The Arabidopsis repressor of light signaling, COP1, is regulated by nuclear exclusion: Mutational analysis by bioluminescence resonance energy transfer

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Bioluminescence resonance energy transfer (BRET) between Renilla luciferase and yellow fluorescent protein has been adapted to serve as a real-time reporter on protein–protein interactions in live plant cells by using the Arabidopsis Constitutive photomorphogenesis 1 (COP1) protein as a model system. COP1 is a repressor of light signal transduction that functions as part of a nuclear E3 ubiquitin ligase. COP1 possesses a leucine-rich nuclear-exclusion signal that resides in a domain implicated in COP1 dimerization. BRET was applied in conjunction with site-directed mutagenesis to explore the respective contributions of the nuclear-exclusion and dimerization motifs to the regulation of COP1 activity in vivo. One specific mutant protein, COP1L105A, showed increased nuclear accumulation but retained the ability to dimerize, as monitored by BRET, whereas other mutations inhibited both nuclear exclusion and COP1 dimerization. Mutant rescue and overexpression experiments indicated that nuclear exclusion of COP1 protein is a rate-limiting step in light signal transduction.

The etiolation response of Arabidopsis seedlings germinating in darkness is mediated in part by the Constitutive photomorphogenesis 1 (COP1) protein, a repressor of light-regulated development. COP1 functions in the nucleus (1) by targeting light-regulatory transcription factors for ubiquitin-mediated degradation by the proteasome (2–5). COP1 possesses E3 ubiquitin ligase activity, presumably in conjunction with the COP1-interacting proteins CIP8 and SPAI (4–7). Accordingly, cop1 mutant plants show transcriptional misregulation of numerous light-inducible genes in darkness (8).

A single bipartite nuclear localization signal is responsible for COP1 nuclear localization and function (1, 9). Within the nucleus, COP1 localizes to subnuclear speckles (10), which also may contain ubiquitination targets and regulators of COP1 activity (4, 11, 12). However, COP1 is subject also to nuclear export: Mutant rescue and overexpression experiments indicated that nuclear exclusion of COP1 protein is a rate-limiting step in light signal transduction.

Materials and Methods

General Procedures. Conditions for plant growth, far-red light treatments (14 µmol m−2 sec−1), Agrobacterium-mediated transformation, quantitative fluorescence microscopy, and immunoblotting were as described (1, 9). Site-directed mutagenesis was carried out on a wild-type (WT) GFP-COP1 expression plasmid (9), driven by the cauliflower mosaic virus 35S promoter, by using overlap-extension PCR (19). For the COP1L105A mutation, a fragment upstream and including the Leu-105→Ala mutation was amplified by using the oligonucleotides 5′-aag gga atc aca gaa ttc-3′ and 5′-gag ttc tgg ccc tta ggg cca gta gtt-3′. The two PCR products were mixed and merged by reamplification using the peripheral two primers, and the resulting product was subcloned. The mutated segment was reexcised with HpaI and NsiI and used to replace the corresponding COP1WT segment in the GFP-COP1 expression plasmid. Other site-directed mutations were generated in a likewise fashion and confirmed by DNA sequencing. T-DNAs (portion of tumor-inducing plasmid that is transferred from Agrobacterium to plant cells) harboring GFP-COP1 fusion transgenes were introduced first into Arabidopsis heterozygous for the cop1-5 T-DNA insertion allele (20). Transgenic progeny were examined.

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Abbreviations: COP1, Constitutive photomorphogenesis 1; CC, coiled coil; BRET, bioluminescence resonance energy transfer; RLUC, Renilla luciferase; YFP, yellow fluorescent protein.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AY189980 (35S:RLUC) and AY189881 (35S:YFP)].

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for functional complementation of the cop1-5 allele and for the effect of the transgene on hypocotyl elongation in a WT COP1 background. See Determination of Nuclear Fluorescence Levels in Transiently Transformed Onion Epidermal Cells, which is published as supporting information on the PNAS web site. This determination was done by quantifying pixel intensity after correcting for background GFP levels.

