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The role of EARLY FLOWERING 3 and PHYTOCHROME B interaction in response to red light in *Arabidopsis thaliana*

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Thesis presented to obtain the degree of Doctor in Science. Area: Plant Physiology and Biochemistry

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SUMMARY

RESUMO

O papel da interação entre o EARLY FLOWERING 3 e PHYTOCHROME B na resposta a luz vermelha em *Arabidopsis thaliana*

O ciclo circadiano é um mecanismo interno complexo responsável por traduzir sinais ambientais em mudanças fisiológicas e metabólicas promovendo a adaptação das plantas. Devido à sua complexidade, há uma lacuna no conhecimento entre percepção luminosa e resposta à luz, além disso, não se sabe ao certo como os genes estão operando. Trabalhos anteriores mostram que o PHYTOCHROME B estabiliza o EARLY FLOWERING 3, no entanto, este estudo pode ser parcialmente comprometido pela presença de metabólitos derivados da fotossíntese, que são capazes de resetar o relógio. Este trabalho visa compreender o papel da interação EARLY FLOWERING 3 e PHYTOCHROME B na resposta à luz vermelha em *Arabidopsis thaliana* sob inibição da fotossíntese. Para isso foram utilizadas as linhagens transgênicas E1.06, *elf3-2* , YHB, YHB ABCDE, e phyB ABCDE. As sementes de Arabidopsis foram semeadas em meio MS-agar e MS-agar com sacarose a 1% e deixadas na câmara de crescimento por 5 dias a 22ºC sob ciclos de 12:12 L/D de 60 µmol m^{-2} s^{-1} de luz LED branca antes da captura da imagem circadiana. Após 5 dias, as mudas foram transferidas para placas de MS e Sacarose e pulverizadas com solução contendo luciferina. A inibição da fotossíntese foi feita utilizando soda lime, 3-(3,4-diclorofenil)-1,1-dimetilureia (DCMU) ou dibromotimoquinona (DBMIB) em ambas as placas MS e Sacarose. A imagem circadiana foi concluída ao longo de 5 dias usando uma câmera Andor iKon-M CCD controlada pelo µManager e os dados foram processados usando o ImageJ. Padrões de atividade de luciferase foram ajustados para ondas cosseno usando transformada rápida de Fourier – não lineares mínimos quadrados (FFT-NLLS) para estimar a duração do período circadiano. As conclusões desse trabalho são a proteína ELF3 modula as entradas fotossintéticas no sistema circadiano e, juntamente com o fitocromo B, definitivamente tem um papel na resposta à luz vermelha e na regulação da temperatura. No entanto, ainda não é possível entender perfeitamente a contribuição de ELF3 para a adaptação à variação de temperatura. Além disso, o desempenho superior de YHB ABCDE nos experimentos sob variação de temperatura, poderia ampliar a hipótese sobre a aplicação do fitocromo B na adaptação à temperatura servindo como um mediador chave de informações para o relógio circadiano.

Palavras-chave: Ciclo Circadiano; Complexo noturno; Sinais ambientais; Fitocromos.

ABSTRACT

The role of EARLY FLOWERING 3 and PHYTOCHROME B interaction in response to red light in *Arabidopsis thaliana*

