Modular Domain Structure of Arabidopsis COP1. Reconstitution of Activity by Fragment Complementation and Mutational Analysis of a Nuclear Localization Signal in Planta[1]

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The Arabidopsis COP1 protein functions as a developmental regulator, in part by repressing photomorphogenesis in darkness. Using complementation of a *cop1* loss-of-function allele with transgenes expressing fusions of *cop1* mutant proteins and β-glucuronidase, it was confirmed that COP1 consists of two modules, an amino terminal module conferring a basal function during development and a carboxyl terminal module conferring repression of photomorphogenesis. The amino-terminal zinc-binding domain of COP1 was indispensable for COP1 function. In contrast, the debilitating effects of site-directed mutations in the single nuclear localization signal of COP1 were partially compensated by high-level transgene expression. The carboxyl-terminal module of COP1, though unable to substantially ameliorate a *cop1* loss-of-function allele on its own, was sufficient for conferring a light-quality-dependent hyperetiolation phenotype in the presence of wild-type COP1. Moreover, partial COP1 activity could be reconstituted in vivo from two non-covalently linked, complementary polypeptides that represent the two functional modules of COP1. Evidence is presented for efficient association of the two sub-fragments of the split COP1 protein in Arabidopsis and in a yeast two-hybrid assay.

The regulation of plant development by light is mediated by positive and negative signaling pathways that transduce environmental light signals from phytochromes, cryptochromes, and UV light sensors to the level of gene expression (Khurana et al., 1998). The Arabidopsis constitutive photomorphogenesis 1 (COP1) protein functions as a repressor of photomorphogenesis during seedling germination in darkness by suppressing the expression of light inducible transcripts in the nucleus. COP1 operates in collaboration with other developmental regulators of the COP/DET/FUS group of proteins, where DET and FUS stand for de-etiolated and fusca, respectively (Deng et al., 1991; Fankhauser and Chory, 1997).

Regulation of COP1 activity, which is under negative control by light, appears to involve a light-dependent and tissue-specific relocalization of the COP1 protein across the nuclear envelope, with hypocotyl cell nuclei containing high levels of COP1 in darkness and reduced levels in constant light (von Arnim and Deng, 1994; von Arnim et al., 1997; Osterlund and Deng, 1998). The β-glucuronidase-(GUS) COP1 fusion transgene used to demonstrate the light modulated subcellular localization of COP1 is fully functional in complementing a *cop1* loss-of-function allele, *cop1-5*, suggesting that the modulation of subcellular localization is functionally significant (von Arnim et al., 1997).

The COP1 protein possesses four recognizable structural domains; beginning at the amino terminus, these are a zinc-binding Ring-finger motif, a potential coiled-coil domain (Helix), a central core domain, and a domain of WD-40 repeats (Deng et al., 1992). Nuclear import of COP1 is mediated by a single bipartite nuclear localization signal (NLS) located in the core domain. Nuclear exclusion of COP1 requires a cytoplasmic localization signal (CLS), which overlaps the Helix domain (Stacey et al., 1999).

The COP1 protein may consist of two functional modules. Mild *cop1* mutant alleles are defective in the repression of photomorphogenesis only, but remain viable and fertile. In contrast, severe *cop1* alleles are seedling lethal, accumulate large amounts of anthocyanins during late embryogenesis, and phenotypically resemble loss-of-function alleles of other genes in the *COP/DET/FUS* group. Molecular analysis of an allelic series of *cop1* mutants revealed that a mild allele of *COP1*, *cop1-4*, contains a stop codon at amino acid position 283. The mutation results in a truncated product (COP1-4 or COP1[1-282]) and, unless readthrough occurs, it will prevent synthesis of the COP1 carboxyl terminal domains. In a converse manner, overexpression of COP1-4 results in a mild dominant-negative phenotype (McNellis et al., 1996). Therefore, COP1-4 appears to represent a partly autonomous, amino terminal module (COP1N), which confers a basic function in plant growth and development (McNellis et al., 1994a). Hence the carboxyl

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terminal module of COP1 consisting of the core domain and the WD-40 repeats (COP1C) may extend the function of the COP1N module by enabling the repression of photomorphogenesis. This notion has recently received experimental support because overexpression of COP1(293-675) fused to GUS caused hypocotyl elongation in a wild-type background (Stacey et al., 1999). In addition, truncation of the WD-40 domain abolished the dominant positive phenotype caused by overexpression of full-length COP1 (McNellis et al., 1994b; Torii et al., 1998).

To further delineate the relative functional contributions of the individual COP1 domains, including its NLS, we have studied the complementation and light-dependent overexpression phenotypes of a series of recombinant COP1 alleles with known subcellular targeting properties. Among other data we established that co-expression of the COP1N and COP1C modules from separate polypeptides in Arabidopsis reconstituted almost the entire wild-type COP1 activity. COP1N and COP1C interacted during Arabidopsis immunoprecipitations and in the yeast two-hybrid system, suggesting stable binding between the two non-covalently linked COP1 fragments.

RESULTS

Functional Analysis of COP1 Domains

Previous experiments had shown that full-length COP1 and specific deletion mutants were able to confer a long hypocotyl phenotype during germination of transgenic seedlings under white light conditions (Stacey et al., 1999). To examine whether any of the deletion mutants retained COP1 activity, we tested the capacity of the 35S:GUS-COP1 mutant overexpression constructs to modify the phenotype of the lethal allele *cop1-5*. Based on the severity of the cop1-5 phenotype and the absence of detectable COP1 gene product, *cop1-5* is thought to represent the *cop1* null phenotype (McNellis et al., 1994a). Tables I and II summarize the phenotypes of some of the mutant alleles and transgenes used in this work. Figure 1 summarizes the data and Figure 2 shows representative examples of weak and partial complementation phenotypes after germination in light or darkness. Non-transgenic, wild-type Arabidopsis, as well as the *cop1-5* mutant are shown for comparison. The *cop1-5* mutants expressing intact GUS-COP1 look identical to wild type (Fig. 3; von Arnim et al., 1997).

None of the deletion constructs tested fully complemented the *cop1-5* allele, indicating that all the deleted domains tested here are indispensable for full COP1 activity. To address the extent of overexpression driven by the 35S promoter in our system, we compared the signals for wild-type COP1 and a dilution series of 35S:GUS-COP1 on a western blot that was probed with a polyclonal COP1 antiserum (Fig. 4A). The levels of GUS-COP1 and GUS-COP1(293-675) were approximately 10-fold higher than that of endogenous COP1. Similar expression levels were found for other GUS-COP1 deletion mutants, including GUS-COP1(105-675) (not shown). Therefore, overexpression of the COP1 deletion fragments tested was insufficient to overcome the mutational defect.

