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AtBBX21 and COP1 genetically interact in the regulation of shade avoidance

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SUMMARY

Plants grown at high densities perceive the reduction in the ratio of red (R) to far-red (FR) light as a warning of competition. This light signal triggers morphological responses such as hypocotyl and stem elongation, and acceleration of flowering, which are known collectively as the shade-avoidance syndrome (SAS). Mutations in the photomorphogenic repressor *COP1* suppress the SAS, but how COP1 modulates these responses is uncertain. We identified a new mutant with altered responses to natural shade, named *lhus* (long hypocotyl under shade). *lhus* seedlings have longer hypocotyls than wild-type under a low R:FR ratio, but not under sunlight or darkness. The *lhus* phenotype is due to a mutation affecting a B-box zinc finger transcription factor encoded by At1g75540, a gene previously reported as *AtBBX21* that interacts with *COP1* to control de-etiolation. Mutations in genes encoding other members of this protein family also result in impaired SAS regulation. Under short-term canopy shade, LHUS/BBX21 acts as positive regulator of SAS genes such as *PAR1*, *HFR1*, *PIL1* and *ATHB2*. In contrast, global expression analysis of wild-type and *lhus/bbx21* seedlings revealed that a large number of genes involved in hormonal signalling pathways are negatively regulated by LHUS/BBX21 in response to long-term canopy shade, and this observation fits well with the phenotype of *lhus/bbx21* seedlings grown under a low R:FR ratio. Moreover, the *bbx21 bbx22* double mutation restored the SAS in the *cop1* background. We propose that LHUS/BBX21 and other B-box-containing proteins, such as BBX22, act downstream of COP1, and play a central role in early and long-term adjustment of the SAS in natural environments.

Keywords: shade-avoidance syndrome, T-DNA mutants, B-box zinc finger proteins, *PAR* genes, canopy light, Arabidopsis.

INTRODUCTION

Plants are intimately tied to their environment, and have evolved a network of sophisticated mechanisms to deal with fluctuating biotic (e.g. pathogens, plant neighbours) and abiotic (e.g. light, temperature) conditions. Plant success depends on the plant's ability to translate these signals into specific cellular responses, fine-tuning their growth and development under changing natural environments. Direct sunlight has a high proportion of blue light (B) and red light (R), but the light reflected by neighbouring vegetation is relatively enriched in far-red light (FR) due to the selective absorption of blue and red light by chlorophyll pigments. Reflected or transmitted light from surrounding vegetation reduces the R:FR ratio. This signal is perceived by phytochromes, a family of reversible photoreceptors that exist as two photo-convertible isomers, a biologically inactive R-absorbing Pr form, and a biologically active FR-absorbing Pfr form. A low R:FR ratio reduces the proportion of Pfr in plant tissues, and consequently triggers a set of physiological responses known as the shade-avoidance syndrome (SAS), in order to anticipate plant competition before canopy closure (Ballaré *et al.*, 1987). The SAS involves extension growth of hypocotyls, petioles and stems, reduces branching and induces early flowering (Smith and Whitelam, 1997).

The SAS is a strategy of major adaptive significance in wild and crop communities that involves massive changes in gene expression. Several transcription factors are rapidly up-regulated upon exposure of plants to a low R:FR ratio

(Devlin *et al.*, 2003). The level of transcripts of *ATHB2*, which encodes a homeodomain-leucine zipper (HD-Zip) transcription factor and is a positive regulator of the SAS, is increased under short-term shade (Carabelli *et al.*, 1996). PIF4 and PIF5, two bHLH transcription factors of phytochrome signalling, show increased stability at a low R:FR ratio and also act as positive regulators of the SAS (Lorrain *et al.*, 2008). In contrast, other bHLH transcription factors, such as HFR1, PAR1 and PAR2, are early negative modulators of the SAS and constitute central molecular components of the 'gas-and-brake' mechanism that prevents exaggerated expression of the SAS (Sessa *et al.*, 2005; Roig-Villanova *et al.*, 2007).

An increasing body of evidence has demonstrated that gibberellins (GA) and phytochrome-mediated signalling pathways converge to regulate elongation growth under shade. The DELLA family, a set of proteins that repress GA signalling, are active under a high R:FR ratio but are degraded under a low R:FR ratio, allowing elongation of plant structures (Djakovic-Petrovic *et al.*, 2007; Achard *et al.*, 2009). The GA-stimulated proteolysis of DELLAs is mediated through interactions with SLY1 protein, the F-box subunit of an SCF E3 ubiquitin ligase complex. This interaction results in polyubiquitination and degradation of DELLAs via the 26S proteosome, thereby relieving the growth inhibition of plant structures (Franklin, 2008).

Another branch of the phytochrome signalling network involves CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1), a regulatory protein that represses photomorphogenesis in darkness (Deng *et al.*, 1991), and promotes the SAS under a low R:FR ratio (McNellis *et al.*, 1994). COP1 encodes an E3 ubiquitin ligase that targets transcription factors for degradation (Saijo *et al.*, 2003; Seo *et al.*, 2003). In dark-grown seedlings, COP1 accumulates in the nucleus, where it interacts directly with positive regulators of photomorphogenesis such as LONG HYPOCOTYL 5 (HY5) and LONG HYPOCOTYL IN FAR-RED (HFR1), which, as a result of this interaction, become ubiquitinated and targeted for degradation via the 26S proteosome (Osterlund *et al.*, 2000; Duek *et al.*, 2004). After light perception, multiple photoreceptors, including phytochromes, induce COP1 re-localization to the cytosol (Osterlund and Deng, 1998), and thus promote the expression of photomorphogenic transcription factors in the nucleus. It has been shown that COP1, but not its best characterized target HY5, modulates SAS gene expression in response to simulated shade in light-grown seedlings (Roig-Villanova *et al.*, 2006). Therefore, the mechanism through which COP1 modulates SAS responses remains unknown.

In addition to bHLH and HD-Zip transcription factors, B-box-containing (BBX) proteins have also been shown to act as regulators of light signalling (Kumagai *et al.*, 2008), and some of them appear to operate within a transcriptional complex that interacts with COP1 (Holm *et al.*, 2002; Datta *et al.*, 2006). Indeed, during development of the present work, SALT TOLERANCE HOMOLOG2 (STH2), now known as BBX21 (Khanna *et al.*, 2009), was identified and characterized, and found to play a central role during de-etiolation processes (Datta *et al.*, 2007). BBX21 interacts physically with the HY5 transcription factor, and inhibits hypocotyl growth under continuous blue, red and far-red light (Datta *et al.*, 2007). In addition, BBX22, a homolog of BBX21, interacts physically with COP1 and HY5 (Chang *et al.*, 2008; Datta *et al.*, 2008). BBX22 operates additively with BBX21 in early seedling development, inducing hypocotyl inhibition, anthocyanin accumulation and chloroplast biogenesis (Chang *et al.*, 2008; Datta *et al.*, 2008). BBX21 has been shown to act by two pathways, one dependent on HY5 and the other independent of HY5, suggesting that BBX21 and other BBX proteins may be the components through which COP1 regulates the SAS.

By screening an *Arabidopsis thaliana* T-DNA population of seedlings under a low R:FR ratio, we identified a mutant that we named *lhus* (long hypocotyl under shade). Molecular characterization of the *lhus/bbx21* mutation revealed a previously uncharacterized role for LHUS/BBX21 under shade conditions. We demonstrate that LHUS/BBX21 acts as a positive regulator of early SAS genes, such as *PAR1*, *HFR1*, *PIL1* and *ATHB2*, in response to canopy shade. In contrast, global gene expression analysis of wild-type and *lhus/bbx21* seedlings shows that a large number of genes are negatively regulated by LHUS/BBX21 in response to long-term canopy shade, an observation that fits well with the phenotype of *lhus/bbx21* seedlings grown under a low R:FR ratio. Using a genetic approach, we show that *lhus/bbx21*, in combination with *bbx22*, partially suppresses the cop1 phenotype under a low R:FR ratio, strongly suggesting that both BBX proteins act downstream of COP1 to mediate the SAS. We conclude that BBX proteins play a central role in adjusting plant growth and development in response to shade.

