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Integration of light and circadian signals that regulate chloroplast transcription by a nuclear-encoded sigma factor

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Received: 24 March 2016
Accepted: 28 July 2016

doi: 10.1111/nph.14176

Key words: Arabidopsis thaliana, chloroplasts, circadian rhythms, photobiology, signal transduction.

Summary

• We investigated the signalling pathways that regulate chloroplast transcription in response to environmental signals. One mechanism controlling plastid transcription involves nuclear-encoded sigma subunits of plastid-encoded plastid RNA polymerase. Transcripts encoding the sigma factor SIG5 are regulated by light and the circadian clock. However, the extent to which a chloroplast target of SIG5 is regulated by light-induced changes in SIG5 expression is unknown. Moreover, the photoreceptor signalling pathways underlying the circadian regulation of chloroplast transcription by SIG5 are unidentified.

• We monitored the regulation of chloroplast transcription in photoreceptor and sigma factor mutants under controlled light regimes in Arabidopsis thaliana.

• We established that a chloroplast transcriptional response to light intensity was mediated by SIG5; a chloroplast transcriptional response to the relative proportions of red and far red light was regulated by SIG5 through phytochrome and photosynthetic signals; and the circadian regulation of chloroplast transcription by SIG5 was predominantly dependent on blue light and cryptochrome.

• Our experiments reveal the extensive integration of signals concerning the light environment by a single sigma factor to regulate chloroplast transcription. This may originate from an evolutionarily ancient mechanism that protects photosynthetic bacteria from high light stress, which subsequently became integrated with higher plant phototransduction networks.

Introduction

Plants are sessile autotrophs that require light for photosynthesis within chloroplasts, but experience continuous changes in their light environment. Predictable changes in light conditions arise from day–night cycles, and unpredictable changes include the effects of weather and shading by competitors. Phototransduction pathways and circadian clocks allow plants to anticipate, sense and respond to these environmental changes.

Both predictable and unpredictable changes in light conditions are perceived by photoreceptors, including phytochromes, cryptochromes, phototropins, other blue light-sensing light-oxygen-voltage (LOV)-domain photoreceptors and the UV-B photoreceptor UV RESISTANCE LOCUS8 (UVR8) (Casal, 2013). These elicit changes in gene expression that underlie global alterations in development and physiology (Casal, 2013). The action spectra of photoreceptors are allied closely with the wavelengths of light that are available for photosynthesis (Rockwell et al., 2014), because photoreceptors regulate physiology and development to optimize photosynthetic light harvesting. Phototransduction pathways also synchronize the plant circadian oscillator with the day–night cycles of the environment (Somers et al., 1998). The plant circadian oscillator comprises a network of interlocked transcription/translation feedback loops that produce a cellular estimate of the time of day (Nagel & Kay, 2012), which increases growth and fitness (Harmer et al., 2000; Dodd et al., 2005; Michael et al., 2008).

Chloroplast transcription is regulated by light and the circadian clock (Gamble & Mullet, 1989; Klein & Mullet, 1990; Tsinoremas et al., 1996; Noordally et al., 2013), but knowledge of the mechanisms that integrate these signals is incomplete. Chloroplast genes are transcribed by two types of RNA polymerase: plastid-encoded plastid RNA polymerase (PEP) and nuclear-encoded plastid RNA polymerase (NEP) (Kanamaru et al., 1999). PEP requires a bacterial-type σ70 subunit (sigma factor) to confer promoter specificity and initiate transcription. In higher plants, six sigma factors are encoded by the nuclear genome. It is thought that, during higher plant evolution, sigma factors transferred from the genomes of ancestral chloroplasts to the nuclear genome, and provide a mechanism for nuclear control of the specificity of chloroplast transcription (Kanamaru et al., 1999; Ueda et al., 2013).

Transcripts encoding SIGMA FACTORS5 (SIG5) are regulated by several light signals in mature leaves (Ichikawa et al., 2008;
Onda et al., 2008; Mellethlin et al., 2014) and during de-etiolation (Monte et al., 2004; Pepperman et al., 2006). This involves the cryptochrome, phytochrome and UVR8 photoreceptors (Monte et al., 2004; Brown & Jenkins, 2008; Onda et al., 2008; Mellethlin et al., 2014). SIG5 transcript abundance is also regulated by photosynthesis (Mellethlin et al., 2014), abiotic stress (Nagashima et al., 2004), retrograde signalling (Ankele et al., 2007) and the circadian clock (Noordally et al., 2013). Within chloroplasts, SIG5 regulates transcription of the blue light-responsive promoter (BLRP) of psbD (psbD BLRP), which encodes the light-labile D2 protein of photosystem II (PSII) (Nagashima et al., 2004), and transcripts with less well-characterized promoters (Noordally et al., 2013). psbD BLRP is one of at least four differently sized transcripts that originate from the chloroplast psbD operon in Arabidopsis (Hoffer & Christo-

pher, 1997; Hanaoka et al., 2003; Nagashima et al., 2004). Here, we focused on psbD BLRP because it provides an experimentally tractable readout of chloroplast transcriptional regulation by SIG5.

Although sigma factors are known to be regulated by a variety of light signals, the extent to which this alters the transcription of sigma factor-regulated genes within chloroplasts is not known. We investigated this using nuclear-encoded SIG5 and chloroplast-encoded psbD BLRP as a model. First, we report a series of new findings concerning the regulation of chloroplast transcription and the sigma factor SIG5 by light. Second, we demonstrate that specific light signalling pathways are required for SIG5 to maintain circadian rhythms of transcription of chloroplast psbD BLRP. We conclude that sigma factors integrate and communicate several types of information concerning the light environment to the chloroplast genome.