Among the deletion mutants, GUS-COP1-4 resulted in the most striking complementation, a phenotype reminiscent of yet more severe than that of the *cop1-4* allele regarding hypocotyl elongation, cotyledon expansion, and anthocyanin accumulation. The original *cop1-4* allele carries a stop codon at position 283 of *COP1*. The GUS-COP1-4 transgene allowed survival and seed set of soil grown *cop1-5* mutants. This result demonstrates conclusively that the COP1(1-282) fragment can function independently of the WD-40 domain to confer a basal level of COP1 activity, even when fused to GUS.

In contrast, deletion of the Ring-finger domain from COP1 resulted in a more severe defect than deletion of the WD-40 and core domains. In detail, GUS-COP1(105-675) retained only weak COP1 activity compared with the *cop1-5* allele, yielding a slightly more elongated hypocotyl in darkness. In addition, partial expansion of the cotyledons took place under light conditions (Fig. 2). A weakly ameliorated phenotype was also seen with GUS-COP1(293-675) (Fig. 2) as well as with GUS-COP1(1-392) and GUS-COP1-9 (Fig. 1). In contrast, GUS-COP1(393-675) expressing a cytoplasmic WD-40 domain did not modify the *cop1-5* phenotype at all (Fig. 1). The lack of function of GUS-COP1(1-392) is of interest in comparison with the substantial activity conferred by GUS-COP1(1-282) and suggests that the core domain of COP1 can interfere with the basal activity of the N-terminal domain.

Table I. *Arabidopsis COP1 alleles used in this work (Deng et al., 1992; McNellis et al., 1994a)*

COP1 Allele	Phenotype		Remarks
	Dark-grown seedling	Adult	
COP1	Etiolated	Wild type	Wild type; 675 residues
cop1-4	cop[a], viable	Reduced size, fertile	Expresses COP1N; stop codon at residue 283
cop1-5	cop, lethal	(lethal)	T-DNA insertion at residue 451
cop1-6	cop, viable	Reduced size, fertile	Five residue insertion at position 301 (NLS)

[a] cop, Constitutive photomorphogenesis.

Table II. *Phenotypes of key COP1-transgenes in Arabidopsis*

Transgene	Seedling Phenotype Background		
	cop1-5	*cop1-4*	Wild-type COP1
GUS-COP1	Dark: (not determined)	(not determined)	(not determined)
	Light: (not determined)	(not determined)	Hyperetiolation
GUS-COP1-4 (= 1-282); = COP1N	Dark: cop, viable	(not determined)	Wild type
	Light: cop, viable	(not determined)	Dominant-negative
GUS-COP1 (293-675) = COP1C	Dark: cop, lethal	Wild type or mild cop	Wild type
	Light: cop, lethal	Mild hyperetiolation	Hyperetiolation

Functional Analysis of an NLS

We then asked to what extent defects in the COP1 NLS would compromise GUS-COP1 activity at the phenotypic level. For each of two site-directed mutants in the NLS, which had shown reduced nuclear accumulation of GUS activity (Stacey et al., 1999), two lines were analyzed for the degree of complementation of the *cop1-5* allele, and the level of transgene expression was determined by GUS activity assay (Figs. 1 and 3). The mutants COP1 mut1 and COP1 mut2 and even the double mutant COP1 mut1 mut2 all suppressed the seedling lethality of *cop1-5* when expressed at levels above 4.0 pmol methylumbelliferone min^{-1} μg^{-1} protein, and fertile plants homozygous for the *cop1-5* allele were recovered for all three constructs. This expression level corresponds to the level shown in the immunoblot of Figure 4A. However, the three mutants differed in the extent of complementation. In darkness, COP1 mut1 showed the most complete complementation at the seedling stage, followed by COP1 mut2 and COP1 mut1 mut2. As adults the COP1 mut2-complemented plants were essentially wild type, whereas COP1 mut1 was slightly dwarfed and COP1 mut1 mut2 was severely dwarfed (average heights at maturity were 20 cm for COP1 mut2 and wild type, 12 cm for COP1 mut1, and 4.5 cm for COP1 mut1 mut2).

Lines with reduced expression levels of the NLS mutants COP1 mut2 and COP1 mut1 mut2 showed more severe *cop1*-like phenotypes after germination in darkness. When grown in soil, no viable plants were recovered for a COP1 mut1 mut2 line characterized by a GUS expression level of 0.9 pmol methylumbelliferone min^{-1} μg^{-1} protein (Fig. 3). For COP1 mut1, all lines analyzed had high expression levels. Taken together, these results confirm that COP1 functions in the nucleus to repress photomorphogenesis, and they demonstrate that the NLS is essential. However, a defect in NLS activity can be partially compensated by a high gene expression level.

It seemed plausible that Ser phosphorylation at sites adjacent to the COP1 NLS, located between residues 293 and 314, might play a role in NLS activity and consequently, in the activity of the overexpressed COP1 (Moll et al., 1991; Jans et al., 1995; Jensen et al., 1998). Among those Ser residues that are conserved in COP1 from Arabidopsis (GenBank accession no. P43254), tomato (accession no. L24437), and pea (accession no. P93471), S280 and S288 are closest to the NLS. To eliminate the possibility of

