A Novel Motif Mediates the Targeting of the Arabidopsis COP1 Protein to Subnuclear Foci*

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The constitutive photomorphogenesis 1 (COP1) protein of Arabidopsis thaliana accumulates in discrete subnuclear foci. To better understand the role of subnuclear architecture in COP1-mediated gene expression, we investigated the structural motifs of COP1 that mediate its localization to subnuclear foci using mutational analysis with green fluorescent protein as a reporter. In a transient expression assay, a subnuclear localization signal consisting of 58 residues between amino acids 120 and 177 of COP1 was able to confer speckled localization onto the heterologous nuclear NLS protein from tobacco etch virus. The subnuclear localization signal overlaps two previously characterized motifs, a cytoplasmic localization signal and a putative α-helical coiled-coil domain that has been implicated in COP1 dimerization. Moreover, phenotypically lethal mutations in the carboxyl-terminal WD-40 repeats inhibited localization to subnuclear foci, consistent with a functional role for the accumulation of COP1 at subnuclear sites.

In Arabidopsis thaliana, the constitutive photomorphogenesis 1 (COP1) protein mediates diverse developmental adaptations in response to environmental light signals. When grown under light conditions, Arabidopsis seedlings follow a developmental pathway known as photomorphogenesis, during which aerial portions of the seedling are prepared for photoautotrophic metabolism. When germinating in darkness, however, seedlings use their seed storage reserves to follow an alternative pathway, termed etiolation, in apparent adaptation for phototrophic metabolism. When grown in darkness, Arabidopsis seedlings use their seed storage reserves to follow an alternative pathway, termed etiolation, in apparent adaptation for rapid growth toward a light source (1). Loss of function mutants in the COP1 gene cause constitutive photomorphogenesis, implicating COP1 as a repressor of photomorphogenesis or an activator of etiolation. In cop1 mutants, photomorphogenesis in darkness is mediated at least in part by the transcriptional derepression of light-inducible nuclear genes, indicating that COP1 functions, directly or indirectly, as a transcriptional repressor (1–3).

The COP1 protein contains an amino-terminal zinc binding Ring finger domain (Ring), a coiled-coil domain (Helix), a central core domain, and a carboxyl-terminal domain composed of WD-40 repeats (2, 4). COP1 protein is expressed under both light and dark conditions, and severe cop1 mutants show a seedling-lethal phenotype even under light conditions, suggesting that COP1, besides regulating photomorphogenesis, plays a second fundamental role during late embryogenesis, seedling, and vegetative development (5). A COP1 fragment composed of the Ring finger and Helix domains, the allele COP1–4, can satisfy the need for the latter, light-independent, functions of COP1, but for repression of light-inducible gene expression, the full COP1 protein is required (4).

The regulatory function of COP1 appears to be mediated by interactions with other nuclear proteins, including the COP1-interactive protein-7 (CIP7), a likely transcriptional activator (6), and the basic leucine zipper protein HY5 (7). Given that hy5 mutants display reduced responsiveness to light and that HY5 can bind to light-regulatory promoter elements, COP1 may repress transcription by interfering with light-regulated transcriptional activation (8). Light signals may regulate the activity of COP1 at least in part by modulating the nuclear level of the COP1 protein. A fusion protein between COP1 and β-glucuronidase as a reporter accumulates in the nucleus of Arabidopsis seedling stem (hypocotyl) cells in darkness, yet it is excluded from the nucleus under light conditions (9, 10). The redistribution of the β-glucuronidase-COP1 protein by light is mediated by multiple photoreceptors of the phytochrome and cryptochrome families (11). Cytoplasmic localization of COP1 is mediated by a cytoplasmic localization signal (CLS),1 which counteracts a classical bipartite nuclear localization signal (NLS), located in the central core domain, in a light-dependent manner (12).

