The Integration of Environmental and Circadian Signals to Regulate Transcription from the Chloroplast Genome

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Abstract

In plants, circadian oscillations in gene expression and physiology occur with a period of approximately 24 hrs. The coordination of these oscillations with environmental signals ensures appropriate biological responses depending on the time of day and season, presenting a fitness advantage. There are extensive roles for the transcription factors HY5 and HYH in the regulation of gene transcription in response to environmental and circadian signals. SIG5 is multiple stress-responsive and coordinates nuclear and chloroplast gene expression; ATHB17 regulates *SIG5* transcription in response to salt stress. The work described in this thesis aimed to investigate roles for HY5/HYH in the signalling pathway to *SIG5* and its chloroplast gene target *psbD* BLRP, and to identify whether ATHB17 regulates *SIG5* in response to cold stress. qRT-PCR was used to monitor *SIG5* and *psbD* BLRP transcript accumulation under circadian and diel conditions in mutants of *HY5* and *HYH*. Roles for these transcription factors in the circadian and environmental regulation of chloroplast transcription were confirmed, suggesting redundancy between the two. Analysis of *CCA1* relative expression over a circadian timeseries suggested that HY5/HYH act on the amplitude of *SIG5* expression downstream of *CCA1*. Bioluminescence imaging was also used to assess *SIG5* promoter activity in mutants of these transcription factors, suggesting regulation of *SIG5* promoter activity by HY5/HYH. Furthermore, measurement of *SIG5* transcript abundance following cold stress suggested it is unlikely that ATHB17 regulates *SIG5* in response to cold. This research highlights the existence of both distinct and overlapping roles for HY5/HYH in regulating *SIG5* in response to environmental and circadian cues. However, other factors contribute to the maintenance of *SIG5* and *psbD* BLRP circadian rhythmicity. Therefore, future work should aim to identify these factors, as well as to further understand the distinct and intersecting roles for HY5/HYH in the regulation of *SIG5* and *psbD* BLRP.

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List of abbreviations

℃	Degrees Celcius
µg	Micrograms
µL	Microlitres
µM	Micrometres
µmol	Micromoles
ABA	Abscisic acid
ACE	ACGT-containing element
ACT	ACTIN
ANOVA	Analysis of Variance
ATHB17	ARABIDOPSIS THALIANA HOMEOBOX 17
ATP	Adenosine triphosphate
B	Blue (light)
BBX	B-BOX CONTAINING PROTEIN
bHLH	Basic helix-loop-helix
BLRP	Blue light-responsive promoter
BOA	BROTHER OF LUX ARRHYTHMO
bp	Base pair
bZIP	Basic Leucine Zipper
CBF	C-REPEAT BINDING FACTOR
CCA1	CIRCADIAN CLOCK ASSOCIATED 1
CHE	CCA1 HIKING EXPEDITION
Col-0	Columbia-0
COP1	CONSTITUTIVE PHOTOMORPHOGENIC 1
COR	Cold-responsive gene
CRY	Cryptochrome
DET1	DEETIOLATED 1
dNTP	Deoxynucleoside triphosphate
EC	Evening complex
EE	Evening element
ELF	EARLY FLOWERING PROTEIN
FFT-NLLS	Fast Fourier Transform- Non-Linear Least Squares method
FLC	FLOWERING LOCUS C
FR	Far red (light)
g	Grams
GFP	Green fluorescence protein
GI	GIGANTIA
GUS	β-glucuronidase
H3	Histone 3
HD-ZIP	Homeodomain leucine zipper
h/hr	Hour(s)
HY5	ELONGATED HYPOCOTYL 5

HYH	HY5 HOMOLOGUE
inHg	Inches of mercury
kb	Kilobase
L	Litre
LB	Left border primer
LB	Lysogeny broth
LD	Light-dark
LHY	LATE ELONGATED HYPOCOTYL
LL	Light-light
LNK	NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED GENE
LOV	Light-oxygen-voltage
LP	Left genomic primer
LUC	LUCIFERASE
LUX	LUX ARRHYTHMO
min	Minute
mL	Millilitres
mm	Millimetres
mM	Millimolar
MS	Murashige & Skoog
MS-S	Murashige & Skoog without sucrose supplementation
NASC	Nottingham Arabidopsis Stock Centre
NEP	Nuclear-encoded plastid RNA polymerase
nm	Nanometres
NRT	No reverse transcriptase (cDNA synthesis control)
NTC	No transcript control (cDNA synthesis control)
PCR	Polymerase Chain Reaction
PEG	Plastid-encoded gene
PEP	Plastid-encoded plastid RNA polymerase
pH	Potential of hydrogen
PhANGs	Photosynthesis associated nuclear genes
PHY	Phytochrome
PIF	PHYTOCHROME INTERACTING FACTOR
PRR	PSEUDO RESPONSE REGULATOR
PS (I & II)	Photosystem (I & II)
psi	Pounds per square inch
qRT-PCR	Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction
R	Red (light)
RNA	Ribonucleic acid
ROI	Region of interest
ROS	Reactive oxygen species
RP	Right genomic primer
rpm	Revolutions per minute
RVE8/LCL5	REVEILLE8/LHY-CCA1-LIKE5

SIG	SIGMA FACTOR
SPA	SUPPRESSOR OF PHYA
TAE	Tris-acetate-EDTA buffer
TAIR	The Arabidopsis Information Resource
T-DNA	Transfer DNA
TF	Transcription factor
TOC1	TIMING OF CAB EXPRESSION 1
UV(-B)	Ultraviolet (B) (light)
UVR8	UV-Resistance locus 8
v/v	Volume/volume percentage
Ws	Wassilewskija
WT	Wild type
w/v	Mass/volume percentage
ZTL	ZEITLUPE

Chapter 1 Introduction

Predictable daily and seasonal cycles in environmental factors such as light and temperature occur as a result of the Earth's rotation about its axis. These cycles have acted as selection pressures for the evolution of internal circadian oscillators in the organisms exposed to these conditions. Circadian oscillators enable organisms to appropriately time their responses to stimuli, thereby coordinating behavioural, physiological and developmental processes with the prevailing environment. The functional significance of circadian rhythms presents organisms with an adaptive advantage.

Environmental signals can act as entrainment cues that adjust the timing of the oscillator. In plants, the existence of chloroplasts with genomes distinct from the nuclear genome necessitates the coordination between the expression of these two sets of genes. Chloroplast genes encode essential components of the photosynthetic apparatus. Both predictable and unpredictable (such as changes in weather conditions) environmental fluctuations can have profound effects upon photosynthesis. Therefore, mechanisms have evolved to signal information to chloroplasts about environmental changes and circadian time, in order to acclimate photosynthesis to the fluctuating environment.

We have identified in Arabidopsis that sigma factor proteins communicate information from the nucleus to chloroplasts about light conditions (Belbin et al., 2017), low temperatures (Cano-Ramirez et al., under revision), and circadian time (Noordally et al., 2013). Sigma factors are nuclear-encoded regulators of transcription from the chloroplast genome. It is emerging that this signalling mechanism is important because it increases photosynthetic efficiency in fluctuating light conditions and cold temperatures, and also increases freezing tolerance (Nagashima et al., 2004, Cano-Ramirez et al., under revision). I reason that one site of signal integration will be the promoter of the *SIG5* gene. The upstream regulators that signal circadian and environmental information along this pathway are as yet unknown. The work described in this thesis therefore focuses on roles for various transcription factors in the SIG5-mediated circadian and environmental regulation of chloroplast transcription.

The plant circadian clock

Circadian clocks are cellular oscillators that generate endogenous estimates of the time of day, co-ordinating cellular processes with external 24-hr cycles. Organisms possessing circadian oscillators can anticipate predictable daily and seasonal environmental fluctuations and transitions, thereby coordinating their biological activities with these fluctuations (Panda et al., 2002, Dodd et al., 2014). This organisation of gene expression and biological activity into 24-hr rhythms is ubiquitous among biological organisms, from non-photosynthetic prokaryotes (Eelderink-Chen et al., 2021) and cyanobacteria, to plants and humans, enabling the integration of environmental signals with internal cues to regulate physiological outputs. The widespread and varied nature of internal circadian oscillators and their disparate phylogenetic origins points to the importance of circadian regulation for an individual organism's fitness and survival (Young and Kay, 2001, Dodd et al., 2005, Vaze and Sharma, 2013). The coordination of internal processes with external stimuli facilitates appropriate responses, specific to both time of day and season, thereby providing an adaptive advantage to organisms (Dodd et al., 2005).

The period of these endogenous circadian cycles, i.e., the time taken to complete one cycle, is subsequently approximately ('*circa*') 24 hrs ('*dies*'). This is a defining characteristic of circadian rhythms. An additional defining characteristic of circadian rhythms is that they persist upon transfer to constant conditions (e.g. continuous light) following a period of entrainment (McClung, 2006). This persistence in the absence of external time cues confirms the endogenous nature of these biological rhythms (McClung, 2006).

The time of day of any given biological event, such as the peak of an oscillation, is referred to as its phase. The amplitude refers to the difference between the peak/trough of an oscillation and its midpoint, providing a measure of magnitude. The period of a circadian rhythm has relative uniformity under a range of ambient temperatures, which is termed temperature compensation (Pittendrigh, 1954), rather than having an acceleration at increased temperatures, as is typical with reactions controlled by enzymes (Michael et al., 2003). This enables stable functioning of the clock given changes in cellular metabolism (McClung, 2006).

Environmental time cues, or zeitgebers ('time givers' in German), entrain the circadian clock through so-called input pathways (McClung, 2006). Input pathways communicate information to the central oscillator, entraining the clock to match the phase of the environment (Bell-Pedersen et al., 2005). Output pathways subsequently regulate the rhythm and phase of the expression of genes within the plant. Through direct clock control of processes such as gene transcription (Covington et al., 2008), post-transcriptional regulation (mRNA splicing) (Sanchez et al., 2010) and post-translational modifications (Fujiwara et al., 2008), biochemical and physiological processes such as flowering time (Johansson and Staiger, 2014) and photosynthesis (Dodd et al., 2015) acquire rhythmicity.

Individual plant cells possess their own distinct circadian clocks, the period lengths of which are coordinated by entrainment to external light-dark (LD) cycles (Muranaka and Oyama, 2016). Intercellular circadian communication or coupling has been identified at both the local and long-distance (shoots to roots) levels (Endo et al., 2014, Takahashi et al., 2015, Gould et al., 2018, Chen et al., 2020). Furthermore, shoot apex cells have been suggested to be master synchronisers of cellular clocks in Arabidopsis, conferring robustness to oscillations and facilitating phase adjustments (Takahashi et al., 2015). Local coupling of individual plant cell clocks, which is especially strong in the root tip, facilitates the generation of this robustness of clock rhythms throughout the plant (Gould et al., 2018). However, there exists desynchronisation between plant cell clocks in their periods; this feature has been attributed to the existence of two separate waves of clock gene expression, each travelling up and down the root, respectively, and each facilitated by local cell coupling (Gould et al., 2018).

The circadian clock can gate its sensitivity to environmental time cues at different times of day. Circadian gating refers to the way that the magnitude of a response to a stimulus given at one time of day may differ to that at another time of day (McClung, 2006). This coordination with environmental conditions ensures responses to stimuli remain appropriate, presenting a fitness advantage to plants (Dodd et al., 2005). Arabidopsis plants whose circadian period matches that of the external 24-hr cycle of light and dark are conferred a photosynthetic advantage (Dodd et al., 2005). This coordination facilitates greater carbon fixation, increased leaf chlorophyll content, increased plant growth and enhanced survival (Green et al., 2002, Dodd et al., 2005, Graf et al., 2010).

Circadian clock architecture

Morning-phased oscillator components

The Arabidopsis circadian oscillator has been well-characterised. CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) and its closely related homolog LATE ELONGATED HYPOCOTYL (LHY) are two MYB-like transcription factors whose expression peaks around dawn (Wang and Tobin, 1998, Schaffer et al., 1998). These proteins function synergistically to repress the expression of *TIMING OF CAB EXPRESSION 1* (*TOC1*), also known as *PSEUDO RESPONSE REGULATOR 1* (*PRR1*) (Alabadí et al., 2001). TOC1 – an evening-phased component of the clock – contains a *cis*-regulatory element in its gene promoter known as the evening element (EE). CCA1 and LHY bind to the EE, promoting the deacetylation of *TOC1* histone 3 (H3), thereby repressing its expression (Farinas and Mas, 2011). CCA1/LHY and TOC1 form a negative feedback loop – the first described in plants. As *TOC1* transcripts increase throughout the day, they repress the expression of the morning-phased elements *CCA1* and *LHY* through interaction with *CCA1 HIKING EXPEDITION* (*CHE*) (Pruneda-Paz et al., 2009, Gendron et al., 2012, Hsu et al., 2013, Hsu and Harmer, 2014).

Day-phased oscillator components

Other members of the PRR family (*PRR5*, *7* and *9*) display sequential expression patterns throughout the day (Matsushika et al., 2000) and negatively regulate *CCA1* and *LHY* expression in a partially redundant manner (Nakamichi et al., 2010). *PRR9* is the first of the family to be expressed, displaying peak expression levels shortly after dawn; this is followed in turn by *PRR7*, *PRR5*, *PRR3*, and finally *TOC1* in the evening. *prr5 prr7 prr9* triple mutants display arrhythmic phenotypes, highlighting the importance of these clock components in the functioning of the plant circadian oscillator (Farré and Liu, 2013).

Another family of circadian-regulated genes, which display peak expression levels around subjective noon, have also been implicated in maintaining clock function. The light-inducible LNK1 and LNK2 (NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED GENE 1 and 2) may activate the expression of the clock components *PRR5* and *ELF4*, both of which have late-in-the-day phases, as *lnk1 lnk2* double mutants show significantly reduced *PRR5* and *ELF4* expression levels (Rugnone et al., 2013). The double mutant also displays a long period

phenotype, indicating a significant role for these genes in maintaining oscillator function (Rugnone et al., 2013).

Afternoon-phased oscillator components

REVEILLE 8/LHY-CCA1-LIKE 5 (RVE8/LCL5) is homologous to CCA1 and LHY (Rawat et al., 2011). The RVE family of transcription factors display dawn-phased gene expression which is circadian-regulated, but unlike *cca1* and *lhy* mutants which have short period phenotypes, *rve8* mutants show long period phenotypes (Rawat et al., 2011). RVE8 protein levels peak in the subjective afternoon and, similar to CCA1 and LHY, RVE8 binds to the EE in *TOC1* and other EE-containing clock output genes (including *GI*, *LUX*, *ELF4* and *PRR5*) (Farinas and Mas, 2011, Hsu et al., 2013). RVE8, however, induces the expression of these genes later in the subjective day by increasing H3 acetylation (Farinas and Mas, 2011). RVE4 and RVE6 – the two other members of the RVE family – might act partially redundantly with RVE8 in maintaining the correct period of the clock (Hsu et al., 2013). *rve4 rve6 rve8* triple mutants both have longer period phenotypes than the single *rve8* mutant and fail to display the characteristic afternoon-phased EE binding (Hsu et al., 2013).

Evening-phased oscillator components

TOC1, in combination with other evening-phased transcription factors, including EARLY FLOWERING 3 (ELF3), ELF4, GIGANTEA (GI) AND LUX ARRHYTHMO (LUX), form the Evening Complex (EC), the expression of which is repressed by CCA1/LHY (Hsu et al., 2013). These evening-phased components subsequently repress *PRR9* expression, which peaks just after subjective dawn (Takahashi et al., 2015). Mutations in any of these genes in the evening complex results in arrhythmicity, highlighting their relative importance in the functioning of the clock (Nagel and Kay, 2012). Additionally, these elements all possess an EE in their promoters, which has emerged as an essential component of the clock architecture. The mode of action of the majority of clock components occurs either through regulation of the EE, e.g., by CCA1/LHY repression or RVE4/6/8 activation, or through regulation of other components via the EE contained within clock gene promoters. This reciprocal regulation of clock components maintains the ordered expression of core clock genes and enables the complex functioning of the circadian oscillator.

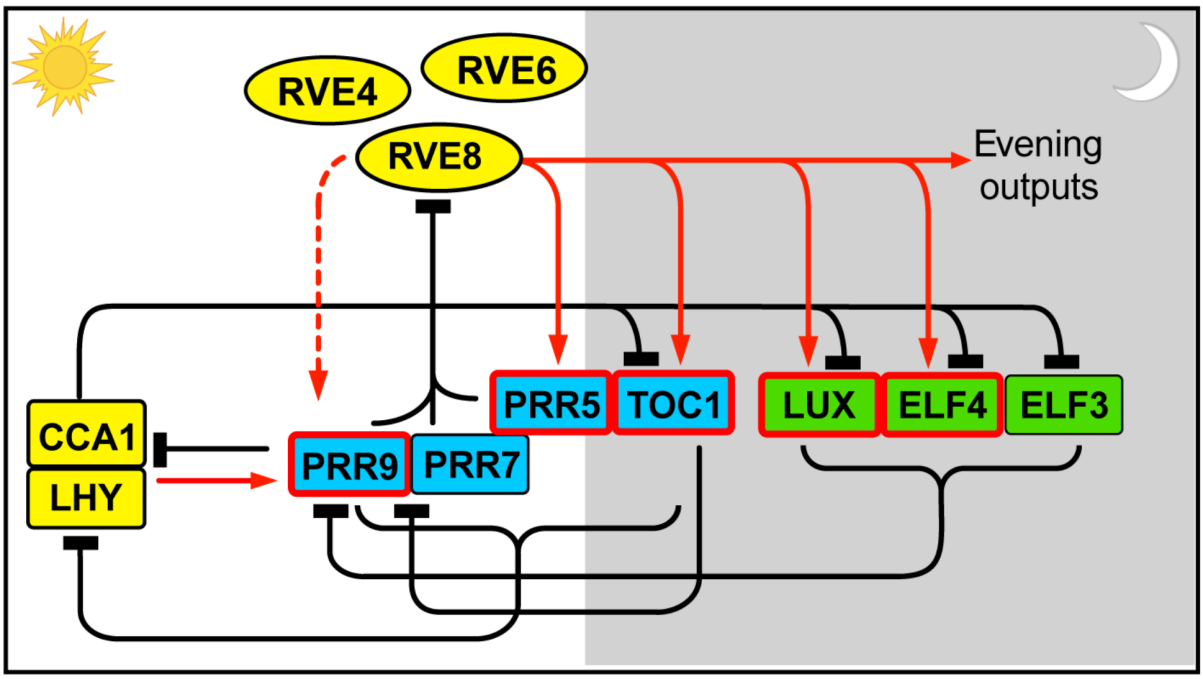

Figure 1.1 Simplified model of the components of the Arabidopsis circadian oscillator and the transcriptional regulation among these. CCA1, LHY (morning-phased) and the RVE components (afternoon-phased) are in yellow, members of the PRR family are in blue and evening complex (EC) components are in green. Sequential expression of clock components throughout the 24-hr day-night cycle is presented from left to right (white area = subjective day; grey area = subjective night). Red boxes indicate genes with evening elements (EEs) in their promoters. Arrows = activation, perpendicular bars = repression, broken red arrow = conditional activation. Reproduced from Hsu and Harmer (2014).

Post-transcriptional regulation

Post-transcriptional regulation also maintains the period of the clock. For example, PRR5, 7 and 9 physically interact with TOPLESS, which is a transcriptional co-repressor (Wang et al., 2013), to negatively regulate *CCA1* and *LHY* expression (Hsu and Harmer, 2014). An additional physical interaction that occurs to regulate clock protein activity includes the interaction between CCA1 and LHY with DET1 (DEETIOLATED 1). DET1 is a central repressor in photomorphogenesis and associates with the promoter regions of CCA1/LHY target genes to direct the transcriptional repression of these. DET1 is hence required for the transcriptionally repressive function of CCA1/LHY (Lau et al., 2011). Further, ZEITLUPE (ZTL), which is a F-box protein with a blue light photo-sensing LOV (light, oxygen, voltage) domain and is involved in targeting clock components for proteasomal degradation, physically interacts with GI, stabilising both proteins (Kim et al., 2007).

Alternative splicing of *CCA1*, *LHY*, *PPR5*, *PRR7*, *PRR9*, *TOC1*, *RVE4* and *RVE8* transcripts has also been identified (Hsu and Harmer, 2014). A naturally occurring splice variant of *CCA1*,

which produces a protein termed CCA1β, can interact with full-length CCA1 (CCA1α) to form non-functional heterodimers, thereby interfering with the role of CCA1 (Seo et al., 2012). CCA1β lacks the MYB-like DNA-binding domain found in CCA1α, and overexpression of CCA1β results in a short period phenotype not dissimilar to *cca1 lhy* double mutants (Seo et al., 2012). Therefore, this splice variant can modify the pace of the clock via its interaction with CCA1α. Furthermore, phosphorylation of the PRRs has been suggested to maintain their stability (Hsu and Harmer, 2014), and CCA1 phosphorylation is required to control its promoter binding activity (Daniel et al., 2004, Portolés and Más, 2010).

The coupling of the circadian clock with physiological and developmental processes

Many core components of the circadian oscillator directly regulate clock outputs, such as plant growth, metabolism, photoperiodic control of flowering time, and biotic and abiotic stress responses. These outputs are additionally able to feed back into the circadian oscillator to further modulate circadian function and to ensure responses to the environment are continuously fine-tuned. Here, I will discuss the coupling of the clock with growth, metabolism and abiotic stress outputs.

Elongation growth

Many growth responses in plants are regulated by the circadian oscillator. A well-studied clock-controlled growth response is hypocotyl elongation in seedlings. Under constant light conditions, there is a rhythm in the elongation rate of the hypocotyl, indicating that it is a circadian-controlled response (Dowson-Day and Millar, 1999). However, under constant darkness, this rhythmic elongation does not occur, meaning light is necessary for this growth response to occur (Nozue et al., 2007). Various pathways involved in hypocotyl elongation are regulated by the clock and contribute to this rhythmic response. PHYTOCHROME-INTERACTING FACTOR 4 (PIF4) and PIF5 (two basic helix-loop-helix (bHLH) transcription factors that promote growth) integrate circadian signals with light, hormone signalling, temperature cues and sucrose to regulate hypocotyl growth (Feng et al., 2008, Stewart et al., 2011, Hsu and Harmer, 2014).

In the evening, evening complex proteins directly repress *PIF4* and *PIF5* expression, thereby contributing to their circadian regulation. *PIF4* and *PIF5* expression peak at the end of the night/in the early morning, correlating with the phase of maximum hypocotyl elongation. Light regulation of PIF4 and PIF5 abundance also occurs via PHYB, which promotes their degradation under red light conditions, thereby maintaining PIF4 and PIF5 inactivation throughout the day (Lorrain et al., 2008). High levels of sucrose additionally stabilise PIF5 (Stewart et al., 2011), thus the control of the hypocotyl elongation response via the internal circadian oscillator together with external environmental cues presents an interesting external coincidence model in Arabidopsis. Further, the proteins DE-ETIOLATED 1 (DET1) and CONSTITUTIVE PHOTOMORPHOGENESIS 1 (COP1) promote the elongation growth of hypocotyls at increased temperatures, whereas ELONGATED HYPOCOTYL 5 (HY5) negatively regulates PIF4-mediated hypocotyl growth through its competitive binding to G-box elements in PIF4 target gene promoters (Gangappa and Kumar, 2017, Toledo-Ortiz et al., 2014).

Abiotic stress

Low temperatures have pervasive effects upon plants, with photosynthesis and biological membranes being particularly susceptible to the effects of cold temperatures. Mechanisms have evolved to enable temperate plants to acclimate to the cold, thereby protecting physiological and developmental processes by the development of freezing tolerance through exposure to low, non-freezing temperatures (Chinnusamy et al., 2007).

C-REPEAT BINDING FACTOR 1 (CBF1), CBF2 and CBF3 are circadian-regulated, cold-induced transcription factors that act downstream of cold-regulated (COR) genes and are involved in cold acclimation. The cold induction of these genes is conserved across plants and the expression and cold induction of *CBF*s is gated by the clock, so that induction is greater during the subjective day than the subjective night (Fowler et al., 2005, Dong et al., 2011, Chew and Halliday, 2011). The clock proteins CCA1, LHY, TOC1, PRR5 and PRR7 have all been identified to bind to CBF gene promoter regions, hence modulating their expression (Nakamichi et al., 2012, Dong et al., 2011). Each of these clock components impacts freezing tolerance in different ways: CCA1 and LHY promote freezing tolerance (Dong et al., 2011), whereas the PRRs repress *CBF* expression and thus may act to inhibit the freezing tolerance

response (Nakamichi et al., 2009). Further, under long day conditions, PHYB, PIF4 and PIF7 repress *CBF* expression, whereas under short day conditions, there is a de-repression of *CBF* transcription (Lee and Thomashow, 2012), providing a mechanism by which cold acclimation and subsequent freezing tolerance coincides with winter. This circadian regulation of the cold induction of genes provides plants with seasonal information, whereby cold temperatures during the day are distinctive from those during the night, enabling the appropriate seasonal responses.

Zhao et al. (2016) identified that *SIG5* is not regulated by CBFs. Further, other members of the Dodd lab have identified that SIG5 does not regulate the CBF cold response pathway. There also appears to be an independence of the low-temperature regulation of HY5 from CBFs (Catalá et al., 2011). Hence, CBFs have not been addressed further in this thesis.

Metabolism

Photosynthesis is a metabolic process that is regulated by the circadian clock, with both nuclear and chloroplast genes associated with photosynthesis having circadian regulation (Hennessey and Field, 1991). As described previously, SIG5 – a nuclear-encoded protein comprising a subunit of PEP – is involved in coordinating the circadian regulation of these two genomes. Following import of SIG5 into the chloroplast, it generates circadian rhythms in chloroplast genes with photosynthetic functions (Noordally et al., 2013). The accumulation of starch occurs as a consequence of photosynthetic carbon fixation throughout the day. These starch reserves are subsequently broken down during the night to aid in plant growth and metabolic processes. There is tight control over the rate of starch degradation during the night, such that 95% of starch reserves are utilised by the time of anticipated dawn (Graf et al., 2010). Thus, the rate of nocturnal starch degradation is also under circadian control. This is beneficial to plants as it maintains the availability of carbohydrate until the next anticipated dawn, thereby ensuring continued growth and productivity throughout the night (Graf et al., 2010).

Clock-controlled metabolic outputs can also act as inputs to the clock, entraining the oscillator to the environment. An example includes the rhythmic accumulation of sucrose from photosynthesis. Sucrose produced via photosynthesis can entrain the clock by defining a ‘metabolic dawn’, thereby regulating the expression of clock components (Haydon et al.,

2013). As *prr7* mutants appear insensitive to the clock-resetting effects of sucrose, particular roles for PRR7 have been assigned to this response (Haydon et al., 2013). Additionally, roles for numerous PIFs have been suggested in mediating sucrose signalling to the circadian oscillator (Shor et al., 2017). Metabolic processes are therefore both controlled by the circadian oscillator and the outputs of these processes can feedback into the clock to maintain robust circadian rhythms.

The integration of circadian and environmental signals

The adjustment of the phase of the circadian oscillator in plants to match that of the environment enables the generation of an accurate estimate of the time of day by the circadian oscillator. This process of phase adjustment is termed entrainment. Various environmental signals, comprising temperature, light quantity/quality and metabolic products, are zeitgebers that entrain the phase of the Arabidopsis circadian clock to the phase of the environment (Somers et al., 1998, Millar, 2004, Haydon et al., 2013).

Light

Light is one of the strongest and most consistent of the entrainment cues for the plant circadian oscillator, and influences the oscillator in multiple ways (Somers et al., 1998). Several circadian clock components are involved in the modulation of these light input pathways. Plant chlorophyll and carotenoids absorb light between the wavelengths of 550 and 700 nm and below 480 nm, thus green (480 to 550 nm) and far red (FR) (>700 nm) light are reflected. A low red to far red (R:FR) ratio indicates to plants both actual shade and the threat of shade, triggering a shade avoidance response. This developmental response enables the plant to escape shading by competitors (Franklin, 2008).

Families of photoreceptors perceive and monitor light throughout the day, and fluctuations in light can be both predictable (i.e., day-night cycles) and unpredictable (e.g., weather conditions/shading by competitors). Cryptochromes, phototropins and LOV-domain photoreceptors such as ZTL absorb blue light, phytochromes are sensitive to R/FR light and UVR8 detects UV-B (Somers et al., 1998, Kim et al., 2007). Exposure of plants to the full spectrum of white light under normal conditions means that information about different

wavelengths of light from each of these photoreceptors will combine to entrain the circadian oscillator.

Phytochromes are activated by R light, which converts the inactive Pr form to the active Pfr form. FR light converts phytochromes back from the Pfr to the Pr form, thereby inactivating them. There are five phytochromes in Arabidopsis: phytochromes A-E (PhyA-PhyE). PhyA detects low-intensity R light and is responsible for signalling to the circadian oscillator and for mediating developmental responses under continuous FR light (Wenden et al., 2011). PhyB, phyD and phyE perceive high intensity R light for clock control (Quail et al., 1995, Somers et al., 1998, McWatters and Devlin, 2011). In the *phyA* mutant, the free-running period of the clock is lengthened relative to the wild type (WT) under low intensities of R light (<1.0 µmol m^{-2} s^{-1}); this period lengthening occurs at high fluence rates (>5.0 µmol m^{-2} s^{-1}) in the *phyB* mutant (Somers et al., 1998). In wild type Arabidopsis plants, the period length of the endogenous circadian oscillator decreases with higher fluence rates (Aschoff, 1960). Research suggests that this response is dependent upon the phytochromes, as phyB overexpression leads to shortened period lengths relative to the wild type in a fluence rate-dependent manner (Somers et al., 1998). Furthermore, the period lengths of *phyABCDE* quintuple mutants are less responsive to increasing fluence rates of R light than wild type Arabidopsis, displaying longer and shorter periods than wild type at low and high fluence rates, respectively (Hu et al., 2013). Thus, it has been suggested that the Pfr form slows the pace of the clock, whereas the Pr form accelerates the pace (Hu et al., 2013). PhyB additionally interacts with core components of the circadian oscillator, displaying differential binding to these clock components under R and FR light (Yeom et al., 2014). This may be a mechanism by which information about light quality is transferred to the clock, enabling the adjustment of the circadian period depending on the ratio of R:FR light (Yeom et al., 2014).

