The constitutive photomorphogenic 1 (COP1) protein of Arabidopsis functions as a molecular switch for the seedling developmental fates: photomorphogenesis under light conditions and skotomorphogenesis in darkness. The COP1 protein contains a cysteine-rich zinc-binding RING finger motif found in diverse groups of regulatory proteins. To understand the role of the COP1 RING finger in mediating protein-protein interaction, we have performed a yeast two-hybrid screen and isolated a novel protein with a RING-H2 motif, a variant type of the RING finger. This protein, designated COP1 Interacting Protein 8 (CIP8), is encoded by a single copy gene and localized to cytosol in a transient assay. In addition to the RING-H2 motif, the predicted protein has a C4 zinc finger, an acidic region, a glycine-rich cluster, and a serine-rich cluster. The COP1 RING finger and the CIP8 RING-H2 domains are sufficient for their interaction with each other both in vitro and in yeast, whereas neither motif displayed significant self-association. Moreover, site-directed mutagenesis studies demonstrated that the expected zinc-binding ligands of the RING finger and RING-H2 fingers are essential for their interaction. Our findings indicate that the RING finger motif, in this case, serves as autonomous protein-protein interaction domain. The allele specific effect of cop1 mutations on the CIP8 protein accumulation in seedlings indicates that its stability in vivo is dependent on the COP1 protein.

Zinc ions provide structural integrity to many regulatory proteins, most often through cysteine and histidine ligands that form a tetrahedral geometry. The RING finger motif is a cysteine-rich zinc-binding domain defined by the consensus sequence: CX$_2$C$_{x-3}$C$_{x-10-13}$HX$_{1-3}$C$_{x-4}$C$_{x-49}$C$_{x}$C (C$_{x}$HC$_x$). A unique feature of this motif is that the consensus residues coordinate two zinc ions in a “cross-braced” fashion (1, 2). Thus, the RING finger forms one integrated structural unit, rather than forming two tandem zinc finger modules. Proteins containing RING finger domains are found in viruses and all eukaryotes, including yeast, plants, and animals. They have diverse cellular functions, including oncogenesis, viral gene expression, signal transduction, peroxisome biogenesis, DNA repair and recombination, and membrane vesicle sorting (2). The RING finger motif has been shown in many cases to play a role in mediating protein-protein interactions. However, the precise role of the RING finger, as well as specific interactive target motifs of the RING finger, have yet to be clearly defined.

The Arabidopsis COP1 protein serves as a repressor of photomorphogenesis. In the dark, the COP1 protein localizes to the nucleus and represses photomorphogenesis by inhibiting transcription factors that promote light-inducible gene expression (3–5). Light stimuli abrogate the nuclear localization of COP1 and allows seedlings to pursue photomorphogenic development (6). COP1 contains an N-terminal RING finger motif (7, 8), which has been demonstrated to bind two zinc ions (9). The RING finger motif is followed by a coiled-coil motif, which is common among a subgroup of RING finger proteins (10) and a WD40 repeat domain in the C-terminal half. Our previous domain-deletion analysis of COP1 suggested that the RING finger domain had a supportive role in the self-association and light-regulated nucleocytoplasmic partitioning of COP1 (11).

In an effort to understand the function of the COP1 RING finger domain, we have performed a yeast two-hybrid screen to identify interacting proteins. Our analysis uncovered a novel protein, named CIP8 (COP1 interacting protein 8), with a RING-H2 module, a variant type of the RING finger with the fourth cysteine ligand substituted by a histidine. Mutational analysis revealed that the COP1 RING finger and the CIP8 RING-H2 finger are both necessary and sufficient for mediating this protein-protein interaction. The fact that CIP8 protein accumulation was compromised specifically in severe cop1 mutants indicates that COP1 plays a role in regulating CIP8 protein level.

EXPERIMENTAL PROCEDURES

Materials and Growth Conditions—The yeast strain EGY48–0 (12, 13) was used for yeast two-hybrid analysis. Arabidopsis thaliana wild type and cop1–4 mutants are in Columbia (Col) background and cop1–5

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mutant is in Wassilewskijae background. All other cop1 alleles and fus mutants are in Landsberg erecta background (8). The plant growth conditions were described previously (8).

