

# Discrete Domains Mediate the Light-Responsive Nuclear and Cytoplasmic Localization of Arabidopsis COP1

Minviluz G. Stacey, Stephanie N. Hicks, and Albrecht G. von Arnim<sup>1</sup>

Department of Botany, University of Tennessee, HBB 437, Knoxville, Tennessee 37996-1100

The Arabidopsis CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1) protein plays a critical role in the repression of photomorphogenesis during Arabidopsis seedling development. We investigated the control of COP1 partitioning between nucleus and cytoplasm, which has been implicated in the regulation of COP1 activity, by using fusion proteins between COP1 and  $\beta$ -glucuronidase or the green fluorescent protein. Transient expression assays using onion epidermal cells and data from hypocotyl cells of stably transformed Arabidopsis demonstrated that COP1 carries a single, bipartite nuclear localization signal that functions independently of light. Nuclear exclusion was mediated by a novel and distinct signal, bordering the zinc-finger and coiled-coil motifs, that was able to redirect a heterologous nuclear protein to the cytoplasm. The cytoplasmic localization signal functioned in a light-independent manner. Light regulation of nuclear localization was reconstituted by combining the individual domains containing the nuclear localization signal and the cytoplasmic localization signal; the WD-40 repeat domain of COP1 was not required. However, phenotypic analysis of transgenic seedlings suggested that the constitutively nuclear-localized WD-40 repeat domain was able to mimic aspects of COP1 function, as indicated by exaggerated hypocotyl elongation under light conditions.

## INTRODUCTION

Seedling development in flowering plants involves a choice between photomorphogenesis, which is the pathway followed under light conditions, and etiolation, which is the pathway followed upon germination in darkness. Typical of dicotyledonous plants, etiolated Arabidopsis seedlings display an elongated hypocotyl, unexpanded cotyledons, which remain closely appended to each other, and an arrest of shoot apical meristem development. In contrast, seedlings during photomorphogenesis have a short hypocotyl, expanded and photosynthetic cotyledons, and continued cell proliferation and differentiation in the shoot apical meristem. The decision to follow one or the other pathway is reversible and controlled by photoreceptors of the phytochrome and cryptochrome families (reviewed in Quail et al., 1995; von Arnim and Deng, 1996; Cashmore, 1997; Chory, 1997).

The molecular genetic and physiological mechanisms underlying the switch between photomorphogenesis and etiolation are being unraveled. Arabidopsis mutants defective in the biosynthesis of brassinosteroids show partial deetiolation in darkness (Li et al., 1996; Szekeres et al., 1996),

suggesting that brassinosteroid hormones play an important role in orchestrating the etiolation response. In addition, mutagenesis has defined 10 genes, now grouped together as *CONSTITUTIVE PHOTOMORPHOGENIC/DEETIOLATED/FUSCA* (*COP/DET/FUS*) genes, which share the mutant phenotype of constitutive photomorphogenesis, or deetiolation, besides profuse anthocyanin accumulation during late embryogenesis. The wild-type *COP/DET/FUS* alleles promote the etiolation pathway by repressing photomorphogenesis in darkness. The majority of the *COP/DET/FUS* genes operate by directly or indirectly repressing the transcription of light-inducible genes (Miséra et al., 1994; Kwok et al., 1995). How exactly the brassinosteroid-mediated pathway and the *COP/DET/FUS* pathway are integrated with each other remains to be shown, but partial rescue of severe *cop1* mutants by brassinosteroid treatment has suggested that the two pathways are separable (Szekeres et al., 1996).

At the current stage of biochemical analysis, the *COP/DET/FUS* proteins fall into two subgroups. COP9 and FUS6 are representative of one subgroup, because they copurify as subunits of a 500-kD nuclear-localized protein complex, which may also include COP8 (Chamovitz et al., 1996; Staub et al., 1996). More recently, mammalian homologs of COP9 and FUS6 were also discovered in a protein complex of similar size (Seeger et al., 1998; Wei et al., 1998). The

<sup>1</sup> To whom correspondence should be addressed. E-mail vonarnim@utk.edu; fax 423-974-0978.

second subgroup of COP/DET/FUS proteins includes COP1 and DET1, which are nuclear-targeted proteins whose interactions with the COP9-containing complex remain to be resolved (Pepper et al., 1994; von Arnim and Deng, 1994).

Overexpression of the COP1 protein in transgenic Arabidopsis induces features of the etiolation pathway under light conditions, most notably an elongated hypocotyl, suggesting that COP1 is able to repress a subset of photomorphogenic responses autonomously (McNellis et al., 1994b). On the other hand, downregulation of light-inducible gene expression during the circadian day–night cycle does not require wild-type COP1, which is consistent with a role for COP1 in directing long-term responses to light, such as etiolation and dark adaptation (Deng et al., 1991; Deng and Quail, 1992; Millar et al., 1995).

COP1 may serve as a transcriptional regulator by inhibiting the transcriptional activator COP1 interactive protein 7 (CIP7; Yamamoto et al., 1998) or by interacting with DNA binding proteins, for instance, the basic leucine zipper protein HY5 (Oyama et al., 1997; Ang et al., 1998). The COP1 protein contains an N-terminal zinc binding RING finger domain, a C-terminal domain composed of WD-40 repeats, and within the intermediate region, a domain with the potential to adopt a coiled-coil structure (Deng et al., 1992; Lovering et al., 1993; McNellis et al., 1994a). The N-terminal portion of COP1, which alone is retained in the mild *cop1-4* allele, is sufficient to perform a basal set of functions that prevent seedling lethality, but the C terminus is required for the repression of light-inducible genes (McNellis et al., 1994a).

Fusion proteins between COP1 and the  $\beta$ -glucuronidase (GUS) reporter enzyme have suggested a mechanism for the regulation of COP1 activity. The GUS–COP1 protein was detected in hypocotyl cell nuclei after germination in darkness, but its nuclear level is reduced after germination in continuous white light or other light conditions, suggesting that light inactivates COP1 by preventing its nuclear accumulation (von Arnim and Deng, 1994; Osterlund and Deng, 1998). The *GUS–COP1* transgene complements a lethal *cop1* allele and is therefore functionally equivalent to wild-type *COP1* (von Arnim et al., 1997).

Resolving the molecular determinants for the light regulation of COP1 nucleocytoplasmic partitioning should provide new insights into the workings of a fundamental light signaling pathway. Here, we have investigated the nucleocytoplasmic partitioning of COP1 by using mutagenesis of *COP1*. A transient yet quantitative assay for nuclear protein localization based on the green fluorescent protein (GFP; von Arnim et al., 1998) has been employed, and we have also localized GUS–COP1 fusion proteins in transgenic plants. Our data indicate that light regulation of COP1 subcellular localization is achieved by the cooperation between one nuclear localization signal (NLS), which acts constitutively when tested independently, and a novel cytoplasmic localization signal (CLS) that is spatially separable from the NLS. We also demonstrate that the C terminus of COP1, including the WD-40 domain, elicits a long hypocotyl pheno-

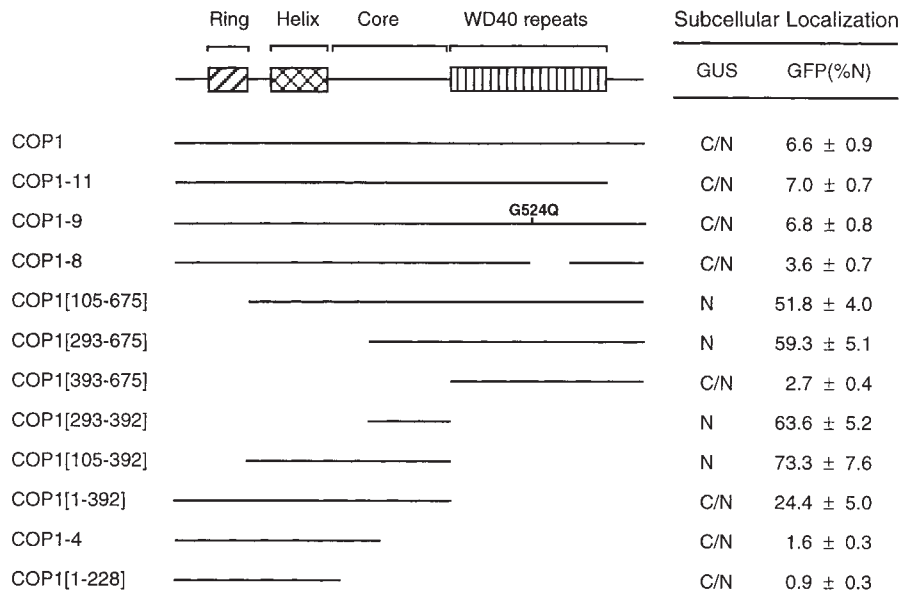
type, supporting the role of the WD-40 domain in the repression of photomorphogenesis in Arabidopsis.