The COP1 protein displays a characteristic localization to discrete subnuclear sites under a variety of experimental conditions. Immunofluorescence labeling has highlighted COP1 in discrete subnuclear regions in wild-type Arabidopsis cells. Moreover, both β-glucuronidase-COP1 and green fluorescent protein (GFP)-COP1 fusion proteins accumulate in subnuclear foci when expressed in transgenic Arabidopsis or in transiently transformed onion epidermal cells (13, 7). Like the nucleus of animal cells (14, 15), the plant cell nucleus is a highly structured organelle (16). For example, telomeres appear to be located preferentially at the nuclear periphery (17). Ribosomal gene transcription and ribosome preassembly are sequestered into nucleoli, organelles that are further subdivided into domains (18, 19). Certain splicing components are concentrated in subnuclear granules or speckles, some of which are immunologically related to coiled bodies (20–23). In animal cell nuclei, numerous proteins are distributed in diverse “micro-punctate” patterns, which may be functionally relevant in the regulation of gene expression (e.g. Refs. 24, 25). However, the subnuclear compartmentalization of the plant nucleus and its role in gene expression are comparatively poorly understood.

We reasoned that a mutational analysis of the structural requirements in COP1 for localization to nuclear foci may shed...
light on the biological role of the foci for COP1 function and on
the cooperation between the cytoplasmic, nuclear, and sub-
nuclear targeting signals within COP1. Using primarily fusion
proteins between COP1 mutants and green fluorescent protein,
we found that a short subfragment of the COP1 coiled-coil
domain confers localization to foci on a heterologous protein,
that a domain responsible for the formation of cytoplasmic
inclusion bodies can be separated from the subnuclear localiza-
tion signal, and that three phenotypically lethal mutations in
the WD-40 domain of COP1 interfere with the subnuclear
targeting of GFP-COP1. Our data represent the first muta-
tional analysis of the subnuclear targeting of a plant protein.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Alleles of COP1—Standard procedures
were followed for recombinant DNA work (26). Protein expression plas-
mids were constructed on the basis of pAVA319, pAVA121, and
pAVA120 (13), plasmids that harbor a GFP fusion cassette driven by
the cauliflower mosaic virus 35S promoter. Insertions of COP1 frag-
ments in between GFP and the NIa protein from tobacco etch virus (27)
were made by amplification of subfragments from a COP1 cDNA using
anchored polymerase chain reaction and cloning of the fragments to
pRTL2-GFP-NIa (13). The fusions lacking the NIa cDNA have been
described previously (12). The structures of proteins expressed from
COP1 mutant alleles are diagrammed in Fig. 1 (4).

Transient Assay—Fusion proteins were expressed using particle
gun-mediated DNA delivery in onion epidermal cells (12) and imaged by
epifluorescence microscopy with a MicroMax CCD camera (Princeton
Instruments (12, 13)). The position of the nucleus and the nucleoli was
confirmed under bright-field illumination. Between 40 and 150 cells/
construct were examined for nuclear foci in at least two independent
experiments. Two patterns of subnuclear localization were most com-
mon, either an even distribution (“soluble”) or a confinement of all
visible fluorescence to subnuclear foci (“speckles only”). In some cases,
foci were accompanied by soluble protein (“speckles and soluble”). A few
expression constructs resulted in a large fraction of cells that displayed
nuclear granules that were much larger than the typical foci and of
irregular size (“aggregates”). If not mentioned otherwise, fusions were
made to a GFP with wild-type fluorescence properties. For double-
labeling experiments, one cDNA was fused to wild-type GFP in
pAVA319, and the second one was fused to the S65T mutant of GFP in
pAVA121. Equal amounts of each plasmid were co-transformed. Under
blue excitation, both wild type and S65T mutant are detected, whereas
under UV light only the wild-type GFP is visible.

RESULTS

Deletion Analysis of COP1 Localization to Nuclear Foci—We
first addressed which structural elements of COP1 are required
for its targeting to subnuclear foci. To this end, fusion proteins
between the GFP and a series of COP1 mutants (12) were
analyzed for their subnuclear localization pattern in a tran-
sient expression assay in onion epidermal cells. The quantita-
tive data are summarized in Fig. 1, and representative nuclear
expression patterns are shown in Fig. 2. Consistent with pre-
vious results (12), wild-type COP1 localized to subnuclear foci (speckles), rounded structures of approximately 1-μm diameter, which were distributed evenly throughout the nucleus (Fig. 2A). In 18% of the cells, faint dispersed (soluble) localization of COP1 was also seen. When compared with a bright-field image of the same cell (Fig. 2B), no obvious association of GFP-COP1 with the nucleoli could be discerned. Deletion of the Ring finger domain (COP1ΔRing, Fig. 2C) or of the COP1 amino terminus (COP1[105–675], Fig. 2D) resulted in brighter and more numerous foci when compared with wild-type COP1, as would be expected for these mutants, which lack the intact cytoplasmic localization signal of COP1 (12).