RESULTS

Identification and characterization of the *lhus/bbx21-101* mutant under shade

To isolate SAS signalling mutants, we screened a collection of 65 000 Arabidopsis lines containing T-DNA insertions, and selected seedlings with longer hypocotyls than wild-type under an acetate filter in a glasshouse (i.e. simulated canopy). We selected a mutant with a longer hypcotyl than wild-type under simulated canopy shade, and a normal phenotype under sunlight and darkness (Figure 1a and Figure S1). The mutant was named *lhus* (long hypocotyl under shade). In this mutant, the T-DNA was inserted into the 5′ UTR of the *LHUS* gene (At1g75540) at nucleotide position -120 from the ATG start position (Figure 1b). At1g75540 is a gene that had previously been reported as *STH2* (*SALT TOLERANCE HOMOLOG2*) (Datta *et al.*, 2007)

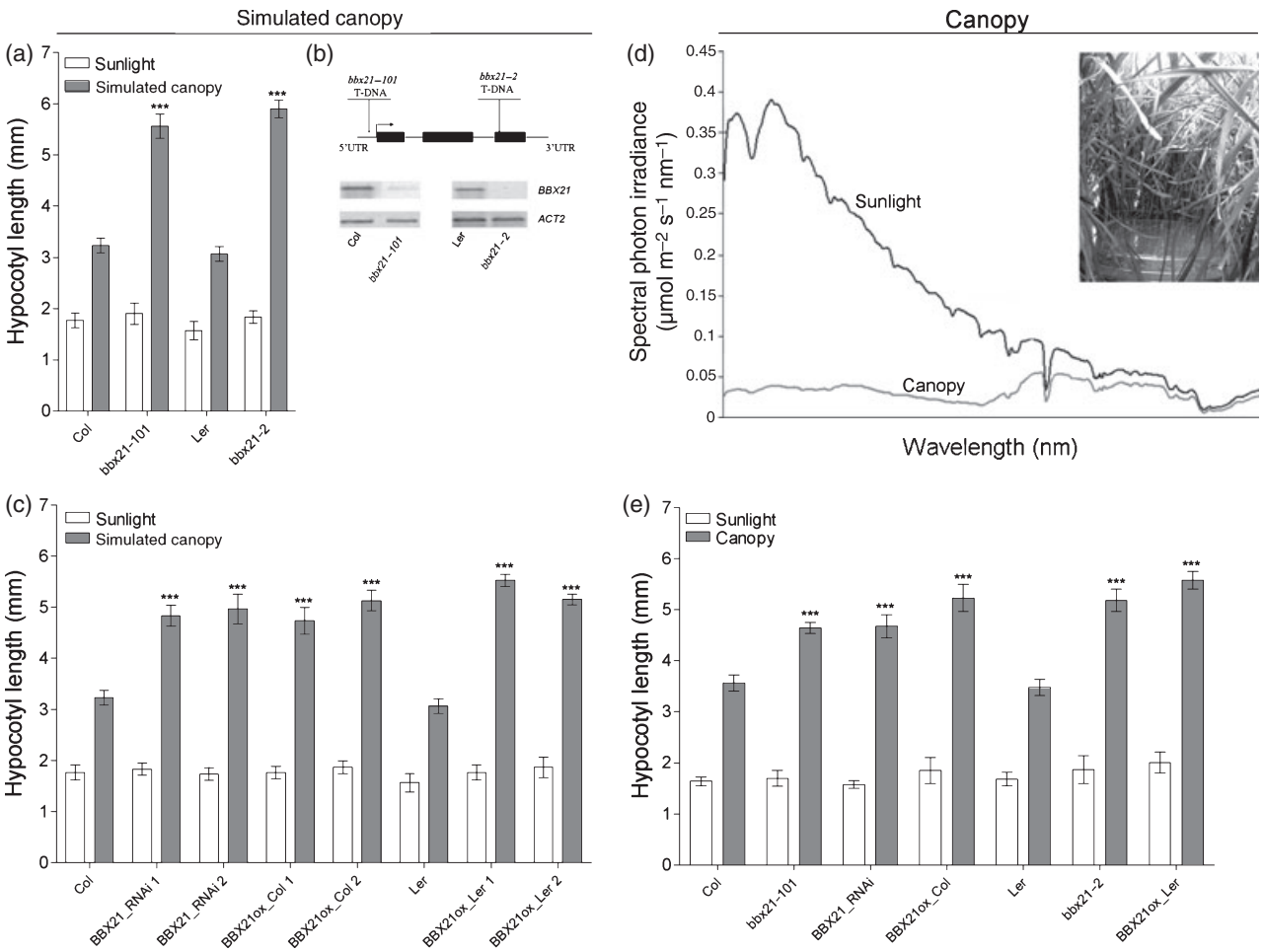

Figure 1. BBX21 is a negative regulator of hypocotyl inhibition under simulated shade and canopy shade.
(a) Hypocotyl length of wild-type, *bbx21-101* and *bbx21-2* seedlings grown under sunlight or simulated canopy shade for 4 days in a glasshouse.
(b) *BBX21* gene structure and location of T-DNA insertions for *bbx21-101* and *bbx21-2*. Filled boxes represent exons and lines represent introns. The expression of *BBX21* transcripts was analysed by RT-PCR in wild-types, *bbx21-101* and *bbx21-2*. *ACTIN2* is shown as a load controling.
(c) Hypocotyl lengths of wild-type, *bbx21-2*, RNAi lines under-expressing *BBX21* (*BBX21*_RNAi 1 and 2) and *BBX21* over-expressing lines (*BBX21ox*_Col 1 and 2, and *BBX21ox*_*Ler* 1 and 2) seedlings grown under sunlight or simulated canopy shade.
(d) Spectral photon fluences of sunlight and canopy shade. The inset photograph shows the canopy shade conditions in the present work.
(e) Hypocotyl lengths of wild-type, *bbx21-2*, *BBX21*_RNAi, *BBX21ox*_Col and *BBX21ox*_*Ler* seedlings grown under sunlight or canopy shade (6 days sunlight or 2 days sunlight + 4 days canopy shade). Values are means ± SE ($n \geq 15$). Asterisks indicate values that are significantly different from wild-type under the same light treatment ($P < 0.001$).

that encodes a BBX protein. It is now known as *AtBBX21* (Khanna *et al.*, 2009). Our mutant allele was named *Atbbx21-101* because it is different from the *Atbbx21-1* mutant, which has the insertion at position -43 in the 5′ UTR (Datta *et al.*, 2007). We confirmed the function of LHUS/BBX21 under simulated canopy shade using another allele of *BBX21* in the Landsberg *erecta* background (*bbx21-2*, Figure 1a). *LHUS/BBX21* expression in *lhus/bbx21-101* was significantly reduced compared with wild-type (Figure 1b). To confirm the *lhus/bbx21* phenotype, we constructed transgenic plants with reduced levels of *LHUS/BBX21* using RNA interference technology. As expected, reduced expression of *LHUS/BBX21* resulted in seedlings with longer hypocotyls than wild-type under simulated canopy shade, but not under sunlight and darkness (RNAi, Figure 1c and Figure S1). Transgenic plants over-expressing the full-length *LHUS/BBX21* gene under the control of the 35S promoter also displayed an enhanced hypocotyl length under simulated canopy shade, and these effects were independent of the background (Figure 1c and Figure S1).

