CIRCADIAN REGULATION OF PHOTOSYNTHETIC EFFICIENCY UNDER FLUCTUATING ENVIRONMENTAL CONDITIONS

MSc by Research

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Abstract

In natural environments, plants experience constantly changing conditions. These environmental fluctuations lead to damage of the photosynthetic apparatus. This means that maintaining photosynthetic efficiency is underpinned by an ongoing cycle of damage and repair. This repair is supported by multiple layers of regulation – from rapidly induced response mechanisms to underlying circadian regulation – allowing appropriate responses to changes in condition intensity and duration be elicited. The SIGMA FACTOR 5 (SIG5) transcriptional pathway drives expression of genes in the chloroplast genome involved in photosynthesis. Previous studies have shown there is rapid induction of *SIG5* expression in response to tissue exposure to multiple abiotic stress conditions. Further, in response to treatments of short-term cold and light conditions induction of *SIG5* has been shown to be under circadian regulation.

This thesis provides evidence that circadian regulation influences both the response and recovery of photosynthetic efficiency to short-term exposure to cold and high light conditions. In addition, this thesis shows this circadian regulation is in part mediated by the SIG5 transcriptional response pathway. Moreover, this regulation by the circadian oscillator and SIG5 varies with leaf developmental type, suggesting a differing vulnerability of leaf types to cold and high light conditions. Understanding how photosynthetic efficiency is regulated under different abiotic environmental conditions could be informative of how the photosynthetic fitness of ecosystems, both agricultural and natural, may be shaped by the changing climate.

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1 Introduction

In natural environments, plants experience constantly changing conditions. These environmental fluctuations lead to damage of the photosynthetic apparatus. This means that maintaining photosynthetic efficiency is underpinned by an ongoing cycle of damage and repair. This repair is supported by multiple layers of regulation – from rapidly induced response mechanisms to underlying circadian regulation – allowing appropriate responses to changes in condition intensity and duration be elicited.

Anthropogenic activity is driving changes in climate, including an increased intensity and frequency of previously uncommon extreme weather events (Cohen, Pfeiffer and Francis, 2018; Lamichhane, 2021). Beyond increasing the frequency of conditions which drive photooxidative damage, these weather events could represent times of disruption of expected diel changes in temperature (Cano-Ramirez et *al.*, 2022). This could drive mis-entrainment of the circadian oscillator, since it is entrained to both diel cycles in light and temperature (Hsu and Harmer, 2014). Mis-regulation of processes by the circadian oscillator has been shown to greatly reduce seed viability and vegetative biomass, and delay flowering time (Dodd *et al.*, 2005; Green *et al.*, 2002; Wang and Tobin, 1998). Therefore, understanding relevance of the circadian oscillator in contributing to maintenance of photosynthesis under different abiotic environmental conditions could be informative of how the photosynthetic fitness of ecosystems, both agricultural and natural, may be shaped by the changing climate. This chapter will focus on the current relevant literature regarding plant photosynthesis and the circadian oscillator, to provide a context for the experiments in the subsequent chapters.

1.1 Photosynthesis, photoinhibition, and photoprotective mechanisms

Photosynthesis is the process of converting intercepted light energy into chemical energy, and in plants, occurs within the chloroplasts. The reactions of photosynthesis can be divided into two major steps; the light-dependent and light-independent reactions (Fig. 1.1) (Nelson and Ben-Shem, 2004). The light-dependent reaction occurs at the thylakoid membranes of the chloroplasts and begins with light/excitation energy interception by peripheral photosynthetic pigment molecules (Nelson and Ben-Shem, 2004). This excitation energy is then transferred between pigment molecules until it reaches a reaction centre pigment. Here, the excitation energy enters into the electron transfer chain (ETC) – a series of protein complexes and

mobile electron carrier molecules – where it drives the reduction of ADP^+ into ATP and $NADP^+$ into NADPH (Fig. 1.1) (Anderson and Chow, 2002). These products are then used as electron donors for in the Calvin cycle carbon fixation in the light-independent reaction which occurs in the stroma (Fig 1.1).

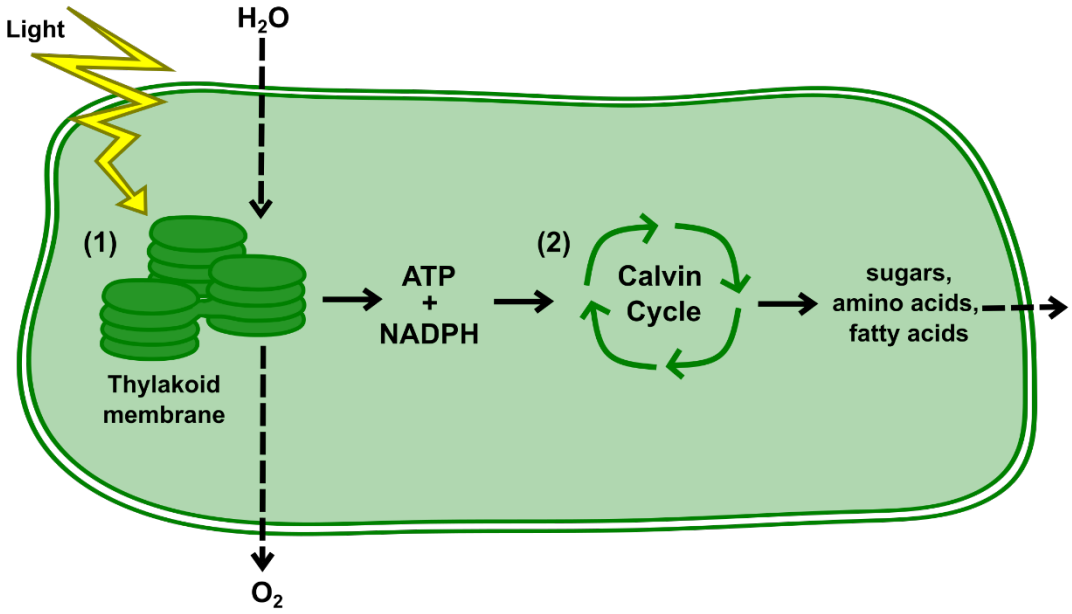

Figure 1.1 The light-dependent and light-independent reactions in the chloroplast. (1) Light/excitation energy is intercepted at the grana, where it drives photochemistry in the light-dependent reaction to generate ATP and NADPH. (2) ATP and NADPH act as electron donors in the light-independent reaction to fix carbon.

1.1.1 ROS generation and photooxidation

Whilst many changes driven by fluctuations in environmental conditions can reduce capacity of the photosynthetic system, ubiquitous across different environmental conditions is the accumulation of reactive oxygen species (ROS) (Asada, 2006). ROS is generated when excess excitation energy leads to overreduction of the photosynthetic electron transport chain. This ROS causes irreversible damage to the photosynthetic apparatus through photooxidation (Gururani et al., 2015).

In photosynthesis, the majority of photooxidation of proteins and lipids occurs at the thylakoid membranes as this is the location of excess excitation energy. Much of this damage to photosynthetic proteins is irreversible. Therefore, repair and recovery of rate of photosynthesis

requires the replacement of proteins. One major site of photooxidative damage is the ETC, primarily PSII, and within this, the core protein D1 (Chen *et al.*, 2020; Gururani, Venkatesh and Tran, 2015). In fact, D1 protein turnover represents such a significant proportion of overall protein turnover in the chloroplasts that despite D1 only representing 0.1% of total protein in the chloroplasts, its synthesis represents around 50% of total protein turnover (Barber and Andersson, 1992; Anderson and Chow, 2002). Replacement of PSII subunits is a multistep process requiring removal of the PSII complex from the grana of the thylakoid membrane, disassembly, selective degradation of damaged subunits, reassembly with newly synthesised subunits, and finally re-insertion into the grana (Nixon et al., 2010).

Beyond ROS accumulation, there are also abiotic condition-specific causes of ROS accumulation. For instance, cold/chilling conditions impede plant productivity by altering the thermodynamic state of the cell. This reduces enzymatic activities and changes the structure and stability proteins and lipids (Miura and Furumoto, 2013). There are many ways which these changes reduce the rate of photosynthetic, for example, increasing membrane viscosity of the thylakoid membranes restricts diffusion of plastoquinone. This slows electron transfer from PSII to PSI, leading to overexcitation of PSII, generation of ROS, and eventual photoinhibition (Ruelland *et al.*, 2009; Miura and Furumoto, 2013).

1.1.2 Photoprotective mechanisms

In the chloroplasts, photoproduced ROS are reduced by ROS scavenger enzymes. The process of ROS reduction is aptly named the Water-Water cycle due to the initial use of water from PSII as an electron donor, and later the production of water (Asada, 1999). The Water-Water cycle involves multiple types of ROS scavenger enzymes which act in sequence, and are differentially localised with in the chloroplast. For instance, CuZn-superoxide dismutase (CuZn-SOD) and Fe-SOD are mainly found bound to PSI, whilst peroxiredoxin Q is found associated with PSII (Asada, 2006). Another enzyme class, ascorbate peroxidases, exist as both thylakoid (tAPX) and stroma (APX) localised forms. In Arabidopsis, both APX and tAPX are encoded by a single gene, where each isoform is produced through alternative splicing (Yoshimura et al., 2002).

Another ever-present mechanism which acts to limit ROS driven photoinhibition is non-photochemical quenching (NPQ) of chlorophyll fluorescence (Kromdijk, 2022). NPQ involves dissipation of excess excitation energy at singlet chlorophyll in PSII and is made of up many different processes which contribute to overall NPQ. These processes include qE (pH-depending quenching), qT (state-transition quenching), and qI (quenching related to

photoinhibition), and were elucidated by the different molecular components involved in each (Yarkhunova *et al.*, 2018; Kress and Jahns, 2017; Lu *et al.*, 2022). Not only do these processes differ in their molecular components, these different aspects of NPQ have different rates of upregulation. The most rapidly inducible is qE, which acts to reduce ROS accumulation within seconds to minutes through activation of qE protein effectors that increase dissipation of energy from the light-harvesting complex II (Kress and Jahns, 2017; Lu et al., 2022). Other components, such as qI – which involves the downregulation and photoinactivation of PSII – have much longer induction times but are also able to have more lasting effects on reducing ROS accumulation (Yarkhunova *et al.*, 2018; Lu et al., 2022). Beyond the several types of NPQ, when there is a great excess of excitation energy, i.e., under high light conditions, changes in orientation and distribution of chlorophyll proteins act to increase NPQ further (Gururani et al., 2015).

Whilst the previously discussed mechanisms act to limit ROS accumulation under prolonged unfavourable conditions, irreversible photoinhibition is inevitable. Replacement of the damaged photosystem subunits and recovery of photosynthetic efficiency requires transcriptional changes (Nagashima et al., 2004). The core proteins of PSII which are frequently damaged by ROS, D1 and D2, are encoded by the chloroplast genome. There are two types of RNA-polymerases in the chloroplasts of higher plants, these are nuclear-encoded plastid RNA polymerase (NEP) and plastid-encoded plastid RNA polymerase (PEP) (Shiina et al., 2005). Transcription of genes transcribed by PEP requires binding of nuclear-encoded sigma factors to PEP to form the holoenzyme, recognize the promoter, and allow the initiation of gene transcription (Marder et al., 1987; Cuitun-Coronado and Dodd, 2020). Most genes involved in photosynthesis-related genes are transcribed by PEP, including D1 and D2 which are encoded by chloroplast genes *psbA* and *psbDC* respectively (Noordally et al., 2013) (Marder et al., 1987). Therefore increased transcription of these genes first requires increased abundance of the relevant sigma factor (Marder et al., 1987; Cuitun-Coronado and Dodd, 2020). Both psbA and psbDC are transcribed when PEP is bound with SIGMA FACTOR 5 (SIG5), whilst for psbA SIG5 is functionally redundant with SIG1 (Nagashima et al., 2004; Noordally et al., 2013).

1.2.3 Photosynthetic efficiency as a measure of photosynthesis

Photosynthetic efficiency can be defined as the proportion of intercepted light that drives photochemistry. It is frequently used to quantify the capacity of a photosynthetic system as it is indicative of, under many conditions, of the overall rate of photosynthesis (Maxwell and Johnson, 2000). It is not possible to measure the portion of light that drives photochemistry.

However, driving of photochemistry is only one of three possible forms which intercepted light can become (Fig. 1.2) (Maxwell and Johnson, 2000). Often excitation energy which reaches the reaction centres cannot be used for photochemistry. This energy is, either dissipated as heat, through processes such as NPQ, or due to the return of chlorophyll from an excited to non-excited stated, re-emitted as chlorophyll fluorescence (Fig1.2) (Kress and Jahns, 2017; Maxwell and Johnson, 2000). The capacity of the reaction centres to accept excitation energy varies with the current state of the photosynthetic system. For instance, capacity is reduced by photooxidative damage to subunits in the photosystems, which limits the rate at which excitation energy can be passed on from the reaction centres. This means that the amount of energy re-emitted as fluorescence increases when the capacity of reaction centres to accept electrons is reduced (Kromdijk, 2022). As the wavelength of the re-emitted fluorescence is longer than the wavelength of the light intercepted, if the wavelength of the intercepted light is known, the proportion of light re-emitted as fluorescence can be determined (Maxwell and Johnson, 2000). By considering the proportion of intercepted light which is re-emitted as fluorescence, it is possible to estimate photosynthetic performance under a given condition almost instantaneously. For discussion of the mechanics of chlorophyll fluorescence measurement, see Materials and Methods.

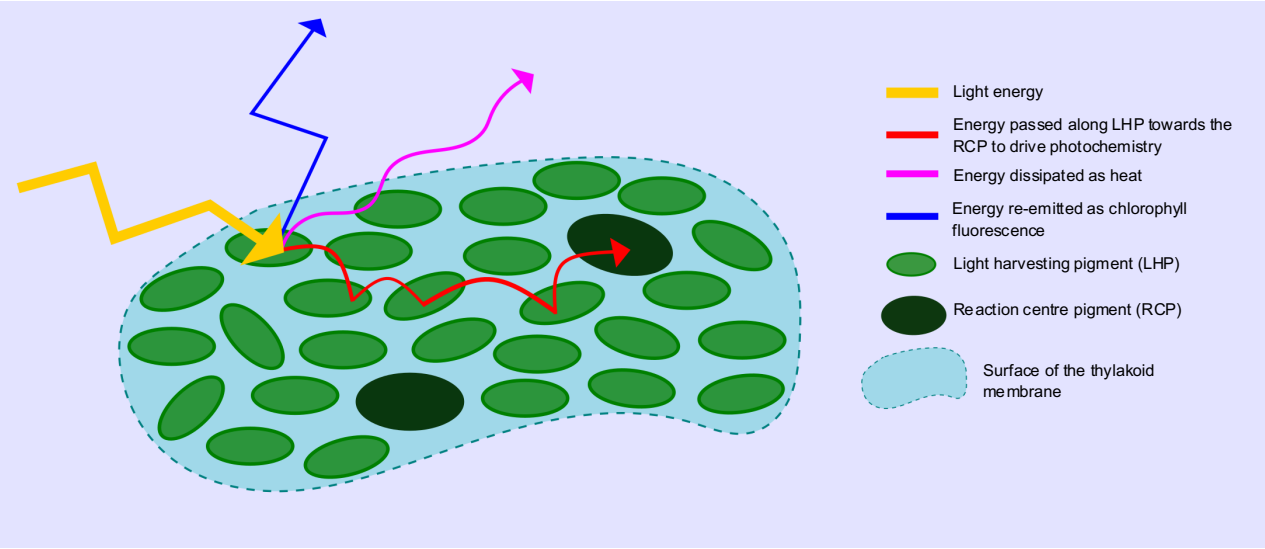

Figure 1.2 Three fates of intercepted light/excitation energy. Light/photon energy that is intercepted by a light harvesting pigment on the surface thylakoid membrane will be converted into one of three fates. Fate 1: energy is passed along light harvesting pigments towards the reaction centre pigment to drive photochemistry. Fate 2: energy is dissipated as heat. Fate 3: energy is re-emitted as chlorophyll fluorescence. Whilst depicted here to occur at the same pigment as light interception, Fate 2 and 3 can occur at any point between interception and acceptance at the reaction centre pigment.

1.3 The plant circadian oscillator

The Arabidopsis circadian oscillator is a network of interlocking transcription-translation feedback loops that generates free-running rhythms in gene expression (Fig. 1.3) (Hsu and Harmer, 2014; Hotta et al., 2007). The phase of these emergent rhythms is entrained by external cycles in light and temperature cues and internal cycles in sugar metabolism to maintain rhythms with a robust near 24 h period (Green *et al.*, 2002; Hsu and Harmer, 2014).

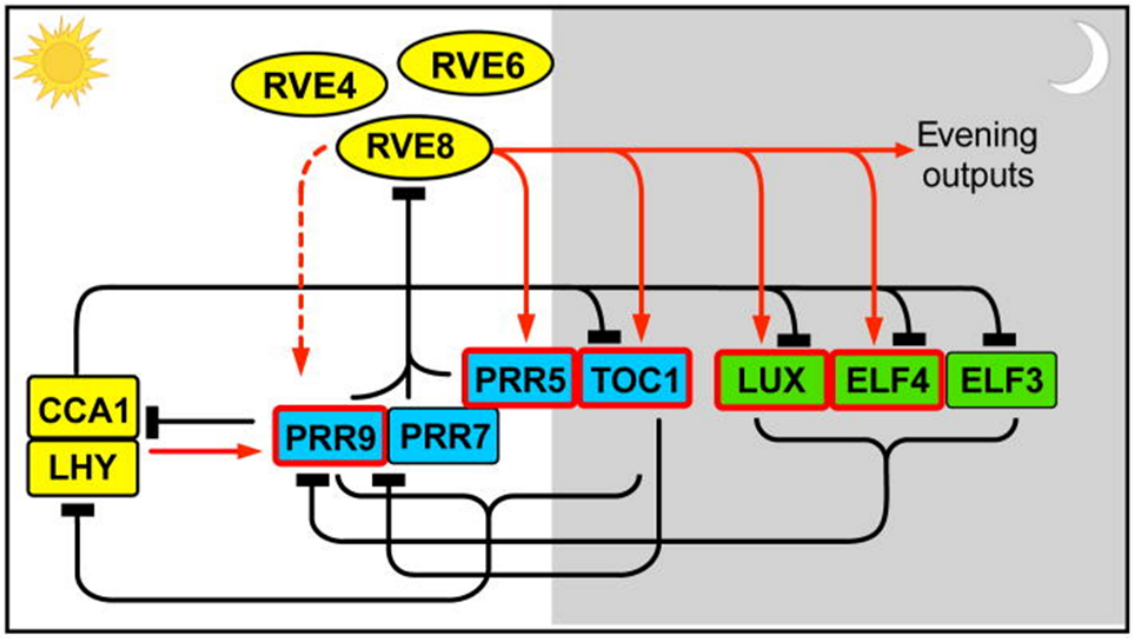

Figure 1.3 Schematic of the circadian oscillator transcription-translation feedback loop showing relative timing of action of each component. Taken from Hsu and Harmer (2014)**.** Subjective day is represented by white area, subjective night is represented by grey area. Solid red arrows indicate activation, dashed red represent conditional activation. Black line with bars represent repression.

One feedback loop in the circadian oscillator involves the MYB-like transcription factors CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) and ELONGATED HYPOCOTYL (LHY) (Fig. 1.3). CCA1 and LHY physically interact with each other and peak in transcript and protein levels in the morning (Lu et al., 2009). CCA1 and LHY repress expression of TIMING OF CAB EXPRESSION (TOC1) through binding to an element in the TOC1 promoter called the evening element (EE) (Rawat et al., 2011). This leads TOC1 expression to peak around dusk. To close this loop, TOC1 reciprocally represses expression of CCA1 and LHY via the transcription factor CCA1 HIKING EXPEDITION (CHE) (Pruneda-Paz et al., 2009).

An interlocking loop of the oscillator involves REVEILLE 8 (RVE8) (Fig 1.3) (Rawat et al., 2011). RVE8 is a MYB-like transcription factor which activates TOC1 through binding to the EE. RVE8 also activates the expression of LUX ARRHYTHMO (LUX), PSUEDO-REPONSE REGULATOR 5 (PRR5), EARLY FLOWERING 3 (ELF3) and ELF4 through the same mechanism (Hsu et al., 2013). LUX, ELF3, and ELF4 interact to form the 'evening complex' that acts to repress expression of PRR9 (Nusinow *et al.*, 2011). Similarly, TOC1 represses the expression PRR9 as well as PRR7. Along with PRR5, PRR7 and PRR9 act to repress the expression of the RVE8, completing another feedback loop of the circadian oscillator (Hsu et al., 2013). In this loop, other members of the RVE family, RVE4 and RVE6 act redundantly to the function of RVE8 (Hsu et al., 2013).

There are further interactions between the discussed components of the circadian oscillator including the conditional activation of expression of PRR9 by RVE8. CCA1 and LHY also promote the expression of PRR9, which in turn, represses the expression of CCA1 and LHY (Fig. 1.3) (Nakamichi et al., 2010).

Circadian rhythms of the oscillator function to regulate many processes, so that activity is matched to diel changes in the environment. Many vital plant functions are regulated by the circadian oscillator; for instance, rate of starch degradation at night is proportional to expected night length (Graf et al., 2010). Moreover, a wild-type circadian oscillator is required for correct growth and development – biomass accumulation, stomatal opening, and flowering, among other essential processes are disturbed by arrhythmic/altered circadian oscillators (Paajanen et al., 2021). Thus, lack of a wild-type circadian oscillator, such as in the short-period mutant *toc1-1*, long period mutant *ztl-1*, and arrhythmic period mutant *CCA1-ox,* causes aspects of plant fitness to be significantly reduced (Dodd et al., 2005). The lack of a wild-type circadian oscillator in each of these genotypes is driven by loss or function or miss expression of a 'core' component of the oscillator (Fig. 1.3).

1.3.2 Circadian Gating

One type of circadian regulation is "circadian gating" (Hotta *et al.*, 2007; Paajanen, Dantas and Dodd, 2021). This is the process whereby the sensitivity of a pathway to a stimulus is regulated by the circadian oscillator, such that a stimulus of identical magnitude provided at different times of day produces a response whose magnitude depends on the time of day. Therefore, circadian gating can lead to graded responses to stimuli, this is simplified into two scenarios in Figure 1.4 – a time when there is a strong response and a time when there is a weak response. Circadian gating of responses has been shown for many different essential

plant processes. For instance, during early establishment, hypocotyl growth of *Cosmos bipinnatus* was shown to be most responsive to wind stimuli when it was applied during the day (Gaal and Erwin, 2005). Furthermore, the rate of induction of CHLOROPHYLL A/B BINDING PROTEIN 2 (CAB2) expression, a protein involved in light harvesting, by light is dependent on the time of day (Millar and Kay, 1996). There is also circadian gating of induction of genes involved in the rapid shade-avoidance such that induction in response to low-red;far-red light is greatest during the day (Salter et al., 2003). Similarly, the responsiveness of stomatal aperture to changes in light intensity was shown to the greatest during the first half of the subjective day, whilst the greatest response to cold was during the day (Gorton, Williams and Assmann, 1993; Dodd *et al.*, 2006). In each of these instances, circadian gating enables changes to the sensitivity to the system to be altered across the day such that plants only respond to a stimulus when it is advantageous to do so (Hotta et al., 2007).

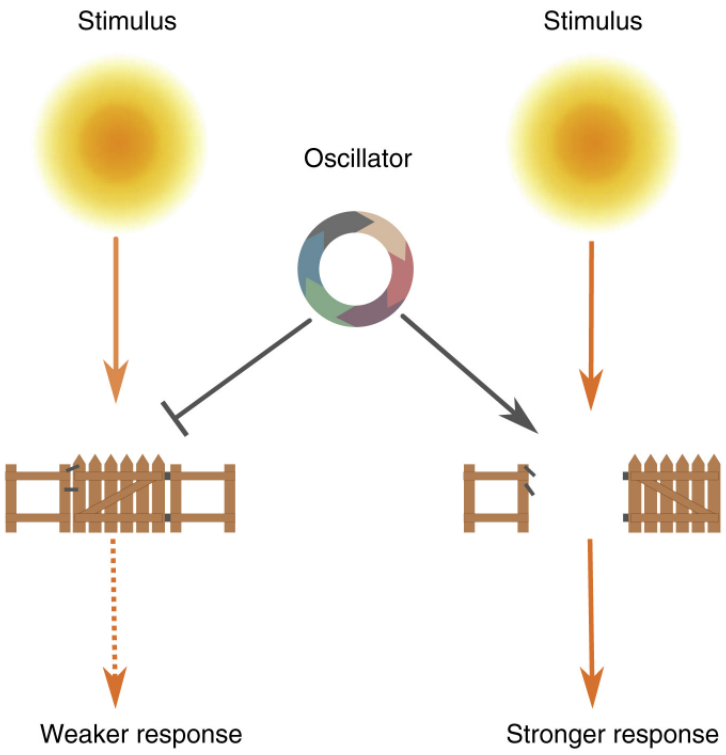

Figure 1.4 Diagrammatic representation of circadian gating. Reproduced from (Paajanen et al., 2021).Circadian gating can be defined is when the sensitivity of a pathway to a stimulus is regulated by the circadian oscillator, such that a stimulus of identical magnitude provided at different times of day produces a response whose magnitude depends on the time of day. The lefthand side of the diagram represents a scenario when the gate is “closed” meaning the circadian oscillator is regulating the system such that there is only a weak response to the stimulus. The right hand side of the diagram represents

a scenario when the gate is “open” meaning the oscillator has primed the system to respond strongly to the stimulus.

1.3.2 Circadian control of photosynthesis

Circadian rhythms are evident across many aspect of photosynthesis (Dodd et al., 2014). In Arabidopsis, circadian oscillations have been shown in a wide range of chlorophyll fluorescence parameters, such as, F_v/F_m, Y(II), NPQ (Litthauer *et al.*, 2015; Yarkhunova *et al.*, 2018). Rhythms in Y(II) have also been shown in marimo and *Marchantia polymorpha*, but are absent in *Picea abies* (Cano-Ramirez *et al.*, 2018; Cuitun-Coronado *et al.*, 2022; Gyllenstrand *et al.*, 2014). Similarly, circadian rhythms have also been shown in another output parameter, delayed fluorescence (Gould et al., 2009). Circadian regulation of F_v/F_m may enable plants to maintain a greater level of F_v/F_m, as in genotypes with altered free-running periods, *toc1* (<20hrs) and *ztl* (~28hrs), F_v/F_m is significantly reduced compared to wild-type (Dakhiya et al., 2017). Whilst the mechanisms which underly these rhythms in output variables of photosynthesis are yet to be elucidated, they are thought to reflect oscillations in components of photochemistry, for instance, in the activity of PSII (Dodd et al., 2014). Beyond circadian gating of *CAB2* expression, many genes encoding components of the light harvesting complex have circadian rhythms in promoter activity and transcript abundance (Millar et al., 1992). Oscillations in post-translational modifications of D1, a subunit of PSII, have also been shown in *Spirodela*, despite no rhythms in transcript abundance of D1 being evident (Booij-James et al., 2002).

In addition to light harvesting, in *Arabidopsis*, mangoes, and *Phaseolus vulgaris* there are oscillations in the rate of CO_2 assimilation, showing aspects of the carbon fixation are also under circadian regulation (Dodd *et al.*, 2005; Allen *et al.*, 2000). This rhythm is thought to be distinct from rhythms in stomatal conductance, as in *Phaseolus vulgaris,* rhythms in CO_2 assimilation occur when stomatal conductance and intracellular [CO_2] is constant. Similarly, there is clear circadian regulation of transcript levels of components of the Calvin cycle for carbon fixation, and, in pathways of degradation of fixed carbon (Farré and Weise, 2012; Graf *et al.*, 2010).

1.4 Sigma factor 5

SIG5 is the sigma factor most commonly associated with regulating chloroplast gene expression in response to changes in abiotic conditions. At the chloroplast genome, SIG5

binds to PEP to induce expression of *psbD* blue light-responsive promoter (*psbD* BLRP) (Nagashima et al., 2004). This operon contains three genes, *psbD*, *psbC* and *psbZ*, which encode the proteins D2, CP43 and a YCF9 protein respectively (Nagashima et al., 2004). Each of these proteins are components of PSII induction of SIG5 to drive expression of *psbD* BLRP has been shown in tissue exposed to a wide range of conditions, for instance; low temperature, high light, salt and high osmolarity (Fig 1.5A,B) (Nagashima et al., 2004).

