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Genetic and Molecular Analysis of an Allelic Series of *cop*1 Mutants Suggests Functional Roles for the Multiple Protein Domains

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The Arabidopsis protein COP1, encoded by the *CONSTITUTIVE PHOTOMORPHOGENIC* locus 1, is an essential regulatory molecule that plays a role in the repression of photomorphogenic development in darkness and in the ability of light-grown plants to respond to photoperiod, end-of-day far-red treatment, and ratio of red/far-red light. The COP1 protein contains three recognizable structural domains: starting from the N terminus, they are the zinc binding motif, the putative coiled-coil region, and the domain with multiple WD-40 repeats homologous to the β subunit of trimeric G-proteins (G_β). To understand the functional implications of these structural motifs, 17 recessive mutations of the *COP*1 gene have been isolated based on their constitutive photomorphogenic seedling development in darkness. These mutations define three phenotypic classes: weak, strong, and lethal. The mutations that fall into the lethal class are possible null mutations of *COP*1. Molecular analysis of the nine mutant alleles that accumulated mutated forms of COP1 protein revealed that disruption of the G_β-protein homology domain or removal of the very C-terminal 56 amino acids are both deleterious to COP1 function. In-frame deletions or insertions of short amino acid stretches between the putative coiled-coil and G_β-protein homology domains strongly compromised COP1 function. However, a mutation resulting in a COP1 protein with only the N-terminal 282 amino acids, including both the zinc binding and the coiled-coil domains, produced a weak phenotypic defect. These results indicated that the N-terminal half of COP1 alone retains some activity and a disrupted C-terminal domain masks this remaining activity.

INTRODUCTION

Ambient light conditions play a critical role in determining the patterns of higher plant development (Mohr and Shropshire, 1983; Kendrick and Kronenberg, 1986). The most dramatic developmental changes in response to light can be seen at the seedling stage. When grown under normal light conditions, Arabidopsis plants undergo photomorphogenic development. These light-grown plants characteristically have short hypocotyls, open, expanded cotyledons and leaves, and photosynthetically active chloroplasts, and they express light-regulated genes, such as the chlorophyll *a/b* binding protein gene and the gene for the small subunit of ribulose bisphosphate carboxylase/oxygenase, at high levels (Gilmartin et al., 1990; Thompson and White, 1991). In the dark, however, Arabidopsis plants exhibit a very different developmental pattern known as skotomorphogenesis. These etiolated plants have long hypocotyls, closed, unexpanded cotyledons, and apical hooks. Their light-inducible genes are expressed at very low levels, and their plastids develop into etioplasts that possess no chlorophyll and are not photosynthetically competent (Kirk and Tilney-Bassett, 1978). Dark-grown plants are able to initiate photomorphogenesis as soon as they receive light stimuli (Mohr and Shropshire, 1983; Kendrick and Kronenberg, 1986), and this process is extremely rapid. Changes in gene expression occur within minutes of the perception of light signals (Kaufman et al., 1986), and plastids visibly begin chloroplast development within an hour of light perception (Kirk and Tilney-Bassett, 1978).

Plants also respond to light quality and quantity in more subtle ways throughout their lives. Two well-characterized plant responses, the shade avoidance and end-of-day far-red responses, illustrate the ability of the plant to respond to light quality (Kendrick and Kronenberg, 1986; Smith and Whitelam, 1990). The shade avoidance response is the capability of the plant to accelerate stem elongation in response to a low ratio of red to far-red light, a condition commonly encountered under a canopy. The end-of-day far-red response is the capability of the plant to respond to changing light quality during the day.

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For example, Arabidopsis seedlings will respond to a far-red pulse at the end of the photoperiod with rapid hypocotyl elongation. Photoperiodic induction of flowering is an example of a plant response to light duration: many plants are able to sense the day/night length and use this information to control the switch between vegetative and reproductive growth phases (Kendrick and Kronenberg, 1986).