**Yeast Two-hybrid Screen and Protein Analysis—**A yeast two-hybrid screen was performed using an Arabidopsis light-green seedling cDNA library (Stratagene, La Jolla, CA) as bait. The library was cloned into the yeast expression vector pJG4-5 (Novagen, Madison, WI) and transformed into the expression plasmids harboring mutated forms of COP1 was detected by protein gel blot analysis using anti-LexA polyclonal antibodies. The sample was harvested. After brief centrifugation, SDS-PAGE buffer was added to the beads, and the samples were boiled and separated by SDS-PAGE. The proteins in the gel were visualized by silver staining. After protein concentration analysis, an equal volume of 4x SDS-PAGE loading buffer was added to the beads. The beads were washed extensively with binding buffer. SDS loading buffer was added to the beads, and the samples were boiled and separated by SDS-PAGE. The protein gel immunoblot analysis was followed a previously described procedure (14) except that the affinity-purified CIP8 antibodies were used at 1:300 dilution.

**Results**

**Site-directed Mutagenesis—**The RING finger domains of COP1 and CIP8 were mutated by the polymerase chain reaction (PCR) method. To mutate the RING-H2 motif of CIP8, a fragment was amplified with a combination of primers T7 and BD4–2x3x3 (5'CAT GCC ATG GCA CAA TAG CAT CTC CAG CTT AAG CTC CAG CCG GTA TCT TCT TAC C-3'), digested with NcoI and EcoRI, and inserted into the EcoRI site of pSH18–34. The plasmid was transformed into the E. coli strain BL21 and induced by 1 mM isopropyl-β-D-thiogalactopyranoside for 4 h. The recombinant MBP-CIP8 protein was purified and used for affinity purification on SAM resin (New England Biolabs). Six copies of the histidine codon were added after the DNA fragment was ligated to the pRTL2-Baphi vector. The MBP-CIP8 versus anti-cIP8 polyclonal antibodies, a recombinant maltose binding protein (MBP)-CIP8 His fusion protein was produced from a modified pMal-c vector (New England Biolabs). Four copies of the histidine codon were added after the cloning site in the pMal-c vector by ligating overlapping oligomers into the SalI/HindIII sites of pJG4–5, which added a BamHI site to the 5' end and a SalI site to the 3' end. The cloned fragments were used for affinity purification on anti-CIP8 antibodies as described previously (15).

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COP1—We first determined whether the CIP8 fragment interacts with a full-length COP1 (Fig. 1A). The CIP8-COP1 interaction was significant, but reduced by about 3-fold compared with the CIP8-N282 interaction (Fig. 1B, lanes 1 and 2). To identify the COP1 domain responsible for the interaction with CIP8, a deletion analysis of the COP1-N282 fragment was carried out in the yeast two-hybrid system. Deletion of the RING finger region abolished the interaction, whereas in contrast, elimination of the coiled-coil region (Coil) and sequence flanking the RING finger domain resulted in stronger interaction based on the β-galactosidase activity (Fig. 1B). Thus, the COP1 RING finger domain per se (amino acids 39–103) is sufficient for interaction with CIP8. The surrounding sequence, including the coiled-coil region domain, seems to attenuate the interaction. Similar trends were observed when a reciprocal combination of the bait and the prey were tested (Fig. 1B, lanes 6–9). It should be noted that the protein gel immunoblot analysis using anti-LexA antibodies did not reveal any significant differences in expression levels of the truncated COP1 constructs (data not shown). This eliminates the possibility that the different activities in the yeast two-hybrid assay were caused by changed expression levels of the proteins.

**CIP8 RING-H2 and COP1 RING Motifs Are Sufficient to Confer Direct Interaction in Vitro**—To confirm that the CIP8 RING-H2 and COP1 RING finger domains interact directly, a recombinant fusion protein between the maltose-binding protein and the RING finger motif of COP1 (MCOP1RF) was produced and purified. It was then used to test for the ability to bind to the RING-H2 domain-containing protein (CIP8RF) encoded by the partial CIP8 cdNA in vitro (see “Experimental Procedures”). As shown in Fig. 2, the amylose beads (preincubated with 1 mg/ml bovine serum albumin) were able to pull down CIP8RF in the presence of MCOP1RF (lane 3). However, the same beads incubated without other proteins (Fig. 2, lane 1) or with maltose-binding protein alone (Fig. 2, lane 2) were unable to pull down CIP8RF. Thus the CIP8 RING-H2 and COP1 RING motifs interact directly in vitro.