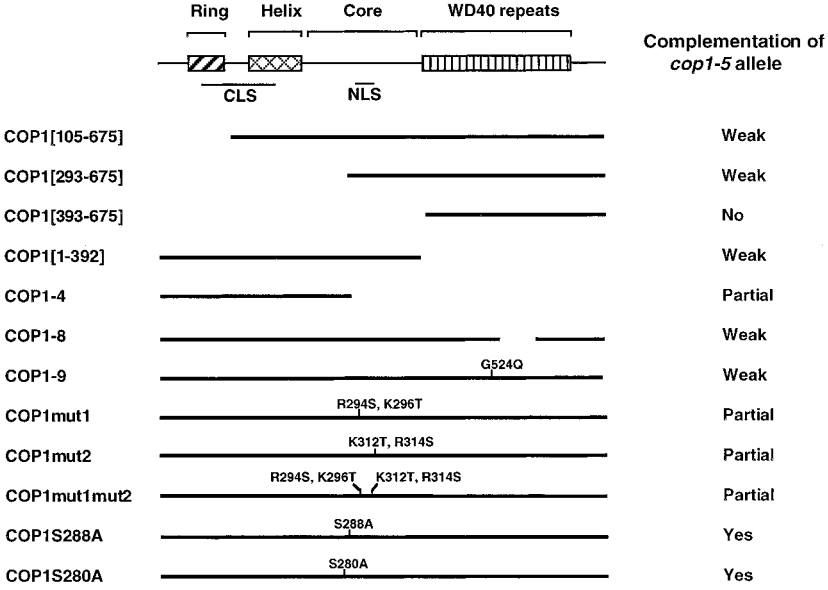

Figure 1. Structure of *COP1* mutants. The capacity of the respective mutants to complement the *cop1-5* mutation is indicated on the right. All COP1 mutants were expressed as GUS-fusions. Yes, Full complementation, i.e. a wild-type phenotype; Partial, plants that retained a mild cop1 phenotype, but produced seeds (e.g. Fig. 2, COP1[1-282]); Weak, a slight amelioration of the *cop1-5* seedling phenotype (Fig. 2, COP1[293-675]), which was insufficient to overcome lethality of *cop1-5*. The structural domains of COP1 are indicated by patterned boxes. CLS and NLS denote the cytoplasmic and nuclear localizaton signal of COP1, respectively.

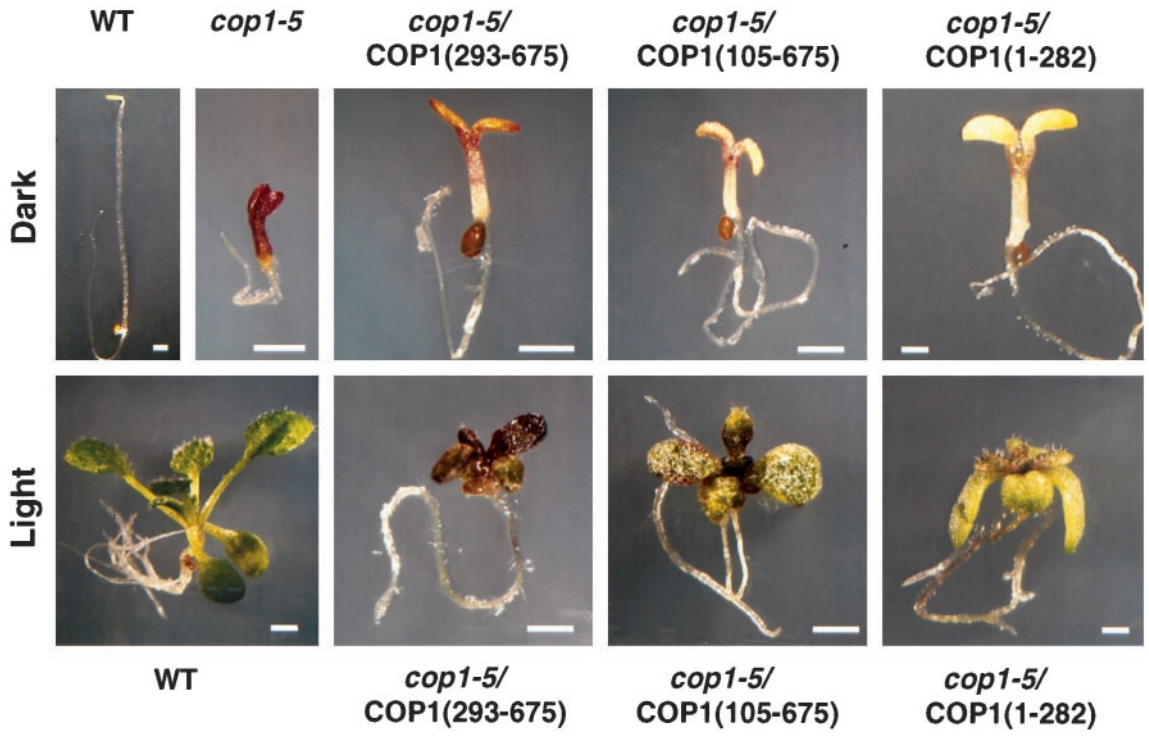

Figure 2. Complementation phenotypes of *cop1-5* homozygous plants expressing COP1 mutant proteins. A *cop1-5* mutant seedling and *COP1* wild-type plants (WT) are shown for comparison. The *cop1-5* mutant looks identical in light and darkness. All COP1 mutant proteins were expressed as GUS fusions. Seedlings were germinated on agar medium for 5 d in darkness (top row) or for 12 d in the light (bottom row). Bars = 1 mm.

phosphorylation at these sites, the Ser residues were changed to Ala. The site-directed mutants S280A and S288A were fully capable of complementing the *cop1-5* allele (Fig. 1) and caused hypocotyl elongation similar to intact GUS-COP1 in a wild-type background (not shown). Therefore, we do not favor a model in which COP1 NLS activity is controlled through phosphorylation at adjacent sites. It obviously remains possible that phosphorylation at nonconserved residues or at more remote residues modulates COP1 NLS activity.

Fragment Complementation between COP1N and COP1C Modules of COP1

COP1-4 alone provides a basal function of COP1, which allows the plant to reach the flowering stage, but renders it constitutively photomorphogenic. COP1(293-675), on the other hand, promotes hypocotyl elongation in a wild-type background and thus acts as a repressor of photomorphogenesis (Stacey et al., 1999). We asked whether co-expression of COP1-4, encoding COP1(1-282), and GUS-COP1(293-675) as separate polypeptides would reconstitute full COP1 activity in transgenic Arabidopsis. When germinating in darkness, *cop1-4* mutant seedlings expressing the GUS-COP1(293-675) protein showed wild-type hypocotyl length (Fig. 5, top), and approximately one-half of the seedlings had closed cotyledons, whereas the remainder lacked an apical hook and had slightly open cotyledons. A non-transgenic *cop1-4* seedling is shown for comparison. Furthermore, GUS-COP1(293-675) transgenic *cop1-4* plants formed a larger rosette, were taller, and produced more seeds than *cop1–4* controls (Fig. 5, D–F).

However, despite the striking complementation seen at the seedling stage, not all *cop1-4* mutant plants carrying a GUS-COP1(293-675) transgene were complemented at the rosette stage. When rosette leaves were stained for GUS activity, we noted that a complemented phenotype correlated with a high transgene expression level, whereas a *cop1-4* like phenotype, i.e. small, rounded rosette leaves, correlated with silenced GUS expression (Fig. 6, top row).

