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Original Article

Osmotic stress at the barley root affects expression of circadian clock genes in the shoot

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ABSTRACT

The circadian clock is an important timing system that controls physiological responses to abiotic stresses in plants. However, there is little information on the effects of the clock on stress adaptation in important crops, like barley. In addition, we do not know how osmotic stress perceived at the roots affect the shoot circadian clock. Barley genotypes, carrying natural variation at the photoperiod response and clock genes *Ppd-H1* and *HvELF3*, were grown under control and osmotic stress conditions to record changes in the diurnal expression of clock and stress-response genes and in physiological traits. Variation at *HvELF3* affected the expression phase and shape of clock and stress-response genes, while variation at *Ppd-H1* only affected the expression levels of stress genes. Osmotic stress up-regulated expression of clock and stress-response genes and advanced their expression peaks. Clock genes controlled the expression of stress-response genes, but had minor effects on gas exchange and leaf transpiration. This study demonstrated that osmotic stress at the barley root altered clock gene expression in the shoot and acted as a spatial input signal into the clock. Unlike in Arabidopsis, barley primary assimilation was less controlled by the clock and more responsive to environmental perturbations, such as osmotic stress.

Key-words: *Hordeum vulgare*; *Eam8*; *HvELF3*, *Ppd-H1*.

INTRODUCTION

Abiotic stresses such as drought, salinity and heat are among the greatest problems facing agriculture today. Under stress, plants undergo a series of morphological, physiological, biochemical and molecular changes that adversely affect growth and productivity, but also improve adaptation and survival (Ingram & Bartels 1996; Harb *et al.* 2010). Adaptive responses to stress can be grouped into three major classes: (1) osmotic homeostasis; (2) stress damage control and repair; and (3) growth control (Zhu 2002). Drought stress signalling 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-Shinaozaki & 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 signalling 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 (1) osmotic homeostasis and control stomatal opening and examples include the LIGHT HARVESTING CHLOROPHYLL *A*/*B* BINDING PROTEIN (LHCB; Xu *et al.* 2012) and PHYTOCHROME B (PHYB; González *et al.* 2012), (2) in stress detoxification and scavenging of REACTIVE OXYGEN SPECIES (ROS), where the CATALASES (*CAT*) and PEROXIDASES (*APX*) work and (c) in growth control. In the latter, the phytochrome-interacting factor-like proteins (PIF) have a known role (Todaka *et al.* 2012). The network action of these genes has been unravelled 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 *et al.* 2011; Sanchez *et al.* 2011). The circadian clock is an autonomous oscillator that produces endogenous biological rhythms with a period of about 24 h. 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: (1) 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, (2) the inhibition of PSEUDO RESPONSE REGULATOR genes (*PRR*) by the EC early at night and (3) the inhibition of

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LHY/*CCA1* by *TIMING OF CAB EXPRESSION1* (*TOC1*) in the morning (Pokhilko *et al.* 2012; Bujdoso & Davis 2013). Furthermore, the evening-expressed *GIGANTEA* (*GI*) protein was modelled 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 & Davis 2009; Boikoglou *et al.* 2011). The central oscillator controls a large fraction of the Arabidopsis transcriptome, and this notably includes genes from the plant hormone and stress-responsive pathways (Covington *et al.* 2008; Staiger *et al.* 2013). The clock thus modulates sensitivity and response to daily changes in temperature, water availability and irradiance in a process termed 'gating' (Nakamichi *et al.* 2009; Robertson *et al.* 2009; de Montaigu *et al.* 2010; Wilkins *et al.* 2010). *TOC1*, for example, was shown to control the diurnal expression of a putative ABA receptor, ABA-RELATED/H SUBUNIT OF THE MAGNESIUM-PROTOPORPHYRIN IX CHELATASE/ GENOMES UNCOUPLED 5 (ABAR/CHLH/GUN5), and thus TOC1 was shown to influence the 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 (Green *et al.* 2002; Dodd *et al.* 2005; Sanchez *et al.* 2011; Yerushalmi *et al.* 2011). For example, Dodd *et al.* (2005) showed that Arabidopsis mutants not in phase with day/night cycles produced less chlorophyll, fixed less carbon and had lower biomass than wild-type plants under 24 h day cycles. Together, emerging evidence in Arabidopsis has implicated the clock as a major gating regulator of the abiotic stress response.

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 an excellent 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. Additionally, Campoli *et al.* (2012b) have shown that many circadian clock genes are structurally conserved between barley and Arabidopsis, and their circadian expression patterns suggested conserved functions. Interestingly, 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 harbours 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* cause photoperiod insensitivity and early flowering by up-regulating *Ppd-H1* under long and 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 cereal physiological traits.

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, we tested (1) whether natural variation at *Ppd-H1* and *HvELF3* controlled the diurnal expression of stress-response genes and diurnal changes in physiology under stress conditions, and (2) whether osmotic stress applied to the root acted as an input signal to the shoot circadian clock and thus changed diurnal patterns of physiological traits. Our results showed that osmotic stress acts as an input into the barley circadian clock, which in turn controls expression of stress-response genes, but had a more modest effect on physiological performance under stress.

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), and the spring barley Triumph and a derived introgression line Triumph-IL (provided by D. Laurie, JIC, Laurie *et al.* 1995) were used in this study. Scarlett and Triumph carry a mutation in the CCT domain of *Ppd-H1* and are late flowering under long days (LD; Turner *et al.* 2005). The introgression line S42-IL107 and Triumph-IL harbour the photoperiod-responsive *Ppd-H1* allele introgressed from wild and winter barley, respectively, and are early flowering under LD. In addition, we have used the spring barley Bowman and a derived introgression line Bowman(*eam8.w*)

carrying a mutation in the *EAM8*/*HvELF3* gene (Faure *et al.* 2012). Scarlett, S42-IL107, Bowman and Bowman(*eam8.w*) were analysed for diurnal expression of core clock and stress-response genes, and for diurnal fluctuation in physiological traits under control and osmotic stress conditions. To verify expression differences observed between Scarlett and S42-IL107, Triumph and Triumph-IL were analysed for stress-gene expression at 24, 48 and 72 h (Zeitgeber T4) after stress application. Germinated seeds were placed in 1.5 mL pierced microcentrifuge tubes (Eppendorf, Hamburg, Germany), filled with 0.5% agar and transferred to a half-strength Hoagland nutrient solution (Hoagland & Arnon 1950). The nutrient solution was renewed every 3–4 d. Plants were grown for 8–10 d in a growth chamber under LD (16 h/8 h light/dark) at an irradiance of 300 μmol m^{-2} s^{-1}, an air temperature of 20/18 °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 (Roth, Karlsruhe, Germany), which corresponded to –0.8 MPa osmotic potential.