## RESULTS

### Separable Domains Mediate Nuclear and Cytoplasmic Localization of COP1

We first sought to define the minimal domain of COP1 capable of mediating nuclear localization. To this end, a series of COP1 deletion mutants was constructed, as diagrammed in Figure 1. The COP1 mutant proteins were expressed as C-terminal fusions to GUS or GFP by using quantitative imaging in a transient expression assay in onion epidermal cells, and the subcellular localization of each fusion protein was determined. Figure 1 summarizes the quantitative data, which are expressed as the percentage of total cellular fluorescence that is found in the nucleus. Representative fluorescence micrographs of labeled cells are shown in Figure 2. Based on nuclear and cytoplasmic fluorescence intensities, 6% of the GFP–COP1 protein was localized to the nucleus of onion epidermal cells (Figures 1 and 2A). This moderate level of nuclear localization is consistent with previous results (von Arnim and Deng, 1994; von Arnim et al., 1998). Confocal microscopy confirmed the intranuclear localization of GFP–COP1 (Figure 3A). Deletion of amino acid residues 393 to 675, which contain the WD-40 repeats (COP1[1–392]; Figure 2B), or deletion of residues 1 to 104, which contain the RING finger domain (COP1[105–675]; Figure 1), did not prevent nuclear localization of GUS or GFP. Nuclear localization was also retained upon deletion of the RING finger together with the helical domain (COP1[293–675]; Figure 2C) and upon deletion of the RING finger and the WD-40 repeats (COP1[105–392]; Figure 1), suggesting that the central core domain is responsible for nuclear localization. Consistent with this hypothesis, three deletions that eliminated the central core domain (COP1[393–675], Figures 2D and 3D; COP1-4, Figure 2G; and COP1[1–228], Figure 1) abolished nuclear localization. Furthermore, both GUS and GFP were nuclear localized after fusion to the central core domain (COP1[293–392]; Figure 2F). Therefore, all major NLSs in COP1 must reside in the central core domain between residues 293 and 392.

Certain COP1 deletion mutants resulted almost exclusively in nuclear localization, whereas the wild-type COP1 fusion was only weakly karyophilic, suggesting that we had deleted elements necessary for cytoplasmic localization. To define the cytoplasmic localization domain more systematically, we determined the extent of nuclear localization of the GUS and GFP fusions by qualitative GUS histochemical staining and by quantitative GFP fluorescence measurements, respectively. Deletion of the RING finger alone (COP1[105–675]), or the RING finger together with the heli-



**Figure 1.** COP1 Contains Elements Mediating Its Nuclear and Cytoplasmic Localization.

Shown is the structure of COP1 mutant proteins and their subcellular localization as fusions to GUS or GFP in onion epidermal cells. The RING finger (Ring), putative coiled-coil (Helix), and WD-40 domains (WD40 repeats) are indicated by different symbols. The nuclear localization of GUS activity was determined qualitatively. C/N denotes predominantly cytoplasmic staining without nuclear enrichment. N denotes predominantly nuclear staining. The percentage of nuclear GFP fluorescence was determined quantitatively, as described in Methods; averages and standard errors from eight to 12 cells per data point are presented.

cal domain (COP1[293–675]), caused an approximately ninefold increase in nuclear localization (Figure 1).

Conversely, deletion of the entire WD-40 domain (COP1[1–392]) resulted in only a fourfold increase in nuclear localization, and only in the context of the GFP–reporter gene construct. Three mutants of the WD-40 repeat domain, which included an exon-skipping mutant (COP1-8), a premature stop codon mutant (COP1-11), as well as a point mutant (COP1-9; McNellis et al., 1994a), retained a moderate level of nuclear accumulation (Figures 1 and 2). For the weakly nuclear COP1-8 protein as well as for COP1-9, intranuclear localization was confirmed by confocal microscopy (Figure 3). As is evident from comparing Figure 2A with Figures 2E, 2H, and 2I, and from the corresponding confocal images (Figures 3A to 3C), each of the three mutations within the WD-40 domain drastically increased the solubility of the GFP–COP1 protein in the cytoplasm and reduced the punctate nuclear staining, which is caused by COP1 localizing to characteristic subnuclear particles (“speckles”; Ang et al., 1998; von Arnim et al., 1998). The result for COP1-9 is particularly significant, because COP1-9, a point mutant and probably a complete loss-of-function allele of COP1 (McNellis et al., 1994a), partitioned between the nucleus and cytoplasm similar to the wild-type protein. Therefore, in onion cells, speckle formation is regulated independently from nucleocytoplasmic partitioning of COP1. Taken together,

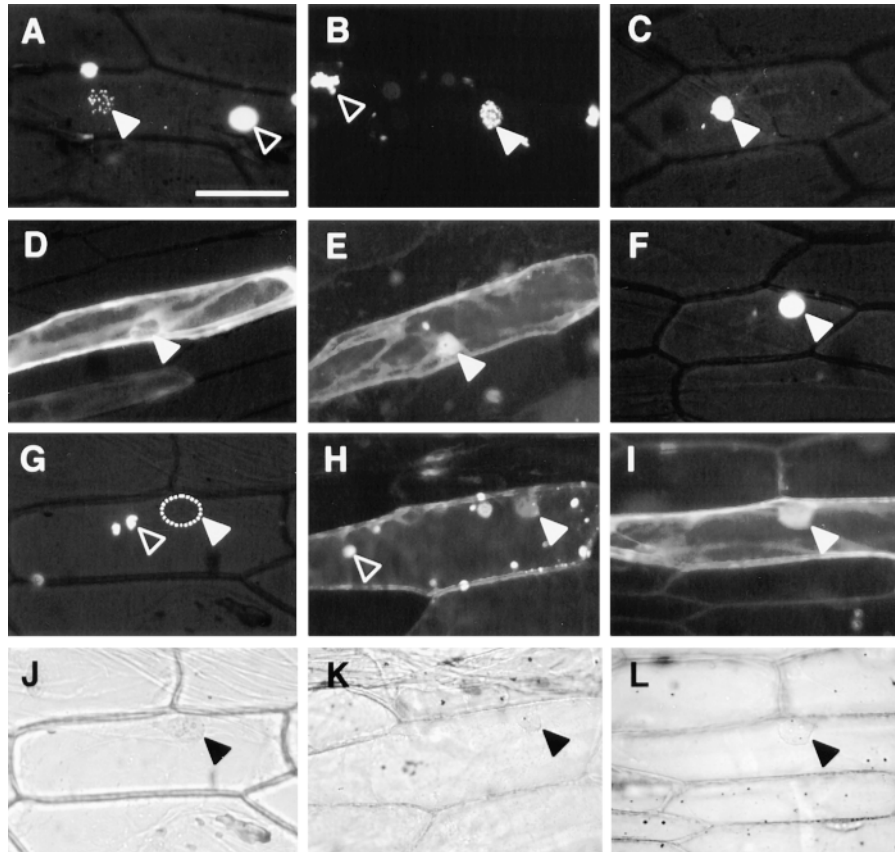
these data suggest that the major determinant for cytoplasmic localization of COP1 resides in the N-terminal portion of COP1. However, elements of the WD-40 domain may contribute to cytoplasmic localization.

**Identification of the NLS of COP1**

To understand the regulation of COP1 nucleocytoplasmic partitioning by light, it was essential to identify the NLSs in COP1. Classical NLSs consist of clusters of basic residues. There are only two clusters of basic amino acids within the core domain, which is responsible for nuclear localization. It was hypothesized that each of these basic clusters might contribute to the nuclear localization of COP1, according to the concept of multiple or bipartite NLSs (Dingwall and Laskey, 1991). Therefore, we incorporated the amino acid changes shown in Figure 4 into either or both of the basic clusters by site-directed mutagenesis. Each mutation was introduced into the predominantly nuclear mutant protein COP1(105–675) and expressed as a fusion to GFP or GUS in the transient onion cell assay. As summarized in Table 1 and Figures 5A to 5D, mutation of either or both basic clusters caused a drastic reduction in nuclear localization, indicating that the two basic clusters function cooperatively as a bipartite NLS.

