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Research

BBX16 mediates the repression of seedling photomorphogenesis downstream of the GUN1/GLK1 module during retrograde signalling

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Summary

• Plastid-to-nucleus retrograde signalling (RS) initiated by dysfunctional chloroplasts impact photomorphogenic development. We have previously shown that the transcription factor GLK1 acts downstream of the RS regulator GUN1 in photodamaging conditions to regulate not only the well established expression of photosynthesis-associated nuclear genes (*PhANGs*) but also to regulate seedling morphogenesis. Specifically, the GUN1/GLK1 module inhibits the light-induced phytochrome-interacting factor (PIF)-repressed transcriptional network to suppress cotyledon development when chloroplast integrity is compromised, modulating the area exposed to potentially damaging high light. However, how the GUN1/GLK1 module inhibits photomorphogenesis upon chloroplast damage remained undefined.

• Here, we report the identification of *BBX16* as a novel direct target of GLK1. *BBX16* is induced and promotes photomorphogenesis in moderate light and is repressed via GUN1/GLK1 after chloroplast damage. Additionally, we showed that BBX16 represents a regulatory branching point downstream of GUN1/GLK1 in the regulation of *PhANG* expression and seedling development upon RS activation.

• The *gun1* phenotype in lincomycin and the *gun1*-like phenotype of *GLK1OX* are markedly suppressed in *gun1bbx16* and *GLK1OXbbx16*.

• This study identified BBX16 as the first member of the BBX family involved in RS, and defines a molecular bifurcation mechanism operated by GLK1/BBX16 to optimise seedling de-etiolation, and to ensure photoprotection in unfavourable light conditions.

Introduction

To cope with their sessile condition, plants need to optimise their growth and development in response to changes in their habitat. Light is a critical environmental component necessary for photosynthesis and for the regulation of growth and development (Arsovski *et al.*, 2012). Required as a primary source of energy and as an informative cue, light also represents a challenge for plant life when in excess. Plants have therefore evolved exquisite methods for light sensing and signalling to allow the appropriate adaptive response. Light of different wavelengths is perceived by different photoreceptors. Phytochromes sense red and far-red light (600–750 nm), whereas cryptochromes, phototropins, and Zeitlupes perceive blue and UVA (320–500 nm) and UVR8 senses UVB (Galvão & Fankhauser, 2015). Light perception by photoreceptors can be complemented by chloroplasts, which act as sensors of environmental changes and contribute to responses in high light (Chan *et al.*, 2016).

One of the most dramatic developmental transitions in plants is de-etiolation, in which a germinating seedling experiences light for the first time (Arsovski *et al.*, 2012; Gommers & Monte, 2018). When germinating in the dark, skotomorphogenic seedlings growing heterotrophically exhibit fast-growing hypocotyls, unexpanded and appressed cotyledons with etioplasts, and the formation of an apical hook to protect the apical meristem from damage. In the light, de-etiolated or photomorphogenic seedlings adapt their morphology to enhance light capture for photosynthesis, which involves inhibition of hypocotyl elongation, hook unfolding, stimulation of cotyledon separation and expansion, and the formation of the photosynthetic apparatus and fully functional chloroplasts.

Distinct transcriptomic landscapes underlay the skotomorphogenic and photomorphogenic programmes, regulated by a suite of positive- and negative-acting factors (Ma *et al.*, 2001; Jiao *et al.*, 2005; Pham *et al.*, 2018; Shi *et al.*, 2018; Jing & Lin, 2020). Major positive regulators are HFR1, HY5/HYH and LAF1 (Lau & Deng, 2012; Xu *et al.*, 2015, 2016), whereas phytochrome-interacting factors (PIFs) act as major negative-

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acting factors of photomorphogenesis (Castillon *et al.*, 2007; Leivar & Quail, 2011; Leivar & Monte, 2014). PIFs (PIF1, PIF3-8) are basic helix–loop–helix (bHLH) transcription factors (Toledo-Ortiz *et al.*, 2003) that bind to G-box (CACGTG) and PBE (CACATG) DNA elements in the dark to inhibit or activate the expression of light-induced or light-repressed genes, respectively (Leivar *et al.*, 2009; Zhang *et al.*, 2013; Pfeiffer *et al.*, 2014). The quadruple mutant *pifq* lacking PIF1, PIF3, PIF4 and PIF5 displays a partial constitutively photomorphogenic phenotype in the dark, suggesting that PIFs promote skotomorphogenesis (Leivar *et al.*, 2008; Shin *et al.*, 2009). Upon illumination, phytochromes become active and trigger PIF inactivation and degradation through the 26S proteasome-mediated pathway, allowing seedlings to initiate light-regulated gene expression and follow a photomorphogenic programme of development (Leivar *et al.*, 2008, 2009; Pham *et al.*, 2018). Additional transcription factors involved include the GOLDEN2-LIKE 1 (GLK1) and GLK2 (Chen *et al.*, 2016) and members of the B-box family (BBX) (Khanna *et al.*, 2009; Gangappa & Botto, 2014; Su *et al.*, 2015; Song *et al.*, 2020a). Whereas GLKs target genes involved in chlorophyll biosynthesis, light harvesting and electron transport are necessary for chloroplast development (Fitter *et al.*, 2002; Waters *et al.*, 2008, 2009; Oh & Montgomery, 2014; Zubo *et al.*, 2018), some BBX members have been described as general positive regulators of photomorphogenesis (e.g. BBX4/COL3, BBX11, BBX20/BZS1, BBX21/STH2 and BBX22/LZF1) (Datta *et al.*, 2006, 2007, 2008; Chang *et al.*, 2008; Fan *et al.*, 2012; Xu *et al.*, 2018; Job & Datta, 2021), and some as negative regulators (e.g. BBX18/DBB1a, BBX19/DBB1b, BBX24/STO, BBX25/STH, BBX28, BBX29, BBX30, BBX31 and BBX32/EIP6) (Datta *et al.*, 2006; Khanna *et al.*, 2006; Indorf *et al.*, 2007; Kumagai *et al.*, 2008; Holtan *et al.*, 2011; Wang *et al.*, 2011, 2015; Gangappa *et al.*, 2013; Lin *et al.*, 2018; Heng *et al.*, 2019b; Song *et al.*, 2020b; Ravindran *et al.*, 2021). In addition, the role in photomorphogenesis of BBX23/MIDA10 appears to be organ specific (positive for hypocotyl elongation) (Zhang *et al.*, 2017) and negative for hook unfolding (Sentandreu *et al.*, 2011). The protein stability of several of these transcription factors (e.g. HY5, LAF1, HFR1, BBX21, BBX22 and others) is directly modulated by the COP1/SPA complex acting as an E3 ubiquitin ligase, which interacts and targets them for degradation via the 26S proteasome pathway in darkness (Yi & Deng, 2005; Hoecker, 2017).

In Arabidopsis, chloroplast biogenesis during seedling de-etiolation depends on the expression of chloroplast proteins encoded by the nuclear genome (*c.* 2000–3000) (Li & Chiu, 2010) (anterograde regulation) that are imported into the chloroplast following synthesis in the cytosol (Jung & Chory, 2010). In turn, chloroplasts can communicate with the nucleus through retrograde signalling (RS) to regulate nuclear gene expression according to chloroplast status (Kleine *et al.*, 2009; Jarvis & López-Juez, 2014). This coordination between the nucleus and chloroplast genomes ensures optimised photosynthetic capacity and growth (Ruckle *et al.*, 2007; Hills *et al.*, 2015; Martín *et al.*, 2016). Moderate light intensities during de-etiolation induce expression of the PIF-repressed target gene *GLK1* (Martín *et al.*, 2016), and GLK1 subsequently promote photosynthetic apparatus formation by directly inducing the expression of nuclear-encoded photosynthetic genes (*PhANGs*) such as those from the *LHCb* gene family (Waters *et al.*, 2009). Under photodamaging conditions, however, RS is activated (Ruckle *et al.*, 2007; Estavillo *et al.*, 2011; Kindgren *et al.*, 2012) leading to the repression of *GLK1* expression and downregulation of *PhANGs* (Waters *et al.*, 2009; Martín *et al.*, 2016). The use of drugs such as lincomycin specifically inhibits plastid translation and activates RS and repression of *PhANG* expression (Oelmüller *et al.*, 1986; Sullivan & Gray, 1999). *Genomes uncoupled* (*gun*) mutants exhibit *PhANG* derepression in response to these drugs, and have helped elucidate components of RS-like tetrapyrroles such as heme, and GUN1 (Koussevitzky *et al.*, 2007; Chan *et al.*, 2016). Importantly, RS has been shown to impact light-regulated seedling development in high light environments to prevent photodamage, through a GUN1-mediated mechanism that is still not well defined (Ruckle *et al.*, 2007; Martín *et al.*, 2016). It is also currently unknown whether light regulation of seedling development and *PhANG* expression after RS activation operate through the same components.

