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ORIGINAL ARTICLE

Self-transcriptional repression of the Arabidopsis NAC transcription factor ATAF2 and its genetic interaction with phytochrome A in modulating seedling photomorphogenesis

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Abstract

Main conclusion **The NAC transcription factor ATAF2 suppresses its own transcription via self-promoter binding. ATAF2 genetically interacts with the circadian regulator CCA1 and phytochrome A to modulate seedling photomorphogenesis in *Arabidopsis thaliana*.**

Abstract ATAF2 (ANAC081) is a NAC (NAM, ATAF and CUC) transcription factor (TF) that participates in the regulation of disease resistance, stress tolerance and hormone metabolism in *Arabidopsis thaliana*. We previously reported that ATAF2 promotes Arabidopsis hypocotyl growth in a light-dependent manner via transcriptionally suppressing the brassinosteroid (BR)-inactivating cytochrome P450 genes *BAS1* (*CYP734A1*, formerly *CYP72B1*) and *SOB7* (*CYP72C1*). Assays using low light intensities suggest that the photoreceptor phytochrome A (PHYA) may play a more critical role in ATAF2-regulated photomorphogenesis than phytochrome B (PHYB) and cryptochrome 1 (CRY1). In addition, ATAF2 is also regulated by the circadian clock. The core circadian TF CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) physically interacts with ATAF2 at the DNA–protein and protein–protein levels, and both differentially suppress BAS1- and SOB7-mediated BR catabolism. In this research, we show that ATAF2 can bind its own promoter as a transcriptional self-repressor. This self-feedback-suppression loop is a typical feature of multiple circadian-regulated genes. Additionally, *ATAF2* and *CCA1* synergistically suppress seedling photomorphogenesis as reflected by the light-dependent hypocotyl growth analysis of their single and double gene knock-out mutants. Similar fluence-rate response assays using *ATAF2* and photoreceptor (*PHYB*, *CRY1* and *PHYA*) knock-out mutants demonstrate that PHYA is required for ATAF2-regulated photomorphogenesis in a wide range of light intensities. Furthermore, disruption of *PHYA* can suppress the BR-insensitive hypocotyl-growth phenotype of *ATAF2* loss-of-function seedlings in the light, but not in darkness. Collectively, our results provide a genetic interaction synopsis of the circadian-clock-photomorphogenesis-BR integration node involving ATAF2, CCA1 and PHYA.

Keywords ATAF2 · Brassinosteroid · CCA1 · Hypocotyl growth · Photomorphogenesis · Phytochrome A

Abbreviations

BL	Brassinolide
BR	Brassinosteroid
CBS	CIRCADIAN CLOCK ASSOCIATED 1-binding site

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CCA1	CIRCADIAN CLOCK ASSOCIATED 1
CRY1	Cryptochrome 1
EMSA	Electrophoretic mobility shift assay
NAC	NAM ATAF and CUC
PHYA	Phytochrome A
PHYB	Phytochrome B
TF	Transcription factor
Y1H	Yeast one-hybrid

Introduction

A wide range of plant activities are regulated by kingdom-specific transcription factors (TFs), such as the NAM, ATAF and CUC (NAC) TF family (Mathew and Agarwal 2018). Specifically, the four ATAF NAC TF homologs in the model plant *Arabidopsis thaliana* (Kleinow et al. 2009; Christianson et al. 2010), including ATAF1 (ANAC002), ATAF2 (ANAC081), ANAC102 and ANAC032, have been demonstrated to extensively regulate plant defense responses such as abiotic stress tolerance (Lu et al. 2007; Christianson et al. 2009; Wu et al. 2009; D'Alessandro et al. 2018), disease resistance (Delessert et al. 2005; Jensen et al. 2008; Wang et al. 2009a, b; Wu et al. 2009; Allu et al. 2016), abscisic acid biosynthesis (Jensen et al. 2013), leaf senescence (Garapati et al. 2015b; Takasaki et al. 2015; Mahmood et al. 2016a; Nagahage et al. 2020), and metabolism of defense-related compounds (Garapati et al. 2015a; Zhao et al. 2018a; Mahmood et al. 2016b; Sun et al. 2019a). Depending on promoter context, downstream target genes, developmental stages and growth conditions, ATAF TFs can act as either a transcriptional activator or a repressor (Delessert et al. 2005; Wang et al. 2009b; Peng et al. 2015; Mahmood et al. 2016a, 2016b; Nagahage et al. 2018).

In addition to their roles in defense responses, ATAF TFs were also found to regulate plant development and relevant hormonal metabolism. For example, ANAC032 modulates root cell elongation via the MYB30 transcriptional cascade (Maki et al. 2019). ATAF2 can activate the expression of the auxin biosynthetic gene *NIT2* via direct promoter binding (Huh et al. 2012). We previously reported that ATAF2 promotes Arabidopsis hypocotyl growth in a light-dependent manner (Peng et al. 2015) via transcriptionally suppressing brassinosteroid (BR)-inactivating cytochrome P450 genes *BAS1* (*CYP734A1*, formerly *CYP72B1*) (Neff et al. 1999; Turk et al. 2003; Thornton et al. 2011) and *SOB7* (*CYP72C1*) (Nakamura et al. 2005; Takahashi et al. 2005; Turk et al. 2005; Thornton et al. 2010). ATAF2 is also subject to circadian regulation (Peng and Neff 2020) of the core clock TF CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) (Wang and Tobin 1998). CCA1 and ATAF2 physically interacts at both DNA–protein and protein–protein levels to differentially suppress *BAS1* and *SOB7* expression (Peng and Neff 2020).

ATAF2 has extensive promoter motif-binding targets that share a common A/T-rich feature (Wang et al. 2009b, 2012; Huh et al. 2012; Peng et al. 2015). The protein interaction partners of ATAF2 include two AT-hook-motif containing nuclear-localized (AHL) proteins that specifically bind A/T-rich DNA (Zhao et al. 2013). The ATAF2-binding targets on the *BAS1* and *SOB7* promoters are the A/T-rich Evening Element (EE; AAAATATCT) and CCA1-binding site (CBS; AAAAATCT), both of which are well-known binding sites of CCA1 (Michael and McClung 2002; Harmer and Kay 2005; Zhai et al. 2019). There is also a CBS on the promoter of *ATAF2* itself, and CCA1 can bind this site and suppress *ATAF2* expression in the light (Peng and Neff 2020). Since ATAF2 can also bind CBS, it is possible that ATAF2 binds its own promoter to self-regulate transcript accumulation. In this research, we demonstrated that ATAF2 is a self-transcriptional repressor using a promoter-GUS fusion system. The self-suppressing characteristic of ATAF2 is similar to that of CCA1 (Wang and Tobin 1998), which is a common feature for multiple circadian-oscillated genes (Schaffer et al. 1998; Helfer et al. 2011; Adams et al. 2015). We further showed that ATAF2 and CCA1 synergistically suppress seedling photomorphogenesis.