To determine whether the bipartite NLS was required for light-regulated nuclear targeting of COP1 in Arabidopsis hypocotyl cells, each of the NLS mutations was introduced into wild-type COP1 and fused to GUS, and the fusions were expressed in transgenic Arabidopsis plants. These data are summarized in Table 2, and representative micrographs are shown in Figures 5E to 5H. When compared with a control

expressing wild-type COP1 fused to GUS (Figure 5E), mutations in either or both of the basic clusters resulted in the nuclear exclusion of GUS in Arabidopsis hypocotyl cells after germination in continuous darkness (Figures 5E to 5H) or in continuous light (Table 2), confirming that the two basic clusters function as the elements of a bipartite NLS that is required for light-regulated nuclear accumulation of COP1.



**Figure 2.** Subcellular Localization of COP1 Mutants Fused to the GFP.

Shown are onion epidermal cells expressing GFP fusion proteins. Nuclei and cytoplasmic inclusion bodies are highlighted by filled and open arrowheads, respectively. The positions of the nuclei in (G) to (I) are evident from the bright-field images shown immediately below in (J) to (L). The dotted circle in (G) outlines the nucleus.

(A) GFP-COP1.

(B) GFP-COP1(1-392).

(C) GFP-COP1(293-675).

(D) GFP-COP1(393-675).

(E) GFP-COP1-11.

(F) GFP-COP1(293-392).

(G) GFP-COP1-4.

(H) GFP-COP1-8.

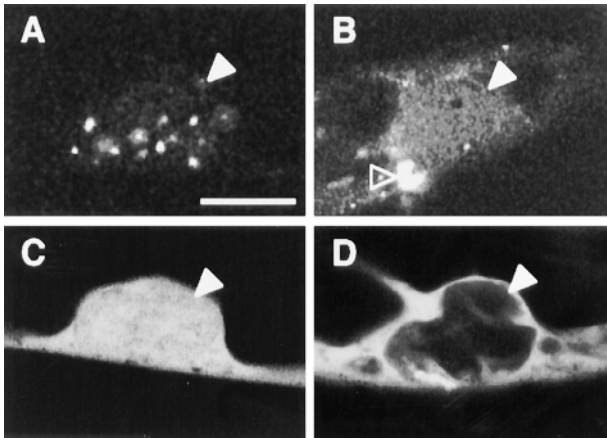
(I) GFP-COP1-9.

(J) Bright-field image of the cell shown in (G).

(K) Bright-field image of the cell shown in (H).

(L) Bright-field image of the cell shown in (I).

Bar in (A) = 50  $\mu$ m for (A) to (L).



**Figure 3.** Confocal Microscopy of GFP-COP1 Mutant Proteins in Onion Epidermal Cells.

Shown are single optical sections through the center of individual nuclei. Nuclei and cytoplasmic inclusion bodies are indicated, as given for Figure 1.

- (A) GFP-COP1.  
 (B) GFP-COP1-8.  
 (C) GFP-COP1-9.  
 (D) GFP-COP1(393-675).

Bar in (A) = 15  $\mu$ m for (A) to (D).

Moreover, the data show that the bipartite NLS is clearly the predominant NLS and likely the only NLS in COP1.

#### A COP1 Domain Mediating the Cytoplasmic Retention of a Heterologous Nuclear Protein

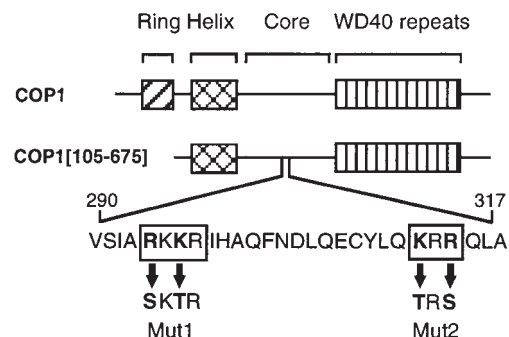
Although our data indicated that deletion of the N-terminal RING finger and helical domains promoted nuclear localization, the mechanism for the increased nuclear accumulation remained unclear. One possibility was that these domains imposed steric constraints, for example, masking the NLS of COP1 or interfering with the nuclear retention of COP1. Alternatively, the RING finger or helical domains might provide an autonomous CLS for either nuclear export or cytoplasmic retention. To test the second hypothesis directly, we fused individual COP1 fragments to the predominantly nuclear Nla protein (Restrepo et al., 1990; von Arnim and Deng, 1994). As shown quantitatively in Figure 6 and with representative micrographs in Figure 7, the chimeric Nla protein carrying the N-terminal 287 amino acids of COP1, COP1(1-287)Nla, localized predominantly, although not exclusively, to the cytoplasm in onion epidermal cells, whereas Nla alone was strongly nuclear (Figures 7A, 7B, 7D, and 7E). The COP1 N terminus, therefore, was able to redirect Nla to the cytoplasm, thus overriding the strong NLS of Nla.

Results obtained using GUS or GFP fusions are entirely consistent (Figures 6, 7D, and 7E). To delineate further the

sequences of COP1 containing the CLS, subfragments of the first 287 residues of COP1 were fused to Nla and expressed as GUS or GFP fusions. The N-terminal RING finger alone was insufficient to direct cytoplasmic localization of Nla (COP1[1-117]Nla; Figures 6, 7G, and 7H). In contrast, the helical domain alone (residues 105-287; Figures 7J and 7K) functioned as a CLS. However, residues 105-287 and 1-287 caused poor solubility of the GFP-Nla fusions, and insolubility may have contributed to their nuclear exclusion. As the next step, we attempted to separate the core CLS from the domain causing insolubility. A fragment overlapping parts of the RING finger and helical domains (residues 67 to 177; Figures 7M to 7O) prevented the nuclear accumulation of Nla, while maintaining solubility. The residual nuclear level of (Figure 6) is similar to the level of bona fide cytoplasmic proteins such as GUS-GFP (von Arnim et al., 1998). We then delineated more closely the sequences required for the CLS between residues 67 and 177 (Figures 6, 7C, 7F, 7I, and 7L). Each of four additional deletions caused a reduction in cytoplasmic localization and an increase in nuclear localization, indicating that for full CLS activity, the complete domain from residue 67 to 177 was required. In summary, our experiments indicate that COP1 contains an extended N-terminal CLS that is spatially separable from the COP1 NLS and from an insolubility determinant in the helical domain. The CLS functions efficiently outside the context of the COP1 protein.

#### Reconstitution of Light-Responsive Nuclear Localization from COP1 Targeting Domains

The GUS-COP1 fusion protein displays light-regulated nucleocytoplasmic partitioning in *Arabidopsis* hypocotyl cells (von Arnim and Deng, 1994; von Arnim et al., 1997; Osterlund and Deng, 1998; Torii et al., 1998). Based on the data



**Figure 4.** COP1 Contains a Single, Essential, Bipartite NLS.

Shown is a diagram of the amino acid sequence of the wild-type COP1 bipartite NLS and amino acid replacements in the mutants Mut1 and Mut2 and the Mut1Mut2 double mutant. Two clusters of basic residues are boxed, and residues altered by site-directed mutagenesis are shown in boldface.

**Table 1.** Subcellular Localization of NLS Mutants in Onion Epidermal Cells<sup>a</sup>

Protein (NLS) <sup>b</sup>	Localization	
	GUS <sup>c</sup>	GFP (%N)
COP1 (105–675)		
(WT)	N	51.8 ± 4.0
(Mut1)	C/N	1.6 ± 0.4
(Mut2)	C/N	3.2 ± 1.3
(Mut1Mut2)	C/N	2.6 ± 0.6

<sup>a</sup> For details, refer to the legend for Figure 1.

<sup>b</sup> WT and Mut refer to the amino acid sequences of the wild-type and mutant NLSs, as depicted in Figure 4.

