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, COP1^{L105A}, 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.

dimerization | photomorphogenesis | nuclear export

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 SPA1 (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 exclusion from the nucleus, mediated by a 110-residue domain referred to as a cytoplasmic localization signal (CLS), which overlaps a predicted α-helical coiled-coil (CC) region (9). The nuclear exclusion of a β-glucuronidase-COP1 fusion protein is enhanced by light (13–15). When fused to β-glucuronidase or GFP, COP1 in fact is predominantly cytoplasmic and accumulates in a cytoplasmic inclusion body.

Testing the hypothesis that the nuclear exclusion of COP1 by the CLS does, in fact, down-regulate COP1 activity in *Arabidopsis* is not trivial, because the CLS overlaps the CC domain, which governs both COP1 dimerization (15) and targeting to nuclear speckles (10). Thus, three aspects of COP1 that are tightly linked to one another are nuclear localization and dimerization and their role in the function of COP1. To test the dimerization of COP1 after mutagenesis within the CLS, we decided to apply bioluminescence resonance energy transfer (BRET) (16). In a BRET experiment, two candidate interaction partners are tagged with the blue-light-emitting *Renilla* luciferase (RLUC) and yellow fluorescent protein (YFP), respectively. Close physical proximity of the RLUC and YFP tags due to protein–protein interaction results in an increase in the yellow-to-blue luminescence ratio. One key advantage of the BRET method is that interactions are detected in live cells in real time. BRET was established originally in bacteria (16) and has since been adapted to mammalian cells, especially for interactions involving G protein-coupled receptors (17, 18). We adapted the BRET assay to plant cells to assess the relative contributions of dimerization and nuclear exclusion to the functional activity of COP1. These data, in conjunction with transgenic experiments, allowed us to conclude that nuclear exclusion of COP1 is a necessary component of the light signal transduction machinery in *Arabidopsis*. These experiments represent application of the BRET protein–protein interaction technique in plants.

Materials and Methods

General Procedures. Conditions for plant growth, far-red light treatments (14 μmol·m^{-2} per sec), *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 COP1^{L105A} mutation, a fragment upstream and including the Leu-105 → Ala mutation was amplified by using the oligonucleotides 5′-aag aat gga atc aaa gtt aac ttc-3′ and 5′-tgc tgc ctt atc tgc agc gaa att agg gta aag ctg att att g-3′. A separate fragment including the Leu-105 → Ala mutation and downstream sequences was amplified with the overlapping (underlined) oligonucleotide 5′-tcg ctg cag ata agg cag caa aga aaa ctt cag ctc ggc atg tg-3′ and 5′-tct cat gtg gcc atg cat ttc-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 *Hpa*I and *Nsi*I and used to replace the corresponding COP1^{WT} 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; CLS, cytoplasmic localization signal; 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 AY189981 (35S:YFP)].

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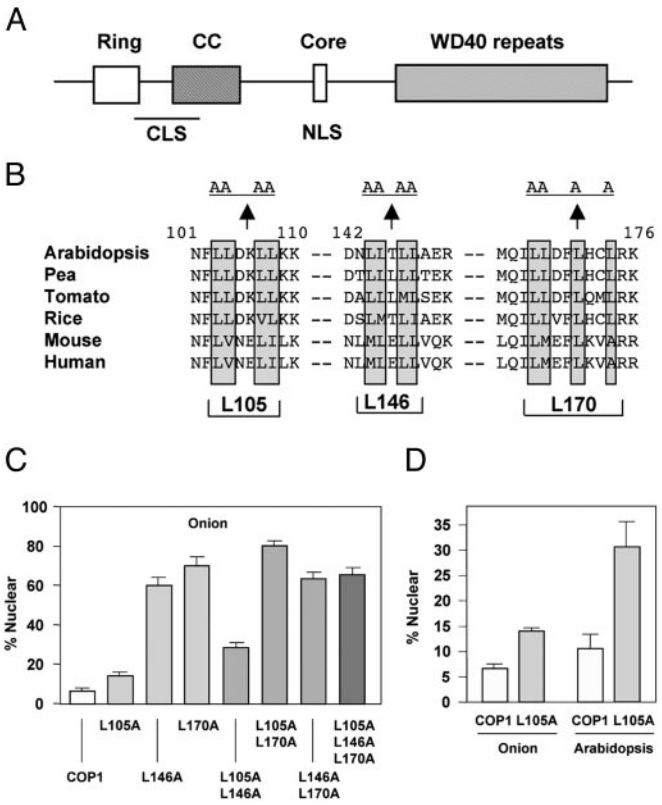