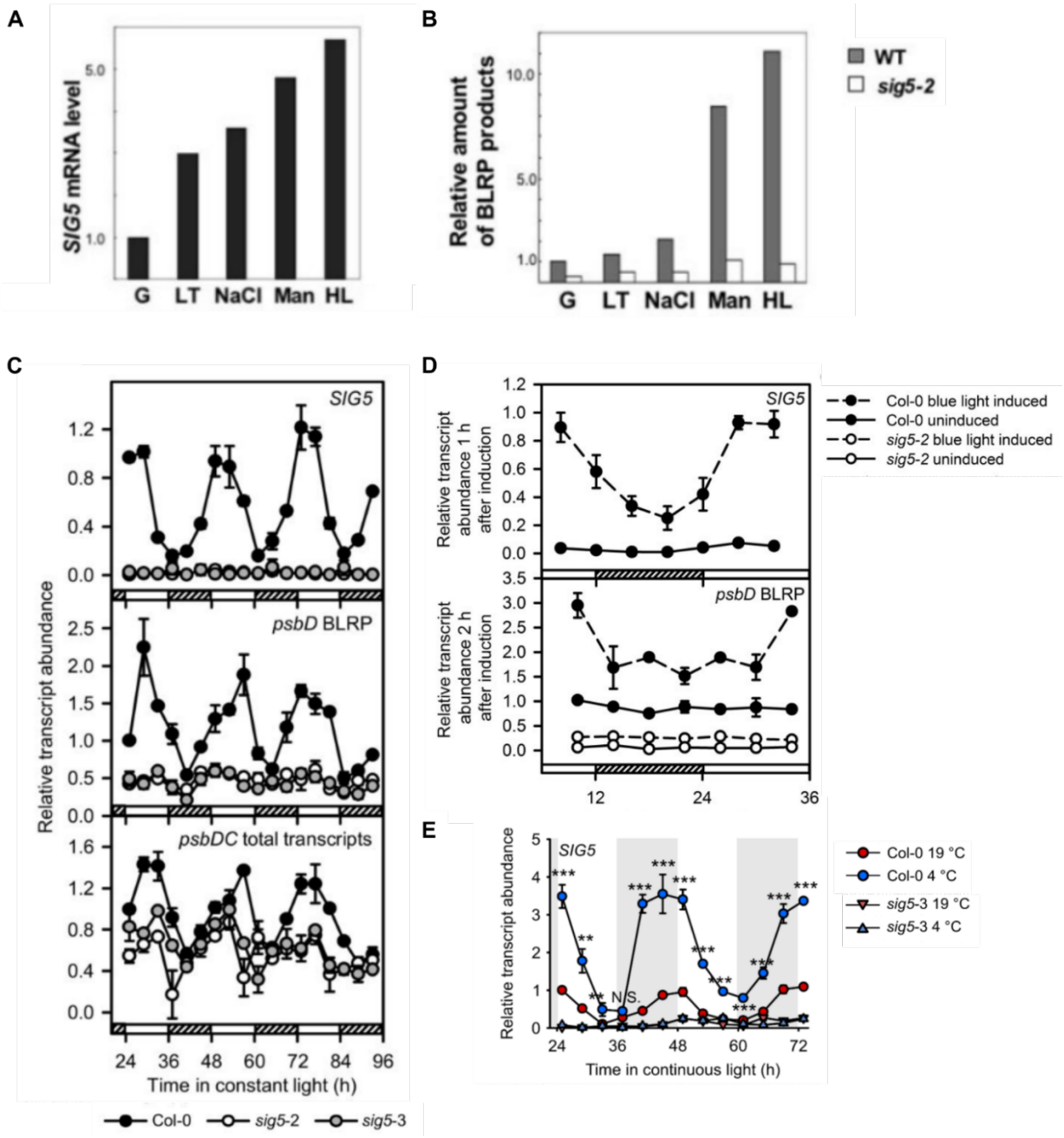

Figure 1.5 Summary of transcriptional responses of SIG5 in response to different abiotic stimuli and circadian regulation of this. (A,B) 10 or 11 day old Col-0 (and *sig5-2*) plants following treatment with growth light (50 $\mu molm^{-2}s^{-1}$, G), low temperature (4ºC, LT), salt (250 mM, NaCl), mannitol (250 mM, Man) or high light (1000 $\mu molm^{-2}s^{-1}$, HL). **(A)** *SIG5* mRNA induction following each treatment relative to G, reproduced from Nagashima *et al.* (2004). **(B)** psbD BLRP mRNA induction following each treatment relative abundance of Col-0 in growth light conditions, reproduced from Nagashima *et al.* (2004). **(C,D,E)** Transcript abundance of *SIG5*, *psbD* BLRP, or *psbDC* of 11 day old Col-0, *sig5-2*, and/or *sig5-3*. Plants grown under 12L/12D cycles then transferred into constant light 24 h before experiments commenced. **(C)** Samples collected from plants held in 19ºC and growth light conditions, reproduced from Noordally *et al.* (2013). **(D)** Plants treated with 50 $\mu molm^{-2}s^{-1}$ blue light or control prior to each timepoint, samples for *SIG5* and *psbD* BLRP collected 1 and 2 h respectively after treatment

commencement, reproduced from Noordally *et al.* (2013). **(E)** Plants treated with 3h 4ºC or 19ºC prior before each sampling timepoint, reproduced from Cano Ramirez (2018).

Beyond induction of expression in response to changes in abiotic conditions, *SIG5* transcription is under circadian regulation and this is thought to drive circadian rhythms in expression of *psbD* BLRP (Fig. 1.5C) (Noordally et al., 2013a). In addition to rhythms in expression, experiments have shown circadian gating of the level of induction of *SIG5* in response to cold and high light (Fig. 1.5D,E) (Cano-Ramirez and Panter, under revision; Noordally *et al.*, 2013). This gating is such that induction of *SIG5* upon challenge with cold or high light is maximal around subjective dawn and midday respectively. Here, peak *SIG5* induction in response to dawn would correspond to the time of day when cold conditions can drive increased photodamage – in the presence of light (Fig. 1.5E) (Cano-Ramirez and Panter, under revision). Similarly, greatest induction of *SIG5* by light treatments falls at midday, the time of day when high light is most likely to occur (Fig. 1.5D) (Noordally et al., 2013a). As discussed, the process of PSII subunit replacement following photooxidation is multistep, making it time and resource intense. Therefore, whilst currently not shown experimentally, gating of *SIG5* transcript induction might drive time-of-day appropriate increases in the proteins encoded by *psbD* BLRP to correspond to the times of day when the photosynthetic apparatus is most at risk of photodamage by the conditions (Cano-Ramirez and Panter, under revision).

1.6 Aims and Scope

The aim of this thesis is to investigate the physiological significance of previously-demonstrated molecular evidence of circadian gating of mechanisms that respond to cold and high light. For this work, the SIG5 response mechanism was focussed on as an experimental model. This larger aim was refined into three over-arching questions, each of which forms the focus of an experimental results chapter.

1) How do photoprotective mechanisms act to support photosynthetic efficiency under increasing durations of cold and/or high light conditions?
2) Are there circadian rhythms or circadian gating in the response and recovery of photosynthetic efficiency following exposure to short-term cold and/or high light conditions?
3) How is the circadian- and SIG5-mediated regulation of photosynthetic efficiency in response to short-term cold and high light conditions influenced by leaf developmental stage?

To investigate these questions, chlorophyll fluorescence approaches were selected as an experimental tool to investigate responses to experimental conditions. *Arabidopsis thaliana* was used as an experimental model plant, because the basis of the circadian oscillator both molecular and genetic have been well established. The findings from my experiments provide informative new insights into how regulation by the circadian oscillator and SIG5 shape the response of photosynthetic efficiency to short-term exposure to cold and/or high light conditions.

2 Materials and Methods

2.1 Plant material and growth conditions

Experiments were carried out using *Arabidopsis thaliana* . Experiments investigating the role of SIG5 used T-DNA insertion mutant *sig5-3* in the Col-0 background (Noordally et al., 2013b). For experiments investigating the role of the circadian oscillator, seeds harbouring single copy T-DNA insert 35S::CCA1 plants in the Col-0 background were included (Wang and Tobin, 1998). For all experiments, seeds were obtained from the same generation of parent plants.

Seeds were surface sterilised with 70% (v/v) ethanol and then with 20% (v/v) sodium hypochlorite for 1 and 10 minutes respectively. Seeds were then washed in sterile water and suspended in 0.1% (w/v) agar. Seeds were plated evenly in an 8x8 grid onto 100 mm square petri dishes containing half strength (w/v) Murashige and Skoog basal salts mixture (Duchefa Biochemie) dissolved in 0.8% (w/v) Agar solution, buffered with a potassium hydroxide solution to pH6.8.

Following seed plating, dishes were wrapped in foil and seeds were stratified for 3 days in darkness at 4 ^{o}C before cultivation. Cultivation occurred in Sanyo MLR-352 plant growth chambers at 19 ^{o}C under 12 h light /12 h dark cycles, with 110 µmol m^{-2} s^{-1} intensity white light, until seedling age was 11 days. For 28 day old plants, seedlings were grown to 11 days old on petri dishes as described, then transplanted into pots of Levington F2 Starter compost (five seedlings per pot), and grown for a further 17 days under the same chamber conditions. Plants were watered with DI water every 4-5 days.

Plants/plates used for timecourse experiment were transferred to continuous light conditions 24 hours before experiments began.

2.2 Cold and high light treatment conditions

Across experiments, plants were subjected to four external treatment conditions; control, high light, cold, and high light and cold. Treatments were designed to be provided within the growth chambers. The conditions of each treatment were:

- Control – 19 ^{o}C and 110 µmol m^{-2} s^{-1} intensity white light provided by growth chamber
- Cold – 4 ^{o}C and 110 µmol m^{-2} s^{-1} intensity white light provided by growth chamber

- High Light – 19 °C and 700 µmol m^{-2} s^{-1} intensity, provided by increased chamber light intensity (200 µmol m^{-2} s^{-1}) supplemented with red and blue light by an LED panel (500 µmol m^{-2} s^{-1}).
- High Light and Cold – 4 °C and 700 µmol m^{-2} s^{-1} intensity, combination of the cold and high light treatments as described.

Spectra of each light treatment (control and high light) are provided in Figure 2.1.

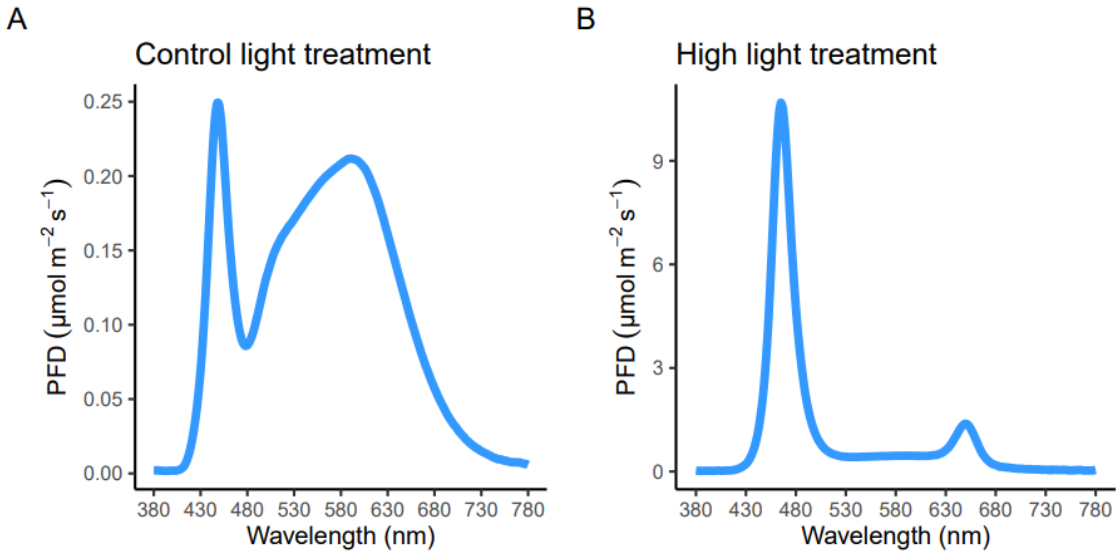

Figure 2.1 Light spectra of control and high light treatments. Measurements were taken using a LI-180 Spectrometer. **(A)** Control light treatment, provided by chamber LED tube lights. **(B)** High light treatment, provided by increased chamber LED tube light intensity supplemented with red and blue light provided by LED panel.

2.3 Measurement of effective PSII quantum yield

Effective PSII quantum yield, Y(II), was measured using an IMAGING PAM MAXI chlorophyll fluorescence system with pulse amplitude modulation (Walz GmbH, Effeltrich, Germany).

2.3.1 Calculation of Y(II)

Y(II) was calculated as $(F'_m - F')/F'_m$, where F'_m represents maximum fluorescence emitted after a saturating pulse of light and F' is the chlorophyll fluorescence emission of the plant tissue. F'_m is measured by exposing light adapted tissue to a saturating light pulse (SP) which closes the reactions centres of the photosynthetic apparatus, maximising the emitted fluorescence. F' is measured as the chlorophyll fluorescence emission of the plant tissue under the actinic light (AT) conditions, meaning Y(II) can be measured at any given light conditions.

To investigate maintenance of Y(II) under treatment conditions, Y(II) was measured, using a saturating light pulse intensity of 845 $\mu mol\ m^{-2}\ s^{-1}$, immediately following the tissue exposure to treatment conditions, meaning AT is equal to the light intensity of each experimental abiotic treatment condition. To investigate rate of recovery in Y(II) following treatment conditions, at each timepoint/treatment length Y(II) was also measured 15, 30, 45, and 60 minutes after the treatment. Recovery occurred in darkness at 19 ^oC under the IMAGING PAM MAXI system under measuring light (MP) pulses (frequency 1 Hz), meaning recovery Y(II) measurements were obtained under dark adapted states of the same length as the recovery time after treatment. Meaning in this case, $F' = F_o$ and $F'_m = F_m$, where F_o and F_m are zero and maximum chlorophyll fluorescence in a dark adapted tissue respectively. Therefore, for recovery measurements, $Y(II) = F_v/F_m$, (where $F_v = F_m - F_o$), another common measure of photosynthetic efficiency. The order of application of light pulses for the measurement of all of these parameters is shown in Figure 2.2.

The IMAGING PAM MAXI system provides images of plant material under the camera with false colour mapping that corresponds to Y(II). Plates of 11 day old seedlings used in experiments also contained late germinating seedlings. In images, these appear as circles of colour that are much smaller than the 11 day old seedlings. Y(II) measurements of these younger seedlings have been excluded from analysis.

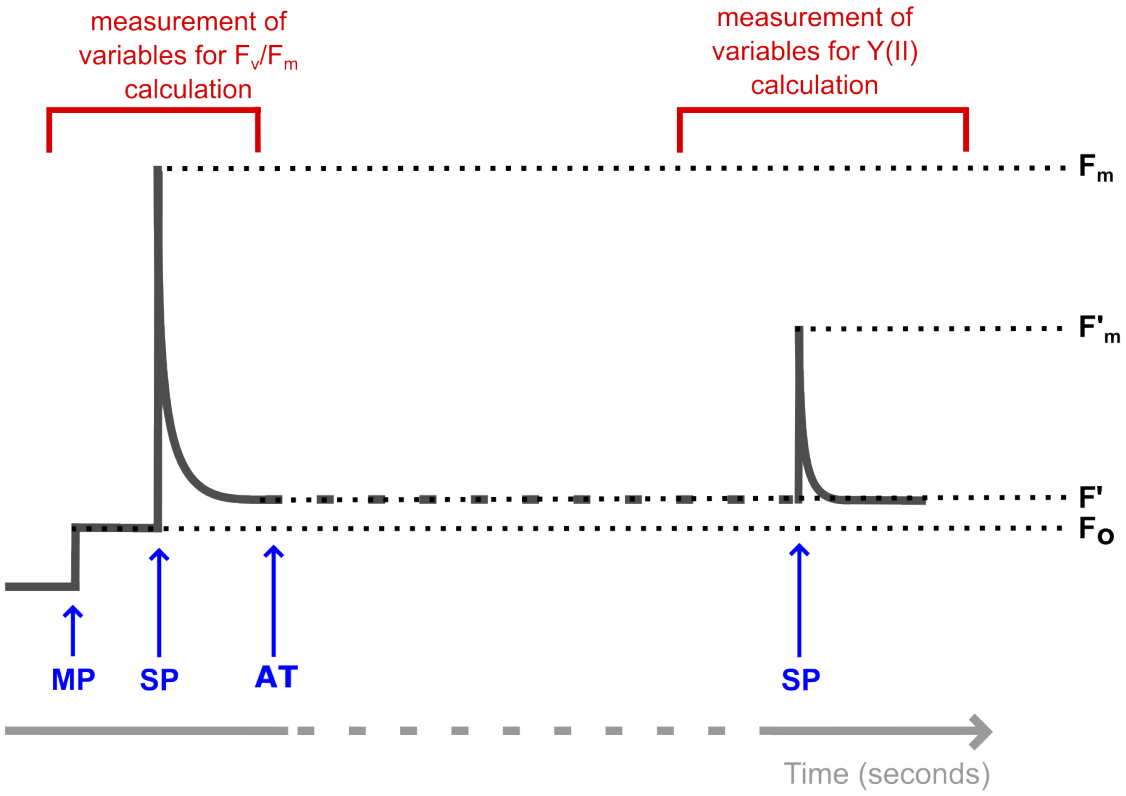

Figure 2.2 Light treatments used for measurement of chlorophyll fluorescence variables. For measurement of variables for the calculation of F_v/F_m, (1) F_o is measured from dark adapted tissue under measuring light pulses (MP), then (2) Fm is measured by exposing the plant to a saturating pulse of light (SP). For measurement of variables for the calculation of Y(II), (1) F is measured as fluorescence after/whilst the tissue is exposed to experimentally chosen light conditions (AT), then (2) F'_m measured by exposing the tissue to a SP. Grey line is a representative trace of the expected direction of change in emitted chlorophyll fluorescence following each light treatment.

2.3.2 Tissue selection for measurement of Y(II)

To investigate the role of leaf developmental stage in the circadian regulation of Y(II), experiments were carried out with both 11 and 28 day old plants. When measuring 11 day old seedlings, cotyledons were for selected, whilst for measurements of 28 day old plants, the widest unobstructed region of the outermost rosette leaves were selected. This selection is shown in Figure 2.3.

A B

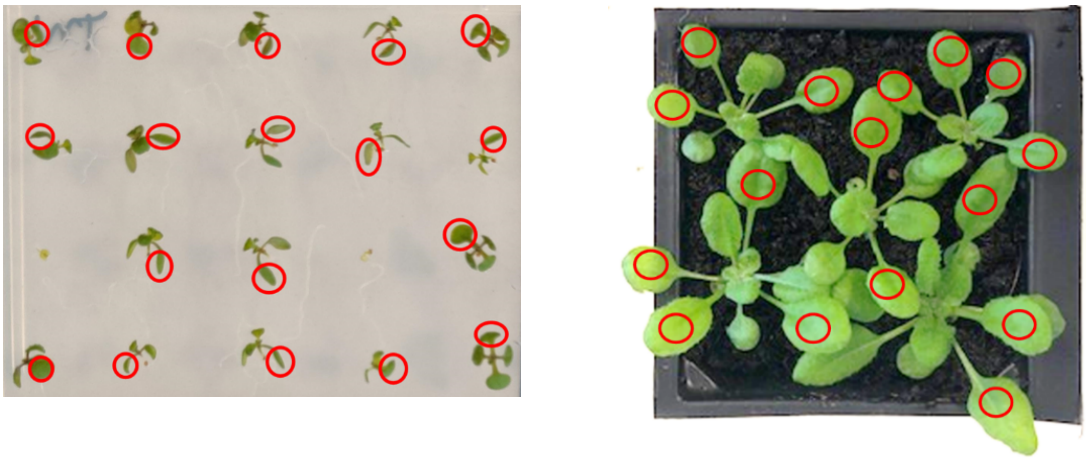

Figure 2.3 Plant tissue selection for effective PSII quantum yield measurements. Red circles indicate regions of leaves that were selected for measurement. In both cases only leaves which were fully exposed were selected. **(A)** cotyledon selection of 11 day old seedlings. **(B)** outer rosette leaf selection of 28 day old plants.

2.3.3 Equipment technical issues

Technical issues with the IMAGING PAM MAXI system controller unit caused occasional miss-flashing of the saturating light pulse at a lower light intensity than required for the measurement of Y(II). This is a known system failure by the manufacturer. At timepoints where this error occurred, there was mismeasurement of Y(II) of all the seedlings/plants. These mismeasurements were easily identifiable in the dataset and manually removed from analysis.

2.4 Data analysis

Data were formatted and analysed with R (v.4.1.3, RStudio, 2022). Plots were produced using R packages ggplot2, ggpubr, ggpattern (Wickham, 2016; Kassambara, 2020; FC and Davis, 2022).

2.4.1 Removal of late germinating seedlings from cotelydon measurements

Data collection of Y(II) of 11 day old seedlings also captured Y(II) of late germinating seeds on the plates. These needed to be removed from the dataset before statistical analysis.

Under the same conditions, late germinating seeds have lower Y(II) than 11 day old seedlings, and recovery in Y(II) upon transfer to the measuring chamber is also reduced. Therefore, late germinating seedlings were outliers in the dataset. As a large number of data points were collected, manual outlier removal was not possible and could introduce experimenter bias. Therefore, using the knowledge of slower recovery from treatments, younger seedling exclusion was conducted using scaled Cook's Distances of the 15 minute recovery Y(II) measurements. To calculate Cook's Distances, first all measurements of each plant genotype under each condition at each timepoint were fitted to a linear regression model. Then the Cook's Distance of each measurement is calculated as the normalised change in this model when that measurement is removed from the model. Therefore, the larger the Cook's Distance, the larger the influence of this individual measurement on the model, and the greater the possibility that this measurement is an outlier in the dataset. Through empirical assessment, it was determined that excluding seedlings having Y(II) values more 2 scaled Cook's Distances below their relative mean was sufficient to remove the late-germinating seedlings from the dataset whilst conserving values recorded from comparable 11 day old seedlings.

In experiments using 28 day old plants, there were no late germinating seedlings as these plants were removed during transplantation of seedlings to compost. Therefore, no outlier removal methods were used for data collected from 28 day old seedlings.

2.4.2 Calculation of rate of recovery

Rate of recovery was for each seedling/leaf calculated using Equation 2.1.

$$[2.1]\ \text{rate of recovery} = \frac{\mathrm{Y(II)_{rx}} - \mathrm{Y(II)_{i}}}{\mathrm{x}}$$

Where x is time in recovery after treatment in minutes, $Y(II)_{rx}$ is Y(II) after following x time in recovery, and $Y(II)_{i}$ is Y(II) immediately after the treatment.

Rate of recovery for each length of time after treatment was calculated using the initial Y(II) measurement following the treatment. This method was chosen to gain insight into the timing and presence of circadian regulation of processes which become active upon removal of the tissue from the treatment conditions, and isolate these rhythms from any which are present in the maintenance of Y(II) under the treatments.

2.4.3 Statistical analysis of duration of treatment experiments

Following outlier removal as previously described, Y(II) and rate of recovery of the seedlings were modelled (Equation 2.2)

$$[2.2]\ \text{model of Y(II)} \leftarrow \text{lmer(Y(II)} \sim \text{Plant Line x Length of Treatment x Treatment Type)}$$

Visual inspection of diagnostic residual plots indicated the models fits were appropriate for the datasets. These models were adapted as appropriate to carry out two-way ANOVA tests on subsets of the data, and subsequent Tukey post-hoc analysis to carry out pairwise comparisons. For each adapted model visual inspection of diagnostic plots was repeats.

2.4.4 Quantitative timecourse analysis

For timecourse analysis, mean Y(II) and rates of recovery were calculated (Equations 2.3 and 2.4). For this, Y(II) and rate of recovery were modelled with mixed effects linear models with fixed effects of “Timepoint”, “Treatment”, and “Plant Line”, and a random effect of “Experiment Repeat”, using the lmer function of R package lme4 (Bates *et al.*, 2015).

$$[2.3]\ \text{model of Y(II)} \leftarrow \text{lmer(Y(II)} \sim \text{Plant Line x Timepoint x Treatment Type} + \text{Experimental Repeat)}$$

$$[2.4]\ \text{model of RecoveryRate} \leftarrow \text{lmer(RecoveryRate} \sim \text{Plant Line x Timepoint x Treatment Type} + \text{Experimental Repeat)}$$

Visual inspection of diagnostic residual plots indicated the models fits were appropriate for the datasets. Therefore, from these models, estimated marginal means and standard error of Y(II) and rate of recovery were calculated, using R package emmeans (Lenth et al., 2022). Similar methods have been used in earlier studies (Simon et al., 2020).

Quantitative timeseries analysis of Y(II) and rate of recovery was carried out using R package MetaCycle (Wu et al., 2019), using as input data the estimated marginal means of Y(II) and rate of recovery calculated as discussed. MetaCycle is a tool that incorporates several algorithms for the identification of periodic data. For analysis, the outputs of the meta2D method was used. The meta2D method integrates the outputs of three other common circadian analysis algorithms, ARSER, JTK_CYCLE, and Lomb-Scargle. By integrating these

three algorithms with the meta2d method, the major limitations of each individual method are avoided.

3 Impact of duration of cold and high light conditions on maintenance of PSII quantum efficiency

3.1 Background

In a natural environment, fluctuations in environmental conditions can have varying durations. For instance, plants may experience high light as both short sunflecks and prolonged direct light on a clear day. Therefore, understanding the influence of condition duration on maintenance of Y(II) is an important aspect of understanding the response of photosynthesis to these conditions in field.

Whilst many different mechanisms act together to maintain Y(II), their rate of induction, and contribution to maintaining Y(II) varies under different conditions.

Fast acting mechanisms include ubiquitous ROS scavenging enzymes, which act rapidly to limit ROS accumulation. The components of NPQ, whilst similarly fast acting, have varying induction times. For example, the induction of qE can occur within seconds to minutes, but induction of qT occurs over a longer timescale taking from 2-15 minutes (Yarkhunova *et al.*, 2018; Kress and Jahns, 2017). Although these mechanisms are sufficient to prevent ROS accumulation during short periods of unfavourable conditions, they are quickly overwhelmed upon prolonged exposure. Therefore, other mechanisms are required to maintain Y(II) under sustained fluctuations in the environment.

Upon prolonged exposure to photooxidative stress, other photoprotective mechanisms become active. Under these conditions, qI, transcriptional changes, alterations in chloroplast protein orientation and distribution, and chloroplast movements, all act to relieve pressure from the rapidly activated systems (Yarkhunova *et al.*, 2018; Kress and Jahns, 2017; Kasahara *et al.*, 2002). However, these processes have longer induction pathways and, therefore, any influence they have on maintaining photosynthetic efficiency becomes apparent after longer periods of altered external conditions. For example, upon exposure to high light, chloroplast movements take between 120-180 minutes (Suetsugu et al., 2016).

Induction times of many mechanisms are specific to the type of stimulus driving the photooxidative stress. For example, the rate of induction and amplitude of responses of the SIG5 pathway appears stimulus specific. Nagashima et al. (2004) showed *SIG5* transcript levels peaked with a five-fold increase within 1 hour of high light irradiation (1000 µmol m^{-2} s^{-1}). In comparison, exposure to cold (4°C) led to a peak in *SIG5* transcript levels three-fold greater than ambient between 2 and 4 hours into the treatment (Nagashima et al., 2004). These results however, conflict with data from the Arabidopsis eFP Browser (https://bar.utoronto.ca), which shows that *SIG5* transcript levels peak with an approximately 4.5 fold increase, 6 hours into cold treatment (Cano-Ramirez and Panter, under revision).

These distinct inductions of *SIG5* transcripts upon exposure to each of cold and high light have been shown to act downstream upon F_v/F_m (Nagashima *et al.*, 2004; Cano-Ramirez and Panter, under revision). Therefore, understanding the role of SIG5 in maintaining chlorophyll fluorescence changes under varying durations of conditions that induce photooxidative stress can provide insights into how responses to these conditions are coordinated, and also the contribution of *SIG5* induction to influence downstream photosynthetic efficiency.

Not only is understanding the response of photosynthetic efficiency under varying durations of cold and/or high light treatments a biologically interesting question, the results of this chapter were also informative for designing experiments in subsequent thesis chapters.

3.2 Results

3.2.1 Methodology and Experimental Design

To investigate the impact of stress condition duration on maintenance of Y(II), Y(II) of 11 day old Col-0 (WT) seedlings was measured after exposure to cold (4°C, 110 µmol$m^{-2}s^{-1}$), high light (19°C, 700 µmol$m^{-2}s^{-1}$), and combined cold and high light (4°C, 700 µmol$m^{-2}s^{-1}$), for 5, 45, 90, 180, or 360 minutes. These treatment lengths were selected to cover a range of induction times of the mechanisms discussed. Experiments commenced 1 h after dawn. Here, it was hypothesised that the greatest reduction in Y(II), as a result of treatment, will occur at intermediate treatment lengths (90 and 180 minutes), as this is when ROS scavenger enzymes and the faster induced components of NPQ will have been overwhelmed, but mechanisms which take longer to act are not yet contributing to maintaining Y(II).

To elucidate the contribution of SIG5 in maintaining Y(II) under difference treatment lengths, *sig5-3* seedlings were included in experiments. It is hypothesised that Y(II) of wild-type and *sig5-3* seedlings will be similar under shorter treatment durations (5-45 minutes); whereas under longer durations (90-360 minutes) there will be a greater reduction in Y(II) in *sig5-3* compared to wild-type due to a loss of SIG5 driven transcriptional changes.