Because we used a filter that establishes a low R:FR ratio to identify *lhus/bbx21*, we wished to determine the function of LHUS/BBX21 under natural canopy shade. Wild-type, *lhus/bbx21* and *LHUS/BBX21* under-expressing and over-expressing seedlings were exposed to sunlight (6 days) or placed under a dense canopy of ryegrass (2 days of

sunlight + 4 days shade) (Figure 1d). Under- or over-expressing lines of *LHUS/BBX21* showed longer hypocotyls than wild-type under the canopy shade but not under sunlight, independently of the background (Figure 1e). These results confirm the prominent role of LHUS/BBX21 in control of the SAS under simulated and natural shade.

The hypocotyl phenotype of *lhus/bbx21-101* seedlings exposed to continuous blue, red and far-red light demonstrates that LHUS/BBX21 regulates de-etiolation processes (Figure S2), as documented previously by Datta *et al.* (2007). LHUS/BBX21 belongs to a sub-family of eight members of the BBX protein family (Khanna *et al.*, 2009). Some members of this family have been reported to be involved in regulation of the circadian clock (Kumagai *et al.*, 2008). We analysed the circadian rhythm of leaf movement in *lhus/bbx21-101*, 35S:LHUS/BBX21 and wild-type seedlings, and determined that LHUS/BBX21 is not involved in the control of clock function (Figure S3) (Kumagai *et al.*, 2008).

BBX proteins act as negative or positive regulators of plant growth under shade

Eight members of the BBX family (BBX18–BBX25) contain B-boxes B1 and B2 only (Khanna *et al.*, 2009). Various members of this sub-family of BBX proteins act as negative or positive regulators of light signalling during seedling growth (Datta *et al.*, 2007, 2008; Indorf *et al.*, 2007; Chang *et al.*, 2008). We analysed the function of six of these eight BBX proteins under canopy shade and simulated shade. *bbx19*, *bbx21* and *bbx22* seedlings showed longer hypocotyls than wild-type under shade, but *bbx18* and *bbx24* seedlings showed the opposite phenotype under the same light conditions (Figure 2a,b). Comparing the phenotype of the mutants, we concluded that BBX21 has a prominent role in the negative regulation of the elongation growth, and that BBX21 and BBX22 have additive effects in the control of hypocotyl elongation under canopy shade and simulated

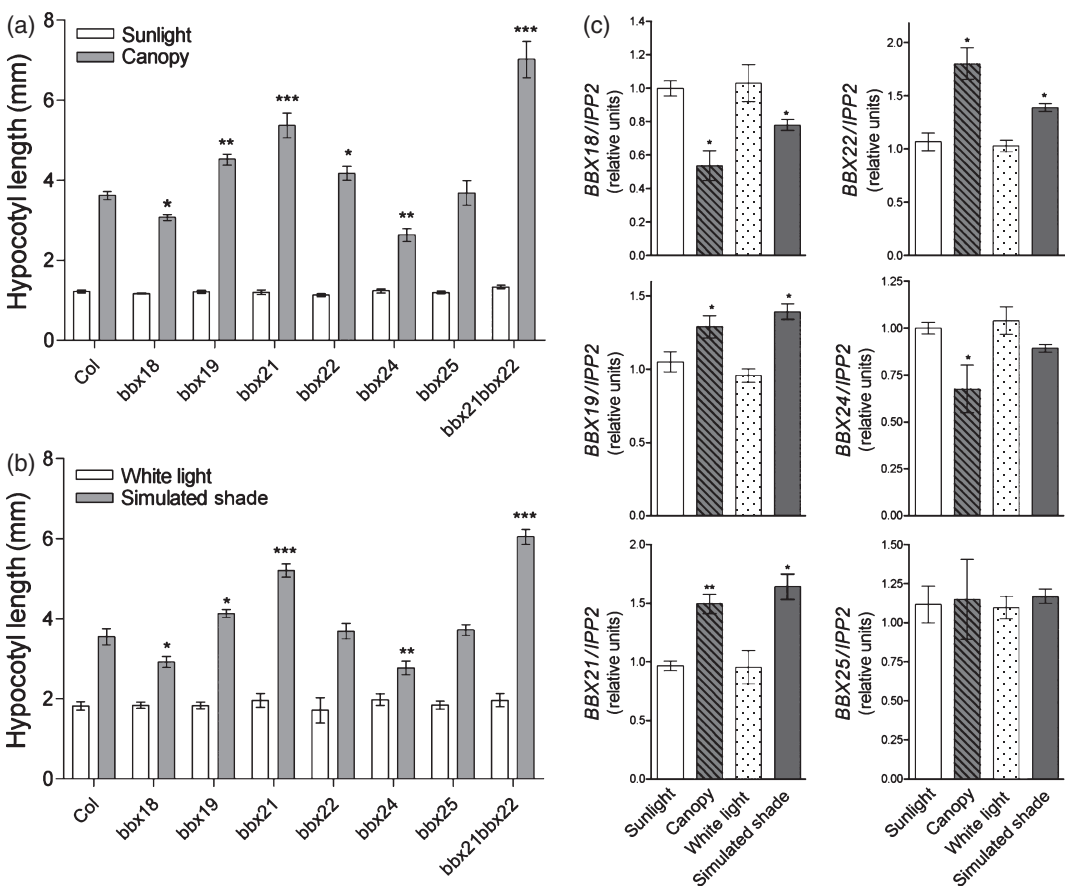

Figure 2. B-box-containing proteins promote or inhibit hypocotyl growth under canopy shade.
(a, b) Hypocotyl lengths of wild-type, *bbx18*, *bbx19*, *bbx21*, *bbx22*, *bbx24*, *bbx25* and *bbx21 bbx22* seedlings grown under canopy shade (a) or simulated shade (b). Values are means ± SE ($n \geq 20$). Asterisks indicate values that are significantly different from wild-type under the same light treatment (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).
(c) Quantitative RT-PCR analysis of *BBX18*, *BBX19*, *BBX21*, *BBX22*, *BBX24* and *BBX25* genes in the wild-type in response to canopy shade and simulated shade. The expression of transcripts was normalized to that of the *IPP2* gene, and data are standardized to wild-type expression under control treatment (sunlight or white light). Values are means ± SE ($n \geq 3$). Asterisks indicate values that are significantly different from wild-type under the same light treatment (*$P < 0.05$, **$P < 0.01$).

shade (Figure 2a,b). In the wild-type, the levels of *BBX21*, *BBX19* and *BBX22* transcripts increased, and those of *BBX18* and *BBX24* transcripts decreased, in response to shade (Figure 2c). We conclude that BBX19, BBX21 and BBX22 function as repressors of plant growth during SAS expression, and BBX18 and BBX24 play an opposite role in SAS signalling.

BBX21 is rapidly up-regulated by shade and promotes the expression of *PAR* genes under short-term canopy shade

We performed quantitative RT-PCR to analyse the expression of *BBX21* under sunlight and canopy shade. The expression analysis was performed after exposing 6-day-old seedlings to sunlight, long-term and short-term canopy shade (Figure 3a). In the wild-type, BBX21 transcript was up-regulated under both short- and long-term canopy shade relative to levels in sunlight, with the expression being higher in seedlings exposed to short-term canopy shade than in those grown under long-term canopy shade (Figure 3b). These results suggest the existence of a feedback regulatory mechanism that fine-tunes *BBX21* expression in shade. Bearing in mind that shade regulates *PAR* gene expression (Sessa *et al.*, 2005; Roig-Villanova *et al.*, 2006), we hypothesized that BBX21 could play a central role in the regulation of *PAR* gene expression, and found that BBX21 is required for full rapid and activation of *HFR1*, *ATHB2*, *PAR1* and *PIL1* expression under short-term canopy shade in Col and L*er* backgrounds (Figure 3c and Figure S4).