Materials and Methods

Plant materials and growth conditions

Seeds of Arabidopsis thaliana (L.) Heynh. were surface sterilized by exposure to 70% (v/v) ethanol for 1 min, 20% (v/v) domestic bleach for 12 min and then washed twice with sterile distilled H2O. Seeds were resuspended in 0.1% (w/v) agar and sown individually onto half-strength (2.15 g l⁻¹) Murashige and Skoog nutrient mix (basal salts without vitamins, pH 6.8; Duchefa Biochemie, Haarlem, the Netherlands) in 0.8% (w/v) agar, without sucrose supplementation. For luciferase imaging, seeds were sown into sterile plastic rings embedded within growth medium (15 seeds per ring) to produce circular regions of luciferase bioluminescence (Love et al., 2004; Noordally et al., 2013; Dodd et al., 2014). Seeds were stratified in the dark for 3 d at 4°C and then cultivated under 12 h : 12 h, light : dark cycles at 19°C and 90 μmol m⁻² s⁻¹ white light (MLR-352; Panasonic, Osaka, Japan). Modified conditions were required for comparable germination and growth of phyABCDE mutants, involving germination in 120 μmol m⁻² s⁻¹ white light (Microclima 1600E; Snijder Scientific, Tillburg, the Netherlands) at 20°C and 16 h : 8 h, light : dark cycles for 5 d, before transfer to standard growth conditions (as earlier). All photoreceptor mutants described, except phyABCDE, were transformed with SIG5-LUCIFERASE+ (Noordally et al., 2013). T3 generation SIG5-LUCIFERASE+ expressing homozygous seedlings were used for all experimentation. Multiple transgenic lines were screened to identify those having comparable luciferase bioluminescence, and then characterized using bioluminescence time course imaging to select lines for experimentation with representative circadian periodicities (Supporting Information Fig. S1). Eleven-day-old seedlings were used for all experiments.

Genotypes were Col-0, Landsberg erecta (L.), sig5-3 (Noordally et al., 2013), phyA-201 (Nagatani et al., 1993), phyB-5 (Nagatani et al., 1993), phyA-201 phyB-5 (Reed et al., 1994), phyABCDE (Hu et al., 2013), cry1-B104 (Bruggemann et al., 1998), cry1-2 (Guo et al., 1998), cry1 cry2 (hrf-1 hfsa-1, El-Assal et al., 2003).

Transcript abundance

Aerial tissue was harvested 11 d after germination, as described previously (Noordally et al., 2013). Total RNA was extracted using a NucleoSpin RNA extraction kit (Macherey-Nagel, Duren, Germany), from which cDNA was synthesized (High Capacity cDNA Reverse Transcription Kit, ThermoFisher, Waltham, MA, USA). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis was performed using Brilliant III Ultra-Fast SYBR Green qRT-PCR Master Mix (Agilent Technologies, Santa Clara, CA, USA, using Agilent Mx3005P qRT-PCR instruments) and the primers described later. Transcript abundance was relative to ACTIN2 (ACT2), an established reference for the study of this pathway (Noordally et al., 2013), and calculated using the ΔΔG method. For light induction experiments, transcript abundance was measured 1 h (SIG5) and 4 h (psbD BLRP) after the start of light treatments, as a time delay exists between the upregulation of SIG5 and psbD BLRP transcripts (Noordally et al., 2013), and these times correspond with maximum SIG5 and psbD BLRP transcript abundance attained after exposure to light of dark-adapted seedlings (Mochizuki et al., 2004; Onda et al., 2008; Noordally et al., 2013). qRT-PCR primers were SIG5 (GTGGTGGAGCTAATAACAGCAGACA (FP), TGGCGAA TAACCGACCTCTCTTTCG (RP)), psbD BLRP (GGAAATC CTGCGATATCTCT (FP), CTCTCTTTCTCTAGGCAGGA AC (RP)) (Mochizuki et al., 2004); LHY (LATE ELONGATED HYPOCOTYL) (ACGAAACAGGTAAGTGGCGACA (FP), TGGGAAACATCTTGAACCGCGTT (RP)) (Noordally et al., 2013); ACT2 (TCAGATGCCCAGAAGTGTTGTTCC (FP), TCAGATGCCCAGAAGTGTTGTTCC (RP)) (Noordally et al., 2013); LHY (LATE ELONGATED HYPOCOTYL) (ACGAAACAGGTAAGTGGCGACA (FP), TGGGAAACATCTTGAACCGCGTT (RP)) (Noordally et al., 2013); ACT2 (TCAGATGCCCAGAAGTGTTGTTCC (FP), CGTGCGATATCTCT (RP), or TGAGAG ATTCAATGCGGCGAGA (FP), TGGCGCGCGCGCGC CGGT (RP) in Fig. 4(c) only (see later)).

Light conditions

Blue (B), red (R) and far red (FR) light manipulations used custom LED panels installed within temperature-controlled growth chambers, and custom Photek LB-1 R/F/R/B LED panels controlled by the bioluminescence imaging system. Photosynthetically active radiation (PAR) and light spectra were quantified...
with a spectroradiometer (Ocean Optics, Dunedin, FL, USA). Peak output wavelengths of R, B and FR LEDs were 660, 470 and 740 nm, respectively (Fig. S2). The R : FR ratio was calculated using PAR integrated from 660 to 670 nm divided by 725–735 nm (Franklin, 2008). Light induction experiments used 25 μmol m$^{-2}$ s$^{-1}$ total photon flux density (PFD) for each light colour treatment, except Fig. 4(c) only (see later), which used 10 μmol m$^{-2}$ s$^{-1}$ per treatment. In all figures, ***, P < 0.001; **, P < 0.01; *, P < 0.05; ns, not statistically significant. All light treatments commenced at zeitgeber time (ZT) 4, using dark-adapted seedlings, because SIG5 has greatest sensitivity to B light pulses at ZT4 (Noordally et al., 2013).

