat treatment in Arta compared to Keel. Elongation factor G has been the subject of intense study due to its role in translation where it is known to catalyze the translocation tRNA within the ribosome. However, more recent studies have proposed an additional role for elongation factor G as a molecular chaperone. Caldas et al. [201] demonstrated that bacterial elongation factor G could increase the refolding of proteins *in vitro* and protect them from thermal denaturation. If such chaperone abilities exist for elongation factor G *in vivo* in plants then this protein would have a dual role in protein synthesis and protecting proteins from heat induced inactivation. Therefore, increased expression of elongation factor G in Arta could convey additional heat tolerance compared to Keel.

Another protein in the first group, a putative aldo-keto reductase, showed a cross-over effect between environments in Arta and Keel i.e. the protein was up-regulated in Keel but down-regulated in Arta under heat stress. The putative aldo-keto reductase family-like protein was identified in the spot 809. Aldo-keto reductases are NADPH dependant oxidoreductases that typically reduce carbonyl compounds [202]. Members of the aldo-keto reductase family are known to play roles in desiccation tolerance in barley embryo [203], and protection against lipid peroxidation in alfalfa [204] and rice [205]. Thus, evidence from literature and up-regulation of aldo-keto reductases by high temperature in Keel

suggest that this uncharacterized protein has potential in conveying tolerance to abiotic stress and warrants further investigation.

While several studies have quantified changes in the proteome of barley seeds [125–129] roots [130] and shoots [131], a review of the current literature did not reveal any studies concerned with proteomic changes in barley leaf samples due to abiotic stress, as in the present study. However, a comparison can be made with the study by Süle et al. [131] that studied the proteomic responses to heat stress in barley shoots since a large portion of shoots contain leaf material. The shoots of two barley genotypes were heat stressed and six proteins were identified as being up-regulated and six proteins as being down-regulated due to the heat treatment. The identified heat responsive proteins up-regulated in the shoots were all of small heat shock protein family with masses ranging from 15-20 kDa with functions as molecular chaperones [206]. In the present study, heat shock proteins indentified in spots 564 and 939 were also up-regulated due to the heat treatment. However, these heat shock proteins were of high molecular weights and are likely to be members of the HSP90 and HSP70 families based on their predicted molecular weights [199]. The small heat shock protein identified by Süle et al. [131] not being discovered in the present study can be explained by differences in experimental setup between the two studies; proteins with molecular weights smaller than 20 kDa were electrophoresed out of the gel bottom in the present study as seen in the molecular weight standard in **Figure 10**. The exclusion of low molecular weight proteins from the gel was a side effect of longer electrophoresis runs which allowed for greater separation of large molecular weight proteins. The focus on higher molecular weight proteins was chosen to maximize the overall number of proteins resolvable on the gel based on preliminary work (data not shown) which indicated that higher molecular weight proteins were present in high numbers on the gel than low molecular weight proteins. Of the proteins reported to be down-regulated by the heat treatment in shoots, the glycolytic enzyme triosephosphate isomerase [178] was also detected to be up-regulated (spot 912) in the present study. In the work by Süle et al. [131], triosephosphate isomerase was identified in one spot that was a mixture of proteins that co-migrated in the gel. The authors then incorrectly concluded that all proteins in this spot were down regulated. The conclusion is incorrect because each protein in the mixture can be up or down-regulated independently of the combined spot intensity [207]. Süle et al. [131] also compared the regulation of proteins between the two genotypes and discovered one protein, S-adenosylmethionine synthetase, to be up-regulated in one genotype under both control and high temperature conditions. S-adenosylmethionine synthetase was identified (spot 711) in the present study to be up-regulated in Keel compared to Arta under control temperatures but not under the high temperature treatment. S-adenosylmethionine synthetase is responsible for the enzymatic conversion of L-methionine into S-adenosyl-L-methionine [208], which is a

precursor for molecules with known roles in heat stress response such as the osmolyte betaine [209] and the plant hormone ethylene [210][211]. The results from Süle et al. only partially confirm ours here; it is evident that S-adenosylmethionine synthetase, an enzyme with roles in responding to heat, can be regulated in a genotype dependant manner.

Altogether, the proteomic data analysis allowed the detection of heat responsive proteins as well as proteins differentially regulated between the genotypes Arta and Keel. Connections between the proteomic changes and the phenotypic changes were drawn as a way to further our understanding of the molecular basis of responses to heat stress. Specifically, an increase in glycolytic proteins was linked to the reduction in the thousand kernel weight. Additionally, the damage of structural components of photosystem II as well as an alteration in the maintenance of Rubisco activity was attributed to the reduction of photosynthetic performance. Proteins that responded to the heat treatment in a genotype dependant manner, such as a putative aldo-keto reductase and elongation factor G, were considered as unique adaptations in heat tolerance between Arta and Keel and warrant further research to understand their transcriptional and post-transcriptional control.

Conclusion

The simulation of heat and drought stress under controlled conditions as they often occur in agronomic settings allowed responses to the two abiotic stresses to be compared in barley on the morphological, physiological and molecular level. The heat and drought treatments applied separately resulted in equal and significant decreases in grain yield compared to control plants. Applied in combination, the stress treatments resulted in greater reductions in yield. Inspection of yield component traits revealed that the reduction in yield under drought stress was due to the development of fewer tillers while the reduction under heat stress was primarily due to reduced kernel weight. In general, more pronounced morphological responses were exhibited by drought treated plants than heat treated plants which was attributed in part to the slower rate and longer duration in which the drought treatment was applied. In contrast, heat treated plants were perturbed more on the physiological level than drought treated plants. Perturbation of physiology was exacerbated in combination treated plants by interacting effects of heat and drought stress. It appeared that morphological plasticity allowed avoidance of physiological stress by acclimation to the drought treatment which was not observed in the heat treatment. Instead, heat treated plants attempted to tolerate the stress by changes on the molecular level. The proteomic analysis revealed 99 protein spots differentially regulated in response to the heat treatment with functions primarily in metabolism and photosynthesis. Differentially regulated proteins with potential roles in the observed morphological and physiological changes under heat stress included

photosynthetic proteins Rusbisco activase B and chlorophyll a-b binding proteins in addition to the glycolytic enzymes fructose-bisphosphate aldolase and glyceraldehyde-3-phosphate dehydrogenase.

