The Plant Cell, Vol. 10, 1083–1094, July 1998, www.plantcell.org © 1998 American Society of Plant Physiologists

RESEARCH ARTICLE

Role of a COP1 Interactive Protein in Mediating Light-Regulated Gene Expression in Arabidopsis

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Arabidopsis seedlings display distinct patterns of gene expression and morphogenesis according to the ambient light condition. An Arabidopsis nuclear protein, CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1), acts to repress photomorphogenesis in the absence of light. The Arabidopsis CIP7 protein was identified by its capability to interact with COP1. CIP7 is a novel nuclear protein that contains transcriptional activation activity without a recognizable DNA binding motif. *CIP7* requires light for its high level of expression, and COP1 seems to play a role in repressing its expression in darkness. Decreasing *CIP7* expression by introducing antisense CIP7 RNA resulted in defects in light-dependent anthocyanin and chlorophyll accumulation. Antisense plants also displayed reduced expression of light-inducible genes for anthocyanin biosynthesis and photosynthesis. However, no defect was observed in light-dependent inhibition of hypocotyl elongation. Taken together, our data indicate that CIP7 acts as a positive regulator of light-regulated genes and is a potential direct downstream target of COP1 for mediating light control of gene expression.

INTRODUCTION

One of the unique features of plants is developmental plasticity. As sessile organisms, plants have developed strategies to sense the ambient environment for optimizing their growth and survival. Perhaps because plant growth depends on light as the energy source, the light environment is one of the most important signals for regulating a seedling's developmental program (reviewed in Kendrick and Kronenberg, 1994; von Arnim and Deng, 1996a). For example, dicotyledonous seedlings germinated in the dark follow an etiolated pattern, with elongated hypocotyls and undeveloped and closed cotyledons. This is necessary so that plants can grow through the soil or fallen leaves to reach the light. Light-grown seedlings have open and green cotyledons with well-developed chloroplasts for photosynthesis. At least three families of photoreceptors are involved in perceiving light and modulating the developmental program. In Arabidopsis, several of those photoreceptors have been well characterized both genetically and physiologically. For example, phytochrome A (PHYA), phytochrome B (PHYB), and cryptochrome1 (CRY1/HY4) have been shown to be responsible for sensing distinct wavelengths of the high-irradiance light signal (Ahmad and Cashmore, 1993; Mohr, 1994; Quail, 1994; Quail et al., 1995). It is well established that the perception of light by photoreceptors can lead to changes in gene expression patterns. This includes genes for photosynthesis and biosynthesis of protective materials against light stress (Thompson and White, 1991; Miller et al., 1994).

Genetic screens for constitutive photomorphogenic or deetiolated development in darkness resulted in the identification of at least 10 pleiotropic Arabidopsis *COP*/*DET*/*FUSCA* (*FUS*) loci (Chory et al., 1989; Deng et al., 1991; Wei and Deng, 1992; Miséra et al., 1994; Wei et al., 1994b; Kwok et al., 1996). All loss-of-function mutations in those genes result in similar pleiotropic photomorphogenic phenotypes in the dark and include activation of light-inducible genes, anthocyanin accumulation, chloroplast development, epidermal cell differentiation, inhibition of hypocotyl elongation, and cotyledon opening. All of these characteristics are observed only in wild-type plants that have been light grown. From genetic analyses, this class of genes seems to act downstream of the multiple photoreceptors, including *PHYA*, *PHYB*, and *CRY1* (Ang and Deng, 1994; Wei et al., 1994a; Kwok et al., 1996; Pepper and Chory, 1997). Among

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this group of genes, four have been molecularly characterized: *COP1*, *DET1*, *COP9*, and *FUS6*/*COP11* (Deng et al., 1992; Castle and Meinke, 1994; Pepper et al., 1994; Wei et al., 1994b), and all were shown to encode nuclear proteins (Pepper et al., 1994; von Arnim and Deng, 1994; Chamovitz et al., 1996; Staub et al., 1996).

Recent analysis suggests that the subcellular localization of COP1 can be regulated by light (von Arnim and Deng, 1994). When COP1 is fused with a reporter protein, β-glucuronidase (GUS), the GUS–COP1 fusion protein was observed mainly in the nucleus in the absence of light. After light perception, the fusion protein was not detectable in the nucleus. The fact that the total amount of the cellular COP1 protein was not changed by light suggests that light caused a specific redistribution of COP1 within the cell. Subsequently, it was demonstrated that the nuclear localization of the GUS–COP1 fusion is diminished in the other nine *COP*/*DET*/*FUS* loci even in the dark (Chamovitz et al., 1996; von Arnim et al., 1997). The fact that COP1 alone can act autonomously to suppress photomorphogenesis and that this ability is highly dependent on its cellular abundance (McNellis et al., 1994b) suggests that COP1 is part of a key regulatory step responsible for repression of photomorphogenesis (Torii and Deng, 1997). Furthermore, the data support the view that COP1 acts within the nucleus to suppress photomorphogenic development in the dark and that the other nine loci are required for its proper nuclear localization or stability. The perception of light results in the inactivation of COP1 and reduction of its nuclear abundance.

An understanding of how COP1 suppresses photomorphogenesis in the nucleus is thus of great interest. COP1 contains several characteristic motifs (Deng et al., 1992; McNellis et al., 1994a). They include a zinc binding motif called a RING finger (Saurin et al., 1996), a putative coiled-coil region (COIL), and WD40 repeats (Neer et al., 1994). Based on the fact that COP1 is a repressor of a set of light-inducible genes (Deng et al., 1991) and shares homology with known RING finger proteins and WD-40 (Deng et al., 1992), it was hypothesized that COP1 may interact with and inhibit the aspecific transcriptional activator(s) for the target promoters or directly contact target promoters to regulate their expression (von Arnim and Deng, 1996b). The recent finding that COP1 interacts with HY5, a basic leucine zipper (bZIP)–type transcription factor and a positive regulator of photomorphogenesis and light-inducible gene expression (Ang et al., 1998; Chattopadhyay et al., 1998), supports the first hypothesis given above. However, the partially deetiolated phenotype observed in null *hy5* mutants suggests that HY5 is only one of the possible COP1 targets. Furthermore, the fact that all of the recognizable domains of COP1 are supposed to be protein–protein interaction modules is also consistent with the possibility that COP1 may interact with multiple targets in mediating its pleiotropic effects. Here, we report the identification and analysis of one such protein, CIP7 (for COP1 interaction protein 7), that most likely plays a role in mediating the light activation of gene expression as a transcription factor and may serve as one of the targets for COP1.