BBX21 and BBX22 interact with COP1 to modulate SAS responses

COP1 is involved in promotion of the SAS (McNellis *et al.*, 1994; Roig-Villanova *et al.*, 2006), and genetic interactions have been reported between COP1 and BBX proteins in the regulation of de-etiolation processes (Datta *et al.*, 2007, 2008). We performed an experiment to evaluate whether BBX proteins are involved in the COP1 signalling branch of the SAS. Hypocotyl growth of *cop1*, *bbx21* and *bbx22* single, double and triple mutants was evaluated under shade. *cop1* seedlings showed an impaired SAS under simulated shade or canopy shade (Figure 4a and Figure S5). Although the phenotypes of both *bbx21 cop1* and *bbx22 cop1* resembled those of *cop1-4* and *cop1-6*, the combination of *bbx21* and *bbx22* in *bbx21 bbx22 cop1-4* seedlings restored the SAS phenotype (Figure 4a and Figure S5). In addition, expression analysis of early SAS genes in single, double and triple mutants also suggests that BBX21, BBX22 and COP1 mediate the early and rapid up-regulation of *HFR1*, *ATHB2* and *PAR1* in response to short-term shade through the same signalling pathway. The observation that the induction of *PAR* gene expression in response to shade in the triple mutant is not lower than that observed in the single *cop1* or *bbx21 bbx22* double mutants (Figure 4c) suggests that the three proteins act as part of a common protein complex or signalling pathway. All the above experiments strongly suggest a role for both BBX proteins in the COP1 signalling branch of the SAS.

Another SAS negative regulator, HFR1, has been shown to interact with COP1, which is responsible for its degradation *in vivo* (Duek *et al.*, 2004). As BBX21, similar to HFR1 (Sessa *et al.*, 2005; Roig-Villanova *et al.*, 2007), is a negative modulator of the SAS that interacts with COP1, we investigated whether HFR1 and BBX21 participate in the same SAS signalling pathway. To evaluate this hypothesis, the phenotype of *hfr1* was evaluated under our simulated shade and canopy conditions. Interestingly, *hfr1* displayed a strong mutant phenotype under simulated shade, but an almost normal phenotype compared with wild-type seedlings under canopy shade (Figure 5a). The lack of an *hfr1* phenotype under our canopy shade conditions, and the normal BBX21 transcript regulation by shade in the hfr1 mutant background, strongly suggests that these two genes operate through independent pathways to regulate the SAS (Figure 5b).

Global expression analysis identifies genes repressed by BBX21 under long-term canopy shade

The observation that BBX21 acted as a positive regulator of *PAR* gene expression was striking, given that BBX21 plays a role as a negative regulator of the SAS based on the phenotype of *bbx21* seedlings. As some *PAR* genes, such as *HFR1* and *PAR1*, act as negative regulators of the SAS, we reasoned that the exaggerated SAS phenotype of *bbx21* seedlings could be the result of an absence of effects on expression of these negative regulators. To understand the molecular basis of the physiological alterations in the SAS in the *bbx21* mutant in more detail, we used Affymetrix ATH1 microarrays to compare the transcriptome of wild-type and *bbx21-2* seedlings exposed to sunlight or long-term canopy shade. A global expression analysis identified a group of genes in the transcriptomes of wild-type and *bbx21* that are up- and down-regulated independently of the light conditions (Table S1). A more detailed analysis was performed for genes that are regulated by BBX21 specifically under canopy shade. The analysis identified 576 genes whose expression was statistically significantly different between wild-type and *bbx21-2* seedlings grown under canopy shade (Figure 6a and Table S2). By cluster analysis, we identified two major groups of genes comprising 146 and 59 genes (clusters 1 and 2, respectively) whose expression increased in the *bbx21* mutant under shade conditions (Figure 6b and Table S2). Clusters 1 and 2 are enriched in genes involved in cell growth and proliferation activities. Functional classifications using GO terms showed that genes involved in transport activity and responses to abiotic and biotic stimuli were over-represented, and genes related to nucleotide binding activity were under-represented, in both clusters (Figure 6c). To identify potential common targets of BBX21 action and hormonal signalling, we analysed the overlap between

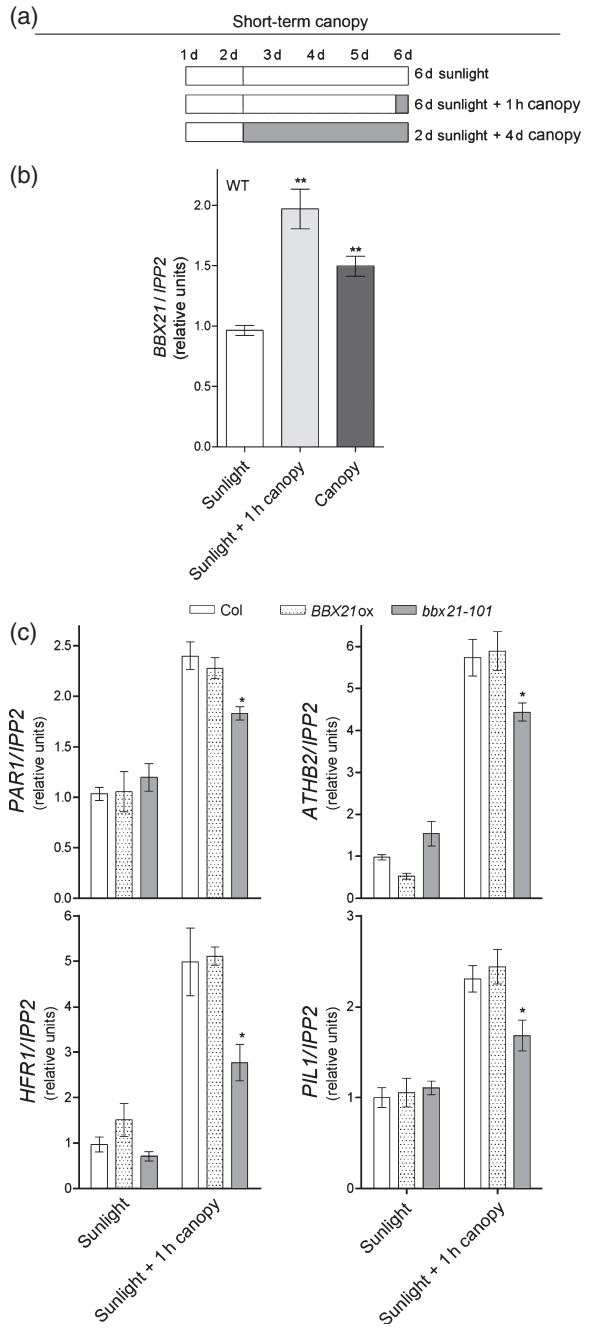

Figure 3. *BBX21* is rapidly up-regulated under canopy shade and its product promotes the expression of *PAR* genes.
(a) Schematic representation of short- and long-term canopy shade.
(b) Quantitative RT-PCR analysis of the expression of *BBX21* in wild-type in response to short or long-term canopy shade.
(c) Quantitative RT-PCR analysis for *PAR* genes (*PIL1*, *HFR1*, *PAR1* and *ATHB2*) in wild-type, *bbx21-101* and *BBX21ox* seedlings grown under sunlight or short-term canopy shade. Values are means ± SE ($n \geq 3$). Asterisks indicate values that are significantly different from sunlight (a) or wild-type under the same light treatment (b) (*$P < 0.05$, **$P < 0.01$).

genes belonging to clusters 1 and 2 and those genes previously identified as hormonally regulated in 7-day-old seedlings (Nemhauser *et al.*, 2006). We found a significant association between genes down-regulated by BBX21 and those up-regulated by auxins (representation factor 3.5, $P < 3.850e\text{-}0.6$), brassinosteroids (representation factor 3.5, $P < 3.218e\text{-}04$) and ethylene (representation factor 3.5, $P < 0.004$). These results suggest that BBX21 acts as a negative regulator of the SAS under long-term canopy shade in conjunction with specific hormonal transcriptional networks.