**BRET Assays.** Luminescence spectra were obtained from 8- to 9-day-old light-grown seedlings in the presence of 10 μM coelenterazine in a FluoroLog (Splex Industries, Metuchen, NJ) spectrofluorimeter with blocked excitation path and with the slit width set at 5 nm. A vector containing 35S promoter and terminator signals suitable for transiently expressing RLUC-fusion proteins (35S:RLUC, GenBank accession no. AY189980) was created by amplifying the RLUC cDNA from pRL-null (Promega). A YFP-tagging vector (35S:YFP, GenBank accession no. AY189981) was constructed similarly by using enhanced YFP cDNA (CLONTECH). 35S:RLUC and a 35S:RLUC-YFP fusion were introduced into transgenic Arabidopsis. COP1 cDNAs were inserted into the pRL-null vector. The cassette including the COP1 cDNA was amplified using the BglII site of the BRET vectors to generate N-terminally tagged COP1 fusions. For BRET assays, 500 ng of each expression plasmid were coated onto tungsten particles and introduced into onion epidermal cells by using the PDS-1000 He particle gun (Bio-Rad). After overnight incubation in darkness and the addition of 1 μM coelenterazine substrate (Biotium, Hayward, CA) in water, yellow-to-blue luminescence ratios were collected on a TD-20 tube luminometer equipped with the dual-color accessory (Turner Designs, Sunnyvale, CA). Typically, four pairs of 10-sec readings were taken through blue (390 ± 30 nm) and yellow (+520 nm long-pass) filters. Readings were averaged and background was subtracted by using a reading from untransformed tissue. Unless otherwise indicated, data presented are from at least three independent transformations.

**Results**

**Sequence Motifs for Nuclear Exclusion of COP1.** To address the hypothesis that COP1 activity is limited by nuclear exclusion, site-directed mutations were introduced into each of three clusters of leucine residues that are found throughout the 110-residue CLS domain of COP1 (Fig. 1 A and B). When expressed as GFP-fusion proteins in transiently transformed onion epidermal cells, all three single mutants as well as double and triple mutants displayed increased nuclear localization (Figs. 1 C and D and 2 A–J). The COP1L105A mutant protein was moderately but significantly more nuclear than WT (P < 0.001, Fig. 1D), whereas most other single, double, and triple mutants were predominantly nuclear (Fig. 1C). These data indicate that nuclear exclusion of COP1 is governed by a multiparticle or extended domain. Notably, although the COP1 fragment encompassing residues 120–177 can target a heterologous nuclear protein to nuclear speckles (10), none of the mutants tested were disrupted in this respect but rather continued to form nuclear speckles similar to WT COP1 (Fig. 2). Next, two representative
GFP-COP1 mutants, COP1_L105A and COP1_L170A, were expressed from stable transgenes in Arabidopsis (Figs. 1D and 2J–L). At similar overall expression levels, the COP1_L105A mutant formed smaller cytoplasmic inclusion bodies than WT COP1 and accumulated in the nucleus to higher levels than WT COP1 (P = 0.003). Similar results were obtained for COP1_L170A (Fig. 2L). Therefore, both the Leu-105 and Leu-170 motifs are important for nuclear exclusion in Arabidopsis.

BRET Assay and COP1 Dimerization. To test which mutations of leucine-rich motifs might compromise COP1 dimerization in vivo, we adopted the BRET assay (Fig. 3A). First, transgenic Arabidopsis expressing an RLUC-YFP fusion protein displayed a bimodal luminescence spectrum, whereas RLUC alone gave a single peak near 480 nm, as expected (Fig. 3B). These data demonstrate unambiguously that RLUC and YFP are compatible partners for BRET in plant cells. Coexpression of RLUC-COP1 and YFP-COP1 in onion epidermal cells yielded an elevated yellow-to-blue ratio, compared with RLUC-COP1 alone (Fig. 3C), indicating BRET between RLUC-COP1 and YFP-COP1 and thus interaction between WT COP1 proteins. When RLUC-tagged COP1 mutants were tested for heterodimerization with WT YFP-COP1, the COP1_L105A mutant,
significantly, retained full interaction, whereas all other mutants tested interacted less well or not at all (Fig. 4A). Consistent results were obtained when the RLUC and YFP tags were swapped between the COP1 partners (Fig. 4C) or when testing for homodimerization of COP1 mutants (Fig. 4D). All BRET signals depended on the presence of a YFP partner protein (Fig. 4B). No BRET was observed with RLUC-COP1 and YFP alone (Fig. 4C, eighth bar).