The hypocotyl growth of Arabidopsis seedlings is suppressed upon photomorphogenesis, which is regulated by multiple light and hormonal signals (Neff et al. 2000). The genetic interactions between the far-red light photoreceptor phytochrome A (PHYA), the red-light receptor phytochrome B (PHYB), and the blue light receptor cryptochrome 1 (CRY1) play a central role in seedling photomorphogenesis (Neff and Chory 1998). BAS1 and SOB7 redundantly modulate photomorphogenesis via inactivating BRs (Turk et al. 2003, 2005), and they have complex genetic interactions with PHYA, PHYB and CRY1 (Sandhu et al. 2012). Our preliminary hypocotyl growth and *ATAF2* transcript analyses in low-fluence white light as well as monochromatic light conditions (10 µmol m^{-2} s^{-1}) suggest that PHYA may be the essential photoreceptor for ATAF2-regulated photomorphogenesis (Peng et al. 2015). Here, we investigated the genetic interactions between ATAF2 and the three major photoreceptors via fluence-rate response assays and demonstrated the necessary role of PHYA in ATAF2-mediated photomorphogenic suppression. Furthermore, PHYA is shown to be essential for the reduced BR sensitivity phenotype of an *ATAF2* loss-of-function mutant in the light, but not in darkness. Collectively, our results provide a genetic interaction synopsis of the circadian-clock-photomorphogenesis-BR integration node involving ATAF2, CCA1 and PHYA.

Materials and methods

Arabidopsis genotypes

The Arabidopsis Columbia (Col-0) ecotype was used as the wild-type control. *ataf2-1* (SALK_136355), *ataf2-2* (SALK_015750) and *cca1-1* (CS67781) T-DNA insertional mutants were obtained from the Arabidopsis Biological Resource Center (ABRC). The original *cca1-1* mutant was identified in the Ws genetic background (Green and Tobin 1999) and later introgressed into Col-0 via six rounds of backcrossing (Yakir et al. 2009). Photoreceptor knock-out mutants *phyB-9* (Reed et al. 1993), *cry1-103* (Liscum and Hangarter 1991) and *phyA-211* (Reed et al. 1994) are all in the Col-0 background. All gene knock-out mutants, *cca1-1 ataf2-2*, *phyB-9 ataf2-1*, *cry1-103 ataf2-1*, and *phyA-211 ataf2-1* double knock-out mutants, and the *ATAF2* overexpression line *ATAF2ox-1* have been previously described and verified (Peng et al 2015; Peng and Neff 2020).

GUS assays

p*ATAF2*::GUS is a transcriptional fusion of GUS with 2-kb ATAF2 promoter (Wang et al. 2009b). GUS histochemical staining of six-day-old p*ATAF2*::GUS/Col-0 and p*ATAF2*::GUS/*ataf2-1* transgenic-segregation seedlings was performed as previously described (Sandhu et al. 2012). GUS-stained seedlings were photographed using the Leica MZ10 F and DFC295 digital microscope/imaging workstation. GUS activity was quantified by measuring the amount of fluorescent 4-methylumbelliferone (MU) produced by GUS-mediated hydrolysis of 4-methylumbelliferyl β-D-glucuronide (MUG) (Sheng et al. 2014). In brief, 10 mg (20–25 seedlings) of plant tissue was ground in 600 µL of GUS extraction buffer. 100 µL of supernatant was mixed with 40 µL of 10 mM MUG and incubated at 37 °C for 1 h. After adding 1 mL of sodium carbonate stop buffer, 200 µL of mixture was added to a black microtiter plate for fluorescence measurement using a fluorometer (Molecular Devices SpectraMax M2). Stop buffer, MUG + stop buffer, and GUS + stop buffer mixtures were used as negative controls. Serial MU dilutions were used to make a standard curve. Five independent replicates were performed for each sample.

Seedling growth

Arabidopsis seedling growth conditions have been described previously (Favero et al. 2016, 2017). Unless otherwise stated, ethanol-sterilized seeds were plated on half-strength Linsmaier-Skoog medium with 10 g/L USA-made Phytagel (Sigma-Aldrich) (Jacques et al. 2020) and 15 g/L sucrose. For the exogenous brassinolide (BL) response assay, gradient concentrations of BL were added to the media with the same volume of ethanol applied to all plates. After four-day stratification at 4 °C in the dark followed by red light treatment to induce germination, seeds were grown at 25 °C in designated dark, continuous white (red:far-red light ratio 1:1; 0–80 µmol m^{-2} s^{-1}) or monochromatic light intensities. Seeds used for each physiological or molecular assay were harvested from the same batch of plants grown in the greenhouse. For comparative fluence-rate response assays, seedlings were grown on plates without sucrose. The growth temperature was lowered to 20 °C in certain assays as indicated.

Hypocotyl measurement

Depending on experimental purposes, hypocotyl lengths of three- (for monochromatic fluence-rate response assays) or four-day-old (for white-light fluence-rate response and BL response assays) Arabidopsis seedlings were measured from scanned seedling images using NIH ImageJ (Schneider et al. 2012). We have previously used four-day-old seedlings for fluence-rate and BR -response assays in white light (Peng et al. 2015; Peng and Neff 2020). Compared to white light, both red and blue light are less efficient in inhibiting hypocotyl growth (Neff and Chory 1998), which leads to a relatively long-hypocotyl phenotype. Thus, three-day-old seedlings were used for monochromatic fluence-rate response assays (Peng et al. 2015) to obtain hypocotyl measurement results that are comparable to those from four-day-old seedlings grown in white light. To minimize the potential impact of non-simultaneous germination, only the measurement result of the thirty tallest seedlings was included for each data point (Fankhauser and Casal 2004). Each assay had three independent replicates showing a similar trend of differences.

Quantitative PCR

Total RNA samples were extracted from six-day-old p*ATAF2*::GUS/Col-0 and p*ATAF2*::GUS/*ataf2-1* seedlings with on-column DNase I digestion to eliminate genomic DNA contamination. First-strand cDNA reverse transcription and quantitative PCR (RT-qPCR) were performed using Bio-Rad iScript followed by SYBR Green Supermix. The Bio-Rad CFX96 Real-Time System and CFX Manager Software were used to generate and analyze qPCR data using the $\Delta\Delta C_T$ method. *UBQ10* (AT4G05320) was used as the reference gene for normalization of *GUS* transcript levels. qPCR primers for *UBQ10* were described previously (Peng et al. 2015). qPCR primers for *GUS* are 5′-CGTCCTGTAGAAACCCCAACC-3′ and 5′-GCTTTCCCACCAACGCTGATC-3′.

Yeast one-hybrid

A Gateway-compatible yeast one-hybrid (Y1H) system (Deplancke et al. 2006) was used to test the interaction between ATAF2 and the CBS-containing DNA fragment (p*ATAF2*-CBS) from its own promoter. p*ATAF2*-CBS sequence and the Y1H procedure were described previously (Peng et al. 2015; Peng and Neff 2020). Interaction between the p*ATAF2*-CBS bait and the ATAF2 prey was demonstrated by increased yeast tolerance to the His3p enzyme inhibitor 3-aminotriazole (3-AT). An empty prey vector was used as the negative control.

Electrophoretic mobility shift assay

N- and C-terminal Hexa-histidine tagged ATAF2 (His-ATAF2 and ATAF2-His) were expressed in the *Escherichia coli* strain Rosetta, respectively. ATAF2 proteins were purified using HisPur Ni–NTA resin followed by imidazole removal using the Slide-A-Lyzer mini dialysis device (Thermo Fisher Scientific). Electrophoretic mobility shift assay (EMSA) was carried out using the fluorescence-based EMSA kit (Invitrogen). Non-denaturing polyacrylamide gel electrophoresis (PAGE) was used to separate the DNA probe (p*ATAF2*-CBS) and DNA–protein complexes (p*ATAF2*-CBS + His-ATAF2 or p*ATAF2*-CBS + ATAF2-His). The Bio-Rad ChemiDoc Touch imaging system was used to scan SYBR Green-stained DNA bands.