**CIP8 Is a Novel RING-H2 Finger Protein with Another C4 Zinc-binding Domain and a Highly Negative Charged Region**—Because the CIP8 RING-H2 fragment isolated by the yeast two-hybrid screen was clearly missing the 5′ portion of the

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**Fig. 1.** The RING finger domain of COP1 interacts with CIP8 in the yeast two-hybrid system. A, diagrams of the three main protein forms used for the analysis. COP1 (8) and N282 (14) are shown with the locations of the RING finger (RING), coiled-coil (Coil), and WD-40 repeat (WD-40) highlighted. The 92-amino-acid C-terminal fragment of CIP8, which has been isolated by an interactive screen, is shown with a RING-H2 motif highlighted. Numbers correspond to the amino acid residues at the beginning and the ending of each motif as well as the last amino acid of each construct. B, interaction of the COP1 domain deletion series with CIP8. In the first five pairs (rows 1–5), the CIP8 fragment fused with LexA DNA-binding domain (closed box) was used as a bait (Bait) to test against different prey (Prey): row 1, full-length COP1; row 2, N282; row 3, RING (11); and row 4, NARING (11) fused to a synthetic activation domain (closed box); or row 5, activation domain only. In the last four pairs (rows 6–9), the interaction assays were carried out with a reciprocal combination of the constructs were shown. The domain-deletion clones of COP1, namely NARING (row 6), N282 (row 7), NACoil (11) (row 8), and RING (amino acids 39–103) (row 9) were fused with LexA and used as baits to test interaction with the CIP8 fragment fused to the activation domain (AD). For unknown reasons, LexA-COP1 could not be stably expressed in yeast and thus is missing in this panel. The graph on the right indicates the relative LacZ reporter activity in yeast cells corresponding to combinations of bait and prey constructs presented in each row. For each pairwise combination, at least ten individual transformants were used to measure the LacZ activity. Error bars represent the standard deviations.

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**Fig. 2.** The RING finger of COP1 interacts directly with the RING H2 finger of CIP8 in vitro. The purified recombinant RING-H2 finger domain protein of CIP8 was incubated in a binding buffer (see “Experimental Procedures”) for 2 h at 4 °C alone as a negative control (lane 1), and with either maltose-binding protein (lane 2) or the maltose-binding protein fused with the RING finger of COP1 (MCOP1RF, lane 3). Amylose beads, which were preincubated with 1 mg/ml bovine serum albumin, were added and allowed to incubate for 30 min. The beads were washed extensively with binding buffer, resuspended in SDS loading buffer, boiled, and loaded onto a 15% SDS-polyacylamide gel. The arrows on the right indicate the positions of MBP, MCOP1RF, and MBB-CIP8RF on the silver-stained gel.
gene, we screened an Arabidopsis cDNA library to recover the full-length clone. Approximately 7 × 10⁶ plaques were screened with ³²P-labeled CIP8 RING-H2 fragment as a probe, and 13 positive clones were isolated. The longest clone (clone 13b) contained 1,266 nucleotides. Further rounds of 5’ rapid amplification of cDNA ends PCR (Stratagene) from the mRNA recovered another 18 nucleotides 5’ upstream of the longest cDNA clone. The entire 1,284 nucleotides encode a single open reading frame of 334 amino acids. Comparison of the cDNA with the Arabidopsis genomic DNA sequence recently available in the GenBank™ data base (accession number AB019236) revealed that the CIP8 gene does not have any intron. Our predicted CIP8 cDNA open reading frame is likely full-length, because an in-frame stop codon is present 63 nucleotides upstream of the predicted translational start codon. The predicted CIP8 protein has several characteristic domains: an N-terminal motif similar to the zinc-binding GATA finger (C₅); a highly acidic region; a glycine cluster immediately before the RING-H2 finger; and a serine-rich cluster at the C terminus (Fig. 3A). The original cDNA clone isolated from the two-hybrid screen only contained amino acids 243–334, encompassing the RING-H2 motif and the C-terminal serine-rich region.