The circadian clock is a complex internal mechanism that is responsible for translating environmental cues into physiological and metabolical changes to increase plant fitness. Due to its complexity, there is a gap in the knowledge between light perception and light response, moreover, which genes are operating and how it happens. Previously work has shown that PHYTOCHROME B acts to stabilize EARLY FLOWERING 3, however, this study could be partially compromised by the presence of metabolites from photosynthesis, internal cues that are capable of resetting the clock. This work aims to understand the role of EARY FLOWERING 3 and PHYTOCHROME B interaction in the response to red light in *Arabidopsis thaliana* under photosynthesis inhibition. To do that it was used the transgenic lines E1.06, *elf3-2* , YHB, YHB ABCDE, and phyB ABCDE. Arabidopsis seeds were sown in MS-agar and MS-agar with 1% Sucrose plates and left in the growth chamber for 5 days at 22 ºC under 12:12 L/D cycles of 60 μmol m^{-2} s^{-1} white LED light before circadian imaging. After 5 days, seedlings were transferred to MS and Sucrose plates and sprayed with a solution containing luciferin. The photosynthesis was inhibited by soda lime, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and dibromothymoquinone (DBMIB) in both MS and Sucrose plates. Circadian imaging was completed over 5 days using an Andor iKon-M CCD camera controlled by μManager and data were processed using ImageJ. Patterns of luciferase activity were fitted to cosine waves using fast Fourier transform–nonlinear least squares (FFT-NLLS) to estimate circadian period length. ELF3 is not necessary for YHB to induce photomorphogenesis. The conclusions are ELF3 protein modulates photosynthetic inputs into the circadian system and together with phytochrome B definitely has a role in the red-light response and temperature regulation. However, is not possible to understand the contribution of ELF3 to temperature entrainment yet. Moreover, the superior performance of YHB ABCDE in the experiments under temperature variation, could expand the hypothesis about the application of phytochrome B in temperature adaptation serving as a key mediator of information to the circadian clock.

Keywords: Circadian clock; Evening complex; Environmental cues; Phytochromes.

1. INTRODUCTION

Circadian clocks are involved in photoperiodic responses in cyanobacteria, fungi, plants, and animals. The circadian system is a self-sustainable mechanism that ensures plant survival and optimal growth by setting environmental changes to fix more carbon, grow faster and hold more chlorophyll than plants without a functional clock (Dodd et al., 2005). Closely to one-third of the *Arabidopsis* transcriptome is controlled by the circadian clock (Michael F. Covington et al., 2008) and it is possible to verify a similar pattern for other plants like rice, papaya, maize, soybean and poplar (Filichkin et al., 2011; K. R. Hayes et al., 2010; Khan et al., 2010; Marcolino-Gomes et al., 2014; Zdepski et al., 2008). The circadian regulation of genes is responsible for a range of physiological processes like flowering time (Lu et al., 2012), phytohormone production and signalling (Covington et al., 2008; Covington & Harmer, 2007), abiotic stress responses (Grundy et al., 2015) and plant-pathogen interactions (Goodspeed et al., 2012).

The evening complex is a core component of the circadian clock and its formed by the transcription factor LUX ARRHYTHMO (LUX), EARLY FLOWERING 3 (ELF3) a scaffold protein, and EARLY FLOWERING 4 (ELF4) a protein of unknown function. These genes overlap, peaking at dusk with greater activity at early night/end of the day (Huang & Nusinow, 2016). Mutations in the evening complex genes impact phenotypic growth, early flowering nevertheless the day length and lead to arrhythmic behaviour (Hicks et al., 1996; Nusinow et al., 2012; Zagotta et al., 1996). One of the EC components, ELF3 was originally identified from a mutant screen to identify lines with accelerated flowering but was quickly noted to be essential for the maintenance of circadian rhythms in constant light (Hicks et al., 1996; Zagotta et al., 1996). Besides that, ELF3 acts as a thermosensor in Arabidopsis (Jung et al., 2020) and has a role in the perception of light as entrainment possible through physical interaction with phytochrome B (phyB) (Liu et al., 2001).

The interaction between phyB and ELF3 has been reported to stabilize ELF3, but other work has suggested that phyB could be repressing ELF3 function within the oscillator (Herrero et al., 2012; Kolmos et al., 2011; Nieto et al., 2015). *elf*3 lines maintain a degree of rhythmicity in constant darkness (when grown on media containing sucrose), with detailed studies suggesting that the loss of circadian rhythms in constant light is caused by the loss of circadian gating of photosensitivity in *elf*3 (McWatters et al., 2000; Thines & Harmon, 2010). ELF3 requires PHYB function in early morphogenesis but not for the regulation of flowering time (Zagotta et al., 1996). This suggests that ELF3 is a component of a PHYB signalling complex that controls early events in plant development but that ELF3 and PHYB control flowering via independent signal transduction pathways. Although previous work indicates

