B-box Proteins in Light-regulated Development in *Arabidopsis*

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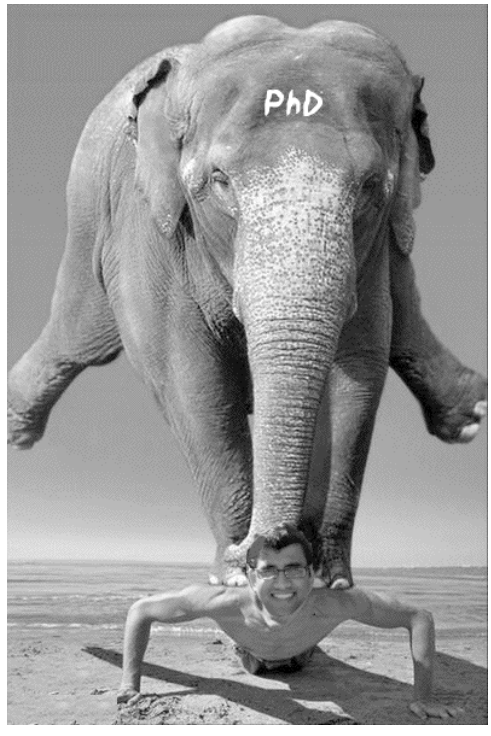


To be conscious that you are ignorant is a great step to knowledge.
Benjamin Disraeli

Stay Hungry. Stay Foolish.
Steve Jobs

To Ma and Baba

Abstract

COP1 and HY5 are two key regulators of light signaling in plants. Proteins interacting with either could therefore be important regulators of light-dependent development. Previous yeast two-hybrid screens, using COP1 or HY5 as bait, identified several putative regulators of light signaling. We isolated T-DNA insertion mutants in three of these genes: *COL3*, *STH2* and *STH3*. Phenotypic characterization of these mutants revealed pigmentation, hypocotyl and root phenotypes suggesting that the genes have a positive role in light-regulated processes. Moreover, study of double mutants with *hy5* and *cop1* confirmed that all of them genetically interact with both *HY5* and *COP1*.

COL3, *STH2* and *STH3* encode proteins containing N-terminal B-boxes. B-boxes are zinc-ligating domains consisting of conserved cysteine and histidine residues. In animals, B-boxes are often found together with a RING finger domain (originally termed A-box) and a coiled-coil domain forming RBCC or tripartite motif (TRIM) proteins. Although RBCC proteins are absent in *Arabidopsis*, there are 32 proteins with N-terminal B-boxes. This thesis deals with the characterization of the B-box containing proteins, COL3, STH2 and STH3 and the study of their role in light-regulated development of plants.

Our results show that the B-boxes play multiple roles in plant development. We found that the B-boxes in COL3 were required for localization of the protein into nuclear speckles. In STH2 and STH3, the 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. Transient transfection assays in protoplasts indicated that functional B-box domains in STH2 and STH3 are required for transcriptional activation. We hypothesize that the B-box proteins might act as co-factors for the transcription factor HY5, regulating light-mediated transcription and development.

COP1 acts as an E3 ubiquitin ligase that targets positive regulators of photomorphogenesis for degradation in the dark. We found that COP1 could ubiquitinate STH3 *in vitro* suggesting that STH3 might be regulated by COP1. Our results show that COL3 co-localizes with COP1 in nuclear speckles and the two proteins interact physically. Moreover, our genetic studies show that *col3*, *sth2* and *sth3* partially suppress *cop1* in the dark. All these interactions allow us to place COL3, STH2 and STH3 in the light-signaling network. Thus, starting from preliminary yeast interaction data, my doctoral work provides genetic, physiological and functional evidence for the role of B-box containing proteins in light-signaling.

List of papers discussed

This thesis is based on the following publications, which will be referred to by their roman numerals:

Paper I

Datta, S., Hettiarachchi, G.H.C.M., Deng, X.W., and Holm, M. (2006).
Arabidopsis CONSTANS-LIKE3 is a positive regulator of red light signaling and root growth.
Plant Cell 18**,** 70-84.

Paper II

Datta, S., Hettiarachchi, C., Johansson, H., and Holm, M. (2007).
SALT TOLERANCE HOMOLOG2, a B-box protein in *Arabidopsis* that activates transcription and positively regulates light-mediated development.
Plant Cell 19**,** 3242-3255.

Paper III

Datta, S., Johansson, H., Hettiarachchi, C., Irigoyen, M.L., Desai, M., Rubio, V., and Holm, M. (2008).
LZF1/SALT TOLERANCE HOMOLOG 3, an *Arabidopsis* B-box protein involved in light-dependent development and gene expression undergoes COP1-mediated ubiquitination.
Plant Cell (in press)

CONTENTS

Abbreviations

bHLH	basic-Helix-Loop-Helix
bZIP	basic-Leucine Zipper
CAB	Chlorophyll A/B-Binding
CCT	Constans, Constans-like, Timing of CAB expression 1
CHI	Chalcone Isomerase
CO	Constans
COL3	Constans-Like 3
COP1	Constitutively Photomorphogenic 1
CRY	Cryptochrome
DET1	De-etiolated 1
HY5	Elongated Hypocotyl 5
HYH	HY5 Homolog
LZF1	Light-regulated Zinc Finger Protein 1
PHOT	Phototropin
PHY	Phytochrome
PIF3	Phytochrome Interacting Factor 3
RBCC	Ring, B-box, coiled-coil
STH1	Salt Tolerance Homolog 1
STH2	Salt Tolerance Homolog 2
STH3	Salt Tolerance Homolog 3
STO	Salt Tolerance
TRIM	Tripartite Motif

Introduction

Role of light in the life cycle of *Arabidopsis*

'In the beginning there was nothing. God said, "Let there be light!" And there was light. There was still nothing, but you could see it a whole lot better' (Ellen DeGeneres). Since time immemorial light has played an inevitable role in the lives of all living organisms on this planet. It provides the ultimate source of biological energy, which is harvested by the photosynthetic organisms to sustain life. Besides being the critical energy source, light regulates several developmental processes throughout the plant's life. The small weed *Arabidopsis thaliana* is a perfect model organism to study the role of light in the life cycle of plants (Figure 1). Plants have evolved complex methods of sensing the quantity, wavelength, direction and duration of light and interpreting these signals to produce the appropriate physiological and developmental response (Sullivan and Deng, 2003).

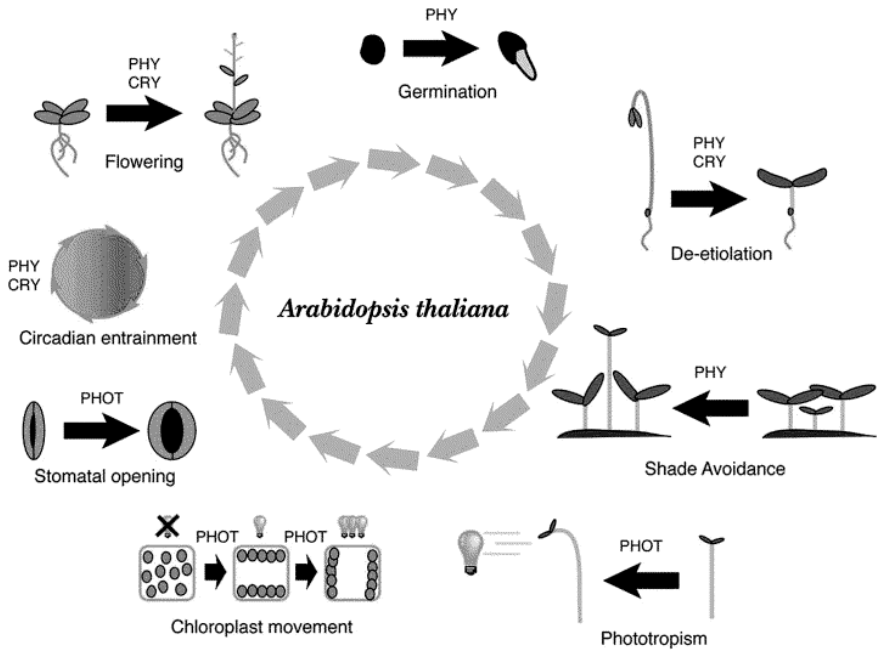


Figure 1. Role of light in the life cycle of *Arabidopsis*. Light regulates several developmental processes throughout the plant's life. PHY, CRY and PHOT represent the photoceptors Phytochrome, Cryptochrome and Phototropin respectively, involved in the different developmental processes. *Adapted from* (Sullivan and Deng, 2003).

The germination of the seed at the onset of the plant life cycle is a light-dependent event. Light, availability of water and temperature together determine the timing of germination. This is followed by the emergence of the seedling from under the soil into light, initiating light-mediated development or photomorphogenesis, which is a developmental process that entails massive light regulation. This thesis mainly deals with this developmental phenomenon, described in detail below. As the growing plant attains photosynthetic capacity, it begins to compete with the neighbouring plants for light. This initiates a developmental mechanism called 'shade avoidance' wherein plants try to grow out from the canopy of overhanging vegetation. Phototropism or the directional curvature of plant organs in response to light is another developmental process in plants controlled by light, more precisely the direction of the incoming light. The bending of the aerial part of the plant towards light is an evolutionary adaptation to optimize photosynthesis. A dramatic example of light intensity affecting a physiological mechanism in the plant is the regulation of chloroplast movement within the plant cell. Plants align their chloroplasts either perpendicular or parallel to the direction of the incoming light based on low or high intensities of the available light respectively. This phenomenon allows the plant to maximize the solar energy capture in low light conditions while prevents bleaching of the photosynthetic organelles when there is too much light. Another vital mechanism in plants that is governed by light is the opening and closing of the stomata. Blue light has been long shown to regulate this fundamental mechanism.

Most organisms show rhythms in metabolism, physiological processes and behaviour in response to the day-night cycle. An internal oscillator called the circadian clock maintains these rhythms. Although these rhythms can persist even in the absence of any external environmental cues, the circadian clock is reset or 'entrained' to be synchronized with the day-night cycle. Cross talk between the light-signaling and circadian pathways regulates several developmental processes in the plant. Towards the end of their life cycle, the time for transition from vegetative to reproductive form to produce flowers is determined by the plant by perceiving the duration of light or day length. Some plants flower when the days are short and are termed short-day plants while others flower when the days are long and are called long-day plants. Thus throughout their life cycle, plants continuously monitor the intensity, spectral composition, direction and periodicity of the ambient light to optimize their growth and development.

Photomorphogenesis, a light-regulated developmental process

The period between seed germination and the formation of the first true leaves is one of the most extensively studied stages of *Arabidopsis* development. After germination, the young seedling must choose between two developmental pathways depending on the availability of light. In the absence of light, the seedling grows heterotrophically, using the seed's resources in an effort to reach light. This etiolated stage is characterized by a long hypocotyl (primary stem), an apical hook and unopened cotyledons (embryonic leaves), features that allow the seedling to grow through a layer of soil and emerge in the light. Once the seedling perceives sufficient light, it de-etiolates, a developmental process that optimizes the body plan of the seedling for efficient photosynthetic growth. During de-etiolation or photomorphogenesis, the rate of hypocotyl growth decreases, the apical

hook opens, cotyledons expand, chloroplasts develop, and a new gene expression program is induced. A complex web of regulation controls photomorphogenesis, which is perhaps not surprising considering the fact that in this brief window of time, a plant matures from an endosperm-dependent embryo to a self-sufficient photoautotroph.

Light perception and signal transduction

The action spectra of light responses provided assays to identify three photoreceptor systems absorbing in the red/far-red, blue/near-ultraviolet, and ultraviolet spectral ranges. Following absorption of light, the signal is transduced to the downstream components of the light-signaling pathway. Molecular genetic studies using the model plant *Arabidopsis* have led to substantial progress in dissecting the signal transduction network. Important gains have been made in determining the function of the photoreceptors, the terminal response pathways, and the intervening signal transduction components.

Photoreceptors

In *Arabidopsis* at least four classes of wavelength-specific photoreceptors have been reported. Red/Far-red light (600-750 nm) is perceived by the 'Phytochrome' family, whereas the 'Cryptochromes' and 'Phototropins' perceive blue/UV-A (320-500 nm) light (Figure 2). UV-B (282-320 nm) is perceived through a yet uncharacterized photoreceptor.

Phytochromes

The phytochrome family in *Arabidopsis* consists of five members, designated as phyA to phyE. While phyA is light-labile, phyB-E are light-stable. Phytochromes are homodimers in solution. Each monomer is a 125-kDa polypeptide and has a linear tetrapyrole chromophore (phytochromobilin) attached to it through a -S- (thioether) bond to the amino acid cysteine (Chen et al., 2004). The phytochrome protein can be divided into two domains: an amino-terminal photosensory (signal input) domain and a carboxy-terminal domain that has been traditionally regarded as a regulatory, dimerization and signal output domain (Bae and Choi, 2008). In dark the phytochromes are in their inactive Pr conformer and are present as soluble, cytoplasmically localized proteins. Upon light irradiation the linker between the C and D rings of the bilin undergoes a Z to E isomerization, photoconverting the Pr to the active Pfr conformation inducing translocation to the nucleus. The nuclear import of phyA is much faster than that of phyB-E (Kircher et al., 2002). Recently it was shown that the proteins FHY1 (Far-red elongated hypocotyl 1) and FHL (Fhy1-like) are required for the nuclear accumulation of the phytochrome A (Hiltbrunner et al., 2006).

PhyA, B and D have been shown to form speckles in the nucleus (Kircher et al., 2002; Kevei et al., 2007). While all of them can form speckles very rapidly (2-3 min) after irradiation, phyB has been shown to form late speckles after continuous irradiation for one-two hours (Gil et al., 2000; Kircher et al., 2002). The late phyB speckles appear to be larger in size and less in number. Nuclear import is not sufficient for speckle formation, and these two processes have different requirements for the amount of Pfr to total phyB

(Chen et al., 2003). While PfrPr heterodimer is sufficient for nuclear import, PfrPfr homodimers favour localization to nuclear speckles.

The chromophore isomerization also leads to structural changes that include the disruption of intramolecular interactions between the N- and C-terminal domains of the phytochrome (Bae and Choi, 2008). This disruption exposes surfaces required for interactions with other proteins. The active far-red absorbing form of phyB was found to interact with a DNA-bound transcription factor PIF3 (Phytochrome Interacting Factor 3), suggesting a rather direct signal transduction where the photoreceptor could act in promoter context (Martinez-Garcia et al., 2000). It has been shown that red light pulses induce transient co-localization of PIF3 with phyB in nuclear speckles and presence of PIF3 is essential for the detection of early phyB speckles (Bauer et al., 2004). The phytochromes interact with several other PIFs (PIF1 interacts with both phyA and B, PIF7 co-localizes with phyB in nuclear speckles) to regulate light-dependent transcription and development (Leivar et al., 2008; Moon et al., 2008; Shen et al., 2008). Recently PIF1 was reported to bind to the G-box element present in the promoter of a key chlorophyll biosynthetic gene regulating the greening process (Moon et al., 2008). Phytochromes and their interacting factors regulate all major developmental transitions such as germination, de-etiolation, and the commitment to flowering. They also fine-tune vegetative development by influencing gravitropism, phototropism, and by mediating the shade-avoidance response (Chen et al., 2004).

Cryptochromes

The identification of an *Arabidopsis* mutant, *hy4*, impaired specifically in blue light perception (Koornneef et al., 1980; Ahmad and Cashmore, 1993) allowed the cloning of the first blue light receptor, CRY1, from plants (Ahmad and Cashmore, 1993). *Arabidopsis* contains two cryptochrome genes *CRY1* and *CRY2* showing strong homology to each other and to bacterial DNA photolyase genes, although they do not possess any photolyase activity. Cryptochromes have a PHR (Photolyase homology region) domain at their N-terminal end, which binds a FAD (Flavin Adenine Dinucleotide) and a pterin chromophore. At the C-terminal end the Cryptochromes have a conserved DAS motif (Lin et al., 1995). The two cryptochromes CRY1 and CRY2 are nuclear in darkness; both are phosphorylated in response to light whereby CRY1 becomes enriched in the cytoplasm whereas the light labile CRY2 is degraded (Shalitin et al., 2002; Shalitin et al., 2003). A more divergent family member, CRY3, is present in the mitochondria and chloroplast (Brudler et al., 2003). The cryptochromes have been shown to play important roles in de-etiolation, resetting the circadian clock and in the induction of flowering. Recent data suggest that the cryptochromes are directly involved in the light-dependent stabilization of the floral-inducing transcription factor CO (Valverde et al., 2004; Liu et al., 2008). The cryptochrome mediated signaling involves several transcription factors like HY5, HYH, GBF1, etc. (Holm et al., 2002; Mallappa et al., 2006).

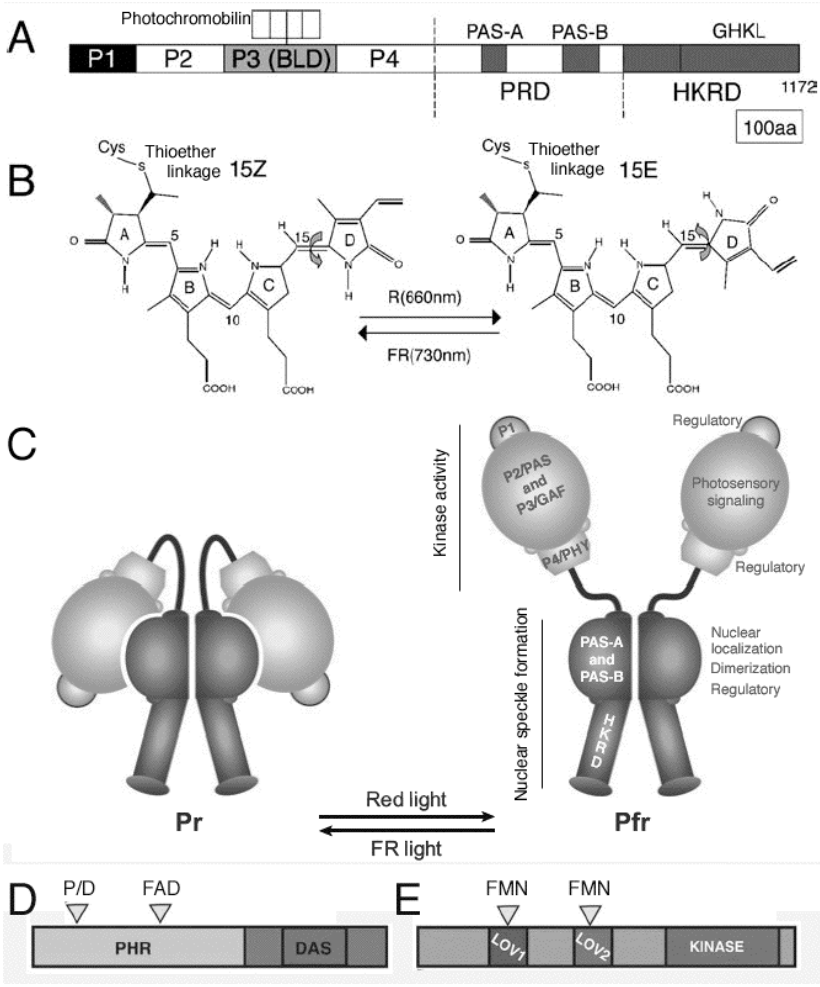


Figure 2. Domain structure of photoreceptors. (A) Domain structure of phytochromes using *Arabidopsis* phyB as a model; (B) Two isomers of phytochromobilin, 15Z (Pr chromophore) and 15E (Pfr chromophore), thioether bond is indicated; (C) The structural change in phytochrome accompanying photoisomerization, the functions associated with the different domains are also indicated; (D) and (E) Domain structure of *Arabidopsis* cryptochrome and phototropin respectively. Triangles represent the attached chromophores. *Redrawn from* (Chen et al., 2004; Bae and Choi, 2008).