RESULTS

Isolation and Characterization of a COP1-Interacting cDNA Clone, *CIP7*

To isolate proteins interacting with COP1, we screened an Arabidopsis cDNA library with a ^{32}P-labeled COP1 protein as a probe. Full-length COP1 fused with a Flag epitope (see Methods), and a heart muscle kinase (HMK) phosphorylation site was expressed in *Escherichia coli* and purified using the Flag affinity resin. The purified protein was labeled with γ–^{32}P-ATP, using HMK to high specific activity, and used to screen 3×10^6 independent cDNA clones of dark-grown Arabidopsis seedlings by a filter binding assay (Matsui et al., 1995). Among the clones recovered, three were confirmed subsequently to specifically bind to the COP1 protein. One clone, CIP7, contains one long open reading frame encoding 671 amino acid residues in frame with *LacZ* in the λgt10 vector. Because this cDNA clone was clearly missing the 5′ end of the mRNA, it was used as a hybridization probe to screen for another size-fractionated cDNA library. Ten cDNA clones were isolated from this second screening, including two independent clones containing the entire open reading frame, as indicated by the presence of stop codons upstream and downstream of the longest open reading frame of 1058 amino acids (Figure 1A). RNA gel blot analysis of *CIP7* revealed a single mRNA species of ~3.9 kb (see Results), whose length is reasonably close to that of the longest cDNA clone (3563 bp). This suggested that the isolated cDNA clone covers most of the *CIP7* transcript. DNA gel blot analysis using the *CIP7* cDNA as a probe for the total Arabidopsis genomic DNA digested with BglII, SacI, and XbaI indicated that CIP7 is encoded by a single-copy gene (data not shown).

The predicted protein sequence of CIP7 revealed several features commonly associated with other transcription factors (Figures 1B and 1C): CIP7 contains a region rich in proline and glutamine, two acidic clusters, and two putative nuclear localization signals. A computer analysis using the PSORT program (http://psort.nibb.ac.jp/) predicted that CIP7 is a nuclear protein. Besides these domains shared with other transcription factors, CIP7 also contains a lysine-rich region and two putative COILs. CIP7 has no strong homology with any proteins in the GenBank and EMBL databases, which indicates that it is a novel protein.

The Predicted Helical Domain of COP1 Is Critical for Mediating Its Interaction with CIP7

To identify the CIP7-interacting domain in COP1, we subjected several COP1 deletion proteins to an in vitro column

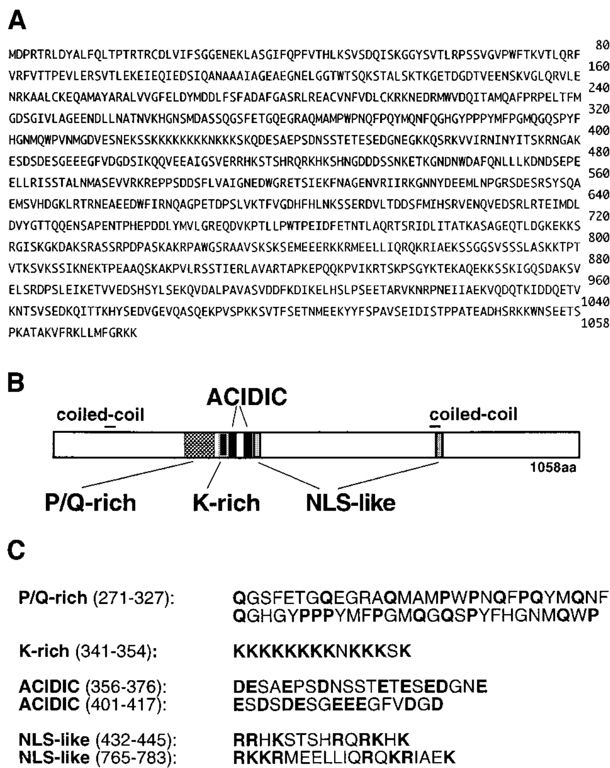

Figure 1. Sequences and Characteristics of the CIP7 Protein.

(A) Complete CIP7 amino acid sequence.

(B) Schematic diagram of the relative locations of seven recognizable sequences or secondary structural motifs. aa, amino acid; NLS, nuclear localization signal.

(C) The primary sequences of the motifs diagrammed in **(B)**. Numbers in parentheses indicate amino acids.

binding assay. The original CIP7 (amino acids 387 to 1058) clone from SK-CIP7 was fused to either the maltose binding protein (MBP) or glutathione *S*-transferase (GST), expressed in *E. coli*, and charged to the amylose or glutathione resin. A series of COP1 deletion proteins labeled with phosphorus was applied to the prepared resin, and we analyzed the COP1 proteins that were retained. After washing the resin to remove nonspecific binding proteins, we subjected the resin to SDS-PAGE and subsequently to autoradiography. Both CIP7 fusions gave the same results, and the results with the GST–CIP7 fusion are shown in Figure 2B. The results indicated that when compared with the GST negative control (lane 6), full-length COP1 (COP1 in Figure 2A) binds to GST–CIP7 with higher specificity (Figure 2B, lane 7). Hence, this result confirms the direct interaction between COP1 and CIP7 in vitro. Several COP1 deletion proteins, including a RING finger–deleted COP1 (COP1ΔZn), the N-terminal 282 amino acids of COP1 without the entire WD-40 repeats (N282), and the N282 domain with RING finger motif or COIL motif deleted (NΔZn and NΔCOIL), were used to delineate the COP1 domain responsible for mediating the CIP7 interaction. The studies revealed that all of the COP1 mutants (Figure 2B, lanes 9, 11, and 13, respectively), except for NΔCOIL (lane 15), bound to GST–CIP7. This result suggests that the COIL region is essential for COP1–CIP7 interaction and that a small portion of COP1 consisting mostly of the coil domain (NΔZn) is sufficient to interact with CIP7. It should be noted that the different forms of COP1 mutant proteins were labeled with slightly different efficiency, and thus, the relative

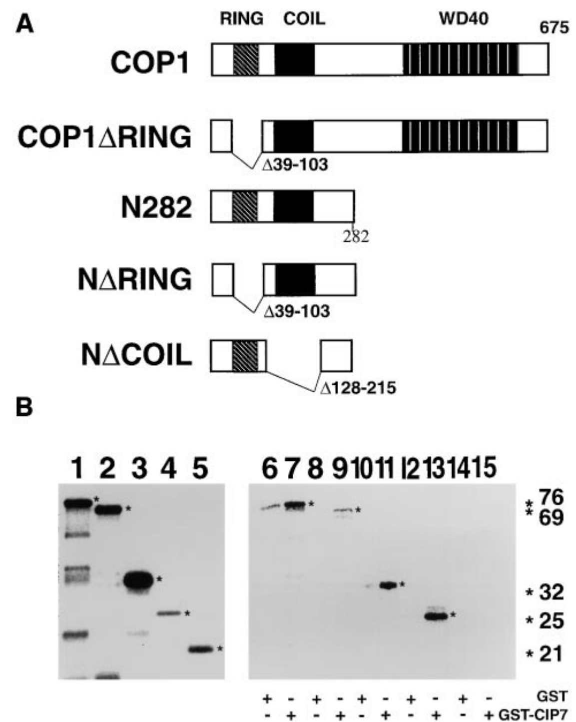

Figure 2. Determination of the CIP7 Interaction Domain of COP1.