Statistical analysis

Two-tailed Student's *t* test was used for two-group comparison. When comparing multiple groups, one-way ANOVA with Tukey's HSD test was applied with significant differences being labeled by different letters. The *P* value significance level was set as 0.05.

Results

ATAF2 binds its own promoter

Since the CBS DNA motif is a binding target of ATAF2 (Peng et al. 2015) and a CBS (− 577 to − 570) can be found on the *ATAF2* promoter (Peng and Neff 2020), we tested the binding of ATAF2 to its own 63-bp promoter fragment p*ATAF2*-CBS (− 598 to − 536; with CBS being the only predicted TF binding site) in a targeted Y1H assay (Fig. 1a). Both 20-mM and 40-mM 3-AT drastically inhibited the growth of yeast harboring the p*ATAF2*-CBS bait and the empty prey vector in a concentration-dependent manner (Fig. 1a). In contrast, the coexistence of p*ATAF2*-CBS bait and ATAF2 prey significantly increased yeast tolerance to the same concentrations of 3-AT (Fig. 1a). These results demonstrate that ATAF2 can bind its own promoter (p*ATAF2*-CBS). The interaction between ATAF2 and p*ATAF2*-CBS was further confirmed in an EMSA assay, demonstrating that both N- and C-terminal 6xHis-fusion ATAF2 proteins (His-ATAF2 and ATAF2-His) can physically bind the p*ATAF2*-CBS DNA probe in a dose-dependent manner (Fig. 1b).

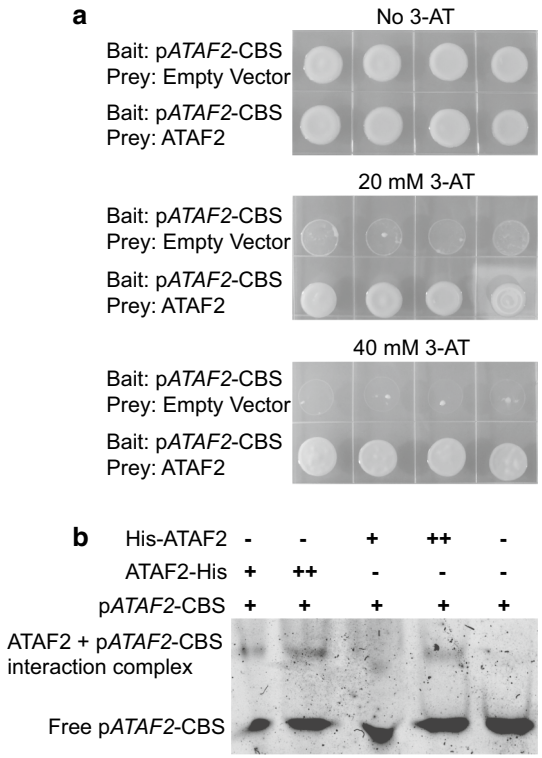

Fig. 1 ATAF2 binds its own promoter. **a** ATAF2 bind its own CBS-containing promoter fragment p*ATAF2*-CBS in a targeted Y1H assay. Enhanced 3-AT tolerance in yeast harboring the *ATAF2*-CBS bait and the ATAF2 prey represented the detection of bait-prey interaction. Four independent clones were shown for each Y1H sample. **b** In an EMSA assay, both His-ATAF2 and ATAF2-His tagged proteins physically bind the p*ATAF2*-CBS DNA probe in a dose-dependent manner

ATAF2 is a self-transcriptional repressor

ATAF2 can be either an activator (Wang et al. 2009b; Huh et al. 2012) or a repressor (Delessert et al. 2005; Peng et al. 2015) of its downstream-regulated genes (Nagahage et al. 2018). To examine whether ATAF2 activates or represses itself, the p*ATAF2*::GUS/Col-0 transgenic line expressing an *ATAF2* promoter-*GUS* transcriptional fusion (Wang et al. 2009b; Peng and Neff 2020) was crossed with the *ATAF2* knock-out mutant *ataf2-1* (Fig. 2a). Homozygous p*ATAF2*::GUS/Col-0 and p*ATAF2*::GUS/*ataf2-1* lines were characterized from F3 segregants, with two lines being selected from each genotype for GUS expression

