Molecular Interaction between COP1 and HY5 Defines a Regulatory Switch for Light Control of *Arabidopsis* Development

Lay-Hong Ang,* Sudip Chattopadhyay,*
Ning Wei,* Tokitaka Oyama,† Kiyotaka Okada,†
Alfred Batschauer,‡ and Xing-Wang Deng*§
*Department of Molecular, Cellular, and
  Developmental Biology
Yale University
New Haven, Connecticut 06520
†Department of Botany
Kyoto University
Kyoto 606-01
Japan
‡Biological Institute II
Albert-Ludwig-University
79104 Freiburg
Germany

Summary

Arabidopsis COP1 acts as a light-inactivable repressor of photomorphogenic development, but its molecular mode of action remains unclear. Here, we show that COP1 negatively regulates HY5, a bZIP protein and a positive regulator of photomorphogenic development. Both in vitro and in vivo assays indicate that COP1 interacts directly and specifically with HY5. The hyperphotomorphogenic phenotype caused by the overexpression of a mutant HY5, which lacks the COP1-interactive domain, supports the regulatory role of HY5–COP1 interaction. Further, HY5 is capable of directly interacting with the *CHS1* minimal promoter and is essential for its light activation. We propose that the direct interaction with and regulation of transcription factors by COP1 may represent the molecular mechanism for its control of gene expression and photomorphogenic development.

Introduction

Light is one of the most influential environmental factors that regulate plant development throughout its entire life cycle (Kendrick and Kronenberg, 1994). The influence of light on plant development is elegantly demonstrated by the light control of early seedling development. Seedlings grown in the dark follow a skotomorphogenic developmental mode and display an etiolated phenotype typified by their elongated hypocotyls and folded cotyledons with apical hooks. In contrast, seedlings grown in the light follow the photomorphogenic developmental mode and display a de-etiolated phenotype, which includes inhibition of hypocotyl elongation, unfolding of apical hooks, expansion of cotyledons, and expression of light-regulated genes such as ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (*RBCS*), chlorophyll a/b binding protein (*CAB*), and chalcone synthase (*CHS*). Equipped with an array of photoreceptors that include phytochromes, blue/UV-A receptors, and

UV-B receptors, plants are able to sense the changes in the quality, quantity, direction, and duration of the light environment and trigger a complex signaling network that allows the plants to adapt for optimal growth (von Arnim and Deng, 1996).

How light signals perceived by the multiple photoreceptors are communicated via specific signal transduction events to bring about the specific changes in gene expression has begun to be unraveled (Barnes et al., 1997). While biochemical complementation studies have implicated the role of trimeric G protein, cGMP, and calcium/calmodulin as intermediates in phytochrome signal transduction (Neuhaus et al., 1993; Romero and Lam, 1993; Bowler et al., 1994), genetic studies have identified two groups of key players in this pathway (Chory, 1993; McNellis and Deng, 1995). Mutations in one group result in constitutive photomorphogenic development in darkness and are defined by at least ten *COP/DET/FUS* loci (Deng et al., 1992; Castle and Meinke, 1994; Miséra et al., 1994; Pepper et al., 1994; Wei et al., 1994a; Kwok et al., 1996) that act downstream of the phytochromes (PHY) and cryptochrome 1 (CRY1) (Chory, 1992; Ang and Deng, 1994; Wei et al., 1994a). The recessive nature of *cop/det/fus* mutations suggests that the wild-type gene products act to repress photomorphogenic development in darkness. In contrast, mutations in the second group result in seedlings that show reduced responses to light stimuli. With the exception of HY5, this group is represented by mutations in either photoreceptors such as PHYA, PHYB, and CRY1 (Quail, 1991; Ahmad and Cashmore, 1993; Furuya, 1993; Kaufman, 1993; Vierstra, 1993), or signaling components specific for PHYA and PHYB pathways (PHYA: FHY1 and FHY3, Whitelam et al., 1993; PHYB: RED1, Wagner et al., 1997). The elongated hypocotyl phenotype of *hy5* in continuous white, far-red, red, and blue light indicates that *HY5* encodes a positive regulator that acts downstream of the PHYA, PHYB, and CRY1 signaling pathways (Koornneef et al., 1980).

All four of the cloned *COP/DET/FUS* genes (*COP1*: Deng et al., 1992; *COP9*: Wei et al., 1994b; *DET1*: Pepper et al., 1994; *FUS6*: Castle and Meinke, 1994) encode novel proteins. In the case of COP1, it consists of three familiar motifs: a ring finger zinc-binding motif, a coiled-coil domain, and multiple WD-40 repeats characteristic of the β subunit of the trimeric G protein (Deng et al., 1992). Recent overexpression studies demonstrated that COP1 acts as a cell-autonomous repressor of photomorphogenic development whose repressive activity is abrogated by light (McNellis et al., 1994b, 1996; Miséra et al., 1994). Consistent with this role, subcellular localization studies of GUS-COP1 fusion protein indicated that COP1 is enriched in the nuclei in darkness, and its nuclear abundance is quantitatively reduced with increasing light intensity and duration (A. von Arnim and X.-W. D., 1994). Moreover, GUS-COP1 is absent from the nuclei of the remaining nine pleiotropic *cop/det/fus* mutants, indicating that these nine other genes are essential for either the translocation or the retention of COP1 in the nuclei (Chamovitz et al., 1996; von Arnim

§To whom correspondence should be addressed.

et al., 1997). However, how COP1 regulates gene expression and represses photomorphogenic development is still not clear. One possibility is that COP1 could act directly on the promoters of light-regulated genes. Alternatively, COP1 could indirectly modulate the activity of other nuclear factors that contact these promoters. Three pieces of indirect evidence support the latter possibility and imply HY5 as a candidate nuclear factor that bridges COP1 to some of its target genes. First, the allele-specific and antagonistic interactions between *hy5* and *cop1* reported previously (Ang and Deng, 1994) suggest potential direct and regulatory interactions between HY5 and COP1. Second, HY5 encodes a bZIP transcription factor (Oyama et al., 1997) and could potentially directly interact with promoter(s) to regulate gene expression. Third, COP1 acts within the nucleus to repress light-activated development and gene expression (von Arnim and Deng, 1994) and hence could contact HY5 in the nucleus.