<sup>c</sup> N indicates nuclear enrichment and C/N denotes cytoplasmic localization without nuclear enrichment.

presented so far, we considered three models that could account for the role of the COP1 domains in the light-regulation of subcellular localization. First, the activity of the NLS may be light responsive. Second, the activity of the CLS may be light responsive, overriding a constitutive NLS in the light but not in darkness. Third, neither the NLS nor the CLS may be light responsive on its own; rather, light responsiveness may be conferred by the cooperation of the two signals in the context of other COP1 domains. To distinguish between the three models, we introduced fusions between GUS and the corresponding COP1 fragments into Arabidopsis by stable transformation and stained transgenic seedlings, grown under light or dark conditions for 5 days, for the subcellular localization of the fusion protein.

We first examined whether the COP1 central core domain containing the bipartite NLS, COP1(293–392), was sufficient for light-regulated nuclear localization. Comparison of GUS localization after germination in continuous light or constant darkness revealed that the central core domain directed constitutively nuclear localization, regardless of the light regime, as shown in Figures 8C and 8D.

Subsequently, we addressed whether the RING finger and helical domains, which together mediate the nuclear exclusion of the Nla protein in onion cells (Figure 6), could impart light-regulated subcellular localization on the Nla protein. It is clear from Figure 9 that the chimeric GUS–COP1(1–287)Nla protein was localized primarily to the cytoplasm under both light and dark conditions (Figures 9A and 9B), whereas GUS–Nla alone was constitutively nuclear (Figures 9E and 9F). The weak perinuclear staining seen in the GUS–COP1(1–287)Nla plants was also observed in seedlings expressing the GUS protein alone (Figures 8S and 8T). In contrast, the RING finger domain alone did not modify the constitutively nuclear localization of Nla (Figures 9C and 9D). This observation, which is consistent with results from onion epidermal cells, suggests that nuclear exclusion of Nla in Arabidopsis requires elements of the COP1 helical domain. Taken together, these data suggest that neither the

NLS domain nor the CLS domain is sufficient to bring about light-regulated nuclear localization. Therefore, although the NLS and the CLS can function outside the context of the COP1 protein, light regulation of their activities is specified within the context of additional COP1 protein domains.

With the goal of delineating a protein element capable of mediating light-regulated subcellular localization, additional domains of COP1 were added onto the constitutive NLS and CLS domains and tested for light-regulated localization in transgenic Arabidopsis. Constitutive nuclear localization was retained upon addition of the WD-40 repeats to the NLS core domain (COP1[293–675]; Figures 8E and 8F). Moreover, the addition of both the WD-40 repeats and the helical domain to the central core domain, as in COP1(105–675), did not prevent constitutively nuclear localization (Figures 8G and 8H). As expected, addition of the helical domain alone to the central core (COP1[105–392]; Figures 8I and 8J) also maintained constitutively nuclear localization.

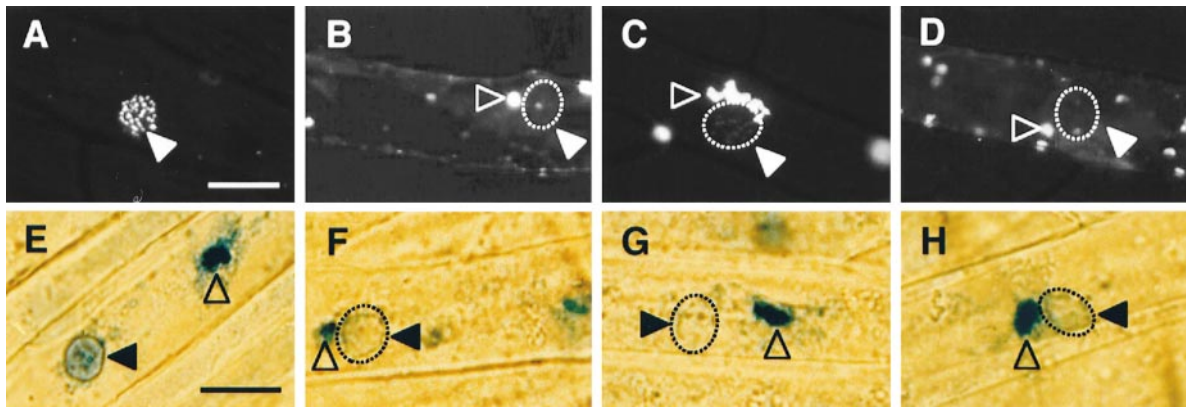
In contrast, COP1-4, which retains only the N terminus, and COP1(393–675), which retains the WD-40 repeats, were unable to localize to the Arabidopsis nucleus when fused to GUS, which is consistent with their lack of an essential NLS located within the central core domain (Figures 8O to 8R). Instead, cytoplasmic staining was evident. Note, again, that perinuclear staining, as seen in Figure 8O for GUS–COP1-4, is also seen with GUS alone (Figure 8S) and does not provide evidence for GUS localization within the nucleus.

Finally, combining the central core domain, which harbors the bipartite NLS, with the N-terminal domain harboring the CLS resulted in a protein that displayed light-regulated nuclear localization in Arabidopsis hypocotyls (COP1[1–392]; Figures 8K and 8L). The nuclear and cytoplasmic levels for COP1(1–392) were similar to those for the full-length COP1 protein (Figures 8A and 8B), although COP1(1–392) did not form cytoplasmic inclusion bodies. Further summarized in Table 3, these results suggest that light-regulated subcellular localization of COP1 requires an interaction between the N terminal CLS domain and the central core domain harboring the NLS and that the WD-40 repeats are not essential.

The point mutation in the WD-40 repeats of the lethal allele COP1-9 did not dramatically affect light-regulated subcellular localization of COP1 (data not shown). However, the COP1-8 mutant showed reduced nuclear accumulation in darkness (Figures 8M and 8N), more closely resembling GUS (Figure 8S) than GUS–COP1 (Figure 8A). Consistent with the weak nuclear uptake of GFP–COP1-8 in onion cells (Figures 2G and 3B), this result confirms that mutations in the WD-40 repeats can interfere with the subcellular localization of COP1.

### Role of COP1 Domains in the Repression of Photomorphogenesis

Overexpression of either COP1 alone or GUS–COP1 results in hypocotyl elongation and reduced cotyledon expansion



**Figure 5.** Subcellular Localization of Site-Directed Mutants of the COP1 NLS.

Hypocotyl cells are shown after germination in darkness for 5 days. Note nuclear exclusion of the mutant proteins. White and black arrowheads indicate the positions of nuclei, and open arrowheads indicate cytoplasmic inclusion bodies. Nuclei are further outlined by dotted circles. (A) to (D) show mutants that were introduced into COP1(105–675) and expressed as GFP fusion proteins in onion epidermal cells. (E) to (H) show mutants that were introduced into wild-type COP1 and expressed as GUS fusions in transgenic Arabidopsis seedlings.

- (A) COP1(105–675).  
 (B) COP1(105–675)Mut1.  
 (C) COP1(105–675)Mut2.  
 (D) COP1(105–675)Mut1Mut2.  
 (E) COP1.  
 (F) COP1–Mut1.  
 (G) COP1–Mut2.  
 (H) COP1–Mut1Mut2.

Bar in (A) = 50  $\mu\text{m}$  for (A) to (D); bar in (E) = 15  $\mu\text{m}$  for (E) to (H).

under light-grown conditions (McNellis et al., 1994b; von Arnim and Deng, 1994). Does GUS–COP1 function in the cytoplasm or the nucleus to cause hypocotyl elongation? Hypocotyl elongation by GUS–COP1 overexpression might be the indirect result of cytoplasmic accumulation of GUS–COP1, if GUS–COP1 caused the release of wild-type COP1 protein from hypothetical cytoplasmic binding sites and its transport to the nucleus, thereby increasing the level of nuclear COP1. Alternatively, a small amount of GUS–COP1 protein present in the nucleus under light conditions may directly mimic COP1 activity in the nucleus.