We have previously shown that the RS and phytochrome pathways converge to antagonistically regulate the PIF-repressed light-induced transcriptional network (Martín *et al.*, 2016). Our findings showed that GLK1 acts downstream of GUN1 to modulate not only *PhANG* expression but also seedling morphogenesis in photodamaging conditions. Specifically, GUN1/GLK1-mediated RS antagonise phytochrome/PIF signalling to inhibit cotyledon separation and expansion when chloroplast integrity is compromised, effectively reducing the area exposed to potentially damaging high light. How this is achieved is still unclear, but does not involve the reaccumulation of PIF proteins in these conditions (Martín *et al.*, 2016), therefore suggesting the participation of yet undefined components (Supporting Information Fig. S1). Here, we address the question of how the GUN1/GLK1 module inhibits photomorphogenesis upon chloroplast damage, and report the identification and characterisation of *BBX16* as a novel GLK1 target. BBX16 promotes photomorphogenesis downstream of PIF and GLK1 in moderate light and is repressed via the GUN1/GLK1 module after chloroplast damage. Additionally, we showed that BBX16 represents a regulatory branching point in the regulation of *PhANG* expression and seedling development upon RS activation.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana wild-type and mutant seeds used in this study have been described previously. *gun1* (*gun1-201*) (Martín *et al.*, 2016), *glk1* and *glk1glk2* (Fitter *et al.*, 2002), *GLK1OX* and *GLK1OX-GFP* (both on the *glk1glk2* background) (Waters *et al.*, 2008) are on the Col-0 background; whereas *col7*, *BBX16OX #10* and *BBX16OX #11* (here renamed as *bbx16-1*, *BBX16OX1* and *BBX16OX2*, respectively) (H. Wang *et al.*, 2013) are on the Col-4 background. *BBX16OX* lines express the *BBX16* open

reading frame under the control of the 35S promoter and were described to overexpress BBX16 *c.* 250-fold (H. Wang *et al.*, 2013). *bbx16-1* is an insertional mutant from the GABI-Kat collection (GABI-639C04) with a T-DNA insertion in the second exon of *BBX16* (H. Wang *et al.*, 2013). A new second BBX16 mutant allele (named *bbx16-2*) was obtained from the SALK collection (SALK_036059), harbouring a T-DNA insertion in the first exon (Fig. S2). *gun1bbx16-1* was obtained by crossing *gun1-201* to *bbx16-1*; wild-type (WT) (Col-0 × Col-4 background), *gun1* and *bbx16* siblings from the cross were selected to be used in the experiments shown in Fig. 4. *GLK1OXbbx16-1* and *GLK1OXbbx16-2* were generated by crossing *GLK1OX* to *bbx16-1* and to *bbx16-2*, respectively. The obtained mutants were selected to maintain the *glk1glk2* background in *GLK1OX*; *GLK1OX* siblings from each cross were selected to be used as controls. Seeds were surface sterilised in 20% bleach and 0.25% sodium dodecyl sulfate (SDS) for 10 min and plated on half-strength Murashige and Skoog (0.5× MS) medium without sucrose, stratified at 4°C in the dark for 4 d, exposed to white light for 3 h to induce germination, and then placed under the specific light conditions indicated in each experiment. For experiments carried out under continuous conditions, plates were placed under white light (5 μmol m^{-2} s^{-1}) or darkness for 3 d unless otherwise indicated. In the text we refer to low light (< 25 μmol m^{-2} s^{-1}), light (100–150 μmol m^{-2} s^{-1}), and high light (> 300 μmol m^{-2} s^{-1}), whereas the specific light intensity used in each experiment is specified in the corresponding figure legend. For lincomycin treatments, the medium was supplemented with 0.5 mM lincomycin (Sigma L6004) (Sullivan & Gray, 1999). Primers sequences used for genotyping are provided in Table S1.

Phenotypic measurements

Hypocotyl length, cotyledon area and cotyledon aperture were measured as described previously (Sentandreu *et al.*, 2011), using NIH Image software (ImageJ; National Institutes of Health). The median was calculated from at least 20 seedlings and the experiments were repeated at least two times with similar results.

Quantitative reverse transcriptase

For quantitative reverse transcriptase (qRT-PCR) analysis, seedlings were grown in the dark or in white light for the indicated time. qRT-PCR was performed as described previously (Khanna *et al.*, 2007) with variations. Briefly, 1 μg of total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) or using the Maxwell® RSC Plant RNA Kit (Promega), treated with DNase I (Ambion) according to the manufacturer's instructions (if extracted with Qiagen kit), and first-strand cDNA synthesis was performed using the SuperScript III reverse transcriptase (Invitrogen) and oligo(dT) as a primer (dT30) or the NZY First-Strand cDNA Synthesis Kit (NZYTech). In all cases, cDNA was then treated with RNase Out (Invitrogen) before being subjected to a 1 : 20 dilution with water, and 2 μl of this mix was used for real-time PCR (Light Cycler 480; Roche) using SYBR Premix Ex Taq (Roche) and primers at a 300 nM concentration. Gene expression was generally measured in three independent biological replicates, and at least two technical replicates were used for each of the biological replicates. *PP2A* (*AT1G13320*) was used for normalisation as described (Shin *et al.*, 2007). Primers sequences used for qRT-PCR are described in Table S2.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) and ChIP-qPCR assays were performed as described previously (Martín *et al.*, 2018) using the previously described *35S::GLK1OX-GFP* line (Waters *et al.*, 2008). Seedlings (3 g) were vacuum infiltrated with 1% formaldehyde and cross-linking was quenched using vacuum infiltration with 0.125 M glycine for 5 min. The tissue was ground, and nuclei-containing cross-linked protein and DNA were purified using sequential extraction with extraction buffer 1 (0.4 M sucrose, 10 mM Tris-HCl pH 8, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM MG132, proteinase inhibitor cocktail); buffer 2 (0.25 M sucrose, 10 mM Tris-HCl pH 8, 10 mM $MgCl_2$, 1% Triton X-100, 5 mM β-mercaptoethanol, 0.1 mM PMSF, 50 mM MG132, proteinase inhibitor cocktail); and buffer 3 (1.7 M sucrose, 10 mM Tris-HCl pH 8, 0.15% Triton X-100, 2 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0.1 mM PMSF, 50 mM MG132, proteinase inhibitor cocktail). Nuclei were resuspended in nuclei lysis buffer (50 mM Tris-HCl pH 8, 10 mM EDTA, 1% SDS, 50 mM MG132, proteinase inhibitor cocktail), sonicated 10 times for 30 s each, and diluted in 10 volumes of dilution buffer (0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8, 167 mM NaCl). Overnight incubation was performed with the corresponding antibody (or with no antibody as control) at 4°C and immunoprecipitation was performed using Dynabeads. Washes were done sequentially in low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8, 150 mM NaCl), high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8, 500 mM NaCl), LiCl buffer (0.25 M LiCl, 1% NP40, 1% deoxycholic acid sodium, 1 mM EDTA, 10 mM Tris-HCl pH 8), and 1× TE. Immunocomplexes were eluted in elution buffer (1% SDS, 0.1 M $NaHCO_3$), de-crosslinked overnight at 65°C in 10 mM NaCl, and then treated with proteinase K. DNA was purified using Qiagen columns and eluted in 100 μl of Qiagen elution buffer, and 2 μl were used for qPCR (ChIP-qPCR) analysis using *BBX16* promoter-specific primers spanning the regions P1 (EMP1180-P1 and EMP1182-P1) and P2 (EMP1175-P2 and EMP1176-P2) containing the predicted binding sites for GLK1 (Waters *et al.*, 2009; Franco-Zorrilla *et al.*, 2014), and a pair of primers inside the *BBX16* gene body as control (EMP869-P3 and EMP1177-P3). Three biological replicates were performed for *35S::GLK1-GFP* (Waters *et al.*, 2008) incubated with or without antibody. Wild-type controls were performed with one replicate of Col-0 seedlings with or without antibody.