Phototropins

Just over a decade ago 'phototropin' was identified as a photoreceptor responsible for directional growth. In *Arabidopsis* there are two phototropins: PHOT1 and PHOT2 (Briggs et al., 2001). While PHOT1 is specialized for low blue light fluence responses, PHOT2 plays a more important role under high fluences. Both proteins contain two LOV (Light, Oxygen, Voltage) domains at the N-terminus and a serine/threonine kinase domain at the C-terminus. The LOV domains are the photosensory domains that noncovalently bind a FMN (Flavin Mononucleotide) molecule. The Phototropins are plasma membrane-associated and upon light stimulation a fraction of PHOT1 is released into the cytoplasm. Light-regulated PHOT1 autophosphorylation appears to be the initial event in phototropin-mediated signaling. The Phototropins regulate several light-dependent responses like phototropism, chloroplast movements and stomatal opening (Briggs and Christie, 2002). Recently a new group of blue light photoreceptors called Zeitlupes, which are F-box proteins containing a LOV domain and Kelch repeats, were identified in *Arabidopsis* (Imaizumi et al., 2003).

Signal integration and transduction

Plants integrate external environmental signals with internal cues to develop a discrete growth response. While the role of different photoreceptors in perceiving different wavelengths of light is quite clear, as the signal moves downstream towards the eventual cell mechanics of expansion, division and differentiation, the picture becomes quite foggy. It is increasingly apparent that there is a constant crosstalk between different signaling pathways creating a network inside the plant. Understanding this complex signaling network will allow us to comprehend the mechanism behind different developmental processes.

Most of the plant hormones have been implicated in photomorphogenic growth, with cytokinin promoting photomorphogenesis, and auxin, brassinosteroids (BRs), and gibberellins (GAs) acting in opposition (Vert et al., 2008). Abscisic acid (ABA) acts in opposition to GAs and BRs in some contexts, yet the ABA response also appears necessary to maintain etiolated growth. Analysis of ethylene response mutants suggests that ethylene can act either to promote or inhibit photomorphogenic growth in a tissue and environment-dependent manner. Photoreceptor responses are also mediated by the circadian clock. In *Arabidopsis*, circadian rhythmicity in hypocotyl growth has been well documented (Dowson-Day and Millar, 1999). Nuclear localization of phyB appears to follow a circadian fluctuation even after plants are shifted to complete dark or continuous light (Nagy et al., 2000). The production of chlorophyll in the chloroplasts is a light-dependent process. Several studies have reported a strong link between chloroplast development and photomorphogenesis. Other studies have shown interactions between regulators of the carbohydrate and the light-signaling pathways. Exposure of the aerial part of seedlings to exogenous sucrose leads to a variety of aberrant light responses in dark-grown plants (Roldan et al., 1999). The role of calcium and cyclic GMP has also been implicated in phytochrome-mediated signaling (Bowler et al., 1994).

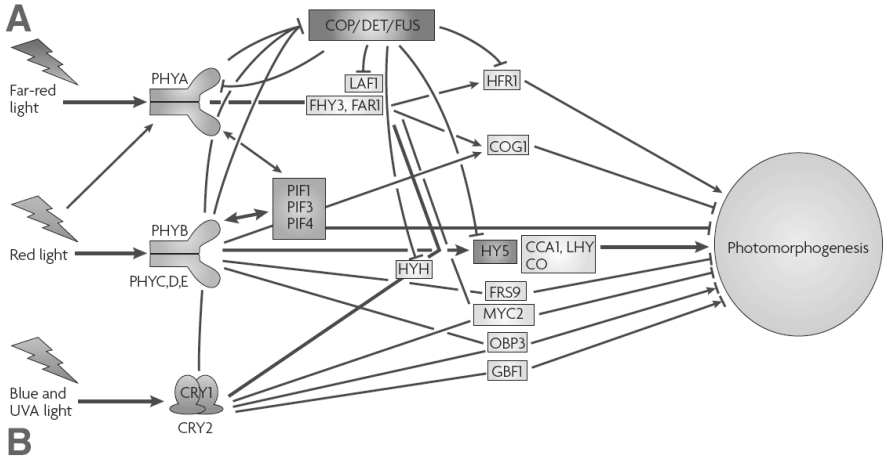
Plants grow in a light environment composed of a mixture of light qualities and quantities, simultaneously activating several signaling pathways. The signals from these pathways appear to be integrated by a wealth of shared downstream components many of which are transcription factors. Microarray studies performed on seedlings grown in dark and moved to monochromatic far-red, red or blue light found that a large fraction of the early affected genes are transcription factors (Tepperman et al., 2001; Jiao et al., 2003; Tepperman et al., 2004). 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.

Transcriptional regulators of light signaling

Molecular genetic approaches have identified several transcription factors acting downstream of a single or a combination of photoreceptors, forming a light-regulated transcriptional network. 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 (Figure 3).

A small subgroup of the basic helix-loop-helix (bHLH) family of transcription factors interacting with the phytochromes have revolutionized the concept of phytochrome regulation of gene expression and have been designated PIFs (phytochrome interacting factors) (Duek and Fankhauser, 2005). PIF3 (Phytochrome Interacting factor 3) was the first member of this subfamily interacting mainly with phyB (Ni et al., 1999). Other bHLH proteins: PIF4, PIF5, PIF7, act as negative regulators of phyB signaling under prolonged red light irradiation, 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). HFR1 on the other hand plays a positive role in both phyA and cryptochrome-mediated signaling (Fairchild et al., 2000; Duek and Fankhauser, 2003) whereas MYC2 acts a repressor of blue and far-red light-mediated de-etiolation (Yadav et al., 2005).

Some transcription factors like FAR-RED IMPAIRED RESPONSE 1 (FAR1) and FAR-RED ELONGATED HYPOCOTYL 3 (FHY3) act specifically downstream of the far-red photoreceptor phyA (Hudson et al., 1999; Wang and Deng, 2002; Hudson and Quail, 2003). Recently it was shown that FAR1 and FHY3 represent transcription factors that act together to modulate phyA signaling by directly activating the transcription of FHY1 and FHL, whose products are essential for light-induced phyA nuclear accumulation and subsequent light responses (Lin et al., 2007). Another transcription factor showing specific phyA-mediated photomorphogenesis is LAF1, which belongs to the R2R3-MYB family of transcription factors (Ballesteros et al., 2001). Two Dof family transcription factors: COGWHEEL 1 (COG1) and OBF4 BINDING PROTEIN 3 (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).



B

Transcription factor family	Members
bHLH	PIF1,3,4,5,7,HFR1,MYC2
bZIP	HY5,HYH,GBF1
MYB	LAF1,CCA,LHY
DOF	COG1,OBP3
Homeodomain	HAT4
Others	FHY1,3,FAR1
B-box	COL3,STO,STH1,2,3

Figure 3. Transcriptional network for seedling photomorphogenesis (A) and members of different transcription factor families involved in the network (B). *Redrawn from* (Jiao et al., 2007))

Members of the bZIP transcription factor family like HY5, HYH and GBF1 are also involved in this networking cascade (Oyama et al., 1997; Holm et al., 2002; Park et al., 2003; Ward et al., 2005; Mallappa et al., 2006). GBF1 regulates blue light signaling, HYH promotes light-dependent development in blue and far-red light whereas HY5 acts as a positive regulator downstream of all the photoreceptors. HY5 not only binds to the promoters of several light-regulated genes but also some circadian regulators like CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) that encode partially redundant MYB transcription factors also involved in photomorphogenesis.

HY5, a positive regulator of photomorphogenesis

Among all the transcription factors acting downstream of the photoreceptors, the most vividly characterized one is the bZIP transcription factor LONG HYPOCOTYL 5 (HY5). Mutations in *HY5* result in an elongated hypocotyl in all light conditions (Figure 4A and

B), 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, chlorophyll and anthocyanin accumulation (Oyama et al., 1997; Holm et al., 2002). Additionally, a role of HY5 in both auxin and cytokinin signaling pathways has been reported (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.

HY5 has been shown to specifically bind the G-Box present in the promoters of several light-inducible genes like chalcone synthase (CHS) and ribulose-biphosphate carboxylase small subunit (RbcS1A) in *in-vitro* gel-shift assays (Ang et al., 1998; Chattopadhyay et al., 1998). A recent ChIP-chip assay revealed that HY5 binds to promoter regions of more than 3,000 genes in the *Arabidopsis* genome *in vivo* (Lee et al., 2007). These included numerous light-regulated genes and transcription factor genes. Interestingly more than 60% of the genes induced early by phyA and phyB (Tepperman et al., 2001; 2004) were found to be HY5 binding targets (Lee et al., 2007), which suggests that HY5 is a high hierarchical regulator of the transcriptional cascade for photomorphogenesis acting downstream to the photoreceptors. However, the fact that 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, irrespective of the light-dark transition or the daily rhythm, suggests that HY5 binding is not sufficient and additional factors are required for HY5-dependent transcriptional regulation (Lee et al., 2007).

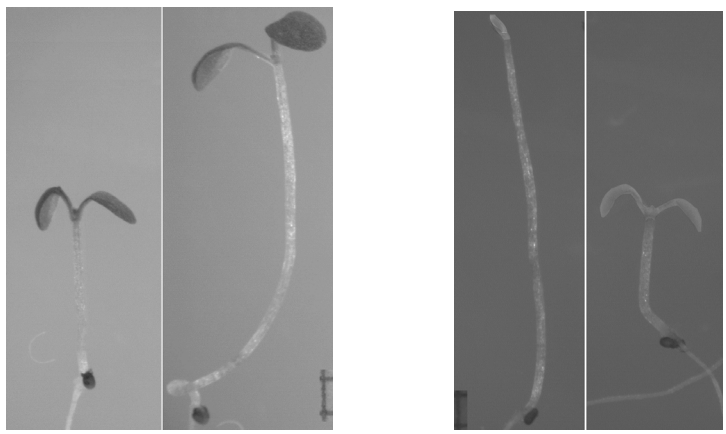


Figure 4. (A) and (B) *hy5* mutant has elongated hypocotyl in the light. Representative Col-0 (wild-type) (A) and *hy5-215* (B) seedlings grown in light for six days. (C) and (D) *cop1* mutant grown in the dark phenocopy light-grown seedlings producing short hypocotyl and expanded, partially differentiated cotyledons. Representative Col-0 (wild-type) (C) and *cop1-6* (D) seedlings grown in dark for six days. Scale bar = 1 mm.

Repressors of photomorphogenesis

Besides the positive factors involved in light signal transduction, repressors of the default photomorphogenic pathway in *Arabidopsis* have also been identified in several genetic screens. In dark, the seedlings become etiolated, a developmentally arrested growth mode characterized by limited root growth, an elongated hypocotyl, closed un-differentiated cotyledons and an apical hook. The developmental arrest seen during etiolated growth is mediated by the COP/DET/FUS proteins, which act as repressors of photomorphogenesis. Mutations in any of the ten *COP/DET/FUS* genes result in a dramatic phenotype in the dark where they phenocopy light-grown seedlings producing short hypocotyl and expanded partially differentiated cotyledons, thus being constitutively photomorphogenic (Figure 4C and D). The recessive nature of these mutations suggests that these genes act as repressors of a default photomorphogenic pathway. Interestingly, the genome expression profiles of dark-grown *cop/det/fus* alleles closely resemble light grown seedlings (Ma et al., 2003). The failure of plants with mutations in the *COP/DET/FUS* genes to arrest photomorphogenic development during etiolated growth suggests that the targets of this pathway are likely to be key regulators of photomorphogenesis. The photomorphogenic development seen in *cop/det/fus* mutants in the dark could not be mediated by photoreceptors since they are activated by light. Furthermore, genetic analysis revealed that *cop1* is epistatic to mutations disrupting phytochrome and CRY1 function in darkness (Ang and Deng, 1994). The photomorphogenic development in dark-grown *cop/det/fus* seedlings is therefore likely caused by loss of COP/DET/FUS repression of factors acting downstream of the photoreceptors.

One of these COP/DET/FUS proteins, COP1, is a major negative regulator of photomorphogenic responses. *cop1* mutants undergo photomorphogenesis in darkness in the absence of photoreceptor activation so that *cop1* seedlings grown in the dark phenocopy light-grown seedlings (Deng et al., 1991). In addition to these roles in seedling development, COP1 also influences photomorphogenesis of adult plants. Although null mutant alleles of *COP1* cause seedling lethality, plants homozygous for weaker *cop1* alleles are viable (McNellis et al., 1994). These plants are early flowering, dwarfed and show reduced apical dominance indicating that *COP1* has pleiotropic effects.

Role of COP1-mediated proteolysis in light signaling

Several studies have shown the role of regulated proteolysis in light signaling. The molecular characterization of the COP1 protein suggests that it acts in a proteolytic pathway aimed at degrading photomorphogenesis promoting factors in the absence of light (Osterlund et al., 2000a). This notion was first introduced in a study attempting to characterize the regulation of HY5. The HY5 protein was found to accumulate to a much higher level in light-grown seedlings and, upon light-to-dark transition, was degraded through proteasome-mediated proteolysis (Osterlund et al., 2000b), a process that usually requires the targeted proteins first to be modified by a chain of ubiquitin. COP1, a RING-finger protein and negative regulator of HY5, had been previously shown to directly

interact and co-localize with HY5 to subnuclear speckles in living plant cells and was immediately suspected to be the HY5 E3 ubiquitin ligase (Ang et al., 1998; von Arnim et al., 1998). This hypothesis was further strengthened by the observations that HY5 degradation during light-to-dark transitions is impaired in *cop1* mutant seedlings, transgenic seedlings expressing HY5 with point mutations at the HY5 COP1-interacting motif, or in COP1 mutants with point mutations in the COP1 WD40 domain abolishing HY5 interaction (Osterlund et al., 2000b; Holm et al., 2001). Moreover, HY5 becomes stabilized in white light when the COP1 protein is excluded from the nucleus (Osterlund et al., 2000b). COP1 was later confirmed to possess intrinsic E3 activity and to ubiquitylate HY5 *in vitro* (Saijo et al., 2003).

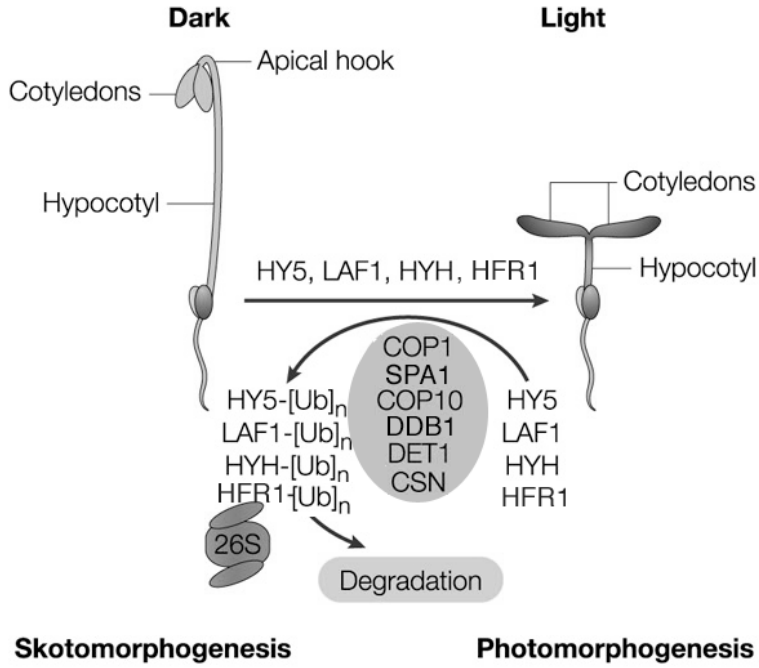


Figure 5. COP1-mediated proteolysis in light signaling. COP1 acts as an E3 ubiquitin ligase and together with SPA1, CDD complex (COP10, DET1 and DDB1) and the COP9 signalosome, targets the positive regulators of photomorphogeneis like HY5, LAF1, HYH and HFR1 for degradation in the dark, promoting skotomorphogenesis. However in the light COP1 activity is suppressed allowing these positive factors to accumulate and promote seedling photomorphogenesis. *Redrawn from* (Sullivan et al., 2003).

COP1 encodes a 76-kD protein with three recognizable domains, the RING finger, coiled-coil and WD40 domains (Deng et al., 1992). The COP1 dependent degradation requires the activity of at least three different protein complexes: a ~700-kD complex containing COP1 and SPA1 (Saijo et al., 2003); a 350-kD CDD complex containing COP10, an E2 ubiquitin conjugating enzyme variant, DAMAGED DNA-BINDING PROTEIN 1 (DDB1), and DET1 (Yanagawa et al., 2004); and the COP9 signalosome (CSN), a nuclear protein complex that activates cullin-containing multisubunit ubiquitin ligases (Cope and Deshaies, 2003; Wei and Deng, 2003). Except for COP1 all the other COP/DET/FUS loci encode polypeptides that are part of either the CDD complex or the eight-subunit COP9 signalosome, which is conserved in both plants and animals.

COP1 has been shown to mediate ubiquitin-dependent degradation of the transcription factors HY5, HYH, LAF1 and HFR1 (Osterlund et al., 2000a; Holm et al., 2002; Seo et al., 2003; Yang et al., 2005). Furthermore, COP1 was found to interact with several photoreceptors like phyA, CRY1 and CRY2 (Wang et al., 2001; Yang et al., 2001; Shalitin et al., 2002; Seo et al., 2004) and can target at least 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). These interactions suggest that COP1 acts as an E3 ubiquitin ligase and targets the positive regulators of photomorphogenesis for degradation in the dark (Figure 5). However, in the light COP1 activity is suppressed allowing these positive factors to accumulate and promote seedling photomorphogenesis.

Discussion of results

COP1 and HY5 are two major regulators of light signaling in plants. Proteins interacting with either could therefore be important regulators of light-dependent development. Previous yeast two-hybrid screens, using COP1 or HY5 as bait, had identified several putative regulators of light signaling (Holm et al., 2001). Analysis of the protein sequences of the identified candidates interacting with HY5, COP1 or both revealed that five of them (COL3, STO, STH1, STH2, STH3) contained tandemly repeated zinc-ligating B-box domains at their N-terminal end. I started my PhD studies with the aim of characterizing these B-box domain containing proteins and studying their role in light-regulated development. My doctoral work is mainly based on the characterization of three B-box containing proteins: COL3, STH2 and STH3. Results from these studies can be found in the papers attached to this thesis (Papers **I**, **II**, **III**). Here I discuss the major research findings from the three papers.

B-box containing proteins in *Arabidopsis*

B-boxes are zinc-ligating domains consisting of conserved cysteine and histidine residues. 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 (Figure 6A). The B-box domain was so called because it was first identified in animal proteins as a second Zn-binding domain in addition to a RING finger domain, which was originally termed an A-box. In *Arabidopsis*, there are 32 B-box containing proteins (Riechmann et al., 2000; Robson et al., 2001; Datta et al., 2008a) (Figure 6B). In contrast to animals, all *Arabidopsis* B-box containing proteins have at least one B-box with an aspartic acid as the fourth zinc-coordinating residue. The consensus sequence of this B-box is shown in Figure 6C. A large subgroup (the 17 COL proteins) of this family contains an additional CCT domain in the C-terminal part of the protein. Within this subgroup COL3 belongs to a subset of eight proteins that contain two tandemly repeated, juxtaposed B-boxes with high homology at their N-terminal part. It was recently reported that the CCT domain of the B-box containing protein CONSTANS (CO) was involved in the formation of a heterotrimeric DNA-binding complex (Wenkel et al., 2006). STH2 and STH3 together with six other proteins form another subgroup, with two tandem-repeated B-boxes spaced by 8-15 amino acids. Members of this subgroup have also been called double B-box zinc finger (DBB) genes (Kumagai et al., 2008). A third subgroup consists of five proteins, which contains only one B-box. Some other variants of B-boxes in *Arabidopsis* contain a glutamic acid or histidine residue instead of the aspartic acid as the fourth zinc-coordinating residue (Figure 6C). The fact that evolution has separated the B-box function from the RING and coiled-coil functions makes *Arabidopsis* an excellent model organism to study B-box function. Moreover, not much is known about the role of B-box proteins in light signaling.