(A) Schematic diagram of the COP1 deletion series used for the CIP7 binding assay.

(B) Relative binding affinity of the COP1 mutant proteins to the GST–CIP7 protein. The radiolabeled COP1 deletion proteins (see **[A]**) for test are shown in lanes 1 to 5. Lane 1 contains COP1; lane 2, COP1ΔRING; lane 3, N282; lane 4, NΔRING; and lane 5, NΔCOIL. These five COP1 deletion proteins were incubated with glutathione–sepharose beads charged individually with GST (lanes 6, 8, 10, 12, and 14) or GST–CIP7 (lanes 7, 9, 11, 13, and 15) and washed extensively. The bound COP1 probes were subjected to SDS-PAGE. The COP1 probes used were COP1 in lanes 6 and 7, COP1ΔRING in lanes 8 and 9, N282 in lanes 10 and 11, NΔRING in lanes 12 and 13, and NΔCOIL in lanes 14 and 15. Asterisks and the numbers at right indicate the predicted positions and sizes (in kilodaltons) of the COP1 proteins.

intensity of the bands in Figure 2B does not necessarily reflect the binding affinity of a given COP1 protein to CIP7.

CIP7 Is a Nuclear Protein

To serve as a downstream target of the nuclear repressor COP1, CIP7 would have to colocalize with COP1 at least in the dark. Indeed, the primary sequence of CIP7 suggests that it is a nuclear protein. To test this localization and to study the possible regulation of its nuclear localization, we analyzed the subcellular localization of CIP7 with the aid of the green fluorescent protein (GFP) (Chalfie et al., 1994). A derivative of GFP, which can be expressed in Arabidopsis (Haseloff et al., 1997) and has a S65T modification for stronger fluorescence (Heim et al., 1995), was fused to a full-length CIP7 clone and placed under the control of the cauliflower mosaic virus (CaMV) 35S promoter for ubiquitous and high expression. The fusion gene, named *GFPS65T–CIP7*, was stably introduced into Arabidopsis. As reported previously, GFP alone accumulates in the nucleus as well as in the cytoplasm (Figures 3A to 3D and 3G; Haseloff and Amos, 1995; Grebenok et al., 1997; Haseloff et al., 1997). This is likely due to the fact that small molecules $<$40 kD can go through the nuclear pore by passive diffusion (Forbes, 1992) and that free GFP is only 27 kD. However, by increasing the molecular mass of GFP to $>$100 kD by fusing it to a neutral protein with no organellar targeting signal, the protein was localized exclusively in the cytoplasm (Grebenok et al., 1997). When GFP was fused to CIP7, with a final size of 141 kD, the fusion protein localized exclusively in the nucleus, demonstrating the presence of a functional nuclear localization signal within the CIP7 sequence (Figures 3E and 3H). Furthermore, its nuclear localization did not seem to depend on the light condition or cell type (Figures 3E, 3F, 3H, and 3I; data not shown). Thus, CIP7 is most likely a constitutively nuclear-localized protein.

CIP7 Contains a Transcriptional Activation Domain

As a possible nuclear target of COP1, CIP7 may be directly involved in regulating transcriptional activity. Also, the primary structure of CIP7 is consistent with its being a transcription factor. Thus, we attempted to analyze whether CIP7 has the ability to regulate transcription. Because CIP7 does not have a recognizable DNA binding domain, the DNA binding domain of LexA was fused to the original CIP7 C-terminal clone (amino acids 387 to 1058) (Figure 4A). We first assayed its ability to activate transcription of a *LacZ* reporter gene under the control of the *LexA* operator in yeast (Figure 4A). The result (Figure 4B) indicates that the LexA–CIP7 fusion protein itself can activate transcription of the reporter gene as much as 53-fold, indicating that this portion of CIP7 (amino acids 387 to 1058) contains a potent transcriptional activation domain in yeast. The positive and negative controls, LexA–GAL4 (amino acids 74 to 881) and LexA–Bicoid (amino acids 2 to 160) (Figures 4A and 4B), respectively, behaved as expected (Ausubel et al., 1987).

To test whether CIP7 can also activate transcription in plant cells, we developed an improved GAL4 system as a plant chimeric transactivation assay (see Methods). Both the full-length CIP7 and the C-terminal half of CIP7 (amino acids 387 to 1058) were fused to the DNA binding domain of GAL4 (amino acids 1 to 147) (Figure 5A) and introduced into tobacco leaves together with a reporter plasmid encoding the luciferase reporter protein under the control of a GAL4 operator (Figure 5A) by using the microprojectile bombardment method. After 2 days of post-transfectional incubation, expression of the reporter gene was determined by enzymatic assay for luciferase activity. The transformation effi-

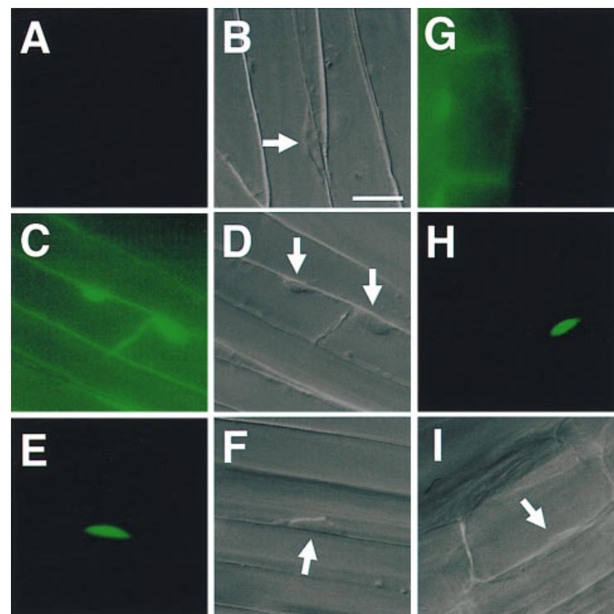

Figure 3. GFP–CIP7 Is a Nuclear Localized Protein.

(A) and **(B)** Hypocotyl cells of wild-type nontransformants used as negative controls.
(C) and **(D)** Hypocotyl cells or root cells of GFP-expressing transgenic plants.
(E) and **(F)** Hypocotyl cells of GFPS65T–CIP7—expressing plants.
(G) Root cells of GFP-expressing transgenic plants.
(H) and **(I)** Root cells of GFPS65T–CIP7—expressing plants.
Green fluorescence under blue light excitation (**[A]**, **[C]**, **[E]**, **[G]**, and **[H]**) or corresponding differential interference contrast of the same field (**[B]**, **[D]**, **[F]**, and **[I]**, except for that of **[G]** from representative transgenic seedlings) is shown. GFP itself freely diffused between the nucleus and the cytosol and accumulated in both compartments (**[C]** and **[G]**), whereas GFPS65T–CIP7 localized exclusively in the nucleus (**[E]** and **[H]**). The magnification is the same for **(A)** to **(I)**, and the bar in **(B)** indicates 16 μm. Arrows mark the positions of the nuclei.