Bioluminescence imaging

Clusters of 10-d-old seedlings surrounded by sterile rings (e.g. Fig. S3) were dosed with 100 μl of 5 mM luciferin (potassium salt of D-luciferin; Melford Laboratories Ltd, Ipswich, UK) 24 h before imaging. Bioluminescence was measured using a Lumintek EM-CCD imaging system (Photek Ltd, St Leonards on Sea, UK) controlled by IMAGE32 software (Photek) and custom control scripts (45-s integrations, EM gain setting 2700). For experiments investigating SIG5::LUCIFERASE induction by light, 11-d-old seedlings were exposed to the light regime specified after dark adaptation for 24 h. Images were captured at 13-min intervals, preceded by a dark delay of 2 min to eliminate chlorophyll autofluorescence from the bioluminescence signal. Sequences of images lasted between 4 and 8 h, depending on the experiment; data on the figures represent peak SIG5::LUCIFERASE activity. Circadian time course imaging of SIG5::LUCIFERASE bioluminescence commenced at ZT0, using 11-d-old seedlings entrained previously to 12 h : 12 h, light : dark cycles. Seedlings were exposed to two 12 h : 12 h, light : dark cycles of the wavelength(s) under investigation before transfer to constant light, to reduce transitory effects. Bioluminescence images were captured approximately every hour. Imaging data were analysed using IMAGE32 software (Photek), with circadian time courses analysed further using the fast Fourier transform-nonlinear least-squares (FFT-NLLS) algorithm within BRASS software (Southern & Millar, 2005), downloaded in 2015 from http://millar.bio.ed.ac.uk. The first 24 h of data in constant light were discarded before FFT-NLLS analysis to remove transient responses to the final dark period.

Inhibitor experiments

For experiments with norflurazon (Sigma-Aldrich), growth medium was supplemented with 5 μM norflurazon and 1% (w/v) sucrose to allow growth in the absence of photosynthesis (e.g. Fig. S3a). For bioluminescence imaging experiments with 3-(3,4-dichlorophenyl)-1,1-dimethyleurea (DCMU, Sigma-Aldrich), 20 μM DCMU was added to the 100 μl of 5 mM luciferin that was dosed onto seedlings. For RNA sampling, 100 μl of 20 μM DCMU was dosed onto seedlings. In both cases, DCMU was dosed onto seedlings 24 h before the start of light treatment. Inhibitors were dissolved in dimethylsulfoxide (DMSO) (working concentrations of DMSO were 0.0025% (v/v) and 0.01% (v/v) with norflurazon and DCMU, respectively), and inhibitor controls contained an equal volume of DMSO without the inhibitor.

Results

We used the regulation of chloroplast psbD BLRP by nuclear-encoded SIG5 as an experimental model. To provide a basis for subsequent experiments, we investigated the accumulation of chloroplast psbD BLRP transcripts in wild-type and sig5-3 loss-of-function plants, under various light conditions, to determine the role of nuclear-encoded SIG5 in the regulation of chloroplast psbD BLRP by light. Like SIG5 transcripts, SIG5 promoter activity and chloroplast-encoded psbD BLRP transcripts were induced most strongly by B light, other treatments including B light, and a combination of R and FR light with R : FR = 0.7 (Fig. 1a,b). SIG5 transcripts and SIG5 promoter activity were not induced by either R or FR light alone (Fig. 1a,b). The transcriptional responses of psbD BLRP were SIG5 dependent because light treatments did not induce psbD BLRP in the sig5-3 loss-of-function mutant (Fig. 1b). The behaviour of SIG5 transcripts (Fig. 1b) was consistent with studies conducted under similar conditions (Mochizuki et al., 2004; Nagashima et al., 2004; Onda et al., 2008; Noordally et al., 2013). The regulation of SIG5 promoter activity by light, measured with SIG5::LUCIFERASE, appeared to account largely for the regulation of SIG5 transcript accumulation (Fig. 1a,b).

SIG5 communicates information concerning light intensity and quality to chloroplasts

We hypothesized that chloroplast transcription is regulated by SIG5 in response to light intensity, as: (1) SIG5 transcript abundance depends on B light intensity (Onda et al., 2008); (2) psbD BLRP is regulated by SIG5 in a dose-dependent manner (Onda et al., 2008); and (3) we found that both B and R + FR light upregulation of psbD BLRP was dependent on SIG5 (Fig. 1b). It is not known whether SIG5 transcription is dependent on the intensity of R light, nor how these fluence responses of SIG5 affect chloroplast transcription. To test this, we applied a range of intensities of either B or R + FR light to seedlings. Treatment with each light intensity commenced at ZT4, using separate batches of seedlings (we did not progressively increase light intensity over time, because that approach would be confounded by circadian gating). In both B and R + FR light, the magnitude of induction of chloroplast psbD BLRP tran-scripts was determined by PFD, and also required SIG5 (Fig. 1c,d). The magnitude of induction of the SIG5 promoter and SIG5 transcript abundance were also determined by PFD (Fig. 1c,d). This suggests that, across the PFD range investigated, regulation of the SIG5 promoter by PFD of both B and R + FR light controlled the accumulation of SIG5 transcripts, causing the magnitude of chloroplast psbD BLRP transcript accumulation to be PFD dependent.
Photoreceptors and retrograde signals underlie the regulation of chloroplast transcription in response to light intensity by SIG5