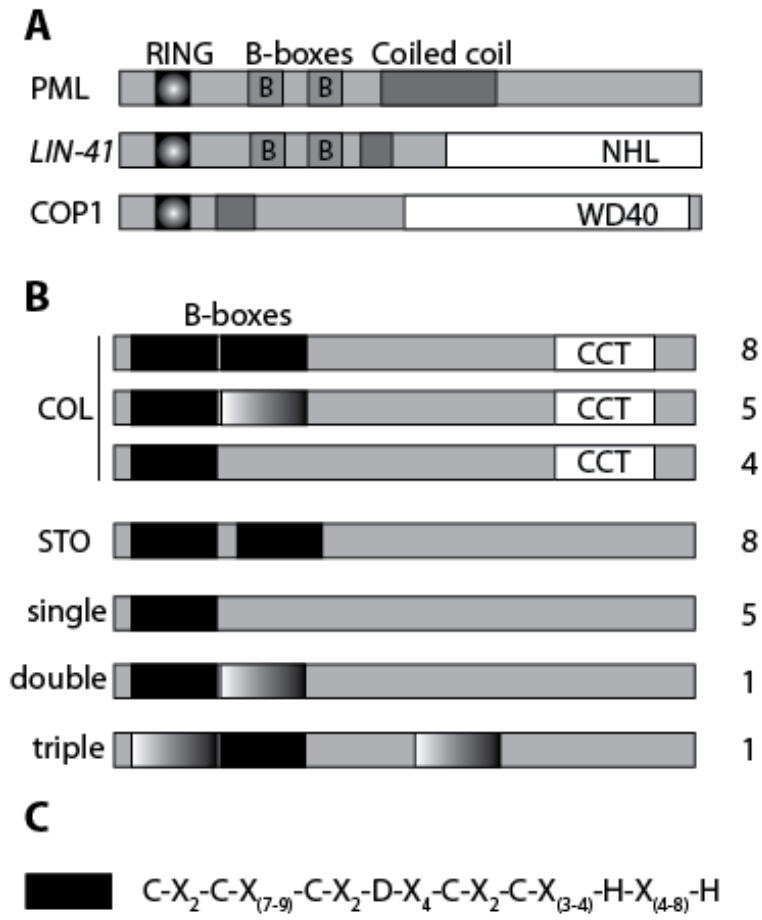


Figure 6. (A) Schematic representation of domains present in PML and LIN-41, two RBCC proteins found in animals, and the RING finger, coiled-coil domain containing COP1 protein. NHL and WD40 are protein interaction domains. (B) Classification and schematic representation of the 32 B-box containing proteins in *Arabidopsis*. Black boxes represent B-boxes present in all *Arabidopsis* proteins, whereas graded boxes indicate a variant of B-box containing glutamic acid or histidine as the fourth zinc-coordinating residue. CCT represents the CO, CO-like, TOC1 domain. Numbers indicate members in each class. (C) Consensus sequence of the B-box found in all *Arabidopsis* proteins containing an aspartic acid as the fourth zinc-coordinating residue.

B-box proteins interact with COP1 and/or HY5

I started by confirming the interactions with HY5 and COP1 in yeast and then checked if similar interactions were seen in plants as well. We found that in yeast COL3 interacted with COP1 while STH2 and STH3 interacted with HY5. Structurally COL3 differs from STH2 and STH3 in having an additional CCT domain, absence of spacer between the two B-boxes and a conserved six amino acid motif at the C-terminal end. Mapping studies showed that a VP (Valine Proline) pair found at the core of this six amino-acid motif in COL3 mediated interaction with the WD40 domain of COP1 (**I**). Previous studies with the B-box proteins STO and STH1 revealed a COP1-interacting motif consisting of a stretch of negative amino acids and a spacer of three amino acids followed by the motif V-P-E/D-Ø-G, where Ø designates a hydrophobic residue (Holm et al., 2001). Although the VP pair at the core of the COP1 interacting motif was found to be critical for the interaction with COP1, the cluster of negative residues in front of the motif also contributed to the interaction. Furthermore, it was recently shown that the B-box containing protein CO interacts with COP1 both *in vitro* and *in vivo* through the C-terminal part of CO and the authors suggested that VP pairs in that region of CO might be dispensable for the interaction with COP1 (Jang et al., 2008). These differences in the interaction specificities suggest a mechanism by which COP1 could presumably differentiate between various B-box containing proteins. In addition, these interactions might possibly bring the RING and coiled-coil domains in COP1 in close proximity to the B-boxes without interfering with the ability of these domains to interact with other proteins.

We found that a GFP-COL3 fusion protein co-localized with COP1 into nuclear speckles in darkness (**I**). This localization required the B-box domains in COL3, indicating a novel function of this domain. COL3 protein with the B-boxes deleted gave a uniform diffused fluorescence throughout the nucleus. However when co-expressed with COP1 the truncated COL3 protein could be recruited into nuclear speckles. Furthermore we found that COP1 could recruit STH2 and STH3 into nuclear speckles (**II**; **III**). COP1 has been previously found to form subnuclear speckles in the dark with HY5, HYH, LAF1, ABI5, HFR1 and phyA, most of which (with the exception of ABI5), are validated substrates for COP1-mediated ubiquitylation and are involved in light signaling (Osterlund et al., 2000a; Holm et al., 2002; Lopez-Molina et al., 2003; Seo et al., 2003; Seo et al., 2004; Yang et al., 2005). It is quite tempting to speculate that these nuclear speckles might be sites for COP1-mediated ubiquitylation and proteolysis. The sequence that targets COP1 to subnuclear speckles has been mapped to a region overlapping the coiled-coil domain (Stacey and von Arnim, 1999). Nevertheless, the WD40 domain, through which COP1 interacts with the majority of its substrates, also seems crucial for speckle formation. Deletion of the entire WD40 domain decreases subnuclear speckles formation, whereas several mutations at the WD40 domain also abolish the subnuclear speckles (Stacey and von Arnim, 1999). Importantly, *cop1* homozygous mutant alleles containing the same mutations show constitutive photomorphogenic phenotypes at the seedling stage and are adult lethal (McNellis et al., 1994). This suggests that these subnuclear speckles formed by COP1 and its substrates might be required for normal *Arabidopsis* development. Moreover it has been shown that the WD40 repeat domain is necessary for hypocotyl

elongation, and when combined with the core domain, it is sufficient (Stacey et al., 1999). Also, it has been reported that increasing fluence rates of red light concomitantly induce a change in the nuclear patterning of phyB and enhance inhibition of hypocotyl elongation rates (Chen et al., 2003). These results indicated that the formation of phyB nuclear speckles play a role in the regulation of phyB-mediated signal transduction, at least at higher fluence, and adds to the possibility of a physiological function of the nuclear speckles. At the moment we can only speculate about the functional importance of these subnuclear structures. A more definite understanding of the physiological significance of these speckles and what signals regulate their assembly and disassembly requires genetic studies (mutants specifically affecting nuclear speckle formation) and biochemical data (identification of components present in the nuclear speckles).

Our results indicated that COL3 could form nuclear speckles even in the light. However the speckles in light look different from those in the dark, being larger in size and less in number. Interestingly the speckles are strikingly similar to those of the late phyB speckles, suggesting the speckles might be the same. COL3 is a positive factor acting downstream of the photoreceptors and might very well be a target of photoreceptor-mediated regulation. It would be interesting to see if there is a relationship between the COL3 speckles and the phytochrome speckles by performing co-localization experiments for which we have already obtained lines harbouring 35S:YFP-PHYB and 35S:CFP-COL3. The interaction between COL3 and COP1 and the fact that COP1 was found to interact with several photoreceptors suggests the possibility of an indirect interaction mediated via COP1. Preliminary results in the laboratory suggest that COL3 interacts with a phytochrome interacting factor. A line of research for the future would be to perform a detailed *in planta* analysis of this interaction.

We isolated T-DNA insertion mutants in each of the three B-box encoding genes *COL3*, *STH2* and *STH3*. Phenotypic characterization of these mutants revealed pigmentation, hypocotyl and root phenotypes, suggesting that these genes have a positive role in light- and HY5-regulated processes. Moreover study of the double mutants with *hy5* and *cop1* confirmed that all of them genetically interact with both *HY5* and *COP1*. An interesting observation about the genetic interaction between the different B-box containing proteins and COP1 was the allele-specific interaction with the different *cop1* alleles. We had used three different alleles of the *COP1* for our studies. While the strong allele *cop1-1*, which has a deletion of 22 amino acids just in front of the WD domain, was used in the genetic studies with *col3*, the other two weak alleles *cop1-4* and *cop1-6* were used in all the three studies. *cop1-4* encodes a 33-kD truncated COPl protein containing only the 282 N-terminal amino acids without the WD40 domain (McNellis et al., 1994). On the other hand *cop1-6* is a temperature-sensitive allele that behaves like *cop1* mutant at 22°C but as wild-type at 30°C (Hsieh et al., 2000). The *cop1-6* mutation changes the splicing junction at the 3'-end of intron 4 that leads to the insertion of five novel amino acids (Cys-Leu-Val-Leu-Trp) between Glu-301 and Phe-302 of the wild-type protein (McNellis et al., 1994). The allele-specific interaction between these two partial loss-of-function weak alleles of *COP1* and genes encoding mutated versions of the different B-box proteins is consistent with a direct interaction between them. It would be interesting to fine-map these interactions to the amino acid level using leads from the molecular details of the

allele-specific interactions. Moreover all the genetic data suggest that the B-box proteins act downstream of COP1 and play antagonistic roles in light-regulated development.

Further evidence of interaction between the B-box proteins and COP1 came from *in vitro* ubiquitylation studies performed on STH3. With the help of our collaborator, Vicente Rubio, we were also able to show that the E3 ubiquitin ligase COP1 can ubiquitinate STH3 *in vitro* and possibly target it for proteolysis (**III**). Recently another B-box containing protein, CONSTANS, was found to act downstream of COP1 and physically interact with it (Liu et al., 2008). The fact that COP1 could ubiquitylate CONSTANS *in vitro* and reduce CO levels *in vivo* suggests the possibility of a common mechanism of regulation for this group of B-box proteins. Furthermore our genetic data showed that COL3, STH2 and STH3 could partially suppress COP1 providing strong evidence for interaction between the B-box proteins and COP1 *in vivo*.

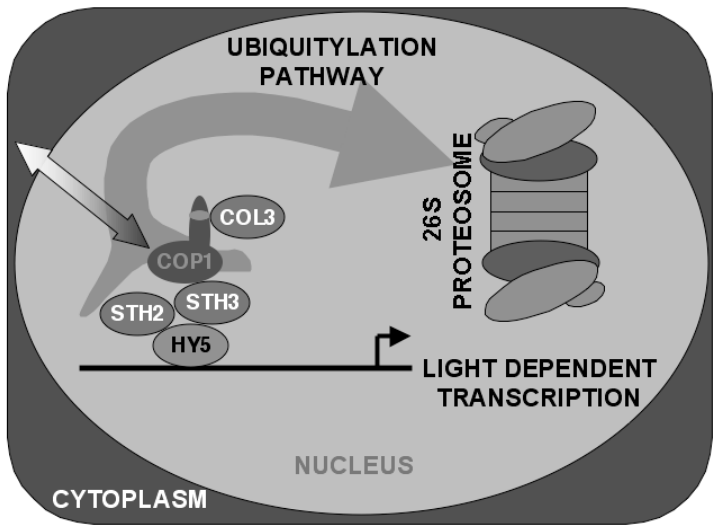


Figure 7. Schematic representation of a plant cell showing the interaction of the B-box proteins COL3, STH2 and STH3, with HY5 and COP1, regulating light-dependent development. While the B-boxes might act as cofactors for the transcription factor HY5 to regulate light-dependent transcription, interaction with COP1 in the dark might target them for proteolysis via the 26S proteosomal pathway thereby creating a balance in their levels inside the plant cell.

We found that the B-box domain in STH2 and STH3 and the bZIP domain of HY5 are important for the interaction between HY5 and the B-box proteins (**II**; **III**). STH3 was also identified as a HY5-regulated transcription factor by another group who named it as Light-regulated Zinc finger protein 1 (LZF1) (Chang et al., 2008). While the structural

disruption of each of the B-boxes in STH2 interfered with interaction the with HY5, in STH3, the intact structure of only the second B-box appeared to be critical for HY5 interaction. Furthermore, STH3 was also found to interact with another bZIP protein HYH, which is a close homolog of HY5. The specific interaction of STH3 with HYH indicates that differences within the B-box domains of closely related STH proteins are important for the specificity of B-box-bZIP interaction. It would be interesting to fine-map the interaction to amino acid residue resolution within the B-box and the bZIP domains. This could provide a handle to examine the putative mini-transcriptional network formed by the B-box and bZIP domain containing proteins.

All these results indicate that the B-box proteins interact both with HY5 and COP1 to positively regulate light-dependent development (Figure 7).

B-box proteins play a role in light-regulated transcription and development

The interaction between the B-box proteins and HY5 suggested that the two proteins might functionally act together. To address the functional relationship between STH2, STH3 and HY5 we examined the activity of STH2 and STH3 in a transient transfection assay in protoplasts using a LUC reporter driven by the *CHI*/*CAB* promoter. We found that the B-box proteins STH2 and STH3 could activate transcription and showed that the B-boxes and a functional G-box element (which is also the HY5 binding site) in the promoter are required for the transcriptional activity.

Light induces massive re-programming of the plant transcriptome, and many of the early light-responsive genes are transcription factors (Ma et al., 2001). HY5 is a high hierarchical regulator of the transcriptional cascades for photomorphogenesis and acts downstream to the photoreceptors (Lee et al., 2007). It is constitutively nuclear, binds to the promoters of light-inducible genes and regulates their expression during photomorphogenesis. The fact that HY5 is constitutively bound to the promoters of a set of genes related to photosynthesis and circadian regulation, such as RbcS1A, CHS, CCA1 and TOC1, irrespective of the light-dark transition or the daily rhythm, suggests that HY5 binding is not sufficient for transcriptional activation and might require some additional cofactors for regulation. Results from our work suggest that STH2, STH3 and possibly other B-box containing proteins could be the additional factors HY5 requires for transcriptional regulation.

Furthermore, the interaction between STH3 and HYH suggests that STH3 might act as a cofactor for other G-box binding proteins such as HYH, regulating HY5 independent processes. The hypocotyl and root phenotypes of the various mutants studied suggest that different combinations of bZIP transcription factors and B-box containing cofactors activate transcription at different levels on different promoters controlling organ-specific light-dependent development. The B-box proteins thus provide an additional layer of complexity in light-regulated transcription.

Our results indicated that the activity of the B-box proteins is dependent on the promoter context. While STH2 activated the CHI-Luc reporter stronger than STH3, the reverse was

true for the CAB-Luc reporter. This suggests that different B-box proteins like STH3 and STH2 regulate distinct sets of target genes. Interestingly STH3 and STH2 together showed an enhanced ability to activate transcription suggesting synergistic regulation of light-dependent promoters. While mutating the G-box in the CAB or CHI promoters resulted in almost complete loss of activation by STH3 or STH2 alone, significant activation could be detected when the two B-box proteins were present together (**III**). A possible explanation for this could be that transcriptional activation is also mediated through sites other than the G-box when STH3 and STH2 are expressed together. Recent results in the laboratory indicate the possibility of the presence of plausible alternate binding targets in the CHI and CAB promoters, which would be a future direction of research in the group.

The mode of action of these transcriptional cofactors could be achieved through stabilizing HY5 containing complexes on promoters, providing activating or repressive surfaces to these transcriptional complexes or providing accessibility to the E3 ubiquitin ligase COP1 to interact with members of the complex. Further studies need to be performed in order to reveal a possible mechanism of action for the B-box containing transcriptional cofactors. Furthermore, in a recent study it was reported that the transcription of five DBB (Double B-box) genes of the STO subfamily were under the control of circadian rhythm reciprocating the fact that the B-box containing proteins perform manifold functions in plants (Kumagai et al., 2008).

Significance of the study of *Arabidopsis* B-box proteins from an evolutionary perspective

It was recently shown that hDET1 and hCOP1 act together to regulate c-Jun (Wertz et al., 2004) and that hCOP1 is a critical negative regulator of p53 where it represents a novel pathway for maintaining p53 at low levels in unstressed cells (Dornan et al., 2004). Thus the conserved COP/DET/FUS pathway appears to play important regulatory roles both in plants and humans. As a matter of fact this regulatory system and its biochemical function was first discovered in *Arabidopsis*. However, the pleiotropic nature of the mutant phenotypes in plants suggests that the full function of the regulatory system remains to be discovered. The identification and characterization of B-box proteins interacting with these regulators offer a handle to further analyze this critical pathway. The fact that the B-box domain of the tumor suppressor protein PML (Promyelocytic Leukemia) is critical for localization to sub-nuclear speckles, similar to *Arabidopsis* COL3, suggests functional conservation of this domain across organisms.

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 like apoptosis, cell cycle regulation and viral response. Recently a number of TRIM/RBCC proteins have been found to play a role in ubiquitylation and the B-boxes proposed to participate in substrate recognition. Other functions of this domain involve localization into nuclear bodies as in the tumor suppressor protein PML, transcriptional regulation and protein-protein interaction (Borden et al., 1996; Beenders et al., 2007).

While RBCC proteins are absent in plants it is interesting that COP1 was found to interact with at least six different B-box containing proteins, namely COL3, CO, STO, STH1, STH2 and STH3. It was recently shown that the coiled-coil domain containing SPA proteins were important for the stability of the B-box containing protein CO (Laubinger et al., 2006). All these interactions between B-box containing proteins and the RING, coiled-coil domain containing COP1-SPA proteins suggest a mechanism of creating a functional equivalent of RBCC protein in an organism that lacks such proteins. Whether these interacting B-box proteins together with COP1 play a role in ubiquitylation and proteolysis or act as a substrate for COP1-mediated degradation, as in the case of STH3, requires more in-depth studies. Elucidation of these biochemical complexes might help unravel the functional intricacies of manifold cellular processes regulated by B-box containing proteins.

Conclusion

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 the B-box proteins interacting with both of these key regulators are candidates to fill the gaps in the regulatory network. Understanding the operation of this complex transcriptional network will allow us to fine-tune the light signaling pathway and modulate plant development leading to increased productivity and yield.

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Arabidopsis CONSTANS-LIKE3 Is a Positive Regulator of Red Light Signaling and Root Growth[W]

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CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1) is an E3 ubiquitin ligase that represses photomorphogenesis in the dark. Therefore, proteins interacting with COP1 could be important regulators of light-dependent development. Here, we identify CONSTANS-LIKE3 (COL3) as a novel interaction partner of COP1. A green fluorescent protein–COL3 fusion protein colocalizes with COP1 to nuclear speckles when transiently expressed in plant cells. This localization requires the B-box domains in COL3, indicating a novel function of this domain. A loss-of-function *col3* mutant has longer hypocotyls in red light and in short days. Unlike *constans*, the *col3* mutant flowers early and shows a reduced number of lateral branches in short days. The mutant also exhibits reduced formation of lateral roots. The *col3* mutation partially suppresses the *cop1* and *deetiolated1* (*det1*) mutations in the dark, suggesting that *COL3* acts downstream of both of these repressors. However, the *col3* mutation exerts opposing effects on *cop1* and *det1* in terms of lateral roots and anthocyanin accumulation, suggesting that *COL3* also has activities that are independent of *COP1* and *DET1*. In conclusion, we have identified COL3 as a positive regulator of photomorphogenesis that acts downstream of COP1 but can promote lateral root development independently of COP1 and also function as a daylength-sensitive regulator of shoot branching.