Cryptochrome 2 (Cry2) absorbs low-fluence blue light, whereas Cry1 responds to light at higher fluences, transmitting this information to the clock (Somers et al., 1998). In cryptochrome mutants, the period of circadian oscillations lengthens under continuous blue light conditions (McWatters and Devlin, 2011). In the *cry1* mutant, this lengthening occurs at both high and low fluence rates of blue light and in the *cry2* mutant lengthening occurs in dim blue light. In the *cry1 cry2* double mutant, the period lengthens severely under a wide

fluence range, indicating redundancy between Cry1 and Cry2 in blue light signalling to the clock (Devlin and Kay, 2000).

The direct physical interaction between GI and ZTL is necessary to stabilise the rhythmic expression of ZTL. This interaction occurs at the LOV domain of ZTL and is enhanced in blue light, thus ZTL mediates blue light signalling to the clock (Kim et al., 2007). ZTL overexpression leads to oscillator arrhythmicity, indicating important and comprehensive roles for the protein in circadian oscillator function and in mediating light input to the clock (Somers et al., 2004).

PHYTOCHROME INTERACTING FACTORS (PIFs) are basic helix-loop-helix (bHLH) transcription factors (TFs) that accumulate throughout the night and are considered key integrators of circadian and environmental signals (Martín et al., 2018). In Arabidopsis, PIFs are involved in inducing the expression of genes related to hypocotyl growth. Physical interaction of PIFs with activated phyB at dawn leads to PIF degradation, thereby enhancing rhythmic hypocotyl elongation under short day conditions (Nozue et al., 2007, Niwa et al., 2009). Recent studies have additionally identified that the PRRs coordinate with the EC to both directly repress *PIF4* and *PIF5* transcription and to regulate PIF activity post-translationally, thereby fine-tuning circadian-regulated photoperiodic growth and contributing to both clock input and output pathways (Soy et al., 2016, Zhu et al., 2016, Martín et al., 2018, Li et al., 2020). The circadian regulation of *PIF4* and *PIF5* has also been suggested to play a central role in the circadian gating of rapid shade avoidance (Franklin, 2008). Their transcript abundance increases towards the end of the dark period, peaking in the middle of the day, and mutants in these genes display reductions in shade avoidance phenotypes under low R:FR (Franklin, 2008). Furthermore, dependent upon *ELF3*, light input to the circadian oscillator is gated around subjective dusk, thereby reducing the sensitivity of the clock to light (Covington et al., 2001).

Sensitivity to UV-B stress is also mediated by both light and the circadian clock, with increased sensitivity to UV-B occurring during the dark period compared with to during the light period (Takeuchi et al., 2014). The rhythm of UV-B-induced gene expression is disturbed in clock mutants (the *prr579* triple mutant, a *PRR7* over-expressor (*PRR7*ox), the *cca1lhy* double mutant and *CCA1*ox), indicating a role for circadian gating in this process (Takeuchi et al., 2014).

Temperature

The circadian period is stable when presented with fluctuating physiologically relevant temperatures – a characteristic termed temperature compensation. In Arabidopsis, the range in which temperature compensation occurs is between 12℃ and 27℃ (Gould et al., 2006). Roles for PRR7, PRR9 and FLC, as well as interactions between CCA1, LHY and GI, have been identified in temperature compensation (Edwards et al., 2005, Salomé and McClung, 2005, Edwards et al., 2006, Gould et al., 2006). Temperature compensation acts as a buffer, ensuring that small fluctuations in temperature do not act as entrainment signals to the circadian oscillator. Under increasing temperatures (17℃ to 27℃), *TOC1* and *GI* are responsible for buffering temperature changes, whereas with decreasing temperatures (17℃ to 12℃), *CCA1* compensates (Gould et al., 2006). The phenomenon of temperature compensation may also be dependent upon blue light and cryptochromes, with light and temperature paths converging at common targets within the central oscillator system (Gould et al., 2013).

Temperature signals present a significant entrainment cue for the circadian clock, enabling plants to adjust their growth and development accordingly. Thermocycles with a 24 hour day-night temperature difference of as little as 4℃ can regulate clock function (McClung and Davis, 2010). Roles for PRR7 and PRR9 have been suggested in temperature entrainment of the clock, acting with partial redundancy to respond to temperature cues (Salomé and McClung, 2005). The integration of temperature signals appears to occur through the EC of the circadian clock (*ELF3*, *ELF4* and *LUX*), which directly regulates the activity of *PRR7*/*9*, *GI* and *LUX* (Mizuno et al., 2014). The EC is proposed to act as a thermosensor, binding DNA with high affinity at low temperatures, and with low affinity at higher temperatures, thereby acting as a temperature-responsive transcriptional repressor (Nusinow et al., 2011, Silva et al., 2020, Jung et al., 2020). This thermal responsiveness requires the presence of a prion domain in ELF3, whereby the length of a polyglutamine repeat embedded within the prion domain determines the magnitude of response (Jung et al., 2020). ELF4 additionally modulates this temperature sensitivity (Silva et al., 2020). Furthermore, phyB shows temperature-dependent inactivation in the dark, meaning the rate of inactivation is relative to temperature. Therefore, phytochromes integrate light and

temperature signals and this environmental information is communicated to the circadian oscillator (Jung et al., 2016, Legris et al., 2016).

Low temperatures represent an abiotic stressor to plants, and abiotic stress responses are closely linked with circadian regulation. Cold acclimation in temperate plants is the process by which, upon exposure to low, non-freezing temperatures, transcriptional, biological and physiological changes occur, resulting in freezing tolerance. Circadian gating of the transcriptional responses of many cold-induced genes occurs in Arabidopsis (Fowler et al., 2005). Photosynthesis is affected by low temperatures, thus communication between the nucleus and chloroplasts about low temperature conditions is essential to optimise photosynthetic efficiency. SIG5 is involved in communicating this information to chloroplasts, with circadian gating of *SIG5* and *psbD* BLRP transcript accumulation occurring in response to chilling (Cano-Ramirez et al., under revision).

The alternative splicing of many clock genes is also regulated by temperature. The production of CCA1β (the truncated form of CCA1) is suppressed in low temperatures, and CCA1a is necessary for freezing tolerance (Seo et al., 2012). This is evident as overexpression of CCA1a in plants results in an enhancement of freezing tolerance, yet overexpressing CCA1β results in freezing hypersensitivity (Seo et al., 2012).

Through the integration of various environmental signals with circadian timing information, plants are therefore able to fine-tune and adapt their responses to converging stimuli.

The chloroplast and chloroplast gene expression

Chloroplasts are organelles that are the site of photosynthesis within plant cells, including Arabidopsis. The endosymbiotic theory suggests that chloroplasts emerged from an endosymbiosis event between a photosynthetic cyanobacteria and a eukaryotic cell. This idea was first proposed by Schimper in 1883, who noted the division of chloroplasts by binary fission, independent of the host cell.

Chloroplasts possess a small (120 to 150 kb) circular genome (the plastome), which is distinct from the nuclear genome and is thought to originate from the prokaryotic ancestor of chloroplasts (Sato et al., 1999). Through endosymbiotic gene transfer, much of the chloroplast genome has been transferred to the nucleus (The Arabidopsis Genome Initiative,

2000), yet the genes remaining in the plastome encode essential components of the photosynthetic apparatus, as well as proteins with transcription-translation functions (Link, 1996). Hence, communication between the nucleus and chloroplast is essential to ensure the coordination of gene expression. Signalling pathways exist to coordinate gene expression between these two genomes at the transcriptional and post-translational levels, communicating information about, for example, changes in light conditions or circadian timing information. Transcription from the chloroplast genome is thus regulated by both environmental signals and the circadian clock (Noordally et al., 2013). Anterograde signalling involves communication from nucleus to chloroplasts; additionally, retrograde signalling enables chloroplasts to regulate nuclear expression of genes encoding photosynthesis-related proteins (Allen et al., 2003).

Plant sigma factors are nuclear-encoded regulators of transcription from the chloroplast genome, first identified in a species of red algae (Tanaka et al., 1996). They are involved in a suite of processes that signal information from the nucleus to chloroplasts during chloroplast biogenesis and in mature chloroplasts. Nuclear-encoded plastid RNA polymerase (NEP) and plastid encoded plastid RNA polymerase (PEP) are the two types of RNA polymerases that transcribe plastid-encoded genes. Both NEP and PEP are required for the development of photosynthetically active chloroplasts.

NEP is a T7/T3 bacteriophage-type single-subunit RNA polymerase encoded by the nuclear genome (Lerbs-Mache, 1993, Hedtke et al., 2000). NEP transcribes various housekeeping genes on the chloroplast genome, including the *rpoB* operon and *accD*, binding to promoters specific to the enzyme, which are generally characterised by a conserved YRT motif (Weihe, 2004). These phage-type RNA polymerases are encoded by the *RpoT* family of nuclear genes. There are three of these genes in dicots (two in monocots), with two of these being NEPs involved in plastid gene transcription (Weihe, 2004).

PEP, conversely, is a multi-subunit eubacteria-type RNA polymerase, whose core proteins are chloroplast-encoded (Tanaka et al., 1996, Allison, 2000). PEP is primarily responsible for transcribing genes involved in photosynthesis (Allison, 2000). In eubacteria, different sigma subunits associate with RNA polymerase to alter its promoter recognition specificity, regulating the transcription of genes in response to environmental and cellular conditions (Ishihama, 2000). Plastid sigma factors function in a similar way. PEP promoter specificity is

conferred when the core PEP proteins associate with a single nuclear-encoded sigma factor, of which there are six (SIGMA FACTOR 1 (SIG1)-SIG6) in the nuclear genome of Arabidopsis (Nagashima et al., 2004, Allison, 2000). Each sigma factor is assigned specific roles, for example SIG2 and SIG6 are involved in the development of chloroplasts (Hanaoka et al., 2003). SIG5 has specific responsibilities in responding to blue light and environmental stresses, thereby enhancing the repair mechanism of the PSII reaction centre following exposure to stressful conditions (Nagashima et al., 2004). As SIG5 is the only stress-inducible sigma factor, and is involved in both light and stress signalling to chloroplasts, I chose to study this sigma factor to further understand the integration of different signalling pathways in the regulation of chloroplast transcription.

SIG5, after associating with PEP, regulates the accumulation of transcripts from the *psbDC* operon on the chloroplast genome. These transcripts encode key components of the photosynthetic machinery, specifically the D2 and CP43 proteins of photosystem II (PSII) (Noordally et al., 2013). *psbD* BLRP (blue light-responsive promoter) is a transcription start site upstream of the *psbD* gene body, and transcription from *psbD* BLRP is regulated by SIG5. Transcription of this gene is induced by both the perception of blue light and a range of environmental stresses. High light, low temperature, high salt and low osmotic conditions all induce *SIG5* and subsequent *psbD* BLRP expression (Nagashima et al., 2004). In both high light and low temperature conditions, photosynthetic efficiency is compromised in *sig5* mutants, highlighting the role of SIG5 in responses to environmental stresses (Nagashima et al., 2004, Cano-Ramirez et al., under revision).

SIG5 is thought to integrate circadian and environmental signals to regulate chloroplast transcription, participating in chloroplast responses to light (Belbin et al., 2017). In response to exposure to UV-B and blue light, the transcript abundance of both *SIG5* and *psbD* BLRP increases. This induction by blue light occurs more strongly during the subjective day than the subjective night, indicating a role for circadian gating in SIG5-mediated blue light regulation of chloroplast transcription (Noordally et al., 2013). *SIG5* transcript abundance peaks around subjective dawn, and *psbD* BLRP abundance peaks shortly after this. Rhythms of transcription are maintained under constant conditions. Circadian rhythms in the transcription of *psbD*, along with additional chloroplast genes encoding components of the

PSI and PSII reaction centres, has been identified to be regulated by SIG5 (Noordally et al., 2013).

SIG5 promoter-interacting transcription factors

A key aim of this work is to understand the pathways involved in signalling circadian and environmental information to chloroplasts to regulate their transcription, with specific focus on SIG5-mediated chloroplast transcription. Here, I introduce current knowledge of transcription factors that regulate *SIG5* transcript accumulation.

HY5/HYH

Two transcription factors that are thought to regulate *SIG5* transcript accumulation are HY5 and HYH. ELONGATED HYPOCOTYL 5 (HY5) and its homolog HY5 HOMOLOG (HYH) are members of the basic leucine zipper (bZIP) family of transcription factors. These proteins act downstream of photoreceptors to modulate the expression of many light-regulated genes in Arabidopsis (Gangappa and Botto, 2016). HY5 is a positive regulator of de-etiolation and photomorphogenesis, acting antagonistically with the CONSTITUTIVE PHOMORPHOGENIC 1-SUPPRESSOR OF PHYA (COP1-SPA) E3 ubiquitin ligase complex in light-mediated development of seedlings (Koornneef et al., 1980, Oyama et al., 1997, Yongqiang Zhang, 2011, Chattopadhyay et al., 1998). This complex directly regulates HY5 and HYH at the protein level, targeting transcription factors involved in activating photomorphogenesis for proteasome-mediated degradation in the dark (Lau and Deng, 2012). This degradation leads to an etiolated growth phenotype and prevents photomorphogenic responses from occurring in the absence of light (Hardtke et al., 2000). Etiolated growth is characterised by rapid hypocotyl elongation with the apical hook and cotyledons tightly closed. Following light illumination, photoexcited photoreceptors (PHYs, CRYs, and UVR8) disrupt the activity of the COP1-SPA complex, enabling the switch to de-etiolated growth and the continuation of photomorphogenesis (Xu, 2019). De-etiolated growth involves the deceleration of hypocotyl elongation, the opening of the cotyledons and chlorophyll accumulation.

Studies have identified HY5 as a master regulator of transcription, with suggestions that the transcription factor can act as a transcriptional activator, repressor or both (Kindgren et al., 2012, Gangappa and Botto, 2016). HY5 controls a network of thousands of genes, with one-

third of the Arabidopsis genome under direct or indirect HY5 control (Lee et al., 2007). Through recent detailed phenotypic and molecular analysis of HY5 activity, Burko et al. (2020) identified that HY5 primarily acts to activate transcription, relying on other factors for this function, as HY5 lacks its own transcription activation domain. Additionally, HY5 is able to promote its own transcription, thereby presenting an example of a positive feedback mechanism (Binkert et al., 2014).

HY5 binds to the promoters of light-inducible genes, binding specifically to G-box, Z-box, C-box, T/G-box, E-box, ACE-box, GATA-box, GC-hybrid and CA-hybrid promoter regions (Chattopadhyay et al., 1998, Zhang et al., 2011, Yadav et al., 2002, Song et al., 2008). By analysis of *hy5* mutant and *HY5* over-expressor lines, roles for the transcription factor beyond the light regulation of development have been identified, including the regulation of chlorophyll biosynthesis, primary and lateral root development, shade responses, flowering time, and many other processes (Oyama et al., 1997, Ang et al., 1998, Nozue et al., 2015). HY5 and HYH additionally interact with other light- and growth-regulated transcription factors, including one another (Holm et al., 2002, Singh et al., 2012, Gangappa et al., 2013, Gangappa and Botto, 2016). The specific function of HYH is often shrouded by studies aiming to elucidate HY5 function, although there seems to be some degree of functional redundancy between the two transcription factors (Brown and Jenkins, 2008, Cano-Ramirez et al., under revision).

A group of proteins named BBXs (B-BOX CONTAINING PROTEINs) have been implicated in promoting or repressing photomorphogenesis by their interaction with HY5. BBXs which promote photomorphogenesis (namely BBX21, BBX22 and BBX23) enhance the transcriptional activity of HY5 (Job et al., 2018, Datta et al., 2008, Xu et al., 2016), whereas BBXs which repress photomorphogenesis (BBX24, BBX25 and BBX28) repress the action of HY5 through direct physical interaction (Xu, 2019, Gangappa et al., 2013, Job et al., 2018, Lin et al., 2018). This suggests that BBXs act as co-regulators of HY5, presenting an example of the interaction of HY5 with other TFs to regulate gene transcription. Further, numerous BBXs have been shown to physically interact with HYH, identifying additional roles for these proteins as HYH co-regulators (Datta et al., 2008, Gangappa et al., 2013).

Following irradiation by UV-B light, *HY5* transcripts accumulate in the nucleus (Oravecz et al., 2006). HY5 and HYH mediate UV-B-induced gene expression via the photoreceptor

UVR8, doing so with partial or complete redundancy, depending on the target gene (Brown and Jenkins, 2008). Microarray analyses suggest the regulation of *SIG5* by UVR8, indicating that HY5/HYH act upstream of the *SIG5* promoter to regulate its transcription in response to UV-B, as *hy5 hyh* double (but not single) mutants display greatly reduced *SIG5* expression. These data thus show complete redundancy of HY5 and HYH in this UVR8-dependent UV-B signalling pathway (Brown and Jenkins, 2008). Additionally, under red and blue light conditions, *SIG5* transcript abundance is greatly reduced in *HY5*-deficient lines, identifying *SIG5* signal transduction as photoreceptor-regulated in a HY5- and COP1-dependent manner (Mellenthin et al., 2014).

A recent study has since identified the *SIG5* promoter as a high-confidence binding target of HY5 by ChIP-seq analysis (Burko et al., 2020). The study used plants expressing HY5-fusion proteins HY5-SDRX and HY5-VP16, which either repress or activate the transcription of HY5 target genes, respectively, and compared these expression patterns to those of *HY5ox* and *hy5*. The seedlings in these experiments were grown dark-adapted for 3 days, meaning the experimental conditions differed from those described in this thesis, and these results were obtained in a different context (Burko et al., 2020).

A similar redundancy between HY5 and HYH occurs in the upregulation of *SIG5* transcripts in response to cold temperatures. Photosynthetic efficiency is enhanced by SIG5 in cold conditions (Cano-Ramirez et al., under revision). HY5 is degraded by COP1 in darkness at ambient temperatures. However, at low temperatures, nuclear depletion of COP1 in the dark occurs, thus HY5 accumulates under low temperature conditions (Catalá et al., 2011). In the *hy5 hyh* double mutant, transcripts of both *SIG5* and its target *psbD* BLRP showed no response to chilling, whereas single *hy5* and *hyh* mutants showed responses no different from wild type. These data indicate that the optimisation of photosynthetic efficiency by SIG5 in cold conditions is mediated by HY5 and HYH in a redundant manner. They also indicate that HY5/HYH may play a role in signal integration at the promoter of *SIG5* (Cano-Ramirez et al., under revision).

ATHB17

ARABIDOPSIS THALIANA HOMEOBOX 17 (ATHB17) is a homeodomain leucine-zipper (HD-ZIP) class II transcription factor, which localises to both the cytoplasm and nucleus (Hymus

et al., 2013). HD-ZIP proteins contain a homeodomain with a leucine zipper which functions as a dimerization motif (Ariel et al., 2007). ATHB17 acts to positively regulate abiotic stress tolerance, and it is suggested that this occurs partly by the positive modulation of *SIG5* transcription (Zhao et al., 2017). Zhao et al. (2017) propose that the pathways controlling responsiveness to salt stress of *SIG5* in *Arabidopsis* is distinct from the SIG5 light-responsiveness pathway. This is because, in response to salt stress, ATHB17 is suggested to be an upstream regulator of *SIG5*, directly binding the HD binding *cis*-elements in the *SIG5* promoter to activate its expression (Zhao et al., 2017). Through regulation of *SIG5*, ATHB17 may positively modulate and coordinate plastid encoded gene (PEG) expression, which are responsive to environmental signals (Zhao et al., 2017). Light induction of SIG5, however, is strongly dependent on blue light and cryptochromes, and requires involvement of HY5 (Mellenthin et al., 2014), suggesting independence of these pathways.

Project aims and objectives

Given the extensive roles identified for HY5 and HYH in regulating gene transcription in response to light and other environmental stimuli, it seems reasonable to hypothesise that these transcription factors may be involved in integrating environmental and circadian signals to regulate chloroplast gene transcription. SIG5 is involved in coordinating nuclear and chloroplast gene expression in response to external and internal cues. The existing literature identifies the *SIG5* gene promoter as a high-confidence HY5 binding target (Burko et al., 2020), thus signal integration by HY5 (and potentially HYH) to regulate chloroplast transcription might occur at this gene promoter. The homology and partial functional redundancy between HY5 and HYH also indicate that these two transcription factors could be acting with partial redundancy to control *SIG5* transcription (Cano-Ramirez et al., under revision). Additionally, the identification of ATHB17 as a positive regulator of abiotic stress tolerance suggests that it may achieve this through direct *SIG5* transcriptional modulation (Zhao et al., 2017). Hence, the work outlined in this thesis (summarised in Figure 1.2) aims to:

1. Identify the roles for HY5, HYH and ATHB17 in the integration of circadian and environmental signals at the promoter of *SIG5*.

2. Understand how these transcription factors contribute to the circadian and environmental regulation of chloroplast transcription.

Techniques used to achieve these aims include qRT-PCR, to monitor *SIG5* and *psbD* BLRP transcript accumulation under circadian and diel conditions, and bioluminescence timecourse imaging, to understand the roles of different transcription factors in the modulation of *SIG5* promoter activity.

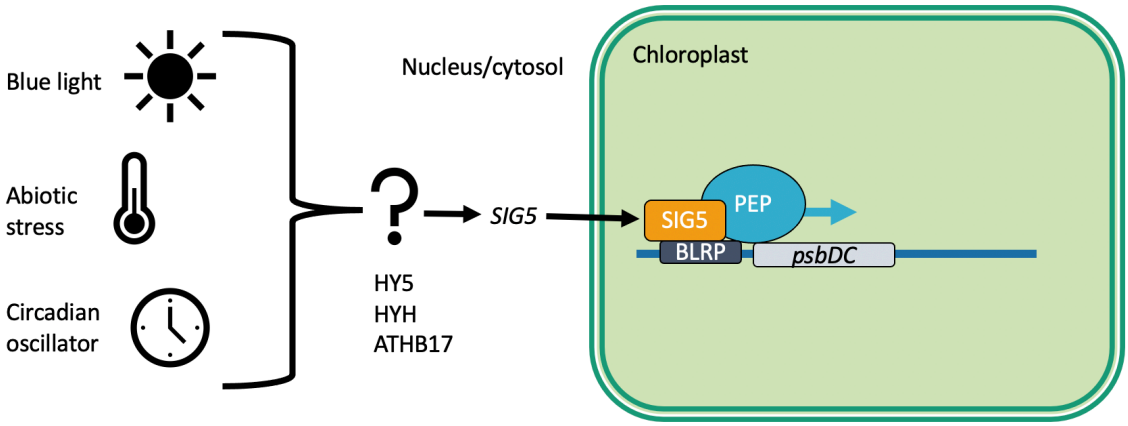

Figure 1.2 Simplified illustration of the proposed pathway through which environmental and circadian signals might be integrated at the promoter of SIG5 to regulate transcription of the *psbDC* operon following the association of SIG5 with PEP. HY5, HYH and ATHB17 are the transcription factors hypothesised to be involved in this signal convergence.

Chapter 2 Materials and Methods

Plant Materials & Growth Conditions

Arabidopsis seed stocks

Wild type *Arabidopsis thaliana* seeds (Wassilewskija (Ws) ecotype), as well as *hyh* (Holm et al., 2001), *hy5-KS50* (*hy5*) (Oyama et al., 1997), *hy5-KS50/hyh* (*hy5 hyh*) (Holm et al., 2001) and *cop1-4* (McNellis et al., 1994) lines (Ws background) were gifted to the Dodd lab by Kerry Franklin (University of Bristol). *athb17* mutant alleles were obtained from Nottingham Arabidopsis Stock Centre (NASC) (homozygous stocks N654410/SALK_134535C and N657998/SALK_095524C (Alonso et al., 2003), Col-0 background). Seeds of the Col-0 background were also used, as well as the *sig5-3* mutant (Col-0 background) (Noordally et al., 2013).

Seed Sterilisation & plating

The method of seed surface sterilisation was adapted from Belbin et al. (2017). 70% (v/v) ethanol was added to Arabidopsis seed for 1 minute, followed by 20% (v/v) sodium hypochlorite for 8-10 minutes, before being washed with sterile dI H_2O twice. Seeds were then resuspended in 0.1% (w/v) agar.

For bioluminescence imaging, the growth medium used was comprised of Murashige and Skoog (MS) (4.41g l^{-1}) nutrient mix (basal salts with vitamins, pH 5.8) in 0.8% (w/v) agar, with 1% (v/v) sucrose supplementation. Sucrose was added to enhance seedling growth in non-circadian experiments. Seeds for use in qRT-PCR were pipetted onto MS (4.41g l^{-1}) nutrient mix (basal salts with vitamins, pH 5.8) in 0.8% (w/v) agar, without sucrose supplementation (MS-S). Seedlings transplanted from soil and adult leaves used as controls in the primary round of the particle bombardment method development were also originally pipetted onto MS-S media. Ws seeds used for the second round of the particle bombardment method optimisation were pipetted onto MS (4.41g l^{-1}) nutrient mix (basal salts with vitamins, pH 5.8) in either 0.5% or 0.6% (w/v) agar, supplemented with 1% sucrose and 0.2% activated charcoal. *athb17* seeds grown for bulking were pipetted onto MS (4.41g l^{-1}) nutrient mix (basal salts with vitamins, pH 5.8) in 0.8% (w/v) agar,

supplemented with 1% (v/v) sucrose. All plates were subsequently sealed with micropore tape and placed in dark conditions at 4℃ for 2 or 3 days to stratify.

Plant growth and entrainment conditions

All plants were cultivated under sterile conditions using Sanyo MLR-352 growth chambers with LED-based lighting tubes. For initial particle bombardment method optimisation and qRT-PCR experiments, seedlings were entrained under 12 hr light/12 hr dark cycles in 80 µmol m^{-2} s^{-1} white light at 19℃ for 11 days, thus experiments started at a seedling age of 11 days. For subsequent particle bombardment and bioluminescence timecourse imaging, seedlings were entrained under these conditions for 2 weeks before being transformed in order to increase the cotyledon surface area for bombardment. For circadian and diel timecourse experiments, seedlings were entrained under either 12 hr light/12 hr dark (forward/phase) conditions or 12 hr dark/12 hr light (reverse/anti-phase) conditions. In circadian experiments, after 11 days, all seedlings were subjected to continuous light conditions for 72 hours, with sampling occurring after 24 hours in continuous light to eliminate any effect of the final dawn upon the rhythm detected. In diel cycle experiments, seedlings were maintained under light/dark conditions throughout the sampling period. For chilling experiments using *athb17* mutant seedlings, plants were subjected to continuous light for 24 hours at 11 days old, before being exposed to 4℃ 1 hr after subjective dawn on the 12^{th} day.

For seed bulking, *athb17* mutants were cultivated under 12h light/12h dark cycles in 80 µmol m^{-2} s^{-1} white light at 19℃ for 10 days before transplanting to soil. Twelve Arabidopsis seedlings from each *athb17* mutant allele (SALK_095524C and SALK_134535C) were transplanted to soil (Levington F2 peat and 4 mm grit with Exemptor® Chloronicotinyl insecticide). Each individual plant was covered with an ARACON (Arasystem) base and tube to collect seeds produced by the inflorescence and to isolate each plant, thereby avoiding contamination. After around 10 weeks, when plants began to senesce, watering of the plants ceased to facilitate seed drying and seeds were harvested. Seeds were collected using paper negative bags and left for a further 4 weeks to dry. Seeds were separated from other plant material using a mesh sieve and stored in 1.5 ml Eppendorf tubes at 4℃.

RNA extraction

Aerial tissue was harvested from 10 seedlings per genotype under sterile conditions for RNA extraction. RNaseZAP (Invitrogen) was used to clean surfaces and equipment. Five seedlings across two Petri dishes were sampled. Three independent biological repeats were performed for each timepoint. Samples were flash-frozen with liquid nitrogen before being stored at -80℃. Frozen samples were homogenised with 5 mm stainless steel beads (QIAGEN) and a TissueLyzer II before total RNA was extracted using the Macherey-Nagel Nucleospin RNA kit, according to the manufacturer's instructions and as described previously (Noordally et al., 2013). The quality and concentration of the extracted RNA was determined through use of a Nanodrop spectrophotometer (Thermo Scientific). Samples with 260/280 and 260/230 ratios above 2.0 were considered sufficiently pure for use in qRT-PCR reactions.

cDNA biosynthesis

cDNA was synthesised from the extracted RNA using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit, as described previously (Belbin et al., 2017). Two control samples – no reverse transcriptase (NRT) and no transcript control (NTC) – were also produced, controlling for reagent contamination and DNA contamination, respectively. Each reaction consisted of 2 μg template RNA in a total volume of 10 μL (the remaining volume consisting of RNase-free water). In the case of low RNA yields, 1 μg of template RNA was added to the cDNA biosynthesis reaction. 10 μL of a master mix, consisting of RT buffer, dNTP mix at 10 mM, RT random primers, nuclease-free water and Multiscribe™ reverse transcriptase was aliquoted into each reaction tube (except for NRT). The tubes were transferred to a thermal cycler, which ran the following program for cDNA synthesis: 10 min at 25℃, 120 min at 37℃, 5 min at 85℃, 4℃ indefinitely. cDNA was then stored at -20℃ before analysis by qRT-PCR.

DNA extraction

DNA was extracted for use in PCR reactions as described in Edwards et al. (1991). DNA quality was determined using a nanodrop.

Polymerase Chain Reaction (PCR)

PCR reactions using DreamTaq polymerase (Thermofisher) were used to genotype *athb17* T-DNA mutant lines (SALK_095524 and SALK_134535) obtained from NASC, thereby confirming the presence of the T-DNA inserts. Primer pairs consisted of left genomic primers (LP) and right genomic primers (RP) for each line, LP and left border (LB) primers, and RP and LB primers. LP and RP primers for ACT2 were used as negative controls. Each reaction consisted of 1 µL template DNA, 0.2 µL DreamTaq polymerase, 2.5 µL DreamTaq buffer, 0.5 µL dNTPs (10mM), 0.5 µL LP/LB primer (10 µM), 0.5 µL RP/LB primer (10 µM) and 19.8 µL nuclease-free water, for a total volume of 25 µL per reaction. The PCR cycling conditions consisted of 3 min at 95℃ (initial activation), followed by 30 cycles of 10 sec at 95℃ (denaturation), 30 sec at 60℃ (annealing) and 1 min at 72℃ (extension).

Gel electrophoresis

PCR products were separated on 1% (w/v) agarose gels at 90 V for 30 min in mini-sub cell GT tanks with 1x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA), and GelRed. For fragment size reference, the 2-Log DNA ladder (New England Biolabs) was also run.

qRT-PCR

A cDNA dilution series (1:10, 1:100, 1:200, 1:400) was initially created to determine the most appropriate dilution for qRT-PCR analysis. 1:100 cDNA dilutions were analysed using qPCRBIO SyGreen Blue Mix (PCR Biosystems) with the addition of primer sets for *SIG5*, *psbD* BLRP and *CCA1* at 10 µM (Table 2.1). The qRT-PCR run consisted of 2 min at 95℃ (polymerase activation), followed by 40 cycles of 5 seconds at 95℃ (denaturation) and 20-30 seconds at 60℃ – 65℃ (annealing/extension). Two/three technical repeats, and three biological repeats, were performed per experiment. Transcript abundance was relative to *ACT2* (Table 1), calculated using the $2^{-\Delta\Delta Ct}$ method (the comparative Ct method) in Excel.

