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SHORT COMMUNICATION

The N-terminal fragment of Arabidopsis photomorphogenic repressor COP1 maintains partial function and acts in a concentration-dependent manner

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Summary

Arabidopsis seedlings exhibit distinct developmental patterns according to their light environment: photomorphogenesis in the light and etiolation or skotomorphogenesis in darkness. COP1 acts within the nucleus to repress photomorphogenesis in darkness, while light depletes COP1 from nucleus and abrogates this repression. COP1 contains three structural modules: a RING finger followed by a coiled-coil domain, and a WD40 repeat domain at the C-terminus. By introducing various domain deletion mutants of COP1 into *cop1* null mutant backgrounds, we show that all three domains are essential for the function of COP1 *in vivo*. Interestingly, a fragment containing the N-terminal 282 amino acids of COP1 (N282) with both the RING finger and coiled-coil modules is sufficient to rescue the lethality of the *cop1* null mutations at low expression level. However, high expression levels of the N282 fragment result in a phenocopy of the *cop1* null mutation. The sensitivity of the seedling to levels of N282 could reflect the importance of the abundance of COP1 for the appropriate regulation of photomorphogenic development.

Introduction

As sessile organisms, plants depend heavily on their ability to perceive and respond to environmental cues. Light is one of the most important factors affecting plant development, as shown by the dramatic difference in morphogenetic patterns in response to different light conditions. In the light, Arabidopsis seedlings develop according to a photomorphogenic program, with short hypocotyls, open cotyledons and expanded, differentiated chloroplasts. In darkness, seedlings adapt an etiolated or skotomorphogenic development, with long hypocotyls and small cotyledons on an apical hook with etioplasts (Kendrick and Kronenberg, 1994; von Arnim and Deng, 1996). A number of the signal transduction pathway components have been identified by screening for etiolated phenotypes in the light or photomorphogenic phenotypes in the dark (Chory *et al.*, 1989; Deng *et al.*, 1991; Koorneef *et al.*, 1980; Misera *et al.*, 1994).

One important component in the light regulatory pathway is Constitutively Photomorphogenic 1 (COP1) which functions as a molecular switch for mediating light control of seedling development. Recessive mutations at the *COP1* locus result in a photomorphogenic phenotype independent of light (Deng *et al.*, 1991). This suggests that COP1 acts as a repressor of photomorphogenesis in the dark. Subcellular localization studies revealed that in the dark, COP1 is enriched in the nucleus and light depletes its nuclear abundance (von Arnim and Deng, 1994). Increased cellular abundance of COP1 caused hypersuppression of photomorphogenesis in the light, supporting the notion that COP1 is an autonomous repressor or a rate-limiting step in the repression of photomorphogenesis (McNellis *et al.*, 1994a). In addition, genetic epistatic analyses indicate that COP1 acts downstream of multiple photoreceptors (Ang and Deng, 1994).

The COP1 protein contains three structural modules, all of which may be involved in protein–protein interactions (Osterlund and Deng, 1999; Torii *et al.*, 1998). The N-terminus contains a RING finger motif found in a large variety of proteins with many different functions ranging from DNA repair and recombination to peroxisome biogenesis (Saurin *et al.*, 1996). In several cases, the RING finger motifs have been shown to be involved in protein–protein interaction (Bellon *et al.*, 1997; Mackay *et al.*, 1998). In the case of COP1, the RING finger specifically interacts with the RING-H2 domain of a novel Arabidopsis protein CIP8 (Torii *et al.*, 1999). Similar to many RING finger-containing proteins, there is a coiled-coil domain following the RING-finger motif (Reddy *et al.*, 1992). The C-terminal half of COP1 is made up of seven WD-40 repeats which were first identified in the β subunit of the heterotrimeric G proteins (Neer *et al.*, 1994). Each WD-40 repeat in the G-protein

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forms a ring-like β propeller structure that acts as a platform for different protein–protein interactions (Gaudet *et al.*, 1996). In Arabidopsis, COP1 mediates its interaction with HY5 through the WD-40 repeat domain (Ang *et al.*, 1998; Torii *et al.*, 1998). The confluence of these many protein–protein interaction domains in COP1 suggests that it can interact with multiple proteins contributing to the pleiotropic phenotype of *cop1* mutants.

