The dawn burst: phytochromes and cryptochromes link light entrainment and photomorphogenesis in the early morning via the BBX family and HY5

mahiarmahjoub[1], martin.balcerowicz[1], sconde[1], Philip Wigge[1], and Daphne[1]

[1]Affiliation not available

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Please note that the supplement

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Abstract

The early morning is an important time period in which light and temperature signals entrain the plant circadian clock and trigger changes in a variety of developmental and physiological processes such as elongation growth and stress responsiveness. Through a high-resolution RNA-seq time series experiment using Arabidopsis seedlings, we demonstrate that there are multiple coordinated bursts of gene expression of transcription factors within the first two hours of light exposure that are both light- and temperature-sensitive. We find that *prr5 prr7 prr9* and *phyA phyB cry1 cry2* mutants both show an overall delay in the expression of morning genes and an elevated expression of genes that are usually expressed at high temperatures. In particular, we find that that phytochromes and cryptochromes induce expression of the photomorphogenesis-promoting bZIP transcription factors HY5 and HYH, as well as multiple BBX transcription factors that are known to interfere with HY5-mediated light responses. It appears that photoreceptors orchestrate multiple transcriptional cascades to tightly control gene expression at the control gene expression at dawn, which suggests that the dawn burst plays an important role in fine-tuning photomorphogenic responses in response to light.

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Introduction

In the early morning, plants need to adapt their transcriptional programmes to respond to biotic and abiotic stresses that primarily occur in the daytime. Many researchers have observed bursts of gene expression shortly after dawn in drought-response genes (Grundy et al., 2015), temperature response genes such as heat shock factor 70 (HSP70) (Dickinson et al., 2018), anthocyanin biosynthesis genes (Seaton et al., 2018) and phytohormone genes (Michael et al., 2008). In the latter case, Michael et al. 2008 observed that dawn expressed phytohormone genes had a G-box motif in their promoters, and indeed there is a large set of genes with G-box promoter motifs that are expressed within one hour of dawn including many that are involved in response to metals (Ezer et al., 2017b). Moreover, Arabidopsis is less susceptible to certain fungal pathogens in the morning, linked to jasmonic acid signalling (Ingle et al., 2015).

For these responses to occur at the appropriate time of day, it is important to have well-calibrated diurnal gene expression cycles. Dawn (and dusk) are critical periods for entrainment of the circadian clock, in response to changes in light (Kinmonth-Schultz et al., 2013; Covington et al., 2001; Edwards et al., 2010; Seo and Mas, 2014), temperature (Michael et al., 2003; Gould, 2006; McClung and Davis, 2010; Salome et al., 2010; Mizuno et al., 2014) and even humidity (Mwimba et al., 2018). The evidence that these transition periods are important for entrainment comes from experiments that show that light or temperature pulses in different times of day cause phase shifts in the circadian clock (Covington et al., 2001; Michael et al., 2003). However, dawn and dusk transitions are not exclusively responsible for entrainment– photosynthesis and sugar production can also gate the circadian clock, producing a second 'metabolic dawn' (Haydon et al., 2013).

There are several proposed mechanisms for circadian entrainment by light and temperature. Red- and blue-light sensors – including phytochrome A (phyA), phyB and cryptochrome 1 (cry1) – are involved in entrainment (Hall et al., 2002; Ω; **?**; **?**). In addition, members of the ZEITLUPE (ZTL) family, including ZTL, FLAVIN-BINDING KELCH F-BOX 1 (FKF1) and LOV-KELCH PROTEIN 2 (LKP2) all have clock-associated functions (Somers et al., 2000; Nelson et al., 2000; Schultz et al., 2001; Somers et al., 2004; Kim et al., 2007; Baudry et al., 2010). While phyB has also been implicated as a temperature sensor (Legris et al., 2016; Jung et al., 2016) and interacts with the Evening Complex (EC) that is part of the evening loop of the circadian clock (Ezer et al., 2017a; Huang et al., 2016), it is unclear whether phyB plays a role in temperature entrainment of the circadian clock. However, there is evidence that HEAT SHOCK PROTEIN 90 (HSP90) may be responsible for temperature entrainment (Davis et al., 2018).

NEW PARAGRAPH, NOT REFERENCED YET: Intriguingly, many of the genes that are involved in entraining the circadian clock are also key genes in photoperiodism and photomorphogenesis, including the phytochromes/cryptochromes and ZTL family members (Jackson, 2009; Kami et al., 2010; Franklin et al., 2005). phyA in particular has been reported as a key sensor of dawn and photoperiod (Seaton et al., 2018). It makes sense that there should be a mechanistic link between photoperiod-dependent processes and circadian entrainment, since both depend on day length. However, it is unclear how entrainment genes (such as phytochromes and cryptochromes) relay information to photomorphogenesis pathways and the circadian clock.

Although there have been a number of studies that demonstrate that there is a burst of gene expression after light stimulus in the morning, the dynamics of this burst have not been fully characterised because time points were not sampled frequently enough. Through a high resolution RNA-seq time series, we find five distinct transcriptional waves within the first two hours of the morning. We characterise how each wave of expression responds to temperature elevation and light signals during the night and subjective day, and how these waves are affected by a light signalling mutant (*phyA phyB cry1 cry2*), a circadian clock

mutant (*prr5 prr7 prr9*), and a temperature response mutant (*hsfa1QK*). Furthermore, we infer a gene regulatory network and validate edges using DNA binding data. We find that HY5 and BBX31 are among the TFs that are predicted to regulate multiple expression waves. Phytochromes and cryptochromes are required for a burst of expression of HY5 and BBX family proteins that fine-tune hypocotyl elongation and photomorphogenesis, suggesting that the dawn bursts may play a role in time-of-day dependent growth response to light stimulus. This work provides unprecedented detail as to how light, temperature, and circadian genes are regulated at dawn, as well as providing evidence of a mechanistic link between morning entrainment and photomorphogenesis.