Leaf sampling and gene expression analysis

Leaf samples from stressed and control samples were harvested 48 h after PEG application at 4 h 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 Supporting Information Table S1 were performed as explained in Campoli *et al.* (2012b).

Physiological and morphological measurements

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 via centrifugation (Eppendorf Centrifuge 5415D) for 2 min at 160 *g*. 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 a 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 (ΔCO_2) stabilized after 5–10 min. Seedling height and biomass yield (dry matter) was measured 2 d after the beginning 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, Darmstadt, Germany) for 2 d either under continuous red light (LED) with different fluency rates or in dark and mean coleoptile length (±SD) of 8–10 seedlings was 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 2009) 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 2009).

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 (Supporting Information Table S2). 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 in the promoter regions were determined by phylogenetic footprinting approach (>70% identity in a 20 bp window; Guo & Moose 2003) using the VISTA tool (Mayor *et al.* 2000). The conserved non-coding sequences 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, we 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 in the shoot 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 (Figs 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*) (Fig. 1, Supporting Information Table S3). Expression of *HvCCA1* was reduced in Bowman(*eam8.w*) compared with 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 with Bowman, for example, expression of *HvGI* peaked at ZT8 in Bowman(*eam8.w*) and at ZT12 in Bowman (Fig. 1b,c). In addition, *Ppd-H1* expression was significantly higher in Bowman(*eam8.w*) than Bowman during the night and the early morning (Fig. 1d). Under stress, expression of *HvPRR59* and *HvPRR95* was significantly lower in Bowman(*eam8.w*) compared with Bowman at peak time ZT8 (Fig. 1f,g). In contrast, circadian clock orthologs did not show differences in expression between Scarlett and S42-IL107 under stress and control conditions (Supporting Information Table S4). Thus, the mutation in *HvELF3* had a greater role in modulating the clock compared with the mutation in *Ppd-H1*.

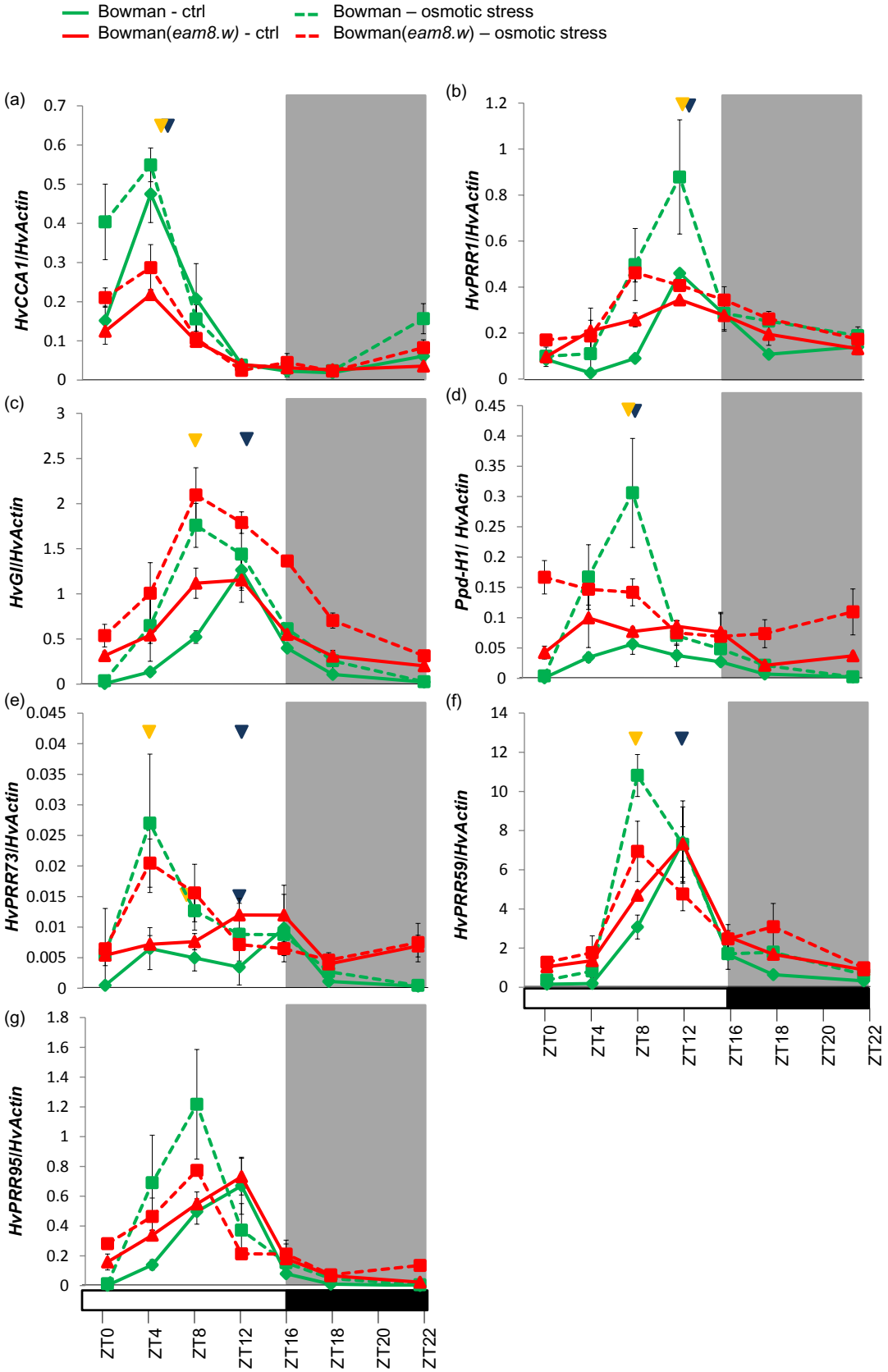
Osmotic stress caused a significant increase in the expression of clock orthologs compared with control conditions in all genotypes. In particular, *Ppd-H1*, *HvPRR73* and *HvPRR95* showed a strong induction of expression under stress compared with control conditions (Figs 1 & 2). For example, *Ppd-H1* showed a 2.5-fold increase in Scarlett and a fivefold increase in Bowman under stress conditions. Furthermore, osmotic stress advanced the phase of clock gene expression compared with 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 (Faure *et al*. 2012; Campoli *et al*. 2012b). Variation at either gene did not change the effects of osmotic stress on the expression of clock genes.

