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SALT TOLERANCE HOMOLOG2, a B-Box Protein in *Arabidopsis* That Activates Transcription and Positively Regulates Light-Mediated Development [W]

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CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1) and ELONGATED HYPOCOTYL5 (HY5) are two major regulators of light signaling in plants. Here, we identify *SALT TOLERANCE HOMOLOG2* (*STH2*) as a gene that interacts genetically with both of these key regulators. *STH2* encodes a B-box–containing protein that interacts physically with HY5 in yeast and in plant cells. Whereas STH2 is uniformly nuclear by itself, it shows a COP1-dependent localization to speckles when coexpressed with COP1. We identified two independent T-DNA insertion lines in *STH2*. Both alleles are hyposensitive to blue, red, and far-red light. The *sth2* mutant, like *hy5*, shows an enhanced number of lateral roots and accumulates less anthocyanin. Analysis of double mutants between *sth2* and *hy5* indicates that *STH2* has both *HY5*-dependent and -independent functions. Furthermore, besides partially suppressing the hypocotyl phenotype of dark-grown *cop1* alleles, *sth2* also suppresses the reduced number of lateral roots and high anthocyanin levels in light-grown *cop1* alleles. Interestingly, we found that STH2 can activate transcription. Transient transfection assays in protoplasts using a LUC reporter driven by the chalcone isomerase promoter show that the B-boxes in STH2 and a functional G-box element in the promoter are required for this activity. In conclusion, we have identified STH2, a B-box protein in *Arabidopsis thaliana*, as a positive regulator of photomorphogenesis and report that the B-box domain plays a direct role in activating transcription in plants.

INTRODUCTION

Throughout their life cycle, plants continuously monitor the quality, quantity, duration, and direction of light in order to optimize their growth and development according to the ambient light environment. The light is sensed by wavelength-specific photoreceptors, of which at least four classes have been reported in *Arabidopsis thaliana*. The phytochromes PHYA to PHYE perceive red/far-red light (600 to 750 nm); the cryptochromes CRY1 to CRY3 and the phototropins PHOT1 and PHOT2 detect blue and UV-A light (320 to 500 nm), respectively; and UV-B light (282 to 320 nm) is perceived by a yet uncharacterized photoreceptor (Sullivan and Deng, 2003). A dramatic example of light signaling can be seen when a seedling emerges from soil into the light. During this developmental transition, light signaling releases a developmental arrest upon which autotrophic growth and adult development can commence. In no or low light, the seedlings develop long hypocotyls with undifferentiated cotyledons forming an apical hook, whereas in light, the seedling undergoes photomorphogeneis or deetiolation, which is characterized by the inhibition of hypocotyl elongation, cotyledon expansion, and greening, leading to the acquisition of photosynthetic capacity. This light-induced developmental transition is primarily mediated by the phytochromes, CRY1, and CRY2 and is accompanied by a massive transcriptional reprogramming. Microarray analysis revealed that up to one-third of the genes in *Arabidopsis* showed changes in expression between seedlings grown in the dark and the light (Ma et al., 2001). Studies performed on seedlings grown in monochromatic far-red, red, or blue light found that a large fraction of the early-affected genes are transcription factors (Tepperman et al., 2001, 2004; Jiao et al., 2003). It has been proposed that activation of a photoreceptor initiates a transcriptional cascade by regulating a group of master transcription factors that, in turn, control the transcriptional reprogramming during photomorphogenesis (Tepperman et al., 2001, 2004).

Genetic screens have identified several transcription factors acting as positive or negative regulators downstream of a specific photoreceptor or set of transcription factors. Studies of far-red light–dependent photomorphogenensis have revealed FAR-RED IMPAIRED RESPONSE1 (FAR1) and FAR-RED ELONGATED HYPOCOTYL3 (FHY3), both of which are novel transposase-related putative transcription factors (Hudson et al., 1999; Wang and Deng, 2002; Hudson and Quail, 2003), whereas LONG AFTER FAR-RED LIGHT1 (LAF1) is homologous with R2R3-MYB transcription factors (Ballesteros et al., 2001). The developmental defects of loss-of-function mutations in *far1*, *fhy3*, and *laf1* are specific to PHYA-mediated photomorphogenesis in response to far-red light. Mutations in LONG HYPOCOTYLS IN FAR-RED LIGHT1 (HFR1), encoding a basic helix-loop-helix protein, show similar light-hyposensitive phenotypes in both far-red and blue light, suggesting a role in both PHYA and CRY signaling (Fairchild et al., 2000; Duek and Fankhauser, 2003).

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Two Dof family transcription factors, COGWHEEL1 (COG1) and OBF4 BINDING PROTEIN3 (OBP3), are involved in red light signaling: COG1 acts as a negative regulator in both red and far-red light (Park et al., 2003), whereas OBP3 has both positive and negative roles in PHYB and CRY1 signaling pathways (Ward et al., 2005). In addition the identification of MYC2, a basic helix-loop-helix protein, and GBF1, a basic domain/leucine zipper (bZIP) protein, revealed that these two factors act as a repressor of blue and far-red light–mediated deetiolation and as a negative and positive regulator of blue light signaling, respectively (Yadav et al., 2005; Mallappa et al., 2006).

The regulation of protein stability has been found to play a key role in the signaling pathways downstream of the photoreceptors. Mutations in a group of at least 10 genes, the *COP*/*DET*/*FUS* genes, result in constitutive photomorphogenesis (Wei and Deng, 1996). The molecular characterization of the COP/DET/FUS proteins suggests that most, if not all, act in a proteolytic pathway aimed at degrading photomorphogenesis-promoting factors in the absence of light (Osterlund et al., 2000). CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1), an E3 ubiquitin ligase, acts in concert with SPA proteins as a dark-dependent repressor of photomorphogenesis (Saijo et al., 2003). The COP1 repression is partly mediated through ubiquitin-dependent degradation of the transcription factors ELONGATED HYPOCOTYL5 (HY5), HYH, LAF1, and HFR1 (Osterlund et al., 2000; Holm et al., 2002; Seo et al., 2003; Yang et al., 2005). Furthermore, COP1 was found to interact with several photoreceptors, such as phyA, phyB, cry1, and cry2 (Wang et al., 2001; Yang et al., 2001; Shalitin et al., 2002; Seo et al., 2004), and can target some of them for degradation, as in the case of phyA (Seo et al., 2004), or regulate its abundance, as in cry2 (Shalitin et al., 2002).

The bZIP transcription factor HY5 is a well-characterized target of COP1 regulation. Mutations in *HY5* result in an elongated hypocotyl in all light conditions, suggesting that *HY5* acts downstream of all photoreceptors (Koornneef et al., 1980; Oyama et al., 1997; Ang et al., 1998; Ulm et al., 2004). The *hy5* mutant also has defects in lateral root formation, secondary thickening in roots, and chlorophyll and anthocyanin accumulation (Oyama et al., 1997; Holm et al., 2002). Recently, it was shown that HY5 plays a role in both auxin and cytokinin signaling pathways (Cluis et al., 2004; Sibout et al., 2006; Vandenbussche et al., 2007), suggesting that HY5 might be a common intermediate in light and hormone signaling pathways. In vitro DNA–protein interaction studies revealed that HY5 binds specifically to the G-box present in the promoters of several light-inducible genes, such as chalcone synthase (CHS) and ribulose bisphosphate carboxylase small subunit (RbcS1A) (Ang et al., 1998; Chattopadhyay et al., 1998). Recently, a modified chromatin immunoprecipitation technique in combination with a whole-genome tiling array (ChIP-chip) revealed that HY5 binds to promoter regions of a large number of annotated genes (Lee et al., 2007). HY5 appears to mediate both the upregulation and downregulation of gene expression by light. Most of the genes subject to HY5 regulation are included among the genes regulated by light and constitute ~20% of all light-regulated genes (Ma et al., 2001). Interestingly, Lee et al. (2007) found that >60% of the early-induced genes by PhyA and PhyB (Tepperman et al., 2001, 2004) are HY5 binding targets, which suggests that HY5 is high in the hierarchy of the transcriptional cascade during photomorphogenesis. However, HY5 binding is not sufficient for transcriptional regulation. HY5 was found to be constitutively bound to the promoters of both light-regulated genes such as CHS and RbcS1A and circadian regulators such as CCA1, LHY, TOC1, and ELF4, suggesting that additional factors are required for HY5-dependent transcriptional regulation (Lee et al., 2007).