INTRODUCTION

The perception of light participates in the gating of key developmental transitions throughout the life cycle of the plant, such as germination of the seed, photomorphogenesis or deetiolation of the seedling, and flowering. Deetiolation is arguably the most dramatic of these light-dependent transitions. Exposure of an etiolated seedling to light results in the inhibition of hypocotyl elongation, the promotion of cotyledon expansion, and the synthesis of a number of pigments, including chlorophyll and anthocyanin, and entails a dramatic transcriptional reprogramming (Ma et al., 2001).

Arabidopsis thaliana has three major classes of photoreceptors: red/far-red-light–responding phytochromes, blue light/UV-A light–responding cryptochromes, and phototropins. The cytoplasmic phototropins primarily regulate processes optimizing photosynthesis, whereas the transcriptional and developmental changes are attributed to the phytochromes and the cryptochromes. The phytochromes, encoded by the five genes *PHYA* to *PHYE*, are cytoplasmic in the dark but translocate into the nucleus in the light (Kircher et al., 2002). The active far-red-light–absorbing form of phyB was found to interact with a DNA-bound transcription factor, suggesting a rather direct signal transduction in which the photoreceptor could act in the promoter context (Martinez-Garcia et al., 2000). The two cryptochromes cry1 and cry2 are nuclear in darkness; both are phosphorylated in response to light, whereby cry1 becomes enriched in the cytoplasm and the light-labile cry2 is degraded. Cryptochromes interact genetically with multiple phytochromes (Neff et al., 2000). phyB and cry2 have been shown to tightly colocalize in vivo (Mas et al., 2000), suggesting that the different photoreceptors might act together to initiate similar developmental pathways. This is consistent with the large overlap in transcription profiles seen in microarray studies of seedlings grown in different monochromatic lights (Ma et al., 2001). Microarray studies performed in far-red light suggest that light initiates a transcriptional cascade in which a large fraction of the early affected genes are transcription factors (Tepperman et al., 2001).

In the absence of light, the seedlings become etiolated, a developmentally arrested growth mode characterized by limited root growth, an elongated hypocotyl, closed undifferentiated cotyledons, and an apical hook. The developmental arrest seen during etiolated growth is mediated by the COP/DET/FUS proteins, which act as repressors of the default photomorphogenic pathway. Mutations in any of these 10 genes result in deetiolated growth in darkness. Dark-grown *cop/det/fus* alleles have genome expression profiles closely resembling those of light-grown seedlings (Ma et al., 2003). Recent results have shown that COP/DET/FUS repression involves protein degradation. CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1), an E3 ubiquitin ligase, mediates ubiquitin-dependent degradation of the transcription factors HY5, HYH, LAF1, and HFR1 as well as the phyA photoreceptor (Osterlund et al., 2000a; Holm et al., 2002; Seo et al., 2003, 2004; Duek et al., 2004; Jang et al., 2005; Yang

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et al., 2005). The COP1-dependent degradation requires the activity of at least three different protein complexes: an ~700-kD complex containing COP1 and SPA1 (Saijo et al., 2003); a 350-kD complex containing COP10, an E2 ubiquitin-conjugating enzyme variant, and DEETIOLATED1 (DET1) (Yanagawa et al., 2004); and the COP9 signalosome, a nuclear protein complex that activates cullin-containing multisubunit ubiquitin ligases (Cope and Deshaies, 2003; Wei and Deng, 2003).

The failure of plants with mutations in the *COP/DET/FUS* genes to arrest development during etiolated growth suggests that the targets of this pathway are likely to be key regulators of photomorphogenic development. To date, several photoreceptors as well as transcription factors have been shown to interact with COP1. phyB, cry1, and cry2 were found to interact with COP1 (Wang et al., 2001; Yang et al., 2001), and cry2 accumulates in *cop1-4* and *cop1-6* mutants (Shalitin et al., 2002), suggesting that COP1 might regulate cry2 abundance. Furthermore, COP1 was recently shown to interact with and ubiquitinate phyA (Seo et al., 2004). These interactions suggest that COP1 could mediate the desensitization and/or termination of signaling through the photoreceptors in the light.

By contrast, the photomorphogenic development seen in *cop/det/fus* mutants in the dark could not be mediated by photoreceptors because they are activated by light. Furthermore, genetic analysis revealed that *cop1* is epistatic to mutations disrupting phytochrome and cry1 function in darkness (Ang and Deng, 1994). The photomorphogenic development in dark-grown *cop/det/fus* seedlings, therefore, is likely caused by the loss of COP/DET/FUS repression of factors acting downstream of the photoreceptors. Four of the previously identified COP1-interacting proteins are transcription factors, and all four are positive regulators of light signaling. HY5 acts as a positive regulator in far-red, red, blue, and UV-B light conditions, HFR1 is a positive regulator in far-red and blue light, whereas LAF1 and HYH promote light-dependent development in far-red and blue light, respectively (Osterlund et al., 2000b; Ballesteros et al., 2001; Holm et al., 2002; Duek and Fankhauser, 2003; Ulm et al., 2004). Despite significant recent progress, only a few downstream regulators of light signaling have been identified, and the functional relationship between them is not well understood.

To date, mutations in only two genes have been found to suppress the phenotypes conferred by both *cop1* and *det1*. One of these is *HY5* (Ang and Deng, 1994; Pepper and Chory, 1997), the first identified target of the COP/DET/FUS pathway. The other gene, *TED3*, encodes a peroxisomal protein, and analysis of the dominant *ted3* mutation revealed that enhanced peroxisomal function partially suppresses weak *cop1* and *det1* alleles (Hu et al., 2002).

Here, we identify COL3 (for CONSTANS-LIKE3) as a COP1-interacting protein. Characterization of a *col3* mutant indicates that *COL3* positively regulates the light-dependent development and formation of lateral roots. Furthermore, *COL3* inhibits shoot elongation and promotes branching of the shoot specifically in short-day conditions. Finally, *col3* can suppress the deetiolated phenotype conferred by both *cop1* and *det1* alleles, and we characterize genetic interactions between *col3* and *hy5*, *cop1*, and *det1*.

RESULTS

COL3 Interacts with COP1 in Yeast Two-Hybrid Assays

COP1 was used as bait in a yeast two-hybrid screen in an effort to identify novel light-signaling components (Holm et al., 2001, 2002). In addition to the previously reported HYH, STH, and STO proteins, the screen identified three cDNAs encoded by the *COL3* gene, At2g24790 (Arabidopsis Genome Initiative, 2000). COL3 is one of the five CONSTANS (CO)-like proteins most closely related to CO (Robson et al., 2001). COL3, like CO, has two N-terminal tandemly repeated B-box domains, a CCT domain in the C-terminal half of the protein and a conserved motif in the C terminus (Figure 1A). The B-boxes show 59% amino acid identity (41 of 85), and the CCT domain shows 91% amino acid identity (39 of 43), between COL3 and CO, respectively. The Zn^{2+}-ligating B-box has been proposed to be a protein interaction domain, but it does not appear to be required for the interaction with COP1 in yeast because all three cDNAs identified in the screen encode a truncated COL3 protein lacking the 75 N-terminal amino acids (Figure 1A).

The COP1 protein used as bait contains three protein-interacting domains: a RING finger, a coiled-coil domain, and a WD40 repeat domain. To further examine the interaction between COP1 and COL3, we used Gal4 DNA binding domain fusions of COP1 proteins identified in three *cop1* alleles, *cop1-4*, *cop1-8*, and *cop1-9*. COP1-4 lacks the WD40 domain, whereas COP1-8 and COP1-9 contain a deletion and an amino acid substitution in the WD40 domain, respectively (Figure 1B) (McNellis et al., 1994). We found that COL3 is unable to interact with either of the COP1 proteins containing deletions or mutations in the WD40 domain, suggesting that the WD40 domain in COP1 is required for the interaction with COL3 (Figure 1C). Previous studies have identified Val–Pro (VP) pairs in the HY5, HYH, STH, and STO proteins that are critical for their interaction with COP1 (Holm et al., 2001, 2002). COL3 contains five VP pairs, and we substituted three of these with Ala (VP91AA, VP204AA, and VP291AA) to examine whether they were involved in the interaction with COP1. The COL3 proteins were all expressed at similar levels in yeast (Figure 1D). As shown in Figure 1C, both the VP91AA and VP204AA COL3 proteins interact with COP1, but the VP291AA substitution renders COL3 unable to interact with COP1, suggesting that, as in the B-box–containing proteins STH and STO, a VP pair in the C terminus is required for the interaction with COP1.

The COL3 Protein Colocalizes with COP1 When Transiently Expressed in Plant Cells

The COP1 protein localizes to nuclear speckles in the dark, and the nuclear abundance of COP1 decreases in the light (von Arnim and Deng, 1994). Furthermore, COP1 has been found to colocalize with several interaction partners, such as HY5, LAF1, HYH, HFR, and the CCT domain of CRY1 (CCT1), in nuclear speckles when expressed in onion (*Allium cepa*) epidermal cells (Ang et al., 1998; Wang et al., 2001; Holm et al., 2002; Seo et al., 2003). Both LAF1 and HFR localize to nuclear speckles when expressed

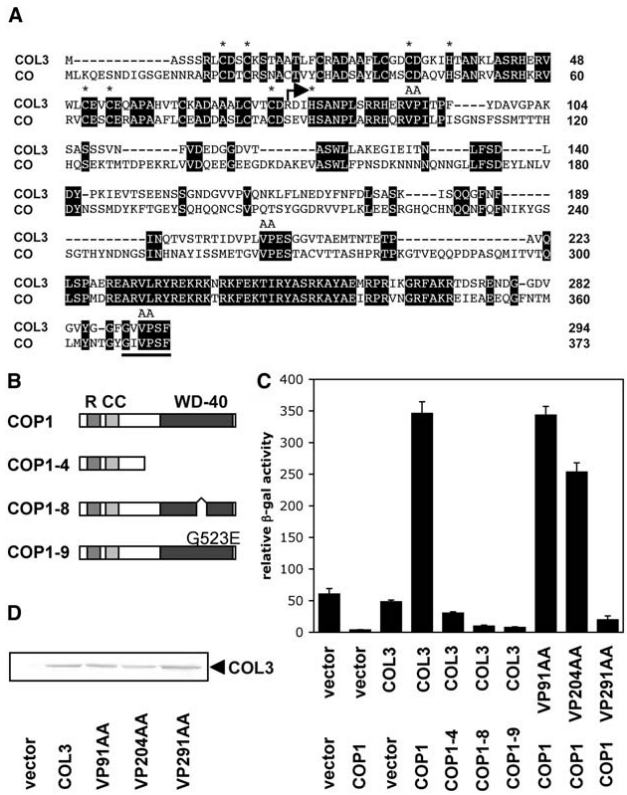

Figure 1. COL3 Interacts with COP1 in Yeast, and a VP Pair in the C Terminus Is Critical for the Interaction.

(A) Alignment of COL3 and CO. Identical amino acids are shaded in black. The arrow indicates the 5′ end of the cDNA identified in the screen, the asterisk indicates the Cys and His residues binding Zn^{2+}, and the underlined amino acids represent a motif conserved among the CO and COL1 to COL5 proteins.

(B) Scheme of the domain structure of COP1 and the mutated COP1 proteins. R, CC, and WD-40 indicate the RING finger, coiled-coil, and WD40 repeat domains present in COP1.

(C) Yeast two-hybrid interactions between the indicated COP1 and COL3 proteins. The Gal4 DNA binding domain–fused COP1 proteins are all expressed at similar levels in yeast (Holm et al., 2002). VP-n-AA indicates the Ala substitution of the three VP pairs at positions 91, 204, and 291, as indicated in **(A)**. Error bars indicate SE ($n = 5$).

(D) Immunoblot showing the similar expression levels of COL3 and substituted COL3 proteins in yeast.

in onion cells, but HY5, HYH, and CCT1 give a diffuse nuclear fluorescence when expressed alone and require coexpression of COP1 for speckle localization. We made two green fluorescent protein (GFP)–COL3 fusion constructs to examine the subcellular localization of COL3: GFP-COL3, containing the entire coding sequence of COL3; and GFP-COL3ΔB, lacking amino acids 1 to 75 encoding the B-box domains (Figure 2A). The GFP-COL3 protein is exclusively nuclear when expressed in onion cells, and it localizes to nuclear speckles both in the dark and in the light (Figure 2B). The speckles were consistently smaller and more numerous in cells incubated in the dark compared with cells incubated in the light (Figure 2B). By contrast, no speckles were observed for GFP-COL3ΔB. The truncated COL3ΔB protein was predominantly nuclear and gave a diffuse nuclear fluorescence both in the dark and in the light (Figure 2C).

Thus, the N-terminal B-boxes in COL3 are required for COL3 to localize to speckles, whereas the C terminus is required for interaction with COP1 in yeast. GFP fusions of HY5, HYH, and CCT1 give diffuse nuclear fluorescence when expressed alone but localize to speckles when coexpressed with COP1. To test whether overexpression of COP1 also can localize GFP-COL3ΔB to speckles, we coexpressed the two proteins and

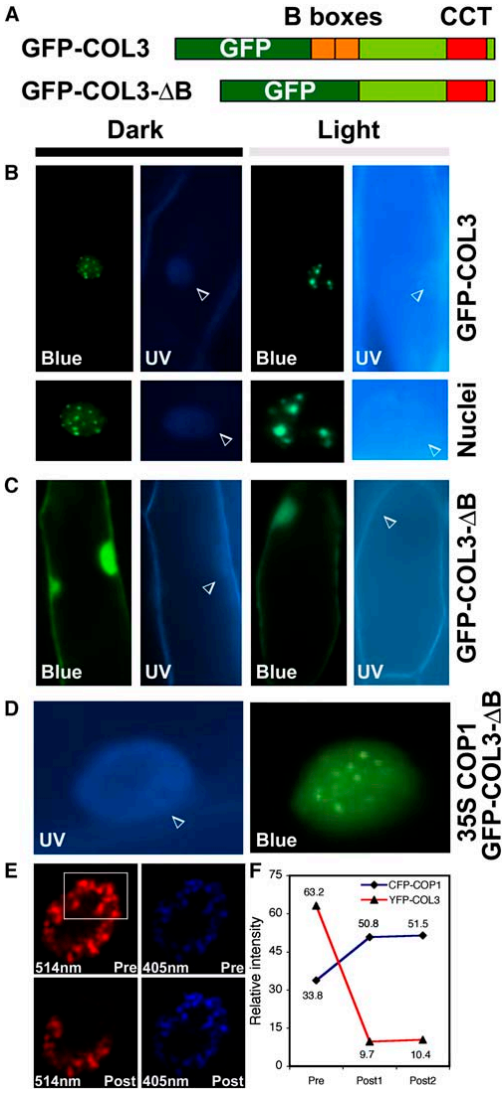

Figure 2. COL3 Localizes to Nuclear Speckles and Colocalizes with COP1 in Onion Cells.

(A) Schemes of the GFP-COL3 and GFP-COL3ΔB constructs.

(B) Onion epidermal cells expressing GFP-COL3 incubated in the dark and light and excited with blue light to induce GFP fluorescence and with UV light to show the positions of nuclei in 4′,6-diamidino-2-phenylindole–stained cells. Enlarged images of the nuclei, showing the size and number of speckles, are shown below. White arrowheads indicate the positions of the nuclei.

found that in these cells GFP-COL3ΔB localized to speckles (Figure 2D).

Because both full-length COL3 and COP1 localize to speckles, we set out to examine whether the two proteins are found in the same subnuclear structures using the fluorescence resonance energy transfer (FRET) technique. To this end, we coexpressed cyan fluorescent protein (CFP)–fused COP1 with yellow fluorescent protein (YFP)–fused COL3 and analyzed FRET by acceptor photobleaching using a confocal microscope. As shown in the top panels of Figure 2E, a nucleus coexpressing YFP-COL3 and CFP-COP1 excited with 514- and 405-nm lasers resulted in the emission of YFP and CFP, respectively, before the 514-nm bleach of the region of interest. After the bleach, emission from YFP-COL3 in the region of interest was reduced dramatically, whereas we saw a clear increase in the emission of CFP-COP1 in the region of interest (Figure 2E, bottom panels), indicating that FRET had occurred. The relative intensities of emissions from CFP-COP1 and YFP-COL3 in the region of interest, before and after bleach, are shown in Figure 2F.

Identification of a T-DNA Insertion Mutation in the *COL3* Locus

To further characterize the role of *COL3* in plants, we screened the *Arabidopsis* knockout collection at Madison, Wisconsin, for T-DNA insertions in the *COL3* gene (Sussman et al., 2000). The collection was screened with primers annealing to sequences 5′ and 3′ of *COL3*, and we identified a T-DNA insertion within the gene (Figure 3A). The same T-DNA insertion was identified with both 5′ and 3′ primers, suggesting that the insertion consists of at least two T-DNAs inserted in a head-to-head orientation. Sequencing of the flanking regions revealed that the T-DNA was inserted in the first exon at nucleotide position 455 from the translational start site. The T-DNA results in the insertion of codons for the amino acids KSTCPAE followed by a stop codon after Glu-151 in COL3.

RNA gel blot hybridization revealed that a truncated mRNA is expressed at wild-type levels in the *col3* mutant (Figure 3B). The truncated mRNA in *col3* was amplified with RT-PCR, and sequencing confirmed that an mRNA fusion between *COL3* and the T-DNA was transcribed (data not shown).

(C) GFP expression of GFP-COL3ΔB (COL3 with the B-box deleted), showing diffused fluorescence in the nuclei under the same conditions as in **(B)**.

(D) Nucleus of a cell coexpressing 35S:COP1 (untagged) and GFP-COL3ΔB, excited with UV and blue light.

(E) and **(F)** FRET between CFP-COP1 and YFP-COL3 analyzed by acceptor bleaching in nuclei ($n = 10$). The top panels in **(E)** show a representative prebleach nucleus coexpressing YFP-COL3 and CFP-COP1 excited with either a 514- or a 405-nm laser, resulting in emission from YFP (red) or CFP (blue), respectively. The white square indicates the region of interest that will be bleached with the 514-nm laser. The bottom panels in **(E)** show the same nucleus after bleaching excited with a 514- or a 405-nm laser. The relative intensities of both YFP and CFP in the region of interest were measured once before and twice after the bleaching, as indicated in **(F)**. An increase in donor fluorescence (blue) is seen only if a protein–protein interaction occurs.

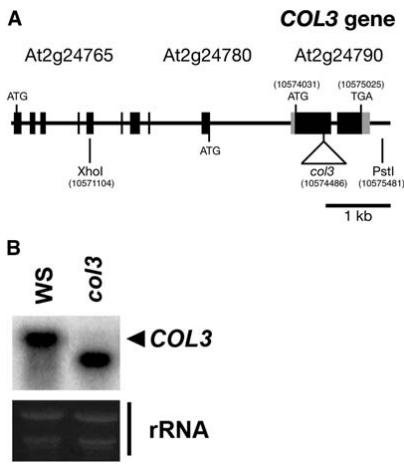

Figure 3. Identification of a T-DNA Insertion in the *COL3* Gene.

(A) Scheme of the *Arabidopsis COL3* gene (At2g24790). The T-DNA insert in *col3* and the *Xho*I and *Pst*I restriction sites used to create the pFP100-*COL3* complementation vector are indicated at positions 10,574,486, 10,571,104, and 10,575,481, respectively, on chromosome II.
(B) RNA gel blot showing *col3* transcript accumulation in wild-type Wassilewskija (Ws) and *col3* seedlings 6 d after germination in continuous white light. rRNA bands are shown to serve as a loading control.