In this study, we have investigated the physical interaction between COP1 and HY5 by in vitro and in vivo binding assays and their effects on light-regulated seedling development. Further, we have studied the direct link between HY5 and *CHS* gene expression. Together, our results indicate that COP1 and HY5 may represent two antagonistic nuclear regulators and that, via direct protein–protein interactions, COP1 negatively modulates the activity of HY5 in regulating gene expression and photomorphogenic development.

Results

HY5 and COP1 Physically Interact In Vitro

To examine the possible physical interaction between HY5 and COP1, an in vitro binding assay was performed. As shown in Figure 1A, the amount of ^{32}P-labeled COP1 retained by the GST-HY5 beads increased proportionally with increasing amounts of COP1 applied. Further, the level of COP1 retained by GST-HY5 beads was significantly higher than the background level of COP1 retained by the control GST beads. Since equal amounts of GST-HY5 and GST were used in the binding experiment, our results indicate a specific and direct protein–protein interaction between COP1 and HY5 in vitro.

The COP1-Interactive Domain of HY5 Is Separable from the DNA-Binding and Dimerization Module

To substantiate the observed in vitro COP1–HY5 interaction and to dissect the specific protein domains involved, a yeast two-hybrid protein–protein interaction assay (Ausubel et al., 1994) was adapted. As illustrated in Figure 1B, chimeric fusion protein of the LexA DNA-binding domain and HY5 (LexA-HY5) activated transcription of the *lacZ* reporter gene in the presence of Gal4 activation domain–COP1 fusion (AD-COP1) but not with Gal4 activation domain (AD) alone, suggesting a direct protein–protein interaction between COP1 and HY5 in yeast cells.

To determine the specific HY5 protein domain that mediates its interaction with COP1, a deletion series of
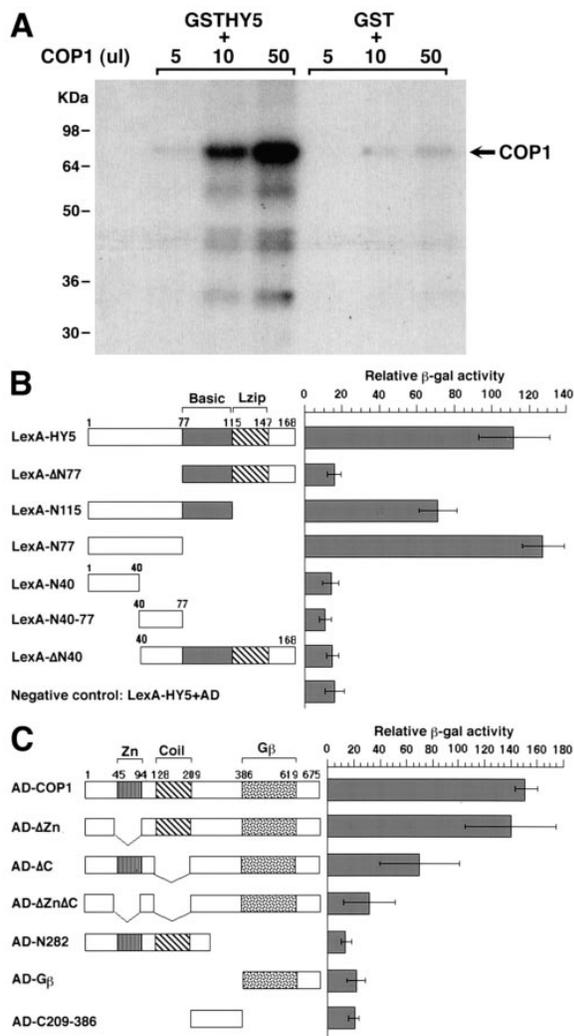
Figure 1. Analysis of COP1 and HY5 Interaction In Vitro and in Yeast

(A) An autoradiograph of a typical in vitro binding experiment where 5, 10, and 50 μl of ^{32}P-labeled COP1 were added to 10 μg of GST-HY5 or GST. The 74 kDa radioactive COP1 product is indicated by the arrow.

(B) The N-terminal 77 amino acids of HY5 are essential and sufficient for COP1 interaction in yeast. Left panel illustrates the LexA-HY5 fusion constructs; the basic region (basic) and leucine zipper (Lzip) domain of HY5 are as indicated. Their respective β-galactosidase activities with AD-full-length COP1 shown in the right panel were averaged from 6–10 individual primary cotransformants; the standard deviations are represented by the error bars.

(C) Overall structure of COP1 is necessary for interaction with HY5 in yeast. Left panel illustrates the AD-COP1 fusion constructs; the zinc-binding ring finger (Zn), the coiled-coil domain (coil) and the WD-40 repeats (Gβ) of COP1 are as indicated. Their corresponding β-galactosidase activities with LexA-full-length HY5 shown in the right panel were averaged from 6–10 individual primary cotransformants; the standard deviations are represented by the error bars.

HY5 was constructed and fused to the LexA DNA-binding domain. As illustrated in Figure 1B, deletion of the N-terminal 77 amino acids, leaving the basic plus leucine zipper (bZIP) domain of HY5 intact, completely abolished its ability to interact with COP1. By contrast, deletion of the leucine zipper domain or the entire bZIP

domain, only marginally affected its interaction with COP1. Further deletion analysis of HY5 indicated that although the N-terminal 40 amino acids were essential for HY5 to interact with COP1, they were not sufficient for mediating COP1 interaction. To ensure that the different degrees of interaction observed were not due to differences in protein expression, Western blot analysis was conducted. All of the LexA-HY5 fusion proteins were expressed at similar levels, except LexA-N77 and LexA-N40, which were slightly higher and could account for the slightly stronger interaction between LexA-N77 and AD-COP1 (data not shown). Together, our data demonstrate that the N-terminal 77 amino acids of HY5 are both necessary and sufficient for mediating direct interaction with COP1. Hence, HY5 consists of at least two distinct functional modules, one for the presumed dimerization and DNA-binding activity (the bZIP domain) and the other for interacting with COP1.