Plants employ an array of photoreceptors to assess environmental light conditions. These receptors include the phytochromes, which absorb mainly red and far-red light (Quail, 1991; Furuya, 1993), the cryptochromes, which absorb blue and UV-A light (Ahmad and Cashmore, 1993), and the UV-B receptors (Mohr, 1986). For some responses, such as light control of seedling morphogenesis, the concerted action of multiple photoreceptors is necessary, whereas for some other responses, only one individual photoreceptor or class of photoreceptors may be involved. For example, the shade avoidance and end-of-day far-red responses are primarily mediated by phytochrome B (Furuya, 1993). Biochemical studies have implicated the involvement of trimeric G-protein(s) and calcium/calmodulin in phytochrome signaling processes controlling gene expression (Neuhaus et al., 1993; Romero and Lam, 1993), and trimeric G-proteins may also be involved in blue-light regulation of gene expression (Warpeha et al., 1991).

Mutant analysis has proven to be a powerful approach to dissect the light signaling mechanisms controlling plant development (Adamse et al., 1988; Chory, 1993; Deng, 1994). Nine Arabidopsis loci have been reported; when mutated, they cause light-grown development in darkness. These are *DEETIOLATED* 1 (*DET*1; Chory et al.. 1989), *DET*2 (Chory et al., 1991), *DET*3 (Cabrera y Poch et al., 1993), *CONSTITUTIVE PHOTOMORPHOGENIC* 1 (*COP*1; Deng et al., 1991), *COP*2, *COP*3, and *COP*4 (Hou et al., 1993), and *COP*9 (Wei and Deng, 1992). Among these mutants, *cop*1, *cop*9, and *det*1 exhibit the most pleiotropic phenotypes during dark-grown seedling development. Previous studies indicate that the *COP*1 gene product plays an important role in the light signal transduction system and that signals from all three classes of photoreceptors converge to regulate COP1 activity (Deng and Quail, 1992). It has been postulated that the COP1 protein acts to repress photomorphogenesis in the dark because mutations at this locus are all strictly recessive and cause constitutive photomorphogenesis (Deng and Quail, 1992).

The *COP*1 locus has recently been cloned (Deng et al., 1992), and the gene encodes a 76.2-kD protein with a zinc finger domain at the N terminus (von Arnim and Deng, 1993), a putative coiled-coil region, and a domain with homology to the β subunit of trimeric G-proteins at the C terminus. The G_β-protein homology domain consists of multiple WD-40 repeats, which are thought to be involved in protein–protein interactions in a number of regulatory proteins (van der Voorn and Ploegh, 1992). Interestingly, COP1 shares homology with $TAF_{II}80$, a subunit of the Drosophila TFIID complex that is required for polymerase II transcription initiation. The homology does not extend to the Zn binding domain of COP1, because $TAF_{II}80$ does not contain such a motif (Dynlacht et al., 1993). Although COP1 is unlikely to be a functional analog of $TAF_{II}80$ in plants, it may have the potential to interact with transcriptional machinery.

A better understanding of the mode of action of COP1 will require detailed analysis of the structural and functional domains of this novel regulatory molecule. Without a practical gene replacement method, we have undertaken an in vivo mutagenesis approach to dissect the structure and function of COP1. This approach consists of three formal steps. The first is the isolation of mutations in the desired gene after random in vivo mutagenesis by phenotypic screens and complementation tests. The availability of a simple genetic screen and the short generation time of Arabidopsis make it possible to obtain a large number of alleles in a reasonable time frame. The second step is to identify mutant alleles that alter the structure of the gene product but accumulate the mutated proteins. Finally, the structural alterations in selected mutant alleles are determined by direct sequence analysis. This mutagenesis approach has distinct advantages over an alternative approach, such as mutagenesis in vitro and analysis of function after reintroduction into the plant genome. First, only mutations compromising COP1 activity will be selected, thereby avoiding the numerous neutral mutations in COP1 that do not alter activity. The second advantage is that the expression patterns of the mutated COP1 forms should closely resemble that of the wild-type gene, because their expression is still under the control of the same regulatory elements. This avoids possible complications, such as ectopic expression, effects of genomic environment of the introduced mutated genes, and epigenetic interactions with the endogenous gene that may be associated with the reverse genetic approach. In this report, a total of 17 *cop*1 mutations were selected by a phenotypic screen after in vivo random mutagenesis. Detailed characterization of the effects of these mutations on light-regulated development and on the COP1 protein itself provides insight into the functional implications of the structural motifs of COP1.