By studying two barley genotypes adapted to different drought prone agronomic settings genotype dependant abiotic stress resistances and possible resistance mechanisms were able to be detected. Arta plants were more prone to inhibition of growth under drought. Keel plants proved to be more resistant to heat induced floret abortion than Arta but was more susceptible heat induced inhibition of photosynthesis. Inclusion of the Arta and Keel in the proteomic analysis allowed fourteen proteins which respond to heat stress in a genotype specific manner, like aldo-keto reductase and elongation factor G, to be identified. Such proteins are potential outputs of heat resistance mechanisms that allow Arta and Keel to thrive in different marginal environments prone to stress. Altogether, the detection of genetic variation in traits important for maintaining yield under abiotic stress will allow these characteristics to be exploited in future crop breeding efforts.

Tables

Table 9. Trait means, minimums (Min) and maximums (Max) measured for Arta and Keel under control or drought conditions at either 21°C or 36°C. Means that are not significantly ($p<0.05$) different share the same letter subscript.

	Control 21°C Arta			Control 21°C Keel			Control 36°C Arta			Control 36°C Keel			Drought 21°C Arta			Drought 21°C Keel			Drought 36°C Arta			Drought 36°C Keel		
Trait	**Mean**	**Min**	**Max**	**Mean**	**Min**	**Max**	**Mean**	**Min**	**Max**	**Mean**	**Min**	**Max**	**Mean**	**Min**	**Max**	**Mean**	**Min**	**Max**	**Mean**	**Min**	**Max**	**Mean**	**Min**	**Max**
GY	11.8 a	6.5	15.1	11.7 a	4.2	18.2	5.6 bcd	2.6	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	19.1 ab	12.7	26.0	21.8 b	10.3	32.0	14.8 ac	8.0	26.0	20.7 ab	14.3	29.7	11.6 c	9.3	14.0	15.6 ac	10.7	22.7	9.7 c	3.3	15.3	11.6 c	4.7	19.3
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
LT_7	23.0 a	17.6	24.4	21.4 a	18.3	23.4	33.2 b	27.6	38.9	34.6 bc	32.2	37.5	22.3 a	18.8	25.1	21.8 a	18.4	24.8	36.4 cd	32.9	39.7	37.2 d	33.5	39.4
RWC_1	90.3 a	87.2	93.3	83.2 ab	65.8	96.4	81.6 abc	55.3	91.2	78.3 bc	67.4	100.0	87.1 ab	75.7	93.6	85.1 ab	70.2	96.2	74.2 c	55.4	92.2	79.2 bc	53.8	94.7
RWC_3	88.5 a	76.1	93.6	87.5 ab	75.1	97.3	76.2 bcd	49.9	88.4	80.5 ab	70.5	87.3	79.8 abc	63.1	90.8	78.3 abc	65.5	93.9	67.3 d	54.8	83.1	72.9 cd	61.0	82.2
RWC_7	82.9 a	77.7	90.2	79.4 ab	69.9	90.4	70.4 bd	63.2	77.4	74.4 ab	63.8	80.2	76.7 ab	58.7	85.2	75.6 ab	58.0	87.7	60.8 cd	43.1	70.5	59.4 c	50.8	69.3
Fv/Fm_1	0.836 a	0.813	0.863	0.835 a	0.814	0.866	0.795 b	0.788	0.801	0.784 bc	0.771	0.792	0.835 a	0.812	0.862	0.826 a	0.806	0.845	0.785 bc	0.751	0.804	0.775 c	0.767	0.785
Fv/Fm_3	0.820 a	0.760	0.838	0.816 ab	0.799	0.821	0.789 bc	0.720	0.829	0.770 c	0.747	0.785	0.829 a	0.806	0.839	0.811 ab	0.795	0.822	0.791 bc	0.724	0.834	0.732 d	0.676	0.765
Fv/Fm_7	0.829 bc	0.818	0.846	0.803 bc	0.765	0.820	0.782 abc	0.716	0.809	0.728 a	0.566	0.782	0.834 c	0.823	0.863	0.815 bc	0.806	0.832	0.761 ab	0.617	0.810	0.564 d	0.322	0.722
PI_1	3.3 a	2.1	5.2	3.5 a	2.7	4.8	1.9 b	1.5	2.3	2.3 bc	1.9	2.7	3.0 ac	1.0	4.4	3.4 a	2.6	4.2	2.0 b	1.5	2.9	2.1 b	1.7	2.6
PI_3	2.5 a	1.9	2.9	3.1 a	2.6	3.5	1.8 b	0.6	3.3	1.8 b	1.2	2.3	2.9 a	2.1	3.7	3.0 a	2.2	3.8	1.8 b	0.7	3.5	1.0 c	0.5	1.4
PI_7	3.0 a	2.2	3.7	2.8 a	2.2	3.4	1.6 b	0.6	2.4	1.4 b	0.3	1.9	3.6 c	2.8	4.8	3.2 ac	2.9	3.8	1.1 b	0.1	1.8	0.3 d	0.0	0.8

Table 10. Summary of the four-way analysis of variance for the traits measured in Arta and Keel genotypes under the two soil water content (SWC) treatments at temperatures (Temp.) of 21°C or 36°C. For each trait, the F-value, coefficient of determination and significance probability is given for each of the three fixed effects (genotype, SWC, and temperature), the random effect of the experimental replication, the interaction between every possible pairing of the fixed effects and the interaction of all three. * p<0.05,** p<0.01, *** p<0.001, n/s non-significant.