The Overall Structure of COP1 Is Crucial for Its Interaction with HY5

In a parallel experiment to dissect COP1's structural requirement for interacting with HY5, a series of COP1 deletions were constructed and fused to the hemagglutinin (HA) epitope–tagged Gal4 activation domain. Since the various AD-COP1 chimeric constructs were expressed at similar protein levels as detected by Western blot analysis (data not shown), their relative strength of interaction with HY5 should be reflected by the respective reporter β-galactosidase activities. Figure 1C showed that while deletion of the ring finger (ΔZn) had no significant effect on HY5–COP1 interaction, deletion of the coiled-coil domain (ΔCoil) reduced COP1's ability to interact with HY5 by half. Further, deletion of both the ring finger and the coiled-coil domain (ΔZnΔCoil) almost completely abolished HY5–COP1 interaction, indicating that the ring finger and the coiled-coil domain were essential for interacting with HY5. However, neither the N-terminal fragment (N282) that contains both the ring finger and the coiled-coil domain, nor the C-terminal fragment (Gβ) that contains all of the WD-40 repeats, nor an internal fragment (amino acid 209–386) was sufficient to mediate the interaction of COP1 with HY5. The apparent lack of specific HY5-interactive domain in COP1 suggests that the overall protein structure of COP1, as well as specific sequences, may be critical for its molecular association with HY5. This is consistent with the observation that functional forms of COP1 may be involved in both intermolecular and intramolecular interaction and folding (McNellis et al., 1996; K. Torii and X.-W. D, unpublished data).

COP1 Can Recruit HY5 into Specific Nuclear Foci in Living Plant Cells

To investigate a possible interaction between COP1 and HY5 in living plant cells, we tagged both proteins with a green fluorescent protein, S65TGFP, and analyzed their subcellular localization patterns in living onion epidermal cells. Punctate green fluorescent speckles were observed in a weak and uniform green fluorescent background in the nuclei of S65TGFP-COP1-expressing onion cells (Figures 2A and 2B). In addition, fluorescent
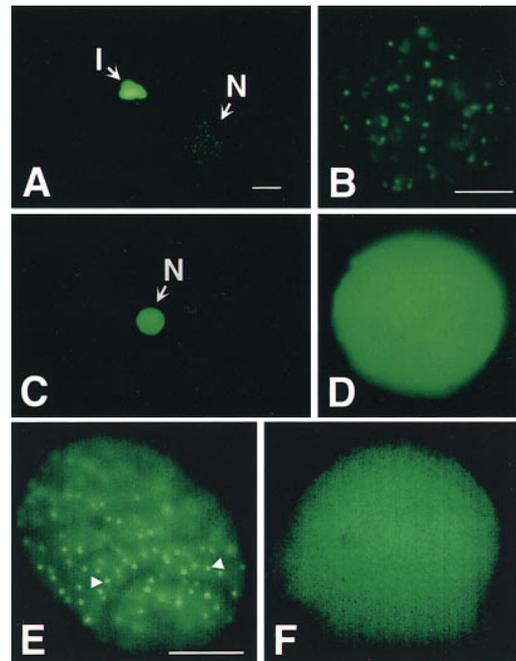
Figure 2. Recruitment of HY5 into COP1 Nuclear Speckles in Living Plant Cells

(A–D) Fluorescent images of S65TGFP-COP1 and S65TGFP-HY5 in onion epidermal cells. In (A), the green fluorescent speckles of S65TGFP-COP1 are clearly evident in the nucleus (N), and the fluorescent S65TGFP-COP1 inclusion body in the cytoplasm is indicated by (I). A close-up of the same nucleus is shown in (B). In (C), Uniform green fluorescence of S65TGFP-HY5 is evident throughout the nucleus (N), and a close-up of the nucleus is shown in (D).

(E) Numerous bright green fluorescent speckles indicated by the triangles are observed among the uniform green fluorescent background in the nucleus of a typical onion epidermal cell coexpressing S65TGFP-HY5 and nontagged COP1.

(F) Nuclear speckle is absent in a typical onion epidermal cell coexpressing S65TGFP and nontagged COP1. The scale bar in (A), which represents 50 μm, pertains to (A) and (C); the scale bar in (B), which represents 20 μm, pertains to (B) and (D); and the scale bar in (E), which also represents 20 μm, pertains to (E) and (F).

inclusion bodies were detected in the cytoplasm, presumably due to an overaccumulation of S65TGFP-COP1. Note that the subcellular localization of S65TGFP-COP1 was essentially the same as that of GUS-COP1 reported previously (von Arnim and Deng, 1994). In contrast, 100% of the onion epidermal cells (n = 50) expressing S65TGFP-HY5 fusion protein displayed a uniformly bright green fluorescent expression pattern exclusively in the nuclei (Figures 2C and 2D). The contrasting nuclear localization patterns of COP1 and HY5 inspired an assay for their in vivo interaction: the recruitment of HY5 into COP1 nuclear speckles. When S65TGFP-HY5 was coexpressed with nontagged full-length COP1 protein in the onion epidermal cells, bright green nuclear speckles were consistently observed throughout the uniform green fluorescent background (Figure 2E). Since uniform nuclear fluorescent signal was observed when S65TGFP-HY5 was expressed alone, the detection of nuclear green fluorescent speckles when S65TGFP-HY5 was coexpressed with the nontagged full-length COP1 would support the recruitment of S65TGFP-HY5 into the nuclear COP1 speckles. To verify the specificity of such
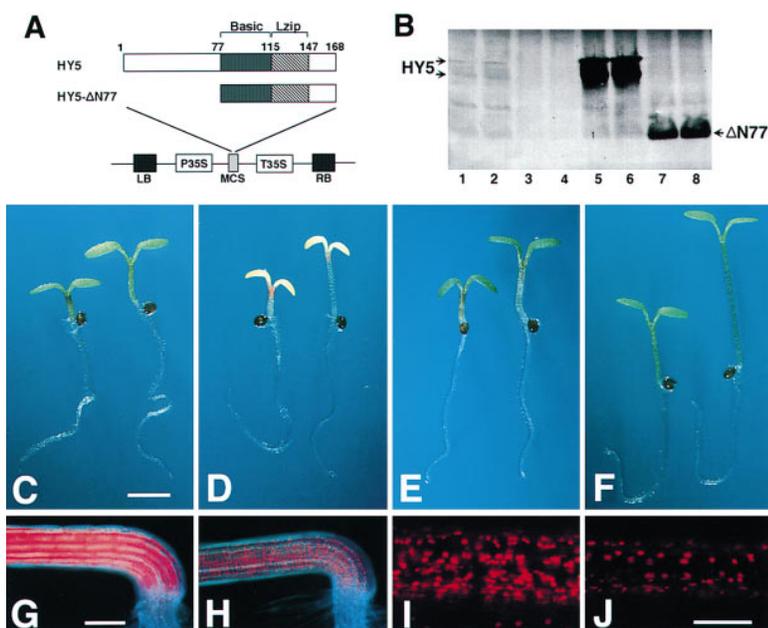
Figure 3. Hyperphotomorphogenic Phenotypes of HY5-ΔN77 Overexpressing Lines

(A) Full-length HY5 and HY5-ΔN77 under CaMV 35S promoter (P35S) and terminator (T35S) were cloned into the multiple cloning sites (MCS) of pPZP222 binary vector as indicated. The left and right borders of the T-DNA are represented by LB and RB, respectively. The basic region (Basic) and leucine zipper domain (Lzip) of HY5 are indicated.