RESULTS

*cop*1 Mutants Define Three Phenotypic Classes

After the identification of four *cop*1 mutant alleles in two phenotypic classes (Deng and Quail, 1992), a new phenotypic class of recessive *cop*1 mutant alleles has been isolated. The new class initially included the T-DNA–tagged allele *cop*1-5 (Deng et al., 1992) and a new ethyl methanesulfonate–induced allele *cop*1-7. Compared with the previously reported *cop*1 mutants belonging to the strong and weak phenotypic classes (Deng and Quail, 1992), the new class of *cop*1 mutants possesses dark purple seed color in addition to constitutive photomorphogenic development in darkness. Because the dark purple seed color was the basis for the *fusca* mutant screen (Muller, 1963; Miséra, 1993; Castle and Meinke, 1994), complementation tests

between *cop*1 and available *fusca* mutations were performed. Genetic complementation tests between the *fusca*1 mutations and the *cop*1 mutations clearly indicated that they are allelic (X.-W. Deng, unpublished data), and, therefore, the 10 available *fusca*1 alleles (Miséra, 1993) were renamed *cop*1-8 to *cop*1-17 in this report. Table 1 summarizes all *cop*1 mutant alleles in the three phenotypic classes, and examples from each class are shown in Figure 1. All *cop*1 mutant alleles in the new phenotypic class share the following features. Both dark- and light-grown homozygous mutant seedlings are severely retarded in their development, with significantly reduced cell elongation in their hypocotyls and reduced cell enlargement in their cotyledons (Figures 1B and 1F). Both mature seeds (Figures 1I and 1J) and seedlings (Figures 1B and 1F) homozygous for the mutations exhibit dark purple coloration in their hypocotyls and cotyledons due to high levels of anthocyanin accumulation. Most important, homozygous mutants are adult lethal. Occasionally, some mutant individuals will develop up to three pairs of tiny true leaves (Figure 1K) before senescence. Not a single mutant plant has been able to survive to reproduce. The severity of this class of mutations suggests that they may represent *cop*1 null mutations, and we grouped them as the lethal class of *cop*1 mutations. The lethality caused by these recessive *cop*1 mutations indicated that *COP*1 is an essential gene for Arabidopsis.

In contrast, mutations in both the strong (*cop*1-1, *cop*1-2, and *cop*1-3) and the weak (*cop*1-4 and *cop*1-6) classes lead to progressively milder phenotypes and are not adult lethal (Deng and Quail, 1992; Figures 1C, 1D, 1G, and 1H), although mutations in both classes, particularly in the strong class, cause severe size reduction of light-grown mature plants and greatly reduce seed set (Deng and Quail, 1992). Dark-grown seedlings homozygous for both strong and weak *cop*1 mutations exhibit light-grown characteristics, including patterns of cell differentiation and gene expression (Deng et al., 1991; Deng and Quail, 1992). However, dark-grown seedlings homozygous for weak *cop*1 mutations have longer hypocotyls than their light-grown siblings (Figures 1D and 1H) and light-grown wild-type seedlings (Figure 1E), although they are clearly shorter than etiolated wild-type seedlings (Figure 1A). On the other hand, both dark- and light-grown strong *cop*1 mutant seedlings have short hypocotyls of similar length (Figures 1C and 1G). This indicated that the mutants of the weak class are still capable of reacting to light to a certain degree. Based on the above observations and the fact that the phenotypes of *trans* heterozygotes for representative *cop*1 mutation pairs of different classes more closely resemble those of parental mutants homozygous for the weaker *cop*1 mutations (Deng and Quail, 1992; data not shown), we concluded that the order of severity of the three classes of *cop*1 mutations are the lethal class, the strong class, and the weak class.