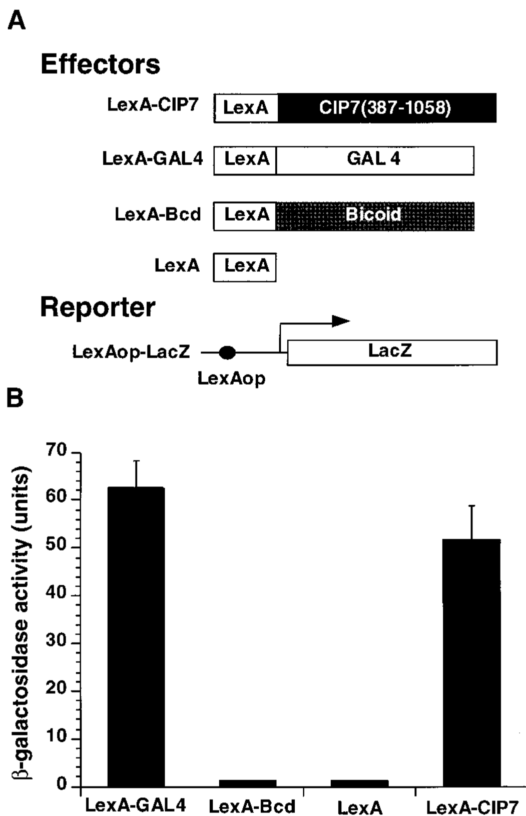

Figure 4. CIP7 Exhibits Transcriptional Activation Activity in Yeast.

(A) Diagrams of the effector proteins tested and the reporter construct.

(B) CIP7 can confer LexA-dependent transactivation to the reporter gene.

The reporter plasmid LexAop–LacZ (*LexA* operator sequence in the promoter of the reporter *LacZ*) was introduced into a yeast strain (EGY48-0) together with effector plasmids encoding LexA (pEG202), LexA–GAL4 (amino acids 74 to 881), LexA–Bicoid (amino acids 2 to 160), or LexA–CIP7 (amino acids 387 to 1058), respectively. LacZ expression of the transformants was determined by directly assaying LacZ enzymatic activity. The LexA–CIP7 fusion activated the reporter gene to 53-fold higher activity levels than did LexA alone. The error bars indicate the standard deviation of the mean from at least four independent repeats.

ciency was monitored by introducing another reporter construct, *GUS*, driven by a constitutive CaMV 35S promoter derivative (Figure 5A), together with the effector and the reporter constructs. The result shown in Figure 5B indicates that although full-length CIP7 did not elevate reporter activity, the C-terminal half of CIP7 (amino acids 387 to 1058), which activated transcription in yeast, can also activate transcription in tobacco. However, because of the relatively large variation of the transient assay system, it was not possible to obtain definitive evidence for light regulation of the observed CIP7 transactivating activity (data not shown). Nevertheless, the result indicates that CIP7 contains a transcriptional activator domain between amino acids 387 to 1058 that can function in plants as well as in yeast. It is possible that in the full-length protein, the activation domain could be masked somehow by a regulatory domain or improper folding. Similar phenomena have been reported elsewhere (Ma et al., 1988; Tamaoki et al., 1995).

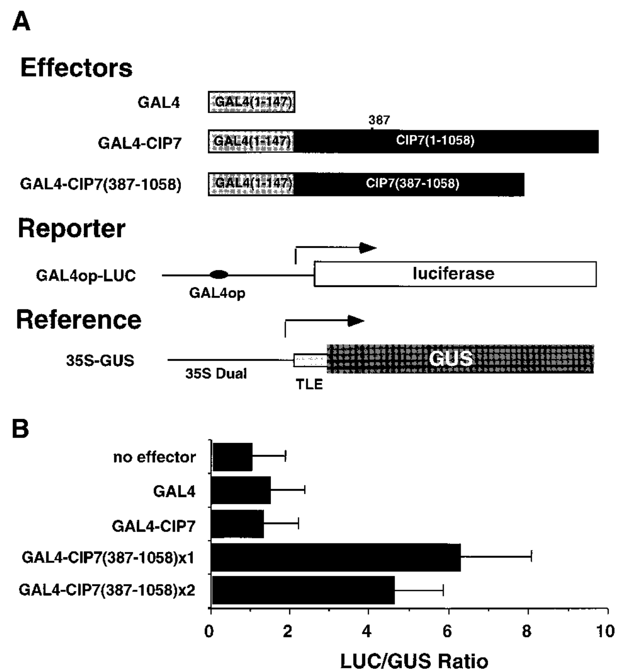

Figure 5. CIP7 Exhibits Transcriptional Activation Activity in Plant Cells.

(A) Diagrams of the effector proteins tested and reporter and reference constructs.

(B) GAL4-dependent chimeric transactivation assay in tobacco.

The reporter and reference plasmids, together with an individual effector, were introduced by microprojectile bombardment into tobacco leaves. The reporter gene promoter contains 17 copies of the GAL4 binding site (GAL-4op). The reference plasmid (which has two copies of the 35S promoter, marked as 35S Dual) serves to monitor the transformation efficiency. Transient expression of the reporter and reference genes was determined by enzymatic assays, and reporter expression was normalized to the reference expression as luciferase (LUC)/GUS ratio (right). Whereas the GAL4 fusion with CIP7 full length (amino acids 1 to 1058) had no effect on transcription, the fusion with a C-terminal half of CIP7 (amino acids 387 to 1058) activated the reporter expression up to sixfold in this system. Two concentration levels of the effector constructs (×1 and ×2) were used for GAL4–CIP7 (amino acids 387 to 1058). The error bars indicate the standard deviation of the mean from at least four independent repeats. op, operator sequence; TLE, translation leader enhancer sequence.

Expression of CIP7 Is Regulated by Light and COP1

To examine the direct involvement of CIP7 in the light regulation of gene expression, we first tested whether its own expression is regulated by light. *CIP7* mRNA levels in 5-day-old light- and dark-grown seedlings were analyzed by RNA gel blotting. The results showed that CIP7 is highly regulated by light (Figure 6). The *CIP7* mRNA level was very low in dark-grown seedlings and elevated at least 20-fold in the light (Figure 6A). This result implies that *CIP7* expression is light regulated and more abundant in the light, which is consistent with a possible role as a positive regulator of photomorphogenic development. Because COP1 is involved in repressing light-inducible genes in darkness (Deng et al., 1991), we examined whether CIP7 expression is also affected by a *cop1* mutation. As shown in Figure 6A, *CIP7* mRNA levels were partially elevated in the dark-grown *cop1* seedlings and were approximately three- to fivefold higher than those in dark-grown wild-type seedlings. This increase in *CIP7* mRNA could not have been due to unequal loading, because the rRNA levels in each lane are very similar (Figure 6, bottom). This moderate activation of *CIP7* in the dark-grown *cop1* mutants is somewhat similar to that observed for the chlorophyll *a*/*b* binding protein (*Cab*) genes (Deng et al., 1991). This suggests that COP1 is involved in maintaining the low-level expression of *CIP7* in darkness.