Previous studies have shown circadian rhythms regulate or are involved in maintenance of Y(II), also referred to as F'q/F'm (Litthauer et al., 2015). Therefore, to mitigate for any underlying circadian influences, for each treatment duration, Y(II) of a seedlings exposed to control conditions (19ºC, 110 $\mu molm^{-2}s^{-1}$) was measured.

3.2.2 Reduction in maintenance of Y(II) is dependent on duration of the treatment

Across the dataset of wild-type seedlings, maintenance of Y(II) is influenced by both the treatment conditions, and the duration of condition (ANOVA, $p_{treatment} < 2x10^{-16}$, $p_{duration} < 2x10^{-16}$) (Fig. 3.1). Furthermore, the interaction between treatment type and duration also drove changes in maintenance of Y(II) (ANOVA, $p_{treatment:duration} < 2x10^{-16}$). This suggests, in agreement with a wealth of literature, that presence and intensity of cold and high light conditions impacts the ability of the system to maintain photosynthetic efficiency (Mishra *et al.*, 2011; Kasahara *et al.*, 2002; Cano-Ramirez and Panter, under revision). To investigate the nature of interactions between maintenance of Y(II) and each treatment, the data was subset by treatment condition (control + cold and/or high light).

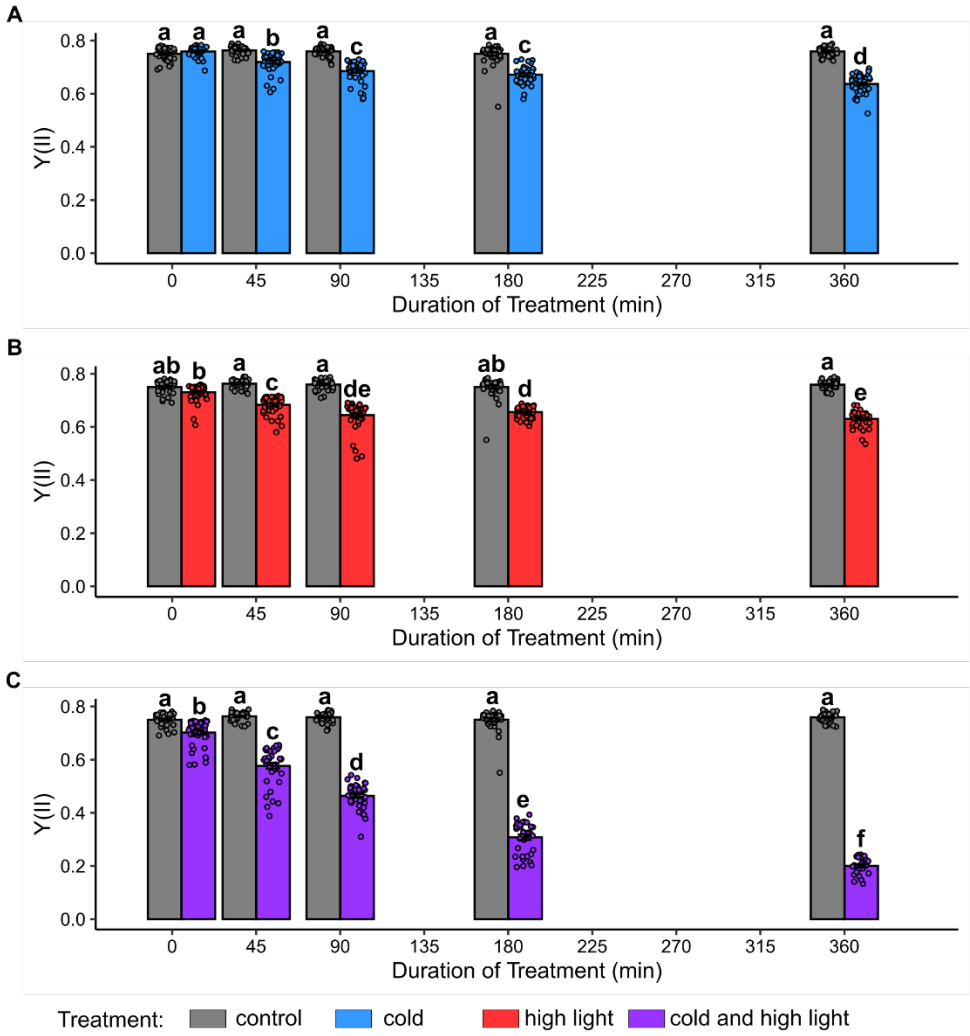

Figure 3.1 Impact of treatment duration on maintenance of Y(II) depends of treatment type. Data from 11 day old Col-0 (WT) seedlings exposed to **(A, B, C)** control (19°C, 110 μmolm^{-2}s^{-1}), **(A)** cold (°C, 110 μmolm^{-2}s^{-1}), **(B)** high light (19°C, 700 μmolm^{-2}s^{-1}), **(C)** combined cold and high light (4°C, 700 μmolm^{-2}s^{-1}) treatments for 5, 45, 90, 180, or 360 minutes. Error bars represent mean ± SE calculated from two independent experimental repeats. N=8-20 individual plants within each independent repeat. Lower case letters represent significantly different results, $p<0.05$ (Tukey HSD comparisons).

3.2.2.1 Maintenance of Y(II) under cold or high light conditions suggests the response pathways act to support Y(II)

For both cold and high light treatments, maintenance of Y(II) was significantly reduced compared to the control treatment (two-way ANOVA, $p_{cold} < 2x10^{-16}$, $p_{high\ light} < 2x10^{-16}$). This suggests that both cold and high light drive photooxidative stress, Y(II). There was also a significant interaction between presence of cold or high light conditions and the duration of

these treatments (two-way ANOVA, $p_{treatment:duration} < 2x10^{-16}$). To elucidate the nature of this interaction, Tukey post-hoc analysis for pairwise comparisons was carried out (Fig. 3.1).

For both cold and high light treatments, there was a general reduction in maintenance of Y(II) with increasing treatment duration (Fig. 3.1A, B). However, this decrease was not proportional to the treatment duration. For treatment durations of 5 minutes, maintenance of Y(II) was comparable between seedlings under control and cold or high light conditions. This could suggest that after this short treatment, the activity of ROS scavenging enzymes and NPQ were sufficient to stem ROS accumulation and prevent significant photodamage.

By 45 and 90 minutes of treatment, there was a significant impact of both cold and high light upon the maintenance of Y(II). The impact of 90 minutes duration was greater than after 45 minutes, for both treatment types. This reduction in Y(II) might be the combined result of increased ROS accumulation from the prolonged treatments overwhelming ROS scavenger enzymes and NPQ, and the treatment's impact the efficiency of scavenger enzymes and NPQ. Impacts of the treatment on the efficiency of photoprotective systems could include, for instance, the reduction in activity of ROS scavenger enzyme caused by low temperatures (Li et al., 2013).

After 180 minutes of cold or high light treatment, the contribution of response mechanisms to mitigate the impact of the treatments on Y(II) can be clearly seen; there is no further reduction in maintenance of Y(II) of either treatment compared to the 90 minute treatment duration. For both treatment types, some of this recovery in Y(II) might be attributed to outputs of transcriptional response pathways. For instance, pathways upregulating *psbD* transcription (e.g. by sigma factors) (Nagashima *et al.*, 2004). For high light, my result differs from a previous study by Kato *et al.* (2012) that found a further decrease in F_v/F_m between high light treatments of 120 minutes and 240 minutes length. These differences might be due to differences in experimental conditions, because Kato *et al.* (2012) measured fluorescence of detached leaves, and used a high light treatment (2500 $\mu molm^{-2}s^{-1}$).

For both cold and high light conditions, after 360 minutes there was a further reduction in maintenance of Y(II) from the level at 180 minutes. For high light, these results have similar behaviour to previous experiments by Kasahara *et al.* (2002), which showed that F_v/F_m reduced by 20% after 1 h high light treatment and extended to a 30% reduction after 5 h. Moreover, this result aligns with the result from Kato *et al.* (2012), suggesting the possible difference in results suggested previously could be a result of the different treatment lengths used in this experiment. This decrease in maintenance of Y(II) could suggest that after 360

minutes, photoprotective processes that sustain Y(II) become overwhelmed by the impact of this prolonged treatment.

3.2.2.2 Exposure to combined cold and high light conditions appears to overwhelm photoprotective mechanisms

In contrast to exposure to cold or high light separately, exposure to a combined cold and high light treatment reduced Y(II) after just five minutes (Fig. 3.1C). In fact, under the combined treatment of cold and high light, Y(II) of treatments of all durations differed significantly from their relative control. The impact of the treatment on maintenance of Y(II) increased with each treatment length (Fig. 3.1C). This reduction in Y(II) at all treatment lengths suggests that the intensity of simultaneous cold and high light is much greater than exposure to either cold or high light conditions alone, driving rapid accumulation of ROS, and potentially overwhelming the action of photoprotective mechanisms. This greater impact of combined cold and high light aligns well with knowledge that electron transfer in the thylakoid membranes driving greater photooxidative stress than would otherwise be observed at a given light intensity (Miura and Furumoto, 2013).

3.2.3 In the presence of cold or high light the contribution of SIG5 to maintenance of Y(II) was limited

Alongside significant influences of treatment condition and duration on maintenance of Y(II), when wild-type and *sig5-3* seedlings were considered together, there was an influence of genotype on maintenance of Y(II) (ANOVA, $p_{genotype} < 2x10^{-16}$). Furthermore, there were significant interactions between genotype and both treatment condition, and duration of treatment contributed to differences in maintenance of Y(II) observed (ANOVA, $p_{genotype:treatment} = 0.0128$, $p_{genotype:duration} = 0.0425$). To investigate these interactions further, the dataset was subset by treatment condition and duration of treatment then analysed using Tukey post-hoc analysis for pairwise comparisons (Fig. 3.2). Under control conditions, maintenance of Y(II) for each treatment duration did not differ between wild-type and *sig5-3* (one-way ANOVA, $p = 0.159$). Therefore, comparison between wild-type and *sig5-3* under the cold and/or high light conditions can be made, without the need for comparison between relevant measurements under control conditions alongside.

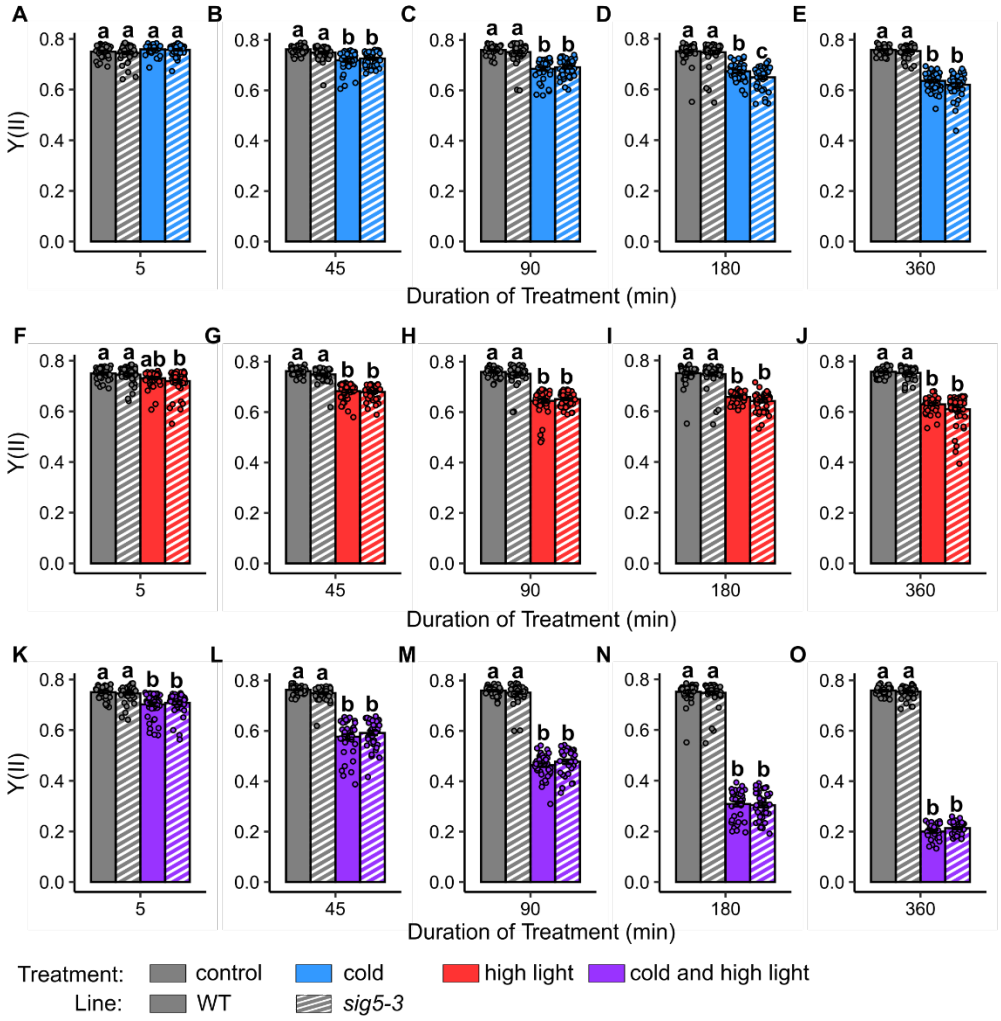

Figure 3.2 Role of SIG5 in maintenance of Y(II) across treatment types and durations appears minimal. Results obtained from 11 day old Col-0 (WT) and *sig5-3* seedlings exposed to **(A-O)** control (19ºC, 110 µmolm^{-2}s^{-1}), **(A-E)** cold (4ºC, 110 µmolm^{-2}s^{-1}), **(F-J)** high light (19ºC, 700 µmolm^{-2}s^{-1}), **(K-O)** combined cold and high light (4ºC, 700 µmolm^{-2}s^{-1}) treatments for 5 (A, F, K), 45 (B, G, L), 90 (C, H, M), 180 (D, I, N), or 360 (E, J, O) minutes. Error bars represent mean ± SE calculated from two independent experimental repeats. N=8-20 individual plants within each independent repeat. Lower case letters represent significantly different results, $p<0.05$ (Tukey HSD comparisons). Post-hoc analysis was carried out independently for each plot.

3.2.3.1 Behaviour of *sig5-3* under cold conditions reflected observations of previous studies

Under cold conditions of durations 5-90 minutes, Y(II) in *sig5-3* was maintained at a level similar to that of wild-type plants (Fig. 3.2A). However, following exposure to cold conditions for 180 minutes, Y(II) of *sig5-3* was reduced compared to WT. Whilst this result suggests a role of SIG5 in maintaining Y(II) under cold conditions, it differs a previous observation by

Cano-Ramirez *et al.* of chlorophyll fluorescence of *sig5-3* under cold (Cano-Ramirez and Panter, under revision). That study found F_v/F_m of *sig5-3* was not significantly reduced below the level of wild-type following 180 minutes of cold treatment. However, this difference from my results may be due to the additional dark adaptation step involved in measuring F_v/F_m vs measuring Y(II). Similar to Cano-Ramirez *et al.*, when cold treatment length was extended, to 360 minutes, the reduction in *sig5-3* below wild-type was lost.

3.2.3.2 Under high light conditions *sig5-3* performed similar to WT

For high light no significant differences were found between maintenance of Y(II) of wild-type and *sig5-3* genotypes (Fig. 3.2B). This result suggests the induction of *SIG5* by treatment with high light, shown by previous experiments, may not contribute significantly to maintaining downstream photosynthetic efficiency (Noordally *et al.*, 2013; Belbin *et al.*, 2017). Additionally, this result differs from those of experiments by Nagashima *et al.* (2004) which found a difference *sig5-2* plants had reduced F_v/F_m compared to wild-type (Nagashima et al., 2004). However, these results may not be directly comparable as the previous study used a different mutant allele, different conditions for the high light treatment (1000 $\mu molm^{-2}s^{-1}$ intensity and 4 h treatment duration), and had a dark adaptation step before measurements were taken.

3.2.3.3 Intensity of combined cold and high light conditions limited influence of SIG5 on maintenance of Y(II)

Similar to results under high light conditions, there was no significant difference in Y(II) between wild-type and *sig5-3* arising from any treatment duration of combined cold and high light conditions. Again, this suggests a limited role of the previously shown induction of SIG5 in response to separate cold and high light conditions (Noordally *et al.*, 2013; Belbin *et al.*, 2017; Cano-Ramirez and Panter, under revision). However, given that the *sig5-3* mutation affected Y(II) under cold conditions, this result could instead reflect the intensity of the combined cold and high light treatment. In other words, the ability of this treatment to drive photooxidative stress is such that any actions of SIG5 to support a greater maintenance of Y(II) in wild-type than *sig5-3* are lost.

3.3 Discussion

Photooxidative stress reduces photosynthetic efficiency and plant fitness (Kromdijk, 2022). To limit this, plants have evolved mechanisms to reduce damage to the photosynthetic apparatus. Some of these processes involve long term ultrastructural and developmental changes. However, in natural environments, plants may be exposed to a specific driver of photooxidative stress for only a short period of time making adaptive structural changes unnecessary. Therefore, plants have evolved mechanisms which make short term alterations to the system to minimise irreversible damage and maintain photosynthetic efficiency. Studies have shown that the induction of many of these response pathways is specific to both environmental conditions and is intensity dependent (Nagashima *et al.*, 2004; Cano-Ramirez and Panter, under revision; Belbin *et al.*, 2017). This chapter presented evidence that these mechanisms act to support photosynthetic efficiency under increasing durations of cold and high light conditions and that the intensity of combined cold and high light overwhelms the effectiveness of these processes.

3.3.1 Under increasing durations of cold or high light conditions, the contribution of photoprotective mechanisms in maintaining Y(II) is evident

A key result of this chapter is the lack of decrease in the level of Y(II) maintained from 90 to 180 minutes of cold or high light. This result differs from experiments by Kato *et al.* (2012) which found Y(II) continues to decrease under prolonged high light treatment. However, this difference is likely due to many dissimilarities in experimental design, including but not limited to the used of detached leaves, different treatment durations and high light irradiance in Kato *et al.* (2012). For high light, this result differs from what is seen at protein level, where D1 protein levels continually decline from 1 to 3 h of high light irradiance (Zhang et al., 2010). Therefore, these results provide new insights into the contribution of photoprotective mechanisms, aside from photosystem protein turnover, in maintaining photosynthetic efficiency. These results also show that interpretation of this kind of data is incredibly dependent of experimental conditions, making comparisons between studies challenging.

Overall, assigning importance of different photoprotective mechanisms to explaining the results of this chapter is limited by the number of interconnected processes which can work to maintain Y(II). However, these results – that varying induction times of different mechanisms

influences photosynthetic efficiency under cold and high light – provide ample justification for more resource-intensive biomolecular studies to investigate the underlying mechanisms.

3.3.2 Well established induction of SIG5 in response to cold and high light may not contribute significantly in maintaining Y(II) under short-term exposure

As discussed, previous studies have considered the impact of cold or high light treatments on chlorophyll fluorescence and the role of SIG5 in this process (Nagashima *et al.*, 2004; Cano-Ramirez and Panter, under revision). However, direct comparison of the results of this chapter with those studies is hampered experimental design – previous studies have used the parameter F_v/F_m to measure chlorophyll fluorescence. Measuring F_v/F_m requires a dark adaption stage between exposure the treatment and measurement (Fig. 2-2). Therefore, the decision to measure Y(II) over F_v/F_m in this chapter's experiments was driven by a desire to gain the best insight into photosynthetic efficiency of the system under the treatment condition, without this being confounded by the influence of the dark adaption stage (Guadagno et al., 2018).

This difference in the response of different chlorophyll fluorescence parameters may explain a result from this chapter. Here, there was a significant difference between Y(II) of *sig5*-3 and wild-type under after 3 h of cold conditions. This could suggest the role of SIG5 in supporting photosynthetic efficiency differs between maintenance under cold *versus* recovery immediately after the treatment. This hypothesis aligns with results of Nagashima *et al.*, (2004), which identified impaired recovery in chlorophyll fluorescence of *sig5-2* compared to wild-type following a high light treatment. For this reason, recovery of Y(II) after the treatments was considered in experimental design within my subsequent chapters.

Apart from the difference that was present after 3 h cold treatments, these experiments found Y(II) of *sig5-3* and wild-type under each treatment condition to be similar. This suggests that the induction of SIG5 transcription upon exposure to many different conditions may not contribute significantly to maintaining Y(II) (Cano-Ramirez and Panter, under revision ; Noordally *et al.*, 2013). To consolidate this conclusion and confirm SIG5 activity is not the driving factor behind the increase in Y(II) observed between the 90 and 180 minute treatment durations, it would be informative to compare SIG5 transcript abundance in parallel with and under exactly the same conditions as the measurements of Y(II). This would allow a direct

comparison of *SIG5* transcript levels under these conditions with the observed changes in Y(II).

3.3.3 Combined cold and high light represents a scenario of great photooxidative stress and irreversible photodamage

The results of this chapter have also provided experimental evidence that a combination of cold and high light conditions represents a scenario of great photooxidative stress for the photosynthetic apparatus (Fig. 3.1C). This supports other studies that have shown that cold conditions reduce the irradiance at which photoinhibition occurs (Miura and Furumoto, 2013). Many factors likely contribute to this, for example, prolonged cold increases membrane viscosity which in turn restricts diffusion of plastoquinone, disrupting electron transfer (Li et al., 2013). Beyond this study, another has shown that there are differences in gene upregulation when tissue is exposed to cold and dark conditions *vs* cold and light conditions (Soitamo et al., 2008). This difference in gene expression again suggests response to combined cold and high light differs from exposure to cold alone.

3.3.4 Conclusions

This chapter provides experimental evidence that mechanisms acting to maintain photosynthetic efficiency under photooxidative stress have varying induction times. This could suggest that maintenance of Y(II) under short fluctuations in environmental conditions is limited by the rate of induction of these mechanisms. Further, experiments of this chapter demonstrate the effectiveness of these mechanisms in maintaining Y(II) is rapidly reduced under scenarios that drive substantial photooxidation, such as combined cold and high light conditions. Over the last century, there has been an acceleration in the frequency and intensity of previously uncommon weather events, for instance, late spring frosts (Lamichhane, 2021). Therefore, understanding the effectiveness of photoprotective mechanisms in mitigating reductions in plant fitness under these conditions is increasingly important.

Not only does combined cold and high light occur during late spring frosts, because under natural conditions they also occur to a lesser intensity regularly over the diel cycle at dawn. Considering the results of this chapter in this context, circadian regulation of relevant gene expression and induction of photoprotective mechanisms could increase plant fitness by

aligning activity of these processes to the diel cycles in cold and high light, potentially increasing plant fitness. Therefore, circadian regulation of maintenance of Y(II) under cold and/or high light was studied in the subsequent Chapters 4 and 5.

4 Circadian regulation of maintenance and recovery in effective PSII quantum yield following cold and high light conditions

4.1 Background

The role of the circadian oscillator in driving circadian rhythms in many aspects of photosynthesis is well established (Dodd et al., 2014). However, the importance of this regulation in supporting plant fitness is less well understood. To investigate this, Y(II) of seedlings was measured as an indicator of photosynthetic efficiency, which ultimately could be an indicator of a component of plant fitness (Molina-Montenegro et al., 2013).

4.1.1 Circadian regulation of maintenance photosynthetic efficiency is well established

Circadian rhythms in maintenance of Y(II) – also referred to in literature as F'_q/F'_m – have previously been investigated. Under experimental control conditions, rhythms in Y(II) have been shown to occur in *Arabidopsis*, marimo and, *Marchantia polymorpha* (Litthauer *et al.*, 2015; Cano-Ramirez *et al.*, 2018; Cuitun-Coronado *et al.*, 2022). Moreover, in *Arabidopsis*, rhythms have been identified in other photosynthetic parameters under steady state, e.g. F_v/F_m, and delayed fluorescence (Yarkhunova *et al.*, 2018; Dakhiya *et al.*, 2017).

Alongside rhythms in chlorophyll fluorescence parameters, transcript abundance of *SIG5* and its downstream targets are under circadian control (Noordally et al., 2013b). Furthermore, *SIG5* transcription is induced by exposure to multiple stress conditions (Nagashima et al., 2004) and in response to both cold and high light treatments; this induction has been shown to be circadian gated (Belbin et al., 2017) (Cano Ramirez, under review).

In natural environments light intensity changes with a diel cycle, increasing from dawn to a peak during the day and then declining towards dusk. Similarly, temperature changes with a diel cycle reaching a minimum prior to dawn (Cano-Ramirez et al., 2022). Therefore, the coincidence of cold and light conditions also occurs with diel cycles, with both conditions coinciding after dawn. Under these conditions, the system is at great risk of photoinhibition

because the slowing of electron transfer by cold reduces the threshold of excitation energy causing photodamage (Miura and Furumoto, 2013). Thus, circadian regulation might provide a process that anticipates these fluctuations in conditions, thus improving the efficiency of the system and contributing to plant productivity. Therefore, the hypothesis that Y(II) might exhibit distinct circadian rhythms under cold and high light conditions, compared to control conditions. By understanding the role circadian regulation on maintenance Y(II) under fluctuating conditions it might be possible to gain further insights into why there is circadian regulation of photosynthesis overall.

Whilst there was a limited role of SIG5 in maintaining Y(II) under cold and/or high light conditions (Chapter 3) during time-independent measurements of Y(II), SIG5 may have a role in circadian regulation of maintenance of Y(II). Moreover, the peak in *SIG5* transcript abundance 1 h after dawn coincides with the time of day when plants are most likely to experience cold and high light conditions (Cano-Ramirez and Panter, under revision). Combined with gating of *SIG5* induction in response to cold and high light, this might enable maximum upregulation of transcription of core PSII subunits at times of day to when damage is most likely to occur, optimising maintenance of photosynthetic efficiency across the day.

4.1.2 Understanding of the role of circadian regulation in recovery of photosynthetic efficiency is limited

During periods of increased photooxidative stress, alterations occur to the photosynthetic system to limit damage. Some of these changes work to increase maintenance of Y(II) under the driver of photooxidative stress. However others, with the goal of limiting long term damage to the system, reduce Y(II). For instance, NPQ competes with Y(II) (Fig. 1.1). This means increases in NPQ to dissipate excess energy and limit ROS accumulation, in turn, reduces Y(II) (Kromdijk, 2022; Maxwell and Johnson, 2000). Therefore, upon the removal of the driver of photooxidative stress, mechanisms such as NPQ must be relaxed to increase the efficiency of the system under the new conditions. In spite of photoprotective mechanisms, following prolonged photooxidative stress, irreversible damage to the photosynthetic apparatus is inevitable (Chen et al., 2020). In addition, under oxidative stress replacement of photosystem proteins is limited. For instance, oxidative conditions inhibit de novo synthesis of the subunit D1, which is a major site of irreversible photooxidation of PSII (Nishiyama *et al.*, 2001; Chen *et al.*, 2020). Therefore, replacement of most damaged proteins is likely to occur once the drivers of oxidative stress have been lessened.

Studies investigating the recovery in chlorophyll fluorescence following exposure to photooxidative stress are limited in number. The premise of one study, by Gray *et al.* (2003), was to consider recovery in chlorophyll fluorescence following cold treatments and how cold-acclimation impacts this. However, given the light intensity used (2200 $\mu molm^{-2}s^{-1}$), this could instead be viewed as a study into recovery following cold and high light exposure. This study found that even 80h after exposure to their experiment treatment, F_v/F_m of plants was still reduced compared pre-treatment values. In contrast to Gray *et al.* (2003), another study Khanal *et al.* (2017) found F_v/F_m of plants treated with 4 h of 1750 $\mu molm^{-2}s^{-1}$ had recovery to a level similar to control plants after 24 h. This inconsistency in results combined with the limited number of studies considering recovery of chlorophyll fluorescence following photooxidative stress highlights the need for comprehensive studies to investigate this process. The role of sigma factors in recovery following photooxidative stress has been previously explored to a limited extent. Nagashima *et al.* (2004) suggested *sig5-2* had impaired recovery in F_v/F_m following a high light treatment compared to wild-type. However, drawing conclusions from the results of this study is limited due to a lack of any statistical analysis and rather noisy data.

As previously discussed, many drivers of photooxidative stress such as cold and high light may cycle in intensity with a 24 h period. Therefore, at certain times of day the system is more likely to require the activity of mechanisms for recovery from this stress. Whilst little work aimed to understand the role of circadian regulation in recovery in photosynthesis following photooxidative stress, many studies have suggested links between the circadian oscillator and cell redox state (Simon *et al.*, 2019; Jiménez, Sevilla and Martí, 2021; Lai *et al.*, 2012). As photooxidative stress involves accumulation of ROS, this circadian regulation of cell redox state could act downstream to influence recovery of photosynthetic efficiency following photooxidative stress. Reviews such as that by Guadagno, Ewers and Weinig (2018) have proposed measuring Y(II) would be useful for investigating this because the dynamic nature of Y(II) allows small changes in chlorophyll fluorescence to be detected.

4.2 Results

4.2.1 Methodology and Experimental Design

As experimental design differs between previous studies showing circadian rhythms in chlorophyll fluorescence parameters, first it had to be established that rhythmicity of Y(II) under free running conditions could be identified. For this, Y(II) was measured from the cotyledons of 11 day old Col-0 (wild-type), and 35S::CCA1 (CCA1-ox) seedlings. Prior to each measurement, seedlings were exposed to 3 h treatments of control conditions (19^{o}C and 110 $\mu molm^{-2}s^{-1}$), with measurements obtained repeatedly over a 44 h time period. Prior to the start of the experiment treatment, all seedlings were held in free-running (constant light and temperature, 110 $\mu molm^{-2}s^{-1}$) conditions for 24 h.

