

Functional dissection of *Arabidopsis* COP1 reveals specific roles of its three structural modules in light control of seedling development

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***Arabidopsis* COP1 acts as a repressor of photomorphogenesis in darkness, and light stimuli abrogate the repressive ability and nuclear abundance of COP1. COP1 has three known structural modules: an N-terminal RING-finger, followed by a predicted coiled-coil and C-terminal WD-40 repeats. A systematic study was undertaken to dissect the functional roles of these three COP1 domains in light control of *Arabidopsis* seedling development. Our data suggest that COP1 acts primarily as a homodimer, and probably dimerizes through the coiled-coil domain. The RING-finger and the coiled-coil domains can function independently as light-responsive modules mediating the light-controlled nucleocytoplasmic partitioning of COP1. The C-terminal WD-40 domain functions as an autonomous repressor module since the overexpression of COP1 mutant proteins with intact WD-40 repeats are able to suppress photomorphogenic development. This WD-40 domain-mediated repression can be at least in part accounted for by COP1's direct interaction with and negative regulation of HY5, a bZIP transcription factor that positively regulates photomorphogenesis. However, COP1 self-association is a prerequisite for the observed interaction of the COP1 WD-40 repeats with HY5. This work thus provides a structural basis of COP1 as a molecular switch.**

Keywords: *Arabidopsis*/coiled-coil/photomorphogenesis/RING-finger/WD-40 repeats

Introduction

Arabidopsis seedlings display contrasting developmental patterns in the presence and absence of light. Under normal light conditions, seedlings follow photomorphogenic development characterized by inhibition of hypocotyl elongation, development of expanded cotyledons, biogenesis of chloroplasts and expression of light-inducible genes. In darkness, seedlings etiolate, displaying elongated hypocotyls, closed and unexpanded cotyledons, and apical hooks. Also, the light-inducible genes are repressed, and plastids develop into non-photosynthetic

etioplasts in the darkness. This developmental commitment is plastic and reversible; the etiolated seedlings can respond dynamically to incoming light stimuli and initiate photomorphogenesis (for review see Chory, 1993; McNellis and Deng, 1995).

It is not fully understood how light stimuli perceived by multiple photoreceptors are transduced and integrated to affect developmental programs. Genetic screens of *Arabidopsis* seedlings, based on either etiolated phenotypes under light conditions or photomorphogenic phenotypes in complete darkness, have identified a large number of the light-signal transduction components involved in controlling seedling development (for review see McNellis and Deng, 1995). Mutant seedlings with reduced light-responsiveness display characteristic long hypocotyl (*hy*) phenotypes. This class of mutants defines positive regulators of photomorphogenesis including photoreceptors (e.g. *phyA*, *phyB* and *hy4*) and components acting downstream of specific photoreceptor (e.g. *shy1*, *shy3* and *red1*) or multiple photoreceptors (e.g. *hy5*). (Chory, 1992; Whitelam *et al.*, 1993; Wagner *et al.*, 1997). The recent molecular identification of HY5 as a bZIP transcription factor may provide a tool to bridge the light-signal transduction pathway to the control of gene expression (Oyama *et al.*, 1997). The second class of mutants includes those that display constitutive photomorphogenesis, namely constitutive photomorphogenic (*cop*), de-etiolated (*det*) and fusca (*fus*) mutants (reviewed by Wei and Deng, 1996). Genetic studies indicate that their gene products function as negative regulators acting downstream of multiple photoreceptors, including *phyA*, *phyB* and the blue-light receptor CRY1 (Ang and Deng, 1994; McNellis and Deng, 1995). While a subset of these mutants are implicated in playing a role in phytohormone signaling (Chory and Li, 1997; Kraepiel and Miginiac, 1997), 10 of the pleiotropic and essential *COP/DET/FUS* loci are believed to be responsible for mediating the suppression of photomorphogenic seedling development in darkness (Wei and Deng, 1996).

The molecular identification of four *COP/DET/FUS* genes, namely *COP1*, *COP9*, *DET1* and *FUS6* (*COP11*) provides an opportunity to understand the molecular mechanisms of repression of photomorphogenesis (Deng *et al.*, 1992; Castle and Meinke, 1994; Pepper *et al.*, 1994; Wei *et al.*, 1994). *COP9*, *DET1* and *FUS6* encode novel α -helical-rich proteins that constitutively localize in the nucleus (Pepper *et al.*, 1994; Wei *et al.*, 1994; Chamovitz *et al.*, 1996; Staub *et al.*, 1996). *COP9* has been found to be a part of an eight-subunit protein complex consisting of *COP9*, *FUS6* (*COP11*), presumably *COP8* and others (Wei *et al.*, 1994, 1998; Chamovitz *et al.*, 1996; Wei and Deng, 1996, 1998). *COP1*, on the other hand, appears to function as an autonomous repressor of photomorphogenesis based on previous experiments in modulating

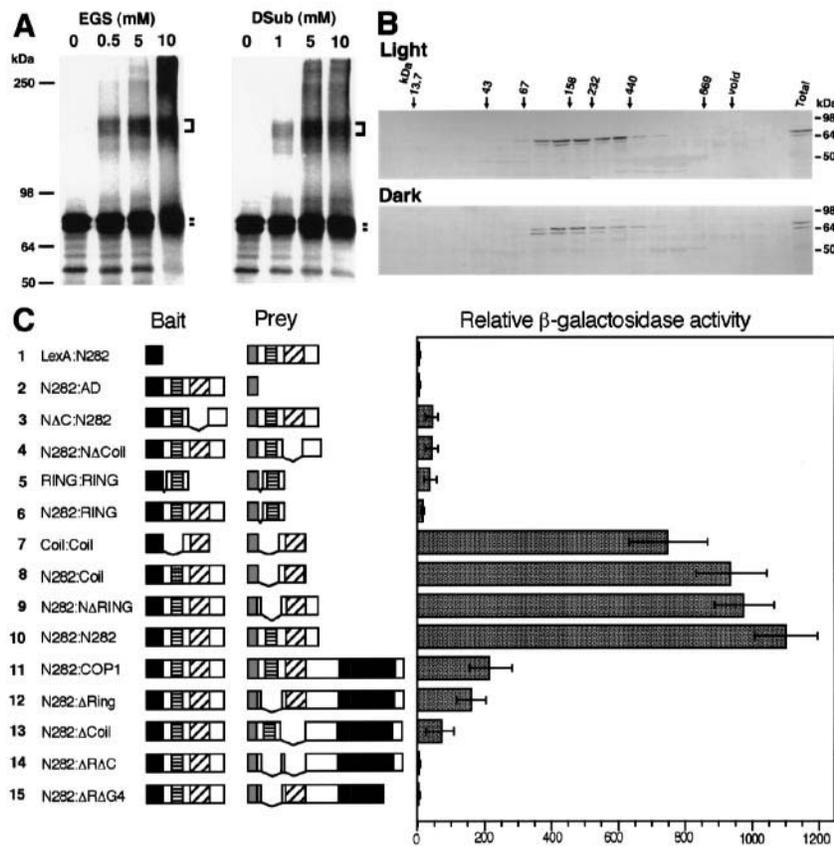


Fig. 1. COP1 dimerizes *in vitro* and *in vivo* through the Coil domain. (A) *In vitro* cross-linking analysis of COP1. The oligomeric nature of radiolabeled flag-COP1 (indicated by bars at the right side of each panel) was analyzed by using chemical cross-linker EGS and Dsub. Brackets on the right-hand side of each panel indicate the positions of cross-linked products that correspond to dimers. *In-vitro*-translated flag-COP1 yielded two bands with close molecular masses, the lower of which may be due to inappropriate translation initiated from an endogenous methionine of COP1. (B) The gel-filtration profiles of the COP1 protein extracted from wild-type seedlings grown under continuous white light or in darkness for 6 days. The proteins in the fractions were separated by SDS-PAGE and examined by protein gel immunoblot. The molecular masses (in kDa) estimated by protein standards are listed above the appropriate fractions. For experimental details, see Materials and methods. (C) Delimitation of COP1 self-association domain utilizing the yeast two-hybrid system. For baits (Bait), COP1 domain fragments were fused with the LexA DNA-binding domain (LexA). For preys (Prey), the COP1 domain fragments were fused with the synthetic transcription activation domain (AD). Numbers and names on the left indicate a pairwise combination of bait and prey constructs in each row. The graph on the right indicates the relative LacZ reporter activity in yeast cells corresponding to combinations of bait and prey constructs presented in each row. For each pairwise combination, at least eight individual transformants were used to measure the LacZ activity. Error bars represent the standard deviations. Combination 1 (LexA: N282) and 2 (N282:AD) serve as negative controls.