(B) Western blot analysis of six-day-old light-grown wild-type (lanes 1 and 2), hy5-1 (lanes 3 and 4), transgenic Arabidopsis overexpressing full-length HY5 (lanes 5 and 6), and HY5-ΔN77 (lanes 7 and 8) on a 15% SDS–PAGE gel and probed with anti-HY5 antibodies. Full-length HY5 and HY5-ΔN77 proteins detected were indicated by the arrows.

(C–F) Compare the hyperphotomorphogenic phenotype of the HY5-ΔN77 overexpressing line (left) and a wild-type sibling (right) grown under continuous white light (C), far-red light (D), blue light (E), and red light (F).

(G and H) Compare the level of chloroplast autofluorescence in the hypocotyl of an HY5-ΔN77 overexpressing line (G) and the wild-type seedling (H) grown under continuous red light for six days.

(I and J) Compare the level of chloroplast autofluorescence in the roots of an HY5-ΔN77 overexpressing line (I) and the wild-type seedling (J) grown in continuous white light for 3 weeks.

The scale in (C), which represents 2 mm, pertains to (C)–(F); The scale bar in (G), which represents 0.5 mm, pertains to (G) and (H); and the scale bar in (J), which represents 0.2 mm, pertains to (I) and (J).

interaction, S65TGFP itself was coexpressed with the same nontagged full-length COP1. 100% (n = 50) of the cells showed a uniform fluorescent pattern in the nuclei and none displayed the nuclear speckles (Figure 2F), indicating that the nuclear fluorescent speckles were indeed due to the specific association of S65TGFP-HY5 with the nuclear COP1 speckles. Hence, these data strongly support the in vivo interaction between HY5 and COP1 in living plant cells.

The COP1-Interactive Domain of HY5 May Function as a Regulatory Module

The clear separation of the COP1-interactive domain from the putative DNA-binding and dimerization domain for HY5 (Figure 1B) raised the possibility that HY5 consists of two distinct functional modules: the bZIP domain that presumably interacts with target gene promoters and/or other transcription factors to regulate gene expression, and the N-terminal 77 amino acids that interact with its negative regulator, COP1. A direct prediction from this model would be that the overexpression of a mutant HY5 (HY5-ΔN77) that lacks the COP1-interactive domain will uncouple COP1's negative regulation on HY5 activity, and hence lead to a hyperactive HY5. To test this prediction, two constructs that constitutively overexpress either the full-length HY5 or the mutant HY5 (HY5-ΔN77) were stably introduced into Arabidopsis (Figure 3A).

As predicted, the overexpression of the mutant HY5 (HY5-ΔN77) created a hyperphotomorphogenic phenotype in the light-grown seedlings. The dramatic reduction in the hypocotyl lengths and the elevated accumulation of anthocyanin at the upper hypocotyls were clearly evident in the HY5-ΔN77 overexpressing seedlings

grown in continuous white, far-red, blue, and red light (Figures 3C–3F). In addition, precocious development of chloroplasts was observed in the hypocotyls and the roots of 6-day-old and 3-week-old light-grown seedlings, respectively (compare Figure 3G with 3H and Figure 3I with 3J). By contrast, overexpression of the full-length HY5 caused no significant effect on hypocotyl and cotyledon development. In addition, precocious development of chloroplasts was also not observed in the roots (data not shown). Since the protein level of HY5-ΔN77 was similar to, if not lower than that of full-length HY5 in transgenic Arabidopsis (Figure 3B), the observed phenotypes of the HY5-ΔN77 overexpressing lines could best be explained by its higher activity in activating photomorphogenic development than full-length HY5. Further, the ability of HY5-ΔN77 to partially rescue the elongated hypocotyl phenotype of the hy5 null mutant (data not shown) indicates that HY5-ΔN77 itself is physiologically active. It is however impossible to confirm whether HY5-ΔN77 is quantitatively more active than the wild-type HY5 due to the extremely low level of HY5-ΔN77 protein in the hy5 mutants, possibly due to transgene silencing (data not shown). Interestingly, no deviant phenotype was observed when the full-length HY5 and the truncated HY5-ΔN77 overexpressing lines were grown in darkness (data not shown). This result suggests that a hyperactive HY5 alone may not be sufficient to activate photomorphogenic development (see Discussion).

COP1 and HY5 Act Antagonistically to Regulate Lateral Root Development

To further elucidate the nature of HY5–COP1 interaction, we examined their roles in regulating lateral root development. Consistent with previous studies (Ang and Deng,
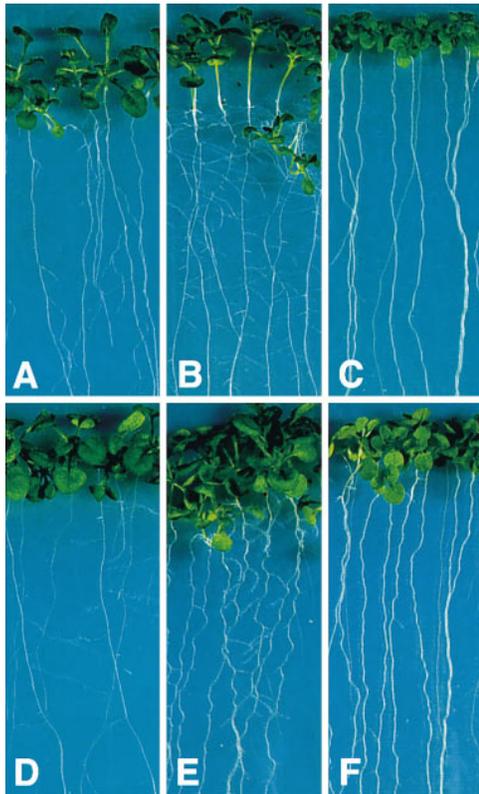
Figure 4. Antagonistic Interaction between HY5 and COP1 in Regulating Lateral Root Development

The seedlings were grown on vertical agar plates for 2 weeks under a cycling white light regime. The root phenotype of (A) wild-type, (B) *hy5-1*, (C) *cop1-6*, (D) *hy5-1/cop1-6* double mutant, and transgenic *Arabidopsis* overexpressing (E) full-length COP1 and (F) N282 fragment of COP1 are shown.