The reduction of the BRET signal seen with specific COP1mut alleles such as COP1L105A and the triple mutant was not simply caused by an inadvertent drop in expression of the YFP partner, because no significant increase of the BRET signal was detected when the YFP-COP1mut/RLUC-COP1 plasmid ratio was increased by a factor of 3 (Fig. 4E). Together these results indicate that the Leu-105→ Ala mutation partially inhibits nuclear exclusion but not COP1–COP1 interaction.

Complementation Test and Gain-of-Function Assay. The dimerizing COP1L105A allele and the COP1L170A allele, as a representative allele with poor dimerization, were tested for functionality by transgenic rescue of the null allele, cop1-5. The COP1L105A allele was able to rescue the cop1 mutant (Table 1). The cop1 mutants carrying the COP1L105A transgene were etiolated fully in darkness, i.e., rescue was complete (data not shown). In contrast, no rescue was observed with the COP1L170A allele among 11 families tested. These results may suggest that the ability of COP1 to dimerize is important for COP1 function. Hypocotyl length has served previously as an indicator of COP1 activity in a gain-of-function assay (1, 21). The GFP-COP1L105A transgene caused an elongated hypocotyl under constant far-red light but not in darkness, compared with a similar level of GFP-COP1WT expression (Fig. 5), suggesting that the increased nuclear accumulation of this mutant (Fig. 2A) is functionally significant. Likewise, the COP1L170A mutant also caused increased hypocotyl elongation, although it did not complement cop1-5. Clearly, hypocotyl elongation correlated with increased nuclear localization. The hypocotyl elongation phenotype of COP1L170A may arise because this mutant continues to accumulate in nuclear speckles and still may be able to interact with nuclear targets that are effectors of COP1 activity despite its reduced tendency to dimerize (Fig. 4). The GFP-tagged WT and mutant COP1 proteins accumulated to similar levels in the specific lines compared in this experiment as judged by fluorescence microscopy (Fig. 2 J–L) and immunoblotting (Fig. 5C). In summary, these data suggest that increased nuclear targeting of COP1 promotes etiolation and that nuclear exclusion of COP1 protein is a rate-limiting step in light signal transduction.

Discussion

The COP1 protein of Arabidopsis is a developmental regulator that is essential for etiolation during seedling development in darkness and other processes thereafter. COP1 seems to be tightly regulated at the level of nuclear accumulation. First, COP1 with a site-directed mutation of its single nuclear localization signal enters the nucleus inefficiently, causing constitutive photomorphogenesis (1). Second, COP1 possesses a powerful CLS motif that drives its exclusion from the nucleus (9). Here, we show that site-directed mutations in the CLS cause increased nuclear accumulation in both onion cells and Arabidopsis. Considering the fine structure of the CLS (Fig. 1), the Leu-146 and Leu-170 motifs function in a bipartite fashion in that both are necessary together for efficient nuclear exclusion. Here, we set out to test the hypothesis that an increase in nuclear localization by interfering with nuclear exclusion of COP1 would enhance COP1 activity. Our results obtained with the COP1L105A mutant substantiate this hypothesis by demonstrating that a moderate but significant defect in nuclear exclusion, which was observed in both onion and Arabidopsis cells, causes an increase

Table 1. Complementation of the cop1–5 null allele by GFP-COP1 transgenes

<table>
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<tr>
<th>Transgene</th>
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<tr>
<td>None</td>
<td>56</td>
<td>247</td>
<td>3.4</td>
<td>No</td>
</tr>
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</table>

The cop1 phenotype was scored, based on purple seed color (20), in the progeny of cop1–5+/GFP-COP1+/ dihybrid plants. Complementation is indicated by a reduction in the fraction of purple seedlings from 1/4 to 1/16.