	GY			BM			HI			PH			Pedex			SN			AS			GS		
Genotype (G)	1.39	0.005		0.38	0.002		5.72	0.019	*	40.98	0.235	***	6.03	0.067	*	14.12	0.084	***	33.85	0.173	***	1.57	0.018	
SWC (S)	73.11	0.257	***	125.88	0.567	***	14.63	0.047	***	36.05	0.207	***	8.87	0.098	**	53.49	0.320	***	2.05	0.010		7.82	0.092	**
Temp. (T)	108.30	0.381	***	17.59	0.079	***	165.19	0.536	***	4.15	0.024	*	0.34	0.004		8.73	0.052	**	62.73	0.320	***	0.40	0.005	
Replication	23.29	0.082	***	0.21	0.001		47.98	0.156	***	3.91	0.022		0.39	0.004		17.29	0.103	***	12.67	0.065	***	2.88	0.034	
G*S	0.19	0.001		0.05	0.000		0.67	0.002		1.82	0.010		1.66	0.018		0.47	0.003		0.32	0.002		0.79	0.009	
G*T	0.28	0.001		2.23	0.010		0.17	0.001		2.42	0.014		1.52	0.017		0.10	0.001		6.16	0.031	*	0.40	0.005	
S*T	4.44	0.016	*	1.57	0.007		1.78	0.006		2.75	0.016		0.04	0.000		0.02	0.000		6.56	0.033	*	0.01	0.000	
G*S*T	2.45	0.009		3.21	0.014		1.15	0.004		11.03	0.063	**	0.21	0.002		1.87	0.011		0.87	0.004		0.16	0.002	

	TKW			DM			WU			WUE			LT_1			LT_3			LT_7			RWC_1		
Genotype (G)	18.26	0.048	***	4.65	0.020	*	9.95	0.018	**	6.67	0.034	*	0.98	0.000		0.81	0.000		0.00	0.000		1.22	0.008	
SWC (S)	12.53	0.033	***	0.42	0.002		425.98	0.769	***	6.12	0.031	*	74.42	0.023	***	5.29	0.002	*	14.23	0.009	***	1.34	0.008	
Temp. (T)	271.44	0.716	***	7.70	0.033	**	0.00	0.000		106.07	0.542	***	2921.3	0.922	***	2436.8	0.956	***	1329.7	0.856	***	22.83	0.142	***
Replication	0.32	0.001		148.01	0.634	***	42.48	0.077	***	0.08	0.000		81.26	0.026	***	31.81	0.012	***	111.79	0.072	***	57.56	0.357	***
G*S	2.40	0.006		0.03	0.000		3.75	0.007		0.05	0.000		0.57	0.000		0.55	0.000		0.10	0.000		3.97	0.025	
G*T	0.07	0.000		1.13	0.005		0.54	0.001		0.78	0.004		1.18	0.000		1.60	0.001		8.380	0.005	**	2.52	0.016	
S*T	2.33	0.006		0.25	0.001		0.02	0.000		3.09	0.016		4.49	0.001	*	0.04	0.000		17.56	0.011	***	0.62	0.004	
G*S*T	0.67	0.002		0.11	0.000		0.08	0.000		1.87	0.010		14.93	0.005	***	0.80	0.000		1.33	0.001		0.23	0.001	

	RWC_3			RWC_7			Fv_Fm_1			Fv_Fm_3			Fv_Fm_7			PI_1			PI_3			PI_7		
Genotype (G)	1.00	0.008		0.09	0.000		8.19	0.018	**	29.77	0.118	***	43.23	0.146	***	3.28	0.020		0.04	0.000		16.11	0.031	***
SWC (S)	21.35	0.176	***	31.05	0.163	***	7.70	0.017	**	3.39	0.013		13.84	0.047	***	0.97	0.006		0.58	0.003		1.46	0.003	
Temp. (T)	25.11	0.207	***	64.2	0.337	***	326.2	0.732	***	111.4	0.443	***	97.87	0.331	***	84.42	0.513	***	114.59	0.520	***	381.84	0.741	***
Replication	0.01	0.000		15.37	0.081	***	29.87	0.067	***	11.57	0.046	**	8.93	0.030	**	3.22	0.020		3.45	0.016		2.05	0.004	
G*S	0.01	0.000		0.24	0.001		0.46	0.001		8.38	0.033	**	9.23	0.031	**	0.11	0.001		10.07	0.046	**	4.09	0.008	*
G*T	2.79	0.023		1.37	0.007		0.81	0.002		9.51	0.038	**	20.680	0.070	***	0.160	0.001		11.80	0.054	**	1.07	0.002	
S*T	0.03	0.000		5.56	0.029	*	0.66	0.001		4.51	0.018	*	20.04	0.068	***	0.65	0.004		8.33	0.038	**	37.13	0.072	***
G*S*T	0.05	0.000		1.52	0.008		0.62	0.001		1.99	0.008		11.09	0.037	**	0.85	0.005		0.350	0.002		0.390	0.001	

Table 11. Temperature responsive proteins quantified by DIGE and identified via mass spectrometry. For each identified protein, the corresponding spot number (No.) is given in addition to the Uniprot protein name and accession number. Proteins are grouped according to their biological function. Based on the Mascot searches, the predicted molecular weights (MW), isoelectric points (pI), scores, and percent sequence coverage (SC%) are reported. Regulation factor, the log2 fold change in protein expression are given for plants grown in 36°C over plants grown in 21° (36/21). Spots also found to be differentially regulated between genotypes are in dark grey.