1994; Oyama et al., 1997), mutations in *COP1* and *HY5* resulted in contrasting root developmental defects (Figure 4). Lateral root initiation was severely inhibited in *cop1-6*, a weak *cop1* mutant (Figure 4C), as compared to the wild-type (Figure 4A). By contrast, a dramatic increase in the number and the length of the lateral roots was observed in the *hy5-1* mutant (Figure 4B). Further, *hy5-1/cop1-6* double mutants developed an increased number and length of their lateral roots resembling the *hy5-1* single mutants (Figure 4D), indicating that *hy5-1* can suppress the root defects of *cop1-6*. These data suggest that HY5 acts to repress lateral root initiation and elongation, while COP1 plays a positive role in lateral root development, possibly by negatively regulating the repressive activity of HY5.

To gain further insights into the antagonistic roles of HY5 and COP1 in lateral root development, we examined the effects of overexpressing full-length COP1 (McNellis et al., 1994b) and an N-terminal fragment of COP1 (N282, McNellis et al., 1996) on root development in transgenic plants. Consistent with a positive regulatory role, the overexpression of full-length COP1 led to an increased number of lateral roots as well as an increased number and length of root hairs when compared to the wild type (Figure 4E). In contrast, the overexpression of a dominant-negative form of COP1, N282, resulted in short primary roots that lacked lateral roots (Figure 4F). Since N282 was unable to interact with HY5 (Figure 1C) but capable of interacting with full-length COP1 (McNellis et al., 1996), its effect on root development could be best explained by its unproductive intermolecular interaction with the endogenous COP1 and thus compromising COP1's ability to physically interact with HY5 and negatively regulate HY5. The resulting lack of down-regulation of HY5 activity would lead to a hyperactive HY5 and hence hyperrepression of lateral root development. Taken together, the data would be consistent with a direct protein–protein interaction between COP1 and HY5 in regulating root development. However, this does not exclude other possible forms of regulatory interaction between HY5 and COP1.

HY5 Is Essential for Light Activation of the Chalcone Synthase Gene Promoter

To examine whether HY5 is directly involved in mediating light-regulated gene expression, we chose to analyze the effect of HY5 on the expression of chalcone synthase gene (*CHS*), which encodes the first committed step for anthocyanin biosynthesis. *CHS* gene expression and anthocyanin accumulation are highly inducible by light stimulus (Martin, 1993) and are regulated by COP1 and HY5 in a contrasting fashion (Figure 3; Ang and Deng, 1994). To examine the effect of HY5 on *CHS* gene expression, two *CHS* promoter-glucuronidase (GUS) reporter constructs were introduced into a null *hy5* mutant background, and the expression patterns of the transgenes were analyzed. The two promoters used were the mustard *CHS1* full-length promoter and its minimal light-responsive promoter fragment (Unit 1) fused to a basal 35S promoter (Kaiser and Batschauer, 1995). Both promoters, when fused with GUS reporter, displayed proper expression patterns with regard to tissue specificity and light regulation in transgenic *Arabidopsis* (Kaiser and Batschauer, 1995; Batschauer et al., 1996). In this study, very low activity was observed for both *CHS1* full-length and Unit 1 promoters in the dark-grown wild-type and *hy5* seedlings, with GUS staining restricted to the cotyledons (Figures 5A and 5D). In the light, however, clear elevation of *CHS* promoter activity in the cotyledons and most dramatically in the roots was detected in the wild-type but not in the *hy5* mutants (Figures 5B and 5E). Further quantitative GUS assays showed a 3- to 5-fold induction of the *CHS1* and U1 promoter activity in the wild-type, while the light inducibility of both promoters was completely abolished in the *hy5* mutants (Figures 5C and 5F). These results confirmed the essential role of HY5 in mediating light activation of *CHS* gene promoter, a sharp contrast to the repressive role of COP1 in repressing *CHS* gene expression (Deng et al., 1991). The antagonistic roles of HY5 and COP1 in regulating *CHS* gene expression suggest that COP1 may repress *CHS* gene expression by directly interacting with HY5 and down-regulating HY5's ability to activate *CHS* gene expression. The fact that *hy5* mutation caused similar effects on *CHS1* and U1 promoter activity strongly suggests that HY5 regulates *CHS* promoter activity through the minimal light-responsive promoter unit, Unit 1, which is both necessary and sufficient for the light inducibility of *CHS* gene expression.
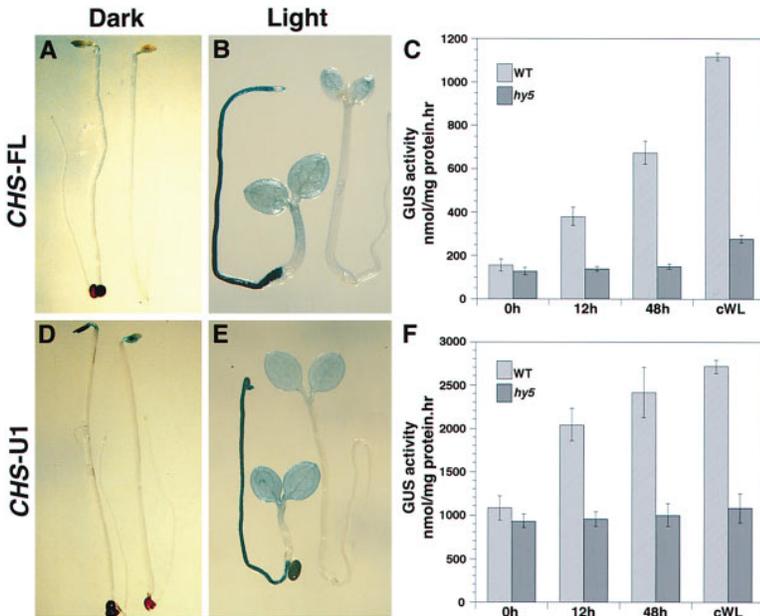
Figure 5. HY5 Is Essential for Light Induction of *CHS* Gene Expression