that phyB and ELF3 signal via partially overlapping pathways, these studies are confounded by the consequences of photosynthesis that induce metabolic signaling in response to irradiation. To understand the interaction between ELF3 and phyB in response to red light in *Arabidopsis thaliana*, it was used a rhythmic expression of an always active version of phytochrome B-Y276H (YHB) to re-examine whether phyB signalling into the circadian system requires ELF3 in isolation from photosynthesis.

2. DEVELOPMENT

2.1. BIBLIOGRAPHIC REVIEW

2.1.1. The circadian clock

Circadian rhythms are present in all living organisms. It organises processes such as gene transcription, mitosis, feeding, and rest at different times of day and night. Living species from bacteria to plants and humans exhibit cycles in physiology and behaviour over periods as short as seconds (e.g., in the case of cardiac pace-making cells) and up to as long as months (e.g., in the case of seasonal oscillators) (Wulund & Reddy, 2015). The circadian clock is defined by three parameters: period, phase, and amplitude. The period is the necessary time for a cycle to be completed, measured as the time between two consecutive maximum (peaks) or minimal (valleys) periods. Usually, the period lasts approximately 24 hours. The phase is defined as any point in the cycle that is known for its relation to the rest of the cycle. Finally, the amplitude is considered as the distance between the peak and the valley. The amplitude of a biological rhythm may often vary, while the period remains unchanged (McClung, 2006). The circadian rhythms are endogenously generated and self-sustaining, so they persist under constant environmental conditions, typically constant light (or dark) and constant temperature. Under these controlled conditions, the organism is deprived of external time cues and has a free-running period of 24 h. The circadian system is also temperature compensated, meaning the period remains constant under a range of ambient temperature (Pittendrigh, 1954).

The circadian clock is assembled by an oscillator that generates the rhythms in response to external stimuli such as light and temperature (McClung, 2019). This oscillator is compounded by a network of positive and negative transcriptional translational feedback regulators that are interlocked (Harmer, 2009). LATE ELONGATED HYPOCOTYL (LHY) and CIRCADIAN CLOCK ASSOCIATED-1 (CCA1) peak in the morning, and act to repress the expression of a pseudo response regulator (PRR1, also known as TIMING OF CAB-1, or TOC1) during the day. As LHY/CCA1 protein levels decline in the evening, TOC1 accumulates and acts to repress transcription from their respective promoters. TOC1 transcription is then down-regulated late at night by an Evening Complex (EC) composed of three proteins, LUX and EARLY FLOWERING (ELF) 3 and 4 and this enables the transcription of LHY and CCA1 at the following dawn.

CCA1, LHY and PSEUDO RESPONSE REGULATOR9 (PRR9) are activated by light (Ito et al., 2003; Rugnone et al., 2013; Z. Y. Wang & Tobin, 1998), while PRR9 and PRR7 are important for entrainment to temperature (Mizuno et al., 2014; Salomé & McClung,

2005). CCA1 and LHY repress the expression of PRR9, PRR7, PRR5 and PRR1 (or TIMING OF CAB EXPRESSION1 - TOC1), with TOC1 having the peak expression close to dusk. In turn, the PRRs repress CCA1/LHY expression (Hsu & Harmer, 2014). GIGANTEA (GI) regulates ZEITLUPE (ZTL) protein stability in a blue-light-enhanced manner and its subsequent regulation of TOC1 (Kim et al., 2013; Kim et al., 2007). In the dark, ZTL and GI dissociate and ZTL promotes TOC1 and PRR5 proteasomal degradation(Fujiwara et al., 2008; W. Y. Kim et al., 2007). The expression of GI is negatively regulated by the EC, TOC1, and CCA1/LHY, thereby closing another loop (Huang et al., 2012; Mizuno et al., 2014a).