The T-DNA line was backcrossed into the wild type (Ws) and crossed into *hy5-ks50*, *cop1-1*, *cop1-4*, *cop1-6*, and *det1-1* alleles. Analyses of these crosses revealed a single T-DNA locus cosegregating with the phenotype conferred by *col3*. To confirm that any observed phenotypes were indeed caused by disruption of the *COL3* gene, we introduced a 4377-bp genomic construct containing the *COL3* gene and 2927-bp 5′ and 456-bp 3′ sequences into the *col3* mutant as well as into each of the *col3* double mutants. For these genomic complementation experiments, we used the pFP100 vector, which allowed analysis in the T1 generation (Bensmihen et al., 2004).

COL3 Is a Positive Regulator of Light Signaling

To examine whether *COL3* is involved in light responses, *col3* seedlings were germinated in different fluences of blue, red, and far-red light. The *col3* seedlings did not differ significantly from wild-type seedlings in blue or far-red light (see Supplemental Figure 1 online) but had longer hypocotyls in high-fluence red light (Figures 4A and 4B). The finding that *col3* is specifically hyposensitive to high-fluence red light suggests that *COL3* acts as a positive regulator of the phytochrome-mediated inhibition of hypocotyl elongation. T1 transgenic *col3* seedlings transformed with pFP100-*COL3* (*col3COL3*) displayed hypocotyl lengths similar to wild-type plants (Figure 4B), indicating that a functional *COL3* gene could complement the phenotype conferred by *col3* in red light. Analysis of a segregating *col3* population revealed that the *col3* mutation is recessive. The complementation experiments and the recessive nature of the *col3* mutation are consistent with *col3* being a loss-of-function mutation.

We then examined *col3* seedlings grown in white light under different daylength conditions. We found no significant difference between wild-type and *col3* seedlings in constant light or under long-day conditions (16 h of light/8 h of dark) (see Supplemental Figure 2 online), but *col3* showed reduced inhibition of hypocotyl elongation in short-day conditions (8 h of light/16 h of dark) (Figure 4C). Also, this phenotype was complemented in T1 transgenic *col3* seedlings transformed with pFP100-*COL3* (Figures 4C and 4D).

The *hy5* mutation resulted in reduced inhibition of hypocotyl elongation in all light conditions. We generated a *col3 hy5* double mutant and examined the hypocotyl length in different light conditions. In all conditions tested, *col3 hy5* behaved like the *hy5* mutation (Figures 4B and 4C; see Supplemental Figure 2 online).

col3 Plants Flower Early in Both Long and Short Days

CO, the founding member of the CO-like family, was identified as a factor promoting flowering in long days (Putterill et al., 1995). To examine whether *COL3* affects flowering time, we compared *col3* with *co-2* grown in short days (8 h of light/16 h of dark) and long days (16 h of light/8 h of dark), respectively. We found that *col3* plants flower earlier than wild-type plants in both short and long days (Figure 5). The early flowering seen in long-day-grown *col3* plants is opposite that seen in *co* (Figure 5B) but similar to the early flowering seen in mutations in the genes encoding two COP1-interacting proteins, *hy5* and *hyh* (Holm et al., 2002), whereas neither *laf1* nor *hfr1* affects flowering time (Fankhauser and Chory, 2000; Ballesteros et al., 2001).

COL3 Regulates Lateral Organ Formation

When grown in short-day conditions, *col3* plants were taller and their primary shoots had fewer lateral branches than those of wild-type plants (Figures 6A and 6B). Neither the wild type nor *col3* produced secondary shoots under our short-day growth conditions. The elongated shoot and reduced branching phenotypes were observed only in short-day conditions: no significant difference in either height or branching was seen between *col3* and the wild type under long-day conditions (see Supplemental Figure 3 online). These results suggest that *COL3* promotes the formation of branches and inhibits the growth of the primary shoot specifically during short days.

The observation that the *col3* mutation affects the growth of the shoot prompted us to examine whether *col3* has any effect on root growth. To this end, we germinated *col3* and wild-type seeds on vertical plates in constant white light and measured the growth of the primary root. As shown in Figure 6C, *col3* seedlings had shorter primary roots than wild-type seedlings. The difference in root length was most pronounced at day 7 after germination and decreased at later time points. Interestingly, we found that *col3* seedlings produced fewer lateral roots than wild-type seedlings (Figure 6D). T1 *col3* seedlings transformed with pFP100-*COL3* displayed wild-type primary root length and number of lateral roots (Figures 6C and 6D), indicating that the

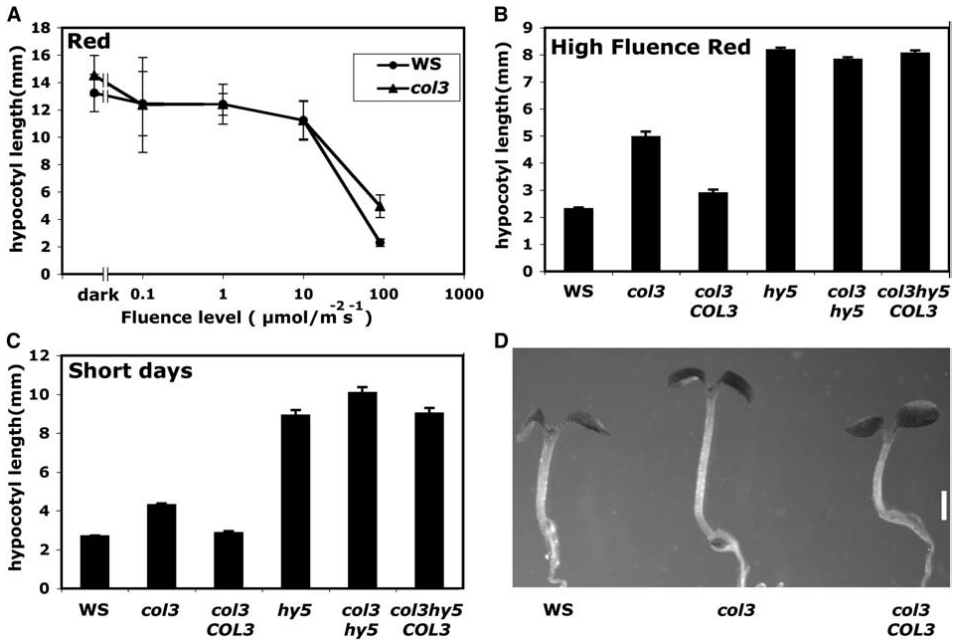

Figure 4. *col3* Seedlings Have Longer Hypocotyls When Grown under High-Fluence Red Light or Short Days.

(A) Fluence response curve of wild-type (Ws) and *col3* seedlings grown under continuous monochromatic red light. The experiment was performed twice with similar results. The graph depicts one of these experiments. Error bars represent SE ($n = 30$).

(B) Bar graph showing the difference in hypocotyl length between Ws, *col3*, *col3COL3*, *hy5*, *col3 hy5*, and *col3 hy5COL3* seedlings grown under high-fluence red light (90 μmol·m^{-2}·s^{-1}). *col3COL3* and *col3 hy5COL3* represent T1 *col3* and *col3 hy5* seedlings transformed with pFP100-*COL3*. Error bars represent SE ($n = 18$).

(C) Hypocotyl lengths of the indicated seedlings grown on plates under short-day conditions. Error bars represent SE ($n = 18$).

(D) Representative Ws, *col3*, and *col3COL3* seedlings grown on plates under short-day conditions.

COL3 gene complemented both phenotypes. Because the reduction of lateral branches in the shoot was seen in short-day conditions only, we examined lateral root formation in both short and long days, but similar results were obtained in all three light conditions, indicating that the lateral root phenotype, unlike the branching phenotype, is independent of daylength.

Both COP1 and the COP1-regulated transcription factor HY5 affect lateral root formation. The *cop1* mutation reduces the number of lateral roots, whereas the *hy5* mutation enhances both the initiation and elongation of lateral roots (Oyama et al., 1997; Ang et al., 1998). In addition to the lateral root phenotype, *hy5* seedlings show altered gravitropic and touching responses, enhanced cell elongation in root hairs, and reduced greening and secondary thickening of the root. We examined whether *col3* affects any of these processes, but we found no difference in gravitropic responses, greening, secondary thickening, or root hair elongation between *col3* and the wild type (data not shown).

To examine the genetic relationship between *col3* and *hy5* on lateral root formation, *col3 hy5-ks50* double mutant seedlings were analyzed. The double mutants were indistinguishable from *hy5* (Figure 6D), suggesting that *hy5* is epistatic to *col3* with respect to lateral root formation.

col3 Acts as a Suppressor of Both *cop1* and *det1*

We generated double mutants between *col3* and the *cop1* alleles *cop1-1*, *cop1-4*, and *cop1-6* as well as with *det1-1* to examine the genetic relationships between these genes. *col3* seedlings germinated in the dark were indistinguishable from wild-type plants (Figures 7A and 7B). However, when the *col3 cop1-1*, *col3 cop1-4*, *col3 cop1-6*, and *col3 det1-1* double mutants were germinated in the dark, we found that the double mutants had longer hypocotyls than either the *cop1* or *det1* single mutant, indicating that *col3* can partially suppress the hypocotyl phenotype of *cop1* and *det1* in the dark (Figures 7A and 7B). T1 *col3 cop1-1*, *col3 cop1-4*, *col3 cop1-6*, and *col3 det1-1* seedlings transformed with pFP100-*COL3* displayed hypocotyl lengths similar to those of the *cop1* and *det1* single mutants (Figure 7B),

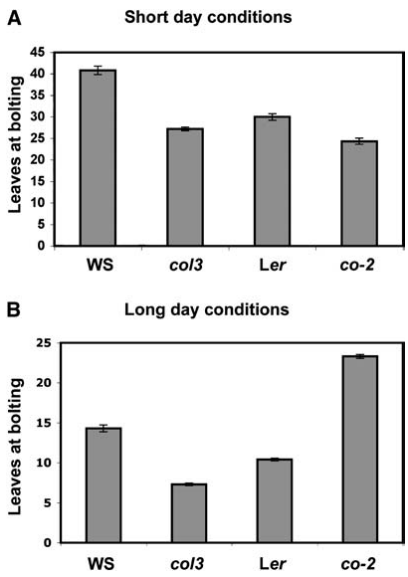

Figure 5. Unlike *co*, *col3* Flowers Early in Both Long and Short Days.

Graphs showing the number of rosette leaves at bolting for wild-type Ws, *col3*, wild-type Landsberg *erecta* (*Ler*), and *co* plants grown under short-day conditions (8 h of light and 16 h of dark) **(A)** and long-day conditions (16 h of light and 8 h of dark) **(B)**. Error bars represent SE ($n = 15$).

indicating that a functional *COL3* gene could reverse the *col3*-dependant suppression.

Deetiolated *cop1* seedlings that have been germinated in darkness are sensitive to high-fluence light, and most of them are unable to green and will die upon transfer to white light (Ang and Deng, 1994). This COP1-dependent block-of-greening phenotype follows an allelic series and becomes more pronounced the longer the seedlings have been grown in the dark. We found that a higher percentage of *col3 cop1-4* (85%) and *col3 cop1-6* (57%) seedlings were able to green when germinated in the dark for 6 d and then transferred to light for 6 d compared with the *cop1* single mutant (19 and 19%, respectively) (Figure 7C). The difference was smaller between *cop1-1* and *col3 cop1-1* (54% compared with 75%), suggesting that *col3* acts as an allele-specific suppressor of this *cop1* phenotype (Figure 7C). We found a reduced competence to green also in *det1-1* (33% were able to green), the *col3 det1-1* double mutant displayed slightly improved greening (50%), and the *col3* suppression of *det1-1* was similar in magnitude to the suppression of *cop1-1*.

In conclusion, the *col3* mutation, like the *hy5* mutation, can suppress the hypocotyl phenotypes of both *cop1* and *det1* in the dark. Furthermore, similar to *hy5* and *hyh*, *col3* acts as an allele-specific suppressor of the COP1-dependent block-of-greening phenotype.

col3 Exerts Opposing Effects on *cop1* Alleles and *det1-1* in Terms of Emerged Lateral Roots under High-Fluence Red Light

Although the *hy5* mutation enhances the formation of lateral roots, both *col3* and *cop1* show reduced numbers of lateral roots. We consistently found a higher number of lateral roots formed in red light. To facilitate the analysis of the emergence of lateral roots in the double mutants, we performed the experiments in red light. Similar results, albeit with fewer lateral roots, were seen in white light.

In red light, *col3*, *cop1-1*, *cop1-4*, *cop1-6*, and *det1-1* all showed reduced numbers of emerged lateral roots (Figure 8). When analyzing the double mutants, we found that *col3* enhanced the phenotypes of *cop1-1* and *cop1-6*, whereas no significant difference was seen between *col3*, *cop1-4*, and *col3 cop1-4* (Figure 8), suggesting that *col3* acts as an allele-specific enhancer of the lateral root phenotype in *cop1*. Surprisingly, *col3* suppresses the lateral root phenotype of *det1-1* (Figure 8). The reduced number of lateral roots in *col3*, the *col3* enhancement of *cop1-1* and *cop1-6* lateral root phenotypes, and the suppression of *det1-1* lateral root phenotypes were complemented in T1 seedlings carrying a functional *COL3* gene (Figure 8), indicating that the phenotypes were caused by the *col3* mutation.

Thus, although *col3* partially suppresses the dark phenotype of weak *cop1* and *det1* alleles alike, we found very different genetic interactions in lateral root formation. Although the *col3* mutation partially suppresses the reduced formation of lateral roots in the *det1-1* mutant, it acts as an allele-specific enhancer of *cop1-1* and *cop1-6*.

col3 Has Reduced Levels of Anthocyanin and Has Opposite Effects on Anthocyanin Accumulation in *cop1* and *det1*

We found that the *col3* seedlings were slightly paler than wild-type seedlings during the first days after emergence from the seed coat. We assayed chlorophyll and anthocyanin contents of the seedlings to ascertain whether *col3* affects the accumulation of either of these pigments. We found no significant difference in chlorophyll content; however, *col3* seedlings had approximately half the amount of anthocyanin compared with the wild type in both red and white light (40 and 46%, respectively) (Figures 9A and 9B). The difference was more pronounced at day 4 after germination and had decreased to 9% at day 7. The reduction in anthocyanin levels in 4-d-old red light–grown *col3* seedlings was complemented in T1 seedlings transformed with pFP100-*COL3*, indicating that the phenotype was caused by the loss of the *COL3* gene (Figure 9A).

The *hy5* mutation causes reduction in both chlorophyll and anthocyanin levels during deetiolation (Holm et al., 2002). Because *hy5* is epistatic to *col3* with respect to lateral root formation, we analyzed anthocyanin levels in the *col3 hy5* double mutant. However, we found that the effect of the *col3* and *hy5* mutations was additive in red light, whereas the anthocyanin levels of *col3 hy5* were intermediate between those of the *col3* and *hy5* mutants in white light, suggesting that *col3* and *hy5* regulate anthocyanin accumulation independently (Figures 9A and 9B).

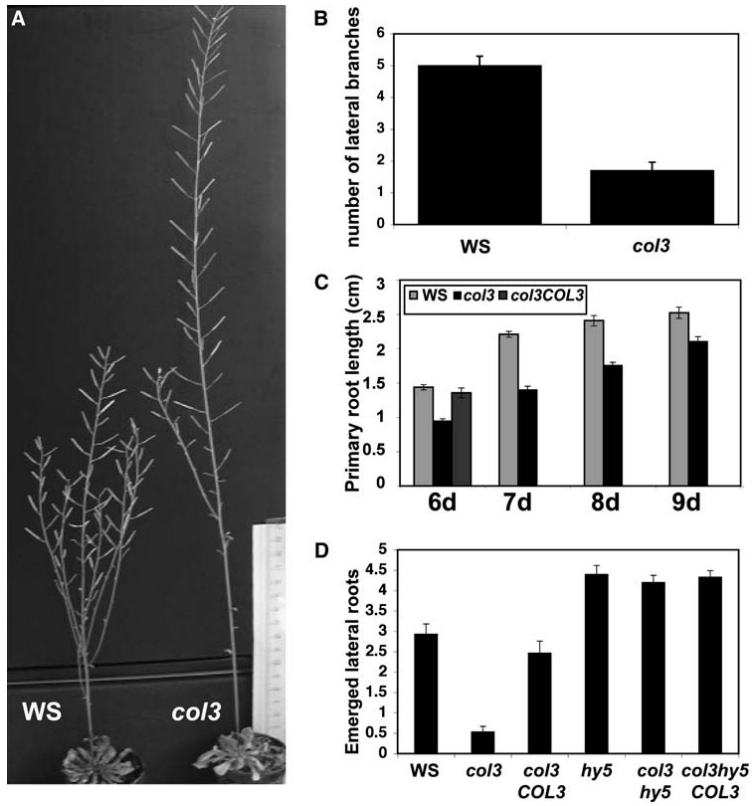

Figure 6. *COL3* Regulates Lateral Organ Formation as the Mutant Exhibits Reduced Branching in Both the Shoot and the Root.

(A) Representative Ws and *col3* plants showing lateral branching in the shoot grown under short-day conditions for 70 d.

(B) Number of lateral branches arising from the main shoot in 70-d-old Ws and *col3* plants grown under short-day conditions. Error bars represent SE ($n = 15$).

(C) Primary root length in Ws, *col3*, and *col3COL3* plants grown on vertical plates in continuous white light for the indicated number of days. Error bars represent SE ($n = 22$).

(D) Number of emerged lateral roots in Ws, *col3*, *col3COL3*, *hy5*, *col3 hy5*, and *col3 hy5COL3* seedlings grown under continuous white light for 10 d. Error bars represent SE ($n = 15$).

Both *cop1* and *det1* have increased expression of chalcone synthase, the first committed enzyme in the anthocyanin biosynthetic pathway (Chory and Peto, 1990; Deng et al., 1991). To further characterize the genetic relationship between *col3*, *cop1*, and *det1*, we analyzed anthocyanin accumulation in *col3 cop1-6* and *col3 det1-1*. As seen in Figures 9C and 9D, *cop1-6* and *det1-1* have increased levels of anthocyanin in 4-d-old seedlings germinated in red or white light. The *cop1* mutation has 12.1- and 4.5-fold increases in anthocyanin content compared with the wild type in red and white light, respectively. The *det1-1* mutation results in 8.0- and 1.7-fold higher anthocyanin content than the wild type in red and white light, respectively. Double mutant analysis revealed that *col3* has opposite effects on anthocyanin accumulation in the *cop1* and *det1* seedlings. In the case of *col3 cop1-6*, the anthocyanin content was reduced to 53 and 44.7% of *cop1-6* levels in red and white light, respectively (Figures 9C and 9D). By contrast, *col3 det1-1* had higher anthocyanin content than the *det1-1* single mutant, 9.6- and 3.2-fold above wild-type levels in red and white light, respectively (Figures 9C and 9D). These results suggest that although the *col3* mutation can suppress the accumulation of anthocyanin in both red light– and white light–grown *cop1* seedlings, it enhances the anthocyanin accumulation in *det1-1*, particularly in white light.