No.	Protein Name	UniRef100	MW	pI	Score	SC%	36/21
	Metabolic process						
349	Glycolate oxidase	Q3L1H0	111.4	7.0	227.2	2.8	1.35
365	Predicted protein	F2D4I5	43.8	9.0	363.1	19.7	1.65
402	Serine-glyoxylate aminotransferase	Q3S2I1	135.3	7.0	210.6	2.4	1.27
487	Glycine decarboxylase P subunit	O22575	202.3	6.9	117.2	1.1	1.19
491	Glycine decarboxylase P subunit	O22575	202.3	6.9	115.0	7.0	1.49
575	Predicted protein (Fragment)	F2DEY7	73.5	5.4	181.9	4.9	-1.34
578	Methionine synthase 1 enzyme	Q4LB13	215.8	6.9	190.0	10.6	-1.22
780	MRNA-binding protein	Q7X998	162.3	7.0	95.6	1.3	1.37
804	Glyceraldehyde-3-phosphate dehydrogenase	F4HNZ6	88.9	7.0	205.2	3.3	1.54
816	Glyceraldehyde-3-phosphate dehydrogenase	F4HNZ6	88.9	7.0	86.6	1.5	1.46
820	Predicted protein	F2DT74	39.6	9.1	108.5	5.3	1.15
828	Predicted protein	F2DT74	39.6	9.1	77.2	5.3	-1.37
877	2-Cys peroxiredoxin BAS1, chloroplastic	Q96468	23.3	5.4	122.4	6.7	1.52
878	2-Cys peroxiredoxin BAS1, chloroplastic	Q96468	23.3	5.4	106.5	6.7	1.23
912	Triosephosphate isomerase	F2DTB2	32.3	7.9	113.4	5.0	1.32
977	Serine hydroxymethyltransferase	F2D3S8	56.1	8.8	135.1	4.9	-1.37
	Carbohydrate metabolic process						
327	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	P26517	122.3	7.0	154.6	2.4	1.29
330	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	P26517	48.3	7.0	49.0	2.6	1.68
713	Predicted protein	F2CTY2	44.2	4.9	131.4	3.7	-1.17
758	Phosphoribulokinase, chloroplastic	P26302	104.4	6.9	140.0	18.3	-1.22
766	Phosphoglycerate kinase, chloroplastic	P12782	99.4	7.0	152.3	1.5	-1.47
788	Fructose-bisphosphate aldolase	F2ELD1	41.9	7.5	304.6	10.8	1.22
791	Malate dehydrogenase	A3KLL4	69.5	6.9	181.0	20.6	-1.34
	Protein metabolic process						
623	Putative zinc dependent protease	Q2PEV7	191.4	7.0	92.0	1.4	-1.43
641	RuBisCO large subunit-binding protein subunit beta	Q43831	100.6	7.0	119.2	1.4	3.14
644	RuBisCO large subunit-binding protein subunit beta	Q43831	118.3	7.0	178.9	2.2	2.63
647	RuBisCO large subunit-binding protein subunit beta	Q43831	100.6	7.0	117.7	1.4	1.99
662	RuBisCO large subunit-binding protein subunit alpha	P08823	113.6	7.0	91.5	1.5	2.20
668	RuBisCO large subunit-binding protein subunit alpha	P08823	113.6	7.0	204.1	2.7	2.01

Table 11 continued.

No.	Protein Name	UniRef100	MW	pI	Score	SC%	36/21
	Nucleobase-containing compound metabolic process						
181	Germin-like protein 2a	Q0GR10	21.8	5.7	132.8	10.8	1.48
184	Germin-like protein 2a	Q0GR10	21.8	5.7	152.1	10.8	1.68
711	S-adenosylmethionine synthase	F2CRM1	42.7	5.4	64.1	3.8	-1.34
752	Os03g0315800 protein	Q0DSD6	132.6	7.0	73.1	5.6	-1.15
844	Predicted protein	F2DLP9	107.1	5.8	32.3	2.0	1.22
	Lipid metabolic process						
511	Lipoxygenase	A1XCI1	92.1	7.0	44.2	1.2	-2.72
515	Lipoxygenase	A1XCI1	92.1	7.0	52.9	1.2	-2.21
519	Lipoxygenase	F2E2Z8	96.7	6.2	106.0	12.8	-2.72
	Catabolic process						
315	Aminomethyltransferase	Q01KC0	137.0	6.9	208.5	2.1	1.33
	Carbon utilization						
162	Carbonic anhydrase, chloroplastic	P40880	85.1	6.9	219.3	2.8	-1.37
	Photosynthesis						
97	Oxygen-evolving enhancer protein 2, chloroplastic	Q00434	96.0	6.9	44.9	0.9	1.46
182	Chlorophyll a-b binding protein 1B-21, chloroplastic	Q9SDM1	26.4	5.8	59.6	4.1	1.21
221	Oxygen-evolving enhancer protein 2, chloroplastic	Q00434	96.0	6.9	91.5	7.0	1.29
323	Peroxisomal (S)-2-hydroxy-acid oxidase GLO1	Q10CE4	72.9	7.0	153.0	15.1	1.30
324	Peroxisomal (S)-2-hydroxy-acid oxidase GLO1	Q10CE4	72.9	7.0	131.8	2.2	1.45
325	Peroxisomal (S)-2-hydroxy-acid oxidase GLO1	Q10CE4	72.9	7.0	141.0	19.5	1.54
846	Predicted protein	F2CRK1	34.4	5.6	306.6	11.6	1.29
847	Chloroplast oxygen-evolving enhancer protein 1	A5JV93	68.7	7.0	198.6	3.7	1.55
851	Predicted protein	F2CRK1	34.4	5.6	199.0	11.6	1.63
853	Chloroplast oxygen-evolving enhancer protein 1	A5JV93	68.7	7.0	48.1	2.5	-1.37
866	Chlorophyll a/b-binding protein WCAB	O24401	78.9	6.9	106.0	10.3	1.48
867	Chlorophyll a/b-binding protein WCAB	O24401	66.8	7.0	54.2	6.0	1.66
870	Chlorophyll a-b binding protein of LHCII type III, chloroplastic	P27523	91.6	7.0	108.6	1.2	1.75
873	Predicted protein	F2D9M7	29.3	9.4	64.0	4.1	1.82
900	Predicted protein	F2D9M7	29.3	9.4	89.2	4.1	1.47
911	Chlorophyll a-b binding protein 1B-21, chloroplastic	Q9SDM1	26.4	5.8	38.4	4.1	1.63

Table 11 continued.

