The Effect of Natural Variation at *Ppd-H1* and *HvELF3* on Responses to Osmotic Stress in Barley (*Hordeum vulgare*)

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Abstract

The circadian clock is an important timing system that, in part, controls stress adaptation in Arabidopsis thaliana. In the model crop barley, the clock orthologs *Ppd-H1* and *HvELF3* are important regulators of photoperiod response and flowering. However, little is known about additional effects of the clock on plant performance and stress adaptation in barley. Therefore, the objectives of this study were i) to analyse the effects of natural variation at the barley photoperiod response and clock genes *Ppd-H1* and *HvELF3* on response to osmotic stress and ii) to test whether osmotic stress at the root acted as an input signal to the shoot circadian clock and thus changed diurnal patterns of physiological traits.

The first chapter describes changes in gene expression and physiology under polyethylene glycol induced osmotic stress in seedlings of two spring barley cultivars carrying a natural mutation in *Ppd-H1* and two derived introgression lines with the wild type *Ppd-H1* allele. Analysis of performance at three consecutive days under stress revealed that the natural mutation in *Ppd-H1* resulted in reduced cell membrane injury and increased photosynthetic activity and concomitant lower expression of stress-responsive and senescence-activated genes as compared to the introgression lines with the wild type *Ppd-H1* allele. In the second chapter, I analysed diurnal changes of clock and stress -expression and of leaf water relations and gas exchange in two pairs of genotypes varying at *Ppd-H1* and *HvELF3.* Variation at *HvELF3* affected the phase and shape of the clock and stress-gene expression profiles, whereas variation at *Ppd-H1* modified the expression levels only of stress genes. Osmotic stress upregulated expression of clock and stress-response genes and advanced their expression peaks. Expression differences in clock genes did not have strong effect on the diurnal expression of physiological traits.

Taken together, this thesis demonstrates that osmotic stress at the barley root altered clock gene expression in the shoot and acted as a spatial input signal into the clock. *Ppd-H1* controlled stress-induced senescence, while variation at *HvELF3* did not affect senescence related traits, and had minor effects on gas exchange under stress. Unlike in Arabidopsis, barley primary assimilation was less controlled by the clock and more responsive to environmental perturbations, such as osmotic stress.

Zusammenfassung

Die circadiane Uhr ist ein interner Zeitmesser, der unter anderem die Anpassung an Stress in der Modelpflanze Arabidopsis thaliana koordiniert. In der Modell-Getreidepflanze Gerste spielen die Uhrgene *Ppd-H1* und *HvELF3* eine wichtige Rolle für die Regulierung der Blüte in Abhängigkeit der Photoperiode. Allerdings ist der Einfluss der circadianen Uhr auf die agronomische Leistung und Anpassung an Stress in Gerste noch wenig erforscht. Das Ziel dieser Arbeit ist es, i) den Einfluss natürlicher Variation an *Ppd-H1* und *HvELF3* auf das Verhalten von Gerstenkeimlingen unter osmotischen Stress zu untersuchen, ii) den Effect von osmotischem Stress an der Wurzel auf die Expression von Uhrgenen und physiologischer Merkmale im Blatt zu untersuchen.

Im ersten Teil meiner Arbeit beschreibe ich die Effekte von Variation an *Ppd-H1* auf die Expression von Stress-induzierten Genen und physiologische Merkmale unter osmotischem Stress, induziert durch Polyethylenglycol. Zwei Genotypen mit verschiedenen Allelen für *Ppd-H1* zeigten unter osmotischem Stress Unterschiede in der photosynthetischen Aktivität und Zellmembranpermeabilität, und in der Expression von Stress- und Seneszenz- induzierten Genen. Im zweiten Teil meiner Arbeit gehe ich näher auf die Interaktion von osmotischem Stress und diurnalen Änderungen circadianer Rhythmen ein. Hierfür wurden diurnale Genexpressionsprofile der Uhrgene und Stress-induzierter Gene erfasst, sowie Änderungen physiologischer Parameter unter osmotischem Stress untersucht. Osmotischer Stress führte zu einer Induktion von Genen der circadianen Uhr und der Stress-Signalwege, sowie zu einer Verschiebung ihrer diurnalen Expressionsprofile. *HvELF3* beinflusste die Expressionsphase und Form von Uhr- und Stressgenen, während *Ppd-H1* nur die Expressionshöhe von Stressgenen regulierte. Diese Veränderungen korrelierten nicht mit Änderungen in den betrachteten physiologischen Parametern.

Zusammenfassend zeigen meine Ergebnisse, dass Ppd-H1 neben seiner Rolle in der photoperiodischen Regulation der Blüte pleiotrope Funktionen in der osmotischen Stressantwort und der Stress-induzierten Seneszenz übernimmt. Des Weiteren führte osmotischer Stress an den Wurzeln zur Änderung der Genexpresssion der circadianen Uhr im Spross. Im Unterschied zu Arabidopsis scheint der Primärmetabolismus in Gerste weniger stark von der circadianen Uhr als von äusseren Umwelteinflüssen, wie osmotischer Stress, kontrolliert zu sein.

Abbreviations

ABA	Abscicic acid
ABI5	ABA-insensitive5
ABRE	ABA responsive element
APX1	Ascorbate peroxidase1
ARF1	ADP-rybosylation factor 1
AT	Time after stress
CAT1	Catalase 1
CCA1	Cicadian clock -associated1
CCAF	Circadian clock factor
DRE	Drought responsive element
DREB1	Drought responsive binding1
DRF1	Drought responsive factor 1
EC	Evening complex
EE	Evening element
EL	Electrolyte leakage
Fv/Fm	Maximum quantum yield of PSII
GA	Gibberlicc acid
GI	Gigantia
HRGP	Hydroxyproline-Rich Glycoprotein
LD	light dark
LHY	Late elongated hypocotyl
LREM	Light response element motif
LT	Leaf temperature
MDA	Malondialdehyde
PEG	Poly ethylene glycol
PI	Performance index
PIF	Phytochrome interacting factor
PRR	PSEUDO-RESPONSE REGULATOR
PSII	Photosystem II
RWC	Relative water Content
SAM	Shoot apical meristem
TF	Transcript factor
TOC1	Timing of CAB1
ZT	Zeitgeber time

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Chapter One- The Effect of Natural Variation at *Ppd-H1* on Responses to to Osmotic in Barley

Introduction

Drought is the most important abiotic constraint to plant survival and global crop productivity. Water deficit affects plant physiological, biochemical, as well as molecular processes (Harb *et al*., 2010). Its impact on plants differs depending on developmental stage of the plant, duration and severity of the stress and the ability of plant to adapt to drought stress. Responses to drought stress at different growth stages could thus provide a basis for developmental strategies to adapt and respond to drought stress (Vurayai, *et al*.,2011). Plants respond to drought stress through changes in morphology, physiology, and metabolism in different organs of the plant (Chaves *et al*., 2002). At the cellular level, plant responses to water deficit may result from cell damage, whereas responses at tissue and organ level may be correspond to adaptive mechanisms (Cellier *et al*., 1998). The adaptive strategies to cope with the prevailing drought stress include drought escape, drought avoidance and drought tolerance. An important drought escape mechanism is rapid phenological development which allows reproduction outside the dry season. Drought avoidance describes the maintenance of a high tissue water potential through reduced transpiration or improved water uptake. Finally, drought tolerance allows the plant to survive and reproduce in the presence of a low water content in the plant, through accumulation of osmolytes, antioxidants, and other protective proteins (Ingram and Bartels, 1996; Chavez *et al*., 2003).

As an escape strategy, the pattern of crop development is an important trait for adaptation to dry environments. Quantitative Trait Loci (QTL) studies showed that genomic regions associated with improved yield under drought coincided with major flowering genes in barley and wheat(Quarrie *et al*., 2006; von Korff *et al*., 2008; McIntyre *et al.*, 2009; Rebetzke *et al*., 2008, Rollins *et al*. 2013). Studies suggest that flowering time genes have pleiotropic effects on plant architecture, yield structure and even shoot sodium accumulation (Taeb, *et al*., 1992; Kurepa, *et al*. 1998; Lens, *et al*., 2008; Kinoshita et al. 2011; Rollins *et al*. 2013). However, not much is known about the direct or indirect effects of flowering time genes on genes and pathways other than those involved in the control of development.

Photosynthesis is one the plant processes that is affected primarily by drought stress. The effects of drought stress on photosynthesis can be direct through the reduction of CO_2 diffusion (Flexas *et al*., 2012) or indirectly through alteration of photosynthetic metabolism (Cornic and Fresneau, 2002) that arises from imbalances of electron transport (Bartoli *et al*., 2000). Furthermore, these imbalances of electron transport enhance the accumulation of reactive oxygen species (ROS) such as superoxide radicals, singlet oxygen, and hydroxyl radicals (Cruz de Carvalho, 2008). Hence, the increased ROS would damage proteins, lipids and DNA (Mittler *et al*., 2002). For example, peroxidation of lipids, commonly taken as an indicator of oxidative stress, disrupts the membrane integrity of the plant cell. This means that essential solutes leak out from the organelles and from the cell and cause the damage of membrane function and metabolic imbalances (Blokhina *et al*., 2003). In addition, drought stress could also damage oxygen-evolving complex of photosystem II and reaction centers (Subrahmanyam *et al*., 2006). Therefore, in order to examine the extent of damage in the photosynthetic apparatus chlorophyll fluorescence measurements have become a widely used method to study the functioning of the photosynthetic activity and are used as an indicator of the plant's response to drought stress (Massacci *et al*., 2008). Furthermore, drought induced senescence in plants plays an important role in plant survival. This drought-induced leaf senescence under stress contributes to the remobilization of nutrient material to the new tissues (Munne-Bosch and Alegre, 2004). ROS are regulators of leaf senescence (Zentgraf and Hemleben, 2008) and their production is also known to be increased under drought stress. Understanding this connection between development/ senescence and stress response might be crucial to decipher the genetic and molecular control of stress responses in plants.

Studies on the model plant Arabidopsis revealed genes and gene networks of drought responses in plants, and these were classified in regulatory and functional (response) genes (McCue and Hanson, 2002; Shinozaki and Yamaguchi-Shinozaki, 2007; Harb *et al*., 2010). Nakashima *et al*., (2009) showed that gene networks regulating drought responses are conserved between dicots and monocots. The common drought stress signaling pathway are comprised of abscisic acid (ABA)-dependent and ABA-independent pathways (Shinozaki and Yamaguchi-Shinozaki, 2007). Key genes for these ABA-dependent/independent pathways have been identified in Arabidopsis , such as *DROUGHT-RESPONSIVE-BINDING PROTEIN2* (*DREB2*)/*C-REPEAT BINDING FACTOR* (*CBF*) and *ABA-RESPONSIVE ELEMENT BINDING PROTEINS* (*AREB*)

(Bartels and Sunkar, 2005; Sakuma *et al*., 2006). These transcription factors induce downstream functional genes, which are involved in cellular homeostasis to mitigate the effects of stress (Ramanjulu and Bartels, 2002). Orthologous genes involved in these regulatory pathways were also identified in barley (Tondelli *et al*., 2006; Guo *et al*., 2009), but the functions of most of these genes have yet to be identified. Despite the existence of common regulatory mechanisms between monocots and dicots, some stress-inducible genes such as *DREB2* like genes in barley are induced both by ABA and drought stress (Xue and Loveridge, 2004), suggesting that drought signaling pathways might be different in barley as compared to the model plants Arabidopsis and rice.

Barley is one of the most drought tolerant crops which is cultivated in various parts of the world and is an ideal model crop for drought stress studies (Eshghi *et al*., 2010). Mediterranean barley which is adapted to terminal drought is characterized by a rapid development under long day (LD) conditions (von Korff *et al*., 2008). Early flowering under LD in barley is primarily controlled by the photoperiod response gene *Ppd-H1* (Turner *et al*., 2005). Barley genotypes carrying the mutated recessive *ppd-H1* allele are late flowering whereas the dominant *Ppd-H1* allele causes early flowering under LD (Campoli *et al*., 2012). A single nucleotide mutation in the CCT domain of *Ppd-H1* resulting in an amino acid change is causative for this difference in sensitivity to LD (Turner *et al.,* 2005). Natural variation at this gene is adaptive and shows a specific geographical distribution. The photoperiod sensitive winter barley genotypes (ancestral) are predominant in Mediterranean areas and represents an adaptation to terminal drought and heat. In contrast, the derived photoperiod insensitive allele was selected in spring barley cultivars grown in temperate Northern European areas as an adaptation to longer growing seasons (Cockram *et al*., 2007). *Ppd-H1* encodes a *PSEUDO-RESPONSE REGULATOR* (*PRR*) gene, most similar to the circadian clock gene *PRR7* in Arabidopsis (Turner *et al*., 2005). The circadian clock is an autonomous oscillator that produces endogenous biological rhythms with a period of about 24 hours. The Arabidopsis circadian clock consists of core oscillators that connect morning and evening phases. The central core feedback loop comprises two *MYB* genes *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*) which is expressed in the morning and represses transcription of the evening expressed *PRR* gene *TIME OF CAB EXPRESSION1* (*TOC1*) (Wang and Tobin, 1998; Alabadi *et al*., 2001). The expression of *CCA1*/*LHY* declines in the evening releasing the repression of *TOC1* in the evening. The

morning feedback loop comprises of *PRR7* and *PRR9*, the transcription of these genes is promoted by *CCA1*/*LHY* and the subsequent accumulation of *PRR7* and *PRR9* proteins down regulates the transcripts of *CCA1*/*LHY* genes (Farre' *et al*., 2005). This loop also involves, *EARLY FLOWERING 3* (*ELF3*), *EARLY FLOWERING 4* (*ELF4*) and *LUX ARRYTHMO* (*LUX*) which promotes *CCA1* and *LHY* expression and represses *PRR7* and *PRR9* (Doyle *et al*., 2002; Hazen *et al*., 2005). The evening feedback loop involves *GIGANTIA* (*GI*) regulates negatively *TOC1* expression (Pokhilko *et al*., 2012).

The circadian clock is implicated in regulation of stress responses in plants (Matsui *et al*., 2008; Mizuno and Yamashino, 2008). For example, studies in Arabidopsis have shown that the circadian clock is an important mechanism controlling stress adaptation in plants by coordinating their metabolism and development with predicted daily and seasonal changes of the environment (Green *et al*, 2002; Michael *et al*, 2003; Dodd *et al*, 2005). In line to these, global transcriptome analysis identified several stress-responsive genes that are controlled by circadian clock in Arabidopsis such as genes providing protection against cold stress, oxidative and heat stress (Covington *et al.*, 2008; Lai *et al*., 2012). For example, constitutive expression of *DREB1*s/*CBF*s genes were observed in triple *PRR* mutants (*PRR5*, *PRR7* and *PRR9*), which reveals a direct link between clock genes such as *PRR*s to the stress inducible transcription factors *CBF*s (Nakamichi *et al*., 2009). This suggests a relationship between *PRR* genes and stress-inducible *DREB*/*CBF* genes in plants. Despite the prominent role of *Ppd-H1* for photoperiod response and adaptation, its effect on stress adaptation through clock dependent or independent control of stress responsive genes has not yet been analyzed in barley.

Furthermore, recent studies indicated that the circadian clock is also involved in the control of seed germination (Penfield and King, 2009). It is believed that circadian clock is arrested in dry seeds, but the start of seed imbibition could set the phase and synchronize Arabidopsis circadian clock (Zhong *et al*., 1998). Circadian clock and seed germination are sensitive to environmental stimuli and can be modulated by light and temperature (McClung *et al*., 2006). Because of the common environmental factors setting these two events, there is a possible interaction between clock and seed germination. Moreover, metabolism of the plant hormones such as ABA and gibberilic acid (GA) are under the control of the clock, including genes important in germination control, suggesting that this is the mechanism through which the clock controls germination

(Michael *et al*. 2008; Penfield and Hall, 2009). In agreement to these ideas, a previous study has indicated the interaction of central seed dormancy regulator *ABA-INSENSITIVE3* (*ABI3*) and *TOC1* in Arabidopsis (Kurup *et al*., 2000), indicating the involvement of circadian clock in seed germination through hormone balance control. In addition, a recent study by Penfield and Hall, (2009) demonstrated that mutations in the circadian clock genes *CCA1*/*LHY*, *GI* and *LUX* altered seed germination in Arabidopsis. Their study also indicated the importance of clock genes for the normal ABA and GA responses in seeds. However, the effects of other circadian clock genes including *PRR7* and *PRR9* on seed germination remain to be demonstrated. Furthermore, there is less information available on how alteration of circadian clock genes affects seed dormancy and germination in cereals.

Different experimental procedures have been developed for mimicking drought stress which differed in terms of intensity and dynamics. Polyethylene glycol (PEG) is a ionically neutral osmotically active polymer and has been widely used to induce osmotic stress by decreasing the water potential of the nutrient solution (Murillo-Amador *et al*., 2002). With this method a water deficit can be uniformly applied to all plants and higher molecular weight of PEG such as 8000 or more does enter into the plant roots and is not toxic to plant cells (Verslues *et al*., 2006). Hence, many drought/osmotic stress experiments have used PEG to understand physiological, metabolism and molecular changes drought stress (Kumar *et al*., 2011; Marcin'ska *et al*., 2013; Yang *et al*., 2011) and to identify tolerant cultivars in different crops (Badiane *et al*. 2004; Nodichao, 2010).

The objective of this study was to understand the role of natural genetic variation at major photoperiod response genes and circadian clock ortholog *Ppd-H1* on plant performance under osmotic stress. The second objective of the present study was to study the effects of natural variation at *Ppd-H1* on seed germination under ABA. The plant performance was analyzed by measuring physiological responses and transcript changes under short-term PEG-induced osmotic stress conditions and by assessing seed germination under different concentrations of ABA.

Materials and Methods

Barley genotypes

The spring barley cultivars Scarlett and Triumph and derived introgression lines S42-IL107 and Triumph-IL in the background of Scarlett and Triumph, respectively, were used in this study. S42-IL107 was generated by crossing the spring barley Scarlett with the wild barley accession ISR42-8, the introgression line was then obtained after repeated selection and backcrossing to Scarlett (von Korff *et al*., 2004; Schmalenbach *et al*., 2011); Triumph-IL was obtained from the cross of Triumph and the winter barley Igri and was kindly provided by David Laurie (John Innes Center, Norwich). Scarlett and Triumph carry the same mutation in the CCT domain of *Ppd-H1* and are late flowering under LD (Turner *et al*. 2005). The introgression lines S42-IL107 and Triumph-IL harbor the photoperiod-responsive *Ppd-H1* allele introgressed from wild barley and winter barley, respectively, and are early flowering under LD (Campoli *et al*., 2012).

Growth conditions

All chemical were purchased from Carl Roth GmbH unless stated otherwise. Seeds of all genotypes used in this study were surface-sterilized with 6 % Sodium hypochlorite solution for 30 min and stratified at +4°C for 2-3 days (d) on wet filter paper in the dark. The stratified seeds were pre-germinated at room temperature in the dark for 1 d. The germinated seeds were then placed in seed-holders which were filled with agar (Merck, Germany). After 2 d, seedlings were transferred to the hydroponics system which consisted of half-strength Hoagland nutrient solution (3.5 mM $Ca(NO_3)_2$, 2.5 mM KNO_3, 1 mM KH_2PO_4, 1 mM $MgSO_4.7H_20$, 0.5 mM Fe–EDTA, 0.023 mM H_3BO_3, 0.004 mM $MnCl_2$-$4H_2O$, 0.47 mM $ZnSO_4$-$7H_2O$, 0.12 mM $CuSO_4$-$5H_2O$, 0.006 mM Na_2MoO_4), as described by (Hoagland and Arnon, 1950). Plants were kept for 8-10 d in a climatic chamber at irradiance of 300 µmol/m^2/s and air temperature 20 °C during day and 16 °C at night time. The nutrient solution was changed every three to four days.

Experimental set up

Short-term osmotic stress experiments were conducted in 16 h photoperiod length (LD) using cultivars Scarlett and Triumph as well as introgression lines S42-IL107 and Triumph-IL. Osmotic stress was applied after seedlings reached the two leaf stage. In order to generate uniform osmotic stress conditions in the roots of the plants, seedlings were subjected to water

deficit by replacing the normal nutrient solution with one supplemented with 20 % PEG 8000 (Fluka, Germany) which corresponds to -0,8 MP osmotic potential. The media of the control plants were also replaced with freshly prepared Hoagland's nutrient solution that corresponded to -0.2 Mpa. According to Hsiao (1973) the stress induced by PEG for 24, 48 and 72 h represents mild, moderate and severe water stress, respectively.

Each experiment was repeated three times. Physiological measurements and leaf sampling for RNA were conducted at three time points i.e. 24 h, 48 h and 72 h after stress. All measurements were conducted from second leaf from the bottom and samples were collected between ZT4-ZT6 in order to minimize the diurnal effects on sampling. In each experiment, RWC, photochemical efficiency and leaf temperature were analysed from 8-9 plants per genotype, treatment and time point. Measurements of proline content and MDA accumulation were conducted using three replicates per genotype, treatment and time point, each pool of three leaves was considered as one biological replicate. Leaf samples for total RNA extraction were also collected at three different time points using three replicates each of which consists of two pooled leaf samples. The leaf samples collected for RNA were immediately frozen in liquid nitrogen and stored -80 ^{o}C until processed.

Determination of relative water content (RWC)

Leaf relative water content (RWC) was measured on the second emerged leaf. Water content was estimated according to Turner (1981) and was calculated from the equation: RWC = (FM - DM)/(SM - DM) X 100, where FM is the fresh mass of the leaves, SM is the mass at full water saturation, measured after submerging the leaves for 24 h in the dark in distilled water at +4 °C, and DM is the mass after drying the leaves for 24 h at 70 °C.

Photochemical efficiency

Photochemical efficiency was estimated using chlorophyll fluorescence measurements. Chlorophyll fluorescence was measured on the middle part of the abaxial side of the second fully developed intact leaf after dark-adaptation for 20 min with an *in situ* portable fluorometer Handy Plant Efficiency Analyzer (PEA) (Hansatech, King's Lynn, Norfolk, UK) as described by Humbeck *et al*. (1996). Fluorescence was induced using 3000 µmol photons m $^{-2}s^{-1}$ flash of actinic light persisting for 1 s on dark adapted leaves. The induction curves were analyzed using the PEA plus software (Hansatech,UK). The chlorophyll fluorescent parameters calculated

include Fv/Fm, performance index (PI) and Area. Where, Fv/Fm ratio measures the efficiency of excitation energy captured by open PSII reaction centers representing the maximum capacity of light-dependent charge separation (Krause and Weis, 1991). Performance index (PI) encompasses three components; the force of the light reactions, the force of the dark reactions and the efficiency of light trapping by the light harvesting complex. The area above the fluorescence curve between Fo and Fm (Area) is proportional to the pool size of electron acceptors Qa on the reducing side of PSII.

Leaf temperature

Leaf temperature (LT) was measured on the second emerged leaf. LT was measured using Optris LS LT portable infrared thermometer (Optris, USA) set to close focus mode and with the emissivity set 0.99. Temperature measurements were taken prior to sampling and measured from the middle portion of the blade.

Electrolyte leakage

Electrolyte leakage was measured according to Szalai *et al*., (1996). Uniform leaf discs from nine plants from each genotype per condition were pooled and placed in a glass vial (20 ml). The leaf discs were then washed three times in deionised water to remove electrolytes adhered on the leaf surface. Then 10 ml deionised water was added to the vial, capped and incubated in the dark for 24 h at room temperature. The conductance was measured using a conductivity meter (Horiba, Ohio). After the initial measurement (i), the vials were autoclaved for 15 min to kill the leaf tissue and to achieve 100 % electrolyte leakage. After cooling, the final conductivity reading (ii) was taken. The measurement was then represented as percentage of (i/ii) *100. These two measurements were carried out individually for all samples from both the control (non-stress) and stress treatments every 24 h for 3d after stress application.

Quantification of proline

Plant material (0.1-0.2 g fresh weight of leaf) was collected on ice and stored at -80 °C for further processing. The proline content was estimated according to the method of Bates *et al.* (1973). Leaf samples were extracted with 3% sulphosalicylic acid, extracts (200µl) were held for 1 hour in boiling water after adding 200 µL acidic ninhydrin and 200 µL glacial acetic and the reaction was terminated in an ice bath. The reaction mixture was extracted with 400 µL toluene

mixed for 60 seconds. The chromophore containing toluene was aspirated from aqueous phase. The proline content was then measured by spectrophotometer (Synergy 4, Biotek, Germany) by reading at 520 nm against toluene blank. The standard curve was prepared from a 10X dilution series of L-proline (Sigma Aldrich, Germany) with 0.1 to 100 µg ml-1 concentrations. Free proline content was determined from standard curve and calculated following Bates *et al*., (1973):

$$\left[\frac{\frac{(\mu g\ proline\ \ /ml\ X\ ml\ \ toluene\ \ )}{115.5 \mu g/\mu mole}}{g\ sample\ /5}\right] = \mu mol/g \text{ fresh weight}$$

Leaf Malondialdehyde (MDA) measurement

The level of lipid peroxidation Malondialdehyde (MDA) was measured following the modified method of Heath and Packer (1968) with 0.2 g of fresh leaf per plant of the second leaf. The leaf material ground in liquid nitrogen and homogenized with 2 ml solution of 0.1 % Trichloracetic acid (TCA). The homogenate was centrifuged at 1000xG for 15 min. Then 2 ml of the supernatant was mixed with 2 ml of TCA and 2 ml of Thiobarbituric acid (TBA). The samples were incubated at 95 °C for 30 min and immediately transferred to ice for 5 min, followed by centrifugation at 1000 g for 1 min. The supernatant absorbance was read at 532 nm, and values correspond to non specific absorption 600 nm were subtracted. MDA concentration was calculated using its molar extinction coefficient (ϵ= 155 mM-1 cm-1)

Apical meristem measurement

The development of plants was monitored by dissecting and scoring the shoot apical meristem three day after application of osmotic stress in 3-5 replicate plants according to the Waddington scale (Waddington *et al*., 1983).

Germination test

Seeds of all genotypes were surface-sterilized with 6 % sodium hypochlorite solution for 30 min and rinsed with sterile water. The sterilized seeds were placed in 10cm Petri dishes lined with two sheets of Whatman No.1 filter paper, saturated either with 3ml distilled water (control) or by adding different concentrations of 3ml ABA (± Cis,trans-abscisic acid, Sigma, Germany). Germination tests for all samples were immediately conducted in the dark at room temperature.

Three replications of 40 seeds were used per genotype and treatment conditions. After 3d, germinated seeds (those where the coleoptile had emerged through the hull) were counted and expressed as a germination percentage. The experiment was repeated three times.

RNA extraction, cDNA synthesis and real time qRT-PCR

Total RNA was extracted from 100 mg of tissue using TRIZOL® reagent (Invitrogen,Germany) following manufacturer's instructions, except for the addition of RNaseH, followed by a DNase treatment (final volume 100 µL). First strand cDNA synthesis was performed on 4 µL of total RNA using 100 U of SuperScriptTM II RT (Invitrogen, Germany) and 500 ng of poly-T primer and following manufacturer's recommendations (final volume 40 µL). The resulting cDNA was diluted 1:4 in nuclease-free water and stored in aliquots at −20 °C.

Real-Time quantitative PCRs (qRT-PCR) were performed on cDNA samples using gene-specific primers (Supplementary Table 1). Amplifications were performed using 4 µL of cDNA, 0.5 U of GoTaq DNA polymerase (Promega), 0.2 mM dNTP, 2.5 mM MgCl2, 0.2 µM each primer, and 0.5 µL of EvaGreen (Biotium) in a final volume of 10 µL. Reactions were performed in a LightCycler480 (Roche) with the following amplification conditions: 95 °C for 5 min, 45 cycles of 95 °C (10 s), 60 °C (10 s) and 82 °C (10 s). Appropriate non-template controls were included in each 384-well PCR. Dissociation analysis was performed at the end of each run and the melting curves for each primer pair showed a single peak confirming the specificity of the reaction. The standard curves were prepared from a dilution series of plasmids containing the target fragments and subjected to qRT-PCR analysis with the respective cDNA samples. Starting amounts for each data point were calculated based on the titration curve for each target gene and the reference (*HvActin*) gene using the LightCycler480 Software (Roche; version 1.5).

Design and validation of qRT-PCR primers

Drought/osmotic responsive genes in barley were identified either through public data base searches from NCBI (http://www.ncbi.nlm.nih.gov/) and literature searches or via BLAST searches of known drought responsive genes to barley EST, Contigs, and mRNA sequences in NCBI, IPK Barley Blast Server (http://webblast.ipk-gatersleben.de/barley/viroblast.php) and HARVEST: Barley databases. Specific primer pairs for qRT-PCR were designed by using Primer3 (http://primer3.wi.mit.edu) (Supplementary Table S1). The specificity and efficiency of

the primers were tested by carrying out preliminary qRT-PCR assays on a pool of cDNAs from stress and control samples of different concentrations. Primers for candidate gene were then selected based the presence of single peak by melting curve analysis and absence of non-specific products or primer-dimer artifacts. The specificity of the amplicons was also checked by electrophoresis on 2 % agarose gel and sequencing of the PCR products in order to confirm that the product sequence was the same as the target candidate gene.

Cloning of PCR product for standard curve in qRT-PCR

Amplification of stress response genes was conducted from cDNA of stress samples using GoTaq® DNA polymerases (Promega, Germany). The PCR amplifications were performed using 5 µL of cDNA, 0.5 U of GoTaq DNA polymerase, 5 µL 5x GoTaq buffer, 1 µL 2 mM dNTP, 0.5 µL 10 mM of each primer, and 12.9 µL sterile H_2O in a final volume of 25 µL Reactions were performed in a PCR Cycler (Eppendorf, Germany) with the following amplification conditions: 95 °C for 3 min, 35 cycles of 95 °C (10 s), 60 °C (30 s) and 72 °C (30 s). After detection of specific PCR amplicons, the PCR products were cloned into the pCR®2.1-TOPO® vector using T TOPO® TA Cloning® Kit according to manufacturers recommendation (Invitrogen, USA). The recombinant plasmids were extracted by the Nucleospin ® Plasmid purification kit (Mascherey-Nagel, Germany). The extracted recombinant plasmids were sequenced. The obtained sequences were queried online by using the BLAST service at the NCBI. Dilutions of purified plasmid DNA were used to construct gene specific calibration curves. These calibration curves were used for calculation of each candidate and reference gene concentration in qRT-PCR.

Statistical analysis

Statistical analyses were carried out with SAS version 9.1 (SAS Institute Inc, 2003). The procedure LSMEANS was used to calculate adjusted means and standard deviations for each trait. A multifactorial analysis of variance (ANOVA) was performed for each trait with a mixed general linear model using the PROC GLM procedure:

$$Y_{ijkl} = \mu + G_i + T_j + R_k + P_l + GT_{ij} + GP_{il} + TP_{jl} + GTP_{ijl} + R_{ijkl.}$$

where µ is overall mean, G_i is the fixed effect of the i-th genotype, T_j is the fixed effect of the j-th treatment, R_k is the random effect of the k-th replication , P_l is the fixed effect of the l-th time point, GT_{ij} is the fixed interaction of the i-th genotype with j-th treatment, GP_{il} is the fixed

interaction of the i-th genotype with l-th time point, TP_{jl} is the fixed interaction of the j-th treatment with l-th time point ,GTP_{ijl} denotes the interaction effects of i-th genotype with j-th treatment and l-th time point, R_{ijkl} is the residual effect. Pearson correlations coefficients between trait values were calculated with the least squares means for stress and control plants separately.

Results

Effect of short-term osmotic stress on physiological responses of genotypes varying at *Ppd-H1*

The present study was conducted to examine whether the natural mutation in the CCT domain of the major photoperiod response gene *Ppd-H1* and pseudo response regulator homolog (*PRR7)* affected performance under osmotic stress. The effects of short-term osmotic stress on physiological performance, relative water content (RWC), leaf temperature (LT) and proline content, were analysed in the spring barley cultivars Scarlett/ S42-IL107 and Triumph/Triumph-IL at 24h, 48h and 72h after beginning of the stress treatment (AT). A significant reduction of RWC was observed in Scarlet and S42-IL107 under stress compared to control conditions at all time points (Fig. 1A), while in Triumph and Triumph-IL the reduction of RWC was only significant 72h AT (Fig. 1B). The lowest RWC was observed under stress 72h AT, when it was 86 % for Scarlett(*ppd-H1*), 85 % for S42-IL107(Ppd-H1), 85 % for Triumph*(ppd-H1*) and 75 % for Triumph-IL(*Ppd-H1*). The RWC of control plants ranged between 91 % and 94 % in all genotypes. Although no significant differences were observed between Scarlett(*ppd-H1*) and S42-IL107(*Ppd-H1*) at all time points under control and stress conditions, a significant difference in RWC was observed between Triumph(*ppd-H1*) and Triumph-IL(*Ppd-H1*) in stressed plants 72h AT (Supplementary Table S2,S3,S4 and S5). LT was significantly increased in stressed plants compared to control plants in all genotypes (Fig. 1, C and D). No significant differences between genotypes differing at *Ppd-H1* was recorded for LT. Proline content increased gradually during the stress treatment and was significantly higher under stress compared to control conditions in all genotypes 48h and 72h AT, with an up to three-fold increase of proline content in stress as compared to control conditions (Fig. 1, E and F). No significant differences in proline accumulation were observed between genotypes differing at

the *Ppd-H1* locus. Taken together short term osmotic stress affected RWC, LT and proline content, but variation at *Ppd-H1* did not cause differences in these physiological traits.

Cell-membrane stability affected by short-term osmotic stress and by natural genetic variation at *Ppd-H1*

Osmotic stress commonly leads to the production of reactive oxygen species (ROS) which in turn affect membrane integrity (Blokhina *et al.*, 2003). In order to test for the effects of osmotic stress and variation at *Ppd-H1* on ROS production and membrane integrity in barley, lipid peroxidation was analyzed as was the percentage of electrolyte leakage (EL) under osmotic stress. Changes in lipid peroxidation levels under osmotic stress were quantified by measuring malondialdehyde (MDA) content. MDA levels were increased in stressed plants in comparison to control plants 48h and 72h AT in all genotypes (Fig. 2, C and D). In general, MDA production was elevated in Scarlett(*ppd-H1*) and S42-IL107(*Ppd-H1*) compared to Triumph(*ppd-H1*) and Triumph-IL(*Ppd-H1*) under osmotic stress. The production of MDA was higher in S42-IL107(*Ppd-H1*) (0.52) than Scarlett(*ppd-H1*) (0.38) and in Triumph-IL(*Ppd-H1*) (0.31) than in Triumph(*ppd-H1*) (0.22) under osmotic stress 72h AT. The analysis of variance also showed significant genetic differences in MDA production (Supplementary Table S2 and S4), indicating a genotype dependent accumulation of MDA under osmotic stress.