Statistical analysis

Cotyledon angle and hypocotyl length differences between all genotypes across the two conditions Light and Light Linc were analysed using the Kruskal–Wallis test to assess the significance of global variation in a nonparametric dataset. After significant result of the omnibus test (Kruskal–Wallis) was found, a post-hoc Dunn test was performed to identify significantly different pairs of genotypes taking into account the global variation across the two conditions. Significantly different pairs of genotypes were represented by letters. Subsequently, we sought to find different genotypes focusing in the single condition Light Linc. Therefore, a pairwise Mann–Whitney test was used and the significant effect was represented with asterisks.

To identify differences at the gene expression level between all genotypes taking into account the global variation across the two conditions Light and Light with Linc (unless otherwise indicated), and given the parametric nature of the gene expression measurements, data were analysed using ANOVA. Upon a significant result of the omnibus test (ANOVA), a post-hoc Tukey test was performed to identify significant differences between pairs of genotypes. Significantly different pairs of genotypes were represented by letters.

To find different genotypes within the single condition Dark, Light or Light Linc, a *t*-test was performed and asterisks in specific samples indicated statistically significant differences between each mutant and its respective wild-type seedlings.

Results

BBX16 is a PIF-repressed gene that is induced by light in a GLK1-dependent manner

To elucidate how the PIF/GLK1 and GUN1/GLK1 modules regulated cotyledon development under different light conditions, we aimed to identify genes downstream of GLK1 that might be involved in the regulation of photomorphogenesis. We reasoned that plausible candidates would need to meet the following criteria: (1) be a light-induced gene in a GLK1-dependent manner and PIF repressed in the dark; (2) promote cotyledon development under moderate light; (3) be a high light- and lincomycin-repressed gene via the GUN1/GLK1 module; (4) display reduced sensitivity to RS-inducing treatments when overexpressed in seedlings, preventing RS repression of cotyledon development. Additionally, to verify the importance of the selected candidate (represented as X); (5) genetic removal of *X* in *gun1* and *GLK1OX* mutants should suppress their phenotype in lincomycin at least partly (Fig. S1).

To begin our search, we made use of previous data describing genes directly targeted and upregulated by GLKs (Waters *et al.*, 2009). We observed that these targets (119 in total) not only included chloroplast-localised photosynthetic genes (the main focus of Waters and colleagues' work). Significantly, we observed among them an enrichment of genes encoding for BBX transcription factors, with four of the described 32 BBX family members being present in the list of 119 genes (*P*-value: 2.46 e-05). Moreover, three of these BBX were members of subclass III, which is composed by four members (BBX14–BBX17). Different BBX proteins have been involved in several aspects of light-regulated development (Gangappa & Botto, 2014). In particular, BBX16/COL7 has been described to play a role in shade responses (H. Wang *et al.*, 2013; Zhang *et al.*, 2014), and was considered a good candidate for further characterisation.

To start to evaluate this candidate, *BBX16* expression was analysed in dark-grown and light-grown wild-type, GLK1-deficient *glk1* and *glk1glk2* (Fitter *et al.*, 2002), and *GLK1*-overexpression *GLK1OX* (Waters *et al.*, 2008) seedlings. *BBX16* was strongly upregulated in light-grown wild-type seedlings compared with dark, and this induction required GLK1 (Fig. 1a). *BBX16* is a PIF-repressed gene, although not described as a direct target

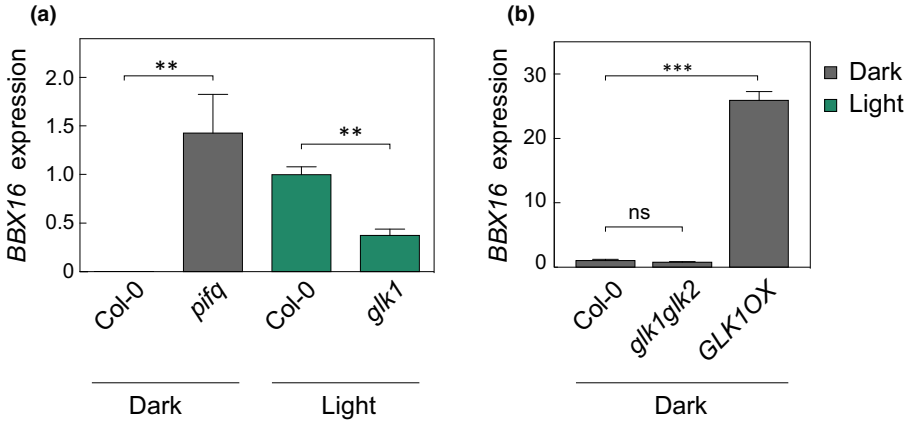

Fig. 1 *BBX16* is a phytochrome-interacting factor (PIF)-repressed gene whose expression is induced by light in a GOLDEN2-LIKE 1 (GLK1)-dependent manner. (a, b) Transcript levels of *BBX16* analysed using quantitative reverse transcription polymerase chain reaction (qRT-PCR) in (a) 3-d-old Col-0, *pifq* and *glk1* and (b) Col-0, *glk1glk2* and *GLK1OX* Arabidopsis seedlings grown in the dark or in continuous white light (5 μmol m^{-2} s^{-1}) as indicated. Values were normalised to *PP2A*, and expression levels are expressed relative to Col-0 light set at one. Data are the means ± SE of biological triplicates ($n = 3$) and asterisks indicate statistically significant differences between each mutant and its respective wild-type (WT) seedlings (*t*-test; **, $P < 0.01$; ***, $P < 0.001$; ns, nonsignificant).

(Pfeiffer *et al.*, 2014). As such, in *pifq* etiolated seedlings, *BBX16* expression showed high levels of expression compared with the wild-type (Fig. 1a). Interestingly, the expression of the other *BBX* in the same clade showed a similar pattern except for *BBX17* (Fig. S3), suggesting that BBX14 and BBX15 might share some function with BBX16. Furthermore, *GLK1* overexpression in the dark induced *BBX16* expression (Fig. 1b). Together, these results indicated that, during seedling establishment, *BBX16* is a PIF-repressed gene in the dark that is light-induced in a GLK1-mediated manner. Therefore, the identified BBX16 met our first criterion (Fig. S1) and was considered for further genetic and molecular analyses.

BBX16 promotes cotyledon development during seedling de-etiolation

Next, to evaluate the role of BBX16 during de-etiolation, we analysed the previously described *bbx16* T-DNA insertion mutant line *col7* (referred here as *bbx16-1* for clarity), a newly characterised *bbx16-2* line (see the Materials and Methods section and Fig. S2), and two overexpressing *BBX16* lines (*OX1* and *OX2*) (H. Wang *et al.*, 2013). Under 3 d of continuous low light conditions, deficiency of *BBX16* in the *bbx16* mutants led to significantly reduced cotyledon area compared with the wild-type, whereas cotyledons in *BBX16-OX1* and *OX2* were more expanded (Fig. 2a,b). *BBX16-OX1* and *OX2* also showed slightly shorter hypocotyls (Fig. 2c). In addition, dark-grown OX lines displayed faster cotyledon aperture compared with the wild-type after light exposure (Fig. 2d). Together, these results indicated that BBX16 contributed to the promotion of early photomorphogenesis with a role in cotyledon development (and therefore fulfilled the second criterion, Fig. S1), and a possible minor contribution to the inhibition of hypocotyl elongation.