COP1 cellular activity. For instance, overexpression of full-length COP1 causes reduced light responsiveness (McNellis *et al.*, 1994b), while overexpression of a dominant-negative mutant form of COP1 results in hypersensitivity to the light and a partial de-etiolation in darkness (McNellis *et al.*, 1996). Cell-biological studies using a fusion protein of COP1 with a reporter β -glucuronidase (GUS) protein revealed that the light-regulated nucleocytoplasmic partitioning of COP1 may be one of the mechanisms by which light negatively regulates the repressors of photomorphogenesis (von Arnim and Deng, 1994).

COP1 encodes a protein with a novel combination of three structurally recognized domains, namely an N-terminal RING-finger domain, a putative coiled-coil (Coil) region and C-terminal WD-40 repeats (Deng *et al.*, 1992; McNellis *et al.*, 1994a). The RING-finger domain comprises eight metal ligands with a consensus of C3HC4, and binds two zinc atoms in a unique tetrahedral 'cross-brace', thus forming one integrated structural unit (von Arnim and Deng, 1993; for review see Berg and Shi,

1996; Borden and Freemont, 1996; Saurin *et al.*, 1996). The Coil region is predicted to be an α -helical structure capable of forming a superhelix (Lupas, 1996). The WD-40 motif is ~40 amino acids in length and contains a highly conserved tryptophan-aspartate (WD) sequence (for review see Neer *et al.*, 1994). Proteins with RING-finger or WD repeats are involved in a wide variety of processes, including gene repression, oncogenesis and signal transduction (for review see Neer *et al.*, 1994; Borden and Freemont, 1996; Saurin *et al.*, 1996). Studies in other systems have implicated roles in protein-protein interactions for all three modules, suggesting that the pleiotropic role of COP1 may be achieved through interactions with multiple proteins. However, the specific functional roles of the COP1 modules have not been addressed.

Here we report the functional dissection of COP1 domains by the utilization of a combination of reverse-genetic, biochemical and cell-biological approaches. Our study reveals the distinct but overlapping functions of COP1 domains in the light control of seedling develop-

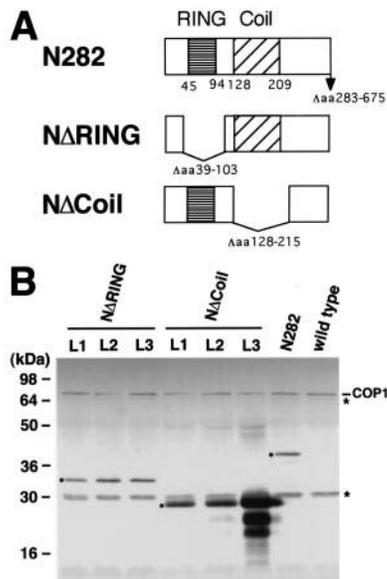


Fig. 2. The COP1 protein N-terminal fragments used and their expression in transgenic plants. (A) Diagrams of the constructs used for the overexpression analysis. N282 (McNellis *et al.*, 1996), N Δ RING and N Δ Coil are shown in comparison, with the locations of the RING-finger (RING) and Coil (Coil) highlighted. aa, amino acids. (B) Protein gel blot analysis of three representative N Δ RING and N Δ Coil transgenic lines compared with the wild type and the N282 transgenic line (L2: McNellis *et al.*, 1996). Total proteins of \sim 12.5 μ g were loaded on each lane. Protein blots were probed with anti-COP1 antibodies. Overexpressed N282, N Δ RING and N Δ Coil protein bands are marked with dots. Protein bands corresponding to the endogenous COP1 and a non-specific reaction band at 30 kDa are marked with asterisks on the right-hand side of the gel. Molecular mass markers (in kDa) are indicated on the left.

ment, and provides a structural basis for COP1 as a molecular switch.

Results

COP1 forms a dimer in vitro and in vivo

We have reported that the N282 fragment of COP1, which contains both the RING-finger and Coil regions, possesses the ability to interact with the full-length COP1 in a yeast two-hybrid system, implying that COP1 may function as a homodimer or multimer (McNellis *et al.*, 1996). To understand better the COP1 self-association, we performed a chemical cross-linking analysis of *in vitro* translated COP1 protein in solution. An epitope-tagged FLAG-COP1 protein was translated *in vitro* and then cross-linked with either dimethylsuberimidate (DSub) or ethylene glycobis (succinimidylsuccinate) (EGS). Figure 1A shows monomeric FLAG-COP1 (78 kDa) and the cross-linked products resolved by SDS-PAGE and detected by fluorography. Cross-linking with either Dsub or EGS generated a band with an apparent molecular size of \sim 160 kDa, clearly indicating dimer formation *in vitro* (Figure 1A).

To confirm the presence of COP1 dimers *in vivo*, a gel-filtration analysis was performed using protein extracts from light- or dark-grown *Arabidopsis* wild-type seedlings. As shown in Figure 1B, the endogenous *Arabidopsis* COP1 protein fraction peaked at the \sim 160 kDa region, very close to the size of the cross-linked dimer *in vitro*. However, the COP1 peak was very broad and contained

a broad shoulder toward the larger molecular size fractions (Figure 1B). Therefore it is possible that a portion of COP1 may present as homo-oligomers or in heterogeneous associations with other molecules *in vivo*. Further, the limited resolution of the gel filtration cannot rule out the presence of a minor amount of COP1 as monomer, since the shoulder of the COP1 peak at the side of smaller molecular weight extended toward the 70 kDa region. Although the relative amount and distribution of the COP1 doublet bands (due to partial degradation in the extracts) is somewhat variable from experiment to experiment, the gel-filtration profiles of both the light- and dark-grown seedlings were essentially identical (Figure 1B), indicating that light does not affect COP1 self-association *in vivo*.

COP1 dimerizes through the Coil domain

To delimit further the COP1 dimerization domain within the N282 fragment of COP1, a series of deletion mutants of N282 were constructed and analyzed using the yeast two-hybrid assay (Figure 1C). The result indicates that the Coil domain of COP1 was shown to be both necessary and sufficient for self-association (Figure 1, C3, C4, C7–12, C10). However, the deletion of the Coil from the N282 fragment still retained some weak and reproducible interactions slightly higher than those of negative controls (see Figure 1, C1–C4), suggesting the presence of residual COP1 self-association within the rest of the N-terminal region. The RING-finger domain seems responsible for this residual activity since this domain showed weak interaction with itself (Figure 1, C5). A supportive role for the RING-finger in COP1 intramolecular association became evident among the constructs with an intact C-terminus (Figure 1, C11–C14): deletion of the Coil domain still retained a weak interaction (Figure 1, C13), while the deletion of both the RING-finger and the Coil domains completely abolished the interaction (Figure 1, C14). The disruption of the WD-40 repeats also perturbed the intramolecular interaction (Figure 1, C15), possibly due to a conformational hindrance caused by a misfolded WD-40 repeats domain. Protein gel immunoblot analysis did not reveal any significant differences in expression levels of those domain-deletion constructs (data not shown). Thus, it rules out the possibility that the different activities in the yeast two-hybrid interaction assay were caused by different expression levels of the proteins.