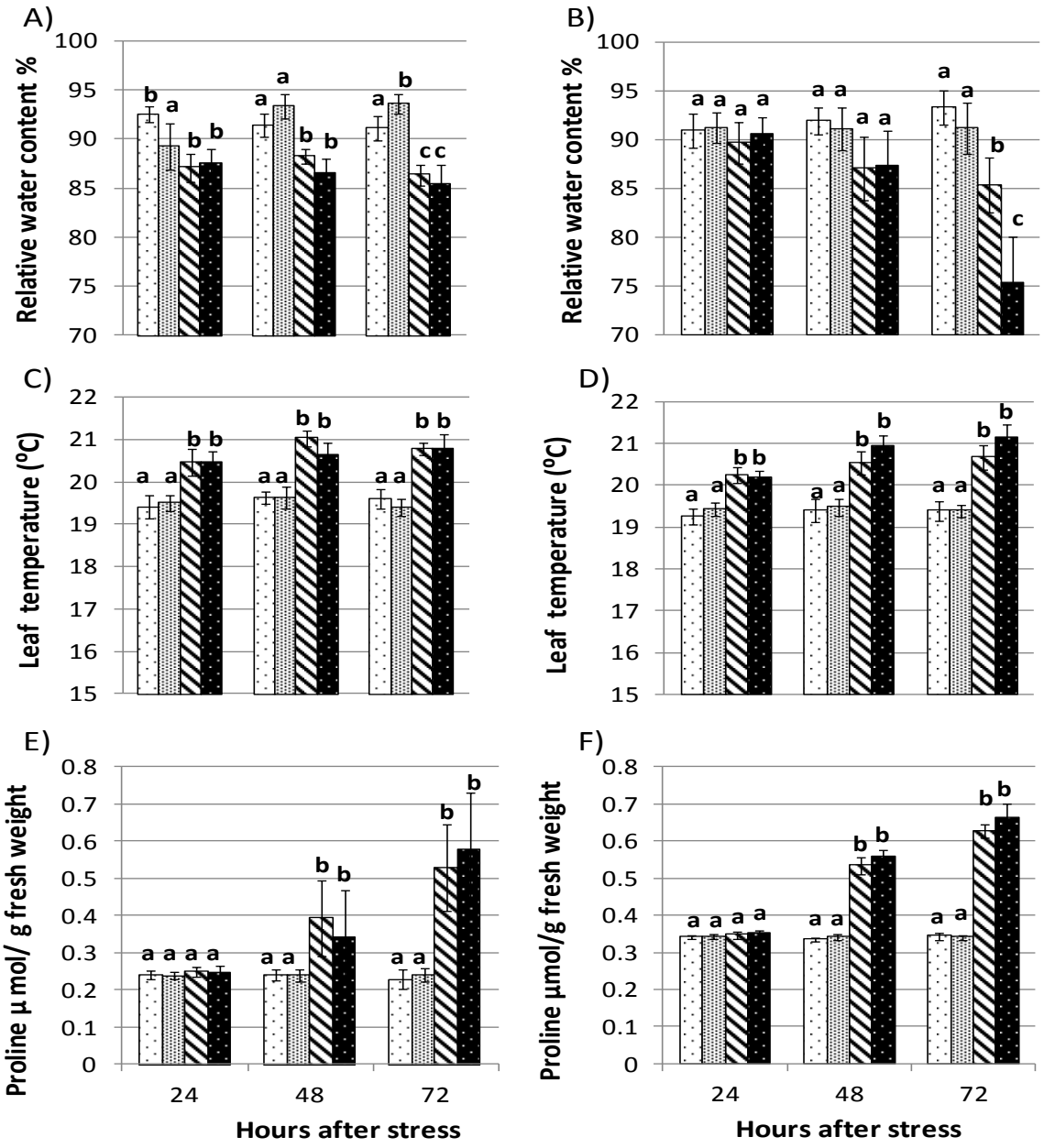


Figure 1. Effects of osmotic stress and variation at *Ppd-H1* on physiological performance in barley. Seedlings were grown in hydroponics under long days (16h light) for 10 days. Seedling were immersed in 20 % PEG at the two leaf stage or kept under control conditions for 3 days and physiological traits were measured at a 24h interval. Scarlett(*ppd-H1*) control , Scarlett(*ppd-H1*) stress , S42-IL107(*Ppd-H1*) control and S42-IL107(*Ppd-H1*) stress are shown in left panel and Triumph(*ppd-H1*) control , Triumph(*ppd-H1*) stress , Triumph-IL(*Ppd-H1*) control and Triumph-IL(*Ppd-H1*) are shown in the right panel. A) and B) Relative water content, C) and D) Leaf temperature and E) and F) Proline accumulation. Different letters indicate significant differences at $p \leq 0.05$ using least square means. Means ± standard deviation (Sd) (n = 3) are shown.

Like MDA, EL was significantly increased in stressed plants compared to control plants 48h and 72h AT in Scarlett(*ppd-H1*) and S42-IL107(*Ppd-H1*) (Fig. 2A). Triumph(*ppd-H1*) and Triumph-IL(*Ppd-H1*) showed significantly higher EL under osmotic stress compared to control conditions at all time points (Fig. 2B). The increase in EL under osmotic stress was significantly higher in S42-IL107(*Ppd-H1*) as compared to Scarlett 72h AT and in Triumph-IL(*Ppd-H1*) as compared to Triumph at all time points. The analysis of variance demonstrated a significant effect of the genotype by time point interaction between Scarlett(*ppd-H1*) and S42-IL107(*Ppd-H1*) and between Triumph(*ppd-H1*) and Triumph-IL(*Ppd-H1*) (Supplementary Table S2 and S4). EL was thus affected by osmotic stress and by genetic variation between the spring barley genotypes Scarlett and Triumph and their respective introgression lines.

Taken together, the gradual increases in MDA and EL under osmotic stress indicated the generation of free radicals and subsequent effects on cell membrane integrity. In addition, the increased production of MDA and EL in the introgression lines S42-IL107(*Ppd-H1*) and Triumph-IL(*Ppd-H1*) compared to Scarlett(*ppd-H1*) and Triumph(*ppd-H1*) showed genotype dependent accumulation of ROS and differences in cell membrane stability under osmotic stress.

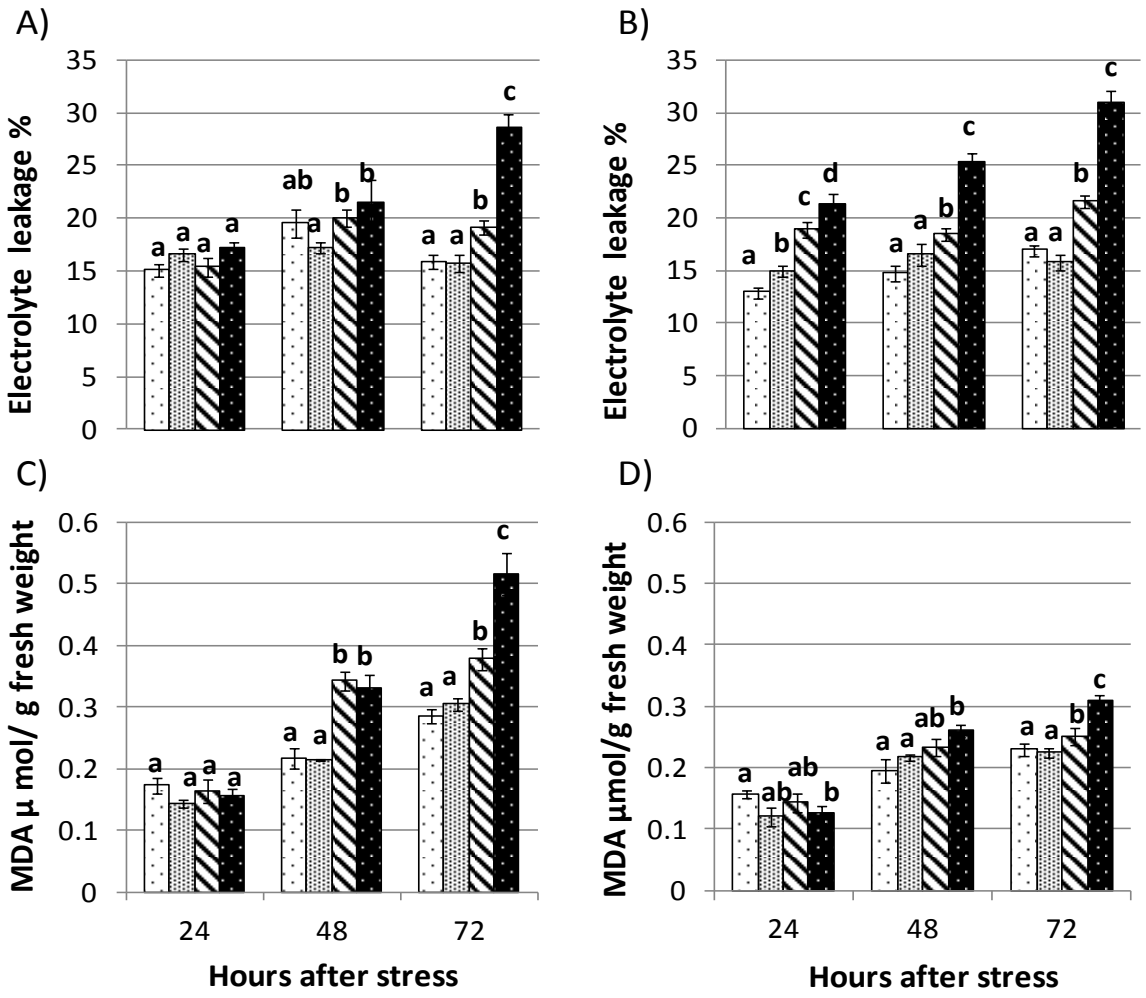


Figure 2. Effects of genetic variation (*Ppd-H1)* and osmotic stress on ROS production and cell membrane injury in barley seedlings grown under PEG-induced or under control conditions. Seedlings were grown in hydroponics under long days (16h light) for 10 days. Roots of seedlings at the two leaf stage were immersed in 20 % PEG to induce osmotic stress or were kept under control conditions for 3 days and A) and B) electrolyte leakage (EL) and C) and D) malondialdehyde (MDA) were measured at 24h intervals in Scarlett(*ppd-H1*) control , Scarlett(*ppd-H1*) stress , S42-IL107(*Ppd-H1*) control and S42-IL107(*Ppd-H1*) stress are shown in left panel and Triumph(*ppd-H1*) control , Triumph(*ppd-H1*) stress , Triumph-IL(*Ppd-H1*) control and Triumph-IL(*Ppd-H1*) are shown in the right panel. Different letters indicate significant differences at $p \leq 0.05$ using least square means. Means ± standard deviation (Sd) (n = 3) are shown.

Chlorophyll fluorescence is affected by natural genetic variation at *Ppd-H1* osmotic stress

To understand the effects of osmotic stress on photosynthesis activity, I measured the chlorophyll fluorescence transients under short-term osmotic stress. The fluorescence transients recorded included maximum quantum efficiency of photosystem II (Fv/Fm), the area above the fluorescence curve between initial fluorescence (Fo) and maximum fluorescence (Fm) (Area) and Performance index (PI). Osmotic stress had no significant effects on the expression of chlorophyll fluorescence parameters at all time points, except for 72h AT, when S42-IL107 and Triumph-IL showed a significant decrease in all three chlorophyll fluorescence parameters under osmotic stress as compared to control conditions. At the same time, no significant decrease in chlorophyll fluorescence were observed in their respective parental lines Scarlett and Triumph, except Area in Triumph and PI in Scarlett, under osmotic stress (Fig. 3, A, C and E). (Supplementary Table S2,S3,S4 and S5). Furthermore, ANOVA revealed interaction effects of genotype by treatment for all three chlorophyll fluorescence parameters in Triumph(*ppd-H1*) and Triumph-IL(*Ppd-H1*) while in Scarlett(*ppd-H1*) and S42-IL107(*Ppd-H1*) only PI had significant interaction effect (Supplementary Table S3 and S5).

Altogether, the results show that the chlorophyll fluorescence parameters were affected by osmotic stress 72 AT only in the introgression lines carrying a dominant *Ppd-H1* allele.

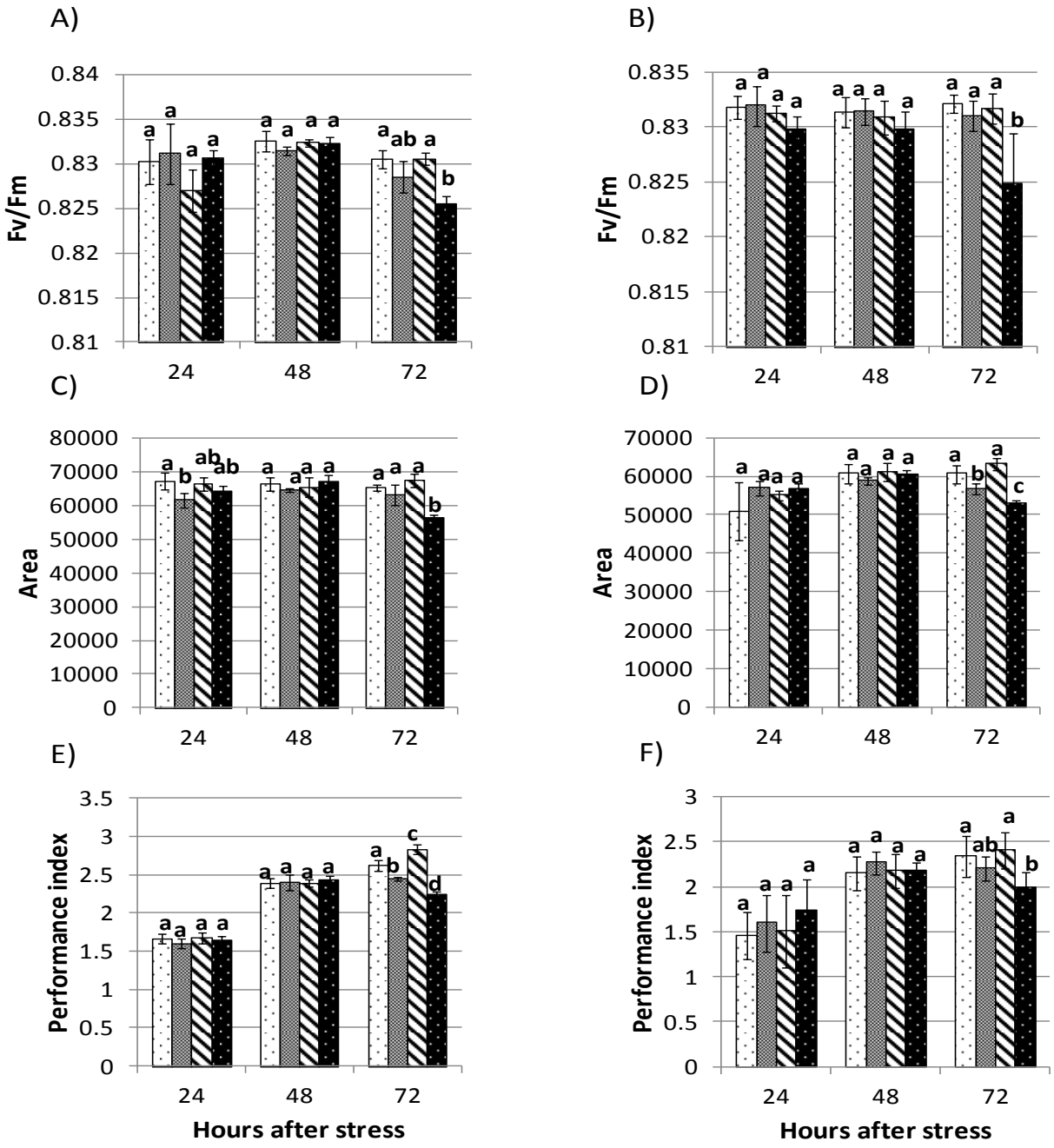


Figure 3. Effect of short-term PEG-induced osmotic stress on chlorophyll fluorescence. Seedlings were grown in hydroponics under long days (16h light) for 10 days. Roots of seedlings at the two leaf stage were immersed in 20% PEG to induce osmotic stress or were kept under control conditions for 3 days and A) and B) Fv/Fm and C) and D) Area, E) and F) Performance index were measured at 24h interval from Scarlett(*ppd-H1*) control , Scarlett(*ppd-H1*) stress , S42-IL107(*Ppd-H1*) control and S42-IL107(*Ppd-H1*) stress are shown in left panel and Triumph(*ppd-H1*) control , Triumph(*ppd-H1*) stress , Triumph-IL(*Ppd-H1*) control and Triumph-IL(*Ppd-H1*) are shown in the right panel. Different letters indicate significant differences at $p \leq 0.05$ using least square means. Means ± standard deviation (Sd) (n = 3) are shown.

Association between physiological parameters in osmotic-stress

In order to identify the relationship among physiological parameters, Pearson correlation coefficients (r) were calculated across all genotypes and time points, but separately for control and stress conditions as shown in Table 1. The results demonstrated that most of the physiological traits had higher correlation coefficients under stress than control conditions. Under stress, RWC was positively correlated with PI (0.26) and negatively with EL (-0.38), LT (-0.33), Proline (-0.3) and MDA (-0.26), but not under control condition. LT was positively correlated with EL (0.33) and MDA (0.29) under stress, while it was only positively correlated under control condition with proline (0.21). Proline accumulation was negatively correlated with chlorophyll fluorescent parameters both under stress and control conditions, while it was strongly and positively correlated with EL (0.54) and MDA (0.47) under stress conditions. Chlorophyll fluorescence parameters (Fv/Fm, Area and PI) were strongly and negatively correlated either with EL or MDA under stress condition. In addition, MDA was positively correlated with EL (0.38) under stress which indicates the oxidative damage of lipid and membrane permeability under stress conditions. Altogether, the strong associations between RWC and other physiological traits might show that responses of the physiological traits were determined by the water status of the leaves. In addition, the significant links between photosynthetic efficiency responses (Fv/Fm, Area and PI) and cell membrane stability indicators (EL and MDA) indicate that the stability of cell membrane influences the photochemical efficiency of the cell.

Table 1. Pearson correlation coefficients for physiological traits measured across all genotypes under stress (above the diagonal) or control conditions (below the diagonal)

	RWC	LT	Proline	Fv/Fm	Area	PI	EL	MDA
RWC		**-0.33***	**-0.3***	0.16	0.16	**0.26***	**-0.38***	**-0.26***
LT	-0.15		0.2	-0.21	-0.16	0.1	**0.33***	**0.29***
Proline	0.11	**0.21***		-0.21	**-0.43***	**-0.34***	**0.54****	**0.47****
Fv/Fm	0.12	0.01	0.15		**0.24***	-0.02	**-0.37***	-0.11
Area	0	**-0.22***	**-0.36***	0.07		0.15	**-0.45****	0.05
PI	0.13	-0.01	**-0.26***	0.03	**0.34***		0.15	**-0.56****
EL	0.02	0.19	-0.18	-0.08	0.08	0.19		**0.38***
MDA	0.05	-0.02	**0.29***	-0.02	**-0.26***	0.18	0.11	

Significant (*= $p<0.05$, **= $p<0.001$) coefficients are underlined. EL electrolyte leakage, RWC relative water content, Proline proline content, Fv/Fm maximum quantum efficiency of Photosystem II ,Area the area above fluorescence curve between Fo and Fm, PI performance index ,LT leaf temperature and MDA malondialdehyde

Changes of shoot apical meristem under short-term osmotic stress

Plant development and growth are affected by drought stress (Harb *et al*.,2010). In order to monitor the developmental changes under short-term osmotic stress, the development stage of shoot apical meristems (SAM) of seedlings were scored 3 d after the start of stress based on the Waddington developmental scale (Fig. 4;Waddington *et al*. 1983). The developmental stage of SAM was advanced in the introgression lines carrying *Ppd-H1* both under control and stress conditions compared to Scarlett and Triumph. However, the development of SAM was not affected by short-term stress.

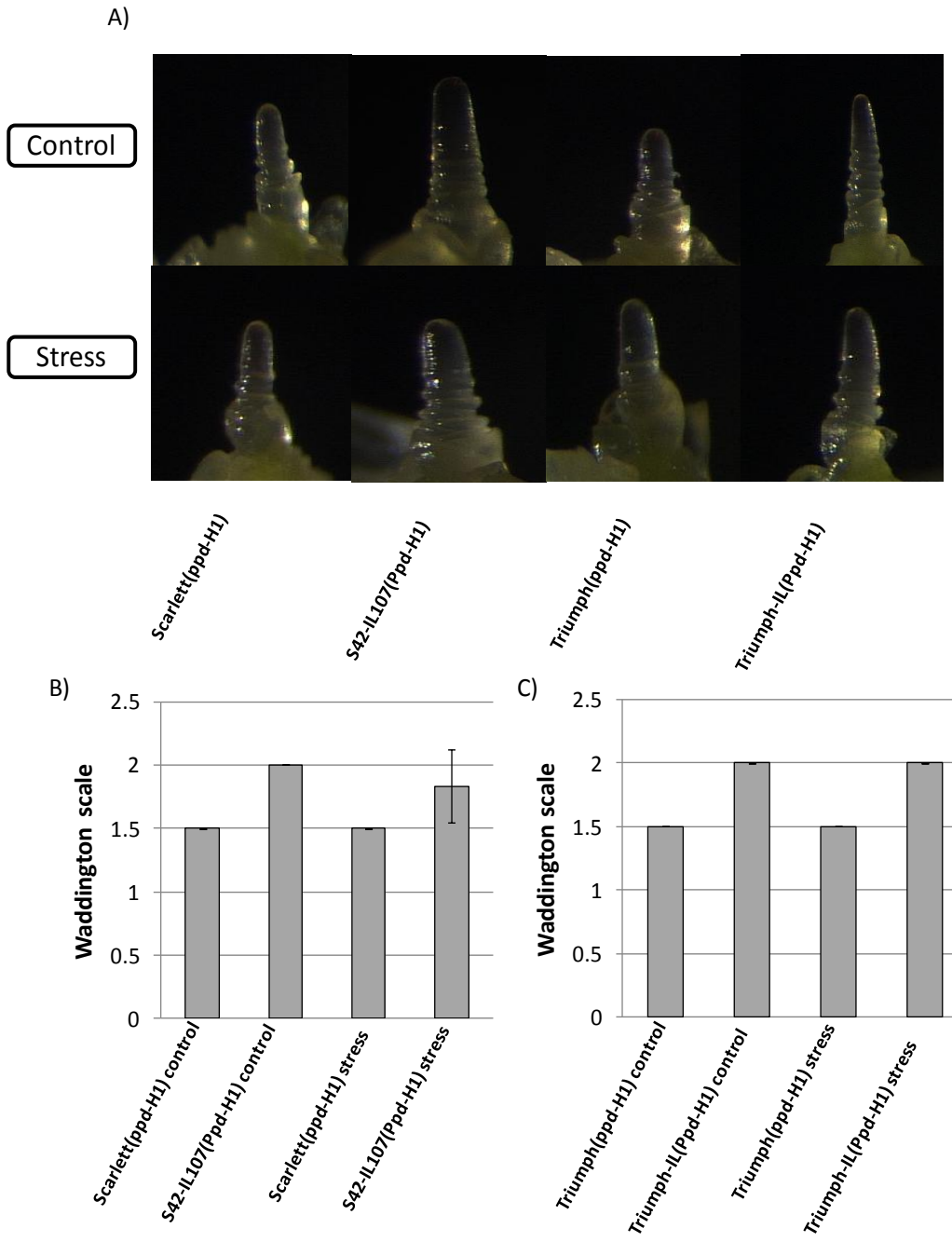


Figure 4. Shoot apical meristem in Scarlett/S42-IL107 and Triumph/ Triumph-IL. Seedlings were grown in hydroponics under long days (16h light) for 10 days. Roots of seedlings at the two leaf stage were immersed in 20 % PEG to induce osmotic stress or were kept under control conditions for 3 days. A) The picture of the meristem after 72h control/stress. Development of the shoot apical meristem under control/stress treatments in B) Scarlett and S42-IL107 and C) Triumph and Triumph-IL. Means ± standard deviation (Sd) (n = 3) are shown.

Expression of genes involved in osmotic stress signaling pathway in genotypes differing at *Ppd-H1* under osmotic stress

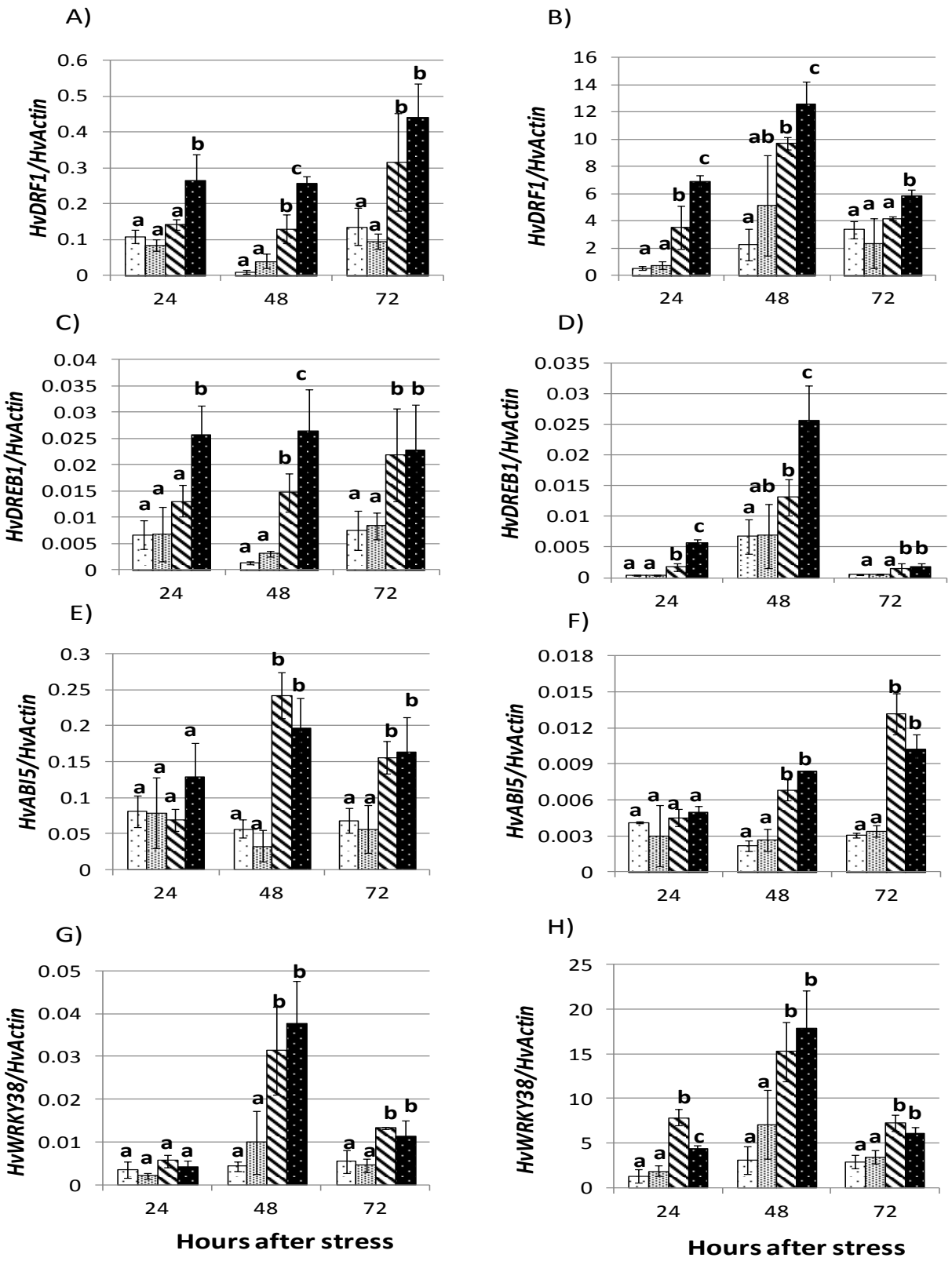
Natural variation at *Ppd-H1* affected photosynthesis parameters, generation of free radicals and cell membrane integrity. In addition, it has been shown that variation at *Pseudo Response Regulator* genes, homologous to *Ppd-H1* in barley controlled the expression of stress response genes (*DREB/CBF* genes) in *Arabidopsis thaliana* (Dong *et al*., 2011; Nakamichi *et al*., 2009). Therefore, the expression of representative genes induced by drought/osmotic stress *HvDRF1 and HvDREB1* (drought responsive element binding protein 2 like genes (*DREB2*), *HvABI5 (*ABA-responsive gene*), HvWRKY38* (ABA-responsive *WRKY* family gene), *HvA22* (ABA-induced late embryogenesis abundant protein) were tested under osmotic stress and control conditions (Shinozaki and Yamaguchi-Shinozaki *et al*.,2007; Todaka *et al*., 2012). As the introgression lines S42-IL107 and Triumph-IL carry other genes from the donor parents (Schmalenbach *et al*.,2011;Turner *et al*.,2005), I have tested the transcript profile of the stress response gene involved in cell wall extension hydroxyproline-rich glycoprotein (*HvHRGP*) (Sujeeth *et al*.,2012), from the introgressed genes in S42-IL107 (Supplementary Table S21). Moreover, the expression of *PHYTOCHROME INTERACTING FACTOR* (*PIF*), which is recently linked to abiotic stress as a negative regulator of *DREB* genes, was also tested (Kidokoro *et al*.,2009). Expression of these stress induced genes was monitored under the same conditions as used for measurements of physiological traits.

The expression of both *DREB2* like genes was elevated in stressed plants as compared to control plants in all genotypes. Under osmotic stress, S42-IL107(*Ppd-H1*) and Triumph-IL(*Ppd-H1*) showed higher levels of *HvDRF1* and *HvDREB1* transcripts 24h and 48h AT compared to Scarlett(*ppd-H1*) and Triumph(*ppd-H1*) (Fig. 5A, B, 5C and D). Under control conditions, the expression of *HvDRF1* and *HvDREB1* was not significantly different between genotypes differing at *Ppd-H1*.

The expression of *HvABI5* was significantly different between control and stress conditions 48h and 72h AT in all genotypes. However, no differences in expression of *HvABI5* were detected between genotypes differing at *Ppd-H1* both in control and stress conditions (Fig. 5 E and F). Significant differences in the expression of *HvWRKY38* between stress and control conditions were observed at 48h and 72h AT in Scarlett and S42-IL107, and in Triumph and Triumph-IL

24h, 48h and 72h AT(Fig. 5 G and H). No significant differences of *HvWRKY38* expression were recorded between genotypes under stress or control conditions. In contrast to other genes studied, the barley *PIF3* like gene (*HvPIL3*) was down regulated in stressed plants compared to control plants (Fig. 5 I and J). In Scarlett(*ppd-H1*) and S42-IL107(*Ppd-H1*) the reduction of *HvPIL3* transcript level was shown under stress at all time points, except for 24h AT when *HvPIL3* expression was not significantly different between stress and control conditions in S42-IL107 (Fig. 5I). In Triumph(*ppd-H1*) a significant difference in *HvPIL3* expression between control and stress conditions was observed 48h AT, while Triumph-IL(*Ppd-H1*) showed significant differences in *HvPIL3* expression between control and stress conditions at 24h and 48h AT. The mRNA levels of the drought and ABA-inducible gene *HvA22* was significantly higher in stress compared to control condition in all genotypes(Fig. 5, K and L). A significant difference in *HvA22* expression was observed under stress conditions for S42-IL107 compared to Scarlett at 72h AT and for Triumph-IL compared to Triumph at 48h AT. *HvHRGP* was higher expressed under stress than control conditions with no differences between genotypes,(Fig. 5 M and N).

Taken together, osmotic stress increased expression of stress-response genes and decreased the expression of *HvPIL3*. Genotypes carrying the wild type *Ppd-H1* allele showed a higher expression of *HvDRF1*, *HvDREB1* and *HvABI5* under osmotic stress compared to spring barley lines with the mutated *ppd-H1* allele.