The repression of light-inducible gene expression during dark adaptation is another physiological process regulated by COP1 (Deng et al., 1991). Thus, we examined *CIP7* expression during dark adaptation (Figure 6B). Five-day-old seedlings grown under continuous light were transferred to darkness for 2 days and then subjected to RNA gel blot analysis. Seedlings grown in the light for 7 days were included as controls. The results indicate that during dark adaptation, there is a decrease in *CIP7* expression, and this response is similar to those of many other light-regulated genes.

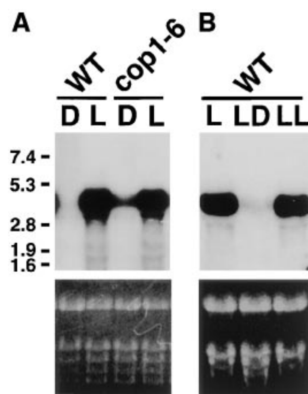

Figure 6. CIP7 Expression Is Regulated by Light and COP1.

(A) Equal amounts of total RNA from 5-day-old seedlings of wild type (WT) and *cop1-6* mutants grown under dark (D) and continuous light (L) were subjected to RNA gel blot analysis by using an antisense CIP7 riboprobe.

(B) Similar mRNA gel blot with total RNA samples from 5-day-old wild-type seedlings grown under continuous light (L) and then transferred to the dark (LD) or left in the light (LL) for 2 days.

Numbers at left indicate positions of size markers in kilobases.

Partial Suppression of CIP7 Expression by the Antisense Transgene Results in Decreased Anthocyanin and Chlorophyll Accumulation

To determine the physiological role of CIP7 in light regulation, we placed the C-terminal half of the *CIP7* cDNA in antisense orientation under the control of CaMV 35S promoter and stably introduced it into Arabidopsis. A total of 22 independent transgenic lines was generated and exhibited similar phenotypes in the T_2 generation. Two lines, αCIP7-22a and αCIP7-27d, were used for detailed analysis. Although no detectable effect was observed in hypocotyl elongation or cotyledon expansion of antisense plants grown in far-red, red, blue, and white light or darkness (Figure 7A and data not shown), the anthocyanin accumulation at the upper end of the hypocotyl just below the cotyledons, known as the anthocyanin ring, was reduced in the white light– and blue light–grown antisense lines when compared with the wild-type seedlings (data not shown). A quantitative analysis of white light–grown transgenic seedlings revealed 32.5 and 39.9% reduction of the total anthocyanin accumulation in αCIP7-22a and αCIP7-27d lines, respectively (Figure 7B). This effect is similar to that of the COP1 overexpression line but opposite that of the *cop1* mutants.

To examine whether chloroplast development, which is influenced by COP1 (Deng et al., 1991), was also affected in the antisense lines, we measured chlorophyll accumulation. As shown in Figure 7C, chlorophyll accumulation in αCIP7-22a and αCIP7-27d lines was reduced to 71.2 and 68.5%, respectively, of the wild-type level. This reduced chlorophyll content is consistent with slightly pale green cotyledons in the white light– and blue light–grown transgenic seedlings. Here again, CIP7 appears to play a role opposite that of COP1.

Antisense *CIP7* Transgenic Plants Display Defects in the Light Control of Gene Expression

To test the effects of the antisense *CIP7* transgene on the light control of gene expression, we harvested light-grown seedlings of the wild type, αCIP7-22a, and αCIP7-27d and subjected them to RNA gel blot analyses. As shown in Figures 7D and 7E, relative to the wild-type sibling, *CIP7* expression was suppressed to 61.1 and 34.9% in αCIP7-22a and αCIP7-27d, respectively. This indicates that in the αCIP7-27d line, *CIP7* expression is more suppressed than

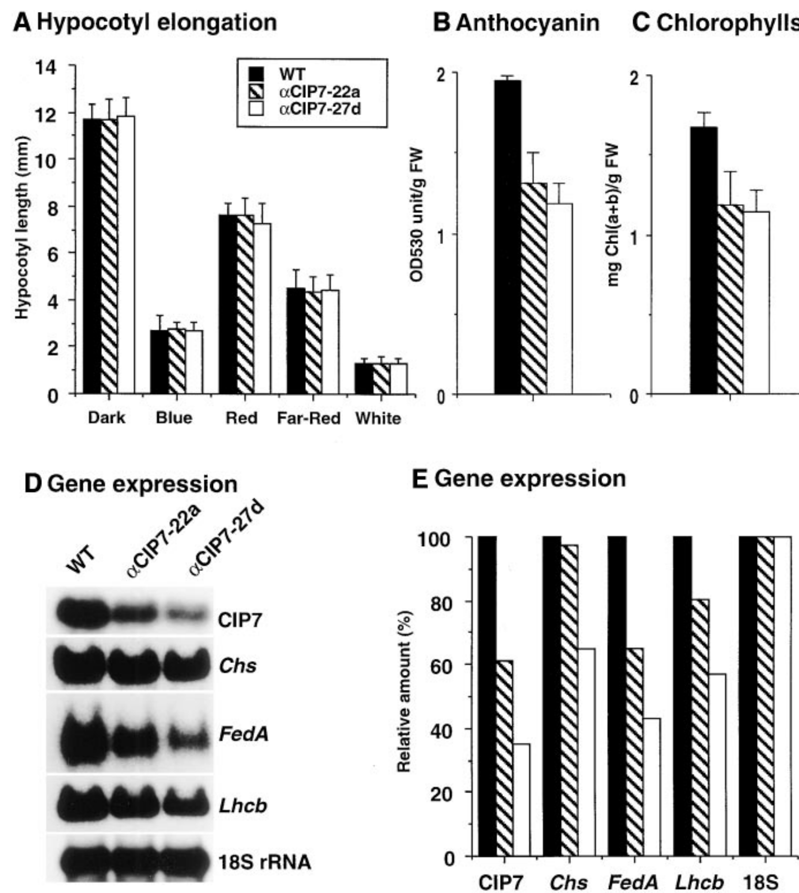

Figure 7. *CIP7* Antisense Transgenic Lines Display Reduced *CIP7* mRNA Levels, Lower Light–Induced Gene Expression, and a Reduction in Anthocyanin and Chlorophyll Levels.

(A) Hypocotyl elongation under various light conditions. Twenty-five to 50 seedlings were grown for 5 days under far-red light or 6 days in darkness or red, blue, or white light. After these growth periods, the hypocotyls were measured. Error bars indicate the standard deviation. WT, wild type.

(B) and **(C)** Anthocyanin and chlorophyll (Chl) accumulation. The average of three experiments and the standard deviations are shown. Chlorophyll *a*/*b* ratios are 3.10 (wild type), 3.08 (αCIP7-22a), and 3.06 (αCIP7-27d). FW, fresh weight.