Plant responses to light, including the transcription of SIG5, are mediated by photoreceptors and photosynthesis (Onda et al., 2008; Mellenthin et al., 2014). It is not known which of these light response pathways underlies the light intensity-dependent transcriptional response that we identified for SIG5 and chloroplast psbD BLRP (Fig. 1c,d). Therefore, we investigated this question with a combination of photoreceptor mutants and photosynthetic inhibitors. Regulation of SIG5 and psbD BLRP has been reported to involve the photoreceptors phytochrome A (phyA), cryptochrome 1 (cry1) and cry2 (Thum et al., 2001; Ichikawa et al., 2004; Onda et al., 2008; Mellenthin et al., 2014). Although phyA was required for SIG5 induction by R + FR light and phyB may suppress SIG5 transcript accumulation (Fig. S4b), SIG5 was not regulated by R or FR light when applied alone (Fig. 1a,b; Mochizuki et al., 2004; Onda et al., 2008; Noordally et al., 2013). A single report demonstrating SIG5 induction in de-etiolated seedlings by R or FR light alone used sucrose-supplemented growth media (Mellenthin et al., 2014). SIG5 transcripts were induced by B light in the phyA mutant, presumably as a result of cryptochrome-mediated regulation of SIG5 (Fig. S4d). However, as the regulation of SIG5 by R and FR light is atypical for phytochrome signalling, we reasoned that additional mechanisms act alongside phytochromes to regulate chloroplast transcription by SIG5 in response to the intensity of R + FR light.

We investigated the involvement of retrograde signalling in the control of chloroplast transcription by SIG5 in response to PFD. B light activation of SIG5::LUCIFERASE was unaltered by norflurazon, which inhibits carotenoid biosynthesis, leading to photobleaching (e.g. Fig. S3a). By contrast, norflurazon inhibited the upregulation of SIG5::LUCIFERASE by R + FR light (Figs 2a, S3a). We also investigated the effect of DCMU, an inhibitor of photosynthetic electron transport between PSII and plastoquinone (PQ), on light activation of SIG5-mediated signals to chloroplasts. First, we determined the minimum effective dose for the inhibition of photosynthesis by DCMU under our experimental conditions using modulated PSI chlorophyll fluorescence (Imaging-PAM, M. Walz, Germany). Seedlings grown exactly as for bioluminescence imaging and RNA sampling were dosed with 0, 5, 10, 15, 20, 35 or 50 µM DCMU (mixed with and without luciferin for Col-0 SIG5::LUCIFERASE and L. erv, respectively) and dark adapted for 24 h before determination of Fees and Fm (intensity setting 1, frequency 4). Actinic light (107 µmol m–2 s–1) was switched on for 10 min, after which the effective quantum yield of PSII (YII) was calculated as (Fm' – Fm')/Fm', where Fm' is the maximum fluorescence emission from the light-adapted seedling after a saturating pulse, and Fm' is the chlorophyll fluorescence emission from light-adapted seedlings. Based on these data, we used DCMU at a concentration of 20 µM, and luciferin did not alter the efficacy of DCMU.

DCMU treatment reduced R + FR light induction of SIG5::LUCIFERASE by 42%, whereas induction of SIG5::LUCIFERASE
LUCIFERASE by B light was insensitive to DCMU (Fig. 2b). Together, these results indicated that a retrograde signal arising from photosynthetic electron transport was required for the regulation of the SIG5 promoter by R + FR but not B light.

We used this information to investigate the contribution of phytochrome and photosynthetic signals to the regulation of chloroplast transcription in response to the intensity of R + FR light. There was some variation in the sensitivity of psbD BLRP transcripts to R + FR light; the PFD threshold for significant BLRP from 0.03 to 0.01 (Fig. 2c,d). SIG5 and psbD BLRP were generally not induced significantly in phyA mutants at any PFD relative to dark controls (Fig. 2c,d), demonstrating that this response of SIG5 to light intensity was dependent on phyA. A single exception was that, in phyA, psbD BLRP was induced by R + FR light at 40 µmol m⁻² s⁻¹ in the absence of DCMU, and this response was abolished when DCMU was added (Fig. 2d). Across the PFD range tested, DCMU reduced the slope estimate ($r^2$) of the R + FR PFD response of SIG5 from 0.46 to 0.14, and of psbD BLRP from 0.03 to 0.01 (Fig. 2c,d). The absence of an effect of DCMU on B light activation of SIG5::LUCIFERASE (Fig. 2b) suggests that the DCMU sensitivity of R + FR light induction of SIG5::LUCIFERASE is a specific signalling response rather than a nonspecific consequence of DCMU-induced oxidative damage. Overall, these data indicate that, although R + FR light activation of psbD BLRP by SIG5 is dependent on phyA, a photosynthetic signal underlies the quantitative response of the pathway to R + FR light intensity.

Regulation of SIG5-mediated signalling to chloroplasts by the proportions of red and far red light

As R or FR light alone had little effect on chloroplast psbD BLRP transcription by SIG5, but R and FR light in combination induced this pathway (Fig. 1a,b), we reasoned that chloroplast psbD BLRP might be regulated by the relative proportions of R and FR light in a SIG5-dependent manner. In nature, R:FR light conditions provide plants with information concerning vegetational shade or the threat of vegetational shade, because vegetation absorbs R light and transmits and reflects FR light. The balance of R and FR light also affects plants because R and FR light preferentially excite PSII and PSI, respectively, altering the energy balance across the photosynthetic electron transport system and the redox state of the PQ pool (Pflanzschmidt et al., 1999; Bonardi et al., 2005).