A second mild *cop1* allele, *cop1-6*, which contains a five-residue insertion within the NLS (McNellis et al., 1994a), was not complemented by GUS-COP1(293-675), even when the transgene expression level was high (Fig. 6). Therefore, the interaction between the COP1 amino terminus and GUS-COP1(293-675) was not only transgene expression dependent, but also allele specific. In summary our results clearly demonstrate that a substantial fraction of COP1 activity can be recovered by expressing the two functional modules of COP1 as two separate polypeptides. Henceforth the two modules are referred to as COP1N and COP1C.

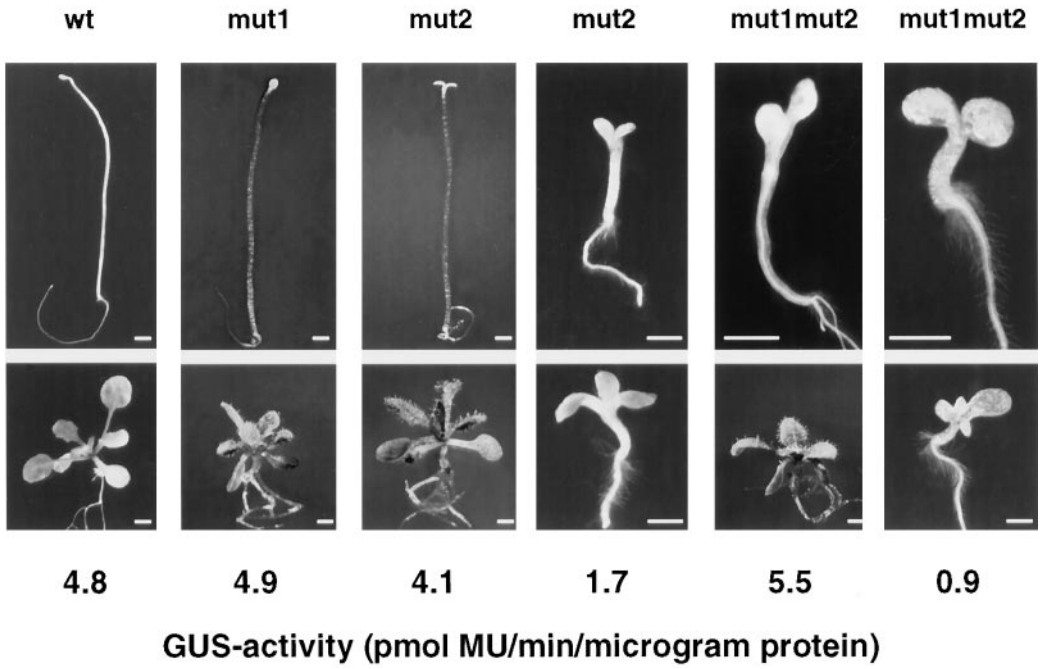

Figure 3. Complementation phenotypes of *cop1-5* homozygous plants expressing the indicated NLS mutants of COP1 or wild-type COP1 (wt). All proteins were expressed as GUS fusions. The relative transgene expression levels are indicated by the GUS activities (picomoles of methylumbelliferone per minute per microgram protein) given below the panels. Seedlings were germinated on agar medium for 5 d in darkness (top row) or for 12 d in the light (bottom row). Bars = 1 mm. Note that the unmutated GUS-COP1 transgene complements the *cop1-5* allele to the level of wild-type Arabidopsis.

The hyperetiolation phenotype conferred by overexpression of GUS-COP1(293-675) in the wild-type COP1 background, which can be seen under white light and far-red light (Stacey et al., 1999), was partly suppressed in the *cop1-4* mutant (Fig. 7). Under blue light, red light, or darkness, the GUS-COP1(293-675) transgene did not cause substantial hypocotyl elongation in wild-type *COP1* plants. Therefore, the requirement of wild-type COP1 under these conditions could not be tested. However, even under red light and blue light the amelioration of the cop1-4 phenotype by GUS-COP1(293-675) can be seen clearly (Fig. 7). The dependence of the hypocotyl elongation under white light and far-red on the presence of wild-type COP1 suggests that the hypocotyl elongation caused by COP1C overexpression may be mediated by the wild-type COP1 protein.

The COP1N and COP1C Modules Interact

The allele-specific complementation between COP1N and COP1C suggested that the two modules of COP1 interact physically in the cell. In this case one might find evidence for a stable complex between the two proteins. In an alternate manner, the two modules might carry out their respective cellular functions independently, but in this case one would not expect to detect a physical association between the two proteins. We tested COP1N and COP1C for a physical interaction in three assays, by co-immunoprecipitation, by colocalization, and in the yeast two-hybrid assay.

Immunoprecipitates were prepared from light-grown *cop1-4* mutant seedlings transgenic for GUS-COP1(293-675). The antibodies used were a polyclonal antiserum raised against the COP1(1-287) protein or a monoclonal antibody that recognizes an N-terminal epitope within COP1. As expected, on western blots, neither antiserum recognized the GUS-COP1(293-675) protein directly (Fig. 4A). The anti-COP1N immunoprecipitates were gel fractionated, immunoblotted, and probed with an anti-GUS antiserum to reveal any coprecipitated GUS-COP1(293-675) protein (Fig. 4B). GUS-COP1(293-675) was co-immunoprecipitated from light-grown *cop1-4* seedling extracts in an antibody-dependent fashion, suggesting that COP1N and COP1C associate with each other in Arabidopsis. Co-immunoprecipitation was also evident in extracts from dark-grown seedlings (not shown). No signal was detected in anti-COP1 immunoprecipitates from transgenic seedlings harboring the wild-type COP1 gene, suggesting that any association between wild-type COP1 and COP1(293-675) may be unstable under our immunoprecipitation conditions. The absence of a stable interaction in the wild type further confirmed that the anti-COP1 antibodies did not recognize the COP1 carboxyl terminus of GUS-COP1(293-675) directly.