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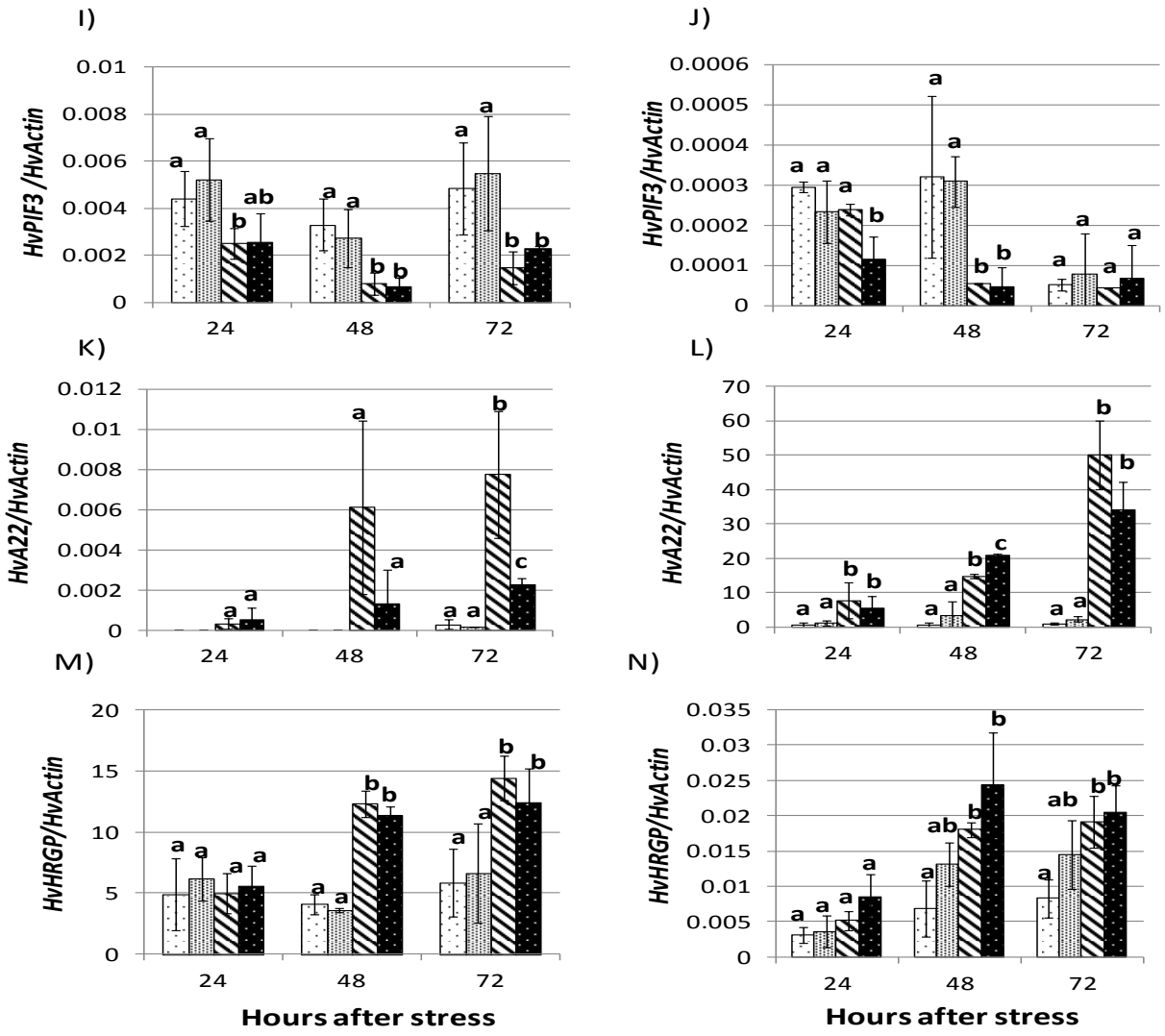

Figure 5. Effects of osmotic stress and variation at *Ppd-H1* on the expression of genes involved in stress response in barley. Seedlings were grown in hydroponics under long day (16h light) for 10 days. Roots of seedlings at the two leaf stage were immersed in 20% PEG to induce osmotic stress or were kept under control conditions for 3 days A) and B) *HvDRF1* expression, C) and D) *HvDREB1* expression, E) and F) *HvABI5* expression, G) and H) *HvWRKY*38 expression, I) and J) *HvPIL3* expression, K) and L) *HvA22* expression and M) and N) *HvHRGP* levels were analysed at 24h intervals from Scarlett(*ppd-H1*) control , Scarlett(*ppd-H1*) stress , S42-IL107(*Ppd-H1*) control and S42-IL107(*Ppd-H1*) stress are shown in left panel and Triumph(*ppd-H1*) control , Triumph(*ppd-H1*) stress , Triumph-IL(*Ppd-H1*) control and Triumph-IL(*Ppd-H1*) stress are shown in the right panel. Different letters indicate significant differences at $p \leq 0.05$ using least square means. Means ± standard deviation (Sd) (n = 3) are shown.

Differential expression of ROS scavenging and senescence activated genes under osmotic stress between genotypes differing at *Ppd-H1*

To test whether the differences observed in cell membrane damage by production of ROS were due to differences in ROS scavenging, I examined expression of ROS scavenging genes, *HvApx1* (ascorbate peroxidase) and *HvCAT1*(catalase),and senescence activated genes *HvARF1*(ADP ribosylation factor 1-like protein) and *HvGR-RBP1* (glycine-rich RNA-binding protein) (Ay *et al*., 2008; Parott *et al*., 2012). The expression levels of *HvApx1* and *HvCAT1* were elevated under osmotic stress and increased proportional to the duration of the stress treatment, except for expression levels of *HvCAT1* in Scarlett and S42-IL107 which peaked 48h and declined at 72h AT (Fig.6). Under osmotic stress, the expression levels of *HvApx1* were higher in the introgression line S42-IL107 than in Scarlett at 24h and 72h AT, and in Triumph-IL compared to Triumph 48h and 72h AT (Fig. 6, A and B). The expression of *HvApx1* was not significantly different between genotypes under control condition, except between Triumph and Triumph-IL at 48h AT (Fig. 6, A and B). The expression of *HvCAT1* was higher in Triumph-IL than in Triumph 72 h AT, but no significant genetic differences were detected between Scarlett and S42-IL107 (Fig. 6, C and D).

Under osmotic stress, the induction of *HvARF1* was higher in S42-IL107 than Scarlett at 48h AT, and in Triumph-IL compared to Triumph 24h and 74h AT (Fig. 6, E and F). *HvGR-RBP1* showed no significant induction under stress in all genotypes compared to control conditions (Fig. 6, G and H). Taken together, genetic variation in the expression of ROS scavenging gene *HvApx1* and the senescence activated gene *HvARF1* was observed under osmotic stress condition but not in control conditions.

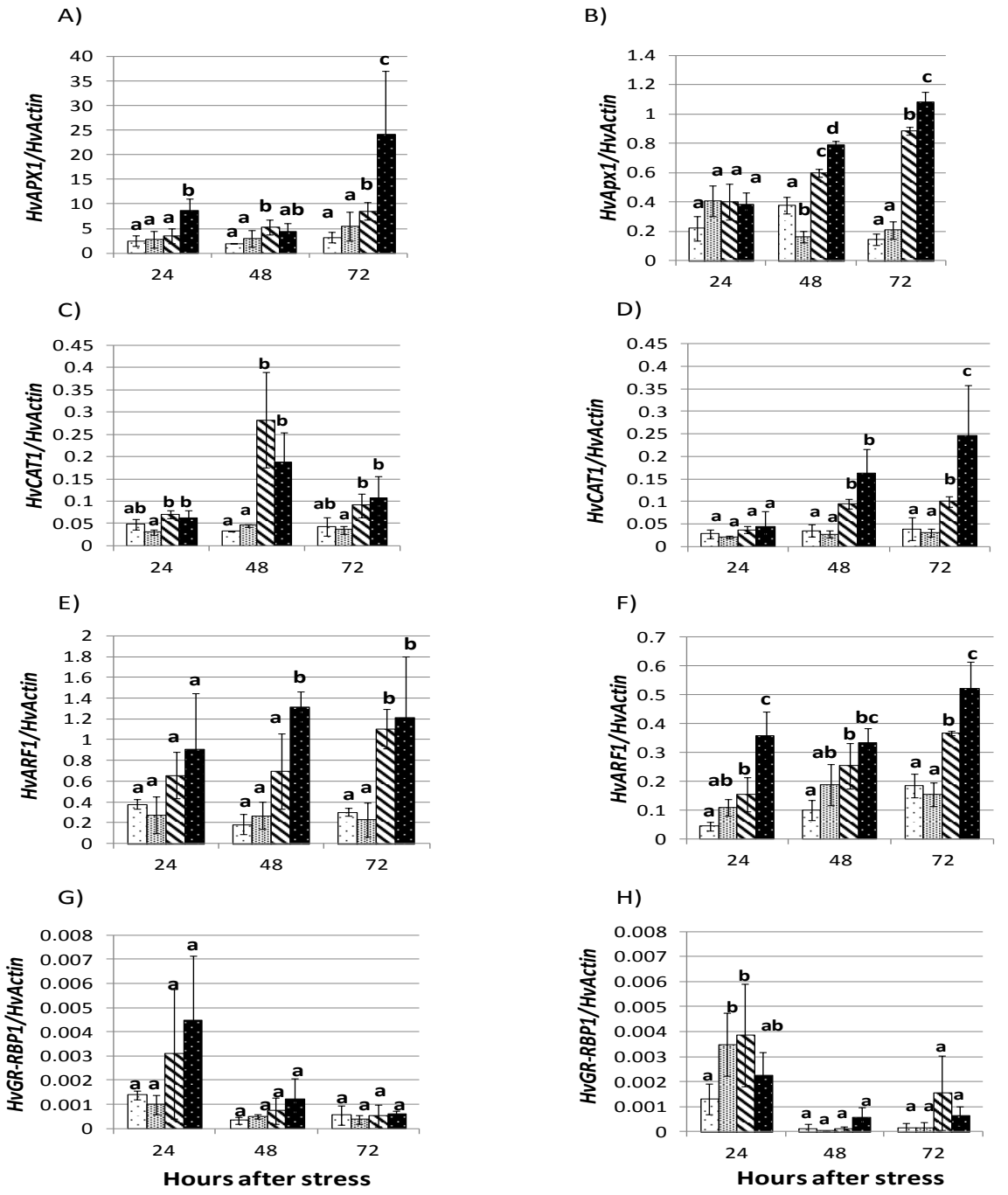

Figure 6. Effects of osmotic stress and variation at *Ppd-H1* on the expression of genes involved in ROS scavenging and leaf senescence. Seedlings were grown in hydroponics under long days (16h light) for 10 days. Roots of seedlings at the two leaf stage were immersed in 20% PEG to induce osmotic stress or were kept under control conditions for 3 days. A) and B) *HvAPX1* expression, C) and D) *HvCAT1* expression, E) and F) *HvARF1* expression and G) and H) *HvGR-RBP1* expression levels were analysed at 24h intervals from Scarlett(*ppd-H1*) control ⬚ , Scarlett(*ppd-H1*) stress ▨ , S42-IL107(*Ppd-H1*) control ▩ and S42-IL107(*Ppd-H1*) stress ■ are shown in left panel and Triumph(*ppd-H1*) control ⬚, Triumph(*ppd-H1*) stress ▨ , Triumph(*Ppd-H1*) control ▩ and Triumph(*Ppd-H1*) ■ are shown in the right panel. Different letters indicate significant differences at $p \leq 0.05$ using least square means. Means ± standard deviation (Sd) (n = 3) are shown.

Correlation between candidate drought stress gene expression under osmotic-stress

To determine co-expression of genes, pairwise Pearson correlation coefficients (r) were calculated for drought-responsive genes across genotypes of all time points and treatments combined. The strongest significant correlations were detected between *HvABI5* and *HvHRGP* (0.88),followed by between *HvHRGP* and *HvAPx1* (0.86) and between *HvDRF1* and *HvWRKY38* (0.83). Senescence activated gene *HvARF1* was positively correlated with stress induced genes such as with *HvAPx1* (0.73), *HvABI5* (0.64) and with *HvHRGP* (0.65), indicating strong association between senescence activated genes and stress-responsive genes. Furthermore, there was strong correlation between ABA-induced *HvABI5* with *HvDRF1* (0.42) and with *HvDREB1* (0.70) suggesting the induction of both ABA-dependent and ABA-independent in osmotic stress. *HvPIL3* was negatively correlated with most of stress-responsive genes including with *HvCAT1* (-0.44), *HvARF1* (-0.43) and *HvDRF1* (-0.39). Similarly *HvABI5* was also negatively correlated with *HvWRKY38* (-0.30).

Table 2. Pearson correlation coefficients for osmotic-stress induced gene expression measured across all genotypes ,conditions and time points

	HvDRF1	*HvDREB1*	*HvABI5*	*HvWRKY38*	*HvPIL3*	*HvA22*	*HvHRGP*	*HvApx1*	*HvCAT1*	*HvARF1*	*HvGR-RBP1*
HvDRF1		0.13	**0.42***	**0.83****	**-0.39***	0.27	**-0.49****	**0.30***	0.06	-0.2	-0.18
HvDREB1	0.13		**0.70***	0.16	**-0.39***	0.09	**0.51****	**0.45***	**0.48****	**0.52****	0.09
HvABI5	**0.42***	**0.70***		**-0.30***	-0.12	0.02	**0.88****	**0.64****	0.2	**0.67****	0.2
HvWRKY38	**0.83****	0.16	**-0.30***		**-0.32***	**0.62****	**-0.36***	-0.24	-0.05	-0.19	-0.07
HvPIL3	**-0.39***	**-0.39***	-0.12	**-0.32***		-0.27	0.01	-0.16	**-0.44***	**-0.43***	0.05
HvA22	0.27	0.09	0.02	**0.62****	-0.27		-0.03	-0.07	0.16	0.03	0.2
HvHRGP	**-0.49****	**0.51****	**0.88****	**-0.36***	0.01	-0.03		**0.86****	0	**0.65****	0.15
HvApx1	**0.30***	**0.45***	**0.64****	-0.24	-0.16	-0.07	**0.86****		0.24	**0.73****	0.13
HvCAT1	0.06	**0.48****	0.2	-0.05	**-0.44***	0.16	0	0.24		**0.42****	-0.17
HvARF1	-0.2	**0.52****	**0.67****	-0.19	**-0.43***	0.03	**0.65****	**0.73****	**0.42****		0.2
HvGR-RBP1	-0.18	0.09	0.2	-0.07	0.05	0.2	0.15	0.13	-0.17	0.2	

Significant (*=p<0.05, **=p<0.001) coefficients are underlined.

Seed germination affected by allelic variations at *Ppd-H1* under exogenous ABA application

To investigate the effects of the stress hormone ABA on germination of barley seeds differing at *Ppd-H1*, seeds of Scarlett, Triumph and the respective introgression lines were germinated on increasing concentrations of ABA. Increasing concentrations of ABA reduced the germination of barley seeds in all genotypes after 3 days of imbibition. Interestingly, the introgression lines S42-IL107 and Triumph-IL were more sensitive to ABA application particularly to ABA concentrations of 50 µM and 100 µM compared to the cultivars Scarlett and Triumph (Fig. 7).The germination percentage of S42-IL107 and Triumph were 40 % and 15 % respectively, while Triumph and Scarlett cultivars had a germination percentage 60 % and 80 % respectively under 50 µM ABA concentrations. Under a concentration of 100 µM ABA, seeds from both introgression lines did not germinate, but the seeds from Triumph and Scarlett cultivars showed germination percentages of 35% and 20% respectively. These results suggest a hypersensitivity of the introgression lines carrying *Ppd-H1* to exogenous ABA application.

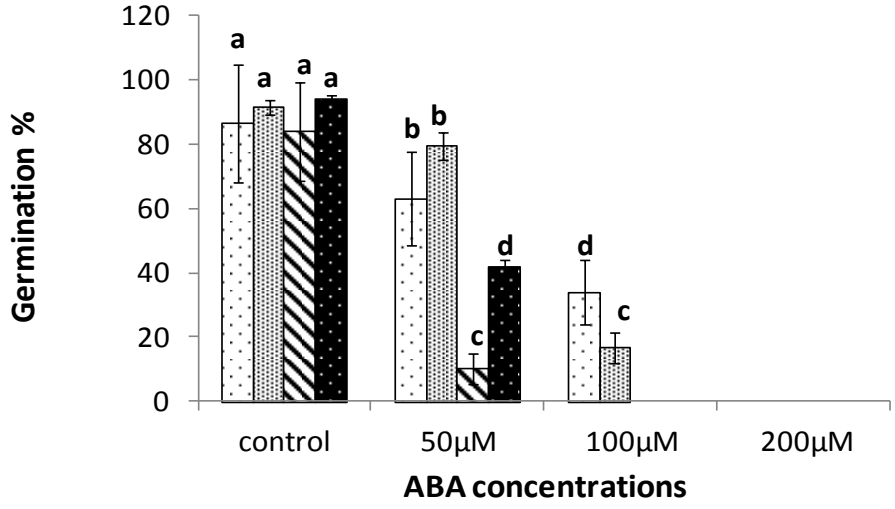

Figure 7. The effect of ABA on seed germination of barley genotypes differing at *Ppd-H1*. Germinated seeds of Triumph(*ppd-H1*), Scarlett(*ppd-H1*), Triumph-IL(*Ppd-H1*) and S42-IL107(*Ppd-H1*) were counted after three days of imbibition either in ABA solution of different concentrations or in water (control). Means ± standard deviation of three independent experiments are shown. Different letters indicate significant differences at $p \leq 0.05$ using least square means.

Discussion

Short term Osmotic stress affects water status of barley seedlings

Osmotic stress causes dehydration in plant tissues which in turn affects various physiological traits in plants (Farooq *et al*., 2009). Physiological parameters such as relative water content (RWC), leaf temperature (LT) and proline content are among the traits used to describe how the plant responds to the water deficit (Anjum *et al*.,2011). In the present study, the application of PEG-induced short-term osmotic stress enabled me to evaluate the effects of variation at *Ppd-H1* on physiological responses in barley seedlings. These results showed that the earliest responses to osmotic stress, observed already 24h AT, were a reduced RWC, increased LT, electrolyte leakage and MDA, followed by changes in proline content 48h AT. Changes in photosynthesis were only observed 72h AT (Fig. 1, 2, 3). Similar observations were reported by Kocheva *et al*., (2005), who showed a significant reduction in the water status of the leaves in barley seedlings subjected to PEG-induced osmotic stress, while the photosystem II was only weakly affected. An increased LT was among the earliest responses to osmotic stress and thus supported previous findings that stomata closure and reduced leaf transpiration are among the earliest plant responses to water deficit (Chavez *et al*., 2003). A decrease in RWC was followed by an increase in proline 48h AT. The accumulation of osmolytes, such as proline, decreases the cell osmotic potential and thus helps the plant to maintain the cell homeostasis and improves drought tolerance. (Armenguad *et al*., 2004; Hong *et al*., 2000; Nayar *et al*., 2003; Sharma *et al*., 2011). The fastest physiological responses were thus drought avoidance strategies, such as reduced transpiration, followed by strategies to improve drought tolerance through proline production. Finally, reduced photosynthesis rates 72h after stress application indicated damage of the cell due to increasing stress. Increased damage of the cell was indicated by an increased EL and thus reduced cell membrane stability under osmotic stress at 48h AT and thus before the reduction of phototsynthesis parameters (Fig 2.). Indeed, the observed negative correlation between EL and chlorophyll fluorescence parameters (Fv/Fm, Area and PI) (Table 1.) suggested that the cell membrane integrity is important to sustain photosynthesis during osmotic stress. Cell membrane stability was likely affected by the production of ROS as indicated by increased levels of MDA under osmotic stress (Fig 2.). The increase in MDA levels was gradual and depended on the length of stress duration, suggesting a progress of oxidative damage. Reactive oxygen species (ROS) are known to be enhanced by drought stress when excited electrons move to oxygen

molecules rather than being used for carbon assimilation (Moradi and Ismail, 2007). ROS can also cause serious damage by oxidizing multiple cellular components including cellular membranes (Noctor *et al*., 2002). Therefore, plants develop a mechanism of detoxification of ROS by inducing scavenging enzymes and protecting compatible solutes such as proline (Chinnusamy *et al*., 2007). The positive correlations obtained in this study between proline with EL and MDA suggest that proline might be involved in ROS scavenging and membrane protection (Table 1). The higher correlation coefficients between photosynthesis parameters and EL, MDA and proline compared to RWC, suggested that oxidative damage was the primary cause for a reduction in photosynthesis and not the reduced RWC in cells.

Several studies have shown that osmotic stress accelerates leaf senescence which exhibits similar symptoms as those recorded under stress in this study. Merewitz *et al*., (2011) showed that delayed leaf senescence resulted in reduced electrolyte leakage (EL) and higher chlorophyll fluorescence parameters in transgenic bentgrass under water stress. In addition, membrane stability and the disruption of light harvesting and electron transfer complex in chloroplast is associated with leaf senescence. In addition, Rivero *et al*., (2007) found that accelerated leaf senescence was affected by environmental stress such as drought. Therefore, these results suggested that osmotic stress accelerated leaf senescence in the present experiment.

Photosynthesis is among plant processes that are affected by drought/osmotic stress as a result of reduced CO_2 diffusion, due to stomatal closure and enhanced oxidative stress damage under water deficit conditions (Chavez *et al*., 2009). The present study indicated that the maximal quantum efficiency of photosystem II (Fv/Fm) was significantly reduced in stressed plants after 72h stress as compared to controls. In addition, the PI was significantly reduced under stress which suggests that both the light-dependent and light-independent mechanisms, e.g. carbon fixation, were damaged or inhibited due to stress and reduced gas exchange. Thus, the osmotic stress had a detectable effect of photoinhibition and the availability of carbon dioxide was probably limited by stomata closure (Fig. 3). Previous reports have indicated that PI is more sensitive than Fv/Fm suggesting that the dark reaction of photosynthesis is more sensitive than the light reaction to short-term water deficit (Strasser *et al*., 1995; Živčák *et al*., 2008). However, the finding from this study suggests that under PEG induced osmotic stress, both the light and dark reaction show similar reactions. The observed reductions in the chlorophyll fluorescent

parameters might indicate permanent damage to the aforementioned photosynthetic apparatus by short-term osmotic stress.

The present study showed that RWC was positively correlated with chlorophyll fluorescent parameters indicating that the observed responses under short term osmotic stress are a function of the water status of the seedlings. Previous studies have shown that chlorophyll fluorescence parameters are not affected under mild drought stress. Rollins *et al*. (2013) described that chlorophyll fluorescence parameters are only affected when the RWC dropped below 80% in adult barley plants.

Expression of stress-responsive genes under PEG-induced osmotic stress in barley

The plant's response to drought is accompanied by the induction of genes involved in protection of cells from stress such as LEA protein, ROS scavenging genes and transcription factors (TFs) that are involved in signal transduction and gene modulation (Lata and Prasad, 2011). All analysed candidate genes were regulated by osmotic stress. The majority of stress responsive genes in particular TFs were upregulated between 24-48h AT and were downregulated again 72h AT. Early and transient up-regulation of the TFs suggest their involvement early stress response (signal), while the expression of ROS scavenging and LEA genes continued to increase between 24 and 72h AT (Fig. 5 and 6).

In the present study, osmotic stress enhanced the expression of *DREB2* like genes (*HvDRF1* and *HvDREB1*). It is known that *DREB2s* are AP2/ERF containing TFs and are involved in ABA-independent drought stress signaling in Arabidopsis and rice (Shinozaki and Yamaguchi-Shinozaki, 2007). These TFs interact with a *cis*-acting DRE elements and activate downstream genes involved in drought stress responses and drought tolerance in plants. Several studies have shown the induction of *DREB2*-type genes in different plant species for example *TaDREB1* (Shen *et al*., 2003) and *Wdreb2* (Egawa *et al*., 2006) in wheat, *HvDRF1* (Xue and Loveridge, 2004) and *HvDREB1* (Xu *et al*., 2009) in barley and *ZmDREB2A* (Qin *et al*., 2007) in maize under stress. The results from this study thus confirm previous results and suggest that the PEG treatment induced ABA-independent stress response pathways.

Similar to DREB2 like genes, *HvABI5* was induced by osmotic stress.Casaretto and Ho, (2003) demonstrated that *HvABI5* is an important basic leucine zipper (bZIP) transcriptional activator involved in the activation of ABA-induced gene expression by binding the promoter regions containing ABRE *cis*-acting elements. Upregulation of *HvABI5* under osmotic stress thus suggested the induction of ABA signaling pathways under PEG. This is in line with previous studies in grasses which showed the induction of OsbZIP46 rice homologous to *ABI5*, *HvABI5* in barley and *WABI5* in wheat by drought and ABA (Casaretto and Ho, 2003; Kobayashi *et al*., 2008; Tang *et al*., 2012).

In the present study, *HvWRKY38* was up-regulated by osmotic stress. Barley *HvWRKY38* is an ortholog of *AtWRKY40* in Arabidopsis, which is activated by drought, cold as well as by ABA (Mare`et al. 2004; Xie *et al*.,2007). Earlier reports have indicated that *AtWRKY40* directly targets a number of AP2/ERF and bZIP TFs by binding to W box sites of the promoters in *ABI4*, *ABI5*, *ABF4*, and *DREB1A* genes and thereby negatively regulating ABA signaling (Rushton *et al*., 2012). In addition it has been demonstrated that *AtWRKY40* is placed upstream of bZIP transcription factor of *ABI5* (Shang *et al*., 2010). In the present study, *HvWRKY38* and *HvABI5* expression was negatively correlated. *HvWRKY38* may thus have similar targets as *AtWRKY40* in Arabidopsis.

PHYTOCHROME-INTERACTING FACTOR (*PIFs*) are a basic helix loop helix (bHLH) family TFs that directly interacts with phytochromes under specific light conditions (Castillon *et al*., 2007). However, recent studies linked *PIF*s to stress responses. For example, *PIF7* was demonstrated to be a repressor of the stress-responsive *DREB1* gene in Arabidopsis (Kidokoro *et al*., 2009), and a *PIF* like gene in rice (*OsPIL1*/*OsPIL13*) is involved in internode elongation under drought stress (Todaka *et al*., 2012). Similar to these findings, the present study showed that the *PIF3* like gene in barley (*HvPIL3*) was down-regulated under stress. Moreover, the correlation analysis revealed negative association of *HvPIL3* gene with *DREB2* like genes under stress suggesting it may have negative regulatory function similar to Arabidopsis *PIF7* gene. Since *PIF* like genes are strongly regulated by stress are important for plant growth, it would be important to elucidate their mechanism of action so that they can incorporated in future drought tolerance breeding applications.

The present study showed a clear induction of *HvA22* under osmotic stress, while its expression was low or not detected under control condition. Previous reports showed that LEA protein *HvA22* is regulated by environmental stress and by developmental cues (Sivamani *et al*., 2000 ; Shen *et al*., 2001), which provides cell tolerance to seed desiccation and environmental stresses. Therefore, the induction of *HvA22* under stress suggests its important role in protecting cells from damage.

ROS scavenging and senescence activated genes were altered by osmotic stress

ROS production is a common phenomenon during osmotic stress in plants due to perturbation of photosynthesis. This increased ROS production in the cell is counteracted by antioxidant enzymes such as peroxidases, catalases and dismutases and their induction is correlated with the severity of stress (Grene, 2002; Miao *et al*., 2006; Miller *et al*., 2010). Similarly, the present study showed that the expression of ROS scavenging genes *HvAPX1* and *HvCAT1* were increased and their induction correlated with increased levels of lipid peroxidation (MDA) and electrolyte leakage (EL) observed under osmotic stress. Interestingly, the senescence activated gene *HvARF1* (Ay *et al*., 2008) was upregulated under osmotic stress even before the ROS scavenging genes. *ARF1*s (ADP ribosylation factor-1 proteins) regulate membrane trafficking, organelle structure and vesicle transport processes and has thus a potential role in the senescence process. Therefore, the induction of *HvARF1* by osmotic stress support the findings that senescence related processes are an immediate response to osmotic stress even at the seedling stage.

Variation at *Ppd-H1* affects osmotic stress in barley

The present study revealed genetic differences in physiological and molecular responses to osmotic stress, suggesting that variation at *Ppd-H1* affected osmotic stress responses. In particular, the wild type *Ppd-H1* allele was associated with higher levels of EL and MDA 48h AT and reduced photosynthesis rates 72h AT, while RWC and LT were not significantly different between genotypes. This indicates that variation at *Ppd-H1* affect ROS production and the expression of ROS scavenging genes. Consistent with these observations the expression of the ROS scavenging gene *HvAPX1* was strongly induced in the introgression lines with a dominant *Ppd-H1* allele. In addition, the strong negative correlation between chlorophyll

fluorescence parameters and electrolyte leakage (Table 1.) suggested that increasing oxidative damage affected photosynthesis activity negatively. This effect was more pronounced in the introgression lines with the wild type *Ppd-H1* allele. Induction of the senescence-activated gene *HvARF1* in the introgression lines indicated that *Ppd-H1* controlled stress induced senescence consistent with the increased EL in the introgression lines.

Ppd-H1 might control stress-response either a) through its putative function in the barley circadian clock, or b) through clock independent functions or c) as a major photoperiod response and developmental gene. Finally, the linkage drag effect of other genes found in the introgression lines might also be responsible for the observed variation. Two different introgression lines with the *Ppd-H1* allele derived from very different genetic backgrounds, a wild and a cultivated winter barley were used to control for background genes in the introgressions. We tested *HvHRGP* as the only gene within the introgression, annotated as stress responsive and differing between Scarlett and S42-IL107 (Supplementary Table S11). The expression of *HvHRGP* was not significantly different between genotypes varying at *Ppd-H1* under both control and stress conditions (Fig. 4 M and N) indicating the observed differences may not be due to this gene. But still other genes, not yet functionally characterised and present in the introgressions might have a role in the stress responses.

In Arabidopsis, the circadian clock is implicated in the adaptation to environmental stresses (Sanchez *et al*., 2011), suggesting that the clock may also control stress adaptation in barley. A recent study found that ROS-responsive genes, hydrogen peroxide production and scavenging were under circadian control and controlled by the circadian clock gene *CCA1* in Arabidopsis (Lai *et al*. 2012). The circadian clock thus affected the transcriptional regulation of ROS-responsive genes, ROS homeostasis, and tolerance to oxidative stress. *Ppd-H1* is a barley ortholog of the *PRR* gene family from the core oscillator in Arabidopsis. The potential role of *Ppd-H1* in controlling stress-response through its presumed function in the circadian clock is discussed in detail in chapter 3. Here, I would only like to point out that the natural mutation in the CCT domain of *Ppd-H1* does not alter the circadian rhythm or expression of any core clock orthologs in barley (Campoli *et al*. 2012). *Ppd-H1* may thus affect osmotic stress responses independently of its function in the clock. In line to this, a recent report by Liu *et al*. (2013) showed that *PRR7* directly binds to and regulates several stress-responsive genes in Arabidopsis such as the ROS detoxifying gene superoxide dismutase and the transcription factors

*CBF*s/*DREB*s .Furthermore, Nakamichi *et al*. (2009) demonstrated that *PRR9*, *PRR7* and *PRR5* in Arabidopsis are involved in a mechanism that initiates a stress response by mediating cyclic expression of the stress response genes *DREB1*/*CBF*. In the present study, the expression of stress responsive genes such as *DREB2* like genes (*HvDRF1*and *HvDREB1*) were strongly up-regulated in the introgression lines carrying *Ppd-H1* compared to genotypes carrying the mutated *ppd-H1* allele under stress. This differential induction of stress-responsive genes might display differences in the binding and thus regulatory efficiency between the two alleles. In contrast to the Arabidopsis *prr* mutants with abolished gene function (Nakamichi *et al*., 2009), *ppd-H1* is a hypomorphic allele with reduced functionality (Campoli *et al*., 2012). The mutation in *ppd-H1* correlates with lower expression levels of *HvFT1*, the barley ortholog of Flowering Locus T in Arabidopsis, and delayed flowering under long day conditions. The developmental difference is already expressed at early stages of development where the meristem stage of the introgression lines carrying *Ppd-H1* was advanced compared to genotypes carrying *ppd-H1* (Fig.4). This developmental difference between introgression lines and genotypes carrying *ppd-H1* might also influence the osmotic stress responses. Parrot *et al*., (2012) showed that early flowering is linked to an early senescence as evidenced from induction of senescence regulated genes. Senescence is driven primarily by endogenous factors but is also influenced by environmental conditions, such as abiotic stress. Reproductive changes and abiotic stresses can lead to the accelerated production of ROS and the subsequent onset of cell death (Ahmed *et al*. 2009). Generally, the increase in oxidative stress occurs with increase in plant age, especially in chloroplasts. As such it is interesting to note that variation at *Ppd-H1* affected lipid peroxidation and expression of *HvAPX1* already at an early seedling stage. Genotypes with a wild type *Ppd-H1* allele are adapted to environments with terminal drought as an accelerated development represent a drought escape strategy. Second, these genotypes also show a different response to osmotic stress and this might be an indirect consequence of the advanced development or an independent effect of *Ppd-H1* on response to oxidative stress. I can only speculate that enhanced senescence under stress could be beneficial because i) it affects nutrient remobilization during stress, so that newly growing parts of the plant would benefit from the mobilized nutrients, ii) a reduction in the growing biomass might minimize water loss through transpiration, thus contributing to the maintenance of water balance of the plant. In conclusion, variation at *Ppd-H1* affected stress induced leaf senescence and induction of ROS scavenging genes. However, further

physiological, molecular and genetic studies would be required to determine how *Ppd-H1* affects adaptation to water deficit conditions in barley.

Ppd-H1 sensitivity to ABA-mediates seed germination

Here I report the possible link of ABA and a circadian clock gene *Ppd-H1* in seed germination. ABA plays a key role in broad array of developmental processes and triggers plant responses to several environmental stimuli including water deficit (Fujita *et al*., 2011). In addition, ABA is also known as repressor of seed germination by stabilizing the dormant state (Holdsworth *et al*., 2008). The present study indicated that the application of exogenous ABA resulted in reduced seed germination (Fig. 7) as demonstrated by Ramagosa *et al*., (2001) and Bradford *et al*., (2008). Interestingly, the introgression lines carrying *Ppd-H1* showed an increased sensitivity to ABA, as seen by the reduced germination of introgression lines under ABA. In addition, germination was not altered between genotypes varying at *Ppd-H1* in non-ABA treated seeds, suggesting the underlying endogenous ABA level did not significantly differ between the genotypes.

A recent report reported that circadian clock is linked to seed germination in Arabidopsis (Penfield and Hall, 2009). They showed that the double mutant *lhy cca1* and single mutant *gi* had a reduced dormancy. The circadian clock might thus control dormancy and seed germination. The metabolism of hormones such as ABA and GI, which are involved in seed germination, is known to be controlled by circadian clock (Yakir *et al*.,2007). In addition, the Arabidopsis *PRR7* gene was shown to be involved in the regulation of ABA-responsive genes (Liu *et al*., 2013). Like in Arabidopsis, the clock ortholog *Ppd-H1* might have a role in ABA-mediated seed germination possibly via ABA signaling pathways.

Seed germination is a very complex process that involves both genetic and environmental factors. The genetic factors determine the balance between GA and ABA pathways which is critical in determining seed germination (Jacobson *et al*., 2002). However, I tested only the effects of exogenously applied ABA. Future studies on the role of *Ppd-H1* should consider its effect on the giberellic acid (GA) pathway to obtain a clearer picture of the interactions between *Ppd-H1* and seed germination in cereal crops.

Conclusion

PEG-induced short-term osmotic stress caused reductions in the leaf relative water content, in electrolyte leakage and lipid peroxidation, which are indicative of oxidative stress and this resulted in a decrease of the photosynthesis rates. Variation at *Ppd-H1* affected stress induced senescence at the seedling stage, germination under ABA, and expression of ABA dependent genes and genes involved in senescence processes. It is interesting to observe that *Ppd-H1*, known as a major regulator of flowering under long days, also affected germination and stress-induced senescence. Coordination of these key developmental processes is important for successful adaptation to different environments. Plant hormones play an important role to coordinate developmental events in the plants (Gray, 2004). *Ppd-H1* might thus affect important life cycle events through affecting hormone signaling in plants. Future studies should focus on the role of *Ppd-H1* in controlling ABA and GA signaling in plants.