Fig. 1. Site-directed mutagenesis of the COP1 CLS causes increased nuclear localization. (*A*) Domain structure of COP1. CLS, residues 67–177; NLS, nuclear localization signal. (*B*) Blocks of leucines within the CLS were replaced by alanines in the context of full-length GFP-COP1. Five COP1 homologs are listed to illustrate the level of conservation within and beyond the leucine-rich motifs: *Arabidopsis*, GenBank accession no. P43254; pea, P93471; tomato, AAC98912; rice, AB040053; mouse, AF151110; human, AF508940. The mutants are referred to by a single residue, for example $COP1^{L105A}$, even though four adjacent residues were converted to alanine. Unless otherwise indicated, all COP1 proteins were tagged with GFP at the COP1 N terminus. (*C* and *D*) The percentage of cellular protein that is nuclear-localized was determined for GFP-tagged COP1 WT and mutants by using quantitative fluorescence microscopy (9) in transiently transformed onion epidermal cells or *Arabidopsis* roots as indicated. Bars denote standard errors from 15–20 cells. Experiments were repeated three times with similar results.

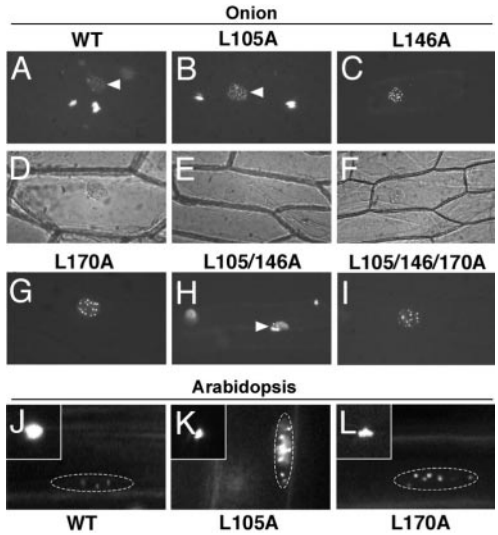

Fig. 2. (*A–I*) Fluorescence micrographs of onion epidermal cells expressing GFP-COP1 and its mutated derivatives. Three representative bright-field images (*D–F*) of the cells shown in *A–C* demonstrate identification of the nucleus. WT, wild-type COP1. Numbers refer to the amino acid coordinates of the mutated clusters of leucine residues. In cells with prominent cytoplasmic inclusion bodies, the nucleus is highlighted by an arrowhead. Note that all mutant proteins retained the ability to form nuclear speckles. (*J–L*) Nucleocytoplasmic localization of WT GFP-COP1 (WT) and two GFP-COP1 mutants in root epidermal cells of transgenic *Arabidopsis*. Note the enhanced nuclear accumulation and decreased size of cytoplasmic inclusion bodies (*Inset*) of the $COP1^{L105A}$ and $COP1^{L170A}$ mutants when compared with WT COP1. The stippled ellipse outlines the nucleus.