Results

There is a burst of gene expression of DNA binding proteins at dawn

While previous research found groups of genes that had peak expression at dawn, we wondered whether the dawn burst was a more widespread phenomenon and whether regulatory proteins also exhibited a peak in expression (**Table S1**).

Overall, 39% of DNA binding proteins have peak gene expression immediately before or an hour after dawn (**Figure S1**). We find that there is a significant enrichment for ABA and ethylene-linked DNA binding proteins that have maximal expression in this time period compared to other DNA binding proteins (58% and 55%, $p<0.005$ in both cases using a Fisher Exact test with Bonferroni correction), which is consistent with the observations in Michael et al, 2008 (**Figure 1Ai,ii**). There is also an enrichment for DNA-binding proteins that are associated with GO terms related to light (53%, $p<0.02$) and stress (55%, $p<0.002$)– see **Figure 1Aiii,iv**. However, we found no significant enrichment for auxin-associated DNA binding proteins or circadian clock genes (**Figure S1**).

Consistent with the role of phytochromes in regulating the dawn peak (Michael et al., 2008), we observe that DNA binding factors that have peak expression immediately before dawn have increased expression in a *phyABCDE* quintuple mutant both before and after dawn (Figure 1B). In contrast, genes that have peak expression in the hour after dawn do not have perturbed expression in *phyABCDE*. This suggests that phytochromes may play a more important role in inhibiting nighttime genes than activating morning genes. We observe the same trends in *elf3-1*, which is consistent with evidence that phytochromes interact with ELF3 (Ezer et al., 2017a) (**Figure S2**).

S1: gene expression in all DNA binding proteins, auxin, and the circadian clock (DONE)

S2: the scatterplots in elf3-1 and phyABCDE in each of the GO term categories (DONE)

Table S1: dawn peak genes within each GO category (DONE)

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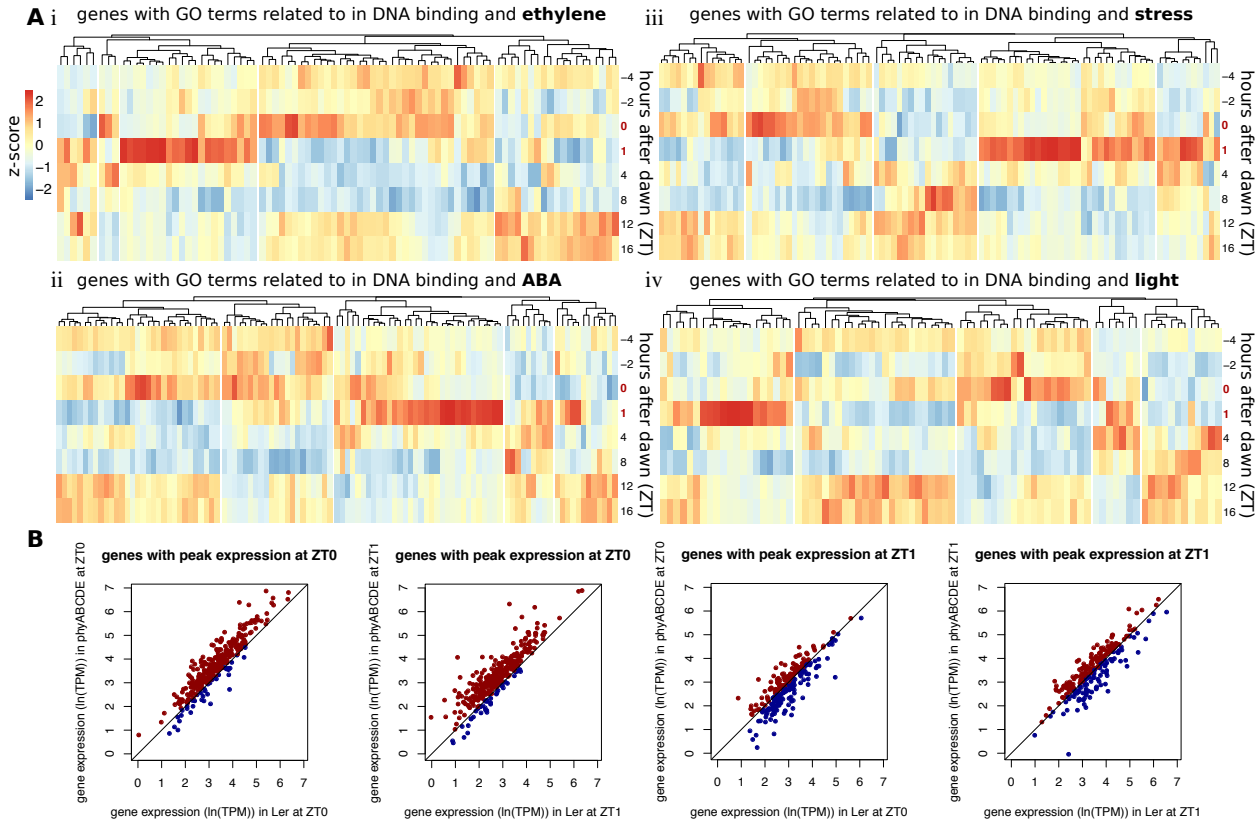