Chapter Two- Osmotic Stress at the Barley Root Affects Expression of Circadian Clock Genes in the Shoot

Introduction

Abiotic stresses such as drought, salinity and heat are amongst the greatest problems facing agriculture today. Under stress, plants undergo a series of morphological, physiological, biochemical and molecular changes which adversely affect their growth and productivity, but also improve adaptation and survival (Ingram and Bartels, 1996; Harb *et al*., 2010). Adaptive responses to stress can be grouped into three major classes (a) osmotic homeostasis; (b) stress damage control and repair; and (c) growth control (Zhu, 2002). Drought stress signaling pathways have been classified at the molecular level into abscisic acid (ABA)-dependent and ABA-independent pathways that regulate the expression of stress-responsive genes (Yamaguchi-Shinozaki and Shinaozaki, 2005). The ABA RESPONSIVE ELEMENT BINDING PROTEINS, such as the AREB/ABF regulons are involved in ABA-dependent gene expression and regulate downstream genes harbouring ABA RESPONSIVE ELEMENTS (ABRE) in their promoters. ABA independent stress signaling is mediated by the *DEHYDRATION RESPONSIVE ELEMENT BINDING PROTEIN2* (*DREB2*), which binds to downstream genes that contain DROUGHT RESPONSIVE ELEMENTS (DRE) in their promoters (Agarwal *et al*., 2006). In both the ABA-dependent and ABA-independent pathways, transcription factors such as *DREB2*, *ABI5* and *WRKY* bind to specific *cis*-elements and induce stress-responsive genes. These genes have roles in a) osmotic homeostasis and control stomatal opening, e.g. the LIGHT HARVESTING CHLOROPHYLL A/B BINDING PROTEIN (LHCB) (Xu *et al*.,2012) and PHYTOCHROME B (PHYB) (Gonzalez *et al*., 2012) b) in stress detoxification and scavenging of REACTIVE OXYGEN SPECIES (ROS), e.g. CATALASES (*CAT*) and PEROXIDASES (*APX*) and c) in growth control, e.g. the phytochrome-interacting factor-like proteins (PIF) (Todaka *et al*., 2012). These genes and their functions have been unraveled in the model plant Arabidopsis, however, much less is known about their functions in crop plants which commonly grow under stress in the field.

The circadian clock is an important system that controls stress adaptation in plants by coordinating their metabolism and development with predicted daily and seasonal changes of the environment (Kant *et al*., 2008; Dong, Farré and Thomashow, 2011; Sanchez, Shin and Davis,

2011). The circadian clock is an autonomous oscillator that produces endogenous biological rhythms with a period of about 24 hours. Conceptually, a circadian system can be divided into three parts: the central oscillator, input and output pathways. In the model plant *Arabidopsis thaliana*, the central oscillator is composed of three negative feedback loops: (a) the inhibition of evening complex (EC) genes *EARLY FLOWERING 3* (*ELF3*), *EARLY FLOWERING 4* (*ELF4*), and *LUX ARRHYTHMO* (*LUX*) by the rise of *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*) late at night, (b) the inhibition of *PSEUDO RESPONSE REGULATOR* genes (*PRR*) by the EC early at night, and (c) the inhibition of *LHY*/*CCA1* by *TIMING OF CAB EXPRESSION1* (*TOC1*) in the morning (Pokhilko *et al*., 2012; Bujdoso and Davis, 2013). Furthermore, the evening-expressed *GIGANTEA* (*GI*) protein was modeled as a negative regulator of the EC, which in turn inhibits *TOC1* expression (Pokhilko *et al*., 2012). The internal circadian rhythms are entrained to external conditions by daily changes in light and temperature (Boikoglou and Davis 2009, Boikoglou *et al*., 2011). The central oscillator controls of a large fraction of the Arabidopsis transcriptome, in particular genes from the plant hormone and stress-responsive pathways (Covington *et al*., 2008; Staiger *et al*., 2013). The clock thus modulates or "gates" plant hormone sensitivity and response to daily changes in temperature, water availability and irradiance (Robertson *et al*., 2009; de Montaigu, Tòth and Coupland, 2010, Nakamichi *et al*., 2009, Wilkins, Bräutigam and Campbell, 2010). *TOC1*, for example, was shown to control the diurnal expression of the putative ABA receptor, ABA-RELATED/ H SUBUNIT OF THE MAGNESIUM-PROTOPORPHYRIN IX CHELATASE/ GENOMES UNCOUPLED 5 (ABAR / CHLH / GUN5), and thus stomatal aperture and dehydration response in Arabidopsis (Shen *et al*., 2006; Legnaioli *et al*., 2009; Castells *et al*., 2010). In addition, the PSEUDO RESPONSE REGULATOR arrhythmic triple Arabidopsis mutant *prr9*/*prr7*/*prr5* showed increased levels of *DREB1* or C-repeat binding protein (*CBF*) and a correlated higher resistance to drought, salinity and cold stresses (Nakamichi *et al*., 2009). Other studies have shown that a close match between the length of the internal circadian and the external daily cycles represented a selective advantage (Dodd *et al*., 2005, Green *et al*., 2002, Yerushalmi, Yakir and Green, 2011; Sanchez *et al*., 2011). For example, Dodd *et al*., (2005) showed that arrhythmic Arabidopsis mutants produced less chlorophyll, fixed less carbon and had lower biomass than wild-type plants under 24 h day cycles.

Several studies have suggested a strong interdependence and reciprocal interactions between the stress-response hormone ABA and circadian clock regulatory systems (Robertson *et al*., 2009). For example, ABA controlled the expression of *TOC1* in the presence of a functional ABAR/CHLH/GUN5 protein and impacted the amplitude, period and phase of *CCA1* expression (Hanano *et al*., 2006; Legnaioli *et al*., 2009). Despite evidence on the hormonal control of the circadian clock, little is known about the role of abiotic stress at the roots as an input signal to the shoot clock.

Although the clock regulates plant performance and stress adaptation in Arabidopsis, much less is known about its functions in important cereal crop plants. Barley represents a good crop model to study the effects of the clock on performance and stress adaptation, because it has extensive genetic variation for resistance to abiotic stresses. Campoli and colleagues (2012b) have shown that many circadian clock genes are structurally conserved between barley and Arabidopsis, and their circadian expression patterns suggested conserved functions. However, phylogenetic analyses revealed that independent duplications/deletions of clock genes occurred throughout the evolution of eudicots and monocots (Takata *et al*., 2010; Campoli *et al*., 2012b). Barley carries only a single ortholog for *CCA1* and five *PRR* orthologs designated as *HvPRR1* orthologous to *TOC1*, *HvPRR73*/*HvPRR37* corresponding to *AtPRR7* and *AtPRR3*, and *HvPRR59*/*HvPRR95* corresponding to *AtPRR5* and *AtPRR9* (Campoli *et al*., 2012b). Functional analyses of clock genes in barley have shown that circadian clock genes play an important role in photoperiod response and flowering time. For example, the *PRR* gene *Ppd-H1* (*HvPRR37*) is the major photoperiod response gene in barley and induces early flowering under long photoperiods. A natural recessive mutation in the CCT domain of *Ppd-H1* (*HvPRR37*) causes photoperiod insensitivity and late flowering in cultivated spring barley (Turner *et al*., 2005). In addition, recent studies have shown that mutations in *HvELF3* and *HvLUX1* caused photoperiod insensitivity and early flowering by upregulating *Ppd-H1* under non-inductive short day conditions (Faure *et al*., 2012; Zakhrabekova *et al*., 2012; Campoli *et al*., 2013). In contrast to the *Ppd-H1* variant in spring barley, which does not affect the expression of circadian clock genes (Campoli *et al*., 2012b); a non-functional *HvELF3* allele severely compromised the expression of clock oscillator genes (Faure *et al*., 2012). Despite the strong evidence that clock genes control photoperiod response and thereby adaptation in cereals, it has not yet been reported if allelic variation in the clock affects other physiological traits in cereals.

The objectives of the present study in barley were to better understand how abiotic stress applied to the root affected the shoot clock, and how genetic variation in clock genes affected stress adaptation. For this, I tested 1) whether natural variation at *Ppd-H1* and *HvELF3*, respectively, controlled the diurnal expression of stress-response genes and diurnal changes in physiology under stress conditions, and 2) whether osmotic stress to the root acted as an input signal to the shoot circadian clock and thus changed diurnal patterns of physiological traits.

Materials and Methods

Plant material and growth conditions

The spring barley cultivar Scarlett and an introgression line S42-IL107 (von Korff *et al*., 2004, 2006; Schmalenbach *et al*., 2011) were used in this study. Scarlett carries a mutation in the CCT domain of *Ppd-H1* and is late flowering under long days (LD) (Turner *et al*., 2005). The introgression line S42-IL107 harbors the photoperiod-responsive *Ppd-H1* allele introgressed from wild barley and is early flowering under LD. In addition, I have used the spring barley cultivar Bowman and two derived introgression lines, Bowman(*eam8.k*) and Bowman(*eam8.w*), carrying natural mutations in the *EAM8*/ *HvELF3* gene (Faure *et al*. 2012, Zakhrabekova *et al*., 2012). Bowman(*eam8.k*) and Bowman(*eam8.w*) were generated by crossing the spring barley Bowman with the genotypes Kinai5 and Early Russian, respectively. Bowman(*eam8.k*) has a partial deletion of the gene, while Bowman(*eam8.w*) has a C-to-T point mutation in exon 2, both of which lead to the production of a truncated protein (Faure *et al*., 2012). To assess the physiological response to osmotic stress seedlings varying at *HvELF3*/*EAM8* (Bowman, Bowman(*eam8.k*) and Bowman(*eam8.w*) were analyzed at 24h, 48h and 72h (Zeitgeber T4) after stress application.

In addition, Scarlett, S42-IL107, Bowman and Bowman(*eam8.w*) were analyzed for diurnal expression of core clock and stress-response genes, and for diurnal fluctuation in physiological traits under control and osmotic stress conditions.

Germinated seeds were placed in 1.5 ml pierced Eppendorf tubes, filled with 0.5% agar and transferred to a half-strength Hoagland nutrient solution (Hoagland and Arnon, 1950). The nutrient solution was renewed every 3-4 d. Plants were grown for 8-10 d in a growth chamber

under LD (16h/8h light/dark) at an irradiance of 300 µmol m^{-2} s^{-1}, an air temperature of 20/16 °C (day/night) and a relative humidity of 50 - 60%.

Osmotic stress application

Osmotic stress was applied after seedlings reached the two-leaf stage. In order to generate uniform osmotic stress conditions in the roots of the plants, seedlings were moved from a Hoagland nutrient solution (-0.2 Mpa) to one supplemented with 20 % PEG 8000 (Fluka, Germany), which corresponded to -0.8 Mpa osmotic potential.

Leaf sampling and gene expression analysis

Leaf samples from stressed and control samples were harvested 48h after PEG application at 4h intervals starting from the onset of light (ZT0) to lights off ZT16 and in addition at ZT18 and ZT22 during the dark phase. For all genotypes and treatment condition, three biological replicates of two pooled plants were sampled per time point. Total RNA extraction, cDNA synthesis, and qRT-PCRs using gene-specific primers as detailed in Supplementary Table S1 were performed as explained in materials method of chapter 1.

Physiological and morphological measurements

Measurements of relative water content (RWC), leaf temperature (LT), electrolyte leakage (EL), proline content, Malondialdehyde content (MDA) and chlorophyll fluorescence parameters from Bowman, Bowman(*eam8.k*) and Bowman(*eam8.w*) plants were conducted at 24h. 48h and 72h under osmotic stress and control conditions as described in materials and methods of chapter 1.

Diurnal measurements of leaf osmotic potential, gas exchange (stomata conductance, leaf transpiration rate and carbon exchange rate) and leaf temperature were taken 48 h after start of the osmotic stress treatment. Measurements were taken under osmotic stress and control conditions at 4 h intervals starting from ZT0 to ZT16 and in addition at ZT18 and ZT22 during the night phase. Each parameter was measured on the second leaf from three replicate plants per genotype and treatment conditions.

Samples for measurement of the leaf water potential were collected from the middle part of the second leaf and frozen immediately in liquid nitrogen and stored at –20 °C. Samples were thawed and placed in 1 ml microtubes that had a fine hole at the base and the sap was then

extracted after centrifuging (Eppendorf Centrifuge 5415D, Hamburg, Germany) for 2 min at 1400 rpm. The osmolality of expressed sap samples was measured with an Osmomat 030 freezing point depression osmometer (Gonotec GmbH, Berlin, Germany). Gas exchange and leaf temperature were measured using portable photosynthesis system (LI-6400, LI-COR, Inc., Lincoln, NE, USA). Gas exchange measurements were conducted with CO_2 concentration set at 400 µmol.mol^{-1} by means of a CO_2 mixer and CO_2 tank and the light intensity was set to the condition of the chamber using a red-blue (10%) source (LI-6400-02B; LI-COR, Inc.). Measurements were taken when readings for CO_2 exchange (ΔCO2) stabilized after 5-10 min. Seedling height and biomass yield (dry matter) was measured 2 d after the begin of the treatment from 12-15 seedlings comprised of shoot and leaf material from each genotype and condition after drying the seedlings for 2 d at 70 ˚C. For measurements of coleoptile lengths, seedlings were grown on 1% (w/v) agar (Merck, Germany) for 2 d either in continuous red light (LED) with different fluency rates or in dark and mean coleoptile lengths (± SD) of 8-10 seedlings were determined.

Statistical analysis

Significant differences in gene expression and physiological responses were calculated using a general linear model in the SAS software, version 9.1 (SAS Institute 2003) with the factors genotype, treatment, time point and first and second order interaction effects. Significant differences in expression between genotypes and treatments were calculated for each time point based on three biological replicates. Pairwise correlation coefficients between gene expression data were calculated across genotypes and treatments using Pearson correlation coefficients (SAS Institute 2003).

Comparative analysis of *cis*-acting regulatory elements

Comparative *in silico* analyses of promoter regions of the core clock and stress-responsive genes were conducted to identify *cis*-acting regulatory elements conserved between barley, maize, sorghum, rice and Brachypodium. First, homologs of barley stress-response and clock genes were identified in maize, sorghum, rice and Brachypodium by using predicted polypeptide sequences of a given barley protein as a query in BLAST searches in the Phytozome database (http://www.phytozome.net). The most similar sequences were selected based on e-values and score (Supplementary Table S18). Subsequently, the promoter regions of barley genes and

corresponding homologs from the grass species including 3 kb sequences upstream of the transcription start site were retrieved using the Ensemble barley genome database (http://plants.ensembl.org/Hordeum_vulgare) and the Phytozome database for maize, sorghum, rice and Brachypodium. Conserved non-coding sequences (CNS) in the promoter regions were determined by phylogenetic footprinting approach (>70% identity in a 20-bp window; Guo and Moose, 2003) using the VISTA tool (Mayor *et al*., 2000). The CNSs were then searched for putative transcription factor binding motifs in barley using the GENOMATIX database (http://www.genomatix.de/).

Results

Osmotic stress at the root acts as an input into the shoot circadian clock

In order to examine whether osmotic stress acts as input to the circadian clock in barley, I studied diurnal changes of the circadian-clock genes *HvCCA1*, *HvPRR1*, *HvGI*, *Ppd-H1* (*HvPRR37*), *HvPRR73*, *HvPRR59*, and *HvPRR95* under control and osmotic stress conditions. To test whether genetic variation at *HvELF3* and *Ppd-H1* affected the input of osmotic stress into the barley circadian clock, diurnal expression was compared between Bowman and Bowman(*eam8.w*), and between Scarlett and S42-IL107.

Circadian clock genes showed a diurnal pattern of expression under control and stress conditions (Fig. 1, 2). *HvCCA1* peaked in the morning, followed by *Ppd-H1* and *HvPRR73* in the middle of the day, and *HvPRR1*, *HvGI*, *HvPRR59* and *HvPRR95* in the evening of the long day. Expression of clock orthologs was significantly different between Bowman and Bowman*(eam8.w*) (Supplementary Table S12). Expression of *HvCCA1* was reduced in Bowman*(eam8.w*) compared to Bowman at peak times during the day under stress and control conditions (Fig. 1A). *HvPRR1* and *HvGI* expression increased earlier in Bowman(*eam8.w*) compared to Bowman, for example, expression of *HvGI* peaked at ZT8 in Bowman(*eam8.w*) and at ZT12 in Bowman (Fig. 1 B, 1C). In addition, *Ppd-H1* expression was significantly higher in Bowman*(eam8.w*) than Bowman during the night and the early morning (Fig. 1E). Under stress, expression of *HvPRR59* and *HvPRR95* was significantly lower in Bowman(*eam8.w*) compared to Bowman at peak time ZT8. In contrast, circadian-clock orthologs did not show differences in expression between Scarlett and S42-IL107 under stress and control conditions (Supplementary Table S13). Thus the

mutation in *HvELF3* had a greater role in modulating the clock compared to the mutation in *Ppd-H1*.

Osmotic stress caused a significant increase in the expression of clock orthologs compared to control conditions in all genotypes. In particular, *Ppd-H1*, *HvPRR73* and *HvPRR95* showed a strong induction of expression under stress compared to control conditions (Fig. 1, 2). For example, *Ppd-H1* showed a 2.5-fold increase in Scarlett and a 5-fold increase in Bowman under stress conditions. Furthermore, osmotic stress advanced the phase of clock gene expression compared to control conditions in all genotypes. *HvPRR73* peaked at ZT4 under stress conditions and at ZT8 under control conditions. Similarly, *HvGI*, *HvPRR59* and *HvPRR95* peaked at ZT8 under stress conditions and at ZT12 under control conditions. Osmotic stress also affected the shape of the expression amplitude of *HvCCA1*, *HvPRR1*, *HvGI* and *Ppd-H1*. The expression of these genes increased earlier under stress than control conditions, and decreased at the same time (*HvCCA1*, *HvPRR1*) under both treatments or even later under stress (*HvGI*, *Ppd-H1*). The expression peaks for these genes were thus broader under stress than control conditions.

Taken together, these results indicate that osmotic stress applied at the root altered the expression of circadian clock genes in the barley shoot. Osmotic stress advanced the expression phase of evening expressed clock genes and affected the shape of the expression peaks of several clock genes. Variation at *HvELF3* controlled the expression of clock genes, while variation at *Ppd-H1* did not, as previously reported (Campoli *et al*., 2012b, Faure *et al*., 2012). Variation at either gene did not change the effects of osmotic stress on the expression of clock genes.

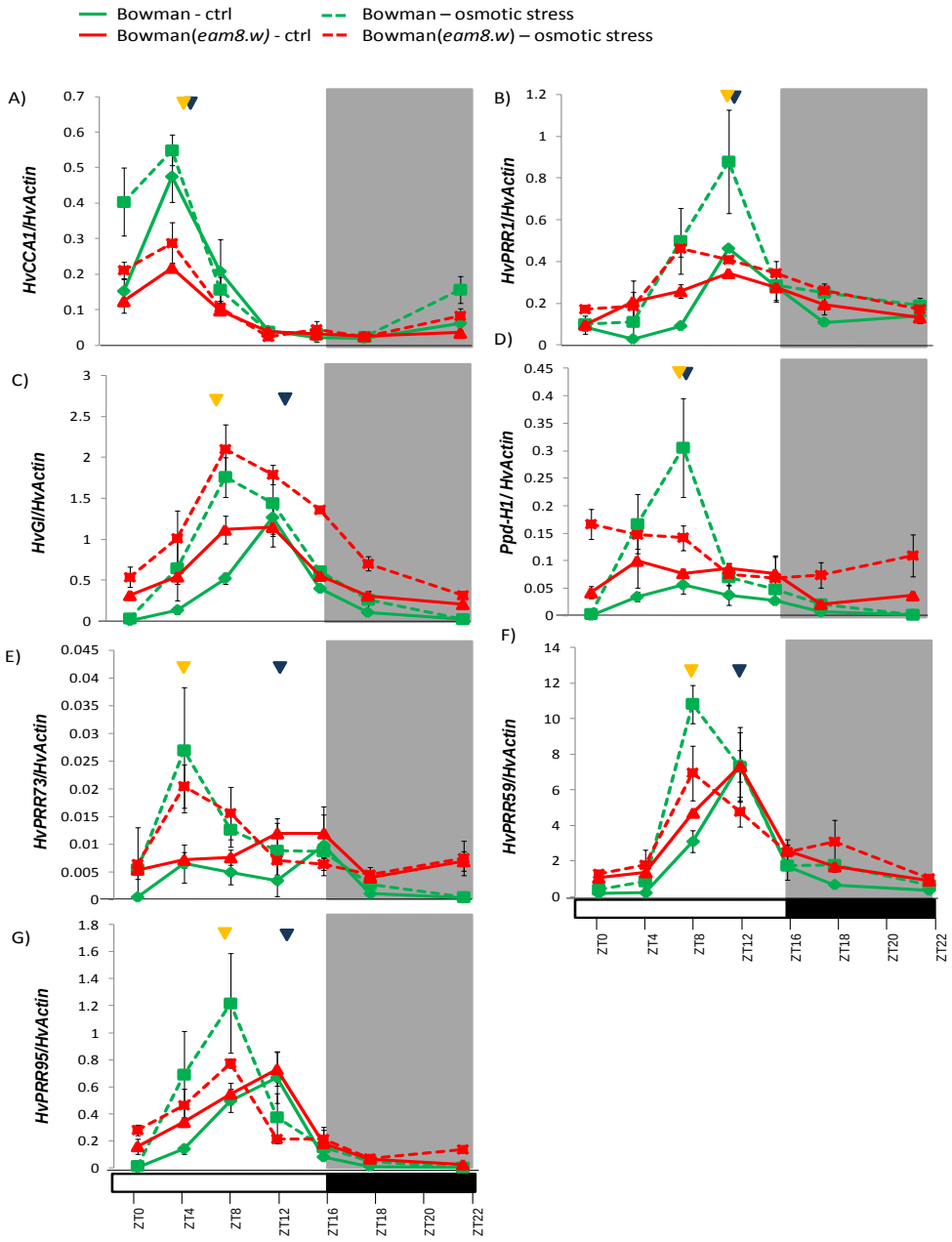

Figure 1. Diurnal expression of circadian clockgenes in barley under control (solid line) and osmotic stress (dashed line) in the spring barley Bowman (green) and introgression line Bowman(*eam8.w*) (red). Seedlings of both genotypes were grown in hydroponics for ten days in long day (16h/8h, light/dark). Leaf samples for total RNA were collected after 48h of osmotic stress (20% PEG) or under control conditions at 4 h intervals during the day time (including samples taken in the dark 2 h before and after light on and off). Transcript accumulation was measured by qRT-PCR analysis of target genes normalized to *HvActin*. Arrows indicate peak time of expression under control (blue) and stress (orange) conditions. Values are means ± SD of three biological replicates. Black bars and shaded regions indicate the night period.

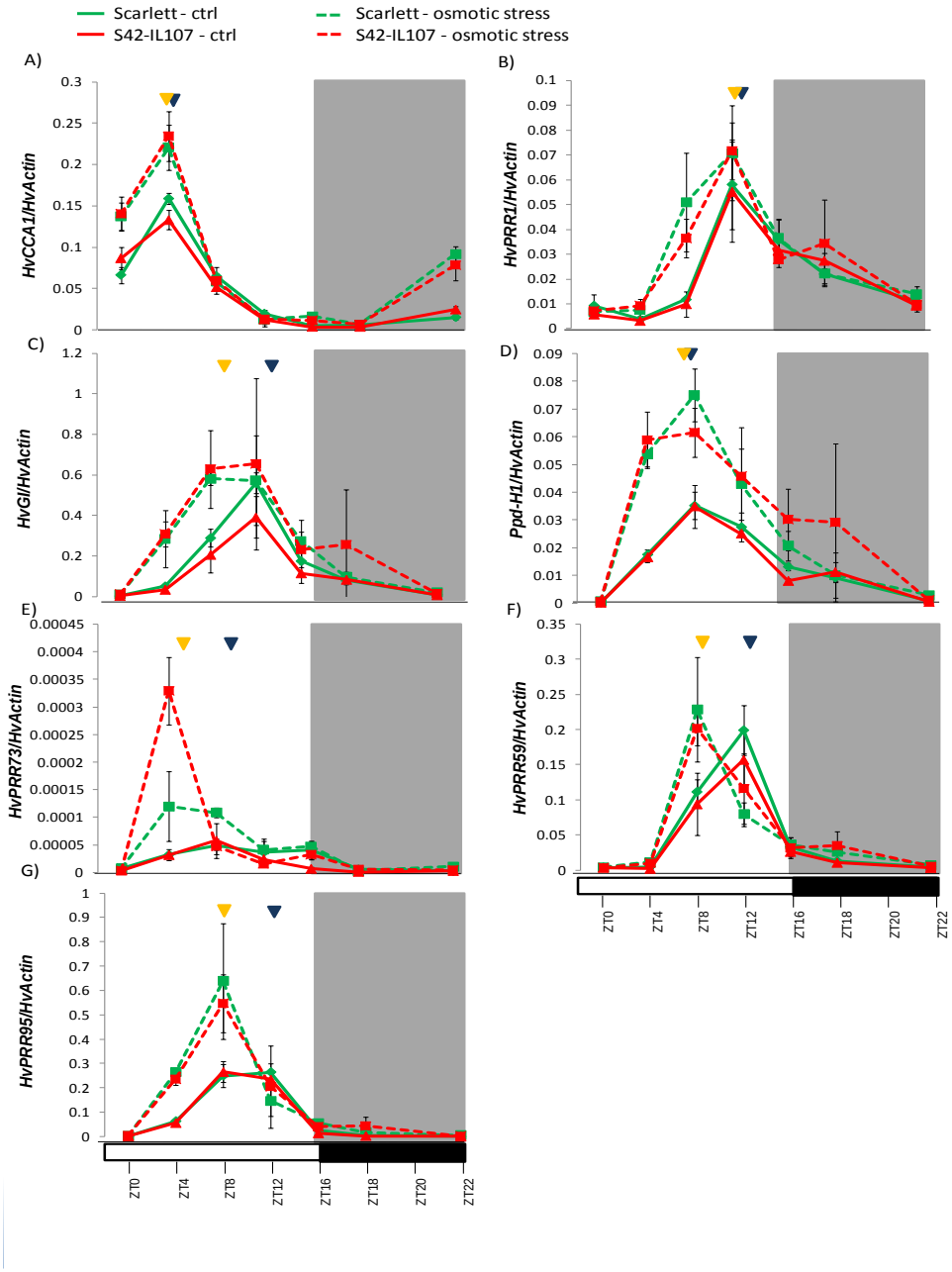

Figure 2. Diurnal expression of circadian ortholog genes in barley under control (solid line) and osmotic stress (dashed line) conditions in the spring barley Scarlett (green) and the introgression line S42-IL107 (red). Seedlings of both genotypes were grown in hydroponics for ten days in long day (16h/8h, light/dark). Leaf samples for total RNA were collected after 48 h of osmotic stress (20% PEG) or under control conditions at 4 h interval during the day time (including samples taken in the dark 2 h before and after light on and off). Transcript accumulation was measured by qRT-PCR analysis of target genes normalized to *HvActin*. Arrows indicate peak time of expression under control (blue) and stress (orange) conditions. Values are means ± SD of three biological replicates. Black bars and shaded regions indicate the night period.

Osmotic stress affects the levels and peak phases of stress gene expression

Since many stress-response genes are clock regulated in Arabidopsis, I examined whether variation in clock-gene expression affected expression of stress-response genes (Fig.3, 4). I tested diurnal expression of representative genes controlling signaling, response and sensitivity to drought and ABA as well as genes involved in ROS scavenging. These included the drought- and ABA-induced transcription factors *HvDREB1*, *HvDRF1* (dehydration responsive factor 1), *HvABI5* (ABA-response gene) and *HvWRKY38*. In addition, the expression of *HvPHYB* (*PHYTOCHROME B*), *HvLHCB, HvCAT1* (catalase), *HvAPX1* (ascorbate peroxidase) and the senescence activated *HvARF1* (ADP ribosylation factor 1-like protein) was measured, which are involved in ABA-induced responses such as stomatal closure and ROS homeostasis in Arabidopsis (Gonzalez *et al*., 2012; Xu *et al*., 2012). Finally, the expression of a *PIF3* like gene (*HvPIL3*) was measured, homologous to the phytochrome-interacting factor-like protein, *OsPIL1*, which acts as a key regulator of growth under drought in rice (Todaka *et al*., 2012).

Under control conditions, the majority of genes peaked at the end of the day (ZT12) (Fig. 3, 4). Under stress, the expression levels of the majority of genes were significantly increased, with the exception of *HvLHCB* and *HvPIL3,* which showed significantly reduced expression levels under stress compared to control conditions in all genotypes. Furthermore, the expression peaks of *HvABI5*, *HvDRF1*, *HvWRKY38*, *HvAPX1*, *HvCAT1*, *HvLHCB* and *HvPHYB* were advanced under stress in all genotypes. *HvAPX1* For example, the expression peaks of *HvABI5*, *HvDRF1*, *HvCAT1* and *HvPHYB* were shifted from ZT12 under control to ZT8 under stress conditions in Bowman and Scarlett (Fig 3, 4). Together, stress often altered the rhythmic peak of diurnal transcript accumulation and advanced the phase.

Genotype-dependent expression was observed in *HvPIL3* and *HvLHCB* under control and stress conditions. In contrast, the remaining stress-response and signaling genes showed genetic differences under stress, but not under control conditions. Under control condition, *HvPIL3* and *HvLHCB* exhibited a strong genetic difference in the phase of expression. *HvPIL3* expression peak was shifted from ZT8 in Bowman to ZT0 in Bowman*(eam8.w*); and *HvLHCB* peak expression was altered from ZT12 to ZT8 (Fig. 3H, 3J). In Scarlett and S42-IL107, both genes peaked at the same time during the day, but Scarlett showed significantly higher expression levels of *HvPIL3* and *HvLHCB* at peak time of expression compared to S42-IL107 (Fig.4H, J). In

Bowman(*eam8.w*), the expression of *HvABI5*, *HvDRF1*, *HvDREB1*, *HvWRKY38* and *HvAPX1* showed a reduced amplitude and a broader peak shape which extended into the night compared to Bowman (Fig.3A-E). In contrast, *HvCAT1* exhibited a higher expression peak in Bowman(*eam8.w*) than Bowman at ZT8 under stress (Fig.3F). *HvPHYB* expression levels were comparable between Bowman and Bowman(*eam8.w*), but the *HvPHYB* expression in Bowman peaked 4h earlier than in Bowman(*eam8.w*) under stress. S42-IL107 showed higher expression levels of the *DREB2*-like genes *HvDRF1* and *HvDREB1*, the ROS scavenging genes, and *HvPHYB* compared to Scarlett (Fig.4). This higher expression of stress genes was also observed in Triumph compared to Triumph-IL after stress application at the time point 24, 48 and 72h, as shown for *HvDREB1*, *HvDRF1*, *HvCAT1* and *HvAPX1* in chapter 1.

In summary, variation at *HvELF3* resulted in differences in the diurnal expression patterns, the phase and shape of peak expression, while variation at *Ppd-H1* affected only the levels of expression.

In order to examine diurnal co-expression of core clock and stress-responsive genes, pair wise Pearson correlation coefficients were calculated across genotypes (Bowman, Bowman(*eam8.w),* Scarlett and S42-IL107) and treatments (control and stress). Within core clock genes, *HvGI*, *HvPRR1* and *HvPRR59* showed the highest correlation of expression patterns (R > 0.8). Expression of *HvPRR37* was most highly correlated with expression of *HvPRR95* (R=0.82) and *HvPRR73* (R=0.72) (Table 1). Stress response genes most closely correlated with clock genes were *HvABI5*, *HvARF1*, and *HvCAT1* which showed the highest positive correlation with *HvPRR1* and *HvGI*. Among the stress-response genes, the highest correlations of above 0.8 were observed between *HvAPX1*, *HvDRF1* and *HvPHYB*. Finally, *HvPIL3* and *HvLHCB*, as the only two genes down-regulated under stress (Fig 3H, J, 4H, J), showed positive correlation coefficients of 0.72. Evening expressed clock genes were thus highly correlated with stress-response genes.

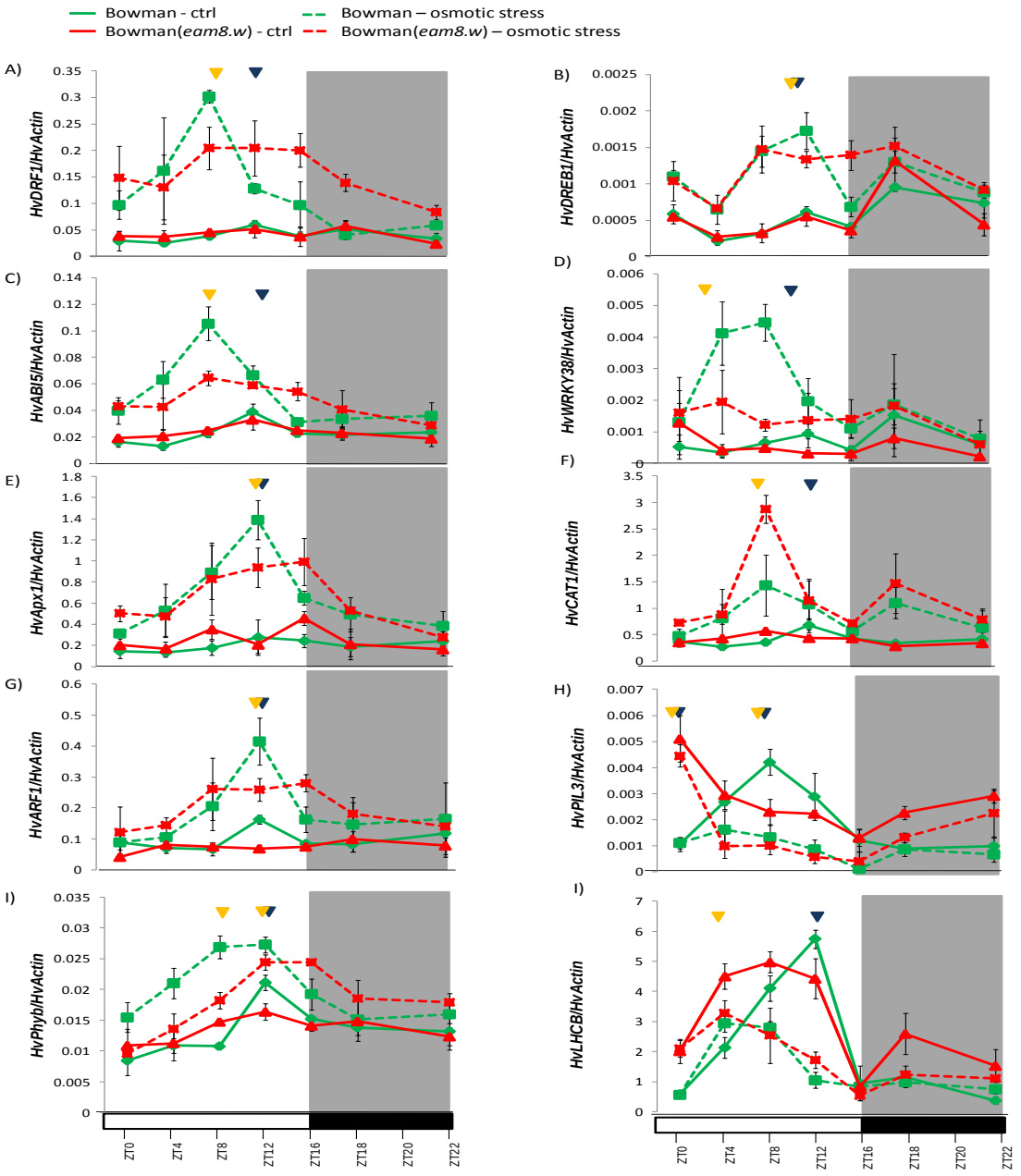

Figure 3. Diurnal expression of stress response genes under control (solid line) and osmotic stress (dashed line) conditions in the spring barley Bowman (green) and the introgression line Bowman(*eam8.w*) (red). Seedlings of both genotypes were grown in hydroponics for ten days in long day (16h/8h, light/dark). Leaf samples for total RNA were collected after 48h of osmotic stress (20% PEG) or under control conditions at 4 h interval during the day time (including samples taken in the dark 2 h before and after light on and off). Transcript accumulation was measured by qRT-PCR analysis of target genes normalized to *HvActin*. Arrows indicate peak time of expression under control (blue) and stress (orange) conditions. Values are means ± SD of three biological replicates. Black bars and shaded regions indicate the night period.