Beyond this, to investigate whether there is circadian regulation of maintenance of Y(II) under cold and/or high light conditions, separate to circadian regulation under control conditions. Therefore, using the same experimental design as the above experiment, Y(II) of seedlings of each genotype was measured following exposure to 3 h treatments of either cold (4^{o}C and 110 $\mu molm^{-2}s^{-1}$), high light (19^{o}C and 700 $\mu molm^{-2}s^{-1}$), or combined cold and high light (4^{o}C and 700 $\mu molm^{-2}s^{-1}$). 3 h long treatments with these conditions were selected, because previous experiments have shown that this duration was sufficient for detection of the impact of adaptive mechanisms. Moreover, this is the shortest duration of cold treatment necessary to drive significant increases in SIG5 transcript levels (Cano Ramirez, under review). Seedling age for experiments was chosen to be in line with previous studies investigating SIG5 transcript levels under cold and high light conditions (Cano Ramirez, under review; Belbin *et al.*, 2017).

To elucidate the involvement of SIG5 in the maintenance of Y(II), *sig5-3* seedlings were also included in experiments. Here it was hypothesised that in the presence of the stress treatments wild-type will out-perform *sig5-3* at only the certain times of day. These times of day of under-performance would correspond to times of greatest induction of SIG5 activity in response to the stress conditions, representing the times when SIG5 is required to maintain the photosynthetic apparatus under the conditions.

To test the hypothesis that there could be circadian regulation recovery in Y(II) following photooxidative stress, immediately after initial measurements of Y(II) seedlings were held in darkness and Y(II) was measured again, 15, 30, 45, and 60 minutes later (as the seedlings

are held in darkness before the recovery measurements, Y(II) under this condition is equal to F_v/F_m). From each of these durations of recovery, a rate of recovery in Y(II) was calculated.

4.2.2 Circadian regulation of maintenance of Y(II) under cold and/or high light conditions

Estimate marginal means of the response of Y(II) to each treatment at each timepoint are displayed, grouped by treatment type, in Fig. 4.1A-D. These means of response of Y(II) to each treatment have been repeated in Fig. 4.2A, C ,E, to allow for visual comparison between mean Y(II) in response to each stress treatment with Y(II) under control conditions. To consider whether there was circadian gating of the response of Y(II) to each stress conditions, the difference between mean Y(II) under control and each stress treatment at each timepoint was calculated and are shown in Fig. 4.2B ,D ,F. To elucidate differences between the responses of Y(II) of *sig5-3* and CCA1-ox *vs* wild-type, the difference between these was calculated for each treatment, this is shown in Fig. 4.3.

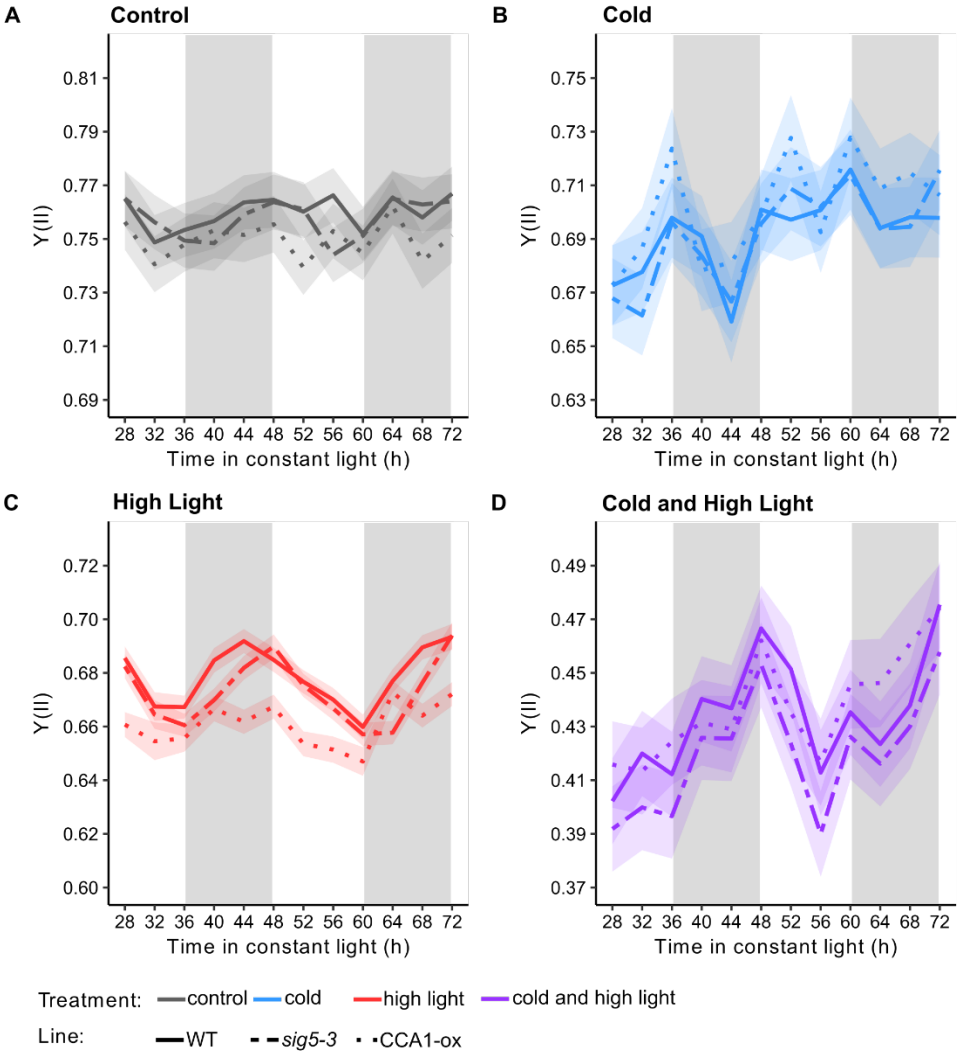

Figure 4.1 Impact of treatment conditions on circadian features of effective PSII quantum yield (Y(II)) are treatment type specific. Measurements of 11 day old cotyledons of Arabidopsis thaliana seedlings. Prior to measurement plants were exposed to 3 h treatments of **(A)** control (19ºC, 110 µmolm^{-2}s^{-1}), **(B)** cold (4ºC, 110 µmolm^{-2}s^{-1}), **(C)** high light (19ºC, 700 µmolm^{-2}s^{-1}), **(D)** combined cold and high light (4ºC, 700 µmolm^{-2}s^{-1}). Plots show estimated marginal mean ± SE for three independent experimental repeats (two for CCA1-ox), calculated using a mixed effects linear model which adjusted values for the influence of random effect experimental repeat. N = 8-20 individual plants within each independent repeat. Clear areas of panels indicate subjective day, grey areas of panels indicate subjective night. Note, y-axes of each plot are to the same scale but cover different ranges to allow visual inspection of rhythmicity of the traces.

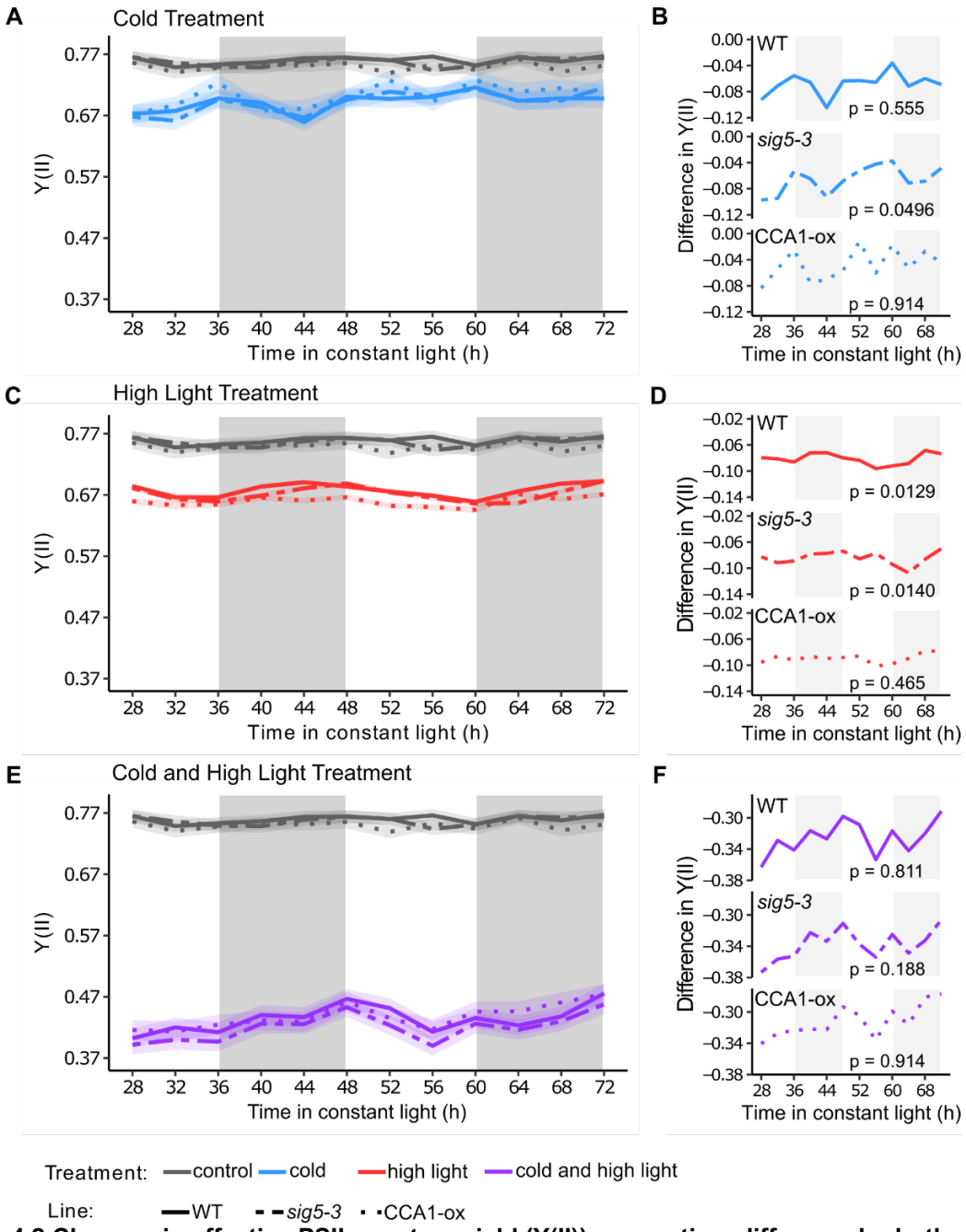

Figure 4.2 Changes in effective PSII quantum yield (Y(II)) across time differs under both control and cold and/or high light conditions. Measurements of 11 day old cotyledons of Arabidopsis thaliana seedlings. Prior to measurement plants were exposed to 3 h treatments of **(A,B)** cold (4°C, 110 $\mu molm^{-2}s^{-1}$), **(C,D)** high light (19°C, 700 $\mu molm^{-2}s^{-1}$), **(E,F)** combined cold and high light (4°C, 700 $\mu molm^{-2}s^{-1}$), or **(A.F)** control (19°C, 110 $\mu molm^{-2}s^{-1}$). **(A,C,E)** estimated marginal mean ± SE for three independent repeats (two for CCA1-ox), calculated using a mixed effects linear model which adjusts values for the influence of random effect experimental replicate. N = 8-20 individual plants within each independent repeat. Data repeated from Fig. 1 for treatment comparison. **(B,D,F)** difference is treatment minus control mean whereby more negative differences reflect a greater impact of treatment on Y(II) compared to control conditions. p-values (meta2D, $p<0.05$) represent a test for statistically significant rhythmicity, shown to three significant figures. Clear areas of panels indicate subjective day, grey areas of panels indicate subjective night.

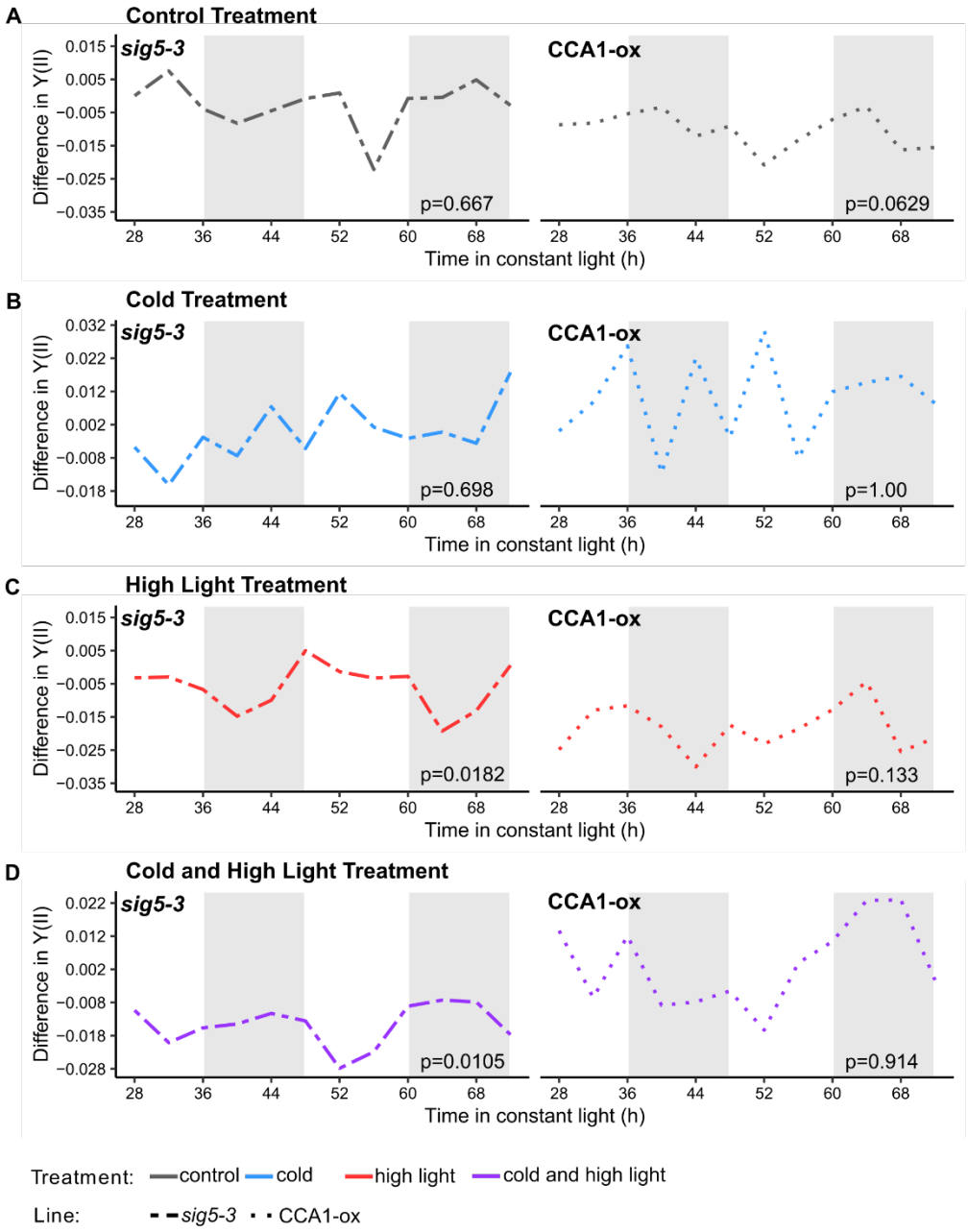

Figure 4.3 Rhythmicity in differences between genotypes suggest role of SIG5 in circadian gating of effective PSII quantum yield (Y(II)) in response to high light. Plots show difference in Y(II) between means of WT and each genotype under treatments of **(A)** control (19ºC, 110 μmolm^{-2}s^{-1}), **(B)** cold (4ºC, 110 μmolm^{-2}s^{-1}), **(C)** high light (19ºC, 700 μmolm^{-2}s^{-1}), **(D)** combined cold and high light (4ºC, 700 μmolm^{-2}s^{-1}) conditions. Estimated marginal means calculated from three independent repeats (two for CCA1-ox) using a mixed effects linear model, adjusting values for the influence of random effect experimental replicate. N = 8-20 individual plants within each independent repeat. Differences calculated as mutant minus WT mean such that a more negative difference represents a greater reduction in Y(II) by the treatment in the mutant than WT. p-values (meta2D, $p<0.05$) represent a test for statistically significant rhythmicity, shown to three significant figures. Clear areas of panels indicate subjective day, grey areas of panels indicate subjective night. Note, y-axes of each plot are to the same scale but cover different ranges to allow visual inspection of rhythmicity of the traces.

4.2.2.1 Rhythmicity in maintenance of Y(II) of *sig5-3* under control conditions suggests underlying circadian regulation

To investigate the maintenance of Y(II) to cold and/or high light conditions, first the maintenance of Y(II) under control environmental conditions was considered. In contrast to previous studies, no significant rhythmicity in Y(II) of wild-type was identified (Fig. 4.1A, Table 4.1) (Litthauer et al., 2015). This might reflect differences in experimental design, including growing and measurement conditions. Similar to wild-type, no rhythm was found in maintenance of Y(II) in CCA1-ox seedlings under control conditions (Table 4.1). Although this might suggest a lack of circadian regulation of maintenance of Y(II), a rhythm was present in Y(II) of *sig5-3*. This suggests SIG5 activity is under regulation by the circadian oscillator such that it is SIG5 acts to support a more constant level of Y(II) across time in wild-type. Hence, when SIG5 activity is lost, a rhythm emerges in *sig5-3*.

Table 4.1 Summary of meta2d quantitative timecourse analysis of maintenance of effective PSII quantum yield (Y(II)) under cold and/or high light conditions. Y(II) of 11 day old cotyledons measured at 12 timepoints across a 44 hour subjective time period, following cotyledon exposure to 3 h treatments conditions. Analysis carried out on estimated marginal mean Y(II), calculated for each timepoint of three (two for CCA1-ox) independent experimental repeats, N=8-20 individual plants within each independent repeat. Estimated marginal means calculated using mixed effects linear models, adjusting means for the influence of experimental repeat as a random effect. P-values obtained from meta2d method analysis for statistically significant rhythmicity. Period, phase, and amplitude estimates are excluded for experiments which did not yield statistically significant rhythms because these measures are derived from poor fits to the data. Results shown to three significant figures. $p<0.05$ *, $p<0.01$ **, $p<0.001$ ***

Treatment	Genotype	p-value	Period est. (h)	Phase est. (h)	Amplitude est. (Y(II))
Control	WT	0.466	–	–	–
(19°C, 110 µmol m^{-2} s^{-1})	*sig5-3*	4.95×10^{-05} ***	20.3	7.21	0.00910
	CCA1-ox	0.811	–	–	–
Cold	WT	0.698	–	–	–
(4°C, 110 µmol m^{-2} s^{-1})	*sig5-3*	0.714	–	–	–
	CCA1-ox	0.936	–	–	–
High Light	WT	0.000159 ***	24.0	22.3	0.0145
(19°C, 700 µmol m^{-2} s^{-1})	*sig5-3*	2.29×10^{-06} ***	24.7	23.9	0.0162
	CCA1-ox	0.112	–	–	–
Cold and High Light	WT	0.188	–	–	–
(4°C, 700 µmol m^{-2} s^{-1})	*sig5-3*	0.0473 *	26.2	19.7	0.0200
	CCA1-ox	0.177	–	–	–

4.2.2.2 Under cold conditions SIG5 may support Y(II) at certain times of day

Rhythmicity was not detected in the maintenance of Y(II) of wild-type, *sig5-3*, or CCA1-ox following exposure to cold conditions (Table. 4.1, Fig. 4.1B). To investigate whether Y(II) in response to short-term cold differed from under control conditions, for each genotype the difference between mean Y(II) under control and cold conditions at each timepoint was calculated (Fig. 4.2B). Of these differences, a rhythm was found in the difference between control and cold treated *sig5-3* (Fig. 4.2B) This could suggest there is circadian gating of maintenance of Y(II) under cold conditions in *sig5-3*, such that the impact of cold conditions on reducing Y(II) of *sig5-3* is smallest at dusk. This result appears inconsistent with the transcript level dynamics, where gating of induction of *SIG5* to cold treatment occurs in wild-type and is lost in *sig5-3* (Cano Ramirez, under review). However, lack of retention of gating of *SIG5* to cold upon the output variable Y(II) could suggest that gating of *SIG5* induction in wild-type works to maintain a constant level of Y(II) under cold conditions across the day by being most active during times of greatest sensitivity to cold. Thus, when SIG5 activity is lost in *sig5-3*, rhythmicity emerges in Y(II).

4.2.2.3 SIG5 is involved in circadian gating of maintenance of Y(II) under high light

Following treatment with short-term high light, rhythms were found in maintenance of Y(II) of both wild-type and *sig5-3*, whilst no rhythm was found in CCA1-ox (Table 4.1), suggesting there is circadian regulation of the maintenance of Y(II) under high light conditions. When the difference between Y(II) in response to high light compared to under control conditions was considered for each genotype, rhythmicity was found in the difference for both wild-type and *sig5-3*, but not CCA1-ox (Fig. 4.2D). For both wild-type and *sig5-3*, high light had the greatest impact on maintenance of Y(II) in the hours around subjective dusk (Fig. 4.2D). Moreover, the estimated amplitude of the rhythmic difference in Y(II) of *sig5-3* is greater than wild-type (wild-type = 0.0108, *sig5-3* = 0.0113, meta2d (Y(II)), suggesting the impact of high light on *sig5-3* is greater at certain times of day. Alongside this difference in amplitude, the rhythm of mean Y(II) of *sig5-3* showed a phase shift of ~1.6 h compared to wild-type (Table 4.1). For these reasons, differences in the rhythms observed in *sig5-3* compared to wild-type were considered. Therefore, the difference between maintenance of Y(II) under high light of wild-type and *sig5-3* at each timepoint was calculated (Fig. 4.3C). In contrast to the difference between wild-type and *sig5-3* under control conditions (Fig. 4.3A), the difference between Y(II) these genotypes in response to high light was rhythmic (Fig. 4.3C). This rhythm was such that under high light

sig5-3 most under-preformed wild-type ~4 h after subjective dusk, whilst, at subjective dawn, *sig5-3* preformed similar to wild-type.

Together these results suggests the presence of circadian regulation of maintenance of Y(II) under high light in both WT and *sig5-3*, which is absent from the arrhythmic genotype CCA1-ox. This regulation is such that maintenance of Y(II) under high light is gated, whereby the system is more sensitive to high light during the day. This is consistent with previous studies that have shown induction of *SIG5* by high light is greatest in the hours approaching dusk and provides a line of evidence that this gating of SIG5 transcript induction in response to high light acts downstream to influence Y(II) (Noordally et al., 2013b).

In addition to the results for rhythmicity in Y(II), from visual inspection, at all timepoints maintenance of Y(II) in response to short-term high light CCA1-ox was reduced compared to WT for all timepoints (Fig. 4.1C). This could suggest underlying circadian regulation of the response to high light which acts to supports a greater level of Y(II) in WT.

4.2.2. Under cold and high light SIG5 may support maintenance of Y(II)

Similar to the control, under cold and high light conditions, rhythmicity was absent from the maintenance of Y(II) of wild-type or CCA1-ox but was found in *sig5-3* (Table 4.1, Fig. 4.1D). To elucidate whether circadian gating was present in the maintenance of Y(II) under cold and high light conditions, the difference between Y(II) control and this condition was calculated (Fig. 4.2E,F). No rhythmicity was detected for these differences in any genotype tested. This suggests there is not circadian gating of maintenance of Y(II) under cold and high light. For completeness, the difference between Y(II) of each genotype and wild-type under was calculated (Fig 4.3D). Interestingly, when the differences between the genotypes were tested for rhythmicity, a rhythm was present in the difference between Y(II) of wild-type and *sig5-3*. This could suggest there is circadian gating of SIG5 activity in wild-type, which works to support maintenance of Y(II) are certain times of day, to maintain a more constant level of Y(II) across the day.

4.2.3 Circadian regulation of rate of recovery in Y(II) after exposure to cold and/or high light conditions

Estimate marginal means of the rate of recovery in Y(II) to each treatment at each timepoint are displayed, grouped by treatment type, in Fig. 4.4. To elucidate differences between the rate of recovery in Y(II) of *sig5-3* and CCA1-ox *vs* wild-type, the difference between these was calculated for each treatment, this is shown in Fig. 4.5. To consider whether there was circadian gating of rate of recovery in Y(II) to each of the stress conditions, the difference between mean rate of Y(II) under control and each stress treatment at each timepoint was calculated and are shown in Fig. 4.6.

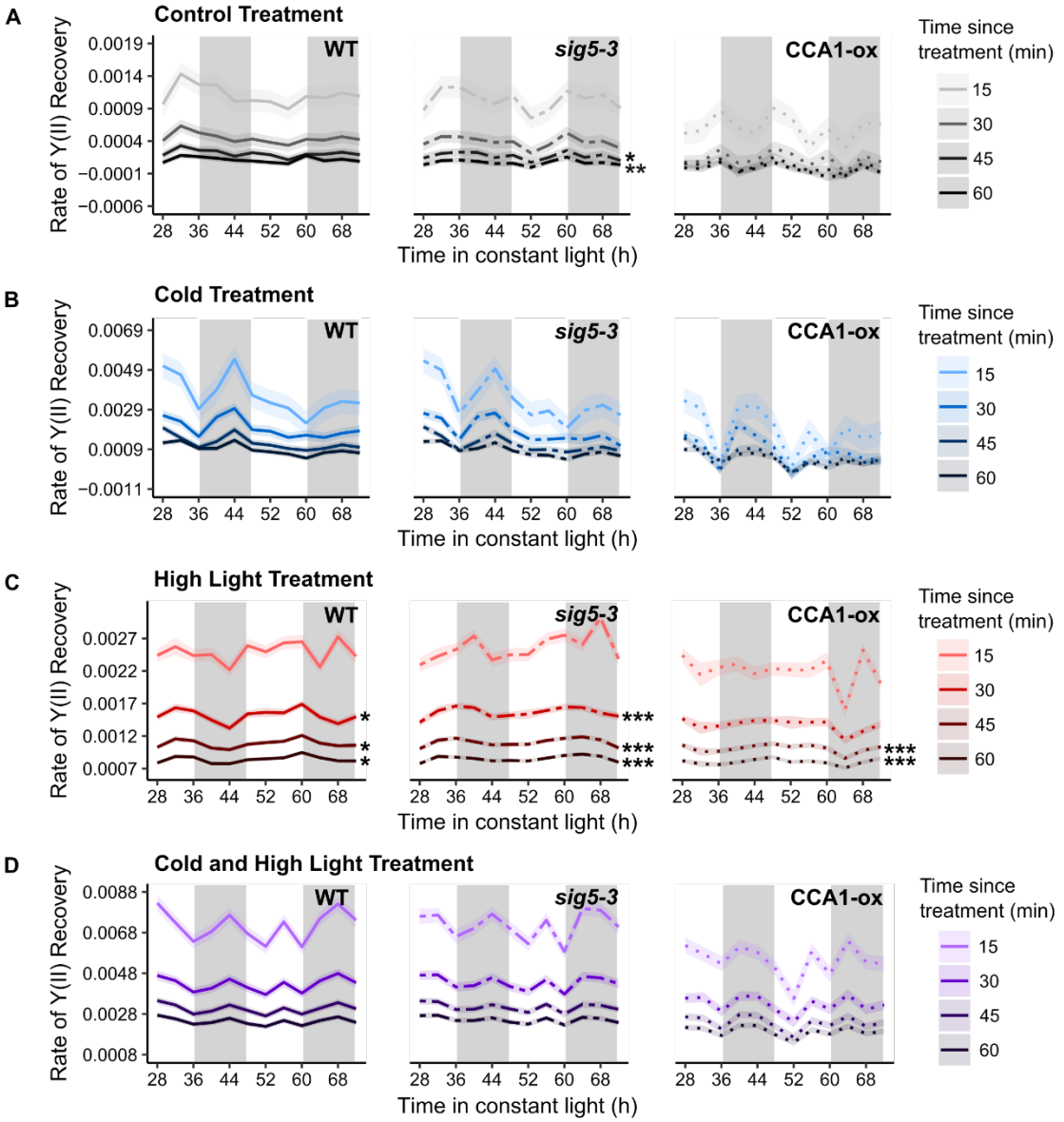

Figure 4.4 Circadian features emerge in rate of recovery in effective PSII quantum yield (Y(II)). Measurements of 11 day old cotyledons of Arabidopsis thaliana seedlings. Prior to measurements plants were exposed to 3 h treatments of **(A)** control (19ºC, 110 µmolm^{-2}s^{-1}), **(B)** cold (4ºC, 110 µmolm^{-2}s^{-1}), **(C)** high light (19ºC, 700 µmolm^{-2}s^{-1}), **(D)** combined cold and high light (4ºC, 700 µmolm^{-2}s^{-1}). At each timepoint, Y(II) measurements were taken 0, 15, 30, 45, and 60 minutes after the treatment. Rate of recovery was calculated for each seedling as ((Y(II) at time x after treatment) – Y(II) at time 0)/ x. Plots show estimated marginal mean ± SE for three independent experimental repeats (two for CCA1-ox), calculated using a mixed effects linear model which adjusted values for the influence of random effect experimental repeat. N = 8-20 individual plants within each independent repeat. p<0.05 *, p<0.01 **, p<0.001 ***, representing results of meta2D method test for statistically significant rhythmicity. Note y-axis scales differ between plots. Clear areas of panels indicate subjective day, grey areas of panels indicate subjective night. Note, y-axes of each plot are to the same scale but cover different ranges to allow visual inspection of rhythmicity of the traces.