Overexpression of the Coil region confers seedling hyperphotomorphogenic development

If the Coil domain has a major role for COP1 dimerization, it may represent the domain primarily responsible for the observed dominant-negative phenotype in N282-overexpressing plants (McNellis *et al.*, 1996). To test this possibility, transgenic *Arabidopsis* plants expressing two N-terminal COP1 mutant forms under the strong CaMV35S promoter were generated. As shown in Figure 2A, N Δ RING and N Δ Coil are essentially the N282 fragment of COP1 lacking the RING-finger or the Coil domain, respectively. Protein gel immunoblot analysis with anti-COP1 antibody of three representative N Δ RING and N Δ Coil transgenic lines suggested that all lines accumulated transgene products at comparable or higher levels than that of the N282 overexpressors (Figure 2B; McNellis *et al.*, 1996). In all transgenic lines examined, the level

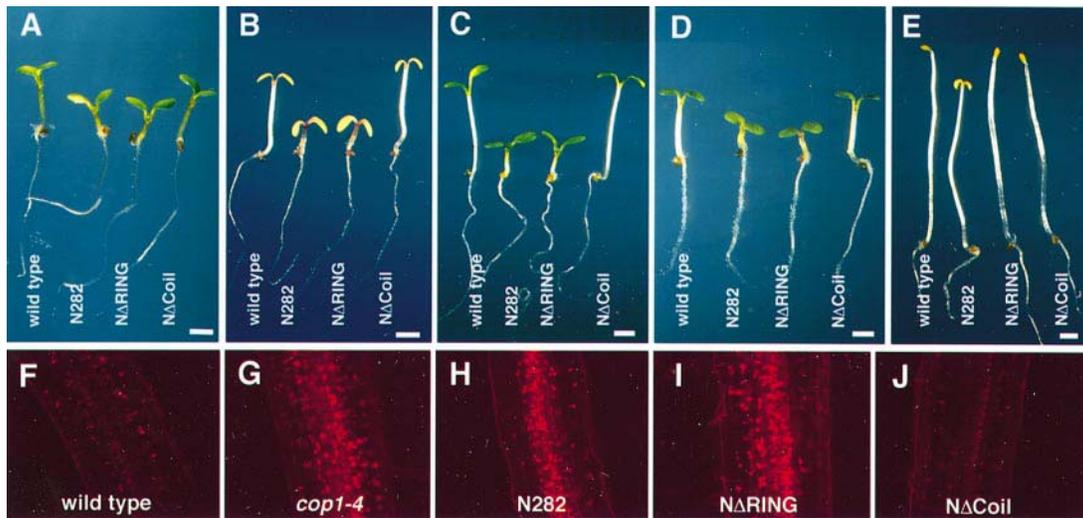


Fig. 3. Phenotypic comparison of N282, NΔRING and NΔCoil transgenic seedlings. (A–E) Morphogenetic comparison of 6-day-old seedlings. Seedlings were grown (A) under continuous white light ($50 \mu\text{mol m}^{-2} \text{s}^{-1}$), (B) under continuous far-red light ($40 \mu\text{mol m}^{-2} \text{s}^{-1}$), (C) under continuous red light ($40 \mu\text{mol m}^{-2} \text{s}^{-1}$), (D) under continuous blue light ($40 \mu\text{mol m}^{-2} \text{s}^{-1}$), and (E) in complete darkness. In each panel, wild type (Columbia), N282 (L2), NΔRING (L2) and NΔCoil (L3) are shown from left to right. The scale bars represent 1 mm. (F–J) Chloroplast autofluorescence from the roots (~ 5 mm below the junction of the hypocotyl) of 6-day-old seedlings. (F) Wild type (Columbia), (G) *cop1-4*, (H) N282 (L2), (I) NΔRING (L2) and (J) NΔCoil (L3). Seedlings were grown under continuous white light conditions ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$).

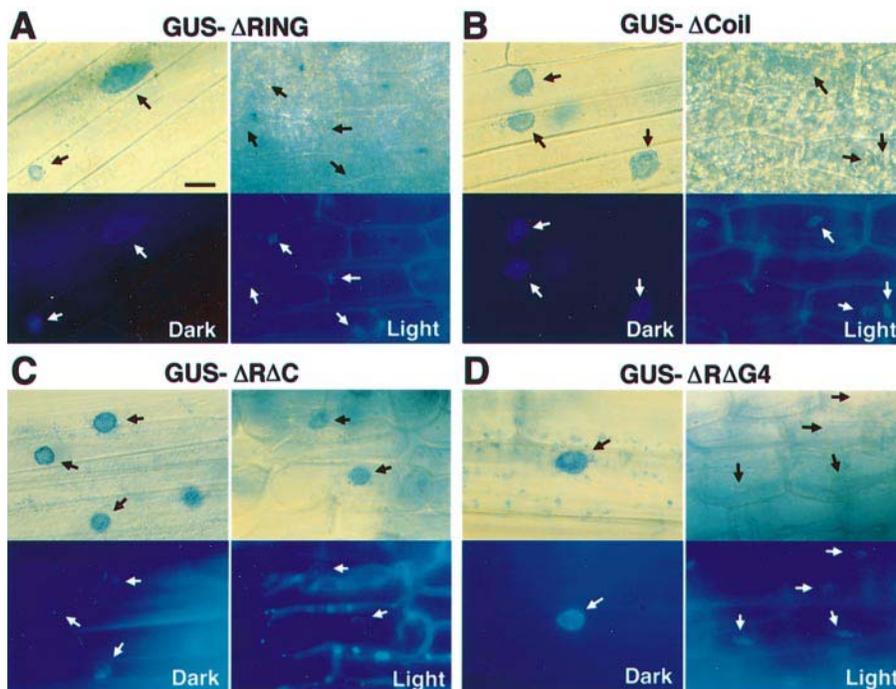


Fig. 4. Representative cellular GUS staining patterns in hypocotyls of transgenic *Arabidopsis*. Five-day-old seedlings transgenic for (A) GUS-ΔRING, (B) GUS-ΔCoil, (C) GUS-ΔRAC and (D) GUS-ΔRAG4 were grown in complete darkness (left-hand panels) or under high-intensity continuous white light ($150 \mu\text{mol m}^{-2} \text{s}^{-1}$; right-hand panels). They were stained for GUS activity without prior fixation and whole-mounted in the presence of DAPI. For both GUS cytochemical staining (top panels) and DAPI staining (bottom panels), the location of representative nuclei are indicated by arrows. The scale bar in (A) represents $10 \mu\text{m}$ and applies to all panels.

of the endogenous COP1 protein appeared to be unaltered (Figure 2B).

Transgenic *Arabidopsis* seedlings were examined under different light conditions and in darkness (Figure 3). The overexpression of NΔRING conferred a dramatic reduction in hypocotyl length when seedlings were grown under continuous white, far-red, red and blue light (Figure 3A–D). In addition, NΔRING seedlings displayed an excessive

accumulation of anthocyanin in the upper hypocotyls (see Figure 3B). Furthermore, the overexpression of NΔRING as well as N282 led to ectopic chloroplast differentiation in seedling roots (Figure 3F–J). Hence, the overexpression of the NΔRING is sufficient to impair the endogenous COP1 function and to enhance the light signaling mediated by multiple photoreceptors. In contrast to the NΔRING overexpressors, NΔCoil overexpressors did not confer any

visible phenotypes, indicating that the RING-finger domain itself is insufficient for causing any dominant-negative effects (Figure 3A–E and J). Although some Δ Coil overexpressors (lines 2 and 3, Figure 2B) contain partially degraded forms (Figure 2B), they could not be the cause for the lacking of the phenotype in those Δ Coil overexpressors since other lines (such as line 1) do not contain the same partial degradation products and also exhibit no phenotype.

The Δ RING overexpressors did not show any de-etiolation phenotype in darkness (Figure 3E). Since the overexpression of N282 conferred a partial de-etiolation in darkness (McNellis *et al.*, 1996), it seems to indicate that an effective functional interference with the endogenous COP1 in darkness requires the additional RING-finger domain. A supportive role for the RING-finger in COP1 dimerization (Figure 1C) is consistent with this observation.

