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LZF1/SALT TOLERANCE HOMOLOG3, an *Arabidopsis* B-Box Protein Involved in Light-Dependent Development and Gene Expression, Undergoes COP1-Mediated Ubiquitination [W]

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B-box containing proteins play an important role in light signaling in plants. Here, we identify *LIGHT-REGULATED ZINC FINGER1/SALT TOLERANCE HOMOLOG3* (*STH3*), a B-box encoding gene that genetically interacts with two key regulators of light signaling, ELONGATED HYPOCOTYL5 (HY5) and CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1). STH3 physically interacts with HY5 in vivo and shows a COP1-dependent localization to nuclear speckles when coexpressed with COP1 in plant cells. A T-DNA insertion mutant, *sth3*, is hyposensitive to high fluence blue, red, and far-red light and has elongated hypocotyls under short days. Analyses of double mutants between *sth3*, *sth2*, and *hy5* suggest that they have partially overlapping functions. Interestingly, functional assays in protoplasts suggest that STH3 can activate transcription both independently and together with STH2 through the G-box promoter element. Furthermore, *sth3* suppresses the *cop1* hypocotyl phenotype in the dark as well as the anthocyanin accumulation in the light. Finally, COP1 ubiquitinates STH3 in vitro, suggesting that STH3 is regulated by COP1. In conclusion, we have identified STH3 as a positive regulator of photomorphogenesis acting in concert with STH2 and HY5, while also being a target of COP1-mediated ubiquitination.

INTRODUCTION

Besides providing the ultimate source of biological energy, light regulates key developmental processes throughout the plant life cycle. Photomorphogenesis is a developmental transition preceding and initiating adult development, when a seedling acquires photosynthetic capacity. Light perceived mainly by phytochromes (phyA and phyB) and cryptochromes (cry1 and cry2) regulates photomorphogenesis by inducing dramatic changes in the transcriptome of the seedling (Ma et al., 2001; Jiao et al., 2007). It has been proposed that the photoreceptors initiate a transcriptional cascade by regulating a set of master transcription factors that in turn control the expression of multiple downstream genes (Jiao et al., 2007).

Recent studies have shown that regulated proteolysis plays a pivotal role in light signaling. The CONSTITUTIVE PHOTOMORPHOGENIC/DEETIOLATED/FUSCA (COP/DET/FUS) proteins act in a proteolytic pathway aimed at degrading photomorphogenesis promoting factors in the absence of light (Osterlund et al., 2000a, 2000b). Specifically, COP1 acts as a RING motif–containing E3 ligase that ubiquitinates positive factors and targets them for degradation via the 26S proteosomal pathway (Osterlund et al., 2000a, 2000b; Holm et al., 2002; Seo et al., 2003; Yang et al., 2005). COP1 was also found to interact with several photoreceptors, such as phyA, cry1, and cry2 (Wang et al., 2001; Yang et al., 2001; Shalitin et al., 2002; Seo et al., 2004), and can even target one 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).

Molecular genetic approaches have identified several transcription factors acting downstream of the photoreceptors and/or being targets for the COP1 degradation. These transcription factors act downstream to a single or a combination of photoreceptors forming a light-regulated transcriptional network. However, some of these factors receive inputs also from circadian, stress, and/or hormonal signals, thus creating signal integration points for a complex set of regulatory circuits. Photomorphogenesis involves transcription factors belonging to a range of families. Members of the basic helix-loop-helix (bHLH) family play a central role in phytochrome-mediated signal transduction (Duek and Fankhauser, 2005). PIF3 (for Phytochrome Interacting Factor 3) was the first member of the bHLH family interacting specifically with activated phyA and phyB (Ni et al., 1999). Interestingly, while PIF3 undergoes phytochrome-mediated phosphorylation followed by ubiquitin-mediated

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degradation, it can in turn regulate PhyB protein abundance during long-term continuous red (Rc) irradiation, suggesting reciprocal interactions between photoreceptors and transcriptional regulators (Park et al., 2004; Al-Sady et al., 2006, 2008). Other bHLH proteins, PIF4, PIF5, and PIF7, act as negative regulators of PhyB signaling under prolonged Rc, while PIF1 negatively regulates seed germination and chlorophyll synthesis (Huq and Quail, 2002; Fujimori et al., 2004; Huq et al., 2004; Leivar et al., 2008). LONG HYPOCOTYL IN FAR-RED1 (HFR1) is an atypical member of this family playing a positive role in both phyA and cryptocrome-mediated signaling as indicated by the hyposensitive phenotype of the *hfr1* mutant under far-red and blue light (Fairchild et al., 2000; Duek and Fankhauser, 2003). On the other hand, MYC2 acts as a repressor of blue and far-red light-mediated deetiolation (Yadav et al., 2005). Members of other transcription factors families involved in this network include LAF1, belonging to the R2R3-MYB family (Ballesteros et al., 2001), the Dof family transcription factors COG1 and OBP3, and several members of the bZIP transcription factor family, such as ELONGATED HYPOCOTYL5 (HY5), HYH, and GBF1 (Oyama et al., 1997; Holm et al., 2002; Park et al., 2003; Ward et al., 2005; Mallappa et al., 2006).

HY5 was the first protein shown to be a target of COP1 regulation. The fact that the *hy5* mutant has elongated hypocotyls under all light conditions suggests that HY5 acts downstream to all the photoreceptors (Lee et al., 2007). Recently, a modified chromatin immunoprecipitation technique where DNA chip hybridization on a microarray was used to detect precipitated DNA revealed >3000 in vivo HY5 targets in the *Arabidopsis thaliana* genome. The class of HY5 binding targets was enriched in light-responsive genes and transcription factor genes (Lee et al., 2007), suggesting that HY5 is a high hierarchical regulator of the transcriptional cascades for photomorphogenesis. Mutation in the *HY5* gene also causes defects in lateral root formation, secondary thickening in roots, and chlorophyll and anthocyanin accumulation (Oyama et al., 1997; Holm et al., 2002). In addition, recent studies showed the involvement of HY5 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. However, while HY5 was found to bind to a large number of promoters, this binding is not sufficient for transcriptional activation, suggesting that HY5 requires additional cofactors for regulation (Lee et al., 2007).

In addition, several proteins containing N-terminal zinc binding B-boxes have been shown to act as transcriptional regulators in response to light and circadian cues. These include CONSTANS (CO), which regulates the expression of the flowering time gene *FT*, CONSTANS-LIKE3 (COL3), and SALT TOLERANCE HOMOLOG2 (STH2) (Putterill et al., 1995; Datta et al., 2006, 2007). All three of these genes also interact genetically with *COP1*. While *co-10* can suppress the early flowering of *cop1-4* in both long- and short-day conditions (Jang et al., 2008), *col3* and *sth2* can suppress the hypocotyl phenotype in dark-grown *cop1* seedlings (Datta et al., 2006, 2007). Interestingly, COP1 was recently shown to degrade CO in the dark, thus shaping the temporal pattern of CO accumulation and its effect on photoperiodic flowering (Jang et al., 2008; Liu et al., 2008). Both CO and COL3 also contain a C-terminal CCT (for CO, CO-LIKE, TOC) domain that shows similarity to the HAP2 subunit of the CCAAT box binding complex, suggesting that they might bind to DNA directly (Wenkel et al., 2006). STH2 lacks a CCT domain and has the tandem repeated B-boxes as its only discernable conserved domains. The B-boxes in STH2 are required both for the ability of STH2 to activate transcription and for its ability to physically interact with HY5, whereas the B-boxes in COL3 were found to be important for localization of the protein into nuclear speckles (Datta et al., 2006, 2007).

Recently, a homolog of STH2, LIGHT-REGULATED ZINC FINGER1 (LZF1)/STH3, was identified as a gene transcriptionally regulated by HY5 (Chang et al., 2008). LZF1/STH3 was found to act as a positive regulator of deetiolation affecting both anthocyanin accumulation and chloroplast biogenesis (Chang et al., 2008). Here, we further examine the function of STH3 and report that LZF1/STH3 can physically interact with HY5. Furthermore, *STH3* genetically interacts with both *HY5* and *COP1*. Characterization of an *sth3* mutant indicates that *STH3* positively regulates light-mediated development primarily independently of *HY5*. Genetic and functional data suggest that STH3 and STH2 function synergistically to activate transcription and regulate light-dependent development. In vitro ubiquitination data suggest that STH3 is ubiquitinated by COP1. In summary, the B-box protein STH3 positively regulates photomorphogenesis both independently and in concert with HY5 and STH2, while at the same time is targeted for proteolysis by the E3 ubiquitin ligase COP1.

RESULTS

LZF1/STH3 Interacts with HY5 in Yeast

The B-box containing proteins SALT TOLERANCE (STO), STH1, and COL3 were identified in a yeast two-hybrid screen using COP1 as bait (Holm et al., 2001, 2002; Datta et al., 2006). Interestingly, two related proteins, STH2 and LZF1/STH3, had previously been identified in a yeast two-hybrid screen using HY5 as bait; however, in each case, the cDNAs had 5′ untranslated region sequences that put them out of frame from the activation domain (T. Oyama, personal communication). STH3 showed highest conservation in the N-terminal B-boxes that had 64, 61, and 55% amino acid identity with those of STO, STH1, and STH2, respectively (Figure 1A). To confirm and further characterize the interaction between HY5 and STH3, we inserted the STH3 cDNA into the pYX242 yeast expression vector and assayed its interaction with HY5 in liquid β-gal assays (Figure 1B). The STH3 protein expressed from the pYX242 vector did not activate transcription together with the Gal4 DNA binding domain (Gal4-DBD) vector control but resulted in a 49-fold increase in β-Gal activity over the vector control when expressed together with Gal4-DBD-HY5.