(D) and **(E)** Autoradiography of an RNA gel blot and its quantitative analysis. Equal amounts of total RNA from 5-day-old light-grown seedlings were analyzed. An 18S rRNA probe was used as a control. The radioactivity of each band in **(D)** was measured by using a PhosphorImager (Fujix BAS2000) and normalized with the corresponding signal of 18S rRNA. The average of two experiments is shown. WT, wild type.

that in the αCIP7-22a line, and in both lines, the suppression level is relatively moderate. This order of suppression of *CIP7* expression in antisense lines (αCIP7-27d > αCIP7-22a) is consistent with the order of reduction in anthocyanin and chlorophyll accumulation (Figures 7D and 7C). On the same order, chalcone synthase (*Chs*), *FedA*, and light-harvesting complex *Lhcb* gene expression was also not fully activated in the light, which is consistent with a similar reduction in pigment accumulation. This result suggests that the partial suppression of *CIP7* expression caused a defect in light-dependent gene activation of at least *Chs*, *FedA*, and *Lhcb*. This suppression may be responsible for the observed reduction in anthocyanin biosynthesis and chloroplast development.

DISCUSSION

Here, we report the characterization of CIP7 and provide evidence supporting its role as a possible downstream target of COP1 in mediating light control of gene expression. First, not only was CIP7 isolated as a COP1 interacting protein but the specific interaction with COP1 was further confirmed by

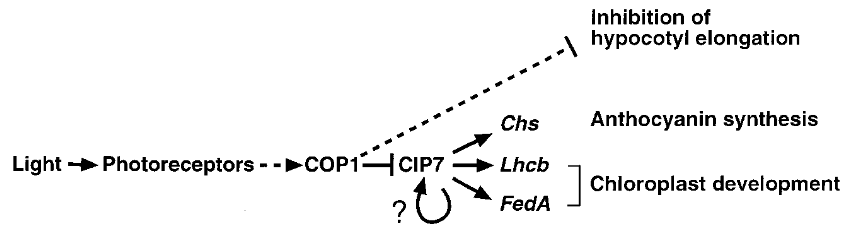

Figure 8. Proposed Role of CIP7 in Light Control of Gene Expression and Development.

It has been proposed that CIP7 may be specifically involved in the regulation of gene expression, including its own expression, in response to light and COP1. However, the CIP7 protein may not be involved in COP1-mediated light inhibition of hypocotyl elongation.

additional in vitro binding assays. Also, the CIP7 protein's nuclear localization and its ability to activate transcription in both yeast and plant cells when brought in proximity to a promoter support the possibility that it may be a nuclear target of COP1. Several lines of additional evidence support CIP7 as being a physiological COP1 target. *CIP7* antisense plants exhibited reduced anthocyanin and chlorophyll accumulation as well as reduced expression of light-inducible genes. This suggests a role for CIP7 in mediating the light control of gene expression. Because these characteristics represent a subset of the phenotypes regulated by COP1, the observation is consistent with the notion that COP1 may mediate part of its light-regulated gene expression through CIP7. Finally, the expression of *CIP7* is regulated by both light and COP1, supporting its direct involvement in light regulation and acting downstream of COP1.

The fact that a reduction in *CIP7* expression in antisense *CIP7* transgenic lines resulted in a partial and contrasting phenotype relative to *cop1* mutants implies antagonistic roles for CIP7 and COP1. It suggests that the interaction of COP1 with CIP7 results in the negative regulation of *CIP7*; for example, COP1 acts to inhibit CIP7 activity (Figure 8). Considering that COP1 is most abundant in the nucleus in darkness and is reduced in proportion to the increase in light intensity, its ability to inhibit CIP7 activity would be maximal in darkness and quantitatively reduced with increasing light signals. Currently, there is no indication of the biochemical nature of this regulatory interaction. At least two types of mechanisms could serve the regulation function. The first is a simple "titration" mechanism in which COP1 sequesters CIP7 in the dark or low light to suppress CIP7 activity, whereas light triggers the quantitative decrease of nuclear COP1, and thus the proportional release and activation of CIP7. The second possibility is that COP1 may transiently interact with CIP7, resulting in either covalent or conformational modifications of CIP7 to repress its activity. Regardless of which mechanism is used, the light dependence of *CIP7* expression could also be utilized to enhance the light-inducible gene expression of other light-regulated genes. It is reasonable to assume that low expression levels of *CIP7* in dark-grown plants, as well as in dark-adapted light-grown plants, are responsible for the initial induction of gene expression right after light perception. Thus, this initial induction would not require new protein synthesis and could be very rapid. With time, light enhances *CIP7* expression, possibly through positive feedback regulation, and thus could dramatically enhance the availability of CIP7.

Because antisense *CIP7* plants have no effect on the light-dependent inhibition of hypocotyl elongation, we suggest that COP1 may also regulate other downstream factors in addition to CIP7. Indeed, several other possible downstream targets have been identified. For example, another clone that was isolated together with CIP7 displays light-regulated expression and contains a transcriptional activation domain, although its physiological role still needs to be confirmed (Y.Y. Yamamoto, M. Matsui, and X.-W. Deng, unpublished results). Also, the genetically defined positive regulator of photomorphogenesis, *HY5*, which has been shown to encode a bZIP protein (Oyama et al., 1997), directly binds to light-responsive promoters and directly interacts with COP1 (Ang et al., 1998; Chattopadhyay et al., 1998). Clearly, HY5 represents another physiological target of COP1 action. The work presented here adds to an emerging picture in which COP1 acts within the nucleus in the dark to directly regulate transcription factors, such as CIP7 and HY5, through protein–protein interactions. The perception of light may lead to the breakdown of those regulatory interactions and nuclear depletion of COP1, thus resulting in altered patterns of gene expression and development.

METHODS

Plant Growth

Surface sterilization of seeds and pretreatment before germination were performed according to Hou et al. (1993). The plated seeds in plastic dishes were kept at 4°C in the dark for 2 to 4 days for vernalization, exposed to light at room temperature for 1 hr, and placed under appropriate light conditions for growth at 22°C. The hypocotyl elongation assays under monochromatic light conditions were described previously (McNellis et al., 1994b). Throughout this study, *Arabidopsis thaliana* ecotype Columbia (Col-0) was used as the wild type.

Isolation and Sequence Analysis of the *CIP7* cDNA

The purification and labeling of the Flag-tagged constitutive photomorphogenic COP1 protein containing a heart muscle kinase (HMK) domain were performed as previously described (Matsui et al., 1995), with minor modifications. Briefly, after expression in BL21(DE3)pLysS cells, the expressed protein was purified with an M_2 anti-Flag monoclonal antibody resin. Purified protein was labeled in vitro with γ–^{32}P-ATP by using the HMK. Labeled protein was separated from unincorporated isotopes by G-50 column chromatography. Reverse protein gel blot screening of an Arabidopsis cDNA library was performed as described previously (Matsui et al., 1995). *CIP7* was one of three specific interacting cDNA clones isolated.