A sequence homology search (17) revealed over 40 Arabidopsis expressed sequence tags and hypothetical proteins that possess RING-H2 domains with significant sequence similarity to the CIP8 RING-H2. As shown in Fig. 3B, six of those motifs (accession numbers AC004521-g3128178, AB015472, AC003000-g2642154, AC002409-g2623297, AC004473-g3249088, and AL022222-g2982466) are aligned with the RING-H2 of CIP8. This indicates that Arabidopsis has a large number of proteins containing the RING-H2 motif. This is consistent with the finding that the nearly complete sequence of the Caenorhabditis elegans genome contains 98 RING finger or RING-H2 proteins (18). Using degenerate PCR, Jensen et al. (19) identified many RING-H2 proteins and suggested that they are more prevalent than the original RING finger motif in Arabidopsis. All of the Arabidopsis genes listed in Fig. 3B possess the RING-H2 domain in the C-terminal half of the gene products. This feature also applies to several representative RING-H2 proteins from animals including the Dro sophila Goliath (M97204; Ref. 20), mouse Praja1 protein (U06944; Ref. 21), and rat neurodegeneration-associated protein (D32249; Ref. 22) (Fig. 3B). The amino acid alignment revealed strictly conserved spacing both at site 1 (between C2 and C3) and site II (between C6 and C7) (Fig. 3B). In addition, amino acid sequences between C6 and C7 are highly conserved with the invariable PWL residues in all Arabidopsis RING-H2 motifs (Fig. 3B). CIP8 displayed no extensive sequence homologies with any known or hypothetical proteins beyond the RING-H2 domain.

The Integrity of the COP1 RING Finger and CIP8 RING-H2 Finger Is Necessary for Their Interaction—To rule out the possibility that the observed interaction between the COP1 RING finger and the CIP8 RING-H2 interaction is due to a general affinity within this group of zinc-binding domains, the self-association of the COP1 RING finger (amino acids 22–117) and CIP8 RING-H2 (amino acids 243–334) domains were examined. As shown in Fig. 4A, the interaction between COP1 RING finger and CIP8 RING-H2 domain is very strong, whereas the self-association in either COP1 RING-finger or CIP8 RING-H2, if any, is close to the background level of detection.

To confirm that it is the zinc-binding RING finger motif per se, and not the remaining flanking amino acids, that is responsible for mediating the COP1-CIP8 interaction, specific mutations were introduced to remove the predicted zinc-coordinating ligands and their effect on protein-protein interaction was analyzed. The RING finger is known to form a cross-brace topology, with the first and the third pairs of ligands forming one zinc-binding site and the second and the fourth pairs forming the other (1). We first substituted Cys-67, His-69, Cys-72, and Cys-75 of the COP1 RING finger fragment (amino acids 22–117) with alanines (Fig. 4B). These mutations destroy the second and the third pairs of the metal binding ligands. Thus the mutated COP1 RING finger should not be able to bind any zinc ion, thereby perturbing the overall structural integrity. As shown in Fig. 3A, disruption of the COP1 RING finger domain completely abolished its ability to interact with CIP8 in the yeast two-hybrid assay. The RING-H2 finger domain of CIP8 was then similarly mutated. Likewise, a substitution of Cys-275, His-277, His-280, and Cys-283 of the CIP8 RING-H2 finger with alanines completely abolished the ability to interact with the COP1 RING finger domain (Fig. 4B).