To map the protein domains responsible for the interaction between HY5 and STH3, we examined the interactions between wild-type and mutated HY5 and STH3 proteins in β-Gal assays. To this end, we used Gal4-DBD fusions of full-length HY5 and two truncated HY5 proteins with either the leucine zipper domain

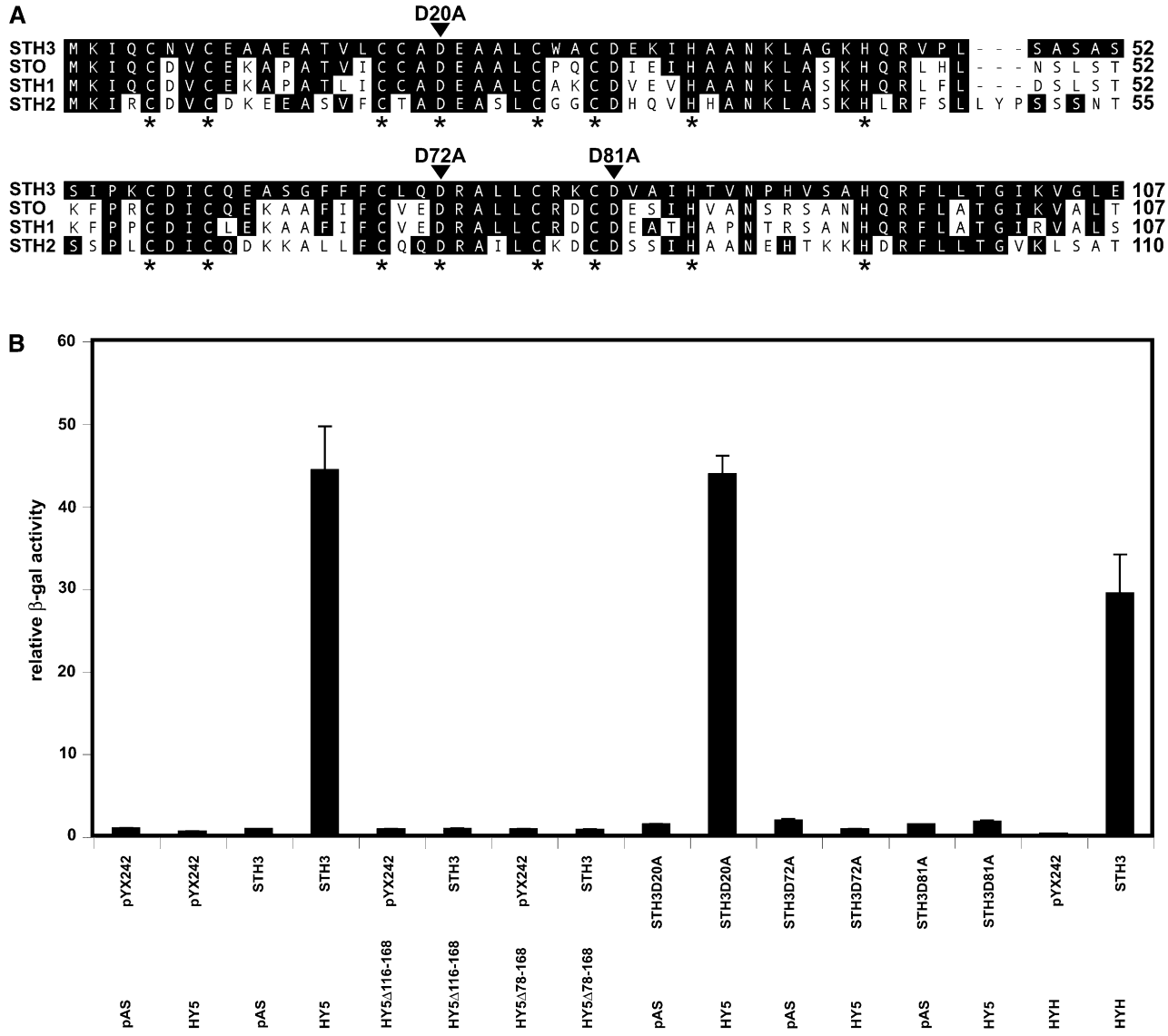

Figure 1. STH3 Interacts with HY5 in Yeast, and the bZIP Domain of HY5 and the Second B-Box in STH3 Are Important for the Interaction.

(A) Alignment of the B-box containing the N-terminal half of STH3, STO, STH1, and STH2. Asterisks indicate zinc ligating conserved Cys, His, and Asp residues, while arrowheads indicate residues mutated in the B-boxes. D-n-A indicates the Ala substitution of the three Asp residues in the B-boxes at positions 20, 71, and 81 (n).

(B) Yeast two-hybrid interactions between indicated STH3 and HY5 proteins. Error bars indicate SE ($n \geq 4$).

(amino acids 115 to 168) or the basic zipper domain (amino acids 77 to 168) deleted. Deletion of the zipper domain or the basic zipper domain in HY5 resulted in a dramatic reduction in β-Gal activity, indicating that the basic zipper domain is required for the interaction with STH3 (Figure 1B, cf. HY5, HY5Δ116-168, and HY5Δ78-168 with STH3). To further characterize the interaction between HY5 and STH3, we individually substituted three conserved Asp residues in the B-boxes of STH3 to Ala (Figure 1A); two of these residues (D20 and D72) 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 STH3-D20A, -D72A, and -D81A (Figures 1A and 1B). Interestingly, we found that D72A and D81A substitutions resulted in a dramatic reduction of β-Gal activity compared with wild-type levels; however, the D20A mutation, despite disrupting a Zn^{2+} ligating residue, did not affect the interaction between STH3 and HY5 (Figure 1B). This suggests that the tandem repeated B-boxes have different functions: while the second B-box in STH3 is required for interaction with HY5, the first B-box is not. Finally, we examined whether STH3 could interact with HYH, the bZIP protein most closely related to HY5 in *Arabidopsis*, in yeast two-hybrid assays.

We found a 30-fold increase in relative β-Gal activity, indicating that STH3 indeed could interact also with HYH (Figure 1B). Together, these results suggest that the basic zipper domain in HY5 and the second B-box in STH3 are important for the HY5–STH3 interaction and that STH3 can interact also with the related HYH protein.

STH3 Is a Nuclear Protein and Colocalizes with HY5 in Plant Cells

HY5 localizes to the nucleus and green fluorescent protein (GFP)-HY5 gives a diffused nuclear fluorescence when expressed in onion epidermal cells, whereas GFP-COP1 localizes to discrete nuclear speckles (Ang et al., 1998). To determine the subcellular localization of the STH3 protein and further characterize its interactions with HY5 and possibly with COP1, we prepared a cyan fluorescent protein (CFP) fusion of STH3 and expressed it in onion epidermal cells. STH3, like HY5, localizes uniformly throughout the nucleus (Figure 2A). Since the proteins interact in yeast assays and both proteins give a diffuse nuclear fluorescence, we proceeded to examine whether they physically interact in the nucleus. To this end, we examined if fluorescence resonance energy transfer (FRET) occurred between the two fusion proteins using the acceptor photobleaching technique. Here, we coexpressed CFP-STH3 with yellow fluorescent protein (YFP)-fused HY5 and excited them with 405- and 514-nm lasers. Both CFP and YFP fluorescence was 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-STH3, as shown in Figures 2A and 2B, indicating that FRET had occurred between the two proteins prior to the bleach. In control photobleaching experiments using the same microscope settings, we did not detect FRET between unfused CFP and YFP or between YFP-STH3 and CFP-STH2 (see Supplemental Figure 1 online).

COP1 has previously been shown to recruit HY5 and several other interacting proteins to nuclear speckles (Ang et al., 1998; Holm et al., 2002; Seo et al., 2003). Since STH3 showed a localization pattern similar to HY5, we wanted to check if and how COP1 affected STH3 localization in vivo. To this end, we coexpressed nontagged COP1 (35S COP1) with YFP-STH3 in onion epidermal cells. Interestingly, upon coexpression of COP1, we found consistent nuclear speckles in addition to a weak uniform fluorescence (Figure 2C). The detection of nuclear speckles when STH3 is coexpressed with untagged COP1 suggests a COP1-dependent recruitment of STH3 into COP1 speckles. Coexpression of CFP-COP1 also resulted in localization of YFP-STH3 into nuclear speckles; however, we did not detect any FRET in these experiments (data not shown).

The T-DNA Insertion Mutant *sth3* Is Hyposensitive to Light

To examine the role of *STH3* in light-regulated development, we obtained a T-DNA insertion line (SALK_105367) in the *STH3* gene (At1g78600) from the SALK collection. The *STH3* gene is located on the bottom arm of chromosome I and contains three exons. We PCR amplified and sequenced the T-DNA flanking sequence and found that the insertion was present in the first intron of the *STH3* gene (Figure 3A). The insertion results in a total loss of *STH3* mRNA (Figure 3A), indicating that it is a null mutation. This null mutant is the same as the *lzf1-1* allele described by Chang et al. (2008) and was named *sth3* to conform with the nomenclature of the previously identified B-box proteins homologous to STO. Homozygous *sth3* plants were obtained and confirmed by PCR-based genotyping since the kanamycin resistance of this mutant had been lost.