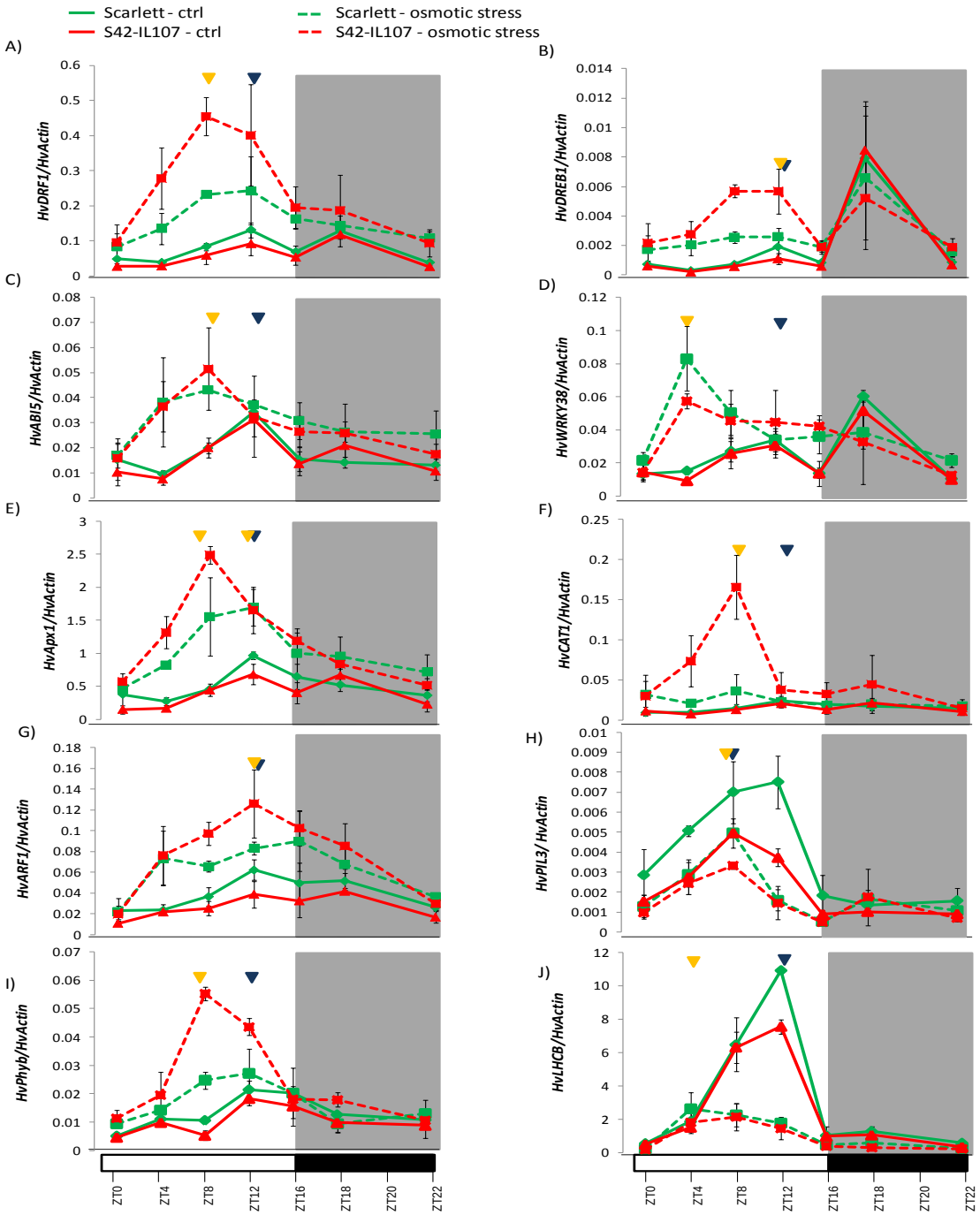

Figure 4. Diurnal expression of stress response genes under control (solid line) and osmotic stress (dashed line) conditions in the spring barley Scarlett (green) and introgression line S42-IL107 (red). Seedlings of both genotypes were grown in hydroponics for ten days in long day (16h/8h, light/dark). Leaf samples for total RNA were collected after 48h of osmotic stress (20% PEG) or under control conditions at 4 h interval during the day time (including samples taken in the dark 2 h before and after lights on and off). Transcript accumulation was measured by qRT-PCR analysis of target genes normalized to *HvActin*. Arrows indicate peak time of expression under control (blue) and stress (orange) conditions. Values are means ± SD of three biological replicates. Black bars and shaded regions indicate the night period.

Table 1. Pearson correlation coefficients between core clock and stress-responsive genes in all genotypes and treatment conditions

Genes	*HvCCA1*	*HvPRR1*	*HvGI*	*HvPRR37*	*HvPRR73*	*HvPRR59*	*HvPRR95*	*HvDRF1*	*HvDREB1*	*HvABI5*	*HvWRKY38*	*HvAPX1*	*HvCAT1*	*HvARF1*	*HvPIL3*	*HvPHYb*	*HvLHCB*
HvCCA1		-0.08	-0.02	**0.33***	**0.49***	-0.03	0.25	-0.05	**-0.28***	0.18	-0.18	-0.21	0.15	-0.03	0.08	-0.14	0.05
HvPRR1	-0.08		**0.8****	**0.51****	**0.55****	**0.87****	**0.51****	0.08	-0.21	**0.66****	**-0.49****	0.07	**0.74****	**0.87****	**-0.25***	**0.28***	0.07
HvGI	-0.02	**0.8****		**0.71****	**0.6****	**0.85****	**0.77****	**0.41***	-0.08	**0.79****	-0.24	**0.31***	**0.75****	**0.76****	-0.07	**0.46***	**0.29***
HvPRR37	**0.33***	**0.51****	**0.71****		**0.72****	**0.64****	**0.82****	**0.4***	-0.12	**0.79****	-0.2	0.17	**0.62****	**0.48***	0.05	**0.3***	0.23
HvPRR73	**0.49***	**0.55****	**0.6****	**0.72****		**0.54****	**0.59****	0.03	**-0.31***	**0.54****	**-0.5***	-0.14	**0.65****	**0.46***	-0.16	0.09	0.12
HvPRR59	-0.03	**0.87****	**0.85****	**0.64****	**0.54****		**0.74****	0.12	-0.2	**0.68****	-0.42*	0	**0.71****	**0.66****	-0.13	0.23	0.21
HvPRR95	0.25	**0.51****	**0.77****	**0.82****	**0.59****	**0.74****		**0.4***	-0.1	**0.73****	-0.09	0.25	**0.51****	**0.38***	0.22	**0.4***	**0.48***
HvDRF1	-0.05	0.08	**0.41***	**0.4***	0.03	0.12	**0.4***		**0.55****	**0.62****	**0.5****	**0.9****	0.14	**0.33***	-0.06	**0.83****	-0.02
HvDREB1	**-0.28***	-0.21	-0.08	-0.12	**-0.31***	-0.2	-0.1	**0.55****		0.06	**0.68****	**0.54****	-0.21	-0.04	-0.13	**0.33***	-0.17
HvABI5	0.18	**0.66****	**0.79****	**0.79****	**0.54****	**0.68****	**0.73****	**0.62****	0.06		-0.06	**0.49****	**0.67****	**0.74****	-0.13	**0.57****	0.14
HvWRKY38	-0.18	**-0.49****	-0.24	-0.2	**-0.5***	-0.42*	-0.09	**0.5****	**0.68****	-0.06		**0.58****	**-0.49***	**-0.31***	0.19	0.24	0.06
HvAPX1	-0.21	0.07	**0.31***	0.17	-0.14	0	0.25	**0.9****	**0.54****	**0.49****	**0.58****		-0.02	**0.3***	-0.02	**0.82****	-0.02
HvCAT1	0.15	**0.74****	**0.75****	**0.62****	**0.65****	**0.71****	**0.51****	0.14	-0.21	**0.67****	**-0.49***	-0.02		**0.75****	**-0.25***	0.17	0.02
HvARF1	-0.03	**0.87****	**0.76****	**0.48***	**0.46***	**0.66****	**0.38***	**0.33***	-0.04	**0.74****	**-0.31***	**0.3***	**0.75****		**-0.34***	**0.44***	-0.07
HvPIL3	0.08	**-0.25***	-0.07	0.05	-0.16	-0.13	0.22	-0.06	-0.13	-0.13	0.19	-0.02	**-0.25***	**-0.34***		-0.07	**0.72****
HvPHYb	-0.14	**0.28***	**0.46***	**0.3***	0.09	0.23	**0.4***	**0.83****	**0.33***	**0.57****	0.24	**0.82****	0.17	**0.44***	-0.07		0.06
HvLHCB	0.05	0.07	**0.29***	0.23	0.12	0.21	**0.48***	-0.02	-0.17	0.14	0.06	-0.02	0.02	-0.07	**0.72****	0.06	

Significant (*= p<0.05, **= p<0.001) coefficients are underlined.

Cis-acting regulatory elements in core clock and drought-responsive genes

As the expression of clock and stress genes was correlated, I analysed the presumed promoter regions, 3000 bp located upstream of the stress-response genes, for the presence of conserved *cis*-elements driven by circadian clock and light factors (Adams and Carre, 2011). Furthermore, since circadian clock genes were induced by osmotic stress, I searched for stress-response elements in the presumed promoter regions (3000 bp) of the circadian clock genes. One to three CNS per promoter sequences were identified across the five grass species in the stress-response and clock genes. Dependent on the lengths of CNSs identified for each gene, the number of *cis*-acting elements differed for each gene (Supplementary Table S2). ABA-responsive elements (ABRE), drought responsive elements (DRE), circadian-clock factors (CCAF), and light-responsive motifs (LREM) were identified in the CNS of drought-responsive genes (Supplementary Table S19). Similarly, the analysis of circadian clock promoters also revealed an enrichment of CCF, LRE, ABRE and DRE (Supplementary Table S19).

Taken together, the identification of conserved regulatory elements in drought-responsive genes suggested that the circadian clock and light regulators are involved in the transcriptional control of stress-response genes. In addition, the identification of stress and ABREs in the promoter sequences of circadian-clock orthologs indicated that their transcription is regulated by stress and ABA signaling factors, which supports results of the expression analysis (Fig. 1, 2).

Diurnal changes of physiological responses to short-term osmotic stress

I have examined whether variation in the expression of circadian clock and stress-response genes, variation affected physiological traits. Physiological traits such as biomass, daily fluctuations in leaf osmotic potential, stomatal conductance, leaf transpiration, net CO_2 uptake and leaf temperature under control and stress conditions were measured. The treatment had the strongest effects on the diurnal variation of physiological traits, while genetic variation had only minor effects on trait expression with the exception of biomass (Fig. 5, 6, Supplementary Tables S14,S15). Osmotic stress caused an increase in the leaf osmotic potential and leaf temperature, and a reduction in the stomatal conductance, leaf transpiration, and net CO_2 uptake compared to control conditions in all tested genotypes (Figure 5). Phenotypic differences between stress and control conditions were generally more pronounced during the light period, in particular towards the end of the light period. Bowman(*eam8.w*) had a significantly higher osmotic potential than

Bowman at ZT4 and ZT12 under control conditions, and at ZT4 under stress conditions. In addition, a significantly lower net CO_2 uptake was observed in Bowman(*eam8.w*) and S42-IL107 compared to their recurrent parents at ZT12 and ZT16 under stress conditions. Furthermore, S42-IL107 exhibited significantly lower stomatal conductance and leaf transpiration rate compared to Scarlett at ZT12 and ZT16 under control conditions.

Total biomass was significantly regulated by treatment and genotype (Fig. 7, Supplementary Table S16). Bowman biomass was 68 ±2.2 mg and 59 ±2.4 mg under control and stress conditions, respectively. Bowman(*eam8.w*) had a lower biomass of 59 ±4.1 mg and 52 ±3.3 mg under control and stress conditions, respectively. Biomass was also significantly lower in S42-IL107 than in Scarlett under stress, but not under control conditions. The introgression lines, varying at *HvELF3* and *Ppd-H1* thus showed a significantly reduced biomass accumulation under stress compared to their recurrent parents. In addition, both lines exhibited a reduction in the net CO2 uptake at the end of the day under stress.

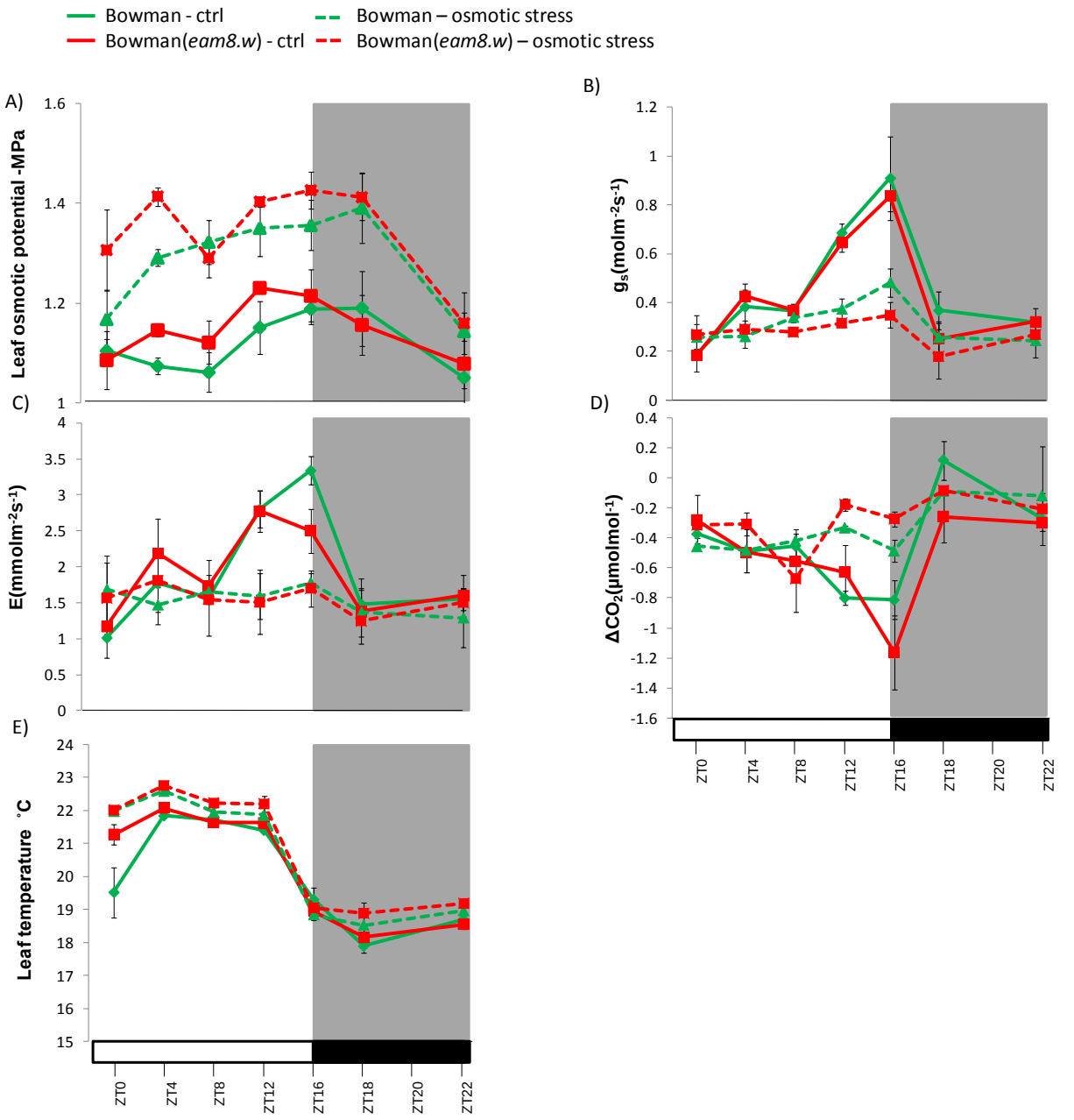

Figure 5. Diurnal expression of physiological traits under control (solid line) and osmotic stress (dashed line) conditions in the spring barley Bowman (green) and the introgression line Bowman(*eam8.w*) (red). A) Leaf osmotic potential, B) Stomata conductance (g_s), C) Leaf transpiration rate (E), D) Net CO2 exchange and E) Leaf temperature. Seedlings were grown in hydroponics for ten days under long day (16h/8h, light/dark). Physiological measurements were taken after 48 h of osmotic stress at 4 h intervals during the day time (including samples taken in the dark 2 h before and after light on and off). Values are means ± SD of three biological replicates. Black bars and shaded regions indicate the night period.

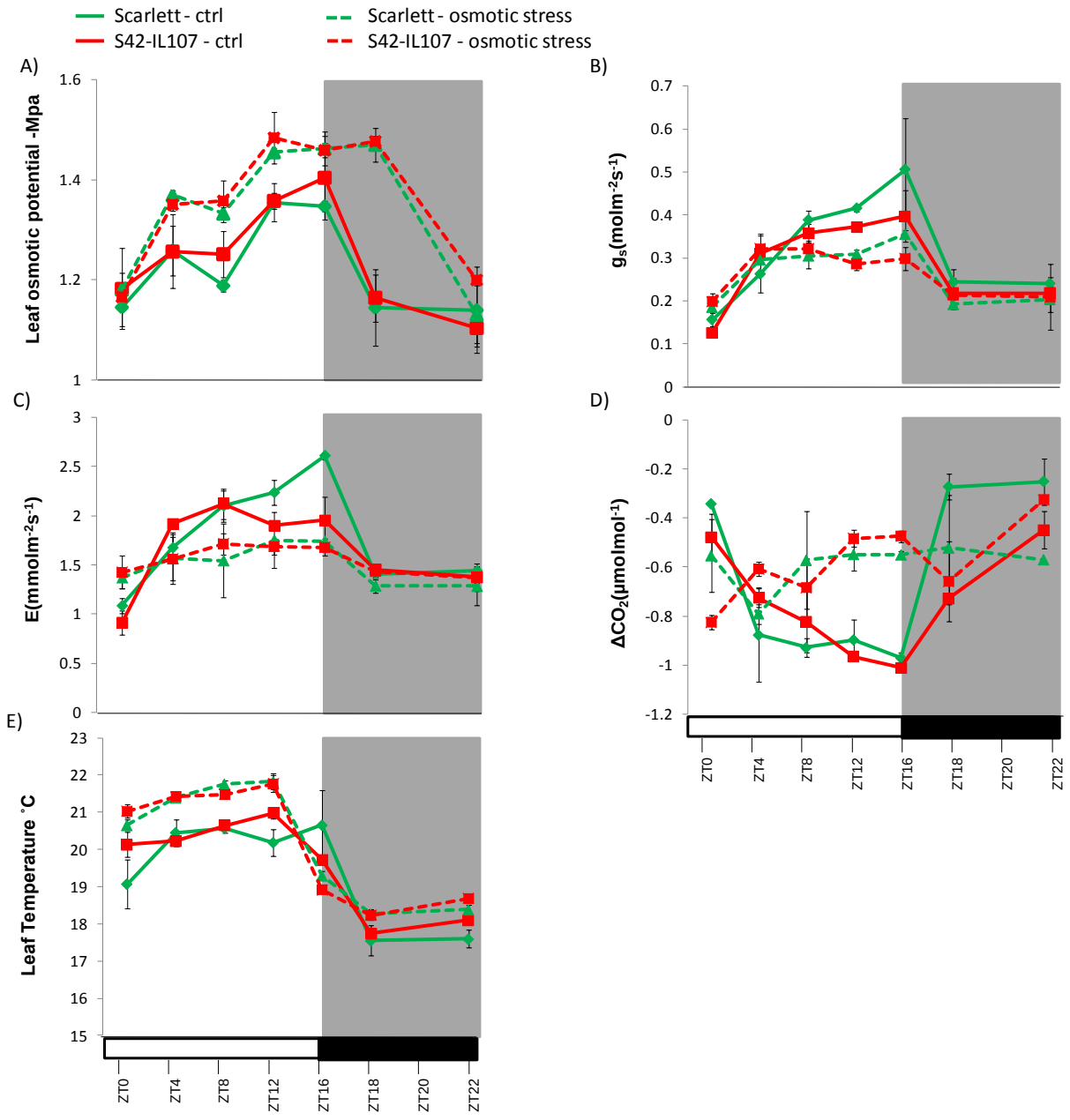

Figure 6. Diurnal expression of physiological traits under control (solid line) and osmotic stress (dashed line) conditions in the spring barley Scarlett (green) and the introgression line S42-IL107 (red). A) Leaf osmotic potential, B) Stomata conductance (g_s), C) Leaf transpiration rate (E), D) Net CO2 exchange and E) Leaf temperature. Seedlings were grown in hydroponics for ten days in long day (16h/8h, light/dark). Physiological measurements were taken after 48 h of osmotic stress at 4 h intervals during the day time (including samples taken in the dark 2 h before and after light switch on and off). Values are means ± SD of three biological replicates. Black bars and shaded regions indicate the night period.

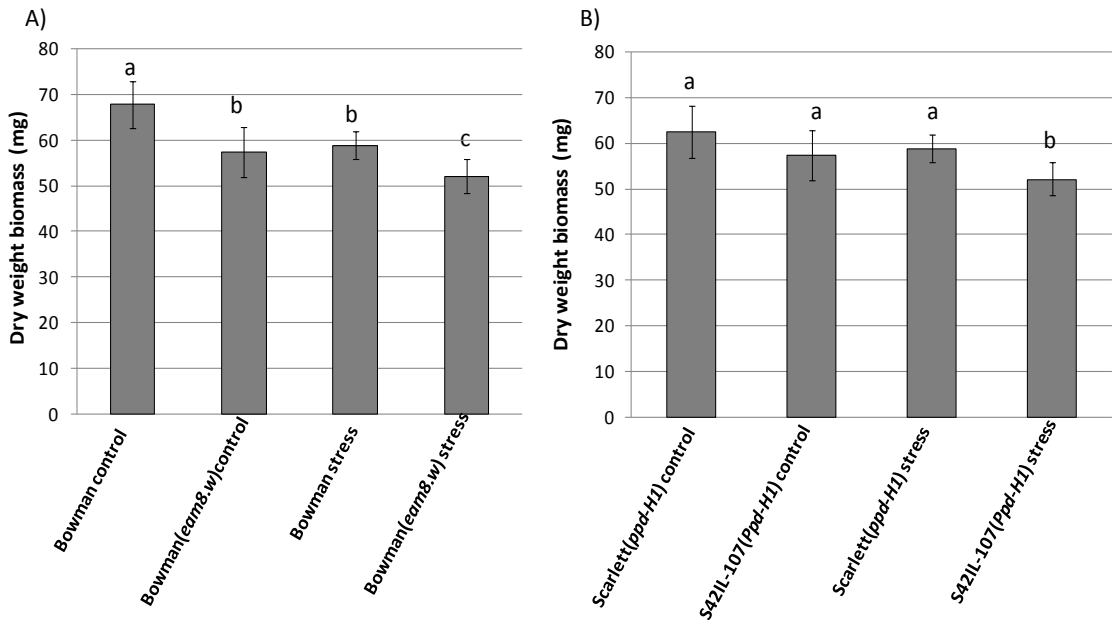

Figure 7. Dry weight biomass in Bowman/ Bowman(*eam8.w*) (A) and Scarlett/ S42-IL107 (B) under control and osmotic stress conditions. Measurements were taken in 12 day old seedlings after 48h of osmotic stress (20% PEG). Values are means ± SD of 12-15 seedlings. Different letters indicate significant differences at $p \leq 0.05$ using least square means.

Variation at *HvELF3* does not affect physiological responses to osmotic stress

As indicated in chapter 1, physiological responses to short-term osmotic stress was analyzed at 24h, 48h and 72h time points after stress to assess the performance of genotypes differing at *Ppd-H1*. Similarly, I tested whether the variation at *HvELF3* affected the physiological responses at different time points subjected to osmotic stress. RWC was significantly reduced under osmotic stress (PEG treated plants) as compared to control conditions 24h after the beginning of the treatment (AT) only in Bowman and 48h and 72h AT in all three genotypes (Fig. 8A). There were no significant genetic differences in RWC between Bowman and Bowman(*eam8.k)* or Bowman*(*e*am8.w*) under control or osmotic stress condition at any time point. Similarly, significant differences in LT were recorded between control and stressed plants of Bowman 24h AT, and in all three genotypes at 48h and 72h AT (Fig. 8B). No significant differences in LT were revealed between Bowman and Bowman(*eam8.k*) or Bowman(*eam8.w*) at any time point.

Osmotic stress thus caused significant differences in relative water content and leaf temperature, but variation in the circadian clock gene *HvELF3* did not affect both traits under control or stress conditions. Electrolyte leakage (EL) was not significantly increased in plants under osmotic stress as compared to control conditions and did not show significant differences between genotypes at any time point (Fig. 8C). In contrast, malondialdehyde (MDA) was significantly increased 48h and 72h AT under osmotic stress as compared to control conditions, but did not show significant differences between genotypes. Proline levels were significantly increased in stressed plants 48h and 72h AT as compared to control plants (Fig. 8E). However, there was no significant difference in proline content between genotypes differing at *HvELF3*. Hence, short-term osmotic stress caused an increase in the accumulation of MDA and proline content, but did not affect EL. No significant differences in MDA and proline content were detected between Bowman and Bowman(*eam8*) lines.

As variation at *HvELF3* affected the diurnal expression of chlorophyll a/b binding proteins, which are important for the light reaction of photosynthesis (Faure *et al*. 2012), I analysed the effects of variation at *HvELF3* on photosynthesis efficiency. Changes of the chlorophyll fluorescence parameter PI under control and osmotic stress was studied in Bowman and Bowman(*eam8*) lines. PI was only significantly decreased in stressed plants of Bowman compared to control plants at 72h AT. At this time point however, the reduction of PI in Bowman was not significantly different from Bowman(*eam8.k*) (Fig. 8F). PI was thus not strongly affected by osmotic stress in Bowman and no significant differences were observed between genotypes.

Altogether, short-term osmotic stress significantly altered RWC, LT, and proline accumulation however; no significant effects of variation at *HvELF3* on these physiological responses were recorded under osmotic stress and control conditions. In addition, osmotic stress had only small effects on photosynthetic activity and cell membrane injury.

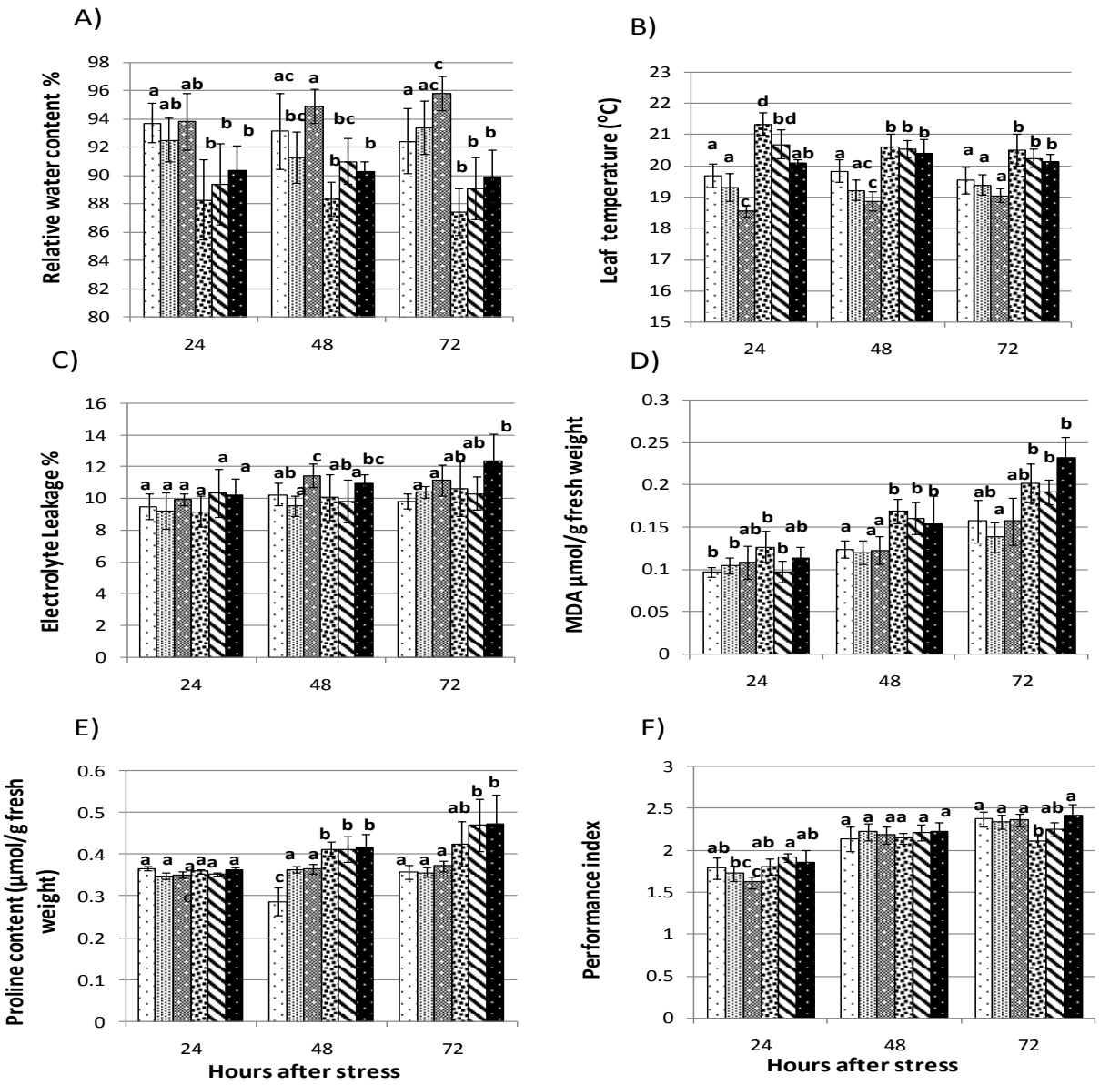

Figure 8. Physiological responses of barley genotypes varying at *HvELF3* to short-term osmotic stress. Bowman control, Bowman(*eam8.k*)control, Bowman(*eam8.w*)control, Bowman stress, Bowman(*eam8.k*)stress and Bowman(*eam8.w*) stress. Seedlings were grown in hydroponics under LD (16h light). After 10 days osmotic stress was induced by immersing the roots of seedlings in PEG. Physiological parameters were measured every 24 h for three consecutive days after onset of stress. A) Relative water content, B) Leaf temperature, C) Electrolyte leakage, D) Malonaldehyde content, E) Proline content and F) Performance index. Values are means ±sd of three independent experiments. Different letters indicate significant difference at $p \leq 0.05$ using least square means.

Variation at *HvELF3 and Ppd-H1* does not affect coleoptile development

In Arabidopsis *elf3* and *prr7* mutants show defects in red light response during hypocotyl elongation (Zagotta *et al*., 2002; Kaczorowski and Quali, 2003). Hence, in order to study the role of *HvELF3 and Ppd-H1* in barley early seedling photomorphogenesis, I compared the length of coleoptiles of Bowman with Bowman(*eam8.w*) and Scarlett with S42-IL107 under different fluence rates of continuous red light and under dark conditions. The coleoptile was significantly elongated in the dark compared to red light in all genotypes (Fig. 9, A and B). The growth response of coleoptile development in red light was similar between Bowman and Bowman(*eam8.w*), likewise there was no genetic variation between Scarlett and S42-IL107 (Fig. 9). This result indicates that variation at *HvELF3and Ppd-H1* do not affect photomorphogenesis.