It is not known whether sigma factor-mediated signals to chloroplasts are regulated by the relative proportions of R and FR light. To test this, we exposed dark-adapted seedlings to R:FR light conditions in the range 0.02–1.4,
and monitored both SIG5 promoter activity and SIG5 and psbD BLRP transcript abundance (Fig. 3a). The magnitude of activation of SIG5, its promoter and chloroplast-encoded psbD BLRP was dependent on the relative proportions of R and FR light (Fig. 3a). SIG5::LUCIFERASE was induced strongly by R:FR in the range 0.46–0.96, and SIG5 transcripts were induced most strongly by R:FR in the range 0.66–1.24 (Fig. 3a). psbD BLRP induction was reduced at very low R:FR and R:FR exceeding 1.2 (Fig. 3a). The psbD BLRP response to R:FR conditions was dependent on SIG5, as psbD BLRP was not induced in sig5-3 (Fig. 3a). This was consistent with Fig. 1(a,b), where SIG5 and psbD BLRP transcript accumulation was low under R or FR light alone, but high under R + FR light (R + FR = R:FR conditions of 0.7).

phyA promoted SIG5 transcription under R + FR light (Fig. S4c), but the smallest induction of SIG5::LUCIFERASE and SIG5 transcript abundance occurred under conditions of very low R:FR (Fig. 3a), when phyA signalling would be expected to be greatest (Martínez-García et al., 2014). We explored this difference by testing the contribution of photosynthesis to R:FR responses of SIG5. DCMU had no effect on the small increase in SIG5::LUCIFERASE at low R:FR, yet inhibited SIG5::LUCIFERASE upregulation at higher R:FR conditions (Fig. 3b). The magnitude of SIG5::LUCIFERASE induction was dependent on the proportions of R and FR light, rather than simply R light intensity, because SIG5 was not induced by R light alone (Fig. 1a,b).

Circadian signalling to chloroplasts by SIG5 is primarily dependent on blue light and cryptochrome

SIG5 communicates circadian timing information from the nuclear-encoded circadian oscillator to circadian-regulated chloroplast transcripts, including psbD BLRP (Nakahira et al., 1998; Ichikawa et al., 2008; Noordally et al., 2013). Specific light conditions and photoreceptors regulate SIG5 induction of psbD BLRP in dark-adapted seedlings (Figs 1, S4), the circadian clock gates transient B light induction of SIG5 and psbD BLRP (Noordally et al., 2013) and, in cycles of B light and darkness, cryptochromes contribute to the transcriptional patterns of a SIG5 orthologue in Physcomitrella (Ichikawa et al., 2004). It is not known which photoreceptor systems or light conditions underlie SIG5-mediated circadian signalling to chloroplasts, and so we investigated this with a combination of photoreceptor mutants and manipulations to the light conditions.

Circadian oscillations of SIG5::LUCIFERASE showed greatest amplitude under continuous B light, lower amplitude under a combination of B, R and FR light, and very low amplitude under continuous R light (Figs 4a,b, S5); see also Fig. S6 for these light conditions plotted separately for Col-0). The relative amplitude error (RAE) from analysis by FFT-NLLS indicates the quality of fit of a sine wave to the experimental data, from 0 (perfect fit) to 1 (no fit), where >0.5 typically reflects arrhythmicity (Xu et al., 2007) (Fig. 4b). Using this measure, SIG5::LUCIFERASE was arrhythmic under both R + FR and FR light alone (Figs 4a,b, S6), but was rhythmic when B light was added to R + FR (Fig. 4a). SIG5::LUCIFERASE has been shown elsewhere to be rhythmic under R + B light (Noordally et al., 2013). Together, these data indicate that robust circadian oscillations of the SIG5 promoter require B light.

To determine the relationship between the arrhythmia of SIG5::LUCIFERASE under R and FR light and circadian oscillator function, we monitored circadian oscillations of CCA1::LUCIFERASE under combinations of R and FR light (Fig. S5b). CCA1::LUCIFERASE was rhythmic under R and R + FR light, but arrhythmic under FR light alone (Fig. S5b). The amplitude of oscillations of CCA1::LUCIFERASE was approximately sixfold greater under R than R + FR light (Fig. S5b). Next, we investigated the role of selected wavelengths in the circadian regulation of SIG5 and psbD BLRP transcripts (Fig. 4c). SIG5 and psbD BLRP were rhythmic under B light and, under R light, there were low-amplitude oscillations of SIG5 but psbD BLRP was arrhythmic (Fig. 4c). Circadian oscillations of LHY indicated that the circadian oscillator remained rhythmic under R light (Fig. 4c). It has been proposed that there is a minimum abundance of SIG5 transcripts that is required for circadian oscillations of psbD BLRP (Noordally et al., 2013), and so the low-amplitude oscillations of SIG5 transcript abundance under R

![Fig. 3](image-url) Relative proportions of red (R) and far red (FR) light regulate SIGMA FACTORS (SIG5)‐mediated signalling to chloroplasts in Arabidopsis thaliana. (a) SIG5::LUCIFERASE bioluminescence and abundance of transcripts encoding SIG5 and psbD light‐responsive promoter (BLRP) in dark‐adapted seedlings exposed to a range of R:F ratios (totalising 25 μmol m⁻² s⁻¹). (b) Role of photosynthesis in R:FR response of SIG5::LUCIFERASE in seedlings treated with 3–(3,4-dichlorophenyl)-1,1-dimethyleurea (DCMU). Analysis by (a) ANOVA and post‐hoc Tukey test comparing lowest R:FR with all other R:FR, (b) two‐sample t‐tests within each R:FR, where: ***, P < 0.001; **, P < 0.01; *, P < 0.05; ns, not significant. Data are mean ± SE; n = 5–12 (SIG5::LUCIFERASE); n = 2–6 (quantitative reverse transcription‐polymerase chain reaction, qRT‐PCR). Light treatments commenced at zeitgeber time 4.
light may have been below this threshold for psbD BLRP transactivation (Fig. 4c).