The homozygous *sth3* mutants were germinated in different fluences of monochromatic blue, red, and far-red light to examine if *STH3* plays a role in light-mediated seedling development. We found that *sth3* had longer hypocotyls under high fluence blue, red, and far-red light (Figures 3B to 3D). In blue light, at a fluence level of 30 μmol/m^2/s, the *sth3* seedlings were 24% longer than the Columbia (Col-0) seedlings, and in high fluence red light, the *sth3* seedlings had 9% longer hypocotyls compared with the wild type (Figures 3B and 3C). Similar phenotypes were

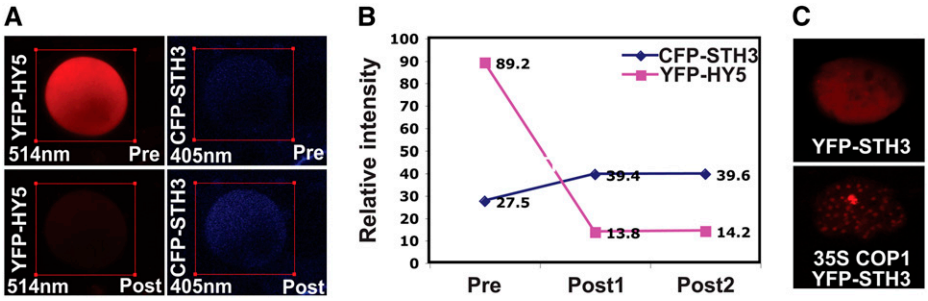

Figure 2. STH3 Interacts Both with HY5 and COP1 in Living Plant Cells.

(A) and **(B)** FRET between YFP-HY5 and CFP-STH3 analyzed by acceptor bleaching technique in nuclei (n = 10). The top panels in **(A)** show a representative prebleach nucleus coexpressing YFP-HY5 and CFP-STH3 excited with either 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 nucleus after bleaching excited with a 514- or a 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 YFP-STH3, excited with 405-nm laser.

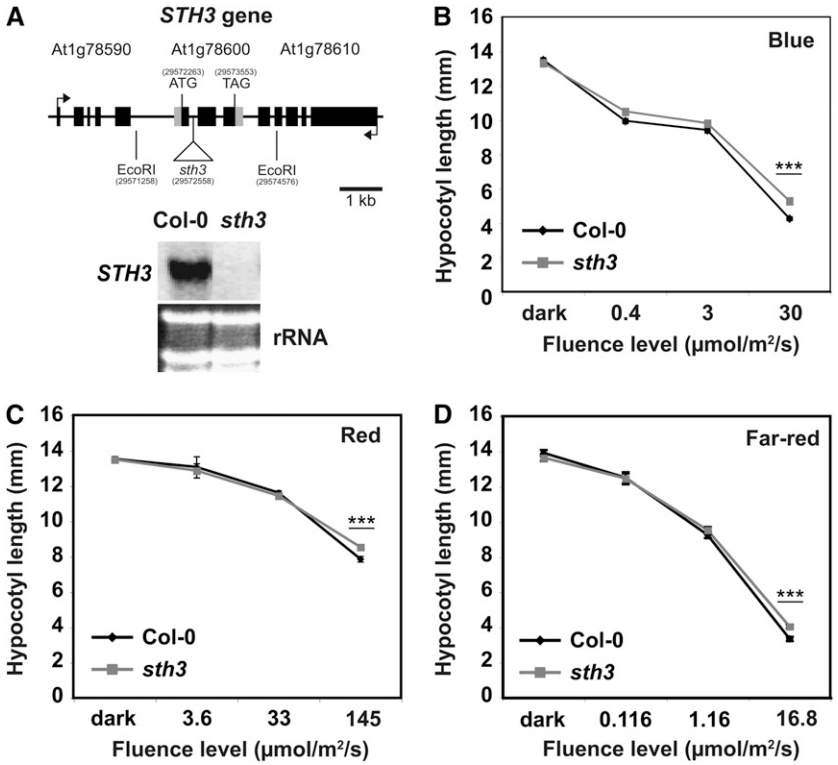

Figure 3. The *STH3* Mutant Is Hyposensitive to High Fluence Blue, Red, and Far-Red Light.

(A) Schematic representation of the *STH3* gene (At1g78600) showing the start (29572263), stop (29573553) and T-DNA insertion (29572558) positions. The two adjoining genes (At1g78590 and At1g78610) and the *Eco*RI sites used to create the pFP100-*STH3* complementation construct are indicated at positions 29571258 and 29574576. Exons and introns are indicated by black boxes and bars, respectively, while the gray boxes indicate the untranslated regions of *STH3*. Arrows indicate the orientation of the adjoining genes. The bottom panel shows an RNA gel blot of RNA from 6-d-old wild-type and *sth3* seedlings grown in continuous white light. Full-length *STH3* was used as the probe. rRNA bands are shown as loading controls.
(B) to **(D)** Fluence response curves of wild-type (Col-0) and *sth3* seedlings grown in continuous monochromatic blue, red, and far-red light, respectively. The experiment was performed at least twice with similar results. The graph depicts one of these experiments. Error bars represent SE ($n \geq 22$) and *** $P \leq 0.001$ for *sth3* relative to Col-0 at the highest fluence in blue, red, and far-red light.

seen under high fluence far-red light, with *sth3* seedlings being 21% longer than the wild type (Figure 3D). At very low fluence levels, the hypocotyl length in the *sth3* mutant was similar to the wild type, suggesting that *sth3* is specifically hyposensitive to higher fluence levels of light. Together, these phenotypes suggest that *STH3* acts as a positive regulator of light-dependent inhibition of hypocotyl elongation.

Genetic Interactions among STH3, STH2, and HY5

We then examined *sth3* seedlings grown in white light under different daylength conditions (Figure 4). We found that the *sth3* mutant showed 21% elongated hypocotyl compared with the wild type under short-day conditions (8 h light/16 h dark). Interestingly, in most light conditions where we saw a phenotype in the *sth3* mutant we had previously seen phenotypes in the *sth2* mutant as well (Datta et al., 2007). We therefore made and examined the *sth2 sth3* double mutant. We found that while the *sth2* single mutant was 21% elongated compared with the wild type, the *sth3 sth2* double mutant seedlings had a more pronounced phenotype with 77% longer hypocotyls than the wild type (Figure 4A). To confirm that any observed phenotypes were indeed due to the disruption of the *STH3* gene, a 3.3-kb genomic *Eco*RI fragment containing the *STH3* gene and 1-kb sequence both upstream and downstream of the gene (Figure 3A) was introduced into the *sth3* mutant as well as each of the *sth3* double mutants. For this we used the pFP100 vector encoding GFP from a seed-specific promoter, thus allowing identification and analysis of transformed seeds in the T1 generation (Bensmihen et al., 2004). The long hypocotyl phenotypes of the *sth3* and *sth3 sth2* double mutants could be complemented in T1 transgenic seedlings transformed with pFP100-*STH3*, indicating that the phenotypes were caused by the T-DNA insertion in *sth3* (Figure 4C). Since STH3 and HY5 physically interact in yeast and in plant cells, we generated a *sth3 hy5* double mutant to examine the genetic interactions between *HY5* and *STH3*. Homozygous *sth3 hy5-215* double mutants were germinated in different light conditions together with the single mutants and the wild type (Figures 4A, 4B, and 4D to 4G). Since *sth3* showed a hyposensitive phenotype under short-day conditions, we checked the *sth3 hy5-215* double mutant phenotype in short days. Interestingly, the double mutant had 12% longer hypocotyls than *hy5-215*

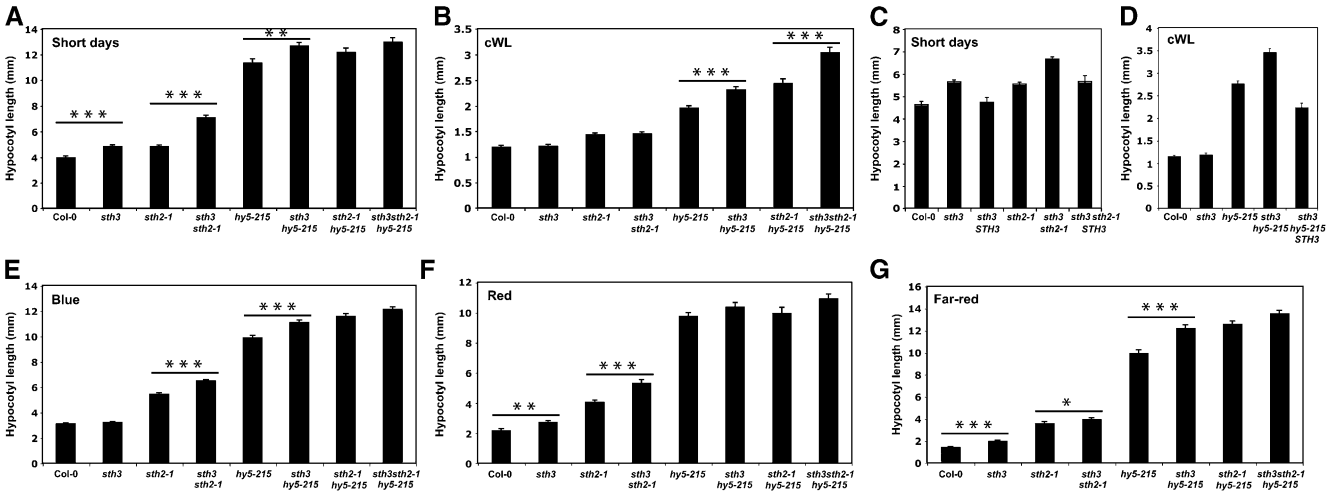

Figure 4. *sth3* Enhances the Long Hypocotyl Phenotype of *sth2* and *hy5.*

(A) and **(B)** Bar graph showing the difference in hypocotyl length between the indicated seedlings grown under short-day conditions **(A)** and constant white light **(B)**.