In this paper, we examine the functional property of mutant proteins with deletions of the RING finger motif (ΔRING), the coiled-coil module (ΔCoil), and the WD40 repeat domain (N282) in the null mutant backgrounds. Our data suggest that all three modules are essential for the complete COP1 activity. In the case of N282, it is sufficient to rescue the lethality of the null mutations and its physiological activity is critically dependent on its protein level.

Results

Both the RING finger and coiled-coil domains are essential for COP1 function

To determine the function of the COP1 domains, the representative transgenes overexpressing the ΔRING and ΔCoil mutant proteins (Torii *et al.*, 1998) were crossed to heterozygous *cop1-5* mutant plants. The F1 plants heterozygous for a single locus transgene and the *cop1-5* mutation were allowed to self fertilize. In the F2 generation, all lines segregated approximately 20% *cop1-5*-like mutant seedlings (Table 1) indicating that the transgenes do not rescue the mutant phenotype. This ratio is slightly lower than 25% due to the low germination rate of the severe *cop1* mutant seeds but is significantly different than the 6.3% expected if the protein was able to complement the mutation. Western blot analysis of the mutants in those F2 progeny confirmed that the COP1 deletion proteins were present (data not shown). The similar phenotypes of mutants with or without the transgenes demonstrated that neither of the mutated proteins are functional (Figure 1a,b).

Since COP1 can form a dimer *in vitro*, we investigated the possibility of intermolecular complementation by two different mutant molecules of COP1 within a dimer. The *cop1-8* mutation and the ΔRING transgene were selected because their mutations were in separate domains. The *cop1-8* mutation disrupts the WD40 repeat domain (McNellis *et al.*, 1994b) whereas in the ΔRING protein it is intact. They both contain the coiled-coil motif, which has been shown to be the dimerization domain (McNellis *et al.*, 1996). As shown in Table 1, the ΔRING protein was unable to complement the *cop1-8* mutant phenotype. Hence the dimerization of two individual COP1 proteins defective in separate domains appears not to be able to reconstitute a functional COP1 activity.

Table 1. F2 segregation of ΔRING and ΔCoil overexpressing transgenes crossed to *cop1-5* or *cop1-8* mutant plants

Cross	Wild-type	Mutant	WT:Mutant ratio
ΔRING × *cop1-5*	152	33	4.6 : 1
ΔRING × *cop1-8*	134	31	4.3 : 1
ΔCoil × *cop1-5*	53	18	2.9 : 1

The F2 segregating seedlings were grown in cycling light for 6 days and scored for phenotype. Mutants were identified by their purple (*fusca*) coloration and diminutive status.

One explanation for the inability of the deletion mutant proteins to rescue these mutant phenotypes could be their improper folding or dimerization. However, in wild-type backgrounds, overexpression of the ΔRING protein or a fusion protein between GUS and the ΔCoil protein results in elongated hypocotyls when grown in the light (Torii *et al.*, 1998). This is similar to the phenotype observed when the full-length COP1 protein is overexpressed (McNellis *et al.*, 1994a). This suggests that the deletion of the specific domains does not affect folding of the remaining part and these mutant proteins are able to dampen the light response to the same extent as the full-length protein. Therefore, it is more likely that the RING finger and coiled-coil domains are essential for the function of COP1.

High levels of the N282 protein result in a cop1 null phenotype in the absence of wild-type protein

The weak *cop1-4* mutation creates a stop codon at amino acid 283 in the *COP1* gene and results in a low level of a truncated protein (N282) (McNellis *et al.*, 1994b). At least two contrasting scenarios can explain the weak phenotype of the *cop1-4* mutants. One possibility is that a low level of COP1 full-length protein is produced due to the translational read-through of the stop codon in the mRNA. Alternatively, the N282 protein could retain partial function and is responsible for the phenotype observed. To differentiate between these possibilities, a transgene overexpressing only the N282 protein without the C-terminal coding sequence after the stop codon (line L2, see McNellis *et al.*, 1994b) was crossed into the *cop1-5* and *cop1-8* mutant backgrounds. If the N282 protein is responsible for the weak *cop1-4* phenotype, overexpressing N282 in a null mutant background should recreate this or even a weaker phenotype. Surprisingly, the progeny of plants heterozygous for the N282 transgene and the null *cop1* mutation segregated with about 25% of the seedlings with a null mutant phenotype (Table 2; Figures 1c and 2a). Western analyses of those mutant seedlings revealed that N282 was indeed highly overexpressed (data not shown).