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

Since many stress-response genes are clock regulated in Arabidopsis, we examined whether variation in clock gene expression affected expression of stress-response genes (Figs 3 & 4). We tested diurnal expression of representative genes controlling signalling, 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, we measured expression of *HvPHYB* (*PHYTOCHROME B*), *HvLHCB, HvCAT1* (catalase), *HvAPX1* (ascorbate peroxidase) and the senescence activated *HvARF1* (ADP ribosylation factor 1-like protein), which are involved in ABA-induced responses such as stomatal closure and ROS homeostasis in Arabidopsis (González *et al*. 2012; Xu *et al*. 2012). Finally, we studied the expression of a PIF like gene (*HvPIL3*), 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; Figs 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 with 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. For example, the expression peaks of *HvABI5*, *HvDRF1*, *HvCAT1* and

Figure 1. Diurnal expression of circadian clock genes in barley under control (solid line) and osmotic stress (dashed line) in the spring barley Bowman (green) and introgression line Bowman(*eam8.w*) (red). Transcript accumulation was measured by qRT-PCR analysis of a) *HvCCA1*, b) *HvPRR1*, c) *HvGI*, d) *Ppd-H1* (*HvPRR37*), e) *HvPRR73*, f) *HvPRR59* and g) *HvPRR95* normalized to *HvActin*. Seedlings of both genotypes were grown in hydroponics for 10 d in long day (16 h/8 h, light/dark). Leaf samples for total RNA were collected after 48 h 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). 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.



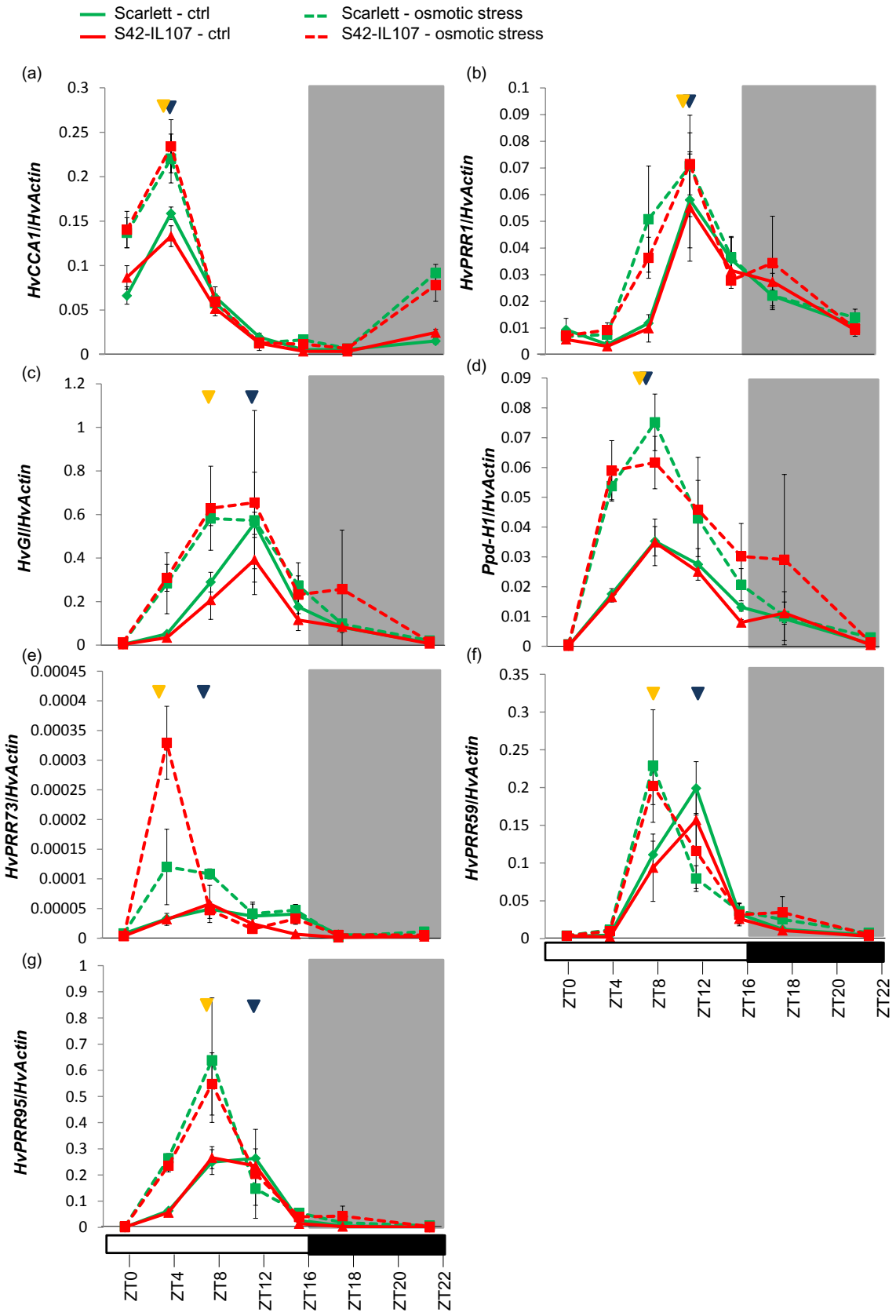


Figure 2. Diurnal expression of circadian clock genes in barley under control (solid line) and osmotic stress (dashed line) conditions in the spring barley Scarlett (green) and the introgression line S42-IL107 (red). Transcript accumulation was measured by qRT-PCR analysis of a) *HvCCA1*, b) *HvPRR1*, c) *HvGI*, d) *Ppd-H1* (*HvPRR37*), e) *HvPRR73*, f) *HvPRR59* and g) *HvPRR95* normalized to *HvActin*. Seedlings of both genotypes were grown in hydroponics for 10 d in long day (16 h/8 h, 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). 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.

HvPHYB were shifted from ZT12 under control to ZT8 under stress conditions in Bowman and Scarlett (Figs 3 & 4). Together, stress applied to the root altered the rhythmic peak of diurnal transcript accumulation of stress genes in the shoot and advanced the phase.