(C) and **(D)** Hypocotyl length of the indicated seedlings showing complementation by the *STH3* gene where *sth3STH3*, *sth2sth3STH3*, and *sth3 hy5-215STH3* represent *sth3*, *sth2 sth3*, and *sth3 hy5-215* seedlings transformed with the pFP100-*STH3* vector containing a 3.3-kb genomic fragment including the *STH3* gene.

(E) to **(G)** Bar graph showing the hypocotyl length of the indicated seedlings grown under high fluence blue light (30 μmol m^{-2} s^{-1}) **(E)**, high fluence red light (120 μmol m^{-2} s^{-1}) **(F)**, and under high fluence far-red light (16.8 μmol m^{-2} s^{-1}) **(G)**. Error bars represent SE ($n \geq 20$ except for *sth3STH3* where $n \geq 13$). *** $P \leq 0.001$, ** $P \leq 0.01$, and * $P \leq 0.05$ for the indicated pair of seedlings. All experiments were performed at least thrice with similar results. Each graph depicts one of these experiments.

(Figure 4A). We further generated the *sth2 sth3 hy5-215* triple mutant and analyzed its phenotypes. In short days, the triple mutant looked similar to either of the double mutants *sth2 hy5-215* and *sth3 hy5-215* (Figure 4A).

When grown in constant white light or under long-day conditions (16 h light/8 h dark), we did not find a significant difference between wild-type and *sth3* seedlings and the hypocotyl length of *sth2 sth3* double mutant was similar to that of *sth2-1*. By contrast, the *sth3 hy5-215* seedlings had 26% longer hypocotyls than the *hy5-215* single mutant seedlings, indicating that both genes had independent effects. However, the absence of phenotypes in *sth3* suggests that the loss of *STH3* alone could be compensated for by *HY5* (Figure 4B). This was very similar to the effect seen in the *sth2 hy5* double mutants previously (Datta et al., 2007). Interestingly, the *sth2 sth3 hy5-215* triple mutant had 12 and 14% longer hypocotyls than either of the double mutants *sth2 hy5* and *sth3 hy5*, respectively, suggesting a synergistic relationship between the three genes (Figure 4B). Also, the long hypocotyl phenotype of the *sth3 hy5* double mutant could be complemented in T1 transgenic *sth3 hy5* seedlings transformed with pFP100-*STH3*; however, here, we observed that the *sth3 hy5 STH3* seedlings were shorter than the *sth3 hy5* seedlings at 2.2 $\pm$ 0.08 mm compared with 2.8 $\pm$ 0.06 mm (Figure 4D).

In high fluence monochromatic blue, red, and far-red light, the *sth3* mutant showed an elongated hypocotyl phenotype although the effect was quite subtle. However, in all the light conditions tested, the *sth3 sth2* double mutant seedlings had longer hypocotyls than either of the single mutants. When grown under high fluence monochromatic blue light, the *sth3 sth2* double mutant had 75 and 19% more elongated hypocotyls than the *sth3-1* and *sth2-1* seedlings, respectively. Under red light, the loss of both *STH2* and *STH3* in the double mutant enhanced the long hypocotyl phenotype of *sth2-1* by 25%, while in far-red light, the double mutant showed twofold enhancement in the hypocotyl length compared with the *sth3-1* single mutant. This indicates that *STH2* can partially compensate for the loss of *STH3* but the loss of both results in an enhanced effect. Together, all these results indicate that *STH3* and *STH2* together play a role in light-mediated signaling and have partially overlapping functions.

We also checked for *sth3 hy5-215* double mutant phenotypes under different monochromatic light conditions and found that in blue, red, and far-red light, the *sth3 hy5* double mutant had 12, 6, and 23% longer hypocotyls than *hy5-215* (Figures 4E to 4G). Additive effects seen in the double mutants between *sth3* and *hy5* under different light conditions indicate that *STH3* and *HY5* might function independently. Analysis of the *sth2 sth3 hy5-215* triple mutant showed that under all the monochromatic light conditions, the triple mutant had longer hypocotyls than either of the double mutants *sth2 hy5* and *sth3 hy5*, although the effect was quite small. (Figures 4E to 4G) All these results together indicate a partially overlapping, partially parallel relationship between *STH2*, *STH3*, and *HY5* in regulating photomorphogenesis.

sth3 Partially Suppresses *cop1* in the Dark

The COP1-dependent localization of STH3 in onion epidermal cells prompted us to examine the genetic relationship between the two mutants. To this end, we generated double mutants

between *sth3*, *cop1-6*, and *cop1-4*. Both *cop1-6* and *cop1-4* are weak viable alleles of *cop1*, *cop1-6* has a splice site mutation that results in a five–amino acid insertion after Gln-301 before the WD40 domain, whereas *cop1-4* has a premature stop codon at amino acid 283 and thus lacks the WD40 domain (McNellis et al., 1994). *cop1* mutants show a very dramatic phenotype in the dark wherein they have short hypocotyls and open expanded cotyledons. On the other hand, *sth3* does not show any significant difference from the wild type when grown in the dark (Figures 5A and 5B). We found that while the *sth3 cop1-4* double mutant behaved as *cop1-4*, the *sth3 cop1-6* double mutant had 36% longer hypocotyls than the *cop1-6* single mutant seedlings when germinated in the dark (Figures 5A to 5D). The suppression was caused by the *sth3* mutation since the hypocotyls of *sth3 cop1-6* seedlings transformed with pFP100-*STH3* were indistinguishable from *cop1-6* seedlings (Figures 5C and 5D). Interestingly,

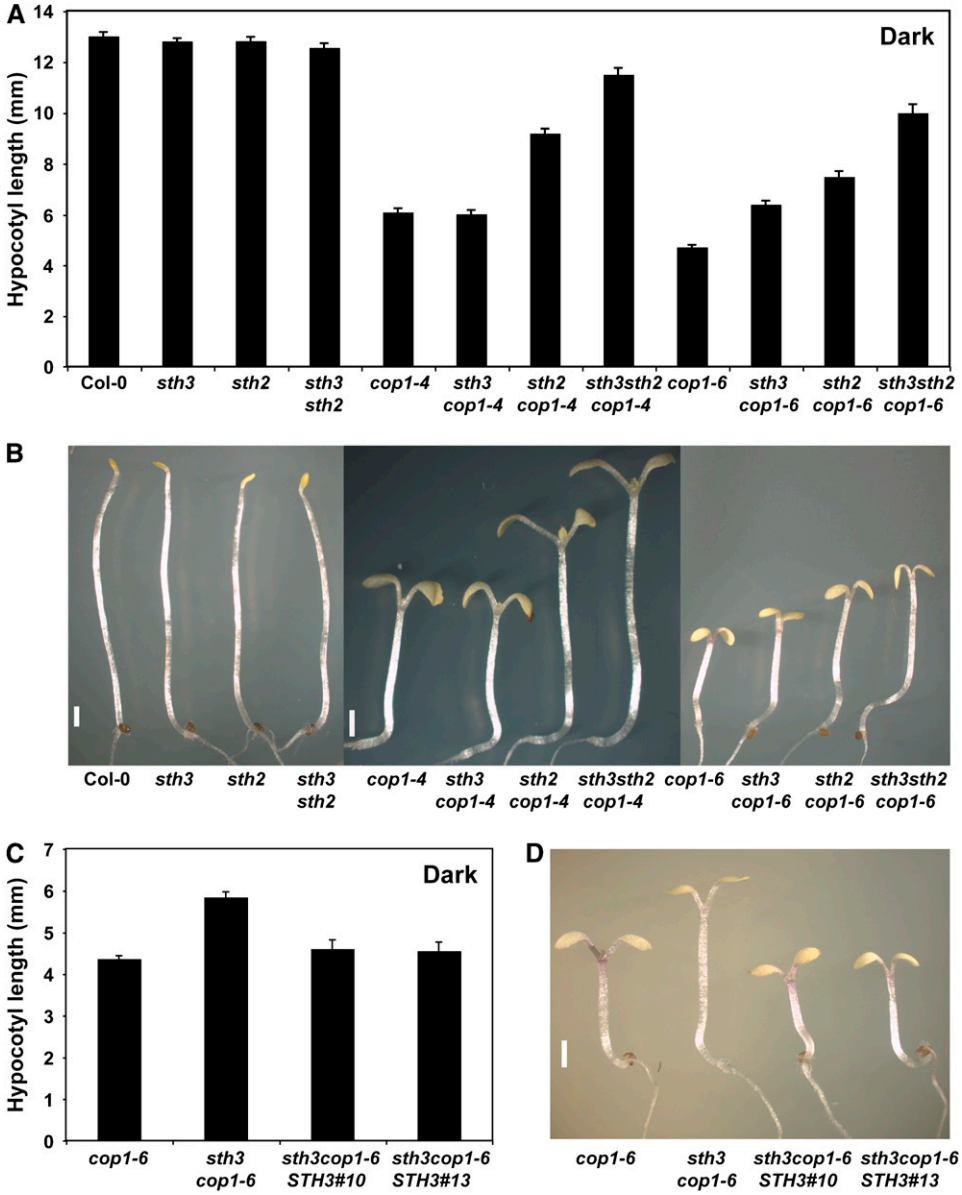

Figure 5. *sth3* Suppresses *cop1* in the Dark Both by Itself and Together with *sth2*.