The RING-finger and the Coil domains act redundantly to mediate light-induced depletion of COP1 from nucleus

Light signals negatively regulate COP1 abundance in the nucleus (von Arnim and Deng, 1994; von Arnim *et al.*, 1997). In darkness, GUS–COP1, a full-length COP1 fused to a reporter β -glucuronidase, predominantly localizes in the nucleus, but the transfer of seedlings from dark to light reduces nuclear abundance of GUS–COP1 (von Arnim and Deng, 1994). To identify the domain(s) that mediates the light responsiveness of COP1, the subcellular localizations of the fusion proteins of GUS fusions with COP1 deletion mutants were examined. For this purpose, the GUS protein was fused to the N-terminus of the COP1 fragments that lack either the RING-finger (GUS– Δ RING), or the Coil (GUS– Δ Coil), or both RING-finger and Coil (GUS– Δ RAC), or the RING-finger and the last repeat of the WD-40 motif (GUS– Δ RAG4). Transgenic *Arabidopsis* plants that express the four constructs under the CaMV 35S promoter were produced and examined.

Figure 4 shows representative GUS staining patterns in hypocotyl cells of dark- or light-grown transgenic *Arabidopsis*. In darkness, all GUS fusion constructs localized in the nucleus and in a similar manner to that described for GUS–COP1 (von Arnim and Deng, 1994), suggesting that the NLS remains functional in all the mutated forms of COP1. This result is consistent with a site-directed mutagenesis study that revealed that the COP1 NLS is contained within amino acids 293–314 (A.G.von Arnim, personal communication). When seedlings were grown under high-intensity continuous white light (150 $\mu\text{mol}/\text{m}^2/\text{s}$), GUS– Δ RING, GUS– Δ Coil and GUS– Δ RAG4 chimeric proteins were excluded from the nucleus (Figure 4A, B and D). In contrast, GUS– Δ RAC displayed a constitutive nuclear localization in hypocotyl cells (Figure 4C), as well as in cotyledon epidermis and mesophyll cells of light-grown seedlings (data not shown). The data suggests that while the deletion of the RING finger or the Coil alone does not significantly affect the light-activated nuclear depletion of COP1, deletion of both domains clearly compromises light regulation of COP1 nucleocytoplasmic partitioning.

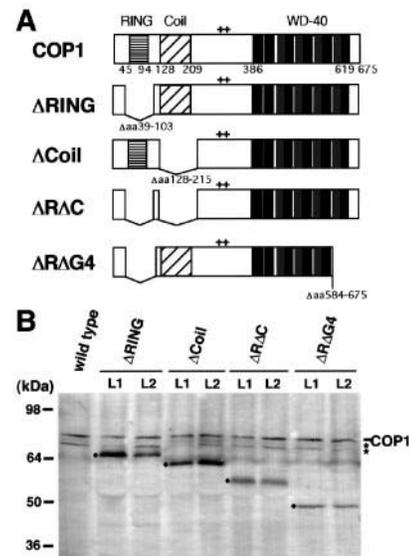


Fig. 5. The COP1 domain-deletion constructs designed to analyze the *in vivo* function of the COP1 C-terminus and their expression in transgenic plants. (A) Diagrams of Δ RING, Δ Coil, Δ RAC and Δ RAG4 in comparison to the full-length COP1 protein. Positions of putative nuclear localization signals (NLS) are indicated by ++. This set of COP1 mutants was also used for GUS fusions (GUS– Δ RING, GUS– Δ Coil, GUS– Δ RAC and GUS– Δ RAG4, respectively) in subcellular localization studies (Figure 4) and phenotypic studies. aa, amino acids. (B) Protein gel blot analysis of the transgenic *Arabidopsis* expressing COP1 domain-deletion fragments that retain the intact C-terminus. Two representative transgenic lines for Δ RING, Δ Coil, Δ RAC and Δ RAG4 are compared with the wild-type Columbia. Total proteins of $\sim 12.5 \mu\text{g}$ were loaded on each lane. Protein blots were probed with anti-COP1 antibodies. Overexpressed Δ RING, Δ Coil, Δ RAC and Δ RAG4 protein bands are marked with dots. Protein bands corresponding to the endogenous COP1 and a non-specific reaction are marked with a bar or asterisks, respectively, on the right-hand side of the gel. Molecular mass markers (in kDa) are indicated at the left.

The C-terminal WD-40 repeat domain has an essential but not self-sufficient role in repressing light inhibition of hypocotyl elongation

To reveal the function of the WD-40 repeats, we first generated transgenic *Arabidopsis* plants overexpressing only the COP1 C-terminal region (amino acids 283–675) which contains the entire WD-40 repeats. All transgenic lines with this construct failed to accumulate any detectable amount of the mutated COP1 (data not shown). Therefore, we generated a new set of COP1 domain deletion constructs which specifically lack the RING-finger (Δ RING) or the Coil (Δ Coil), or both (Δ RAC), in the full-length COP1 context and are driven by the CaMV 35S promoter (Figure 5A). As a negative control, a construct that lacks the last repeat of the WD-40 motif in addition to the RING finger deletion (Δ RAG4) was also generated. The RING-finger was not included in this control construct, since its deletion does not influence the effect mediated by WD-40 repeats (see later). As shown in Figure 5B, protein gel immunoblot analysis of Δ RING, Δ Coil, Δ RAC and Δ RAG4 overexpressors indicated that all COP1 mutant forms accumulate to similar levels and that their expression does not affect the level of endogenous COP1. Therefore, the severity of the phenotypes is most likely due to the effectiveness of the mutated forms of COP1.

Seedling phenotypes of the Δ RING, Δ Coil, Δ RAC and Δ RAG4 overexpressors were examined under continuous

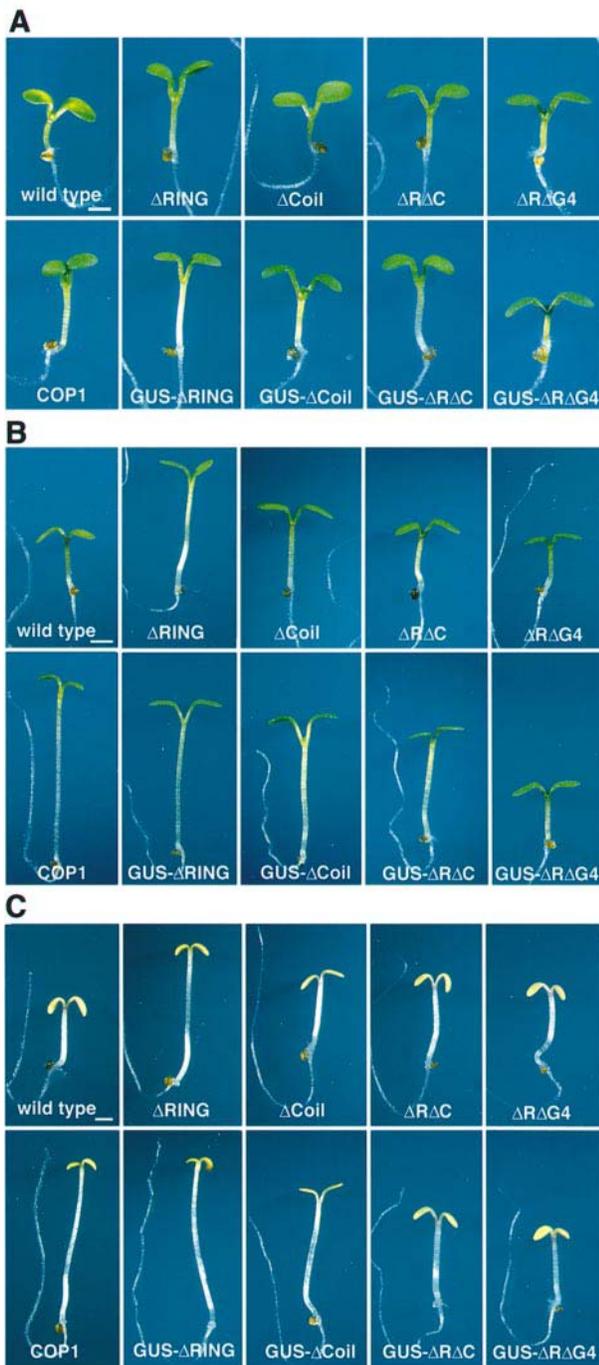


Fig. 6. Morphogenetic comparison of 6-day-old wild-type and transgenic seedlings expressing COP1 domain-deletion fragments and their GUS fusion shown in Figure 5. Seedlings of wild-type (ecotype Columbia) and transgenic *Arabidopsis* that overexpress a full-length COP1 (COP1; McNellis *et al.*, 1994b), Δ RING, Δ Coil, Δ RAC, Δ RAG4, GUS- Δ RING, GUS- Δ Coil, GUS- Δ RAC and GUS- Δ RAG4 were grown under (A) continuous white light conditions ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$), (B) continuous blue light conditions ($12.5 \mu\text{mol m}^{-2} \text{s}^{-1}$) and (C) continuous far-red light conditions. The scale bars represent 1 mm and all seedlings shown in each panel were viewed under the same magnification.