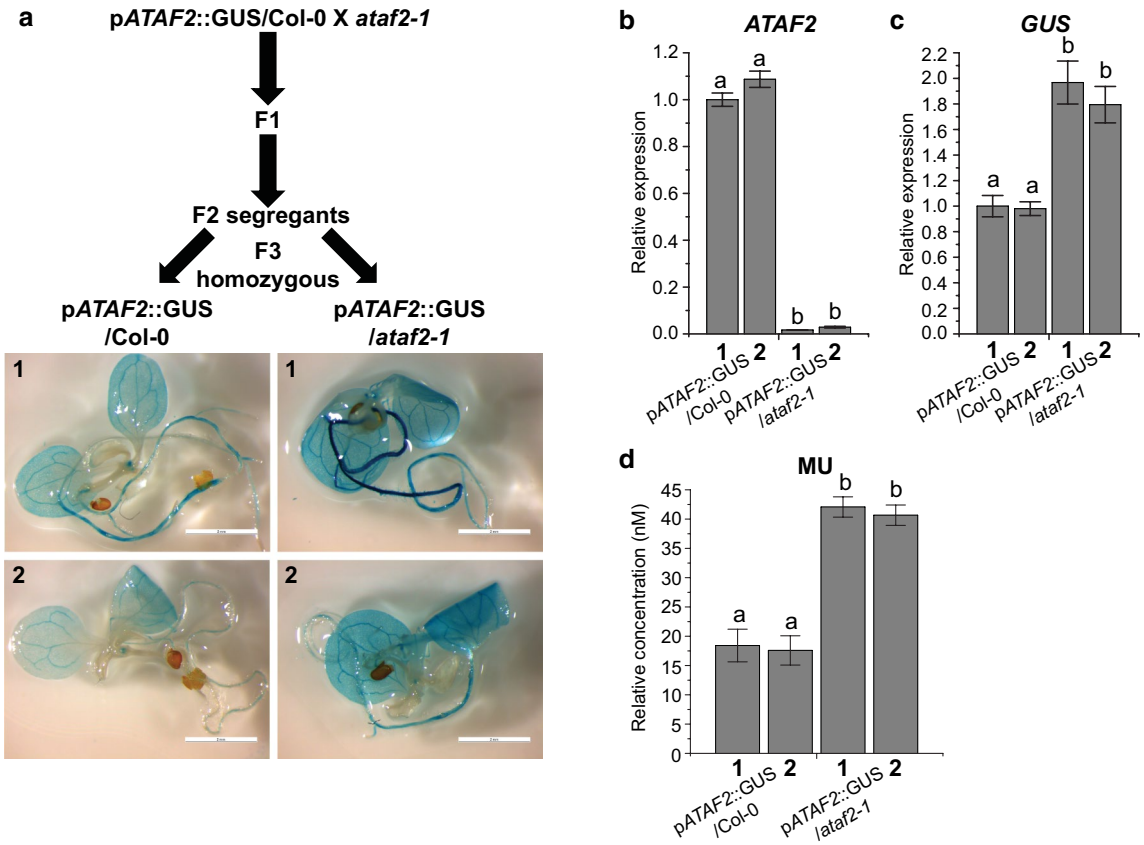

Fig. 2 ATAF2 is a self-transcriptional repressor. **a** The p*ATAF2*::GUS/Col-0 transgenic line expressing *ATAF2* promoter-*GUS* transcriptional fusion was crossed with the *ATAF2* knock-out mutant *ataf2-1*. Homozygous p*ATAF2*::GUS/Col-0 and p*ATAF2*::GUS/*ataf2-1* lines were characterized from F3 segregants, with two lines being selected from each genotype for GUS expression analysis. Six-day-old white-light-grown seedlings were used for GUS staining and RT-qPCR assays. Both p*ATAF2*::GUS/*ataf2-1* lines showed higher GUS protein accumulations than two p*ATAF2*::GUS/Col-0 lines, with no apparent change of spatial expression pattern. Scale bars = 2 mm. **b** RT-qPCR assay confirmed that *ATAF2* expression was disrupted in both p*ATAF2*::GUS/*ataf2-1* lines. **c** RT-qPCR assays confirmed that when compared to p*ATAF2*::GUS/Col-0 lines, both p*ATAF2*::GUS/*ataf2-1* lines exhibited significantly elevated *GUS* transcript accumulation. **d** GUS activity was quantified by its hydrolysis capacity of converting MUG to MU. When compared to p*ATAF2*::GUS/Col-0 lines, both p*ATAF2*::GUS/*ataf2-1* lines exhibited significantly elevated GUS enzyme activity. Each RT-qPCR data point represents the mean value of three biological replicates × three technical replicates ($n = 9$). Each MU concentration data point represents the mean value of five independent replicates. Error bars denote the SE. The significance of differences was determined by one-way ANOVA with Tukey's HSD test. Groups with significant differences ($P < 0.05$) were labeled by different letters

analysis (Fig. 2a). Six-day-old white-light-grown seedling were used for GUS staining and RT-qPCR assays. Both p*ATAF2*::GUS/*ataf2-1* lines showed higher GUS protein accumulation than the two p*ATAF2*::GUS/Col-0 lines, with no apparent change of spatial expression pattern (Fig. 2a). RT-qPCR assays confirmed that when compared to p*ATAF2*::GUS/Col-0 lines, both p*ATAF2*::GUS/*ataf2-1* lines exhibited disrupted *ATAF2* expression (Fig. 2b) and significantly elevated *GUS* transcript accumulation (Fig. 2c). Quantifications of GUS activity in p*ATAF2*::GUS/Col-0 and p*ATAF2*::GUS/*ataf2-1* seedlings also showed consistent results of higher GUS protein accumulation negatively correlated to *ATAF2* disruption (Fig. 2d). These results demonstrate the transcriptional suppression activity of ATAF2 on its own promoter, which is a common feature of multiple circadian-regulated genes (Adams et al. 2015) including CCA1 (Wang and Tobin 1998).

ATAF2 and CCA1 synergistically suppress seedling photomorphogenesis

Arabidopsis seedlings have shorter hypocotyls when undergoing photomorphogenesis. Both ATAF2 and CCA1 are repressors of BR inactivation (Peng et al. 2015; Peng and Neff 2020) and photomorphogenesis (Peng et al. 2015; Zhao et al. 2018b). Therefore, we tested their genetic interaction in a white-light fluence-rate-response assay using four-day-old seedling of Col-0, *ataf2-2*, *cca1-1* and the *cca1-1 ataf2-2* double mutant (Fig. 3a). In low fluence rates (10–40 μmol m^{-2} s^{-1}), the disruption of either *ATAF2* (*ataf2-2*) or *CCA1* (*cca1-1*) resulted

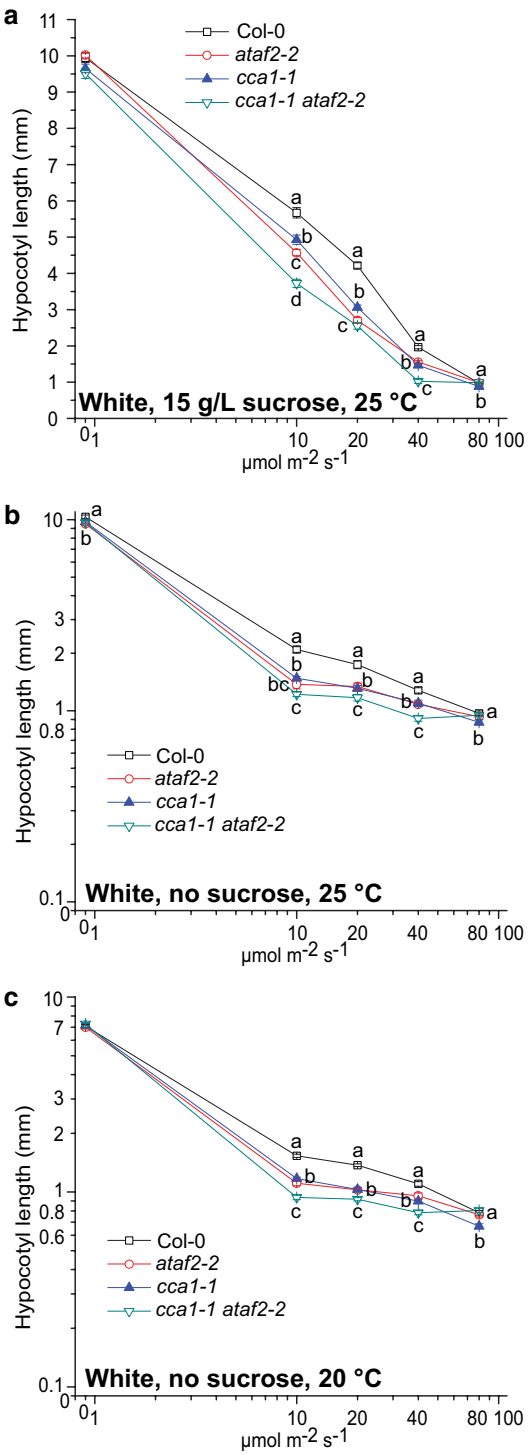

Fig. 3 ATAF2 and CCA1 synergistically suppress seedling photomorphogenesis. Four-day-old seedlings of Col-0, *ataf2-2*, *cca1-1* and the *cca1-1 ataf2-2* double mutant were used in a fluence-rate response assay in continuous white light. **a** In low fluence rates (10–40 μmol m^{-2} s^{-1}), the disruption of either *ATAF2* (*ataf2-2*) or *CCA1* (*cca1-1*) resulted in a short-hypocotyl phenotype, with *cca1-1 ataf2-2* largely exhibiting further suppressed hypocotyl growth. **b** Similar results were observed in the absence of sucrose in the growth media. **c** Similar results were observed in the absence of sucrose and seedlings were grown at 20 °C instead of 25 °C. Each data point represents the average of measurements from 30 seedlings ($n = 30$). Error bars denote the SE. The significance of differences was determined by one-way ANOVA with Tukey's HSD test. Groups with significant differences ($P < 0.05$) at a given fluence rate were labeled by different letters

in a short-hypocotyl phenotype, with *cca1-1 ataf2-2* largely exhibiting further suppressed hypocotyl growth (Fig. 3a). Similar results were observed when sucrose was removed from the media (Fig. 3b) and the plant growth temperature was further lowered to 20 °C (Fig. 3c). These results indicate a genetically synergistic effect of ATAF2 and CCA1 in suppressing seedling photomorphogenesis.