Table 1. Summary of *cop*1 Mutant Alleles and Their Effects on the COP1 Protein

Designation	Allele[a]	Size (kD)	Quantity[b]
Wild type		76	+ +
Weak	*cop*1-4	33	+
	*cop*1-6	76	+ +
Strong	*cop*1-1	74	+ +
	*cop*1-2	74	+ +
	*cop*1-3	76	+
Lethal	*cop*1-5	—	—
	*cop*1-7	—	—
	*cop*1-8 (R348-26)	66	+ +
	*cop*1-9 (Tu3-16)	76	+ + +
	*cop*1-10 (G280-15)	66	+ +
	*cop*1-11 (U17-35)	70	+ + +
	*cop*1-12 (G26-7.8)	—	—
	*cop*1-13 (U88-24)	—	—
	*cop*1-14 (U146-19)	—	—
	*cop*1-15 (U36-14)	—	—
	*cop*1-16 (T251-11)	nd[c]	nd
	*cop*1-17 (G247-5)	nd	nd

[a] For *cop*1 alleles originally identified as *fus*1 mutants, the previous allele assignments are indicated within parentheses.
[b] Symbols: + +, wild-type protein level; +, less than the wild-type protein level; + + +, more than the wild-type protein level; —, none detected.
[c] nd, not done.

*cop*1-6 Mutation Leads to Reproductive Growth and Flowering in Complete Darkness

*cop*1-6, a newly identified weak mutation, exhibits two interesting differences from the other weak allele, *cop*1-4. First, a significant portion of the plastids from the dark-grown *cop*1-6 mutants still have prolamellar bodies, as shown in Figures 2B to 2D. These structures are characteristic of etioplasts and are absent from dark-grown *cop*1-4 mutant plastids (Deng and Quail, 1992). The prolamellar bodies observed in *cop*1-6 are relatively small compared with those found in the etioplasts of wild-type plants (Figure 2A). Second, *cop*1-6 plants grown in total darkness were able to initiate reproductive development, including bolting and flowering, after growing for 3 weeks and producing four to six rosette leaves (Figure 1L), whereas dark-grown *cop*1-1 and *cop*1-4 mutants were only able to develop small flower buds but never bolted (T.W. McNellis and X.-W. Deng, data not shown). True leaf formation and flowering have never been observed for wild-type plants, even after extended growth in darkness (up to 70 days) under the same conditions. A recent report has shown that wild-type Arabidopsis plants grown in liquid culture under continuous shaking can also flower in darkness (Araki and Komeda, 1993), suggesting that the *cop*1-6 mutation somehow circumvents the need for continuous shaking to flower in darkness.