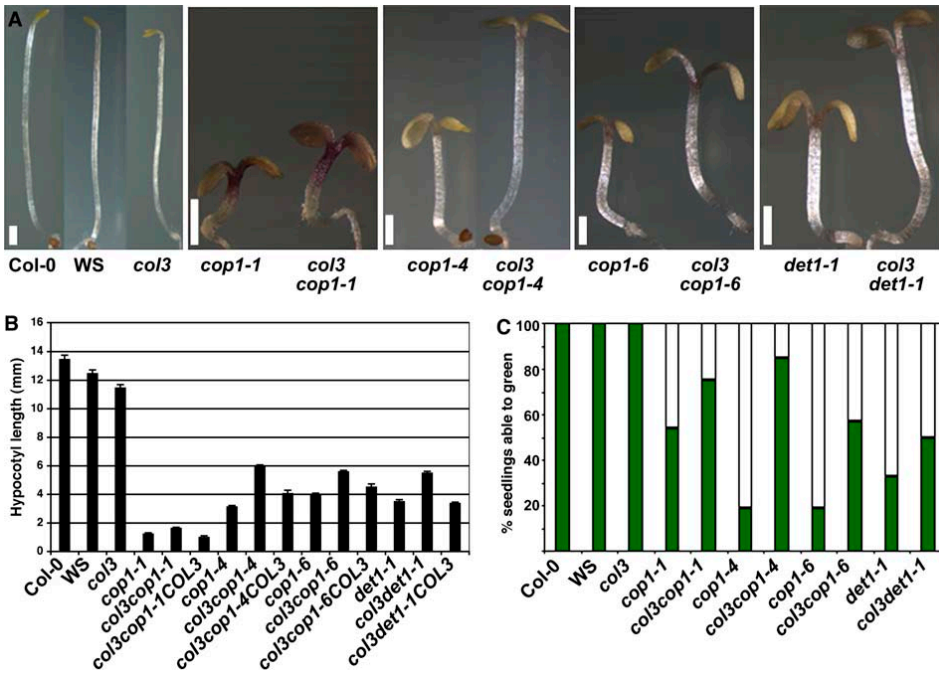

Figure 7. *col3* Suppresses the Phenotypes Conferred by *cop* and *det* in Darkness and in the Light.

(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 ($n \geq 8$).

(C) The *col3* mutation partially suppresses the light-dependent block-of-greening phenotype of the *cop1* and *det1* alleles. Seedlings were germinated in the dark for 6 d and then transferred to constant white light for 6 d. Seedlings with green cotyledons and/or true leaves were scored as able to green, and those with bleached cotyledons and/or true leaves were scored as unable to green ($n = 50$). The number of seedlings able to green is expressed as a percentage of the total number of seedlings.

DISCUSSION

Here, we report the identification of COL3 as a COP1-interacting protein and the characterization of a *col3* loss-of-function mutant. The interaction between COP1 and COL3 that was identified in yeast two-hybrid assays is supported by colocalization and positive FRET signals between the proteins in onion epidermal cells. A functional interaction between *COP1* and *COL3* is further supported by phenotypic and genetic analyses of the *col3* mutant.

Functional Domains in COL3

Analysis of the 16 CO-like proteins in *Arabidopsis* has revealed that the family is divided into three broad groups (Robson et al., 2001; Griffiths et al., 2003). COL3 is included together with CO and COL1 to COL5 in a group that has two B-boxes, a CCT domain, and a conserved six–amino acid motif in the C terminus. In animals, B-boxes are usually found in proteins that also have RING finger and coiled-coil domains. These proteins are often referred to as the RBCC (for RING, B-box, coiled-coil) or tripartite motif family. The RBCC family includes a large number of genes involved in functions such as axial patterning, growth control, differentiation, and transcriptional regulation (Torok and Etkin, 2001). The tumor suppressor Promyelocytic Leukemia (PML) gene, identified at the chromosomal breakpoint in t(15;17)-associated acute promyelocytic leukemia (de The et al., 1991; Kakizuka et al., 1991), encodes what is arguably the best-characterized RBCC protein. PML localizes to the PML nuclear body (NB), a subnuclear structure to which at least 30 proteins have been found to colocalize (Salomoni and Pandolfi, 2002). PML appears to be required for NB formation, because all NB components tested to date acquire an aberrant nuclear localization pattern in $PML^{-/-}$ primary cells, and their normal localization patterns can be restored by expression of PML (Zhong et al., 2000; Lallemand-Breitenbach et al., 2001). The localization of PML to NB requires functional RING finger and B-box domains, because substitutions of Zn^{2+} ligating residues in

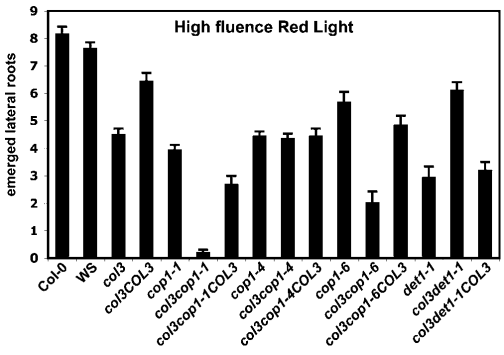

Figure 8. *col3* Exerts Opposing Effects on *cop1* Alleles and *det1-1* in Terms of Emerged Lateral Roots.

Number of emerged lateral roots on the indicated seedlings grown on vertical plates for 8 d under high-fluence red light (90 μmol·m^{-2}·s^{-1}). Error bars represent SE ($n \geq 11$ except for *col3 cop1-1COL3*, for which $n = 3$).

either of these domains disrupt PML NB formation (Borden et al., 1996). In addition, PML NB formation requires the sumoylation of Lys-160 in the first of the two B-boxes of PML, further indicating the role of the B-box domain in NB formation (Zhong et al., 2000; Lallemand-Breitenbach et al., 2001). The B-box is generally considered to mediate protein–protein interactions either directly or indirectly (Torok and Etkin, 2001), and the B-boxes in PML have been shown to interact with the GATA-2 transcription factor (Tsuzuki et al., 2000). The finding that deletion of the B-boxes in COL3 results in uniform nuclear fluorescence suggests that the COL3 B-boxes, like the PML B-boxes, are involved in speckle formation (Figure 2).

However, although RBCC proteins are found in several eukaryotes, they seem to be absent in *Arabidopsis* (Kosarev et al., 2002). In light of this, the interactions between the RING finger and coiled-coil domain containing COP1 protein and the B-box containing COL3, STH, and STO proteins (Holm et al., 2001) are interesting because this could bring the three domains together through protein–protein interaction. The interactions require the WD40 domain in COP1 and the C termini of COL3, STH, and STO, which would leave the RING, coiled-coil, and B-box domains available to interact with other proteins.

The CCT (for CONSTANS, CO-like, and TOC1) domain is a highly conserved basic module of ~43 amino acids often found in association with other domains, such as B-boxes, the response regulatory domain, the ZIM motif, or the DNA binding GATA-type zinc finger. Alleles with mutations in the CCT domain have been identified in both *TOC1* and *CO* (Strayer et al., 2000; Robson et al., 2001), suggesting that the domain is functionally important. The CCT domain contains a putative nuclear localization signal within the second half of the CCT motif and has been shown to be involved in nuclear localization (Robson et al., 2001). The CCT domain probably also has a role in protein–protein interaction, and the CCT domains of CO and TOC1 (also called ABI3-interacting protein1 or APRR1) were found to interact with the *Arabidopsis* transcription factor ABI3 in yeast cells (Kurup et al., 2000). Furthermore, the C-terminal portion of TOC1, including the CCT domain, was found to interact with several basic helix-loop-helix (bHLH) transcription factors, including PIF3 (Yamashino et al., 2003).

Thus, both the B-boxes and the CCT domain appear to mediate protein–protein interactions, and although the domains are found together in the CO-like proteins, each domain is also found in proteins with no other defined domains, suggesting that they can function independently.

In addition to B-boxes and the CCT domain, the CO and COL1 to COL5 proteins contain a conserved six–amino acid motif with the consensus sequence G-I/V-V-P-S/T-F in their C termini. The motif is separated from the CCT domain by 16 to 22 amino acids. The finding that the VP pair in the COL3 motif is required for the interaction with COP1 in yeast suggests a functional role for this motif. The conservation of the motif might indicate that other group members could be COP1-interacting partners and perhaps targets of COP1-mediated degradation. Interestingly, the CO protein is stabilized by light in the evening but degraded by the proteasome in the morning and in darkness (Valverde et al., 2004). However, studies of the COP1-interacting motifs in HY5, HYH, STH, and STO indicate that although the VP pair at the core of the motif is critical, residues before the core contribute to the interaction (Holm et al., 2001), and these residues show little conservation between the CO and COL1 to COL5 proteins. Further studies are needed to determine whether the COP1 interaction with the COL3 motif regulates COL3 protein stability, but the identification of the conserved C-terminal motif in COL3 as a COP1 interaction motif could facilitate the characterization of the motif in the CO and COL1 to COL5 proteins.

COL3 Is a Positive Regulator of Light Signals and Affects Lateral Organ Formation

The T-DNA insertion in the first exon of *COL3* results in a truncated mRNA that, if translated, would produce a protein consisting of the N-terminal amino acids 1 to 151 in COL3 followed by the amino acids KSTCPAE and a translational stop codon. This protein would contain the B-box domains but lack the C-terminal half of the COL3 protein, including the CCT domain.

However, the fact that we could complement all tested *col3* phenotypes by introducing the *COL3* gene (note that we have not tested complementation of the flowering-time phenotypes), together with the recessive nature of the *col3* mutation, indicates that *col3* is a loss-of-function mutation.

Because COL3 was identified as a COP1-interacting protein, we were interested in examining whether the *col3* mutation is defective in any known *COP1*-regulated process(es). Our analysis of the *col3* mutation reveled that this is indeed the case. The *col3* mutation resulted in reduced inhibition of hypocotyl elongation in short-day conditions and in high-fluence red light and in early flowering in both long-day- and short-day-grown plants (Figures 4 and 5). Furthermore, we observed reduced branching of the shoot in short-day-grown plants and found that *col3* seedlings form fewer lateral roots and show reduced accumulation of anthocyanin. These results suggest that *COL3* is

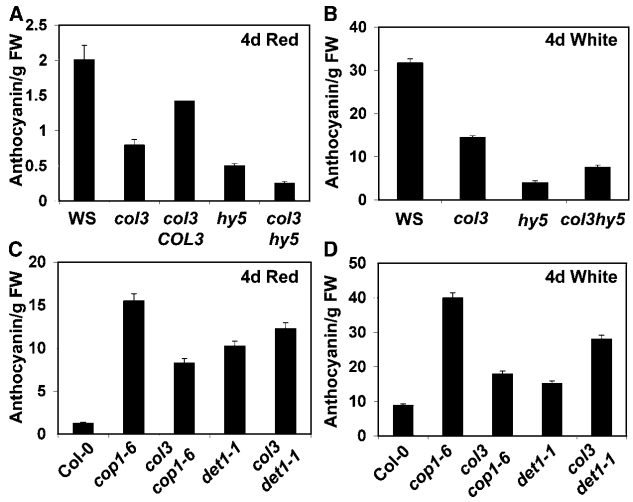

Figure 9. *col3* Has Reduced Levels of Anthocyanin and Has Opposite Effects on Anthocyanin Accumulation in *cop1* and *det1* Mutants.

(A) and **(B)** Anthocyanin content of the indicated seedlings grown for 4 d under continuous red and white light, respectively. Error bars represent SE ($n = 4$ except for *col3COL3*, for which 50 GFP-positive T1 seedlings were compared with 50 GFP-negative siblings in one experiment). FW, fresh weight.
(C) and **(D)** Anthocyanin accumulation in 4-d-old Columbia, *cop1-6*, *col3 cop1-6*, *det1-1*, and *col3 det1-1* seedlings grown under continuous red and white light, respectively. Error bars represent SE ($n = 3$).

a positive regulator of light signaling involved in a subset of the pathways regulated by *COP1*. The fact that COL3 contains the B-box and CCT domains, both of which have been found in other proteins to interact with transcription factors, suggests that COL3 acts as a downstream regulator, possibly in a promoter context.

COP1 has been shown previously to interact with and promote the degradation of the transcription factors HY5, LAF1, HYH, and HFR1 in the dark. By contrast, COP1 positively regulates PIF3 accumulation in darkness (Bauer et al., 2004). All of the transcription factors degraded by COP1 act as positive regulators of light signals of single or multiple wavelengths, whereas the phytochrome-interacting bHLH proteins PIF3, PIF1, PIF4, and PIF5 act mainly as negative regulators of phytochrome signaling (Huq and Quail, 2002; Kim et al., 2003; Fujimori et al., 2004; Huq et al., 2004). However, PIF3 might differentially affect distinct branches of red light signaling, because it acts as a positive factor in anthocyanin and chlorophyll accumulation (Kim et al., 2003; Monte et al., 2004).

To further define and characterize *COL3*, we analyzed genetic interactions between *col3* and *hy5*. In addition to the hypocotyl phenotype, *hy5* seedlings show enhanced initiation and elongation of lateral roots, altered gravitropic and touching responses, enhanced cell elongation in root hairs, reduced greening and secondary thickening of the root, and reduced chalcone synthase expression (Oyama et al., 1997; Ang et al., 1998). Of the spectrum of phenotypes seen in *hy5*, the more subtle phenotypes of *col3* are restricted to reduced inhibition of hypocotyl elongation in short days and red light, reduced anthocyanin accumulation, and lateral root formation. However, surprisingly and in contrast with *hy5*, *col3* mutants have reduced formation of lateral roots. Analysis of *hy5 col3* double mutants revealed that *hy5* is epistatic to *col3* with respect to lateral roots (Figure 6), whereas *HY5* and *COL3* appear to act as independent positive regulators of anthocyanin accumulation (Figure 9).

The flowering-time phenotype seen in *col3* is opposite to the long-day late-flowering phenotype of *co* and similar to the early-flowering phenotype seen in *hy5* and *hyh*. *CO* promotes flowering in response to long days; flowering is induced when *CO* mRNA expression coincides with the exposure of plants to light. Recent results suggest that the daily rhythm of *CO* transcription is refined by photoreceptor-dependent regulation of CO protein levels (Valverde et al., 2004). Light stabilizes the CO protein in the evening, whereas CO is degraded by the proteasome in the morning or in darkness.

The early flowering in *col3* mutants suggests that *COL3* does not act as a promoter of flowering. However, the reduced branching of the shoot seen only in short days suggests that *COL3* has a positive role in this process and that *COL3* might decode a subset of daylength-sensitive outputs.

All six *CO* and *COL1* to *COL5* genes are represented on the ATH1 array, and review of the diurnal experiments performed by Smith et al. (2004) using the genevestigator website revealed that the expression of all six genes shows diurnal changes. The expression of *COL3* peaks at dawn, and the expression profile of *COL3* most closely resembles that of *COL4*.

The ability to translate daylength into physiological responses requires crosstalk between light signals and the circadian clock (Hayama and Coupland, 2003). Interestingly, overexpression of *COL1* in plants shortened the period of two distinct circadian rhythms in a fluence rate–dependent manner, suggesting an effect on a light-input pathway (Ledger et al., 2001). The light-dependent regulation of CO protein levels, together with the diurnal expression and conserved domain structure of all six CO and COL1 to COL5 proteins, make it tempting to speculate that these proteins act at the crossroads of light signals and the circadian clock. However, further genetic and biochemical studies are needed to address this issue.

The finding that *col3* partially suppresses the hypocotyl phenotype of dark-grown *cop1-1*, *cop1-4*, *cop1-6*, and *det1-1* alleles firmly establishes *COL3* as a gene affecting *COP*/*DET*/*FUS*-regulated processes. Surprisingly, although *col3* suppresses both *cop1* and *det1* in darkness, we found that *col3* has different and sometimes opposing effects on *cop1* and *det1* in light-grown seedlings. In the root, where *col3*, *cop1*, and *det1* all show reduced numbers of emerged lateral roots, *col3* enhances the effect of *cop1-1* and *cop1-6* and suppresses the phenotype conferred by *det1-1*. Furthermore, although *col3* suppresses the enhanced anthocyanin accumulation in *cop1-6*, *col3 det1* seedlings have higher anthocyanin content than the *det1-1* mutant.

The different effects seen in the dark and light could be attributable to the reduced nuclear abundance of COP1 in light-grown seedlings. However, it is also possible that although COP1 represses COL3 in the dark, COL3 might be regulated independently of COP1 in the light.

Several lines of evidence suggest that the mechanisms whereby *col3* and *hy5* suppress *cop1* and *det1* in the dark are similar. Both COL3 and HY5 interact physically with COP1 in yeast, and both proteins colocalize with COP1 in onions. COL3 is a nuclear protein (Figure 2), and nuclear localization of the homologous CO protein is required for CO function (Simon et al., 1996). Both *col3* and *hy5* are recessive loss-of-function mutations. The phenotypes of the *col3* mutation suggest that *COL3* promotes photomorphogenesis. The recent mechanistic understanding of the COP/DET/FUS proteins further suggests that COL3 might be targeted for degradation in the dark.

Further biochemical analysis of the COL3 protein is needed to address the mechanism by which COL3 is regulated. Our functional and genetic studies provide a framework from which a more complete understanding of light signaling can be built.

METHODS

Plant Material, Growth Conditions, and Complementation Tests

The *Arabidopsis thaliana col3* and *hy5-ks50* (Oyama et al., 1997) alleles are in the Ws accession, the *cop9-1*, *det1-1*, *cop1-1*, *cop1-4*, and *cop1-6* alleles are in the Columbia accession, and *co-2* (Putterill et al., 1995) is in Landsberg *erecta*. Unless stated otherwise, 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 transferred to light chambers maintained at 22°C with the desired light regime. For the complementation test, a 4.4-kb genomic fragment containing the full-length *COL3* gene was excised from the genomic BAC clone F27A10 and inserted into the *Xho*I and *Pst*I sites of the pBSK+ vector. The *COL3* gene was subsequently excised with *Kpn*I and *Pst*I and inserted into the pFP100 vector (Bensmihen et al., 2004), containing an At2S3:E-GFP:$35S_{ter}$ cassette driving the expression of E-GFP in seeds, to generate pFP100-*COL3*. This construct was used to transform *Agrobacterium tumefaciens* strain GV3101 by the freeze-thaw method, which was then introduced into the *col3*, *col3 cop1-1*, *col3 cop1-4*, *col3 cop1-6*, *col3 det1-1*, and *col3 hy5* mutant plants via the floral dip method (Clough and Bent, 1998). 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 controls.

Yeast Two-Hybrid Methods and Onion Experiments

The yeast strain Y190 (Kim et al., 1997) was used for the two-hybrid screen and for the two-hybrid assays. The conversion of the λ*ACT* cDNA expression library (ABRC number CD4-22) into a pACT library, the two-hybrid screen, and the β-galactosidase assays were performed as described by Holm et al. (2001). The pAVA321-S65TGFP-COL3 and pRTL2-mGFP-ΔCOL3 constructs, containing versions of the GFP (von Arnim et al., 1998), the pAM-PAT-35SS-CFP-COP1 and pAM-PAT-35SS-YFP-COL3, and the pRTL2-COP1 overexpression constructs were introduced into onion (*Allium cepa*) epidermal cells by particle bombardment, incubated, and examined by epifluorescence microscopy as described previously (Holm et al., 2002).

For the FRET–acceptor photobleaching experiments, 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 24 h after particle bombardment using the confocal microscope through a Plan-Neofluor 40×/1.3 oil (differential interference contrast) objective. The multitracking mode was used to eliminate spill-over 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%.

Expression of AD-COL3 and the three VP-substituted COL3 fusion proteins was examined by protein gel blot analysis using polyclonal rabbit antibodies raised against COL3.

RNA Gel Blotting

Total RNA was extracted from seedlings grown in continuous white light for 6 d after their germination using the RNeasy kit (Qiagen). Twenty micrograms of total RNA was loaded for RNA gel blot analysis.

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 transferred to monochromatic light conditions and incubated at 22°C for 6 d in the case of hypocotyl experiments and for 6 to 12 d for the measurement of roots. Red, far-red, and blue lights were generated by light emission diodes at 670, 735, and 470 nm, respectively (model E-30LED; Percival Scientific). Fluence rates were measured with a radiometer (model LI-250; Li-Cor). Unless stated otherwise, all experiments with the roots under red light were performed using a high fluence of 90 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The hypocotyl lengths of seedlings, the lengths of the primary roots, and the numbers of lateral roots were measured/counted using ImageJ software.