Primers

Primer design and validation

Primers for use in genotyping PCR reactions were designed using the SALK T-DNA Express Arabidopsis gene mapping tool.

Primer dissociation curve

The specificity of primers used for qRT-PCR were tested through the addition of a dissociation/melt curve at the end of each qRT-PCR run. These were used to ensure each primer only bound to one specific area of DNA and thus produced a single, specific amplicon. Primers were determined to be acceptable if only a single, clear peak was produced by the dissociation curve analysis.

Primer sequences

Table 2.1 Primer sets used for qRT-PCR reactions, as described previously (Mochizuki et al., 2004, Noordally et al., 2013)

Primer	**Sequence (5' to 3')**
ACT2 Forward	TGAGAGATTCAGATGCCCAGAA
ACT2 Reverse	TGGATTCCAGCAGCTTCCAT
SIG5 Forward	GTGTTGGAGCTAATAACAGCAGACA
SIG5 Reverse	TGTCGAATAACCAGACTCTCTTTCG
psbD BLRP Forward	GGAAATCCGTCGATATCTCT
psbD BLRP Reverse	CTCTCTTTCTCTAGGCAGGAAC
CCA1 Forward	GCACTTTCCGCGAGTTCTTG
CCA1 Reverse	TGACTCCTTTCTTACCCTGTTATTCTG

Table 2.2 Primers used for PCR genotyping reactions – to verify the presence of T-DNA inserts

Primer	**Sequence (5' to 3')**
LBb1.3 (LB)	GCGTGGACCGCTTGCTGCAACT
SALK_095524 LP	CGGGATTAAGGGTATGATTCTG
SALK_095524 RP	TCCATTTCACTGATTGACACG
SALK_134535 LP	TGAACCAACCACCTTTTGAG
SALK_134535 RP	CGGAAAGAGATTAATTAACAGCC
ACT2 LP	CTCCAGCTATGTATGTTGCCATTC
ACT2 RP	CATACTCTGCCTTAGAGATCCAC

DNA Maxi-prep

Plasmid DNA reporter constructs

The SIG5::LUC reporter construct used for particle bombardment and bioluminescence imaging, created from the pGREENII0229 plasmid, was described previously (Noordally et al., 2013). Gold particles coated with pB7WG2.0-GFP were also used, as described previously (Cheval et al., 2019).

Bacterial growth media and conditions

For plasmid DNA purification, *Escherichia coli* containing the SIG5::LUC plasmid were streaked from frozen glycerol stocks onto two LB agar (36g LB Agar L^{-1}) plates before being incubated at 37℃ overnight. The following morning, a single colony was picked from each plate and two starter cultures of 10 mL LB medium containing 50 μg mL^{-1} kanamycin (selective antibiotic) were inoculated in tubes. The tubes were then incubated for 8 hrs at 37℃ with vigorous shaking (250 rpm). After 8 hrs, starter cultures were diluted 1:500 into selective LB medium (containing kanamycin). Cultures were grown at 37℃ for 16 hrs with vigorous shaking (250 rpm).

Plasmid DNA purification

The QIAGEN Plasmid Maxi Kit was used for extraction of SIG5::LUC plasmids from the bacterial culture, according to the manufacturer's instructions (QIAGEN). Yield (DNA concentration) was determined using UV spectrophotometry at 260 nm (Nanodrop spectrophotometer, Thermo Scientific), which is the peak wavelength of nucleic acid absorbance. 600 µL of plasmid was obtained at a concentration of 1.1 µg μL^{-1} (1117.1 ng μL^{-1}).

Particle bombardment

Particle bombardment experiment

SIG5::LUC and pB7WG2.0-GFP reporter constructs were introduced into Arabidopsis cells via particle bombardment. The method was adapted from previously published methods (Sanford et al., 1993, Muranaka et al., 2013). To coat gold particles with the reporter construct, a 25 µL aliquot of 1 nm gold particle suspension (BioRad) was combined with 5 µL of plasmid DNA (1 µg μL^{-1}), 25 µL $CaCl_2$ (2.5 M, Sigma) and 10 µL spermidine (0.1 M, Sigma). After incubation on ice for 30 min, the supernatant was removed and 180 µL 100% ethanol was added to wash the DNA-coated gold particles. The gold particles were then pelleted, resuspended in 100 µL 100% ethanol and stored at -20°C before operation of the gene gun.

The PDS-1000/He particle delivery system (Bio-Rad) was used for microprojectile bombardment of Arabidopsis plants, according to the manufacturer's instructions (vacuum, 26-30 inHg; helium pressure, 1350 psi (rupture disk-determined)). A 5 µL aliquot of the DNA-coated gold preparation was spread onto the downward facing surface of a microcarrier. Arabidopsis plants/plant tissue (seedlings/adult leaves), on 90 mm agar plates, were placed underneath the muzzle of the gun, at the highest position ('floor 2'). DNA-coated gold particles were then fired into the plants/plant tissue. Luciferin (5 mM or 10 mM potassium salt, Melford) was applied to the plants bombarded with the *SIG5::LUC* construct 24 hrs before bioluminescence imaging. Seedlings grown in sterile plastic rings on agar for development of the method were treated with 100 µL luciferin 5mM or 10 mM per well. Luciferin 5 mM was dotted/pooled onto the underside of bombarded adult leaves. Seedlings transplanted onto agar from soil were surrounded with a ring of grease, creating a

compartment for luciferin 5 mM application. For final experimentation, a small spray bottle was used to evenly distribute luciferin 5mM onto the surface of the seedlings.

Fluorescence imaging

As a positive control, the GFP reporter construct pB7WG2.0-GFP was introduced into Arabidopsis plants by particle bombardment. The plants bombarded with the construct were imaged using LeicaM205FA (Leica Microsystems) or ZEISS Axio Zoom.V16 (ZEISS Microscopy) fluorescence stereo microscopes 24 and 96 hrs after bombardment.

Single-cell bioluminescence imaging

A Photek HRPCS intensified CCD photon counting imaging system (Photek) controlled by Image32 imaging software (Photek) was used to image luciferase bioluminescence from plants bombarded with the *SIG5::LUC* construct 24 hrs after luciferin application. Bioluminescence images were taken after at least 2 minutes of dark treatment to allow chlorophyll autofluorescence to decay before bioluminescence measurement. To assess the efficiency of particle bombardment at each stage of the method development, 10-min integrations were conducted, giving single timepoint measurements. For the circadian timecourse experiment, 10-min integrations were conducted every hour for ~3 days in continuous light conditions. Regions of interest (ROIs) and background areas were selected using the Bright Field mode. 3 ROIs of equal area per plate were determined – these were the areas from which the highest bioluminescence signal was detected, thus each may conceivably represent a single seedling with transformed cells (Figure 2.1). Background measurements were taken from areas without plant material present (regions equal in area to the ROIs) to determine the background luminescence signal (Figure 2.1). After trying multiple methods of extracting the data (including both dividing the plates into quarters and extracting data from a single circle within the overall area of the plate) this seemed to be the most reliable method as it somewhat controlled for the inconsistency between plates in their transformation efficiencies (although this could not be completely controlled for). Background photon counts were subsequently deducted from ROI photon counts. Bioluminescence counts were also obtained from the defined ROIs and background regions with no plate present, to control for inconsistencies across the field of view in the background luminescence signal.

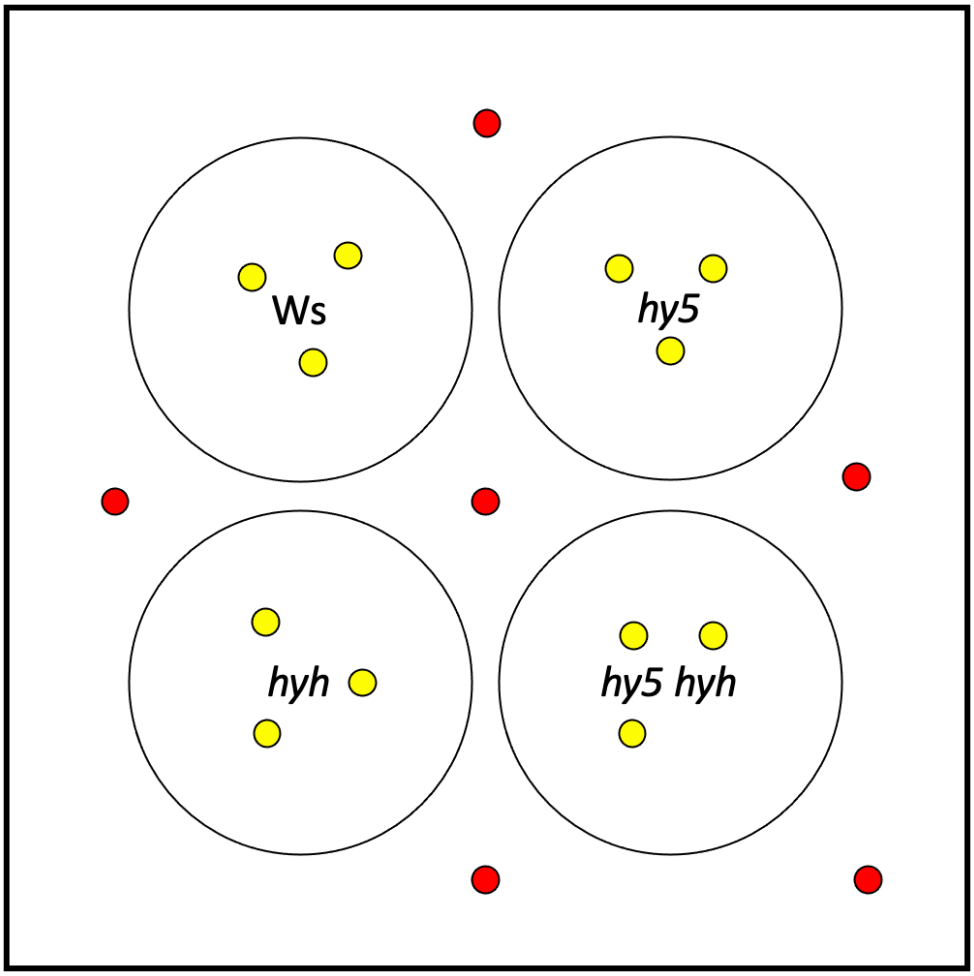

Figure 2.1 Illustration of the layout of the plates of transformed seedlings under the bioluminescence camera. Outer square represents the field of view; larger circles represent the plates of seedlings of the genotypes annotated (Ws, *hy5*, *hyh* and *hy5 h*yh mutants); small yellow circles within larger circles represent areas emitting the greatest bioluminescence signals per plate and thus areas from where bioluminescence counts were obtained; small red circles outside of larger circles represent areas from where background bioluminescence counts were obtained.

Statistical analysis

All statistical analyses were conducted using RStudio version 1.2.5033. Plots were produced using RStudio version 1.2.5033 with ggplot2 and ggpubr packages, with the exception of Figure 3.12 which was produced using Microsoft Excel version 16.48.

Gene expression rhythmicity analysis

BioDare2 (Zielinski et al., 2014) and the R package MetaCycle (Wu et al., 2016) were used as tools to determine the periodicity of each gene over the time series. The function meta2d in MetaCycle was used, which integrates three different algorithms (ARSER, JTK_CYCLE and

Lomb-Scargle) to calculate the p-value, Benjamini–Hochberg q-value (BH.Q value), period, phase, baseline expression value, amplitude (AMP), and relative amplitude (rAMP). The rAMP provides the ratio between the AMP and the baseline expression of a gene, enabling comparison of amplitude values between differently expressed genes. The BH.Q value helps to avoid Type I errors by decreasing the false discovery rate, hence gene expression was determined to be rhythmic if BH.Q < 0.05. In BioDare 2, the Fast Fourier Transform - Non-Linear Least Squares method (FFT-NLLS) was used to analyse the periodicity of the timeseries data.

Promoter motif analysis

The 3 kb sequence of amino acids upstream of *SIG5* and *psbD* were retrieved from the TAIR Araport11 database. The gene nomenclature identifier for *SIG5* is AT5G24120, occurring at the locus chr5: 8157794-8159746; the gene nomenclature identifier for *psbD* is ATCG00270. Analyses of the 3 kb promoter regions were performed using PlantPAN 3.0 to identify known HY5/HYH/ATHB17 transcription factor binding sites.

Chapter 3 The roles of HY5 and HYH in SIG5-mediated circadian signalling to chloroplasts

The circadian regulation of *SIG5* and *psbD* BLRP transcript accumulation

Introduction and aims

A role for SIG5 in the communication of circadian timing information from the nucleus to chloroplasts has been identified (Noordally et al., 2013), but the upstream factors regulating the pathway are not known. The bZIP transcription factors HY5 and HYH are required for the regulation of *SIG5* in response to both UV-B (Brown and Jenkins, 2008) and chilling (Cano-Ramirez et al., under revision), optimising photosynthetic efficiency under these conditions. Here, I tested the hypothesis that these transcription factors contribute to the circadian regulation of *SIG5* transcription. I investigated the contributions of HY5 and HYH to (1) circadian rhythms in the accumulation of transcripts encoding *SIG5* and its chloroplast target *psbD* BLRP and (2) circadian rhythms of *SIG5* promoter activity. The latter aim also involved the development of new methods for transient gene expression assays.

Results

HY5 and HYH are upstream of *SIG5* and *psbD* BLRP in the signalling pathway

HY5 and HYH are known to be involved in signalling to chloroplasts by modulation of *SIG5*, under various different light conditions and in response to cold temperatures (Nagashima et al., 2004, Brown and Jenkins, 2008, Belbin et al., 2017, Catalá et al., 2011, Cano-Ramirez et al., under revision). To confirm that HY5 and HYH are involved in the modulation of *SIG5* transcript accumulation in response to white light, and to inform future, more complex qRT-PCR timecourse analyses, a preliminary experiment was conducted under light-dark (LD) conditions. RNA was extracted from dawn-sampled Arabidopsis seedlings of seven genotypes (wild type (Ws and Col-0), *hy5*, *hyh*, *hy5 hyh* double mutant, *cop1-4* and *sig5*-3 mutants), grown for 11 days, and analysed using qRT-PCR. The qRT-PCR was performed using primers for both *SIG5* and transcripts from its target chloroplast gene *psbD* BLRP (Figure 3.1).

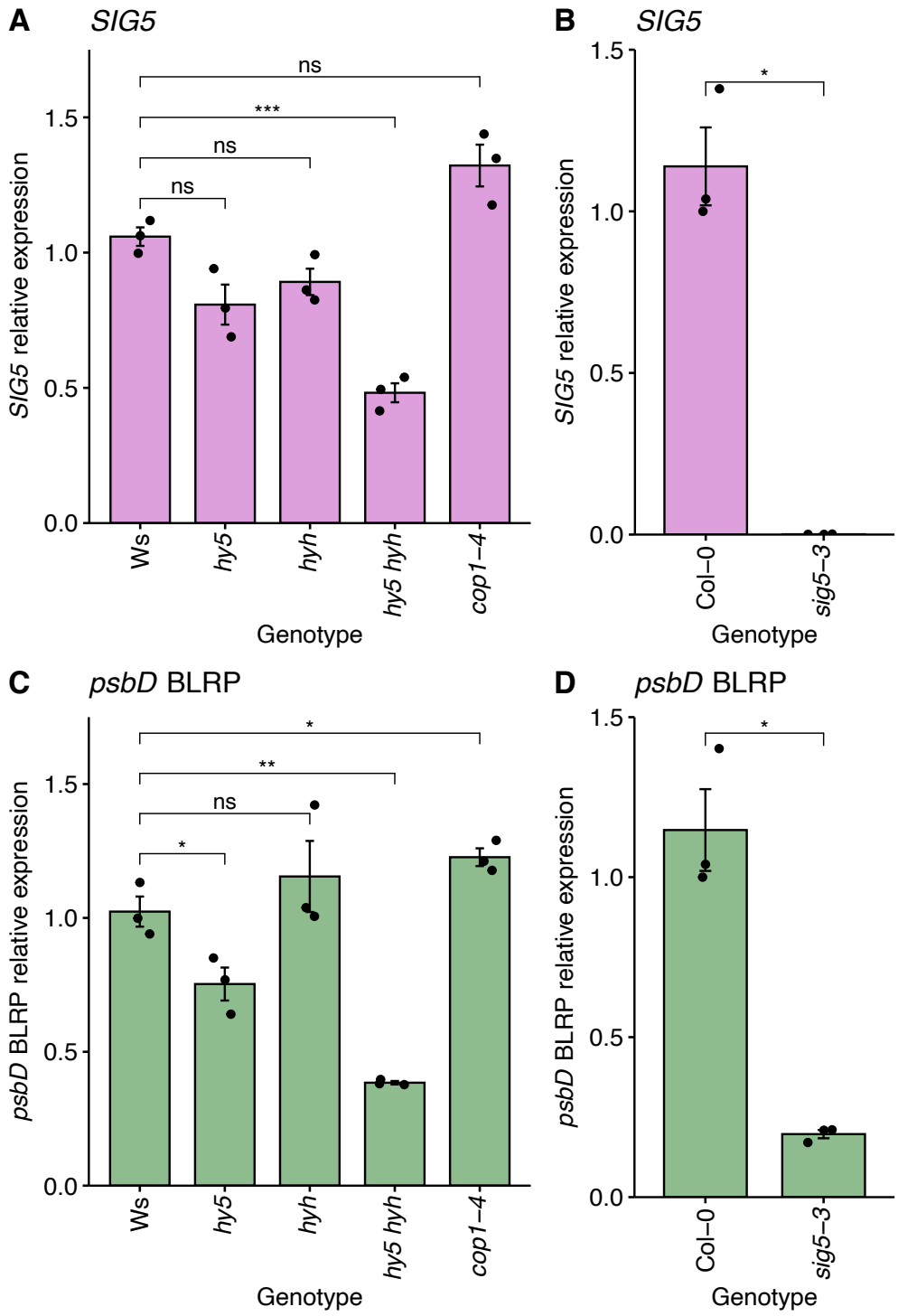

Figure 3.1 Transcript abundance (relative to *ACT2*, determined by qRT-PCR and analysed using the $2^{-\Delta\Delta Ct}$ method) of *SIG5* and its chloroplast gene target *psbD* BLRP in wild type ((A, C) Ws or (B, D) Col-0) and mutant ((A, C) *hy5*, *hyh*, *hy5 hyh*, *cop1-4* and (B, D) *sig5-3*) genotypes. Two/three technical repeats, and three biological repeats, were performed per experiment. Data are the mean of three biological repeats ± SEM (n=3, N≥10 seedlings). Purple = *SIG5* relative transcript abundance; green = *psbD* BLRP relative transcript abundance. Circles on plot represent individual data points (jitter plot). ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ (one-way ANOVA followed by pairwise t-test with Benjamin & Hochberg adjustment in R).

The abundance of *SIG5* transcripts in *hy5*, *hyh*, *hy5 hyh* and *cop1-4* mutants and in the wild type background Ws are shown in Figure 3.1A. Statistically significant differences in *SIG5* transcript abundances were identified between all genotypes using a one-way ANOVA. Pair-wise t-tests with Benjamini & Hochberg adjustment were used to identify the genotypes where *SIG5* transcript abundance differed significantly. In *hy5* and *hyh* single mutants, *SIG5* induction around dawn was not different from the wild type (Figure 3.1A). However, in the *hy5 hyh* double mutant, *SIG5* induction was reduced significantly compared with the wild type (Figure 3.1A). Therefore, HY5 and HYH might act redundantly to regulate the induction of *SIG5* transcripts around dawn. The extent to which HY5 and HYH are involved in light and circadian signalling to chloroplasts is not clear from these data alone, but their role in regulating *SIG5* is confirmed.

Performing the same analyses on the data obtained for *psbD* BLRP transcript abundance involving the same genotypes identified statistically significant differences between Ws and *hy5*, Ws and *hyh*, and Ws and *hy5 hyh* (Figure 3.1C). The most significant reduction in *psbD* BLRP transcript abundance occurs between Ws and *hy5 hyh* ($p < 0.01$). This indicates that, as hypothesised, HY5 and HYH are acting partially redundantly to control the light-induction of both *SIG5* and its subsequent target *psbD* BLRP under 12h light-12h dark conditions, 1 hour after dawn. Nonetheless, transcripts of both *SIG5* and *psbD* BLRP were not absent in either assay in the *hy5 hyh* double mutant, suggesting that at least one other additional factor modulates the expression of *SIG5*.

As expected, *SIG5* transcripts were absent in the *sig5-3* mutant (Figure 3.1B), confirming the mutant genotype. *psbD* BLRP transcript abundance showed a significant reduction in the *sig5-3* mutant compared to the wild type (Col-0) ($p < 0.05$) (Figure 3.1D), but transcripts remained detectable. This indicates that transcripts for this gene may be controlled by a factor additional to SIG5.

Nagashima et al. (2004) used Northern Blotting to observe *SIG5* induction in various mutant genotypes. After 1.5 hours of light exposure, *hy5* mutants showed a partial reduction in *SIG5* transcript abundance compared to wild type; only after 6 hours of light exposure was there a marked reduction in *SIG5* induction. The results of the study concur with the results described here, which report a numerical reduction in *SIG5* transcript abundance in *hy5* mutants sampled 1 hour after dawn. This suggests that conducting transcript analyses in *hy5*

mutants following a longer period of light illumination may reveal more about the relationship between HY5/HYH and *SIG5* expression. Given that these data identify roles for HY5 and HYH in the regulation of *SIG5* and *psbD* BLRP, they suggest that a circadian timecourse experiment using mutants in the same transcription factors would yield interesting and informative results.

HY5 and HYH are involved in the circadian regulation of *SIG5* transcript accumulation

A qRT-PCR timeseries using wild type (Ws), *hy5*, *hyh* and *hy5 hyh* was conducted over a 72-hr period in continuous light. The experimental design for this experiment is shown in Figure 3.2. Following germination, wild type (Ws) and mutant (*hy5*, *hyh*, *hy5 hyh*) seedlings were entrained under 12 hr light/12 hr dark cycles at 19°C for 11 days. Phase plants were in phase with the external LD cycles (i.e. exposed to light during the day and darkness during the night), whereas anti-phase plants were under opposite light conditions (i.e. dark during the day and light during the night), allowing sampling during a 12 h time block. Following entrainment, seedlings were subjected to constant light conditions on the morning of the eleventh day. After 24 h in constant light, plant tissue was harvested 1, 5 and 9 h after subjective dawn/dusk (phase/anti-phase, respectively) for the following two days. Two technical replicates per timepoint per genotype were sampled, and three biological replicates per genotype per timepoint were analysed. Primers for *SIG5* and *psbD* BLRP transcripts were included in the analysis to elucidate the roles of HY5 and HYH in the circadian regulation of *SIG5* and *psbD* BLRP transcript abundance, and hence their role in circadian signalling to chloroplasts.

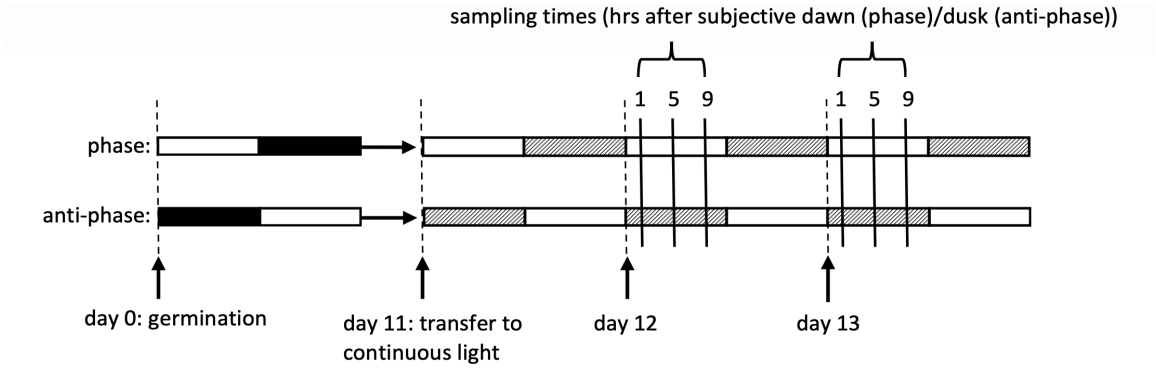

Figure 3.2 Experimental design for circadian timecourse experiment. Plants were entrained under 12-hr light/12-hr dark cycles at 19°C for 11 days before transfer to constant light conditions. Phase plants were in phase with the external LD cycle; anti-phase plants were subjected to dark in the day and light at night. Sampling took place in the 48 hrs following the first 24 hrs in constant light. Sampling of seedlings from each genotype occurred 1, 5 and 9 hrs after subjective dawn for the phase-entrained seedlings, and after subjective dusk for the anti-phase-entrained seedlings. Black boxes = dark conditions; white boxes = light conditions/subjective day; hashed boxes = subjective night.

The relative abundance of *SIG5* transcripts throughout the circadian timeseries is shown in Figure 3.3A. The amplitude of *SIG5* expression is maintained at its highest level in the wild type (Ws). The *hy5* mutant and the *hy5 hyh* double mutant show the most significant decreases in amplitude compared to wild type, with little differences between these throughout the timeseries. The *hyh* mutant displays the smallest decrease in amplitude compared to wild type (Figure 3.3A), suggesting a less significant role for this transcription factor in the signalling pathway than HY5. These results indicate that there may be some partial redundancy occurring between HY5 and HYH in the circadian modulation of *SIG5* transcription. However, HY5 is likely to play a more significant role in this signalling pathway than HYH due to the greater decreases in the amplitude of rhythmic *SIG5* expression in the *hy5* mutants compared with the *hyh* mutants. Furthermore, as the *hy5 hyh* mutant continues to display rhythmic *SIG5* expression in continuous light conditions, the circadian modulation of *SIG5* transcript abundance cannot rely solely on the transcription factors HY5 and HYH, indicating that there must be other factors involved in this process.

BioDare2 was used to process the timeseries data and for period analysis of each of the repeats. The Fast Fourier Transform - Non-Linear Least Squares method (FFT-NLLS) method of period analysis was used. There was no significant difference between the circadian periods of the different groups. All wild type and *hy5 hyh* mutant replicates were rhythmic, whereas only 1 replicate of the 3 *hy5* replicates, and 2 of the 3 *hyh* replicates had rhythmic accumulation of *SIG5* transcripts. It is possible that there was an issue with some of the samples, although NanoDrop spectrophotometer analyses indicated the RNA samples were of sufficient quality and quantity. However, although there is no evidence for this, there is the possibility that there was cross-contamination of RNA samples, due to human error. It's also possible that some root tissue was included in the samples from which RNA was extracted for period analysis. As root expression patterns differ from leaf expression patterns this may have influenced the results here. In order to confirm the results reported

here, this experiment should be repeated, ensuring no root tissue be included in the samples, and ensuring a high quality and concentration of RNA with each sample.

The amplitude analyses of the *hy5* and *hyh* mutants cannot be considered reliable because an amplitude value was not obtained from all repeats (due to some transcripts being identified as arrhythmic through the FFT-NLLS analysis), although visual inspection of the data suggest some rhythmicity of these transcripts. This is why these figures are included in Table 1, but excluded from Figure 3.3B. The amplitude analyses reveal a statistically significant difference between the amplitude of *SIG5* transcript abundance between wild type and the *hy5 hyh* double mutant (Figure 3.3B).

To further validate this analysis, I used the meta2d function in the R package MetaCycle to evaluate the periodicity of the data because this package includes algorithms that are suited to short, relatively noisy datasets such as transcript data. This analysis generated BH.Q values less than 0.05 for all genotypes (wild type, *hy5*, *hyh* and *hy5 hyh*), indicating strong evidence that *SIG5* expression is rhythmic in all genotypes, thus all have been included in Figure 3.3C. The meta2d amplitude analysis generated results similar to those obtained from BioDare 2 for wild type and *hy5 hyh*, confirming the reduction in *SIG5* amplitude in the double mutant.

Taken together, these analyses suggest that HY5 and HYH are involved in inducing *SIG5* transcription, thereby maintaining the amplitude of *SIG5* circadian oscillations. These data cannot reveal much about the relative contributions of HY5 and HYH to the maintenance of *SIG5* amplitude, although the amplitude in these two mutants is numerically lower than the wild type.