ATAF2 loss- and gain-of-function seedlings retain opposite photomorphogenic phenotypes in monochromatic light conditions

We previously reported that *ATAF2* loss- and gain-of-function seedlings exhibit opposite photomorphogenic phenotypes in white light (Peng et al. 2015). In low fluence rates, *ATAF2* knock-out mutants have shorter hypocotyls when compared to the wild type whereas *ATAF2* overexpression lines show longer hypocotyl growth (Peng et al. 2015). *ataf2-1* retains its short-hypocotyl phenotype in 10 μmol m^{-2} s^{-1} of red, blue or far-red light (Peng et al. 2015). Here, we further showed that *ATAF2* loss- (*ataf2-1*) and gain-of-function (*ATAF2ox-1*) seedlings retain their opposite hypocotyl-growth phenotypes in multiple fluence rates of monochromatic red (Fig. 4a), blue (Fig. 4b) or far-red (Fig. 4c) light. These results confirmed that ATAF2-regulated seedling photomorphogenesis is not subject to any single monochromatic light. Fluence rates as low as 0.1 μmol m^{-2} s^{-1} (Fig. 4a,c) were applied for both red and far-red light, as they are primary signals for low fluence rate responses (LFRs). Col-0 seedlings grown in low fluence rates of red light were even slightly taller than dark-grown seedlings (Fig. 4a). This phenotype disappeared with the removal of sucrose from the media (Fig. S1).

PHYA is required for ATAF2-regulated seedling photomorphogenesis

We previously showed that when compared to *phyB-9* and *cry1-103*, the *phyB-9 ataf2-1* and *cry1-103 ataf2-1* double mutants retain their short-hypocotyl phenotype in 10 μmol m^{-2} s^{-1} of red and blue light, respectively (Peng et al. 2015). In contrast, the short-hypocotyl phenotype is largely abolished in *phyA-211 ataf2-1* in 10 μmol m^{-2} s^{-1} of red, blue or far-red light (Peng et al. 2015). These preliminary results suggest that PHYA may be the major photoreceptor for ATAF2-regulated seedling

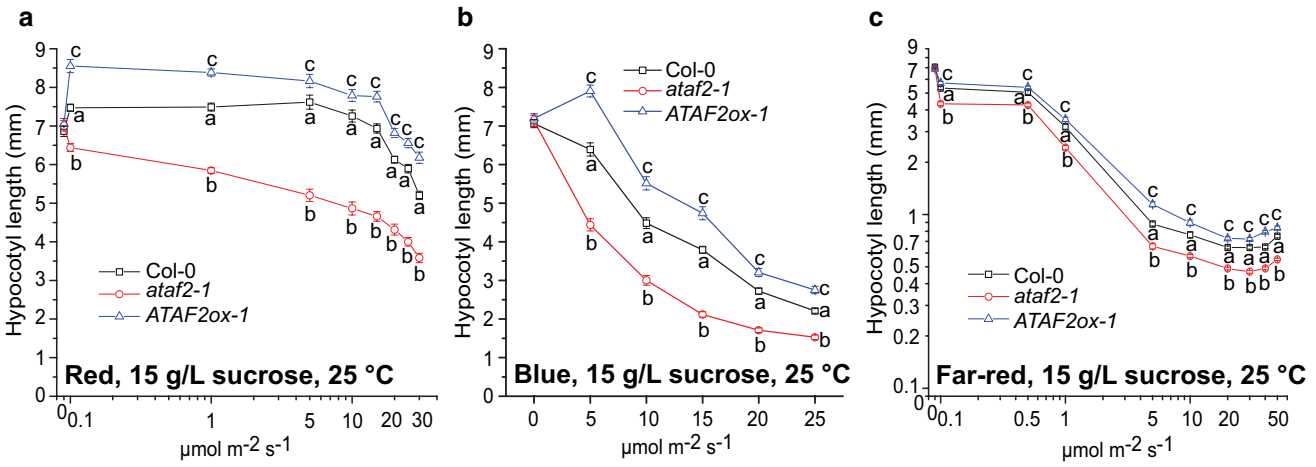

Fig. 4 *ATAF2* loss- and gain-of-function seedlings retain opposite photomorphogenic phenotypes in monochromatic light conditions. **a** *ataf2-1* and *ATAF2ox-1* seedlings retain their opposite hypocotyl-growth phenotypes in multiple fluence rates of monochromatic red light. **b** *ataf2-1* and *ATAF2ox-1* seedlings retain their opposite hypocotyl-growth phenotypes in multiple fluence rates of blue light. **c** *ataf2-1* and *ATAF2ox-1* seedlings retain their opposite hypocotyl-growth phenotypes in multiple fluence rates of monochromatic far-red light. Three-day-old seedlings of Col-0, *ataf2-1*, and *ATAF2ox-1* were used for all three monochromatic fluence-rate response assays. Each data point represents the average of measurements from 30 seedlings ($n = 30$). Error bars denote the SE. The significance of differences was determined by one-way ANOVA with Tukey's HSD test. Groups with significant differences ($P < 0.05$) at a given fluence rate were labeled by different letters

photomorphogenesis. To further test this hypothesis, fluence-rate (0–80 μmol m^{-2} s^{-1}) response assays in white light were performed for *phyB-9 ataf2-1*, *cry1-103 ataf2-1* and *phyA-211 ataf2-1* with appropriate controls (Fig. S2; Fig. 5a). *phyB-9 ataf2-1* hypocotyls were shorter than those of *phyB-9* even in the dark, and this short-hypocotyl phenotype was unchanged in low fluence rates (10 and 20 μmol m^{-2} s^{-1}) of white light (Fig. S2a), which suggests that PHYB is not a major player in ATAF2-mediated photomorphogenesis regulation. Similar trends were observed from hypocotyl growth comparisons between *cry1-103 ataf2-1* and *cry1-103* in the dark and most tested fluence rates (10–60 μmol m^{-2} s^{-1}) of white light (Fig. S2b). Therefore, CRY1, like PHYB, does not have significant genetic interaction with ATAF2 in seedling photomorphogenesis. Unlike PHYB or CRY1, PHYA exhibited strong genetic interaction with ATAF2 in white light (Fig. 5a). Col-0, *ataf2-1*, *phyA-211* and *phyA-211 ataf2-1* seedlings showed similar hypocotyl growth in the dark (Fig. 5a). In low to moderate fluence rates (10–40 μmol m^{-2} s^{-1}) of white light, *ataf2-1* hypocotyls were shorter than those of the other three genotypes, which still showed similar hypocotyl growth (Fig. 5a). Similar results were observed when sucrose was removed from the media (Fig. 5b) and the plant growth temperature was further lowered to 20 °C (Fig. 5c). Thus, disruption of PHYA can abolish the short-hypocotyl phenotype caused by *ATAF2* disruption, demonstrating that PHYA is required for ATAF2-regulated seedling photomorphogenesis in white light. Since the disruption of *ATAF2* confers a short hypocotyl phenotype in far-red light (Fig. 4c) we examined the disruption of *ATAF2* in a *phyA-211* null mutant in the same conditions (Fig. 5d). *phyA-211 ataf2-1* hypocotyls were only slightly shorter than those of *phyA-211* in multiple far-red fluence rates (Fig. 5d), indicating that PHYA activity is necessary for the role of ATAF2 in far-red light.