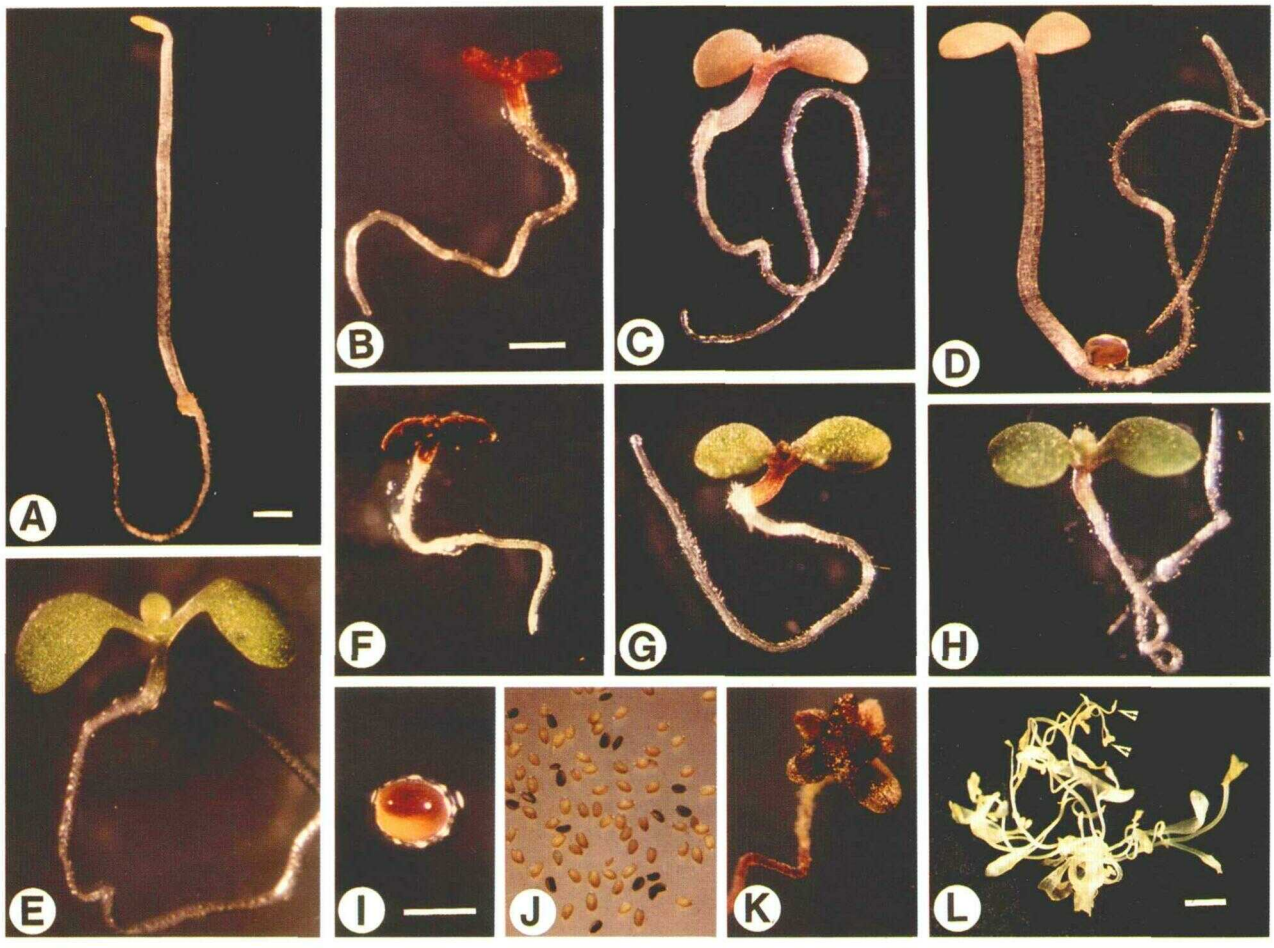

Figure 1. Morphological Comparison of Representative Alleles from Three Classes of *cop*1 Mutants.

(A) and **(E)** Dark- and light-grown wild-type seedlings.
(B) and **(F)** Dark- and light-grown mutant seedlings homozygous for a lethal mutation, *cop*1-8.
(C) and **(G)** Dark- and light-grown mutant seedlings homozygous for a strong mutation, *cop*1-1.
(D) and **(H)** Dark- and light-grown mutant seedlings homozygous for a weak mutation, *cop*1-6.
(I) Imbibed homozygous *cop*1-8 mutant seed.
(J) Segregating seed population from a heterozygous *cop*1-8 parent plant.
(K) A 3-week-old light-grown *cop*1-8 plant.
(L) A 1-month-old dark-grown *cop*1-6 plant that is flowering. Arrows indicate floral structures.
(A) and **(J)** are at the same magnification; the bar in **(A)** = 1 mm. **(B)** through **(H)** and **(K)** are of the same magnification; the bar in **(B)** = 1 mm. The bar in **(I)** = 1 mm; the bar in **(L)** = 1 cm.

*cop*1 Mutations Render Plant Flowering Insensitive to Daylength

The ability of *cop*1-6 mutants to flower in complete darkness prompted us to examine whether the *COP*1 gene is involved in the photoperiodic induction of flowering. For many plant species, the initiation of reproductive development, *i.e.*, floral induction, is greatly influenced by relative day and night length (Kendricks and Kronenberg, 1986). Arabidopsis is a long-day plant, producing fewer rosette leaves and flowering earlier under long-day/short-night conditions than siblings grown under short-day/long-night conditions. Therefore, the number of rosette leaves and days to flowering for wild-type and representative nonlethal *cop*1 mutants grown under continuous light (24 hr per day), long-day (16-hr light/8-hr dark), and short-day (8-hr light/16-hr dark) conditions were examined. Table 2 summarizes these results. Under long-day or continuous light conditions, wild-type plants and all three mutants flowered after producing approximately seven rosette leaves and growing for 3 to 4 weeks. However, under short-day conditions, wild-type plants produced more than 40 rosette leaves and grew for ~75 days before flowering, whereas the three mutant lines