white, blue and far-red light conditions. As shown in Figures 6 and 7, Δ RING seedlings displayed long hypocotyl phenotypes in all light conditions tested, similar to that of the full-length COP1 overexpressor (McNellis *et al.*, 1994b). Δ Coil seedlings displayed a subtle but

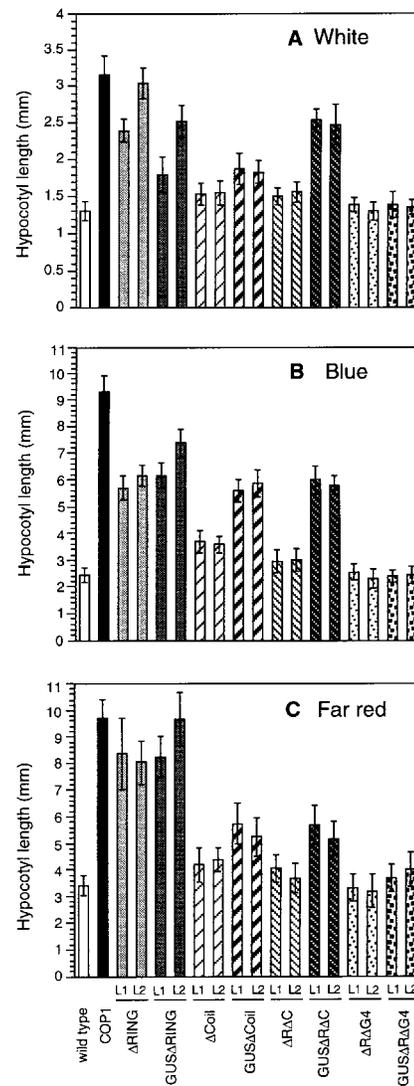


Fig. 7. Comparison of the hypocotyl lengths of wild-type and transgenic *Arabidopsis* seedlings that overexpress full-length COP1, and COP1 domain-deletion overexpressors. Seedlings of wild-type Columbia, COP1 overexpressors and two representative lines for Δ RING, Δ Coil, Δ RAC, Δ RAG4, GUS- Δ RING, GUS- Δ Coil, GUS- Δ RAC and GUS- Δ RAG4 overexpressors were grown under (A) continuous white light, (B) continuous blue light and (C) continuous far-red light conditions, as shown in Figure 6, for 6 days. The hypocotyls of at least 20 seedlings were measured for each line, and the means are shown on the chart. Error bars represent standard deviations.

statistically significant *hy* phenotype only under blue light (Figures 6B and 7B). This weak but reproducible phenotype in the Δ Coil line is consistent with the fact that deletion of the Coil domain still retains a weak self-association in the yeast (Figure 1, C11–C14). Thus, the RING-finger seems dispensable, while the Coil is important but not essential for repressing seedling photomorphogenic development in our transgenic assay. The effect of Δ RING overexpression was completely abolished by an additional disruption of WD-40 repeats (Δ RAG4), indicating a critical role for the intact WD-40 repeat domain in repressing photomorphogenic development. Δ RAC did not confer any detectable phenotypes under any light conditions tested (Figures 6 and 7). Thus, the

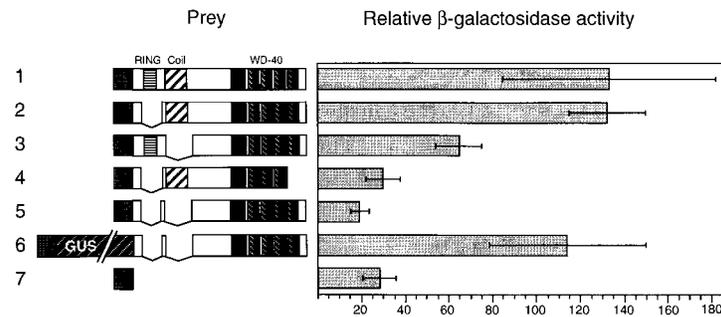


Fig. 8. Interaction between HY5 and COP1 domain-deletion mutant forms in the yeast two-hybrid system. Numbers on the left indicate a pairwise combination of the bait, LexA-HY5 (not shown), with the different prey constructs: 1, AD-COP1; 2, AD-ΔRING; 3, AD-ΔCoil; 4, AD-ΔRAG4; 5, AD-ΔRAC; 6, AD-GUS-ΔRAC; and 7, AD only. Combination 7 represents a negative control. The bar graph on the right shows the relative LacZ activities of the pairwise combinations in corresponding rows. For each pairwise combination, 10 individual transformants were used to measure relative LacZ activity. Error bars represent standard deviations.

C-terminal WD-40 domain alone is not self-sufficient for conferring the repressive activity of photomorphogenesis.

The ΔCoil defect can be largely compensated by the addition of a heterologous self-association protein motif

Since the Coil domain mediates COP1 self-association, the involvement of the Coil domain in repressing seedling photomorphogenesis may be due simply to this structural role. If this is the case, the addition of a new self-association domain to the COP1 ΔCoil mutants may compensate the defects caused by the Coil domain deletion. To test this hypothesis, we examined phenotypic effects of the GUS fusion series with COP1 and its mutated forms, since the GUS protein is capable of self-tetramerization (Jefferson *et al.*, 1987). As shown previously, GUS-COP1 overexpression confers a long hypocotyl phenotype similar to the full-length COP1 overexpressors (von Arnim and Deng, 1994; von Arnim *et al.*, 1997). While GUS-ΔRING and ΔRING overexpressors conferred a similar degree of long hypocotyl phenotype, GUS-ΔCoil and GUS-ΔRAC overexpressors exhibited enhanced seedling long-hypocotyl phenotypes compared with the ΔCoil and ΔRAC overexpressors (Figures 6 and 7). Protein gel blot analysis did not reveal significant differences in the accumulation of transgene products among GUS-ΔCoil, GUS-ΔRAC, ΔCoil and ΔRAC transgenic lines (data not shown). Therefore, our results suggest that fusing a GUS protein can somehow restore the defects of ΔCoil and ΔRAC in repressing photomorphogenesis, probably by providing a new self-association function replacing that of the Coil motif. In contrast, GUS-ΔRAG4 overexpressors failed to restore long hypocotyl phenotypes (Figures 6 and 7), suggesting that the function of the WD-40 repeats cannot be compensated for by the GUS fusion. Therefore, we propose that the WD-40 repeat domain plays a direct role in mediating the repression of photomorphogenesis, while the Coil domain provides the dimerization function of COP1 that is a prerequisite for the proper function of the WD-40 repeats.