To exclude the possibility that the loss of the interaction in yeast two-hybrid assays may be due to instability or low expression levels of the mutated proteins, the mutant protein level was analyzed by protein gel immunoblot. Total proteins were extracted from yeast transformants used for the β-galactosidase activity assay, and the fusion proteins of LexA-RING, LexA-RINGmt, LexA-CIP8, and LexA-CIP8m were detected using anti-LexA antibodies. As shown in Fig. 4C, both the LexA-RING and LexA-RINGmt fusion proteins were detected as a single band with the apparent molecular mass of 36 kDa, whereas LexA-CIP8 and LexA-CIP8m fusion proteins are present as a doublet. Disruption of the RING-H2 domain had no effect on the accumulation of LexA-CIP8 at all, whereas the disruption of the COP1 RING finger domain resulted in a slight decrease in the amount of fusion protein (Fig. 4C). However, it is unlikely that this decrease will account for the over 100-fold difference in β-galactosidase activity (Fig. 4A). Therefore, we conclude that the structural integrity of both the COP1 RING and the CIP8 RING-H2 domains is required for the COP1-CIP8 interaction.

CIP8 Is a Single Copy Gene Whose mRNA Level Is Not Significantly Light-regulated.—To further characterize the CIP8 gene, genomic DNA gel blot analysis was used to determine its gene copy number. The genomic and cDNA sequence revealed that the CIP8 coding region contains single EcoRI and SacI sites but neither XbaI nor XhoI sites. As expected (Fig. 5), when the full-length CIP8 cDNA was used as probe in the DNA gel blot, digestion of Arabidopsis genomic DNA with EcoRI and SacI resulted in two bands, whereas digestion with XbaI and XhoI resulted in single band. The bands match the expected size from the available genomic CIP8 sequence, indicating that CIP8 is a single copy gene.

The expression of CIP8 in Arabidopsis seedlings was examined by the RNA gel blot analysis. A single band of approximately 1.4 kilobases was observed both in light- and dark-grown seedlings. The mRNA size is in agreement with the cDNA length of 1,288 nucleotides, considering that 100 to 200 nucleotides of poly(A) are commonly added to plant mRNAs. The level of the CIP8 transcripts were approximately equal in both light- and dark-grown wild type seedlings (Fig. 5B). To examine the possible effect of COP1 on CIP8 expression, the CIP8 mRNA levels in representative weak (cop1–4) and null (cop1–8) alleles were examined. Similar results were obtained for both alleles, and that of cop1–4 blot is shown in Fig. 5B. Both cop1 mutations led to slightly elevated CIP8 mRNA levels in the dark-grown mutant seedlings, suggesting that COP1 may play a minor role in regulating CIP8 mRNA accumulation.

CIP8 Protein Accumulation Is Affected by Specific cop1 Mutant Alleles.—The protein accumulation pattern of CIP8 in light- and dark-grown wild type and cop1del/fus mutant seedlings were examined by immunoblot analysis using affinity-
FIG. 3. Sequence analysis of CIP8. A, the complete cDNA nucleotide and predicted amino acid sequences of CIP8. The arrow indicates the starting position of the original CIP8 fragment isolated from the yeast two-hybrid screen. The consensus metal-binding ligands in the predicted N-terminal C4 zinc-binding domain and C-terminal RING-H2 domain are highlighted with open circles. The RING-H2 domain is also underlined. Glycine-rich are double underlined and serine/threonine-rich regions are indicated by a dotted line. The acidic domains are boxed. The GenBank™ accession number for the CIP8 cDNA is AF162150.

B, amino acid sequence alignment of the conserved RING-H2 domains from CIP8 and other representative proteins. Amino acids are shown using the one-letter code. Open boxes indicate amino acid residues identical to those of CIP8. The positions of eight predicted metal-binding ligands are marked by an asterisk (*) at the top. See text for reference to each sequence.
purified antibodies against CIP8 (see “Experimental Procedures”). As shown in Fig. 6A, light-grown seedlings appear to have approximately 2-fold higher level of the CIP8 protein in wild type seedlings. This difference is not due to unequal loading, because no change in the level of the proteasome subunit AtS6A in the same samples was observed. Interestingly, severe cop1 mutations (cop1–5, cop1–8, and cop1–9) negatively affect the accumulation of CIP8 in both light- and dark-grown seedlings, whereas the weak cop1–4 mutation has no effect on CIP8 accumulation. The reduction of CIP8 protein level is specific for severe cop1 mutations, because none of the other severe cop1 delttus mutants, whose phenotype is indistinguishable from that of the severe cop1 mutant alleles, show any reduction (Fig. 6B). In addition, the accumulation of the AtS6A protein was unaffecte in the other cop1 delttus mutants (data not shown). Because both weak and null alleles seem to accumulate similar levels of CIP8 mRNA levels (Fig. 5B and data not shown), our results suggest that the stability of CIP8 requires at least some degree of COP1 function.