We previously identified three B-box–containing proteins, COL3, STH (now renamed STH1), and STO, that are able to interact with COP1 (Holm et al., 2001; Datta et al., 2006). We found that COL3 acts as a positive regulator of red light signaling (Datta et al., 2006), and functional SPA proteins were recently found to be important for the stability of the B-box–containing protein CONSTANS (Laubinger et al., 2006). These proteins all contain N-terminal tandem repeated B-boxes. The B-box is a Zn^{2+}-ligating domain constituted of conserved Cys and His residues that has been proposed to be a protein interaction domain (Borden et al., 1995; Torok and Etkin, 2001); however, no interaction partners or functions have been described for this domain. There are 32 B-box–containing proteins in *Arabidopsis*, and although they are listed as transcription factors by Riechmann et al. (2000), the molecular function of the B-box domain is still poorly understood.

Here, we identify SALT TOLERANCE HOMOLOG2 (STH2), a B-box–containing protein that interacts physically with HY5 and interacts genetically with both HY5 and COP1. We found that the B-boxes in STH2 are required for interaction with HY5. We present evidence for the role of STH2 in light-dependent inhibition of hypocotyl elongation, lateral root formation, and anthocyanin accumulation. We also describe the genetic interaction of *sth2* with *hy5* and *cop1*. All of the phenotypic data indicate a positive role of *STH2* in photomorphogenic development. Finally, we provide functional data demonstrating that STH2 can activate transcription and show that the B-boxes in STH2 and a functional G-box element in the promoter are required for this activity. We propose that STH2 interacts with HY5 through the B-box, thus providing transactivating potential to the HY5 transcription factor.

RESULTS

STH2 Interacts with HY5 in Yeast

In an effort to identify putative regulators of light signaling, COP1 was used as bait in a yeast-two hybrid screen (Holm et al., 2001, 2002). STO, STH1, and COL3, all containing B-boxes, were identified in this screen (Holm et al., 2001; Datta et al., 2006). Interestingly, STO as well as a related protein, STH2, had been identified previously in a yeast two-hybrid screen using HY5 as bait; however, in both cases, the cDNAs had 5′ untranslated region sequences that put them out of frame from the activation domain (T. Oyama, personal communication). STH2, like STO and STH1, contains two B-boxes in the N-terminal region, which show 57.3 and 52.7% amino acid identity respectively (Figure 1A). To confirm and further characterize the interaction between HY5 and STH2, we inserted the *STH2* cDNA into the pYX141 yeast expression vector, where the cDNA is expressed from a weak promoter, and assayed its interaction with HY5 in liquid

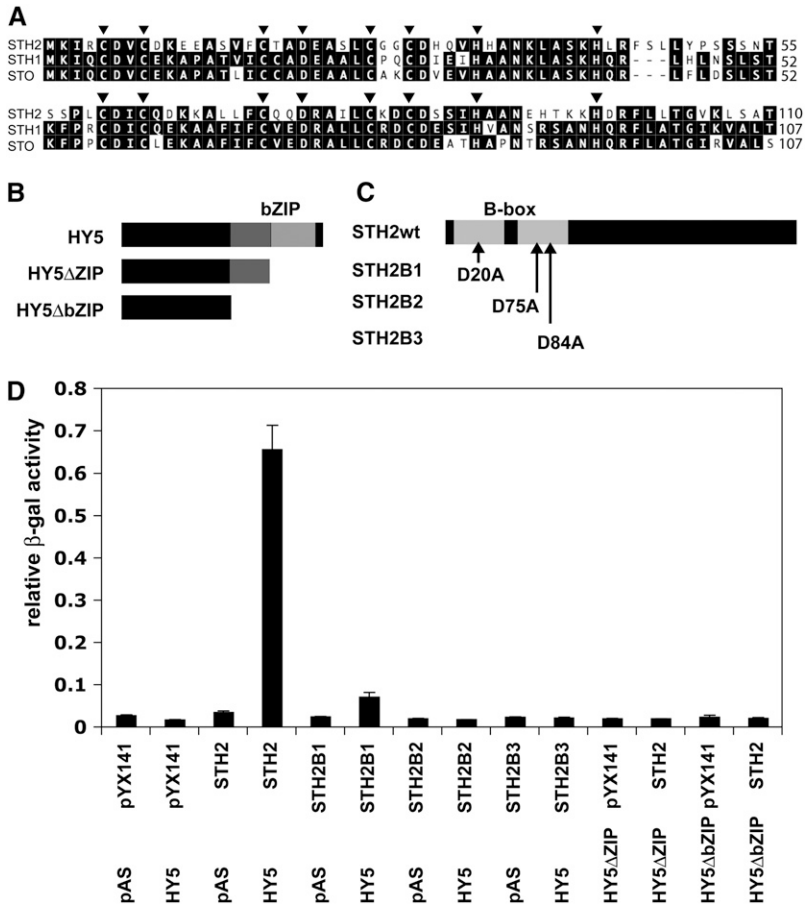

Figure 1. STH2 Interacts with HY5 in Yeast, and the bZIP Domain of HY5 and the B-Boxes in STH2 Are Important for the Interaction.

(A) Alignment of the B-box–containing N-terminal half of STH2, STH1, and STO. Arrowheads indicate the Zn^{2+}-ligating conserved Cys, His, and Asp residues present in the B-box.

(B) and **(C)** Schematic representation of the domain structures of HY5 **(B)** and STH2 **(C)** showing mutations in the bZIP domain and the B-boxes, respectively. D-n-A indicates the Ala substitution of the three Asp residues in the B-boxes at positions 20, 75, and 84 (n).

(D) Yeast two-hybrid interactions between STH2 and HY5 proteins as indicated in **(B)** and **(C)**. Error bars indicate SE ($n = 6$).

β-galactosidase assays (Figure 1D). The STH2 protein expressed from the pYX141 vector did not activate transcription together with the Gal4 DNA binding domain (Gal4-DBD) vector control but resulted in a 24-fold increase in β-galactosidase activity over the vector control when expressed together with Gal4-DBD-HY5.

In order to map the interaction between HY5 and STH2, we examined the interactions between wild-type and mutated HY5 and STH2 proteins in β-galactosidase assays. To this end, we used Gal4-DBD fusions of full-length HY5 and two truncated HY5 proteins with either the Leu zipper domain (amino acids 115 to 168) or the basic zipper domain (amino acids 77 to 168) deleted (Figure 1B). Deletion of the zipper domain or the basic zipper domain in HY5 resulted in a dramatic reduction in β-galactosidase activity, indicating that the basic zipper domain is required for the interaction with STH2 (Figure 1D). To further characterize the interaction between HY5 and STH2, we individually substituted three conserved Asp residues in the B-boxes of STH2; two of the residues, Asp-20 and Asp-75, correspond to Zn^{2+}-ligating residues in the B-box protein MID1 (Massiah et al., 2007), and substitution of these is likely to disrupt the structure of the B-box. The substituted proteins were named STH2-B1, -B2, and -B3, respectively (Figures 1C and 1D). We found that all three substitutions resulted in dramatic reductions of β-galactosidase activity compared with wild-type levels (Figure 1D). Together, these results suggest that the basic zipper domain in HY5 and the B-boxes in STH2 are important for the HY5–STH2 interaction.

STH2 Is a Nuclear Protein and Colocalizes with HY5 in Plant Cells

HY5 gives a diffused nuclear fluorescence when expressed in onion (*Allium cepa*) epidermal cells (Ang et al., 1998) (Figure 2A). In order to determine the subcellular localization of the STH2 protein, we prepared a Cyan Fluorescent Protein (CFP) fusion of STH2 and expressed it in onion epidermal cells. STH2, like HY5, localizes uniformly throughout the nucleus (Figure 2A). Since both

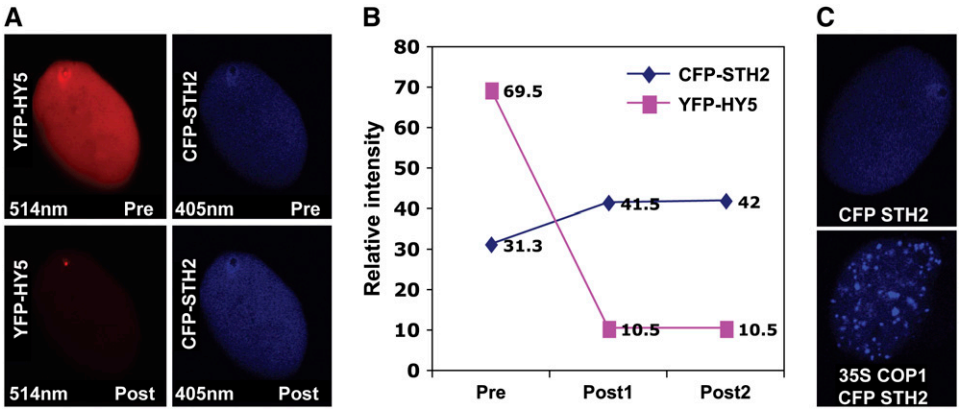

Figure 2. STH2 Interacts with Both HY5 and COP1 in Living Plant Cells.