Figure 1: **DNA binding proteins exhibit a burst of expression at dawn. (A) The gene expression pattern of DNA binding proteins in ethylene (i), ABA (ii) , stress (iii), and light (iv) were clustered based on their expression in 8 time points under short day conditions in Col-0 at 22°C. The time point right before dawn (ZT0) and an hour after dawn (ZT1) are highlighted for clarity. Similar figures for 'auxin', 'circadian clock' and 'all DNA binding' are found in Fig S1. (B) DNA-binding proteins whose genes have maximal expression at ZT0 (right before dawn) or ZT1 (an hour after dawn) in Col-0 at 22°C were identified, based on the analysis in (A). We compare the expression levels of these genes in *phyABCDE* backgrounds compared to Ler (the background strain for *phyABCDE*). Similar figures comparing *elf3-1* and Col-0 are found in Fig S2.**

There are multiple waves of gene expression after light exposure at dawn

While many regulatory genes have peak expression at the onset of light, we do not know the precise timing of this burst, so a high-resolution time series experiment was performed at the onset of light, with 25 time points sampled at 22°C (**Table S2**).

To visualise this data, we clustered the gene expression values from the 22°C time series, focussing on genes with GO terms associated with DNA binding– see **Methods** and **Table S3**. The dawn burst consists of multiple coordinated waves of gene expression of genes associated with DNA binding. While there are genes whose expression levels decrease (clusters 1-2) or increase (clusters 3-4) over the time course, there are clearly three distinct bursts of expression at 16-24 minutes (clusters 7-8), 18-45 minutes (clusters 9-10), and 45-105 minutes (clusters 5-6), see **Figure 2A**.

These clusters contain many genes that are relevant to light signalling, temperature response, and immu-

nity. Clusters 7-8 include many genes that are related to temperature, such as *HEAT SHOCK FACTOR A1A* (*HSFA1A*) and immunity genes such as the WRKY transcription factor *WRKY33* and *BASIC LEUCINE ZIPPER 10* (*bZIP10*). This is consistent with other research that suggests that plants may be less sensitive to certain types of infections at dawn (Ingle et al., 2015; Wang et al., 2011). Interestingly, clusters 5-6 include *HEAT SHOCK FACTOR B2B* (*HSFB2B*) that suppresses the heat shock response and inhibits *HSFA1A* (Ikeda et al., 2011). The clusters of genes that are expressed at 45-105 minutes include genes that appear to be downstream of red (*RVE7, HY5, JMJ22, PNT1*) and blue (*MYC2, MYC4, CIB2, CRY3*) light signalling.

The dawn gene expression waves are sensitive to temperature and light

Since these clusters contained many genes associated with temperature and light signalling pathways, we investigated the effect of temperature and light perturbations on these expression waves. We repeated the high resolution RNA-seq experiment at an elevated but ambient temperature of 27°C (**Table S2**), and observed that genes continue to peak at the same time points, but the earlier expressed genes (clusters 1-2 and 7-8) have elevated expression and the later expressed genes (clusters 3-6) have lower expression than at 22°C, see **Figure 2A**. Please recall that the clusters were determined using the 22°C time series only.

Previously, Rugnone et al. 2013 found sets of genes that were induced or repressed by light, and moreover they identified genes whose sensitivity to light was dependent on whether the light treatment occurred at night or during the subjective day (after an extended night). We compare their gene lists with ours in **Figure 2B**, also see **Table S4**. There was significant enrichment for light repressed genes among early expressing genes in clusters 1-2 (p-value $<$ 1e-14 for genes that were repressed by light either during the day or in a time-neutral manner, based on Fisher exact test with Bonferroni correction). In contrast, those genes that were expressed late in the time series or that peaked at 45-105 minutes or 18-45 minutes tended to be light induced. Previously, we noticed that there were a number of key light signalling genes in clusters 5-6 and we find that over a third of genes within this group are light induced. Intriguingly, the clusters of genes that includes *HSFA1A* (clusters 7-8) are not enriched for light induced genes, except for a slight enrichment for genes whose expression is induced by light at night (p-value $<$0.037).