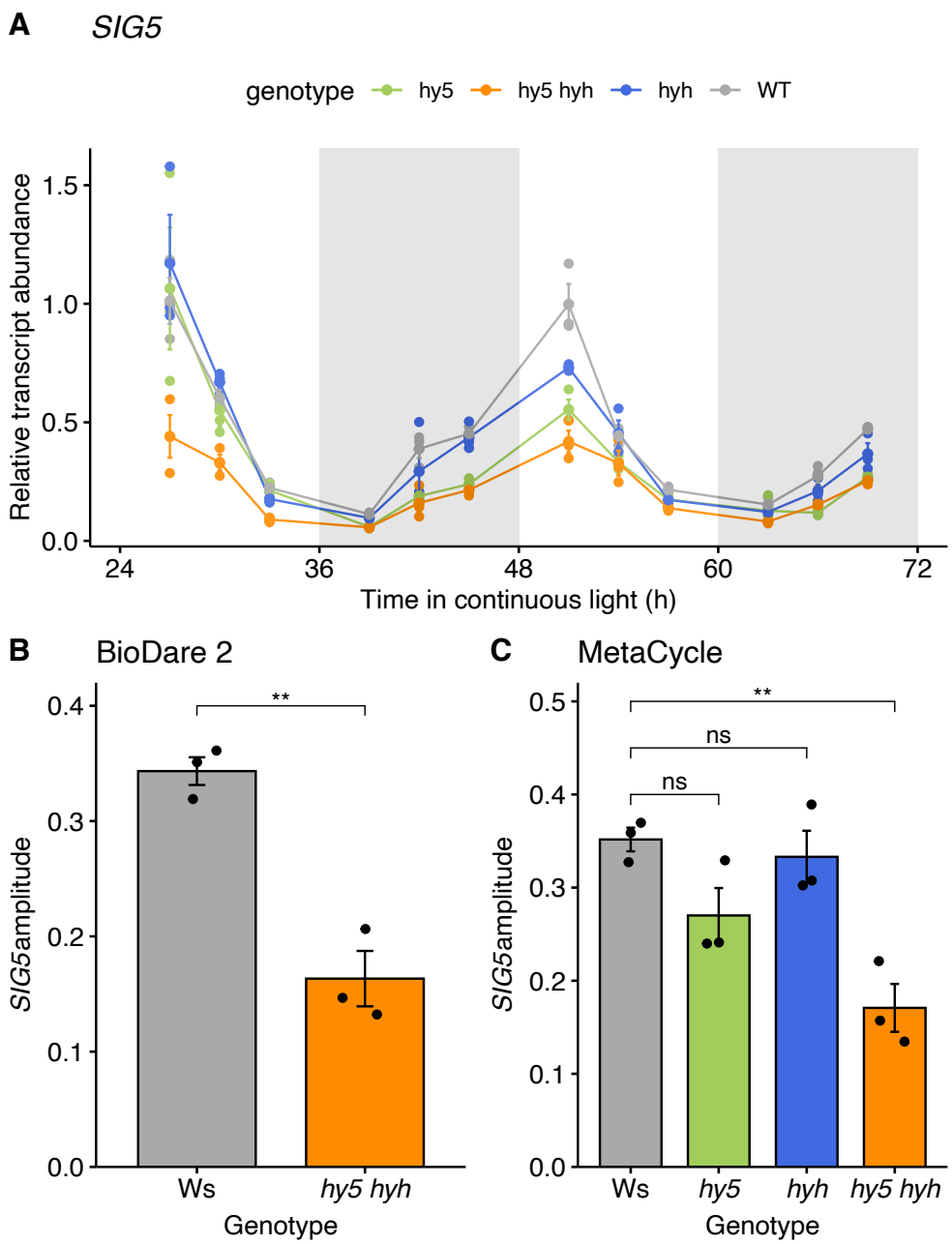

Figure 3.3 HY5 and HYH may act with partial redundancy in the circadian regulation of *SIG5* transcription. (A) Relative transcript abundance of *SIG5* in wild type (Ws), *hy5*, *hyh* and *hy5 hyh*. (B) Amplitude of *SIG5* oscillations in wild type and *hy5 hyh*, calculated using the FFT-NLLS method in BioDare2. (C) Amplitude of *SIG5* oscillations in wild type, *hy5*, *hyh* and *hy5 hyh*, calculated using the meta2d function in the R package MetaCycle. *SIG5* transcript abundance was analysed by qRT-PCR in wild type, *hy5*, *hyh* and *hy5 hyh* seedlings over a 48-hr period in continuous light. Seedlings were grown under 12 h:12 h LD conditions for 11 days then subjected to 24 hrs continuous light before sampling occurred. Sampling occurred 1 hr after subjective dawn and continued every 4 hrs thereafter. Data are represented as means of three independent biological replicates, circles on plot indicate individual data points. Error bars indicate SEM (n=3, N≥10 seedlings). (A) Grey shaded areas indicate subjective night; white areas indicate subjective day. (B) ** represents a statistically significant difference of at least $p<0.01$ (t-test in R). (C) ns = not significant, ** represents a statistically significant difference of at least $p<0.01$ (pairwise t-test with Benjamin & Hochberg adjustment in R).

Table 3.1 BioDare2 periodicity analysis of relative *SIG5* transcript abundance over a 48-hr timeseries in wild type (Ws), *hy5*, *hyh* and *hy5 hyh* using the FFT-NLLS method. Table shows the number of replicates determined to show rhythmic *SIG5* expression per genotype, as well as *SIG5* period and amplitude estimates for each genotype. Rhythmicity was determined if cos waves could be found to fit the data.

Genotype	N (rhythmic)	Period	Period Std	Period SEM	Amplitude	Amplitude Std	Amplitude SEM
Ws	3	24.34	0.48	0.28	0.34	0.02	0.01154701
hy5	1	23.94	0	0	0.22	0	0
hyh	2	23.83	0.32	0.23	0.3	0	0
hy5 hyh	3	24.25	1	0.58	0.16	0.04	0.02309401

psbD BLRP is a chloroplast gene that is a regulatory target of SIG5. Analysing the relative expression of this transcript under continuous light conditions in plants with mutations in the genes of interests reveals roles for the mutated genes in the circadian regulation of chloroplast gene transcription, downstream of SIG5. Figure 3.4 shows the relative expression of *psbD* BLRP over 72 hours in continuous light conditions in wild type (Ws), *hy5*, *hyh* and *hy5 hyh* seedlings. The overall rhythmicity and amplitude of *psbD* BLRP expression damps more quickly in all the genotypes analysed than does *SIG5* expression, indicating that the maintenance of rhythmic expression of *psbD* BLRP is more dependent upon external light cues than *SIG5* rhythmicity. MetaCycle was used to analyse the rhythmicity of this dataset over BioDare2 due to the short and noisy nature of the dataset. The periodicity analysis using the meta2d function in Metacycle gave BH.Q values less than 0.05 for all genotypes except *hyh* (Table 3.2), indicating *psbD* BLRP expression is rhythmic in all but the *hyh* mutant.

Table 3.2 MetaCycle (meta2d) periodicity analysis of *psbD* BLRP transcripts over a 48-hr timeseries in wild type (Ws), *hy5*, *hyh* and *hy5 hyh*. Meta2D analysis provides p-values and BH.Q values, along with estimates of period, phase, baseline gene expression, amplitude (AMP) and relative amplitude (rAMP; the ratio between baseline and AMP). Expression profiles with BH.Q values <0.05 were determined to be rhythmic.

Genotype	p-value	BH.Q	Period	Phase	Baseline	AMP	rAMP
WT	0.001	0.001	22.552	5.763	0.942	0.237	0.237
hy5	0.005	0.006	25.749	3.411	0.704	0.183	0.183
hyh	0.266	0.266	22.586	5.036	0.871	0.209	0.209
hy5 hyh	0.001	0.001	22.772	8.042	0.604	0.106	0.106

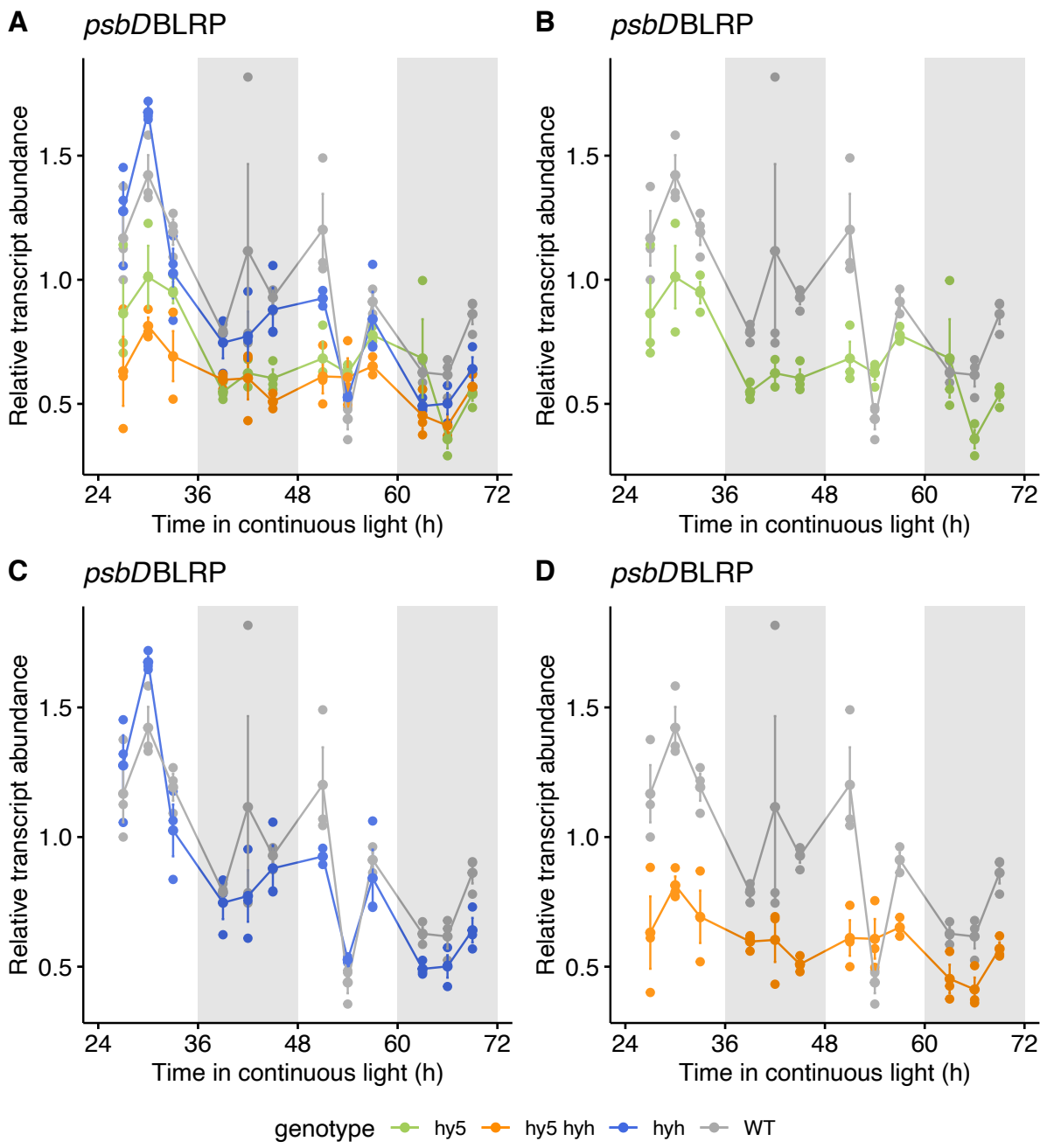

Figure 3.4 The circadian regulation of *psbD* BLRP transcript abundance may depend on HY5 and HYH. (A) Transcript abundance analysis of *psbD* BLRP by qRT-PCR in wild type, *hy5*, *hyh* and *hy5 hyh* seedlings. Comparison of *psbD* BLRP transcript abundance between (B) wild type and *hy5* mutants, (C) wild type and *hyh* mutants and (D) wild type and *hy5 hyh* mutants. Measurements taken every 5 hrs in continuous light over 72 hrs (following a period of 24 hrs in continuous light). Data expressed as means of three independent biological repeats. Error bars indicate SEM (n=3, N≥10 seedlings).

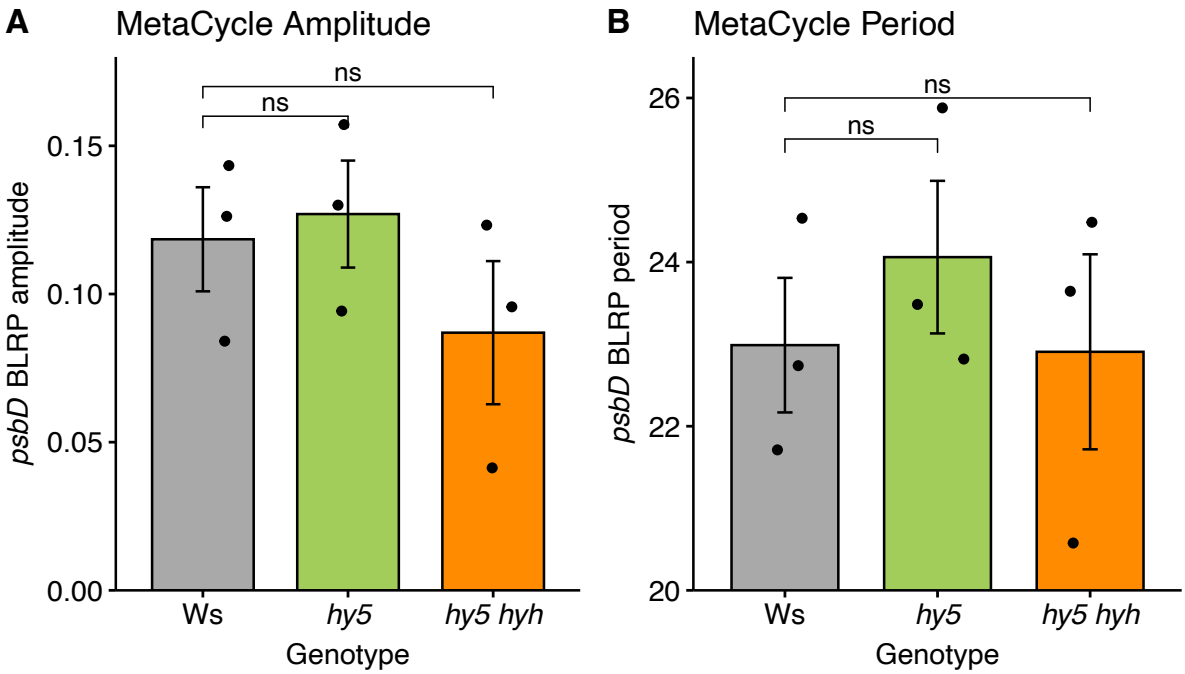

Figure 3.5 Analysis of *psbD* BLRP (A) amplitude and (B) period in wild type (WT), *hy5* and *hy5 hyh* over a 48-hr timeseries using the meta2d function in the R package Metacycle. Estimated values for *hyh* are excluded from the figure as the analysis outputted a BH.Q value > 0.05 for this mutant, indicating that transcripts are not rhythmic in *hyh*. Data are represented as means of three independent biological replicates, circles on plot indicate individual data points. Error bars indicate SEM (n=3, N≥10 seedlings). ns = not significant (pairwise t-test with Benjamin & Hochberg adjustment in R).

Again, the data may suggest more significant roles for HY5 than for HYH in this signalling pathway, as the transcript abundance of *psbD* BLRP shows a smaller decrease in the *hyh* mutant compared to wild type than in the *hy5* mutant (Figure 3.4A, B, C). However, it is unlikely that this difference is significant, given that no significant difference was detected between the *SIG5* amplitudes in the wildtype and *hy5*/*hy5hyh* mutants (Figure 3.5). Note that the meta2d analysis did not detect rhythmicity in *psbD* BLRP transcripts in the *hyh* mutants, hence estimates of amplitude and period in *hyh* were not included in Figure 3.5. This suggests independent roles for HYH in the circadian regulation of *psbD* BLRP.

In the *hy5 hyh* mutant, the data seem to flatline (Figure 3.4D), and Figure 3.5 shows that the amplitude of expression in the double mutant is lower than in wild type. The amplitude of *hy5* expression is intermediate between wild type and *hy5 hyh* (Figure 3.5A), supporting the argument that HY5 and HYH act with partial redundancy in the circadian regulation of this pathway. There is thus some consistency concerning the roles for these transcription factors between the data for *SIG5* and for *psbD* BLRP expression, as we would expect, given that

psbD BLRP expression is regulated by SIG5. There are, however, inconsistencies between the expression of *SIG5* and *psbD* BLRP in each of the genotypes over the timeseries. For example, the data for *psBD* BLRP is much noisier than *SIG5*, which could reflect a lower quality of biological samples, or the possibility of contamination of RNA. Alternatively, it could be truly representative of *psbD* BLRP expression patterns in continuous light, and could reflect a lower level of stability or consistency in *psbD* BLRP compared to *SIG5* expression under these conditions. To confirm the results reported here and clarify the reasons for these inconsistencies, it would be beneficial to repeat these timecourse experiments described.

HY5 and HYH contribute to the maintenance of *CCA1* period

Transcription of the circadian oscillator genes *CCA1*, *LHY*, *PRR9* and *ELF4* is modulated by light conditions, enabling entrainment of the circadian clock to the prevailing environmental conditions (Li et al., 2011, Hajdu et al., 2018). The transcription factor HY5 binds to ACGT-containing elements (ACEs) in the promoters of light-responsive genes, including central clock genes such as *ELF4*, *PRR5* and *LUX* (Lee et al., 2007, Li et al., 2011), hence mediating light (specifically blue light) signalling to the central oscillator (Hajdu et al., 2018). Furthermore, previous studies have identified a physical interaction between CCA1 and HY5 (Andronis et al., 2008) as well as the association of HY5 with the majority of clock and clock-associated genes (Hajdu et al., 2018). In mutants of *HY5* and *HYH*, the period of oscillations in *CCA1* has been shown to shorten, particularly in continuous blue light conditions (Hajdu et al., 2018), identifying these transcription factors as components of input pathways to the central circadian oscillator.

With aims to understand the position of HY5 and HYH in the circadian signalling pathways to chloroplasts and identify their relative importance in mediating light signalling to the clock, *CCA1* transcript abundance was assayed by qRT-PCR over 72 hours in continuous light conditions. There was little observable difference in *CCA1* relative expression levels between the wild type and mutant plants (Figure 3.6A). However, in the *hy5 hyh* mutant, rhythms of *CCA1* accumulation might have a slightly shorter circadian period compared with the wild type and single *hy5* and *hyh* mutants. I used the FFT-NLLS method in Biodare2 to measure the periods of each of the mutants (Figure 3.6B). This analysis identified a shorter period phenotype for *hy5 hyh*, differing significantly from wild type ($p < 0.05$), whereas

single *hy5* and *hyh* mutants had periods no different to wild type. This result is consistent with previous studies, which have reported a slight shortening of *CCA1* period under continuous white and blue light (Hajdu et al., 2018). This suggests that HY5 and HYH play redundant roles in modulating the pace of the circadian oscillator. Furthermore, the amplitude of *CCA1* oscillations is not reduced in the *hy5 hyh* mutant, whereas the amplitude of *SIG5* oscillations is reduced (Figure 3.5). HY5 and HYH therefore act on the amplitude of *SIG5* oscillations between CCA1 and SIG5.

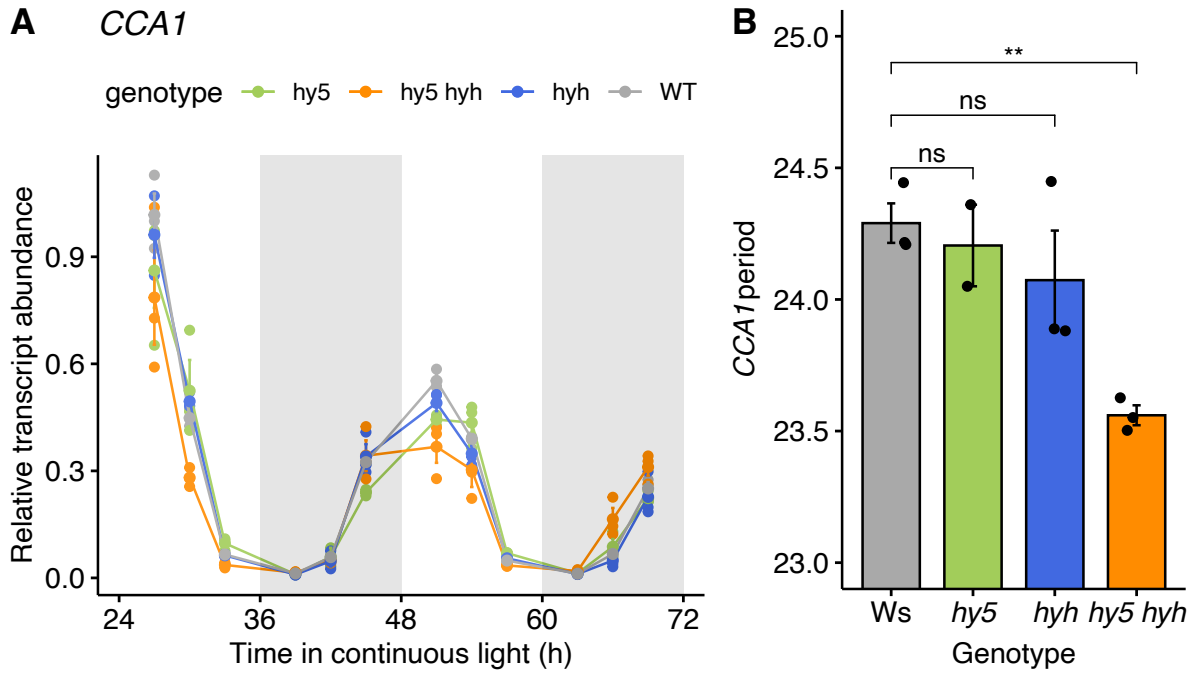

Figure 3.6 HY5 and HYH may influence the period of *CCA1* oscillations in a redundant manner. (A) *CCA1* transcript analysis in wild type (Ws), *hy5*, *hyh* and *hy5 hyh* mutant Arabidopsis seedlings. (B) Period analysis of *CCA1* oscillations using Biodare 2. Measurements were taken every 5 hrs in continuous light over 72 hrs (following a period of 24 hrs in continuous light). Data are expressed as means of three independent biological repeats, circles on plot indicate individual data points. Error bars indicate SEM (n=3, N≥10 seedlings). ns = not significant, ** = p<0.01 (one-way ANOVA followed by pairwise t-test with Benjamin & Hochberg adjustment in R).

CCA1 oscillations strongly correlate with *SIG5* oscillations

There appears to be a direct relationship between the expression of *CCA1* and *SIG5* under constant light conditions, suggesting that *SIG5* might occur downstream of *CCA1* in the circadian signalling pathway. I produced scatterplots in R of *CCA1* and *SIG5* relative expression in Ws, *hy5*, *hyh* and *hy5 hyh* to illustrate this, alongside performing Pearson correlation coefficient analyses (Figure 3.7).

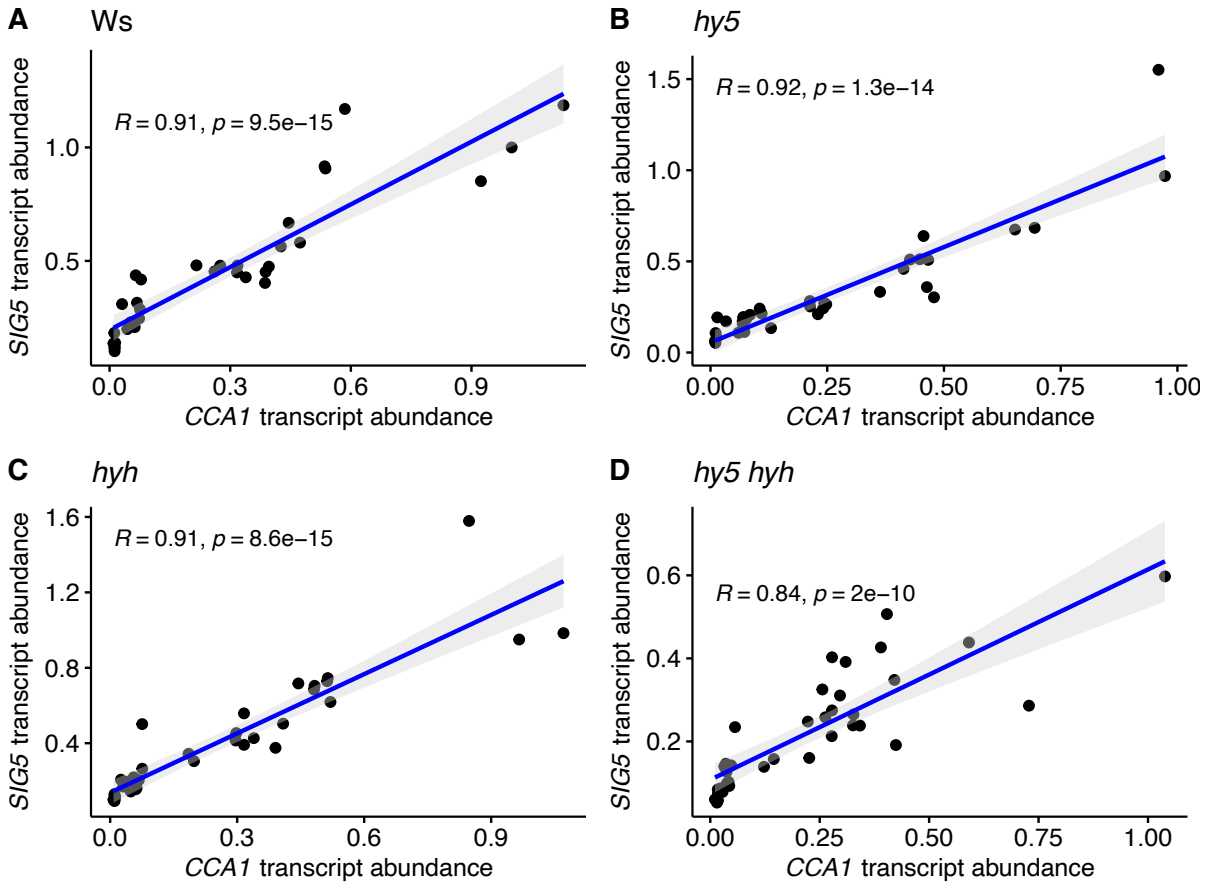

Figure 3.7 Scatterplots of *CCA1* and *SIG5* relative transcript abundance showing Pearson correlation coefficients (R values) in (A) wild type (Ws), (B) *hy5* mutants, (C) *hyh* mutants and (D) *hy5 hyh* double mutants under continuous light conditions.

The correlation coefficients between *CCA1* and *SIG5* relative expression for the wild type (Ws), *hy5* and *hyh* are all high and almost identical (R=0.91, R=0.92 and R=0.91, respectively), yet the correlation between the expression of *CCA1* and *SIG5* in the *hy5 hyh* double mutant is slightly lower (R=0.84). Although this remains a strong correlation with a very significant p-value, the slight decrease in the strength of the correlation (R parameter) in the double mutant suggests HY5 and HYH may act with redundancy (alongside other factors) in contributing to the correlation between *CCA1* and *SIG5* gene expression, therefore acting downstream of CCA1 and upstream of *SIG5* in this signalling pathway. Hence, the analysis suggests a weaker coupling between the circadian oscillator and *SIG5* transcript accumulation in the double *hy5 hyh* mutant. This lower correlation could relate to the *CCA1* period shortening phenotype in the *hy5 hyh* mutant described previously, as this phenotype is not observed in *SIG5* expression in *hy5 hyh* mutants. However, overall, HY5/HYH appear to have minimal impact on the period of *SIG5* oscillations downstream of

CCA1, suggesting these transcription factors primarily act on the amplitude of *SIG5* oscillations downstream of the circadian oscillator.

The circadian regulation of *SIG5* promoter activity

Introduction and aims

The generation of stable transgenic plants expressing luciferase under the control of circadian-regulated gene promoters has been reported in various plant experimental models, including Arabidopsis (Millar et al., 1992, Nakamichi et al., 2004). Transient expression techniques such as particle bombardment have the potential for application to any plant species, enabling the analysis of circadian-regulated gene promoter activity (Miwa et al., 2006, Muranaka et al., 2013).

The overall aim of this work was to develop and use transient expression to investigate the roles of various factors in the circadian regulation of *SIG5* promoter activity, avoiding the need to make stable transformants. Particle bombardment was used as a method of single cell transient transformation of Arabidopsis seedlings. The method uses gold particles which have been coated with the transgene of interest (in this case, *SIG5::LUC*). The coated particles are fired into the plant cells using the delivery system and can be incorporated into the nuclear DNA by a mechanism which is not yet understood. The construct, by this mechanism, thus becomes expressed by the plant cell.

In the presence of oxygen and ATP, luciferase catalyses the emission of bioluminescence from its substrate luciferin. By ligation of the *LUCIFERASE+* coding sequence to the *SIG5* -2460 bp upstream sequence in a plasmid (pGREENII0229), the *SIG5* promoter controls the expression of the luciferase gene in the *SIG5::LUC* construct (Noordally et al., 2013). In this way, circadian oscillations of *SIG5* promoter activity could be monitored in *hy5*, *hyh* and *hy5 hyh* mutants by bioluminescence imaging. Roles for these transcription factors in the circadian regulation of *SIG5* promoter activity could thus be investigated. The intention of the work described here was to develop the method used to bombard seedlings to obtain sufficient transformation efficiency, and to use the method to successfully transform seedlings with the reporter construct.

Method development

Optimisation of the bombardment pressure and distance from the gun for maximum transformation efficiency

The protocol required optimisation to detect luciferase bioluminescence. Particle bombardment of Arabidopsis seedlings with a luciferase construct has been reported in other laboratories (Kanesaka et al., 2019), but the technique required development in order for it to work efficiently for the purposes described here. The initial optimisation was designed to determine the optimum particle gun pressure and distance of plant tissue from the gun for the most efficient transformation. 11-day old seedlings that had been grown on MS (4.41 g L^{-1}) nutrient mix (basal salts with vitamins, pH 5.8) in 0.8% (w/v) agar, without sucrose supplementation (MS-S), were bombarded. The seeds were pipetted inside plastic tubing that had been cut into rings and partially embedded in the agar (Figure 3.8). I reasoned that this might enable easy application of luciferin and create a dense and stable lawn of cotyledons that would be less likely to be displaced by the particles upon firing.

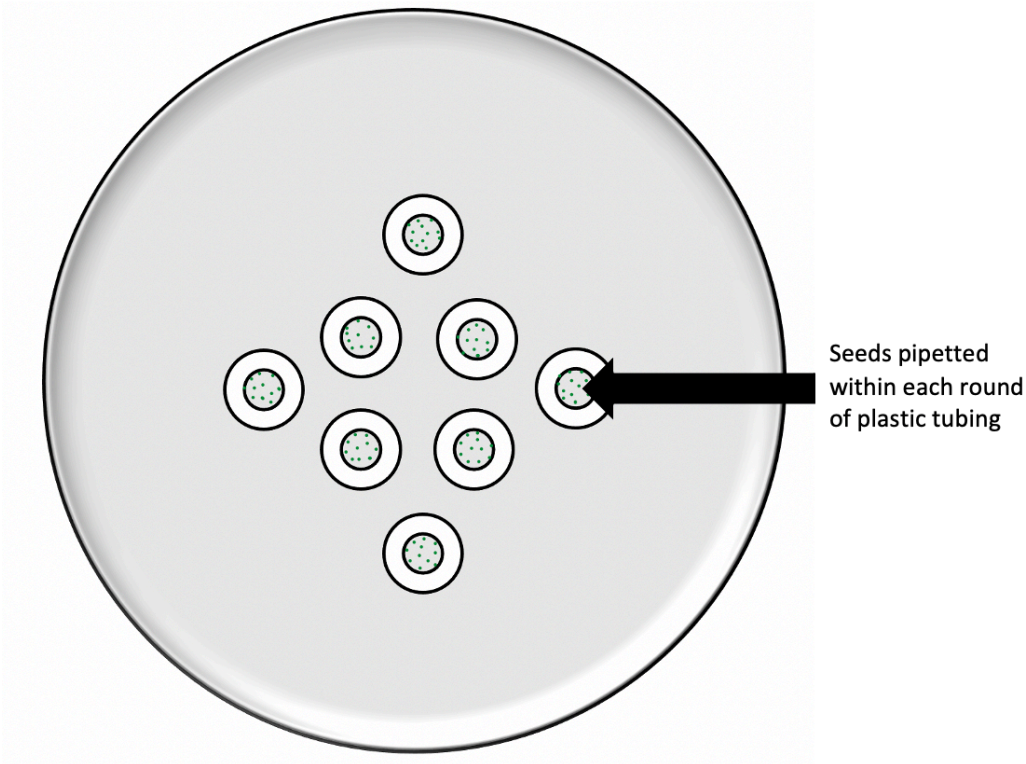

Figure 3.8 Illustration of seedlings cultivated in sterile plastic rings embedded in MS media in 90 mm plates for particle bombardment experiments. 8-15 seeds were pipetted per ring (represented by green dots).