(A) and **(B)** FRET between YFP-HY5 and CFP-STH2 analyzed by acceptor bleaching in nuclei ($n = 10$). The top panels in **(A)** show representative prebleach nuclei coexpressing YFP-HY5 and CFP-STH2 excited with a 514- or a 405-nm laser, resulting in emission from YFP (red) or CFP (blue), respectively. The total nucleus was bleached with the 514-nm laser. The bottom panels in **(A)** show the same nuclei after bleaching excited with a 514- or 405-nm laser. The relative intensities of both YFP and CFP inside the nucleus were measured once before and twice after the bleaching, as indicated in **(B)**. An increase in donor fluorescence (blue) is seen only if a protein–protein interaction occurs.
(C) Nucleus of a cell coexpressing 35S:COP1 (untagged) and CFP-STH2, excited with a 405-nm laser.

proteins give a diffused nuclear fluorescence, we proceeded to examine whether they interact physically within the nucleus. To this end, we examined whether fluorescence resonance energy transfer (FRET) occurred between the two fusion proteins using the acceptor photobleaching technique. Here, we coexpressed CFP-STH2 with Yellow Fluorescent Protein (YFP)–fused HY5 and excited them with 405- and 514-nm lasers. Both CFP and YFP fluorescence were detected before the bleach (Figure 2A, top panels). After raising the intensity of the 514-nm laser, the YFP fluorescence from the acceptor, YFP-HY5, was bleached (Figure 2A, bottom panel). The bleaching of the acceptor resulted in an increased emission from CFP-STH2, as shown in Figures 2A and 2B, indicating that FRET had occurred between the two proteins prior to the bleach.

COP1 has previously been shown to be able to recruit HY5 as well as several other interacting proteins to nuclear speckles (Ang et al., 1998; Holm et al., 2002; Seo et al., 2003). Since STH2 showed a similar localization pattern to HY5, we wanted to determine whether STH2 interacts with COP1 in vivo. To this end, we coexpressed unfused COP1 (35S:COP1) with CFP-STH2 in onion epidermal cells. A weak uniform fluorescence with consistent nuclear speckles was seen (Figure 2C). Since STH2 by itself gives a uniform fluorescence, the detection of nuclear speckles when coexpressed with untagged COP1 suggests the recruitment of STH2 into COP1 speckles. Coexpression of CFP-COP1 also resulted in the localization of YFP-STH2 into nuclear speckles; however, we did not detect any FRET in these experiments (see Supplemental Figure 1 online).

Identification of Two *sth2* Alleles

In order to examine the role of *STH2* in light-regulated development, we obtained T-DNA insertions in the *STH2* gene (At1g75540) from the SALK collection. The *STH2* gene is located on the bottom arm of chromosome I and contains three exons. We identified two T-DNA insertions in the *STH2* gene (Figure 3A). The first insertion is located in the 5′ untranslated region at nucleotide position −43 from the start ATG and results in a total loss of *STH2* mRNA (Figure 3B), indicating that it is a null mutation. This null mutant is in the Columbia-0 (Col-0) accession and was named *sth2-1*. The second allele, *sth2-2*, is in the

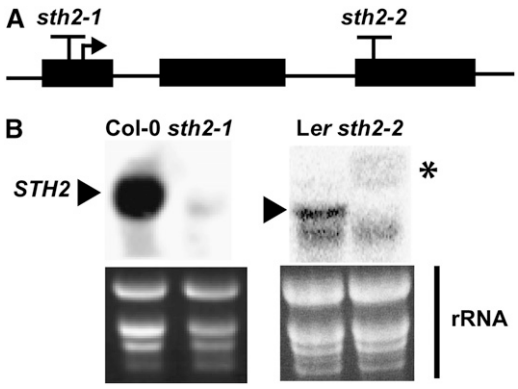

Figure 3. T-DNA Insertion Mutants in *STH2*.

(A) Schematic representation of the *STH2* gene (At1g75540). The arrow indicates the position of the start Met, and T indicates the T-DNA insertion positions.
(B) RNA gel blot showing *sth2* transcript accumulation in wild-type (arrowhead) and *sth2* seedlings at 6 d after germination in continuous white light. Full-length STH2 was used as the probe. rRNA bands are shown as loading controls. The asterisk indicates the larger transcripts seen in *sth2-2*.

Landsberg *erecta* (L*er*) accession. There, the T-DNA insertion is present in the third exon and the RNA gel blot revealed weak signals from larger transcripts, possibly *STH2*–T-DNA fusion transcripts, in *sth2-2* (Figure 3B), suggesting that *sth2-2* is a knocked down allele. Both mutant lines *sth2-1* and *sth2-2* were backcrossed into their corresponding wild-type backgrounds, Col-0 and L*er*, respectively, and *sth2-1* was also crossed into *hy5-215*, *hy5-ks50*, *cop1-4*, and *cop1-6*. The backcrossed F1 plants were indistinguishable from wild-type plants, and we observed a 3:1 (wild type:*sth2*) segregation in the F2 plants, in which the selection markers cosegregated with the phenotypes. This suggests that the mutations are recessive in nature and that single T-DNA loci were responsible for the observed phenotypes in *sth2-1* and *sth2-2*.

sth2 Is Hyposensitive to Blue, Red, and Far-Red Light

The homozygous *sth2* mutants were germinated at different fluences of monochromatic blue, red, and far-red light to examine whether *STH2* plays a role in light-mediated seedling development. We found that *sth2* had longer hypocotyls in blue light and that the effect became more pronounced at higher fluence levels (Figure 4). At a fluence level of 9 $\mu mol \cdot m^{-2} \cdot s^{-1}$, the *sth2-1* seedlings were 65.6% longer than the Col-0 seedlings and the