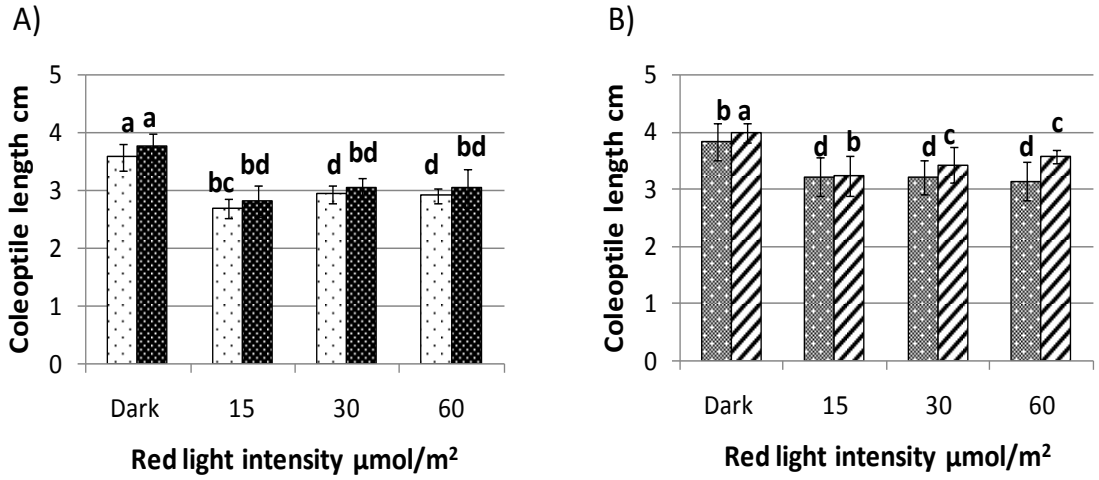

Figure 9. Coleoptile lengths of barley seedlings grown in the dark or under continuous red light. A) Coleoptile length of Bowman and Bowman(*eam8.w*) B) coleoptile length of Scarlett and S42-IL107. Germinated seeds were grown in petri dish containing agar for 2 days in different intensities of continuous red light or dark. Coleoptile length was measured using a calibrated ruler. Values are means ± SD of 8-10 seedlings. Different letters indicate significant difference at $p \leq 0.05$ using least square means.

Discussion

Reciprocal interaction between clock and stress-response genes

The effects of daily changes in light and temperature on the clock are well characterized (Millar, 2004; Boikoglou *et al*., 2011). However, much less is known about the effects of other less predictable environmental signals on the circadian clock. A major factor for plant growth and productivity is water availability and changes in water relations have profound effects on the plant metabolism (Chavez *et al*., 2002; Sanchez *et al*., 2013). Here, I show that osmotic stress applied at the barley roots affected expression of clock and stress genes in the shoot. Osmotic stress upregulated the expression of clock genes and advanced the expression peaks of evening expressed clock genes compared to control conditions irrespective of the genotype (Fig. 1 and 2). Interestingly, ABRE and DRE elements were identified in clock gene promoters (Supplementary Table S10). Similarly, Lai *et al*., (2012) demonstrated that ROS, an important secondary messenger during stress (Foyer and Noctor, 2005), acted as an input to the circadian clock. In addition, ABA signaling was shown to interact with the clock (Robertson *et al*., 2009; Legnalioli et al., 2009). For example, increased levels of ABA lengthened the free-running period of the clock and reduced *CCA1* mRNA levels in Arabidopsis (Hanano *et al*., 2006). By contrast, in barley, osmotic stress upregulated expression of circadian clock genes and advanced their expression peaks. Therefore, osmotic stress in barley affected the expression of clock genes differently than ABA in Arabidopsis. Genetic variation at *HvELF3 and Ppd-H1* did not affect the stress-response of clock genes, possibly because several entry points of stress into the clock exist, as suggested by the presence of stress-response *cis*-elements in several clock genes. The mechanisms by which osmotic stress and ABA regulate circadian periodicity still await unraveling.

Stress response genes, encoding *HvABI5* and *HvDRF1* binding to ABRE and DRE motifs in the clock gene promoters, were upregulated under osmotic stress. Upregulation of stress-response genes was thus likely upstream of the observed induction of clock genes under osmotic stress. It is noteworthy that osmotic stress also advanced the expression peaks of stress-response genes in all four genotypes, similar to the expression shifts observed for the clock genes under osmotic stress. Differences in the phase and expression levels of clock genes under osmotic stress suggested that clock genes were controlled by stress response genes, possibly encoding the

transcription factors *HvABI5* and *HvDRF1*. The presence of ABRE and DRE motifs in the promoters of clock genes solidified our hypothesis (Supplementary Table S13). On the other hand, differences in the phase and levels of clock gene expression due to osmotic stress or variation at *HvELF3* were reflected in the expression patterns of stress-response genes. Co-regulation of stress-response and clock genes and the presence of circadian clock motifs in the promoters of stress-response genes suggested that the clock controls the expression of stress-response genes in barley. Thus I propose a reciprocal feedback mechanism between the barley clock and stress response in barley.

Variation at *Ppd-H1* affects the expression levels of stress-response genes

In contrast to *HvELF3*, genetic variation at *Ppd-H1* did not affect diurnal expression patterns of clock and stress-response genes. Interestingly it did alter the expression levels of several stress-response genes (Fig. 4, Chapter 1 Fig. 5). These results suggested that the ancestral *Ppd-H1* allele caused an increased expression of stress-response genes under osmotic stress. In Arabidopsis, PRR proteins have been described as transcriptional repressors presumably binding to EE or G-box elements in the promoters of target genes for plant growth, light signaling and stress-response (Liu *et al*., 2013). Nakamichi *et al*., (2009) showed that the *DREB* genes were upregulated in the *prr975* mutant and expression of *DREB* genes was gated by *PRR* genes. The mutation in *Ppd-H1* of Scarlett and Triumph is likely to affect DNA binding qualities and has been associated with reduced expression levels of *HvFT1* and delayed flowering (Turner *et al*., 2005; Campoli *et al*., 2012a). Similarly, the mutated *Ppd-H1* allele in spring barley correlated with reduced expression levels of stress-response genes, such as *DREB*-like genes (Fig. 4). In addition, potential PRR-binding motifs, such as circadian clock factors and G-box motifs were identified in the conserved regions of the promoters of *HvDRF1*, *HvDREB1*, *HvABI5*, *HvAPX1*, *HvPHYB*, *HvLHCB* and *HvWRKY38*, suggesting that *Ppd-H1* controls expression of stress-response genes in barley (Supplementary Table S19). However, in contrast to PRRs acting as repressors in Arabidopsis, the ancestral *Ppd-H1* allele was associated with higher expression of stress-response genes. In Arabidopsis, PRR functions were revealed using the *prr975* mutant or lines over-expressing individual *PRR* genes exhibiting strong effects on the circadian clock. This study considered a natural mutation with apparently reduced functionality, but with no effect on its own expression or on expression of other clock genes. The different nature of the mutations may explain differences in the downstream responses of PRR genes in barley and Arabidopsis.

Because, the expression of clock genes was not different between the spring barley cultivars and introgression lines, we concluded that variation at *Ppd-H1* affected the expression of stress-response genes independently of its role in the barley clock.

Expression of *Ppd-H1* was reduced at peak times, but elevated during the night in Bowman(*eam8.w*) as compared to Bowman consistent with the role of *HvELF3* as a repressor of *Ppd-H1* during the night (Faure *et al*., 2012). Like *Ppd-H1*, stress-response genes like *HvDRF1* and *HvABI5* (Fig. 2) showed a higher expression during the night in Bowman(*eam8.w*) than Bowman. *HvELF3* expression may thus control expression of stress-response genes through changing diurnal expression of *Ppd-H1*.

Diurnal pattern of physiological traits do not correlate with diurnal changes in gene expression

Photosynthesis rate, net CO2 exchange and stomatal opening are under circadian control in Arabidopsis and clock mutants are strongly compromised in growth, physiology and metabolism (Kant *et al*., 2008; Dong *et al*., 2011). In addition, it has been shown that *ELF3* and *PRR* genes control stomatal opening and water relations in Arabidopsis (Nakamichi *et al*., 2009, Kinoshita *et al*., 2011). Hence I hypothesized that in barley physiological traits might be also under the control of the circadian clock. Variation at *Ppd-H1* affected stress induced senescence, electrolyte leakage, MDA and chlorophyll fluorescence as discussed in chapter 1. In contrast to variation at *Ppd-H1*, variation at *HvELF3* did not affect these senescence related traits (Fig. 8), supporting the hypothesis that *Ppd-H1* acted independently of its role in the circadian clock. Variation at both genes, *Ppd-H1* and *HvELF3* did not influence diurnal fluctuations in leaf water potential, stomatal conductance and gas exchange (Fig. 5, 6). Similarly, Izawa *et al*., (2011) reported that a mutation in the rice ortholog of the clock gene GIGANTEA did not affect net photosynthesis rates under field conditions, and the authors concluded that the photosynthesis- and growth-related primary assimilation were maintained under light-dark cycles despite defects in a clock gene and marked changes in the global transcriptome. In contrast, Edwards *et al.* (2011) showed that in field-grown *Brassica rapa* circadian period was correlated with photosynthesis, stomatal conductance and gas exchange, suggesting that physiology is regulated by the clock and synchronized with daily light cycles. Differences in the effects of the circadian clock on photosynthesis- and growth-related primary assimilation between monocots and dicots

may be due to differences in plant architecture and control of growth. Poiré *et al*., (2010) showed that growth is under circadian control and follows a diel pattern in dicots, while growth in monocots is mainly controlled by environmental fluctuations in water availability and temperature, suggesting that external cues are dominant over endogenous signals for the control of primary assimilation in monocots.

Although diurnal patterns of physiological traits were not affected by changes in clock genes, osmotic stress and variation at *HvELF3* and *Ppd-H1* affected biomass (Fig. 7).

A phytochrome-interacting factor-like protein *OsPIL1* has recently been identified as a key regulator of reduced growth under drought (Todaka *et al*., 2012). Similar to *PIF* like genes in rice and Arabidopsis (Nusinow *et al*., 2012), I found that osmotic stress downregulated *HvPIL3*, while the mutation in *HvELF3* upregulated *HvPIL3* expression at night and advanced its expression peak. Since *ELF3*, *PhyB* and *PIF*s are known to control photomorphogenesis in Arabidopsis (Zagotta *et al*., 1996, Liu *et al*., 2001; Soy *et al*., 2012), I analysed coleoptile lengths in the dark and under different fluence rates of red light. Unlike in Arabidopsis, our data indicated that neither *HvELF3*, nor expression differences in *HvPIL3* and *HvPHYB* between Scarlett and S42-IL107, affected barley seedling photomorphogenesis (Fig. 9). Similarly, Yang *et al*., (2012) found that the rice *oself3* mutant was not affected in photomorphogenesis, suggesting that the genetic control of photomorphogenesis is different between Arabidopsis and monocots.

Conclusion

This study demonstrated that osmotic stress at the root altered circadian clock genes in the barley shoot and thus acted as a spatial input signal into the clock. In contrast to Arabidopsis, barley growth and primary assimilation was less controlled by the clock and more responsive to environmental perturbations, such as osmotic stress. A strong response to unpredictable environmental changes may be adaptive in marginal environments which are often characterized by random climatic fluctuations, while a circadian control of the plants metabolism may confer optimal adaptation in environments with predictable diurnal changes. In this context it is interesting to note that grasses, including barley, are among the most stress resistant plants and adapted to wide array of environments. Alternatively, differences in plant architecture and

growth may explain variation in the control of assimilation between monocots and dicots, as meristematic tissue is well protected and covered at the base of the plant in monocots, but exposed to the environment in dicots.

References

Adams S., Carré IA. (2011). Downstream of the plant circadian clock: output pathways for the control of physiology and development.Essays in Biochemistry 49, 53–69.

Agarwal P., Agarwal P., Reddy M., Sopory S. (2006). Role of DREB transcription factors in abiotic and biotic stress tolerance in plants. Plant Cell Rep 25: 1263-1274.

Alabadi D., Oyama T., Yanovsky MJ., Harmon FG., Mas P., Kay SA. (2001).Reciprocal regulation between TOC1 and LHY/CCA1 within the Arabidopsis circadian clock. Science 293: 880–883.

Anjum S., Xiel X., Wang L.,Saleem M., Man C.,Lei W. (2011). Morphological, physiological and biochemical responses of plants to drought stress. African Journal of Agricultural Research.6(9): 2026-2032.

Armengaud P.,Thiery L., Buhot N., Grenier-De March G., Savouré A. (2004). Transcriptional regulation of proline biosynthesis in *Medicago truncatula* reveals developmental and environmental specific features. Physiol Plant 120: 442–450.

Ay N., Clauss K., Barth O., Humbeck K.(2008). Identification and characterization of novel senescence-associated genes from barley (Hordeum vulgare) primary leaves. Plant Biol (Stuttg). 1:121-135.

Badiane, F.A., D. Diouf, D., Sane, O. Diouf, V. Goudiaby, and N. Diallo.(2004). Screening cowpea Vigna unguiculata (L.) Walp. Varieties by inducingwater deficit and RAPD analyses. Afr. J. Biotechnol.3: 174-178.

Bartels D., Sunkar R. (2005). Drought and salt tolerance in plants. Crit. Rev. Plant. Sci. 24(1): 23-58.

Bartoli CG., Pastori GM., Foyer CH.(2000). Ascorbate bioshynthesis in mitochondria is linked to the electron transport chain between complexes III and IV.Plant Physiology123**,**335–343.

Bates L.S., Waldran R.P., Teare I.D. (1973). Rapid determination of free proline for water stress studies. Plant Soil 39: 205-208.

Blokhina O., Virolainen E., Fagerstedt,K.V.(2003). Antioxidants, oxidative damage and oxygen deprivation stress. Ann. Bot. 91: 179-194.

Boikoglou E., Ma Z., von Korff M., Davis AM., Nagy F., Davis S. (2011). Environmental memory from a circadian oscillator: the Arabidopsis thaliana clock differentially integrates perception of photic versus thermal entrainment. Genetics 189:655-664.

Boikoglou, E., and S. J. Davis. (2009). Signaling in the Circadian Clock, pp. 261–285 in Signaling in Plants, edited by F. B. S.Mancuso. Springer, Berlin.

Bradford K., Benech-Arnold R., Daniel Coˆme D., Corbineau F.(2008). Quantifying the sensitivity of barley seed germination to oxygen, abscisic acid, and gibberellin using a population-based threshold model. Journal of Exp. Bot.59(2):335–347.

Bujdoso, N. and Davis S. J. (2013). Mathematical modeling of an oscillating gene circuit to unravel the circadian clock network of higher plants. Front Plant Sci. 4: 3.

Callister, A.N., S.K. Arndt and M.A. Adams. (2006). Comparison of four methods for measuring osmotic potential of tree leaves.Physiol. Plant. 127:383–392.

Campoli C., Drosse B., Searle I., Coupland G., von Korff M. (2012a). Functional characterisation of HvCO1, the barley (Hordeum vulgare) flowering time ortholog of CONSTANS. Plant J 69, 868-880.

Campoli C., Pankin A., Casao CM., Davis SJ., von Korff M. (2013). HvLUX1 is a candidate gene underlying the early maturity 10 locus in barley: phylogeny, diversity, and interactions with the circadian clock and photoperiodic pathways. New Phytologist doi: 10.1111/nph.12346

Campoli C., Shtaya M., Davis S., von Korff M. (2012b). Expression conservation within the circadian clock of a monocot: natural variation at barley Ppd-H1 affects circadian expression of flowering time genes, but not clock orthologs. BMC Plant Biol. 12:97. doi: 10.1186/1471-2229-12-97

Casaretto J., Ho T. (2003). The transcription factors HvABI5 and HvVP1 are required for the abscisic acid induction of gene expression in barley aleurone cells. Plant Cell 15: 271–284.

Castells E., Portoles S, Huang W, Mas P. (2010). A functional connection between the clock component TOC1 and abscisic acid signalling pathways. Plant Signal Behav 5:409–411

Cellier, F., Conéjéro, G., Breitler, J., and Casse, F. (1998). Molecular and physiological responses to water deficit in drought-tolerant and drought-sensitive lines of sunflower. Accumulation of dehydrin transcripts correlates with tolerance. Plant physiology 1(116): 319-328.

Chaves M., Pereira J., Maroco, J., Rodrigues, M., Ricardo, C., Osorio, M, Carvalho, I., Faria, T. and Pinheiro, C. (2002). How plants cope with water stress in the field: photosynthesis and growth. Ann. Bot. 89, 907–916.

Chaves M.M., Flexas J, Pinheiro C. 2009. Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. Annals of Botany;103, 551–560.

Chaves M., Maroco JP., Pereira JS. (2003). Understanding plant responses to drought – from genes to the whole plant. Functional Plant Biology. 30:239-264.

Chinnusamy V., Zhu J., Zhu JK. (2007). Cold stress regulation of gene expression in plants. Trends in Plant Science 12, 444–451.

Cockram J., Jones H., Leigh FJ., O'sullivan D., Powell W., Laurie DA., Greenland AJ.(2007). Control of fl owering time intemperate cereals: genes, domestication, and sustainable productivity.J Exp Bot 58: 1231-1244.

Covington M., Maloof J., Straume M., Kay S., Harmer S. (2008). Global transcriptome analysis reveals circadian regulation of key pathways in plant growth and development. Genome Biol 9: R130.

Cruz de Carvalho MH.(2008). Drought stress and reactive oxygen species:Production, scavenging and signaling. Plant Signal Behav. 3(3): 156–165.

de Montaigu A., Tóth R., Coupland G. (2010). Plant development goes like clockwork.Trends Genet 26:296–306.

Dodd A., Salathia N., Hall A., Kevei E., Toth R., Nagy F., Hibberd JM., Millar AJ., Webb A. (2005). Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. Science 309:630–633.

Dong M.A., Farré E.M., Thomashow M.F. (2011). Circadian clock-associated 1 and late elongated hypocotyl regulate expression of the C-repeat binding factor (CBF) pathway inArabidopsis. PNAS.108: 7241–7246.

Dong MA., Farré EM., Thomashow MF.(2011). Circadian clock-associated 1 and late elongated hypocotyl regulate expression of the C-repeat binding factor (CBF) pathway in Arabidopsis.PNAS.108(17):7241-7246.

Doyle MR., Davis SJ., Bastow RM., McWatters HG., Kozma-Bognar L., Nagy F., Millar AJ., Amasino RM. (2002). The ELF4 gene controls circadian rhythms and flowering time in Arabidopsis thaliana. Nature 419: 74–77.

Edwards CE, Ewers BE, Williams DG, Xie Q, Lou P, Xu X, McClung CR, Weinig C.(2011). The genetic architecture of ecophysiological and circadian traits in Brassica rapa. Genetics.189:375-390.

Egawa C., Kobayashi F., Ishibashi M Nakamura T., Nakamura C., Takumi S. (2006). Differential regulation of transcript accumulation and alternative splicing of a DREB2 homolog under abiotic stress conditions in common wheat. Genes and Genetic Systems 81: 77–91.

Eshghi E.,Ojaghi J.,Rahimi M.,Salayeva S.(2010). Genetic characteristic of grain yield and its componenet in Barley (Hordeum vulgare L.) under Normal and Drought conditions. J Agric. & Environ. 9(5): 519-528.

Farooq M., Wahid A., Kobayashi N., Fujita D., Basra SMA. (2009). Plant drought stress: effects, mechanisms and management. Agron. Sustain. Dev. 29: 185-212.

Farre EM., Harmer SL., Harmon FG., Yanovsky MJ., Kay SA. (2005). Overlapping and distinct roles of PRR7 and PRR9 in the Arabidopsis circadian clock. Curr Biol 15: 47–54.

Faure S., Turner AS., Gruszka D., Christodoulou V., Davis SJ., von Korff M., Laurie D. (2012).Mutation at the Circadian Clock Gene EARLY MATURITY 8 Adapts Domesticated Barley (Hordeum vulgare) to Short Growing Seasons.PNAS.

Flexas J., Barbour MM., Brendel O., Cabrera HM., Carriquí M., Díaz-Espejo A., Douthe C., Dreyer E., Ferrio JP., Gago J., Gallé A., Galmés J., Kodama N., Medrano H,Niinemets Ü., Peguero-Pina JJ., Pou A., Ribas-Carbó M., Tomás M, Tosens T., Warren CR.(2012). Mesophyll diffusion conductance to CO2 : an unappreciated central player in photosynthesis. Plant Science 193–194, 70–84.

Foyer C., and Noctor G.(2005). Oxidant and antioxidant signalling in plants: A re-evaluation of the concept of oxidative stress in a physiological context. Plant Cell Environ.28:1056–1071.

Fujita Y., Fujita M., Shinozaki K., Yamaguchi-Shinozaki K.(2011). ABA-mediated transcriptional regulation in response to osmotic stress in plants. J. Plant Res.124:509-525.

González C., Ibarra S., Piccoli P., Botto J., Boccalandro H. (2012). Phytochrome B increases drought tolerance by enhancing ABA sensitivity in Arabidopsis thaliana.Plant Cell and Env.35 (11):1958-1968.

Gray WM. (2004). Hormonal Regulation of Plant Growth and Development. PLoS Biol 2(9): e311. doi:10.1371/journal.pbio.0020311.

Green RM., Tingay S., Wang Z-Y., Tobin EM. (2002). Circadian rhythms confer a higher level of fitness to Arabidopsis plants. Plant Physiol 129:576–584.

Grene R.(2002). Oxidative Stress and Acclimation Mechanisms in Plants. Arabidopsis Book.1:e0036.

Guo H., Moose S. (2003). Conserved noncoding sequences among cultivated cereal genomes identify candidate regulatory sequence elements and patterns of promoter evolution. Plant Cell 15:1143–1158.

Guo P., Baum M., Grando S., Ceccarelli S., Bai G., Li R., von Korff M., Vershney R., Graner A., Valkoun J. (2009). Differentially expressed genes between drought-tolerant and drought-sensitive barley genotypes in response to drought stress during the reproductive stage. J Exp Botany. 60: 3531-3544.

Hanano S., M. A. Domagalska, F. Nagy, and S. J. Davis. (2006). Multiple phytohormones influence distinct parameters of the plant circadian clock. Genes Cells 11: 1381–1392.

Harb A., Krishnan A., Ambayaram M., Pereira A. (2010). Molecular and physiological analysis of drought stress in Arabidopsis reveals early responses leading to acclimation in plant growth. Plant physiology 3(154): 1254-1271.

Harmer S., Hogenesch J., Straume M., Chang H., Han B., Zhu T., Wang X., Kreps J., Kay S. (2000). Orchestrated transcription of key pathways in Arabidopsis by the circadian clock.Science 290: 2110–2113.

Hazen SP., Schultz TF., Pruneda-Paz JL., Borevitz JO., Ecker JR., Kay SA. (2005). LUX ARRHYTHMO encodes a Myb domain protein essential for circadian rhythms. Proc Natl Acad Sci USA 102: 10387–10392.

Heath, R.L. and L. Packer. (1968). Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch. Biochem. Biophys. 125: 189-198.

Hoagland, D.R. and D.I. Arnon. (1950). The water-culture method for growing plants without soil'. California Agricultural Experiment Station Circular 347:1-32.

Holdsworth MJ., Bentsink L., Soppe WJ. (2008) Molecular networks regulating Arabidopsis seed maturation, after-ripening, dormancy and germination. New Phytol 179(1):33–54.

Hong ZL., Lakkineni K., Zhang ZM., Verma DPS. (2000). Removal of feedback inhibition of delta(1)-pyrroline-5-carboxylate synthetase results in increased proline accumulation and protection of plants from osmotic stress. Plant Physiol 122: 1129–1136.

Hsiao TC. (1973). Plant Responses to Water Stress.Annual Review of Plant Physiology. 24: 519-570.

Humbeck K, Quast S, Krupinska K. (1996). Functional and molecular changes in the photosynthetic apparatus during senescence of flag leaves from field-grown barley plants. Plant, Cell and Environment. 19:337–344.

Ingram J., and Bartels D. (1996). The molecular basis of dehydration tolerance in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47, 377–403.

Izawa T., Mihara M., Suzuki Y., Gupta M.,Itoh H.,Nagano A.,Motoyama R.,Sawada Y., Yano M.,Hirai M.,Makino A.,Nagamura Y. (2011). Os-GIGANTEA confers robust diurnal rhythms on the global transcriptome of rice in the field. Plant Cell 23, 1741–1755.

Jacobsen JV., Pearce DW., Poole AT., Pharis RP., Mander LN. (2002). Abscisic acid, phaseic acid and gibberellin contents associated with dormancy and germination in barley. Physiol Plant 115 428–441.

Kant P., Gordon M., Kant S., Zolla G., Davydov O., Heimer YM., et al (2008). Functional-genomics-based identification of genes that regulate Arabidopsis responses to multiple abiotic stresses. Plant Cell Environ.31:697–714.

Kidokoro S., Maruyama K., Nakashima K., Imura Y., Narusaka Y., Shinwari ZK., Osakabe Y., Fujita Y., Mizoi J., Shinozaki K., Yamaguchi-Shinozaki K. (2009). The phytochrome-interacting factor PIF7 negatively regulates DREB1 expression under circadian control in Arabidopsis. Plant Physiol.151(4):2046-2057.

Kinoshita T., Ono N, Hayashi Y., Morimoto S., Nakamura S., Soda M., Kato Y., Ohnishi M, Nakano T., Inoue S., Shimazaki K. (2011). FLOWERING LOCUS T regulates stomatal opening. Curr. Biol.21:1232-1238.

Kobayashi F., Maeta E., Terashima A., Takumi S.(2008). Positive role of a wheat HvABI5 ortholog in abiotic stress response of seedlings. Physiol Plant.134(1):74-86.

Kocheva K., Busheva M., Gerrgiev G.,Lambrev P.,Golstev V. (2005). Influence of short-term osmotic stress on the photosynthetic activity of barley seedlings. BIOLOGIA PLANTARUM.49 (1): 145-148.

Krause GH and Weiss E. (1991). Chlorophyll fluorescence and photosynthesis; the basics. Ann. Rev. P.l Physiol. P.l Molec. Biol. 42:313-349.

Kumar R.R., Karajol K. and Naik, G.R. (2011) Effect of polyethylene glycol induced water stress on physiological and biochemical responses in pigeonpea (Cajanus cajan L. Millsp.). Recent Research in Science and Technology., 3(1):148-152.

Kurepa J.,Smalle J.,Montagu M., Inze D.(1998). Oxidative stress and longevity in Arabidopsis:the late-flowering mutant gigantean is tolerant to paraquat. The plant journal.14(6):759-764.

Kurup, S., Jones, H.D., and Holdsworth, M.J. (2000). Interactions of the developmental regulator ABI3 with proteins identified from developing Arabidopsis seeds. Plant J. 21: 143–155.

Lai A., Doherty C., Mueller-Roeber B., Kay S., Schippers J., Dijkwel P. (2012). CIRCADIAN CLOCK-ASSOCIATED 1 regulates ROS homeostasis and oxidative stress responses.PNAS.

Lata C., Prasad M.(2011). Role of DREBs in regulation of abiotic stress responses in plants. J Exp Bot. 62(14):4731-47348.

Laurie DA., Prachett N., Bezant JH., Snape JW. (1995). RFLP mapping of five major genes and eight quantitative trait loci controlling flowering time in a winter × spring barley (Hordeum vulgare L.) cross. Genome. 38:575–585.

Legnaioli, T., Cuevas J., and Mas, P. (2009). TOC1 functions as a molecular switch connecting the circadian clock with plant responses to drought. EMBO J. 28, 3745–3757.

Lens F., Gennen J., Vanneste S., Rohde A., Beeckman T. (2008). Flowering-time genes modulate meristem determinacy and growth form in Arabidopsis thaliana.Nature genetics 40: 1489-1492.

Liu T.,Carlesson J.,Takeuchi T.,Newton L ,Farre E.(2013).Direct regulation of abiotic responses by the Arabidopsis circadian clock component PRR7. The plant journal.

Liu, X., Covington M., Fankhauser C., Chory J., and Wagner D. (2001). ELF3 encodes a circadian clock-regulated nuclear protein that functions in an Arabidopsis PHYB signal transduction pathway. Plant Cell 13,1293–1304.

Marcin´ska I., Czyczyło-Mysza I., Skrzypek E., Filek M., Grzesiak S., Grzesiak M., Janowiak F., Hura T., Dziurka M,Dziurka K., Nowakowska A., Quarrie S.(2013). Impact of osmotic stress on physiological and biochemical characteristics in drought-susceptible and drought-resistant wheat genotypes. Acta Physiol Plant.35:451–461.

Mare` C., Mazzucotelli E., Crosatti C., Francia E., Stanca AM.,Cattivelli L. (2004). Hv-WRKY38: a new transcription factor involved in cold- and drought-response in barley.Plant Mol Biol 55:399–416.

Massacci A., S.M. Nabiev L., Pietrosanti S.K., Nematov T.N., Chernikova K., Thor and J. Leipner. (2008). Response of the photosynthetic apparatus of cotton (Gossypium hirsutum) to the onset of drought stress under field conditions studied by gas-exchange analysis and chlorophyll fluorescence imaging. Plant Physiol. Biochem.46: 189–195.

Matsui A., Ishida J., Morosawa T., Mochizuki Y., Kaminuma E., Endo TA., Okamoto M., Nambara E., Nakajima M., Kawashima M., Satou M, Kim J-M., Kobayashi N., Toyoda T., Shinozaki K., Seki M. (2008). Arabidopsis transcriptome analysis under drought, cold, high-salinity and ABA treatment conditions using a tiling array. Plant Cell Physiol 49: 1135–1149.

Mayor C., Brudno M., Schwartz J.R., Poliakov A., Rubin E.M., FrazerK.A., Pachter L.S., and Dubchak, I. (2000). VISTA: Visualizing global DNA sequence alignments of arbitrary length. Bioinformatics 16: 1046–1047.

McClung C.R. (2006). Plant circadian rhythms. Plant Cell 18**:** 792–803.

McCue K., Hanson A. (2002). Drought and salt tolerance: Toward understanding and application. Trends in Biotech. 10: 358-362.

Mclntyre CL., Mathews KL., Rattey A., Chapman SC., Drenth J., Ghaderi M., Reynolds M., Shorter R. (2010). Molecular detection of genomic regions associated with grain yield and yield-related components in an elite bread wheat cross evaluated under irrigated and rainfed conditions. Theor. Appl. Genet 120: 527-41.

Merewitz EB., Gianfagna T., Huang B.(2011). Protein accumulation in leaves and roots associated with improved drought tolerance in creeping bentgrass expressing an ipt gene for cytokinin synthesis. J Exp Bot. 62(15):5311-5333.

Miao Y., Lv D., Wang P.,Wang X., Chen J.,Song C. (2006). An Arabidopsis Glutathione Peroxidase Functions as Both a Redox Transducer and a Scavenger in Abscisic Acid and Drought Stress Responses. The Plant Cell.18(10): 2749-2766.

Michael T., Salome´ P., Yu H., Spencer T., Sharp E., Alonso J.,Ecker J., McClung C. (2003). Enhanced fitness conferred by naturally occurring variation in the circadian clock. Science 302:1049–1053.

Michael T.P., Breton G., Hazen S.P., Priest H., Mockler T.C., Kay S.A., and Chory J. (2008). A morning-specific phytohormone gene expression program underlying rhythmic plant growth. PLoS Biol. 6**:** e225.

Millar A.J.(2004). Input signals to the plant circadian clock. J. Exp. Bot. 55: 277–283.

Miller G., Suzuki N., Ciftci-Yilmaz S., Mittler R. (2010). Reactive oxygen species homeostasis and signalling during drought and salinity stresses. Plant Cell Environ.33(4):453-467.

Mittler R. (2002).Oxidative stress, antioxidants and stress tolerance. Trends in Plant Science.7(9):405–410.

Mizuno T., Yamashino T. (2008). Comparative transcriptome of diurnally oscillating genes and hormone-responsive genes in *Arabidopsis thaliana*: insight into circadian clock-controlled daily responses to common ambient stresses in plants. Plant Cell Physiol 49: 481–487.

Moradi F., Ismail AM.(2007). Responses of photosynthesis, chlorophyll fluorescence and ROS-scavenging systems to salt stress during seedling and reproductive stages in rice. Ann Bot.99(6):1161-1173.

Munné-Bosch S., Alegre L. (2004). Die and let live: leaf senescence contributes to plant survival under drought stress. Functional Plant Biology 31, 203-216.

Murillo-Amador B., R. Lopez-Aguilar C. Kaya J. Larrinaga-Mayoral and A. Flores-Hernandez. (2002). Comparative effects of NaCl and polyethylene glycol on germination, emergence and seedling growth of cowpea. J. Agron. Crop Sci.188: 235-247.

Nakamichi N., Kusano M, Fukushima A., Kita M., Ito S, Yamashino T.,Saito K., Sakakibara H., Mi zuno T. (2009). Transcript profiling of an Arabidopsis PSEUDO RESPONSE REGULATOR arrhythmic triple mutant reveals a role for the circadian clock in cold stress response. Plant Cell Physiol. 50: 447–462.

Nakashima K., Ito Y., Yamaguchi-Shinozaki K. (2009). Transcriptional Regulatory Networks in Response to Abiotic Stresses in Arabidopsis and Grasses.Plant Phys. 149(1):88-95.

Nayyar H, Walia DP (2003) Water stress induced proline accumulation in contrasting wheat genotypes

Noctor G., Veljovic-Jovanovic S., Driscoll S., Novitskaya L., Foyer CH.(2002). Drought and oxidative load in the leaves of C3 plants: a predominant role for photorespiration?. Ann Bot.89 Spec No:841-850.

Nusinow D., Helfer A., Hamilton E., King J., Imaizumi T., Schultz T., Farré E., Kay S. (2011).The ELF4-ELF3-LUX complex links the circadian clock to diurnal control of hypocotyl growth. Nature 2011;475:398-402.

Parrott DL., Downs EP., Fischer AM. (2012). Control of barley (Hordeum vulgare L.) development and senescence by the interaction between a chromosome six grain protein content locus, day length, and vernalization. J Exp Bot.63(3):1329-1339.

Penfield S., Hall A. (2009). A Role for Multiple Circadian Clock Genes in the Response to Signals That Break Seed Dormancy in Arabidopsis.The Plant Cell. 21(6):1722-1732.

Poire´ R., Wiese-Klinkenberg A., Parent B., Mielewczik M.,Schurr U., Tardieu F., Walter A. (2010). Diel time-courses of leaf growth in monocot and dicot species: endogenous rhythms and temperature effects. Journal of Experimental Botany 61, 1751–1759.

Pokhilko A., Fernández A., Edwards K., Southern M., Halliday K., Millar A. (2012). The clock gene circuit in Arabidopsis includes a repressilator with additional feedback loops. Mol Sys Biol 8: 574.

Qin F., Kakimoto M., Sakuma Y., Maruyama K., Osakabe Y., Tran LS., Shinozaki K., Yamaguchi-Shinozaki K. (2007). Regulation and functional analysis of ZmDREB2A in response to drought and heat stresses in Zea mays L. Plant J. 50(1):54-69.