To clarify the role of the COP1 domains in the repression of photomorphogenesis and the likely site of action of the GUS–COP1 fusions, we measured the hypocotyl lengths of seedlings overexpressing GUS–COP1 mutant proteins after germination in continuous white light. As shown in Table 3, the constitutively nuclear GUS–COP1(293–675) protein, comprising the core domain with the NLS as well as the WD-40 repeats, resulted in even more pronounced hypocotyl elongation than did wild-type GUS–COP1 in the light. An elongated hypocotyl was also observed in seedlings expressing GUS–COP1(105–675). On the other hand, overexpression of cytoplasmic GUS–COP1(393–675), containing only WD-40 repeats, or the cytoplasmic GUS–COP1-4 protein, compris-

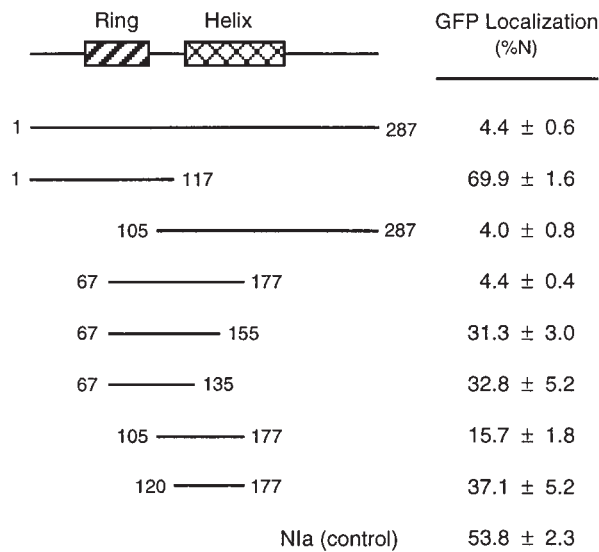
ing the N-terminal RING finger and coiled-coil domains, did not cause hypocotyl elongation (Table 3). These data suggest that GUS–COP1 may function in the nucleus, not the cytoplasm, to promote hypocotyl elongation. The WD-40 repeat domain is necessary for hypocotyl elongation, and when combined with the core domain, it is sufficient. Consistent with a nuclear function, the NLS mutants Mut1,

**Table 2.** Subcellular Localization of COP1 NLS Mutants after Expression as GUS Fusions in Hypocotyl Cells of Transgenic Arabidopsis Plants

Protein <sup>a</sup> (NLS)	GUS Localization <sup>b</sup>	
	Light	Dark
COP1		
(WT)	C	N
(Mut1)	C	C
(Mut2)	C	C
(Mut1Mut2)	C	C

<sup>a</sup>Abbreviations are as given in footnote b of Table 1.

<sup>b</sup>N indicates nuclear enrichment and C denotes cytoplasmic localization.



**Figure 6.** COP1 Contains a CLS.

Fragments from the COP1 N terminus were inserted between GFP and Nla, and the fusion proteins were expressed in the onion epidermal cell assay. Amino acid coordinates of the wild-type COP1 protein are given. The percentage of nuclear localization (%N) is expressed as given for Figure 1. The GFP-Nla protein served as a control.

Mut2, and Mut1Mut2 did not cause hypocotyl elongation, and neither did overexpression of GUS-COP1(1–392), which showed wild-type nucleocytoplasmic partitioning.

## DISCUSSION

Nucleocytoplasmic partitioning plays an important role in diverse cellular signaling processes and has been implicated in the regulation of photomorphogenesis in plants (Harter et al., 1994; von Arnim and Deng, 1994; Sakamoto and Nagatani, 1996; Terzaghi et al., 1997). For instance, the photoreceptor phytochrome B possesses C-terminal elements that can target GUS to the nucleus. This result is consistent with immunological detection of phytochrome B in nuclear and cytoplasmic fractions (Sakamoto and Nagatani, 1996). The G-box binding factors (GBFs), which bind to the G-box DNA sequence elements present in the promoters of many light-regulated nuclear genes, also appear to partition between the cytoplasm and the nucleus (Harter et al., 1994), and the nuclear level of at least one GBF, GBF2, may be increased by blue light (Terzaghi et al., 1997). On the other hand, the nuclear level of the repressor of photomorphogenesis COP1 is diminished under light conditions in Arabidopsis. These data suggest bidirectional trafficking of

transcriptional regulators when seedlings are exposed to light, implicating at least two distinct cellular mechanisms for the regulation of nucleocytoplasmic partitioning.

In this work, we have characterized the protein sequence motifs within COP1 that contribute to its subcellular partitioning, that is, its nuclear localization after germination in darkness and its nuclear exclusion after germination in the light. Besides defining the major NLS in COP1, we have identified and characterized an example in the plant kingdom of a protein domain that confers cytoplasmic localization onto a heterologous, nuclear-targeted protein. Subsequently, how the domains directing either nuclear or cytoplasmic localization work together to bring about the light-regulated partitioning of COP1 in Arabidopsis hypocotyl cells was examined. As discussed in detail below, our data suggest that light-regulated partitioning is mediated by the cooperation of a constitutive CLS and a constitutive bipartite NLS in the context of additional COP1 domains.

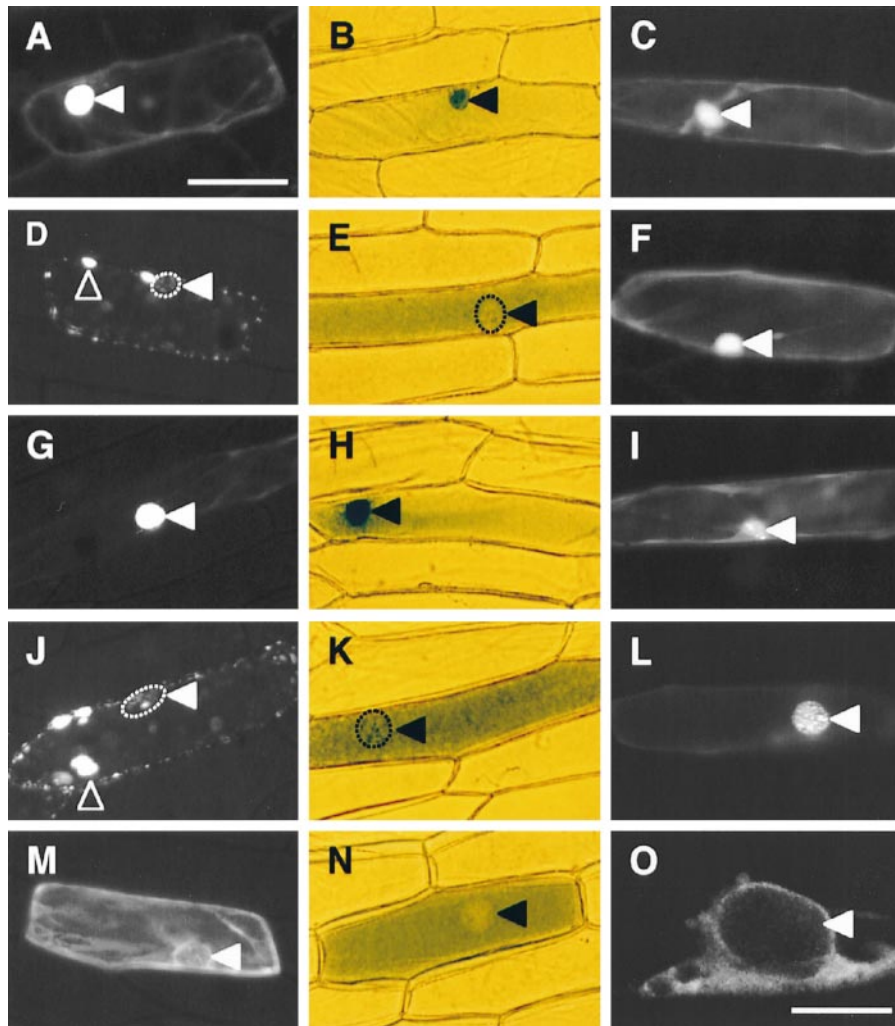
## Role of the WD-40 Repeats

Mutations disrupting the precise conformation of the WD-40 repeats can interfere with all known aspects of COP1 function (McNellis et al., 1994a), although no autonomous function had been ascribed to this domain. Here, we discovered that the C-terminal WD-40 repeat domain was not essential for light-regulated partitioning between nucleus and cytoplasm, because COP1(1–392), which lacks this domain, was capable of directing light-responsive subcellular localization (Figure 8). However, we found constitutive nuclear localization of a fragment comprising the core domain and the WD-40 repeats (GUS-COP1[293–675]). Overexpression of this fragment resulted in pronounced hypocotyl elongation under light conditions (Table 3). The lack of a hypocotyl elongation phenotype in seedlings transgenic for the WD-40 repeats alone, GUS-COP1(393–675), clearly shows that the core domain is important for the hypocotyl elongation phenotype, most likely by providing the NLS for the WD-40 repeats. It is noteworthy that the phenotype of COP1(293–675) is more pronounced than that of wild-type COP1, whether fused to GUS (Table 3) or not (McNellis et al., 1994b). The strong dominant-positive phenotype might be due to the lack of a CLS in COP1(293–675), which might prevent the inactivation of the protein under light conditions.