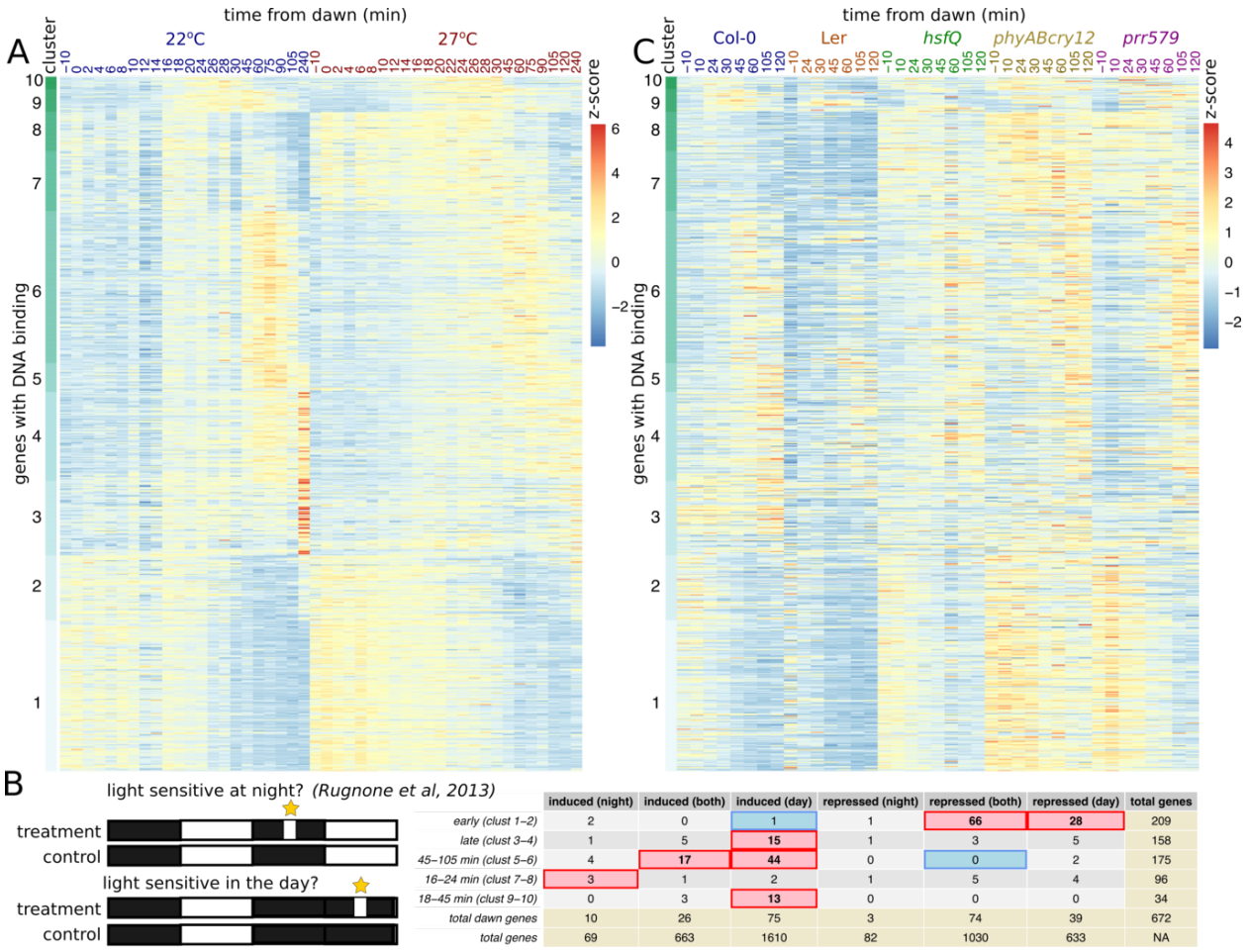
These results suggests that there are multiple coordinated bursts of gene expression at dawn that are differentially regulated by temperature and light, and moreover many of the dawn expressed genes are regulated by light in a time-of-day dependent manner.

Table S2: TPM tables for high resolution experiments (DONE)

Table S3: DNA binding genes within each cluster (DONE)

Table S4: adjusted p-values for light sensitivity comparisons (DONE)

Not necessary, but might add if reviewers insist: Table S5: lists of genes within each interacting pair



	induced (night)	induced (both)	induced (day)	repressed (night)	repressed (both)	repressed (day)	total genes
early (clust 1–2)	2	0	1	1	**66**	**28**	209
late (clust 3–4)	1	5	**15**	1	3	5	158
45–105 min (clust 5–6)	4	**17**	**44**	0	0	2	175
16–24 min (clust 7–8)	3	1	2	1	5	4	96
18–45 min (clust 9–10)	0	3	**13**	0	0	0	34
total dawn genes	10	26	75	3	74	39	672
total genes	69	663	1610	82	1030	633	NA

Figure 2: Multiple waves of gene expression at dawn. Genes encoding DNA binding proteins were clustered into 10 groups using CLUST based on their gene expression pattern at 22°C in Col-0. (A) The gene expression pattern of these clusters is shown for Col-0 at both 22°C and 27°C. (B) Previous experiments by Rugnone et al, 2013 identified genes that are sensitive to light in a time-of-day dependent way. Many of these genes that were identified as being light induced or repressed in the day and night were also found to be expressed within the clusters in (A). We use a table instead of a Venn Diagram for clarity. A coloured block indicates that there is a p-value < 0.05 based on a Fisher exact test with Bonferroni correction (red if it is more overlap than expected and blue if there is less than expected). Bold indicates that the p-value <0.001. (C) The gene expression pattern for those clusters in (A) for additional strains at 22°C. Note that this experiment used fewer time points and also sequencing was done with Lexogen instead of Illumina. Due to space, the names of the strains were abbreviated as follows: *hsfA1QK* was labelled as *hsfQ*, *phyA phyB cry1 cry2* was labelled as *phyABcry12*, and *prr5 prr7 prr9* was labelled as *prr579*.

The dawn gene expression waves are delayed in both phyAphyBcry1cry2 (red and blue light signalling) and prr5prr7prr9 (circadian) mutants

DNA binding proteins appear to have multiple coordinated bursts of expression at dawn and these bursts are either light or temperature dependent or both. To characterise the gene regulatory mechanisms that underly these changes, we performed RNA-seq on a series of mutant strains that perturbed red- and blue-light

sensitivity (*phyA phyB cry1 cry2*, Ler background), the circadian clock (*prr5 prr7 prr9*, Col-0 background) and temperature sensitivity (*hsfA1QK*, **Col-0 background**), all of which have all been previously characterised **(Yanovsky et al., 2000; Liu et al., 2013; Yoshida et al., 2011)**. These were sampled at the most informative time points according to NITPicker **(CITE)**. We observe the observe the same patterns of expression in this subset of time points as we were able to see in the original time series in Col-0 (**Figure 2C**). We also find that the genes within each of these clusters have perturbed patterns of expression in the mutant strains (**Figure 2C**). In particular, the early expressed genes were up-regulated in both the *prr5 prr7 prr9* and *phyA phyB cry1 cry2* mutants and were expressed for a larger portion of the time series, clusters 5-6 (which include many light-responsive genes) are expressed later in the time series (at 105-120 minutes instead of 45-60 minutes) in the *prr5 prr7 prr9* and *phyA phyB cry1 cry2* mutants and at higher expression levels. Clusters 7-8 (clusters that we previously found to have higher expression at elevated temperatures) were found to have higher expression levels in *hsf1aQK*, *prr5 prr7 prr9*, and *phyA phyB cry1 cry2* compared to Col-0 and Ler. Finally, the gene expression within clusters 9-10 was less coordinated in the mutants than the WT and there wasn't as consistent a pattern of up- or down-regulation.