Unfortunately, this dense lawn was not created as the seedlings did not grow as well as anticipated. Nonetheless, the seedlings were bombarded at either 1350 psi or 1100 psi at two different distances from the particle gun, termed floor 1 and floor 2 (Figure 3.9). They were transformed with either the *SIG5::LUC* construct or, as a positive control for the transformation process, a GFP construct. To determine whether other developmental stages or leaf positions would undergo more efficient transformation, detached adult leaves were also bombarded, as well as 11-day old soil-grown seedlings transplanted onto 90 mm MS plates, with their cotyledons pressed flat against the agar (Figure 3.10). Previous experiments by members of the Faulkner lab (JIC) had established successful bombardment using both methods.

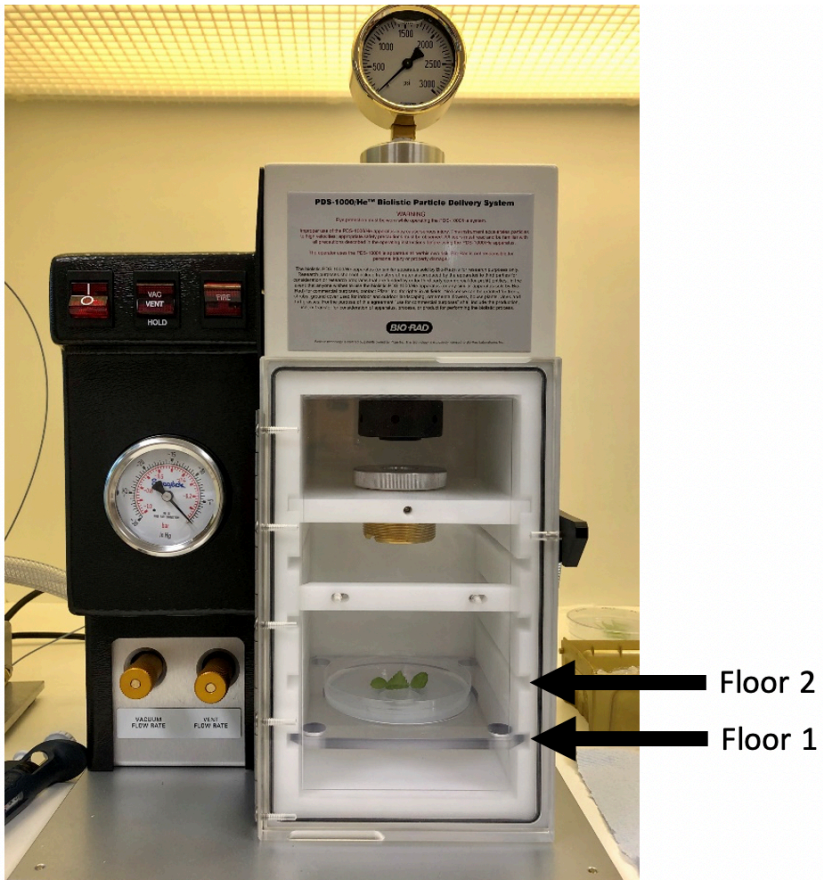

Figure 3.9 The PDS-1000/He particle delivery system (Bio-Rad) used for microprojectile bombardment of Arabidopsis plants, with floors noted.

With the plate positioned under the Photek HRPCS intensified CCD photon counting imaging system (Photek), luciferase bioluminescence counts were measured from the region of interest (ROI (containing plant material)) and from the background area where no plant material was present. The plate was then removed, and bioluminescence counts were

obtained from the same defined regions with no plate present, accounting for spatial and temporal variation in the background signal.

Imaging of bombarded plants with photon counting imaging revealed that the most efficient transformation with the *SIG5::LUC* construct occurred in the soil-grown seedlings and detached adult leaves. The most efficient transformation rates for adult leaves occurred at a lower pressure (1100 psi) and at a relatively further distance from the gun (floor 1). Conversely, for soil-grown seedlings the most efficient rates occurred at a higher pressure (1350 psi) and relatively closer to the gun (floor 2).

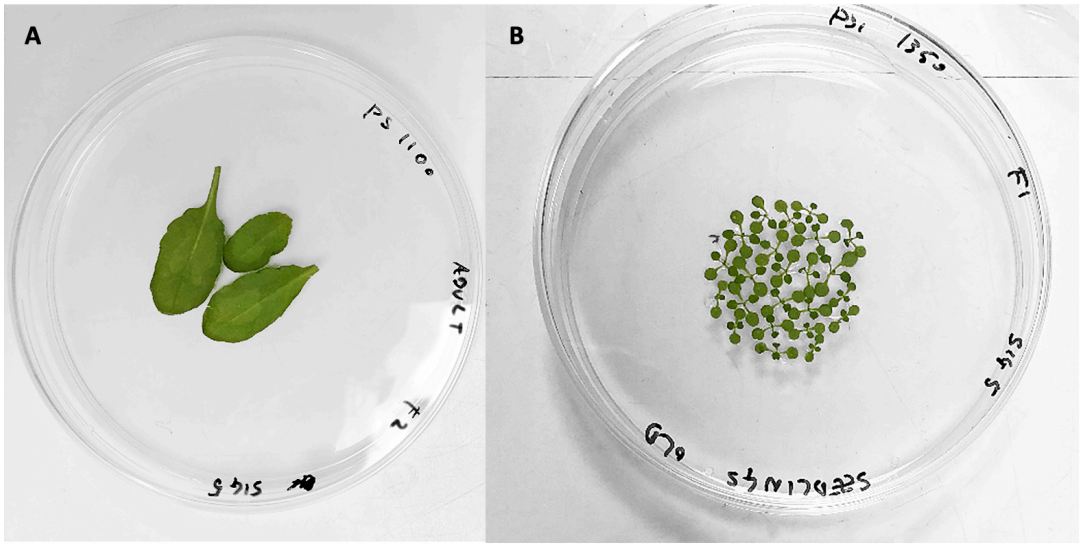

Figure 3.10 Positioning of (A) adult leaves and (B) soil-transplanted seedlings for optimisation of particle bombardment. (A) Adult leaves removed from plant and placed on agar plates in central bombardment zone (underside facing up). (B) 2-week-old seedlings grown on soil and transplanted to agar plates in central zone.

Both adult leaves and soil-grown seedlings presented greater bioluminescence counts than the MS-grown seedlings, as well as greater differences between the control background and ROI counts. Imaging of the GFP-transformed seedlings confirmed this lower transformation rate of MS-grown seedlings, with fluorescence detected from only a few cells per cotyledon. Figure 3.11 shows an example of the images of the GFP-transformed plants 96 hours post-bombardment, obtained from the ZEISS Axio Zoom.V16 (ZEISS Microscopy) fluorescence stereo microscope.

Regardless of the low efficiency of transformation, this preliminary optimisation identified that that the most efficient transformation of seedlings grown on MS medium occurred at a higher pressure and at a greater distance from the gun (1350 psi; floor 2) (Figure 3.12).

Seedlings on floor 1 bombarded at 1100 psi exhibited bioluminescence at the lower limit of detection, as evidenced from the negative background-subtracted counts (Figure 3.12B), indicating that these conditions were the least efficient for successful transformation via particle bombardment.

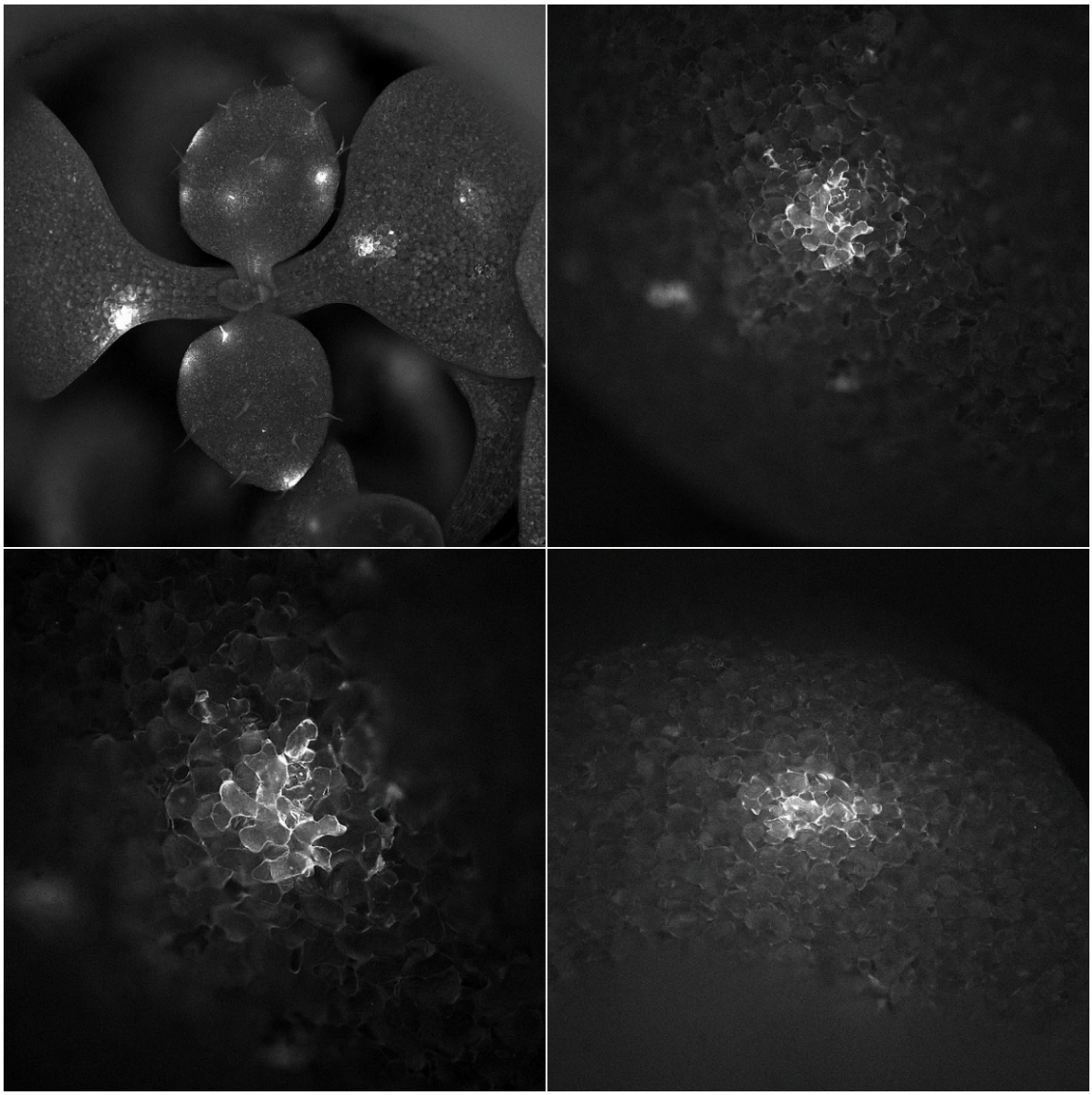

Figure 3.11 Images of Arabidopsis seedlings grown on MS medium transformed with pB7WG2.0-GFP (GFP reporter construct) via particle bombardment. Seedlings were imaged 96-hrs after bombardment using ZEISS Axio Zoom.V16 (ZEISS Microscopy) fluorescence stereo microscope.

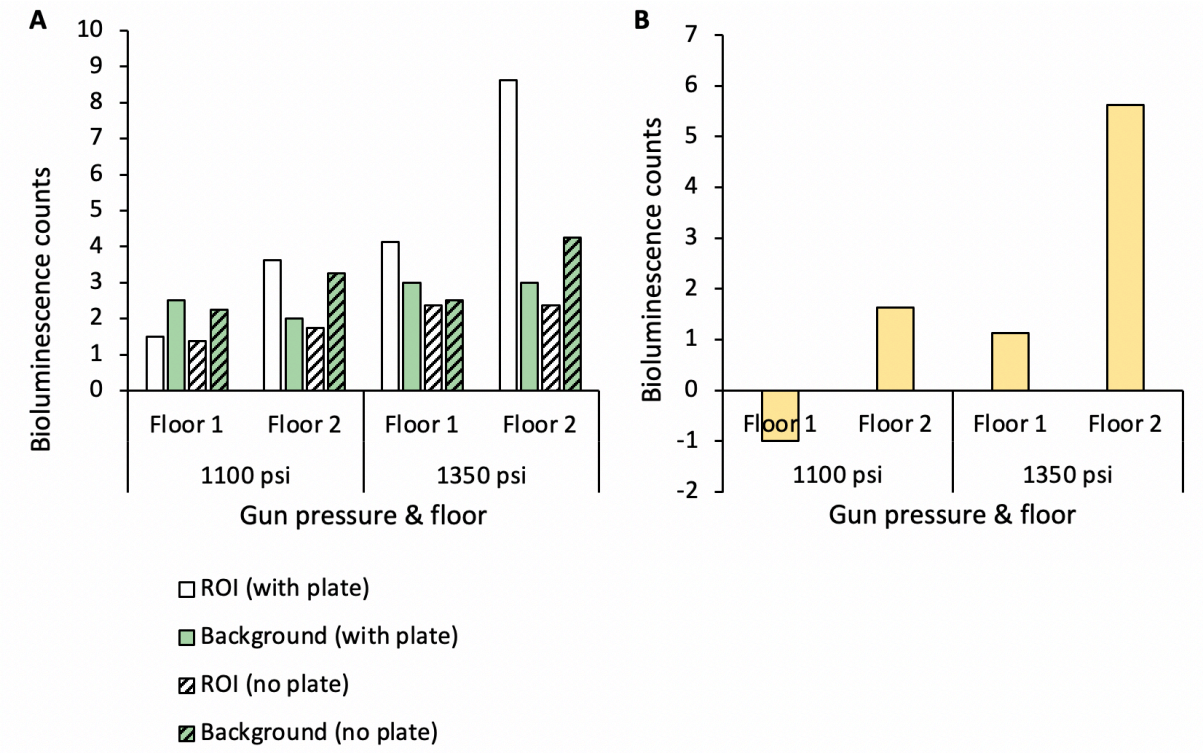

Figure 3.12 Bioluminescence counts from 10-min integrations of 11-day old seedlings grown on MS medium bombarded with the *SIG5::LUC* reporter construct using a Photek HRPCS intensified CCD photon counting imaging system (Photek) controlled by Image32 imaging software (Photek). (A) Counts recorded from regions of interest (with plant material present) and background regions (with no plant material present) with the plate positioned under the camera ('ROI (with plate)' and 'Background (with plate)', respectively). Counts also taken from the same defined regions (ROI and background) but without the plate present ('ROI (no plate)' and 'Background (no plate)'), accounting for variation in the background bioluminescence signal across the image. (B) Background-subtracted bioluminescence counts (i.e. counts from 'ROI (with plate)' minus counts from 'Background (with plate)'). Negative values indicate the bioluminescence signal was at/below the limit of detection.

Optimisation of plant growth and bombardment conditions

The primary aim associated of this aspect of the method development was to produce seedlings with cotyledons positioned flat against the agar, affording a level surface for bombardment. This was conducted because Arabidopsis leaves typically have a curved surface, which might have caused the variation between the agar-grown and soil-grown (transplanted) seedlings in their transient expression efficiencies. Additionally, the poor growth of the seedlings within the plastic tubing might have explained the low efficiency.

As a next step, seeds were pipetted onto a layer of media and then covered with another layer of soft media above the seeds (both layers containing either 0.5% or 0.6% (w/v) agar). I reasoned that the hypocotyls might project through the upper layer, opening their

cotyledons to lie flat on top of the agar. Questions that arose from this idea centred around controlling the timing of the opening of cotyledons, i.e., what measures could be implemented to prevent the cotyledons from opening before completely penetrating the top layer of agar? The solution I proposed was to add 0.2% (w/v) activated charcoal to the media, thereby reducing light penetration to encourage seedling elongation (Figure 3.13). Therefore, the media used in this second optimisation experiment was MS with agar, 0.2% (w/v) charcoal and 1% (w/v) sucrose. The sucrose was added to increase seedling growth. Either 0.5% or 0.6% (w/v) agar was added to the media to enable identification of the most suitable concentration for hypocotyls to penetrate and grow through.

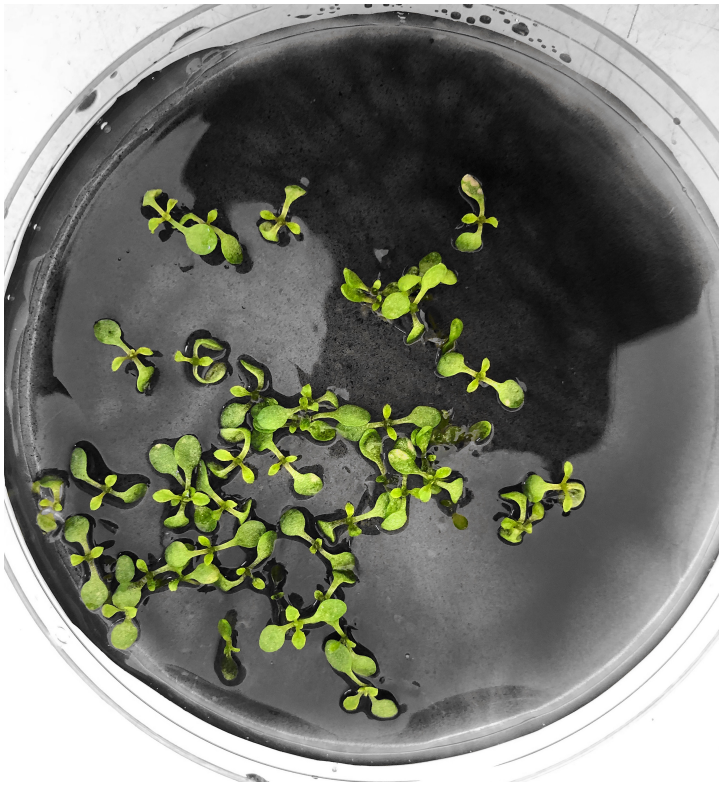

Figure 3.13 11-day old seedlings grown on MS media with 0.2% (w/v) charcoal.

The concentration of charcoal added to the media was too low, as 0.2% (w/v) continued to transmit light. Therefore, some of the cotyledons opened before fully penetrating the upper layer of agar. Additional unforeseen outcomes included extensive root growth (as can be seen in Figure 3.14), larger cotyledons, and displacement of the seeds when applying the top layer of agar. Extensive root growth, specifically fewer, longer lateral roots, occurs as a consequence of adding activated charcoal to Arabidopsis growth media (Caffaro et al., 2013).

The seedling growth and displacement of the seeds from the centre of the plates required the transplantation of seedlings to fresh agar plates. The extensive root length made this difficult, although this was attempted with some success. 9-10 seedlings were transplanted and positioned on five fresh plates in the central bombardment area, pressing the cotyledons flat against the agar (Figure 3.14). The seedlings on two of the charcoal media plates were maintained on their original plates for bombardment (Figure 3.13). These particular plates were chosen as a proportion of the seedlings on them grew as expected, with cotyledons opening just above the top layer of agar.

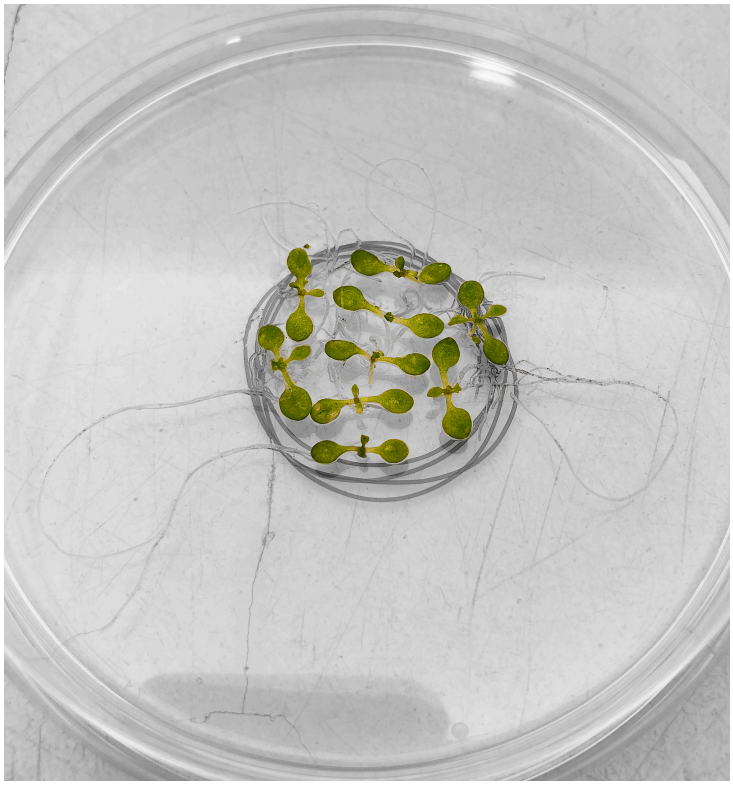

Figure 3.14 11-day old seedlings transplanted from MS media with 0.2% (w/v) charcoal to agar plates for bombardment. Seedlings developed extensive roots. Black circle in the centre of the plate shows the central firing zone of the gun.

One plate of transplanted seedlings was bombarded with the GFP construct as a positive control. The other 4 plates were transformed with the *SIG5::LUC* construct. Similarly, one of the original charcoal plates was bombarded with the GFP construct and the other with the *SIG5::LUC* construct. The pressure was set to 1350 psi and the seedlings were positioned on floor 2. However, a manufacturing error with the rupture disks (which rupture at different

pressures to determine the bombardment pressure) was subsequently identified, which meant that they ruptured at nearer to 1100 psi.

24 hours after the application of luciferin, *SIG5::LUC*-bombarded seedlings were imaged with the Photek HRPCS intensified CCD photon counting imaging system (Photek). 10-min integrations failed to detect differences between the bioluminescence counts between the ROIs and background, thus the bombardment was determined to be unsuccessful.

It is possible that this failed because the rupture disks were faulty, because the photon counts were similar to those obtained previously with seedling bombardment at 1100 psi. Alternatively, the imaging system may have been insufficiently sensitive to detect the bioluminescence signal, because some fluorescence signal was detectable from the seedlings bombarded with the GFP (control) construct. Nonetheless, this line of enquiry was abandoned.

Summary of method development

This protocol development provided insight into the most suitable conditions for transformation of Arabidopsis seedlings via particle bombardment for the transient expression of a luciferase construct. The seedlings transformed with the *SIG5::LUC* construct produced the strongest bioluminescence signal when bombarded at 1350 psi (a high pressure) and relatively close to the particle gun (floor 2). Attempts to optimise the growth conditions of the plants to facilitate flattening of the cotyledons, thus providing a flat surface area for bombardment, were largely unsuccessful. Unfortunately, the COVID-19 pandemic meant that the time available for further exploration of this method idea was limited. It was decided that the efficiency of transformation was sufficient without attempting to optimise the cotyledon positioning, so the results from the initial method optimisation experiments were used to inform the design of the final experimental method, which is detailed in the Methods chapter (Section 2.10).

Results

The activity of the *SIG5* promoter is greater in wild type than in *hy5*, *hyh* and *hy5 hyh* mutants under free-running circadian conditions

To evaluate the relative contributions of HY5 and HYH to the circadian regulation of *SIG5* promoter activity, the *SIG5* promoter cloned in a luciferase-containing vector was transiently expressed in Ws wild type background, *hy5*, *hyh* and *hy5 hyh* mutant seedlings. The activity of the promoter in each genotype was analysed with bioluminescence imaging under continuous light conditions. 2-week-old plants were transformed and imaged using the particle bombardment method that I had gone some way to develop.

Bioluminescence imaging in continuous light conditions was projected to occur over a period of 6 days. Unfortunately, the camera software crashed ~55 hours after measurements started and, due to the time remaining, I was unable to repeat the experiment. Whilst the results are not as robust as I hoped, they do provide new insights into the relative contributions of HY5 and HYH to the circadian regulation of *SIG5* promoter activity.

The background-subtracted bioluminescence counts for each of the genotypes used are shown in Figure 3.15. There are clear oscillations in *SIG5* promoter activity in the wild type seedlings (Ws), which display the highest bioluminescence counts throughout the timeseries. The greatest promoter activity occurred around subjective dawn, consistent with previous studies (Noordally et al., 2013, Belbin et al., 2017). The oscillations in *SIG5* promoter activity in each of the mutants are not clear, although there may be evidence of some rhythmicity in the *hyh* mutant. Nonetheless, the overall bioluminescence signal is much reduced in each of the mutants compared to wild type, with both the *hy5* and *hy5 hyh* mutants demonstrating the lowest levels of *SIG5* promoter activity throughout the timecourse. This is consistent with the data obtained from analysis of transcript abundance (Figure 3.3), which supports the hypothesis that the role of HY5 in this pathway is more important than the role of HYH.

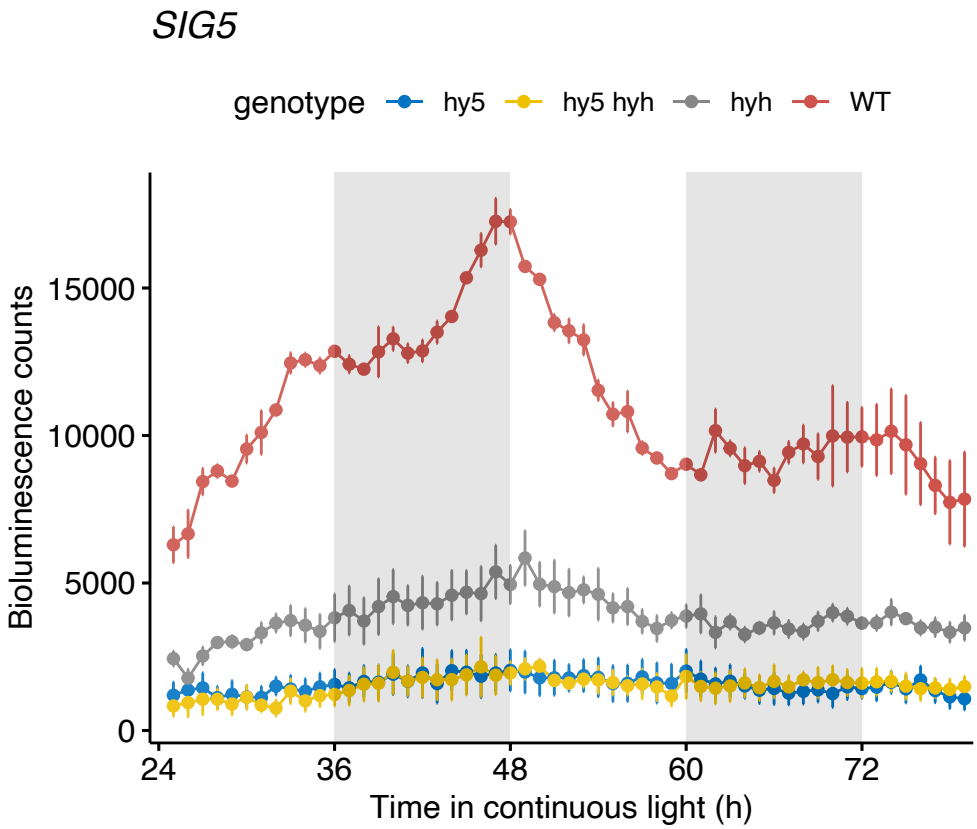

Figure 3.15 Background-subtracted bioluminescence counts from seedlings transformed with the *SIG5::LUC* construct via particle bombardment. Three regions of interest per genotype were determined and background bioluminescence counts were subsequently subtracted from these. Circles represent mean values; error bars represent SEM measurements for each datapoint.

Discussion

Plants are exposed to varying environmental conditions, and the circadian oscillator enables the temporal coordination of physiological and developmental features with this external environment. It is important that alongside nuclear gene expression, chloroplast gene expression is also entrained to the prevailing environmental conditions and hence accurately coordinated with nuclear gene expression. The chloroplast target of SIG5 – *psbD* BLRP – represents one promoter that drives the *psbDC* operon which encodes essential components of the photosynthetic apparatus. Therefore, this pathway presents an example whereby coordination between nuclear and chloroplast gene expression occurs. We speculate that this might optimize the rate of production of new photosystem components according to the time of day, so maintaining an optimum rate of photosynthesis.

Through the work in this chapter, I have identified a mechanism by which HY5 and HYH contribute to the circadian regulation of chloroplast gene expression, potentially by acting

upstream of SIG5 in this pathway. Under free-running conditions, *SIG5* transcript abundance is decreased in the *hy5 hyh* double mutant (Figure 3.3). Previous unpublished work has identified redundancy between HY5 and HYH in modulating the expression of *SIG5* in response to low temperatures (Cano-Ramirez et al., under revision). A similar redundancy may occur in the circadian control of both *SIG5* transcript accumulation and *SIG5* promoter activity by HY5 and HYH, although it is possible that the roles of HY5 in this pathway are more pronounced than those of HYH. Although the transcript abundance of both *SIG5* and its chloroplast gene target *psbD* BLRP are slightly reduced in the single *hy5* and *hyh* mutants compared to wild type, there are greater reductions seen in *hy5* than *hyh* mutants (Figure 3.3). Similarly, the activity of the *SIG5* promoter in the *hy5* mutant under free-running circadian conditions is more reduced than in the *hyh* mutant, with promoter activity in the *hy5* mutant displaying bioluminescence signals no different to the *hy5 hyh* mutant (Figure 3.15).