We observed genotype-dependent expression of *HvPIL3* and *HvLHCB* under control and stress conditions. In contrast, the remaining stress-response and signalling 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. The *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,j). 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 with 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 with 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 with Scarlett (Fig. 4). This higher expression of stress genes was also observed in Triumph compared with Triumph-IL after stress application at the time points 24, 48 and 72 h, as shown for *HvDREB1*, *HvDRF1*, *HvCAT1* and *HvAPX1* in Supporting Information Fig. S1.

In summary, variation at *HvELF3* resulted in differences in the diurnal expression patterns of clock-controlled genes, which included their phase and shape of peak expression, whereas allelic variation at *Ppd-H1* apparently only affected the levels of expression.

In order to examine diurnal co-expression of core clock and stress-responsive genes, pairwise Pearson correlation coefficients were calculated across genotypes (Bowman, Bowman(*eam8.w*), Scarlett and S42-IL107) and treatments (control and stress; Table 1). 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 (Figs 3h,j & 4h,j), showed positive correlation coefficients of 0.72. Evening-expressed clock genes were thus highly correlated with stress-response genes.

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

As the expression of clock and stress genes was correlated, we analysed the presumed promoter regions. For this, we examined 3000 bp located upstream of the stress-response genes for the presence of conserved *cis*-elements driven by circadian clock and light factors (Adams & Carré 2011). Furthermore, since circadian clock genes were induced by osmotic stress, we searched for stress-response elements in the presumed promoter regions (3000 bp) of these genes. One to three conserved non-coding sequences per promoter sequences were identified across the five grass species in the stress-response and clock genes. Dependent on the lengths of conserved non-coding sequences identified for each gene, the number of *cis*-acting elements differed for each gene (Supporting Information Table S2). We found ABA-responsive elements (ABRE), drought-responsive elements (DRE), circadian-clock factors (CCF) and light-responsive motifs (LRE) in the conserved non-coding sequences of drought-responsive genes (Supporting Information Table S5). Similarly, the analysis of circadian clock promoters also revealed an enrichment of CCF, LRE, ABRE and DRE (Supporting Information Table S5). 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 ABREs in the promoter sequences of circadian clock orthologs indicated that their transcription is regulated by stress and ABA signalling factors, which supports results of the expression analysis (Figs 1 & 2).

Diurnal changes of physiological responses to short-term osmotic stress

We examined whether variation in the expression of circadian clock and stress-response genes affected physiological traits. We measured under control and stress conditions biomass, daily fluctuations in leaf osmotic potential, stomatal conductance, leaf transpiration, net CO_2 uptake and leaf temperature. The treatment had the strongest effects on the diurnal variation of physiological traits, while genetic

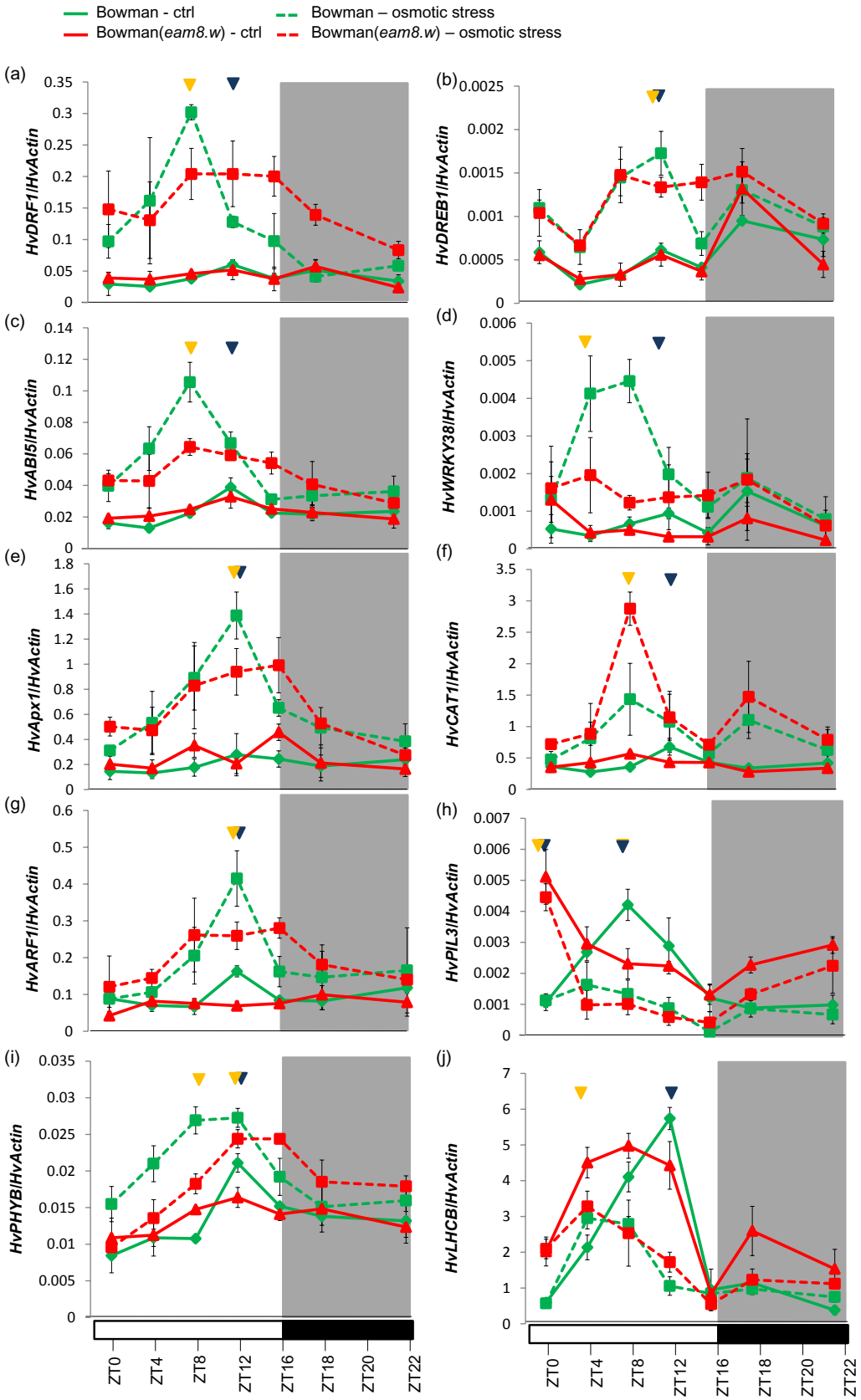


Figure 3. Diurnal expression of stress-response genes under control (solid line) and osmotic stress (dashed line) conditions in the spring barley Bowman (green) and the introgression line Bowman(*eam8.w*) (red). Transcript accumulation was measured by qRT-PCR analysis of a) *HvDRF1*, b) *HvDREB1*, c) *HvABI5*, d) *HvWRKY38*, e) *HvAPX1*, f) *HvCAT1*, g) *HvARF1*, h) *HvPIL3*, i) *HvPHYB* and j) *HvLHCB* normalized to *HvActin*. Seedlings of both genotypes were grown in hydroponics for 10 d 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). 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.