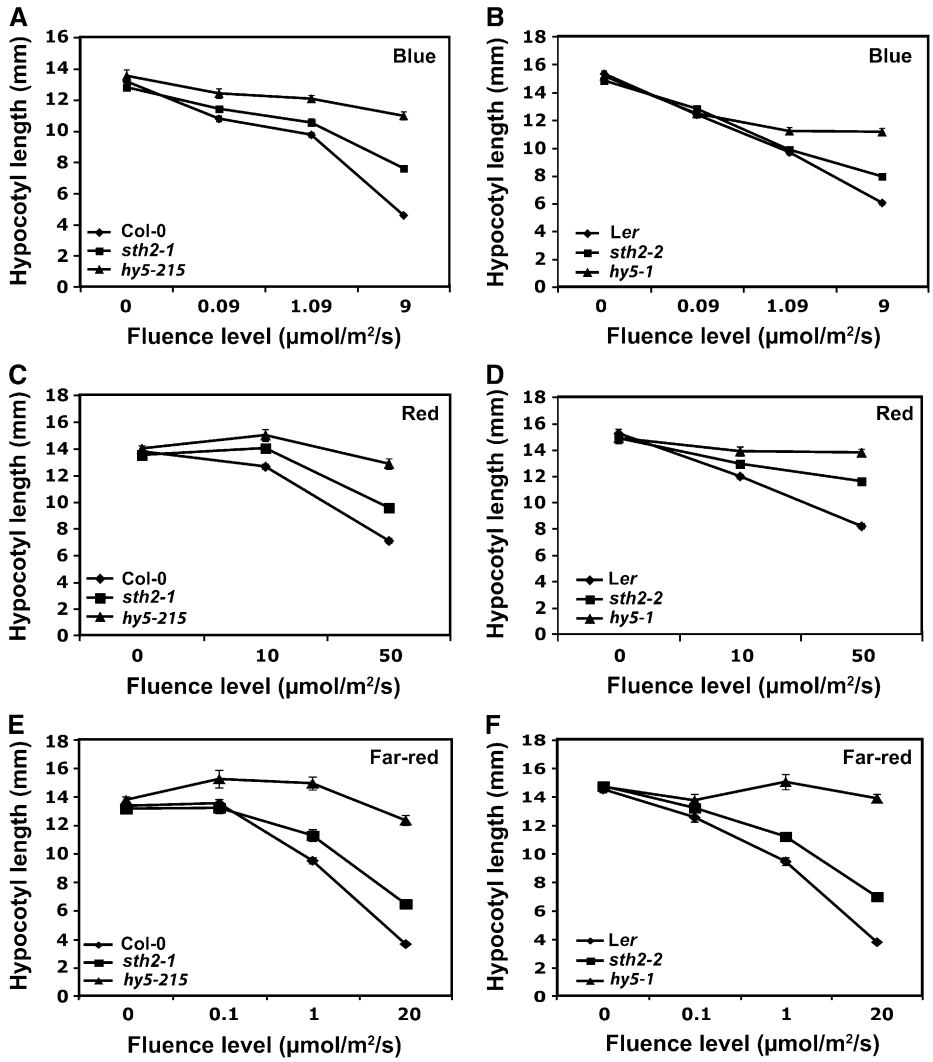

Figure 4. *sth2* Is Hyposensitive to Blue, Red, and Far-Red Light.

(A) and **(B)** Fluence response curves of wild-type (Col-0), *sth2-1*, and *hy5-215* seedlings **(A)** and wild-type (L*er*), *sth2-2*, and *hy5-1* seedlings **(B)** grown under continuous monochromatic blue light.

(C) to **(F)** Fluence response curves of the same seedlings grown under continuous monochromatic red light (**[C]** and **[D]**) and under far-red light (**[E]** and **[F]**).

The experiments were performed twice with similar results. The graphs depict one of these experiments. Error bars represent SE ($n \geq 16$).

sth2-2 seedlings compared with L*er* seedlings had 31.4% longer hypocotyls (Figures 4A and 4B). At very low fluence levels (0.09 $\mu mol \cdot m^{-2} \cdot s^{-1}$), the *sth2* mutant was indistinguishable from wild-type plants, suggesting that *sth2* is specifically hyposensitive to higher fluence levels of blue light. Similar effects were seen in monochromatic red light (Figures 4C and 4D). At high fluence levels (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$), both alleles had longer hypocotyls than the corresponding wild-type plants and the difference in hypocotyl length became lower with reduction in the fluence levels. Under far-red light, at a fluence level of 20 $\mu mol \cdot m^{-2} \cdot s^{-1}$, the *sth2-1* mutant seedlings had 76.7% longer hypocotyls than the Col-0 seedlings, whereas the *sth2-2* seedlings were 84.3% longer compared with L*er* seedlings.

The homolog of STH2, STO, was first identified as an *Arabidopsis* protein that could ectopically rescue the salt sensitivity of calcineurin-deficient yeast (Lippuner et al., 1996) and later shown to enhance root growth tolerance to high salinity upon overexpression in *Arabidopsis* (Nagaoka and Takano, 2003). Therefore, we examined the root growth of *sth2-1* in high-salt medium; however, as has been reported for *sto* T-DNA and RNA interference lines (Indorf et al., 2007), we saw no distinguishable salt tolerance or sensitivity in the *sth2-1* loss-of function allele (see Supplemental Figure 2 online). Together, these phenotypes suggest that *STH2* acts as a positive regulator of light-dependent inhibition of hypocotyl elongation.

The Long Hypocotyl Phenotype of *sth2* Is Partially Dependent on *hy5*

We tried to examine whether daylength had any effect on the seedlings. To this end, we germinated homozygous *sth2* seedlings along with their corresponding wild types under long-day (16 h of light/8 h of dark) and short-day (8 h of light/16 h of dark) conditions. While we did not see a significant effect under long days, in short days *sth2* seedlings were slightly longer than the wild-type seedlings (Figure 5A).

Since *hy5* has long hypocotyls in all light conditions, we wanted to check the phenotype of the *sth2 hy5* double mutant. We generated homozygous *sth2 hy5-215* and *sth2 hy5-ks50* double mutants and germinated them in the different light conditions together with the single mutants and the respective wild types (Figure 5). While both *sth2-1* and *hy5-215* are in the Col-0 accession, *hy5-ks50* is in Wassilewskija. Interestingly, under all light conditions tested, the *sth2-1 hy5-ks50* seedlings had longer hypocotyls than the *hy5-ks50* single mutant seedlings. However, owing to the mixed genetic background of *sth2-1 hy5-ks50*, we decided to focus on the effects seen in the isogenic cross *sth2-1 hy5-215*. In short days and in red light, the *sth2 hy5-215* double mutants were almost indistinguishable from *hy5*, indicating that under these conditions *hy5* is epistatic to *sth2* (Figures 5A and 5B). By contrast, in blue and far-red light, the

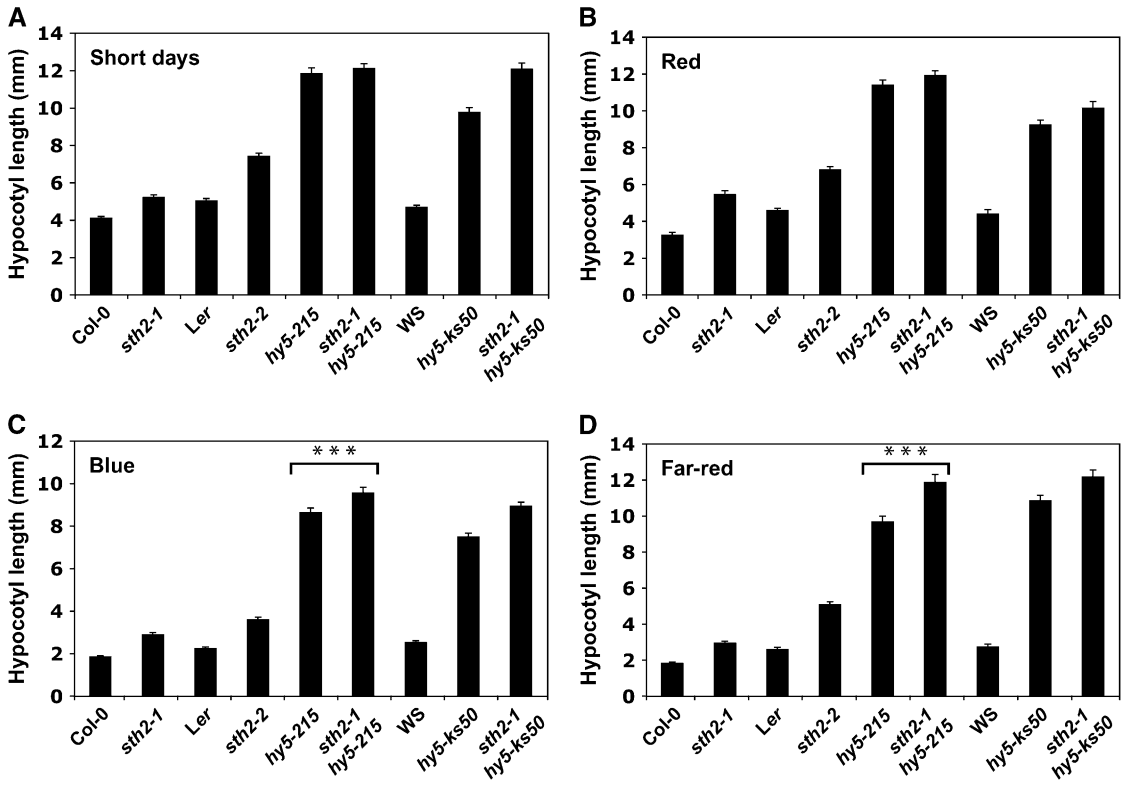

Figure 5. The Long Hypocotyl Phenotype of *sth2* Is Partially Dependent on *hy5*.