With only slight effects of period shortening observed in the *hy5 hyh* double mutant, *CCA1* expression appears to be otherwise unaffected by mutations in *HY5* and *HYH* (Figure 3.6). This is consistent with reports in the literature (Hajdu et al., 2018, Andronis et al., 2008). Oscillator components with evening elements (EEs) present in their gene promoters are known to be direct targets of HY5; roles for the transcription factor in decreasing the transcription of *PRR5*, *LUX* and *BOA* have been reported (Hajdu et al., 2018, Andronis et al., 2008). Mathematical modelling by Hajdu et al. (2018) predicted an elevation in *CCA1* transcript levels in the *hy5 hyh* double mutant, which is not consistent with the biological data. The positive effects of HY5 on *CCA1* transcript levels may be balanced by the influence of HY5 on EE-containing genes which act to decrease *CCA1* transcript levels, thereby masking the predicted increase in *CCA1* transcript abundance (Hajdu et al., 2018).

In the *hy5 hyh* double mutant, my results showed a shortening in the circadian period length of *CCA1* oscillations compared with the wild type and single *hy5* and *hyh* mutants (Figure 3.6B). Previous work has reported shorter period phenotypes for all clock genes in mutants of HY5 and HYH under blue light conditions (Hajdu et al., 2018). In red light conditions, this period shortening was not observed, and in white light, there was some shortening, but not as pronounced as in blue light (Hajdu et al., 2018). Furthermore, there is inconsistency in the literature regarding period shortening in *hy5* mutants: some papers report period

shortening (Andronis et al., 2008, Haydon et al., 2013, Hajdu et al., 2018) whereas others report none (Li et al., 2011). The fact that my experiments were conducted under white light conditions may account for the only slight reductions in *CCA1* period length over the timecourse because, taken together, the literature indicates that this phenotype depends upon the spectrum of light. It may be the case that under blue light conditions this period shortening phenotype that I have described would be more pronounced, as is reported in Hajdu et al. (2018). SIG5-mediated circadian regulation of chloroplast transcription is largely dependent upon blue light signalling (Belbin et al., 2017); thus, it is possible that this blue light dependence translates to a degree of HY5/HYH dependence.

Interestingly, the circadian period of *SIG5* oscillations was not shortened in any of the mutants, which might explain the weaker correlation between *CCA1* and *SIG5* expression in the *hy5 hyh* mutant compared to wild type (Figure 3.7). This suggests that HY5 and HYH may be interacting with both the *CCA1* and *SIG5* promoters through different pathways, or that the convergence of other factors on the *SIG5* promoter modulate its expression, thereby masking the correlation between *SIG5* and *CCA1* expression. Furthermore, despite correlation between *SIG5* and *CCA1* phase of expression in wild type, CCA1 does not appear to interact directly with the *SIG5* promoter (Nagel et al., 2015). This is consistent with the idea that several pathways feed into *SIG5* expression regulation, and additional factors act downstream of *CCA1* to modulate *SIG5*.

An alternative explanation for this decoupling of expression is that *CCA1* and *SIG5* are predominantly expressed in different cell types. There is a decoupling of *CCA1* expression between different cell types following a period of continuous light exposure, indicating cell-type-specific expression (Yakir et al., 2011). It could therefore be the case that the mutants used in this study affect the period of *CCA1* in, for example, the epidermis, whereas *SIG5* is mainly expressed in the mesophyll, thereby explaining the weaker correlation between *CCA1* and *SIG5* expression in the *hy5 hyh* mutant.

A physical interaction occurs between CCA1 and HY5 proteins to regulate the expression of *LIGHT-HARVESTING CHLOROPHYLL A/B* (*Lhcb*) genes (Andronis et al., 2008). HY5 binds to the G-box element in *Lhcb* gene promoters and interacts with bound CCA1 to regulate the circadian responsiveness of *Lhcb* genes. A similar physical interaction may be occurring at the *SIG5* promoter to maintain its amplitude of expression under free-running circadian

conditions. This could explain the reduced *SIG5* amplitude in the *hy5* and *hy5 hyh* mutants compared to WT in continuous light conditions (Figure 3.3). It is possible that this physical interaction could also occur between HYH and CCA1. This idea has not been explored but could explain the increased disruption to *SIG5* oscillations in the double *hy5 hyh* mutant compared to the single *hy5* mutant. It would be informative to investigate whether there is a physical interaction occurring between CCA1 and HY5/HYH at the *SIG5* promoter, by conducting a yeast two-hybrid screen, similar to the experiment described by Andronis et al. (2008).

Physical interaction between HY5 and HYH can also occur in vivo; this HY5-HYH heterodimer has been reported to bind to the G-box of the *RBCS-1A* promoter (Holm et al., 2002). It is suggested that this heterodimer is important for the transcription of downstream HY5 and HYH targets, thus the absence of both the homo- and hetero-dimer in the *hy5 hyh* mutant could further explain the large reduction in *SIG5* transcript accumulation in Figure 3.3. Holm et al. (2002) also show that the presence of the HY5 protein is essential for HYH protein to accumulate, although the opposite is not true. This could explain the greater reduction of *SIG5* transcript abundance observed in *hy5* than *hyh*, as protein (but not mRNA) levels of HYH may be reduced in the *hy5* mutant, alongside HY5 protein levels.

PHYTOCHROME INTERACTING FACTORS (PIFs) are bHLH transcription factors that act downstream of photoreceptors to repress photomorphogenic development (Leivar and Quail, 2011). The role of PIFs is therefore antagonistic to the roles of HY5 and HYH in promoting photomorphogenic responses (Lau and Deng, 2010, Leivar and Quail, 2011). Similar to HY5, PIFs interact with G-box elements in target gene promoters and are light-regulated. However, PIFs are degraded upon light exposure, whereas HY5 and HYH are stabilised by light. Toledo-Ortiz et al. (2014) suggest that HY5 and PIFs act antagonistically and in tandem with the circadian oscillator to co-regulate common target genes through their interaction with G-box promoter motifs. It is possible that *SIG5* gene expression regulation is occurring through this mechanism to integrate environmental signals with circadian timing information. Lee et al. (2007) identified *SIG5* as a direct binding target of HY5. Furthermore, Dubreuil et al. (2017) used a MEME analysis to scan for G-box elements in the *SIG5* promoter, which they identified, thereafter suggesting *SIG5* as a potential target of PIF3. In agreement with Dubreuil et al. (2017), the promoter analysis I conducted using

PlantPAN 3.0, exploring the 3 kb region upstream of the *SIG5* translational start site, identified a G-box element in the *SIG5* promoter (Table 3.2). This confirms the possibility of this interaction occurring at the *SIG5* promoter, yet further work will be needed to confirm its occurrence.

Table 3.3 PlantPAN 3.0 analysis of the 3kb upstream region of the SIG5 transcriptional start site, searching for G-box motifs (CACGTG).

Gene	Motif	Position (distance from transcription start site)	Strand
SIG5	CACGTG (G-box)	1798	+
SIG5	CACGTG (G-box)	1798	-

Belbin et al. (2017) suggest that phytochromes are necessary for *SIG5* responses to relative proportions of R and FR light, but that the circadian regulation of *SIG5* and *psbD* BLRP expression are primarily dependent upon cryptochromes and blue light signalling. Given that we know HY5 and HYH regulate *SIG5* expression in response to light (Nagashima et al., 2004, Brown and Jenkins, 2008, Mellenthin et al., 2014), it may be that HY5 and PIFs coordinate the expression of *SIG5* throughout the day in response to different light wavelengths. Belbin et al. (2017) propose that the relative proportions of R and FR light may be important signals for SIG5 around dawn, thus phytochrome- and PIF-mediated R and FR signalling to *SIG5* may play a more significant role in maintaining cycles of *SIG5* expression under light-dark cycles than under circadian conditions. Cryptochrome- and HY5/HYH-mediated blue light signalling to *SIG5* may be more significant for maintaining the accumulation of *SIG5* over the entire 24-hr period, therefore playing a greater role in the circadian regulation of *SIG5* transcription. In order to test this, identical experiments could be conducted under R light and B light, in both light-dark and continuous light conditions, examining the expression of SIG5 in mutants of HY5/HYH and PIFs under each of these light conditions.

To summarise, the work described in this chapter provides insights into the involvement of HY5 and HYH in the circadian regulation of *SIG5* and *psbD* BLRP expression. This is evident in the reduction of both *SIG5* and *psbD* BLRP transcript abundance in mutants of these genes in continuous light conditions, alongside reduced *SIG5* promoter activity in *hy5*, *hyh* and *hy5*

hyh mutants. The nature of these interactions, and the involvement/requirement of other transcription factors and proteins such as PIFs for the regulation of *SIG5* expression by HY5/HYH should be further explored. Furthermore, future work might elucidate further the relative contributions of HY5 and HYH to this pathway, revealing the overlapping and distinct roles for each of these transcription factors. Future research should aim to clarify specific roles for HYH, and whether the reduction of *SIG5* transcript abundance in *hy5 hyh* is due to a redundancy between the two transcription factors and/or due to their functioning as both homodimers and heterodimers. Coimmunoprecipitation assays, such as those described by Holm et al., 2002, could be used to investigate this further.

Chapter 4 Involvement of HY5, HYH and ATHB17 in light and cold temperature signalling to chloroplasts

Introduction and aims

Changes in light conditions are detected by photoreceptors, which include phytochromes, cryptochromes, phototropins and the UV-B photoreceptor UVR8. These environmental fluctuations can be both predictable and unpredictable, and trigger alterations in gene expression throughout the plant that lead to physiological and developmental changes. These changes occur to optimise photosynthesis under fluctuating light conditions, and ensure that flowering occurs during the most appropriate season. Phototransduction pathways also entrain the circadian oscillator to light-dark cycles, which is important for optimum plant growth, fitness and survival (Dodd et al., 2005).

The bZIP transcription factors HY5 and HYH act downstream of photoreceptors, regulating the expression of numerous downstream target genes in response to light. The expression of *SIG5* is photoreceptor- and HY5-dependent (Mellenthin et al., 2014). In this study, the authors analysed *SIG5* expression in *hy5* mutants, by exposing 10 day old seedlings, dark-adapted for 24hrs, to monochromatic blue or red light for 24 hrs, then measuring relative *SIG5* transcript levels (Mellenthin et al., 2014). Under both red and blue light conditions, *SIG5* transcript abundance is significantly lower in *hy5* mutant Arabidopsis seedlings than in wild type seedlings (Mellenthin et al., 2014). Hence, it is likely that the promoter of *SIG5* is amongst the regulatory targets of HY5 (and HYH) (Burko et al., 2020). Furthermore, specific, UV-B-induced *SIG5* transcript accumulation is mediated redundantly by HY5 and HYH (Brown and Jenkins, 2008). Therefore, HY5 and HYH might act upstream of *SIG5* and downstream of photoreceptors in the light-regulation of *SIG5* gene expression, under specific experimental light conditions, leading to the regulation by light wavelengths of certain chloroplast-encoded genes. These experiments were all conducted under the context of specific wavelengths of light, hence further experimentation under white light conditions, described in this thesis, furthers our understanding of this signalling pathway.

Under natural conditions, plants are also subjected to a range of abiotic stresses, including salinity, low/high temperatures and high light, which have implications for their growth and

survival. These environmental stresses impose limitations to the geographical area within which a plant species can survive. When plants adapted to temperate regions are continuously subjected to low, non-freezing temperatures, they undergo a process called cold acclimation, leading to the development of freezing tolerance. This acclimation process triggers a range of molecular and metabolic responses in the plant, enabling them to survive after exposure to freezing temperatures (Thomashow, 1999, Knight and Knight, 2012). Exposure to freezing temperatures without prior cold acclimation results in cellular dehydration, ice nucleation, membrane disruption and subsequent death (Crosatti et al., 2013). Photosynthetic efficiency is reduced upon exposure to low temperatures via the production of singlet oxygen (the predominant reactive oxygen species (ROS) in plastids), leading to membrane lipid peroxidation (Crosatti et al., 2013, Ramel et al., 2012, Barclay and McKersie, 1994). Therefore, low temperature signalling to chloroplasts is important to enable rapid responses in both photosynthetic metabolism and machinery, facilitating adaptation to the fluctuating environment (Crosatti et al., 2013). However, the mechanisms underlying low temperature information signalling to chloroplasts are not particularly well understood.

In response to low, non-freezing temperatures, *SIG5* is induced strongly, having the greatest transcriptional response to chilling amongst the Arabidopsis sigma factors (Nagashima et al., 2004, Cano-Ramirez et al., under revision). SIG5 is responsible for communicating circadian timing information to chloroplasts, leading to the rhythmic transcription of *psbD*, and in response to chilling, *psbD* BLRP transcripts accumulate in the wild type, but not the *sig5-3* mutant (Noordally et al., 2013, Nagashima et al., 2004, Cano-Ramirez et al., under revision). Therefore, SIG5 integrates low temperature and circadian timing information, and communicates this to chloroplasts. It has been identified that the transcription factors HY5 and HYH act upstream of this signalling pathway to regulate the cold induction of *SIG5* (Cano-Ramirez et al., under revision). It was therefore my aim to identify whether other transcription factors that are known to regulate *SIG5* might be positioned upstream of the low temperature response of SIG5-mediated signalling to chloroplasts.

Another known regulator of *SIG5* is ATHB17, which is responsive to ABA and multiple stress treatments (Zhao et al., 2017). Zhao et al. (2017) suggest that ATHB17 acts upstream of *SIG5* in the salinity stress-responsive pathway, modulating its transcription to regulate

chloroplast gene expression in response to salt stress. Low temperature conditions induce *SIG5* and subsequent *psbD* BLRP gene expression, and the efficiency of photosynthesis in these conditions is reduced in *sig5* mutants (Nagashima et al., 2004).

The work described here aims to further investigate both the distinct and overlapping roles for HY5 and HYH in the regulation by light of *SIG5*, and downstream *psbD* BLRP gene expression. Furthermore, this research aims to identify whether ATHB17 could be acting upstream of *SIG5* to regulate responses to cold stress. Through these investigations, I hope to identify positions for each of these transcription factors in the signalling pathway(s) leading to the circadian, light and cold regulation of *SIG5* and *psbD* BLRP transcription, by measuring the abundance of these transcripts in mutants of candidate genes under light-dark conditions, and following cold exposure. Hence, the work aims to further our understanding of how nuclear and chloroplast gene expression are coordinated in response to light and cold stress.

Results

HY5 and HYH act upstream of *SIG5* in the light signalling pathway

To examine roles of HY5 and HYH in the light-regulation of *SIG5* and *psbD* BLRP gene expression, wild type, *hy5*, *hyh* and *hy5 hyh* mutants were grown under light-dark cycles for 11 days. The relative transcript abundance of *SIG5* and *psbD* BLRP was measured over a 24-hr period.

Light strongly induced *SIG5* transcript accumulation, reaching peak transcript abundance shortly after dawn (Figure 4.1), consistent with previous reports (Nagashima et al., 2004). Strongest induction occurred in the wild type (Ws) plants, although this induction still occurred to a lesser extent in *hy5*, *hyh* and *hy5 hyh* mutants (Figure 4.1). The weakest induction of *SIG5* transcripts occurred in the *hy5 hyh* double mutant, suggesting that HY5 and HYH may be acting with partial redundancy to induce *SIG5* upon light exposure. However, partial maintenance of *SIG5* induction in response to light in the *hy5 hyh* double mutant indicates there are potential additional factors that regulate this signalling pathway (i.e., HY5 and HYH are not the only transcription factors that induce *SIG5* in response to light).

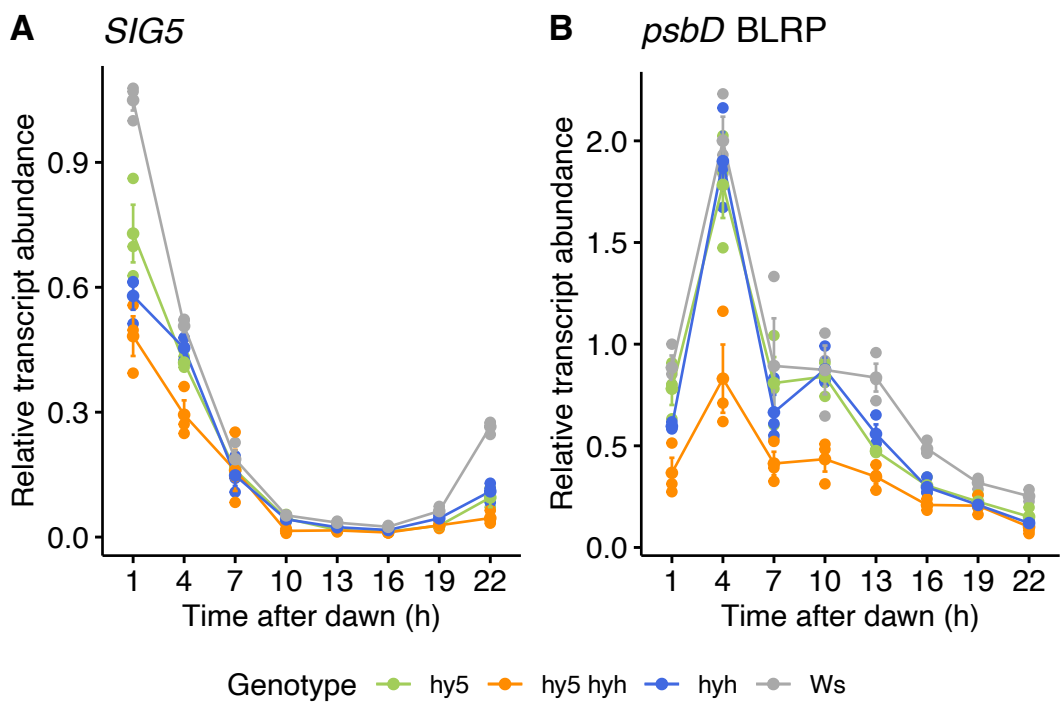

Figure 4.1 HY5 and HYH may act redundantly to induce *SIG5* and subsequent *psbD* BLRP expression in response to light. (A) *SIG5* and (B) *psbD* BLRP transcript abundance measured by qRT-PCR every 3 hours over a 24-hr period (12 h light/12 h dark) in wild type (Ws), *hy5*, *hyh* and *hy5 hyh* double mutant seedlings. Data are expressed as means of three independent biological replicates. Error bars indicate SEM (n=3, N≥10 seedlings).

psbD BLRP is a chloroplast-encoded regulatory target of SIG5. Its relative expression peaks later in the day than *SIG5* and declines at a slower rate (Figure 4.1). This later peak occurred in both the wild type and mutants included in the analysis. However, the peak abundance appears to be much lower in the *hy5 hyh* double mutant than in the wild type and the single *hy5* and *hyh* mutants, consistent with the pattern seen for *SIG5* expression (Figure 4.1). To confirm the observations of *SIG5* and *psbD* BLRP peak expression in each of the mutants, the meta2d function in the R package MetaCycle was used to analyse the periodicity of the data. Transcripts were classed as rhythmic if the BH.Q statistic from MetaCycle was less than 0.05. All genotypes had rhythmic *SIG5* oscillations, yet none of the genotypes had rhythmic oscillations in *psbD* BLRP transcripts. Irrespective of this, the lower peak of *SIG5* expression in all mutants (*hy5*, *hyh* and *hy5 hyh*) compared to wild type (Ws) is clear (Figure 4.2A). The peak expression of *psbD* BLRP was also confirmed to be markedly lower in the *hy5 hyh* double mutant than in the wild type (Figure 4.2B). In the single *hy5* and *hyh* mutants, the periodicity analysis identified no reduction in *psbD* BLRP peak expression

compared to wild type, although the identification of the transcripts as being arrhythmic means it is difficult to infer much from this analysis.

Therefore, HY5 and HYH might be acting with redundancy to regulate chloroplast gene expression in response to light, via SIG5. The data also indicate that other unidentified factors are involved in the light-regulation of *SIG5* and subsequent *psbD* BLRP transcription, as transcription of neither *SIG5* nor *psbD* BLRP is zero in the *hy5 hyh* double mutant.

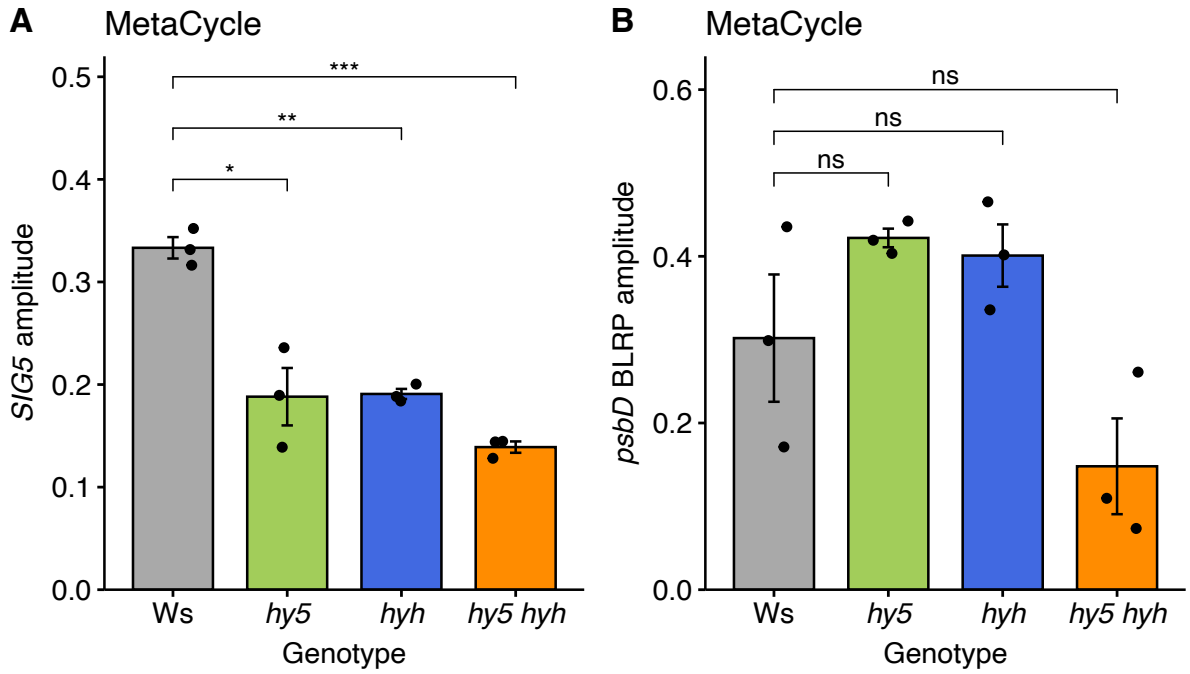

Figure 4.2 Comparison of (A) *SIG5* and (B) *psbD* BLRP peak expression in wild type (Ws), *hy5*, *hyh* and *hy5 hyh* mutants over 24 hrs in 12-hr light/12-hr dark conditions using the meta2d function in the R package MetaCycle. Transcript abundance measured by qRT-PCR every 3 hours over a 24-hr period of LD in wild type (Ws), *hy5*, *hyh* and *hy5 hyh* double mutant seedlings. Data are expressed as means of three independent biological repeats, circles on plot indicate individual data points. Error bars indicate SEM (n=3, N≥10 seedlings). ns = not significant, ** = p<0.01 *** = p<0.001 (one-way ANOVA followed by pairwise t-test with Benjamin & Hochberg adjustment in R).

HY5 and HYH might provide light input to the circadian oscillator

It is possible that HY5 and HYH affect the amplitude of *SIG5* transcript accumulation directly. Alternatively, or additionally, they might reduce the amplitude of the circadian clock which, in turn, reduces the amplitude of the oscillation of *SIG5* transcripts. To test this idea, accumulation of the circadian clock transcript *CCA1* was also measured in this experiment. *CCA1* expression peaks shortly after dawn, having an expression pattern similar to *SIG5*, suggesting common regulatory factors for *CCA1* and *SIG5* under light-dark conditions.

Similar to *SIG5* expression, the amplitude of *CCA1* oscillations appears to be reduced in the *hy5 hyh* double mutant compared to wild type, although this difference isn't statistically significant (Figure 4.3B). Hajdu et al. (2018) report that, through regulation of *HY5* transcript accumulation, light quality signals act to entrain the circadian oscillator.

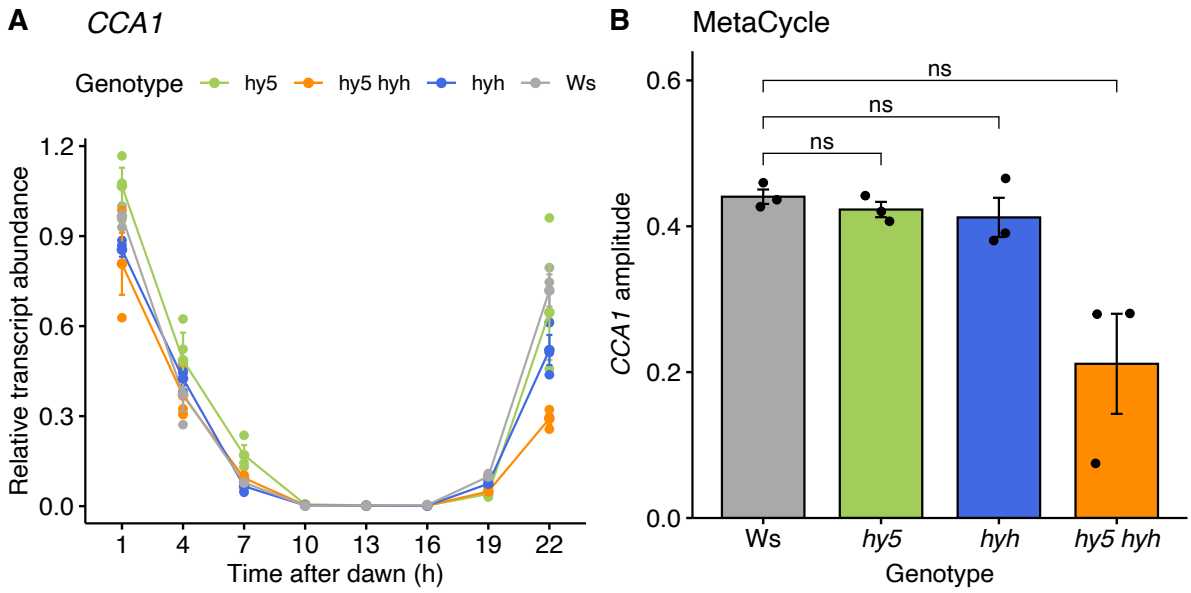

Figure 4.3 (A) *CCA1* relative transcript abundance, measured by qRT-PCR every 3 hrs over a 24-hr period (12 h light/12 h dark) in wild type (Ws), *hy5*, *hyh* and *hy5 hyh* double mutant seedlings. (B) Analysis of *CCA1* amplitude using the meta2d function in the R package MetaCycle. Data are expressed as means of three independent biological replicates, circles on plot indicate individual data points. Error bars indicate SEM (n=3, N≥10 seedlings). ns = not significant (one-way ANOVA followed by pairwise t-test with Benjamin & Hochberg adjustment in R).

CCA1 and *SIG5* transcript abundance strongly correlate in diel conditions

Under continuous light conditions, a strong correlation between *CCA1* and *SIG5* oscillations is maintained between the wild type and mutants in *HY5* and *HYH* (Figure 3.7). This suggested that HY5/HYH do not regulate the period of *SIG5* oscillations downstream of CCA1. A similar correlation analysis was conducted between *CCA1* and *SIG5* transcripts under light/dark conditions. It was my aim to examine whether this strong correlation was maintained under light/dark conditions in wild type and mutants, to highlight whether HY5/HYH are required to maintain the coordination of *SIG5* and *CCA1* transcription under these conditions.

The strength of the correlation between *CCA1* and *SIG5* expression is not weakened in any of the mutants – the p-values indicate a very statistically significant relationship between *CCA1* and *SIG5* in all the genotypes (Figure 4.4). In continuous light conditions, *CCA1* and *SIG5* expression are most strongly correlated in wild type, *hy5* and *hyh*, and display a marginally weaker correlation in *hy5 hyh* (Figure 3.7). The fact that the strong relationship is maintained under diel cycles in *hy5 hyh* might suggest that diel cycles of regulation of *SIG5* by the circadian oscillator are independent from HY5 and HYH under these conditions. It is likely that HY5/HYH act only on the maintenance of *SIG5* amplitude, rather than periodicity.

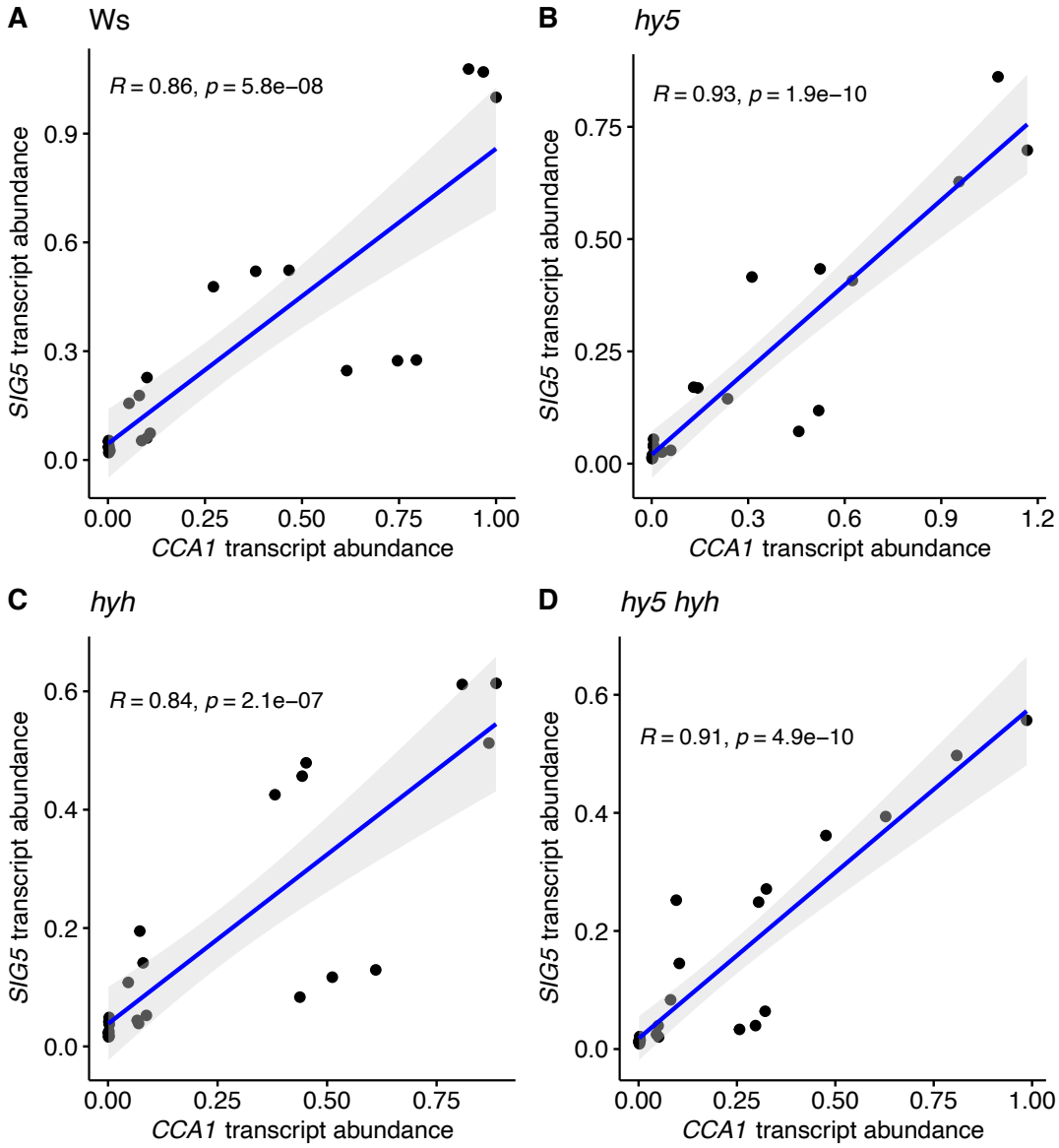

Figure 4.4 Relationships between *CCA1* and *SIG5* relative transcript abundance showing Pearson correlation coefficients (R values) in (A) wild type (Ws), (B) *hy5* mutants, (C) *hyh* mutants and (D) *hy5 hyh* double mutants under light-dark conditions.