**CIP8 Protein Is Cytosol Localized in Onion Epidermal Cells after TransientExpression**—To reveal its cellular localization and possible light regulation, a GUS-CIP8 fusion protein construct was generated and transiently expressed in onion epidermal cells through particle bombardment. As shown in Fig.

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**Fig. 4.** The zinc-binding capacity of the COP1 RING finger and the CIP8 RING-H2 domains are necessary for their interaction in yeast. A, the C-terminal CIP8 fragment (row 1) and the COP1 RING domain (row 2) were tested for self-association. Rows 3–6, mutational analysis of the RING and RING-H2 domains. RING and CIP8 constructs with intact RING and RING-H2 domains, and RINGmt and CIP8mt represent that of mutated versions (see panel B). The Bait and Prey constructs represent those indicated protein domains fused to the LexA DNA-binding domain and a synthetic activation domain, respectively. The graph on the right indicates the relative LacZ reporter activity in yeast cells corresponding to combinations of bait and prey constructs presented in each row. For each pairwise combination, at least ten individual transformants were used to measure the LacZ activity. B, the COP1 RING and the CIP8 RING-H2 domains. Among the eight proposed metal-coordinating residues, four amino acids (Cys-67, His-69, Cys-72, and Cys-75 for COP1 and Cys-275, His-277, His-280, and Cys-283 for CIP8) were mutated to alanines and are highlighted in **capital letters**. The rest of the conserved ligand residues are **underlined**. C, protein gel immunoblot analysis of yeast transformants used in the two-hybrid analysis in panel A (rows 3–6). Equal numbers of yeast cells at mid to late log phase were loaded in each lane, and immunoblotting was performed using anti-LexA antibodies. Molecular mass markers are indicated in kilodaltons (kd).

**Fig. 5.** DNA and RNA blot analyses of CIP8. A, DNA gel blot analysis of CIP8. About 1 µg total DNA was used for each lane. The DNA was digested with the indicated restriction enzymes to completion and subjected to blot analysis with the full-length CIP8 DNA as a probe. The relative positions of size markers (in kilobases (kb)) are indicated on the left. B, RNA gel blot analysis of CIP8. RNA levels of CIP8 in 6-day-old light-grown (L) and dark-grown (D) seedlings of the wild type (ecotype Columbia; WT) and cop1–4 mutants were examined. Equal amounts of total RNA (20 µg) were loaded in each lane. The membrane was re-probed with the 18 S rRNA probe to confirm equal loading.

**Fig. 6.** The CIP8 protein accumulation is responsive to light and diminished in severe cop1 mutants. A, the upper panel shows a protein immunoblot for CIP8 using extracts from 6-day-old seedlings of wild type (lanes 1 and 2), cop1–5 (lanes 3 and 4), cop1–4 (lanes 5 and 6), cop1–8 (lanes 7 and 8), and cop1–9 (lanes 9 and 10). Lanes 1, 3, 5, 7, and 9 are from seedlings grown in complete darkness. 20 µg of total protein was loaded in each lane. The bottom panel is an identical blot probed with an antibody against AtS6A, a member of the Arabidopsis proteasome complex as a loading control. B, CIP8 is accumulated in other photomorphogenic mutants whose phenotype is similar to that of the cop1 severe mutants. In all lanes, 15 µg of total protein was loaded from 6-day-old light-grown (lanes 1, 3, 5, 7, and 9) and dark-grown (lanes 2, 4, 6, 8, and 10) seedlings. Mutants shown are fus2 (lanes 1 and 2), fus4 (lanes 3 and 4), fus9 (lanes 5 and 6), cop9 (lanes 7 and 8), and cop10 (lanes 9 and 10). Note that fus2, fus9, and cop10 mutations also diminished the light and dark difference of CIP8 protein levels.
Interaction of RING Finger and RING-H2 Motif

7. A and B, GUS-CIP8 has a typical cytosolic localization pattern in both light and dark conditions, similar to that of the GUS protein alone (Fig. 7, G and H). As expected, GUS-COP1 exhibited a light-regulated nuclear localization (Fig. 7, C and D) and GUS-N1a exhibited a constitutive nuclear localization (Fig. 7, E and F) under the same assay conditions. Therefore, CIP8 is likely a cytosolic protein.