Bar graphs show the differences in hypocotyl length between the indicated seedlings grown under short-day conditions **(A)**, high-fluence blue light (14 $\mu mol \cdot m^{-2} \cdot s^{-1}$) **(B)**, high-fluence red light (60 $\mu mol \cdot m^{-2} \cdot s^{-1}$) **(C)**, and high-fluence far-red light (77 $\mu mol \cdot m^{-2} \cdot s^{-1}$) **(D)**. Error bars represent SE ($n \geq 17$). *** $P \leq 0.001$ for *sth2-1 hy5-215* relative to *hy5-215*. WS, Wassilewskija.

sth2 hy5 double mutant had significantly longer hypocotyls than *hy5-215*, 12.8 and 22.3%, respectively (Figures 5C and 5D). These results suggest that while the long hypocotyl phenotype of *sth2* is dependent on a functional HY5 protein in short days and red light, it is independent of HY5 in blue and far-red light.

sth2 Partially Suppresses *cop1* in the Dark

Since we had seen a COP1-dependent localization of STH2 in onion epidermal cells, we wanted to examine the genetic relationship between the two mutants. To this end, we generated double mutants between *sth2-1* and two different *cop1* alleles, *cop1-4* and *cop1-6. cop1* mutants show a very dramatic phenotype in the dark wherein they have short hypocotyls and open expanded cotyledons. *sth2*, on the other hand, does not show any significant difference from the wild type when grown in the dark. Interestingly, the *sth2 cop1* double mutants, when germinated in the dark, had longer hypocotyls than the *cop1* single mutants (Figures 6A and 6B). However, there was some difference in the extent of suppression between the different alleles of *cop1*. While the *sth2 cop1-4* double mutant seedlings were 39.4% longer than *cop1-4* seedlings, *sth2 cop1-6* showed an increase of 30.3% over the *cop1-6* single mutant. These results indicate that *sth2* acts as a weak allele-specific suppressor of *cop1* in the dark.

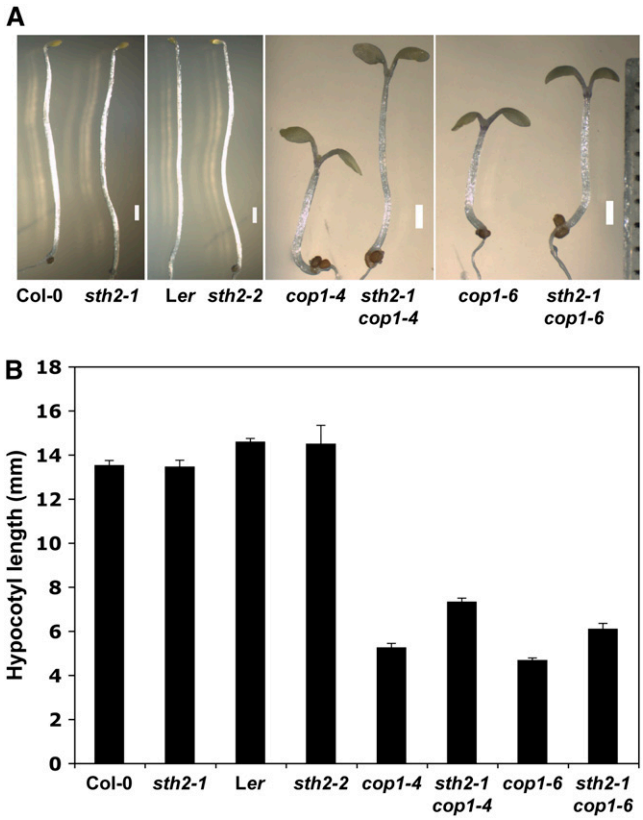

Figure 6. *sth2* Partially Suppresses *cop1* in the Dark.

(A) Wild-type and mutant seedlings (as labeled) grown in the dark for 6 d. Bars = 1 mm.

(B) Hypocotyl lengths of the indicated seedlings grown in the dark for 6 d. Error bars represent SE ($n \geq 21$).

sth2 Has an Enhanced Number of Emerged Lateral Roots

Examination of publicly available microarray data (www.genevestigator.ethz.ch) showed high expression of *STH2* in the radical, indicating a possible role of the gene in root development. Previous studies have shown that several regulators of light signaling, such as *COP1*, *HY5*, *HYH*, and *COL3*, have an effect on the formation of lateral roots (Oyama et al., 1997; Holm et al., 2002; Datta et al., 2006). In order to determine whether *STH2* also plays a role in lateral root development, seedlings were germinated on vertical plates and grown for 8 or 9 d under different light conditions. In all light conditions tested, we found that the *sth2* mutants had more emerged lateral roots than the corresponding wild types. Since the effect was more pronounced under white light in short-day conditions, we performed all experiments in this condition (Figure 7). In order to understand the genetic relationship with *hy5* and *cop1* with respect to the lateral root phenotype, we studied the *sth2 hy5* and *sth2 cop1* double mutants. Interestingly, the *sth2-1* mutant has 67.6% more lateral roots than *hy5-215*, but *hy5* is epistatic to *sth2* in this phenotype. By contrast, *cop1* mutants have a reduced number of lateral roots compared with the wild type. *sth2* can suppress this phenotype in the *sth2 cop1* seedlings; however, while the *sth2 cop1* double mutants have significantly more lateral roots than wild-type plants, they have fewer than the *sth2* single mutant. Interestingly, the suppression seen in the *sth2 cop1-4* double mutant was more than that seen in *sth2 cop1-6*, again indicating an allele-specific genetic interaction. In conclusion, *sth2* like *hy5* has an enhanced number of emerged lateral roots and *hy5* is epistatic to *sth2* with respect to lateral roots; furthermore, *sth2* can suppress the reduced number of lateral roots in the *cop1* mutants.

sth2 Accumulates Less Anthocyanin

hy5 and *cop1* show altered levels of anthocyanin accumulation, while *hy5* has reduced levels, and *cop1* accumulates more anthocyanin. Since *sth2* was found to interact genetically with both *hy5* and *cop1*, we decided to determine whether *sth2* affects anthocyanin accumulation. To this end, seedlings were grown in different light conditions for 3 d. We saw that the *sth2* seedlings looked significantly paler than the wild-type plants when grown under blue light, but the apparent difference was less pronounced in red or white light. Anthocyanin levels were measured in both alleles of *sth2* grown for 3 d in different light conditions. Indeed, seedlings of both *sth2* alleles accumulated less anthocyanin than the corresponding wild-type seedlings under all light conditions tested. While in *sth2-1* the levels were 47.5% of Col-0, *sth2-2* showed 53.6% accumulation with respect to L*er* under blue light, and the corresponding levels were 65.6% of Col-0 for *sth2-1* and 60.9% of L*er* for *sth2-2* in white light (Figures 8A and 8C).

To further investigate the genetic relationships between *sth2*, *hy5*, and *cop1*, anthocyanin levels were measured in the double mutant seedlings (Figures 8A to 8D). Similar to the effect seen in

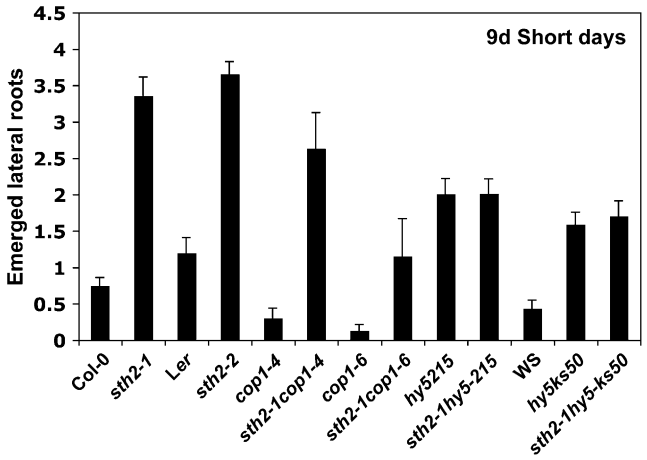

Figure 7. *sth2* Has an Enhanced Number of Emerged Lateral Roots.

Number of emerged lateral roots on the indicated seedlings grown on vertical plates for 9 d under short-day conditions. Error bars represent SE ($n \geq 14$). WS, Wassilewskija.

lateral roots, we found that the anthocyanin levels in the *sth2 hy5* double mutants were close to the levels present in the *hy5* single mutants, indicating an epistatic relationship between *hy5* and *sth2* with respect to the accumulation of this pigment. On the other hand, the enhanced accumulation of anthocyanin in the *cop1* mutants was suppressed in the *sth2 cop1* double mutants, again similar to the effect seen in the roots. In conclusion, *STH2*, like *HY5*, positively regulates the anthocyanin levels in light and can partially suppress the enhanced anthocyanin accumulation in *cop1*.