The WD-40 repeat domain mediates the functional interaction between COP1 and HY5, a positive regulator of photomorphogenesis

COP1 represses photomorphogenic development by directly interacting and negatively regulating specific tran-

scription factors that are responsible for promoting photomorphogenic development (Ang *et al.*, 1998; Yamamoto *et al.*, 1998). Since HY5 plays a role in the light regulation of hypocotyl elongation, we examined whether the repressive effect of COP1 is mediated through HY5 and whether the WD-40 repeat domain of COP1 is responsible for the regulatory protein-protein interaction. As shown in Figure 8, the strength of the interaction of HY5 and a series of COP1 domain deletion mutants in the yeast two-hybrid assay is directly correlated to the hypocotyl length conferred by the overexpression of the corresponding COP1 mutant forms (Figures 6 and 7). For example, HY5 showed a similar degree of interaction with full-length COP1 and ΔRING, reduced interaction with ΔCoil and no interaction with ΔRAC or ΔRAG4 (Figure 8).

To examine the specific roles of the WD-40 repeats and the Coil domains in mediating the COP1 and HY5 interaction, we further analyzed HY5 interactions with the GUS-ΔRAC mutant. Similar to the observed phenotypic effects of GUS-ΔRAC and ΔRAC in transgenic seedlings (Figures 6 and 7), the GUS-ΔRAC fusion protein restored the ability of the COP1 mutant form to interact with HY5 (Figure 8). The result further substantiated the notion that the C-terminal WD-40 repeats of COP1 play a direct role in mediating interaction with HY5 while the dimerization of COP1, which is mediated by the Coil domain, is required for this interaction.

Discussion

In this study, we have dissected the functional roles of the three known COP1 domains in the control of *Arabidopsis* seedling development by light. Our results unveiled the distinct but cooperative nature of the functional modules of COP1. As summarized in Figure 9, the Coil region is most probably the dimerization domain, while the N-terminal RING-finger plays a supportive role in COP1 intramolecular interaction. Both the RING-finger and the Coil domains are important for mediating the light-triggered depletion of COP1 from the nucleus. With proper self-association, the C-terminal WD-repeats are essential for mediating the repression of photomorphogenesis, possibly by directly interacting with and negatively regulating HY5.

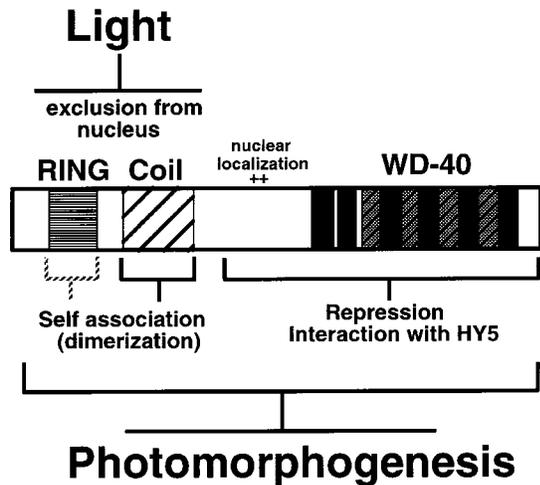


Fig. 9. Working model for COP1 domain functions in the light control of *Arabidopsis* seedling development. The COP1 NLS locates between the Coil and the WD-40 repeat domains. Light signals perceived by multiple photoreceptors are transduced through signaling intermediate(s) that diminish COP1 nuclear accumulation acting through both the RING-finger and the Coil domain. A combination of the RING-finger and the Coil domains is necessary for COP1 self-association, which is a prerequisite for *in vivo* repressive activity of COP1. HY5 is one of the target transcription factors whose activity is inhibited by COP1.

The structural basis of COP1 dimerization

Our results showed that COP1 acts as a dimer *in vivo*, and that the COP1 dimer can also be detected *in vitro* by chemical cross-link analyses (Figure 1A and B). Experiments using the yeast two-hybrid assay have further defined the Coil domain of COP1 as necessary and sufficient for mediating COP1 self-interaction (Figure 1C). Evidence for a minor role for the RING-finger domain in dimerization is also observed in yeast, which is consistent with a recent view of RING-finger as a protein-protein interaction motif (for review see Berg and Shi, 1996; Borden and Freemont, 1996; Saurin *et al.*, 1996). Indeed, involvement of the RING-finger domain in dimerization was recently implicated. For example, a resolved crystal structure of the dimerization domain of RAG1, a V(D)J recombinant-activating protein, demonstrated an involvement of the RING-finger domain in dimerization (Bellon *et al.*, 1997).

The respective roles of the RING-finger and the Coil domain in COP1 dimerization are consistent with the dominant-negative phenotypes of the transgenic seedlings overexpressing COP1 mutant forms that contain these domains. While the N282 fragment overexpressors (which possess both the RING-finger and the Coil domains) displayed hypersensitive photomorphogenic responses in both dark and light-grown seedlings (Figure 3E; McNellis *et al.*, 1996), the Δ RING overexpressors did not exhibit seedling de-etiolation in darkness (Figure 3E). A possible explanation is that the adjacent RING-finger is required for an effective COP1 self-association *in vivo*. This would also best explain the severe dominant-negative phenotype generated by overexpression of the GUS-COP1-9 mutant protein reported recently (Zhou *et al.*, 1998). In the cop1-9 mutant protein, one-and-a-half WD-40 repeats are missing while intact RING-finger and Coil domains are present (McNellis *et al.*, 1994b).

The structural basis of light regulation of COP1 nuclear accumulation

COP1 displays a light-dependent nuclear-cytoplasmic partitioning (von Arnim and Deng, 1994). The reduced nuclear abundance of COP1 under light is presumably a way of preventing COP1 from repressing photomorphogenic development, thus stably maintaining photomorphogenic development commitment. The analysis of GUS fusions with the COP1 domain deletion mutant series revealed that the RING-finger and the Coil domains may have redundant functions in mediating the light induced depletion of COP1 from the nucleus. Although the mechanism is not known, light could deplete nuclear COP1 abundance by two general means: prevention of import in combination with nuclear COP1 degradation, or active COP1 export. Since there is no precedence for a single protein having two independent domains for mediating nuclear export, it may be more reasonable to speculate that light causes the prevention of nuclear import of COP1. The notion that the RING-finger domain is a protein-protein interaction motif (for review see Berg and Shi, 1996; Borden and Freemont, 1996; Saurin *et al.*, 1996) would also be consistent with this hypothesis. Recently, specific COP1 interactive proteins were identified that associate with either the RING-finger domain (K.U.Torii and X.W.Deng, unpublished) or the Coil domain (Matsui *et al.*, 1995; Yamamoto *et al.*, 1998). One such protein, CIP1, has been shown to colocalize with the cytoskeletal structure (Matsui *et al.*, 1995). These interactive proteins could be potential candidates involved in the cytoplasmic retention of COP1. However, our results cannot rule out the possibility that the RING-finger and the Coil domains may each contribute to the structural integrity necessary for the cytoplasmic retention of COP1, while the deletion of both would simply eliminate the conformation or structure necessary for proper cellular localization.

Previous studies revealed that the pleiotropic *COP/DET/FUS* loci are required for the proper nuclear localization of COP1 in darkness (Chamovitz *et al.*, 1996; von Arnim *et al.*, 1997). Our results indicate that none of the RING-finger, Coil or WD-40 repeat modules are specifically involved in associating with other COP/DET/FUS proteins, as the deletion of any of the domains failed to compromise the nuclear localization of COP1 in darkness (Figure 4). It could be that the other COP/DET/FUS proteins act through other regions of COP1, such as NLS or a specific individual WD-40 repeat(s). For example, each WD-40 repeat of the yeast TUP1 is capable of interacting with different partners (Komachi *et al.*, 1994). The molecular basis for COP1 interacting with other COP/DET/FUS proteins, both direct and indirect, will be sought out in future work.