PHYA is essential for ATAF2-regulated BR homeostasis in the light

ATAF2 suppresses photomorphogenesis at least partially via repressing BR inactivation (Peng et al. 2015). Therefore, we tested the genetic interaction between PHYA and ATAF2 in seedling responses to exogenous brassinolide (BL) treatments (Fig. 6). BL promotes seedling hypocotyl growth in the light but the effect switches to inhibition in the dark (Turk et al. 2003). In 80 μmol m^{-2} s^{-1} of white light, *ataf2-1* seedlings were less sensitive to BL treatments when compared to the wild type (Col-0) (Fig. 6a), which has been reported previously (Peng et al. 2015). This reduction of BL sensitivity was attenuated (at 10 nM BL treatment) or even abolished (at 100 and 1000 nM BL treatments) when PHYA was disrupted (Fig. 6a). These results suggest that PHYA is essential for ATAF2-regulated BR homeostasis in the light. In contrast, the disruption of PHYB (Fig. S3a) or CRY1 (Fig. S3b, c) did not abolish the reduction of BL sensitivity caused by *ATAF2* disruption. However, *phyA-211 ataf2-1* seedlings showed a BR-response phenotype similar to that of *ataf2-1* in the dark (Fig. 6b), demonstrating that PHYA has no significant impact on ATAF2-regulated BR homeostasis in the absence of light.

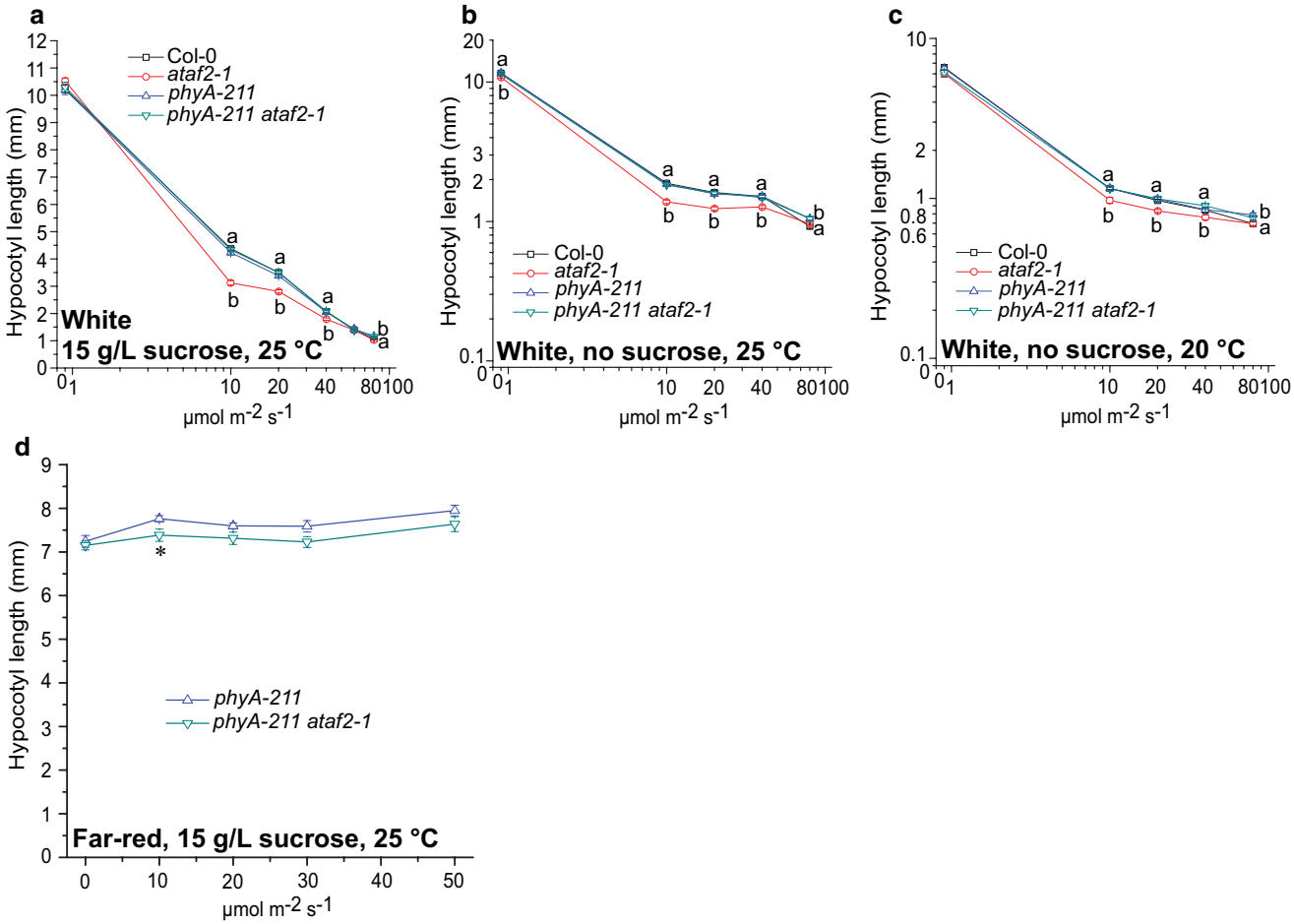

Fig. 5 PHYA is required for ATAF2-regulated seedling photomorphogenesis. **a** A fluence-rate (0–80 µmol m^{-2} s^{-1}) response assay in white light was performed for Col-0, *ataf2-1*, *phyA-211* and *phyA-211 ataf2-1* seedlings. All four genotypes showed similar hypocotyl growth in the dark. In low to moderate fluence rates (10–40 µmol m^{-2} s^{-1}) of white light, *ataf2-1* hypocotyls were shorter than those of the other three genotypes, which still showed similar hypocotyl growth. **b** Similar results were observed in the absence of sucrose in the growth media. **c** Similar results were observed in the absence of sucrose and seedlings were grown at 20 °C instead of 25 °C. **d** *phyA-211 ataf2-1* hypocotyls were only slightly shorter than those of *phyA-211* in multiple far-red fluence rates. The difference was significant at 10 µmol m^{-2} s^{-1}. Four- and three-day-old seedlings were used for white and far-red light fluence-rate response assays, respectively. Each data point represents the average of measurements from 30 seedlings ($n = 30$). Error bars denote the SE. In **a–c**, the significance of the differences was determined by one-way ANOVA with Tukey's HSD test. Groups with significant differences ($P < 0.05$) at a given fluence rate were labeled by different letters. In **d**, two-tailed Student's *t*-test was used to determine the significance of differences. *$P < 0.05$

Discussion

Circadian oscillation of ATAF2 is modulated by its self-transcriptional suppression and physical/genetic interactions with CCA1

Multiple Arabidopsis core circadian regulators and their targeting proteins, such as the central morning loop components CCA1 (Wang and Tobin 1998; Adams et al. 2015) and LATE ELONGATED HYPOCOTYL (LHY) (Schaffer et al. 1998; Adams et al. 2015), as well as an evening-expressed TF LUX ARRHYTHMO (LUX)/PHYTOCLOCK1 (PCL1) (Hazen et al. 2005; Onai and Ishiura 2005; Chow et al. 2012), share a common feature of self-binding to its own promoter as a transcriptional repressor, which forms a negative auto-regulatory feedback loop and leads to a circadian oscillation expression pattern (Helfer et al. 2011; Nagel et al. 2015). In this research ATAF2 is identified as a self-transcriptional repressor (Figs. 1, 2), which is consistent with its previously confirmed circadian oscillation expression pattern (Peng and Neff 2020).