DISCUSSION

The RING Finger Motif as Protein-Protein Interaction Domain—This study clearly demonstrated a role of the COP1 RING finger as an autonomous protein-protein interaction module. In the case of COP1, this RING-finger specifically interacts with the RING-H2 domain of CIP8. It has been shown that the COP1 RING finger domain binds two zinc ions (9). Here we demonstrated that abolishing the zinc-binding capability of the COP1 RING finger domain via specific point mutations that change the consensus metal-coordinating residues results in a loss of its ability to interact with the RING-H2 domain of CIP8. Likewise, reciprocal mutations in the RING-H2 motif of CIP8 also resulted in a loss of its ability to interact with the COP1 RING finger domain. In addition, an analysis of the interaction between purified protein fragments containing these domains indicates that the RING finger modules interact directly. Our data clearly support the conclusion that the zinc-binding motifs are necessary and sufficient for the observed interaction. In addition, it implies that the tertiary structure of the respective domains as a result of zinc binding is essential for the protein-protein interaction.

Our finding adds to the accumulating evidence that the RING finger types of zinc-binding motifs are protein-protein interaction modules (23). For instance, yeast two-hybrid screens using an N-terminal fragment encompassing the RING finger domain of the breast/ovarian cancer susceptibility gene product (BRCA1) as a bait recovered a novel RING finger protein BARD1 (24) and a novel ubiquitin hydrolase BAP1 (25).

In both cases, a tumorigenic mutation that replaces a metal-coordinating ligand of the BRCA1 RING finger with a glycine abolished the BRCA1-BARD1 and BRCA1-BAP1 interactions (24, 25). A Drosophila dosage compensation gene product, MSL2, contains an N-terminal RING finger (26). MSL2 forms a tight protein complex with two other dosage compensation gene products, MSL1 and MSL3. The intact RING finger domain of MSL2 was shown to be critical for the MSL2-MSL1 interaction in yeast (27). Furthermore, the yeast Ste5 protein, a “scaffold” for the pheromone response pathway, contains a RING-H2 domain. Mutations in the Ste5 RING-H2 domain disrupted the Ste5-Ste4 interaction, which is a prerequisite for oligomerization of Ste5 (28). However, it is not clear in either of those cases whether the RING finger domains are sufficient for mediating the observed protein-protein interaction, nor is it clear which domain in the respective interaction partners are responsible for the interaction.

In some cases, the RING finger-containing domains appear to associate with another RING finger-containing protein, thus implying a possibility of it serving as either homo- or heterodimerization motifs. A homodimerization domain of the recombination-activating protein (RAG1) encompasses the RING finger (29, 30). Recently, BRCA1 N-terminal region encompassing the RING finger was shown to homodimerize in solution (31). However, in both cases, the protein-protein interaction seems to require sequences or motifs in addition to the RING finger domain. For instance, formation of a stable dimerization interface between the RAG1 RING finger domains requires an adjacent C2-H2 zinc finger (29). In the case of BRCA1, progressive truncations of the C-terminal sequence flanking the RING-finger domain gradually diminished the interaction with1 BARD1 (24). A recent report indicated that a domain encompassing the RING finger of the mouse oncoprotein, Bmi-1, heterodimerizes with the RING finger domain of dinG/RING1B in yeast (32). However, the C-terminal flanking sequence of Bmi-1 (53 amino acids) and dinG (74 amino acids) in addition to the RING finger modules are essential for the protein-protein interactions (32). The COP1-CIP8 interaction reported here is strictly heterodimeric, because neither domain displayed significant self-association in vitro or in yeast (data not shown). In contrast to the other cases, a removal of the C-terminal flanking sequence of the COP1 RING finger resulted in a marked increase of its interaction with CIP8 (Fig. 1B). Therefore the interaction surface most likely resides within the COP1 RING finger domain. Likewise, the original CIP8 fragments isolated by our yeast two-hybrid screen retains only ten extra amino acids N-terminal to the first cysteine ligand residual of the RING-H2 domain. Therefore, the rest of the N-terminal region of CIP8 is clearly not required for mediating the observed strong interaction with COP1. However, a possible supporting role of the additional 37 amino acids C-terminal to the RING-H2 in CIP8 cannot be ruled out at this point. No recognizable structure feature was found in this C-terminal region except relative richness in serine residues.