An early morning gene regulatory network

Next, we wished to identify possible regulatory links by inferring a gene regulatory network. Because genes change their expression rapidly at dawn, it is likely that there is a time delay between the time the gene is expressed and the time the protein is expressed. For this reason, we applied a time delay network inference method called dynGenie3 (CITE). Because all large-scale network inference algorithms have large false-positive rates, we wished to confirm as many edges as possible using available DNA binding data (via DAP-seq from O'Malley et al. 2016).

Among TFs in our network with available binding data, we found that 34.3% of our high confidence edges (top 1% highest scoring edges) were consistent with DAP-seq. In contrast, we only observed a 12.6% overlap with DAP-seq data among edges in our network that received a score of '0' using dynGenie3 among TF with available DAP-seq data. This suggests that dynGenie3 is successfully enriching for biologically plausible edges.

From now on, we only consider the subset of the dynGenie3 network that is consistent with DAP-seq data. This network is shown in Figure 4A: the network naturally forms a U-shape, with early expressed genes primarily on the left side of the U and late expressed genes primarily on the right side. These genes are enriched for photoperiodism-related GO terms, including GO terms associated with light sensing, heat response and circadian rhythms (Fig. 3B) .

A number of transcription factors that are known to play a role in light signalling and photomorphogenesis sit at the base of the U, linking the early and late expressed genes; these include *ELONGATED HYPOCOTYL 5* (*HY5*), *B-BOX DOMAIN PROTEIN 31* (*BBX31*),*CYCLING DOF FACTOR 5 (CDF5)* and *REVEILLE 1 (RVE1)* (Figure 4A). These transcription factors are all involved in light-controlled hypocotyl elongation (Chattopadhyay et al., 1998; Rawat et al., 2009; Ω; Heng et al., 2019). While *RVE1* and *CDF5* expression is under strong circadian control and peaks at dawn (Rawat et al., 2009; Henriques et al., 2017), *HY5* and *BBX31* are light-induced genes (Oyama et al., 1997; Heng et al., 2019) and their transcript levels display a strong peak post dawn in our time course data (Fig. X?). Based on the timing of their expression and their position within our network, RVE1 and CDF5 may promote expression of growth-related genes before dawn, while HY5 and BBX31 appear to coordinate the down-regulation of early dawn genes as well as the induction of subsequent transcriptional waves.

HY5 is one of the most thoroughly characterised components of the light signalling pathway and, aside from its role in promoting photomorphogenesis, has been found to affect the circadian clock, temperature responses, chlorophyll and anthocyanin biosynthesis as well as nutrient uptake (Gangappa and Botto, 2016).

In agreement with its multifaceted roles, we find genes related to light signalling (*PHYTOCHROME INTERACTING FACTOR 4/PIF4*, *EARLY LIGHT INDUCIBLE PROTEIN 1/ELIP1*, *BBX16*), multiple heat shock genes, the core circadian clock gene *TIMING OF CAB EXPRESSION 1 (TOC1)* and several UDP glucosyltransferase genes involved in flavonoid metabolism among its predicted targets (Fig. 3C). In contrast to HY5, BBX31 represents a negative regulator of photomorphogenesis (Heng et al., 2019). Its targets do not show strong enrichment for specific biological processes, but include the related BBX transcription factors *BBX18* and *BBX30* as well as *HY5* itself (Fig. 3D). It has previously been shown that HY5 represses *BBX31* transcription (Heng et al., 2019), but based on our data a reciprocal regulation of *HY5* by BBX31 may occur as well,