T-DNA mutations in *ATHB17* do not impact the induction of *SIG5* and subsequent *psbD* BLRP transcription around dawn

athb17 mutant seed stocks obtained from NASC (SALK_095524/*athb17-1* and SALK_134535/*athb17-2*) were initially genotyped using a PCR to confirm the presence of a T-DNA insertion and determine the genetics of each mutant (i.e., whether homozygous or heterozygous). This genotyping PCR confirmed the presence of the T-DNA insertion in both *athb17-1* and *athb17-2*, identifying both lines as homozygous (both chromosomes of each line contain the insertion). Reactions with LB and RP primers gave products for both mutant lines, but not for the wild type, confirming the presence of the T-DNA insertion but not confirming the homo-/heterozygosity of each. Reactions with LP and RP primers confirmed that both lines are homozygous, as a PCR product was present for the wild type but not for either mutant (if the lines were heterozygous, we would also expect a product with these primers).

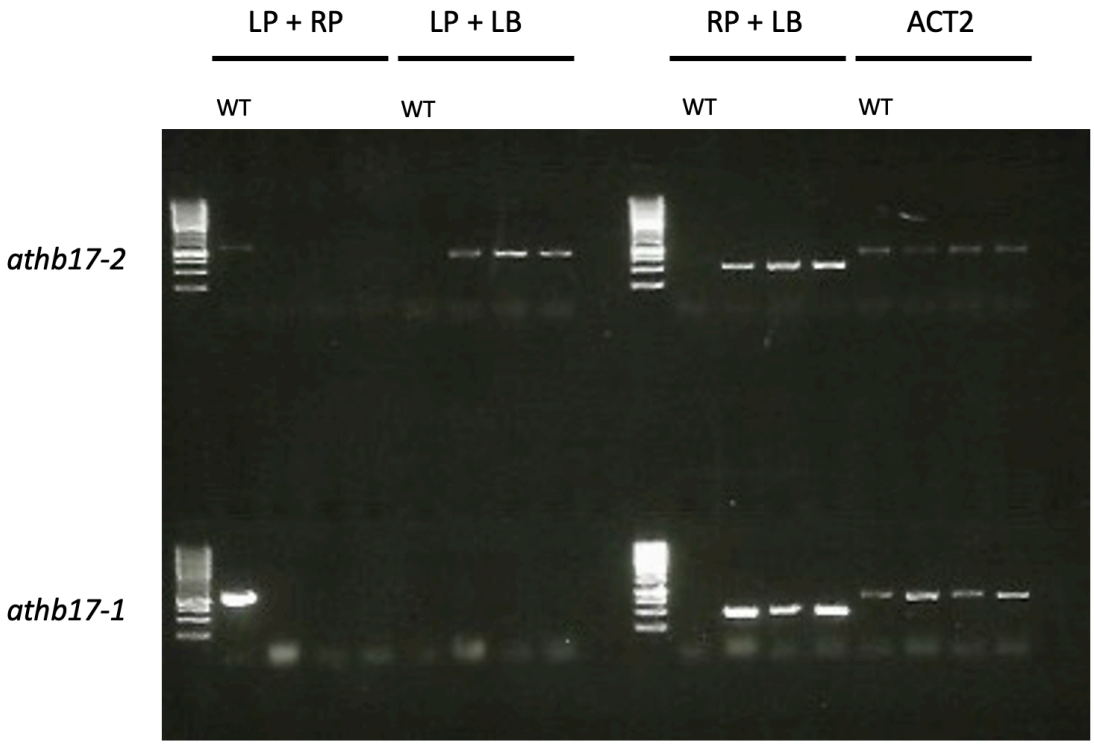

Figure 4.5 *athb17-1* and *athb17-2* confirmed as being homozygous T-DNA insertion mutants by genotyping PCR. For each set of primers, the far left well contains DNA from wild type seedlings, and the following three wells contain DNA from mutant seedings (*athb17-1* or *athb17-2*, as indicated). Reactions with right primer (RP) and left border (LB) primer sets gave a product for both mutants (but not wild type), indicating the presence of the T-DNA insertion. Reactions with left primer (LP) and right primer (RP) sets gave a product for wild type but neither of the mutants, confirming the homozygosity of each line.

SIG5 transcript abundance peaks just after dawn. To initially identify whether ATHB17 plays a role in this dawn-induction of *SIG5* transcription, wild type, *athb17-1* and *athb17-2* mutant seedlings grown under 12-hr light/12-hr dark conditions were sampled around dawn at 11 days old. *SIG5* and *psbD* BLRP transcript abundance in the wild type and mutants was then measure by qRT-PCR. *SIG5* was induced in all genotypes equally, hence ATHB17 is not involved in the light induction of *SIG5* (nor *psbD* BLRP) transcription (Figure 4.6).

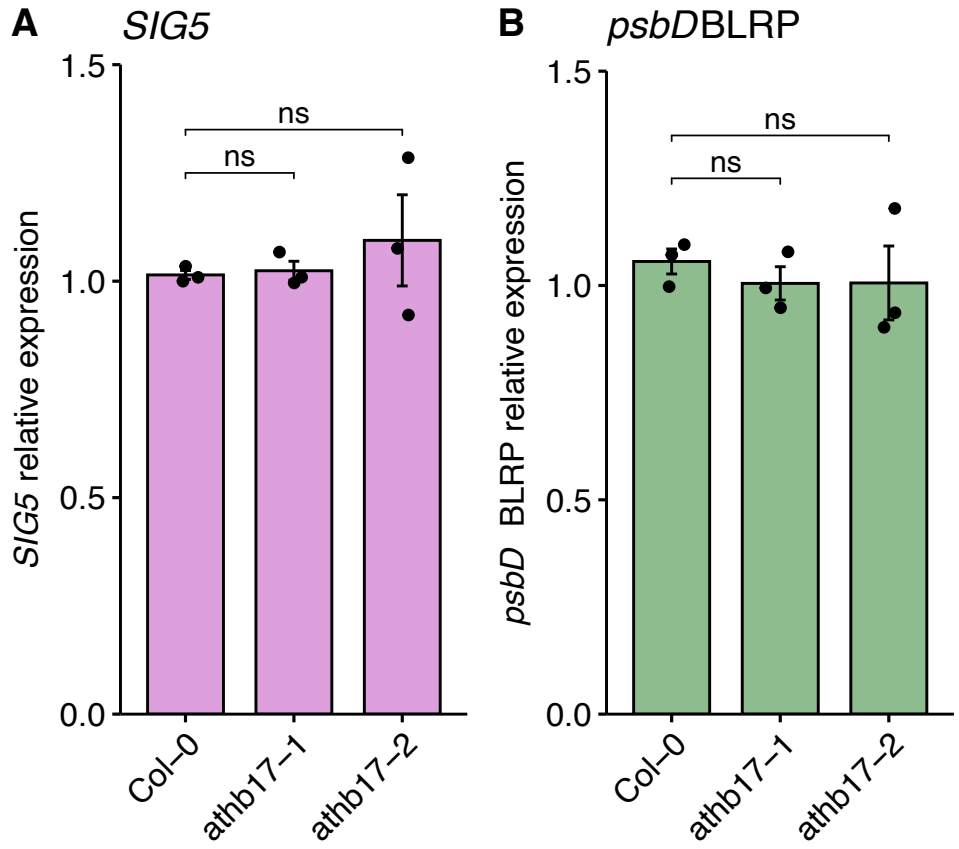

Figure 4.6 ATHB17 is not involved in the light-induction of (A) *SIG5* or (B) *psbD* BLRP transcription around dawn. Transcript abundance of (A) *SIG5* and (B) *psbD* BLRP determined by qRT-PCR in 11-day-old wild type (Col-0) and *athb17* mutant seedlings (SALK_095524/*athb17-1* and SALK_134535/*athb17-2*) sampled 1 hr after dawn. Data are expressed as means of three independent biological repeats. Error bars represent SEM (n=3, N≥10 seedlings). ns, not significant (two-tailed t-test in R).

ATHB17 is not involved in SIG5-mediated low temperature signalling to chloroplasts

To investigate potential roles for ATHB17 in signalling low temperature information to chloroplasts through SIG5, wild type (Col-0) and *athb17* mutant seedlings (SALK_095524/*athb17-1* and SALK_134535/*athb17-2*) were subjected to a low temperature treatment and *SIG5* and *psbD* BLRP induction were assessed.

After three hours of cold (4°C) exposure, given to seedlings one hour after dawn, *SIG5* transcript accumulation was measured by qRT-PCR. As expected, *SIG5* was induced strongly in response to cold exposure in the wild type (Figure 4.7A); this is consistent with prior research (Nagashima et al., 2004, Cano-Ramirez et al., under revision). Compared to the control treatment (ambient, 19°C), *SIG5* induction increased around three-fold in all three genotypes (Figure 4.7A). Previous work had demonstrated that three hours of cold exposure was sufficient to observe the effects of chilling on *SIG5* transcription (Cano-Ramirez et al., under revision). As *SIG5* was strongly induced in both *athb17* T-DNA insertion mutants used, this suggests ATHB17 does not participate in the response of *SIG5* transcripts to cold under these experimental conditions.

After 5 hours of cold exposure, *psbD* BLRP relative transcript abundance was measured by qRT-PCR. Measurements of *psbD* BLRP expression were taken later than for *SIG5* induction to allow time for transmission of the low temperature signal (Noordally et al., 2013, Belbin et al., 2017). The data show no statistically significant difference in *psbD* BLRP induction between the cold and ambient conditions after 5 hours of continuous cold exposure (Figure 4.7B), although there was a numerical increase in the transcript abundance. This is true in the wild type seedlings and both *athb17-1* and *athb17-2* mutants. The absence of a significant response of *psbD* BLRP transcripts is not consistent with the increased *SIG5* induction following cold exposure (Figure 4.7A), or with previous work which demonstrates the cold-induction of *psbD* BLRP (Nagashima et al., 2004, Cano-Ramirez et al., under revision). It is possible that the measurements were taken too soon after cold exposure or the cold treatment of the seedlings was uneven, therefore yielding variable data. Furthermore, it has been identified that a chilling treatment of 9 hours before sampling is required for the cold-induction of *psbD* BLRP transcription (when relative transcript abundance differs significantly to ambient control) (Cano-Ramirez et al., under revision). Hence, a longer period of time between chilling and sampling for the measuring of *psbD* BLRP transcript abundance by qRT-PCR might be considered before complete conclusions can be drawn concerning the role of ATHB17 in the response of chloroplast *psbD* BLRP to chilling.

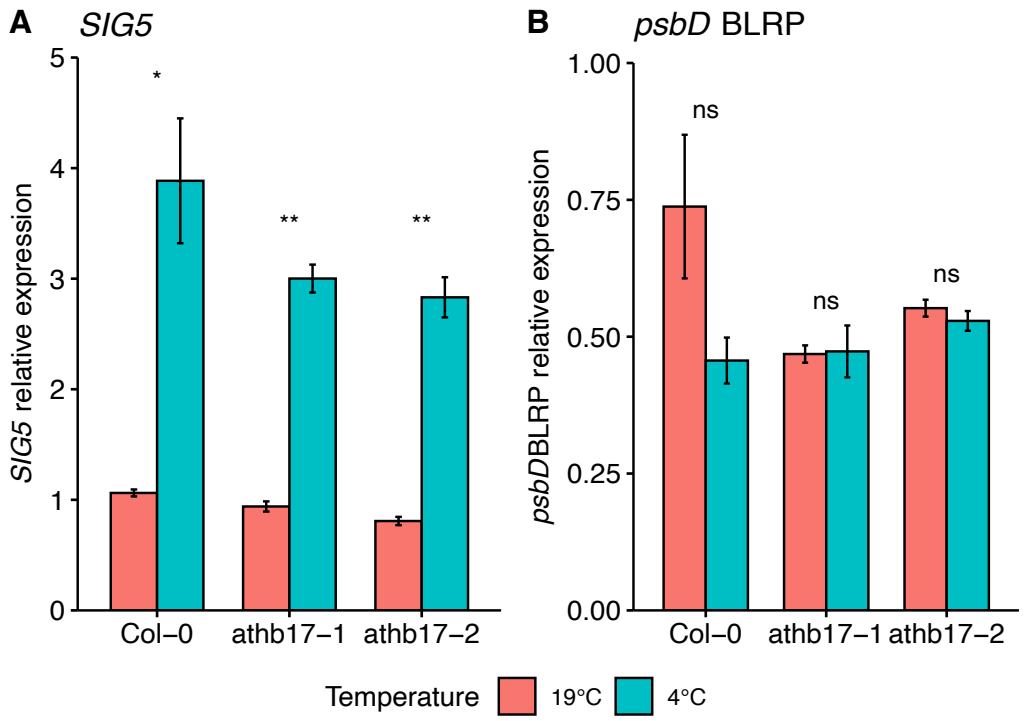

Figure 4.7 ATHB17 is not involved in inducing *SIG5* and *psbD* BLRP in response to cold stress. (A) *SIG5* is induced strongly in response to low temperature in all genotypes; (B) subsequent *psbD* BLRP induction does not occur after 5 hours of cold exposure. Transcript abundance of (A) *SIG5* and (B) *psbD* BLRP determined by qRT-PCR in wild type (Col-0) and *athb17* mutant seedlings (SALK_095524/*athb17-1* and SALK_134535/*athb17-2*). 11-day-old seedlings were exposed to (A) 3 hours or (B) 5 hours of cold (4°C) before sampling. Data are expressed as means of three independent biological repeats. Error bars represent SEM (n=3, N≥10 seedlings). Statistical significance is of chilling treatments (4°C) compared with ambient temperature control (19°C). ns, not significant, *, $p < 0.05$, **, $p < 0.01$ (two-tailed t-test in R).

Discussion

HY5 transcription is tightly regulated by light conditions. HY5, HYH, B-BOX DOMAIN PROTEIN 21 (BBX21) and CALMODULIN 7 (CAM7) all directly bind to the *HY5* promoter in response to light, thereby upregulating its transcription (Holm et al., 2002, Binkert et al., 2014, Xu, 2019, Abbas et al., 2014). In the dark, HY5, HYH and other promoters of photomorphogenesis are degraded by the COP1-SPA-E3 ubiquitin ligase complex (Holm et al., 2002, Lu et al., 2015), and the regulation of HY5 phosphorylation by light controls HY5 protein stability and activity (Hardtke et al., 2000). Hence, HY5 is a key transcription factor in light signalling pathways, and its homologue HYH has been implicated in having overlapping roles with HY5.

The work described in this chapter identifies HY5 and HYH, but not ATHB17, as acting upstream of *SIG5* in the light-responsive signal transduction pathway. It is logical that ATHB17 is not involved in light signalling as, under non-stressed conditions, it is primarily expressed in the root (Park et al., 2013, Zhao et al., 2017). Only under stressful conditions is *ATHB17* upregulated in the leaves (Zhao et al., 2017). Nonetheless, I decided to test this for myself, to confirm these conclusions under my specific experimental conditions, and to enable direct comparisons between the involvement of HY5/HYH and ATHB17 under these conditions.

Previous work has identified roles for HY5 in controlling *SIG5* transcription in response to light (Mellenthin et al., 2014, Nagashima et al., 2004), hence my work helps to elucidate roles for HYH in this signalling pathway. Nonetheless, the data confirm that other factors are involved in inducing *SIG5* and *psbD* BLRP expression in response to light, as transcription of the genes still occurs, albeit to a lesser extent, in the *hy5 hyh* double mutant. In Section 3, I suggested that HY5 and HYH might act with redundancy to regulate *SIG5* and subsequent *psbD* BLRP gene transcription under free-running circadian conditions. It is possible that a similar redundancy occurs in the light-regulation of *SIG5* transcription, as transcripts of both *SIG5* and *psbD* BLRP have the lowest level of expression in the *hy5 hyh* double mutant compared to wild type and *hy5* and *hyh* single mutants (Figure 4.2).

Furthermore, HY5 and HYH might contribute to the maintenance of *CCA1* expression in response to light conditions, as the amplitude of *CCA1* expression under LD conditions appears to decrease in the *hy5 hyh* double mutant compared to wild type (Figure 4.3B). Particularly, the pre-dawn induction of *CCA1* is not as pronounced in *hy5 hyh* than in the wild type and single mutants (Figure 4.3A). This could be due to a direct or indirect interaction of these transcription factors with *CCA1*. For example, HY5/HYH are known to bind EEs in the promoters of evening-expressed oscillator genes, thereby decreasing their levels of transcription. In the double *hy5 hyh* mutant, this reduction of the transcription of these genes by HY5 and HYH cannot occur, leading to an increase in abundance of transcripts with EEs in their gene promoters. These proteins generally act to repress *CCA1* transcription, thus there could be an increased repression of *CCA1* transcription in the *hy5 hyh* mutant, which would explain the results in Figure 4.3. However, the short length of the

timecourse conducted under diel conditions (only 24 hours) makes it difficult to confidently conclude this.

Transcription factors are important for the communication of information between nucleus and chloroplasts. SIG5 is a multiple stress-responsive sigma factor that acts to control transcription from the *psbDC* operon on the chloroplast genome that encodes PS II core proteins, hence SIG5 might act to repair PS II in response to stress. This is suggested by reduced photosynthetic efficiency in *sig5* mutants compared with the wild type when exposed to very high light (Nagashima et al. 2004). ATHB17 regulates *SIG5* in response to salt stress, binding directly to the *SIG5* promoter to modulate its transcription (Zhao et al., 2017). ATHB17 has been identified to be multiple stress-responsive (being induced by ABA, NaCl, mannitol and paraquat) (Zhao et al., 2017), but whether this transcription factor is involved in regulating *SIG5* in response to cold temperatures had not been investigated before now.

Our current understanding identifies the bZIP transcription factors HY5 and HYH as required for low temperature responses of *SIG5* transcripts (Cano-Ramirez et al., under revision). Here, I identify that ATHB17 does not regulate the expression of *SIG5* in response to cold. Nonetheless, obtaining measurements of *psbD* BLRP expression 5 hours after the cold treatment may have still been too soon to see any downstream effects of the treatment on the expression of this chloroplast gene. Work conducted by Cano-Ramirez et al. (under revision) indicates that a chilling treatment of 9 hours is required to see a significant difference in *psbD* BLRP relative transcript abundance between the chilling treatment and ambient control in the wild type. As my data show no difference in *psbD* BLRP transcripts between the cold and ambient conditions in the wild type after 5 hours of cold exposure (Figure 4.7), further experiments should be conducted in which the time between chilling and sampling for *psbD* BLRP transcript analysis is extended, confirming the conclusions drawn here about the contribution of ATHB17 to the chilling response of *psbD* BLRP transcription.

ATHB17 expression is induced significantly in the leaves upon exposure to the stressful conditions listed above (Zhao et al., 2017). It is likely, given the data presented in this chapter, that this transcription factor is not induced in the leaves in response to low temperature stress, and hence communicates information to *SIG5* about specific, rather

than broad, stress signals. This specificity of stress signalling may be important for SIG5 to integrate light, temperature, stress, and circadian information from different transcription factors, generating appropriate responses in the chloroplast. Using an Arabidopsis eFP browser at bar.utoronto.ca (Winter et al., 2007), I identified from a microarray analysis of the global transcriptome stress response in Arabidopsis that *ATHB17* is upregulated in neither the shoot nor the root in response to cold stress (Kilian et al., 2007) (Figure 4.8)

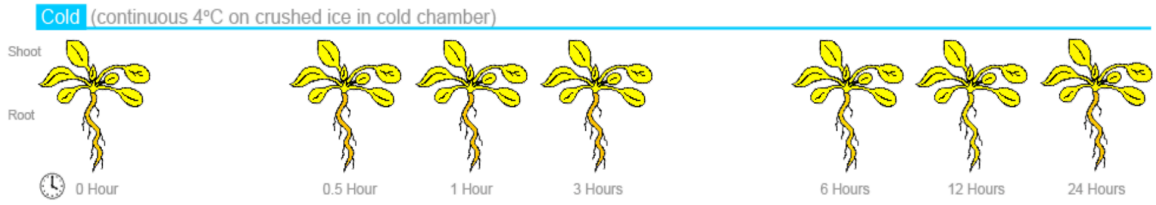

Figure 4.8 Expression of *ATHB17* in the root/shoot over a 24-hr period of cold exposure in wild type. Data obtained from a microarray analysis of the global transcriptome stress response, conducted by Kilian et al. (2007). Figure reproduced from bar.toronto.ca Arabidopsis eFP browser (Winter et al., 2007).

To confirm the data from the global transcriptome microarray analysis by Kilian et al. (2007), that *ATHB17* is not upregulated in the leaves upon chilling stress, a GUS reporter assay using an *ATHB17* promoter-GUS reporter construct, analogous to that conducted in Zhao et al. (2017), could be used, assessing the expression pattern of *ATHB17* following cold exposure.

Based on the identified role of HY5 in cold signalling to SIG5 (Cano-Ramirez et al., under revision), other potential novel candidates to test in the low temperature signalling cascades modulating *SIG5*/*psbD* BRLP transcription include BBX proteins. BBX proteins can both enhance (Job et al., 2018, Datta et al., 2008, Xu et al., 2016) and repress (Xu, 2019, Gangappa et al., 2013, Job et al., 2018, Lin et al., 2018) the transcriptional activity of HY5, suggesting BBXs act as co-regulators of HY5 and HYH. Furthermore, given the antagonistic roles of HY5 and PIFs in their regulation of target gene expression (Gangappa and Kumar, 2017, Toledo-Ortiz et al., 2014), the role of PIFs in low temperature signalling to SIG5 and *psbD* BLRP should also be investigated.

HY5 and HYH contribute to the light-dependent accumulation of *SIG5* transcripts (Figure 4.1) (Nagashima et al., 2004, Mellenthin et al., 2014), although they are not the only factors responsible for this light regulation (Figure 4.1). There is independence of the stress and light responsiveness of *SIG5* (Nagashima et al., 2004), thus different transcription factors

and signal transduction pathways appear to converge to modulate *SIG5* transcription in response to different stresses and to variations in the light environment (Zhao et al., 2017, Cano-Ramirez et al., under revision, Belbin et al., 2017). This convergence of signalling processes at the promoter of *SIG5* supports the idea that *SIG5* is an integrator of signals that enables the chloroplast to adapt to changing and converging stimuli.

The *hy5 hyh* double mutant fails to completely eradicate rhythms in *SIG5* expression under free-running circadian conditions – the amplitude of expression is simply reduced compared to the wild type (Figure 3.3). *ATHB17* transcripts are rhythmic under certain conditions (Mockler et al., 2007), so ATHB17 might be responsible for maintaining the rhythmic expression of *SIG5*, as the transcription factor has been identified to bind the *SIG5* promoter (Zhao et al., 2017). In order to test this, a circadian timecourse experiment similar to that conducted in Section 3.1 could be conducted, using mutants in ATHB17, as well as a triple *hy5 hyh athb17* mutant. If the rhythmicity of *SIG5* transcripts were conserved in the triple mutant, a factor other than HY5, HYH or ATHB17 is responsible for maintaining this rhythm. However, if *SIG5* rhythmicity were absent in the triple mutant, ATHB17 contributes to the maintenance of these rhythms in *SIG5* transcription. As ATHB17 is not induced in the leaves under normal, non-stressed conditions, and its expression seems to be mainly localised to the roots, there may be limited roles for ATHB17 in this, but it would be an interesting avenue to rule out, nonetheless.

In summary, the work in this chapter provides insights into potential roles for HYH in light signalling to *SIG5* and *CCA1*, and there is still much work to be done to identify the separate contributions of HY5 and HYH to these pathways. For example, it would be informative to conduct similar experiments to those described in this chapter, but under different wavelengths of light, such as red and blue light, to further understand the relative contributions and roles of HY5/HYH in light signalling to *SIG5* and *CCA1*. I also investigated whether ATHB17 might regulate *SIG5* in response to cold stress, and my data indicates that ATHB17 does not have a role in this process. It seems likely that ATHB17 communicates information to *SIG5* about specific stress signals, hence induction only occurs in response to these specific stress signals (Zhao et al., 2017). Further work should confirm that *ATHB17* Is not induced in the leaves upon exposure to cold stress, using GUS reporter assays. Circadian timecourse experiments using *athb17* mutants would also be useful to explore further

potential roles for ATHB17 in its circadian regulation of *SIG5* transcription. The role of other potential novel candidates in this low temperature signalling pathway, such as BBX proteins and PIFs, should also be investigated.

Chapter 5 General Discussion

Circadian rhythms in biological processes are pervasive throughout living organisms, persisting in plants, animals, fungi, cyanobacteria and non-photosynthetic prokaryotes (Eelderink-Chen et al., 2021). These 24-hr cycles, generated through cellular circadian oscillators, enable the accurate prediction of changes to environmental stimuli, and hence the appropriate coordination of biological processes with these fluctuations. Circadian oscillators present both a daily and seasonal advantage to organisms, important for their fitness, growth and survival (Young and Kay, 2001, Dodd et al., 2005).

In plants, the coordination of circadian oscillations with environmental signals, such as light and temperature, facilitates appropriate responses to these signals depending on both the time of day and season. As photosynthesis is essential for plant growth and survival, the coordination of chloroplast transcript accumulation with both the circadian oscillator and environmental stimuli is required to maximise plant fitness. The expression of chloroplast genes with photosynthetic functions is controlled by the association of sigma factors (nuclear-encoded proteins) with the RNA polymerase PEP. This association confers chloroplast promoter specificity to PEP, leading to the transcription initiation of specific chloroplast genes. Sigma factors are believed to have evolved through gene transfer from the ancestral chloroplast genome during higher plant evolution (Kanamaru et al., 1999). They provide a means by which chloroplast gene expression can be specifically fine-tuned to environmental and circadian stimuli.

SIG5 regulates the accumulation of transcripts from the *psbDC* operon on the chloroplast genome in response to both blue light and environmental stresses. Here, I focussed on SIG5 regulation of the blue-light responsive promoter of *psbD* (*psbD* BLRP), which encodes the light-labile D2 protein of PSII (Nagashima et al., 2004). SIG5 is important in stress tolerance in plants, and integrates circadian cues with a range of environmental signals to regulate chloroplast gene transcription (Nagashima et al., 2004, Cano-Ramirez et al., under revision).

Extensive roles for HY5 and HYH have been identified in the regulation of target gene transcription in response to a range of stimuli (Lee et al., 2007, Cano-Ramirez et al., under revision). *SIG5* is a high confidence binding target of HY5 (Burko et al., 2020), and the transcript abundance of *SIG5* has been shown to be greatly reduced in HY5-deficient lines

(Mellenthin et al., 2014). Recent work has identified that HY5 and HYH act with redundancy to regulate the transcription of *SIG5* in response to cold, hence contributing to this abiotic stress tolerance conferred by SIG5 (Cano-Ramirez et al., under revision). Given that HY5 and HYH act upstream of SIG5 in various pathways modulating *SIG5* transcription, I tested the hypothesis that these two transcription factors are involved in regulating the circadian rhythmicity of *SIG5* transcription. I also aimed to further examine these signal transduction pathways to elucidate the distinct, complementary, and overlapping roles for HY5 and its homologue HYH in both light and circadian signalling to *SIG5*.

Tolerance to abiotic stresses is important to prevent photosynthetic machinery from being damaged. The relevancy of low temperature signals at different times of day is determined by information from circadian oscillators and by light quality signals, through processes such as circadian gating (Fowler et al., 2005, Hotta et al., 2007, Cano-Ramirez et al., under revision). Hence, the integration of these signals is paramount to ensure the correct responses of chloroplast gene transcription. *SIG5* is responsive to all of these stimuli and integrates various signals to generate appropriate chloroplast responses. For example, in the dark, the accumulation of *psbD* BLRP transcripts in response to chilling does not occur; low temperature signalling to this chloroplast gene by SIG5 occurs only in light conditions (Cano-Ramirez et al., under revision). SIG5 is essential for maintaining photosynthetic efficiency following chilling (Cano-Ramirez et al., under revision), hence understanding the factors upstream of this signalling pathway is important for our overall understanding of the chloroplast chilling response. As ATHB17 is a multiple stress-responsive transcription factor and regulates *SIG5* transcription in response to salt stress (Zhao et al., 2017), this transcription factor may also be involved in communicating low temperature information to SIG5.

The roles of HY5 and HYH in SIG5-mediated circadian signalling to chloroplasts

SIG5 is involved in communicating circadian timing information to the chloroplast genome, and HY5/HYH are involved in modulating *SIG5* transcription (Nagashima et al., 2004, Brown and Jenkins, 2008, Catalá et al., 2011, Belbin et al., 2017). I identified roles for the bZIP

transcription factors HY5 and HYH in the circadian regulation of *SIG5* and *psbD* BLRP transcription by conducting circadian qRT-PCR timecourses, outlined in section 3.1.