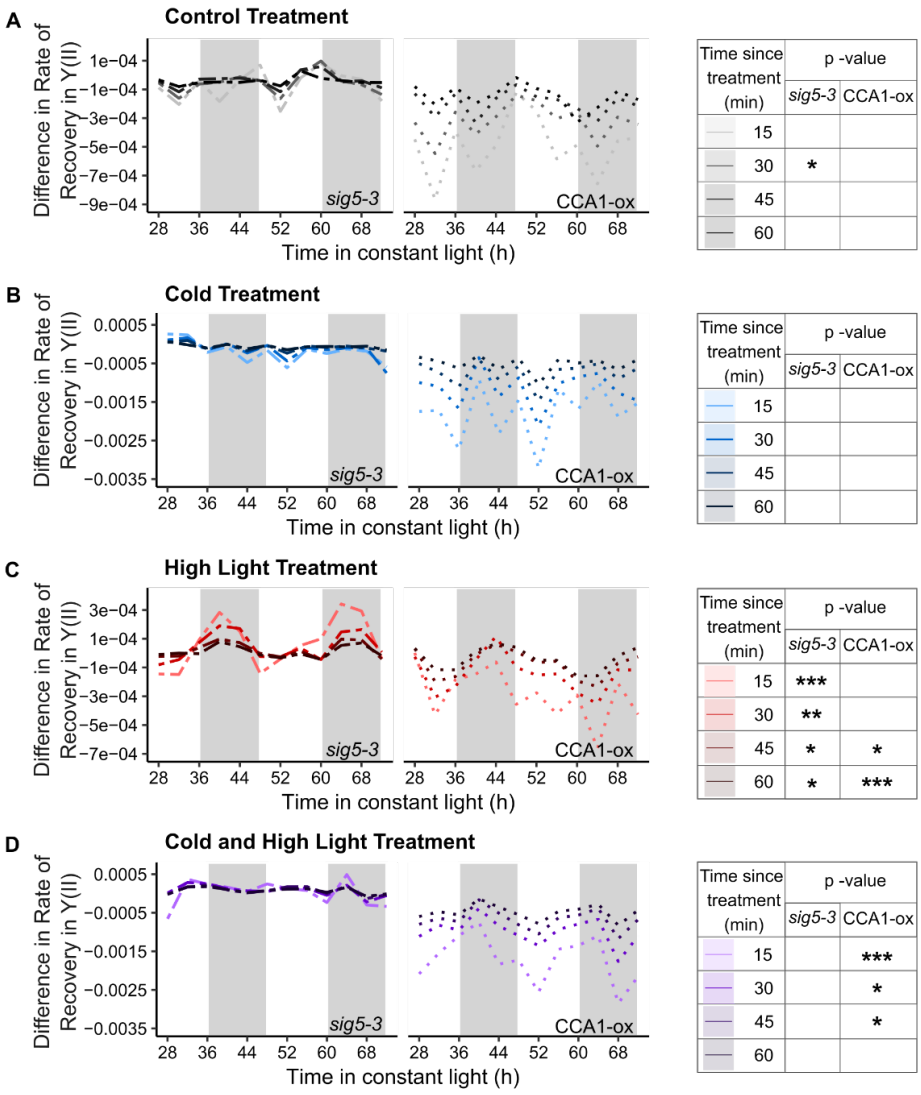

Figure 4.5 Differences between WT and genotype suggests circadian involvement in recovery in effective PSII quantum yield (Y(II)) following treatments. Measurements of 11 day old cotyledons of Arabidopsis thaliana seedlings. Prior to measurements plants were exposed to 3 h treatments of **(A)** control (19ºC, 110 µmolm^{-2}s^{-1}), **(B)** cold (4ºC, 110 µmolm^{-2}s^{-1}), **(C)** high light (19ºC, 700 µmolm^{-2}s^{-1}), **(D)** combined cold and high light (4ºC, 700 µmolm^{-2}s^{-1}). At each timepoint, Y(II) measurements were take 0, 15, 30, 45, and 60 minutes after the treatment. Rate of recovery calculated for each seedling as ((Y(II) at time x after treatment) – Y(II) at time 0)/ x. Estimated marginal means of rates of recovery at each timepoint calculated using mixed effects linear models, adjusting means for influence of random effect experimental repeat. Three (two for CCA1-ox) independent experimental repeats were carried out, N=8-20 individual plants within each independent repeat. Timecourse analysis was carried out on calculated difference between means of WT and genotype under each treatment at each timepoint (difference = mutant – WT), whereby greater differences reflect greater impact of the conditions on rate of recovery in Y(II) of mutant over WT. $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***, represent meta2D method test for statistically significant rhythmicity, integrated in legend for clarity. Note, y-axes of each plot are to the same scale but cover different ranges to allow visual inspection of rhythmicity of the traces. Clear areas of panels indicate subjective day, grey areas indicate subjective night.

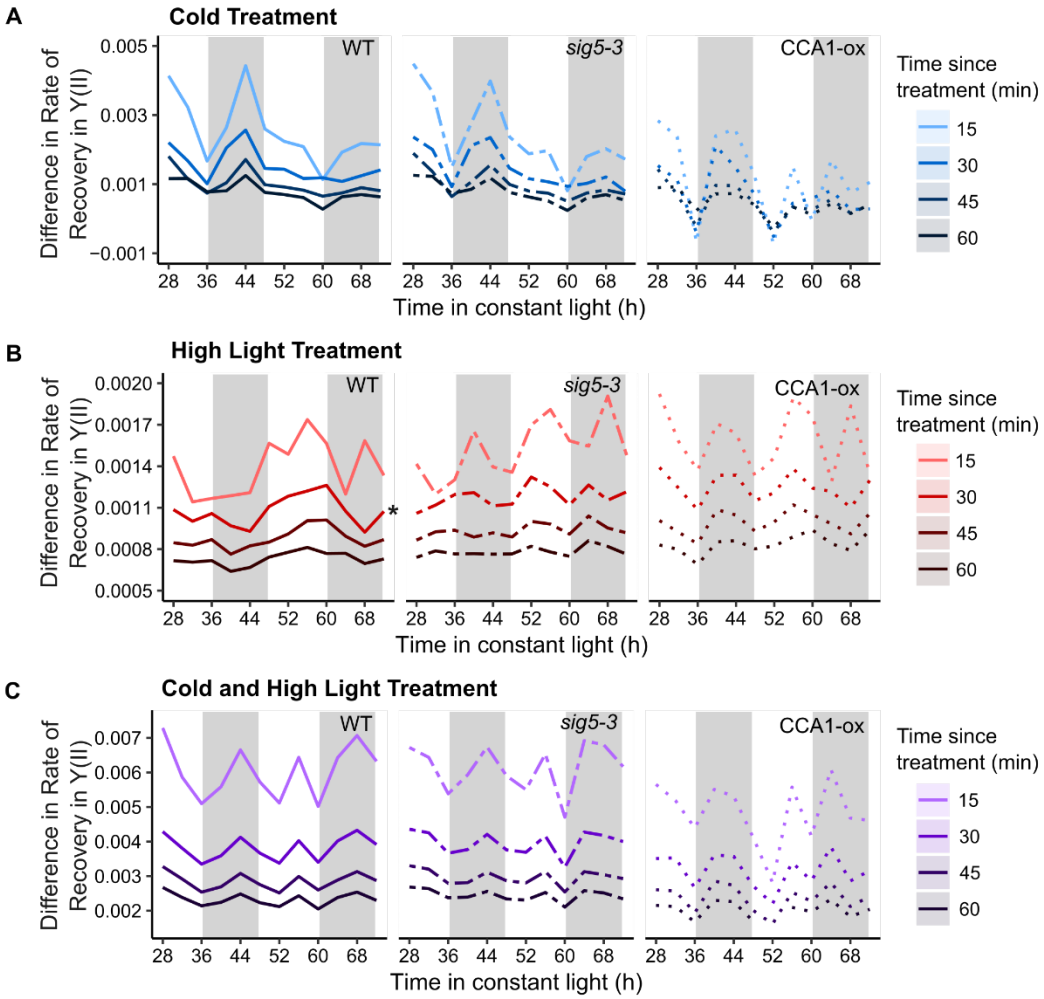

Figure 4.6 Minimal apparent circadian gating of recovery in effective PSII quantum yield (Y(II)) following cold and/or high light treatments. Measurements of 11 day old cotyledons of Arabidopsis thaliana seedlings. Prior to measurements plants were exposed to 3 h treatments of **(A)** control (19ºC, 110 µmolm^{-2}s^{-1}), **(B)** cold (4ºC, 110 µmolm^{-2}s^{-1}), **(C)** high light (19ºC, 700 µmolm^{-2}s^{-1}), **(D)** combined cold and high light (4ºC, 700 µmolm^{-2}s^{-1}). At each timepoint, Y(II) measurements were take 0, 15, 30, 45, and 60 minutes after the treatment. Rate of recovery was calculated for each seedling as ((Y(II) at time x after treatment) – Y(II) at time 0)/ x. Estimated marginal means of rates of recovery at each timepoint were calculated using mixed effects linear models, adjusting means for the influence of experimental repeat as a random effect. Three (two for CCA1-ox) independent experimental repeats were carried out, N=8-20 individual plants within each independent repeat. Timecourse analysis was carried out the calculated difference between means under control and cold and/or high light treatments at each timepoint (difference = treatment – control), whereby larger differences reflect a greater rate of recovery after treatment compared to recovery after control conditions. p<0.05 *, p<0.01 **, p<0.001 ***, representing results of meta2D method test for statistically significant rhythmicity. Note, y-axes of each plot are to the same scale but cover different ranges to allow visual inspection of rhythmicity of the traces. Clear areas of panels indicate subjective day, grey areas of panels indicate subjective night.

4.2.3.1 SIG5 many have a limited role in circadian regulation of recovery/dark adaptation after exposure control conditions

In this chapter, measurements of Y(II) 15, 30, 45, and 60 minutes after the environmental stress treatment are considered 'recovery' measurements, and from these the rate of recovery of Y(II) following treatments was calculated. Following exposure to the control conditions, the rate of recovery could alternatively be considered the rate of dark adaptation. Dark adaptation is a common step in experiments measuring chlorophyll fluorescence and involves the relaxation of NPQ. Therefore, first circadian regulation of this dark adaptation was considered.

Similar to maintenance of Y(II), no rhythm was detected in the mean rates of recovery in Y(II) for wild-type or CCA1-ox (Fig 4.4A ,Table A.1). However, rhythmicity was found in the recovery rates calculated from later recovery measurements of *sig5-3* (45 and 60 minutes after transfer to dark) (Fig 4.4A ,Table A.1). Visual inspection of these rhythmic rates shows recovery is lowest 4 hours after subjective dawn, this then increases across the day to peak at the greatest rate around subjective dusk (Fig. 4.4A). This could suggest a role of SIG5 besides from a stress response element. When the difference between of mean rate of recovery of wild-type and *sig5*-3 was considered (Fig. 4.5A, Table A.3), a rhythm was found in the rate 30 minutes after transfer to dark conditions, further supporting a role of SIG5 in dark adaptation.

4.2.3.2 Circadian rhythms in recovery did not emerge following cold conditions

The rate of recovery in Y(II) following exposure to cold conditions was not rhythmic in any of the genotypes (Fig. 4.4B, Table A.1). This result carried through to difference between rate of recovery between control and cold treatment for each genotype (Fig. 4.6A, Table A.2), and the difference between rate of recovery between wild-type and *sig5-3* and CCA1-ox under after exposure to cold conditions (Fig. 4.5B, Table A.3). This suggests there is not circadian gating of the rate of recovery in Y(II) following exposure to cold conditions. This could mean that rhythms and circadian gating of *SIG5* transcript induction following cold conditions do not contribute to downstream recovery/replacement of the photosynthetic apparatus following the end of cold conditions (Cano-Ramirez and Panter, under review).

4.2.3.3 SIG5 is involved in circadian regulation of recovery following high light conditions

Following high light treatments, there were circadian rhythms in the rates of recovery of all three genotypes (Fig. 4.4C, Table A.1). For wild-type and *sig5-3* this rhythm was such that rate of recovery is greatest around subjective dusk. Rate of recovery following high light then decreased across the subjective night, being lowest around subjective dawn.

A rhythm was also present in the later mean rates of recovery of CCA1-ox (Fig. 4.4C, Table A.1). This is surprising because CCA1-ox is an arrhythmic period mutant where circadian rhythms of expression of both the circadian clock, and clock output genes, are disrupted. Therefore, one might expect a rhythm in Y(II) to be absent. Circadian oscillator characteristics differ between leaf cell types such that mesophyll cells have a much lower amplitude oscillation of some clock components compared to other tissues (Endo et al., 2014). Therefore, the rhythm observed in CCA1-ox could be a consequence of constitutive expression of CCA1 under the 35S driving CCA1 expression in tissues where it would otherwise not be present. In addition to the rhythmicity of recovery that was present in CCA1-ox, CCA1-ox also had a slower mean rate of recovery following high light than wild-type across the whole timeseries, suggesting that correct circadian oscillator function is essential for effective recovery following high light conditions.

To investigate whether these rhythms represented circadian gating of rate of recovery in Y(II) following high light, the difference between mean recovery rate under the control and high light treatments was calculated (Fig. 4.6B, Table A.2). Surprisingly, only one significant rhythm in these differences was detected – the rate of wild-type recovery of the wild-type, 30 minutes after removal from high light. This suggests the presence of circadian gating of rate of recovery in Y(II) such that recovery from high light driven photooxidative stress is faster during the subjective day. Further, lack of a rhythm in any of the *sig5-3* differences could indicate SIG5 involvement in the circadian gating of this recovery.

To further elucidate the contribution of SIG5 and the circadian oscillator to the rhythms in rate of recovery, the difference between mean rates in wild-type *vs sig5-3* or CCA1-ox were determined (Fig 4.5C). Here, the difference between wild-type and *sig5-3* was rhythmic at all durations of recovery (Table A.3). Visual inspection of the difference suggests that *sig5-3* had a lower rate of recovery from high light wild-type compared with the wild-type during the subjective day. This is consistent with results of Nagashima *et al.* (2004) which showed a lower recovery rate F_v/F_m in *sig5-2* following high light treatment compared to wild-type.

However, during the night *sig5-3* out-performed the wild-type, wild-type with a greater rate of recovery from the photooxidative stress. From the data in Fig. 4.1C, during the subjective night *sig5-3* maintained a lower rate of Y(II) than wild-type. As recovery from photooxidative stress involves many mechanisms, rather than indicating that loss of functional SIG5 directly increases recovery rate at certain times of day, i.e. SIG5 in wild-type plants inhibits recovery, this result could instead suggest in that loss of active SIG5 leads to the upregulation of other recovery mechanisms. The difference between the rate of recovery of WT and CCA1-ox in response to short-term high light was rhythmic (Fig. 4.5C, Table A.3). This could suggest that constitutive expression on CCA1 disrupts a component of the circadian regulation of recovery in Y(II) following short-term cold, but not another component.

4.2.3.4 Circadian regulation of recovery following exposure to cold and high light is likely

The mean rate of recovery following treatment with cold and high light conditions was not rhythmic for any genotypes tested (Fig. 4.4D, Table A.1). Similarly, the difference between rate of recovery following control *vs* combined high light and cold was not rhythmic for any of the genotypes used (Fig. 4.6C, Table A.2). However, there was a in the difference between rate of recovery following cold and high light of wild-type and CCA1-ox, such that recovery following cold and high light is more reduced in CCA1-ox compared to wild-type in the hours either side of subjective dawn (Fig. 4.5D, Table A.3). This suggests that in wild-type plants oscillator functions to support a greater rate of recovery of Y(II) around subjective dawn. However as with the rhythmicity under high light, this result could also be a consequence of constitutive expression of CCA1-ox under the 35S promoter (Endo et al., 2014).

4.2.2 Summary of results

The statistical analysis for significant rhythmicity of all measurements has been summarised in Table 4.2

Table 4.2 Summary of results of test rhythmicity. Statistical analysis was carried out using meta2D method of MetaCycle. Significant rhythms have been indicated $p < 0.05$. Significant rhythm in rate of recovery of Y(II) has been indicated if one of more of the times since treatments rates of recovery timeseries yielded a statistically significant rhythm

Treatment	Parameter	Genotype	Maintenance of Y(II) significantly rhythmic?	Recovery in Y(II) significantly rhythmic?
Control ($19^{o}C$, 110 $\mu molm^{-2}\,s^{-1}$)	Mean	WT		
		sig5-3	✓	✓
		CCA1-ox		
	Difference from WT	*sig5-3*		✓
		CCA1-ox		
Cold ($4^{o}C$, 110 $\mu molm^{-2}\,s^{-1}$)	Mean	WT		
		sig5-3		
		CCA1-ox		
	Difference from control	WT		
		sig5-3		
		CCA1-ox		
	Difference from WT	*sig5-3*		
		CCA1-ox		
High light ($19^{o}C$, 700 $\mu molm^{-2}\,s^{-1}$)	Mean	WT	✓	✓
		sig5-3	✓	✓
		CCA1-ox		✓
	Difference from control	WT	✓	✓
		sig5-3	✓	
		CCA1-ox		
	Difference from WT	*sig5-3*	✓	✓
		CCA1-ox		✓
Cold and High Light ($7^{o}C$, 700 $\mu molm^{-2}\,s^{-1}$)	Mean	WT		
		sig5-3	✓	
		CCA1-ox		
	Difference from control	WT		
		sig5-3		
		CCA1-ox		
	Difference from WT	*sig5-3*	✓	
		CCA1-ox		✓

4.3 Discussion

It is well established that proper functioning of the circadian oscillator is essential for enhanced plant fitness (Dodd *et al.*, 2005). Further circadian rhythms are evident in the environmental responses of *SIG5*, a component involved in responding to changes in environmental conditions (Cano-Ramirez and Panter, under review; Noordally *et al.*, 2013). However, the connection between this circadian regulation of *SIG5* and plant fitness is less well understood (Cano-Ramirez and Panter, under review). This chapter provides evidence that circadian oscillator function, and the circadian regulation of *SIG5*, act to increase photosynthetic efficiency under both control, high light, and conditions. Further, this chapter provides preliminary evidence that maintenance and recovery of Y(II) may each be under distinct circadian regulation.

4.3.1 There is underlying regulation of maintenance of Y(II) by both the circadian oscillator and SIG5

This chapter shows clear evidence that SIG5 is involved in circadian regulation to support Y(II) under control conditions (Table 4.2). This conclusion is drawn from the rhythm which emerges in maintenance of Y(II) of *sig5-3* which was not present in wild-type. This could indicate that rhythms in expression of *SIG5* shown by Noordally *et al.* (2013) act to maintain a more constant level of Y(II) across the day in wild-type plants, thus when activity of SIG5 is lost in *sig5-3*, a rhythm in Y(II) emerges. However, these measurements of maintenance of Y(II) under control conditions differed from results of Litthauer *et al.* (2015). It is possible this discrepancy can be attributed to differences in experimental design, the major difference being that Litthauer *et al.* (2015) measured Y(II) under a lower light intensity than the plant growth light. This suggests either the detectability or presence of rhythms of Y(II) in Arabidopsis can depend upon light conditions.

4.3.2 Circadian regulation supports maintenance of Y(II) under photooxidative stress conditions

Another interesting finding of this chapter was the presence of tight circadian regulation and gating of maintenance of Y(II) in response to treatment with high light (Table 4.2). In this experiment there was a reduction in Y(II) in response to short-term high light, in *sig5-3* specific times of day and an overall reduction in CCA1-ox compared to wild-type. This result suggests Y(II) in *sig5-3* is an intermediate between what was observed in wild-type and CCA1-ox. This differences in maintenance of Y(II) across genotypes supports the model proposed by Cano-

Ramirez and Panter (under review), which positions SIG5 as an anterograde signal which conveys information from the circadian oscillator to regulate gene expression in the chloroplast. In addition, this finding, of reduced Y(II) in CCA1-ox is reminiscent of experiments by Yarkhunova et al. (2018) who found reduced F_v'/F_m' in both the short-period mutant *toc1* and long-period mutant *ztl*. This overall reduction maintenance of Y(II) in CCA1-ox compared to wild-type indicates that underlying circadian regulation supports a greater level of Y(II) in response to short-term high light at all times. This finding of reduced functioning of CCA1-xo aligns with the characterisation of CCA1-ox by Dodd *et al.* (2005) which showed CCA1-ox to have a reduced rate of net CO_2 fixation and reduced ariel biomass compared to wild-type. Together, these findings provide evidence of the overall importance of circadian regulation of photosynthesis in supporting components of plant fitness.

In contrast, in the response of Y(II) to 3 h treatments of cold, and of combined cold and high light, limited evidence of rhythmicity in the response of Y(II) was found (Table 4.2). However, Cano-Ramirez and Panter (under review) have shown rhythms in the amplitude of induction *SIG5* transcripts and its downstream targets in response to exposure to short-term cold. Therefore, lack of rhythmicity in Y(II) could indicate that exposure to cold conditions drives post-translational regulation of *SIG5* targets in the chloroplast which modifies circadian regulation of Y(II) which is seen under control conditions and in response to short-term high light. To investigate this, experiments of this chapter should be repeated and include tissue collection simultaneous to Y(II) measurements. Tissue could be sampled for *SIG5* transcript abundance, and protein abundance of the chloroplast proteins whose transcription is solely regulated by SIG5, for instance D2 or CP43 (Nagashima et al., 2004). If cold induced post-translational regulation was present, whilst rhythms of *SIG5* would be present under separate cold and/or high light, it would be expected that rhythms in protein levels would only be observed in tissue treated with high light.

4.3.3 Rate of recovery in Y(II) did not always reflect of maintenance of Y(II) suggesting distinct circadian regulation of these two processes

Rhythms in maintenance of Y(II) were often carried through to also be observed in recovery from the environmental manipulation, although this was not always the case (Table 4.2). Under control, high light, and combined cold and high light conditions, rhythmicity in some parameters emerged only in the recovery phase.

One of these differences between response and recovery was under control conditions. Here, a rhythm in the difference in dark adaptation (recovery) between wild-type and *sig5-3* which was not found in the difference between Y(II) of each genotype measurement immediately after removal form control conditions. This could suggest involvement of SIG5 in dark adaptation separate to any role in maintaining Y(II) under control conditions. Another difference found when comparing response *vs* recovery in Y(II) was in the difference between wild-type and CCA1-ox for each of these. For both the high light, and combined cold and high light treatment, rhythmicity was found in the difference between rate of recovery of each genotype but not in the difference between the response of each genotype to the treatments. This could suggest, as hypothesised by many, that circadian regulation of cell redox state influences recovery photosynthetic efficiency following oxidative stress (Guadagno et al., 2018). These differences between maintenance and rate of recovery in Y(II) could indicate a separation in the circadian regulation of maintenance *vs* rate of recovery of Y(II). This highlights the importance of considering the impact of experimental design on experiments using chlorophyll fluorescence to investigate responses to photooxidative stress conditions, because the circadian regulation of recovery during dark adaptation steps may confound results (Nagashima *et al.*, 2004; Kato *et al.,* 2012; Khanal *et al.,* 2017; Cano-Ramirez and Panter, under revision).

To further investigate the distinction between circadian regulation of the maintenance and recovery of Y(II) and elucidate molecular processes underlying the rhythms in Y(II), experiments of this chapter could be repeated such that other parameters are measured in place of Y(II). Because the existing literature on the circadian regulation of recovery of photosynthetic efficiency in response to environmental cues is limited, there are many directions in which follow up experiments could take to better understand this process. For instance, the same equipment used to measure Y(II) can also be used to measure Y(NPQ) and Y(NO), these being regulated and non-regulated energy dissipation at PSII respectively. Because other aspects of NPQ, for example expression of *psbs*, are under to circadian control it is also possible the down-regulation of NPQ contributes to the rhythmicity observed in Y(II) (Covington *et al.*, 2008; Kromdijk, 2022). Therefore, measuring these parameters would provide insight into whether rhythms observed in the difference in rate of recovery of Y(II) between wild-type and CCA1-ox following high light or cold and high light conditions was driven by circadian regulation of in the rate of downregulation of NPQ. In addition to other fluorescence measurements, tissue could be collected at each timepoint and time after transfer to darkness. *De novo* synthesis of D1 is thought to be inhibited by ROS accumulation, implying a distinction between protein turnover during maintenance under photooxidative stress compared to recovery from it (Chen et al., 2020). Therefore, collected tissue could be

sampled for D1 and/or D2 protein abundance to investigate whether there is circadian regulation in the rate of protein turnover following the alleviation of the treatments differs. Meanwhile, to obtain a indirect measure of ROS accumulation in the tissue at each timpoint, transcript levels of ROS reporter genes could be measured using RT-qPCR (Lai *et al.*, 2012).

4.3.4 Conclusions

This chapter has provided experimental evidence that a wild-type circadian oscillator improves photosynthetic efficiency under steady control conditions, and in response to sudden exposure to high light. Further, experiments in this chapter contributed to evidence of that SIG5 acts as an anterograde signal to convey the circadian regulation underlying this from the nucleus to the chloroplast, in agreement with previous studies considering *SIG5* transcript abundance (Noordally et al., 2013b). In contrast, experiments showed that rhythms in *SIG5* transcript abundance following treatment with cold do not drive circadian rhythms in maintenance of Y(II) (Cano-Ramirez and Panter, under revision). Together with the loss of rhythmicity in the combined cold and high light treatment, this feature of SIG5 under cold suggests the presence of downstream cold induced regulation of targets of SIG5 in the chloroplast.

Beyond this, the preliminary evidence of possible distinct circadian regulation in the rate of recovery in Y(II) highlights the need for a review of the experimental procedures used in existing literature which measured chlorophyll fluorescence to ensure circadian regulation of dark adaptation could not in fact be underlying differences in fluorescence which have been attributed to other processes.

One of the many changes in climate driven by changes in earth systems is an increase in the intensity and frequency of late spring frosts (Lamichhane, 2021). Late springs frosts are defined as frosts which occur later into the spring season, after germination of herbaceous plants has occurred (Lamichhane, 2021). During these times, cold conditions persist later into the day, thereby increasing the overlap of cold conditions and increasing light intensity in the morning. The findings of this study suggest cold conditions interacts with the circadian regulation of maintenance of photosynthetic efficiency in response to exposure to high light. Therefore, this result could indicate a possible consequence of increased frequency and intensity of late spring frosts in future climates on photosynthetic efficiency. However, whilst this chapter provides preliminary evidence to support this hypothesis, to further test this, experiments with a focus on closer replicating the natural environment should be carried out.

5 Influence of leaf developmental stage on circadian regulation on Y(II)

5.1 Background

In Arabidopsis, the first two leaves to emerge are the cotyledons. These leaves are functionally distinct from the subsequently emerging true leaves. Compared to true leaves, the simplicity of leaf expansion of cotyledons makes them a useful model for fundamental studies (Tsukaya et al., 1994). However, for the same reasons, the results obtained from cotyledons cannot necessarily be generalised to represent the behaviour across a plant lifecycle. This is because differences in both aspects of the photosynthetic system and the circadian oscillator have been identified.

Under control conditions, expression of photosynthesis-related genes, the abundance of photosystem subunits, and F_v/F_m were comparable between cotyledons and the first and second emerging true leaves (Shi et al., 2020). However, differences were identified in expression of PSII assembly factors (Shi et al., 2020). Therefore, under drivers of photooxidative stress, differences in Y(II) of cotyledons and true leaves may emerge as rate of PSII turnover is increased (Gururani et al., 2015). Similarly, differences in the functioning of the circadian oscillator across leaf age have also been identified. Here the period of expression of both core clock and clock output genes was shown to be 1 hour shorter in older leaves (Kim et al., 2016).

In addition to differential expression PSII assembly factors, *SIG5* transcript expression varies across leaf developmental stages (Nagashima et al., 2004). Here GUS-staining assays found that SIG5 expression in cotyledons of 9 day old plants is lower than expression in fully expanded leaves of 18 day-old plants. Therefore, the relative contributions of SIG5 might differ between cotyledons and mature leaves.

Together, these studies suggested that it would be informative to extend the experiments in Chapter 4 to examine the response of Y(II) to cold and high light conditions at additional stages of the development of the Arabidopsis rosette.

5.2 Results

5.2.1 Methodology and Experimental Design

To determine the similarities and differences between the circadian regulation of Y(II) of cotyledons and true leaves, a time course of Y(II) of fully expanded outer rosette leaves of 28 day old wild-type plants was measured using the same experimental protocol as for Chapter 4. At each timepoint, Y(II) of 16 leaves from 4-5 plants of Col-0 wild-type was measured after exposure to treatments of 3 h experimental control or cold and high light conditions. Each group of seedlings received a single 3 h treatment.