Under lincomycin treatment, inhibition of cotyledon separation involves GUN1-mediated repression of *BBX16*

Next, *BBX16* expression was analysed under conditions in which chloroplast integrity was compromised by lincomycin treatment,

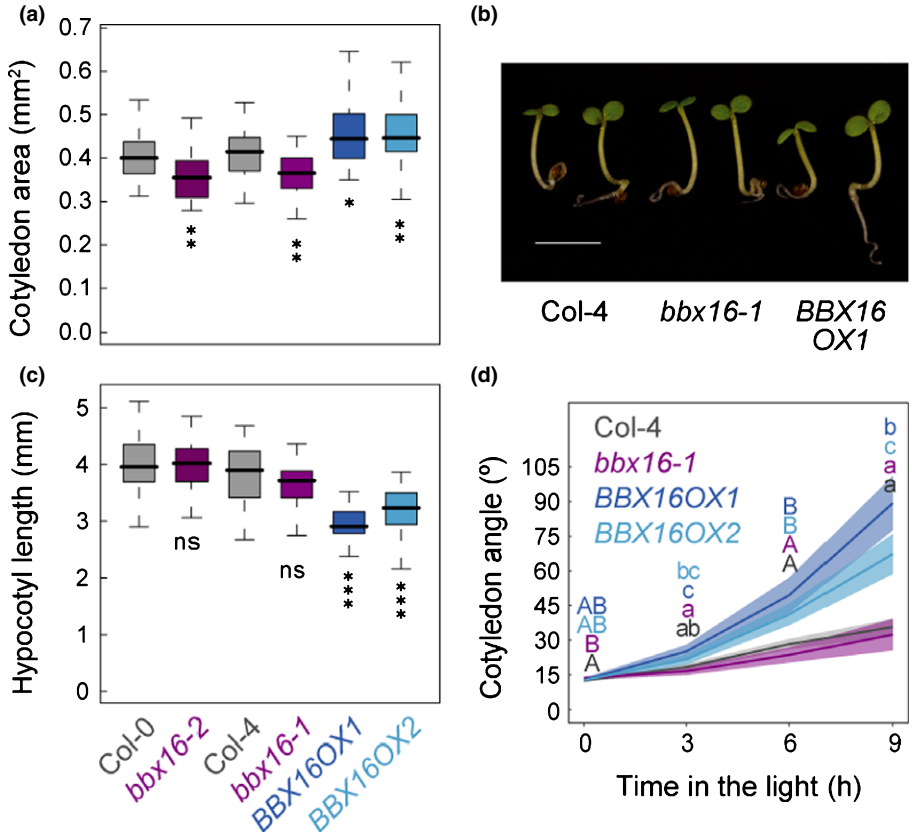

Fig. 2 *BBX16* regulates cotyledon development during early seedling development in continuous light. (a) Boxplot representation of the cotyledon area of *BBX16* loss-of-function (*bbx16*) and gain-of-function (*BBX16OX1* and *OX2*) Arabidopsis mutants grown for 3 d under continuous white light (5 μmol m^{-2} s^{-1}). (b) Visual phenotypes of Arabidopsis seedlings grown as detailed in (a). Bar, 2.5 mm. (c) Boxplot representation of the hypocotyl length of seedlings grown as detailed in (a). (d) Quantification of the cotyledon angle of 2-d-old dark-grown wild-type (WT), *bbx16* and two *BBX16* Arabidopsis overexpressor lines transferred to white light (20 μmol m^{-2} s^{-1}) for the indicated hours (h). The thick lines and shaded areas represent the median and the 95% confidence interval of at least 60 seedlings, respectively. Letters denote the statistically significant differences between genotypes using Dunn's test at each time point ($P < 0.05$). (a, c) Boxplots indicate the median (centre line), interquartile range (box limits), and minimum and maximum values (whiskers). Data represent the median of at least 20 seedlings and asterisks indicate statistically significant differences between each mutant and its respective WT seedlings (Mann–Whitney test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, nonsignificant).

an inhibitor of chloroplast translation that specifically damages the chloroplast under both dark and light conditions (Sullivan & Gray, 1999). When the chloroplast is perturbed, activation of RS induces downregulation of *GLK1* expression in a GUN1-mediated manner, impacting cotyledon development (Martín *et al.*, 2016). We hypothesised that, under these conditions, repression of *GLK1* should also result in the repression of *BBX16* expression as a downstream effector of GLK1 (criterion 3, Fig. S1). Notably, lincomycin treatment prevented de-repression of *BBX16* in dark-grown *pifq* (Fig. 3a). Moreover, the light-induced expression of *BBX16* shown in Fig. 1 was strongly inhibited in response to lincomycin in wild-type seedlings (Fig. 3b,c), similarly to the reported inhibition of *PhANGs* and *GLK1* expression (Martín *et al.*, 2016). Importantly, the inhibition of *BBX16* expression in lincomycin was only partial in *GLK1OX* (Fig. 3b), similar to the *gun1* mutant (Fig. 3c). Compared with *gun1*, *BBX16* expression in lincomycin was not significantly affected in a *gun1glk1* double mutant (Fig. S4), suggesting that the