In contrast, deletion of both the Ring finger and Helix domains (COP1[293–675], Fig. 2H) resulted in dispersed (soluble) nuclear localization and a complete loss of nuclear foci. Moreover, the fusion consisting of the Helix and the central core domain (COP1[105–392], Fig. 2E) retained localization to foci, albeit at a reduced frequency (Fig. 1). Removal of the Helix from this protein completely abolished foci formation (COP1[293–392], Fig. 2G). The helix domain, therefore, is necessary for foci formation and may contain the structural element for COP1 targeting to foci. The Ring finger domain, on the other hand, did not contribute to foci formation, and the WD-40 repeats were not absolutely required.

Three different mutations within the WD-40 repeats resulted in the loss of nuclear foci and the appearance of dispersed nuclear protein (COP1–11, Fig. 2F; COP1–9, Fig. 2J; COP1–8, Fig. 2, K and L), whereas the distribution of the proteins between nucleus and cytoplasm was unaltered, as reported previously (12). When present in the endogenous COP1 gene, each of the three mutations causes a lethal loss-of-function phenotype in Arabidopsis seedling development (4), which is consistent with the possibility that the foci contribute to COP1 function in the nucleus. Given that the foci formed by wild-type COP1 very likely represent large nuclear assemblies, and given that COP1 is known to dimerize (32), we asked whether expression of the COP1–9 or COP1–11 mutants may disrupt the localization of wild-type COP1 to nuclear foci. To test this, we co-expressed the S65T mutant of GFP-COP1 with either COP1–9 or COP1–11 fused to wild-type GFP. However, typical nuclear foci composed of GFP-S65T-COP1 were detected under blue illumination in the presence (Fig. 3C) and in the absence (Fig. 3A) of GFP-COP1–9. The typical soluble distribution of GFP-COP1–9 protein was also evident in co-transformed cells (Fig. 3C), as in cells expressing GFP-COP1–9 alone (Fig. 3B). Therefore, COP1–9 did not appear to be recruited to the nuclear foci formed by GFP-S65T-COP1. Likewise, no dominant-negative effect on COP1 nuclear foci was exerted by COP1–11 (Fig. 3D). These results are consistent with the recessive phenotypes of the cop1–9 and cop1–11 alleles in Arabidopsis (4).

Deletion of the entire WD-40 domain reduced the fraction of cells showing foci (COP111–392; Fig. 2F) and increased the level of dispersed nuclear protein and of irregularly shaped nuclear aggregates (Fig. 1). However, the WD-40 domain alone (COP1[293–675]) did not localize to foci. Taken together, although the WD-40 domain appears to be insufficient for targeting to nuclear foci, its integrity is important for localization to the foci.

The Helix Domain Functions as an Autonomous Determinant for Localization to Subnuclear Foci—To test the hypothesis that a specific domain can confer localization to foci and to further delineate the responsible domain, we tested whether individual COP1 fragments were able to direct a heterologous nuclear protein, the tobacco etch virus NiA protein, to subnuclear foci. COP1 fragments overlapping the Helix domain as

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Fig. 3. Co-transformation of wild-type and mutant COP1 proteins. Images were taken under blue excitation to visualize both GFP<sup>S65T</sup>-COP1 (nuclear foci) and GFP-COP1–9 or GFP-COP1–11 (soluble cytoplasmic and nuclear protein and cytoplasmic inclusion bodies). A, GFP<sup>S65T</sup>-COP1; B, GFP-COP1–9; C, GFP<sup>S65T</sup>-COP1 and GFP-COP1–9; D, GFP<sup>S65T</sup>-COP1 and GFP-COP1–11. Filled arrows point to nuclei, and open arrows indicate cytoplasmic inclusion bodies.