To validate the microarray data, we analysed the expression of genes involved in various biological processes by quantitative RT-PCR. We confirmed the pattern of expression of *AuxRE*, *ATHB52*, *FQR*, *AUX1*, *GRP3S* and *FIN219* under sunlight and canopy shade (Figure 7 and Table S2). Thus, BBX21 is a negative regulator of genes associated with auxin (*AuxRE*, *AUX1 FQR1*), light (*ATHB52*, *FIN219*) and cold stress (*GRP3S*) signalling pathways, suggesting that BBX21 plays a role in the cross-talk between endogenous and environmental signals regulating expression of the SAS. In wild-type, *ATHB2*, *PAR1* and *HFR1* expression levels increased between 1.8- and 2.3-fold under long-term canopy shade compared to sunlight, and this correlates well with the higher expression of these genes under short-term simulated shade and canopy shade (Figure S6). In the *bbx21* mutant, increased expression of the above genes was also observed under long-term canopy shade, in contrast to the lower induction seen in response to short-term shade. The above results indicate that the long-term response to shade results from a complex interplay between signalling cascades, which must be integrated to regulate the SAS.

DISCUSSION

Transcription factors play a central role in induction of the SAS (Franklin, 2008). The HD-Zip and bHLH families of transcription factors have been reported to mediate responses to changes in the R:FR ratio (Carabelli *et al.*, 1996; Salter *et al.*, 2003; Lorrain *et al.*, 2008). ATHB2 and ATHB4 are two HD-Zip transcription factors whose expression increases in response to a low R:FR ratio, and which promote the SAS by direct interaction with regulatory DNA sequences that control the SAS transcriptome (Carabelli *et al.*, 1996). The mechanisms by which phytochromes regulate the expression of these two HD-Zip genes is not completely understood, but appear to require the activity of bHLH transcription factors (Lorrain *et al.*, 2008). Phytochromes modulate the stability of bHLH transcription factors such as PIF4 and PIF5, which are positive regulators of SAS gene expression. Under a high R:FR ratio, phytochromes promote the phosphorylation and subsequent degradation of PIF4 and PIF5. Under a low R:FR ratio, it has been proposed that phytochromes exit from the nucleus, and PIF4 and PIF5 are therefore stabilized, contributing to the promotion of cell elongation, and, at least in part, directly or indirectly inducing *ATHB2* expression (Lorrain *et al.*, 2008). A low R:FR ratio induces the expression of other bHLH

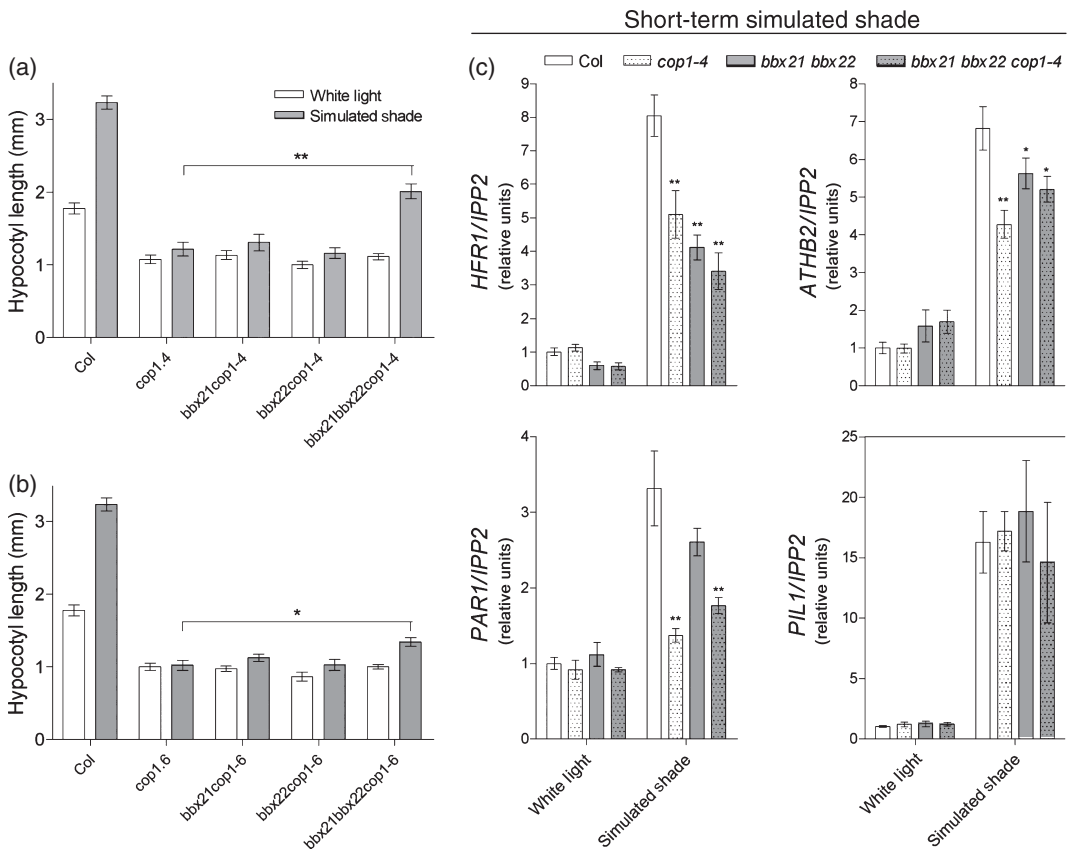

Figure 4. BBX21, together with BBX22, restore the SAS phenotype in the *cop1* background.
(a,b) Hypocotyl length of wild-type, *cop1*, *bbx21 cop1*, *bbx22 cop1* and *bbx21 bbx22 cop1* seedlings grown under simulated shade. Values are means ± SE ($n \geq 15$). Asterisks indicate values that are significantly different from *cop1* under the same light treatment (*$P < 0.05$, **$P < 0.01$).
(c) Quantitative RT-PCR analysis for *PAR* genes (*PIL1*, *HFR1*, *PAR1* and *ATHB2*) in wild-type, *cop1-4*, *bbx21 bbx22* and *bbx21 bbx22 cop1-4* seedlings grown under short-term simulated shade. Quantitative ± SE ($n \geq 3$). Asterisks indicate values that are significantly different from wild-type under the same light treatment (*$P < 0.05$, **$P < 0.01$).

transcription factors, such as HFR1, PAR1 and PAR2, which act as negative regulators of the SAS, constituting a 'gas-and-brake' mechanism that fine-tunes SAS responses (Sessa *et al.*, 2005; Roig-Villanova *et al.*, 2006). In addition to the above signalling mechanisms, phytochromes also regulate the SAS through the photomorphogenic repressor COP1. Although several proteins that interact with COP1 have been identified, the proteins through which COP1 acts to promote growth in response to shade remained uncertain until now.