In addition to the observations that both CCA1 (Peng and Neff 2020) and ATAF2 itself (Figs. 1, 2) can bind the *ATAF2* promoter as repressors in the light, CCA1 and ATAF2 also physically interact at the protein level (Peng and Neff 2020). It is possible that CCA1 and

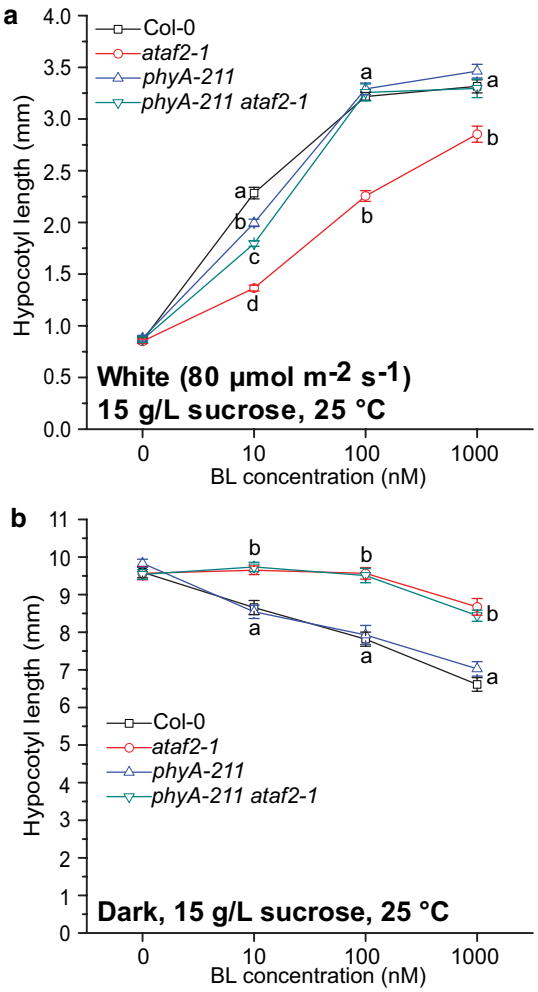

Fig. 6 PHYA is essential for ATAF2-regulated BR homeostasis in the light. **a** In 80 µmol m^{-2} s^{-1} white light, *ataf2-1* seedlings showed reduced sensitivity to BL treatments when compared to Col-0. This BL-reduced-sensitivity phenotype was attenuated (at 10 nM BL treatment) or even abolished (at 100 and 1000 nM BL treatments) when PHYA was disrupted. **b** *phyA-211 ataf2-1* seedlings showed a BR-reduced-sensitivity phenotype similar to that of *ataf2-1* in the dark. Four-day-old seedlings were used for both BL-response assays (light and dark). Each data point represents the average of measurements from 30 seedlings ($n = 30$). Error bars denote the SE. The significance of differences was determined by one-way ANOVA with Tukey's HSD test. Groups with significant differences ($P < 0.05$) at a given BL concentration were labeled by different letters

ATAF2 coordinately suppress *ATAF2* transcription at least partially as a hetero-complex, since the disruption of *CCA1* (Peng and Neff 2020) and *ATAF2* (Fig. 2) similarly increase *ATAF2* and p*ATAF2*–driven *GUS* transcript accumulation to about two-fold, respectively. *CCA1* and *ATAF2* exhibit distinct circadian oscillation patterns (Peng and Neff 2020). The *CCA1* expression peak appears around dawn, whereas *ATAF2* transcript accumulation keeps increasing after dawn and peaks right before sunset (Peng and Neff 2020). The continuous reduction of *CCA1* expression in the light period is consistent with the corresponding increase of *ATAF2* transcript accumulation, as CCA1 is a repressor of *ATAF2* transcription. Additional negative auto-regulatory feedback of ATAF2 itself may help to constrain its oscillation range within two to three-fold (Peng and Neff 2020). Unlike CCA1, ATAF2 does not reciprocally regulate *CCA1* expression (Peng and Neff 2020), which is consistent with the wider oscillation range of *CCA1* (Peng and Neff 2020).

ATAF2/CCA1-regulated seedling photomorphogenesis is sucrose- and thermo-independent

The photomorphogenic phenotype caused by the disruption of *ATAF2* or *CCA1* does not depend on sucrose or relatively warm (25 °C) temperature (Figs. 3, 5). We have previously added 15 g/L sucrose to the medium and grow seedlings at 25 °C in our fluence-rate response assays (Peng et al. 2015; Peng and Neff 2020). This approach integrates more signaling pathways to the modulation of seedling growth but still stays below the threshold temperature (~ 27 °C) for triggering thermomorphogenesis in Arabidopsis (Casal and Balasubramanian 2019). Sucrose has complex effects on hypocotyl growth depending on light condition, seedling age and multiple photoreceptor and hormonal signaling pathways (Zhang et al. 2010, 2015; Liu et al. 2011; Simon et al. 2018; Zhao et al. 2020). In our experimental setting, sucrose significantly promotes hypocotyl growth in relatively low fluence rates (10–40 µmol m^{-2} s^{-1}) of white light (Figs. 3, 5). Adding sucrose in the medium can make subtle hypocotyl-growth phenotypes become more visible.

In our assay, low-fluence-rate red light makes Col-0 seedlings slightly taller than their dark-grown counterparts (Fig. 4a). This phenotype is sucrose-dependent since it can be suppressed by the removal of sucrose from the growth media (Fig. S1). Similar to white light assays, sucrose promotes hypocotyl growth in low-fluence-rate red light (Fig. 4a; Fig. S1) in our experimental setting, which can partially explain the observation in Fig. 4a. Additional factors may also contribute to the long-hypocotyl phenotype observed in low-fluence-rate red light. Compared to white and blue light, red light is most effective in stimulating seed germination (Fankhauser and Casal 2004) and least efficient in inhibiting hypocotyl elongation (Neff and Chory 1998). Both low fluence rates and red light result in longer hypocotyls as compared to high fluence rates and white/blue light, respectively. In our experimental setting, dark-grown seeds are pretreated with red light for 2–4 h to induce subsequent germination in the dark. This treatment may cause a delay in germination and hypocotyl growth when compared to seeds/seedlings continuously exposed to red light.

The synergistic suppression effect of ATAF2 and CCA1 on seedling photomorphogenesis may be at least partially associated with BR homeostasis

Beyond circadian oscillation, both ATAF2 and CCA1 are repressors of seedling photomorphogenesis (Peng et al. 2015; Zhai et al. 2020). ATAF2 promotes seedling elongation in a fluence-rate dependent manner, with its loss- and gain-of-function mutants exhibiting opposite hypocotyl length phenotypes (Peng et al. 2015). Similarly, *CCA1* overexpression lines confer a long-hypocotyl phenotype (Wang and Tobin 1998), whereas gene knock-out mutants have shorter hypocotyls when compared to the wild type (Zhao et al. 2018b; Zheng et al. 2018; Peng and Neff 2020). In our fluence-rate response assay, *cca1-1 ataf2-2* seedlings have shorter hypocotyls than either single mutant in low to moderate fluence rates of white light (Fig. 3), which demonstrates that ATAF2 and CCA1 synergistically suppress seedling photomorphogenesis. We previously reported that ATAF2 and CCA1 have additive effects in suppressing the expression of the BR-inactivating gene *SOB7* in the light (Peng and Neff 2020). Therefore, BR homeostasis may at least partially account for the synergistic suppression of ATAF2 and CCA1 on seedling photomorphogenesis.

PHYA is required for ATAF2-regulated hypocotyl growth and BR homeostasis in the light

Though *ATAF2* transcript accumulation keeps increasing during the light period of a circadian light/dark cycle (Peng and Neff 2020), *ATAF2* expression is suppressed by continuous light or by the dark-to-light transition in seedling photomorphogenesis (Peng et al. 2015). These observations suggest that *ATAF2* undergoes a different set of molecular and genetic regulations during photomorphogenesis. Our fluence-rate-response assays reveal that ATAF2 suppresses photomorphogenesis in all three monochromatic (red, blue and far-red) light conditions (Fig. 4), and the presence of PHYA is essential for the short-hypocotyl phenotype caused by *ATAF2* disruption (Fig. 5). In addition to being a far-red light photoreceptor, PHYA also responds to red (Tepperman et al. 2006; Franklin et al. 2007) and blue (Chun et al. 2001) light signals, which explains the observation that ATAF2 is consistently functional in regulating photomorphogenesis in red, blue and far-red light (Fig. 4).