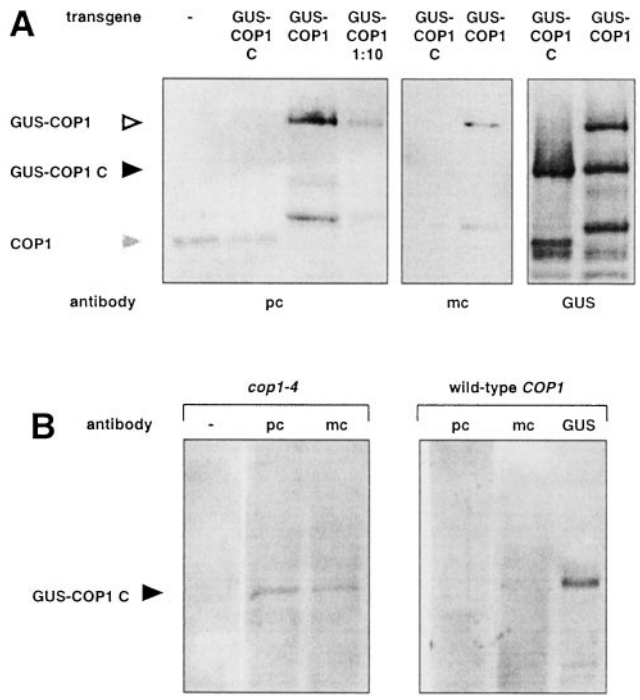

Figure 4. Western-blot data and co-immunoprecipitation between COP1-4 and GUS-COP1(293-675) (GUS-COP1C). A, Equal amounts of total protein from wild-type non-transgenic seedlings (−) or from seedlings expressing GUS-COP1C or GUS-COP1 were separated by SDS-PAGE, blotted, and probed with a polyclonal COP1 antiserum (pc), a COP1 monoclonal antibody (mc), or a GUS antibody (GUS). The monoclonal antibody recognizes an N-terminal epitope absent from GUS-COP1C. Migration positions of the COP1 proteins are indicated. Less than full-length bands are due to protein degradation. For the GUS-COP1 sample, a 1:10 dilution was also loaded to compare the expression levels of GUS-COP1 and wild-type COP1. The GUS-COP1 extracts were from *cop1-5* mutant seedlings and therefore lack the wild-type COP1 signal. The affinity of the monoclonal antibody is insufficient for western-blot detection of wild-type COP1. B, Immunoprecipitates from *cop1-4* mutant or *COP1* wild-type seedlings transgenic for GUS-COP1C were prepared using the polyclonal serum (pc) or the monoclonal antibody (mc), which recognizes COP1-4, but not COP1C. Antibody was omitted from controls (−). The immunoprecipitates were separated, blotted, and probed with an anti-GUS antiserum. Note that GUS-COP1C was coprecipitated with each anti-COP1 antibody from *cop1-4* mutant extracts. One control immunoprecipitation was conducted with an anti-GUS antiserum (GUS).

The GUS-COP1(293-675) module (COP1C) carries a strong NLS, whereas COP1-4 (COP1N) does not (Stacey et al., 1999). We asked whether an association between the two COP1 modules might be indicated by increased nuclear uptake of COP1N when co-expressed with COP1C. When a green fluorescent protein (GFP) fusion of COP1-4 was co-expressed with GUS-COP1(293-675) in onion epidermal cells, approximately one-half of all cotransformed cells clearly showed nuclear GFP fluorescence, specifically in nuclear foci (Fig. 8, top panels). The nuclear foci are characteristic for nuclear COP1 proteins harboring the subnuclear localization signal located within COP1-4 (Stacey and von Arnim, 1999). When GFP-COP1-4 was expressed alone, a small number of nuclear foci were detected in only one out of 20 transformed cells, but all other cells lacked nuclear foci (Fig. 8, C and D). This result is consistent with a physical association between GFP-COP1-4 and GUS-COP1(293-675), an association that is sufficiently stable to promote cotransport into the nucleus of GFP-COP1-4 as directed by the NLS of GUS-COP1(293-675).

We also addressed the hypothesis that the interaction between COP1N and COP1C was mediated by other plant cellular proteins by testing whether COP1(1-287) and COP1(293-675) associate with each other in the yeast two-hybrid assay as a heterologous system (Table III). Co-expression of a LexA fusion of COP1(1-287) and an activation domain fusion of COP1(293-675) did result in strong induction of reporter gene expression. The interaction was as strong as the known interaction between two COP1(1-287) fragments (McNellis et al., 1996), which served as a positive control. Neither COP1(1-287) nor COP1 (293-675) was able to activate transcription on its own or in combination with control partners, indicating that the interaction is most likely direct. Taken together, these data suggest that fragment complemen-

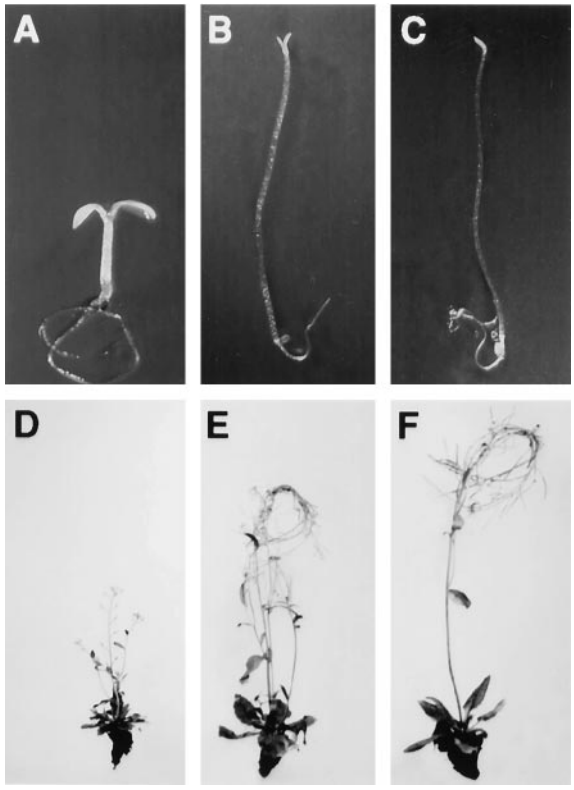

Figure 5. Complementation of the *cop1-4* allele by GUS-COP1(293-675). Transgenic seedlings or non-transgenic controls were germinated in darkness for 5 d (top) or grown under light conditions for 5 weeks (bottom). The two transgenic seedlings shown in B and C represent the range of variation seen among complemented seedlings. A and D, *cop 1-4*; B, C, and E, *cop 1-4*, GUS-COP1(293-675); F, wild type.