Quarrie SA., Quarrie SP., Radosevic R. (2006). Dissecting a wheat QTL for yield present in a range of environments: From the QTL to candidate genes. J Exp Bot .11:2627-2637.

Ramanjulu S., Bartels D. (2002). Drought- and desiccation-induced modulation of gene expression in plants. Plant Cell Environ 25: 141–151.

Rebetzke GJ., Condon AG., Farquhar GD., Appels R., Richards RA. (2008). Quantitative trait loci for carbon isotope discrimination are repeatable across environments and wheat mapping populations. Theor Appl Genet. 118:123–137.

Rivero R., Kojima M., Gepstein A., Sakakibara H.,Mittler R.,Gepstein S.,Blumwald. (2007). Delayed leaf senescence induces extreme drought tolerance in a flowering plant.PNAS.104(49): 19631–19636.

Robertson F., Skeffington A., Gardner M., Webb A. (2009). Interactions between circadian and hormonal signalling in plants. Plant Mol Biol 69: 419–427.

Rollins J., Habte E., Templer S., Colby T., Schmidt J., von Korff M. (2013). Leaf proteome alterations in the context of physiological and morphological responses to drought and heat stress in barley (Hordeum vulgare L.). Journal of Ex Bot. 6(64) 3201-3212.

Romagosa I., Prada D., Moralejo M.,Sopena A., Muñoz P., Casas A.,Swanston J., Molina-Cano J. (2001). Dormancy, ABA content and sensitivity of a barley mutant to ABA application during seed development and after ripening. Journal of Exp. Bot. 52(360): 1499-1506.

Rushton PJ., Somssich IE., Ringler P., Shen QJ. (2010). WRKY transcription factors. Trends Plant Sci. 15(5):247-258.

Sakuma Y., Maruyama K., Osakabe Y., Qin F., Seki M., Shinozaki K., Yamaguchi-Shinozaki K. (2006). Functional analysis of an *Arabidopsis* transcription factor, DREB2A, involved in drought-responsive gene expression. Plant Cell 18 1292–1309.

Sanchez A., Shin J., Bujdoso N., Obata T., Neumann U., Du S., Ding Z., Davis A., Shindo T., Schmelzer E., Sulpice R., Nunes-Nesi R., Stitt M, Fernie A., Davis S. (2013). TIME FOR COFFEE is an Essential Component in the Maintenance of Arabidopsis thaliana Metabolic Homeostasis.The Plant journal.

Sanchez, A., Shin, J., and Davis, S. J. (2011) Stress and the plant circadian clock. Plant Signaling and Behavior 6: 1-9.

SAS Institute.(2008). The SAS System for Windows, release 9.2. Cary NC: SAS Institute.

Schaffer R., Landgraf J., Accerbi M., Simon V., Larson M., Wisman E. (2001). Microarray analysis of diurnal and circadian-regulated genes in Arabidopsis. Plant Cell 13: 113–123.

Schmalenbach I., March TJ., Bringezu T., Waugh R., Pillen K. (2011). High-Resolution Genotyping of Wild Barley Introgression Lines and Fine-Mapping of the Threshability Locus thresh-1 Using the Illumina GoldenGate Assay. Genes, Genomes Genetics. doi:10.1534/g3.111.000182.

Shang Y., Yan L., Liu ZQ., Cao Z., Mei C., Xin Q,. Wu FQ., Wang XF., Du SY., Jiang T., et al. (2010).The Mg-chelatase H subunit of Arabidopsis antagonizes a group of WRKY transcription repressors to relieve ABA-responsive genes of inhibition. Plant Cell 22: 1909–1935.

Sharma S., Villamor J., Verslues P. (2011). Essential Role of Tissue-Specific Proline Synthesis and Catabolism in Growth and Redox Balance at Low Water Potential. Plant Physiol. 157(1): 292–304.

Shen Q., Chen CN., Brands A., Pan SM., Ho TH. (2001). The stress- and abscisic acid-induced barley gene HvA22: developmental regulation and homologues in diverse organisms. Plant Mol Biol.45(3):327-340.

Shen Y., Wang X., Wu F., Du S., Cao Z., Shang Y., Wang X., Peng C., Yu X., Zhu S., Fan R., Xu Y., Zhang D. (2006). The Mg-chelatase H subunit is an abscisic acid receptor. Nature 443: 823–826.

Shen YG., Zhang WK., He SJ., Zhang JS., Liu Q., Chen SY.(2003). An EREBP/AP2-type protein in Triticum aestivum was a DRE-binding transcription factor induced by cold, dehydration and ABA stress. Theor Appl Genet. 106(5):923-930.

Shinozaki K., Yamaguchi-Shinozaki K (2007) Gene networks involved in drought stress response and tolerance. J Exp Bot 58 221–227.

Sivamani E., Bahieldin1 A., Wraith JM., Al-Niemi T., Dyer WE., Ho TD., Qu R. (2000). Improved biomass productivity and water use efficiency under water deficit conditions in transgenic wheat constitutively expressing the barley HVA1 gene. Plant Sci.155(1):1-9.

Soy J., Leivar P., Gonzalez-Schain N., Sentandreu M., Prat S., Quail PH., Monte E. (2012). Phytochrome-imposed oscillations in PIF3-protein abundance regulate hypocotyl growth under diurnal light-dark conditions in Arabidopsis. Plant J . 71(3):390-401.

Staiger D.(2013). The circadian clock goes genomic. Genome Biology.14:208

Strasser R.J., Srivastava A., Govindjee. (1995). Polyphasic chlorophyll a fluorescence transients in plants and cyanobacteria. Photochem. Photobiol., 61: 32–42.

Subrahmanyam D., Subash N., Haris A., Sikka AK. (2006). Influence of water stress on leaf photosynthetic characteristics in wheat cultivars differing in their susceptibility to drought. Photosynthetica 44:125-129.

Sujeeth N.,Kini R.,Shailasree S.,Wallaart E., Shetty S.,Hille J.(2012). Characterization of a hydroxyproline-rich glycoprotein in pearlmillet and its differential expression in response to the downymildew pathogen Sclerospora graminicola. Acta Physiol Plant.34:779–791.

Szalai G, Janda T, Páldi E, Szigeti Z. (1996). Role of light in the development of post-chilling symptoms in maize. J Plant Physiol 148:378-383.

Taeb M., Koebner RMD., Forster B.P, Law CN.(1992). Association between genes controlling flowering time and shoot sodium accumulation in the Triticeae. Plant and Soil146, 117–121.

Takata N., Saito S., Saito CT., Uemura M. (2010). Phylogenetic footprint of the plant clock system in angiosperms: evolutionary processes of pseudo-response regulators. BMC Evol Biol 2010, 10:126.

Tang N., Zhang H., Xianghua L., Xiao J, Xiong L.(2012). Constitutive Activation of Transcription Factor OsbZIP46 Improves Drought Tolerance in Rice. Plant Physiol. 2012 April; 158(4): 1755–1768.

Todaka D., Nakashima K., Shinozaki K., Yamaguchi-Shinozaki K. (2012). Toward understanding transcriptional regulatory networks in abiotic stress responses and tolerance in rice. PNAS.

Tondelli A., Francia E., Barabaschi D., Aprile A., Skinner JS., Stockinger EJ., Stanca AM., Pecchioni N. (2006). Mapping regulatory genes as candidates for cold and drought stress tolerance in barley. Theoretical and Applied Genetics 112: 445–454.

Turner A., Beales J,. Faure S., Dunford RP., Laurie D. (2005). The pseudo-response regulator Ppd-H1 provides adaptation to photoperiod in barley.Science 310: 1031–1034.

Verslues PE., Agarwal M., Katiyar-Agarwal S., Zhu JH., Zhu JK. (2006). Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. Plant J 45: 523–539.

von Korff M., Grando S., Del Greco. A., This D,. Baum M., Ceccarelli S.(2008). Quantitative trai loci associated with adaptation to Mediterranean dryland conditions in barley. Theor Appl Genet.117 (5):653-669.

von Korff M., Wang H., Léon J., Pillen K. (2004). Development of candidate introgression lines using an exotic barley accession (Hordeum vulgare ssp. spontaneum) as donor. Theor Appl Genet, 109:1736–1745.

von Korff M., Wang H., Léon J., Pillen K. (2006). AB-QTL analysis in spring barley: II. Detection of favourable exotic alleles for agronomic traits introgressed from wild barley (H. vulgare ssp. spontaneum). Theor Appl Genet 112:1221–1231.

Vurayai R., Emongor V and Moseki B. (2011). Effect of water stress imposed at different growth and development stages on morphological traits and yield of Bambara Groundnuts (Vigna subterranea L. Verdc). American journal of Plant Physiology.6(1) 17-27.

Waddington SR., Cartwright PM., Wall PC. (1983) .A quantitative scale of spike initial and pistil development in barley and wheat. Ann Bot (Lond) 51:119–130.

Wang Z., Tobin E. (2001). Constitutive Expression of the CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) Gene Disrupts Circadian Rhythms and Suppresses Its Own Expression. Cell.93(7):1207-1217.

Wilkins O.,Brautigam K.,Campbell M. (2010). Time of day shapes Arabidopsis drought transcriptomes.The Plant J. 65(5):715-727.

Xie Z., Zhang ZL., Hanzlik S., Cook E, Shen QJ.(2007). Salicylic acid inhibits gibberellin-induced alpha-amylase expression and seed germination via a pathway involving an abscisic-acid-inducible WRKY gene. Plant Mol Biol.64(3):293-303.

Xu Y.,Liu R., Yan L., Liu Z., Jiang S., Shen Y., Wang X. ,Zhang D. (2012). Light-harvesting chlorophyll a/b-binding proteins are required for stomatal response to abscisic acid in Arabidopsis. J Exp Bot.63 (3):1095-1106.

Xu ZS., Ni ZY., Li ZY., Li LC., Chen M., Gao DY., Yu XD., Liu P., Ma YZ.(2009). Isolation and functional characterization of HvDREB1-a gene encoding a dehydration-responsive element binding protein in Hordeum vulgare. J Plant Res. 122(1):121-130.

Xue GP., Loveridge CW.(2004). HvDRF1 is involved in abscisic acid-mediated gene regulation in barley and produces two forms of AP2 transcriptional activators, interacting preferably with a CT-rich element. Plant J. 37(3):326-339.

Yakir E., Hilman D., Harir Y.,Green R. (2007). Regulation of output from the plant circadian clock.FEBS Journal.274(2):335-345.

Yamaguchi-Shinaozaki K. and Shinozaki K. (2005).Organization of cis-acting regulatory elements in osmotic- and cold-stress responsive promoters. Trends Plant Sci.10:88-94.

Yang Y., Peng Q., Chen G., Li X., Wu C. (2012). OsELF3 Is Involved in Circadian Clock Regulation for Promoting Flowering under Long-Day Conditions in Rice. Molecular plant.6 (1): 202-215.

Yang ZB., Eticha D., Rotter B., Rao IM., Horst WJ. (2011).Physiological and molecular analysis of polyethylene glycol-induced reduction of aluminium accumulation in the root tips of common bean (*Phaseolus vulgaris*) New Phytologist.192:99–113.

Yerushalmi S., Yakir E., Green R. (2011). Circadian clocks and adaptation in Arabidopsis. Mol Ecol 20:1155–1165.

Zagotta M,, Hicks K., Jacobs C., Young J., Hangarter R., and Meeks-Wagner D. (1996). The Arabidopsis ELF3 gene regulates vegetative photomorphogenesis and the photoperiodic induction of flowering.Plant J. 10: 691–702.

Zakhrabekova S., Gough SP., Braumann I., Müller AH., Lundqvist J., Ahmann K., Dockter C., Matyszczak I., Kurowska M., Druka A., Waugh R., Graner A., Stein N., Steuernagel B., Lundqvist U., Hansson M.(2012). Induced mutations in circadian clock regulator Mat-a facilitated short-season adaptation and range extension in cultivated barley. PNAS 109, 4326-4331.

Zentgraf U., Hemleben V. (2008). Molecular cell biology: are reactive oxygen species regulators of leaf senescence? Lüttge U, Beyschlag W, Murata J, editors. , , Progress in Botany.69:117–138.

Zhong H.H., Painter J.E., Salomé P.A., Straume M., and McClung, C.R. (1998). Imbibition, but not release from stratification, sets the circadian clock in *Arabidopsis* seedlings. Plant Cell 10**:** 2005–2017.

Zhu J. K. (2002) Salt and drought stress signal transduction in plants. Annual Review of Plant Physiology andPlant Molecular Biology, 53:247–273.

Živčák M., Brestič M., Olšovská K., Slamka P. (2008). Performance index as a sensitive indicator of water stress in Triticum aestivum L. PLANT SOIL ENVIRON. 54(4): 133–139.

Supplementary Tables

Supplementary Table S1. List of primers used for qRT-PCR.

Primer name	Accession code	Forward primer (5'-3')	Reverse primer (5'-3')	References
HvDRF1	AF521303	GAAGTTGACCCGGTGACTGT	GCTCATCTCAGCATCATGGA	Xue *et al*.2003
HvDREB1	DQ012941	TCAACTTCCCAGAGCATTCC	CACAGTCCCTGCAGACTCAA	Xu *et al*.2009
HvABI5	AY150676	CGCGCTGAAGTATTGAAACA	CACCAGAACGTTGCAGCTTA	Kobashi *et al*.2008
HvWRKY38	AY541586	CCGTCAAAGCCTGCGCAGACAAAGC	ATGTTACAACCTCCCTCGCCG	Xiong *et al*.2009
HvAPX1	AS006358	CGCCCTCTT GTGGAGAAATA	CGCGCATAGTAGCAGCAGTA	Shi *et al*.2001
HvCAT1	AF021938	TGGACGGATGGTACTGAACA	GTGCCTTTGGGTATCAGCAT	Skadsen *et al*.1995
HvARF1	AJ508228	AGCTCCACAGGATGCTGAAT	TCCCTCGTACAACCCTTCAC	Ay *et al*.2008
HvLHCB	X63197	TCTGAGGGTGGTCTCGAT TA	CAACAAGACCCATGAGAAGG	Brandt *et al*. 1992
HvPHYB	AK365283	CTTGCGCACCAACTATCAGA	CTCCATGACACACCGTCAAC	Szucs *et al*. 2006
HvPIL3	AK359117	AGCTCATACCCCACTGCAAC	CATCCACATCATCTGCACCT	Faure *et al*.2012
HvCCA1	HQ850270	CCTGGAATTGGAGATGGAGA	TGAGCATGGCTTCTGATTTG	Campoli *et al*.2012
HvPRR1	HQ850268	TGTCTTTCCTCGGAAATTGG	TGTCAGACATCCCTGGAACA	Campoli *et al*.2012
HvGI	AY740524	TCAGTTAGAGCTCCTGGAAGT	GGTAGTTTGGGCTTTGGATG	Campoli *et al*.2012
HvPRR37	AY970703	GATGGATTCAAAGGCAAGGA	GAACAATTGGCTCCTCCAAA	Campoli *et al*.2012
HvPRR73	JQ791230	GCGCCGTAGAGAATCAGAAC	CATGTCGGGTACAGTCATCG	Campoli *et al*. 2012
HvPRR59	JQ791228	CAGAACTCCAGTGTCGCAAA	TGCTGTTGCCAGAGTTGTTC	Campoli *et al*. 2012
HvPRR95	JQ791232	GAAATTCCGCATGAAAAGGA	TTCCGCATCTTCTGTTGTTG	Campoli *et al*. 2012

Supplementary Table S2. Analysis of variance of physiological traits over all time points of Scarlett(*ppd-H1*) and S42-IL107(Ppd-H1)

Traits	Statistics	Genotype	Treatment	Time point	Experiment	Genotype *Treatment	Genotype *Time point	Genotype*Experiment	Genotype*Treatment*Time point	Genotype *Treatment*Time point*Experiment
RWC	F-value	0.37	56.99**	0.59	4.64*	1.15	1.02	1.08	2.58*	3.34**
	R^2	0	0.26	0.01	0.04	0.01	0.01	0.01	0.05	0.31
LT	F-value	0	111.2**	1.97	11.8*	0.12	0.34	2.45	0.66	1.37
	R^2	0	0.4	0.01	0.08	0	0	0.02	0.01	0.1
EL	F-value	14.85*	49.86**	24.56**	2.12	21.14**	7.8*	1.61	13.41**	2.39*
	R^2	0.04	0.15	0.14	0.01	0.06	0.05	0.01	0.16	0.14
Fv/Fm	F-value	0.88	0.29	3.14*	1.17	0	0.3	2	1.4	0.69
	R^2	0.01	0	0.1	0.02	0	0.01	0.03	0.09	0.11
Area	F-value	0.08	4.96*	1.28	0.02	0.05	0.42	0.02	1.79	0.61
	R^2	0	0.08	0.04	0	0	0.01	0	0.11	0.1
PI	F-value	0	14.53*	162.85**	0.06	0.87	0.04	1.8	5.93*	1.16
	R^2	0	0.03	0.76	0	0	0	0	0.06	0.03
MDA	F-value	1.02	35.78**	45.11**	0.34	12.43*	8.97*	0.25	5.43*	2.07
	R^2	0	0.19	0.22	0.02	0.09	0.06	0.01	0.03	0
Proline	F-value	0.13	129.94**	39.31**	1.76	0	1.91	0.69	22.21**	1.45
	R^2	0	0.32	0.19	0.01	0	0.01	0	0.22	0.07

EL- electrolyte leakage, RWC- relative water content, Proline- proline content, Fv/Fm -maximum quantum efficiency of photosystem II, Area - area over the curve between Fo and Fm, PI -performance index ,LT- leaf temperature and MDA- malondialdehyde.

Supplementary Table S3. Analysis of variance of physiological traits after 24, 48 and 72h stress of Scarlett (*ppd-H1*) and S42-IL107(Ppd-H1)

Traits		RWC		LT		Proline		Fv/Fm		Area		PI		MDA		EL	
source of variation/Statistics	Time	F-value	R^2	F-value	R^2	F-value	R^2	F-value	R^2	F-value	R^2	F-value	R^2	F-value	R^2	F-value	R^2
Genotype	24h	1.11	0.03	0.44	0	0.28	0.01	0.31	0.02	0.04	0	0.06	0	1.02	0.06	5.59	0.26
	48h	0	0	0.43	0	1.7	0.02	0.09	0	0.04	0	0.03	0	0.14	0	0.08	0
	72h	0.44	0	0.01	0	1.41	0.01	1.45	0.05	1.12	0.04	0.06	0	7.81*	0.13	23.29**	0.15
Treatment	24h	4.86*	0.27	35.99**	0.31	6	0.12	0.55	0.03	1.54	0.09	0.25	0.02	0.01	0	0.42	0.01
	48h	21.42**	0.35	51.52**	0.44	38.19**	0.39	0.57	0.01	0	0	0.1	0	37.73**	0.66	5.83*	0.3
	72h	89.41**	0.41	31.12**	0.46	91.71**	0.75	6.91*	0.25	8.69*	0.3	52.64**	0.54	29.85**	0.51	75.73**	0.48
Experiment	24h	2.99	0.08	12.18*	0.43	8.97	0.37	0.34	0.02	1.99	0.12	1.26	0.08	0.25	0.01	0.54	0.03
	48h	5.84*	0.19	6.85*	0.29	6.59*	0.13	1.95	0.05	0.26	0.02	2.81	0.13	0.14	0	1.56	0.04
	72h	1.36	0.03	2.89	0.09	1.82	0.03	0.5	0.02	1.65	0.06	9.93	0.1	0.56	0.01	0.92	0.01
Genotype *Treatment	24h	2.48	0.06	0.16	0	0	0	0.09	0.01	0.11	0.01	0.17	0.01	0.34	0.02	2.54	0.12
	48h	3.56	0.06	1.16	0.01	1.51	0.02	0.25	0.01	0.48	0.03	0.13	0.01	0.08	0	2.85	0.1
	72h	9.99*	0.29	0.33	0	0.45	0	1.41	0.05	3.05	0.1	10.24*	0.11	4.48*	0.08	25.72**	0.16
Genotype* Experiment	24h	0.52	0.02	2.86	0.05	0.84	0.03	0.28	0.02	0.12	0.01	0.16	0.01	2.07	0.12	0.29	0.01
	48h	0.42	0.01	2.94	0.05	1.22	0.02	8.58*	0.22	0.12	0.01	3.12	0.14	0.26	0	3.66*	0.3
	72h	2.94	0.05	0.64	0.02	0.25	0	0.84	0.03	0.2	0.01	1.27	0.01	0.09	0	1.99	0.03
Genotype* Treatment* Experiment	24h	0.42	0.04	0.44	0.02	0.6	0.05	0.77	0.1	0.16	0.02	0.49	0.06	0.53	0.06	1.77	0.1
	48h	0.59	0.04	0.61	0.02	4.27	0.17	6.46*	0.34	0.76	0.09	0.85	0.08	1.61	0.06	2.52	0.14
	72h	6.72*	0.12	1.6	0.1	0.42	0.01	1.24	0.09	0.28	0.02	5.06	0.1	0.03	0	0.91	0.02

EL- electrolyte leakage, RWC- relative water content, Proline- proline content, Fv/Fm -maximum quantum efficiency of photosystem II, Area - area over the curve between Fo and Fm, PI -performance index ,LT- leaf temperature and MDA- malondialdehyde.

Supplementary Table S4. Analysis of variance of physiological traits over all time points of Triumph(*ppd-H1*) and Triumph (*Ppd-H1*)

Traits	Statistics	Genotype	Treatment	Time point	Experiment	Genotype *Treatment	Genotype *Time point	Genotype *Experiment	Genotype*Treatment*Time point	Genotype *Treatment*Time point*Experiment
RWC	F-value	1.4	20.2**	3.59*	2.79	0.49	2.49	0.39	4.2*	1.1
	R^2	0.01	0.14	0.05	0.04	0	0.03	0.01	0.11	0.15
LT	F-value	3.54	198.23**	7.04*	21.23**	1.44	0.51	2.19	3.32	1.46
	R^2	0.01	0.52	0.04	0.11	0	0	0.01	0.04	0.08
EL	F-value	67.91**	299.72**	33.23**	1.22	40.15**	2.49	1.5	9.96**	0.55
	R^2	0.11	0.51	0.11	0	0.07	0.01	0.01	0.07	0.02
Fv/Fm	F-value	8.73*	6.93*	1.26	4.44	5.65*	1.33	7.79*	2.22	4.06*
	R^2	0.04	0.03	0.01	0.04	0.03	0.01	0.07	0.04	0.39
Area	F-value	0.26	1.9	6.63*	0.12	1.66	0.37	0.62	4.2	2.07
	R^2	0	0.01	0.09	0	0.01	0.01	0.01	0.12	0.29
PI	F-value	0.2	0.46	86.73**	3.29	1	1.09	0.1	5.4*	1.07
	R^2	0	0	0.59	0.02	0	0.01	0	0.07	0.07
MDA	F-value	3.45	1.35	200.95**	4.42*	9.19*	12.37**	1.84	7.75**	1.65
	R^2	0.01	0.01	0.63	0.02	0.06	0.12	0.02	0.05	0.05
Proline	F-value	0.2	369.68**	85.05**	2.55	0.5	0.03	0.49	42.03**	0.7
	R^2	0	0.48	0.22	0.01	0	0	0	0.22	0.02

EL- electrolyte leakage, RWC- relative water content, Proline- proline content, Fv/Fm -maximum quantum efficiency of photosystem II, Area - area over the curve between Fo and Fm, PI -performance index ,LT- leaf temperature and MDA- malondialdehyde.

Supplementary Table S5. Analysis of variance of physiological traits after 24, 48 and 72h stress of Triumph(*ppd-H1*) and Triumph(*Ppd-H1*)

Traits		RWC		LT		Proline		Fv/Fm		Area		PI		MDA		EL	
source of variation/Statistics	Time	F-value	R^2	F-value	R^2	F-value	R^2	F-value	R^2	F-value	R^2	F-value	R^2	F-value	R^2	F-value	R^2
Genotype	24h	0.18	0.01	0.22	0	0.34	0.01	2.63	0.05	0.47	0.01	0.65	0.02	6.54*	0.17	13.95*	0.11
	48h	0.01	0	2.06	0.02	1.6	0.01	1.25	0.02	0.35	0.01	0.36	0.01	12.1*	0.1	35.5**	0.24
	72h	3.44	0.07	1.8	0.01	0.02	0	5.08*	0.06	0.1	0	1.23	0.02	12.16*	0.12	20.88*	0.09
Treatment	24h	0.39	0.01	41.35**	0.54	1.96	0.04	0.62	0.01	2.02	0.04	2.68	0.08	0.24	0.01	86.7**	0.7
	48h	5.39*	0.11	57.54**	0.54	284.93**	0.9	0.27	0.01	0.63	0.02	1.56	0.04	36.38**	0.3	71.07**	0.49
	72h	17.94*	0.36	115.17**	0.61	180**	0.9	7.78*	0.09	24.26**	0.42	22.25**	0.33	37.95**	0.39	154.42**	0.64
Experiment	24h	0.07	0	1.8	0.05	2.14	0.57	8.32*	0.32	1.85	0.08	1.89	0.11	3.14	0.16	0.93	0.02
	48h	4.7	0.19	7.04*	0.13	1.24	0.03	9.94	0.39	2.76	0.19	5.66	0.27	9.11*	0.32	0.67	0.01
	72h	1.24	0.05	17.18**	0.18	1.07	0.01	1.65	0.14	0.06	0	0.53	0.02	3.33	0.07	0.25	0
Genotype *Treatment	24h	0.05	0	0.65	0.01	0.07	0	0.89	0.02	0.77	0.02	0.23	0.01	0.85	0.02	0.08	0
	48h	0.03	0	0.96	0.01	0.57	0	1.07	0.02	0.11	0	1.26	0.03	0.22	0	12.2*	0.08
	72h	1.98	0.04	4	0.02	0.61	0	4.91*	0.06	5.36*	0.09	6.26*	0.09	13.9*	0.14	44.31**	0.18
Genotype*Experiment	24h	2.13	0.14	0.87	0.02	1.6	0.06	1.32	0.05	2.15	0.09	0.94	0.06	3.16	0.16	0.17	0
	48h	0.67	0.03	2.99	0.06	0.01	0	1.01	0.04	0.02	0	0.65	0.03	1.17	0.09	1.78	0.02
	72h	0.08	0	0.22	0	0.01	0	2.33	0.18	0.24	0.01	4.52*	0.14	4.18*	0.09	0.18	0
Genotype*Treatment*Experiment	24h	0.35	0.05	1.18	0.06	1.27	0.1	1.32	0.1	2.89	0.25	0.41	0.05	0.76	0.08	0.5	0.02
	48h	2.91	0.23	0.53	0.02	1.03	0.01	1.24	0.1	0.35	0.05	0.89	0.09	1.13	0.04	0.91	0.03
	72h	0.41	0.03	2.56	0.05	0.48	0.01	4.13*	0.2	1.25	0.09	0.92	0.06	0.06	0	0.64	0.01

EL- electrolyte leakage, RWC- relative water content, Proline- proline content, Fv/Fm -maximum quantum efficiency of photosystem II, Area - area over the curve between Fo and Fm, PI -performance index ,LT- leaf temperature and MDA- malondialdehyde.

Supplementary Table S6. Analysis of variance of drought-responsive gene expression over all time points in Scarlett(*ppd-H1*) and S42-IL107(*Ppd-H1*)

Gene	Source of Variation/Statistics	Genotype	Treatment	Replication	Time point	Genotype *Treatment	Genotype* Time point	Genotype*Treatment*Time point
HvDREB1	F-value	0.17	34.69**	0.92	1.13	0.04	4.47*	2.68
	R^2	0	0.45	0.02	0.03	0	0.12	0.14
HvDRF1	F-value	2.07	83.67**	3.49*	20.54**	8.11*	0.86	3.11*
	R^2	0.01	0.47	0.04	0.23	0.05	0.01	0.07
HvABI5	F-value	0.39	27.83**	1.92	10.95*	0.01	3.14	5.69*
	R^2	0	0.27	0.04	0.21	0	0.06	0.22
HvWRKY38	F-value	0.68	23.32**	3.37	15.77**	1.09	0.04	4.64*
	R^2	0.01	0.23	0.07	0.31	0.01	0	0.18
HvPIL3	F-value	0.43	38.34**	2.5	7.36*	0	0.55	0.41
	R^2	0.01	0.46	0.06	0.18	0	0.01	0.02
HvRB1	F-value	0.3	8.87*	2.53	8.91*	1.09	0.11	1.85
	R^2	0.01	0.15	0.08	0.3	0.02	0	0.12
HvARF1	F-value	0.72	45.43**	0.39	0.79	6.53*	0.98	1.02
	R^2	0.01	0.61	0.01	0.02	0.03	0.03	0.06
HvAPX1	F-value	7.59*	19.76**	1.1	10.75*	5.43*	3.73*	5.1*
	R^2	0.07	0.19	0.02	0.21	0.03	0.07	0.2
HvCAT1	F-value	2.09	19.36*	0.01	12.13*	4.42*	1.04	6.2*
	R^2	0.02	0.2	0	0.25	0.05	0.02	0.25
HvHRGP	F-value	4.11	3.2	1.49	9.66*	0	4.91*	0.63
	R^2	0.07	0.05	0.05	0.32	0	0.16	0.04

Supplementary Table S7. Analysis of variance of drought-responsive gene expression after 24,48 and 72 h stress in Scarlett(*ppd-H1*) and S42-IL107(*Ppd-H1*)

Gene	Source of Variation/Statistics	Genotype			Treatment			Replication			Genotype *Treatment		
		24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
HvDREB1	F-value	5.79*	6.25*	0.03	22.9*	12.78*	16.53*	0.62	0.36	1.51	5.56*	4.42*	0
	R^2	*0.14*	*0.18*	*0*	*0.55*	*0.26*	*0.67*	*0.03*	*0.06*	*0.12*	*0.13*	*0.19*	*0*
HvDRF1	F-value	0.01	5.39*	0.85	14.74*	106.29**	31.8*	0.97	2.39	1.72	4.64*	6.67*	3.18
	R^2	*0*	*0.04*	*0.02*	*0.56*	*0.87*	*0.7*	*0.07*	*0.01*	*0.08*	*0.14*	*0.04*	*0.07*
HvABI5	F-value	2.86	2.26	0.08	1.79	5.96*	21.93*	0.38	0.39	1.88	3.05	0.03	2.06
	R^2	*0.2*	*0.15*	*0*	*0.12*	*0.4*	*0.71*	*0.05*	*0.05*	*0.12*	*0.21*	*0*	*0.07*
HvWRKY38	F-value	2.76	0.02	1.01	6.03*	16.25*	27.54*	0.71	2.78	1.05	0	0.79	0.17
	R^2	*0.17*	*0*	*0.03*	*0.37*	*0.59*	*0.75*	*0.09*	*0.2*	*0.06*	*0*	*0.03*	*0*
HvPIL3	F-value	0.3	0.3	1.05	7.96*	14.26*	22.23*	0.3	0.57	4.73	0.21	0.14	0.01
	R^2	*0.02*	*0.01*	*0.03*	*0.53*	*0.65*	*0.57*	*0.04*	*0.05*	*0.24*	*0.01*	*0.01*	*0*
HvRB1	F-value	0.21	1.12	0.12	5.83	3.38	0.29	1.3	1.01	0.65	0.68	0.35	0.22
	R^2	*0.01*	*0.09*	*0.02*	*0.38*	*0.26*	*0.06*	*0.17*	*0.16*	*0.26*	*0.04*	*0.03*	*0.04*
HvARF1	F-value	0.14	5.47*	0.24	5.22*	26.95*	26.96*	0.18	1.04	0.63	0.79	5.15*	0.17
	R^2	*0.01*	*0.14*	*0.01*	*0.42*	*0.68*	*0.8*	*0.03*	*0.03*	*0.04*	*0.06*	*0.08*	*0.01*
HvAPX1	F-value	4.05	0.02	4.54*	8.24*	10.87*	8.5*	0.11	1.81	0.58	5.44*	1.42	5.08*
	R^2	*0.18*	*0*	*0.18*	*0.36*	*0.5*	*0.44*	*0.01*	*0.16*	*0.06*	*0.24*	*0.06*	*0.11*
HvCAT1	F-value	3.22	1.05	0.05	14.99*	7.87*	9.63*	0.67	0.14	0.38	0.44	4.44	0.38
	R^2	*0.12*	*0.06*	*0*	*0.58*	*0.42*	*0.57*	*0.05*	*0.01*	*0.05*	*0.02*	*0.24*	*0.02*
HvHRGP	F-value	6.28*	1.26	0.13	0.17	7.06*	7.6*	0.56	0.28	1.56	0.15	0.22	0.51
	R^2	*0.46*	*0.14*	*0.01*	*0.01*	*0.34*	*0.5*	*0.08*	*0.06*	*0.2*	*0.01*	*0.02*	*0.03*

Supplementary Table S8. Analysis of variance of drought-responsive gene expression over all time points in Triumph(*ppd-H1*) and Triumph(*Ppd-H1*)

Gene	Source of Variation/Statistics	Genotype	Treatment	Replication	Time point	Genotype *Treatment	Genotype*Time point	Genotype*Treatment*Time point
HvDREB1	F-value	10.58*	43.05**	0.53	79.63**	10.1*	4.54*	10.53**
	R^2	0.04	0.14	0	0.54	0.03	0.03	0.14
HvDRF1	F-value	9.96*	89.86**	0.34	27.15**	3.64	2.15	4.52*
	R^2	0.05	0.45	0	0.27	0.02	0.02	0.09
HvABI5	F-value	0.21	83.85**	2.26	10.19*	2.23	0.95	1.56
	R^2	0	0.6	0.03	0.15	0.02	0.01	0.04
HvWRKY38	F-value	0	14.54*	1.74	20.19**	1.35	1.21	3.15*
	R^2	0	0.16	0.04	0.44	0.01	0.03	0.14
HvPIL3	F-value	0.02	62.85**	3.01	19.4**	2.67	0.45	1.62
	R^2	0	0.45	0.04	0.28	0.02	0.01	0.05
HvRB1	F-value	4.42*	0.15	0.05	23.1**	5.08*	0.28	2.55
	R^2	0.05	0	0	0.55	0.06	0.01	0.12
HvARF1	F-value	23.09**	100.68**	0.23	18.2**	7.79*	1.03	2.95*
	R^2	0.11	0.5	0	0.18	0.04	0.01	0.06
HvAPX1	F-value	4.63*	271.14**	2.02	19.07*	9.14*	3.28	35.32**
	R^2	0.01	0.55	0.01	0.08	0.02	0.01	0.29
HvCAT1	F-value	6.42*	56.2**	6.63*	10.42*	18.26*	2.23	5.36*
	R^2	0.04	0.36	0.08	0.13	0.12	0.03	0.14
HvHRGP	F-value	40.81**	19.99*	4.4	13.98*	0.03	2.83	5.11*
	R^2	0.28	0.14	0.06	0.19	0	0.04	0.14

Supplementary Table S9. Analysis of variance of drought-responsive gene expression after 24,48 and 72 h stress in Triumph(*ppd-H1*) and Triumph(*Ppd-H1*)

Gene	Source of Variation/Statistics	Genotype			Treatment			Replication			Genotype *Treatment		
		24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
HvDREB1	F-value	64.98*	5.79*	0.22	180.09**	22.9*	14.53*	1.73	0.62	1.04	64.09*	5.56*	0.09
	R^2	0.2	0.14	0.01	0.57	0.55	0.63	0.01	0.03	0.09	0.2	0.13	0
HvDRF1	F-value	11.78*	4.75*	0.01	78.27**	31.88**	7.04	0.52	0.47	0.7	9.42*	6.54*	2.2
	R^2	0.11	0.11	0	0.73	0.73	0.48	0.01	0	0.1	0.09	0.02	0.15
HvABI5	F-value	0.01	0.84	4.78	11.02*	100.4*	192.85**	0.87	0.04	1.38	1.16	1.19	7.49*
	R^2	0	0.01	0.02	0.58	0.93	0.9	0.09	0	0.01	0.06	0.01	0.04
HvWRKY38	F-value	26.14*	0.59	1.99	95.17**	6.44*	4.35*	0.98	0.18	0.51	17.7*	0.03	0.6
	R^2	0.18	0.05	0.15	0.66	0.56	0.34	0.01	0.03	0.08	0.12	0	0.05
HvPIL3	F-value	0.07	0.74	0.58	43.92**	13.89*	21.39*	3.27	0.11	2.58	1.34	1.38	1.26
	R^2	0	0.03	0.02	0.76	0.63	0.64	0.11	0.01	0.15	0.02	0.06	0.04
HvRB1	F-value	1.16	3.76	3.09	0.29	0.87	0.88	1.51	0.23	0.68	4.56	3.18	0.44
	R^2	0.1	0.28	0.29	0.02	0.07	0.08	0.25	0.03	0.13	0.38	0.24	0.04
HvARF1	F-value	21.66*	2.46	3.94	53.56*	11.46*	48.35*	3.97	0.08	0.05	6.57*	0.01	4.96*
	R^2	0.23	0.13	0.06	0.57	0.6	0.78	0.08	0.01	0	0.07	0	0.08
HvAPX1	F-value	1.12	8.17*	10.68*	1.56	235.26**	486.3**	0.05	1.24	0.19	1.68	58.54*	5.73*
	R^2	0.13	0.03	0.02	0.18	0.76	0.96	0.01	0.01	0	0.2	0.19	0.01
HvCAT1	F-value	0	4.13	3.36	2	77.89*	25.29*	0.31	2.75	1.68	0.47	13.83*	8.48*
	R^2	0	0.04	0.07	0.28	0.74	0.56	0.09	0.05	0.07	0.07	0.13	0.19
HvHRGP	F-value	32.26*	19.13*	2.52	0.83	8.89*	12.5*	3.93	1.8	0.45	5.92	3.07	0.98
	R^2	0.62	0.47	0.11	0.02	0.22	0.55	0.15	0.09	0.04	0.11	0.08	0.04

Supplementary Table S10. Analysis of variance of germination percentage under ABA using genotypes differing at *Ppd-H1*

Sources of Variation	Scarlett/S42-IL107		Triumph/TriumphIL	
	F-value	R^2	F-value	R^2
Model		0.79		0.94
Genotype	42.36**	0.32	43.72**	0.09
Treatment	17.42**	0.39	116.3**	0.75
Replication	0.07	0	0.68	0.01
Genotype*Treatment	3.29*	0.07	14.33**	0.09

Supplementary Table S11. Introgressions in S42-IL107 from wild type based on barley genome zipper (Schmalenbach *et al*.,2011)..