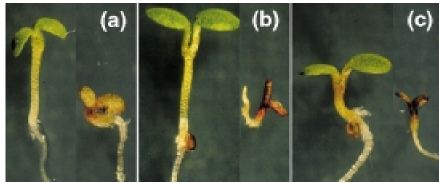

Figure 1. Seedling phenotype of the deletion mutant proteins in wild-type and *cop1* null mutant background.
The 6-day-old continuous light-grown seedlings overexpressing (a) ΔRING, (b) ΔCoil and (c) N282 proteins. In each panel, two representative seedlings carrying the overexpressing mutant transgene in the wild-type (left) and the *cop1-5* mutant background (right) are shown. All pictures are in the same scale.

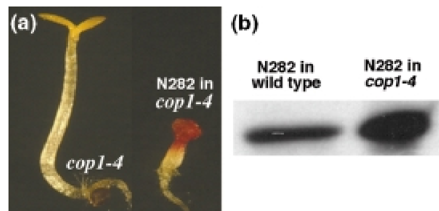

Figure 2. Overexpression of N282 in the *cop1-4* background mimics a null *cop1* mutant phenotype.
(a) Representative *cop1-4* mutant seedlings without (left) or with (right) the N282 transgene. The seedlings were grown in complete darkness for 3 days. Both seedlings are pictured at the same scale.
(b) Western blot analysis of N282 accumulation in the wild-type and *cop1-4* mutant seedlings carrying the same transgene. Approximately 15 μg total protein of seedling extract was loaded onto a 12% SDS polyacrylamide gel.

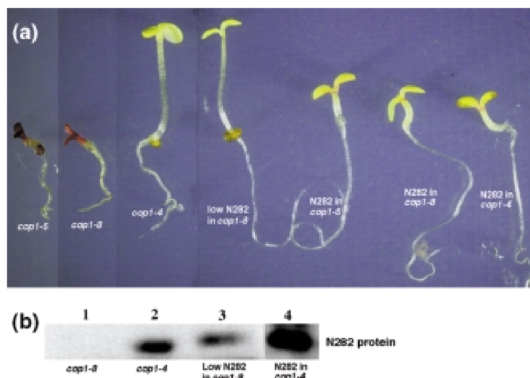

Figure 3. N282 protein acts in a strictly concentration-dependent manner.
(a) Phenotype comparison of 6-day-old dark-grown *cop1* mutant seedlings with or without N282 protein. From left: *cop1-5*, *cop1-8*, *cop1-4*, *cop1-8* expressing a low level of N282, *cop1-5* expressing a high level of N282, *cop1-8* expressing a high level of N282, and *cop1-4* expressing a high level of N282. All seedling were photographed at the same magnification. The slightly healthier seedlings seen in the *cop1* mutants expressing high levels of N282 was most likely due to hybrid vigor since they resulted from crosses of two different ecotypes.
(b) The Western analysis of N282 protein accumulation in representative mutant lines shown in (a). Lanes 1, *cop1-8*; lane 2, *cop1-4*; lane 3, *cop1-8* mutant carrying the transgene with reduced expression level of N282, and lane 4, *cop1-4* with the N282 overexpressing transgene. Approximately 30 (lanes 1–3) or 15 (lanes 4) μg total protein were analyzed in a 12% SDS polyacrylamide gel. A longer exposure time was used to visualize the N282 protein band for lanes 1–3.