BBX21 interacts genetically with *HY5* and *COP1*, two master regulators of light signalling in plants (Datta *et al.*, 2007). BBX21 interacts physically with HY5 in yeast and plant cells, regulating, at least partially, physiological responses such hypocotyl inhibition, accumulation of anthocyanin and repression of lateral roots (Datta *et al.*, 2007). However, *hy5* seedlings exposed to a low R:FR ratio show normal activation of SAS genes, suggesting that HY5 is not involved in the modulation of the SAS by COP1, or that its function can be compensated for by other related transcription factors (Roig-Villanova *et al.*, 2006). Furthermore, epistatic effects between *HY5* and *BBX21* were detected for mutant seedlings exposed to continuous R but not continuous FR (Datta *et al.*, 2007), suggesting that BBX21 could interact with COP1, in a HY5-independent manner, to regulate the SAS. In fact, BBX21 is a negative regulator of the SAS phenotype in response to long-term shade (Figures 1, 2 and 4). At the transcriptional level, BBX21 promotes the expression of early SAS genes (Figure 3) and represses several genes under long-term canopy shade (Figures 6 and 7). BBX21 acts as positive regulator of *PAR1*, *HFR1*, *PIL1* and *ATHB2* expression under short-term canopy shade, all of which were previously identified as genes that are up-regulated early by a low R:FR ratio in simulated shade (Carabelli *et al.*, 1996; Salter *et al.*, 2003; Duek *et al.*, 2004; Roig-Villanova *et al.*, 2006). Interestingly, the impaired induction of *PAR* genes in response to short-term shade in the *cop1* mutant is similar to that observed in the *bbx21 bbx22* double mutant. Furthermore, the *bbx21 bbx22 cop1* triple mutant does not show a stronger phenotype than the single or double

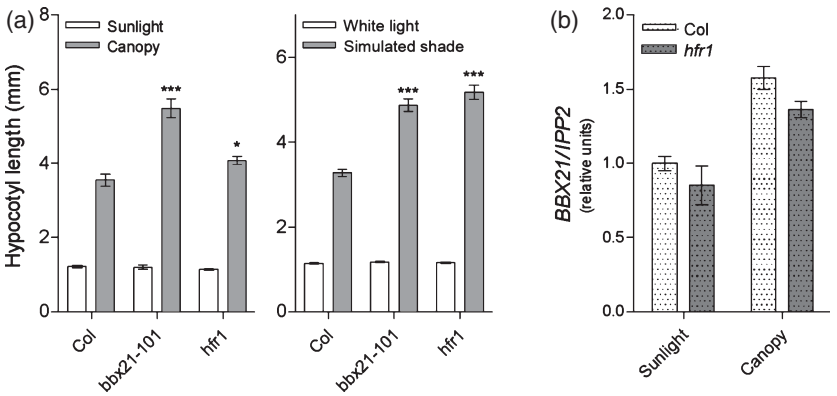

Figure 5. BBX21 and HFR1 act in different SAS signalling pathways.
Hypocotyl length of wild-type, *bbx21-101* and *hfr1* seedlings grown under canopy shade (a) or simulated shade (b). Asterisks indicate values that are significantly different from wild-type under the same light treatment (*$P < 0.05$, ***$P < 0.001$).
(c) Quantitative RT-PCR analysis of the expression of *BBX21* in wild-type and *hfr1* in response to short or long-term canopy shade. Values are means ± SE ($n \geq 3$).

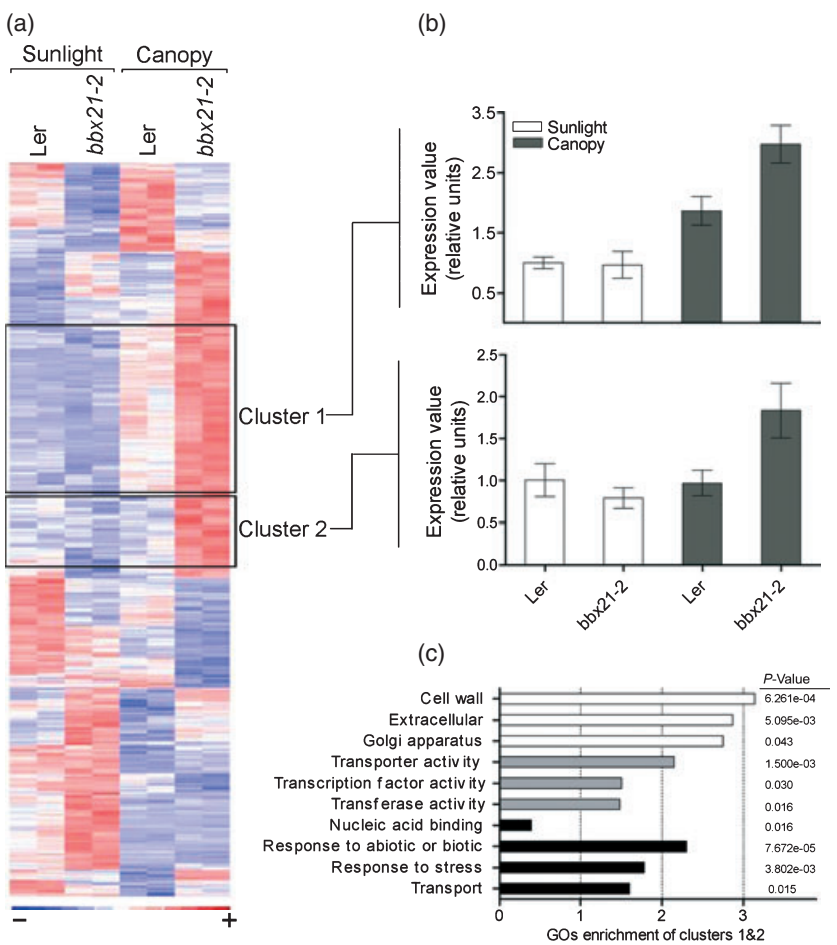

Figure 6. Global expression analysis of wild-type and *bbx21-2* seedlings grown under sunlight and canopy shade.
(a) The dCHIP program was used to define two clusters of genes that showed similar expression under sunlight and higher expression in *bbx21-2* compared with wild-type under canopy shade (clusters 1 and 2).
(b) Mean expression values for genes represented in clusters 1 and 2.
(c) Gene Ontology (GO) assignments for genes of clusters 1 and 2. The graph shows the functional categories of the genes over- or under-represented in clusters 1 and 2 (cellular components, white bars; biological processes, grey bars; molecular functions, black bars). The *P* value is shown for each component.

mutants, as far as *PAR* gene expression is concerned, suggesting that all these proteins act as part of a single molecular complex or signalling pathway during early responses to shade (Figure 4c). Previously Roig-Villanova *et al.* (2006) showed that COP1 regulates the expression of *ATHB2*, *PAR1* and *PIL1* under short-term simulated shade. Here we demonstrate that the regulation and adjustment of the SAS through COP1 involves genetic interactions with *BBX21* and *BBX22* that modulate the expression of *PAR* genes.