Fig. 5. Hypocotyl length in 5-day-old Arabidopsis seedlings expressing 35S:COP1-mutant proteins in a WT COP1 background. (A) Two representative transgenic lines per mutant construct were germinated in constant far-red light. Bars indicate standard errors. (B) Germination in constant darkness. All proteins were tagged with GFP. (C) Total protein (20 μg) from seedlings of the GFP-COP1 transgenic lines shown in A and B was fractionated by SDS/PAGE and immunoblotted to detect the GFP-COP1 protein by using an antiserum against GFP (arrowhead). A cross-reacting band (asterisk) serves as a loading control.

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in hypocotyl elongation. Significantly, the BRET data highlighted that the COP1<sub>1</sub> mutant continued to dimerize normally, whereas mutations in the Leu-146 and Leu-170 motifs interfered with dimerization. These data are fully consistent with the definition of the CC domain as the dimerization interface via domain-deletion constructs and yeast two-hybrid assays (15). Mutant alleles with increased nuclear localization, including COP1<sub>1</sub> and COP1<sub>2</sub>, were more active than WT COP1 in promoting hypocotyl elongation under constant far-red light, a hallmark of COP1 activity during seedling etiolation. Compared with previous transgenes that were more highly expressed than those used here (1), at the moderate expression levels used here COP1<sub>WT</sub> did not cause hypocotyl overgrowth, complementing an earlier analysis of the COP1 nuclear import signal (1).

Numerous nuclear-exclusion/export signals have been defined cell biologically, and in many cases mutation of the signal will affect the cellular function of the protein in cell-culture experiments. However, proof that a specific alteration in a nuclear-exclusion signal can alter the biological function of a protein at the level of the whole organism has been scarce and has not been presented for plants. In one of only a few examples, mutations in a nuclear export signal of the human adenomatous polyposis cancer (APC) protein seem to affect its ability to control the turnover of the transcription factor β-catenin, leading to cancer formation (22).

Does the COP1 CLS direct nuclear export or nuclear protein turnover? COP1 autoubiquitination activity, which may lead to subsequent turnover, may be stimulated by dimerization (4). If COP1 were poised for turnover specifically in the nuclear compartment, the increased nuclear level of COP1 mutant proteins in our experiments might be caused by reduced dimerization, reduced autoubiquitination, and thus increased protein stability. This hypothesis predicts that poorly dimerizing COP1 mutant proteins would accumulate to overall higher cellular levels than WT COP1, with equal or higher cytoplasmic levels, in addition to higher nuclear levels. However, this prediction was not met by our transient expression and localization data, because cytoplasmic fluorescence of the dimerization defective mutants was lower than with WT COP1 (data not shown). On the other hand, the leucine-rich nature of the CLS, its location in a CC domain (23–25), and the fact that COP1 (data not shown) were more active than WT COP1 might stimulate or inhibit its nuclear exclusion. Interestingly, conditional dimerization has been implicated on numerous occasions in promoting nuclear import or inhibiting export, as exemplified by p53 (25). In contrast, the involvement of conditional dimerization in promoting nuclear exclusion remains to be confirmed (28). In the case of COP1, there was a general correlation between dimerization (Fig. 4) and nuclear exclusion (Fig. 1C) across seven different COP1 alleles, suggesting that the nuclear-exclusion signal of COP1 functions well in the context of a COP1 dimer. However, whether WT COP1 ever exists as a monomer in vivo is not clear. At least four known interaction partners bind to COP1 through the CC domain (29, 30), and any one of them might inhibit COP1 dimerization. Most recently, COP1 was observed in a 700-kDa complex in Arabidopsis that may contain COP1 dimers as well as other regulators of COP1 activity, such as SPA1 (5). In future experiments, the BRET technique may lend itself to testing, under in vivo conditions, whether the dimerization of COP1 is influenced by the presence of COP1 partner proteins.

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