We investigated the photoreceptors that underlie circadian oscillations of SIG5::LUCIFERASE. Under continuous B light, the amplitude of circadian oscillations of SIG5::LUCIFERASE was reduced substantially in cry1 and cry1 cry2 relative to the wild-type (Figs 4d, S5), indicating that circadian oscillations of SIG5 in B light were predominantly dependent on cry1. Under R light, the circadian amplitude of SIG5::LUCIFERASE was reduced slightly, but significantly, relative to the wild-type in phyA, phyB and phyA phyB (Fig. 4e). This suggests that phyA and phyB made small contributions to the amplitude of circadian oscillations of SIG5 promoter activity, but were not essential for its rhythmicity. SIG5::LUCIFERASE was arrhythmic in all genotypes in FR and R + FR light (Fig. 4f,g). Under R + FR + B light, circadian oscillations of SIG5::LUCIFERASE required cry1, because SIG5::LUCIFERASE was arrhythmic in cry1 and had reduced rhythmic robustness in cry1 cry2 (RAE = 0.42 ± 0.3; Fig. 4h) relative to other treatments. The greater amplitude of SIG5::LUCIFERASE oscillations in cry2 relative to the wild-type in B and R + FR + B light suggests that there was antagonism between cry1 and cry2 in the circadian regulation of SIG5 (Fig. 4d,h). In the presence of B light, phyA and phyB appeared to antagonize the circadian amplitude of SIG5::LUCIFERASE oscillations (Fig. 4d,h), possibly explaining why SIG5::LUCIFERASE had lower circadian amplitude in R + FR + B than B light alone (Fig. 4d,h).

The dynamics of SIG5::LUCIFERASE under light–dark cycles of five light conditions revealed two features within the daily regulation of the SIG5 promoter under B light (Fig. 5). Under light–dark cycles of B light, SIG5 promoter activity was induced rapidly following dawn (Fig. 5a, feature marked ‘A’), with a
second more slowly acting feature present during the middle of the photoperiod (Fig. 5a, feature marked ‘B’). The ‘spike-shoulder’ dynamics were absent from the daily regulation of \textit{SIG5} transcription under other light conditions tested (Fig. 5a). Under B light–dark cycles, the more slowly acting feature was absent in the \textit{cry1} and \textit{cry1 cry2} mutants, but present in \textit{cry2} (Fig. 5b), suggesting that the feature arose from \textit{cry1} activity. In addition, under light–dark cycles, there was clear anticipation of dawn by \textit{SIG5::LUCIFERASE} under B + R + FR light conditions, but this was absent under B light alone (Fig. 5).

**Discussion**

We present new information concerning a mechanism that integrates light and circadian cues to regulate chloroplast transcription. We first examined the dynamics of this pathway during transition from dark to light, and subsequently investigated the involvement of light conditions in circadian regulation of the pathway. Previous studies have demonstrated that nuclear-encoded transcripts of the chloroplast RNA polymerase subunit \textit{SIG5} are induced in Arabidopsis by B light, R light and UV-B (Monte \textit{et al.}, 2004; Brown & Jenkins, 2008; Onda \textit{et al.}, 2008; Mellenthin \textit{et al.}, 2014). Likewise, orthologues of Arabidopsis \textit{SIG5} in rice, \textit{Physcomitrella} and \textit{Marchantia} are light induced (Ichikawa \textit{et al.}, 2004; Kubota \textit{et al.}, 2007; Kanazawa \textit{et al.}, 2013). Although the sigma factor \textit{SIG5} appears to be conserved amongst land plants (Kanazawa \textit{et al.}, 2013), in cyanobacteria and a species of red alga other sigma factors are light induced (Imamura \textit{et al.}, 2003; Fujii \textit{et al.}, 2015). Here, we demonstrated in Arabidopsis that light-induced changes in sigma factor transcript abundance lead to transcriptional changes in chloroplasts in response to various light signals. We also identified specific light signalling pathways underlying the circadian regulation of chloroplast transcription by \textit{SIG5}. A general interpretation is that information concerning the light environment is integrated by, and communicated to, chloroplasts by nuclear-encoded sigma factors. \textit{SIG5} appears to communicate information to the chloroplast genome concerning light intensity and light quality (Figs 1, 3), and this information is combined with B light and cryptochrome-dependent circadian timing cues (Fig. 4). An area for future investigation is to determine the role of the multiple transcription start sites (TSSs) within the \textit{psbDC} operon in signal integration, as the transcription or activity of other sigma factors is regulated by light conditions and the circadian oscillator (Onda \textit{et al.}, 2008; Puthiyaveetil \textit{et al.}, 2008, 2011; Shimizu \textit{et al.}, 2010; Noordally \textit{et al.}, 2013), and other \textit{psbDC}TSSs are light regulated depending on the developmental stage (Hoffer & Christopher, 1997).

Circadian signalling to chloroplasts by \textit{SIG5} requires specific light signalling pathways