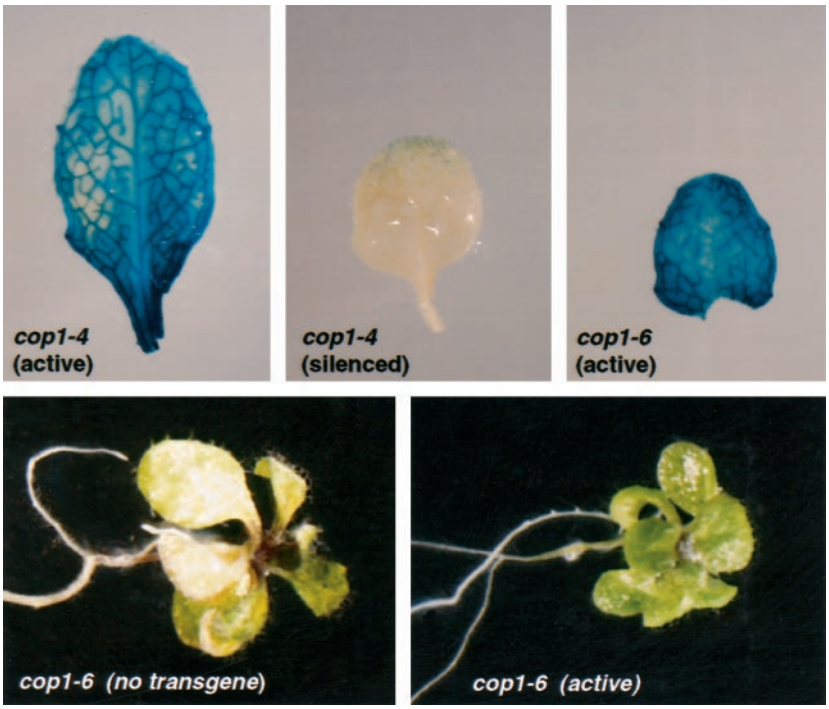

Figure 6. Complementation of *cop1-4,* but not of *cop1-6,* by GUS-COP1(293-675). Rosette leaves were stained for GUS activity (top). Complementation of the *cop1-4* mutant was abolished by silencing of the transgene. Bottom, Unstained plants are shown to demonstrate the lack of complementation of the *cop1-6* phenotype.

tation of COP1 activity is mediated by direct binding of the fragments to each other.

DISCUSSION

In this study we have clarified the modular structure-to-function relationships of the Arabidopsis COP1 protein. Using functional reconstitution in transgenic Arabidopsis we discovered four noteworthy features. First, site-directed mutations of the COP1 nuclear localization signal, although compromising COP1 activity, were less detrimental than anticipated based on prior cell biological assays if expressed at high levels from the cauliflower mosaic virus 35S promoter. Second, deletion of the COP1 amino terminus including the Ring-finger domain abolished COP1 function when assayed in a *cop1-5* mutant background, confirming that the Ring-finger motif is necessary for COP1 activity (Stoop-Meyer et al., 1999). Third, we demonstrated that COP1 function could be reconstituted in vivo from a split protein, i.e. from two separately expressed coding regions that represent the N- and C-terminal modules of COP1. Fourth and finally, the hyperetiolation phenotype displayed by light-grown seedlings that express a COP1 carboxyl-terminal fragment may be mediated by the wild-type COP1 protein.

The Hyperetiolation Phenotype May Be Mediated by Wild-Type COP1

Overexpression of the WD-40 domain with its associated NLS in the core domain confers hyperetiolation in a wild-type background (Stacey et al., 1999). Given that all deletion mutants, as well as the point mutant COP1-9, proved to be non-functional when assayed in a *cop1-5* mutant background, it is clear that the hyperetiolation phenotype does not depend on wild-type activity of the particular COP1 fragment. In a converse manner, the COP1 NLS mutants COP1 mut1 and mut2, which retained substantial COP1 activity, failed to cause hyperetiolation (Stacey et al., 1999). These data are easily explained if we postulate that the COP1 mutants cause hyperetiolation not by autonomously activating the etiolation pathway, but by interfering with the inactivation of endogenous wild-type COP1. This hypothesis is consistent with the observation that the overexpression

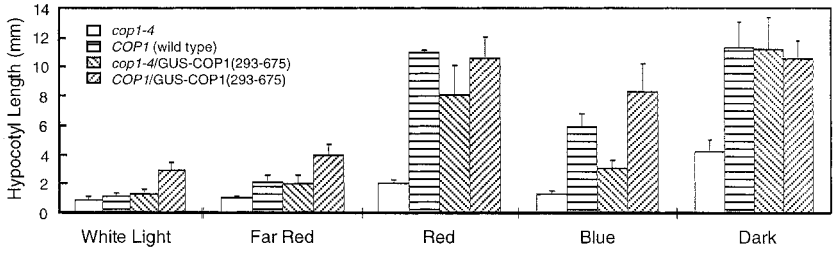

Figure 7. Hypocotyl lengths of *cop1-4* mutant or wild-type *COP1* seedlings with or without the GUS-COP1(293-675) transgene after germination for 5 d under the indicated constant light conditions. Error bars represent standard deviations.

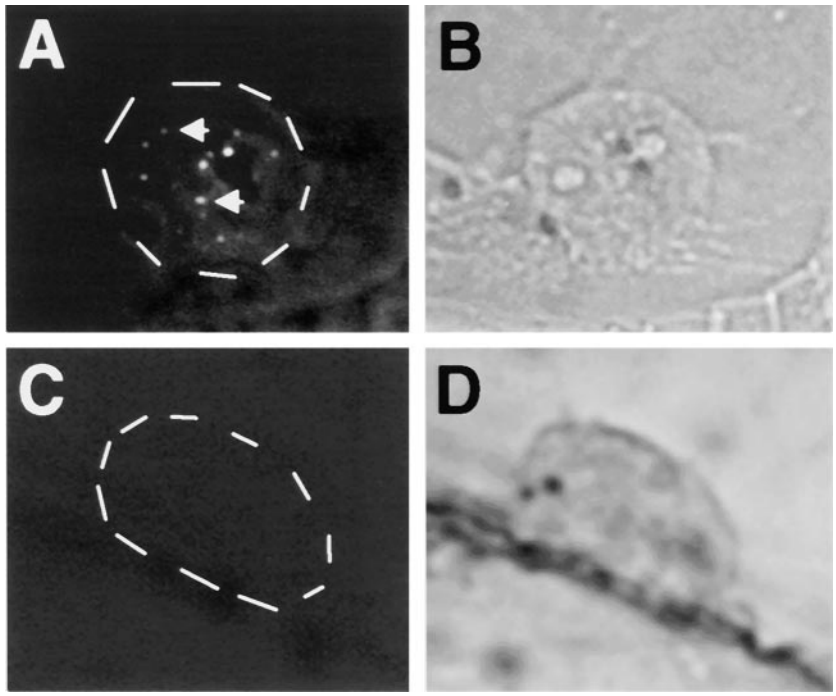

Figure 8. Cotransformation of onion epidermal cells with GFP-COP1-4 and GUS-COP1(293-675). The representative nuclei shown demonstrate that GFP-COP1-4 localizes to nuclear foci when co-expressed with GUS-COP1(293-675), but not when expressed alone. Left, GFP fluorescence; right, brightfield images of the same cells. Broken lines demarcate the nuclei. The cytoplasmic GFP-COP1-4 protein is concentrated in cytoplasmic inclusion bodies, which are outside the field of view shown here. A and B, GFP-COP1-4 and GUS-COP1(293-675); C and D, GFP-COP1-4 alone.

phenotype conferred by GUS-COP1(293-675) under white light and far-red light was reduced in the *cop1-4* mutant (Fig. 7).