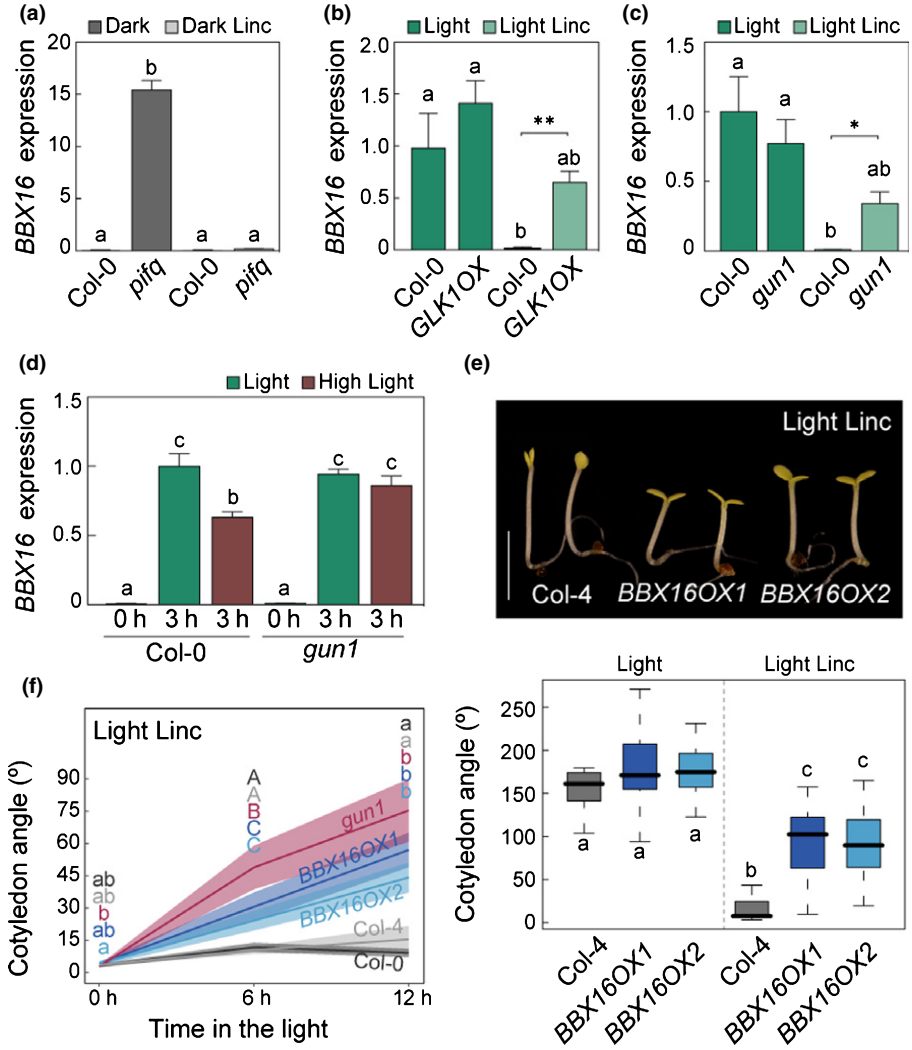

Fig. 3 Downregulation of *BBX16* mediated by the GUN1/GLK1 module is necessary to repress cotyledon development under lincomycin treatment. (a) Transcript levels of *BBX16* from RNA sequencing of Arabidopsis wild-type (WT) Col-0 and *pifq* seedlings grown for 3 d in the dark in the absence or presence of lincomycin (Martín *et al*., 2016). (b, c) Transcript levels of *BBX16* analysed using quantitative reverse transcription polymerase chain reaction (qRT-PCR) in 3-d-old light-grown (5 μmol m^{-2} s^{-1}) Arabidopsis Col-0 and *GLK1OX* seedlings (b), and Col-0 and *gun1* seedlings (c), in the absence or presence of lincomycin. (d) *BBX16* expression levels in 3-d-old dark-grown Arabidopsis WT and *gun1* mutant seedlings (time 0 h) exposed to 3 h of high light (310 μmol m^{-2} s^{-1}) compared with light (130 μmol m^{-2} s^{-1}). (b–d) Values were normalised to *PP2A*, and expression levels are expressed relative to Col-0 light (b, c) or Col-0 light 3 h (d), set at one. Data are the means ± SE of biological triplicates ($n = 3$). (a–d) Letters denote the statistically significant differences using Tukey's test ($P < 0.05$), and asterisks in specific samples indicate statistically significant differences between each mutant and its respective WT seedlings (*t*-test; *, $P < 0.05$; **, $P < 0.01$). (e) Visual phenotypes (top) and cotyledon angle quantification (of at least 40 seedlings) (bottom) of Arabidopsis WT and *BBX16OX* seedlings grown as in (b). Representative seedlings grown in presence of lincomycin are shown in the photograph. Bar, 2.5 mm. Boxplots indicate the median (centre line), interquartile range (box limits), and minimum and maximum values (whiskers). Letters denote the statistically significant differences among genotypes by Dunn's test ($P < 0.05$). (f) Quantification of the cotyledon angle of 2-d-old dark-grown Arabidopsis WT Col-0, *gun1*, WT Col-4, *bbx16-1* and two *BBX16OX* lines transferred to white light (10 μmol m^{-2} s^{-1}) for the indicated times in the presence of lincomycin. The thick lines and shaded areas represent the median and the 95% confidence interval of at least 20 seedlings, respectively. Different letters denote statistically significant differences between genotypes by Dunn's test at each time point ($P < 0.05$). Linc, lincomycin.

inhibition of *BBX16* expression downstream of GUN1 might require GLK1 and additional factors.

The biological relevance of these findings using lincomycin was assessed by testing *BBX16* expression under high light conditions, which causes GUN1-mediated inhibition of cotyledon separation (Martín *et al.*, 2016). Induction of *BBX16* in high light in the wild-type was reduced compared with normal light (Fig. 3d), suggesting that high light damage partially inhibits *BBX16* induction, in agreement with recent transcriptomic data obtained under high light stress (Huang *et al.*, 2019). This effect was not observed in *gun1* mutants (Fig. 3d), indicating that this repression was mediated by GUN1. These results are in accordance with previously observed inhibition of *GLK1* under similar conditions (Martín *et al.*, 2016) and suggested that the light induction of *BBX16* downstream of GLK1 is repressed in conditions in which RS is active and inhibits GLK1 function.

Next, we tested whether the transcriptional repression of *BBX16* in response to RS might contribute to the inhibition of seedling deetiolation upon chloroplast damage previously observed (Martín *et al.*, 2016). Indeed, *BBX16OX* lines grown for 3 d in plates containing lincomycin under light were less sensitive to lincomycin and were able to de-etiolate, showing a cotyledon aperture that was similar to that of wild-type seedlings without lincomycin (Fig. 3e). Similarly, in a de-etiolation experiment using 2-d-old dark-grown seedlings transferred to light in the presence of lincomycin, *BBX16OX* lines showed reduced sensitivity to lincomycin like *gun1*, and displayed higher cotyledon angles compared with the wild-type (Fig. 3f). These results indicated that BBX16 also fulfilled criteria 3 (high light and lincomycin-repressed (via GUN1/GLK1)) and 4 (OX seedlings display reduced sensitivity to RS) (Fig. S1), and provided strong support that RS-imposed GUN1/GLK1-mediated repression of *BBX16* was necessary for the inhibition of cotyledon development under conditions in which the chloroplast is damaged.

Importantly, to provide conclusive support for this pathway, we next tested the genetic interactions between GLK1, GUN1 and BBX16 (criterion 5, *gun1-X* and *GLK1OX-X* mutants confirm X contribution to the pathway) (Fig. S1). Genetic removal of *BBX16* in *GLK1OXbbx16* and *gun1bbx16-1* mutants allowed us to determine the contribution of the endogenous *BBX16* to the cotyledon phenotypes of *GLK1OX* and *gun1* in lincomycin (Figs 4, S5). Markedly, the *gun1*-like phenotype of *GLK1OX* in lincomycin was clearly suppressed in *GLK1OXbbx16* (Figs 4a, S5c). Similarly, the *gun1bbx16-1* double mutant showed strong

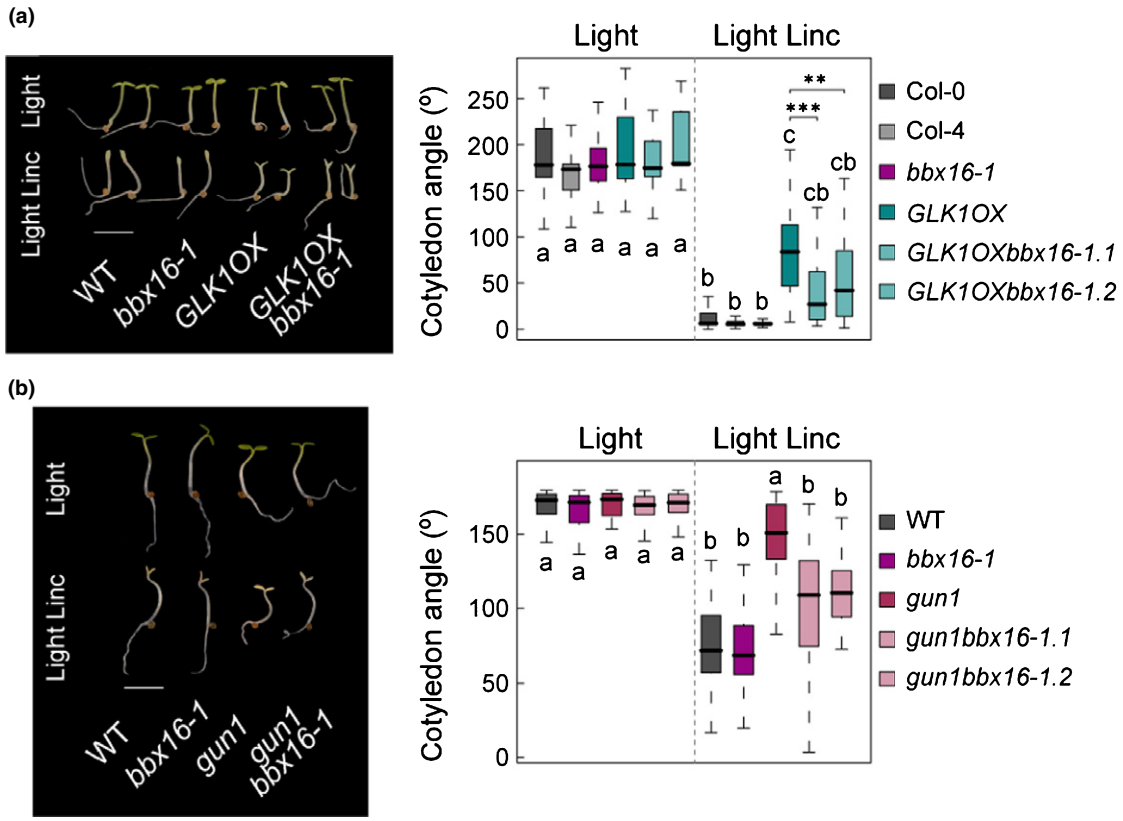

Fig. 4 Genetic removal of *BBX16* partially suppresses the *gun1* and *GLK1OX* open cotyledon phenotype in the presence of lincomycin. (a) Visual phenotypes (left) and quantification of cotyledon angle (right) of 3-d-old light-grown (5 μmol m^{-2} s^{-1}) Arabidopsis Col-0, Col-4, *bbx16-1*, *GLK1OX* and *GLK1OX bbx16-1* seedlings in the presence or absence of lincomycin. (b) Visual phenotypes (left) and quantification of cotyledon angle (right) of 2-d-old dark-grown Arabidopsis WT, *bbx16-1*, *gun1*, and *gun1bbx16-1* seedlings transferred to light (10 μmol m^{-2} s^{-1}) for 24 h in the presence or absence of lincomycin. (a, b) Bars, 2.5 mm. Letters denote the statistically significant differences among genotypes by Dunn's test ($P < 0.05$), and asterisks indicate statistically significant differences between each *GLK1OX bbx16-1* mutant and *GLK1OX* seedlings (Mann–Whitney test; **, $P < 0.01$; ***, $P < 0.001$). Boxplots indicate the median (centre line), interquartile range (box limits), and minimum and maximum values (whiskers). Linc, lincomycin.