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 (Spex 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 *Bgl*II 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-20/20 tube luminometer equipped with the dual-color accessory (Turner Designs, Sunnyvale, CA). Typically, four pairs of 10-sec readings were taken through blue (390 $\pm$ 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–I*). The $COP1^{L105A}$ mutant protein was moderately but significantly more nuclear than WT ($P < 0.001$, Fig. 1*D*), whereas most other single, double, and triple mutants were predominantly nuclear (Fig. 1*C*). These data indicate that nuclear exclusion of COP1 is governed by a multipartite 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

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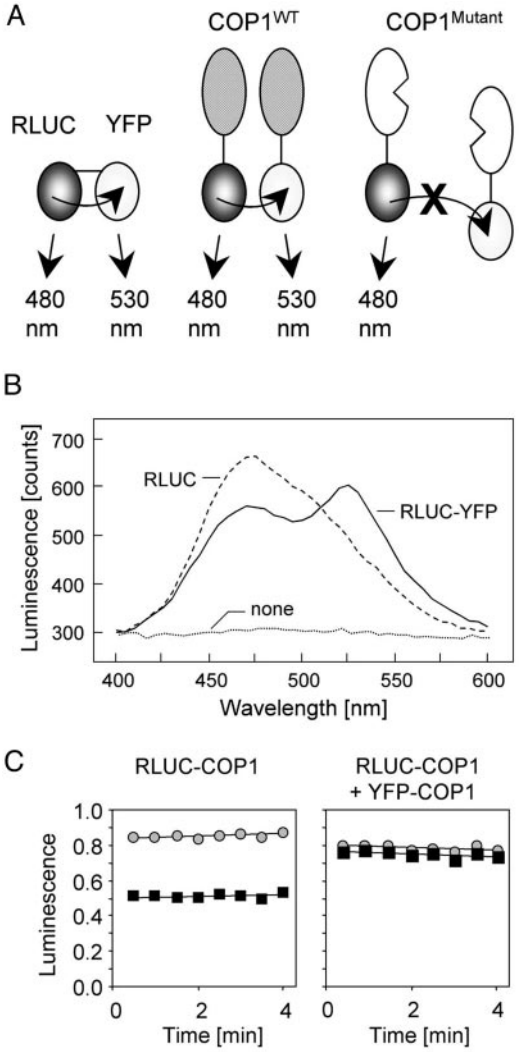

Fig. 3. BRET. (*A*) Overview to illustrate the principle of distance-dependent BRET as exemplified by the RLUC-YFP fusion protein or the dimerization of COP1. (*B*) Luminescence spectra of *Arabidopsis* seedlings expressing the indicated transgene products. (*C*) Raw luminescence from transiently transformed onion epidermal cells was recorded in a representative experiment through blue (gray circles) and yellow (black squares) filters over a time course of eight 10-sec readings for RLUC-COP1 alone (*Left*) and RLUC-COP1 coexpressed with YFP-COP1 (*Right*).