Fig. 4. Structure of GFP fusion proteins and delineation of the domain causing subnuclear foci. Portions of the COP1 amino-terminal domain as outlined by their amino acid coordinates were fused to the tobacco etch virus NiA protein, and subnuclear localization was determined and displayed as described for Fig. 1. The extent of the CLS domain from residues 67 to 177 is indicated.
Two distinct structural elements in COP1 are important for nuclear targeting. The Helix domain, specifically the 58-residue segment from 120 to 177, specifies a structural element that can confer speckled localization to the heterologous NiA protein outside the context of the COP1 protein. We refer to this domain as a subnuclear localization signal (SNLS).

We had previously observed that certain COP1 fusion proteins had a tendency to form large cytoplasmic inclusion bodies and nuclear aggregates (12). Proteins containing the carboxy-terminal half of the helical domain were particularly prone to cytoplasmic and nuclear aggregation (Fig. 1). In the experiments described here, we found that the domain responsible for localization to nuclear foci is distinct from, although adjacent to, the domain most likely responsible for aggregation. Therefore, the formation of nuclear foci is a distinct process from aggregation.

To address whether the foci formed by GFP-SNLS-NiA are distinct from those formed by GFP-COP1 we carried out a double-labeling experiment. When GFPS65T-COP1 and GFP-SNLS-NiA were coexpressed, the number of foci formed by GFP-SNLS-NiA was reduced to that normally observed for GFP-COP1, and we did not observe any foci containing only GFPS65T-COP1 alone (Fig. 6). Therefore there appeared to be complete colocalization between COP1 and SNLS-NiA.

**DISCUSSION**

A variety of nuclear factors can dynamically localize to discrete subnuclear domains or foci rather than being randomly dispersed throughout the nucleus (reviewed in Refs. 14 and 15). These factors include catalytic protein complexes involved in replication (33), transcription (e.g. ribosomal RNA transcription by polymerase I (16)), and splicing (34), as well as regulatory proteins that control these processes, for instance *Drosophila* Polycomb (35) and MSL-2 (25). In plants, pioneering studies have confirmed the general notion derived from studies in animals that the plant nucleus possesses a well defined architecture (17, 19, 20). However, few regulatory proteins have been examined closely for their subnuclear distribution. The COP1 protein, a fundamental plant nuclear regulator encoded by a member of the pleiotropic COP1/DET/FUS genes, exhibits a characteristic localization to discrete nuclear foci besides a faint diffuse distribution (7, 12, 13). In transient co-expression assays, native COP1 is able to redistribute the basic leucine zipper protein HY5 into nuclear foci, suggesting that the COP1 protein in the foci must be in an at least partly native configuration (7). Here, we have begun to delineate the structural motifs that mediate the distribution of COP1 between a soluble form and a form associated with nuclear foci.

![Fig. 6. Co-localization of GFP-COP1 and GFP-COP1(120–177)NiA.](image)
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FIG. 7. Protein sequence comparison of COP1 domains harboring the subnuclear localization signal and the cytoplasmic localization signal. Clusters of leucine residues are boxed, and the extent of the Ring finger and coiled coil domains are illustrated by bars. Protein sequences available under GenBank accessions numbers P43254 (Arabidopsis), L24437 (tomato (Lycopersicon esculentum)), and P93471 (pea (Pisum sativum)) were aligned with CLUSTALW (30) and tested for coiled-coil potential (31). The symbols beneath the alignment symbolize the degree of similarity: minus, identity; colon, strong conservation; period, moderate conservation.

Localization to nuclear foci, namely the WD-40 domain and the Helix domain. The WD-40 domain may play an indirect role, because it did not mediate foci formation alone. However, a loss or a reduction in foci formation was observed for three GFP-COP1 fusions mutated in the WD-40 domain, COP1-8, COP1-9, and COP1-11. The COP1-9 allele has a G524Q missense mutation in a residue conserved among three plant COP1 homologs from Arabidopsis, pea (Zhao et al. (36)), and tomato. COP1-8 is an exon-skipping mutant, and COP1-11 has a premature stop codon. All three are loss-of-function alleles (4). Neither mutant disrupted the localization of wild-type GFP-COP1 in our transient assay, consistent with the recessive nature of the mutations.