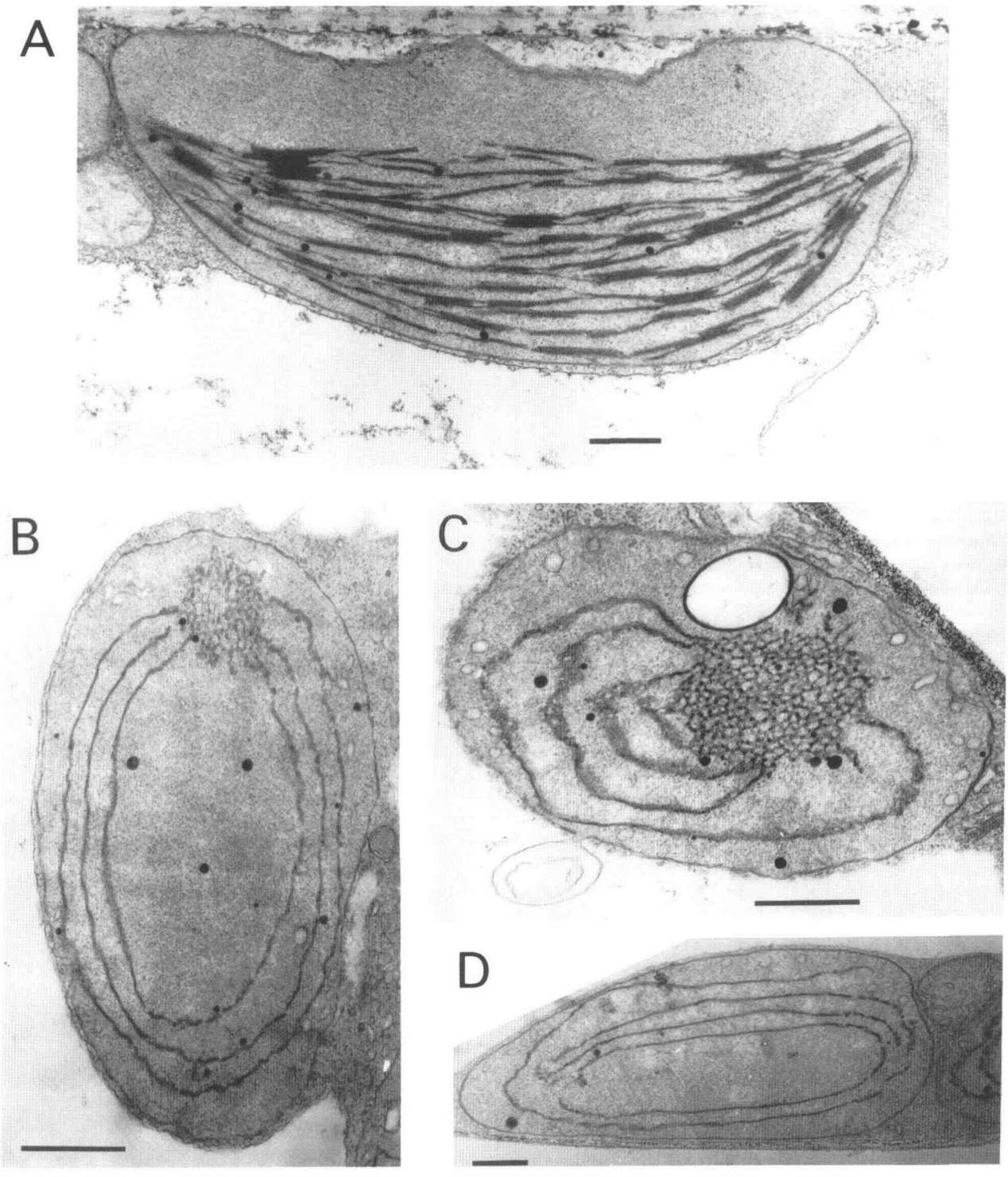

Figure 2. Plastids of Light- and Dark-Grown *cop*1-6 Seedlings.

(A) Representative chloroplast from a 15-day-old light-grown *cop*1-6 seedling.
(B) to **(D)** Representative plastids of 15-day-old dark-grown *cop*1-6 seedlings.
Bars = 500 nm.