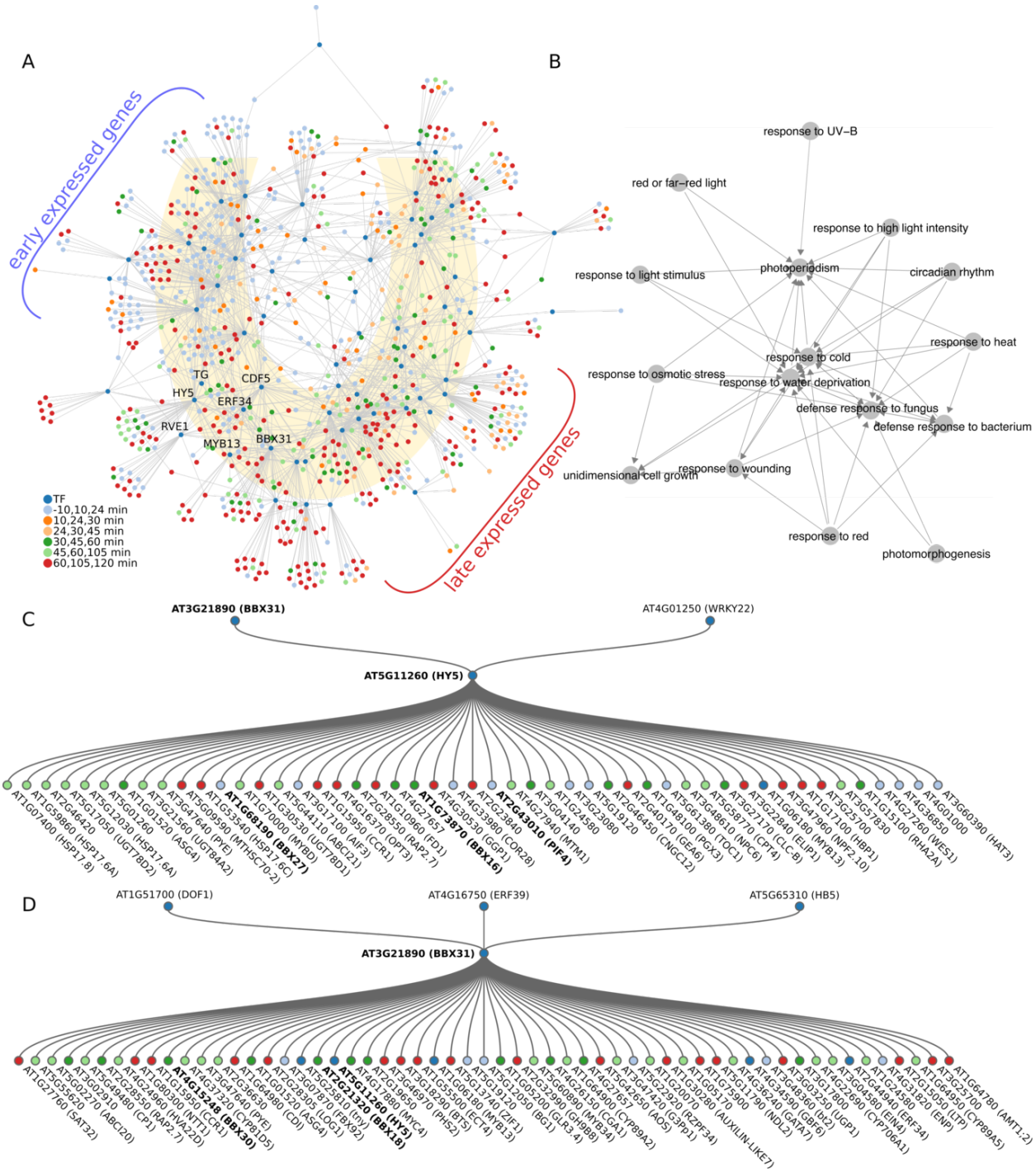


Figure 3: Figure 3: Analysis of higher confidence morning gene network. (A) This depicts the complete network of gene regulatory relationships that are predicted by dynGenie3 and consistent with DAP-seq data. All genes that regulate at least one downstream target are coloured dark blue, while other genes are colour-coded by the time period in which they have maximal expression (as expressed as the average gene expression in 3 adjacent time points). (B) This graph depicts enrichment for pairs of GO terms within the network, as determined by the PAFway package. The specific upstream and downstream targets predicted for HY5 (C) and BBX31 (D) are shown. Genes that are discussed in the text are highlighted in bold for clarity. The colour key is the same as in (A).

Figure 4: Gene expression profiles for genes related to key subnetwork.

Phytochromes and cryptochromes control morning photomorphogenesis pathways via BBX family proteins and HY5

A regulatory network of BBX transcription factors, HY5 and the related bZIP transcription factor HY5 HOMOLOGUE (HYH) has been postulated to control a large portion of the light-regulated transcriptome (Xu, 2019). We observe that multiple members of this network feature strongly during the dawn burst: not only *HY5* and *BBX31*, but also *HYH*, *BBX16*, *BBX24/SALT TOLERANCE (STO)*, *BBX25/SALT TOLERANCE HOMOLOG (STH)*, *BBX30* and *BBX32* transcript levels peak within an hour after dawn (Fig. 4). These peaks are completely abolished in the *phyA phyB cry1 cry2* mutant background, but only partially reduced in *phyA phyB* and *cry1 cry2* double mutants (Supplemental Fig. X). This implies that both sets of photoreceptors act in parallel to induce *HY5*, *HYH* and the *BBX* genes (hereafter referred to as BBX Set A). Another set of *BBX* genes (BBX Set B), comprised of *BBX20* and *BBX21*, does not exhibit a dawn burst in expression, but their transcript levels are strongly upregulated in the photoreceptor mutant (Fig. 4; Supplemental Figure X). Interestingly, apart from BBX16 all transcription factors included in Set B have been shown to negatively regulate photomorphogenesis (Indorf et al., 2007; Holtan et al., 2011; Gangappa et al., 2013a,b; Job et al., 2018; Heng et al., 2019), while HY5, HYH as well as BBX20 and BBX21 represent photomorphogenesis-promoting transcription factors (Chattopadhyay et al., 1998; Holm et al., 2002; Fan et al., 2012; Job et al., 2018). Our results suggest that these factors constitute a highly orchestrated transcriptional network that controls photomorphogenic responses at dawn downstream of phytochrome and cryptochrome photoreceptors (Fig. 4B?)

Discussion

A high resolution time series is required to capture fast transcriptional dynamics in the early morning

While previous researchers have noted that there is a peculiar burst of gene expression in the early morning, they were unable to fully characterise the transcriptional dynamics in this time period because the time points were not sampled frequently enough. We were able to identify multiple coordinated waves of gene expression **(Figure 5A)**. This included a cluster containing HSFA1A (cluster 7-8) that had elevated expression at 27°C and a second cluster that had a large number of light induced genes and that decreased its expression levels at the higher ambient temperature (cluster 5-6). A large proportion of transcription factors have perturbed expression in the early morning, and most of these genes are perturbed in similar ways in both the circadian and light sensing mutants (**Figure 5B**). Specifically, we observed (i) elevated levels of temperature-responsive genes and (ii) a time delay in the expression in most clusters. In general, it is a useful strategy to conduct a few high resolution time course experiments, and then use this data to select informative time point experiments for future experiments.