The WD-40 repeats mediate the repression of photomorphogenic development through an interaction with HY5, a positive regulator of photomorphogenesis

The overexpression of the COP1 mutant forms that retain intact WD-40 repeats caused reduced seedling responses to light (Figures 6 and 7). There are at least two possible explanations for this observation. First, the WD-40 repeat region is an autonomous module responsible for mediating repression of photomorphogenesis. Thus, the more of this

domain that is available, the more is the suppression of photomorphogenesis. Secondly, the intact WD-40 repeat region in the mutated COP1 forms are somehow interfering with an upstream factor(s) that mediates light inactivation of COP1. The fact that the hypocotyl-length phenotype conferred by the COP1 domain-deletion overexpressors showed a correlation with the ability of the corresponding COP1 fragments to interact with HY5 in the yeast two-hybrid system (Figures 6, 7 and 8) would be strictly consistent with the first explanation. Although not mutually exclusive with the second possibility, the observation implies that the WD-40 repeat module of COP1 is required for *in vivo* repression of HY5 activity through a direct interaction (Ang *et al.*, 1998). Further, COP1 dimerization is a prerequisite for the functional interaction of the COP1 WD-40 repeat domain and HY5. This is evident since GUS- Δ RAC overexpressor seedlings display an elongated hypocotyl phenotype due to the ability of GUS to confer self-association (Figures 6 and 7; Jefferson *et al.*, 1987) and to restore the ability to interact with HY5 (Figure 8). Taken together, these results indicate that the C-terminal WD-40 repeats are responsible for conferring repression of photomorphogenic seedling development, at least in part by directly interacting with and negatively regulating HY5. It should be noted that all of our experiments were performed in the wild-type background. Our preliminary observations suggest that neither Δ RING nor Δ Coil mutant proteins can rescue the seedling lethality of the *cop1* null-allele (C.D. Stoop-Myer and X.-W. Deng, unpublished). This is consistent with the results that both the RING-finger and the coiled-coil have multiple functions.

The WD-40 repeats have been found in several nuclear proteins that function as transcriptional repressors, such as *Drosophila* extra sex comb (Esc), yeast Tup1, Hir1 and Met30 (Keleher *et al.*, 1992; Sherwood *et al.*, 1993; Gutjahr *et al.*, 1995; Thomas *et al.*, 1995). These WD-40 proteins do not appear to bind DNA directly, but instead achieve their repressive activities by interacting with sequence-specific DNA-binding transcription factors. Two distinct repression mechanisms have been proposed. One is that the WD-40 proteins repress transcription by interfering with the basal transcriptional machinery, as proposed for Tup1 and Hir1 (Komachi *et al.*, 1994; Tzamaris and Struhl, 1995; Spector *et al.*, 1997). One mechanism would be to directly displace the member of the TFIID complex, as proposed for Esc (Gutjahr *et al.*, 1995). In such a case, the sequence-specific DNA-binding proteins may be simply required for recruiting the WD-40 proteins to the target promoters (Tzamaris and Struhl, 1995; Spector *et al.*, 1997). The second possible mechanism is that of WD-40 proteins sequestering sequence-specific transcriptional activators. One such example is yeast Met30, which represses the transcription of sulfur metabolic genes by associating with and inhibiting the bZIP transcriptional activator Met4 (Kuras and Thomas, 1995; Thomas *et al.*, 1995; Kuras *et al.*, 1996). COP1 could act in a mechanism similar to that of Met30 by sequestering HY5 or masking the ability of HY5 to activate transcription.

In conclusion, the work presented here allowed us to assign specific functional roles to the three conserved COP1 domains in the light control of seedling development. It demonstrated that modulating the activity of COP1 domains or modules can alter seedling developmental fate

to being either more photomorphogenic, such as for N282 and the Coil overexpressors, or more skotomorphogenic, such as for Δ RING and Δ Coil overexpressors. Therefore, our study provides a structural basis for COP1 functioning as an autonomous molecular switch. Obviously, continuous efforts to identify molecules that interact functionally with COP1 protein modules, either the downstream targets that are repressed by COP1 or upstream factors that modulate COP1 nucleocytoplasmic partition by light, will be critically important for our understanding fully the molecular mechanism of how COP1 mediates the light control of seedling development.

Materials and methods

Plant materials and growth conditions

Plant growth conditions were exactly as described by McNellis *et al.* (1994a) unless otherwise described in the text. Full-length COP1, N282 and Δ RING overexpressors are in the No-O ecotype, and all other transgenic plants are Columbia ecotype. Wild-type seedlings of both ecotypes were used as controls, while only one (Columbia unless stated otherwise) is shown in most Figures. To optimize phenotype examination, light intensities were changed in some experiments as described in the text.

Construction of COP1 domain cassettes

To facilitate the further cloning procedures, all of the COP1 domain-deletion constructs were first cloned into the modified pBluescript KS vector (pKSm; Deng *et al.*, 1992). pKS- Δ RING was constructed by replacing the *Bam*HI-*Xba*I fragment of pKS-COP1 with that of pMAL Δ Zn (von Arnim and Deng, 1993). pKS- Δ RING was cleaved with *Bam*HI and *Msc*I, and inserted into pKS-N282 (McNellis *et al.*, 1996) to generate pKS-N Δ RING. To construct pKS-N Δ Coil, two sets of primers, T7 and CCNT3 (5' CCG CTC GAG CCG AAA CTG ATC CAA GGG CGA 3'), and CCCT5 (5' CCG CTC GAG AAG TTG CGG ATG CTC GGA GA 3') and T3, were used to amplify the coding regions N-terminal (amino acids 1–127) and C-terminal (amino acids 216–282) of the Coil domain, respectively. Both fragments were cloned into the pKSm vector to generate pKS- Δ C.nt and pKS- Δ C.ct, respectively. pKS- Δ C.nt insert was cleaved by *Xho*I and inserted into the *Xho*I-digested pKS- Δ C.ct to generate pKS-N Δ Coil. To generate pKS-N $\Delta\Delta$, the PCR was performed using N Δ RING as a template with a primer combination T7 and NT-*Sal*3 (5' A CGC GTC GAC CCC AAA CTG ATC CAA GGG CGA 3'). The amplified fragment was cloned into the pKSm vector to generate pKS- $\Delta\Delta$.nt. pKS- $\Delta\Delta$.nt was cut with *Sal*I, and the released fragment was inserted into the *Xho*I-cleaved pKS Δ C.ct to generate pKS-N $\Delta\Delta$. The *Bam*HI-*Msc*I fragments of pKS-N Δ Coil and pKS-N $\Delta\Delta$ were replaced with that of pKS-COP1 to generate pKS- Δ Coil and pKS- $\Delta\Delta$, respectively. To generate pKS-Coil and pKS-RING, the PCR was performed using pKS-N282 as a template with primer combinations Coil5 (5' CAT GCC ATG GAT AAG CTA TTG AAG AAA ACT 3') and Coil3 (5' CCG CTC GAG TTA GTC CCT AGC TCG GTA TAA ATC 3'), or RING5 (5' CAT GCC ATG GTT GGT GAA GGT GCT AAT CGT 3') and RING3 (5' CCG CTC GAG TTA TGA CAC ATG CCG AGC TGA AGT 3'), respectively. The amplified fragments were digested with *Nco*I and *Xho*I, and inserted into pKSm. For pKS-Coil and pKS-RING, leucine at 104 and serine at position 21, respectively, were replaced with methionine. The sequences of all clones constructed using the PCR methods have been confirmed by sequencing the resulting clones. pKS-*cop1-10* was generated by replacing the *Xba*I and *Xho*I-digested fragment with that of pTA-*fus1-4* (McNellis *et al.*, 1994a). The *Bam*HI-*Xba*I fragment of pKS-*cop1-10* was then replaced with that of pKS- Δ RING to generate pKS- Δ RAG4, which lacks the RING-finger and the last repeat of WD-40.

Construction of expression cassettes and stable transformation of Arabidopsis

To construct a 35S- Δ RING expression cassette, pMAL Δ Zn was digested with *Eag*I and *Hind*III, end-blunted with Klenow enzyme and used to replace the GUS gene in pRTL2-GUS (Restrepo *et al.*, 1990). The GUS gene was excised from pRTL2-GUS by cutting the plasmid with *Nco*I and *Bam*HI, and blunt-ended with Klenow. 35S- Δ RING expression constructs were ligated as *Hind*III fragments into the binary plant

transformation vector pBIN19. *Arabidopsis* plants of the No-O ecotype were transformed using the tissue culture procedure as described in McNellis *et al.* (1994b).