Transgenic *Arabidopsis* expressing *CHS1* full-length promoter (FL) or Unit 1 (U1) fused with GUS reporters were grown in the dark (A and D) or white light (B and E) for 6 days before the seedlings were stained for GUS activity. In each panel, a wild type is shown on the left and an *hy5* mutant is shown on the right. (C) and (F) compare the light inducibility of the *CHS1* full-length promoter and Unit 1, respectively. Four-day-old dark-grown wild-type and *hy5* seedlings were exposed to 0, 12, or 48 hr of white light or grown in continuous white light for six days before the GUS activities were quantitated. The quantitative GUS activity shown is a mean from four independent experiments, and the error bars represent the standard deviations.

HY5 Directly Binds to the Light-Responsive Unit of Chalcone Synthase Promoter

To test whether HY5 directly bind to the minimal light-responsive region of the *CHS1* promoter mentioned above, a gel mobility retardation assay using recombinant GST-HY5 protein and a 180 bp *CHS1* promoter fragment containing Unit 1 as a probe was carried out. Indeed, HY5 specifically bound to the *CHS1* promoter to form a slower mobility protein–DNA complex (Figure 6, lanes 1–3). Furthermore, the association of HY5 with the *CHS1* probe was abolished when challenged with an excess of unlabeled Unit 1 (Figure 6, lanes 4–6), suggesting that the HY5 binding site was located within the Unit 1 region. Since Unit 1 of the *CHS1* promoter was reported to contain at least two essential and highly conserved protein binding sites, which include a G-box-like motif (Feldbrugge et al., 1997), we seek to define the specific HY5 binding site by competition with unlabeled light-responsive elements (LREs) such as G, GATA, and GT1 (Puente et al., 1996). The binding of HY5 to the *CHS1* promoter was abolished when challenged with an excess of unlabeled synthetic consensus G-box tetramer (Figure 6, lanes 7–9), but not with tetramers of two other distinct light-responsive promoter elements, GATA and GT1 (Figure 6, lanes 10 and 11, respectively). This data is further supported by the finding that HY5 can specifically bind to the labeled G-box sequences in vitro by both gel mobility retardation and footprinting assays (S. C. and N. W., unpublished data). Hence, our results suggest that HY5 is capable of specifically binding to the *CHS* promoter via direct contact with the essential G-box motif.

Discussion

The Antagonistic Interactions between COP1 and HY5 Define a Molecular Switch for Light Control of *Arabidopsis* Developmental Patterns and Gene Expression

Our data support the capability and physiological significance of direct protein–protein interactions between COP1 and HY5 during plant development. The results further suggest that the key repressor, COP1, may directly interact with and regulate the transcription factors that are responsible for mediating light-regulated gene expression and development. The direct interactions between COP1 and HY5 were confirmed by three independent assay systems: the in vitro protein affinity assay (Figure 1A), the in vivo yeast two-hybrid assay (Figures 1B and 1C), and the ability of COP1 to recruit HY5 into distinct nuclear foci in living onion cells (Figure 2). It is worth mentioning that although the functional role of the nuclear COP1 speckles during plant development
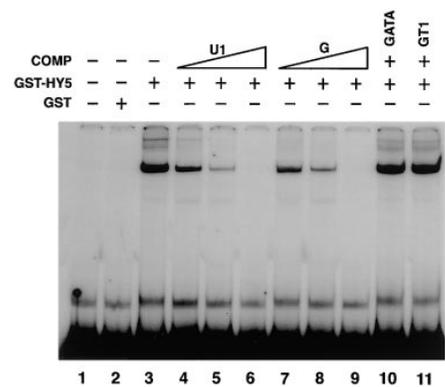
Figure 6. HY5 Binds Specifically to the Light-Responsive Unit 1 Region (U1) of a *CHS1* Promoter

A 180 bp fragment of the *CHS1* promoter containing U1 (Kaiser and Batschauer, 1995) was used as a probe. The amount of proteins added in the reactions were: lane 1, none; lane 2, 4 μg of GST; and lanes 3–11, 0.8 μg of GST-HY5. The amount of unlabeled competitors were: 80, 160, and 320 ng of U1 fragment and G-box tetramer (4G) in lanes 4–6 and lanes 7–9, respectively; and 320 ng of GATA and GT1 tetramers, in lanes 10 and 11, respectively. An autoradiograph of a typical mobility retardation assay is shown, and the DNA–protein complex is indicated by an arrow. DNA sequences of the promoter elements used are as follows: G = TGACACGTGGCA; GATA = AAGATAAGATT; GT1 = TGTGTGGTTAATATG.

is not clear at this point, the existence of nuclear COP1 speckles is physiologically relevant because similar patterns were detected using anti-COP1 antibodies in wild-type *Arabidopsis* protoplasts (A. von Arnim and X.-W. D., unpublished data). Furthermore, GFP-COP1 fusion proteins are functional in *Arabidopsis*, as evident by their ability to rescue a *cop1* null mutation (A. von Arnim and X.-W. D., unpublished data).

The physiological significance of the physical interaction between COP1 and HY5 during plant development is supported by the ability of the mutant HY5 (HY5-ΔN77), which no longer interacts with COP1, to evade COP1's negative regulation and result in hyperphotomorphogenic phenotypes (Figure 3). The antagonistic effects of HY5 and COP1 on lateral root development and *CHS* gene expression (Figures 4 and 5; Deng et al., 1991) further support the importance of HY5–COP1 interaction in regulating morphogenic development and gene expression. While our results support a role of protein–protein interaction between HY5 and COP1 in regulating lateral root development, they do not exclude alternative regulatory interactions between HY5 and COP1.