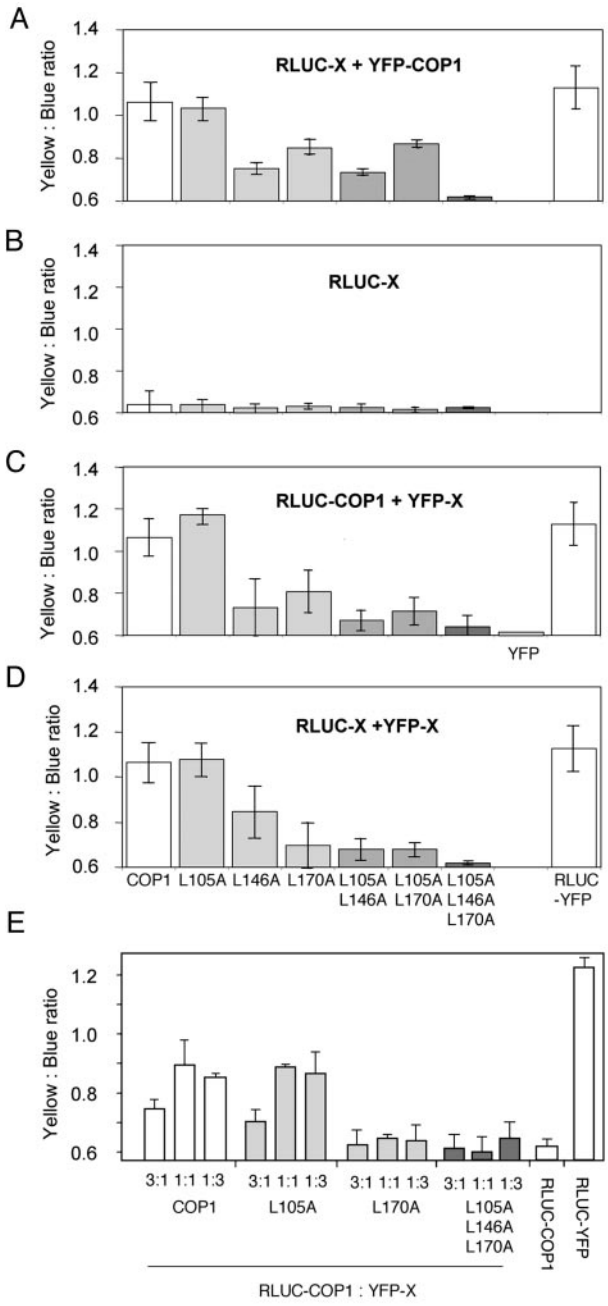

Fig. 4. Dimerization of COP1 mutant proteins as determined by BRET assay. The indicated COP1 WT or mutant proteins were fused to RLUC and YFP and coexpressed in onion epidermal cells. The RLUC-YFP fusion protein served as a positive control. (*A*) Mutant COP1 proteins tagged with RLUC and WT COP1 tagged with YFP. Bars denote standard deviations from three experiments. (*B*) RLUC-mutant fusion partner alone. (*C*) Heterodimerization of WT COP1 tagged with RLUC and mutant COP1 tagged with YFP. (*D*) Homodimerization (RLUC mutant with YFP mutant). The data for WT COP1-COP1 and for RLUC-YFP are the same for *A*, *C*, and *D*. (*E*) Selected combinations of RLUC-COP1 and the indicated YFP-COP1 mutant proteins were coexpressed at plasmid ratios including 3:1, 1:1, and 1:3 (RLUC/YFP). Averages from two independent experiments are shown.

GFP-COP1 mutants, $COP1^{L105A}$ and $COP1^{L170A}$, were expressed from stable transgenes in *Arabidopsis* (Figs. 1*D* and 2 *J–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. 2*L*). 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. 3*A*). 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. 3*B*). 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. 3*C*), 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,

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

Transgene	*cop1*	WT	Ratio	Complementation
$COP1^{WT}$	46	710	14.4	Yes
$COP1^{L105A}$	21	439	19.9	Yes
$COP1^{L170A}$	37	160	3.3	No
None	56	247	3.4	No

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 seeds from 1/4 to 1/16.

significantly, retained full interaction, whereas all other mutants tested interacted less well or not at all (Fig. 4*A*). Consistent results were obtained when the RLUC and YFP tags were swapped between the COP1 partners (Fig. 4*C*) or when testing for homodimerization of COP1 mutants (Fig. 4*D*). All BRET signals depended on the presence of a YFP partner protein (Fig. 4*B*). No BRET was observed with RLUC-COP1 and YFP alone (Fig. 4*C*, eighth bar).

The reduction of the BRET signal seen with specific $COP1^{mut}$ alleles such as $COP1^{L170A}$ 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-$COP1^{mut}$/RLUC-COP1 plasmid ratio was increased by a factor of 3 (Fig. 4*E*). 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 $COP1^{L105A}$ allele and the $COP1^{L170A}$ allele, as a representative allele with poor dimerization, were tested for functionality by transgenic rescue of the null allele, *cop1-5*. The $COP1^{L105A}$ allele was able to rescue the *cop1* mutant (Table 1). The *cop1* mutants carrying the $COP1^{L105A}$ transgene were etiolated fully in darkness, i.e., rescue was complete (data not shown). In contrast, no rescue was observed with the $COP1^{L170A}$ 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-$COP1^{L105A}$ transgene caused an elongated hypocotyl under constant far-red light but not in darkness, compared with a similar level of GFP-$COP1^{WT}$ expression (Fig. 5), suggesting that the increased nuclear accumulation of this mutant (Fig. 2*K*) is functionally significant. Likewise, the $COP1^{L170A}$ 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 $COP1^{L170A}$ 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. 5*C*). 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 $COP1^{L105A}$ 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