Although the molecular mechanism through which COP1 interacts with BBX21 to regulate the SAS is unknown, we hypothesize that COP1 and BBX21 may be part of a molecular complex that regulates SAS signalling at the transcriptional level. BBX21 co-localizes with COP1 in nuclear speckles, and, although they do not interact directly,

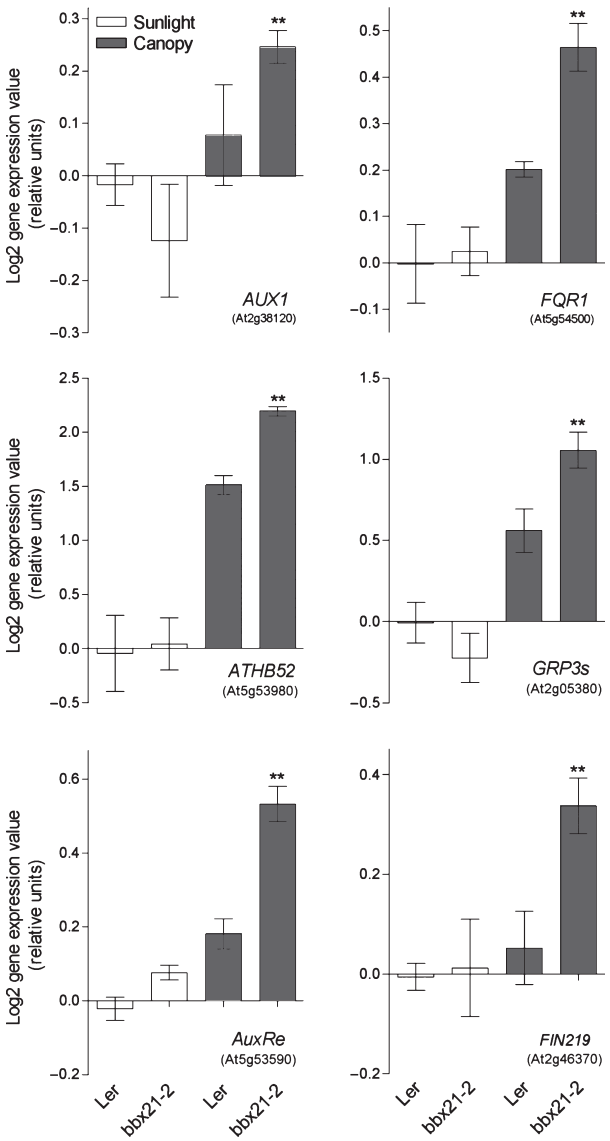

Figure 7. Confirmation by quantitative RT-PCR of the ATH1 Affymetrix microarray output.
Quantitative RT-PCR analysis was performed for selected genes of cluster 1 (*ATHB52, FQR1, AuxRe, AUX1* and *GRP3s*) and cluster 2 (*FIND219*). The expression levels of each gene were normalized to that of the *IPP2* gene, and each treatment was standardized to wild-type expression under sunlight. Values are means ± SE ($n \geq 3$). Asterisks indicate values that are significantly different from wild-type under canopy shade (**$P < 0.01$).

they appear to be part of the same molecular complex through interactions with common partners (Datta *et al.*, 2007, 2008). It is worth noting that, whilst BBX19, BBX21 and BBX22 inhibit the SAS under long-term shade, other members of the BBX protein family such as BBX18 and BBX24 have positive effects on the SAS (Figure 2). In addition, loss of function and over-expressing lines of BBX21 showed increased hypocotyl response to shade compared with wild-type seedlings (Figure 1), suggesting that the mutant phenotypes probably result from a failure to form an accurate complex when expression of the target protein is either abolished or it is over-expressed. Such effects are common when the protein is part of a molecular complex (Pineiro *et al.*, 2003; Chen *et al.*, 2007). When searching for FIN219-interacting proteins in phyA signalling using a yeast two-hybrid approach, Chen *et al.* (2007) showed that gain of function and partial loss of function of FIP1 (FIN219-interacting protein 1) resulted in seedlings with larger hypocotyls than wild-type under continuous FR. We propose that various members of the BBX protein family could be part of the same molecular complex, and that the precise stoichiometric relationship between the BBX proteins that are part of this complex is critical for its proper function.

The central role of BBX21 in the SAS signalling network is not only supported by the molecular and physiological phenotypes of *bbx21* and *BBX21* over-expressing and under-expressing lines. We also show that *BBX21* transcript levels increase twofold immediately after transferring wild-type seedlings to shade, and later decay in plants exposed to prolonged shade. This rapid early induction and subsequent decay of *BBX21* expression in response to canopy shade, together with the positive and negative effects of BBX21 on SAS gene expression in response to short- and long-term canopy shade, strongly suggests that BBX21 is part of the so-called 'gas-and-brake' mechanism, in which a negative feedback loop controls the magnitude of shade-avoidance responses (Sessa *et al.*, 2005). Previously identified components of the 'gas-and-brake' mechanism are HFR1 and PIL1, whose transcripts increase in response to a low R:FR ratio, and with the corresponding proteins repressing molecular and physiological responses to long-term shade (Sessa *et al.*, 2005; Roig-Villanova *et al.*, 2006).

The SAS involves a massive change in gene expression when Arabidopsis seedlings are exposed to a low R:FR ratio (Devlin *et al.*, 2003). Comparing global gene expression in wild-type and *lhus/bbx21* seedlings, we identified a high proportion of genes whose expression was down-regulated by BBX21 under canopy shade but not under sunlight. This group showed statistically significant over-representation of genes previously shown to be up-regulated in response to auxin, brassinosteroids and ethylene, three hormones previously shown to mediate or modulate the SAS (Roig-Villanova *et al.*, 2007; Tao *et al.*, 2008; Pierik *et al.*, 2009). Interestingly, hormones such as auxin and ethylene regulate the SAS independently of DELLA proteins. These observations suggest that phytochromes regulate the SAS through at least two pathways, one involving bHLH transcription factors, such as PIF4, that interacts with GA signalling, and the other involving COP1, BBX21 and BBX22, which appears to interact with auxin, brassinosteroid and ethylene signalling pathways. These pathways may in turn interact, as PIF4 and PIF5 are required for full induction of ATHB2 (Lorrain

et al., 2008), and this transcription factor is known to modulate auxin signalling.

In light of this evidence, we propose that the SAS signalling network is modulated by BBX transcriptional factors that act within the COP1 signalling pathway, regulating the expression of other transcription factors in a positive manner and at an early stage. Under long-term canopy shade, BBX21 down-regulates the expression of a higher proportion of genes to avoid an exaggerated SAS response through the action of hormonal signalling networks.

EXPERIMENTAL PROCEDURES

Plant material

lhus/bbx21-101 was isolated from a population of activation-tagged lines of *A. thaliana* generated in the Col background (CS21995). The *bbx21-2* (GT-5-101527), *bbx22*, *bbx24*, *bbx25*, *cop1-4* and *cop1-6* were obtained from the Arabidopsis Biological Resource Center. *bbx21 bbx22*, *bbx21 cop1-4*, *bbx21 cop1-6*, *bbx22 cop1-4*, *bbx22 cop1-6*, *bbx21 bbx22 cop1-4* and *bbx21 bbx22 cop1-6* have been described previously by Datta *et al.* (2007). *bbx18-2 and bbx19-1* have been described previously by Kumagai *et al.* (2008).

The T-DNA insertion site in *bbx21-101* was determined by thermal asymmetrical interlaced (TAIL) PCR followed by DNA sequencing (Liu *et al.*, 1995). The T-DNA insertions in *bbx21-101* and *bbx21-2* were confirmed by PCR using specific primers (Table S3). To generate transgenic lines over-expressing *LHUS/BBX21*, the coding sequence of *LHUS/BBX21* was amplified by PCR from genomic *A. thaliana* DNA using the primers 35s172for and 35s172rev (Table S3). The full-length DNA was cloned into the pDONOR221 plasmid (Invitrogen, http://www.invitrogen.com), and inserted into the pB7WG2 binary plasmid under the control of the CaMV 35S promoter using Gateway technology (http://www.invitrogen.com), and then introduced into *Agrobacterium tumefaciens* (GV3101). To generate RNAi lines under-expressing *LHUS/BBX21*, the 5′ UTR of *LHUS/BBX21* was amplified by PCR from genomic *A. thaliana* DNA using the primers RNAi172for and RNAi172rev (Table S3). The DNA was cloned into pDONOR221 (http://www.invitrogen.com), inserted into the pH7GWIWWG2 plasmid (Karimi *et al.*, 2002) using Gateway technology (http://www.invitrogen.com), and then introduced into *A. tumefaciens* (GV3101). These constructs were used to transform Col and L*er* plants by the floral-dip method (Bechtold and Pelletier, 1998). Transgenic seedlings were selected on kanamycin-containing medium, and the expression levels of *LHUS/BBX21* in the T_3 generation were determined by semi-quantitative PCR using specific primers (Table S3). Transgenic phenotypes were confirmed in the T_4 and T_5 generations.