Initially, in order to inform these complex circadian timecourses, I sampled wild type, *hy5*, *hyh*, *hy5 hyh*, *cop1-4* and *sig5-3* seedlings for *SIG5* and *psbD* BLRP relative transcript abundance around dawn in 11-day-old Arabidopsis seedlings. *SIG5* relative expression was significantly lower in *hy5 hyh* than in wild type (Figure 3.1A). No other mutants presented transcript levels significantly different to wild type. Nonetheless, I identified a numerical reduction in *SIG5* transcript abundance between wild type and single *hy5* and *hyh* mutants, suggesting that measurements taken at different timepoints throughout the day could describe a significant relationship between *SIG5* transcript abundance and HY5/HYH. These initial findings provided an indication of some redundancy between the roles of HY5 and HYH in their control of *SIG5* transcription, signifying that circadian timecourse experiments in mutants of these transcription factors would further clarify their roles in the circadian modulation of *SIG5* transcription. Further, *SIG5* transcripts were absent in *sig5-3*, confirming the mutant genotype (Figure 3.1B).

The transcript abundance of *psbD* BLRP was significantly lower in *hy5* and *hy5 hyh*, and significantly higher in *cop1-4*, than in the wild type (Figure 3.1). Again, these results indicated a level of redundancy between HY5 and HYH in *psbD* BLRP transcription regulation, suggesting a dominant role for HY5 in this redundancy. Nonetheless, as transcripts of both *SIG5* and *psbD* BLRP remained present in *hy5 hyh*, albeit at a low level, factors other than HY5 and HYH are involved in modulating their transcription. Furthermore, in the *sig5-3* mutant, *psbD* BLRP transcripts were also present, while *SIG5* transcripts were absent. This indicated that factors other than SIG5, by either interacting with or acting independently of SIG5, are involved in regulating *psbD* BLRP transcription. Hence, it is possible that HY5/HYH are additionally acting independently of, or downstream of, SIG5 to regulate *psbD* BLRP transcription.

Next, I designed and implemented circadian qRT-PCR timeseries experiments using *hy5*, *hyh* and *hy5 hyh* mutants, sampling for *SIG5* and *psbD* BLRP transcripts. The experimental design (Figure 3.2) involved sampling over a 48-hr period for *SIG5* and *psbD* BLRP in both phase and anti-phase plants, which, taken together, yielded 72-hr timeseries. The data identified that HY5 and HYH redundantly regulate the transcription of *SIG5* and *psbD* BLRP under free-

running circadian conditions (Figure 3.3; Figure 3.4). The amplitude of expression in the *hy5 hyh* double mutant was significantly lower than wild type and single *hyh* mutants, but comparable to *hy5* mutants, for both *SIG5* and *psbD* BLRP (Figure 3.3; Figure 3.5). Hence, the roles for HY5 in the circadian signalling to *SIG5* may be greater than the roles for HYH. These data indicate that HY5/HYH might be positioned between the circadian oscillator and *SIG5* in this signal transduction pathway. Alternatively, these transcription factors may facilitate the conferring of rhythmicity to *SIG5* through their association with other factors, such as CCA1 – a central circadian oscillator component.

HY5 regulates processes within the chloroplast such as photopigment biosynthesis (Toledo-Ortiz et al., 2014), and both HY5 and HYH are involved in regulating *SIG5* in response to light (Figure 4.1) (Nagashima et al., 2004, Mellenthin et al., 2014). Hence, it is possible that HY5 and HYH both influence the expression of chloroplast genes through various different mechanisms, and that by regulating *SIG5* transcript accumulation they integrate different environmental signals that are subsequently conveyed to chloroplasts.

psbD BLRP, downstream of SIG5, had a longer period in *hy5* mutants and a later phase in *hy5 hyh* mutants, but maintained rhythmicity in both. However, in *hyh*, transcripts of *psbD* BLRP were arrhythmic (Table 3.2), contrary to the pattern of *SIG5* transcript accumulation. Previous research has identified that the chilling response of *psbD* BLRP occurs only in the presence of light, whereas this response occurs in *SIG5* in both light and dark conditions. Furthermore, the chilling responses of *SIG5* and *psbD* BLRP in *hy5* and *hyh* were different to one another (Cano-Ramirez et al., under revision). Taken together, these findings suggest that HY5, and particularly HYH, influence the expression of *psbD* BLRP downstream of SIG5, doing so in distinctive ways. Therefore, there may be non-redundant roles between HY5 and HYH in this signalling pathway, downstream of SIG5 and upstream of *psbD* BLRP. For example, HY5/HYH could regulate the import of proteins into the chloroplast, affecting the levels of accumulation of SIG5 in the chloroplast, and hence indirectly influencing *psbD* BLRP transcription.

I aimed to confirm the results from the described qRT-PCR transcript analyses by examining the activity of the *SIG5* promoter over a period in continuous light in wild type, *hy5*, *hyh* and *hy5 hyh*. In order to do this, I used microprojectile bombardment to transiently transform Arabidopsis plant tissue with a *SIG5::LUC* construct at the single cell level. The construct was

comprised of the *SIG5* promoter fused to the *LUCIFERASE+* coding sequence (Noordally et al., 2013). When the *SIG5* promoter was activated, cells transformed with the construct emitted bioluminescence, as luciferase catalyses the emission of bioluminescence from luciferin. This could then be detected using a photon counting imaging system.

The protocol required optimisation for it to work for my purposes; this method development is outlined in section 3.2.2. Having developed the method to a level that was satisfactory, i.e., sufficient to generate detectable bioluminescence in wild type Arabidopsis seedlings, I then used this method in a circadian timecourse experiment. The final experimental design is described in section 2.10.

In this experiment, I transformed wild type, *hy5*, *hyh* and *hy5 hyh* with the *SIG5::LUC* construct and imaged the plants for a period of ~55 hours. The bioluminescence signal detected from the wild type seedlings was significantly stronger that that emitted by any of the mutants, and exhibited rhythmic oscillations over the timeseries (Figure 3.15). *SIG5* promoter activity in *hy5* and *hy5 hyh* mutants appeared to be arrhythmic, but there did appear to be some rhythmicity of *SIG5* promoter activity in *hyh*. These results, although not as robust as I had hoped, supported the findings from the qRT-PCR timecourse experiments which suggested redundant roles for HY5 and HYH in the circadian regulation of *SIG5* transcription, as well as a level of HY5 dominance in this regulation. Future work should aim to further develop this method to increase transformation rates, and to repeat the experiment over a longer period. The results from this longer timecourse would reveal more about the roles of HY5 and HYH in the circadian regulation of *SIG5* promoter activity and hence in the transcription of *SIG5*, by demonstrating whether the oscillations in bioluminescence are maintained beyond 55 hours in continuous light.

HY5/HYH provide both input to and output from the circadian oscillator

Further to these experiments, I conducted a circadian qRT-PCR timeseries sampling for transcripts of the circadian oscillator gene *CCA1*, following the experimental design outlined in Figure 3.2. Transcription of circadian oscillator components, such as *CCA1*, *LHY*, *PRR9* and *ELF4*, is light-regulated (Li et al., 2011, Hajdu et al., 2018). HY5 binds to ACEs in the promoters of these and other light-responsive genes, and is therefore involved in light

signalling to the circadian oscillator (Lee et al., 2007). As mentioned, a physical interaction occurs between CCA1 and HY5 at the promoters of certain shared target genes (Andronis et al., 2008). Further, *CCA1* and *SIG5* are both morning-phased and their transcript abundance is tightly regulated under circadian conditions (Figure 3.7), yet it has been reported that CCA1 does not directly interact with the *SIG5* promoter (Nagel et al., 2015). Thus, it was my aim with this work to discover whether HY5/HYH are involved in maintaining the correlation between *CCA1* and *SIG5* oscillations, and to identify positions for these transcription factors in this signalling pathway.

The period of *CCA1* oscillations over the timeseries was slightly shortened in the *hy5 hyh* mutant (Figure 3.6B), which is consistent with the results of previous experiments (Hajdu et al., 2018) and suggests that HY5/HYH provide some light input to the circadian oscillator. Under white light conditions, Hajdu et al. (2018) reported a period shortening of *CCA1* expression in both *hy5* and *hy5 hyh*. The results reported in chapter 3 show a significant reduction in *CCA1* period compared to wild type only in *hy5 hyh* (not in *hy5* or *hyh* single mutants), suggesting HY5 and HYH provide light input to the circadian oscillator in a redundant manner. Under blue light conditions, Hajdu et al. (2018) report a stronger phenotype than under white light conditions, with significant period shortening described in *hy5*, *hyh* and *hy5 hyh* mutants. Hence, they conclude that HY5 and HYH provide light input to the circadian oscillator, primarily regulating the expression of oscillator components under blue light conditions.

Furthermore, SIG5-mediated circadian signalling to *psbD* BLRP has been reported to depend primarily on blue light signalling (Belbin et al., 2017). This is because, under R light conditions, *SIG5* oscillates with low amplitude but *psbD* BLRP is arrhythmic. It has been proposed that there is a threshold level of *SIG5* transcript abundance that must be reached before *psbD* BLRP transcripts oscillate rhythmically (Noordally et al., 2013). Therefore, it is likely that the low amplitude of *SIG5* oscillations under R light is insufficient to generate circadian oscillations in *psbD* BLRP (Belbin et al., 2017). Taken together, the dependence of *SIG5* and *psbD* BLRP rhythmicity on blue light signalling, and the regulation by HY5/HYH of the oscillator under primarily blue light conditions, suggest that *SIG5* and *psbD* BLRP rhythmicity depend on the regulation of oscillator components by HY5/HYH. Future work should focus on understanding if this mutual dependence on blue light for circadian

signalling translates to a dependence of *SIG5*/*psbD* BLRP on HY5/HYH under free-running circadian conditions.

There is a reduced amplitude of *SIG5* oscillations in *hy5 hyh*, but *CCA1* amplitude is unaffected in this mutant. This indicates that HY5/HYH also act downstream of the circadian oscillator to maintain the amplitude of *SIG5* oscillations. Alternatively, as HY5 and CCA1 physically interact (Andronis et al., 2008), it is possible that a physical interaction occurs between HY5/HYH and CCA1 at the promoter of *SIG5* to regulate is expression. It could be the case that CCA1 preferentially interacts with HY5; in its absence, CCA1 may interact with HYH to regulate the promoter activity of downstream targets, such as *SIG5*. This theory would explain the redundancy between HY5 and HYH, as well as the greater decrease in *SIG5* amplitude observed in *hy5* than *hyh*, and hence the greater dependence of *SIG5* rhythmicity on HY5 than HYH.

Furthermore, HY5 and PIFs act antagonistically to co-regulate common target genes through their interaction with G-box elements in gene promoters (Toledo-Ortiz et al., 2014), and *SIG5* is a potential target of PIF3 (Dubreuil et al., 2017). Hence, PIFs, or components of the circadian oscillator such as CCA1, may be maintaining low amplitude oscillations of *SIG5* transcription in the absence of HY5 and HYH.

It would be interesting to examine whether the HY5-CCA1 physical interaction occurs at the promoter of *SIG5* to maintain rhythmicity in the accumulation of *SIG5* transcripts. Leading on from this, it would be informative to understand whether a similar physical interaction can occur between HYH and CCA1 as that described between HY5 and CCA1, and, if so, to compare CCA1 binding affinity to both HY5 and HYH. In order to do this, coimmunoprecipitation assays, followed by western blotting, could be used (Holm et al., 2002).

An alternative explanation for the maintenance of low-amplitude rhythmic *SIG5* transcription in the *hy5 hyh* double mutant is that HY5 and HYH are responsible for the regulation of *SIG5* promoter circadian rhythms, but that another mechanism is controlling the rate of *SIG5 degradation* in a rhythmic manner. All of these theories are plausible, and none are mutually exclusive. Thus, future work should aim to elucidate the mechanism(s)

involved in maintaining rhythmic *SIG5* expression in the *hy5 hyh* mutant, exploring these suggestions as possibilities.

The involvement of HY5/HYH in light/dark signalling to chloroplasts

HY5 and HYH act downstream of photoreceptors, hence are important in the light regulation of gene transcription. Having identified roles for HY5/HYH in the circadian regulation of *SIG5* and *psbD* BLRP, I then aimed to understand the roles of these transcription factors in light signalling to *SIG5*.

SIG5 and *psbD* BLRP transcript analyses under light/dark conditions in mutants of *HY5* and *HYH* indicated that these transcription factors act upstream of *SIG5* in response to light (Figure 4.1; Figure 4.2). This is consistent with previous reports (Nagashima et al., 2004, Mellenthin et al., 2014), and this result is valuable in its contribution to furthering our understanding of the involvement of HYH in this signalling pathway. Transcripts of both the nuclear-encoded *SIG5* and the chloroplast-encoded *psbD* BLRP are less abundant in mutants of *HY5* and *HYH* compared to wild type (Figure 4.1). HY5 and HYH transcription and protein abundance, stability and activity are tightly regulated by light conditions (Holm et al., 2002, Xu, 2019, Lu et al., 2015, Hardtke et al., 2000). The abundance of *SIG5* transcript is lowest in the double *hy5 hyh* mutant; in the single *hy5* and *hyh* mutants, abundance is lower than the wild type but maintained at a slightly higher level than *hy5 hyh* (Figure 4.2). Therefore, as suggested for circadian signalling to *SIG5*, HY5 and HYH might be acting with some redundancy to regulate the transcription of this sigma factor, and subsequently its downstream targets, in response to light (Nagashima et al., 2004, Brown and Jenkins, 2008, Mellenthin et al., 2014).

It is possible that there are distinct roles for HY5 and HYH under different wavelengths of light. *SIG5* is primarily responsive to blue light, and cryptochromes and blue light signalling are especially important for the circadian regulation of *SIG5* and *psbD* BLRP transcription, as discussed (Belbin et al., 2017). However, R and FR light (particularly the ratio of R:FR) are important signals for *SIG5* around dawn (Belbin et al., 2017). Hence, the mediation of R and FR light signalling by phytochromes, PIFs, and HY5/HYH could be important for maintaining appropriate responses of *SIG5* oscillations under diel conditions. Future work should aim to identify whether there is variation between HY5 and HYH in the light quality signals they are

primarily responsive to. Differences between these transcription factors in their interactions with different photoreceptors, and hence their responsiveness to different wavelengths of light, could go some way to explain the distinctive responses in *SIG5* and *psbD* BLRP transcript abundance observed between *hy5*, *hyh* and *hy5 hyh*. This may advance our understanding of the relative contributions of HY5 and HYH to circadian signalling versus light signalling to *SIG5*. The partitioning of responsibility between HY5 and HYH in this way could present a mechanism whereby SIG5 distinguishes between different light quality signals, and hence generates specific, appropriate responses in the chloroplast.

The involvement of ATHB17 in the cold regulation of *SIG5* transcripts

Along with predictable and unpredictable fluctuations in light conditions, plants are subjected to a range environmental stresses. Exposure to stress requires a tight regulation between nuclear and chloroplast gene expression in order to optimise plant growth and development, hence ensuring the greatest chances of survival. The coordination of the nuclear and chloroplast genomes can occur by both anterograde (nucleus to chloroplast) and retrograde (chloroplast to nucleus) signalling, and transcription factors are essential components in both of these signalling types. Nuclear-encoded sigma factors are involved in anterograde signalling to chloroplasts, and *SIG5* is responsive to multiple stresses (Nagashima et al., 2004). Retrograde signalling is also an important mechanism in the coordination of genomes in response to stress. Chloroplasts are able to sense environmental and developmental cues, hence use the process of retrograde signalling to communicate these cues to the nucleus, thereby altering transcriptional, translational and post-translational processes in the nucleus (Chan et al., 2016). For example, abiotic stresses such as high light and cold temperatures trigger the production of metabolites and reactive oxygen species (ROS) in the chloroplast that regulate photosynthesis-associated nuclear genes (PhANGs).

In these experiments, I analysed transcript abundance by qRT-PCR to investigate the roles of the transcription factor ATHB17 in the cold temperature signal transduction pathway leading to the regulation of *SIG5* and subsequently *psbD* BLRP transcription. In unperturbed mammalian cells, ~56-84% of variation in protein levels could be explained by the abundance of mRNA transcripts, whereas translational processes only accounted for ~9% of

protein variability when levels of both were stable for several hours (Li et al., 2014). A later study identified that the combined contribution of rates of both protein translation and degradation to protein abundance is half the contribution of levels of mRNA transcripts (Jovanovic et al., 2015). In response to environmental stress, requiring adaptive responses of protein abundance, 80% of protein level changes can be attributed to transcriptional changes in fission yeast (Lackner et al., 2012). Hence, alterations in gene transcription significantly impact protein levels, so measuring transcript abundance is a highly informative method of understanding adaptive changes in protein abundance.

Both HY5 and HYH are known to regulate the transcription of *SIG5* in response to low temperatures, and are required for low temperature responses of *SIG5* (Cano-Ramirez et al., under revision). *ATHB17* expression is induced in response to a variety of stresses, and the transcription factor is important in the regulation of both PhANGs and *SIG5* in response to salt stress (Zhao et al., 2017). By regulation of *SIG5*, ATHB17 influences the transcription of downstream plastid encoded genes (PEGs) (Zhao et al., 2017). However, it seems likely, given the data presented in chapter 4, that ATHB17 does not regulate *SIG5* expression in response to cold temperatures, as *SIG5* induction occurred in both *athb17* mutant lines following cold exposure (Figure 4.7). Surprisingly, expression of *psbD* BLRP was not significantly different between ambient and cold treatments in either wild type or *athb17* mutant lines. This conflicts with previous work demonstrating an increase in *psbD* BLRP expression corresponding to an increase in *SIG5* expression following cold exposure (Cano-Ramirez et al., under revision).

I obtained measurements of *psbD* BLRP 5 hours after subjecting seedlings to the cold treatment (*SIG5* measurements were obtained after 3 hours of cold treatment). A possible explanation for the unexpected, unchanged *psbD* BLRP expression between ambient and cold temperatures in the wild type is that measurements of *psbD* BLRP were obtained too soon after cold exposure.

There exists a delay between the transcription of a gene and the subsequent expression of a protein, although this delay seems to be protein specific. The delay exists due to the time required to export and translate mRNA transcripts. In mice, around half of proteins with circadian rhythms in their abundance followed mRNA oscillations with over a 6 hour delay, and the length of this delay depended on the time of day (Robles et al., 2014).

Previous work in Arabidopsis reported a delay between the transcription of *SIG5* and the subsequent induction of *psbD* BLRP following cold treatment (Cano-Ramirez et al., under revision). It is possible that the sampling of seedlings for analysis of *SIG5* and *psbD* BLRP in Figure 4.7 were not sufficiently staggered in order to observe the effect of *SIG5* transcription on *psbD* BLRP transcript abundance. Cano-Ramirez et al. (under revision) report that a delay of 6 hours between sampling for *SIG5* and sampling for *psbD* BLRP transcript abundance is required to observe significant downstream effects of altered *SIG5* transcription on *psbD* BLRP transcription in response to cold. In my experiments, I staggered measurements by only 2 hours. Therefore, it would be useful to either sample *athb17* mutants for *SIG5* and *psbD* BLRP transcripts over a timeseries, as in Cano-Ramirez et al. (under revision), or sample for *psbD* BLRP ~6 or more hours after sampling for *SIG5*. Alternatively, it could simply be the case that my data are variable, due to uneven cold treatment of the seedlings. Nonetheless, it is difficult to completely rule out ATHB17 as contributing to the cold regulation of *SIG5* and its downstream targets, given the case presented here. Further work should be conducted to support these findings and conclusions.

Zhao et al. (2017) identify that *ATHB17* is upregulated in the leaves following salt exposure, subsequently upregulating *SIG5* expression. The independence of different stress-responsive pathways, as well as light-responsive pathways, that converge at the promoter of *SIG5* to regulate its transcription, may present a method by which SIG5 is able to control appropriate chloroplast responses. It seems likely that there are many different pathways converging on *SIG5* which are both discrete and complementary in function. The circadian regulation and gating of *SIG5*, mediated by HY5 and HYH, are likely to be vital in enabling suitable responses to environmental stimuli, preventing transient changes in environmental conditions from causing significant alterations in downstream gene expression. SIG5 is hence involved in integrating various environmental and circadian signals, to generate the appropriate transcription of chloroplast genes, and this integration of signals is highly dependent on transcription factors such as HY5, HYH and ATHB17.

Recommendations for future work

Circadian rhythms of *SIG5* transcription are maintained, albeit at a low amplitude, in the *hy5 hyh* double mutant (Figure 3.3). Therefore, other factors and mechanisms contribute to the maintenance of rhythmic *SIG5* gene expression. Given that ATHB17 has been identified to bind the *SIG5* promoter (Zhao et al., 2017) and that its expression is rhythmic under certain conditions (Mockler et al., 2007), it would be interesting to investigate whether this HD-ZIP transcription factor is involved in maintaining the circadian rhythmicity of *SIG5* transcription. A recommendation for future work would therefore to conduct circadian qRT-PCR timecourses, similar to those conducted in chapter 3, in *athb17* mutants, as well as *hy5 hyh athb17* triple mutants. Furthermore, other novel components involved in this signalling pathway could be identified and characterised by a forward genetic screen. Arabidopsis T-DNA insertion lines could be screened to isolate mutants displaying sig5-like phenotypes in the cold. Additionally, the involvement of factors in this pathway that are known to co-regulate HY5, such as BBX proteins and PIFs, should be further explored.

Future work should also aim to understand the distinct and complementary roles for HY5 and HYH in regulating *psbD* BLRP expression downstream, and possibly independently, of SIG5. HY5/HYH might regulate the import into chloroplasts of proteins, such as the TIC/TOC complexes on the inner/outer chloroplast envelope, affecting the passage of other regulators into chloroplasts (Kessler and Schnell, 2009). qRT-PCR timeseries using triple *hy5 hyh sig5*, as well as single and double *hy5 sig5* and *hyh sig5*, mutants, should be conducted. If transcripts of *psbD* BLRP remain present and rhythmic in the triple mutant, then factors other than HY5/HYH and SIG5 are involved in its transcription and circadian regulation, respectively. However, if transcripts of *psbD* BLRP are absent in the *hy5 hyh sig5* triple mutant, then HY5/HYH contribute to the regulation of *psbD* BLRP transcription, independently of SIG5.

SIG5 has been estimated to regulate at least 12% of rhythmic chloroplast transcripts, although there likely exists functional redundancy between the sigma factors (Noordally et al., 2013). Transcription of *SIG1*, *SIG3* and *SIG4* is rhythmic, and the chloroplast gene targets of these sigma factors are also rhythmically expressed. Hence, these sigma factors may represent additional pathways by which environmental and circadian information are

integrated and communicated to the chloroplast. It would be informative to investigate the relationships between transcription factors such as HY5/HYH/ATHB17 and other sigma factors in order to gain a broader understanding of the circadian and environmental regulation of chloroplast gene expression, mediated by sigma factors. Nonetheless, SIG5 is the only stress-inducible sigma factor (Nagashima et al., 2004), so although it is possible that other sigma factors integrate light and circadian signals to regulate chloroplast transcription, SIG5 is likely to be the primary sigma factor involved in controlling chloroplast transcription in response to stress.

Finally, to further support the proposition that ATHB17 is not upregulated in response to cold stress, a GUS reporter assay should be used. By fusing the *ATHB17* promoter to a GUS reporter construct, the expression pattern of this transcription factor throughout the seedling following cold exposure could be analysed. A similar experiment was conducted by Zhao et al. (2017) to analyse the promoter activity of *ATHB17* in response to salt and other stresses. A transcriptome microarray analysis indicated that, over a 24-hr period of cold exposure, there is no upregulation of the transcription factor throughout the plant (Figure 4.8) (Kilian et al., 2007), hence this GUS reporter assay would support these findings.

Conclusions

The work described in this thesis provides evidence of redundancy between the bZIP transcription factors HY5 and HYH in the circadian regulation of *SIG5* and *psbD* BLRP transcription. HY5/HYH act upon the amplitude of *SIG5* oscillations downstream of CCA1. However, another factor (potentially CCA1 directly) regulates the periodicity of *SIG5* oscillations, thereby maintaining the functional connection between *SIG5* and *CCA1* oscillations under free-running circadian conditions. The results also suggest that there may be roles for HY5/HYH in regulating *psbD* BLRP transcription downstream of SIG5.

Additionally, the data indicate that redundancy occurs in the regulation by light of *SIG5* by HY5/HYH, although light quality is also a consideration in this modulation (Belbin et al., 2017). Blue light and cryptochromes are important for the circadian regulation of *SIG5*, whereas R/FR light and phytochromes are important in the anticipation of dawn and hence in light/dark signalling to *SIG5*. Future work should therefore be conducted under varying

light wavelengths to understand how HY5 and HYH each respond to different light quality signals.

HY5/HYH have been identified as important in the cold stress responsive pathway to *SIG5* (Cano-Ramirez et al., under revision). Although ATHB17 regulates *SIG5* transcription in response to salt stress (Zhao et al., 2017), it is unlikely that this HD-ZIP transcription factor is involved in regulating *SIG5* in response to cold stress. However, further work should be conducted to confirm its lack of involvement, considering the suggestions made of increasing the delays to sampling after cold exposure.

The convergence of different signalling pathways at the promoter of *SIG5* presents a method by which a single sigma factor can integrate information about a varied environment and alter chloroplast gene expression in response to a myriad of cues. In doing so, processes in the chloroplast are coordinated with nuclear gene expression and optimised to the prevailing environmental conditions, depending on the time of day and season. Figure 5.1 illustrates some of the signalling pathways that are integrated to contribute to the SIG5-mediated regulation of *psbD* BLRP transcripts under ambient conditions, additionally highlighting potential avenues for further exploration.

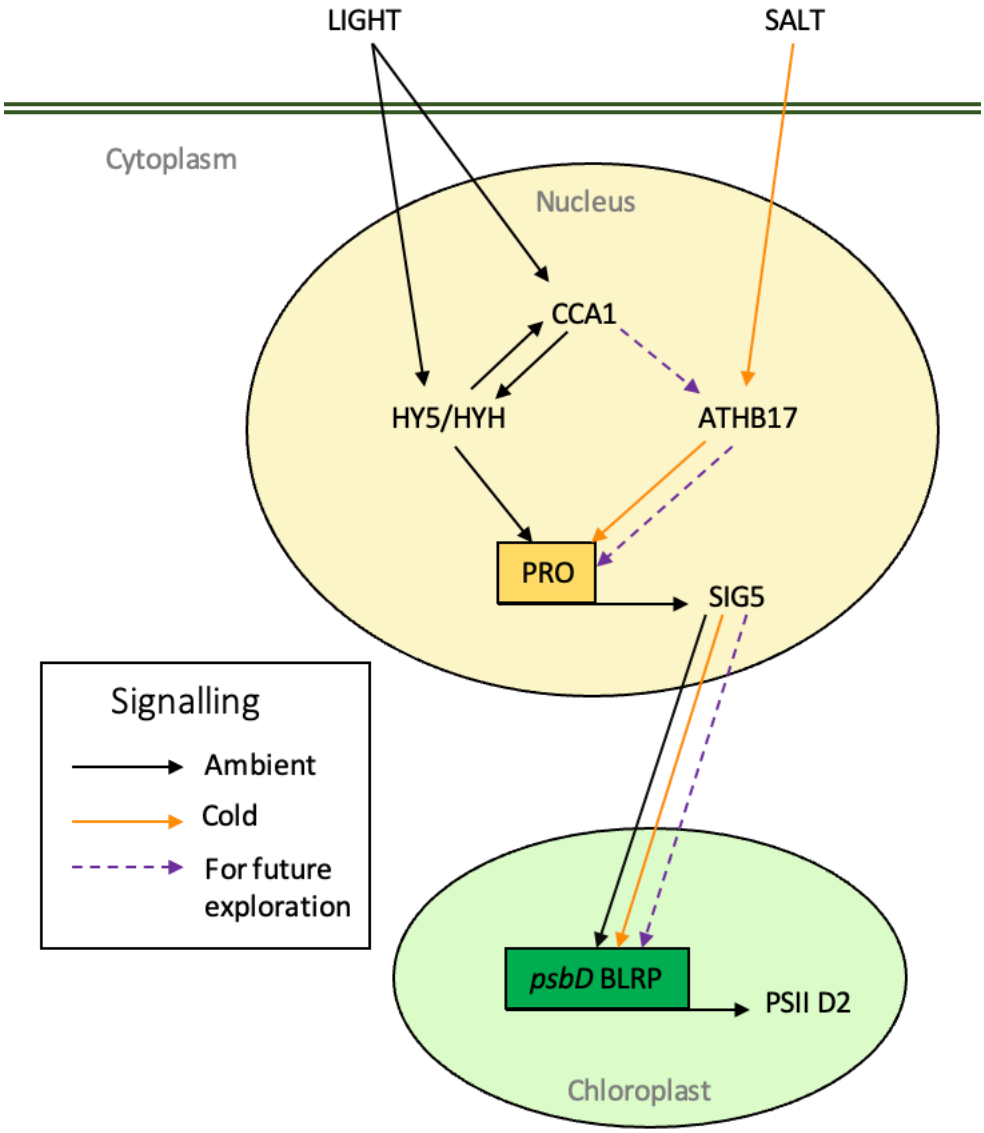

Figure 5.1 The potential mechanisms regulating *SIG5* and *psbD* BLRP transcription under ambient conditions. Light regulates both the circadian oscillator and HY5/HYH signalling. HY5/HYH likely act both upstream and downstream of CCA1, contributing to the circadian rhythmicity of *SIG5* transcripts by maintaining the amplitude of *SIG5* oscillations. There may be a physical interaction between HY5/HYH and CCA1 in the regulation of *SIG5* transcription, and the circadian oscillator might directly regulate *SIG5*, although this suggestion requires further exploration. Under salt stress, ATHB17 is upregulated to activate transcription of *SIG5* and enhance photosystem (PS) repair (Zhao et al., 2017). SIG5 regulates the transcription of *psbD* BLRP on the chloroplast genome, which encodes the D2 protein of PSII. HY5/HYH might have additional mechanisms of regulating *psbD* BLRP independently of SIG5. ATHB17 might also be involved in maintaining the rhythmicity of *SIG5* oscillations – this suggestion should be explored further. PRO = promoter.

Overall, future work should aim to further separate the distinct and overlapping functions of HY5 and HYH, and to identify the additional factors involved in the circadian regulation of *SIG5*, as well as the transcription and circadian regulation of *psbD* BLRP. It would be interesting to investigate if there is a dependence of HY5/HYH on other proteins such as PIFs for this specific modulation of *SIG5* transcription. Furthermore, it is possible that the observed reductions in *SIG5* transcription in the double *hy5 hyh* mutant compared to the single mutants is because HY5 and HYH can function as both homodimers and heterodimers. Hence, future work should aim to clarify whether the observed effect on *SIG5* transcription in the double mutant is due to this characteristic of HY5 and HYH, or, as previously suggested, due to a redundancy between the two transcription factors.

Chapter 6 References

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