To consider the differential expression of SIG5 at different developmental stages, *sig5-3* plants were included in this experiment. This was to test the hypothesis that differential expression of *SIG5* at these developmental stages might result in differing contributions of SIG5 in maintaining Y(II) between cotyledons and true leaves.

As in Chapter 4, the influence of plant age on circadian regulation of rate of recovery following photooxidative stress. Therefore, following measurements immediately after the treatments, plants were held in darkness and Y(II) was measures 15, 30, 45, and 60 minutes after the treatments (as the seedlings are held in darkness before the recovery measurements, Y(II) under this condition is equal to F_v/F_m). From these measurements, rate of recovery in Y(II) was calculated. These rates of recovery and follow up MetaCycle analysis are shown in Appendix B, Figures B1-3 and Tables B1-3.

Results of cotyledons are derived from Chapter 4.

5.2.2 Circadian oscillator contribution to maintaining Y(II) differs with developmental stage under control conditions

Under control conditions the contribution of circadian regulation of Y(II) in wild-type differed between leaf developmental stages (Fig. 5.1A, Table 5.1). In true leaves a rhythm emerged in maintenance of Y(II) which was not detected in cotyledons (Fig. 5.1A). This rhythm in true leaves was such that Y(II) under control conditions was greater during the subjective night, peaking at subjective dawn. This could suggest a greater contribution of the circadian oscillator to maintaining Y(II) in true leaves.

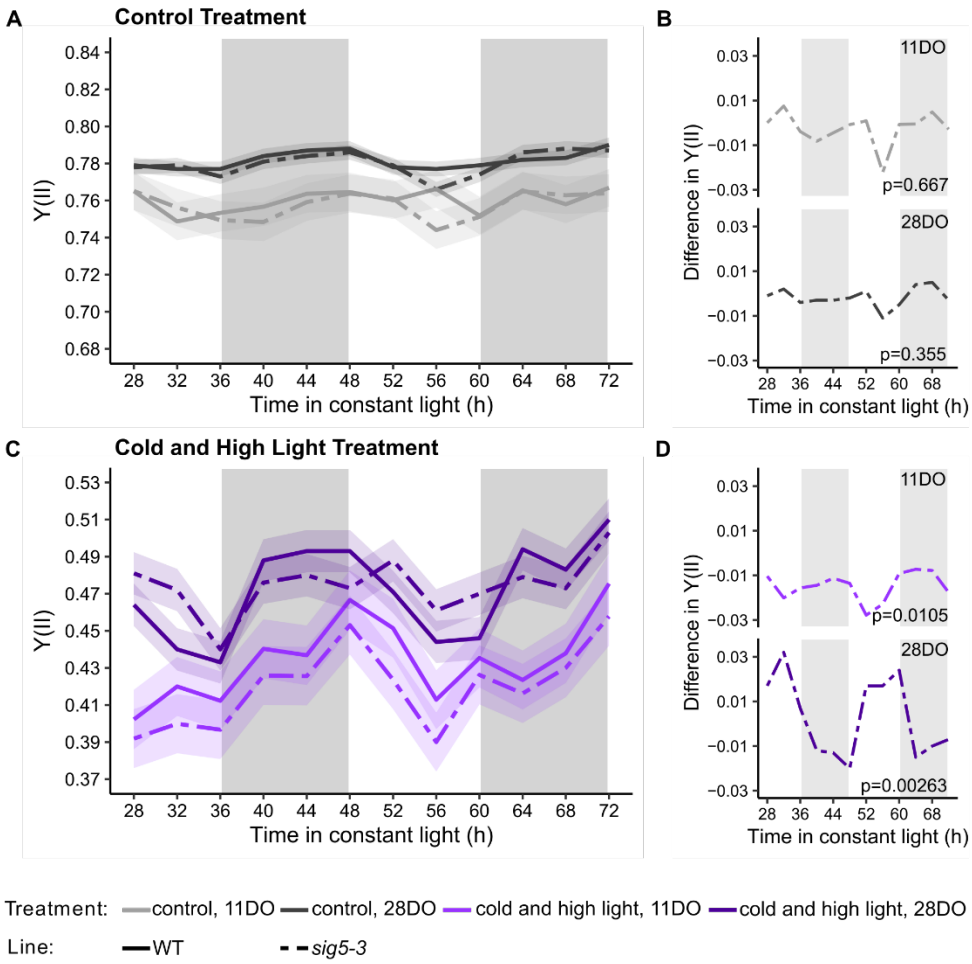

Figure 5.1 Circadian regulation and gating of effective PSII quantum yield (Y(II)) differs between leaf developmental stages under cold and high light conditions. Measurements of 11 day old cotyledons of Arabidopsis thaliana seedlings (11DO) and fully expanded outer rosette leaves (28DO). Genotypes used were Prior to measurement plants were exposed to, **(A)** control (19ºC, 110 µmolm^{-2}s^{-1}) or **(C)** cold and high light (4ºC, 700 µmolm^{-2}s^{-1}) conditions. **(A,C)** mean ± SE for three independent repeats, calculated using a mixed effects linear model which adjusts values for the influence of random effect experimental replicate. 11DO, N = 8-20 individual plants within each independent repeat 28DO, N= 16 individual leave of 4-5 plants within each independent repeat. **(B,D)** difference between WT and *sig5-3* means (WT minus *sig5-3*), whereby greater differences reflect a greater impact of the treatment on Y(II) of *sig5-3* than WT. p-values (meta2D, p<0.05) represent a test for statistically significant rhythmicity, shown to three significant figures. Clear areas of panels indicate subjective day, grey areas of panels indicate subjective night. Data for cotyledons are derived from Chapter 4.

Table 5.1 Summary of meta2d quantitative timecourse analysis of maintenance of effective PSII quantum yield (Y(II)) under cold and high light conditions. Y(II) of 11 day old cotyledons and 28 day old full expanded leaves, measured at 12 timepoints across a 44 hour subjective time period, following tissue exposure to 3h treatments conditions. Analysis carried out on mean Y(II) calculated for each timepoint of three independent experimental repeats, cotyledons, N = 8-20 individual plants within each independent repeat true leaves, N= 16 individual leave of 4-5 plants within each independent repeat. Means calculated using mixed effects linear models, adjusting means for the influence of experimental repeat as a random effect. P-values obtained from meta2d method analysis for statistically significant rhythmicity. Period, phase, and amplitude estimates are excluded for experiments which did not yield statistically significant rhythms because these measures are derived from poor fits to the data. Results shown to three significant figures. Results from cotyledons repeated from Chapter 4. $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***. Data for cotyledons are derived from Chapter 4.

Treatment	Genotype	Age (days)	p-value	Period est. (h)	Phase est. (h)	Amplitude (Y(II))
Control	WT	11	0.466	–	–	–
(19°C, 110 µmol m^{-2} s^{-1})		28	0.000648 ***	26.4	18.9	0.00544
	sig5-3	11	4.95 x10^{-05}***	20.3	7.21	0.00910
		28	0.000713 ***	23.4	22.0	0.00799
Cold and High Light	WT	11	0.188	–	–	–
(4°C, 700 µmol m^{-2} s^{-1})		28	0.00140**	25.4	20.5	0.0285
	sig5-3	11	0.0473 *	26.2	19.7	0.0200
		28	0.182	–	–	–

To investigate the contribution of SIG5 to this circadian regulation, the rhythmicity of *sig5-3* plants under control conditions was considered (Fig. 5.1A, Table 5.1). In contrast to the wild-type, both cotyledons and true leaves of *sig5-3* were rhythmic. In the context of the wild-type results, this could suggest that SIG5 contributes more heavily to circadian regulation in cotyledons. However, from inspection of Fig. 5.1A, the features of the rhythms in true leaves of wild-type and *sig5-3* differed, and this is represented in the period, phase and amplitude estimates of these rhythms (Table 5.1). This could suggest differences in SIG5 expression with leaf age shown by Nagashima *et al.* (2004) leads to differing contributions of SIG5 in maintaining Y(II) between cotyledons and true leaves. To further elucidate the difference between these rhythms in wild-type and *sig5*-3 true leaves, the difference between the mean Y(II) at each timepoint was calculated (Fig. 5.1B). There was not a rhythm in this difference. There was also not a significant rhythm in the difference between Y(II) of wild-type and *sig5*-

3 of cotyledons (Fig. 5.1B). One interpretation is that differences in circadian clock outputs between leaf ages (Kim *et al.* (2016)) may act downstream to influence maintenance of Y(II) however, the contribution of SIG5 to this is less clear.

Aside from the circadian features of maintenance of Y(II) in the leaf types and genotypes, visual inspection of the data indicates that maintenance of Y(II) for both plant lines was consistently greater in true leaves than cotyledons at all time points (Fig. 5.1A). This provides another line of evidence that differences in expression of genes related to photosynthesis between leaf developmental stages (Shi, Chen and Hou (2020)) may drive greater photosynthetic efficiency in true leaves.

5.2.3 Leaf developmental stage influences circadian regulation of maintenance of Y(II) under cold and high light

In true leaves of wild-type plants, a rhythm was present in the maintenance of Y(II) under combined cold and high light conditions which was not found in cotyledons (Fig. 5.1C and Table 5.1). The rhythm in true leaves was such that Y(II) was maintained to a greater level under the treatment of cold and high light when the treatment was given during the night, with the greatest level of Y(II) being maintain under the treatment at dawn (Fig. 5.1C). To investigate whether this difference in rhythmicity of maintenance of Y(II) under high light and cold reflected represented differences between the true leave leaves and cotyledons in this condition, the difference between Y(II) under control and cold and high light was calculated for each leaf type (Fig. 5.2A). This difference was rhythmic in true leaves but not in cotyledons (Fig. 5.2B). The rhythm of this difference showed maintenance of Y(II) of true leaves was increasingly impacted by cold and high light across the subjective day, up until the greatest impact at dusk, meanwhile the treatment had the smallest impact in reducing Y(II) at dawn. This suggests there is circadian gating of the maintenance of Y(II) in true leaves, such that the system is most able to maintain Y(II) under cold and high light at dawn. Further, this circadian regulation was not found in cotyledon. This suggests the differences in expression of circadian clock genes and outputs, and PSII assembly components, shown by Kim *et al.* (2016) and Shi, Chen and Hou (2020) respectively, may act downstream to influence maintenance of Y(II) under photooxidative stress conditions.

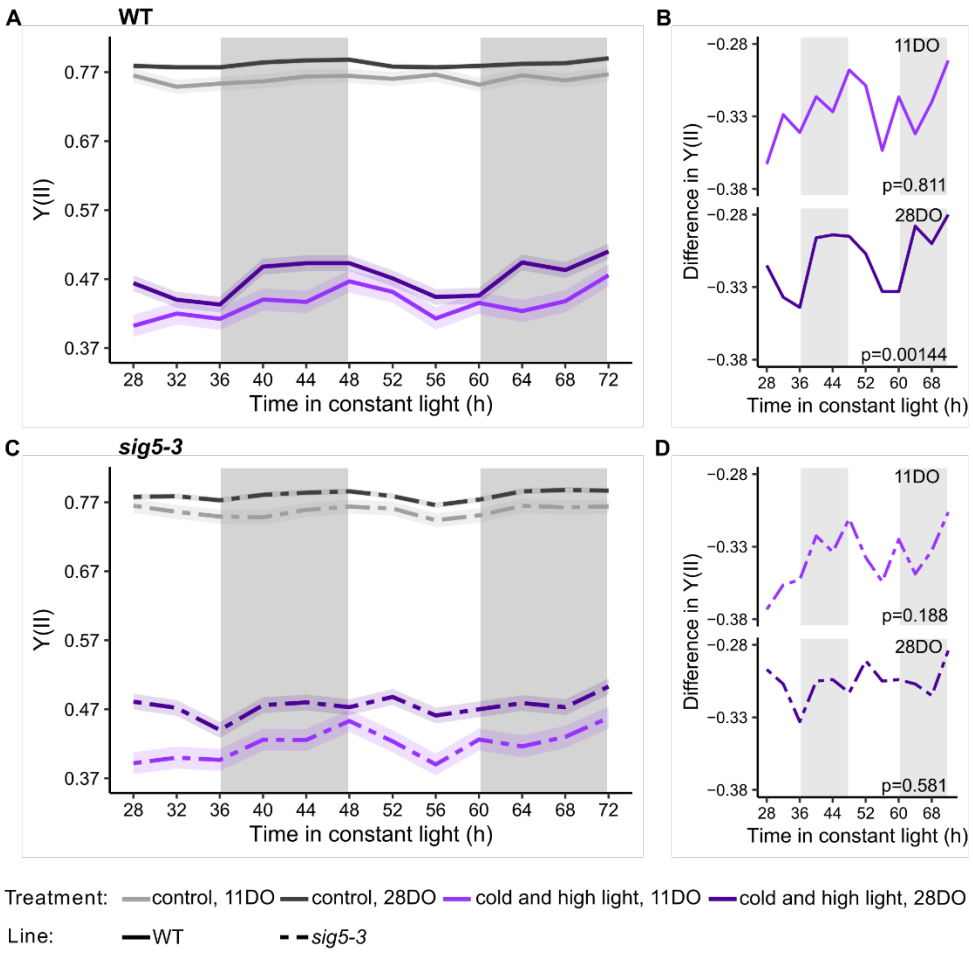

Figure 5.2 Circadian regulation and gating of effective PSII quantum yield (Y(II)) differs between leaf developmental stages under cold and high light conditions. Measurements of 11 day old cotyledons of Arabidopsis thaliana seedlings (11DO) and fully expanded outer rosette leaves (28DO). Prior to measurement plants, **(A)** Col-0 wild-type (WT) and **(B)** *sig5-3*, were exposed to 3h treatments of **(A,C)** control (19°C, 110 µmolm^{-2}s^{-1}) or cold and high light (4°C, 700 µmolm^{-2}s^{-1}). **(A,C)** mean ± SE for three independent repeats, calculated using a mixed effects linear model which adjusts values for the influence of random effect experimental replicate. 11DO, N = 8-20 individual plants within each independent repeat 28DO, N= 16 individual leave of 4-5 plants within each independent repeat. **(B,D)** difference between treatment and control means (treatment minus control), whereby greater differences reflect a greater impact of treatment on Y(II) compared to control conditions. p-values (meta2D, $p<0.05$) represent a test for statistically significant rhythmicity, shown to three significant figures. Clear areas of panels indicate subjective day, grey areas of panels indicate subjective night. Data for cotyledons are derived from Chapter 4.

5.2.4 The role of SIG5 in maintaining Y(II) under cold and high light varies between cotyledons and true leaves

To elucidate the role of SIG5 in this circadian regulation, maintenance of Y(II) of *sig5-3* plants under cold and high light was considered (Fig. 5.2C). In contrast to the wild-type, a rhythm was found in maintenance of Y(II) in *sig5-3* plants under cold and high light in cotyledons but not true leaves. To test whether this difference represented a variation in circadian regulation in *sig5-3*, the difference between Y(II) under control and cold and high light conditions was considered for each leaf type.

In these differences no significant rhythm was detected (Fig. 5.2D). For true leaves, this represents a loss of the circadian gating of Y(II) present in wild-type, suggests involvement of SIG5 in this gating. To test this further, the difference between Y(II) of wild-type and *sig5-3* under cold and high light was calculated. This difference was rhythmic for both cotyledons and true leaves (Fig. 5.1D). This suggests that SIG5 contributes to maintenance of Y(II) under cold and high light in both leaf developmental types. However, the features of these rhythms differ. In true leaves loss of functional SIG5 caused the greatest reduction in maintenance of Y(II) compared to wild-type during the night, with the greatest impact at dawn. Despite this, surprisingly during the subjective day, true leaves of *sig5-3* maintained a greater level of Y(II) under cold and high light than wild-type. This could suggest that at certain times of day SIG5 is involved in negative regulation of components of photosynthesis, thus when it is function is removed in sig5-3, Y(II) is increased. Meanwhile, in cotyledons maintenance of Y(II) under cold and high light was consistently below wild-type (Fig. 5.1D). Again in contrast to true leaves, the rhythm in the difference between wild-type and *sig5-3* showed loss of functional SIG5 drove cold and high light conditions to have the greatest impact in reducing Y(II) during the day.

In addition to differences in the phase of the differences between wild-type and *sig5-3* for each leaf type, the amplitude of the rhythm was much greater in true leaves than cotyledons (Fig. 5.1D). This could suggest the overall importance of SIG5 activity in maintaining Y(II) under cold and high light is larger in true leaves than cotyledons.

Together, these results suggest that differences in SIG5 expression according to leaf age (Nagashima *et al.* (2004)) could drive to differences in contribution and features of SIG5 regulation of maintenance of Y(II) under cold and high light.

4.2.5 Summary of results

For clarity, the statistical analysis for significant rhythmicity of all measurements has been summarised in Table 5.2.

Table 5.2. Summary of results of test rhythmicity. Statistical analysis was carried out using meta2D method of MetaCycle. Significant rhythms have been indicated $p < 0.05$. In 'Parameter', 'Difference from WT' refers to the difference between mean Y(II) of WT and *sig5-3* at each timepoint, and, 'Difference from control' refers to the difference between mean Y(II) under control and cold and high light

Treatment	Parameter	Genotype	Age (days)	Maintenance of Y(II) significantly rhythmic?
Control	Mean	WT	11	
(19°C, 110 µmolm^{-2} s^{-1})			28	✓
		sig5-3	11	✓
			28	✓
	Difference from WT	*sig5-3*	11	
			28	
Cold and High Light	Mean	WT	11	
(4°C, 700 µmolm^{-2} s^{-1})			28	✓
		sig5-3	11	✓
			28	
	Difference from control	WT	11	
			28	✓
		sig5-3	11	
			28	
	Difference from WT	*sig5-3*	11	✓
			28	✓

5.3 Discussion

Young seedlings are often used in experiments to simplify and accelerate experimental procedure. However, the first two emerging leaves, the cotyledons, are functionally distinct from all subsequently emerging true leaves. Therefore, results obtained from experiments using young seedlings, which often only have cotyledons, may not be representative of all leaves across a plant's lifespan. The aim of this chapter was to build on evidence from the literature that suggests there are differences in the regulation – circadian and otherwise – of the photosynthetic system between cotyledons and true (Kim *et al.,* 2016; Shi, Chen and Hou, 2020). The experiments of this chapter have provided evidence that differences between cotyledons and true leaves drive differences in maintenance of Y(II) and its circadian regulation under both experimental control conditions, and in response to combined cold and high light conditions.

5.3.1 Both circadian regulation of Y(II) under control conditions, and under photooxidative stress conditions differs between leaf developmental types

Results of this chapter suggest the circadian oscillator plays a major role in the regulation of maintenance of Y(II) under control conditions in both cotyledons and true leaves (Table 5.2). Moreover, experiments in this chapter show this regulation differs between cotyledons and true leaves, such that circadian rhythms in maintenance of Y(II) were evident in true leaves whilst cotyledons maintained a more constant level of Y(II) across time (Table 5.2). These results provide evidence that differential expression of photosynthesis genes shown by Shi, Chen and Hou (2020) between cotyledons and true leaves influences photosynthetic efficiency downstream. Further, the differences in circadian regulation across leaf types shown provides evidence that suggests tissue specific modification expression of core clock components could influence plant fitness (Endo et al., 2014).

This difference in circadian regulation of Y(II) in cotyledons and true leaves may reflect the relative importance of maintaining optimal photosynthetic efficiency in these leaf types. In a natural environment, contribution of cotyledons towards total plant productivity is rapidly overtaken by true leaves (Turgeon, 1989). However, seedling establishment is reliant on the productivity of cotyledons. Therefore, lack of circadian rhythms in Y(II) may allow the cotyledons to maximise photosynthetic efficiency by allowing the system to exploit given light conditions regardless of what would be expected for the time of day.

Another interesting result of this chapter was the presence of circadian gating of maintenance of Y(II) under cold and high light conditions in true leaves which was absent in cotyledons (Table 5.2). This result could indicate differences in expression of PSII assembly factors in cotyledons vs true leaves, identified by Shi, Chen and Hou (2020), reflect differences in the circadian regulation of these components. To investigate this, a timeseries of transcript abundance of PSII assembly factors in cotyledons and true leaves could be performed. Overall, the lack of tight circadian regulation of Y(II) in response to cold and high light in cotyledons could indicate the presence of a more constitutively active photoprotective system compared to true leaves. This mirrors the increased risk to overall plant fitness caused by irreversible photooxidative stress to a cotyledon.

For all experiments, the lack of inclusion of plants from a genotype with a dysfunctional circadian oscillator represents a limitation of experiments in this chapter which hinders the ability to draw conclusions on how these differences relay to differences in circadian regulation between leaf developmental types. This is because differences in rhythmicity between leaf types which could indicate differences in the significance of circadian regulation to maintaining Y(II) could equally represent differences in the type of regulation by the oscillator. Moreover, it limits the ability to understand how differences in circadian regulation act downstream to influence components of plant fitness. Therefore, experiments of this chapter could be supplemented by inclusion of a genotype such as CCA1-ox, which has an arrhythmic-period circadian oscillator (Wang and Tobin, 1998).

5.3.2 Contribution of SIG5 to circadian regulation differs between leaf developmental types

Variation in circadian regulation was found when comparing Y(II) cotyledons and true leaves of *sig5-3* both in maintenance of Y(II) under control conditions, and, response of Y(II) to treatments of cold and high light conditions (Table 5.2). Whilst time-independent experiments have shown that *SIG5* expression differs with leaf age, differences between the circadian rhythms of *SIG5* at different leaf developmental stages have not been considered (Nagashima et al., 2004). Therefore, similar to overall circadian regulation, how these differences relay mechanistically to the function of SIG5 in different leaf developmental type is unclear. However, this evidence of differing roles of SIG5 in leaf developmental types could point to a role for SIG5 outside of 'stress' response processes. For instance, other sigma factors, such as SIG6, have been shown to regulate chloroplast development, a process where differences between cotyledons and true leaves is well established (Ishizaki *et al.*, 2005; Shimada *et al.*, 2007). To consider a role of SIG5 in chloroplast biogenesis, a possible future experiment could

involve phenotypic characterisation of dark-grown *sig5*-3 before and after transfer into light conditions.

5.3.3 Conclusions

This chapter has shown clear experimental evidence that photosynthetic efficiency and its circadian regulation differ between leaf developmental stages both under control and cold and high light conditions. Whilst the results of this chapter provide few insights into the underlying mechanisms, they do provide a basis and motivation for future work in multiple fields. In addition, my results highlight the importance of considering plant age and/or leaf developmental type when designing experiments and drawing comparisons between existing literature.

In addition to this work helping to improve our experimental design, these results could provide insights into plants in natural environments. These results suggest a differing vulnerability of leaf developmental types to cold and high light conditions. Late spring frosts, where there is combined cold and high light conditions, are occurring with increased frequency and intensity as a consequence of climate change (Lamichhane, 2021). Therefore, understanding how different developmental stages respond to these conditions, we can better predict the impact of a changing climate in plant fitness.

6 General Discussion

In natural environments, the abiotic conditions are constantly fluctuating. These fluctuations drive photooxidation of the photosynthetic apparatus, which reduces photosynthetic efficiency. Therefore, an ongoing cycle of damage and repair underpins maintaining photosynthetic efficiency in natural environments. The occurrence of previously uncommon extreme weather events are increasing in frequency and intensity due to changes in the earth systems driving climate change (Cohen, Pfeiffer and Francis, 2018; Lamichhane, 2021). Therefore, understanding how photosynthetic efficiency is regulated under different abiotic environmental conditions could be informative of how the photosynthetic fitness of ecosystems, both agricultural and natural, may be shaped by the changing climate. The work of this thesis has shown that circadian regulation shapes both the response and recovery of photosynthetic efficiency to short-term exposure to cold and high light conditions. Further, I have shown this circadian regulation is in part mediated by the SIG5 transcriptional response pathway.

6.1 Contribution of SIG5 to photosynthetic efficiency was only evident in timeseries experiments

Chapter 3 considered the role of SIG5 in supporting maintenance of Y(II) following seedling exposure to increasing duration of cold, high light, or cold and high light conditions. *SIG5* transcript induction 1-6 h following exposure to multiple abiotic stress conditions is well established in the literature (Fig. 1.5) (Nagashima *et al.*, 2004; Belbin *et al.*, 2017; Cano-Ramirez and Panter, under revision). Therefore, it was hypothesised that an impact of SIG5 on supporting maintenance of Y(II) would be observed – through a significant reduction in Y(II) of *sig5-3* compared to wild-type – only in the longer treatment durations. Surprisingly this was not the case. Across Chapter 3, the role of SIG5 in supporting Y(II) found for any of the treatment conditions was limited. However, interestingly, in both Chapters 4 and 5, timeseries experiments showed clear evidence that SIG5 supports Y(II) under both control conditions and upon treatment with short-term cold and/or high light. This difference in finding between experiments may be explained be considering the rhythms in Y(II) of wild-type and *sig5-3* observed in Chapter 4. Experiments in Chapter 3 commenced 1 h after dawn. However, from inspection of Fig. 4.1C, it can be seen during the subjective day response of Y(II) to short-term high light was similar between wild-type and *sig5*-3. Therefore, lack of difference between wild-type and *sig5-3* in Chapter 3 could be attributed to the time of day the experiments were conducted. Overall, these results highlight the importance of considering the influence of circadian oscillator when investigating novel pathways.

6.2 The circadian oscillator regulates of the response of Y(II) to short-term cold and/or high light

Timeseries experiments of Chapter 4 show circadian regulation is involved, to varying extents, in the maintenance of photosynthetic efficiency both under control conditions, and in response to short-term treatments of cold, high light, and cold and high light conditions. These results provide evidence to suggest circadian rhythms in *SIG5* transcript abundance in response to high light and cold shown by Noordally *et al.* (2013) and Cano-Ramirez and Panter (under revision) respectively, contribute to supporting photosynthetic efficiency under photooxidative stress conditions.

This circadian regulation was most evident in experiments with high light, where there was clear evidence of circadian gating of Y(II) in response to treatments of high light which is in part mediated by SIG5. Note, the timing of greatest contribution of SIG5 to supporting Y(II) in response to short-term high light differs from the time of greatest *SIG5* induction in response to light treatments in Noordally *et al.* (2013) which was around the middle of the day. This difference may be attributed to the time lag between induction of *SIG5* and the changes in chloroplast protein composition it drives – Cano-Ramirez *et al.* (2022) showed in a natural population of *Arabidopsis* the time lag between induction of *SIG5* and induction of *psbD* BLRP alone was 4 h. Alternatively, this difference in timing between the greatest contribution of SIG5 to supporting Y(II) in response to short-term high light compared to the previously shown time of greatest *SIG5* induction could also be a result of post-translational regulation of SIG5 targets. Results of Chapter 5 also showed clear evidence of circadian gating in true leaves, here, in the response of Y(II) to short-term treatments of cold and high light. However, whilst the results of Chapters 4 and 5 clearly indicated a role of SIG5 the circadian regulation of the response of Y(II) to short term treatments of drivers of photo-oxidative stress, the rhythmicity of Y(II) in *sig5-3* in some treatments suggests circadian regulation of Y(II) is not solely mediated through SIG5.

It may be possible to elucidate the behaviour of the other component(s) that contribute to circadian regulation of Y(II) under each experimental condition using the current datasets of Chapters 4 and 5. For this, the rhythms of Y(II) of cotyledons of each genotype in response to treatments of high light, from Fig. 4.1, have been used as an example (Fig. 6.1). Firstly, by considering the rhythms of Y(II) of CCA1-ox and *sig5-3* in response to the treatments, the contribution of the circadian oscillator and SIG5 to the maintenance of Y(II) in response to the treatments in wild-type can be determined (Fig. 6.1A,B). Then, by considering the difference between the contribution of circadian oscillator and SIG5, a model of the predicted contribution

of the unknown component(s) could be made (Fig. 6.1B,C). The identity of this other component(s) involved in mediating circadian regulation of Y(II) in response to short-term treatments of photooxidative stress can then be determined by comparing the model of the predicted activity of the component(s) against the transcript abundance of known photosynthetic regulators from publicly available timeseries dataset (Covington et al., 2008).