The isolated cDNA was subcloned into pBluescript SK+ (Stratagene, La Jolla, CA) to make SK-CIP7 and subsequently was used as a DNA probe to screen another cDNA library (Kieber et al., 1993) as well as a λ genomic library (Voytas et al., 1990), according to a standard method (Sambrook et al., 1989). Two long cDNA insert clones, λ7C26 and λ7C32, and several genomic clones, including λ7G4, were isolated. λ7C26 and λ7C32 were subcloned into the XbaI-SalI site of pBluescript KS+ to generate yy37 and yy38, respectively, and these two clones were sequenced using the dideoxy chain termination method (Sambrook et al., 1989) with T7 DNA polymerase. The cDNA inserts in the original λ vector were amplified by polymerase chain reaction (PCR) and subjected to restriction digestion analysis and direct sequencing to confirm that there was no mutation during subcloning. Combined sequence analysis of SK-CIP7, λ7C26, λ7C32, and the genomic clone led to a near full-length cDNA sequence for *CIP7* (GenBank accession number AB012912).

Column Binding Assay of the CIP7 and COP1 Interaction

A Flag–COP1 deletion series with HMK site was constructed (K.U. Torii, T.W. McNellis, and X.-W. Deng, unpublished results), and the fusion proteins produced in *Escherichia coli* were purified and labeled with γ–^{32}P-ATP, as described above. The CIP7 protein was fused to the maltose binding protein (MBP) or glutathione *S*-transferase (GST). The MBP–CIP7 or GST–CIP7 proteins were expressed in *E. coli* XL1-Blue. After the cells were broken by sonication, the fusion proteins were applied to amylose or glutathione resins, and nonbound *E. coli* proteins were removed by washing the resins five times with PBS buffer. For the column binding assay, equal amounts of the CIP7 fusion protein, MBP, or GST (in the charged resins) were mixed with ^{32}P-labeled COP1 or COP1 deletion proteins in the binding buffer (10 mM Hepes, pH 7.9, 1 mM KCl, 0.4 mM $MgCl_2$, 60 mM NaCl, 0.8 mM EDTA, and 8% glycerol). After incubation for 10 hr in rotating tubes at 4°C, the resin was washed five times with 300 μL of ice-cold PBS and 0.05% Nonidet P-40. The final pellet of resin was resuspended in 10 μL of protein loading buffer, boiled for 5 min, and applied to a 10% polyacrylamide gel. After electrophoresis, gels were dried and exposed to the imaging plate of a Fujix BAS2000 image analyzer (Tokyo, Japan).

RNA and Genomic DNA Gel Blot Analyses

Wild-type and *cop1-6* mutant seedlings (in the Col-0 ecotype) (McNellis et al., 1994a) were grown on 0.8% agar with GM media (Valvekens et al., 1988). Seedlings were grown in the dark or in continuous white light (150 to 200 μE m^{-2} sec^{-1}) at 22°C for 5 days. Seedlings were harvested after freezing quickly by pouring liquid N_2 onto the plates. Collected hypocotyls and cotyledons were ground using a mortar and pestle, and total RNA was extracted as described previously (Yamamoto et al., 1995). Equal amounts of total RNA from each sample were separated by agarose gel electrophoresis and blotted onto nylon membranes (Hybond-N; Amersham), according to a standard method (Sambrook et al., 1989). Equal loading of the RNA was verified by ethidium bromide staining as well as by rehybridizing the blots with an 18S rDNA probe (Deng et al., 1991). The SK-CIP7 plasmid was linearized, and the antisense riboprobe with phosphorus-32 was prepared by in vitro transcription (Yamamoto et al., 1995). Preparation of chalcone synthase (*Chs*), *FedA*, and light-harvesting chlorophyll complex (*Lhcb*) probes was described previously (Deng et al., 1992). Hybridization and washing of the membranes were performed according to Sambrook et al. (1989). The hybridized signal was visualized by autoradiography. The 3.9-kb band detected by the antisense *CIP7* riboprobe as shown in Figure 6 was resistant to RNase treatment (Deng et al., 1992), indicating that the signal is specific for the *CIP7* probe (data not shown). The radioactivity of each band in Figure 7 was counted using a PhosphorImager (model BAS2000; Fujix). All RNA gel blot analyses were repeated at least twice to confirm the reproducibility of the results.

Genomic DNA from ecotype Landsberg *erecta* was isolated according to Hauge and Goodman (1992). Two micrograms of the isolated DNA was digested with restriction enzymes, separated in an 0.8% agarose gel, blotted onto nylon membranes (Hybond-N), and hybridized with the ^{32}P-labeled SK-CIP7 probe prepared by the random hexanucleotide priming method (Sambrook et al., 1989). The blots were exposed to x-ray film to visualize the signals.

Construction of Binary Vectors for Overexpression in Plants

Plant transformation vectors for constitutive high expression were derived by replacing the BamHI-SacI fragment containing the *GUS* reporter of pBI221 (Jefferson et al., 1987) with a pair of annealed oligonucleotides (5′-GATCCCCGGGTACCACAGTCGACAGAGCT-3′ and 5′-CTGTCGACTGTGGTACCCGGG-3′). This introduced a multiple cloning site to make vector yy43. The HindIII-EcoRI fragment of yy43 was introduced into pPZP122 (Hajdukiewicz et al., 1994) to generate pPZPY122. The order of restriction sites in the introduced expression cassette in pPZPY122 is HindIII-SphI-PstI, PCaMV 35S, XbaI-BamHI-SmaI-KpnI-SalI-SacI-NOS terminator, and EcoRI. Another plant transformation vector containing the same overexpression cassette from yy43, namely, pPZPY112, was made from pPZP112 (Hajdukiewicz et al., 1994). This vector contains a kanamycin resistance marker instead of the gentamycin version.

Subcellular Localization of Green Fluorescent Protein (GFP)–CIP7

To avoid the instability difficulties associated with the *CIP7* full-length cDNA in plasmids, we introduced introns from the *CIP7* gene. A genomic fragment corresponding to amino acid +1 to +242 (the fragment contains the first six introns of the gene; GenBank accession number AB012913) was amplified by high-fidelity PCR, with λ7G4 as the template DNA. The gene-specific primers used were 5′-CCTCTAGAGAATTCAGATCTACAATGGATCCAAGAACAAGAC-TTGAC-3′ and 5′-GTCACCCATGAACGTTAATTCAGGC-3′. The amplified fragment was digested with XbaI and AvrII and introduced into