## Nuclear Localization

We have identified a single NLS in COP1 that consists of two clusters of basic amino acids, separated by 14 residues. Because mutations in each of the two basic clusters reduced NLS activity, this motif is not only structurally but also functionally bipartite (Dingwall and Laskey, 1991). Many plant transcriptional regulators that have been studied





**Figure 7.** COP1 Fragments Redirect Nia to the Cytoplasm.

Onion epidermal cells expressing GFP and GUS fusions of chimeric COP1/Nia proteins are shown. Numbers within parentheses refer to the amino acid positions of wild-type COP1, as diagrammed in Figure 6. (A), (C), (D), (F), (G), (I), (J), (L), (M), and (O) show epifluorescence microscopy of GFP fusions, and (B), (E), (H), (K), and (N) show bright-field images of cells stained for GUS activity. (O) is a confocal image at a three-fold higher magnification compared with (A) to (N). Filled and open arrowheads indicate nuclei and cytoplasmic inclusion bodies, respectively. In (D), (E), (J), and (K), nuclei are outlined by dotted circles.

(A) and (B) Nia.

(C) COP1(67–155)Nia.

(D) and (E) COP1(1–287)Nia.

(F) COP1(67–135)Nia.

(G) and (H) COP1(1–117)Nia.

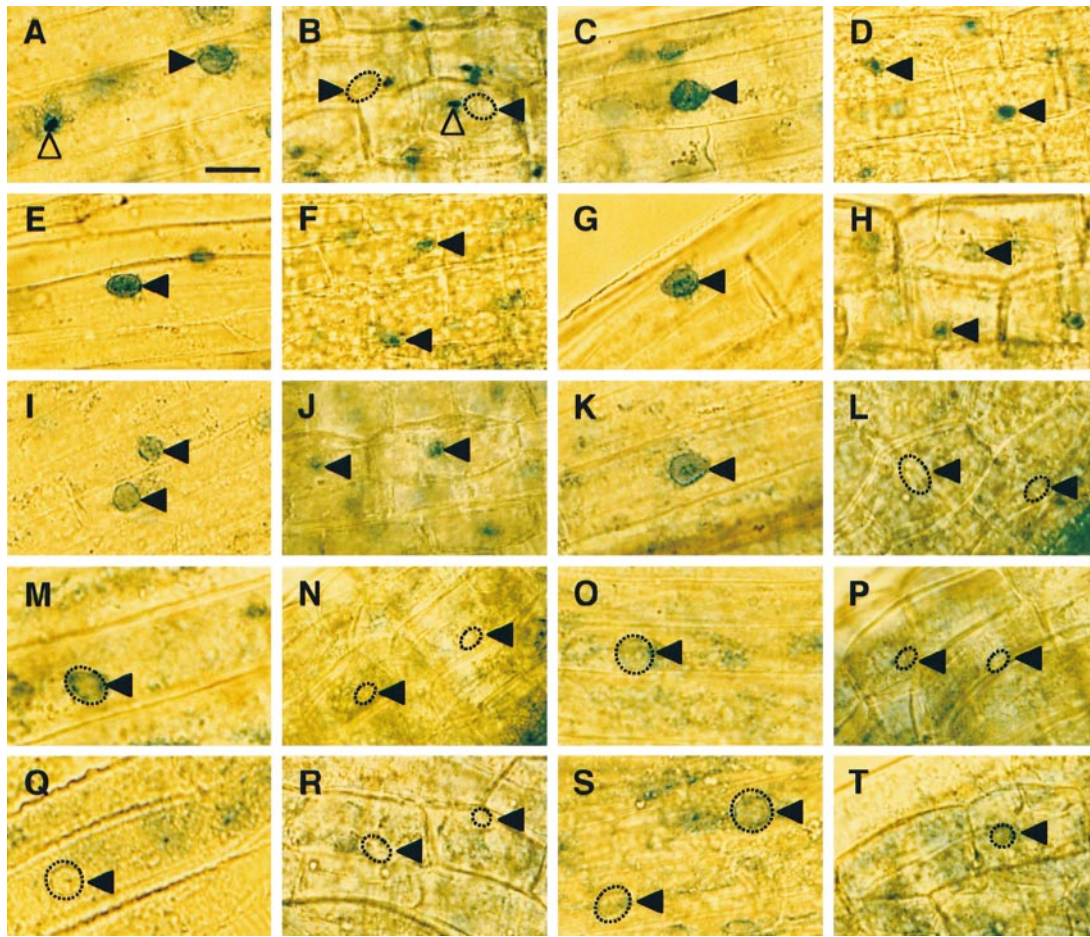
(I) COP1(105–177)Nia.

(J) and (K) COP1(105–287)Nia.

(L) COP1(120–177)Nia.

(M) to (O) COP1(67–177)Nia.

Bar in (A) = 50  $\mu\text{m}$  for (A) to (N); bar in (O) = 15  $\mu\text{m}$ .



**Figure 8.** Light Responsiveness of GUS-COP1 Mutant Fusion Proteins in Hypocotyl Cells of Transgenic Arabidopsis.

Seedlings transformed with the indicated fusion proteins were germinated for 5 days in continuous white light or continuous darkness and stained for GUS activity. Segments are arranged in pairs, with (A), (C), (E), (G), (I), (K), (M), (O), (Q), and (S) showing the dark-grown sample, and (B), (D), (F), (H), (J), (L), (N), (P), (R), and (T) showing the light-grown sample. The positions of the nuclei are indicated by black arrowheads. In (B), a cytoplasmic inclusion body is highlighted by an open arrowhead. Dotted circles outline the nucleus. Note that the cytoplasmic pigment tends to bind to the surface of the nucleus, especially in dark-grown samples, as is evident in (G), (H), and (J).

(A) and (B) GUS-COP1.

(C) and (D) GUS-COP1(293-392).

(E) and (F) GUS-COP1(293-675).

(G) and (H) GUS-COP1(105-675).

(I) and (J) GUS-COP1(105-392).

(K) and (L) GUS-COP1(1-392).

(M) and (N) GUS-COP1-8.

(O) and (P) GUS-COP1-4.

(Q) and (R) GUS-COP1(393-675).

(S) and (T) GUS.

Bar in (A) = 15  $\mu$ m for (A) to (T).

contain more than one NLS (Varagona et al., 1992; Shieh et al., 1993; Dehesh et al., 1995). However, it is unlikely that additional NLSs exist within COP1, although we cannot entirely rule out this possibility. The WD-40 repeats do not contain consensus NLS-like motifs (Görlich and Mattaj, 1996) and

did not drive nuclear localization of GUS or GFP. Likewise, the RING finger domain is devoid of NLS-like motifs and contributed to cytoplasmic rather than nuclear localization. The helical domain contains clusters of basic residues (data not shown), which might possibly function as an NLS if

taken out of the sequence context of COP1. However, both within COP1 and when fused to Nla, their potential activity as an NLS was outweighed by the cytoplasmic localization signal located within this domain. Finally, mutations in the bipartite NLS within the core domain diminished nuclear localization of GUS in Arabidopsis and of GUS and GFP in onion cells. NLS mutants of COP1 did not cause a hypocotyl elongation phenotype, indicating that COP1 must reside in the nucleus to mediate this overexpression phenotype (Table 3).