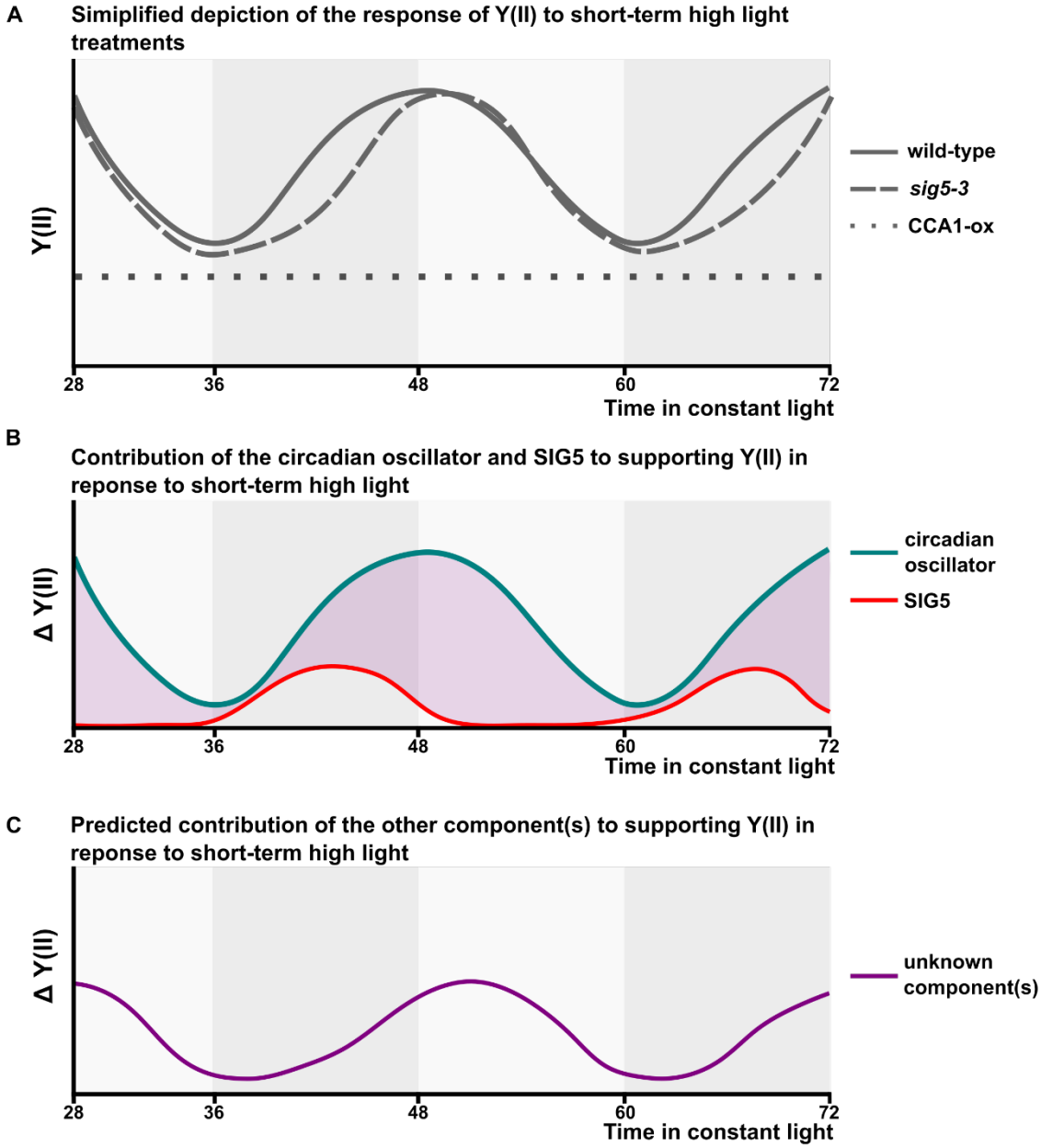

Figure 6.1 Revealing the nature of other component(s) involved in circadian regulation of Y(II) in response to short-term treatments of photooxidative stress. Y(II) of each genotype in response to treatments of high light, from Fig. 4.1, has been used as an example. **(A)** Simplified depiction of Y(II) of wild-type, *sig5-3*, and CCA1-ox in response to treatments of short-term high light (Fig 4.1C). **(B)** Estimated contribution of the circadian oscillator and SIG5 to supporting maintained of Y(II) in response to high light, derived from (A). Purple shaded area depicts the circadian regulation which cannot be

explained by SIG5 activity. **(C)** Predicted activity of unknown component(s) which the mediate circadian regulation of Y(II).

6.3 Variation in circadian regulation between cotyledons and true leaves could reflect functional differences of these leaf types in natural environments

Chapters 4 and 5 showed that under control conditions there were circadian rhythms in in maintenance of Y(II) of true leaves which were not present in cotyledons (Table 4.1, Table 5.2). This result may suggest a greater importance of maximising photosynthesis in cotyledons through maintaining a more constitutively active photosynthetic system, enabling cotyledons to utilise any light they intercept, regardless of the time of day. In addition, circadian gating of maintenance of Y(II) in response to short-term cold and high light conditions was present in true leaves which was absent in cotyledons (Table 5.2). A more constitutively active response system to cold and high light in cotyledons could reflect the greater risk to overall plant survival caused by the loss of an individual cotyledon to photooxidative stress conditions, compared to the loss of a true leaf.

For both experimental abiotic conditions, these results provide another line of evidence that features of the circadian oscillator differ with leaf age and tissue type (Kim *et al.*, 2016; Endo *et al.*, 2014). Similarly, the role of SIG5 in supporting the maintenance of Y(II) differed between cotyledons and true leaves for both experimental conditions, aligning with results of Nagashima *et al.* (2004) which showed variation in *SIG5* expression with leaf age. But, these results contrast with Shi, Chen and Hou (2020) who found few differences in aspects of photosynthesis, such as expression of photosynthesis related genes, between cotyledons and true leaves. However, this difference in results may be explained by considering the age of the true leaves used by Shi, Chen and Hou (2020). In Shi, Chen and Hou (2020), cotyledons were compared to the 1st and 2nd emerging true leaves. However whilst developmentally distinct, the functional importance of these trues leaves is similar to the cotyledons – they are individually responsible for making major contributions to total photosynthesis (Turgeon, 1989). This means that similar to cotyledons, the loss of the 1st and 2nd emerging true leaves could pose a threat to overall plant survival. This is in contrast to the true leaves measured in Chapter 5 which were of 28 day old plants which had many leaves meaning the loss of an individual leaf would represent a much smaller risk to overall plant survival than the loss of the 1st and 2nd emerging true leaves. Therefore, the differences between cotyledons and true leaves found in this work which were absent in Shi, Chen and Hou (2020) could represent differences in the functional role of these leaves in natural environments, rather than structural

differences. These results are wider reaching than plant chronobiology, as they highlight the importance of considering both leaf developmental type and the functional role of each leaf in natural environments in experimental design and analysis.

6.4 Circadian regulation of recovery in photosynthetic efficiency appears distinct from regulation under a given condition

A search of existing literature found relatively few studies that considered recovery in chlorophyll fluorescence following changes in conditions. Similarly, there did not appear to be any studies which considered circadian regulation of rate of recovery of aspects of photosynthesis. Therefore, in addition to measuring Y(II) immediately after tissue exposure to the experimental treatment conditions, in Chapters 4 and 5, at each timepoint Y(II) was also measured 15, 30, 45, and 60 minutes after the treatment and calculated a rate of recovery in Y(II). These extra measurements revealed a novel finding – there was a clear distinction between the circadian regulation of maintenance of Y(II) and circadian regulation of recovery in Y(II). This difference between parameters held true across both the different leaf development types and experimental treatment conditions considered (Table 4.2, Table 5.2, Table B.1).

From an output variable such as Y(II), which is influenced by a large range of inputs, ability to gain deeper insight into exactly what mechanism is underlying this differences is limited (Guadagno, Ewers, and Weinig, 2018). However, it is possible this difference can be attributed to circadian regulation of a process which is only active upon the removal of drivers of photooxidative stress. One possible mechanism which becomes active only upon the release of drivers of photooxidative stress is the downregulation of NPQ (Kromdijk, 2022). To investigate this, experiments of Chapters 4 and 5 could be repeated, where in place of Y(II), Y(NPQ) could be measured. Y(NPQ) is a measure of regulated heat dissipation by NPQ. Therefore, rhythmicity analysis of Y(NPQ) across time would show whether there is circadian regulation of the rate of relaxation of NPQ following the removal of tissue from short-term treatments of drivers of photooxidative stress. Another straightforward follow-up experiment to consider other possible mechanisms is exploration of existing data bases of transcript abundance, such as those in Covington *et al.* (2008), to identify if any genes involved just in recovery processes exhibit circadian rhythms.

The lack of existing molecular studies of this process may reflect the increased labour and resource expense of an experiment considering rate of recovery in an aspect of photosynthesis, namely the collection of multiple samples to cover a recovery time period. However, the prevalence of differences between the circadian regulation of maintenance of Y(II) and recovery in Y(II) found in this study provide ample justification for such follow-up experiments to be carried out. For instance, experiments by Nishiyama *et al.* (2001) suggest de novo synthesis of D1 in inhibited by oxidative stress conditions, suggesting a large portion of D1 is synthesised following the removal of the driver of photooxidative stress. Therefore, a timeseries experiment similar to those in Chapters 4 and 5 could be carried out where leaf tissue is collected and sampled for D1 protein abundance and expression of ROS accumulation marker genes. Through rhythmicity analysis, it could be determined whether there is circadian regulation of the rate of protein synthesis in the chloroplasts.

6.5 Conclusions

To the field of plant and circadian sciences, this thesis contributes the following novel findings:

a) The circadian oscillator has a role in regulating the response of photosynthetic efficiency in response to exposure to short-term cold and high light conditions.
b) There is circadian regulation of mechanisms involved in the maintenance of photosynthetic efficiency in response to exposure to short-term high light and cold and high light conditions. This regulation is such that circadian gating of photosynthetic efficiency under these conditions is evident.
c) Components of circadian regulation of recovery in photosynthetic efficiency following exposure to short-term photo-oxidative stress conditions are distinct from the circadian regulation of photosynthetic efficiency whilst under these conditions.
d) Leaf developmental type influences both the circadian regulation of photosynthetic efficiency both under control conditions and in response to exposure to short term cold and high light conditions.

References

Allen, D.J., Ratner, K., Giller, Y.E., Gussakovsky, E.E., Shahak, Y., Ort, D.R., 2000. An overnight chill induces a delayed inhibition of photosynthesis at midday in mango (Mangifera indica L.). J Exp Bot 51, 1893–1902.

Anderson, J.M., Chow, W.S., 2002. Structural and functional dynamics of plant photosystem II. Philos Trans R Soc Lond B Biol Sci 357, 1421–1470.

Asada, K., 2006. Production and scavenging of reactive oxygen species in chloroplasts and their functions. Plant Physiol 141, 391–396.

Asada, K., 1999. THE WATER-WATER CYCLE IN CHLOROPLASTS: Scavenging of Active Oxygens and Dissipation of Excess Photons. Annu. Rev. Plant. Physiol. Plant. Mol. Biol. 50, 601–639.

Barber, J., Andersson, B., 1992. Too much of a good thing: light can be bad for photosynthesis. Trends in Biochemical Sciences 17, 61–66.

Bates, D., Mächler, M., Bolker, B., Walker, S., 2015. Fitting Linear Mixed-Effects Models Using lme4. Journal of Statistical Software 67, 1–48.

Belbin, F.E., Noordally, Z.B., Wetherill, S.J., Atkins, K.A., Franklin, K.A., Dodd, A.N., 2017. Integration of light and circadian signals that regulate chloroplast transcription by a nuclear-encoded sigma factor. New Phytologist 213, 727–738.

Booij-James, I.S., Swegle, W.M., Edelman, M., Mattoo, A.K., 2002. Phosphorylation of the D1 Photosystem II Reaction Center Protein Is Controlled by an Endogenous Circadian Rhythm. Plant Physiol 130, 2069–2075.

Cano Ramirez, D., 2018. Integration of circadian and environmental information during signalling to chloroplasts (Ph.D.). University of Bristol.

Cano-Ramirez, D.L., Fraine, T.S. de, Griffiths, O.G., Dodd, A.N., 2018. Photosynthesis and circadian rhythms regulate the buoyancy of marimo lake balls. Current Biology 28, R869–R870.

Cano-Ramirez, Nishio, Muranaka, de Barros Dantas, Honjo, Sugisaka, Kudoh, Dodd, 2022. Circadian and environmental signal transduction in a natural population of Arabidopsis. bioRxiv, 2022.09.10.507414

Cano-Ramirez, Panter, under revision. Integration of low temperature and circadian signals by a plant sigma factor.

Chen, J.-H., Chen, S.-T., He, N.-Y., Wang, Q.-L., Zhao, Y., Gao, W., Guo, F.-Q., 2020. Nuclear-encoded synthesis of the D1 subunit of photosystem II increases photosynthetic efficiency and crop yield. Nat. Plants 6, 570–580.

Cohen, J., Pfeiffer, K., Francis, J.A., 2018. Warm Arctic episodes linked with increased frequency of extreme winter weather in the United States. Nat Commun 9, 869.

Covington, M.F., Maloof, J.N., Straume, M., Kay, S.A., Harmer, S.L., 2008. Global transcriptome analysis reveals circadian regulation of key pathways in plant growth and development. Genome Biology 9, R130.

Cuitun-Coronado, D., Rees, H., Colmer, J., Hall, A., Dantas, L.L. de B., Dodd, A.N., 2022. Circadian and diel regulation of photosynthesis in the bryophyte Marchantia polymorpha. Plant, Cell & Environment, 45, 2381– 2394

Dakhiya, Y., Hussien, D., Fridman, E., Kiflawi, M., Green, R., 2017. Correlations between Circadian Rhythms and Growth in Challenging Environments. Plant Physiology 173, 1724–1734.

Dodd, A.N., Jakobsen, M.K., Baker, A.J., Telzerow, A., Hou, S.-W., Laplaze, L., Barrot, L., Scott Poethig, R., Haseloff, J., Webb, A.A.R., 2006. Time of day modulates low-temperature Ca2+ signals in Arabidopsis. The Plant Journal 48, 962–973.

Dodd, A.N., Kusakina, J., Hall, A., Gould, P.D., Hanaoka, M., 2014. The circadian regulation of photosynthesis. Photosynthesis Research 119, 181–190.

Dodd, A.N., Salathia, N., Hall, A., Kévei, E., Tóth, R., Nagy, F., Hibberd, J.M., Millar, A.J., Webb, A.A.R., 2005. Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. Science 309, 630–633.

Endo, M., Shimizu, H., Nohales, M.A., Araki, T., Kay, S.A., 2014. Tissue-specific clocks in Arabidopsis show asymmetric coupling. Nature 515, 419–422.

Farré, E.M., Weise, S.E., 2012. The interactions between the circadian clock and primary metabolism. Current Opinions Plant Biology 15, 293–300.

FC, M., Davis, T.L., authors, ggplot2, 2022. ggpattern: “ggplot2” Pattern Geoms.

Gaal, T.V., Erwin, J.E., 2005. Diurnal Variation in Thigmotropic Inhibition of Stem Elongation. Hort Technology 15, 291–294.

Gorton, H.L., Williams, W.E., Assmann, S.M., 1993. Circadian Rhythms in Stomatal Responsiveness to Red and Blue Light. Plant Physiol 103, 399–406.

Gould, P.D., Diaz, P., Hogben, C., Kusakina, J., Salem, R., Hartwell, J., Hall, A., 2009. Delayed fluorescence as a universal tool for the measurement of circadian rhythms in higher plants. Plant J 58, 893–901.

Graf, A., Schlereth, A., Stitt, M., Smith, A.M., 2010. Circadian control of carbohydrate availability for growth in Arabidopsis plants at night. Proceedings of the National Academy of Sciences 107, 9458–9463.

Gray, G.R., Hope, B.J., Qin, X., Taylor, B.G., Whitehead, C.L., 2003. The characterization of photoinhibition and recovery during cold acclimation in Arabidopsis thaliana using chlorophyll fluorescence imaging. Physiologia Plantarum 119, 365–375.

Green, R.M., Tingay, S., Wang, Z.-Y., Tobin, E.M., 2002. Circadian Rhythms Confer a Higher Level of Fitness to Arabidopsis Plants. Plant Physiology 129, 576–584.

Guadagno, C.R., Ewers, B.E., Weinig, C., 2018. Circadian Rhythms and Redox State in Plants: Till Stress Do Us Part. Frontiers in Plant Science 247-9.

Gururani, M.A., Venkatesh, J., Tran, L.S.P., 2015. Regulation of Photosynthesis during Abiotic Stress-Induced Photoinhibition. Molecular Plant 8, 1304–1320.

Gyllenstrand, N., Karlgren, A., Clapham, D., Holm, K., Hall, A., Gould, P.D., Källman, T., Lagercrantz, U., 2014. No Time for Spruce: Rapid Dampening of Circadian Rhythms in Picea abies (L. Karst). Plant and Cell Physiology 55, 535–550.

Hadley Wickham, 2016. ggplot2: Elegant Graphics for Data Analysis.

Hotta, C.T., Gardner, M.J., Hubbard, K.E., Baek, S.J., Dalchau, N., Suhita, D., Dodd, A.N., Webb, A. a. R., 2007. Modulation of environmental responses of plants by circadian clocks. Plant, Cell & Environment 30, 333–349.

Hsu, P.Y., Devisetty, U.K., Harmer, S.L., 2013. Accurate timekeeping is controlled by a cycling activator in Arabidopsis. Elife 2, e00473.

Hsu, P.Y., Harmer, S.L., 2014. Wheels within wheels: the plant circadian system. Trends Plant Science 19, 240–249.

Ishizaki, Y., Tsunoyama, Y., Hatano, K., Ando, K., Kato, K., Shinmyo, A., Kobori, M., Takeba, G., Nakahira, Y., Shiina, T., 2005. A nuclear-encoded sigma factor, Arabidopsis SIG6, recognizes sigma-70 type chloroplast promoters and regulates early chloroplast development in cotyledons. The Plant Journal 42, 133–144.

Jiménez, A., Sevilla, F., Martí, M.C., 2021. Reactive oxygen species homeostasis and circadian rhythms in plants. Journal Experimental Botany 72, 5825–5840.

Kasahara, M., Kagawa, T., Oikawa, K., Suetsugu, N., Miyao, M., Wada, M., 2002. Chloroplast avoidance movement reduces photodamage in plants. Nature 420, 829–832.

Kassambara, A., 2020. ggpubr: “ggplot2” Based Publication Ready Plots.

Kato, Y., Sun, X., Zhang, L., Sakamoto, W., 2012. Cooperative D1 Degradation in the Photosystem II Repair Mediated by Chloroplastic Proteases in Arabidopsis. Plant Physiology 159, 1428–1439.

Khanal, N., Bray, G.E., Grisnich, A., Moffatt, B.A., Gray, G.R., 2017. Differential Mechanisms of Photosynthetic Acclimation to Light and Low Temperature in Arabidopsis and the Extremophile Eutrema salsugineum. Plants (Basel) 6, 32.

Kim, H., Kim, Y., Yeom, M., Lim, J., Nam, H.G., 2016. Age-associated circadian period changes in Arabidopsis leaves. Journal of Experimental Botany 67, 2665–2673.

Kress, E., Jahns, P., 2017. The Dynamics of Energy Dissipation and Xanthophyll Conversion in Arabidopsis Indicate an Indirect Photoprotective Role of Zeaxanthin in Slowly Inducible and Relaxing Components of Non-photochemical Quenching of Excitation Energy. Frontiers in Plant Science 8.

Kromdijk, J., 2022. Relaxing non-photochemical quenching (NPQ) to improve photosynthesis in crops, in: Sharwood, R. (Ed.), Burleigh Dodds Series in Agricultural Science. Burleigh Dodds Science Publishing.

Lai, A.G., Doherty, C.J., Mueller-Roeber, B., Kay, S.A., Schippers, J.H.M., Dijkwel, P.P., 2012. CIRCADIAN CLOCK-ASSOCIATED 1 regulates ROS homeostasis and oxidative stress responses. Proceedings of the National Academy of Sciences 109, 17129–17134.

Lamichhane, J.R., 2021. Rising risks of late-spring frosts in a changing climate. Nature Climate Change 11, 554–555.

Lenth, R.V., Buerkner, P., Herve, M., Jung, M., Love, J., Miguez, F., Riebl, H., Singmann, H., 2022. emmeans: Estimated Marginal Means, aka Least-Squares Means.

Li, X., Jiang, H., Liu, F., Cai, J., Dai, T., Cao, W., Jiang, D., 2013. Induction of chilling tolerance in wheat during germination by pre-soaking seed with nitric oxide and gibberellin. Plant Growth Regulation 71, 31–40.

Litthauer, S., Battle, M.W., Lawson, T., Jones, M.A., 2015. Phototropins maintain robust circadian oscillation of PSII operating efficiency under blue light. The Plant Journal 83, 1034–1045.

Lu, D., Zhang, Y., Zhang, A., Lu, C., 2022. Non-Photochemical Quenching: From Light Perception to Photoprotective Gene Expression. International Journal of Molecular Sciences 23, 687.

Lu, S.X., Knowles, S.M., Andronis, C., Ong, M.S., Tobin, E.M., 2009. CIRCADIAN CLOCK ASSOCIATED1 and LATE ELONGATED HYPOCOTYL Function Synergistically in the Circadian Clock of Arabidopsis. Plant Physiology 150, 834–843.

Maxwell, K., Johnson, G.N., 2000. Chlorophyll fluorescence—a practical guide 10.
Millar, A.J., Kay, S.A., 1996. Integration of circadian and phototransduction pathways in the network controlling CAB gene transcription in Arabidopsis. Proceedings of the National Academy of Sciences 93, 15491–15496.

Millar, A.J., Short, S.R., Chua, N.-H., Kay'I, S.A., 1992. A Novel Circadian Phenotype Based on Firefly Luciferase Expression in Transgenic Plants 13.

Mishra, A., Mishra, K.B., Höermiller, I.I., Heyer, A.G., Nedbal, L., 2011. Chlorophyll fluorescence emission as a reporter on cold tolerance in Arabidopsis thaliana accessions. Plant Signaling & Behavior 6, 301–310.

Miura, K., Furumoto, T., 2013. Cold Signaling and Cold Response in Plants. International Journal of Molecular Sciences 14, 5312–5337.

Molina-Montenegro, M.A., Salgado-Luarte, C., Oses, R., Torres-Díaz, C., 2013. Is Physiological Performance a Good Predictor for Fitness? Insights from an Invasive Plant Species. PLOS ONE 8, e76432.

Nagashima, A., Hanaoka, M., Shikanai, T., Fujiwara, M., Kanamaru, K., Takahashi, H., Tanaka, K., 2004. The Multiple-Stress Responsive Plastid Sigma Factor, SIG5, Directs Activation of the psbD Blue Light-Responsive Promoter (BLRP) in Arabidopsis thaliana. Plant and Cell Physiology 45, 357–368.

Nakamichi, N., Kiba, T., Henriques, R., Mizuno, T., Chua, N.-H., Sakakibara, H., 2010. PSEUDO-RESPONSE REGULATORS 9, 7, and 5 are transcriptional repressors in the Arabidopsis circadian clock. Plant Cell 22, 594–605.

Nelson, N., Ben-Shem, A., 2004. The complex architecture of oxygenic photosynthesis. Nature Reviews Molecular Cell Biology 5, 971–982.

Nishiyama, Y., Yamamoto, H., Allakhverdiev, S.I., Inaba, M., Yokota, A., Murata, N., 2001. Oxidative stress inhibits the repair of photodamage to the photosynthetic machinery. EMBO Journal 20, 5587–5594.

Nixon, P.J., Michoux, F., Yu, J., Boehm, M., Komenda, J., 2010. Recent advances in understanding the assembly and repair of photosystem II. Annals Botant=y 106, 1–16.

Noordally, Z.B., Ishii, K., Atkins, K.A., Wetherill, S.J., Kusakina, J., Walton, E.J., Kato, M., Azuma, M., Tanaka, K., Hanaoka, M., Dodd, A.N., 2013a. Circadian control of chloroplast transcription by a nuclear-encoded timing signal. Science 339, 1316–1319.

Noordally, Z.B., Ishii, K., Atkins, K.A., Wetherill, S.J., Kusakina, J., Walton, E.J., Kato, M., Azuma, M., Tanaka, K., Hanaoka, M., Dodd, A.N., 2013b. Circadian Control of Chloroplast Transcription by a Nuclear-Encoded Timing Signal. Science 339, 1316–1319.

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

Paajanen, P., Dantas, L.L. de B., Dodd, A.N., 2021. Layers of crosstalk between circadian regulation and environmental signalling in plants. Current Biology 31, R399–R413.

Pruneda-Paz, J.L., Breton, G., Para, A., Kay, S.A., 2009. A functional genomics approach reveals CHE as a component of the Arabidopsis circadian clock. Science 323, 1481–1485.

Rawat, R., Takahashi, N., Hsu, P.Y., Jones, M.A., Schwartz, J., Salemi, M.R., Phinney, B.S., Harmer, S.L., 2011. REVEILLE8 and PSEUDO-REPONSE REGULATOR5 form a negative feedback loop within the Arabidopsis circadian clock. PLoS Genet 7, e1001350.

Ruelland, E., Vaultier, M.-N., Zachowski, A., Hurry, V., 2009. Chapter 2 Cold Signalling and Cold Acclimation in Plants, in: Advances in Botanical Research. Academic Press, pp. 35–150.

Salter, M.G., Franklin, K.A., Whitelam, G.C., 2003. Gating of the rapid shade-avoidance response by the circadian clock in plants. Nature 426, 680–683.

Shi, Y., Chen, J., Hou, X., 2020. Similarities and Differences of Photosynthesis Establishment Related mRNAs and Novel lncRNAs in Early Seedlings (Coleoptile/Cotyledon vs. True Leaf) of Rice and Arabidopsis. Frontiers in Genetics 11.

Shiina, T., Tsunoyama, Y., Nakahira, Y., Khan, M.S., 2005. Plastid RNA polymerases, promoters, and transcription regulators in higher plants. Int Rev Cytol 244, 1–68.

Shimada, H., Mochizuki, M., Ogura, K., Froehlich, J.E., Osteryoung, K.W., Shirano, Y., Shibata, D., Masuda, S., Mori, K., Takamiya, K., 2007. Arabidopsis Cotyledon-Specific Chloroplast Biogenesis Factor CYO1 Is a Protein Disulfide Isomerase. Plant Cell 19, 3157–3169.

Simon, N.M.L., Graham, C.A., Comben, N.E., Hetherington, A.M., Dodd, A.N., 2020. The Circadian Clock Influences the Long-Term Water Use Efficiency of Arabidopsis1 [OPEN]. Plant Physiology 183, 317–330.

Simon, N.M.L., Litthauer, S., Jones, M.A., Dodd, A.N., 2019. Interactions Between Circadian Rhythms, ROS and Redox, in: Panda, S.K., Yamamoto, Y.Y. (Eds.), Redox Homeostasis in

Plants: From Signalling to Stress Tolerance, Signaling and Communication in Plants. Springer International Publishing, Cham, pp. 67–84.

Soitamo, A.J., Piippo, M., Allahverdiyeva, Y., Battchikova, N., Aro, E.-M., 2008. Light has a specific role in modulating Arabidopsis gene expression at low temperature. BMC Plant Biology 8, 13.

Suetsugu, N., Higa, T., Gotoh, E., Wada, M., 2016. Light-Induced Movements of Chloroplasts and Nuclei Are Regulated in Both Cp-Actin-Filament-Dependent and -Independent Manners in Arabidopsis thaliana. PLOS ONE 11, e0157429.

Tsukaya, H., Tsuge, T., Uchimiya, H., 1994. The cotyledon: A superior system for studies of leaf development. Planta 195, 309–312.

Turgeon, R., 1989. The Sink-Source Transition in Leaves. Annual Review of Plant Physiology and Plant Molecular Biology 40, 119–138.

Wang, Z.-Y., Tobin, E.M., 1998. Constitutive Expression of the CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) Gene Disrupts Circadian Rhythms and Suppresses Its Own Expression. Cell 93, 1207–1217.

Wu, G., Anafi, R., Hogenesch, J., Hughes, M., Kornacker, K., Li, X., Carlucci, M., 2019. MetaCycle: Evaluate Periodicity in Large Scale Data.

Yarkhunova, Y., Guadagno, C.R., Rubin, M.J., Davis, S.J., Ewers, B.E., Weinig, C., 2018. Circadian rhythms are associated with variation in photosystem II function and photoprotective mechanisms. Plant, Cell & Environment 41, 2518–2529.

Yoshimura, K., Yabuta, Y., Ishikawa, T., Shigeoka, S., 2002. Identification of a cis element for tissue-specific alternative splicing of chloroplast ascorbate peroxidase pre-mRNA in higher plants. J Biol Chem 277, 40623–40632.

Zhang, D., Kato, Y., Zhang, L., Fujimoto, M., Tsutsumi, N., Sodmergen, Sakamoto, W., 2010. The FtsH Protease Heterocomplex in Arabidopsis: Dispensability of Type-B Protease Activity for Proper Chloroplast Development. The Plant Cell 22, 3710–3725.