The positive arms of the clock are represented by three groups of proteins: LWD1 (LIGHT-REGULATED WD 1) and LWD2 in the morning; and LNK1 to LNK4 (NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED 1) together with RVE4 (REVEILLE 4), RVE6, and RVE8 acting at midday (Hsu et al., 2014; Rugnone et al., 2013; Y. Wang et al., 2011). LWD1 activates the transcription of CCA1 (Wu et al., 2016). LWD1/LWD2 also promote the expression of PRR9, PRR5, and TOC1 (Wang et al., 2011). RVE8, an MYB-like transcription factor, associates with LNK1 and LNK2 to bind TOC1 and PRR5 promoters (Pérez-García et al., 2015; Xie et al., 2014). RVE4, RVE6, and RVE8 have been shown to induce the expression of the EC components and PRRs (Farinas & Mas, 2011; Hsu et al., 2013; Rawat et al., 2011; Xie et al., 2014). The latter represses RVE8 expression, and promoters of the LNK genes are bound by LUX, closing another loop of the TTFL (Mizuno et al., 2014). Myb-like REVEILLE8 (RVE8) is a transcriptional activator and dynamically interacts with NIGHT LIGHT–INDUCIBLE AND CLOCK-REGULATED1 (LNK1) and LNK2 to activate transcription of evening-phased genes (ELF3, ELF4 and LUX) (Xie et al., 2014).

In plants, different organs, tissues, and cells have specific oscillators with distinct entrainment and self-sustaining mechanisms, leading to differences in free-running periods (W. W. Chen et al., 2020; Endo et al., 2014; James et al., 2008; Nimmo et al., 2020; Takahashi et al., 2015). Therefore, coordination of timing across plant tissues is crucial. In Arabidopsis, ELF3, ELF4, and LUX are central in coordinating timing between shoots and roots, and are especially important for transferring light signals from shoots into roots (Nimmo et al., 2020). A recent study showed that although it is most highly expressed in the shoot vasculature, the ELF4 protein moves rapidly from shoots to roots (Chen et al., 2020). Interestingly, low temperature promotes the movement of ELF4, resulting in a slow-paced oscillator in root tissues, while high-temperature conditions block the movement of ELF4, resulting in a fast-paced oscillator in root tissues (Chen et al., 2020). Therefore, the temperature-dependent movement of ELF4 establishes a shoot-to-root connection and determines the length of the circadian period in roots (Chen et al., 2020). In addition, the protein interaction phases between ELF3, ELF4, and LUX in roots are longer than those in shoots (Li et al., 2020).

2.1.2. The evening complex and ELF3 functions

The EC is localized to the nucleus (Herrero et al., 2012; X. L. Liu et al., 2001), where it functions to mediate nighttime repression of key clock genes TOC1, LUX, GI, and PRR7 and 9 (Dixon et al., 2011; Kolmos et al., 2011; Mizuno et al., 2014), and indirectly promotes the expression of the morning oscillators CCA1 and LHY (Dixon et al., 2011; Kolmos et al., 2011). Well-founded evidence has demonstrated the indispensable role of the ELF4–ELF3 LUX- complex in maintaining circadian rhythms and coordinating growth and development in *Arabidopsis thaliana* (Huang & Nusinow, 2016). The EC also represses the expression of thermo morphogenesis, promoting genes such as PIF4, and limiting the period of temperature-induced growth (Box et al., 2015). The loss-of-function mutation in any of the EC components (*elf3*, *elf4*, or *lux*) causes an arrhythmic phenotype (Doyle et al., 2002; Hazen et al., 2005; Hicks et al., 1996). This clock arrhythmia is accompanied by many phenotypes, such as inappropriate cellular elongation in response to environmental cues and early flowering regardless of day length (Khanna et al., 2003; Nozue et al., 2007; Zagotta et al., 1996).