To establish whether the high level of N282 is responsible for the *cop1* null phenotype observed and to rule out further any read-through of the stop codon in *cop1-4* mutant mRNA, the N282 transgene was introduced into homozygous *cop1-4* mutants by a genetic cross to increase the level of N282 protein in those mutants. The progeny of plants heterozygous for the *cop1-4* mutation and the N282 transgene segregated the wild-type seedlings, N282 overexpressing seedlings in a wild-type background (see Figure 1c, left) and *cop1-4* seedlings. However, among the 25% mutant seedlings that were presumably homozygous for the *cop1-4* mutation, there were clearly two populations. A smaller fraction of the mutants were strictly *cop1-4*-like mutants, whereas a large number had the *cop1* null phenotype (Table 2). Protein level analysis showed that the null mutants have a high level of N282 protein (Figure 2b). It is not clear as to why the null mutants exhibited a slightly higher than the parental N282 overexpressing line used in the cross (Figure 2b), although the mutants are expected to have greatly reduced photosynthetic proteins in the total protein extract. Two related conclusions can be drawn from these results. First, the high level of N282 in the absence of wild-type COP1 is responsible for the null mutant phenotype. Second, translational read-through of the *cop1-4* mutant mRNA does not account for its phenotype. If it did, the seedlings that were homozygous for the *cop1-4* mutation and contained the N282 transgene would still have a *cop1-4* phenotype because of the low abundance of full-length COP1 protein.

Table 2. F2 segregation of N282 overexpressing plants crossed to *cop1-5, cop1-8* and *cop1-4* mutant plants

Cross	Wild-type	N282	cop1-4	Null mutants (fusca)
N282 × *cop1-5*	11	174	0	30
N282 × *cop1-8*	6	77	0	20
N282 × *cop1-4*	12	83	13	20

The F2 segregating seedlings were grown in cycling light for 6 days and scored for phenotype. The null mutants (*fusca*) mutants were identified by their purple coloration and diminutive status, while N282 and *cop1-4* mutant phenotype were scored as described previously (Ang and Deng, 1994; McNellis *et al.*, 1996). In brief, the *cop1-4* mutants have extreme short hypocotyl and smaller cotyledon under light (Ang and Deng, 1994) and photomorphogenic development in darkness (Figure 2a, left). The N282 overexpression phenotype in wild-type background has short hypocotyl and normal size cotyledons in the light (Figure 1c, left), but open cotyledons and normal long hypocotyl in the darkness (McNellis *et al.*, 1996). In addition, the adult *cop1-4* plants are severely dwarf-like while the N282 overexpressing wild-type plants are normal in status.

The level of N282 protein is critical for its functionality in the absence of wild-type protein

In the process of examining N282 and *cop1-8* crosses, occasionally some lines lost the dominant negative phenotype associated with the overexpression of N282 in the wild-type background (McNellis *et al.*, 1996). This implied a loss of the ability to overexpress the N282 transgene. However, if the transgene was still expressed at low levels, those lines could be used to recreate the *cop1-4* mutant phenotype if introduced into a *cop1* null mutant background. Hence, we carefully examined the phenotype of the mutant progeny. In one such population, presumably from a parent plant that was homozygous for the N282 transgene and heterozygous for the *cop1-8* mutation, 25% of the seedlings exhibited phenotypes very similar to the *cop1-4* mutant (Figure 3a) and no *cop1-8* mutants were found. Western blot analysis of these mutants verified that they had levels of N282 protein equivalent to the *cop1-4* mutant seedlings (Figure 3b). Again, in all lines where the N282 was still overexpressed, *cop1-8* mutants with a high level of N282 had a null mutant phenotype (Figure 3). These recreated *cop1-4*-like seedlings further substantiate that the low level of N282 is responsible for the weak phenotype observed in the *cop1-4* mutants.

Discussion

The studies reported here confirm an interesting feature of COP1 activity. In previous studies with the *cop1* mutant alleles, it was revealed that minor mutations, such as small deletions or even a point mutation, in the C-terminal WD40 repeat region result in a severe phenotype similar to the *cop1* null mutants (McNellis *et al.*, 1994b). These seedlings have a severely retarded seedling development and die after the seedling stage. However, truncation of the protein by a stop codon at amino acid 283, which presumably deletes the entire WD40 repeat domain, results in a weak phenotype (*cop1-4*). Seedlings carrying the *cop1-4* mutation can complete the life cycle similar to wild-type plants. The weak phenotype observed in the *cop1-4* mutants is intriguing and implies that when the C-half of the COP1 was deleted, the N-terminal 282 amino acids of COP1 maintain some residual functionality which is sufficient to allow the completion of the plant life cycle.