variation had only minor effects on trait expression with the exception of biomass (Figs 5 & 6, Supporting Information Tables S6 & S7). 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 with control conditions in all tested genotypes (Fig. 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 with their recurrent parents at ZT12 and ZT16 under stress conditions. Furthermore, S42-IL107 exhibited significantly lower stomatal conductance and leaf transpiration rate compared with Scarlett at ZT12 and ZT16 under control conditions.

Total biomass was significantly regulated by treatment and genotype (Fig. 7, Supporting Information Table S8). Bowman showed a biomass of 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. Since ELF3 controls photomorphogenesis and hypocotyl growth in Arabidopsis, we analysed coleoptile lengths in the dark and under different fluency rates of red light. No significant differences in coleoptile lengths between genotypes were observed (Supporting Information Fig. S2, Table S9). Together, the introgression lines, varying at *HvELF3* and *Ppd-H1* showed a significantly reduced biomass accumulation under stress compared with their recurrent parents. In addition, both lines exhibited a reduction in the net CO_2 exchange at the end of the day under stress.

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 reported on the effects of other less predictable environmental signals on the circadian clock, such as abiotic stresses. A major factor for plant growth and productivity is water availability and changes in water relations have profound effects on the plant metabolism (Chaves *et al.* 2002; Sanchez *et al.* 2013). Here, we show that osmotic stress applied at the barley roots affected expression of clock and stress genes in the shoot. Osmotic stress up-regulated the expression of clock genes and advanced the expression peaks of evening expressed clock genes compared with control conditions irrespective of the genotype (Figs 1 & 2). Interestingly, ABRE and DRE elements were identified in clock gene promoters (Supporting Information Table S5). Similarly, Lai *et al.* (2012) demonstrated that ROS, an important secondary messenger during stress (Foyer & Noctor 2005), acted as an input to the circadian clock. In addition, ABA signalling was shown to interact with the clock (Legnaioli *et al.* 2009; Robertson *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 up-regulated 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-responsive *cis*-elements in several clock genes. The mechanisms by which osmotic stress and ABA regulate circadian periodicity still await unravelling.

Stress-response genes, encoding *HvABI5* and *HvDRF1* binding to ABRE and DRE motifs in the clock gene promoters, were up-regulated under osmotic stress. Co-regulation of clock and stress-response genes was suggested by the high correlation coefficients of, for example, *HvPRR1*, *HvGI*, *Ppd-H1* (*HvPRR37*) with *HvABI5*, *HvCAT1* and *HvARF1* (Table 1). It is interesting to note that these genes share common *cis*-acting elements, notably those preferentially bound by *HvABI5* (ACGT-box, Casaretto & Ho 2003) in barley (Supporting Information Table S5). In addition, osmotic stress 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, for example, those encoding the transcription factor *HvABI5*. Significant correlations between clock and stress genes, an increase of clock gene expression under osmotic stress and the occurrence of stress-responsive elements in the promoters of clock genes suggested that clock genes were controlled by stress-response genes. Future studies using barley material overexpressing *HvABI5* and other stress-induced transcription factors as described in Casaretto & Ho (2003) and Xue & Loveridge (2004) are needed to test if expression of clock