The Biological Role of the COP1 NLS

NLSs have been delineated in a substantial number of plant proteins, often using transient expression assays. Moreover, the effect of mutations within an NLS on the biological function of the protein has been tested in the case of viral and bacterial proteins, specifically for *Agrobacterium* proteins involved in T-DNA transfer and tumor formation (Gelvin 1998; Relic et al., 1998). However, there are few if any instances in which the biological requirement for a NLS has been evaluated critically for a plant-encoded protein at the organism level (Liu et al., 1996). One classical bipartite NLS consisting of two clusters of basic residues had been defined for COP1 using cell biological assays (Stacey et al., 1999). Here we showed that at moderate expression levels, site-directed mutants of the COP1 NLS, which eliminated either one or both basic clusters, failed to complement the *cop1-5* allele to various degrees (Fig. 3). However, at high expression levels, mutation of either basic cluster alone still allowed almost complete complementation and even mutation of both basic clusters yielded only an intermediate *cop1*-like phenotype in dark-grown seedlings or in soil-grown plants (Fig. 3). From transient expression assays we estimated that each NLS mutation reduced the nuclear accumulation at least 10-fold, compared with the wild type (Stacey et al., 1999). It is clear that the functional complementation assay used here appears to be more sensitive than previous visual assays. Furthermore, throughout this work the functional assay revealed a strict correlation between integrity of the NLS and COP1 activity, indicating that activity of COP1 depends on its nuclear localization (Fig. 3). Our results provide experimental support for the notion that even large proteins lacking a cytologically recognizable NLS and containing a CLS may gain access to the nucleus under only mild overexpression conditions, possibly by cotransport with other proteins.

Table III. *Yeast two-hybrid assay*

Bait	Prey	β-Galactosidase[a]
		units
LexA:COP1(293-675)	AD:COP1(1-287)	117.0 ± 22.8
LexA:COP1(293-675)	AD	0.9 ± 0.5
LexA:bicoid	AD:COP1(1-287)	0.4 ± 0.3
LexA:COP1(1-287)	AD:COP1(1-287)	58.3 ± 31.3

[a] Mean levels of β-galactosidase activity from four independent cotransformants are shown with their standard deviation.

Reconstitution of COP1 Activity from Protein Sub-Fragments

The mild phenotype of the *cop1-4* allele, which carries a stop codon at position 283, suggested that the encoded protein (COP1N) was capable of performing a basal function of COP1 in regulating plant growth independently of the COP1C module (McNellis et al., 1994a). However, it could not be excluded that residual, but quantitatively insufficient expression of the COP1C module occurred in *cop1-4* mutants, by readthrough of the *cop1-4* stop codon or

by spurious translation initiation at Met M292 of COP1. We showed here that expression of a recombinant GUS-COP1-4 protein rescued the phenotype of the *cop1* null allele *cop1-5* to a level slightly below that of the *cop1-4* mutant. This result argues against readthrough and suggests that COP1N most likely functions independently of the rest of the protein in providing a basal element of COP1 activity.

The ability of GUS-COP1-4 to complement the *cop1-5* allele was surprising because GUS-COP1-4 protein was thought to be predominantly cytoplasmic (Stacey et al., 1999). However, a low efficiency of nuclear uptake may have been compensated by the approximately 10-fold overexpression obtained for a 35S-driven COP1 transgene product. Consistent with this notion, when the expression level of GUS-COP1-4 was reduced by gene silencing (Fig. 6), the transgene failed to complement *cop1-5* .

A genetic interaction between the COP1N module and the COP1C module had been suggested because mutations within the C-terminal portion of the WD-40 domain abolished the basal activity of the COP1N module, resulting in a severe, seedling-lethal phenotype (McNellis et al., 1994a). Moreover, such WD-40 mutations disrupted the targeting of the COP1 protein to discrete nuclear foci, which is a function of the COP1N module (Stacey and von Arnim, 1999). Considering the recessive nature of the WD-40 mutations, they must act in cis, maybe through a protein-to-protein contact between the COP1N and COP1C modules. These results prompted us to seek experimental evidence for or against a direct interaction between the COP1N and COP1C modules. Coexpression of COP1N and a fragment representing the COP1C module (GUS-COP1[293-675]) from unlinked genes reconstituted almost full COP1 activity during seedling development (Fig. 5). This result alone does not prove that COP1N and COP1C interact physically in vivo because it remained possible that the two modules carry out their functions independently in the cell. However, additional data consistent with a physical interaction between COP1N and COP1C came from experiments demonstrating co-immunoprecipitation and colocalization, and from the yeast two-hybrid assay. Therefore, we favor the hypothesis that COP1N and COP1C form a stable complex, possibly a heterodimer, in Arabidopsis.

Even though COP1C interacted with the COP1N module encoded by the *cop1-4* allele, COP1C did not interact efficiently with wild-type COP1 in immunoprecipitations and COP1C also did not complement the *cop1-6* allele. This result is not surprising if one considers that COP1N is already covalently linked to a COP1C module in the wild-type *COP1* and *cop1-6* alleles. The native COP1C fragment may compete effectively with the free GUS-COP1(293-675) protein for the same interface in COP1N. The biological significance of the interaction between the COP1N and COP1C modules is not yet clear. We are exploring the hypothesis that the interaction may provide a means to regulate the activities of the three known subcellular localization signals in COP1, a nuclear, a cytoplasmic, and a subnuclear localization signal (Stacey and von Arnim, 1999; Stacey et al., 1999).