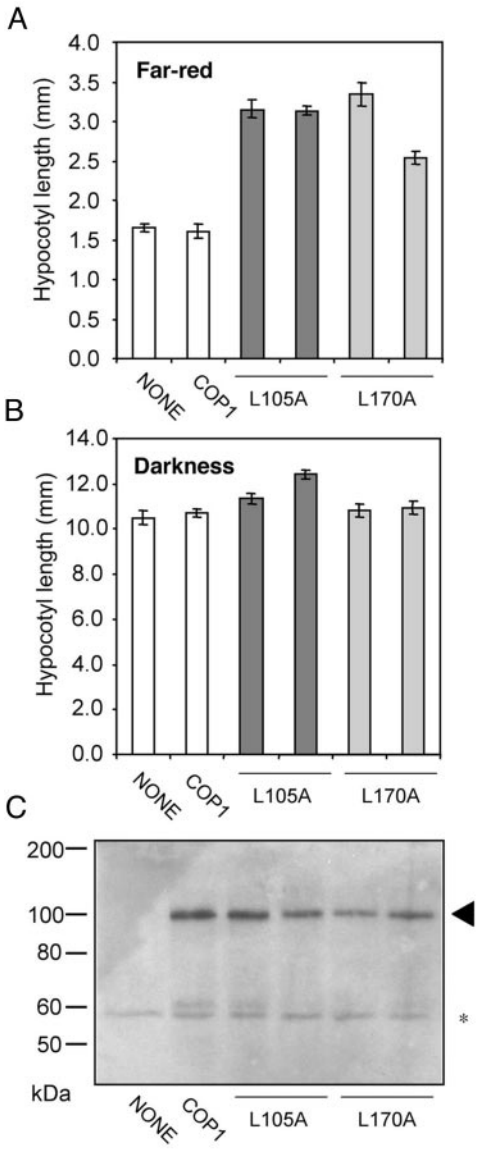

Fig. 5. Hypocotyl length in 5-day-old *Arabidopsis* seedlings expressing 35S:COP1-mutant proteins in a WT COP1 background. (*A*) Two representative transgenic lines per mutant construct were germinated in constant far-red light. Bars indicate standard errors. (*B*) Germination in constant darkness. All 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 $COP1^{L105A}$ 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 $COP1^{L105A}$, 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 $COP1^{WT}$ did not cause hypocotyl elongation. Together, these results suggest that under the conditions tested, COP1 nuclear exclusion is rate-limiting in controlling hypocotyl elongation, 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 mouse COP1 is excluded from the nucleus by a leptomycin B-sensitive nuclear export signal that corresponds to the *Arabidopsis* Leu-146 motif (26) all add weight to the notion that the CLS drives nuclear export, rather than nuclear turnover, of COP1.

We have applied the BRET technique in conjunction with site-directed mutagenesis to address how dimerization of COP1 might affect its function and subcellular localization. Similar to fluorescence resonance energy transfer, BRET yields real-time *in vivo* data, but in contrast to fluorescence resonance energy transfer, BRET measurements are better adapted to plant tissue, because problems arising from photobleaching and autofluorescence are minimized. Photobleaching by fluorescence excitation is irrelevant with a bioluminescent photon donor (27). Moreover, autofluorescence is managed easily as long as (*i*) nonpigmented tissue such as onion epidermal cells is used or (*ii*) delayed fluorescence from chlorophyll in pigmented tissue is allowed to decay. Moreover, BRET is not prone to false-positive results when the energy acceptor, here YFP-COP1, accumulates beyond the level of saturation of its RLUC-partner. The BRET data readily confirmed the COP1–COP1 interaction deduced earlier from gel-filtration and yeast two-hybrid assays (15). The overlap between domains for nuclear exclusion (residues 67–177) and dimerization (residues 105–211) (9, 15) raises the question of whether multimerization of 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. 1*C*) 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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