However, it is important to note that we have characterised the gene expression changes that result from the kind of sudden onset of light that occur in growth chambers, rather than those that are found in natural conditions, such as those investigated in Annunziata et al. (2018). Nevertheless, there are a number of benefits of this approach. Firstly, this allows us to more directly compare our results to other studies of

biotic and abiotic responses in the early morning that were also performed in artificial growth chambers with sudden onset of light (Grundy et al., 2015; Dickinson et al., 2018; Ingle et al., 2015; Ezer et al., 2017b; Cortijo et al., 2018; Michael et al., 2008; Seaton et al., 2018). Secondly, this kind of study allows us to directly address how the transcriptome responds to a *light stimulus* in the morning. Specifically, since we know the exact time that plants were first able to detect light, we can measure the exact time delay between this exposure and a change in gene expression. Finally, farming in artificial lights is becoming common, and it is important to understand how crops respond to lighting conditions that are reminiscent of growth chambers (Ibaraki, 2016; Gupta and Agarwal, 2017; Olvera-Gonzalez et al., 2013).

Phytochromes and cryptochromes coordinate light responses in the early morning via HY5 and BBX transcription factors

Our time course data allowed us to infer a high-confidence transcriptional network that controls the dawn burst in gene expression. HY5 and BBX family proteins appear at the core of this network downstream of phytochrome and cryptochrome photoreceptors and are likely to coordinate the early and late waves of gene expression in the early morning. Notably, the photoreceptors induce both positive (*HY5*, *HYH*) and negative (*BBX* Set A) regulators of light responses, while they simultaneously repress other photomorphogenesis-promoting factors (*BBX* Set B). *HY5* and *HYH* are not only induced by light at the transcriptional level, but the respective proteins are also stabilised through light-induced inactivation of the CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1)/SUPPRESSOR OF PHYA-101 (SPA) repressor complex (Osterlund et al., 2000; Holm et al., 2002; Saijo et al., 2003). Boosting HY5 and HYH protein levels via both mechanisms will trigger light responses in the early morning, but this process may require fine-tuning through other factors including BBX proteins. BBX24, BBX25 and BBX32 interfere with HY5 transcriptional activity through direct interaction (Holtan et al., 2011; Gangappa et al., 2013a; Job et al., 2018), while BBX30 and BBX31 act downstream of HY5 to promote elongation growth (Heng et al., 2019). BBX20 and BBX21 on the other hand promote HY5 function by increasing *HY5* transcript level and post-translationally enhancing HY5 activity (Job et al., 2018; Xu et al., 2018; Wei et al., 2016), but these effects seem to be largely suppressed at dawn. Another layer of complexity is added by the fact that the COP1/SPA complex also promotes degradation of several BBX proteins (Indorf et al., 2007; Yan et al., 2011; Xu et al., 2016; Gangappa et al., 2013a).

HY5 and BBX proteins interdependently tune photomorphogenic responses such as the reduction in elongation growth after dawn, but may exert additional functions independently of each other. BBX proteins have not yet been implicated in other HY5-mediated responses such as temperature signaling or biosynthesis of secondary metabolites. On the other hand, BBX30, BBX31 and BBX32 act as negative regulators of the floral transition independently of HY5 through the repression of *FLOWERING LOCUS T* (*FT*) (Graeff et al., 2016; Tripathi et al., 2017). Strong induction of these genes in the early morning may be involved in gating *FT's* responsiveness to inductive signals in the photoperiodic control of flowering.

In summary, our results reveal a large transcriptional regulatory network that controls the dawn burst of gene expression. Phytochrome and cryptochrome photoreceptors coordinate a substantial portion of this network to adjust photomorphogenesis, photoperiodism and clock entrainment in accordance with the plant's light environment

Materials and Methods

Plant strains used

Plant growth conditions

RNA-seq: High resolution time series

Quant-seq: Low resolution time series

Bioinformatics analysis of RNA-seq data

The Illumina RNA-seq data was analysed using the same bioinformatics pipeline used in (Ezer et al., 2017a), using Trimmomatic-0.32 to trim reads (Bolger et al., 2014), Tophat for mapping to the TAIR10 annotated genome (Trapnell et al., 2009), HTseq-count to find the raw counts after duplicates were removed (Anders et al., 2015), and Cufflinks to calculate Fragments Per Kilobase Million (FPKM), which was then converted into Transcripts Per Million (TPM) (Trapnell et al., 2013). The time points for the Lexogen Quant-seq experiment were selected using NITPicker (Ezer and Keir, 2019).

The Lexogen Quant-seq data was analysed using the Integrated Data Analysis Pipeline on Bluebee® platform, which maps the reads to the genome using the STAR Aligner (Dobin et al., 2013) and counts reads using HTseq-count (Anders et al., 2015). Quant-seq expression values are expressed as Reads per Million (RPM), because Quant-seq does not require normalisation by gene length.

Clustering

Clustering of the 24-hour time series (Figure 1) were drawn using hierarchical clustering (default parameters of hclust in R).

Clustering of the high resolution time gene expression time series data (in FIgure 2) was performed using the CLUST algorithm (Abu-Jamous and Kelly, 2018), using recommended settings for RNA-seq TPM data as per the reference manual (i.e. log2, Z- and quantile normalisation of TPM values).

The clustering in Figure 2A was performed only on the 22°C high resolution time series data. The other gene expression time series in Figure 2A and 2B were drawn using the same gene order. The z-scores were calculated across all samples within each row of Figure 2A and Figure 2B.