Experimental conditions

For all experiments, seeds were sown in clear plastic boxes on 0.8% agar/water, and incubated in darkness at 4°C to reduce dormancy and homogenize germination. After 4 days of incubation at 4°C, imbibed seeds were exposed to an R pulse and incubated in darkness for the following 24 h at 25°C to induce germination.

For the simulated shade experiments, seeds contained in plastic boxes were exposed to a low R:FR ratio using Paolini filters (Paolini 2031, Buenos Aires, Argentina) within a glasshouse with a controlled mean temperature of approximately 22°C, a long-day photoperiod (15 h) and natural radiation (Photosynthetically Active Radiation (PAR) 85 μmol m^{-2} sec^{-1}, R:FR 0.02). Seedlings with long hypocotyls were selected after 5 days. Growth in sunlight treatment used as a control (PAR 400 μmol m^{-2} sec^{-1}, R:FR 1.2).

For the canopy shade experiments, seeds contained in plastic boxes were exposed to sunlight for 2 days (PAR 400 μmol m^{-2} sec^{-1}, R:FR 1.2), and transferred to ryegrass canopy shade (PAR 40 μmol m^{-2} sec^{-1}, R:FR 0.1) for 4 days. Control plants were kept in sunlight for 6 days.

For the simulated shade experiments, we used a light growth chamber with mercury lamps (General Electric, http://www.ge.com, HR175/R/DX/FL39 mercury 33026; PAR 100 μmol m^{-2} sec^{-1}, R:FR 3.4) under a long-day photoperiod (16 h light + 8 h dark) at 22°C. Seedlings were grown for 2 days in white light at an R:FR ratio of 3.4, and then exposed to a low R:FR ratio (0.35) for 4 days (simulated shade). The low R:FR ratio was obtained by use of incandescent lamps (Philips, http://www.philips.com, R19-100R20/FL/S) covered with Paolini filters (Paolini 2031), and placed laterally in the growth chamber. Control plants were kept in the growth chamber at R.FR 3.4. The spectral photon fluences for sunlight and shade conditions were obtained using a Li-Cor integrating quantum radiometer/photometer (Li-188B; LiCor Corp., http://www.licor.com). PAR and R:FR ratios were measured using a SKR-1850SS2 light sensor attached to a SpectroSense2 datalogger (Skye Instruments Ltd, http://www.skyeinstruments.com/).

Gene expression analysis

We used 100 mg of fresh leaf tissue, which was harvested and frozen immediately in liquid nitrogen, for the RNA expression analysis. Total RNA was extracted using an RNeasy plant mini kit (Qiagen, http://www.qiagen.com). Crude RNA preparations were treated with 10 units of RNase-free DNase I (Promega, http://www.promega.com), and the samples were purified according to the RNeasy plant mini kit protocol.

For semi-quantitative RT-PCR studies, cDNA was synthesized from 1.5 μg of DNA-free RNA template using an oligo(dT) primer and SuperScript II reverse transcriptase (http://www.invitrogen.com). *LHUS/BBX21* amplification was performed using RT172for and RT172rev primers, and *ACTIN2* (At3g18780) was used as a control (Table S3). PCR products were detected on 0.8% agarose gels infiltrated with ethidium bromide.

Real-time PCR analysis was performed on an optical 96-well plate using SYBR Green PCR master mix (Applied Biosystems, http://www.appliedbiosystems.com) and an ABI PRISM 7500 real-time PCR system (http://www.appliedbiosystems.com). The thermal cycle used was 95°C for 15 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec and 72°C for 35 sec. Gene-specific primer pairs (Table S3) were designed using Beacon Designer 7.0 (http://www.premierbiosoft.com). The genes evaluated in this study were *BBX18* (At2g21320), *BBX19* (At4g38960), *BBX21* (At1g75540), *BBX22* (At1g78600), *BBX24* (At1g06040), *BBX25* (At2g31380), *HFR1* (At1g02340), *PAR1* (At2g42870), *ATHB2* (At4g16780), *PIL1* (At2g46970), *GRP3s* (At2g05380), *ATHB52* (At5g53980), *AUX1* (At2g38120), *AuxRe* (At5g53590), *FQR1* (At5g54500) and *FIN219* (At2g46370). *ACTIN8* (At1g49240) and *IPP2* (At3g02780) were used to normalize expression levels.

Microarray data analysis

We used a factorial experimental design comprising two genotypes (L*er* and *bbx21-2*) and two light treatments (sunlight and long-term canopy shade). RNA material was extracted from 6-day-old seedlings exposed for 6 days to sunlight, or for 2 days to sunlight followed by 4 days under ryegrass canopy shade. Two replicates per treatment were performed. Affymetrix Arabidopsis ATH1 GeneChips® were used (http://www.affymetrix.com/). RNA was

prepared, labelled and hybridized to the arrays in accordance with the manufacturer's instructions. Data were normalized by multiplying the value of each gene by the mean of each chip, divided by the average intensity of all mean values. Significantly differentially expressed genes were identified by performing profile analysis using Significance Analysis of Microarrays (Tusher *et al.*, 2001) with a δ value of 1.22, which corresponds to a false discovery rate of 4.12%. Genes with 'absent' calls and a signal of <50 units in all replicate experiments were filtered out. A test filter was performed to work only with those genes for which the ratio of expression showed at least a 1.3-fold change between L*er* and *bbx21-2*. Clusters were generated using DNA-Chip Analyzer (dChip) (Li and Wong, 2003; http://www.dchip.org). Statistical significance of the overlap between two groups of genes was calculated as the representation factor, which is the number of overlapping genes divided by the expected number of overlapping genes drawn from two independent groups (http://elegans.uky.edu/). Genes of clusters 1 and 2 were analysed using the Classification SuperViewer Tool of the Bio-Array Resource (http://bar.utoronto.ca).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Hypocotyl length in darkness and mRNA expression for wild-type, *bbx21* and *BBX21ox* and *BBX21*_RNAi transgenic seedlings.

Figure S2. B, R and FR fluence–response curves for hypocotyl length in wild-type, *bbx21* and *BBX21ox* transgenic seedlings.

Figure S3. Circadian rhythm of leaf movement in wild-type, *bbx21-2* and *BBX21ox* transgenic seedlings.

Figure S4. Quantitative RT-PCR analysis of PAR genes in wild-type and *bbx21-2* seedlings grown under sunlight or short-term canopy shade.

Figure S5. Hypocotyl length of wild-type (L*er*), *cop1-4*, *bbx21 cop1-4*, *bbx22 cop1-4* and *bbx21 bbx22 cop1-4* seedlings grown under sunlight and canopy shade.

Figure S6. Microarray expression values of PAR genes for wild-type and the *bbx21* mutant under sunlight and long-term canopy shade.

Table S1. Genes that are up- or down-regulated by BBX21 under sunlight and canopy shade.

Table S2. Genes that are expressed differentially between wild-type and *bbx21* under canopy shade, and clusters 1 and 2 of genes repressed by BBX21 under canopy shade.

Table S3. Primers used in this work.

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