(A) Hypocotyl lengths of the indicated seedlings grown in the dark for 6 d. Error bars represent SE ($n \geq 20$).
(B) Wild-type and mutant seedlings (as labeled) grown in the dark for 6 d. Scale for *cop1-6* mutants is the same as in Col-0. Bars = 1 mm.
(C) Bar graph showing the hypocotyl length of the indicated seedlings where *sth3cop1-6 STH3#10* and *sth3cop1-6 STH3#13* represent two different lines generated by transforming *sth3 cop1-6* with pFP100-*STH3.* Error bars represent SE ($n \geq 18$).
(D) Seedlings (as labeled) grown in the dark for 6 d. Bar = 1 mm.

although *sth3* did not suppress *cop1-4*, the triple mutant *sth2-1 sth3 cop1-4* strongly suppressed the dark phenotype of *cop1-4*, and the *sth2-1 sth3 cop1-6* triple mutant showed a stronger suppression of the *cop1-6* phenotype (Figures 5A and 5B). These results indicate that *sth3* act as a suppressor of *cop1* in the dark and shows that the loss of both B-box proteins STH2 and STH3 enhance the suppression of *cop1* in the dark (Figures 5A and 5B). Furthermore, the results suggest that STH3 and STH2 might be targets of COP1 degradation, and the finding that the length of the hypocotyls in the triple mutants approaches that of the wild type suggests that their misregulation in *cop1* is an important factor for the short hypocotyls of *cop1* in the dark.

sth3 Suppresses the *cop1* Anthocyanin Accumulation in the Light

Both *hy5* and *cop1* show altered levels of anthocyanin accumulation. While *hy5* has reduced levels, *cop1* accumulates more anthocyanin than the wild type. Since *sth3* was found to genetically interact with both *hy5* and *cop1*, we decided to examine the role of *sth3* in anthocyanin accumulation. To this end, seedlings were grown in different light conditions for 3 d and then their anthocyanin levels were measured (Figure 6). The pattern of anthocyanin accumulation looked quite similar for the different mutants in all the light conditions; however, the strongest phenotypes were seen in constant red light. As observed by Chang et al. (2008), the anthocyanin accumulation in the *sth3* seedlings did not differ significantly from that of the Col-0 wild-type under high fluence red light (Figure 6). While the *sth2* mutant accumulated 51% less anthocyanin than the wild type, the levels were 61% lower in the *sth3 sth2* double mutant. Anthocyanin levels were measured in the *sth3 hy5* double mutant seedlings and the levels were found to be close to the levels present in *hy5* single mutants (see Supplemental Figure 2 online). Furthermore, while on one hand the enhanced accumulation of anthocyanin in the *cop1-4* mutant was suppressed in the *sth3 cop1-4* double mutants, there was hardly any difference in the levels of anthocyanin accumulation between *cop1-6* and *sth3 cop1-6*. The levels of anthocyanin were 33% lower in *sth3 cop1-4* compared with *cop1-4*. While *sth2 cop1-4* had 24% less anthocyanin than *cop1-4*, the loss of both *STH2* and *STH3* resulted in a 5.3-fold decrease in the levels compared with *cop1-4.* In case of the other allele, *cop1-6*, the levels of anthocyanin were 18 and 33% lower in *sth2 cop1-6* and *sth2 sth3 cop1-6* compared with *cop1-6*, respectively (Figure 6). All these results reaffirm the genetic interaction between the two genes.

STH3 Can Activate Transcription

The phenotypes of *sth3*, *sth2*, and the double mutant *sth2 sth3* under different light conditions indicated that STH3 and STH2 might act together to regulate light-mediated signaling. We decided to investigate a possible functional interaction between the two proteins on a promoter in vivo. For this, we used a transient transfection assay in *Arabidopsis* protoplasts using the enzyme luciferase under the control of the Chalcone Isomerase (CHI) promoter as a reporter (Datta et al., 2007). We chose this promoter because the *sth3* mutant showed anthocyanin accumulation defect and 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 ProCHI:LUC reporter was transfected into protoplasts along with Pro35S:RnLUC where Renilla luciferase is expressed from a 35S promoter to serve as an internal control for transformation efficiency and the effectors Pro35S:STH3 and Pro35S:STH2 either singly or both together (Figure 7A). As shown in Figure 7B, STH3 activated transcription 74% more than the empty 35S vector from a 610-bp fragment of the CHI promoter. While the activation by STH2 alone was ninefold above the basal level, STH2 and STH3 together showed an enhanced ability to activate, the levels being 20-fold higher than the empty vector. Since we had previously seen that STH2 was acting through the G-box promoter element (Datta et al., 2007), we decided to look at the effect of mutating the G-box on activation by STH3, both alone and together with STH2. We found that mutating the G-box core from CG to TA resulted in negligible activation by STH3, quite similar to STH2, suggesting that STH3 might be acting through this G-box.

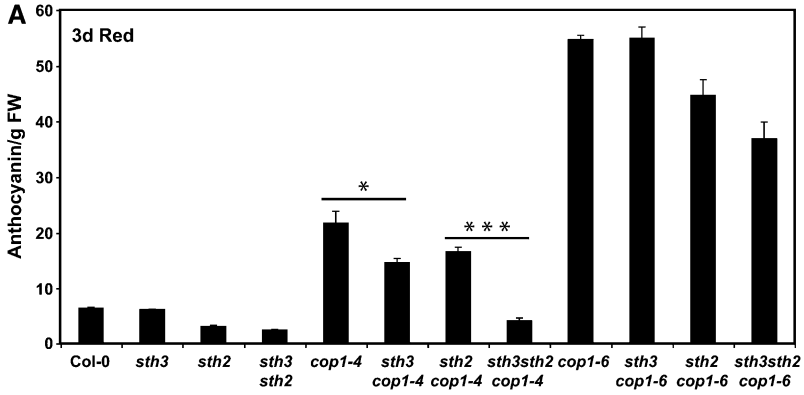

Figure 6. *sth3* Suppresses the Anthocyanin Accumulation in *cop1* Both by Itself and Together with *sth2.*

Anthocyanin content of the indicated seedlings grown for 3 d under continuous red light. *** $P \leq 0.001$ and * $P \leq 0.05$ for the indicated pair of seedlings. The experiment was performed twice with similar results. The graph depicts one of these experiments. Error bars represent SE ($n = 3$). FW, fresh weight.

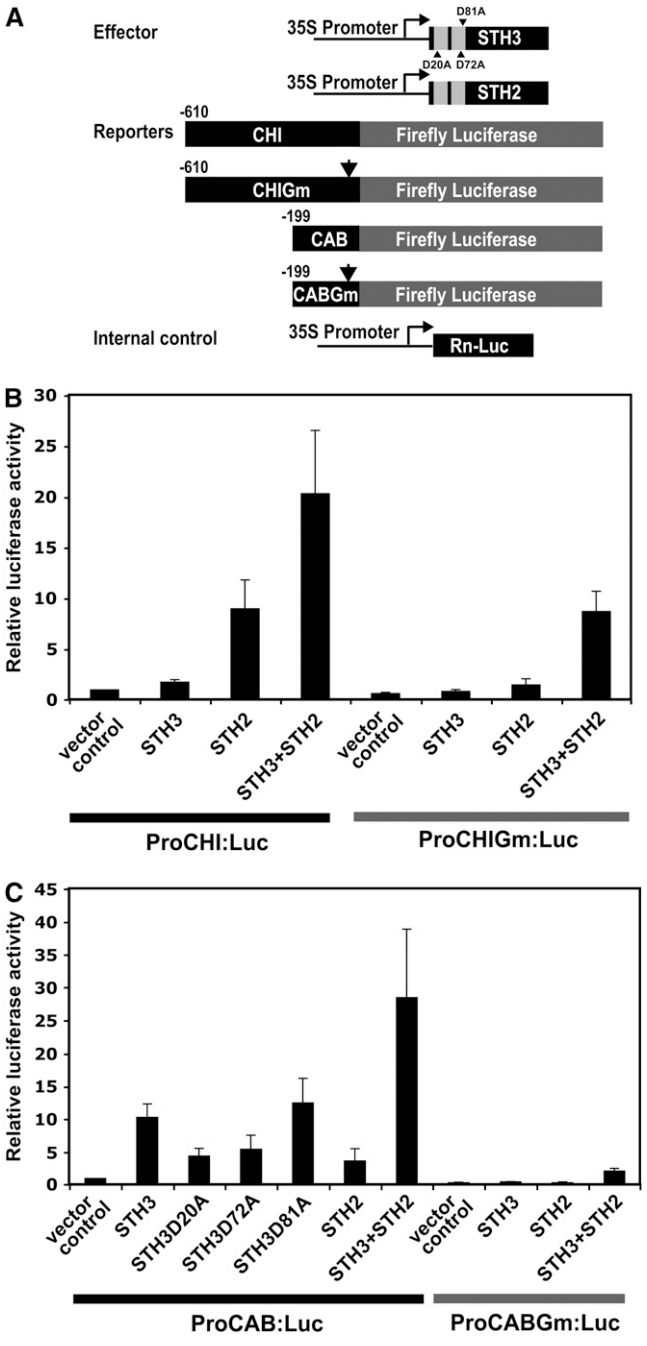

Figure 7. STH3 Can Activate Transcription Both by Itself and Together with STH2.