Table 2. Effects of *cop*1 Mutations on the Photoperiodic Induction of Flowering[a]

	Continuous Light		Long Day[d] (16-hr L/ 8-hr D)		Short Day (8-hr L/ 16-hr D)	
Line	RL[b]	DTF[c]	RL	DTF	RL	DTF
wt (Col-0)[e]	7.7	20	7.5	25.1	44	74.5
	(0.3)	(0.7)	(0.2)	(0.5)	(1.7)	(2.5)
*cop*1-1	6.7	26.6	7.2	28.1	7.0	34
	(1.0)	(1.8)	(0.2)	(0.8)	(0.3)	(1.7)
*cop*1-4	7.7	25	7.1	26.7	7.1	37
	(0.9)	(0.7)	(0.2)	(1.4)	(0.2)	(1.1)
*cop*1-6	7.0	25.5	6.8	28.3	7.2	38
	(0.2)	(1.1)	(0.2)	(1.4)	(0.4)	(2.2)

[a] Standard deviations are given within parentheses.
[b] RL, rosette leaves.
[c] DTF, days to flowering.
[d] L, light; D, dark.
[e] Col-0, Columbia ecotype.

continued to produce only seven rosette leaves and flower earlier than the wild-type plants. The increase in the number of days to flowering seen in the *cop*1 mutants grown under short-day conditions may simply be due to slower growth, because the plants have less time to perform photosynthesis. Therefore, the number of rosette leaves that a plant develops before bolting is considered to be a better indicator of flowering timing than the number of days to flowering. These results show that the mutant plants examined flowered as if they were growing under long-day conditions regardless of the actual daylength, indicating that COP1 is involved in plant responses to photoperiod signals.

*cop*1 Mutants Are Impaired in Their Ability to Respond to Light Quality Signals

We have examined the effects of the *cop*1 mutations on the plant's ability to respond to light quality signals by using the shade avoidance and end-of-day far-red responses as examples. Under a canopy, the low ratio of red to far-red light induces hypocotyl or stem elongation in many plant species, thus helping the plants to grow out from under the canopy and into direct sunlight. This is known as the shade avoidance response, which is mediated primarily by phytochrome B (Furuya, 1993). Figure 3A shows hypocotyl lengths of wild-type and *cop*1 mutants homozygous for three representative nonlethal alleles grown in white light and white light supplemented with far-red light to mimic a canopy environment. None of the *cop*1 mutant alleles showed additional hypocotyl elongation under far-red light–enriched conditions, whereas the wild-type seedlings displayed more than a twofold increase in hypocotyl elongation. This indicates that mutations in COP1 impair the shade avoidance response.

As shown in Figure 3B, wild-type Arabidopsis plants grown for 4 days in a 10-hr light/14-hr dark photoperiod with a 15-min pulse of far-red light at the end of each day developed much longer hypocotyls than siblings grown under the same conditions but without the short end-of-day far-red light pulse. This is the end-of-day far-red response. All three *cop*1 mutant alleles examined showed no response to this end-of-day far-red treatment, indicating that the *cop*1 mutations eliminate the ability of plants to undergo the end-of-day far-red response. Clearly, COP1 is involved in plant responses to light quality signals.

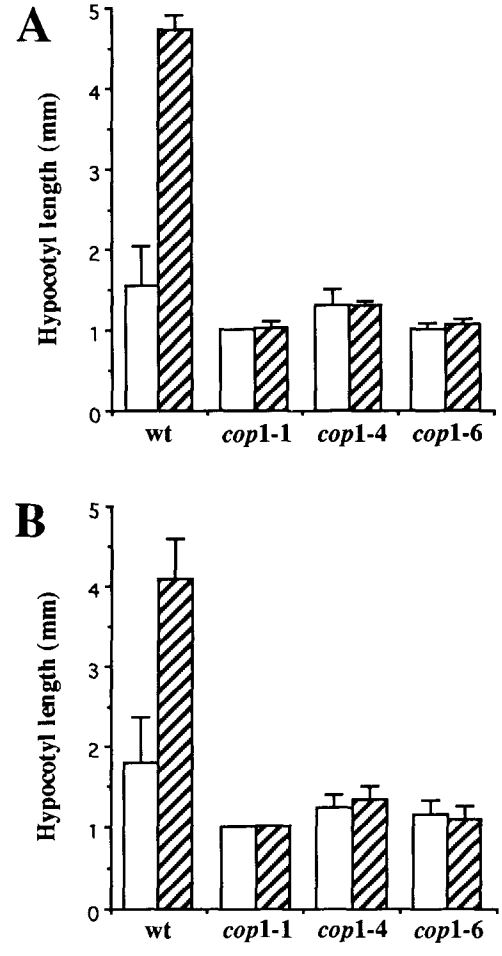

Figure 3. The Shade Avoidance and End-of-Day Far-Red Responses in *cop*1 Mutants.