No.	Protein Name	UniRef100	MW	pI	Score	SC%	36/21
	Response to abiotic stimulus						
566	Transketolase, chloroplastic	Q7SIC9	155.2	7.0	146.0	14.4	1.68
570	Transketolase, chloroplastic	Q7SIC9	155.2	7.0	217.0	18.1	-1.26
586	ATP-dependent zinc metalloprotease FTSH 1, chloroplastic	Q5Z974	80.0	6.9	74.2	9.2	2.69
622	'putative 2,3-bisphosphoglycerate-independent phosphoglycerate mutase'	Q5KQH5	165.5	7.0	69.5	5.1	-1.26
721	RuBisCo activase A, chloroplastic	Q40073	133.8	6.9	239.4	2.2	-1.25
724	RuBisCo activase A, chloroplastic	Q40073	133.8	6.9	189.9	2.2	-1.29
750	Isocitrate dehydrogenase	Q9XHX4	63.7	7.0	38.3	1.9	-1.21
763	RuBisCo activase A, chloroplastic	Q40073	133.8	6.9	277.8	2.2	-1.54
765	RuBisCo activase small isoform	E3WDK8	47.1	8.6	167.0	36.5	-1.80
768	RuBisCo activase A, chloroplastic	Q40073	133.8	6.9	213.4	2.2	-1.88
773	RuBisCo activase B, chloroplastic	Q42450	47.2	8.6	90.2	4.5	4.62
774	RuBisCo activase B, chloroplastic	Q42450	47.2	8.6	375.5	15.5	6.65
799	Putative oxidoreductase, zinc-binding	Q7EYM8	121.9	7.0	277.8	3.6	1.52
	Response to stress						
468	Predicted protein	F2D8I0	47.4	6.2	42.0	2.5	-1.28
527	ATP-dependent Clp protease ATP-binding subunit clpA homolog CD4B, chloroplastic	P31542	173.3	6.9	124.0	7.9	-2.07
564	Chloroplast heat shock protein 70	A4ZYQ0	134.0	7.0	170.2	1.8	1.85
585	Predicted protein	F2D884	71.1	4.9	92.4	4.2	2.44
672	ATP synthase subunit alpha, mitochondrial	P0C520	47.0	7.0	75.9	3.5	1.24
680	ATP synthase subunit alpha, mitochondrial	P0C520	47.0	7.0	79.9	3.5	1.84
921	Ascorbate peroxidase	Q945R5	27.6	5.0	133.2	7.0	1.44
939	Heat-shock protein	Q43638	103.9	7.0	122.8	2.2	2.90
	Translation						
363	Predicted protein	F2D6W5	37.4	9.1	57.6	15.0	-1.39
533	Elongation factor EF-G	Q9SI75	61.3	6.9	64.2	16.0	2.41
703	Eukaryotic initiation factor 4A	P41378	143.9	6.9	121.0	4.8	1.73

Table 11 continued.

No.	Protein Name	UniRef100	MW	pI	Score	SC%	36/21
	Transport						
59	YLP	Q9ZR97	87.1	7.0	91.5	2.1	1.28
473	Chloroplast inner envelope protein, putative, expressed	Q7XD45	112.4	7.0	35.8	0.9	-1.30
592	Vacuolar proton-ATPase	Q9FS11	197.2	7.0	77.0	0.6	2.54
594	Vacuolar proton-ATPase	Q9FS11	197.2	7.0	89.2	0.6	1.52
659	ATP synthase subunit alpha, chloroplastic	A1E9I8	55.3	6.3	173.0	8.3	1.33
669	ATP synthase subunit alpha, chloroplastic	A1E9I8	55.3	6.3	595.5	17.5	1.28
671	ATP synthase subunit alpha, chloroplastic	A1E9I8	55.3	6.3	175.8	10.5	1.68
677	ATP synthase subunit alpha, chloroplastic	A1E9I8	55.3	6.3	159.9	8.1	1.36
678	ATP synthase subunit alpha, chloroplastic	A1E9I8	55.3	6.3	322.7	10.7	1.81
679	ATP synthase subunit alpha, chloroplastic	A1E9I8	55.3	6.3	238.1	5.2	1.42
747	Protein TIC110, chloroplastic	O24293	97.2	6.9	77.4	0.9	-1.19
769	ATP synthase subunit gamma, chloroplastic	P0C1M0	108.6	7.0	129.2	1.8	-1.22
790	ATP synthase subunit gamma, chloroplastic	P0C1M0	108.6	7.0	161.2	1.8	1.09
83	Predicted protein	F2CQ27	25.1	6.8	97.8	33.9	1.43
891	ATP synthase subunit alpha, chloroplastic	A1E9I8	181.6	6.9	119.3	1.1	1.42
	Unknown						
357	Predicted protein (Fragment)	F2ECE5	40.8	9.4	99.1	5.7	-1.13
359	Predicted protein (Fragment)	F2ECE5	40.8	9.4	195.8	8.1	-1.38
859	Cp31BHv	O81988	102.8	7.0	243.8	2.8	1.42
862	Harpin binding protein 1	Q5QJB5	100.3	7.0	76.9	1.2	1.35
988	Probable plastid-lipid-associated protein 3, chloroplastic	Q7XBW5	111.5	7.0	132.6	2.1	1.43

Table 12. Genotype dependent proteins quantified by DIGE and identified via mass spectrometry. For each identified protein, the corresponding spot number (No.) in Figure 10 is given in addition to the Uniprot protein name and accession number. Proteins are grouped according to their biological function. Based on the Mascot searches, the predicted molecular weights (MW), isoelectric points (pI), Mascot scores, and percent sequence coverage (SC%) are reported. Regulation factor, the log2 fold changes in protein expression are given for Keel plants over Arta plants (K/A). Spots also found to be temperature responsive are in dark grey.