The bZIP domain of HY5 bears some similarity to the bZIP proteins that bind to DNA sequences containing an ACGT core motif, which are present in many *cis*-acting elements in the promoters of various stimulus-reponsive genes in plants (Oyama et al., 1997). Those ACGT core containing *cis*-acting elements include the TGACGT/C motif and the CACGTG palindromic G-box-like motif (Menkens and Cashmore, 1994). Since the palindromic G box and G-box-like elements are commonly found in the promoters of light-regulated genes such as *RBCS* and *CHS* (Menkens and Cashmore, 1994), we chose to investigate the interaction between HY5 and a G-box motif. In this study, we demonstrated that, in contrary to the repressive role of COP1, HY5 played a positive role in mediating light-activated anthocyanin accumulation and *CHS* gene expression (Figures 3 and 5). Further, HY5 specifically binds to the minimal light-responsive promoter unit (Unit 1) of the *CHS1* promoter via the G-box motif in vitro (Figure 6). Hence, the direct interaction of HY5 with both COP1 and the light-responsive promoter element suggests that COP1, HY5, and the light-responsive promoter element(s) may constitute a molecular cascade for mediating light control of gene expression. The very fact that both COP1 and HY5 act downstream of the multiple photoreceptors (McNellis and Deng, 1995) suggests that light signals perceived by the multiple photoreceptors are transduced via specific pathways to inactivate COP1 and/or activate transcription factors such as HY5. At this time, the available evidence cannot critically distinguish whether light signals regulate COP1 and HY5 independently, or affect one first that then regulates the other via protein–protein interaction. Further studies to examine the light control of cell- and tissue-specific changes in COP1–HY5 interactions in wild-type and various photomorphogenic mutant backgrounds are necessary to clarify this important issue.

The constitutive nuclear localization of HY5 as revealed by GFP-tagged HY5 in transgenic *Arabidopsis* (L.-H. A. and X.-W. D., data not shown) dictates the requirement of COP1 in the nucleus to regulate HY5's

activity. Consistent with this requirement, COP1 is most abundant in the nuclei of hypocotyl cells in darkness (von Arnim and Deng, 1994), where it could presumably inhibit HY5 and thus repress photomorphogenesis. With increasing light intensity, the nuclear abundance of COP1 is quantitatively decreased and the inhibitory effect on HY5 activity is reduced accordingly. Hence, the photomorphogenic developmental program is derepressed and these seedlings develop a de-etiolated phenotype. It should be noted that under normal laboratory conditions where light intensity ranges between 20 and 100 μmol/m²/s, a low but significant amount of COP1 persists in the nuclei and thus the inhibitory effect of COP1 on HY5 is expected. This would explain why the truncated HY5 that lacks the COP1-interactive domain could evade COP1's inhibition and result in hyperactivation of photomorphogenic development in seedlings grown under laboratory light-growth conditions (Figure 3).

The Photomorphogenic Repressor, COP1, Is Likely to Act on Multiple Transcription Factors to Mediate the Light Control of Seedling Development

Although the direct and functional interaction between COP1 and HY5 suggest that HY5 is an important link for achieving COP1-mediated light control of gene expression and photomorphogenic development, two major observations indicate that HY5 is not the sole partner. First, all *hy5* mutations, including nulls, only resulted in partial skotomorphogenic (etiolated) development in the light, suggesting that additional genes are involved in activating photomorphogenic development. Second, the overexpression of a constitutively active HY5 (HY5-ΔN77) only resulted in hyperphotomorphogenic responses in the light but not in darkness, indicating that a hyperactive HY5 alone is not sufficient to activate photomorphogenesis in darkness. Consistent with this notion, HY5 lacks the proline-rich and the acid-rich domains that are responsible for transcriptional activation activity in several well-characterized bZIP transcription factors (Schindler et al., 1992; Menkens and Cashmore, 1994) and is unable to activate transcription in yeast by itself (Figure 1B).

Based on this evidence, we proposed a working model shown in Figure 7. In the dark, COP1 may interact with multiple transcription factors such as HY5, X, and Y to inactivate their transcriptional activity, possibly by disrupting their contacts with the respective light-responsive promoter elements (LRE1 and LRE2) or altering their active conformations. Hence, it is not surprising that an elevated HY5 activity alone, such as overexpression of HY5-ΔN77, could not lead to photomorphogenic development in darkness since the parallel factors, X and Y, remained inactivated by COP1 in darkness. In the light, when the nuclear abundance of COP1 is reduced, the multiple transcription factors become active and proceed to activate transcription of the target genes. Consistent with this hypothesis, mutations in *COP1* resulted in pleiotropic phenotypes that include constitutive expression of light-regulated genes such as *RBCS*, *CHS*, and *CAB* in darkness (Deng et al., 1991).

Adding to this complexity, the potential to form heterodimers among the transcription factors would provide
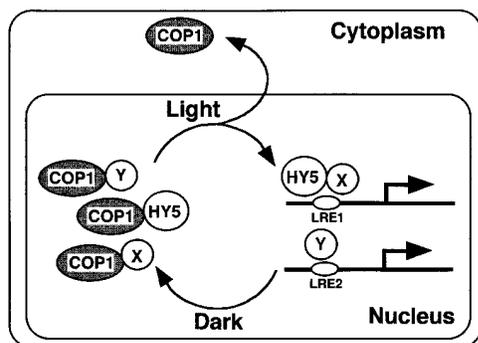
Figure 7. A Working Model that Illustrates the Antagonistic Roles of COP1 and HY5 in Light Control of Gene Expression

LREs represent light-responsive promoter elements. We propose that COP1 interacts with HY5 as well as other yet unidentified transcription factors (X and Y) in the dark or low intensity light to prevent these transcription factors from activating the target gene expression, while in high intensity light, as most of the COP1 is evacuated from the nucleus, these transcription factors become activated and act in concert to activate gene expression through specific LREs and thus lead to photomorphogenic development.

ample opportunities for cross talks and interactions among these parallel transcription factors. While HY5 is unable to activate transcription autonomously, HY5 may heterodimerize with other bZIP transcriptional activators (as indicated by X in Figure 7) to activate gene expression and morphogenic development. It is thus reasonable to suggest that the antagonistic interactions between COP1 and a series of transcription factors such as HY5, X, and Y may constitute a molecular switch that enables the plants to respond to changes in the light environment and adopt the most appropriate program for their growth and survival.