The GUS-COP1 and GFP-COP1 fusion proteins, as well as wild-type COP1, accumulate in subnuclear particles ("speckles") in Arabidopsis, and speckle formation does not appear to interfere with the function of the fusion proteins (von Arnim et al., 1997, 1998). Moreover, COP1 can recruit its signaling partner, HY5, to subnuclear speckles (Ang et al., 1998), suggesting a role for the speckles in mediating COP1 activity. It is possible that the speckles marked by COP1 correspond to one of several subnuclear domains characterized in non-plant cell types (Lamond and Earnshaw, 1998). Here, we present evidence that speckle formation is controlled independently from nuclear uptake, because the COP1-9 point mutation in the WD-40 domain reduced nuclear speckle formation without changing the overall distribution of the protein between nucleus and cytoplasm. The exact domain responsible for targeting to nuclear speckles remains to be determined; however, it appears to be distinct from the WD-40 domain (M.G. Stacey and A.G. von Arnim, unpublished data).

### COP1 Reveals a Novel CLS

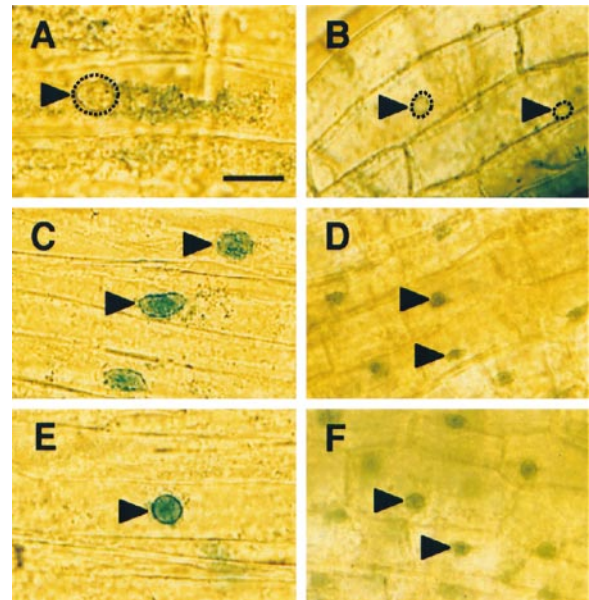
To date, no signal mediating active nuclear exclusion, whether operating by cytoplasmic retention or by nuclear export, has been defined for any plant protein. By fusing individual COP1 fragments onto the heterologous nuclear Nla protein, we showed that the COP1 N terminus contains a CLS. In the context of COP1, we operationally designate as a CLS the minimal motif that is sufficient for redirecting the nuclear Nla protein to the cytoplasm. It is noteworthy that the CLS was functional in the context of two different reporter proteins, GUS and GFP.

The shortest fully active CLS is located within residues 67 to 177 (Figures 6 and 7), overlapping the RING finger and helical domains of COP1. The RING finger domain (residues 1 to 105) did not specify a CLS per se. Its deletion resulted in strongly increased nuclear localization in the context of full COP1, both in onion cells (Figure 1) and in transgenic Arabidopsis (Figure 8G), but it had no effect in the context of the chimeric COP1(1-287)Nla protein (Figure 6), suggesting a conformational role for the RING finger. Therefore, we propose that the core CLS resides between residues 105 and 177. This domain was able to reduce nuclear localization of GFP-Nla from >54 to 16%. The region between residues 177 and 287 may aid in cytoplasmic localization in some

protein contexts (e.g., cf. COP1[105-177]Nla with COP1 [105-287]Nla in Figure 7). The region between 67 and 105 appears to boost the effectiveness of the core CLS, and this function is essential in yet another set of protein contexts (e.g., cf. COP1[105-392] with COP1[1-392] in Figure 1).

We have no evidence that COP1 possesses autonomous CLSs besides the one between residues 67 and 177. However, domains beyond the CLS may contribute to cytoplasmic localization. For example, deletion of the WD-40 repeats resulted in increased nuclear localization of GFP in onion cells, and a deletion of residues 67 to 104, which rendered the CLS less effective, was compensated by including residues 177 to 289 (Figure 6).

Deletion of the core RING finger alone (residues 39 to 103) or the coiled-coil domain alone (residues 128 to 215) did not abolish light-regulated subcellular localization of COP1 in transgenic Arabidopsis, but deletion of both domains did (Torii et al., 1998). These data are consistent with our mapping of the CLS motif and further highlight the possibility that moderate deletion of the core CLS motif can be compensated by other domains of COP1.



**Figure 9.** Nla Protein Becomes Constitutively Cytoplasmic When Fused to the COP1 CLS-Containing Domain.

Transgenic seedlings were germinated for 5 days in continuous darkness ([A], [C], and [E]) or continuous white light ([B], [D], and [F]) and stained for GUS activity. Nuclei are indicated by arrowheads and, in (A) and (B), by dotted lines.

(A) and (B) GUS-COP1(1-287)Nla.

(C) and (D) GUS-COP1(1-117)Nla.

(E) and (F) GUS-Nla.

Bar in (A) = 15  $\mu$ m for (A) to (F).

**Table 3.** Overexpression Phenotype of COP1 Mutant Proteins in Light-Grown Arabidopsis Seedlings

Protein	Deleted/Mutated Domain	Localization <sup>a</sup>		Hypocotyl Length <sup>b</sup> (mm)
		Light	Dark	
GUS-COP1	Wild type	C	N	2.8 ± 0.04
GUS-COP1(105-675)	Ring	N	N	3.4 ± 0.04
GUS-COP1(293-675)	Ring, helix	N	N	3.9 ± 0.10
GUS-COP1(393-675)	Ring, helix, core	C	C	1.1 ± 0.04
GUS-COP1(1-392)	WD-40 repeats	C	N	1.1 ± 0.04
GUS-COP1-4	Core, WD-40 repeats	C	C	1.2 ± 0.04
GUS-COP1(105-392)	Ring, WD-40 repeats	N	N	2.0 ± 0.06
GUS-COP1(Mut1)	NLS	C	C	1.7 ± 0.05
GUS-COP1(Mut2)	NLS	C	C	1.6 ± 0.06
GUS-COP1(Mut1Mut2)	NLS	C	C	1.6 ± 0.05
GUS	None	C	C	1.5 ± 0.04
GUS-Nla	None	N	N	1.5 ± 0.04

<sup>a</sup>The subcellular localization of GUS activity in light-grown or dark-grown Arabidopsis, as summarized from Figure 8, is shown for comparison. Abbreviations are as given in footnotes a and b of Table 2.

<sup>b</sup>Means and standard errors of hypocotyl lengths were determined from 50 Arabidopsis seedlings grown in continuous white light.

We considered whether the predominantly cytoplasmic localization of COP1 and many of its mutants may be caused by their sequestering into cytoplasmic inclusion bodies, because inclusion body formation may conceivably prevent an interaction of COP1 with the nuclear import machinery. Several pieces of data argue against this possibility. First, the COP1-9 point mutant and, to a lesser degree, COP1-8 and COP1-11 were less prone to inclusion body formation than was wild-type COP1, but their increased solubility did not translate into increased nuclear accumulation. Second, GFP-COP1(1-393) formed cytoplasmic inclusion bodies but showed nuclear uptake (Figure 2B). Third, the COP1-GUS protein, a reciprocal fusion between GUS and COP1, which unlike GUS-COP1 did not form inclusion bodies, was nevertheless primarily cytoplasmic in onion epidermal cells and Arabidopsis hypocotyl cells (von Arnim and Deng, 1994). Fourth, the GFP-Nla fusion to the COP1 CLS between residues 67 and 177 was soluble yet clearly cytoplasmic (Figure 7). Clearly, the CLS did not function simply by causing inclusion body formation. However, insolubility may have contributed to the cytoplasmic localization of a subset of fusion proteins in our experiments. Specifically, COP1(105-287)Nla was insoluble and cytoplasmic, whereas COP1(105-177)Nla was soluble and less cytoplasmic (Figures 6 and 7), suggesting that the segment from 177 to 287 causes insolubility. Residues 105 to 177 represent a compromised CLS (Figure 6), and addition of an insolubility determinant may have compensated for the deletion in the CLS.