Flowering-Time Experiments

For short-day and long-day measurements, seeds were sown on soil, cold-treated for 3 d at 4°C, transferred to a light chamber (model

AR-36; Percival Scientific) maintained at 22°C, and grown under a 16-h-light/8-h-dark photoperiod for long days and an 8-h-light/16-h-dark photoperiod for short days. Flowering time was determined by counting the number of rosette leaves at bolting.

Anthocyanin Measurements

For the anthocyanin determinations, seedlings at 4 d after germination were 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 anthocyanin was determined by spectrophotometric measurements of the aqueous phase ($A_{530} - A_{657}$) and normalized to the total fresh weight of tissue used in each sample.

Block of Greening

Seedlings were germinated in the dark for 6 d and then transferred to constant white light for 6 d. Seedlings with green cotyledons and/or true leaves were scored as able to green, and those with bleached cotyledons and/or true leaves were scored as unable to green. The fluence level for white light was maintained at 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Accession Numbers

The COL3, COP1, DET1, and HY5 Arabidopsis Genome Initiative locus identifiers are At2g24790, At2g32950, At4g10180, and At5g11260, respectively.

Supplemental Data

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

Supplemental Figure 1. *col3* Seedlings Do Not Differ Significantly from the Wild Type in Blue or Far-Red Light.

Supplemental Figure 2. *col3* Behaves like the Wild Type and *col3 hy5* Seedlings Do Not Differ Significantly from *hy5* under Constant White Light and Long-Day Conditions.

Supplemental Figure 3. Branching in *col3* and Wild-Type Plants Is Similar under Long-Day Conditions.

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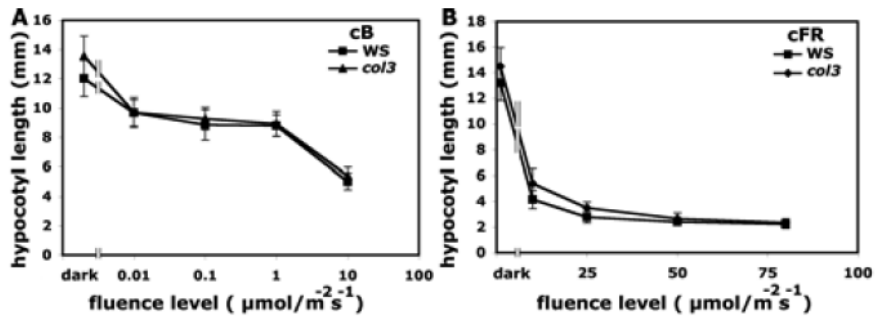
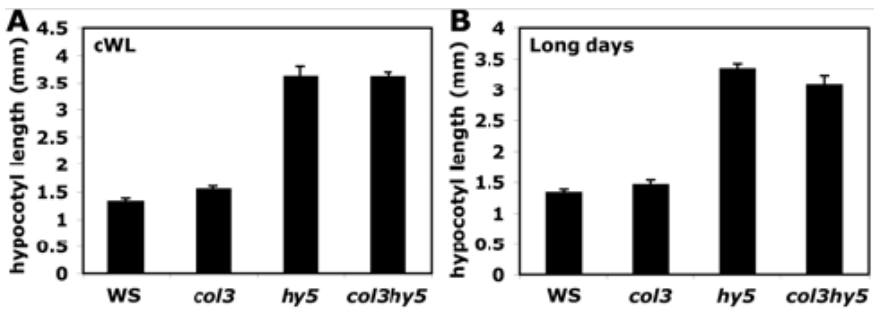
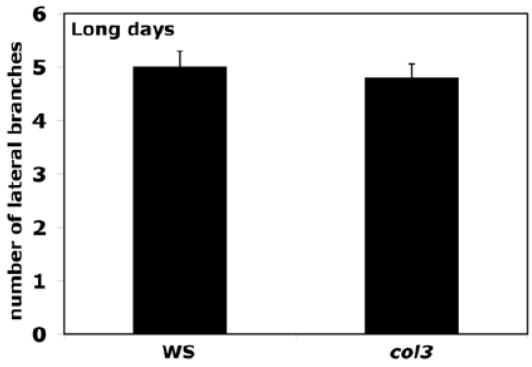
Supplemental Data



Supplemental Figure 1. *col3* seedlings do not differ significantly from the wild-type (WS) in blue or far-red light. Fluence response curves of WS and *col3* seedlings grown under continuous monochromatic blue (A) and far-red (B) light. Error bars represent SE, n=30.



Supplemental Figure 2. *col3* behaves like the wild-type (WS) and *col3hy5* seedlings do not differ significantly from *hy5* under constant white light and long-day conditions. Bar graph showing the hypocotyl lengths of the indicated seedlings grown under constant white light (A) and long days (B). Error bars represent SE, n=30.



Supplemental Figure 3. Branching in *col3* and wild-type (WS) plants is similar under long-day conditions. Bar graph showing the number of lateral branches arising from the shoot in wild-type and *col3* plants grown under long days. Error bars represent SE, n=15.

II

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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

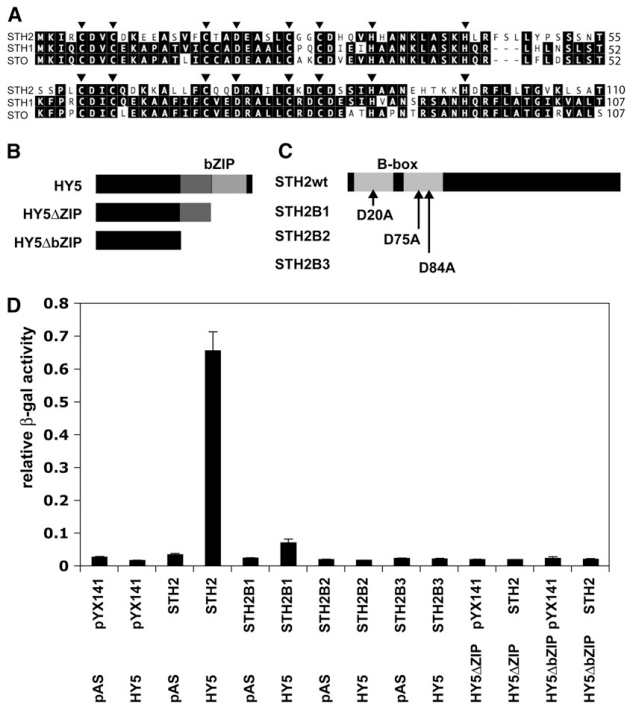

Figure 1. STH2 Interacts with HY5 in Yeast, and the bZIP Domain of HY5 and the B-Boxes in STH2 Are Important for the Interaction.

(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

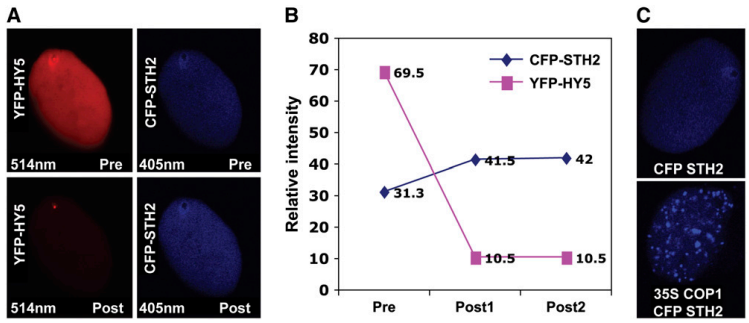

Figure 2. STH2 Interacts with Both HY5 and COP1 in Living Plant Cells.

(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

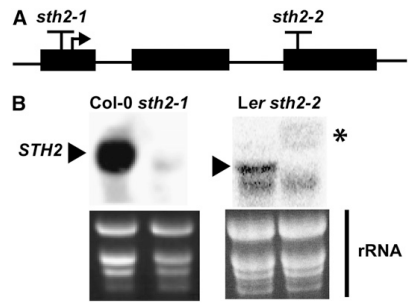

Figure 3. T-DNA Insertion Mutants in *STH2*.

(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

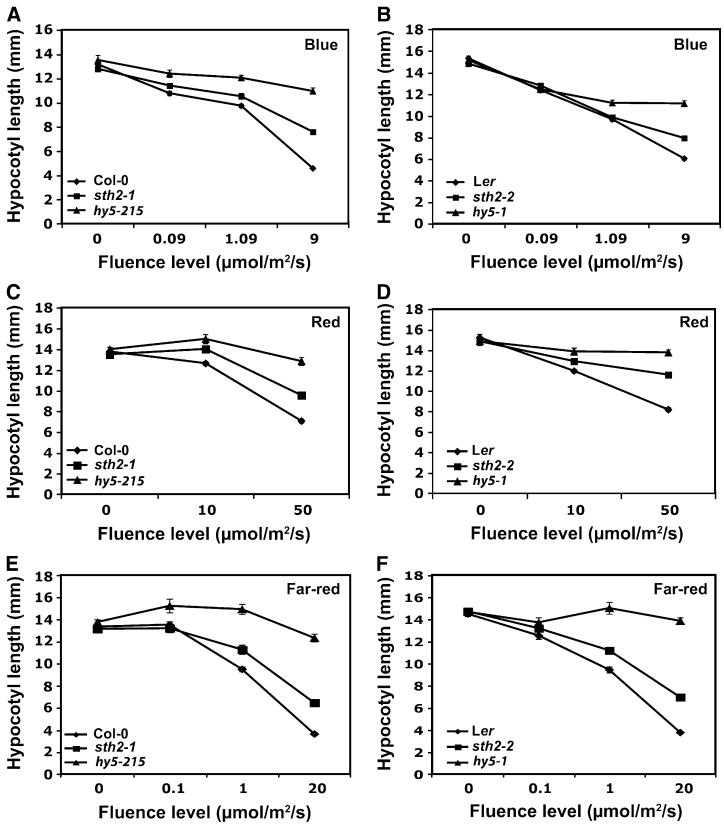

Figure 4. *sth2* Is Hyposensitive to Blue, Red, and Far-Red Light.

(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

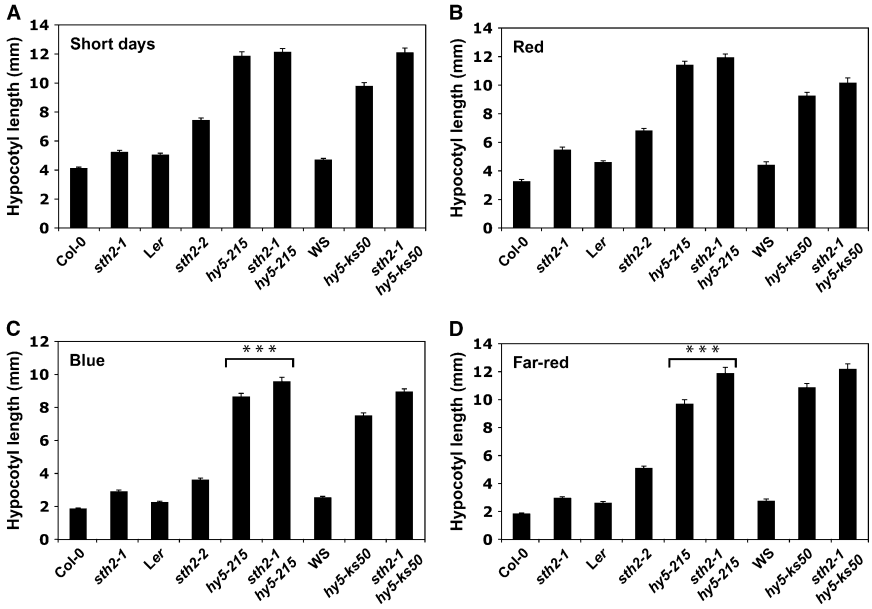

Figure 5. The Long Hypocotyl Phenotype of *sth2* Is Partially Dependent on *hy5*.

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.

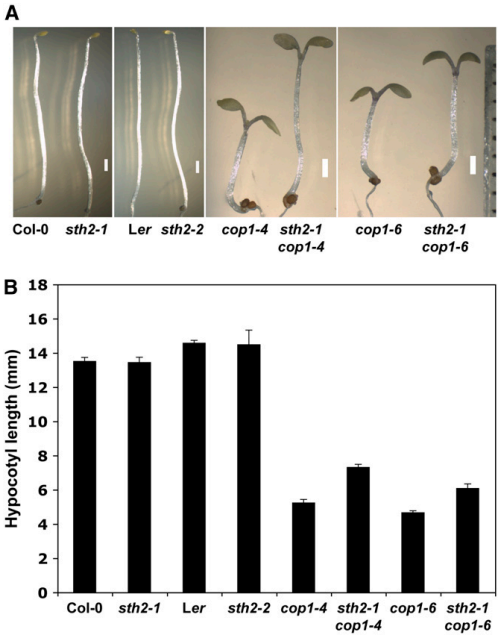

Figure 6. *sth2* Partially Suppresses *cop1* in the Dark.

(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

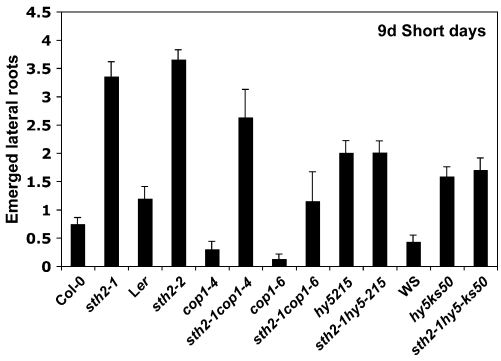

Figure 7. *sth2* Has an Enhanced Number of Emerged Lateral Roots.

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

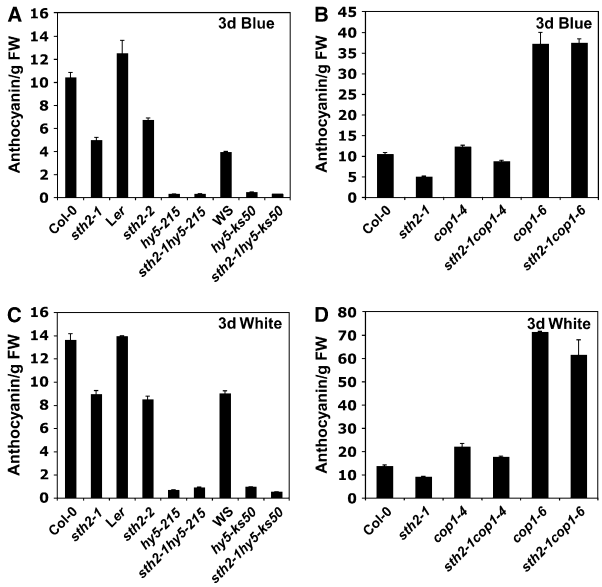

Figure 8. *sth2* Accumulates Less Anthocyanin.

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

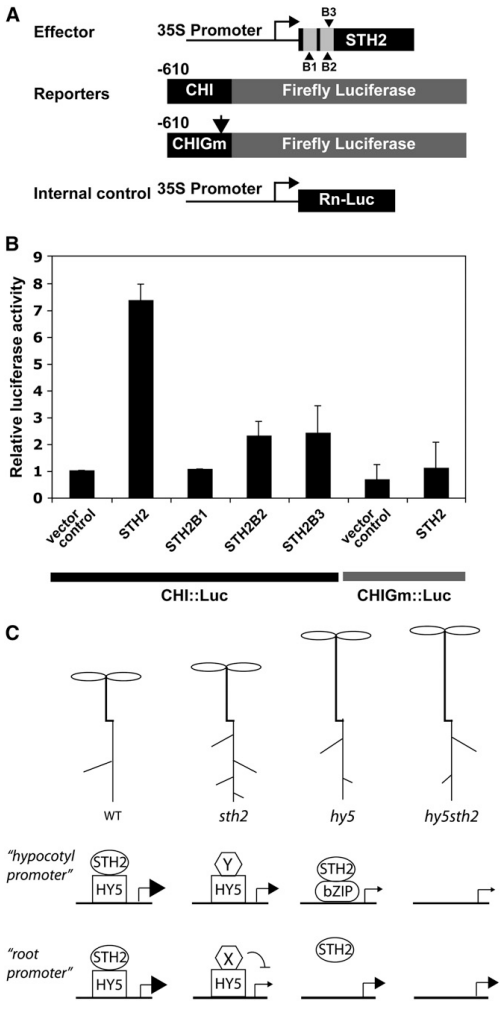

Figure 9. STH2 Can Activate Transcription.

(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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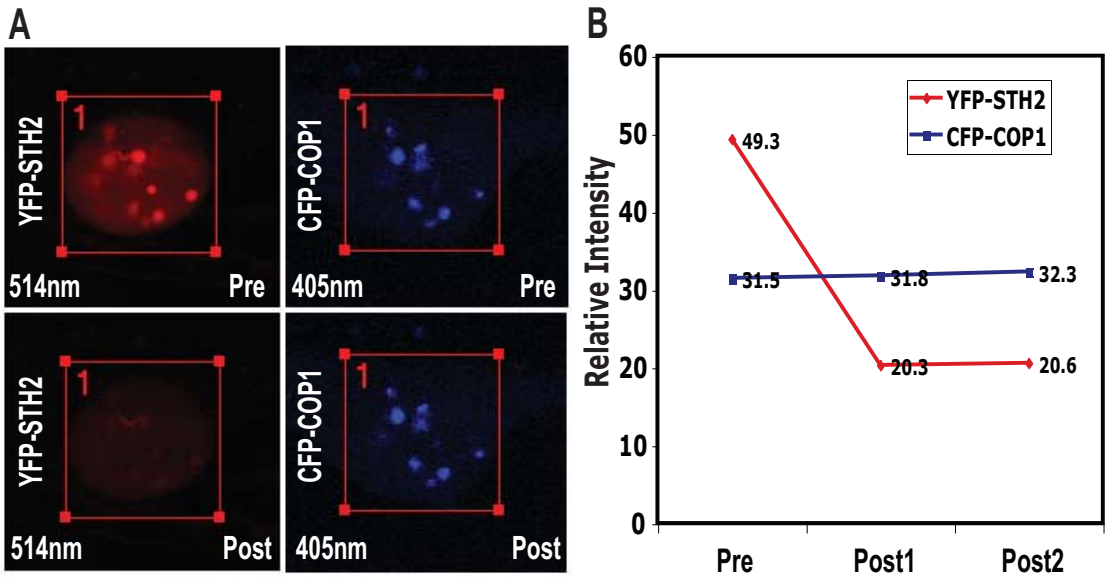
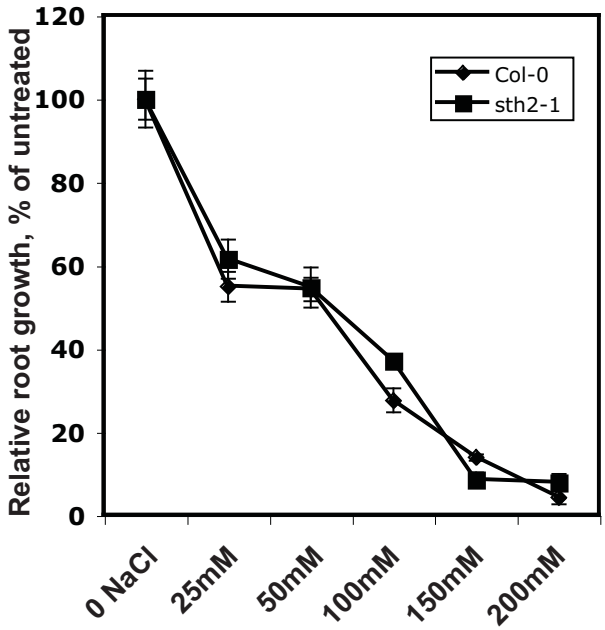
Supplemental Figures. Datta et al. (2007). SALT TOLERANCE HOMOLOG 2 (STH2), a B-box protein in *Arabidopsis* that activates transcription and positively regulates light-mediated development.