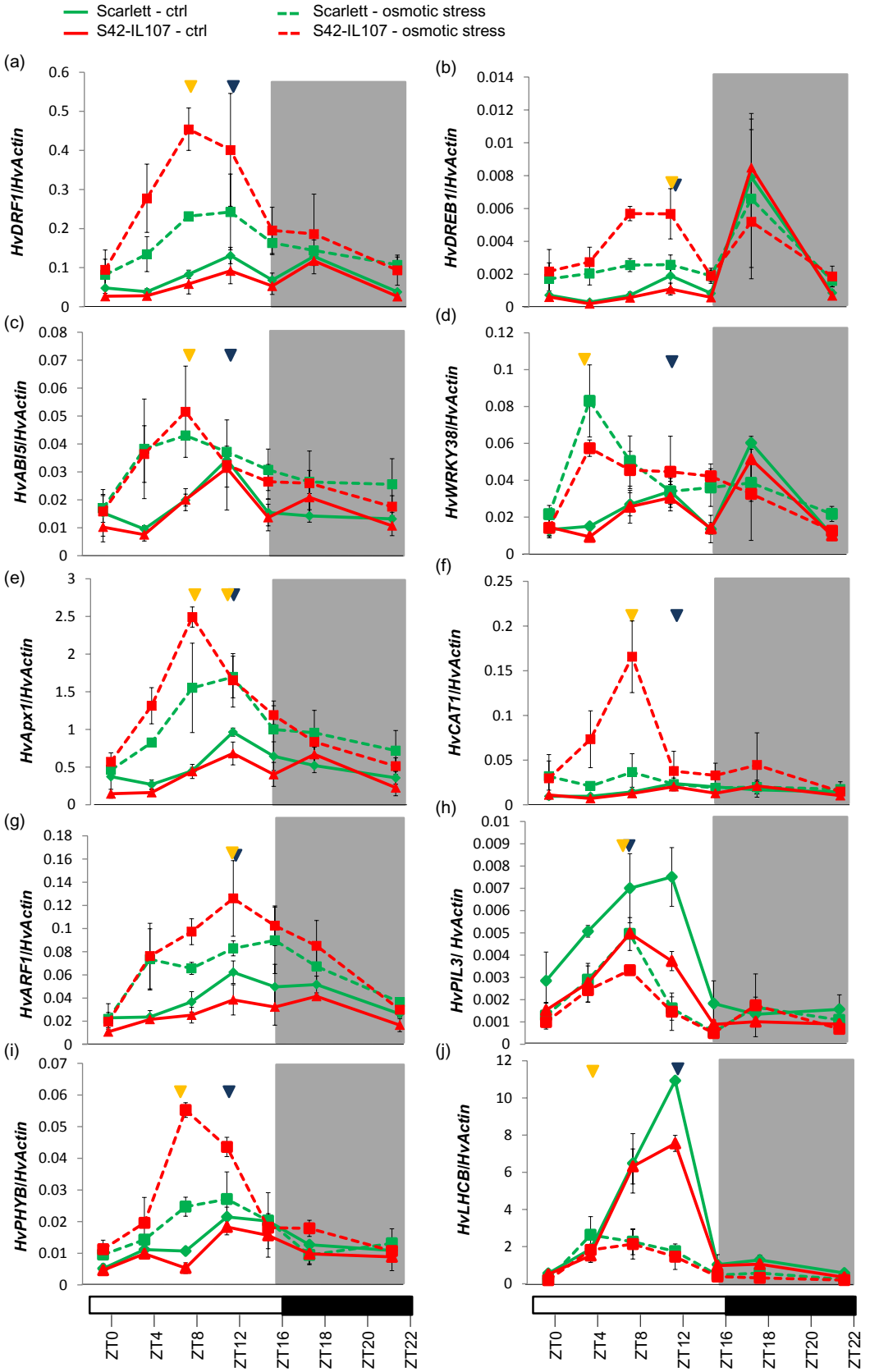


Figure 4. Diurnal expression of stress-response genes under control (solid line) and osmotic stress (dashed line) conditions in the spring barley Scarlett (green) and introgression line S42-IL107 (red). Transcript accumulation was measured by qRT-PCR analysis of a) *HvDRF1*, b) *HvDREB1*, c) *HvABI5*, d) *HvWRKY38*, e) *HvAPX1*, f) *HvCAT1*, g) *HvARF1*, h) *HvPIL3*, i) *HvPHYB* and j) *HvLHCB* normalized to *HvActin*. Seedlings of both genotypes were grown in hydroponics for 10 d in long day (16 h/8 h, 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 lights on and off). 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.

genes is controlled by transcription factors such as *HvABI5* or *HvDRF1*. 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. We thus 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, Supporting Information Fig. S1). 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 signalling and stress response (Liu *et al*. 2013). Nakamichi *et al*. (2009) showed that *DREB* genes were up-regulated in the *prr975* mutant and expression of *DREB* genes was gated by *PRR* genes. The mutation in *Ppd-H1* of Scarlett and Triumph encodes a change in the CCT DNA-binding region and is thus likely to affect DNA-binding qualities. This polymorphism 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 (Supporting Information Table S5). However, in contrast to PRRs acting as repressors in Arabidopsis, we found that 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 overexpressing individual *PRR* genes exhibiting strong effects on the circadian clock. We studied a natural mutation with apparently reduced functionality, but with no detectable 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 with Bowman, and this is 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, such as *HvDRF1* and *HvABI5* (Fig. 3), 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 CO_2 uptake 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). We thus hypothesized that in barley, physiological traits might be also under the control of the circadian clock.

Osmotic stress reduced leaf water potential, stomatal conductance and net CO_2 uptake, and increased leaf temperature. Small differences in leaf temperature between control and osmotic stress conditions may have affected expression levels of clock genes. However, it is well known that circadian clocks maintain robust and accurate timing over a broad range of physiological temperatures, a characteristic termed temperature compensation. Expression studies of core clock genes in *Arabidopsis thaliana* showed relatively small changes in peak expression levels of clock genes between different ambient temperatures. For example, Gould *et al*. (2013) reported that an increase in ambient temperature from 12 to 17 °C caused a strong increase in *PRR9* expression, while expression levels of *CCA1* and *TOC1* did not change. A consistent increase in expression levels of all tested clock genes under osmotic stress in barley thus differed from ambient temperature-induced changes in clock gene expression in Arabidopsis.

Osmotic stress affected the levels of trait expression, but no differences in the phase or shape of diurnal trait expression were observed between genotypes or treatments (Figs 5 & 6). Similarly, Izawa *et al*. (2011) reported that a mutation in

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 in bold.

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. 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 in rice (Todaka *et al*. 2012). Similar to *PIF*-like genes in rice and Arabidopsis (Nusinow *et al*. 2011), we found that osmotic stress down-regulated *HvPIL3*, while the mutation in *HvELF3* up-regulated *HvPIL3* expression at night and advanced its expression peak. Since ELF3, PhyB and PIFs are known to control photomorphogenesis in Arabidopsis (Zagotta *et al*. 1996; Liu *et al*. 2001; Soy *et al*. 2012), we analysed coleoptile lengths of barley 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 (Supporting Information Fig. S2). 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. ELF3 in barley thus appears to control cereal growth, but not through its effect on photomorphogenesis as described in Arabidopsis.

CONCLUSION

Our study demonstrated that osmotic stress at the root altered the pattern of circadian clock gene expression 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. Circadian control of the plant's 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 extreme environments with unpredictable climatic events. Alternatively, differences in plant architecture and growth may explain variation in the control of assimilation between monocots and dicots, as meristematic tissue is