STH2 Can Activate Transcription through the G-Box on the Chalcone Isomerase Promoter

Since we found that STH2 and HY5 proteins interact and the phenotypes of *sth2* suggest a role in HY5-regulated processes, such as the accumulation of anthocyanin, we decided to investigate a possible functional interaction between the two proteins on a promoter in vivo. For this, we set up a transient transfection assay in *Arabidopsis* protoplasts (Yoo et al., 2007) using the chalcone isomerase (CHI) promoter as a reporter. CHI is the second committed enzyme in the anthocyanin biosynthetic pathway. The CHI promoter has a G-box, and the expression of CHI has been reported to show strong HY5 and COP1 dependence (Cluis et al., 2004). The CHI-LUC reporter was transfected into protoplasts along with a 35S-RnLUC internal control and 35S-*STH2* or an empty 35S vector. As shown in Figure 9B, STH2 activated transcription at 7.35-fold above the basal levels from a 610-bp fragment of the CHI promoter. To examine the role of the B-boxes in this activity, we tested the three STH2 proteins with amino substitutions in their B-boxes (Figure 9A) and found an almost complete abolishment of activation in the case of the B1 mutation and reductions in activation by 68.8 and 67.3% in the B2 and B3 mutations, respectively, indicating that the B-boxes play a direct role in transcriptional activation. Furthermore, mutating the G-box core from CG to TA resulted in negligible activation by STH2, suggesting that STH2 might act through this G-box (Figures 9A and 9B). Thus, STH2 can activate transcription, and the B-boxes in STH2 and a functional G-box element in the promoter are required for the transcriptional activity of STH2.

DISCUSSION

We demonstrate that the STH2 protein interacts with HY5 in yeast and living plant cells and that STH2 shows a COP1-dependent localization to nuclear speckles. To further analyze *STH2*, we identified independent alleles in the Col-0 and L*er* accessions. While *sth2-1* is a null allele, the *sth2-2* allele retains residual *STH2* expression. The T-DNA insertion in *sth2-2* is in the third exon and deletes the last 117 codons of *STH2*. The *STH2* transcript in *sth2-2* is thus both truncated and reduced in level. Furthermore, both alleles showed very similar phenotypes, strongly supporting the notion that the phenotypes are a result of loss and reduction of *STH2* expression. The characterization of these alleles revealed hyposensitivity to blue, red, and far-red light and reduced accumulation of photoprotective anthocyanin pigments. Furthermore, *sth2* showed an enhanced number of emerged lateral roots. The phenotypes of *sth2* firmly establish *STH2* as a positive regulator of light signaling during seedling photomorphogenesis and indicate that *STH2* is involved also in root system architecture. Interestingly, in all conditions in which we found phenotypes in the *sth2* alleles, the effect was similar, albeit weaker, than what is seen in *hy5* alleles, except for the lateral root phenotype, suggesting that *STH2* may be involved in *HY5*- and/or *COP1*-regulated processes.

Functional Relationship between STH2, HY5, and COP1

The interaction between STH2 and HY5 requires both the DNA binding and dimerizing bZIP domain in HY5 and each of the two B-boxes in STH2. The finding that the interaction in yeast is lost upon deletion of the Leu zipper in HY5 suggests that the last 53 amino acids in HY5 are required for interaction with STH2 or, alternatively, that STH2 interacts with a HY5 dimer. The recently published structure of the B-box protein MID1 found the B-box to bind two Zn^{2+} ions and suggested that the previous structure of the B-box protein XNF7 (Borden et al., 1995) may have been incorrectly determined (Massiah et al., 2007). In MID1, the coordination of zinc atoms appears to be the main stabilizing force holding the domain together (Massiah et al., 2007). The residues in MID1 that correspond to the three substituted residues in STH2 are solvent-exposed; however, Asp-20 and Asp-75 in the first and second B-boxes of STH2, respectively, correspond to Asp-190 in MID1, which is a zinc-ligating residue. The D20A and D75A substitutions in STH2, therefore, are likely to severely disrupt the structure of the respective B-boxes in STH2. The finding that disruption of each of the B-boxes in STH2 interferes with the interaction with HY5 in yeast suggests that the structural integrity of both domains is important for HY5 interaction.

The interaction between STH2 and HY5 was further supported by positive FRET signals between CFP-STH2 and YFP-HY5 in

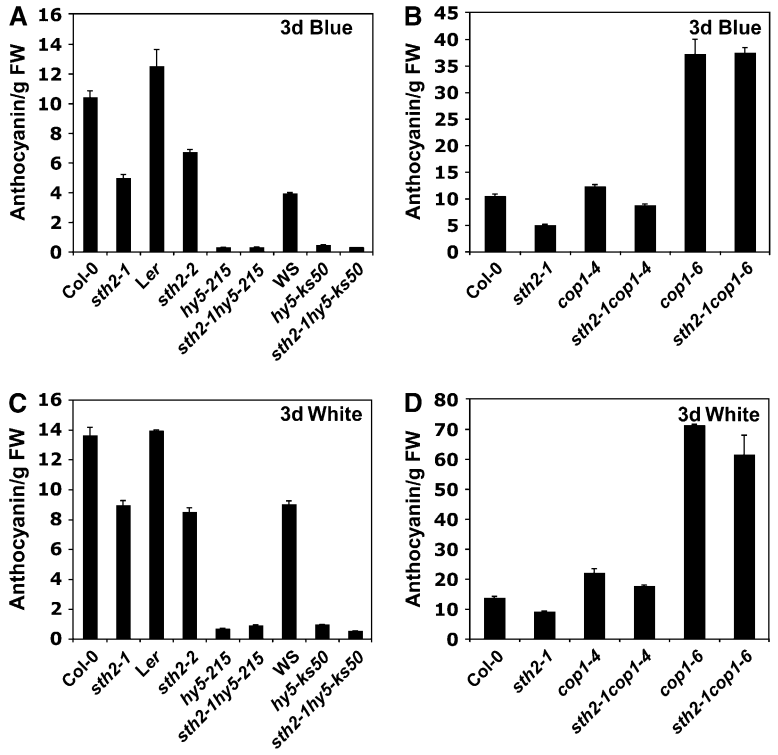

Figure 8. *sth2* Accumulates Less Anthocyanin.

Anthocyanin content of the indicated seedlings grown for 3 d under continuous blue light (**[A]** and **[B]**) or constant white light (**[C]** and **[D]**). Error bars represent SE ($n = 3$). FW, fresh weight; WS, Wassilewskija.

the nuclei of onion epidermal cells. Interestingly, while both STH2 and HY5 showed a diffuse nuclear fluorescence when expressed in onion cells, STH2, like HY5, localized to discrete nuclear speckles upon coexpression of COP1. Thus, although we did not see any interaction between STH2 and COP1 in yeast two-hybrid assays, the onion experiments provide support for an interaction between COP1 and STH2. However, the COP1-dependent localization of STH2 to speckles could be the result of an indirect interaction. One possibility might be that COP1 overexpression localizes HY5 to speckles and this, in turn, causes the accumulation of STH2 protein in these speckles.

The interaction between STH2 and HY5 suggests that the two proteins might act together, and if the activity of STH2 depends exclusively on HY5, *hy5* would be expected to be epistatic to *sth2*. Our efforts to establish the genetic relationship between the two were dampened by the relatively weak phenotypes of *sth2*; however, in blue and far-red light, the hypocotyls were clearly longer in the *sth2 hy5* double mutants than in *hy5-215* (Figures 5C and 5D). This suggests that *STH2* has activities that are independent of *HY5*. By contrast, while the *sth2* alleles produced more lateral roots than the *hy5* alleles, the *sth2 hy5* seedlings had similar numbers of lateral roots as the *hy5* alleles, suggesting that the lateral root phenotype of *sth2* is dependent on a functional HY5 protein (Figure 7). Likewise, the anthocyanin levels were very similar between the *sth2 hy5* and the *hy5* alleles, suggesting that the anthocyanin phenotype of *sth2* is also dependent on *HY5* (Figures 8A and 8C). The finding that *sth2* is able to partially suppress *cop1* phenotypes (Figures 6 and 7) further strengthens the notion of *STH2* acting in *HY5*- and *COP1*-regulated processes; however, at this point, we cannot determine whether the *cop1* suppression is *HY5*-dependent or not.