Gene list curation for network inference

A number of criteria were used to generate a gene list for performing network inference.

Firstly, all lowly or non-expressing genes were not included in the network inference analysis. The criteria for removal each sample were: [1] rowSum(RPM_jt) $<$ 7 x nt_j x 1.05 (RPM_jt is RPM value and n^T_j the total number of time-points for gene j at timepoint t) and [2] genes that had $<$5 time-points where the TPM_j $<$ 7. Genes with at least one time-point containing TPM = 0 were removed (requirement of dynGENIE3 package).

The final gene list used for network inference was obtained from the following sources: (1) GO categories, (2) consensus cluster and (3) DE analysis. A gene only needed to meet one of these three criteria in order to be included in the analysis.

First, all genes that had GO categories that were of biological interest to us were initially included. Specifically, this referred to genes that had Biological Process GO terms that included the words 'stress', 'light', 'auxin', 'abscisic', 'ethylene', 'circadian', as well as all genes that had the Molecular Function 'DNA binding').

Secondly, we selected for genes that had similar expression patterns as other genes in the data set. The reason we chose this criteria was that we did not want to include lots of genes that had extremely noisy patterns of expression. Clustering of the time-course gene expression was performed for each sample Col-0, Ler, *prr579*, *phyAB*, *HsfQk* at 22°C using the CLUST algorithm using recommended settings. Recall that the CLUST algorithm filters genes that do not cluster well with any of the clusters. Any gene that appeared in any of the clusters detected by the CLUST algorithm were included in the analysis.

Thirdly, differential expression analysis of of gene was performed based on the time-course using the odp method from the package edge (R Bioconductor) (Storey et al., 2005). Significant genes were chosen based on having an adjusted p-value (q-value) < 0.05. The following WT and mutant pairs at 22°C were compared: Col-0 vs *prr579*, Col-0 vs *hsfQk* and Ler vs *phyAphyB*.

This analysis produced a gene list of length 6795, which was too expansive for dynGenie3. The filtered gene list was ranked based on decreasing CV (sd/mean) with the top 1500 chosen for network inference.

Network inference and analysis

Network inference on the curated gene list was performed using dynGENIE3 R package (Huynh-Thu and Geurts, 2018). For each gene, the time-course gene expression was supplied from the following samples: Col-0/Ler/*prr579/phyABcry12/HsfQk* at 22°C and Col-0/Ler/*prr579/phyABcry12* at 27°C. For downstream analysis of network, only edges that had scores within the top 1% and that were consistent with DAP-seq data (O'Malley et al., 2016) were included. GO term enrichment within the network was determined using the PAFway package (CITE). Figures were drawn using the network visualisation tools developed for Ara-BOX-cis (Ezer et al., 2017b).

End of Methods: bits of text from Matthew that I had re-organised above are included below for future reference:

Text from Matthew that I re-ordered for clarity:

In the consensus cluster, genes that failed to be paired with at least one other gene in all clusters within Col-0, Ler, *prr579*, *phyAB*, *HsfQk* at 22 oC samples were removed and the remaining included. From the DE analysis, genes with significant DE were included. Within the resulting candidate list, genes of lowly or no expression across the samples Col-0, Ler, *prr579*, *phyAB*, *HsfQk* at 22 oC were removed from the list.

& consensus clustering analysis

What Matthew wrote: Clustering of the time-course gene expression was performed for each sample Col-0, Ler, *prr579*, *phyAB*, *HsfQk* at 22 oC using the CLUST algorithm (CITE). Recommended settings for RNA-seq TPM data were used as per manual [ref] i.e. log2, Z- and quantile normalisation of TPM values. Then, a symmetrical consensus cluster *nxn* (n being number of all genes clustered) was obtained by recording the number of times pairs of genes appear in the same cluster across the various samples. Data was visualised using clustered (hierarchical) heatmap.

Differential expression analysis

The analysis was performed between the following WT and mutant pairs at 22 oC : Col-0 vs *prr579*, Col-0 vs *hsfQk* and Ler vs *phyAphyB*. Prior to each analysis, lowly or un-expressed genes across both samples were removed. The criteria for removal each sample included: [1] rowSum(TPM_jt) $<$ 7 x nt_j x 1.05 (TPM_jt is TPM value and nˆT_j the total number of time-points for gene j at timepoint t) and [2] genes that had <5 time-points where the TPM_j $<$ 7. Differential expression analysis of of gene was performed based on the timecourse using the odp method from the package edge (R Bioconductor) (CITE). Significant genes were chosen based on having an adjusted p-value (q-value) $<$ 0.05.

Any "personal communications" relating to unpublished data should be incorporated within the main text, in the following format: (Author Initial(s) and Surname, personal communication, Month and Year). Authors should have permission from anyone named in this way and should be aware that a supporting letter will sometimes be requested.

Within the Materials and Methods and/or figure legends, we encourage authors to provide complete information about their experiments, analyses, or data collection to ensure that readers can easily understand what was measured and analysed, and can accurately perform the relevant protocols.

In cases where a new method within the submission would benefit from step-by-step protocols in addition to the methods described in the article, we would encourage authors to also consider submitting a detailed protocol to Bio-protocol.

On first mention, please provide details of any manufacturers in the following format: company name, city, country (or state, if based in the United States).

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Competing interests

At this stage we request that the corresponding author provides a statement of financial and non-financial competing interests on behalf of all authors. Examples include paid employment or consultancy, stock ownership, patent applications, personal relationships with relevant individuals, and membership of an advisory board.

Example reference (Nicholson et al., 2015).

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