suppression of the open cotyledon phenotype of *gun1* (Fig. 4b). Together, we concluded that BBX16 is a promoter of cotyledon photomorphogenesis in moderate light that is targeted by the GUN1/GLK1 module under high light conditions to protect the seedling by reducing the exposed cotyledon surface.

GLK1 associates with the promoter of *BBX16*

To further understand the mechanism by which the light environment impacts development through the GLK1 regulation of *BBX16* expression, we aimed to test whether *BBX16* was a direct downstream target of GLK1 during de-etiolation. Interestingly, analysis of the promoter region of *BBX16* revealed two CCAATC motifs, described as putative GLK1 binding sequences using Waters *et al.* (2009) based on the enrichment in the promoter regions of GLK1 targets. These two motifs were 2101 bp (Motif 1) and 767 bp (Motif 2) upstream of the transcriptional start site (TSS) (Fig. 5a). Chromatin immunoprecipitation (ChIP) followed by qPCR in light-grown seedlings expressing GLK1-GFP (Waters *et al.*, 2008) detected strong specific binding of GLK1 to the *BBX16* promoter specifically in the region that spanned Motif 2 (P2), whereas no binding was detected in the region containing Motif 1 (P1) or a control sector within the gene body (P3) (Fig. 5b). This result indicated that *BBX16* is indeed a direct target of GLK1 during seedling de-etiolation. Interestingly, we observed that the region spanning Motif 2 also contained an AGATTCT sequence in the reverse strand, identified as a potential GLK1 binding site using protein-binding microarrays (Franco-Zorrilla *et al.*, 2014). It is currently unknown whether the two binding elements in the region spanning Motif 2 are necessary for GLK1 association with the *BBX16* promoter.

BBX16 mediates regulation of only some GLK1-regulated *PhANG* genes

GLKs are key regulators of *PhANGs* (Waters *et al.*, 2009; Zubo *et al.*, 2018). To test whether BBX16 participates in the downregulation of *PhANG* expression in response to retrograde signals, we next studied the expression of the described RS-regulated *PhANGs LCHB1.4*, *LHCB.2.2*, *CA1*, *RBCS1A* and *RBCS3B* (Waters *et al.*, 2009), in low light-grown wild-type, *bbx16*, *BBX16OX*, *gun1*, *GLK1OX* and *GLK1OXbbx16-1* seedlings. In the absence of lincomycin, *LCHB1.4* and *LHCB.2.2* expression was similar to that of the wild-type in all lines tested except in *GLK1OX*, in which expression of both genes was upregulated as described (Waters *et al.*, 2009), and in *BBX16-OX*, in which *LHCB.2.2* expression was approximately two-fold higher compared with the wild-type (Fig. 6). In response to lincomycin, expression levels in *gun1* and *GLK1OX* lines were derepressed in accordance with Waters *et al.* (2009), whereas expression in *BBX16-OX* seedlings was similar to that of the wild-type (Fig. 6). In clear contrast, expression of *CA1*, *RBCS1A* and *RBCS3B* was similar to that of the wild-type in all lines in the absence of lincomycin, but interestingly their expression in *BBX16OX* in the presence of lincomycin was derepressed compared with the wild-type, similarly to *gun1* (Fig. 6). Together, these results can be interpreted to suggest that BBX16 does not mediate the regulation of the *LCHB1.4* and *LHCB.2.2* upon chloroplast damage, whereas BBX16OX exhibits a *gun*-like phenotype for some *PhANGs* such as *CA1*, *RBCS1A* and *RBCS3B*. This difference may be indicative of branching in signalling downstream of GLK1, whereby GLK1-mediated regulation of some *PhANGs* might be indirect through transcriptional regulation of *BBX16* and possibly other factors. Indeed, whereas *LCHB1.4* and *LHCB.2.2* were described as GLK1 primary targets, *CA1*, *RBCS1A* and *RBCS3B* failed to meet the criteria to be considered in this group (Waters *et al.*, 2009). Importantly, *CA1*, *RBCS1A* and *RBCS3B* transcript levels in lincomycin were similar in *GLK1OX* and *GLK1OXbbx16* (Fig. 6). This was in contrast with the clear suppression of the *GLK1OX* cotyledon phenotype in *GLK1OXbbx16* shown above (Fig. 4), suggesting that for *PhANG* expression the contribution of endogenous BBX16 under these conditions might be relatively small.

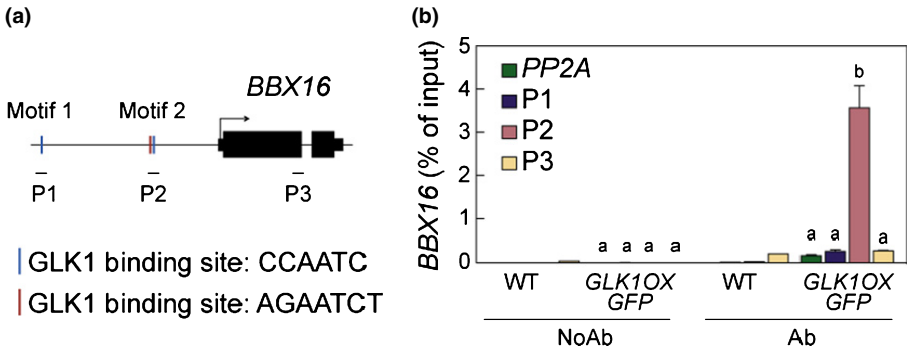

Fig. 5 GOLDEN2-LIKE 1 (GLK1) binds to the *BBX16* promoter. (a) Schematic representation of the *BBX16* promoter and gene body. GLK1 binding sites (CCAATC and AGAATCT) (Waters *et al.*, 2009; Franco-Zorrilla *et al.*, 2014) are indicated with vertical lines in the promoter, and the regions recognised by primer pairs P1, P2 and P3 used in chromatin immunoprecipitation-quantitative polymerase chain reaction (ChIP-qPCR) are underlined (Supporting Information Table S2). (b) GLK1 binding to the *BBX16* promoter in 3-d-old white light (5 μmol m^{-2} s^{-1}) grown Arabidopsis Col-0 and *GLK1OX-GFP* seedlings. Data for *GLK1OX-GFP* correspond to three independent ChIP experiments and error bars indicate the SE. Col-0 controls correspond to one biological replicate. Letters denote the statistically significant differences among *GLK1OX-GFP* samples by Tukey's test ($P < 0.05$). Ab, samples immunoprecipitated with antibody; No Ab, control samples immunoprecipitated without antibody.