No.	Protein Name	UniRef100	MW	pI	Score	SC%	K/A
	Metabolic process						
37	Alanine aminotransferase	Q84UX4	148.5	7.0	205.3	2.7	-1.79
165	Glutathione transferase	Q8VWW3	24.9	5.8	41.3	9.1	-1.35
311	Serine-glyoxylate aminotransferase	Q3S2I1	135.3	7.0	219.4	2.4	-1.83
318	Predicted protein	F2D8L5	42.0	6.5	223.0	38.1	-1.88
347	Glycolate oxidase	Q3L1H0	111.4	7.0	170.0	8.5	-1.59
349	Glycolate oxidase	Q3L1H0	111.4	7.0	227.2	2.8	-1.71
352	Glycolate oxidase	Q3L1H0	111.4	7.0	121.2	1.3	-2.08
355	Glyceraldehyde-3-phosphate dehydrogenase	F4HNZ6	88.9	7.0	241.2	2.7	-1.46
356	Glyceraldehyde-3-phosphate dehydrogenase	F4HNZ6	88.9	7.0	261.1	4.7	1.33
401	Serine-glyoxylate aminotransferase	Q3S2I1	135.3	7.0	213.1	2.3	-1.74
497	Glycine decarboxylase P subunit	O22575	202.3	6.9	124.2	1.1	-1.60
572	Methionine synthase 1 enzyme	Q4LB13	219.5	6.9	244.5	1.6	-1.24
575	Predicted protein (Fragment)	F2DEY7	73.5	5.4	181.9	4.9	-2.10
576	Methionine synthase 1 enzyme	Q4LB13	215.8	6.9	207.0	11.7	-1.65
578	Methionine synthase 1 enzyme	Q4LB13	215.8	6.9	190.0	10.6	-2.39
708	Alanine aminotransferase 2	P52894	52.8	5.9	69.2	2.9	-1.37
736	Glutamine synthetase leaf isozyme, chloroplastic	P13564	152.1	6.9	189.4	2.4	1.43
740	Isocitrate dehydrogenase	F2CQL5	45.8	6.0	54.9	2.2	1.40
743	Glutamate-1-semialdehyde 2,1-aminomutase, chloroplastic	P18492	49.5	6.4	94.1	19.6	-1.23
780	MRNA-binding protein	Q7X998	162.3	7.0	95.6	1.3	-1.61
804	Glyceraldehyde-3-phosphate dehydrogenase	F4HNZ6	88.9	7.0	205.2	3.3	2.04
809	Aldo/keto reductase family-like protein	Q8H4J8	68.3	6.9	103.6	1.9	1.37
820	Predicted protein	F2DT74	39.6	9.1	108.5	5.3	1.36
828	Predicted protein	F2DT74	39.6	9.1	77.2	5.3	-1.40
877	2-Cys peroxiredoxin BAS1, chloroplastic	Q96468	23.3	5.4	122.4	6.7	1.85
912	Triosephosphate isomerase	F2DTB2	32.3	7.9	113.4	5.0	1.26
977	Serine hydroxymethyltransferase	F2D3S8	56.1	8.8	135.1	4.9	-1.42

Table 12 continued.

No.	Protein Name	UniRef100	MW	pI	Score	SC%	K/A
	Carbohydrate metabolic process						
330	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	P26517	48.3	7.0	49.0	2.6	-1.97
336	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	P26517	122.3	7.0	185.8	2.4	-2.06
346	Predicted protein	F2CUW2	46.9	5.9	72.2	3.6	-1.40
351	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	P08477	74.7	7.0	133.0	2.2	-1.50
393	Predicted protein	F2EBQ8	58.5	6.3	32.8	2.8	-1.64
610	Ribulose bisphosphate carboxylase large chain	P05698	53.0	6.2	29.5	2.7	-1.44
713	Predicted protein	F2CTY2	44.2	4.9	131.4	3.7	-1.41
758	Phosphoribulokinase, chloroplastic	P26302	104.4	6.9	140.0	18.3	-1.15
760	Phosphoribulokinase, chloroplastic	P26302	104.4	6.9	77.5	1.0	1.37
766	Phosphoglycerate kinase, chloroplastic	P12782	99.4	7.0	152.3	1.5	-1.79
786	Fructose-bisphosphate aldolase	Q9LLD7	124.9	7.0	431.2	4.3	-1.78
791	Malate dehydrogenase	A3KLL4	69.5	6.9	181.0	20.6	-2.45
795	Fructose-bisphosphate aldolase	A5BDH7	62.1	7.0	52.5	1.3	1.50
	Protein metabolic process						
623	Putative zinc dependent protease	Q2PEV7	191.4	7.0	92.0	1.4	-1.35
644	RuBisCO large subunit-binding protein subunit beta	Q43831	118.3	7.0	178.9	2.2	-1.72
647	RuBisCO large subunit-binding protein subunit beta	Q43831	100.6	7.0	117.7	1.4	-2.63
662	RuBisCO large subunit-binding protein subunit alpha	P08823	113.6	7.0	91.5	1.5	-2.39
668	RuBisCO large subunit-binding protein subunit alpha	P08823	113.6	7.0	204.1	2.7	-2.60
	Nucleobase-containing compound metabolic process						
711	S-adenosylmethionine synthase	F2CRM1	42.7	5.4	64.1	3.8	1.26
752	Os03g0315800 protein	Q0DSD6	132.6	7.0	73.1	5.6	-1.44
	Lipid metabolic process						
519	Lipoxygenase	F2E2Z8	96.7	6.2	106.0	12.8	-1.30
890	Hydroxymethylbutenyl 4-diphosphate synthase	Q672R6	160.1	7.0	77.0	4.0	-2.04
	Catabolic process						
317	Aminomethyltransferase	Q01KC0	137.0	6.9	167.3	1.8	-1.50
319	Aminomethyltransferase	Q01KC0	69.5	6.9	127.3	2.3	-2.40
	Cellular homeostasis						
974	Dihydrolipoyl dehydrogenase	F2E5U7	52.9	8.9	156.2	5.0	-1.27
979	Dihydrolipoyl dehydrogenase	F2E2T3	52.8	8.9	51.4	7.9	-1.79
	Carbon utilization						
162	Carbonic anhydrase, chloroplastic	P40880	85.1	6.9	219.3	2.8	-1.44
210	Carbonic anhydrase, chloroplastic	P40880	35.1	9.9	137.2	9.0	1.63
223	Carbonic anhydrase, chloroplastic	P40880	35.1	9.9	109.0	5.6	-1.17

Table 12 continued.