The *elf3* and *elf4* mutant plants show attenuated free-running rhythmicity in leaf movement, hypocotyl elongation, and diurnal expression of clock-regulated genes such as the chlorophyll a/b-binding protein (CAB), cold-circadian rhythm-RNA binding (CCR), and CCA1 genes (Dowson-Day & Millar, 1999; Hicks et al., 1996; McWatters et al., 2000). Overexpression of ELF3 or ELF4 results in an extended circadian period, shortened hypocotyls, and delayed flowering. Arabidopsis ELF3 is highly expressed in rosette leaves, cauline leaves, inflorescence stems and apices, and siliques (Hicks et al., 2001). In the cotyledons, ELF3 is highly expressed in the vasculature cells and lowly expressed in the mesophyll cells. By contrast, ELF4 is mainly expressed in the vasculature cells (Endo et al., 2014).

Both ELF3 and ELF4 are regulated by light signalling pathways and are induced by light (Kikis et al., 2005; X. L. Liu et al., 2001). However, only the molecular mechanism that ties light regulation into ELF4 expression has been revealed. Three positive transcriptional regulators of the phytochrome A (phyA) light signalling pathway, FAR RED ELONGATED HYPOCOTYL3 (FHY3), FAR-RED IMPAIRED RESPONSE1 (FAR1), and ELONGATED HYPOCOTYL 5 (HY5), directly bind to the FBS and ACE cis-elements within the ELF4 promoter to activate its expression during the day (Li et al., 2011). While photo regulation of ELF3 expression is still poorly understood, more is known about post-translational regulation of ELF3 protein levels. For example, the overexpression of the major red light photoreceptor phyB stabilizes ELF3 proteins, while a light-regulated E3 ubiquitin-ligase CONSTITUTIVE PHOTO MORPHOGENIC1 (COP1) ubiquitinates ELF3 in vitro and negatively regulates the

abundance of ELF3 in vivo (Li et al., 2011; Nieto et al., 2015; Yu et al., 2008). In addition, a B-box containing transcription regulator that regulates photomorphogenesis BBX19, physically interacts with COP1 and ELF3 to promote the COP1-dependent degradation of ELF3 (Wang et al., 2015). Thus, visible light signalling pathways directly regulate the abundance of the EC through transcriptional and post-translational mechanisms.

ELF3 is a key factor antagonizing light input into the clock because overexpressing ELF3 attenuates the sensitivity of the clock to both red and blue light-mediated resetting cues (Covington et al., 2001). Conversely, a weak allele of elf3 (elf3-12) exhibits hypersensitivity to the resetting cue, while a stronger hypomorphic elf3-7 allele orthe null elf3-1 allele results in severe gating and resetting defects (Kolmos et al., 2011; McWatters et al., 2000). Similarly, a loss-of-function mutation in ELF4 also shows hypersensitivity to resetting and the gating of outputs, suggesting that the entire EC participates in the regulation of light input (Harriet G. McWatters et al., 2007). The function of the EC as an integrator of light inputs is consistent with protein-protein interactions between the EC and numerous components of the light signalling pathways (Huang et al., 2016; Huang & Nusinow, 2016; J. Kim et al., 2013; X. L. Liu et al., 2001; Nieto et al., 2015; Yu et al., 2008). The EC also regulates responses to and is regulated by low-intensity non-damaging UV-Blight. ELF4 is highly induced by UV-B light, and null mutants of elf3, elf4, or lux exhibit defects in the gating of UV-B-responsive gene expression (Fehér et al., 2011; Takeuchi et al., 2014). Consistent with the EC possibly directly regulating UV responses, Chl Paralysis showed that ELF4 and LUX are associated with the promoter of a UV-B downstream target gene EARLY LIGHTINDUCIBLE PROTEIN 1 (ELIP1) (Takeuchi et al., 2014). Phytochromes play an important role in regulating the EC (Ezer et al., 2017; Huang et al., 2016) and so temperature sensitivity of DNA binding could potentially be attached to increased thermal reversion of phyB (Legris et al., 2016).