Supplemental Figure 1. STH2 co-localizes with COP1 but does not show FRET.
(A) and **(B)** Absence of FRET between YFP-STH2 and CFP-COP1 as analyzed by acceptor photo bleaching in nuclei (n = 4). The top panels in (**A**) show a representative pre-bleach nucleus co-expressing YFP-STH2 and CFP-COP1 excited with either a 514- or a 405-nm laser, resulting in emission from YFP (red) or CFP (blue), respectively. The area marked with the square 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**). No increase in donor fluorescence (blue) is seen.



Supplemental Figure 2. The loss of *STH2* does not affect tolerance to salt.
The seedlings were germinated on MS plates with 3% sucrose and transferred after 4 days in the light to vertical plates containing 0, 25, 50, 100, 150 or 200 mM NaCl. Primary root length was determined upon transfer and again after 7 days growth in constant white light. Graph represents root length as a percentage of the length on the plate without NaCl, errorbars represent SD, n=5

III

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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* and *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 degradation, it can in turn regulate PhyB protein abundance during long-term continuous red (Rc) irradiation, suggesting reciprocal

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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 cryptochrome-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 (amino acids 115 to 168) or the basic zipper domain (amino acids 77 to 168) deleted. Deletion of the zipper domain or the basic

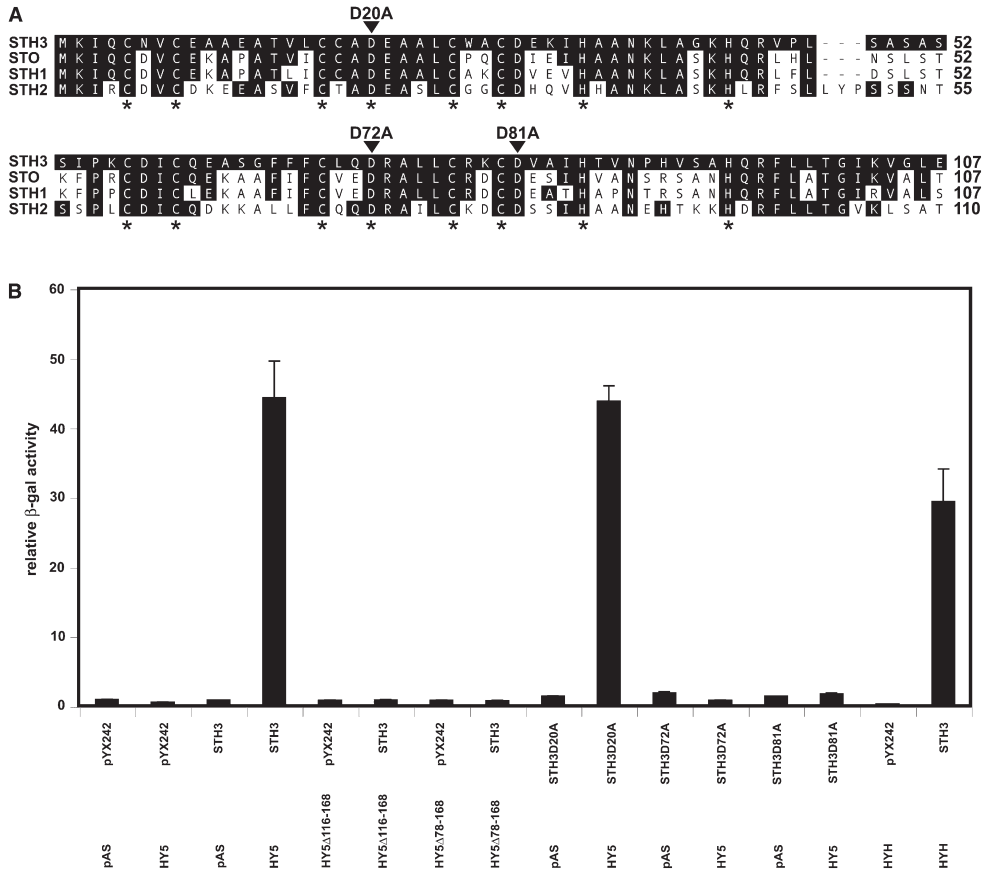

Figure 1. STH3 Interacts with HY5 in Yeast, and the bZIP Domain of HY5 and the Second B-Box in STH3 Are Important for the Interaction.

(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 as indicated. Error bars indicate SE ($n \geq 4$).

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 (Figure 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 seen under high fluence far-red light, with *sth3* seedlings being 21% longer than the wild type (Figure 3D). At very low fluence

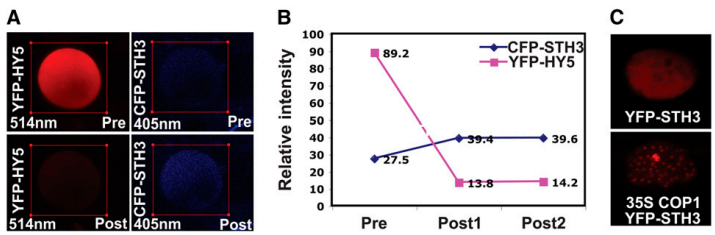

Figure 2. STH3 Interacts Both with HY5 and COP1 in Living Plant Cells.

(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.

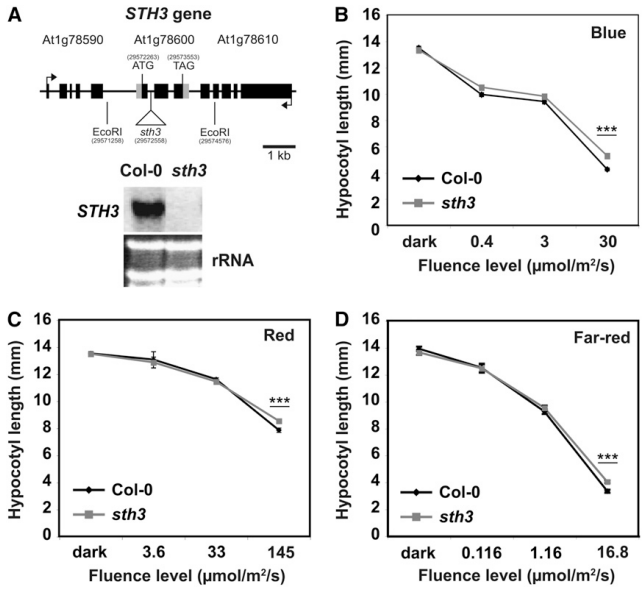

Figure 3. The *STH3* Mutant Is Hyposensitive to High Fluence Blue, Red, and Far-Red Light.

(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.

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*

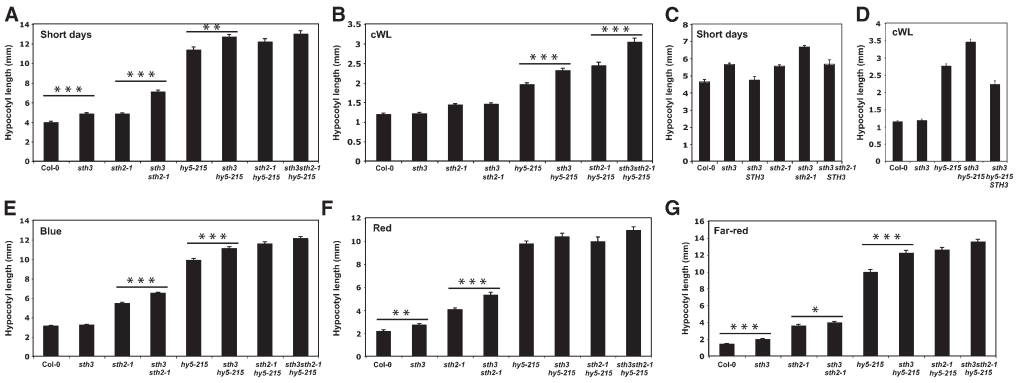

Figure 4. *sth3* Enhances the Long Hypocotyl Phenotype of *sth2* and *hy5.*

(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,

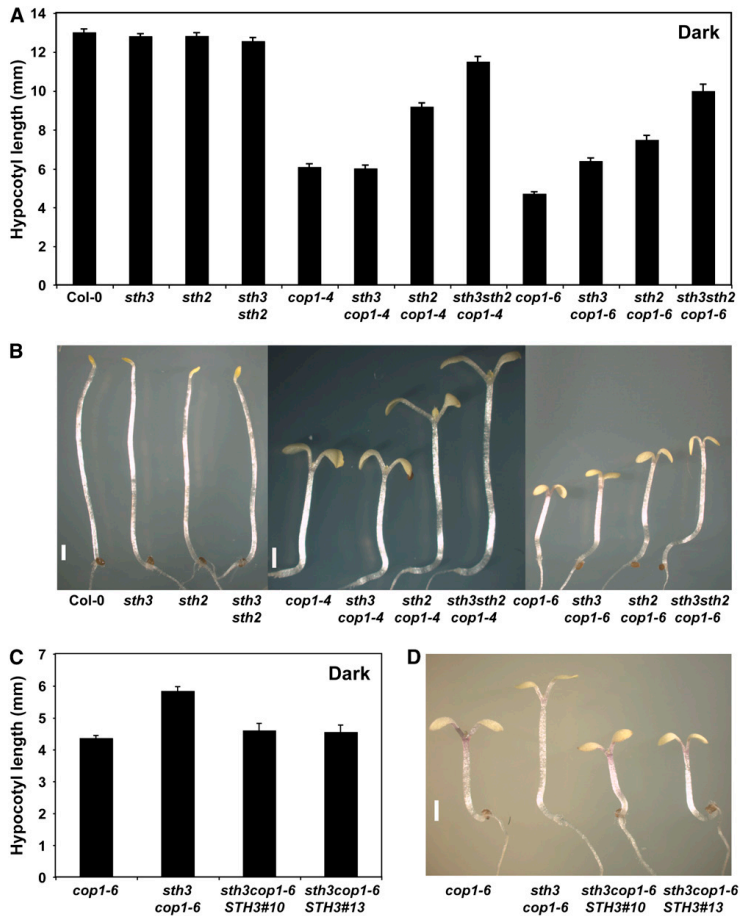

Figure 5. *sth3* Suppresses *cop1* in the Dark Both by Itself and Together with *sth2*.

(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. 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

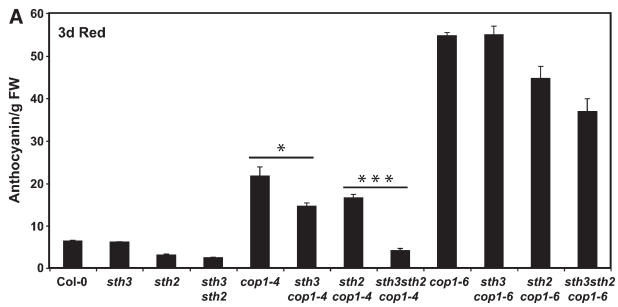

Figure 6. *sth3* Suppresses the Anthocyanin Accumulation in *cop1* Both by Itself and Together with *sth2.*

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.

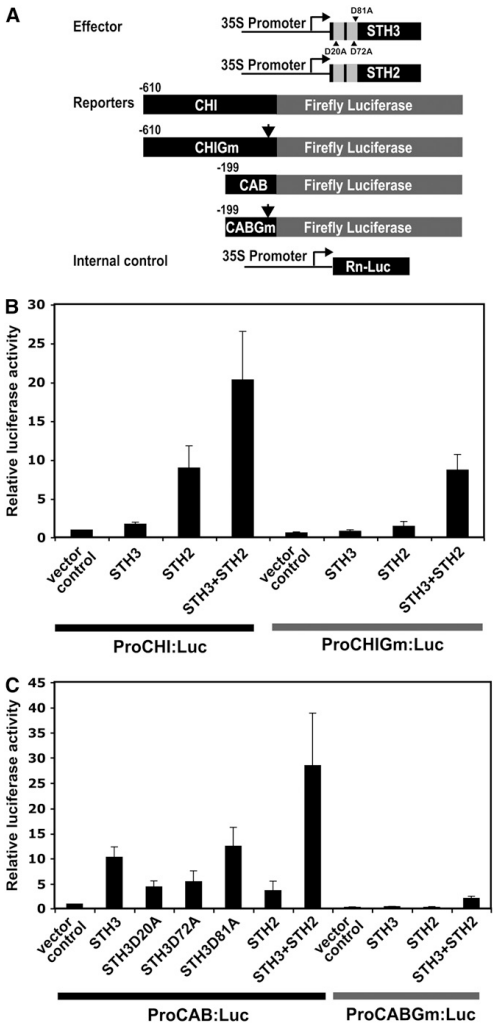

Figure 7. STH3 Can Activate Transcription Both by Itself and Together with STH2.

(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).

resulted in negligible activation by STH3, quite similar to STH2, suggesting that STH3 might be acting through this G-box. 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

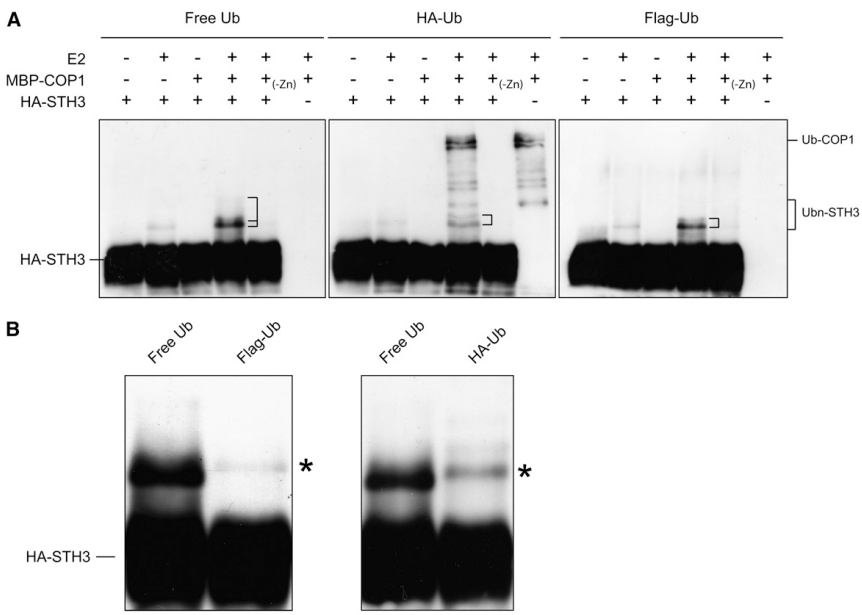

Figure 8. COP1 Ubiquitinates STH3 in Vitro.

(A) HA-STH3 ubiquitination assays were performed using yeast E1 (Boston Biochem), rice Rad6 (E2; Yamamoto et al., 2004), 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.

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* 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′-GTTGCGCCG-CCGAGGCTG-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′-GCTCTA-GAGGTGGTGGTATGAAGATTCAGTGTAACGTTT-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′- AATC-GAAATTCCAACCGACTCAACA-3′) and a 199-bp fragment of the CAB promoter (the region between the sequences 5′-ATAACTTGTGGTCA-CAAAACGC-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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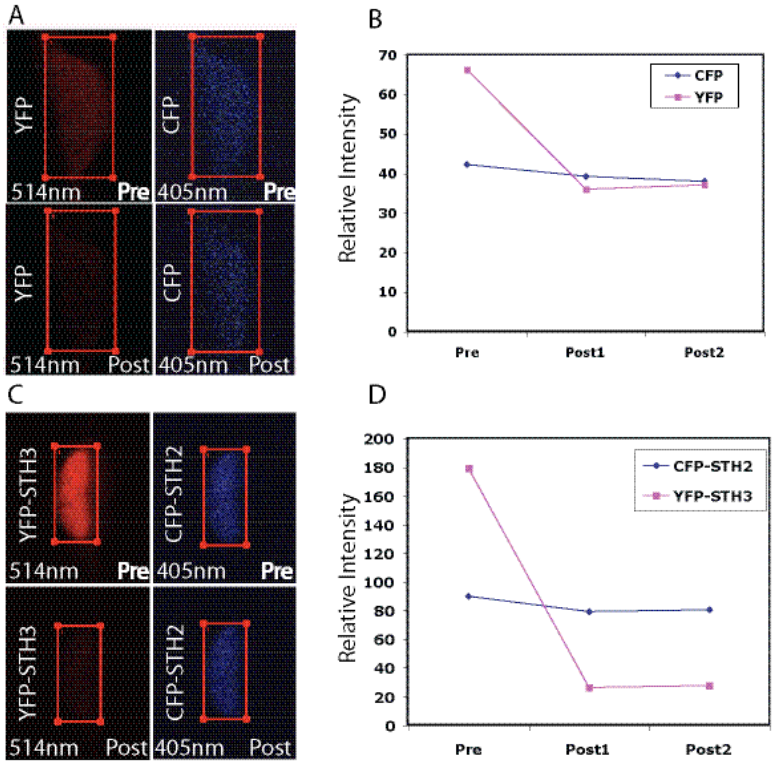
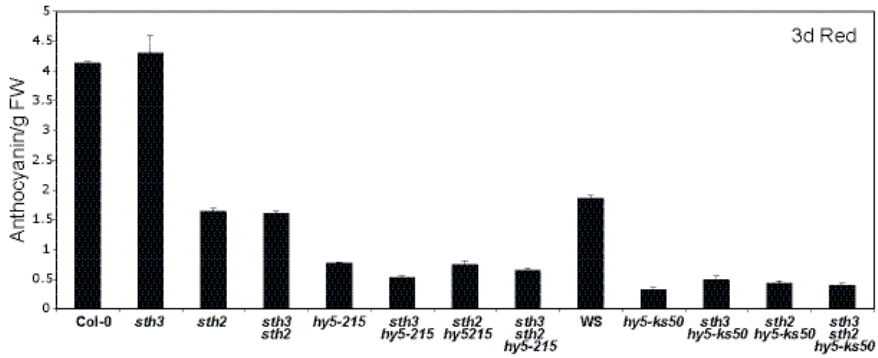
Supplemental Data



Supplemental Figure 1. Absence of FRET between unfused CFP/YFP or CFP-STH2/YFP-STH3. Absence of FRET between unfused CFP/YFP (**A**) and CFP-STH2/ YFP-STH3 (**C**) analyzed by acceptor bleaching in nuclei ($n \geq 13$). The top panels in **A**) and **C**) show a representative pre-bleach nucleus co-expressing YFP and CFP (**A**) or CFP-STH2 and YFP-STH3 (**C**) excited with either a 514- or a 405-nm laser, resulting in emission from YFP (red) or CFP (blue), respectively. The area marked with the square was bleached with the 514-nm laser. The bottom panels in **(A and C)** 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 and D)**. No increase in donor fluorescence (blue) is seen.



Supplemental Figure 2. Anthocyanin levels in *hy5* and *sth3hy5* are close.
Anthocyanin content of the indicated seedlings grown for 3d under continuous red light. Error bars represent SE (n = 3). FW, fresh weight.

Supplemental Dataset 1. **Text file corresponding to the alignment in Figure 1A.**

STH3
MKIQCNVCEAAEATVLCCADEAALCWACDEKIHAANKLAGKHQRVPLSASASSI
PKCDICQEASGFFFCLQDRALLCRKCDVAIHTVNPHVSAHQRFLLTGIKVGLE
STO
MKIQCDVCEKAPATVICCADEAALCPQCDIEIHAANKLASKHQRLHLNSLSTKFP
RCDICQEKAAFIFCVEDRALLCRDCDESIHVANSRSANHQRFLATGIKVALT
STH1
MKIQCDVCEKAPATLICCADEAALCAKCDVEVHAANKLASKHQRLFLDSLSTKF
PPCDICLEKAAFIFCVEDRALLCRDCDEATHAPNTRSANHQRFLATGIRVALS
STH2
MKIRCDVCDKEEASVFCTADEASLCGGCDHQVHHANKLASKHLRFSLLYPSSSN
TSSPLCDICQDKKALLFCQQDRAILCKDCDSSIHAANEHTKKHDRFLLTGVKLSA
T