Unigene	Barley CHR	CHR Arm	SNP Name	Brachypodium e-score	Brachypodium Locus	Brachypodium desccription
U35_15700	B2H	S	2646-1277	1.00E-176	Bradi1g16700.1	protein expressed protein
U35_17375	B2H	S	5880-2547	0	Bradi1g16680.1	protein ubiquitin-activating enzyme, putative, expressed
U35_4459	B2H	S	7766-492	1.00E-109	Bradi1g16820.1	protein PHD-finger family protein, expressed
U35_2854	B2H	S	7747-1056	0	Bradi1g16810.1	protein protein phosphotase protein, putative, expressed
U35_19669	B2H	S	8787-1459	0	Bradi1g16770.1	protein D-alanine--D-alanine ligase family, putative, expressed
U35_18139	B2H	S	7032-201	4.00E-29	Bradi1g17050.4	protein expressed protein
U35_16350	B2H	S	3616-1171	1.00E-128	Bradi1g17090.1	protein nicotianamine synthase, putative, expressed
U35_2416	B2H	S	4490-1344	1.00E-68	Bradi1g68200.1	protein expressed protein
U35_14095	B2H	S	5652-419	0	Bradi1g76700.1	protein GTPase of unknown function domain containing protein, putative, expressed
U35_14190	B2H	S	864-594	4.00E-81	Bradi2g10010.1	protein OsCam1-2 - Calmodulin, expressed
U35_1093	B2H	S	2477-377	1.00E-145	Bradi1g64120.1	protein glycosyl transferase 8 domain containing protein, putative, expressed
U35_1093	B2H	S	2477-910	1.00E-145	Bradi1g64120.1	protein glycosyl transferase 8 domain containing protein, putative, expressed
U35_3824	B2H	S	7144-973	1.00E-170	Bradi1g17160.1	protein expressed protein
U35_1937	B2H	S	5050-1101	1.00E-159	Bradi4g40150.1	protein 2-dehydro-3-deoxyphosphooctonate aldolase, putative, expressed
U35_1841	B2H	S	ABC05236-1-10-217	1.00E-124	Bradi1g17430.1	protein thioredoxin, putative, expressed
U35_2718	B2H	S	6086-690	1.00E-126	Bradi1g17240.1	protein serine hydrolase domain containing protein, expressed
U35_26	B2H	S	ConsensusGBS0155-4	5.00E-91	Bradi1g17460.1	protein stress responsive protein, putative, expressed
U35_20446	B2H	S	ABC12652-1-1-79	2.00E-80	Bradi1g17410.1	protein expressed protein
U35_17092	B2H	S	6471-1139	0	Bradi1g17490.1	protein nucleoside-triphosphatase, putative, expressed
U35_403	B2H	S	816-265	0	Bradi1g17460.1	protein stress responsive protein, putative, expressed
U35_14502	B2H	L	682-767	4.00E-64	Bradi5g17470.1	protein oleosin, putative, expressed
U35_3452	B2H	L	ABC10472-1-2-247	3.00E-78	Bradi3g50490.1	protein ethylene-responsive transcription factor, putative, expressed
U35_15444	B2H	L	2944-1813	0	Bradi5g21710.1	protein aminotransferase, putative, expressed
U35_4022	B2H	L	ABC12363_1_220	1.00E-122	Bradi5g21740.1	protein retrotransposon protein, putative, unclassified, expressed
U35_2689	B2H	L	5347-585	1.00E-137	Bradi3g22640.1	protein EF hand family protein, putative, expressed
U35_19034	B2H	L	ConsensusGBS0272-1	5.00E-56	Bradi5g21570.1	protein expressed protein
U35_4319	B2H	L	7236-1384	1.00E-145	Bradi5g21660.1	protein methyl-CpG binding domain containing protein, putative, expressed

Supplementary Table S12. ANOVA table of core clock and stress-responsive genes in Bowman and Bowman(*eam8.w*).

Gene name	Statistics	Model	Genotype	Treatment	Replication	Time point	Genotype *Treatment	Genotype* Time point	Genotype* Treatment* Time point
HvCCA1	F-value		175.54**	54.46**	2.28	318.83**	4.11*	43.1**	11.27**
	R^2	0.95	0.07	0.02	0	0.71	0	0.1	0.05
HvPRR1	F-value		4.77*	31.49**	1.92	76.67**	30.94**	7.53**	8.52**
	R^2	0.83	0.01	0.04	0	0.57	0.04	0.06	0.13
HvGI	F-value		45.26**	97.8**	1.13	96.53**	0.4	4.02*	5.85**
	R^2	0.86	0.05	0.1	0	0.61	0	0.03	0.07
HvPRR37	F-value		106.93**	102.24**	7.7*	30.57**	0.77	12.58**	10.07**
	R^2	0.82	0.14	0.14	0.02	0.25	0	0.1	0.16
HVPRR73	F-value		1.02	14.1*	1.28	10.26**	0.06	3.02*	2.66*
	R^2	0.52	0	0.06	0.01	0.24	0	0.07	0.13
HvPRR59	F-value		0	37.95**	3.35*	146.98**	21.9**	9.63**	11.41**
	R^2	0.9	0	0.03	0.01	0.69	0.02	0.05	0.11
HvPRR95	F-value		0.17	25.85**	7.36*	112.42**	18.99**	7.61**	14.07**
	R^2	0.87	0	0.02	0.01	0.62	0.02	0.04	0.16
HvDRF1	F-value		11.82*	259.78**	3.12*	14.05**	9.88*	2.26*	7.95**
	R^2	0.78	0.02	0.42	0.01	0.14	0.02	0.02	0.15
HvDREB1	F-value		0.61	180**	1.29	14.3**	1.55	1.78	2.82*
	R^2	0.7	0	0.4	0.01	0.19	0	0.02	0.08
HvABI5	F-value		6.89*	170.68**	3	16.26**	7.81*	4.93*	5.29**
	R^2	0.74	0.01	0.33	0.01	0.19	0.02	0.06	0.12

Gene name	Statistics	Model	Genotype	Treatment	Replication	Time point	Genotype *Treatment	Genotype* Time point	Genotype* Treatment* Time point
HvWRKY38	F-value		10.37*	59.06**	2.51	4.41*	4.53*	3.72*	2.88*
	R^2	0.54	0.03	0.2	0.02	0.09	0.02	0.07	0.12
HvAPX1	F-value		1.93	338.5**	2.25	28.46**	0.87	5.32**	8.29**
	R^2	0.82	0	0.43	0.01	0.22	0	0.04	0.13
HvCAT1			0.93	105.18**	1.65	7.76**	1.12	0.51	3.79**
	R^2	0.61	0	0.31	0.01	0.14	0	0.01	0.13
HvARF1	F-value		0.19	124.47**	0.76	8.18**	2.63	3.66*	4**
	R^2	0.67	0	0.33	0	0.13	0.01	0.06	0.13
HvSRP			5.14*	174.11**	22.59**	34.02**	32.52**	3.64*	3.68**
	R^2	0.8	0.01	0.26	0.07	0.31	0.05	0.03	0.07
HvLHCB	F-value		31.91**	83.14**	2.7	86.68**	6.05*	7.61**	15.47**
	R^2	0.86	0.03	0.08	0.01	0.51	0.01	0.04	0.18
HvPHYB			0.1	65.67**	0.02	12.03**	0.01	1.52	2.72*
	R^2	0.57	0	0.21	0	0.23	0	0.03	0.1
HvPIL3	F-value		63.65**	127.67**	4.85*	27.96**	5.74*	42.58**	3.53*
	R^2	0.84	0.08	0.16	0.01	0.21	0.01	0.32	0.05

Supplementary Table S13. ANOVA table of core clock and stress-responsive genes in Scarlett and S42-IL107.

Gene name	Statistics	Model	Genotype	Treatment	Replication	Time point	Genotype *Treatment	Genotype* Time point	Genotype* Treatment* Time point
HvCCA1	F-value		0.05	196.75**	0.43	547.87**	2.53	2.1	21.85**
	R^2	0.97	0	0.05	0	0.84	0	0	0.07
HvPRR1	F-value		2.01	39.27**	1.77	176.01**	0.11	3.55*	8.38**
	R^2	0.9	0	0.03	0	0.78	0	0.02	0.07
HvGI	F-value		0.01	95.64**	3.01	81.42**	6.25*	4.22*	8.58**
	R^2	0.84	0	0.11	0.01	0.57	0.01	0.03	0.12
HvPRR37	F-value		0	181.09**	0.13	136.78**	1.33	1.86	11.02**
	R^2	0.89	0	0.14	0	0.64	0	0.01	0.1
HVPRR73	F-value		0.59	12.78*	4.14*	17.58**	1.47	1.11	2.56*
	R^2	0.6	0	0.05	0.03	0.38	0.01	0.02	0.11
HvPRR59	F-value		1.57	7.48*	3.09	215.63**	2.85	0.82	20.5**
	R^2	0.92	0	0	0	0.76	0	0	0.14
HvPRR95	F-value		0.54	69.38**	3.49	184**	0.03	0.59	19.01**
	R^2	0.91	0	0.04	0	0.71	0	0	0.15
HvDRF1	F-value		6.18*	105.66**	1.09	10.65**	14.62*	1.2	2.67*
	R^2	0.63	0.02	0.29	0.01	0.17	0.04	0.02	0.09
HvDREB1	F-value		23.87**	63.57**	0.43	31.32**	0.98	8.34**	7.01**
	R^2	0.75	0.04	0.12	0	0.34	0	0.09	0.15
HvABI5	F-value		0.56	80.97**	1.08	15.09**	0	2.05	2.78*
	R^2	0.62	0	0.23	0.01	0.25	0	0.03	0.09

Gene name	Statistics	Model	Genotype	Treatment	Replication	Time point	Genotype *Treatment	Genotype* Time point	Genotype* Treatment* Time point
HvWRKY38	F-value		0	97.65**	0	53.45**	15.68*	16.89**	16.68**
	R^2	0.85	0	0.11	0	0.37	0.02	0.12	0.23
HvAPX1	F-value		9.01*	554.9**	1.63	76.75**	47.16**	16.33**	24.21**
	R^2	0.91	0.01	0.35	0	0.29	0.03	0.06	0.18
HvCAT1	F-value		12.47*	63.88**	1.87	8.86**	16.56**	2.76*	5.34**
	R^2	0.63	0.03	0.17	0.01	0.15	0.05	0.05	0.17
HvARF1	F-value		0.27	233.42**	0.27	43.53**	31.35**	1.45	7.81**
	R^2	0.82	0	0.3	0	0.34	0.04	0.01	0.12
HvSRP	F-value		0.76	119.56**	14.74**	32.82**	0.18	0.79	3.87**
	R^2	0.74	0	0.22	0.06	0.37	0	0.01	0.09
HvLHCB	F-value		10.5*	253.54**	0.79	188.27**	1.63	9.56**	52.19**
	R^2	0.94	0	0.11	0	0.51	0	0.03	0.28
HvPHYB	F-value		6.15*	130.52**	1.02	30.34**	9.93*	3.06*	12.58**
	R^2	0.78	0.01	0.2	0	0.29	0.02	0.03	0.24
HvPIL3	F-value		67.84**	77.76**	5.62*	95.63**	24.77**	3.97*	12.52**
	R^2	0.88	0.06	0.07	0.01	0.54	0.02	0.02	0.14

Supplementary Table S14. ANOVA of physiological responses under osmotic stress in Bowman and Bowman(*eam8.w*).

Factors	Stomatal conductance		CO_2 uptake		Leaf transpiration		Leaf temperature		Leaf osmotic potential	
	F-value	R^2	F-value	R^2	F-value	R^2	F-value	R^2	F-value	R^2
Model		0.93		0.84		0.8		0.99		0.85
Genotype	2.31	0.01	0.23	0	0	0	2.01	0.01	2.34	0.04
Treatment	108.59**	0.16	28.22**	0.13	27.03**	0.11	190.87**	0.04	161.86**	0.46
Time point	61.66**	0.54	12.71**	0.35	15.47**	0.37	643.06**	0.89	15.59**	0.26
Replication	1.4	0	3.89*	0.04	0.09	0	2.64	0	3.95*	0.02
Genotype*Treatment	0.01	0	1.38	0.01	0.14	0	0.51	0	1.96	0.01
Genotype *Time point	2.12	0.02	2.62*	0.07	1.87	0.05	5.71*	0.01	0.81	0.01
Treatment*Time point	21.46**	0.19	6.77**	0.19	10.58**	0.26	17.34**	0.02	2.02	0.03
Genotype*Treatment*Time point	0.23	0	2.19	0.06	0.81	0.02	8.57**	0.01	0.68	0.01

Supplementary Table S15. ANOVA of physiological responses under osmotic stress in Scarlett and S42-IL107

Factors	Stomatal conductance		CO_2 uptake		Leaf transpiration		Leaf temperature		Leaf osmotic potential	
	F-value	R^2	F-value	R^2	F-value	R^2	F-value	R^2	F-value	R^2
Model		0.88		0.81		0.83		0.97		0.88
Genotype	2.21	0.01	1.7	0.01	1.13	0	3.44	0	2.67	0.01
Treatment	13.75*	0.03	0.03	0	0.09	0	96.23**	0.05	79.73**	0.17
Time point	51.4**	0.71	8.47**	0.23	33.22**	0.66	255.93**	0.86	44.02**	0.57
Replication	0.25	0	6.82*	0.06	1.03	0.01	0.19	0	2.37	0.01
Genotype*Treatment	2.06	0	0.06	0	0.57	0	3.94*	0	0.11	0
Genotype *Time point	2.49*	0.03	8.32**	0.23	3.02*	0.06	3.33*	0.01	0.32	0

Factors	Stomatal conductance		CO_2 uptake		Leaf transpiration		Leaf temperature		Leaf osmotic potential	
	F-value	R^2	F-value	R^2	F-value	R^2	F-value	R^2	F-value	R^2
Treatment*Time point	6.51**	0.09	7.87**	0.22	4.23*	0.08	10.93**	0.04	8.77**	0.11
Genotype*Treatment*Time point	0.53	0.01	2.35*	0.06	0.66	0.01	0.57	0	0.93	0.01

Supplementary Table S16. ANOVA of shoot dry weight in Bowman and Bowman(*eam8.w*) and Scarlett and S42-IL107.

Factors	Shoot biomass (Dry weight) Bowman and Bowman(*eam8.w*)		Shoot biomass (Dry weight) Scarlett and S42-IL107	
	F-value	R2	F-value	R2
Model		0.67		0.65
Genotype	17.46*	0.32	10.07*	0.18
Replication	1.66	0.13	0.52	0.06
Treatment	6.77*	0.22	21.86**	0.39
Genotype*Treatment	0.16	0	0.88	0.02

Supplementary Table S17. ANOVA of coleoptile lengths in barley seedlings.

Factors	Coleoptile length of Bowman and Bowman(*eam8.w*)		Coleoptile length of Scarlett and S42-IL107	
	F-value	R2	F-value	R2
Model		0.76		0.6
Genotype	3.77	0.02	13.86*	0.12
Replication	1.03	0.04	1.89	0.12
Light	40.77**	0.69	11.05**	0.3
Genotype*Light	0.3	0.01	2.02	0.05

Supplementary Table S18. Stress-responsive and core clock ortholog genes in barley, Brachypodium, rice, sorghum and maize

Barley		Brachypodium			Rice			Maize			Sorghum			
Gene name	Accession code	Locus name	score	E-Value	Locus name	score	E-Value	Locus name	score	E-Value	Locus name	score	E-Value	Length of CNS in bps
HvDRF1	AF521303	Bd2g29960	387.1	7.50E-108	Loc_Os05g27930	256.9	1.70E-68	GRMZM2G006745		8.30E-60	Sb09g016150		1.70E-44	54
HvDREB1	DQ012941	Bd2g04000	434.5	3.30E-122	Loc_Os01g07120	364.8	3.70E-101	GRMZM5G889719	117.9	8.10E-27	Sb03g004480	305.2	2.40E-83	127
HvABI5	AY150676	Bd4g32090	400.6	7.10E-112	Loc_Os09g28310	369.8	1.40E-102	GRMZM2G157722	290.4	1.30E-78	Sb02g026570	321.2	5.30E-88	62
HvWRKY38	AY541586	Bd3g06070	404.7	4.10E-113	Loc_Os02g08440	364.8	5.40E-101	GRMZM2G120320	330.9	1.00E-90	Sb04g005520	320.5	7.60E-88	94
HvAPX1	AS006358	Bd1g65820	496.1	6.30E-141	Loc_Os03g17690	482.6	1.10E+136	GRMZM2G054300	467.6	3.60E-132	Sb01g038760	467.6	2.80E-132	58
HvCAT1	AF021938	Bd1g29800	805.4	0	Loc_Os06g51150	794.3	0	GRMZM2G088212	788.1	0	Sb10g030840	786.9	0	43
HvARF1	AJ508228	Bd2g53077	375.9	7.00E-105	Loc_Os05g41060	375.9	7.70E-105	GRMZM2G157596	374	3.60E-107	Sb09g023880	375.9	5.80E-105	25
HvLHCB	X63197	Bd1g24760	501.1	2.70E-142	Loc_Os07g37550	475.7	1.30E-134	GRMZM2G057281	500	1.00E-142	Sb02g036380	505.8	8.40E-144	130
HvPHYB	AK365283	Bd1g64360	2210.6	0	Loc_Os03g19590	2144.4	0	GRMZM2124532	2092	0	Sb01g037340	2109	0	73

Barley		Brachypodium			Rice			Maize			Sorghum			
HvPIL3	AK359117	Bd1g13980	165.2	1.90E-41	Loc_Os0394381 0	145.2	2.70E-35	GRMZM2G165042	157.5	6.60E-39	Sb01g013843	164.1	3.10E-41	34
HvCCA1	HQ850270	Bd3g16515	937.2	0	Loc_Os08g0611 0	877.9	0	GRMZM2G014902	792.3	0	Sb07g003870	812	0	119
HvPRR1	HQ850268	Bd3g48880	827	0	Loc_Os02g4051 0	768.5	0	GRMZM2G020081	746.1	0	Sb04g026190	772.1	0	61
HvGI	AY740524	Bd2g05226	2095.9	0	Loc_Os01g0870 0	1994.5	0	GRMZM2G107101	1965.3	0	Sb03g0036500	1955.6	0	50
HvPRR37	AY970703	Bd1g16490	750.7	0	Loc_Os07g4946 0	656.8	0	GRMZM2G033962	507.7	1.20E-143	Sb06g014570	407.9	7.80E-114	42
HvPRR73	JQ791230	Bd1g65910	290.8	3.90E-79	Loc_Os03g1757 0	249.2	1.50E-66	GRMZM2G095727	244.2	5.20E-65	Sb01g038820	240.4	4.80E-64	145
HvPRR59	JQ791228	Bd4g24967	261.9	1.20E-70	Loc_Os11g0593 0	253.8	4.50E-68	GRMZM2G488465	174.5	4.60E-44	Sb05g900366	184.9	2.30E-47	48
HvPRR95	JQ791232	Bd4g36077	292	1.60E-79	Loc_Os09g3622 0	292.7	1.30E-79	GRMZM2G179024	268.1	3.30E-72	Sb05g900366	137.9	4.00E-33	37

Supplementary Table S19. Regulatory elements identified in conserved promoter regions of barley core clock ortholog and stress-responsive genes.

Matrix Family	Detailed Family Information	Sequence	Core clock genes	Stress-responsive genes
P$ABRE	ABA response elements	gctgctgaCGTGgcacc	*HvCCA1*, *HvPRR59*, *HvELF3*	*HvDREB1*, *HvABI5*, *HvWRKY38*, *HvAPX1*, *HvPHYB*, *HvLHCB*
P$AGL1.0	AGL1, Arabidopsis MADS-domain protein AGAMOUS-like 1	tttTTCCctagcaggtgagct	*HvCCA1*	
P$AGP1	AG-motif binding protein 1	gcaGATCcaac	*HvPRR1*, *HvPRR37*	
P$AHBP	HD-ZIP class III protein ATHB9	ggaATGGttgc	*HvCCA1*	*HvABI5*, *HvWRKY38*, *HvCAT1*, *HvARF1*
P$AP1.01	Floral homeotic protein APETALA1	taatacCACAagaagcaatat	*HvCCA1*	
P$AREF	Auxin response element	acgTGTCccacca		*HvAPX1*, *HvPHYB*
P$ASRC	AS1/AS2 repressor complex	tcaTTGAat	*HvPRR37*	
P$ATHB9.01	HD-ZIP class III protein ATHB9	ggaATGGttgc	*HvCCA1*	
P$BRRE	Brassinosteroid (BR) response element	cacgcCGTGcgccagcc	*HvPRR95*	*HvDREB1*
P$CAAT	CCAAT binding factors	aaCCAAtgt		*HvABI5*, *HvLHCB*
P$CBNAC.02	Calmodulin-binding NAC protein	tattGCTTcttgtggtattac	*HvCCA1*	
P$CCAF	Circadian control factors	taaaaaAATAtgtca	*HvGI*, *HvPRR59*, *HvELF3*	*HvDRF1*, *HvDREB1*, *HvABI5*, *HvWRKY38*
P$CDC5	Arabidopsis CDC5 homolog	tcttcAGCGcg		*HvPHYB*
P$CE1F	Coupling element 1 binding factors	gtgcCACCgccgc		*HvDREB1*
P$CE3S	Coupling element 3 sequence	tcaacaCGAGtggcacggc		*HvDREB1*, *HvAPX1*
P$CGCG	Calmodulin binding / CGCG box binding proteins	cgcCGCGtcggcgtcgg	*HvPRR59*, *HvELF3*	
P$CNAC	Calcium regulated NAC-factors	ttctGCTTacatacggcggcc		*HvABI5*
P$DOF1.01	Dof1 / MNB1a - single zinc finger transcription factor	ctagagttAAAGataaa	*HvCCA1*	
P$DOFF	DNA binding with one finger (DOF)	cgcacagaAAAGctacc	*HvPRR37*, *HvELF3*	*HvABI5*
P$DPBF	Dc3 promoter binding factors	gACACgtggcg		*HvAPX1*

Matrix Family	Detailed Family Information	Sequence	Core clock genes	Stress-responsive genes
P$DREB	Dehydration responsive element binding factors	cgcatgcgCCGAcaccgcgca	*HvCCA1*, *HvPRR1*, *HvPRR37*, *HvPRR59*, *HvELF3*	*HvDRF1*, *HvDREB1*, *HvWRKY38*, *HvAPX1*, *HvARF1*, *HvPIL3*
P$EINL	Ethylen insensitive 3 like factors	aTGGAtctt	*HvPRR37*	
P$EREF	Ethylen respone element factors	aTCGAactagcccaagtag	*HvPRR37*	*HvDREB1*
P$ERSE	ER stress-response elements	cccctccggccctccCACG	*HvPRR73*	*HvDREB1*, *HvLHCB*
P$FORC	Fungal and oomycete pathogen response cluster - promoter motif	agaaatGGGCatgctgc	*HvPRR73*	
P$GAGA	GAGA elements	gaggagAGAGaggagaaggggagg	*HvPRR59*	
P$GAPB	GAP-Box (light response elements)	actgATGAatagtgt		*HvPIL3*
P$GARP	Myb-related DNA binding proteins (Golden2, ARR, Psr)	AGATccggc		*HvDREB1*, *HvARF1*
P$GATA.01	Class I GATA factors	ttaaaGATAaagagagg	*HvCCA1*	
P$GBOX	Plant G-box/C-box bZIP proteins	tgaccaTGACgtggagcaagt	*HvPRR73*, *HvPRR59*	*HvDREB1*, *HvABI5*, *HvAPX1*, *HvPHYB*, *HvLHCB*
P$GCCF	GCC-box, ethylene-responsive element (ERE)	aacAGCCgccgcc	*HvPRR1*	
P$GCN4.01	GCN4, conserved in cereal seed storage protein gene promoters	aaaacTGAGtcaacgga	*HvCCA1*	
P$GT1.01	GT1-Box binding factors with a trihelix DNA-binding domain	gtttttGTTAgtctaat	*HvCCA1*	
P$GTBX	GT-box elements	cccaacGTGAatgagta	*HvGI*	*HvABI5*, *HvWRKY38*, *HvCAT1*
P$HEAT	Arabidopsis thaliana class A heat shock factor 1a	ttaaacTTTCaagtact	*HvCCA1*, *HvPRR37*, *HvPRR73*	*HvDRF1*, *HvDREB1*
P$HOCT	Octamer motif of Histone H3, H4 promoters	tccgacgATCCgaggtg		*HvLHCB*
P$HSE.01	Heat shock element	tgaaagcatagAGAAcc	*HvCCA1*	
P$IBOX	Class I GATA factors	ttgaaGATAaagagagg	*HvCCA1*, *HvPRR73*, *HvELF3*	*HvDRF1*, *HvABI5*, *HvCAT1*
P$IBOX.01	I-Box in rbcS genes and other light regulated genes	gaacaGATTagactaac	*HvCCA1*	
P$IDE1.01	Iron-deficiency-responsive element 1 (IDEF1)	aatacttGCAAgttttatgctgattct	*HvCCA1*	
P$L1BX	L1 box, motif for L1 layer-specific expression	ctgCATTaaaaaaatat	*HvGI*, *HvPRR37*	
P$LEGB	Legumin Box family	gatccatGCGTgcttcttgcagattcc	*HvPRR73*, *HvPRR95*	

Matrix Family	Detailed Family Information	Sequence	Core clock genes	Stress-responsive genes
P$LFYB	LFY binding site	gGCCActgggttc		*HvDRF1*, *HvLHCB*
P$LREM	Light responsive element motif, not modulated by different light qualities	gtATCTagaca		*HvABI5*, *HvCAT1*, *HvPIL3*
P$MADS	MADS box proteins	ccgctcCAAAgatggccactg		*HvDRF1*, *HvCAT1*, *HvPIL3*
P$MIIG	MYB IIG-type binding sites	tgGGGGttgattgaa	*HvGI*, *HvPRR59*	*HvDREB1*
P$MSA.01	M-phase-specific activators (NtmybA1, NtmybA2, NtmybB)	gagtcAACGgaattt	*HvCCA1*	
P$MSAE	M-phase-specific activator elements	tagatAACGgggtga	*HvPRR73*	*HvWRKY38*, *HvPIL3*
P$MYB96.01	Myb domain protein 96 (MYBCOV1)	attctagAGTTaaagat	*HvCCA1*	
P$MYBL	CAACTC regulatory elements, GA-inducible	attctagAGTTgaagat	*HvCCA1*, *HvGI*, *HvPRR73*	*HvPIL3*
P$MYBPH3.01	Myb-like protein of Petunia hybrida	caaaaacaGTTGaaagc	*HvCCA1*	
P$MYBS	Zea mays MYB-related protein 1 (transfer cell specific)	ctctcttTATCttcaac	*HvCCA1*, *HvPRR73*, *HvELF3*	*HvPIL3*, *HvPHYB*
P$MYCL	Myc-like basic helix-loop-helix binding factors	gtggagCAAGtggaggcgt	*HvPRR73*	*HvDREB1*, *HvAPX1*
P$NACF	Wheat NAC-domain DNA binding factor (DNA binding site II)	gctgttggatctgcgGACGcctcctcc	*HvPRR1*, *HvPRR73*	*HvDREB1*, *HvABI5*, *HvWRKY38*, *HvCAT1*
P$NCS1	Nodulin consensus sequence 1	aAAAAgttcac		*HvABI5*
P$OCSE	Enhancer element first identified in octopine synthase gene	gaagttaactgtacACTTagg	*HvGI*, *HvPRR95*	*HvARF1*, *HvPHYB*, *HvLHCB*
P$OPAQ	Opaque-2 like transcriptional activators	agtatcataTCAAccaa	*HvGI*, *HvPRR73*	*HvDREB1*, *HvABI5*, *HvCAT1*
P$PALBOXL.01	Cis-acting element conserved in various PAL and 4CL promoters	ttttagtaGGTGagt	*HvCCA1*	
P$PDF2.01	Protodermal factor 2	catggtTAAAttccgtt	*HvCCA1*	
P$PHR1.01	Phosphate starvation response 1	cagaTTATgcaaccatt	*HvCCA1*	
P$PNRE	Plant nitrate-responsive cis-elements	cgatggcgccattatatagagccGAGAgcagag	*HvELF3*	
P$PREM	Motifs of plastid response elements	atggCGACgccgacgccgtggcctctgcctc		*HvWRKY38*, *HvLHCB*
P$PSRE	Pollen-specific regulatory elements	gcacaGAAAagctaccc	*HvPRR37*	
P$RAV1-5.01	5'-part of bipartite RAV1 binding site, interacting with AP2 domain	tgcAACAgacc	*HvCCA1*	

Matrix Family	Detailed Family Information	Sequence	Core clock genes	Stress-responsive genes
P$RAV5	5'-part of bipartite RAV1 binding site	ggcAACAtaca	*HvPRR37*	
P$ROOT	Root hair-specific cis-elements in angiosperms	tcttcttttgactgaCACGtcgcat		*HvABI5*, *HvPHYB*
P$SALT	Salt/drought responsive elements	cctctGTGGgggttg	*HvGI*	*HvDREB1*
P$SBF1.01	SBF-1	agcatggTTAAattccg	*HvCCA1*	
P$SBPD	SBP-domain proteins	gctcCGTAaaacgcacg		*HvPIL3*
P$STK.01	Storekeeper (STK), plant specific DNA binding protein	tacTAAAaaactcaa	*HvCCA1*	
P$TCPF	DNA-binding proteins with the plant specific TCP-domain	caggggCCCGccg	*HvPRR73*	*HvLHCB*
P$TDTF	Transposase-derived transcription factors	ccgtCACCcgctttctc		*HvDREB1*, *HvAPX1*, *HvPHYB*
P$TEFB	TEF-box	tcACGGtcagtcacgtcttct		*HvWRKY38*, *HvCAT1*
P$TERE	Tracheary-element-regulating cis-elements, conferring TE-specific expression	ctcaAAAGgaa		*HvCAT1*
P$TERE.01	Tracheary-element-regulating cis-element	ctgcAAAGtaa	*HvCCA1*	
P$WBXF	W Box family	caaatcTGACcgagagg		*HvDRF1*, *HvDREB1*, *HvABI5*, *HvWRKY38*, *HvCAT1*
P$ZMMRP1.01	Zea mays MYB-related protein 1 (transfer cell specific)	ctctcttTATCtttaac	*HvCCA1*	

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