ELF3 contains a prion-like domain (PrLD) with a high proportion of glutamine residues (polyQ region) (Jung et al., 2020). The PrLD shows variable length between species, with A. thaliana ELF3 (AtELF3) containing a PrLD of 180 amino acids, and Brachypodium distachyon ELF3 (BdELF3) lacking similar sequences. Replacing the PrLD of AtELF3 with the corresponding region from BdELF3 abolished the temperature-dependent DNA binding of AtELF3 (Jung et al., 2020). At high temperatures, AtELF3 forms speckles within the nucleus and this is also dependent on the PrLD. Importantly, PrLD-dependent speckles also form at high temperatures when AtELF3 is expressed in yeast cells, in the absence of other evening complex components. Furthermore, the purified PrLD from AtELF3 spontaneously and reversibly self-associates and forms condensates when in solution, in a temperature-dependent manner (Jung et al., 2020). It appears that this region contributes to phase separation of ELF3, and likely plays a role in temperature sensing (Hayes et al., 2021).

Direct temperature sensing by ELF3 may help to explain a curious observation about phyB at warm temperatures. Under Red light, active phyB accumulates in several large subnuclear foci known as photobodies (Hahm et al., 2020). In red + far red light, phyB is inactivated and disperses to numerous smaller foci. Warm temperature also inactivates phyB and so we might expect it to lead to a similar change in phyB photobodies. However, in direct contrast to Far Red light, warm temperature appears to promote the aggregation of phyB into fewer, larger photobodies (Hahm et al., 2020). Whether there is a link between phyB aggregation and ELF3 PrLD-mediated condensation at warm temperatures remains to be investigated. However, if this is the case, it presents an attractive mechanism whereby plant cells could distinguish between FR and warm temperature signals, based on the inactivation of phyB alone or the inactivation of phyB and ELF3 in conjunction (Hayes et al., 2021).

2.1.3. The phytochromes

Phytochromes were discovered in 1959 as the photoreceptor that mediates plant photomorphogenesis in response to a specific range of visible light (Butler et al., 1959). Phytochromes (phy) are sensitive to both red and far-red light and act by measuring the relative amount of each of these wavelengths. The phytochrome basal state (designated Pr) is sensitive to red light and upon irradiation is converted to a far-red sensitive state (Pfr). Reversion to the Pr form occurs either after far-red light exposure or as a consequence of dark incubation. The relative amounts of each of these forms determine downstream signalling events, with the Pfr form considered to be the active signalling state (Huq & Quail, 2005). Increased ratios of far-red:red light are indicative of shading by overlying vegetation as plants absorb relatively little far-red light compared to other portions of the light spectrum (Franklin & Whitelam, 2007). Responses to such shading include elongation of stems and petioles at the expense of leaf and storage organ development, increased hyponasty and reduced branching (shade avoidance) (Franklin & Whitelam, 2007). The shade avoidance act to reposition the plant into an unshaded position (Franklin & Whitelam, 2005). In the persistence of shaded conditions phytochromes promote flowering to enhance the probability of reproductive success (Franklin & Whitelam, 2005). Phytochromes have a role in regulating the circadian clock as *phy* mutants and lines over-expressing phytochromes display altered circadian rhythms (Somers et al., 1998).

In Arabidopsis, this family of photoreceptors has five members, from phyA to phyE (Fankhauser & Staiger, 2002). Moreover, there are two types of phytochromes, type I is light-labile, type II is light-stable. phyA accumulates in the dark and is rapidly degraded once exposed to light, belonging to type I while the other four members of this family are light-