Since ATAF2-regulated BR homeostasis accounts for the seedling photomorphogenic phenotypes of *ATAF2* mutants (Peng et al. 2015), we tested the genetic interaction between ATAF2 and PHYA in modulating seedling responses to exogenous BL treatments (Fig. 6). Disruption of PHYA attenuates the BR-insensitive phenotype of *ATAF2* knockout seedlings in the light (Fig. 6a) but not in the dark (Fig. 6b), indicating that ATAF2-regulated BR homeostasis only requires PHYA presence in the light. It is reasonable that as a photoreceptor, PHYA does not have genetic interaction with ATAF2 when there is no light input. Down-regulated by light (Cantón and Quail 1999), PHYA accumulates in the dark and plays an important role in the transition of seedlings from dark to light growth (Casal et al. 2014), a process called de-etiolation. The potential functions of PHYA in dark-grown seedlings are still under-investigated (Carlson et al. 2019). However, with regard to genetic interactions with *ATAF2*, *PHYA* is no longer functional in the dark (Fig. 6b). *ATAF2* knock-out seedlings still show reduced BL sensitivity in the dark (Peng et al. 2015; Peng and Neff 2020; Fig. 6b), which may indicate ATAF2-mediated BR homeostasis is regulated by PHYA-independent pathways in the dark, or the increased accumulation of *ATAF2* in the dark (Peng et al. 2015) is sufficient to regulate BR homeostasis with no need for PHYA or other partners. Similar to the opposite observations that *ATAF2* expression is suppressed by continuous light but keeps increasing in the light period of a circadian light/dark cycle, dark-grown seedlings have higher *ATAF2* transcript accumulation whereas *ATAF2* expression is in a continuously decreasing pattern during the dark period of a circadian light/dark cycle (Peng et al. 2015; Peng and Neff 2020). The distinct circadian- and photomorphogenic-regulation of *ATAF2* expression corresponds to its complex genetic interactions with *CCA1* and *PHYA*, respectively.

PHYA has long been associated with BR catabolism in previous research. *bas1-D*, an activation tagging mutant of the BR-inactivating gene *BAS1*, can suppress the long-hypocotyl phenotype caused by the *phyB*-null allele, but not the *phyA*-null-mutation-derived long-hypocotyl phenotype in far-red light (Neff et al. 1999). In addition, the interaction between photomorphogenesis and BAS1-mediated BR-inactivation is found to mainly depend on far-red light, which is primarily sensed by PHYA (Turk et al. 2003). The *bas1-2* null mutation can also suppress the late-flowering phenotype of *phyA-211* in both long day and short day growth conditions (Sandhu et al. 2012). We previously described *PHYA-ATAF2* genetic interactions in low fluence rates (10 μmol m^{-2} s^{-1}) of monochromatic light (Peng et al. 2015). Here we further investigated the genetic interaction between PHYA and ATAF2 in seedling white-light photomorphogenesis (Fig. 5) in a wide range of fluence rates (0–80 μmol m^{-2} s^{-1}) as well as in BR homeostasis regulation (Fig. 6). It is still not clear whether PHYA regulates ATAF2 activities directly via physical interactions or indirectly through its signaling pathway. PHYA has been reported to directly target the promoters of numerous hormone- or stress-responsive genes for subsequent transcriptional regulation, including a NAC TF-encoding gene *ANAC019* (Chen et al. 2014). Therefore, a future direction would be uncovering the molecular mechanism of PHYA-ATAF2 interaction.

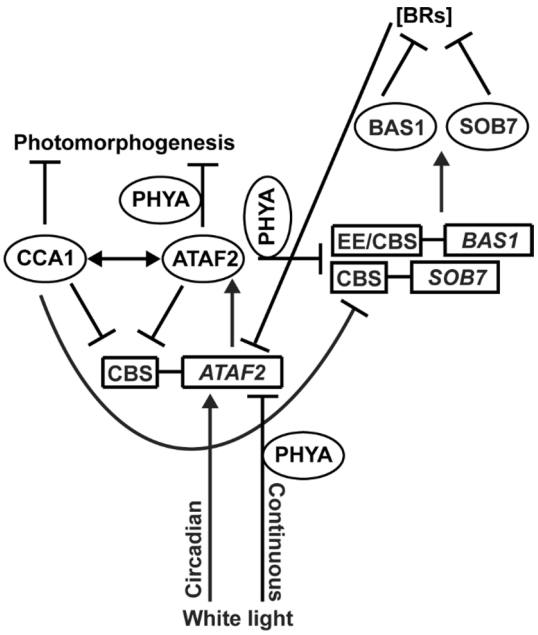

Fig. 7 A model for the molecular and genetic regulations of ATAF2 involving CCA1, PHYA, light and BRs. ATAF2 bind the CBS motif on its own promoter as a self-transcriptional repressor, which forms a negative auto-regulatory feedback loop. CCA1 physically interacts with ATAF2 and also transcriptionally suppresses *ATAF2* expression via promoter binding to CBS. CCA1 and ATAF2 synergistically suppress seedling photomorphogenesis. They also differentially suppress the expression of BR-inactivating genes *BAS1* and *SOB7* via direct binding to the EE/CBS motifs on their promoters. PHYA is require for ATAF2-regulated photomorphogenesis and BR homeostasis in the light. BRs suppress *ATAF2* expression to form a negative feedback regulation loop. *ATAF2* expression increases during the light period of the circadian light/dark cycle but is suppressed by continuous light after the dark-to-light transition of photomorphogenesis

A model for the molecular and genetic regulations of ATAF2 involving CCA1, PHYA, light and BRs

Base on this work and our previous reports (Peng et al. 2015; Peng and Neff 2020), we propose a model to summarize our current understanding on the circadian-clock-photomorphogenesis-BR integration node involving ATAF2, CCA1 and PHYA (Fig. 7). ATAF2 bind the CBS motif on its own promoter as a self-transcriptional repressor, which forms a negative auto-regulatory feedback loop. CCA1 physically interacts with ATAF2 and also transcriptionally suppresses *ATAF2* expression via promoter binding to the CBS motif. CCA1 and ATAF2 synergistically suppress seedling photomorphogenesis. They also differentially suppress the expression of BR-inactivating genes *BAS1* and *SOB7* via direct binding to the EE/CBS motifs on their promoters. PHYA is require for ATAF2-regulated photomorphogenesis and BR homeostasis in the light. BRs suppress *ATAF2* expression to form a negative feedback regulation loop. *ATAF2* expression increases during the light period of a circadian light/dark cycle but is suppressed by continuous light after the dark to light transition of photomorphogenesis. It is worth noting that both ATAF2 (Wang et al. 2009b; Zhao et al. 2013) and CCA1 (Andronis et al. 2008; Lu et al. 2009; Lau et al. 2011; Sun et al. 2019b) have multiple physical interacting partners. This simplified model only focuses on the molecular and genetic regulations of ATAF2 involving CCA1 and PHYA.

Author contribution statement HP and MMN proposed the project and designed the experiments. HP performed most experiments and analyzed the data. JP assisted in isolating and genotyping segregation plants from the cross. YZ performed the GUS quantification experiments. HP and MMN discussed the results and wrote the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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