The mode of action of the CLS is not yet clear. Each of four deletions, two from the N terminus and two from the C terminus, diminished the effectiveness of the CLS. The segment from 155 to 177 appeared to be particularly important, whereas the partial RING finger segment from 67 to 104 made a more moderate contribution. Centered around resi-

dues 105, 146, and 170, COP1 possesses three hydrophobic motifs of five to eight residues with at least four leucine residues per motif. Some nuclear export signals characterized in yeast and mammalian cells are short, leucine-rich motifs (Roth et al., 1998), which is consistent with signal-mediated nuclear export of COP1. However, it is equally plausible that the CLS of COP1 functions in cytoplasmic retention. Interestingly, the putative coiled-coil interaction surface between COP1 and CIP1, which is cytoplasmic and cytoskeleton associated (Matsui et al., 1995), overlaps with the CLS. It is conceivable that CIP1 plays a role in the cytoplasmic retention of COP1.

#### Interplay between an NLS and a CLS Confers Light-Responsive Nuclear Localization

Three models may be advanced to explain the light-responsive nucleocytoplasmic partitioning of COP1. First, the COP1 NLS may be active only in darkness. Second, in the presence of a constitutive NLS, the COP1 CLS may be active only in the light. Third, light regulation may be achieved by the interaction between a constitutive NLS and a constitutive CLS in the context of additional protein domains.

The COP1 NLS functioned effectively in both dark- and light-grown Arabidopsis (Figure 8), which is inconsistent with the first model. The second model, which includes a light-activated CLS, is also not supported by our data, because GUS-COP1(1-287)Nla, incorporating the CLS between 67 and 177, was predominantly cytoplasmic in light-grown as well as in dark-grown seedlings (Figure 9). The third model is the simplest model and is consistent with our data because light regulation of subcellular localization was not mediated by either of the two targeting domains alone.

Rather, light-regulated localization required the combination of the two targeting domains in the context of the RING finger, helix, and core domains of COP1.

Light might regulate the activities of the COP1 NLS and CLS by at least three mechanisms and in conjunction with additional cellular factors. In the context of COP1 protein structure, the NLS may be inactivated under light conditions, and a constitutive CLS then shifts the balance toward nuclear exclusion. Given that typical bipartite NLSs, such as the NLS in COP1, are thought to be recognized in the cytoplasm or on the nuclear envelope before nuclear import (Hicks et al., 1995; Görlich and Mattaj, 1996) and that COP1 is nuclear in darkness, this scenario does not exactly explain how COP1 can become excluded from the nucleus of dark-grown seedlings when exposed to light (von Arnim et al., 1997), unless one invokes an additional nuclear degradation mechanism for COP1. A second scenario proposes that COP1 possesses an additional nuclear retention signal that is inactivated in the light, thereby allowing the CLS to exclude COP1 from the nucleus. The third, and simplest, scenario postulates a CLS that is inactivated in darkness. For example, the CLS may function as a nuclear export signal that is inhibited when shifted from light to darkness; alternatively, the CLS may function as a cytoplasmic retention signal that is inhibited in darkness.

Interactions with other cellular proteins may play a role in the subcellular localization of COP1. Dimerization of COP1 via its coiled-coil domain (Torii et al., 1998) may modulate the activity of the CLS or the NLS. Interaction of COP1 with the cytoplasmic CIP1 (Matsui et al., 1995) or the nuclear HY5 and CIP7 proteins (Ang et al., 1998; Chattopadhyay et al., 1998; Yamamoto et al., 1998) may also play a role. Future experiments will seek to resolve the significance of intramolecular and intermolecular interactions for the regulation of COP1 subcellular targeting.

## METHODS

### Recombinant Plasmids

CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1) mutant cDNAs were cloned as in-frame C-terminal fusions to  $\beta$ -glucuronidase (GUS) and the green fluorescent protein (GFP) via the vectors pRTL2-GUSNla $\Delta$ Bam (Restrepo et al., 1990) and pRTL2-GFP (von Arnim et al., 1998), respectively. Subfragments of COP1 were also cloned into pRTL2-GUS-Nla (Carrington et al., 1991) and pRTL2-GFP-Nla (von Arnim et al., 1998) to create fusions to the C terminus of GUS or GFP and the N terminus of Nla. Gene expression was driven by the cauliflower mosaic virus 35S promoter and terminator signals and enhanced by the translational leader sequence of tobacco etch virus.

The GFP cDNA was version mGFP4 (Haseloff et al., 1997). Standard molecular cloning procedures were used (Sambrook et al., 1989). The majority of COP1 mutant cDNAs were constructed from the COP1 cDNA in KS-COP1 (Deng et al., 1992; McNellis et al.,

1994a) either by employing native restriction sites or by amplification of subfragments using anchored polymerase chain reaction with high-fidelity DNA polymerases. Fusions to GUS or GFP were made via the linker peptide Arg-Ser-Ile-Asp-Lys-Leu-Glu-Phe-Thr. The premature stop codon allele *cop1-4*, the exon skipping allele *cop1-8*, the point mutant allele *cop1-9*, and the C-terminal deletion allele *cop1-11* are ethyl methanesulfonate-induced mutant alleles for which cDNAs were kindly provided by T.W. McNellis and X.-W. Deng (McNellis et al., 1994a). Site-directed mutagenesis was performed with the altered site II mutagenesis kit (Promega). Mutant proteins are designated by the numerical coordinates of their first and last amino acids, given in single-letter code, as numbered in McNellis et al. (1994a). Details of clone construction are available upon request.

### Transient Expression Assay

GUS and GFP fusion proteins were expressed in onion bulb epidermal cells by using particle-mediated DNA delivery (von Arnim and Deng, 1994). Between bombardment and observation, the tissue was incubated in darkness. The location of the nucleus was determined under bright-field illumination. For each GUS fusion, at least 50 transformed cells were observed and scored as follows: strong nuclear enrichment, cytoplasmic without visible nuclear exclusion, and cytoplasmic with nuclear exclusion. For all constructs, the majority of cells fell into one of these categories. For GFP fusions, the relative nuclear and cytoplasmic localization was determined by quantitative fluorescence measurement (von Arnim et al., 1998). Briefly, using IPLab (Signal Analytics, Vienna, VA) software, we analyzed an image acquired with a quantitative imaging device for nuclear and whole-cell fluorescence after background correction, and we calculated the percentage of nuclear fluorescence versus total cell fluorescence. Typically between eight and 12 cells were analyzed for each GFP fusion protein. Confocal sections of 580-nm thickness through the center of the nucleus were acquired on a Leica microscope by using the 488-nm line of the argon laser for excitation.

### Plant Growth Conditions, Arabidopsis Transformation, and Protein Localization

*Arabidopsis thaliana* was grown at 22°C under constant fluorescent illumination of  $\sim 100 \mu\text{mol m}^{-2} \text{sec}^{-1}$  (Sylvania GE F17T8-SP41), using Fafard No. 3 soil mix (Fafard, Agawam, MA) that was sprinkled with a layer of vermiculite. Seedlings were germinated aseptically on GM medium (Valvekens et al., 1988), supplemented as required with 50 mg/L kanamycin sulfate or 100 mg/L gentamycin sulfate. Seeds were germinated for 5 days either in constant darkness or in constant fluorescent white light ( $80 \mu\text{mol m}^{-2} \text{sec}^{-1}$ ; Sylvania GE F32T8-SP35) at 22°C.

For stable transformation of Arabidopsis, we recloned gene expression cassettes as PstI fragments to the binary T-DNA vector pPZP222 (Hajdukiewicz et al., 1994). Arabidopsis ecotype Columbia was transformed by *Agrobacterium tumefaciens*-mediated T-DNA transfer using the vacuum infiltration procedure (Bechtold et al., 1993). Gentamycin-resistant seedlings were selected and allowed to self for seed amplification.

Transgenic Arabidopsis seedlings were stained in GUS assay buffer to visualize GUS activity and were mounted for microscopy in the presence of 1 mg/L 4',6-diamidino-2-phenylindole to visualize the nucleus. At least three transgenic lines and between five and 10

seedlings per line were analyzed for each GUS fusion under both dark and light conditions. Transgenic plants harboring the constructs GUS-COP1, GUS-N1a, or GUS (von Arnim and Deng, 1994) served as controls. In a few transgenic lines, in which a low gene expression level required long staining periods, we lowered the GUS substrate concentration in our highly expressing control lines to attenuate stain development. This way, possible artifacts caused by variable staining times were avoided. Expression in transgenic seedlings of a fusion protein with the correct molecular weight was confirmed by SDS-PAGE and immunoblotting of whole seedling protein extracts with an anti-GUS antibody (Molecular Probes, Eugene, OR; data not shown).

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