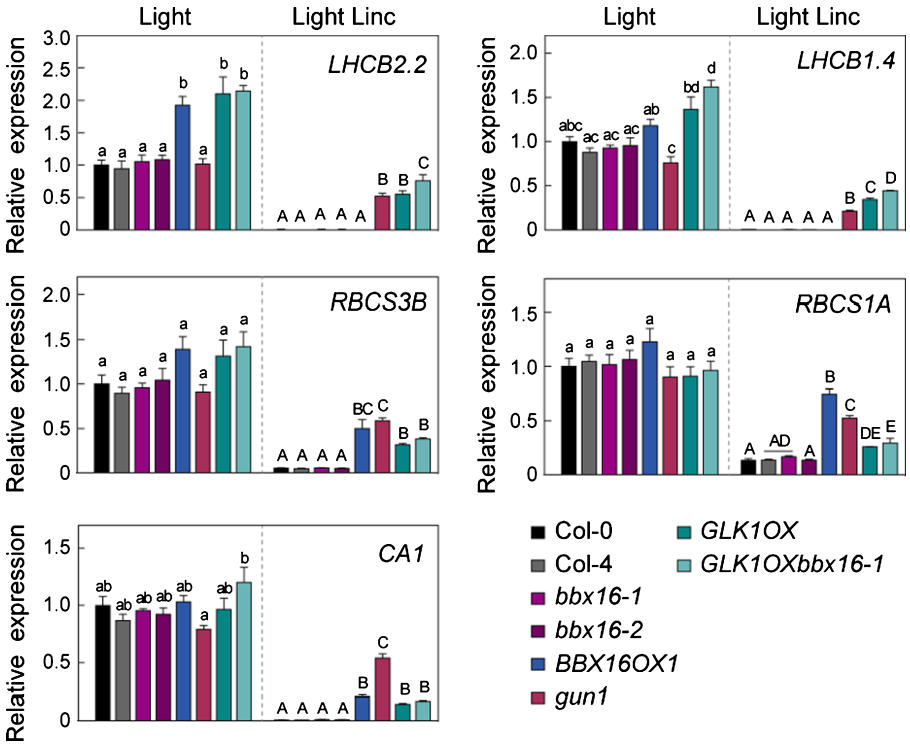

Fig. 6 BBX16 regulation of *PhANG* genes in response to lincomycin. Expression of *LHCB2.2*, *LHCB1.4*, *RBCS3B*, *RBCS1A* and *CA1* was analysed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) in Arabidopsis wild-type (WT), *bbx16*, *BBX16OX*, *gun1*, *GLK1OX* and *GLK1OXbbx16* seedlings grown for 3 d in white light (5 μmol m^{-2} s^{-1}) in the absence or presence of lincomycin. Expression levels relative to Col-0 light are shown. Data are the means ± SE of biological triplicates. Letters denote the statistically significant differences among genotypes by Tukey's test at each condition ($P < 0.05$). Linc, lincomycin. Labelling is indicated by colour, and sequence of represented genotypes is the same within each graph in the Light and Light Linc sections.

Discussion

The establishment of young seedlings after germination is a highly vulnerable process regulated by a myriad of factors, light being one of the most important (Gommers & Monte, 2018). Light induces transcriptional changes of hundreds of genes involved in de-etiolation (Ma *et al.*, 2001), many of them directly regulated by the phytochrome/PIF system, including *GLK1* (Leivar *et al.*, 2009; Pfeiffer *et al.*, 2014). However, because too much light is detrimental for chloroplast function and can hinder establishment, seedlings in potentially photodamaging light initiate RS and inhibit de-etiolation (Ruckle *et al.*, 2007; Martín *et al.*, 2016). This process is mediated by the nuclear-encoded chloroplast-localised PRR protein GUN1, which accumulates preferentially during the early stages of chloroplast biogenesis and under RS conditions (Wu *et al.*, 2018), through a process that is not yet well understood but may require physically interaction with a large number of proteins (Pesaresi & Kim, 2019; Jiang & Dehesh, 2021; Wu & Bock, 2021) involved in plastid translation machinery (Tadini *et al.*, 2016; Marino *et al.*, 2019), tetrapyrrole biosynthesis (Shimizu *et al.*, 2019), RNA editing (Zhao *et al.*, 2019), and plastidial import (Khanna *et al.*, 2009; Wu *et al.*, 2019; Tadini *et al.*, 2020). Given all these putative interactions, GUN1 has been proposed to act as a scaffold protein that promotes protein complex formation (Colombo *et al.*, 2016), and may allow GUN1 to function as an integrator of signals from several RS pathways. Downstream of GUN1, the nuclear-localised GLKs directly regulate *PhANG* expression to inhibit chloroplast development (Waters *et al.*, 2009). The GUN1/GLK1 module has also been shown to be central to the regulation of seedling morphology, although how this takes place was previously unknown (Martín *et al.*, 2016). Here, we show that GLK1 directly induces *BBX16* to promote cotyledon development during seedling de-etiolation in light conditions, sustaining normal photosynthetic activity. By contrast, activation of RS under high light prevents *BBX16* upregulation through GUN1-mediated repression of *GLK1*, and probably other factors, and this keeps the cotyledons underdeveloped to reduce the photosynthetic tissues exposed to light. Therefore, the identification of *BBX16* as a direct target of GLK1 in the regulation of photomorphogenesis defines a new molecular mechanism to optimise development during seedling de-etiolation and to ensure photoprotection of the organism in unfavourable light conditions (Fig. 7).

BBX16 defines a signal branching hub in chloroplast-to-nucleus RS downstream of the GUN1/GLK1 module

Our finding that GLK1 targets *BBX16* to regulate cotyledon development and to possibly regulate some *PhANGs* indirectly, whereas other *PhANGs* are directly regulated by GLK1, establishes a branching point in the regulation of seedling morphology downstream of the GUN1/GLK1 module. This indicates that the signal that GLK1 relays diversifies to specifically regulate different processes central to seedling de-etiolation. Signalling network branching is common in all organisms and contributes to establishing complex responses to a given unique stimulus (Purvis *et al.*, 2008). Interestingly, signal branching was previously described downstream of the PIFs to regulate different organ-specific pathways during seedling de-etiolation (Sentandreu *et al.*, 2011), in which the BBX protein BBX23/MIDA10 was shown to predominantly regulate hook unfolding. Here, whereas direct GLK1 targeting of some *PhANG* genes might allow for fast

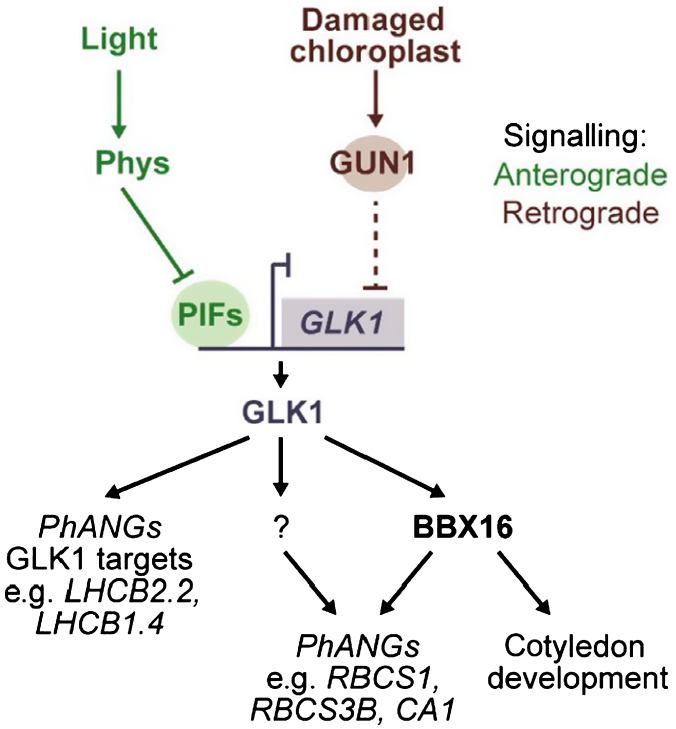

Fig. 7 The GUN1/GLK1 module regulates *BBX16* expression during retrograde signalling. Downstream branching of GOLDEN2-LIKE 1 (GLK1) signalling directly induces two independent transcriptional pathways to regulate expression of (1) photosynthesis-associated nuclear genes (*PhANGs*) such as *LHCB2.2* and *LHCB1.4*; and (2) *BBX16* to implement cotyledon development, and indirect regulation of *PhANGs* such as *CA1*, *RBCS1A* and *RBCS3B*, possibly with involvement of other factors (denoted as ?). In the dark, phytochrome-interacting factors (PIFs) bind to the *GLK1* promoter to directly repress *GLK1* expression. In response to normal light, activated phytochromes (Phys) release PIF repression on the *GLK1* promoter, which triggers *GLK1* transcription. If chloroplast integrity is disrupted by lincomycin or high light, retrograde signals emitted by dysfunctional chloroplasts induce GUN1-mediated repression of *GLK1* expression (and possibly other factors not depicted in the model) by a yet unknown mechanism, preventing *BBX16* and *PhANGs* transcription to block the progression of photomorphogenesis. Arrows and blunt arrows represent positive and negative regulation, respectively, and the dashed arrow represents indirect effects through an unknown intermediate factor(s).

regulation of chloroplast protection to, for example, fluctuations in light conditions, branching of the signal to repress *BBX16* and its target effectors would entail a slower response to arrest cotyledon development only in more sustained high light conditions, a possibility that needs further investigation.