No.	Protein Name	UniRef100	MW	pI	Score	SC%	K/A
	Photosynthesis						
97	Oxygen-evolving enhancer protein 2, chloroplastic	Q00434	96.0	6.9	44.9	0.9	2.14
177	Chloroplast-localized Ptr ToxA-binding protein1	Q5YL57	106.2	7.0	96.3	5.0	1.22
191	Predicted protein	F2D9M7	29.3	9.4	80.1	4.1	1.29
221	Oxygen-evolving enhancer protein 2, chloroplastic	Q00434	96.0	6.9	91.5	7.0	1.86
323	Peroxisomal (S)-2-hydroxy-acid oxidase GLO1	Q10CE4	72.9	7.0	153.0	15.1	-1.39
324	Peroxisomal (S)-2-hydroxy-acid oxidase GLO1	Q10CE4	72.9	7.0	131.8	2.2	-1.54
325	Peroxisomal (S)-2-hydroxy-acid oxidase GLO1	Q10CE4	72.9	7.0	141.0	19.5	-1.57
637	Hydroxyproline-rich glycoprotein-like	Q6ETQ7	132.5	7.0	62.6	4.9	-1.11
846	Predicted protein	F2CRK1	34.4	5.6	306.6	11.6	1.80
847	Chloroplast oxygen-evolving enhancer protein 1	A5JV93	68.7	7.0	198.6	3.7	2.53
849	Chloroplast oxygen-evolving enhancer protein 1	A5JV93	68.7	7.0	89.5	3.0	-1.17
851	Predicted protein	F2CRK1	34.4	5.6	199.0	11.6	2.39
853	Chloroplast oxygen-evolving enhancer protein 1	A5JV93	68.7	7.0	48.1	2.5	-1.43
865	Light harvesting chlorophyll a/b-binding protein Lhcb1	D6RSA1	28.2	5.0	98.7	9.4	1.66
866	Chlorophyll a/b-binding protein WCAB	O24401	78.9	6.9	106.0	10.3	1.57
867	Chlorophyll a/b-binding protein WCAB	O24401	66.8	7.0	54.2	6.0	1.24
870	Chlorophyll a-b binding protein of LHCII type III, chloroplastic	P27523	91.6	7.0	108.6	1.2	1.98
873	Predicted protein	F2D9M7	29.3	9.4	64.0	4.1	2.36
900	Predicted protein	F2D9M7	29.3	9.4	89.2	4.1	1.87
911	Chlorophyll a-b binding protein 1B-21, chloroplastic	Q9SDM1	26.4	5.8	38.4	4.1	2.11
	Response to abiotic stimulus						
566	Transketolase, chloroplastic	Q7SIC9	155.2	7.0	146.0	14.4	1.56
568	Transketolase, chloroplastic	Q7SIC9	155.2	7.0	203.0	18.5	-1.12
570	Transketolase, chloroplastic	Q7SIC9	155.2	7.0	217.0	18.1	-1.76
622	'putative 2,3-bisphosphoglycerate-independent phosphoglycerate mutase'	Q5KQH5	165.5	7.0	69.5	5.1	-1.67
730	RuBisCo activase A, chloroplastic	Q40073	51.0	8.9	240.6	7.8	1.41
748	Glyceraldehyde-3-phosphate dehydrogenase B, chloroplastic	P25857	104.6	6.9	208.0	14.5	-2.00
763	RuBisCo activase A, chloroplastic	Q40073	133.8	6.9	277.8	2.2	-1.20
765	RuBisCo activase small isoform	E3WDK8	47.1	8.6	167.0	36.5	-1.79
768	RuBisCo activase A, chloroplastic	Q40073	133.8	6.9	213.4	2.2	-2.02
773	RuBisCo activase B, chloroplastic	Q42450	47.2	8.6	90.2	4.5	-2.05
799	Putative oxidoreductase, zinc-binding	Q7EYM8	121.9	7.0	277.8	3.6	-3.23
914	Thioredoxin-like protein CDSP32, chloroplastic	Q9SGS4	68.8	7.0	46.7	1.8	-1.45

Table 12 continued.

No.	Protein Name	UniRef100	MW	pI	Score	SC%	K/A
	Response to stress						
91	Os04g0602100 protein	Q0JAF4	59.3	7.0	123.9	3.3	-1.24
527	ATP-dependent Clp protease ATP-binding subunit clpA homolog CD4B, chloroplastic	P31542	173.3	6.9	124.0	7.9	-1.76
529	Predicted protein	F2DXI0	102.0	6.7	84.1	2.4	-1.71
656	Thioredoxin reductase	B0FXK2	52.0	5.3	70.9	12.7	-1.26
680	ATP synthase subunit alpha, mitochondrial	P0C520	47.0	7.0	79.9	3.5	1.85
682	ATP synthase subunit alpha, mitochondrial	P0C520	47.0	7.0	159.3	5.4	-1.20
74	Peroxisome type ascorbate peroxidase	Q94IC3	31.7	8.7	147.0	38.8	-1.25
744	Predicted protein	F2D8I0	47.4	6.2	266.9	9.6	-1.36
939	Heat-shock protein	Q43638	103.9	7.0	122.8	2.2	-1.43
976	Catalase	F2CVM1	56.5	6.6	217.8	6.9	-1.17
980	Catalase	F2CVM1	56.5	6.6	261.1	7.3	-1.38
	Translation						
363	Predicted protein	F2D6W5	37.4	9.1	57.6	15.0	2.26
533	Elongation factor EF-G	Q9SI75	61.3	6.9	64.2	16.0	-1.45
703	Eukaryotic initiation factor 4A	P41378	143.9	6.9	121.0	4.8	-1.65
710	Eukaryotic initiation factor 4A	P41378	68.3	7.0	74.4	2.0	-1.56
723	Elongation factor Tu	Q8W2C3	144.5	6.9	298.2	2.4	-1.34
	Transport						
296	Cytochrome b6-f complex iron-sulfur subunit	F2CZH5	23.7	9.6	103.4	6.8	1.35
297	Cytochrome b6-f complex iron-sulfur subunit	F2CZH5	23.7	9.6	92.8	6.8	-1.24
592	Vacuolar proton-ATPase	Q9FS11	197.2	7.0	77.0	0.6	-1.78
594	Vacuolar proton-ATPase	Q9FS11	197.2	7.0	89.2	0.6	-1.23
661	ATP synthase subunit alpha, chloroplastic	A1E9I8	55.3	6.3	234.0	47.2	-1.24
669	ATP synthase subunit alpha, chloroplastic	A1E9I8	55.3	6.3	595.5	17.5	1.40
671	ATP synthase subunit alpha, chloroplastic	A1E9I8	55.3	6.3	175.8	10.5	1.51
678	ATP synthase subunit alpha, chloroplastic	A1E9I8	55.3	6.3	322.7	10.7	1.42
683	ATP synthase subunit beta, chloroplastic	P00828	53.8	5.0	301.7	8.0	-1.25
747	Protein TIC110, chloroplastic	O24293	97.2	6.9	77.4	0.9	-3.88
769	ATP synthase subunit gamma, chloroplastic	P0C1M0	108.6	7.0	129.2	1.8	-1.61
789	ATP synthase subunit gamma, chloroplastic	P0C1M0	108.6	7.0	65.5	0.8	-1.44
83	Predicted protein	F2CQ27	25.1	6.8	97.8	33.9	-1.62
857	Thioredoxin-like protein CDSP32, chloroplastic	Q84NN4	108.5	7.0	104.0	8.0	-1.19
929	ATP synthase subunit alpha, chloroplastic	A1E9I8	55.3	6.3	98.0	4.0	-1.24
	Unknown						
357	Predicted protein (Fragment)	F2ECE5	40.8	9.4	99.1	5.7	-1.40
359	Predicted protein (Fragment)	F2ECE5	40.8	9.4	195.8	8.1	-2.23
746	Predicted protein	F2DE91	41.6	5.2	97.9	7.7	-1.29
859	Cp31BHv	O81988	102.8	7.0	243.8	2.8	-1.34
862	Harpin binding protein 1	Q5QJB5	100.3	7.0	76.9	1.2	1.49
913	Predicted protein (Fragment)	F2CRX4	20.4	5.3	163.9	16.2	-1.56