the XbaI/AvrII site of yy37 to replace the corresponding cDNA region. Two independent clones, one designated yy117, were subjected to sequence analysis to confirm that no sequencing error had occurred during PCR. The KpnI-XbaI fragment of yy117, which contains the chimeric genomic/cDNA gene for the full-length coding region, was inserted into the XbaI-KpnI site of pPZPY122, resulting in plasmid yy116. The *GFP* gene mGFPS65T, which is designed to be expressed in Arabidopsis by removing the cryptic intron (Haseloff et al., 1997) and has an improved green fluorescence by blue excitation (Heim et al., 1995; AvA118, von Arnim and Deng, unpublished results), was amplified by PCR, using the following primers: 5′-GGGTCTAGAAAACAATGGGTAAAGGAGAAGAACTTTTC-3′ and 5′-GGCATGGATGAACTATACAGATCTAGACCC-3′. The PCR product was digested with XbaI and inserted into the XbaI site of yy116 to make an in-frame fusion of *GFP–CIP7* (GFP at the N terminus). Note that this fusion gene contains the first six introns of the *CIP7* gene. Two independent clones, yy140 and yy141, were used for the transformation of *Agrobacterium tumefaciens* GV3101pMP90 by electroporation, according to the manufacturer′s instructions (Gene Pulser; Bio-Rad). Plasmids from the transformed Agrobacterium strains were subjected to restriction digestion analysis to confirm that no rearrangement of the plasmids had occurred in Agrobacterium. In addition, the recovered plasmids were amplified in *E. coli* and tested in transient assays (Rossi et al., 1993) for identical intracellular localizations of the fusion protein (data not shown). A confirmed yy141 Agrobacterium strain was used for stable transformation of Arabidopsis (ecotype Col-0) by using the vacuum infiltration method (Ronemus et al., 1996).

Live seedlings of transgenic plants were subjected to direct microscopic inspection (Axiophot; Zeiss, Jena, Germany), with filter sets for fluorescein isothiocyanate (CZ910; Chroma Technology Corp., Brattleboro, VT) to visualize the GFP–S65T fusion and GFP. The images were photographed and scanned into digital Photoshop files (Adobe City, CA) in a Macintosh computer for processing and computer printing.

Yeast Transactivation Assay

The EcoRI insert of SK-CIP7 was inserted into the EcoRI site of pEG202 (Ausubel et al., 1987) to make an in-frame fusion with the LexA DNA binding protein. The resulting plasmid, pEGCIP7, as well as pEG202 (LexA only), pRFHMI (LexA–Bicoid), and pSH17-4 (LexA–GAL4), which were used as controls, were each cotransformed into the yeast strain EGY48-0, with pSH18-34 encoding a *LacZ* reporter gene under the control of a *LexA* operator (LexAop–LacZ) (Ausubel et al., 1987). Enzymatic assays for β-galactosidase of the yeast clones containing LexA fusions were performed as described by McNellis et al. (1996).

Plant Transactivation Assay

yy37, which contains the original full-length cDNA insert, was digested with XbaI and BamHI, and the annealed oligonucleotides (5′-CTAGTCGACAGATCTACAATG-3′ and 5′-GATCCATTGTAGATCTGTCGA-3′) were inserted to introduce a multiple cloning site for N-terminal fusions. The resulting plasmid, yy53, was digested with EcoRI to remove a 3′ untranslated region. The resulting plasmid, yy54, was digested with BglII and SalI, and the CIP7 fragment was inserted in-frame after the GAL4 DNA binding domain of the effector vector pMA560 (Ma et al., 1988) to generate a GAL4–CIP7 fusion (amino acids 1 to 1058), yy67. To make GAL4–CIP7 (amino acids 387 to 1058), shown in Figure 5A, the EcoRI fragment of SK-CIP7 was inserted into the EcoRI site of pMA424 (Ma and Ptashne, 1987), and the resulting XhoI-SalI fragment with a portion of GAL4 and CIP7 (amino acids 387 to 1058) was introduced into the XhoI-SalI site of pMA560 (Ma et al., 1988). For the luciferase reporter, a DNA fragment with 17 copies of the GAL4 operator (17mers) followed by a basal 35S promoter (−60/+7) was amplified by PCR from reporter plasmid pMA558 (Ma et al., 1988). The amplified fragment was first inserted into the PstI-XbaI site of pBI221 to make yy76. The 17mer and 35S basal promoter (TATA) fragment was isolated by partial HindIII and BamHI digestion and then inserted into the HindIII and BamHI site of pBIL221. The resulting, final construct was named GAL4op-luciferase (yy96). All of the junctions of the final GAL4 fusions were verified by sequencing. Control effector GAL4 amino acids (1 to 147) and the reference (35S–GUS) are described by Ma and Ptashne (1987) (pMA564) and Restrepo et al. (1990) (pRTL2–GUS), respectively.

The tobacco transcriptional activation assay, based on particle bombardment, was used. Briefly, young mature tobacco leaves from plants (*Nicotiana tabacum* cv Petit Havana, SR1) grown in a greenhouse were surface sterilized and subjected to microprojectile bombardment (von Arnim and Deng, 1994) to introduce the reporter and effector plasmids together with the reference construct. The leaves were incubated for 2 days in the dark at 25°C and then subjected to enzymatic assays.

Preparation and Analysis of Antisense *CIP7* Transgenic Plants

The SalI-XbaI cDNA fragment of the SK-CIP7 clone was inserted into pPZPY122 to make yy87 and was used to stably transform Arabidopsis ecotype Col-0. The observed ratio of gentamycin resistant to the total number of seedlings in the T_2 generation was 76 of 82 and 95 of 102 in αCIP7-22a and αCIP7-27d, respectively. For both anthocyanin and chlorophyll assays and RNA analysis of those T_2 progenies, seedlings were grown on GM media (Valvekens et al., 1988) with or without (RNA analysis only) 1% sucrose and without any antibiotics for selection. The nontransgenic individuals as well as the transgenic seedlings were all pooled; thus, the values obtained are an underestimation of the antisense transgene effect. The 11-day-old seedlings grown under continuous white light (150 $\mu E\ m^{-2}\ sec^{-1}$) were used for anthocyanin quantitation (Chory et al., 1989), and the 13-day-old seedlings grown under a cycling light condition (16-hr light/8-hr dark at 50 $\mu E\ m^{-2}\ sec^{-1}$) were used for chlorophyll quantitation according to Arnon (1949).

ACKNOWLEDGMENTS

We thank Arthur Galston and Mark Osterlund for critical reading of and commenting on the manuscript; Drs. Pal Maliga, Jun Ma, Albrecht von Arnim, Keiko U. Torii, and the Arabidopsis Biological Resource Center (Columbus, OH) for kindly providing plasmids and Arabidopsis DNA libraries; and Dr. Massimo Galbiati for advice on the vacuum infiltration method for Arabidopsis transformation. This work was supported by a National Institutes of Health grant (No. GM47850) to X.-W.D., a grant from the Japan Society for the Promotion of Science (No. JSPS-RFTF96L00601) to M.M., and a grant from

the Human Frontier Science Program (No. RG0043/97). X.-W.D. is a National Science Foundation Presidential Faculty Fellow. Y.Y.Y. was the recipient of a long-term fellowship from the Human Frontier Science Program. L.-H.A. was a recipient of the Joseph F. Cullman Fellowship of Yale University.

Received February 10, 1998; accepted April 21, 1998.

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