BBX16 is the first described BBX protein involved in RS

Our finding that BBX16 is a downstream target of the GUN1/GLK1 module in RS-regulated development identifies the first BBX protein involved in the response to chloroplast damage. This adds to previously described members of the BBX family with regulatory roles in stress-induced signalling pathways, such as BBX24/STO in responses to salt (Nagaoka & Takano, 2003), BBX18 and BBX23 to heat (Q. Wang *et al.*, 2013; Ding *et al.*, 2018), or BBX7 and BBX8 to cold stress (Li *et al.*, 2021). In addition, altered expression levels of *BBX19* were found in *ceh1*, a mutant with high levels of the MEcPP retrograde signal (Xiao *et al.*, 2012), although the significance is still unclear (Wang *et al.*, 2014). Interestingly, a recent bioinformatics analysis of the *BBX* family identified that the promoter region of *BBX16* contains *cis* elements predicted to be abscisic acid, low temperature and drought responsive (Lyu *et al.*, 2020), which could indicate a role for BBX16 in the cross-talk between different stress pathways.

The BBX family in *Arabidopsis thaliana* consists of 32 proteins arranged into five structural groups (I–V) based on the number of B-box motifs (one or two) and the presence or absence of a CCT domain and a VP motif (Robson *et al.*, 2001; Kumagai *et al.*, 2008; Khanna *et al.*, 2009; Gangappa & Botto, 2014). BBX16/COL7 belongs to the Class III clade, the least characterised of the BBX groups, together with BBX14/COL6, BBX15/COL16 and BBX17/COL8, defined by having only one B-box motif (B-box 1) in combination with a CCT domain. The expression patterns shown in Fig. S3 indicated that *BBX14* and *BBX15* respond similarly to *BBX16*. Because functional redundancy is common among members of the same clade within transcription factor families (Soy *et al.*, 2014; Pfeiffer *et al.*, 2014; Zhang *et al.*, 2017; Leivar *et al.*, 2020; Martín *et al.*, 2020) this led us to speculate that BBX14 and BBX15 might share some functional aspects with BBX16. Redundancy within this clade would imply that the *bbx16* mutant still retains functionality and, accordingly, we detected more prominent cotyledon phenotypes in *BBX16-OX* compared with *bbx16*. Future genetic characterisation of single and high order mutant combinations in *bbx14*, *bbx15* and *bbx16* will shed light on possible functional redundancy and address whether BBX14 and BBX15 might also play a regulatory role in response to chloroplast damage. Interestingly, a recent transcriptomic study identified the Class III clade as a potential player in response to high light (Huang *et al.*, 2019). Of future interest will be, as well, to explore whether the BBX family of transcription factors has functionally evolved and diverged to specialise only in the Class III clade in RS regulation, or whether BBX factors from other clades might also be involved.

The domain-function structure of BBX16, a promoter of photomorphogenesis

The domain structure of BBX proteins has important functional implications. B-box domains have been involved in protein–protein interactions and transcriptional regulation, whereas the CCT harbours a nuclear localisation signal (NLS) to mediate nuclear protein transport (Robson *et al.*, 2001), and has also been shown to participate in the association with DNA (Ben-Naim *et al.*, 2006; Tiwari *et al.*, 2010). CCT-containing BBX proteins include CONSTANTS (BBX1/CO), one of the best studied BBX proteins and the founder of the family. In cotyledons, CCT is required to interact with the E3 ubiquitin ligases COP1 and SPA proteins (Laubinger *et al.*, 2006; Jang *et al.*, 2008), whereas the B-box1 domain mediates interaction with BBX19 (Wang *et al.*, 2014). In the regulation of seedling photomorphogenesis,

some BBX proteins are related to the COP1/SPA-HY5 regulatory hub (Gangappa & Botto, 2014; Song *et al.*, 2020a; Xu, 2020). Several of these BBX proteins interact with COP1 and are regulated in a COP1-dependent manner, and/or regulate HY5 transcription, stability or activity (Datta *et al.*, 2006; Chang *et al.*, 2011; Holtan *et al.*, 2011; Jiang *et al.*, 2012; Gangappa *et al.*, 2013; Huang *et al.*, 2014; Wei *et al.*, 2016; Xu *et al.*, 2016; Zhang *et al.*, 2017; Ding *et al.*, 2018; Job *et al.*, 2018; Lin *et al.*, 2018; Bursch *et al.*, 2020). Furthermore, BBX4 has been shown to interact with PIF3 and repress its activity in red light (Heng *et al.*, 2019a), whereas BBX18 and BBX23 have been shown to interact with ELF3 and regulate thermomorphogenesis in Arabidopsis (Ding *et al.*, 2018). Whether BBX16 is regulated by the COP/SPA system, and whether BBX16 regulation of cotyledon development downstream of the GUN1/GLK1 module involves HY5 or other interacting proteins, are matters that await future research. Interestingly, the CCT domain of BBX16/COL7 has been shown to mediate binding to the promoter of the auxin biosynthesis repressor *SUR2* in the regulation of plant architecture under shade conditions in Arabidopsis adult plants (Zhang *et al.*, 2014). In addition, other BBX factors such as BBX20 and BBX32 have been shown to regulate photomorphogenesis through mediating brassinosteroid and strigolactone homeostasis (Wei *et al.*, 2016; Ravindran *et al.*, 2021). Because auxin and other hormones are well known key regulators of photomorphogenesis, and integration of retrograde and hormonal signalling is essential in the adaptation to a myriad of stresses (Jiang & Dehesh, 2021), it will be of interest in the future to explore a connection of RS-mediated control of BBX16 with key regulatory genes in diverse hormone pathways that could impact cotyledon development.

To conclude, this study supports a model whereby *BBX16* is directly targeted by GLK1 to induce cotyledon photomorphogenesis under light conditions favourable for seedling de-etiolation. By contrast, when GUN1-mediated RS is activated, the inhibition of *GLK1*, *BBX16* and *PhANG* expression limits cotyledon and chloroplast development to minimise light damage and optimise photoprotection. The importance of this response is illustrated by studies with *gun1* seedlings exposed to high light that exhibited more photobleached areas in their cotyledons compared with the wild-type controls (Ruckle *et al.*, 2007). This adaptive mechanism would protect an etiolated seedling, which is extremely vulnerable, emerging into excess light such as found on a hot sunny day. This could take place transiently during establishment, allowing the seedling to prevent damage and wait safely for the light to become less strong due to shading or the natural shift in the position of the sun.

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Author contributions

EM, PL, GM and NV conceived the project and planned the experiments. GM and NV performed experiments and analysed the data. All authors wrote the manuscript. NV and GM contributed equally to this work.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Model depicting the criteria we followed to identify putative regulators of cotyledon development downstream of GLK1.

Fig. S2 Molecular characterisation of *bbx16-2*.

Fig. S3 *BBX14*, *BBX15* and *BBX16* are similarly regulated by PIFs and GLK1 in dark and light.

Fig. S4 Characterisation of *gun1glk1*.

Fig. S5 Characterisation of *GLK1OXbbx16*.

Table S1 List of primers used for genotyping.

Table S2 List of primers used for quantitative reverse transcriptase (qRT-PCR).

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