To address the functional relationship between STH2 and HY5, we examined the activity of STH2 in a transient assay using a reporter driven by the *CHI* promoter. STH2 was able to activate the transcription of the reporter, and this ability required functional B-boxes (Figure 9B). Interestingly, the ability of STH2 to activate the CHI reporter was abolished by a two-nucleotide substitution in the core of the HY5 binding G-box. In our hands, expression of HY5 in the protoplasts did not activate the CHI reporter. It is worth noting that Gal4-DBD-HY5 does not activate transcription from the Gal4 upstream activating sequence in yeast and that STH2 can activate transcription in the presence of Gal4-DBD-HY5 without being fused to a yeast activation domain (Figure 1D), suggesting that, at least in yeast, HY5 is unable to activate transcription, whereas the STH2 protein is endowed with a transactivating ability. Based on these results and together with the physical and genetic interactions between STH2 and HY5, we propose a model wherein STH2 act as a transcriptional cofactor that can bind to HY5 and perhaps other G-box binding proteins, providing transcriptional activation to the complex

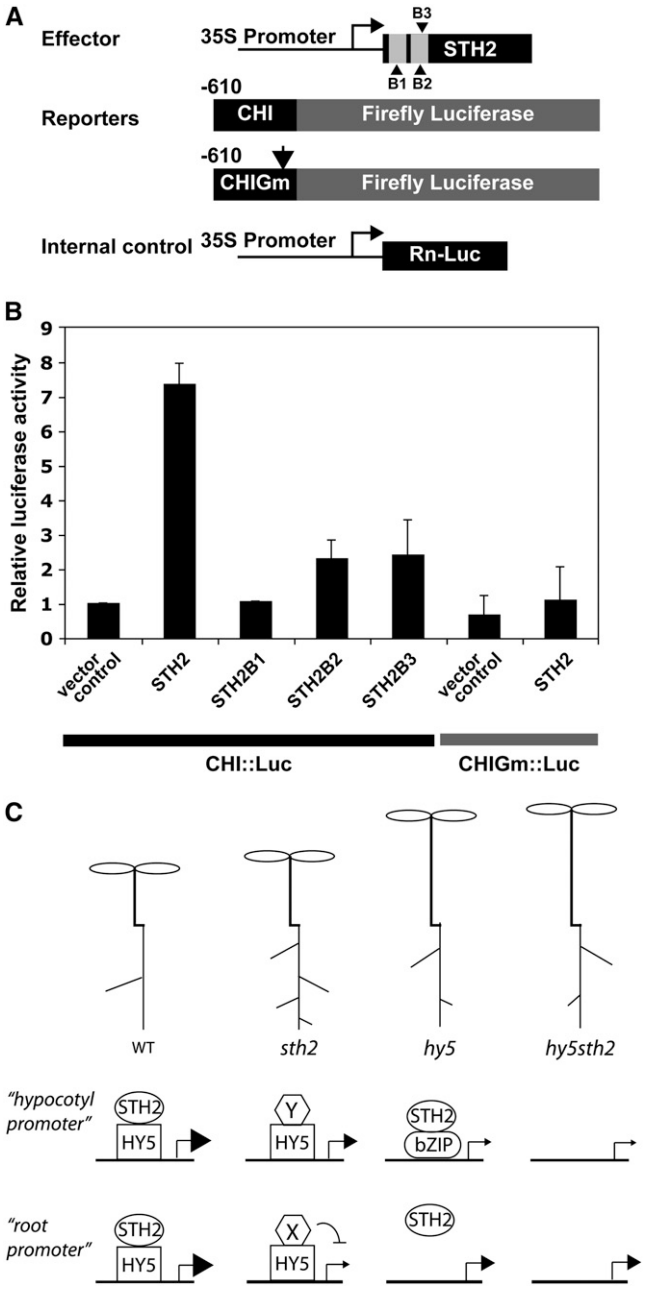

Figure 9. STH2 Can Activate Transcription.

(A) Schematic representation of constructs used in the transient transfection assay in protoplasts. The arrow after the 35S promoter indicates the transcriptional start site, −610 indicates the length of the CHI promoter that was fused to the firefly luciferase to create the reporter construct, and the down arrow indicates the position of the G-box on the CHI promoter that was mutated to create the CHIGm reporter.

(B) Bar graph showing activation by STH2 on the CHI-LUC reporter and the effect of mutating the B-boxes in STH2 or the G-box in the CHI promoter. This experiment was performed four times with similar results. Error bars represent SE ($n = 2$).

(C) Model of the interaction between HY5 and STH2 in a transcriptional network. Both HY5 and STH2 promote the inhibition of hypocotyl elongation and repress the formation of lateral roots. The phenotypes in *sth2*, *hy5*, and *hy5 sth2* are represented schematically, followed by the depiction of hypothetical promoters regulated by HY5 that promote the inhibition of hypocotyl elongation and repress the formation of lateral roots. In the wild type, the HY5 and STH2 proteins strongly activate the hypocotyl promoter, whereas in the *sth2* mutant, HY5 can still activate transcription, possibly through interactions with other B-box proteins (Y). Transcription is reduced in the absence of HY5, although STH2 appears to still have some effect, possibly through interactions with other bZIP proteins such as HYH. In roots, the HY5 and STH2 proteins activate transcription from the root promoter in the wild type. In *sth2*, another B-box–containing protein (X) might take the place of STH2 and repress expression from the promoter; however, in the absence of HY5, STH2 has no effect.

(Figure 9C). If such a complex is formed, it may be possible to examine it in gel shift assays. However, despite extensive efforts, we have been unable to detect a supershift when mixing either bacterially produced STH2 protein or STH2 protein that has been in vitro translated in a wheat germ extract with HY5 protein and G-box–containing DNA, suggesting that such a complex may be too unstable to withstand the gel electrophoreses or that post-translational modifications or additional factors may be required for the formation of the complex.

Role in Transcriptional Networks

It has been proposed that photoactivated light receptors set in motion a transcriptional cascade that promotes light-dependent development (Tepperman et al., 2001, 2004; Jiao et al., 2003). The outcome of this cascade differs depending on the organ of the plant (Ma et al., 2005). Light-responsive elements (LREs) are essential for the light-responsive transcriptional activity of light-regulated promoters (Terzaghi and Cashmore, 1995; Arguello-Astorga and Herrera-Estrella, 1998). However, no single LRE is found in all light-regulated promoters, and it has been suggested that a combination of LREs rather than the single elements confer proper light responsiveness to the promoters (Puente et al., 1996). The HY5 binding G-box is a well-conserved LRE to which several light-responsive transcription factors have been shown to bind or are predicted to bind (Jakoby et al., 2002; Toledo-Ortiz et al., 2003). Recent results suggest that HY5 is positioned high in the hierarchy of transcription factors that form the transcriptional cascade upon light perception (Jiao et al., 2007; Lee et al., 2007). However, light-to-dark transition experiments revealed that the HY5 protein is constitutively bound to light- and circadian-regulated promoters such as *RbcS1A*, *CCA1*, and *LHY*, suggesting that HY5 binding per se might be insufficient for transcriptional regulation and that additional factors are required for proper regulation (Lee et al., 2007).

The phenotypes and genetic interactions of *sth2* indicating a role in HY5-regulated processes, together with the physical interaction between STH2 and HY5 and our finding that STH2 could act as a transcriptional cofactor of HY5, are interesting in this context. The interaction between STH2 and HY5 requires the B-boxes in STH2. In *Arabidopsis*, there are 32 B-box–containing proteins, and STH2, STH1, and STO together with five other

proteins appear to constitute a subgroup of these, with similarly spaced tandem repeated B-boxes with high homology. It is possible that the B-box proteins function as transcriptional cofactors, interacting with DNA binding transcription factors and providing transcription-activating or -repressing activities. Therefore, STH2 and possibly other B-box–containing proteins could be the additional factors HY5 needs for transcriptional regulation. Differences in temporal and spatial expression of the B-box proteins could account for some of the temporal and tissue-specific effects seen in the transcriptional response to light. Thus, STH2 might be one of several cofactors to HY5, and perhaps other G-box binding bZIP proteins, that provide an additional level of regulation. This role might also extend to other B-box factors, such as COL3.