(A) Schematic representation of constructs used in the transient transfection assay in protoplasts. Arrow after the 35S promoter indicates the transcriptional start site, −610 and –199 indicate the length of the CHI and CAB promoters that were fused to the firefly luciferase to create the reporter construct, and downward arrow indicates the position of the G-box mutation in the CHI and CAB promoters to create the CHIGm and CABGm reporters, respectively.

(B) Bar graph showing activation by STH3 both alone and together with STH2 on the ProCHI:Luc reporter and the effect of mutating the G-box in the CHI promoter. Error bars represent SE (n = 3).

(C) Bar graph showing activation by STH3 on the ProCAB:Luc reporter, the effect of mutating the the B-boxes in STH3 (STH3D20A, STH3D72A, and STH3D81A), the combined effect of STH3 and STH2, and the result of mutating the G-box in the promoter (ProCABGm:Luc). Error bars represent SE (n = 3).

However, surprisingly, the mutation did not completely abolish the activation produced by STH3 and STH2 together and the level of activation was still 13.6-fold above the basal level (Figure 7B).

Recently, it was reported that LZF1/STH3 plays a role in plastid development and accumulation of chlorophyll (Chang et al., 2008). We therefore decided to examine the ability of STH3 to activate a reporter driven by a 199-bp chlorophyll *a/b* (CAB) binding protein promoter in *Arabidopsis* protoplasts (Figure 7A). We saw a 10.3- and 3.6-fold activation by STH3 and STH2, respectively, and together the level of activation was enhanced to 28.5-fold above basal level, suggesting a strong synergistic functional relationship between STH3 and STH2 (Figure 7C). Mutating the G-box in the 199-bp CAB promoter resulted in negligible activation by STH3 or STH2 alone. However, similar to the effect seen on the CHI promoter, STH3 and STH2 when present together could activate transcription 6.1-fold above the basal level on the G-box mutated CAB promoter.

To ascertain the role played by the individual B-boxes of STH3 in transcriptional activation, we tested the three amino acid substituted STH3 proteins used in the yeast two-hybrid assays (Figures 1A and 7A). Using these mutated effectors we found that while the level of activation went down from 10.3-fold in the wild-type STH3 to 4.4-fold in D20A and 5.4-fold in D72A, there was no difference in activation in D81A (Figure 7C). Interestingly while the first two Asp residues D20 and D72 are zinc ligating residues and therefore likely to be important for the structural integrity of their respective B-boxes, the third Asp residue D81 does not play such a role. This would indicate that an intact structure of each of the B-boxes is important for transcriptional activation. However, the D81A substitution, found to abolish interaction with HY5 in yeast, does not affect the ability of STH3 to activate transcription. In conclusion, STH3 can activate transcription both independently and together with STH2 through the G-box promoter element, and the zinc ligating Asp residues in both the first and the second B-box are important for the activation.

COP1 Ubiquitinates STH3 in Vitro

COP1 represses photomorphogenesis in darkness mainly by ubiquitinating positive regulators of light signal transduction, such as HY5, LAF1, HFR1, and phyA, resulting in their targeted degradation via the 26S proteasome (Ang et al., 1998; Osterlund et al., 2000b; Holm et al., 2002; Seo et al., 2003; Duek et al., 2004; Yang et al., 2005). Our finding that COP1 recruits STH3 into nuclear speckles in onion epidermal cells (Figure 2C), together with our genetic data showing that STH3 acts downstream of COP1 and that these two proteins play antagonistic roles in the control of light signal propagation (Figures 5 and 6), suggested that COP1 could ubiquitinate STH3 to control its function. To test this hypothesis, we purified bacteria-expressed maltose binding protein (MBP)–tagged COP1 (MBP-COP1) and hemagglutinin

(HA)–tagged STH3 (HA-STH3). Using these fusion proteins, we performed in vitro ubiquitination assays in the presence of different ubiquitin versions: free ubiquitin, HA-ubiquitin, or flagellin (Flag)–tagged ubiquitin. As a result, we found upshifted bands (usually two bands) when MBP-COP1 and HA-STH3 were incubated in the presence of both E1 ubiquitin-activating and E2 ubiquitin-conjugating enzymes, most likely indicating that MBP-COP1 polyubiquitinates HA-STH3 (Figure 8A). The finding that the differences in the size of the shifted bands correlated with the molecular weight of the ubiquitin version used in each case, free ubiquitin (8.5 kD), HA-ubiquitin (9.4 kD), and Flag-ubiquitin (9.3 kD), confirms that the bands correspond to ubiquitinated HA-STH3 products (Figure 8B).

DISCUSSION

This study identifies STH3/LZF1 as a protein that acts in close proximity to STH2 and HY5 to regulate light-dependent development in *Arabidopsis*. We found that STH3 localizes to nuclear speckles in a COP1-dependent manner, that *sth3* acts as a genetic suppressor of *cop1*, and that COP1 can ubiquitinate STH3 in vitro. These results strongly suggest that STH3 acts in the same regulatory pathway as COP1, HY5, and STH2. However, the subtle phenotypes in the *sth3* single mutant coupled with the finding that *sth3* enhanced the phenotypes in both *hy5* and *sth2* mutants suggest that STH3 plays an overlapping and partially independent role compared with HY5 and STH2.

Functional Interactions between STH3, STH2, and HY5

The STH3 protein was identified through its interaction with HY5 in yeast, and the positive FRET signal between CFP-STH3 and YFP-HY5 provided additional support for a physical interaction between these two proteins. We identified and characterized *sth3*, a T-DNA insertion mutant, in an effort to ascertain the role STH3 has in *Arabidopsis*. The *sth3* allele is the same Salk line (*lzf1*) that was recently characterized by Chang et al. (2008). *LZF1*

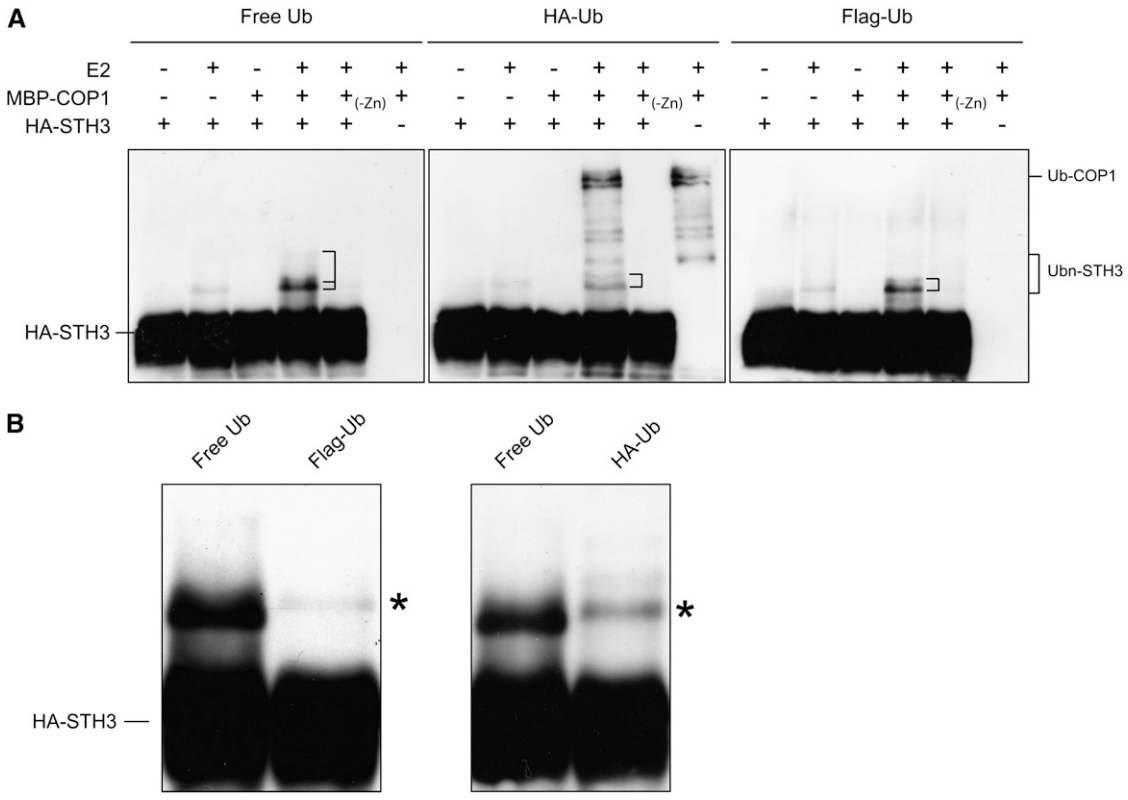

Figure 8. COP1 Ubiquitinates STH3 in Vitro.

(A) HA-STH3 ubiquitination assays were performed using yeast E1 (Boston Biochem), rice Rad6 (E2), and recombinant MBP-COP1 (previously incubated with 20 μM $ZnCl_2$). Nonincubated MBP-COP1 (−Zn) was used as a negative control. Three independent assays were performed using unlabeled ubiquitin (Free Ub; left panel), HA-tagged ubiquitin (HA-Ub; middle panel), and Flag-tagged ubiquitin (Flag-Ub; right panel). Assay conditions were as previously described (Saijo et al., 2003). HA-STH3 was detected using anti-HA antibody (Roche) in all cases. Anti-HA also detected ubiquitinated COP1 (Ub-COP1; middle panel). Brackets indicate the positions for modified STH3 bands in each case.