stable (Franklin & Quail, 2010). Specifically, phyA is considered the primary photoreceptor in the dark, low-intensity red light and pulses of light. Besides that, it is also important for seed germination and seedling de-etiolation. Phy B and other phytochromes have greater importance in mature tissue related to shade avoidance (Schafer and Bowler, 2002). As phyB-E remains stable after illumination these phytochromes control plant responses under low fluence rate and persistent illumination (Wang & Wang, 2015). Low fluence responses are reversible and are determined by the ratio of red and far-red light used to irradiate the plant (Schafer and Bowler, 2002). In the dark, phytochromes are mostly localized in the cytoplasm and can shuttle to the nucleus with pulses of different light qualities (Nagatani, 2004). However, the mechanisms of phyA and phyB-E movement towards the nucleus are different. phyA relies on FHY1 (FAR-RED ELONGATED HYPOCOTYL 1) and FHL (FHY1-LIKE) proteins, FHY1/FHL interact with the active form Pfr phyA in the cytoplasm and transport it into the nucleus rapidly, and once in the nucleus, phyA will be photodegraded or become inactive form Pr phyA and FHY1/ FHL are recycled back to the cytosol (Rausenberger et al., 2011). However, phyB–phyE are located in the nucleus originally by NLS (nuclear-localization signals) at the C-terminal of the proteins (Chen et al., 2005).

At the molecular level, phytochromes regulate transcription by forming a complex with PIFs (PHYTOCHROME INTERACTING FACTORS), a family of basic helix–loop–helix transcription factors. Besides PIFs, phytochromes can also regulate positively acting transcription factors like HY5 (ELONGATED HYPOCOTYL 5) to facilitate photomorphogenesis. Phytochromes have been proved to own multiple connections to the core oscillator at the transcriptional and post-transcriptional levels. *phya* mutants have longer periods than that of wild types in low-fluence red or blue light, and phy A is necessary for resetting the clock mediated by far-red light. *phyb* mutants show longer periods under high-fluence red light, and phy B is involved in the red light resetting clock (Devlin & Kay, 2000; Yanovsky et al., 2001). phyC-E has also been considered to be involved in the red-light signal input to the clock (Devlin & Kay, 2000). Previous studies have proposed that phyB preferentially binds to G-box elements and regulates the expression of *GI* and *PRR9* (Jung et al., 2016). Phy A promotes early evening increases in the expression of the circadian-clock components TIMING OF CAB EXPRESSION 1 (TOC1), PSEUDO RESPONSE REGULATOR 7 (PRR7), EARLY FLOWERING 3 (ELF3), and ELF4, encoding proteins that collectively limit hypocotyl elongation (Fraser et al., 2021). FAR1 (FAR-RED IMPAIRED RESPONSE 1) and FHY3 (FAR-RED ELONGATED HYPOCOTYL 3) are two transcription factors of the phyA signalling pathway, which also could be involved in red-light input to the clock (Allen et al., 2006).

It has been reported that FAR1 and FHY3 are necessary for the amplitude and rhythmic expression of *ELF4* (Li et al., 2011). FAR1, FHY3, and HY5 associate with the ELF4

promoter to induce its expression, and the binding of CCA1 and LHY to that complex on ELF4 locus represses their ability to activate transcription (Li et al., 2011). The activation of ELF4 transcription, mediated by FAR1, FHY3, and HY5 which are regulators of the phyA signalling, represents a light input pathway to the clock (Gangappa & Botto, 2016; G. Li et al., 2011). FHY3 and FAR1 can also activate *CCA1* expression by directly binding to its promoter, while PIF5 inhibits *CCA1* expression directly. Moreover, PIF5 and TOC1 can repress FHY3 and FAR1 activation on CCA1 expression by interacting with them (Liu et al., 2020). The key elements of the light input networks and the central oscillator form multiple interlocked feedforward loops to generate the appropriate time expression pattern for the clock genes (Liu et al., 2020). At the post-transcriptional level, studies have shown that phyB may interact with ELF3, LUX, CCA1, LHY, TOC1, and GI (Yeom et al., 2014). But the output of the interaction between phyB and circadian components remains unclear.

phyB can function as a temperature receptor as well as integrate this signal into the clock network. One indicator is the rate of conversion from the active Pfr form to the inactive Pr form increases at higher temperatures (Jung et al., 2016; Legris et al., 2016). There are numerous links between phytochrome signalling components and clock proteins, maybe temperature regulation of phytochrome function is an integration point. Temperature has also been shown to regulate the activity of the evening complex. At higher temperatures, the association of ELF3 with target promoters is reduced via an unknown mechanism (Box et al., 2015; Ezer et al., 2017; Mizuno et al., 2014). Thus, in warm conditions, evening complex mediated repression of targets such as the clock genes PRR7, PRR9, GI, LUX, and the growth regulating PIF4 is relieved, leading to elevated levels of these transcripts during warm nights.