Experimental Procedures

Plant Materials and Growth Conditions
All *Arabidopsis* lines used are in Columbia ecotype with the exception of *hy5-1* (*Landsberg erecta*), the transgenic lines overexpressing full-length COP1 and N282 (Nossen), and the transgenic lines expressing *CHS1* promoters (Wassilewskija). When necessary, wild-type strains of multiple ecotypes were tested to ensure that a given phenotype is not due to ecotype variation. Plant germination and growth conditions in darkness and under various light sources were the same as previously described (McNellis et al., 1994a; Wei et al., 1994a). Light/dark cycle was 16 hr of white light at 75 μmol/m^2/s and 8 hr of darkness. The continuous red and blue light were at 73 and 15 μmol/m^2/s, respectively. The onion bulbs (Allium cepa) were purchased from local markets.

Recombinant Plasmids
DNA fragments were subcloned into pEG202 and pJG4.5 (Ausubel et al., 1994) to generate LexA-HY5 and AD-COP1 constructs. The HY5 and COP1 deletions generated by PCR amplification were verified by direct DNA sequencing. Full-length HY5 was cloned into pGEX4T-1 (Pharmacia Biotech) to express GST-HY5 fusion protein. To overexpress COP1 and HY5 in plant, DNA fragments were cloned into either pJS203 vector (J. Staub and X.-W. D., unpublished data) to give pRTL2COP1, pRTL2HY5, and pRTL2HY5-ΔN77 or pRTL2S65TGFP vector (A. von Arnim and X.-W. D., unpublished data) to give S65TGFP-COP1 and S65TGFP-HY5. All of the green fluorescent proteins used in this study contained the modified codon usage engineered to mutate the cryptic splicing sites (Haseloff and

Amos, 1995), and the pRTL2 constructs were under 35S CaMV promoters and terminators.

In Vitro Protein–Protein and Protein–DNA Binding Assays
GST and GST-HY5 fusion proteins were expressed in *E. coli* strain (BL21/DE3) and purified using glutathione-agarose beads (Sigma) according to Smith and Johnson (1988). The purification and labeling of the flag-tagged COP1 protein containing a HMK kinase domain with ^{32}P-ATP, as well as the binding assay were performed as previously described (Matsui et al., 1995) with minor modifications. In a typical binding assay, 5, 10, and 50 μl of the ^{32}P-labeled COP1 were mixed with 10 μg of glutathione-agarose beads bound GST-HY5 or GST and incubated at 4°C overnight. After extensive washing, the ^{32}P-labeled COP1 retained by the GST-HY5 and GST beads was size-fractionated on a 10% SDS–PAGE and analyzed by autoradiography.

The protein–DNA binding assay was performed in a final volume of 20 μl containing the binding buffer: 15 mM HEPES (pH 7.5), 35 mM KCl, 1 mM EDTA, 6% glycerol, 1 mM DTT, 0.2 mM MgCl$_2$, and 1 μg of poly dI-dC. We then added 1–1.5 ng of the 3'-end ^{32}P-labeled probes (180 bp fragment of the *CHS* promoter that includes the Unit 1 region: −247 to −68) to 4 μg of GST or 0.8 μg of GST-HY5. The reactions were incubated for 15 min at 25°C and were run in a 7% polyacrylamide gel before the resultant mobility of the probe was visualized by autoradiography.

Transient Expression in Onion Epidermal Cells
Cells in the epidermal layer of onion bulbs were transformed using biolistic bombardment essentially as described (Varagona et al., 1992), except that tungsten replaced gold particles, and 1100 psi rupture discs were employed. After bombardment, onion cell layers were incubated for 48 hr at 22°C in complete darkness. The cell layers were then mounted in water and examined by epifluorescence microscopy.

Yeast Two-Hybrid Assay
LexA-HY5, AD-COP1 recombinant constructs, and a reporter plasmid (pSH18-34) were transformed into a yeast strain EGY48 according to Chen et al. (1992). The selection for transformants and the assay for their β-galactosidase activities were essentially as described previously (McNellis et al., 1996). Relative β-galactosidase activities were calculated according to Ausubel et al. (1994). Expression of LexA-HY5 and AD-COP1 fusion proteins was examined by Western blot using anti-LexA and anti-HA antibodies, respectively.

Generation of *Arabidopsis* Transgenic Lines
pRTL2HY5 expression construct was cloned into the binary plant transformation vector pBIN19 (Bevan, 1984) and introduced into *Arabidopsis* via *Agrobacterium*-mediated root transformation as described (Deng et al., 1991). The pRTL2HY5-ΔN77 expression construct was cloned into the binary plant transformation vector pPZP222 (Hajdukiewicz et al., 1994) and introduced into *Arabidopsis* via *Agrobacterium*-mediated vacuum infiltration (Bechtold and Bouchez, 1995). Transgenic lines containing a single transgene locus per haploid genome for pRTL2HY5 (5) and pRTL2HY5-ΔN77 (4) were generated, and the number of independent lines analyzed for each construct was represented by the number in parentheses. The four transgenic lines that overexpressed HY5-ΔN77 also segregated seedlings with hyperphotomorphogenic phenotype: wild-type in a 3:1 (279:93) ratio among their T2 progeny. Furthermore, an exact cosegregation of the mutant phenotype with transgene was observed: 100% (n = 68) of the seedlings with mutant phenotype possessed the transgene, and 100% (n = 51) of their wild-type siblings did not.

Fluorescence and Confocal Microscopy
Chloroplast autofluorescent images of 6-day-old hypocotyls and 3-week-old roots were taken with a DAPI filter on a Zeiss Axiophot microscope and a rhodamine filter on a Bio-Rad confocal microscope, respectively. Fluorescent images of the various green fluorescent proteins were taken with a 20× objective on a Zeiss Axiophot microscope. The filter set used for S65TGFP was: 480/40

excitation, 505LP dichroic, 535/50 emission (the numbers indicate midpoint wavelength/bandwidth in nm, respectively). Best results were obtained with Ektachrome 64T films (Kodak). Both the DAPI filter and the GFP filter set were from Chroma (Chroma Technology Corp., Brattleboro, VT).

GUS Staining and GUS Activity Measurement

The same procedures as described previously (Puente et al., 1996) were used for GUS histochemical staining and quantitative GUS activity assay. The wild-type and the mutant plants carrying the same transgene were stained with the identical procedure for the same length of time, and the quantitative GUS assay is performed on the aerial portion (which includes hypocotyls and cotyledons) of the seedlings.

Acknowledgments

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