(B) Comparison of band size corresponding to HA-STH3 modified using unlabeled (Free Ub) and Flag-tagged ubiquitin (Flag-Ub; left panel) or HA-tagged ubiquitin (HA-Ub; right panel). As observed, band shifts caused by each type of ubiquitin correlates with their corresponding molecular mass: 8.5 (Free Ub), 9.3 (Flag-Ub), and 9.4 (HA-Ub) kD. Asterisks mark the position of bands corresponding to HA-STH3 modified with a single molecule of Flag-Ub (left panel) or HA-Ub (right panel). Note that modification of HA-STH3 with tagged-Ub peptides is less efficient than that of free Ub.

was identified as a gene transcriptionally regulated by HY5; however, the fact that *hy5* was not epistatic to *sth3* indicates that the activity of LZF1/STH3 cannot be exclusively regulated by HY5 (Chang et al., 2008). Here, we report a detailed phenotypic analysis of *sth3*, *sth3 hy5-215*. Similar to Chang et al., we found that in all the light conditions tested (continuous white, blue, red, far-red light, and in short days), the *sth3 hy5* double mutant had significantly longer hypocotyls than *hy5-215*, indicating that STH3 and HY5 might function independently. STH3 shows 55% amino acid identity with another HY5 interacting protein, STH2, and since both STH3 and STH2 showed similar phenotypes under different light conditions, we tried to determine the individual contributions and the functional relationship between these two genes. Phenotypic analyses indicated that STH2 and STH3 both act as positive regulators of photomorphogenesis but have partially overlapping functions. In most light conditions tested, we found an additive effect in the *sth2 sth3* double mutant compared with either *sth2* or *sth3*, indicating distinct roles and a synergistic relationship between the two B-box protein-encoding genes.

STH2 has previously been reported to activate transcription in transient protoplast assays (Datta et al., 2007). To further examine the functional relationship between the STH3 and STH2 proteins, we examined STH3 and found that STH3 can activate the reporters in these assays and that, as for STH2, the B-boxes in STH3 and a functional G-Box element in the promoter were required for this activity. While the two proteins were able to activate both CHI and CAB reporters, there was a clear difference in that STH2 activated the CHI reporter more strongly than did STH3, whereas the reverse was seen for the CAB reporter. The finding that the activity of the B-box proteins is dependent on the promoter context suggests that STH3 and STH2 and perhaps other family members might regulate distinct sets of target genes. When STH3 and STH2 were tested together, we found that they showed an enhanced ability to activate transcription, suggesting that they regulate light-dependent promoters together. Surprisingly, while mutating the G-box in the CAB or CHI promoter resulted in an almost complete loss of activation by either STH2 or STH3 alone, the mutation did not result in a complete loss of activation produced by STH3 and STH2 together. At present we do not know the reason for this activation; however, it is possible that when the two proteins are expressed together they can act through other promoter elements besides the G-box. All these results indicate that STH3 positively regulates photomorphogenesis both independently and in concert with HY5 and STH2.

Functional Interaction between STH3 and COP1

COP1 is an E3 ubiquitin ligase that plays a key role in the regulation of the light signaling pathway. Genomic expression profiles of viable *cop1* mutants grown in darkness mimic the physiological light-regulated genome expression profiles of wild-type seedlings (Ma et al., 2003). It has been shown that the bZIP proteins HY5 and HYH and the bHLH protein HFR1 bind to the WD-40 domain of COP1, and the MYB domain containing LAF1 interacts with the RING motif of COP1 (Ang et al., 1998; Holm et al., 2002; Seo et al., 2003; Duek et al., 2004; Yang et al., 2005). All these positive regulators of photomorphogenesis are ubiquitinated by COP1 and are thus targeted for degradation by the 26S proteosome.

We found that COP1 can recruit STH3 into nuclear speckles in onion epidermal cells. Furthermore, *sth3* partially suppressed the hypocotyl phenotype and the high anthocyanin levels of dark- and light-grown *cop1-6* and *cop1-4*, respectively, in an allele-specific manner, suggesting genetic interaction between *cop1* and *sth3*. Our in vitro ubiquitination data showed that COP1 functions as an E3 ligase, ubiquitinating STH3 and thus probably targeting it for degradation via the 26S proteasomal pathway. These results fit very well with the genetic interaction between *sth3* and *cop1* that suggested that COP1 could be a negative regulator of STH3. Furthermore, the finding that COP1 can ubiquitinate STH3 in vitro suggests that this could be a common mechanism of regulation for this group of B-box proteins. Recently, CO, a B-box–containing flowering time regulator, was also found to physically interact with COP1 and undergo in vitro ubiquitination and COP1-dependent reduction in levels in vivo (Liu et al., 2008).

The STO Family of B-Box Proteins

There are 32 genes encoding proteins containing B-boxes in *Arabidopsis* (Griffiths et al., 2003; Datta et al., 2008). STH3 together with seven other genes, STO, STH1, STH2, STH4 (At2g21320), STH5 (At4g38960), STH6 (At4g10240), and STH7 (At4g39070), form a clade among the B-box encoding genes in *Arabidopsis* where all the proteins contain tandem repeated B-boxes that are spaced by 8 to 15 amino acids. While STH4 through STH7 remain uncharacterized, it is interesting to note that four of the family members have been implicated in light-dependent development. Analysis of insertion mutants in these genes revealed that STO and STH1 are negative regulators of light signaling (Khanna et al., 2006; Indorf et al., 2007), whereas both STH3 and STH2 are positive regulators (Datta et al., 2007). Moreover, both *sth2* and *sth3* act as suppressors of *cop1* and enhancers of *hy5* mutants, respectively. In addition to these genetic interactions, the STO and STH1 proteins both contain COP1-interacting motifs in their C termini, through which they interact with COP1 in yeast two-hybrid assays (Holm et al., 2001). STH3 and STH2 do not interact with COP1 in the yeast assays and neither contains any obvious COP1-interacting motif. However, both STH2 and STH3 colocalize with COP1 to nuclear speckles. We have previously characterized the B-box protein COL3 that by itself localizes to nuclear speckles and contains a motif in its C terminus through which it can interact with COP1 in yeast (Datta et al., 2006). We found that deletion of the N-terminal B-boxes resulted in loss of speckle localization, but coexpression of COP1 restored the speckled localization of the truncated COL3 protein, suggesting that either the B-box domains or the COP1 interaction motif can target COL3 to these subnuclear structures (Datta et al., 2006). It is therefore possible that the STO and STH1-3 proteins can interact with COP1 either directly as STO and STH1 or indirectly through interactions with common partners (e.g., HY5), as in the case of STH2 and STH3. Either case suggests formation of a larger complex, and the identification and characterization of such complexes could shed light

onto the signaling network during photomorphogenesis and will be important future directions of our studies.

The phenotypes of *sto*, *sth1*, *sth2*, and *sth3* as well as the genetic interactions between *sth2*, *sth3*, *cop1*, and *hy5* together with the finding that both STH2 and STH3 activate transcription from HY5-dependent promoters suggests that at least STO, STH1 to STH3, and possibly also the other clade members act as transcriptional cofactors regulating COP1- and HY5-dependent transcription. This could be achieved through stabilizing HY5 containing complexes on promoters, providing transcriptionally activating or repressive surfaces to such complexes, or helping to present HY5 and other interacting proteins for ubiquitinylation by COP1. Further experiments are needed to discern which if any of these mechanisms are used. These B-box proteins provide an additional layer of complexity in light-regulated transcription where different STH proteins could have different activities and respond to different cues, and the finding that STH3 also interacts with HYH suggests that the STH proteins together with some bZIP proteins might form a small transcriptional network.

Relative Importance of the Two B-Boxes

Although the B-box has been proposed to be a protein interaction domain, its molecular function is not well understood. The B-boxes in the Pro Myelocyte Leukemia (PML) proteins have been shown to be required for its localization to speckles known as PML bodies (Borden et al., 1996). In plants, the B-boxes in COL3 were shown to be required for localization of the protein into nuclear speckles (Datta et al., 2006). Recently, it was shown that the N-terminal B-box–containing part of CO was able to form nuclear speckles, whereas the C-terminal part containing the CCT domain was not (Liu et al., 2008). In STH2, a structurally intact B-box domain was found to be important for interaction with HY5, providing evidence for the role of the B-box domain in protein–protein interaction (Datta et al., 2007). Furthermore, transient transfection assay in protoplasts indicated that a functional B-box domain was required to activate transcription. While some B-box domains exist as single domains, they are often found as tandem repeats. Here, we found that the two B-boxes of STH3 appear to play different roles, while the structure of the first B-box appears to be inessential for HY5 interaction in yeast, and substitutions of Zn^{2+} ligating residues in either B-box reduce the ability of STH3 to activate transcription. By contrast, while the D81A substitution is unlikely to affect the structure of the second B-box, it abolishes interaction with HY5 without affecting the ability of STH3 to activate transcription.