2.2. Plant material and growth conditions

Experiments were conducted in mutants of *Arabidopsis thaliana* carrying the promoter::luciferase system in wild-type (WT), E1.06 as control for YHB mutant, and the transgenic lines *elf3-2* , YHB, YHB ABCDE, and *phyB* ABCDE. The seeds were sterilised using bleach solution (50% (v/v) sodium hypochlorite). Then, it was shaken for three minutes in 1 mL of this solution and kept in the centrifuge for 5 minutes to settle before removing the bleach. Next, the seeds were washed five times in 1 mL sterile dH2O before resuspension in an appropriate volume of sterile dH2O. After that, the seeds were left in the fridge at 5ºC for three days to stratify. 10 arabidopsis seeds of each mutant were sown in MS-agar and MS-agar with 1% Sucrose plates and left in the growth chamber for 5 days at 22ºC under 12:12 L/D cycles of 60 µmol m^{-2} s^{-1} white LED light before circadian imaging. Following, the

seedlings were sprayed with 3-mM D-luciferin in 0.01% (v/v) Triton X-100 as previously described (Litthauer et al., 2015).

The first part of the thesis was designed to understand the role of *elf*3 and YHB in synchronizing the circadian clock under the entrainment of red light without photosynthesis interfering. In order to eliminate signaling molecules from photosynthesis as an input into the circadian system, experiments were performed using CO_2-depleted air completed as previously described (Kircher & Schopfer, 2012). In brief, 5-g soda lime was added to a double-sealed bag enclosing the petri plate on which seedlings had been transferred immediately before circadian imaging. Besides the photosynthesis inhibition by soda lime, it was adopted sensitives inhibitors of photosynthesis: 20 µm of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and 30 µm of dibromothymoquinone (DBMIB) in both MS and Sucrose plates. To do that, the plants were grown in MS or Sucrose plates and then, after 5 days, transferred to MS and Sucrose plates and sprayed with a solution containing luciferin and herbicides. The second part was performed to confirm if the clock can be synchronised by temperature signals rather than light through the interaction phyB and *elf3-2*. Seedlings grow in a chamber at 22ºC during the day and 17ºC during the night under 30 uE of red light. For both experiments, imaging was completed over 5 days using an Andor iKon-M CCD camera controlled by µManager (Edelstein et al., 2010) before data were processed using ImageJ (Schneider et al., 2012). Patterns of luciferase activity were fitted to cosine waves using fast Fourier transform–nonlinear least squares (FFT-NLLS) (Plautz et al., 1997; Zielinski et al., 2014) to estimate circadian period length. The period length was compared 2 by 2 using the t-test in order to understand separately the effects of each photosynthesis inhibitor on genotypes in MS plates and also in the Sucrose plates. For the hypocotyl growth, seeds were irradiated with cool fluorescent white light at 60 µmol m^{-2} s^{-1} for 4 hr before being moved to red light LED as per experimental requirements and grown vertically for 5 days before being imaged and processed using ImageJ (Schneider et al., 2012). The length of hypocotyls was normalized to the average length of a dark-grown control.

3. CONCLUSIONS

ELF3 protein modulates photosynthetic inputs into the circadian system and together with phytochrome B definitely has a role in the red-light response and temperature regulation. However, is not possible to understand the contribution of ELF3 to temperature entrainment yet. Moreover, the superior performance of YHB ABCDE in the experiments under temperature variation, could expand the hypothesis about the application of phytochrome B in temperature adaptation serving as a key mediator of information to the circadian clock.

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