B-Box Function

The B-box domain is found in >1500 proteins from across all multicellular species and some unicellular eukaryotes. In animals, the B-box is often found together with a RING finger (originally termed an A-box) and a coiled-coil domain in the so-called RBCC or tripartite motif proteins. RBCC proteins are involved in diverse cellular processes, and many have been found to be involved in ubiquitination, in which they regulate protein stability, localization, and trafficking and participate in transcriptional regulation (Meroni and Diez-Roux, 2005). In RING E3 ubiquitin ligases, the RING finger has been shown to mediate the interaction with E2 ubiquitin-conjugating enzymes (Lorick et al., 1999); however, while the B-box has been proposed to be a protein–protein interaction domain and has been found to participate in substrate recognition in some RBCC proteins, the function of this domain is still unclear (Torok and Etkin, 2001; Meroni and Diez-Roux, 2005). The B-box has been divided into two subgroups, B-box1 with consensus C-X_2-C-X_{6-17}-C-X_2-C-X_{4-8}-C-X_{2-3}-C/H-X_{3-4}-H-X_{5-10}-H and B-box2 with consensus C-X_{2-4}-H-X_{7-10}-C-X_{1-4}-D/C-X_{4-7}-C-X_2-C-X_{3-6}-H-X_{2-5}-H (Reymond et al., 2001; Massiah et al., 2007). Two zinc atoms are coordinated by the Cys, His, and Asp residues in a RING-like fold (Massiah et al., 2006, 2007), and X can be any amino acid. While all RBCC proteins have a B-box2 domain, some also have a B-box1 domain N-terminal to the B-box2 domain (Reymond et al., 2001). B-boxes can also be found in proteins lacking the RING finger and coiled-coil domains, and in these proteins the B-box is most often of the B-box2 type (Massiah et al., 2006).

Arabidopsis does not have any RBCC proteins but has 32 proteins with N-terminal B-boxes. All of these have at least one B-box with the consensus C-X_2-C-X_{7-9}-C-X_2-D-X_4-C-X_2-C-X_{3-4}-H-X_{4-8}-H, thus closely resembling B-box2, and 23 of the proteins have two B-boxes in tandem, with the second containing a D, E, or H as the fourth zinc-coordinating residue.

The COP1-dependent localization of STH2 to nuclear speckles and the genetic interaction between *sth2* and *cop1* is interesting since it suggests that STH2 and other B-box–containing proteins such as STO, STH1, COL3, and CO might functionally interact with the COP1-SPA proteins. Since COP1 contains RING and coiled-coil domains and the SPA proteins contain coiled-coil domains, such interactions could bring the RBCC domains together through protein–protein interactions, thus creating a functional equivalent of a RBCC protein in an organism that lacks such proteins.

While none of the *Arabidopsis* proteins have the RING or coiled-coil domain characteristic of the RBCC proteins, 17 of the proteins, CONSTANS and COL1 to COL16, have a CCT (CO, CO-like, TIMING OF CAB EXPRESSION1) domain in the C-terminal part of the protein (Griffiths et al., 2003). The recent finding that the CCT domain of CO might be a component of a heterotrimeric DNA binding complex (Wenkel et al., 2006) suggests that plant B-box proteins have evolved to adapt to different functional roles than B-box proteins in animals.

While the phenotypes of *sth2* provide genetic support for an interaction with HY5, our finding that STH2, which contains two N-terminal tandem repeated B-boxes as its only domains, interacts with HY5 through its B-boxes and is able to activate transcription through the G-box provides a functional mechanism for the interaction. These results demonstrate a function for the B-box and suggest that the 15 B-box proteins in *Arabidopsis* that lack CCT domains might be transcriptional cofactors that are recruited to promoters by interactions with DNA binding partners.

Conclusion

We demonstrate here that the STH2 protein interacts with HY5 in yeast and living plant cells and that STH2 shows a COP1-dependent localization to nuclear speckles. The identification and characterization of two *sth2* alleles indicates that STH2 positively regulates anthocyanin accumulation and the inhibition of seedling hypocotyl elongation in red and blue light. The *sth2* mutants show reduced numbers of lateral roots, indicating that STH2 negatively regulates the formation of lateral roots. The genetic characterization of *sth2* revealed that *sth2* acts as a suppressor of *cop1* and that *STH2* plays a positive role in regulating photomorphogenesis, both independently and together with *HY5*. Functional assays in protoplasts suggest that STH2 acts as a transcriptional cofactor able to activate transcription through the G-box promoter element. The B-boxes in STH2 are required both for interaction with HY5 and for the ability of STH2 to activate transcription, thus demonstrating a molecular function for this domain.

METHODS

Plant Materials and Growth Conditions

The *sth2-1*, *hy5-215*, *cop1-4*, and *cop1-6* alleles are in the *Arabidopsis thaliana* Col-0 accession, *sth2-2* is in L*er*, and *hy5-ks50* (Oyama et al., 1997) is in Wassilewskija. Unless stated otherwise, seeds were surface-sterilized and plated on Murashige and Skoog medium supplemented with 0.8% Bactoagar (Difco) and 1% sucrose. The plates were then cold-treated at 4°C for 3 d and transferred to light chambers maintained at 22°C with the desired light regime.

Yeast Two-Hybrid Methods and FRET Experiments

The β-galactosidase assays were performed as described (Holm et al., 2001). For the FRET acceptor photobleaching experiments, the pAM-PAT-35SS-YFP-HY5, pAM-PAT-35SS-CFP-STH2, and pRTL2-COP1

overexpression constructs were introduced into onion (*Allium cepa*) epidermal cells by particle bombardment and incubated, and live cell images were acquired using an Axiovert 200 microscope equipped with a laser scanning confocal imaging LSM 510 META system (Carl Zeiss). Cells were visualized at 20 h after particle bombardment using the confocal microscope through a Plan-Neofluor 403/1.3 oil (differential interference contrast) objective. The multitracking mode was used to eliminate spillover between fluorescence channels. The CFP was excited by a laser diode 405 laser and the YFP by an argon-ion laser, both at low intensities. Regions of interest were selected and bleached with 100 iterations using the argon-ion laser at 100%.

RNA Gel Blotting

Total RNA was extracted from seedlings grown in continuous white light for 6 d after germination using the RNeasy Plant mini kit (Qiagen). Twenty micrograms of total RNA was loaded for the RNA gel blot analysis. A full-length *STH2* open reading frame was used as a probe to detect transcript levels in the wild-type, *sth2-1*, and *sth2-2* backgrounds.

Hypocotyl and Root Experiments

For all monochromatic light assays, plates were cold-treated at 4°C for 3 d and then transferred to continuous white light for 8 h to induce uniform germination. The plates were then transferred to monochromatic light conditions and incubated at 22°C for 6 d in the case of hypocotyl experiments and for 9 d for the measurement of roots. Blue, red, and far-red light were generated by light emission diodes at 470, 670, and 735 nm, respectively (model E-30LED; Percival Scientific). Fluence rates for blue and red light were measured with a radiometer (model LI-250; Li-Cor), and for far-red light we used an opto-meter (40A Opto-Meter; United Detector Technology). The hypocotyl length of seedlings and the number of lateral roots were measured/counted using ImageJ software.

Anthocyanin Measurements

For the anthocyanin determinations, seedlings were harvested at 3 d after putting them in light, weighed, frozen in liquid nitrogen, and ground, and total plant pigments were extracted overnight in 0.6 mL of 1% HCl in methanol. After the addition of 0.2 mL of water, anthocyanin was extracted with 0.65 mL of chloroform. The quantity of anthocyanins was determined by spectrophotometric measurements of the aqueous phase (A_{530} to A_{657}) and normalized to the total fresh weight of tissue used in each sample.

Protoplast Experiments

Arabidopsis mesophyll cell protoplasts were generated and transfected as described previously (Yoo et al., 2007). The reporter used was a 610-bp fragment of the CHI promoter driving firefly luciferase (pPCV814-CHI610). Full-length *STH2* driven by a cauliflower mosaic virus 35S promoter was used as the effecter (pRTL2-STH2). The B-box–mutated STH2 constructs used in this study are the same as those described previously. For detection, the dual luciferase system was used (Promega). Renilla lucerifase driven by a full-length cauliflower mosaic virus 35S promoter was used as an internal control (pRNL).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative database under the following accession numbers: At1g75540 (*STH2*), A5g11260 (*HY5*), At2g32950 (*COP1*), At2g31380 (*STH1*), and At1g06040 (*STO*).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. STH2 Colocalizes with COP1 but Does Not Show FRET.

Supplemental Figure 2. The Loss of *STH2* Does Not Affect Tolerance to Salt.

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