For 35S- Δ Coil, 35S- Δ RAC and 35S- Δ RAG4, the corresponding pKSm cassettes were cleaved with *NcoI* and *BglII*, and ligated into the *NcoI* and *BamHI*-digested pRTL2-GUS. For 35S-NARING and 35S-N Δ Coil, the corresponding pKSm cassettes were digested with *NcoI* and *EcoRV*, and ligated into the pRTL2-GUS vector, which had been cut with *BamHI*, end-blunted and recut with *NcoI*. For 35S-GUS Δ RING, 35S-GUS Δ Coil, 35S-GUS $\Delta\Delta$ 35S-GUS Δ RAG4, the corresponding pKSm cassettes were cleaved with *BglII* and ligated into the *BglII* and *BamHI*-cleaved pRTL2-GUSNla Δ Bam. These pRTL cassettes were then cleaved with either *HindIII* or *PstI* and ligated into pPZP222 (Hajdakiewicz *et al.*, 1994). The resulting clones were then electroporated into *Agrobacterium* strain GV3101 (pMP90). *Arabidopsis* plants of ecotype Columbia were transformed by the vacuum infiltration method (Bechtold *et al.*, 1993). Δ RING transgenic seedlings were selected with Kanamycin (40 μ g/ml; Sigma) and transgenic seedlings for the rest of all constructs were selected with Gentamycin (100 μ g/ml; Sigma).

Five, five, seven, nine, seven and five independent transgenic lines for 35S::NARING, 35S:: Δ RING, 35S::N Δ Coil, 35S:: Δ Coil, 35S:: Δ RAC and 35S:: Δ RAG4, respectively, were confirmed for a single T-DNA insertion and accumulation of the transgene products (data not shown). For COP1 localization analysis, four, five, three and three independent lines for GUS- Δ RING, GUS- Δ Coil, GUS- Δ RAC and GUS- Δ RAG4, respectively, were confirmed for a single T-DNA insertion, GUS activity and for the accumulation of the transgene products (data not shown). For those transgenic lines with phenotypes, the co-segregation of the phenotype and the transgenes were established by a previous procedure (McNellis *et al.*, 1996). Either T3 seedlings homozygous for the transgene or T2 seedlings displaying antibiotic resistance were used for analysis. T2 seeds of one GUS- Δ Coil (line 8) and three GUS- Δ RAC lines segregated one-quarter of *fusca* phenotype seedlings (data not shown). They displayed extreme accumulation of anthocyanins, as do COP1 strong or lethal alleles, and often failed to survive (data not shown). All *fusca* seedlings were gentamycin resistant and had extremely reduced levels of the GUS activity and the transgene accumulation (data not shown). Thus, cosuppression events were suspected for these *fusca* seedlings. A consistent 1:2:1 segregation at T2 and T3 generations for gentamycin-sensitive (wild-type):gentamycin-resistant (overexpressor):gentamycin-resistant (cosuppressor) suggests a dosage-dependent mechanism for cosuppression (data not shown). However, this phenomenon was not studied further, since it was not the primary object of our research. For GUS- Δ RAC lines, overexpressors that comprise one-half of the T2 population were used for further analysis.

Yeast two-hybrid analysis

pKS-RING and pKS-Coil were digested with *EcoRI* and *XhoI*, and the rest of the COP1 domain cassettes in the pKSm vectors were digested with *EcoRI*. Fragments were then ligated into pEG202 or pJG4-5 to generate COP1 domains fused to the LexA DNA binding protein or the synthetic activation domain, respectively (Golemis and Khazak, 1997). Generation of pEG-N282, pJG-N282, pJG-COP1 and pJG- Δ RING was described elsewhere (McNellis *et al.*, 1996; Ang *et al.*, 1998). To generate pJG-GUS- Δ RAC, pRTL-GUS- Δ RAC was cleaved with *NcoI* and *XbaI* and then ligated into *NcoI/XbaI*-digested pKS-COP1. The resultant plasmid was cleaved with *EcoRI* and inserted into pJG4-5. Transformation of yeast strain EGY48-0 (Golemis and Khazak, 1997) with bait, prey and reporter plasmids (pSH18-34), and subsequent β -galactosidase activity assay was performed as described in McNellis *et al.* (1996). For unknown reasons, we could not stably express LexA-COP1 protein, either under *ADH* or inducible *GAL1* promoters (data not shown); therefore, we could not test the full-length COP1-COP1 interaction in yeast.

GUS cytochemical staining and confocal laser scanning microscopy

GUS cytochemical staining of *Arabidopsis* transformants was performed as described in von Arnim and Deng (1994). For the confocal laser scanning microscopy, freshly grown seedlings in GM plates under continuous white light were harvested and directly observed using a Bio-Rad 1024 confocal microscope with the Rhodomin filter.

Protein gel blot, in vitro translation, chemical cross-linking and gel filtration chromatography

Protein immunoblot analysis was performed exactly as described previously (McNellis *et al.*, 1994a). pAR-COP1, a FLAG-tagged COP1 that

has an additional peptide, DDADTKDDDDK, at the N-terminus (Matsui *et al.*, 1995), was transcribed *in vitro* and translated using a reticulocyte lysate system (Promega) and radiolabeled with [³⁵S]methionine (Amersham). For chemical cross-linking, 2 μ l of translation mix (with radiolabeled proteins) was diluted to 18 μ l with a reaction buffer (100 mM potassium phosphate pH 7.7, 100 mM NaCl, 0.1 mM ZnSO₄, 10% glycerol, 0.1% Nonidet P-40, 4 mM DTT) and cross-linked with 2 μ l of freshly diluted Dsub or EGS in dimethyl sulfoxide. For a negative control, 2 μ l of dimethyl sulfoxide was added to the reaction. After 20 min incubation at room temperature, the reaction was quenched by addition of 2 μ l of 1 M glycine (for Dsub) or 200 mM lysine (for EGS) and 22 μ l of 2 \times Laemmli SDS-PAGE buffer. Samples were boiled for 5 min, separated by 6% SDS-PAGE and fluorographed after treatment with Amplify (Amersham).

For gel filtration chromatography, 8-day-old light- or dark-grown seedlings were homogenized with 2 \times gel filtration buffer containing 50 mM Tris-HCl (pH 7.5), 440 mM NaCl, 2 mM MgCl₂, 2 μ M ZnSO₄ and 0.5 mM PMSF, and centrifuged for 5 min at 4°C. Total soluble protein was fractionated through a 25 ml Superdex-200 FPLC column (Pharmacia) with 1 \times gel filtration buffer at a flow rate of 0.5 ml/min. All procedures were carried out at 4°C and, for dark samples, under dim green safe light. Consecutive fractions of 0.5 ml each were collected after the void volume (7.5 ml), concentrated and subjected to 10% SDS-PAGE followed by a protein immunoblot analysis. The mol wt standards for size estimation of the native COP1 protein were as follows: blue dextran (void), thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), BSA (67 kDa), ovalbumin (43 kDa) and ribonuclease A (13.7 kDa) (Sigma).

Acknowledgements

We thank Professor Haig Keshishian for assistance with confocal laser microscopy; Ning Wei for suggestions for experimental designs; Christopher Joseph for sequencing pSK- Δ RAG4; Tokitaka Oyama and Kiyotaka Okada for the *HY5* cDNA; Pal Maliga for pPZP222; Albrecht von Arnim and Chatanika Stoop-Myer for communicating unpublished results; and Ning Wei and Mark Osterlund for reading and commenting on the manuscript. K.U.T. would like to express her sincere appreciation to Steven Clark for his support during preparation of the manuscript, and to Tatsuo Kakimoto for continuous encouragement. This work was supported by grants from NIH (GM47850) and a Human Frontier Science Program to X.-W.D. X.-W.D. is an NSF Presidential Faculty Fellow, and K.U.T. was a recipient of the JSPS Postdoctoral Fellowship for Research Abroad.

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Received June 11, 1998; revised July 27, 1998;
accepted August 11, 1998