Other than the WD-40 domain, the Helix domain was both necessary and sufficient for foci formation, as indicated first by the complete disappearance of foci upon its deletion. Second, the Helix domain, together with the central core domain containing the NLS, formed the minimum COP1 fragment that showed foci. In addition, a 58-amino acid fragment within the Helix, residues 120–177, conferred localization to foci onto the heterologous nuclear-targeted NIA protein. The NIA protein from tobacco etch virus was chosen because it contains a strong context-independent NLS (27). The GFP-NIA fusion, unlike GFP-COP1, was dispersed throughout the nucleoplasm, with some preferential association with nucleoli (37), which may be mediated by an RNA binding activity of the NIA protein (38). The 58-residue fragment, which we refer to as a SNLS, also prevented the characteristic nucleolar enrichment of NIA. Like the GFP-COP1 foci, the foci formed by the GFP-SNLS-NIA fusion were rounded, approximately 1 μm in diameter, and evenly distributed throughout the nucleoplasm, indicating that both are equivalent structures. Moreover, coexpression of the SNLS-NIA fusion with COP1 clearly showed that all the COP1 foci contained the SNLS-NIA protein.

Few proteins have been examined closely for the targeting signals that actively confer localization to subnuclear sites. In the Drosophila Polycomb protein, the chromodomain is able to confer a speckled localization onto β-galactosidase (35). In the human ALL-1 protein, two distinct motifs, both with similarity to Drosophila trithorax, were able to localize covalently linked cytoplasmic pyruvate kinase to nuclear foci (39). In the mammalian protein SP100, targeting to nuclear bodies containing the promyelocytic leukemia protein PML requires a domain thought to include a helical motif (40). The SNLS of COP1 does not show obvious sequence similarity with any of these proteins nor with any other known proteins apart from COP1 orthologs. However, physiologically, COP1 functions as a repressor of gene expression, and the localization to nuclear foci may be instrumental in regulating the access of COP1 to its target sites.

The SNLS between residues 120 and 177 represents a portion of the COP1 CLS (residues 67–177; Ref. 12; Fig. 7), as well as of a COP1 fragment mediating dimerization (residues 105–211 (41)). The Helix domain (coiled-coil: 125–220) also mediates interactions with proteins other than COP1, namely the predominantly cytoplasmic CIP1 protein (42) and the nuclear CIP7 (43), neither of which, however, is known to localize to nuclear foci. It is possible that the SNLS interacts with yet other nuclear proteins to target COP1 to the foci. In addition, COP1-COP1 multimerization may play a role, given that COP1–8 and COP1–11 do not show obvious sequence similarity with any of these proteins

Currently, our data do not allow us to distinguish whether the foci represent a site of active COP1 protein or a dispensable storage site for inactive COP1. However, the observed correlation between loss-of-function and loss of nuclear foci among three mutations in the WD-40 domain is certainly consistent with a functional role of the foci. Perturbations in the subnuclear localization of specific proteins have been implicated in the pathogenesis of human disease (43). For example, the Wilms tumor 1 (WT1) gene product is distributed between a diffuse phase and a punctate phase, the latter containing splicing factors involved in RNA maturation (44). Loss of DNA-binding of WT1 was associated with accumulation in the punctate form (28, 44). Our data on the subnuclear localization of COP1 suggest that subnuclear partitioning of regulatory proteins is not confined to animal cells but also occurs in plants. Recently, a GFP fusion of the phytochrome B photoreceptor was shown to localize to nuclear foci highly reminiscent of those seen for COP1 (29), an intriguing result given that COP1 nuclear localization is negatively regulated by phytochrome B
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(11). Additional examples of subnuclear localization patterns in plant cells are likely to be discovered. Their biochemical characterization with respect to the foci formed by COP1 and by foci seen in animal cells may shed light on the structural basis of gene regulation.

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