By using a transgene which can reproduce this N282 fragment of COP1 without the possibility of translational read-through and thereby recreating a *cop1-4* like phenotype, we can rule out the possibility that the weak phenotype was due to a low degree of translational read-through of the *cop1-4* mutant mRNA. Thus, a low level of the N282 protein maintains significant residual functionality and is capable of rescuing the lethality of the null mutations. It has been shown that the WD40 repeat domain of COP1 is responsible for directly interacting with at least one downstream target which promotes the expression of light inducible genes (Ang *et al.*, 1998). It further substantiates the notion that the function of COP1 is modular in that the N282 fragment carries the functionality for viability, while a mutated or misfolded WD40 repeat region can disrupt this domain specific function. Separation of functions among individual modules of COP1 is possibly achieved by their abilities to interact with distinct functional targets. Indeed, it has been demonstrated recently that a transcriptional regulator (CIP7) interacts with the coiled-coil domain of COP1 while another protein specifically interacts with the RING-finger motif (Torii *et al.*, 1999; Yamamoto *et al.*, 1998).

It is intriguing that the N282 activity is critically dependent on its concentration. At low concentrations, in a level similar to that of the homozygous *cop1-4* mutants, its activity was evident. The mutant plants were viable in both light- and dark-growth conditions and can complete the life cycle, although the stature of light-grown mutant plants is smaller than the wild type. At high concentrations, N282 cannot rescue the lethality of a *cop1* null mutant. This is in contrast to the notion that the dominant negative phenotype caused by N282 overexpression in a wild-type background is exclusively due to its ability to interact with and negatively affect the wild-type COP1 protein (McNellis *et al.*, 1996; Torii *et al.*, 1998). It suggests that the phenotype observed may be a result of the overexpression of N282 itself. In this case, N282 could interact with essential cellular components that are limiting in nature. Therefore, it functions properly only at normal concentrations while excess N282 may simply titrate out some key cellular components, leading to gross malfunctions and lethality. It will be of great interest to determine the nature of those limiting factors in future research.

Experimental procedures

Arabidopsis materials and genetic crosses

The three *cop1* mutant alleles, *cop1-4*, *cop1-8* and *cop1-5* have been described previously (McNellis *et al.*, 1994b). The three COP1 deletion transgenic lines used for crosses were driven by the 35S promoter and reported previously (Torii *et al.*, 1998). The heterozygous *cop1-5* and *cop1-8* mutant plants were used as males for most of the crosses and the successful crosses were assessed by the presence of *fusca* seeds in the F2 progeny. F2 seedlings were sown on growth medium containing 1% sucrose, vernalized for 7 days and scored for phenotype at 6 days with a 16 h light/8 h dark photoperiod or in complete darkness as noted in the text.

Western blot analyses

Western blot analyses were performed on seedlings grown in continuous light or dark for 6 days. Seedlings were harvested, frozen with liquid nitrogen and ground in a mortar and pestle.

Grinding buffer (400 mM sucrose, 50 mM Tris pH 7.5, 10% glycerol, 2.5 mM EDTA, 1 mM PMSF) was added and the extract was centrifuged for 10 min. An aliquot was removed for protein concentration analysis and an equal volume of 4× SDS sample buffer was added. The samples were boiled for 10 min and centrifuged for 10 min before loading onto the gel. Approximately 15 μg of total protein, unless otherwise specified, was separated by SDS-PAGE and transferred to nitrocellulose membranes. The protein concentration was estimated by the Bradford assay (McNellis *et al*., 1994a). The membranes were blocked with 1% block solution (Boehringer-Mannheim) before incubation with antibodies. The blots were washed with TBS (20 mM Tris pH 7.5, 150 mM NaCl) with 0.1% Tween-20. The proteins were detected using 1 : 300 dilution of the α-COP1 antibody and horse radish peroxidase linked secondary antibody (Boehringer-Mannheim) with ECL reagents (Amersham).

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