The circadian oscillator is rhythmic under conditions of B and R light (Somers \textit{et al.}, 1998) (see Fig. 4c for \textit{LHY}), and so the B light dependence of circadian oscillations of \textit{SIG5::LUCIFERASE} (Fig. 4a,c) is a specific feature of \textit{SIG5}-mediated circadian signalling to chloroplasts, rather than a dependence of the circadian oscillator on B light. By contrast, arrhythmia of \textit{SIG5::LUCIFERASE} under continuous FR light appears to arise from arrhythmia of the circadian oscillator, as \textit{CCA1::LUCIFERASE} was arrhythmic under these conditions (Fig. 5b), rather than representing a specific feature of the circadian regulation of \textit{SIG5}. A previous report has indicated that, under continuous FR light, the circadian oscillator is rhythmic with low amplitude and altered phase (Wenden \textit{et al.}, 2011), whereas, under our experimental conditions, \textit{CCA1::LUCIFERASE} was arrhythmic under continuous FR light (Fig. 5b). This difference could be because our experiments were conducted using sucrose-free growth medium, whereas Wenden \textit{et al.} (2011) included 3% sucrose in the growth medium. As FR light has been proposed to act on the circadian oscillator through the evening loop component ELF4 (Wenden \textit{et al.}, 2011) and a long-term effect of sucrose on the circadian oscillator is mediated by the evening loop component GIGANTEA (Dalchau \textit{et al.}, 2011), phytochrome and metabolite signals may interact to provide an input to the circadian oscillator via the evening loop. Circadian oscillations of \textit{SIG5} transcript abundance were approximately coincident with the phasing to subjective day of circadian oscillations of the promoters and transcripts of \textit{cry1}, \textit{cry2} and \textit{phyA-E} (Bognár \textit{et al.}, 1999; Tóth \textit{et al.}, 2001). However, as photoreceptor protein abundance may not cycle under constant light (Bognár \textit{et al.}, 1999; Sharrock & Clack, 2002; Mockler \textit{et al.}, 2003), rhythms of \textit{SIG5} transcript abundance seem unlikely to be a direct consequence of oscillations of photoreceptor transcript abundance.
cry1 cry2 SIG5::LUCIFERASE lacked the longer circadian period identified previously for the CHLOROPHYLL A/B-BINDING PROTEIN2 promoter in cry1 cry2 (Devlin & Kay, 2000). This might be explained by the temperature dependence of the period of cry1 cry2 under conditions that include B light (Gould et al., 2003). Gould et al. (2003) indicated that the experimental temperature of Devlin & Kay (2000) (22°C) would lengthen the period of cry1 cry2 when B and R light are present, whereas the period may be indistinguishable from the wild-type at the lower temperature (19°C) used here (Fig. 4).

Under light–dark cycles, SIG5 integrates several light signals that regulate chloroplast transcription

The presence of two features within the dynamics of SIG5::LUCIFERASE under light–dark cycles (Fig. 5a) suggests that the reduced experimental complexity provided by monochromatic B light alone (as opposed to a more complex spectrum) allowed the separation of light- and circadian-regulated components of SIG5 promoter activity. The more slowly acting feature of SIG5::LUCIFERASE under these conditions (marked ‘B’ on Fig. 5a, b) may be caused by circadian regulation, because this feature requires cry1 (Fig. 5b) and, under continuous B light, cry1 contributes substantially to the amplitude of circadian SIG5::LUCIFERASE oscillations (Fig. 4d,h).

There are several possible explanations for the lack of dawn anticipation by SIG5::LUCIFERASE under B light–dark cycles, compared with clear anticipation of dawn under R + FR + B light (Fig. 5). The degree of dawn anticipation by circadian reporters under light–dark cycles can reflect differences in circadian period, whereby a longer period reduces the extent of dawn anticipation by morning-phased reporters, and a shorter period leads to more obvious anticipation of dawn (Dodd et al., 2014). However, the circadian period of SIG5::LUCIFERASE was not longer under B light than under R + FR + B conditions (Fig. 4d,h), suggesting that period differences might not explain this variation in dawn anticipation. Another possibility is that increased photosynthetic energy availability in R + FR + B light relative to other treatments caused the SIG5 promoter to assume an earlier phase, because increased energy availability can shorten the circadian period (Haydon et al., 2013). We speculate that the anticipation of dawn by SIG5 might be important to ensure appropriate rates of PSII D2 protein accumulation before the onset of photosynthesis. However, to better understand the adaptive significance of these results, it will be important to determine the contribution of the circadian oscillator to the dynamics of this pathway under lighting conditions more representative of natural environments.

Differences between the transcriptional response of SIG5 to specific light conditions during acute induction and circadian free-run provide information about the contribution of circadian regulation to the functioning of this pathway under light–dark cycles, and about the role of specific light conditions around dawn. SIG5 responded strongly to R + FR light in dark-adapted plants (Fig. 1a,b) and under light–dark cycles (Fig. 5a), suggesting that, in nature, R + FR light might be an important regulator of SIG5 around dawn. In comparison, B light and cry1 help to maintain SIG5 transcript accumulation longer term, such as during the circadian free-run (Fig. 4c,d, h) and the second half of the photoperiod (Fig. 5). Therefore, circadian regulation contributes to SIG5 promoter activity during light–dark cycles. Under light–dark cycles, circadian regulation might be particularly important for gating the responses of SIG5 to transient changes in light conditions in order to maintain optimum synthesis of PSII D2 (Noordally et al., 2013).

Regulators of SIG5 transcription in response to light include ELOGNATED HYPOCOTYL5 (HY5) and HY5 HOMOLOG (HYH), which act redundantly to regulate SIG5 transcript accumulation (Nagashima et al., 2004; Brown & Jenkins, 2008; Mellenthin et al., 2014). Abscisic acid also upregulates SIG5 transcripts, but may be without effect on chloroplast psbD under the same conditions (Yamburekno et al., 2015). Although there are a variety of other light- and circadian-regulated cis elements within the SIG5 promoter (Noordally et al., 2013; Mellenthin et al., 2014), it is less clear which pathways underlie the circadian regulation of SIG5. For example, the high mean level of SIG5 promoter activity in B + R + FR compared with B light (Fig. 4a) might reflect convergence on the SIG5 promoter of distinct signals that regulate its activity. This could mean that, under certain lighting conditions, basal SIG5 promoter activity might be increased to a point at which its circadian amplitude becomes reduced or masked. Although circadian oscillations of the SIG5 promoter and SIG5 transcript abundance are morning phased, the dawn-phased oscillator component CIRCADIAN CLOCK ASSOCIATED1 (CCA1) does not appear to bind the SIG5 promoter (Nagel et al., 2015).