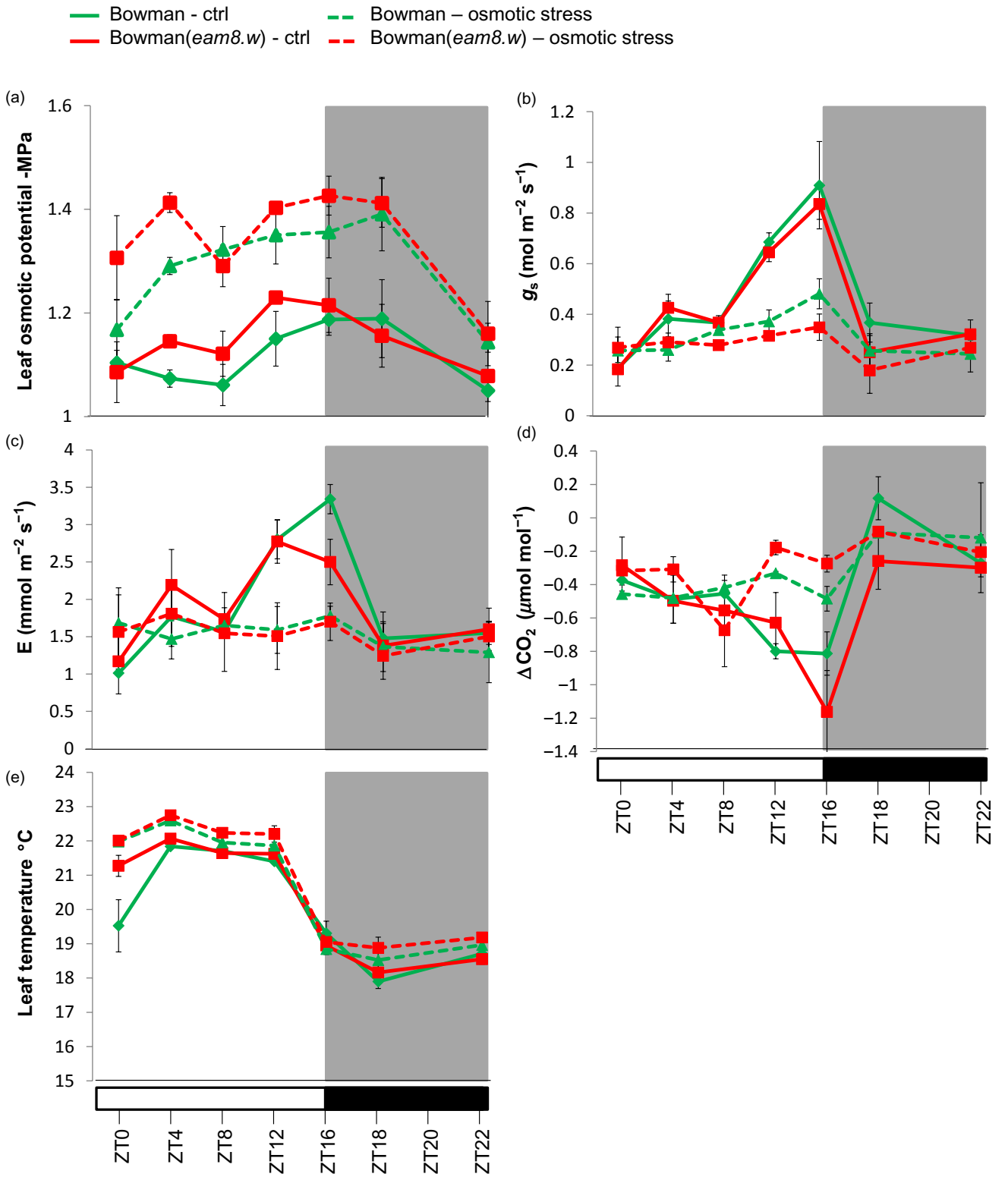


Figure 5. Diurnal expression of physiological traits under control (solid line) and osmotic stress (dashed line) conditions in the spring barley Bowman (green) and the introgression line Bowman(*eam8.w*) (red). (a) Leaf osmotic potential, (b) stomata conductance (g_s), (c) leaf transpiration rate (e), (d) net CO_2 exchange and (e) leaf temperature. Seedlings were grown in hydroponics for 10 d under long day (16 h/8 h, 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.