Numerous cellular functions are performed by protein complexes whose subunits are expressed from separate transcripts. In contrast, reconstitution of activity of a single polypeptide from nonoverlapping sub-fragments ("split protein" and "fragment complementation") has been demonstrated only occasionally. In the majority of cases, functional reconstitution was examined biochemically, often with the goal of delineating protein-folding pathways (Marti, 1998; Goldberg and Baldwin, 1999), or after transient expression (Schmidt-Rose and Jentsch, 1997). Functional reconstitution of split protein activity in vivo has also been accomplished in prokaryotes, at times with high efficiency (Rubin and Levy, 1990; Shiba and Schimmel, 1992). In contrast, to our knowledge efficient functional reconstitution by a split protein in a eukaryotic cell has not been described before. It was possible that such reconstitutions would generally be inefficient and would depend on heterologous, covalently fused, dimerizing partner proteins (Johnsson and Varshavsky, 1994; Remy and Michnik, 1999). In the case of COP1 no heterologous partners may be involved, because the COP1N module was untagged. In addition, even though the GUS-tag, or other cellular proteins, may have stabilized the dimerization between COP1C and COP1N, it remains likely that the interaction between COP1N and COP1C was via a common interface.

MATERIALS AND METHODS

COP1 Mutants

Except for the GUS-COP1S280A and GUS-COP1S288A mutants, the construction of the COP1 mutants has been described previously (von Arnim and Deng, 1994; Stacey et al., 1999). All proteins were expressed as GUS fusions under the control of a double 35S promoter. Mutants are referred to by their amino acid coordinates; for example, COP1(105-675) designates a deletion of residues 1 to 104 in the 675-amino acid COP1 protein. Some mutants are referred to by their given allele numbers, e.g. COP1-4 (McNellis et al., 1994a). The S280A and S288A mutants are Ser-to-Ala substitutions, which were generated with the pAlter system (Promega, Madison, WI). COP1 mut1, COP1 mut2, and COP1 mut1 mut2 have site-directed substitutions within the first, the second, or both basic clusters of the bipartite NLS of COP1, respectively (Stacey et al., 1999). The terms COP1N and COP1C refer to the amino-terminal and carboxyl-terminal modules of COP1, which are represented in this work by COP1-4 or COP1(1-287) and GUS-COP1(293-675), respectively.

Plant Growth and Transgenic Lines

For complementation, *cop1-5* heterozygous plants (Deng et al., 1992) were transformed via *Agrobacterium tumefaciens*-mediated DNA transfer following the vacuum infiltration procedure (Clough and Bent, 1998). In some cases transgenic lines created earlier (Stacey et al., 1999) were crossed to plants that were heterozygous for the loss-of-function allele *cop1-5* or homozygous for the mild allele *cop1-4*. Complementation was scored in F_2 populations segregating for the respective *cop1* allele and a single transgene locus. Full complementation was confirmed by recovering viable seeds from a plant homozygous for the *cop1-5* mutation and testing for homozygosity of a kanamycin resistance marker, which is linked to *cop1-5*.

Seedlings were germinated on agar medium containing one-half-strength Murashige and Skoog salts (Sigma, St. Louis) and 1% (w/v) Suc. For specific light treatments, seedlings were illuminated with Snap-Lite light emitting diodes (Quantum Devices, Barneveld, WI) at the following fluence rates: blue light, 10 μmol m^{-2} s^{-1}; far-red light, 15 μmol m^{-2} s^{-1}; and red light, 10 μmol m^{-2} s^{-1}. Digital images were taken on a stereomicroscope (Olympus SZX12, Olympus, Tokyo) with a camera (Olympus DP10, Olympus) and assembled into composites with Adobe Photoshop software (Adobe Systems, Mountain View, CA).

Immunoprecipitation

Protein extracts were prepared from 6-d-old seedlings by grinding in homogenization buffer {400 mM Suc, 10% [w/v] glycerol, 10 mM KCl, 50 mM Tris [tris(hydroxymethyl)-aminomethane], pH 8.0, and 10 mM EDTA} with 2 μM leupeptin, 10 μg/mL aprotinin, and 0.5 mM phenylmethylsulfonyl fluoride as protease inhibitors and spinning for 2 min. The supernatant was supplemented with one volume of NP-40 buffer (150 mM NaCl, 50 mM Tris, pH 8.0, and 1% [w/v] Igepal [Sigma]). Polyclonal or monoclonal antibody was subsequently added and incubated at 4°C for 2 h. Antibodies used were 1 μg of an affinity-purified anti-COP1 polyclonal antibody raised against COP1(1-287) (McNellis et al., 1994a) or 1 μg of IgG from a monoclonal anti-COP1 cell culture supernatant, which recognizes an N-terminal epitope in COP1 (McNellis et al., 1994b). Immunocomplexes collected on proteinA-agarose beads (Sigma) were washed three times with NP-40 buffer and proteins were eluted by incubation at 95°C with 2× Laemmli SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE on an 8% (w/v) denaturing polyacrylamide gel, electroblotted to polyvinylidene difluoride membrane, and probed with an anti-GUS polyclonal antiserum (Molecular Probes, Eugene, OR). Blots were developed using an alkaline phosphatase-linked secondary antibody and chemiluminescence detection with disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2′-{5′-chloro}tricyclo {3.3.1.$1^{3,7}$}decan]4-yl) (Roche Biochemicals, Basel) as substrate.

Yeast Two-Hybrid Assay and Reporter Assays

The *Eco*RI-*Sal*I polylinker sequence of pEG202 (Golemis et al., 1997) was replaced with an alternative linker formed by annealing the oligonucleotides 5′-AATTCGGATCCA-TGGCCTAGGTAATTAAG-3′ and 5′-TCGACTTAATTAC-CTAGGCCATGGATCCG-3′, thereby forming plasmid pAVA458 with stop codons in all three frames downstream of the linker sequence. pAVA459 was created by replacing the polylinker of pJG4-5 (Golemis et al., 1997) with the alternative linker formed by the oligonucleotides 5′-AATTCAGATCTACCATGGCCTAGGTAATTAAC-3′ and 5′-TCGAGTTAATTACCTAGGCCATGGTAGATCTG-3′. COP1 fragments were sub-cloned to pAVA458 for LexA DNA-binding domain fusions and to pAVA459 for B42 activation domain fusions. Plasmids were cotransformed (Gietz and Schiestl, 1995) into yeast strain EGY148 (Invitrogen, Carlsbad, CA) harboring the LexA:lacZ fusion plasmid pSH18-34. pRFHM1, encoding a LexA-bicoid fusion, and pSH17-4, encoding a LexA-GAL4 fusion served as negative and positive controls, respectively. β-galactosidase assays were carried out according to published methods (Ausubel et al., 1997). At least four independent cotransformants were tested at least three times for each plasmid combination.

GFP-COP1-4 and GUS-COP1(293-675) were co-expressed in onion epidermal cells using particle bombardment (Stacey et al., 1999). GUS activity was assayed fluorimetrically according to the method of Jefferson (1987).

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