Table 13. Proteins differentially regulated due to an interacting effect of temperature and genotype as quantified by DIGE and identified via mass spectrometry. For each identified protein, the corresponding spot number (No.) in Figure 10 is given in addition to the Uniprot protein name and accession number. Proteins are grouped according to their biological function. Based on the Mascot searches, the predicted molecular weight (MW), isoelectric points (pI), Mascot scores, and percent sequence coverage (SC%) are reported. 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), for Keel plants over Arta plants (K/A), 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.

No.	Protein Name	UniRef100	MW	pI	Score	SC%	36/21	K/A	K36/A36	K21/A21
	Metabolic process									
576	Methionine synthase 1 enzyme	Q4LB13	215.8	6.9	207.0	11.7	-1.08	-1.65	-2.03	-1.38
780	MRNA-binding protein	Q7X998	162.3	7.0	95.6	1.3	1.37	-1.61	-1.95	-1.26
809	Aldo/keto reductase family-like protein	Q8H4J8	68.3	6.9	103.6	1.9	1.02	1.37	1.77	1.07
	Carbohydrate metabolic process									
330	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	P26517	48.3	7.0	49.0	2.6	1.68	-1.97	-2.36	-1.49
713	Predicted protein	F2CTY2	44.2	4.9	131.4	3.7	-1.17	-1.41	-1.71	-1.20
	Protein metabolic process									
662	RuBisCO large subunit-binding protein subunit alpha	P08823	113.6	7.0	91.5	1.5	2.20	-2.39	-2.79	-1.76
	Nucleobase-containing compound metabolic process									
711	S-adenosylmethionine synthase	F2CRM1	42.7	5.4	64.1	3.8	-1.34	1.26	-1.08	1.61
	Response to abiotic stimulus									
799	Putative oxidoreductase, zinc-binding	Q7EYM8	121.9	7.0	277.8	3.6	1.52	-3.23	-3.45	-2.95
	Translation									
533	Elongation factor EF-G	Q9SI75	61.3	6.9	64.2	16.0	2.41	-1.45	-1.52	-1.29
703	Eukaryotic initiation factor 4A	P41378	143.9	6.9	121.0	4.8	1.73	-1.65	-1.96	-1.24
	Transport									
592	Vacuolar proton-ATPase	Q9FS11	197.2	7.0	77.0	0.6	2.54	-1.78	-2.30	1.01
594	Vacuolar proton-ATPase	Q9FS11	197.2	7.0	89.2	0.6	1.52	-1.23	-1.44	1.03
83	Predicted protein	F2CQ27	25.1	6.8	97.8	33.9	1.43	-1.62	-1.91	-1.29
	Unknown									
859	Cp31BHv	O81988	102.8	7.0	243.8	2.8	1.42	-1.34	-1.59	-1.06

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Appendix

Abbreviations

2D-PAGE	two dimensional polyacrylamide gel electrophoresis
ABA	abscisic acid
ANOVA	Analysis of variance
Arke	Arta x Keel segregating population
Chr	chromosome
cM	centimorgan
Cys	cysteine

Da	Dalton
DArT	diversity array technology
DIGE	differential in gel electrophoresis
DTT	dithiothreitol
FC	field capacity
FDR	false discovery rate
Fm	fluorescence maximum of induction curve
Fo	fluorescence origin of induction curve
Gbp	gigabasepair
GO	gene ontology
h	hour
kDa	kilodalton
LD	linkage disequilibrium
LOD	logarithm of odds
MALDI	matrix assisted laser deabsorbtion ionization
Met	methionine
mRNA	messenger ribonucleic acid
MS/MS	tandem mass spectrometry
n/s	not significant
NADPH	nicotinamide adenine dinucleotide phosphate
NHS	N-hydroxysuccinimide
NL	non-linear
PCR	polymerase chain reaction
PEV	percent explained variance
PFF	peptide fragmentation fingerprints
pI	Isoelectric point
PMF	peptide mass fingerprint
ppm	parts per million
PSII	photosystem II
Qa	primary electron acceptor
QTL	quantitative trait loci
RF	regulation factor
RIL	recombinant inbred line
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEA	singular enrichment analysis
SSR	simple sequence repeats
SWC	soil water content
TOF	time of flight
tRNA	transfer ribonucleic acid
ZT	zeitgeber time

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"No man is an Island, entire of itself; every man is a piece of the Continent, a part of the main; if a clod be washed away by the sea, Europe is the less, as well as if a promontory were, as well as if a manor of thy friends or of thine own were; any man's death diminishes me, because I am involved in Mankind; And therefore never send to know for whom the bell tolls; It tolls for thee."—John Donne (1571–1631),

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