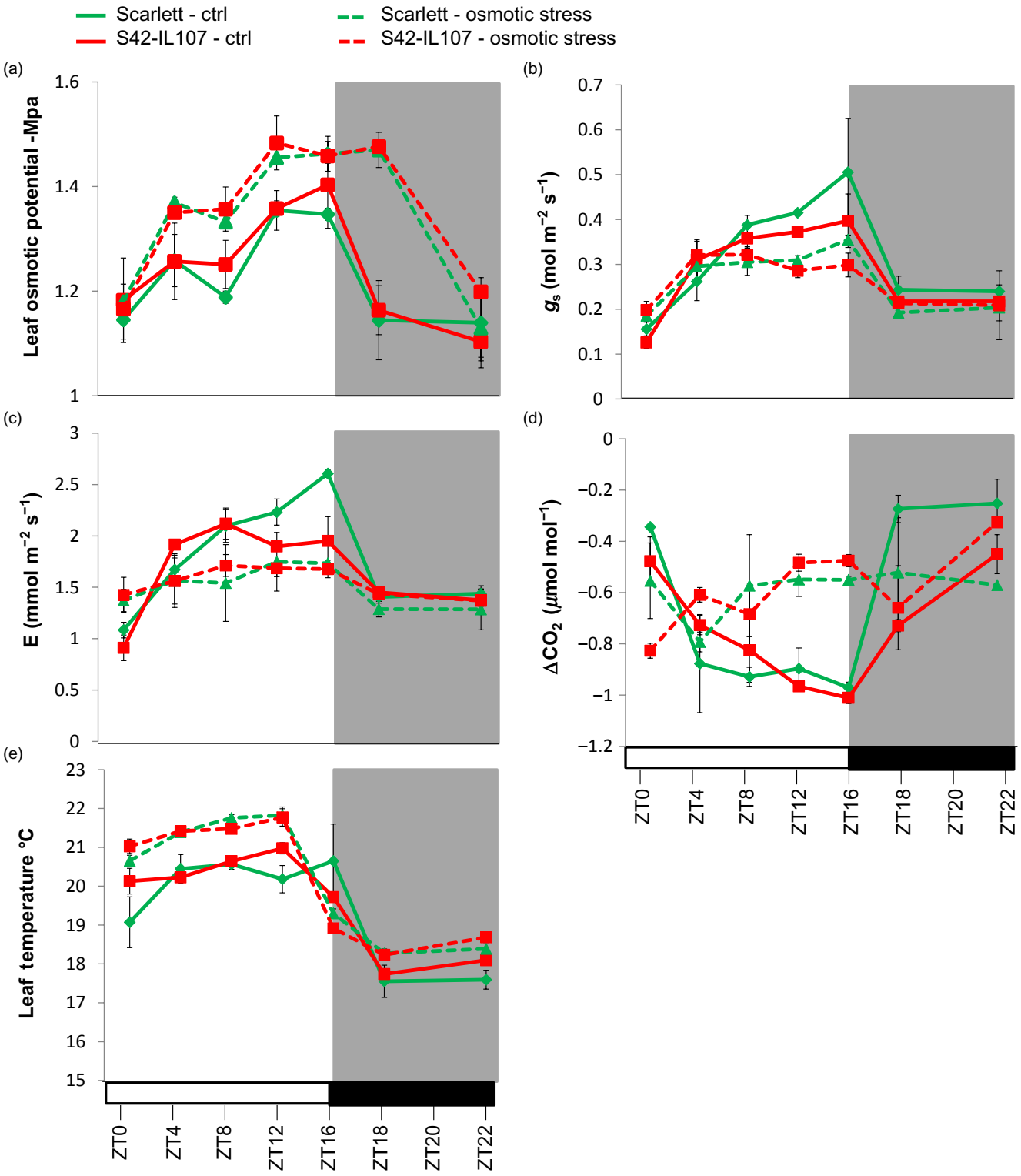


Figure 6. Diurnal expression of physiological traits under control (solid line) and osmotic stress (dashed line) conditions in the spring barley Scarlett (green) and the introgression line S42-IL107 (red). (a) Leaf osmotic potential, (b) stomatal conductance (g_s), (c) leaf transpiration rate (E), (d) net CO_2 exchange and (e) leaf temperature. Seedlings were grown in hydroponics for 10 d in long day (16 h/8 h, 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.

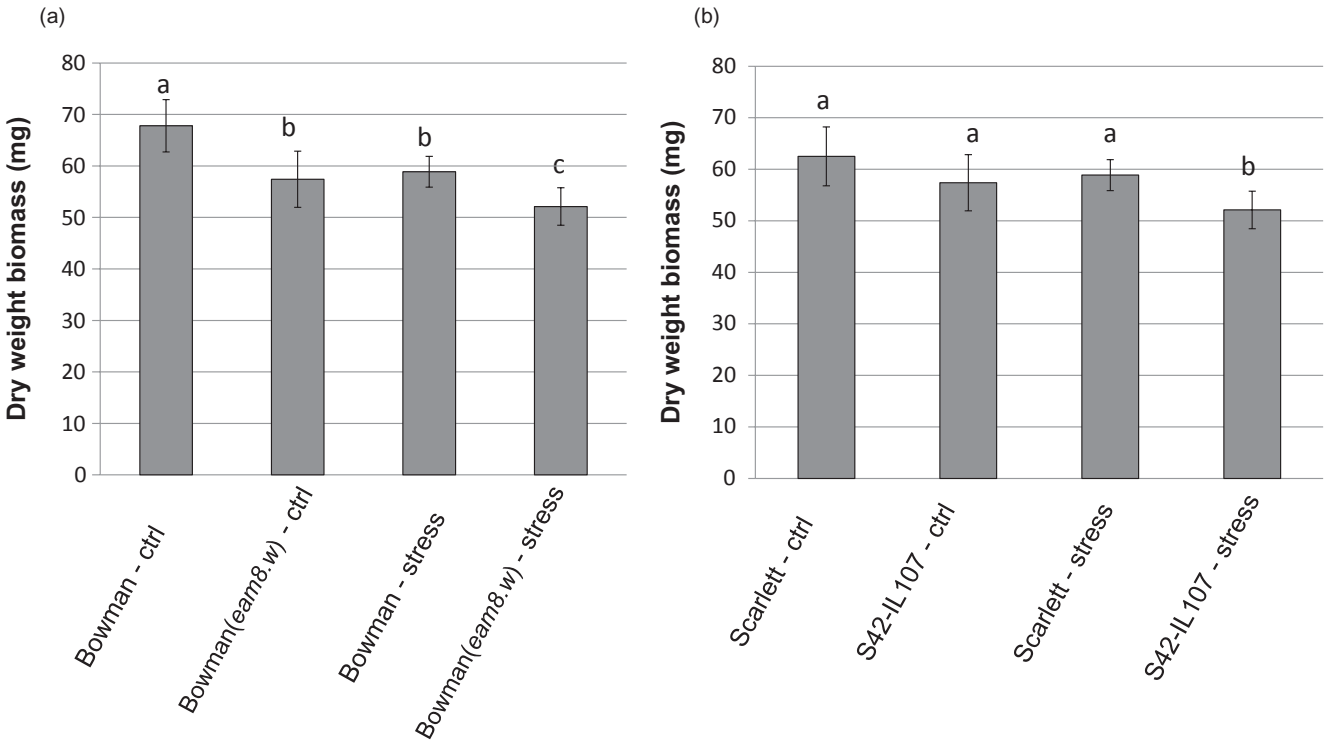


Figure 7. Dry weight biomass in Bowman/Bowman(*eam8.w*) (a) and Scarlett/S42-IL107 (b) under control and osmotic stress conditions. Measurements were taken in 12 day-old seedlings after 48 h of osmotic stress (20% PEG). Values are means ± SD of 12–15 seedlings. Different letters indicate significant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

well protected and covered at the base of the plant in monocots, but exposed to the environment in dicots.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interests.

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SUPPLEMENTARY INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Expression of stress response genes in Triumph and Triumph-IL under PEG-induced osmotic stress. Seedlings were grown in hydroponics under LD conditions for 10 d. At the two leaf stage roots of seedlings were immersed in 20% PEG induced osmotic stress or kept under control conditions for 3 d and A) HvDRF1, B) HvDREB1, C) HvAPX1 and G) HvCAT1 were analysed at 24 h intervals from ⬚ Triumph control, ▩ Triumph-IL control, ▧ Triumph stress, ■ Triumph-IL stress. Transcript accumulation was measured by qRT-PCR from leaf samples collected at ZT4 over three consecutive days. Different letters indicate significant differences at $P \leq 0.05$. Means ± standard deviation (SD) ($n = 3$) are shown.

Figure S2. 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 a Petri dish containing agar for 2 d under 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.

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

Table S2. Stress-responsive and core clock ortholog genes in barley, Brachypodium, rice, sorghum and maize.

Table S3. Analysis of variance for clock and stress-gene expression in Bowman and Bowman (eam8.w).

Table S4. Analysis of variance for clock and stress-gene expression in Scarlett and S42-IL107.

Table S5. Regulatory elements identified in conserved promoter regions of barley core clock ortholog and stress-responsive genes.

Table S6. Analysis of variance for physiological responses under osmotic stress in Bowman and Bowman(eam8.w).

Table S7. Analysis of variance for physiological responses under osmotic stress in Scarlett and S42-IL107.

Table S8. Analysis of variance for shoot dry weight in Bowman and Bowman(eam8.w) and Scarlett and S42-IL107.

Table S9. Analysis of variance for coleoptile lengths in barley seedlings.