A data base search revealed an amazing number of predicted RING-H2 proteins with unknown functions in Arabidopsis. Recently, many more RING-H2 proteins have been identified using degenerate PCR. The abundance of these RING-finger motifs in comparison with the original motif suggests that the RING-H2 may be the more prevalent variant in Arabidopsis (19). Alignment of these RING-H2 domains reveals a high degree of conservation within this subfamily in contrast to the variance found in the RING finger superfamily. The variability in spacing, from 9 to 39 between C2 and C3, and from 4 to 48 between C6 and C7, is believed to reflect a functional diversity among RING finger domains (2). Although it cannot be ruled
out that the uniformity of the RING-H2 family could reflect a statistical bias, this similarity may extend to their structure and ultimately their function. However, a preliminary experiment in the yeast two-hybrid system revealed that the COP1 RING finger domain does not interact with the RING-H2 domain in an Arabidopsis expressed sequence tag clone (accession number ATTS2447) that exhibited the strongest similarity to CIP8 RING-H2 domain (data not shown). Hence, despite the prevalence of RING-H2 motif in Arabidopsis and its high conservation among members, COP1 shows a specificity for the RING-H2 of CIP8.

A Possible Role of CIP8 in COP1-mediated Light Regulation of Arabidopsis Development—The effect of COP1 on the CIP8 expression would support a physiological role of the observed CIP8 and COP1 interaction. First, the CIP8 mRNA seems to be slightly elevated in the dark-grown cop1 mutant seedlings. This would imply a role for COP1 in repressing CIP8 expression in darkness. It has been reported at least in another case (5) that COP1 regulates the expression of its interactive partner, CIP7. Second, the severe cop1 mutants accumulated a greatly reduced amount of CIP8 protein. The reduction of CIP8 protein is not clearly a result of a reduction of CIP8 mRNA levels or a general reduction of cellular proteins (see Figs. 5B and 6A). Further, the reduction of CIP8 is not a consequence of the associated seedling lethality, considering that other photomorphogenic mutants with similar severity and phenotype did not exhibit any reduction of the CIP8 protein (Fig. 6B). Although this indicates that the accumulation of CIP8 is dependent on the structural integrity of COP1, additional studies will be necessary to reveal the exact nature of this possible in vivo role of CIP8 in COP1-mediated development regulation. Our initial attempts to co-immunoprecipitate CIP8 and COP1 were not successful due to the quality of the respective antibodies. Future research, such as isolation of an Arabidopsis knock-out mutant of CIP8 or modulating CIP8 levels by overexpression and antisense strategies, may provide new insights in understanding a role of COP1 RING-CIP8 RING-H2 interaction in Arabidopsis photomorphogenic development.

Our functional dissection of the COP1 protein implied a supportive role for the COP1 RING finger in self-association and light-responsive nucleocytoplasmic partition (11, 33). Considering the fact that the RING finger motif of COP1 is a specific protein-protein interactive motif for RING-H2 motif of CIP8, it may achieve a functional role by its interaction with target proteins, such as CIP8. Previous studies (4, 6) have established that COP1 is enriched in the nucleus in darkness and that light triggered a nuclear depletion. Because CIP8 is likely localized in cytosol, one hypothesis might be that it plays a role in regulating COP1 cellular localization in response to light. It is feasible that CIP8, together with another cytosolic COP1 interactive partner CIP1 (34), may interact with RING finger and coiled-coil motifs of COP1 and regulate the nuclear localization signal or cytosolic localization signal activity of COP1.

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