The response of SIG5 to the proportions of red and far red light may involve photosynthetic retrograde signals

The transcriptional response of SIG5 and psbD BLRP to the relative proportions of R and FR light was atypical of regulation by phytochrome alone (Fig. 3). As FR > c. 700 nm has insufficient quantum energy to drive oxygenic photosynthesis (Chen & Blankenship, 2011), we reasoned that photosynthetic signals might contribute to this R:FR response because SIG5 transcription can be regulated by photosynthesis (Mellenthin et al., 2014). Moreover, there was little alteration in SIG5 promoter activity or transcript abundance across much of the R:FR range that induces shade avoidance responses, except for R:FR conditions typical of deeper shade (R:FR below c. 0.2) (Smith, 1982). sig5-3 has been reported to have shorter hypocotyls and smaller cotyledons than the wild-type in either constant R or FR light, 4 d after germination (Khanna et al., 2006). This was interpreted as a cell expansion defect rather than a photomorphogenic phenotype, potentially caused by increased sensitivity of sig5-3 to light-induced damage (Khanna et al., 2006), which is consistent with the slow recovery of PSII photochemistry after exposure of sig5 mutants to high light (Nagashima et al., 2004).
One interpretation of the response of SIG5 to R and FR light is that, when a large proportion of light is FR, little energy is available to drive oxygenic photosynthesis. Under these conditions, the photosynthetic signal that regulates SIG5 transcription is weak, inhibiting the phyA signal and suppressing SIG5 transcription (Figs 2c, 4b). This would also explain the insensitivity of SIG5 to DCMU under conditions of predominantly FR light (Fig. 3b), because DCMU inhibits photosynthetic electron transport from PSII to PQ, which decreases substantially under predominantly FR light in which PSI is less activated than PSII.

Interestingly, the differing R : FR response profiles of SIG5 and psbD BLRP suggest that there is post-translational regulation of SIG5 activity (Fig. 3). This is also supported by our findings that SIG5 transcripts were not induced by R + FR light in the phyA mutant, whereas psbD BLRP was induced by higher intensity R + FR light in phyA (Fig. 2d); phyA-mediated activation of psbD BLRP by R + FR light required phyB, whereas phyA-mediated activation of SIG5 did not require phyB (Fig. S4b); and B light induced SIG5 through either cry1 or cry2, whereas B light induction of psbD BLRP required both cry1 and cry2 (Fig. S4c). Post-translational regulation might involve phosphorylation of SIG5 protein on one or more of its predicted serine/threonine phosphorylation sites, similar to redox-dependent regulation of SIG1 and chloroplast transcription by PLASTID TRANSCRIPTION KINASE (PTK) and CHLOROPLAST SENSOR KINASE (CSK) (Baena-González et al., 2001; Shimizu et al., 2010). Another possibility is that there is light and/or redox regulation of SIG5 chloroplast protein import (Küchler et al., 2002; Hörmann et al., 2004). In this context, future analysis of SIG5 protein biology may be informative. Although there could also be SIG5-independent regulation of psbD BLRP, this is not supported by an analysis of psbD transcripts accumulating from all TSSs of the chloroplast psbDC operon (Nagashima et al., 2004). In future, it will be informative to determine whether the regulation of chloroplast transcription by sigma factors contributes to photosynthetic adaptation to shade under light conditions more representative of natural environments, and to investigate the nature of the photosynthetic retrograde signal that regulates SIG5 in response to changing light conditions.

Conclusions

The regulation of photosynthesis gene expression by sigma factors in response to light appears to be conserved throughout photosynthetic life. It is possible that this signalling pathway evolved as an adaptation to light stress. In cyanobacteria, sigma factors have an important role in maintaining optimum growth under high light conditions by regulating the expression of photosystem components (Hanaoka & Tanaka, 2008; Pollari et al., 2009). This function appears to have been conserved following the endosymbiosis that led to the evolution of chloroplasts, because the regulation of chloroplast genes by sigma factors is important to maintain photosynthetic efficiency under very high light in Arabidopsis (Nagashima et al., 2004). Our data suggest that, during evolution, this light stress response pathway has become rewired to also underpin subtle and sophisticated responses to the light environment by the integration of a conserved signalling pathway with higher plant photoreceptor systems, retrograde signalling and the circadian clock.

Acknowledgements

Funding was from UK BBSRC (BB/I005811/2, BB/J014400/1), the Lady Emily Smyth Agricultural Research Station (Bristol) and the Wolfson Foundation. We thank Paul Devlin (Royal Holloway), Karen Halliday (Edinburgh), Akira Nagatani (Kyoto University) and Mitsumasa Hanaoka (Chiba University) for advice, Anthony Hall (Liverpool), Steven Penfield (John Innes Centre) and Alex Webb (Cambridge) for donating seeds, Dora Cano-Ramirez for assistance with spectrum measurements, and Kan Tanaka (Tokyo Institute of Technology), Alistair Hetherington (Bristol) and Jill Harrison (Bristol) for critical manuscript review. Thanks are also due to Kyoto University for a Guest Professorship (Joint Usage/Research Program of the Center for Ecological Research) and The Royal Society for awarding a University Research Fellowship.

Author contributions

F.E.B., Z.B.N., K.A.F. and A.N.D. conceived and designed the experiments. F.E.B. and Z.B.N. performed the experiments. F.E.B., Z.B.N., K.A.F. and A.N.D. interpreted the data and wrote the paper.

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**Supporting Information**

Additional Supporting Information may be found online in the Supporting Information tab for this article:

**Fig. S1** Initial circadian period characterization of multiple luciferase lines.

**Fig. S2** Spectra of light treatments used in this study.

**Fig. S3** Efficacy of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and norflurazon under our experimental conditions.

**Fig. S4** Analysis of photoreceptors involved in SIGMA FACTOR5 (SIG5)-mediated regulation of chloroplast *psbD* blue light-responsive promoter (BLRP).

**Fig. S5** Circadian regulation of SIG5::LUCIFERASE by light quality and photoreceptors.

**Fig. S6** Circadian regulation of SIG5::LUCIFERASE in the Col-0 background by light quality.

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