APPENDICES

APPENDIX A

Table A.1. Summary of meta2d quantitative timecourse analysis mean of rate of recovery in effective PSII quantum yield (Y(II)) under cold and/or high light conditions. Y(II) of 11 day old cotyledons measured at 12 timepoints across a 44 hour subjective time period, following cotyledon exposure to 3 h treatments conditions. At each timepoint seedlings were held in darkness and Y(II) was measured 0, 15, 30, 45, and 60 minutes after treatment. Rates of recovery were calculated for each seedling as ((Y(II) at time x after treatment) – Y(II) at time 0)/ x. Timecourse analysis was carried out on estimated marginal mean of rates of recovery in Y(II), calculated for each timepoint from three (two for CCA1-ox) independent experimental repeats. N=8-20 individual plants within each independent repeat. Estimated marginal means calculated using mixed effects linear models, adjusting means for the influence of experimental repeat as a random effect. P-values obtained from meta2d method analysis for statistically significant rhythmicity. Period, phase, and amplitude estimates are excluded for experiments which did not yield statistically significant rhythms because these measures are derived from poor fits to the data. Results shown to three significant figures. p<0.05 *, p<0.01 **, p<0.001 ***

Treatment	Genotype	Time since treatment (min)	p-value	Period est. (h)	Phase est. (h)	Amplitude est. (Rate of recovery in Y(II))
Control (19^oC, 110 µmol m^{-2} s^{-1})	WT	15	0.232	–	–	–
		30	0.975	–	–	–
		45	0.999	–	–	–
		60	0.230	–	–	–
	sig5-3	15	0.136	–	–	–
		30	0.0814	–	–	–
		45	0.0316 *	24.7	12.8	5.38×10^{-05}
		60	0.00601 **	24.3	13.2	4.23×10^{-05}
	CCA1-ox	15	0.987	–	–	–
		30	0.998	–	–	–
		45	0.352	–	–	–
		60	0.849	–	–	–
Cold (4^oC, 110 µmol m^{-2} s^{-1})	WT	15	0.273	–	–	–
		30	0.550	–	–	–
		45	0.178	–	–	–
		60	0.698	–	–	–
	sig5-3	15	0.340	–	–	–
		30	0.282	–	–	–
		45	0.217	–	–	–
		60	0.450	–	–	–
	CCA1-ox	15	0.735	–	–	–
		30	0.469	–	–	–
		45	0.533	–	–	–
		60	0.939	–	–	–
High Light	WT	15	0.932	–	–	–

(19°C, 700 µmol m^{-2} s^{-1})		30	0.0145 *	23.9	8.55	0.000126
		45	0.0263 *	24.0	9.43	7.80×10^{-05}
		60	0.0243 *	26.0	7.59	5.62×10^{-05}
	sig5-3	15	0.511	–	–	–
		30	2.62×10^{-05} ***	23.5	13.9	9.14×10^{-05}
		45	0.000797 ***	26.3	10.1	7.22×10^{-05}
		60	0.00212 ***	24.8	13.0	5.03×10^{-05}
	CCA1-ox	15	0.999	–	–	–
		30	0.216	–	–	–
		45	0.00437 ***	27.8	23.2	6.91×10^{-05}
		60	6.06×10^{-05} ***	27.0	22.0	5.51×10^{-05}
Cold and High Light (4°C, 700 µmol m^{-2} s^{-1})	WT	15	0.860	–	–	–
		30	0.908	–	–	–
		45	0.535	–	–	–
		60	0.969	–	–	–
	sig5-3	15	0.935	–	–	–
		30	0.997	–	–	–
		45	0.999	–	–	–
		60	1.00	–	–	–
	CCA1-ox	15	0.875	–	–	–
		30	0.937	–	–	–
		45	0.739	–	–	–
		60	0.770	–	–	–

Table A.2. Summary of meta2d quantitative timecourse analysis of calculated difference in rate of recovery in effective PSII quantum yield (Y(II)) between seedlings under control vs cold and/or high light treatment conditions. Y(II) of 11 day old cotyledons measured at 12 timepoints across a 44 hour subjective time period, following cotyledon exposure to 3 h treatments conditions. At each timepoint seedlings were held in darkness and Y(II) was measured 0, 15, 30, 45, and 60 minutes after treatment. Rates of recovery were calculated for each seedling as ((Y(II) at time x after treatment) – Y(II) at time 0)/ x. Estimated marginal means of the rates of recovery at each timepoint were then calculated using mixed effects linear models, adjusting means for the influence of experimental repeat as a random effect. Three (two for CCA1-ox) independent experimental repeats were carried out, N=8-20 individual plants within each independent repeat. Timecourse analysis was carried out the calculated difference between means under control and cold and/or high light treatments at each timepoint (difference = cold and/or high light treatment mean – control treatment mean). P-values obtained from meta2d method analysis for statistically significant rhythmicity. Period, phase, and amplitude estimates are excluded for experiments which did not yield statistically significant rhythms because these measures are derived from poor fits to the data. Results shown to three significant figures. $p<0.05$ *, $p<0.01$ **, $p<0.001$ ***

Treatment	Genotype	Time since treatment (min)	p-value	Period est. (h)	Phase est. (h)	Amplitude est. (difference in rate of recovery in Y(II))
Cold ($4^{o}C$, 110 µmol m^{-2} s^{-1})	WT	15	0.292			
		30	0.508			
		45	0.309			
		60	0.465			
	sig5-3	15	0.347			
		30	0.412			
		45	0.132			
		60	0.275			
	CCA1-ox	15	0.802			
		30	0.386			
		45	0.707			
		60	0.984			
High Light ($19^{o}C$, 700 µmol m^{-2} s^{-1})	WT	15	0.711			
		30	0.0277 *	27.5	0.698	0.000125
		45	0.137			

		60	0.166
	sig5-3	15	1.00
		30	0.986
		45	0.964
		60	0.999
	CCA1-ox	15	1.00
		30	0.996
		45	0.976
		60	0.984
Cold and High Light ($4^{o}C$, 700 µmol m^{-2} s^{-1})	WT	15	0.482
		30	0.580
		45	0.630
		60	0.795
	sig5-3	15	0.714
		30	0.917
		45	0.795
		60	0.997
	CCA1-ox	15	0.912
		30	0.965
		45	0.853
		60	0.803

Table A.3 . Summary of meta2d quantitative timecourse analysis of calculated difference in rate of recovery in effective PSII quantum yield (Y(II)) between WT and genotype under each treatment condition. Y(II) of 11 day old cotyledons measured at 12 timepoints across a 44 hour subjective time period, following cotyledon exposure to 3 h treatments conditions. At each timepoint seedlings were held in darkness and Y(II) was measured 0, 15, 30, 45, and 60 minutes after treatment. Rates of recovery were calculated for each seedling as ((Y(II) at time x after treatment) – Y(II) at time 0)/ x. Estimated marginal means of these rates of recovery at each timepoint were then calculated using mixed effects linear models, adjusting means for the influence of experimental repeat as a random effect. Three (two for CCA1-ox) independent experimental repeats were carried out, N=8-20 individual plants within each independent repeat. Timecourse analysis was carried out the calculated difference between means of WT and *sig5-3* or CCA1-ox seedlings under each treatment condition (difference = *sig5-3* or CCA1-ox mean – WT mean). P-values obtained from meta2d method analysis for statistically significant rhythmicity. Period, phase, and amplitude estimates are excluded for experiments which did not yield statistically significant rhythms because these measures are derived from poor fits to the data. Results shown to three significant figures. p<0.05 *, p<0.01 **, p<0.001 ***

Treatment	Genotype	Time since treatment (min)	p-value	Period est. (h)	Phase est. (h)	Amplitude est. (difference in rate of recovery in Y(II))
Control (19ºC, 110 µmol m^{-2} s^{-1})	*sig5-3*	15	0.779			
		30	0.0479 *	21.0	20.1	6.85 $x10^{-05}$
		45	0.0513			
		60	0.809			
	CCA1-ox	15	0.208			
		30	0.893			
		45	0.455			
		60	0.301			
Cold (4ºC, 110 µmol m^{-2} s^{-1})	*sig5-3*	15	0.961			
		30	0.710			
		45	0.996			
		60	0.982			
	CCA1-ox	15	0.995			
		30	0.445			
		45	0.996			
		60	0.999			
High Light (19ºC, 700 µmol m^{-2} s^{-1})	*sig5-3*	15	9.49 $x10^{-05}$ ***	23.7	17.2	0.0002
		30	0.00172 **	25.9	15.6	0.000115
		45	0.0137 *	23.7	17.8	6.64 $x10^{-05}$
		60	0.0218 *	25.5	16.2	4.66 $x10^{-05}$
	CCA1-ox	15	0.944			
		30	0.129			
		45	0.0145 *	27.4	20.3	0.00012
		60	0.000611 ***	26.7	20.1	0.000105

Cold and High Light ($4^{o}C$, 700 µmol m^{-2} s^{-1})	*sig5-3*	15	0.946			
		30	0.765			
		45	0.499			
		60	0.330			
	CCA1-ox	15	0.00441 ***	22.1	17.0	0.000625
		30	0.0239 *	21.6	18.1	0.000302
		45	0.0247 *	20.0	19.9	0.000269
		60	0.134			

APPENDIX B

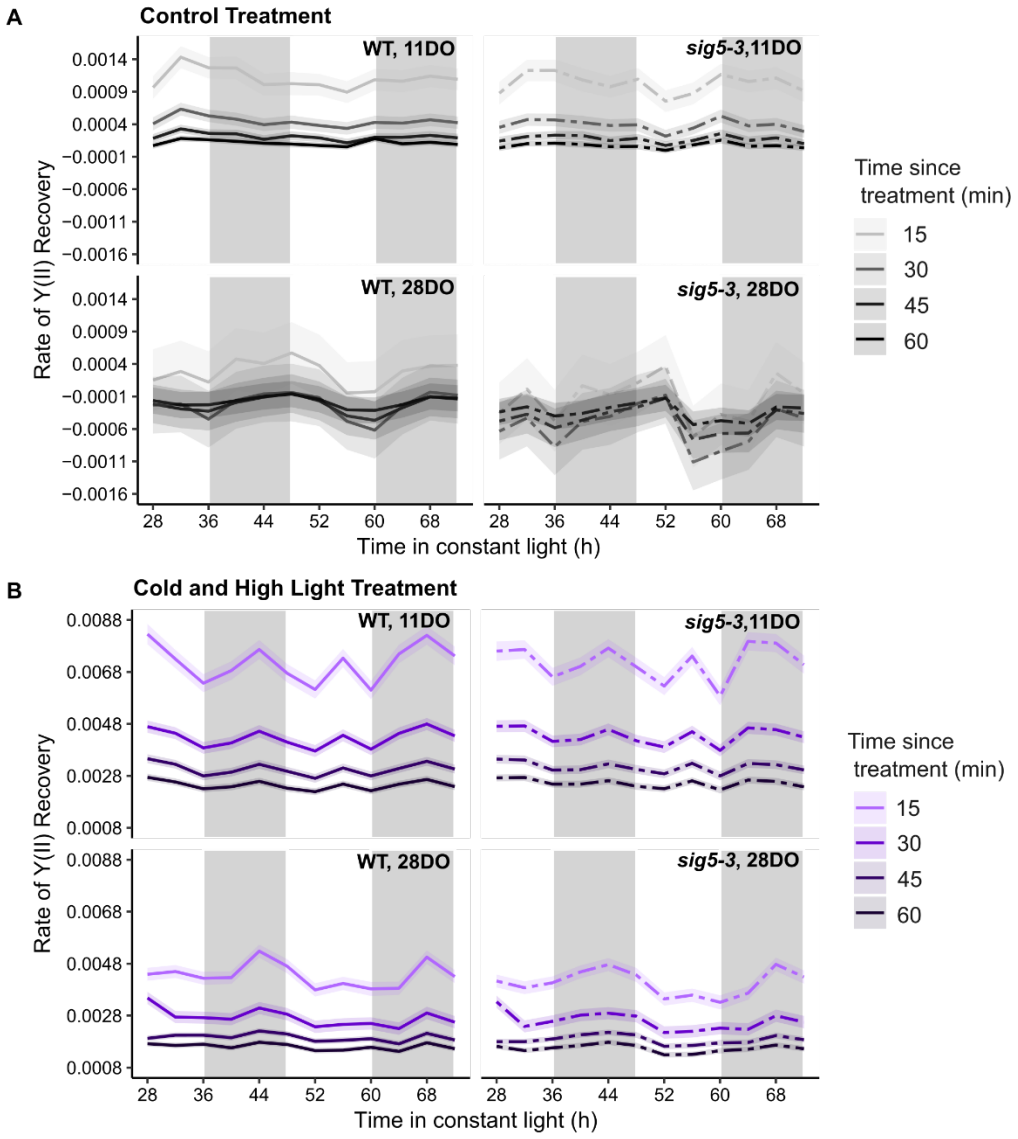

Figure B.1. Circadian features in rate of recovery in effective PSII quantum yield (Y(II)) differs between leaf developmental stages. Measurements of 11 day old cotyledons of Arabidopsis thaliana seedlings (11DO) and fully expanded outer rosette leaves (28DO). Genotypes used were Col-0 wild-type (WT) and *sig5-3*. Following 3 h treatment with **(A)** control (19°C, 110 µmolm^{-2}s^{-1}), **(B)** cold and high light (4°C, 700 µmolm^{-2}s^{-1}). At each timepoint, Y(II) measurements were taken 0, 15, 30, 45, and 60 minutes after the treatment. Rate of recovery was calculated for each seedling as ((Y(II) at time x after treatment) – Y(II) at time 0)/ x. Plots show estimated marginal mean ± SE for three independent experimental repeats, calculated using a mixed effects linear model which adjusted values for the influence of random effect experimental repeat. 11DO, N = 8-20 individual plants within each independent repeat 28DO, N= 16 individual leave of 4-5 plants within each independent repeat. $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ *** representing results of meta2D method test for statistically significant rhythmicity. Data for cotyledons are derived from Chapter 4. Clear areas of panels indicate subjective day, grey areas of panels indicate subjective night.

Table B.1. Summary of meta2d quantitative timecourse analysis mean of rate of recovery in effective PSII quantum yield (Y(II)) under cold and high light conditions. Y(II) of 11 day old cotyledons measured at 12 timepoints across a 44 hour subjective time period, following cotyledon exposure to 3h treatments conditions. Genotypes used were Col-0 wild-type (WT) and *sig5-3*. At each timepoint seedlings were held in darkness and Y(II) was measured 0, 15, 30, 45, and 60 minutes after treatment. Rates of recovery were calculated for each seedling as ((Y(II) at time x after treatment) – Y(II) at time 0)/ x. Timecourse analysis was carried out on estimated marginal mean of rates of recovery in Y(II), calculated for each timepoint from three independent experimental repeats. 11DO, N = 8-20 individual plants within each independent repeat 28DO, N= 16 individual leave of 4-5 plants within each independent repeat. Estimated marginal means calculated using mixed effects linear models, adjusting means for the influence of experimental repeat as a random effect. P-values obtained from meta2d method analysis for statistically significant rhythmicity. Period, phase, and amplitude estimates are excluded for experiments which did not yield statistically significant rhythms because these measures are derived from poor fits to the data. Results shown to three significant figures. $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***. Data for cotyledons are derived from Chapter 4.

Treatment	Genotype	Time since treatment (min)	Age (days)	p-value	Period est. (h)	Phase est. (h)	Amplitude (Y(II))
Control (19^oC, 110 $\mu molm^{-2}s^{-1}$)		15	11	0.232	–	–	–
			28	0.00773 **	26.9	19.5	0.000219
		30	11	0.973	–	–	–
			28	0.000493 ***	23.8	23.5	0.000234
		45	11	0.990	–	–	–
			28	9.15×10^{-06}***	24.2	23.0	0.000165
		60	11	0.230	–	–	–
			28	7.61×10^{-06}***	–	–	–
	sig5-3	15	11	0.136	–	–	–
			28	0.553	–	–	–
		30	11	0.0814	–	–	–
			28	0.0588	–	–	–
		45	11	0.0316 *	24.7	12.8	5.38×10^{-05}
			28	0.0538	–	–	–
		60	11	0.00601 **	24.3	13.2	4.23×10^{-05}
			28	0.0720	–	–	–
Cold and High Light (4^oC, 700 $\mu molm^{-2}s^{-1}$)	WT	15	11	0.860	–	–	–
			28	0.347	–	–	–
		30	11	0.908	–	–	–
			28	0.330	–	–	–
		45	11	0.535	–	–	–
			28	0.801	–	–	–
		60	11	0.969	–	–	–
			28	0.801	–	–	–
	sig5-3	15	11	0.935	–	–	–
			28	0.00485 **	26.4	17.5	0.000621
		30	11	0.997	–	–	–

		28	0.249	–	–	–
	45	11	0.999	–	–	–
		28	0.00756 **	27.7	15.7	0.000210
	60	11	1.00	–	–	–
		28	0.00657 **	23.2	21.3	0.000196

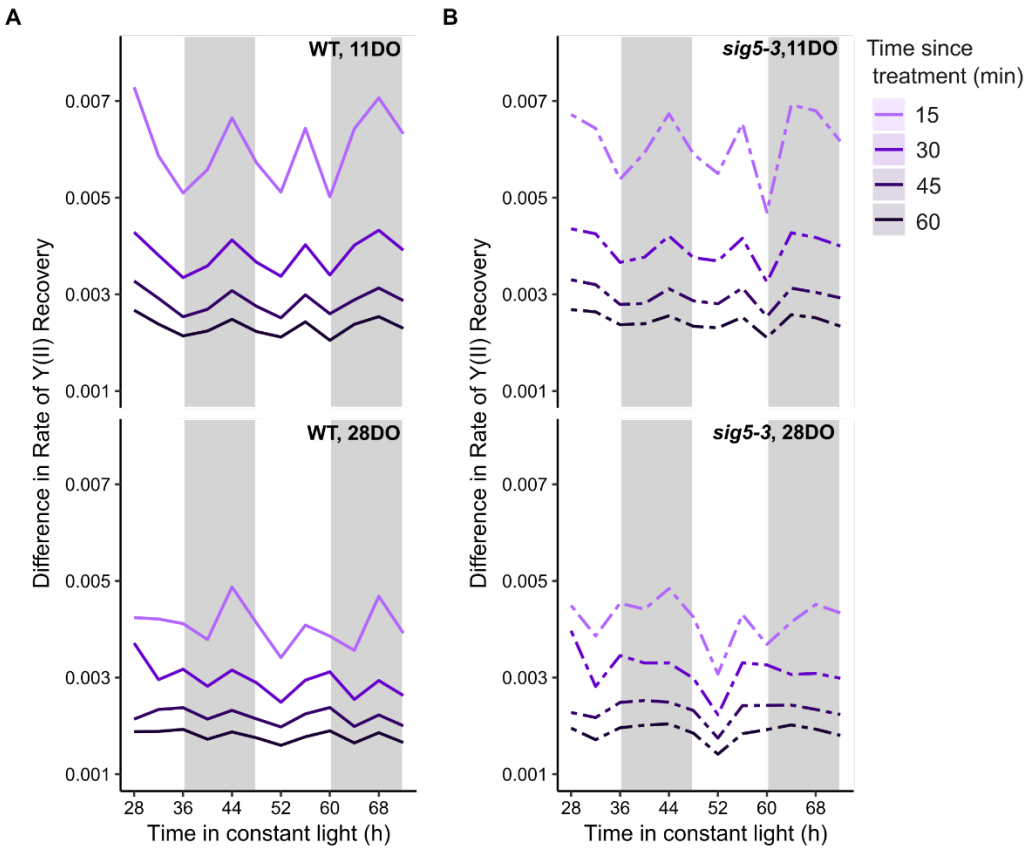

Figure B.2. Differences between recovery in effective PSII quantum yield (Y(II)) under control *vs* cold and high light conditions suggests circadian regulation of recovery. Measurements of 11 day old cotyledons of Arabidopsis thaliana seedlings (11DO) and fully expanded outer rosette leaves (28DO). **(A)** Col-0 wild-type (WT) **(B)** *sig5-3*. At each timepoint, Y(II) measurements were take 0, 15, 30, 45, and 60 minutes after the 3h treatment of control (19ºC, 110 $\mu molm^{-2}s^{-1}$) or cold and high light (4ºC, 700 $\mu molm^{-2}s^{-1}$) treatment conditions. Rate of recovery was calculated for each seedling as ((Y(II) at time x after treatment) – Y(II) at time 0)/ x. Estimated marginal means of rates of recovery at each timepoint were calculated using mixed effects linear models, adjusting means for the influence of experimental repeat as a random effect. Three independent experimental repeats were carried out, 11DO, N = 8-20 individual plants within each independent repeat 28DO, N= 16 individual leave of 4-5 plants within each independent repeat. Plots show the calculated difference between means under control (19ºC, 110 $\mu molm^{-2}s^{-1}$) and cold and high light (4ºC, 700 $\mu molm^{-2}s^{-1}$) treatments at each timepoint (difference = treatment – control), whereby greater differences reflect a greater impact of treatment on rate of recovery in Y(II) compared to control conditions. Data for cotyledons are derived from Chapter 4. Clear areas of panels indicate subjective day, grey areas of panels indicate subjective night.

Table B.2. Summary of meta2d quantitative timecourse analysis of calculated difference in rate of recovery in effective PSII quantum yield (Y(II)) between seedlings under control vs cold and high light treatment conditions. Y(II) of 11 day old cotyledons measured at 12 timepoints across a 44 hour subjective time period, following cotyledon exposure to 3h treatments conditions. Genotypes used were Col-0 wild-type (WT) and *sig5-3*. At each timepoint seedlings were held in darkness and Y(II) was measured 0, 15, 30, 45, and 60 minutes after treatment. Rates of recovery were calculated for each seedling as ((Y(II) at time x after treatment) – Y(II) at time 0)/ x. Estimated marginal means of the rates of recovery at each timepoint were then calculated using mixed effects linear models, adjusting means for the influence of experimental repeat as a random effect. Three independent experimental repeats were carried out. 11DO, N = 8-20 individual plants within each independent repeat 28DO, N= 16 individual leave of 4-5 plants within each independent repeat. Timecourse analysis was carried out the calculated difference between means under control and cold and high light treatments at each timepoint (difference = cold and high light treatment mean – control treatment mean). P-values obtained from meta2d method analysis for statistically significant rhythmicity. Period, phase, and amplitude estimates are excluded for experiments which did not yield statistically significant rhythms because these measures are derived from poor fits to the data. Results shown to three significant figures. $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***. Data for cotyledons are derived from Chapter 4.

Genotype	Time since treatment (min)	Age (days)	p-value	Period est. (h)	Phase est. (h)	Amplitude (Y(II))
WT	15	11	0.482	–	–	–
		28	0.924	–	–	–
	30	11	0.580	–	–	–
		28	1.00	–	–	–
	45	11	0.630	–	–	–
		28	0.769	–	–	–
	60	11	0.795	–	–	–
		28	0.922	–	–	–
sig5-3	15	11	0.714	–	–	–
		28	0.696	–	–	–
	30	11	0.917	–	–	–
		28	0.990	–	–	–
	45	11	0.795	–	–	–
		28	0.00835 **	21.7	18.5	0.000204
	60	11	0.997	–	–	–
		28	0.00807 **	22.1	19.1	0.000182

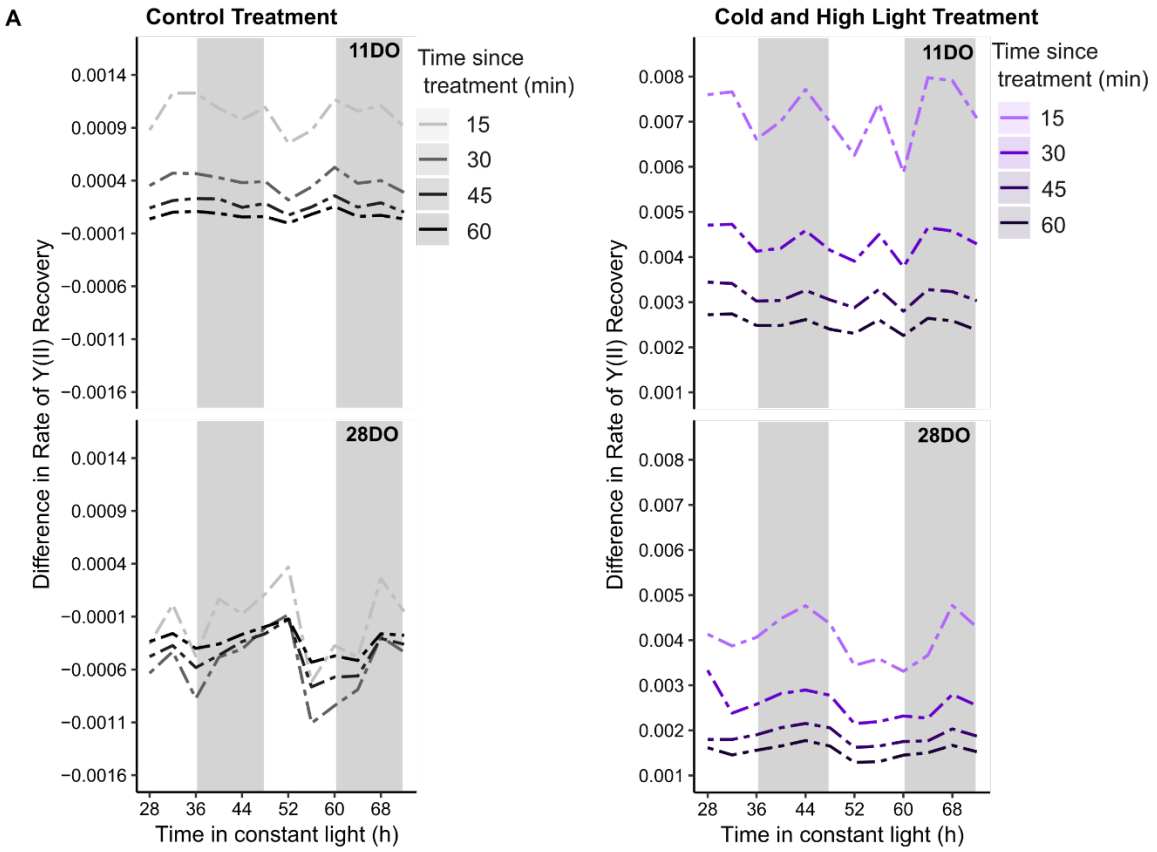

Figure B.3. Differences between WT *vs sig5-3* suggests circadian involvement in recovery in effective PSII quantum yield (Y(II)) following treatments Measurements of 11 day old cotyledons of Arabidopsis thaliana seedlings (11DO) and fully expanded outer rosette leaves (28DO). Following 3 h treatment with **(A)** control (19°C, 110 µmolm^{-2}s^{-1}), **(B)** cold and high light (4°C, 700 µmolm^{-2}s^{-1}). At each timepoint, Y(II) measurements were take 0, 15, 30, 45, and 60 minutes after the treatment. Rate of recovery was calculated for each seedling as ((Y(II) at time x after treatment) – Y(II) at time 0)/ x. Estimated marginal means of rates of recovery at each timepoint were calculated using mixed effects linear models, adjusting means for the influence of experimental repeat as a random effect. Three independent experimental repeats were carried out. 11DO, N = 8-20 individual plants within each independent repeat 28DO, N= 16 individual leave of 4-5 plants within each independent repeat. Plots show the calculated difference between means of WT and *sig5-3* under each treatment condition at each timepoint (difference = *sig5-3* – WT), whereby greater differences reflect a greater impact of the conditions on rate of recovery in Y(II) in the *sig5-3* over WT. Data for cotyledons are derived from Chapter 4. Clear areas of panels indicate subjective day, grey areas of panels indicate subjective night.

Table B.3 . Summary of meta2d quantitative timecourse analysis of calculated difference in rate of recovery in effective PSII quantum yield (Y(II)) between Col-0 wild-type (WT) and *sig5-3* under each treatment condition. Y(II) of 11 day old cotyledons measured at 12 timepoints across a 44 hour subjective time period, following cotyledon exposure to 3h treatments conditions. At each timepoint seedlings were held in darkness and Y(II) was measured 0, 15, 30, 45, and 60 minutes after treatment. Rates of recovery were calculated for each seedling as ((Y(II) at time x after treatment) – Y(II) at time 0)/ x. Estimated marginal means of these rates of recovery at each timepoint were then calculated using mixed effects linear models, adjusting means for the influence of experimental repeat as a random effect. Three independent experimental repeats were carried out. 11DO, N = 8-20 individual plants within each independent repeat 28DO, N= 16 individual leave of 4-5 plants within each independent repeat. Timecourse analysis was carried out the calculated difference between means of WT and *sig5-3* under each treatment condition (difference = *sig5-3* – WT mean). P-values obtained from meta2d method analysis for statistically significant rhythmicity. Period, phase, and amplitude estimates are excluded for experiments which did not yield statistically significant rhythms because these measures are derived from poor fits to the data. Results shown to three significant figures. $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***. Data for cotyledons are derived from Chapter 4.

Treatment	Time since treatment (min)	Age (days)	p-value	Period est. (h)	Phase est. (h)	Amplitude (Y(II))
Control (19^oC, 110 $\mu molm^{-2}s^{-1}$)	15	11	0.779	–	–	–
		28	0.930	–	–	–
	30	11	0.0479 *	21.0	20.1	6.85×10^{-05}
		28	0.871	–	–	–
	45	11	0.0513	–	–	–
		28	0.710	–	–	–
	60	11	0.809	–	–	–
		28	0.397	–	–	–
Cold and High Light (4^oC, 700 $\mu molm^{-2}s^{-1}$)	15	11	0.946	–	–	–
		28	0.937	–	–	–
	30	11	0.765	–	–	–
		28	0.361	–	–	–
	45	11	0.499	–	–	–
		28	0.0288 *	24.1	19.4	0.000119
	60	11	0.330	–	–	–
		28	0.00527 **	23.4	20.1	0.000111