In animals, B-boxes are often found in conjugation with a RING finger domain and a coiled-coil domain forming RBCC or tripartite motif proteins. The RBCC family includes a large number of proteins involved in various cellular processes, such as apoptosis, cell cycle regulation, and viral response. Recently, a number of TRIM/RBCC proteins have been found to play a role in ubiquitination and the B-boxes proposed to participate in substrate recognition. The B-box–containing MID1 protein is a putative E3 ligase that is required for the proteosomal degradation of the catalytic subunit of protein phosphatase 2A (PP2Ac). This function of MID1 is facilitated by direct binding of α4, a regulatory subunit of PP2Ac, to one of the B-boxes, while the second appears to influence this interaction. Structural studies on the tandem B-boxes of MID1 revealed a stable interaction between the B-box domains reminiscent of intermolecular RING heterodimers, suggesting the possibility of an evolutionarily conserved role for B-box domains in regulating functional RING-type folds (Tao et al., 2008). While RBCC proteins are absent in plants, it is interesting that COP1 interacts with at least six different B-box–containing proteins, namely, COL3, CO, STO, STH1, STH2, and STH3 (Holm et al., 2001; Datta et al., 2006, 2007; Liu et al., 2008). All these interactions between B-box–containing proteins and the RING, coiled-coil domain containing COP1 proteins suggest a mechanism of creating a functional equivalent of RBCC protein in an organism that lacks such proteins. The in vitro ubiquitination of STH3 and CO by COP1 points toward a role of these complexes between B-box proteins and the E3 ligase COP1 in regulated proteolysis. Further studies of these biochemical complexes might help unravel the functional intricacies of manifold cellular processes regulated by B-box–containing proteins.

Light-regulated control of development encompasses transcriptional regulation, posttranslational modification, and degradation by regulated proteolysis. As genetic and genomic studies reveal new components of the light-regulated signaling network, a picture of a tug-of-war between the positive and the negative regulators of photomorphogenesis is emerging. HY5 and COP1 are pivotal players in this tussle, and proteins interacting with both of these key regulators are candidates to fill the gaps in the regulatory network. The identification and characterization of STH3 interacting with HY5 and COP1 provide new insights into the mechanisms of this dramatic transcriptional reprogramming and provide a handle to address how the transcriptional network operates to integrate multiple signals during photomorphogenesis.

METHODS

Plant Material, Growth Conditions, and Complementation Tests

The T-DNA insertion line SALK_105367 (*sth3-1*) was obtained from the ABRC. The *sth3*, *hy5-215*, *cop1-4*, and *cop1-6* alleles are in the Col-0 accession. Unless otherwise stated, seeds were surface-sterilized and plated on GM medium supplemented with 0.8% Bactoagar (Difco) and 1% sucrose. The plates were then cold-treated at 4°C for 3 d and then transferred to light chambers maintained at 22°C with the desired light regime. For the complementation test, the pFP100-*STH3* construct, containing a 3.3-kb genomic *Eco*RI fragment (Figure 3A) that included the full-length *STH3* gene, was transformed into homozygous *sth3*, *sth2-1 sth3*, *sth3 hy5-215*, *sth3 cop1-6*, and wild-type Col-0 plants by the modified floral dip method (Logemann et al., 2006). Transgenic T1 seeds were selected using the Leica MZFL III stereomicroscope equipped with a GFP filter. These transgenic seeds were used for phenotypic analyses, with untransformed siblings serving as control.

Constructs and Primers

The full-length *STH3* cDNA was amplified using the primers STH3u, 5′-GGAATTCTCCAAAGAGAAAACGTATGAG-3′ (*Eco*RI site underlined), and STH3d, 5′-CGGGATCCCCCTTATAATCTTCAACCAAA-3′ (*Bam*HI site underlined) and then subcloned as an *Eco*RI-*Bam*HI fragment into the corresponding sites of pYX242 to create pYX242 STH3. All amino acid

substitutions in the B-boxes were made using PCR-based mutagenesis and subsequently subcloned into pYX242 as mentioned before. Primers used for the D20A substitution were STH3B1u, 5′-GTTGCGCCGCCGAGGCTG-3′, and STH3B1d, 5′-CAGCCTCGGCGGCGCAAC-3′, for D72A; STH3B2u, 5′-TGTCTGCAAGCTAGAGCTTTGCTA-3′, and STH3B2d, 5′-TAGCAAAGCTCTAGCTTGCAGACA-3′, and for D81A; STH3B3u, 5′-AGGAAATGTGCTGTTGCAATC-3′, and STH3B3d, 5′-GATTGCAACAGCACATTTCCT-3′.

The same primers were used to make pRTL STH3 and the mutated versions for the protoplast experiment. However, in this case the *Eco*RI-*Bam*HI fragments were first subcloned into an *Eco*RI/*Bam*HI cut pBSK+, which was then digested with *Eco*RI/*Xba*I and subcloned into the corresponding sites in the pRTL vector. For generating the pAM-PAT-35SS-CFP-STH3 construct, full-length STH3 was PCR amplified using the primers STH3 kpn, 5′-AGGGTACCATGAAGATTCAGTGTAACGTTTG-3′, and STH3 xba, 5′-CGTCTAGATTACTAGAACCGTCGCCGCCG-3′, and then subcloned as a *Kpn*I-*Xba*I fragment into the corresponding sites of pAM-PAT-35SS-CFP vector to create pAM-PAT-35SS-CFP-STH3.

To generate pET28c-3HA-STH3 for the ubiquitination assay, full-length *STH3* was PCR amplified using the primers 35s_HA_STH3, 5′-GCTCTAGAGGTGGTGGTATGAAGATTCAGTGTAACGTTT-3′ (*Xba*I site underlined), and STH3d, 5′-CGGGATCCCCCTTATAATCTTCAACCAAA-3′ (*Bam*HI site underlined), and then subcloned as a *Xba*I-*Bam*HI fragment into the corresponding sites of pBHA to create pBHA STH3. This construct was then used as a template for a PCR reaction to amplify three copies of the HA tag fused to STH3 using the primers NheI_HA_fwd, 5′-CTAGCTAGCATGGCATACCCATACGACGT-3′ (*Nhe*I site underlined), and STH3d and subcloned as a *Nhe*I-*Bam*HI fragment into the corresponding sites of pET28c to create pET28c STH3.

Yeast Two-Hybrid Methods and FRET Experiments

The yeast strain Y187 was used for the two-hybrid assays, and the β-galactosidase assays were performed as described by Holm et al. (2001). For the FRET acceptor photobleaching experiments, the pAM-PAT-35SS-YFP-HY5, pAM-PAT-35SS-CFP-STH3, and the pRTL2-COP1 overexpression constructs were introduced into onion epidermal cells by particle bombardment and incubated, and live cell images were acquired using an Axiovert 200 microscope equipped with an LSM 510 META laser scanning confocal imaging system (Carl Zeiss). Cells were visualized 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 their germination using the Rneasy plant mini kit (Qiagen). Twenty micrograms of the total RNA was loaded for the RNA gel blot analysis. A full-length *STH3* open reading frame was used as a probe to detect transcript levels in the wild type and *sth3* mutant backgrounds.

Hypocotyl 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. Blue, red, and far-red light was 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 was measured using ImageJ software.

Anthocyanin Measurements

For the anthocyanin determinations, seedlings were harvested 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 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 thaliana mesophyll cell protoplasts were generated and transfected as described by Yoo et al. (2007). The reporters used were a 610-bp fragment of the CHI promoter (the region between the sequences 5′-TAGAAGCTTTAATAGATAAGAAAAGAAAG-3′ and 5′- AATCGAAATTCCAACCGACTCAACA-3′) and a 199-bp fragment of the CAB promoter (the region between the sequences 5′-ATAACTTGTGGTCACAAAACGC-3′ and 5′-AAACACAAAAAAGTTTCA-3′) driving firefly luciferase (pPCV814-CHI610-Luc and pPCV814-CAB199-Luc). Reporters with G-box mutated in the promoter, pPCV814-CHI610Gm-Luc and pPCV814-CAB199Gm-Luc, were also used in this assay. Full-length *STH3* driven by a cauliflower mosaic virus 35S promoter was used as the effecter (pRTL2-STH3). For detection, the Dual Luciferase system was used (Promega). Renilla lucerifase driven by a full-length cauliflower mosaic virus 35S promoter was used as internal control (pRNL).

In Vitro Ubiquitination Assays

In vitro ubiquitination assays were performed as previously described (Saijo et al., 2003) with minor modifications. Ubiquitination reaction mixtures (60 μL) contained 50 ng of yeast E1 (Boston Biochem), 50 ng of rice His-Rad6 E2, 10 μg of unlabeled (Free Ub), HA-tagged (HA-Ub), or Flag-tagged ubiquitin (Flag-Ub; Boston Biochem), 200 ng HA-STH3, and 0.5 μg MBP-COP1 (previously incubated with 20 μM $ZnCl_2$) in reaction buffer containing 50 mM Tris at pH 7.5, 5 mM $MgCl_2$, 2 mM ATP, and 0.5 mM DTT. MBP-COP1 that was not incubated with $ZnCl_2$ (-Zn) was used as a negative control. After 2 h incubation at 30°C, reaction mixtures were stopped by adding sample buffer, and one-half of each mixtures (30 μL) was separated onto 7.5% SDS-PAGE gels. Ubiquitinated HA-STH3 was detected using anti-HA antibody (Roche).

Accession Number

The STH3 Arabidopsis Genome Initiative locus identifier is At1g78600.

Supplemental Data

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

Supplemental Figure 1. Absence of FRET between Unfused CFP/YFP or CFP-STH2/YFP-STH3.

Supplemental Figure 2. Anthocyanin Levels in *hy5* and *sth3 hy5* Are Close.

Supplemental Data Set 1. Text File Corresponding to the Alignment in Figure 1A.

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