(A) Shade avoidance response. Hypocotyl lengths of wild-type (wt) and *cop*1-1, *cop*1-4, and *cop*1-6 mutant seedlings grown in white light (open blocks) or white light supplemented with far-red light (hatched blocks) for 4 days under continuous illumination are shown.
(B) End-of-day far-red response. Shown are the hypocotyl lengths of wild-type (wt) and *cop*1-1, *cop*1-4, and *cop*1-6 mutant seedlings grown in a 10-hr light/14-hr dark cycle for 4 days without (open blocks) or with (hatched blocks) a 15-min end-of-day far-red treatment.

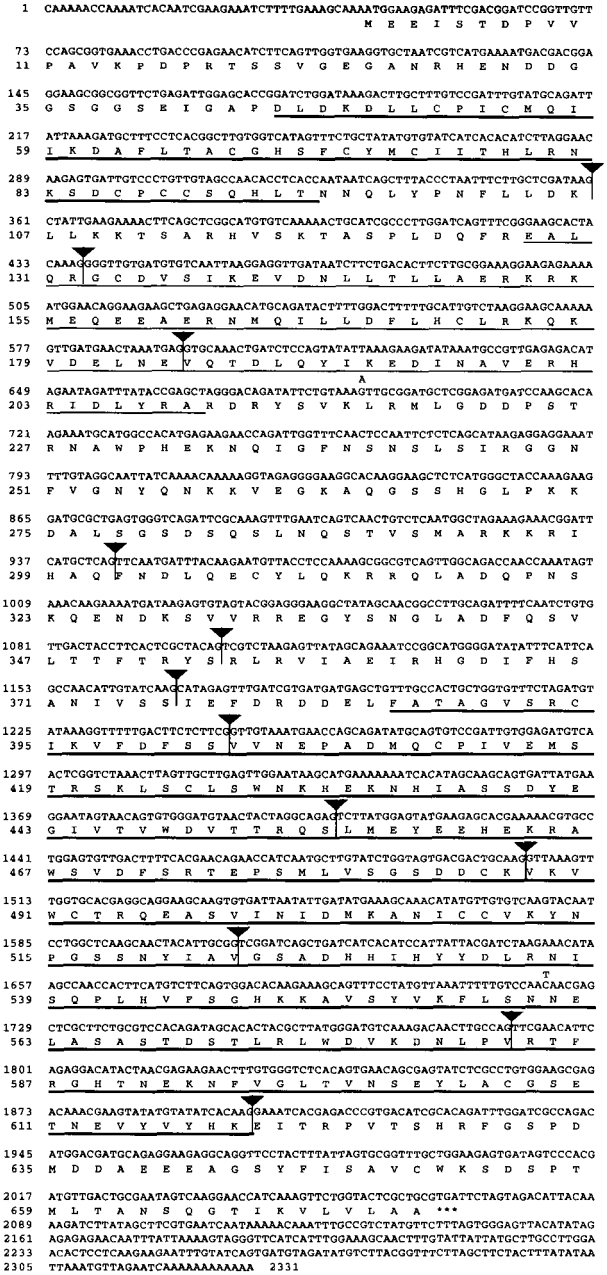

Figure 4. The Sequence and Structural Features of the *COP*1 Gene and Its Encoded Protein.

Shown are the cDNA sequences of the Arabidopsis *COP*1 gene from both Columbia and Landsberg *erecta* ecotypes and the predicted protein sequence. The two ecotypes have identical sequences except at two nucleotide positions (691 and 1722). The different bases found in Landsberg *erecta* are shown above the full-length Columbia sequence (GenBank accession number L24437). The locations of the 12 introns predicted by comparison of cDNA and genomic DNA sequences are marked by triangles. The intron lengths range from 94 bp (intron 5) to 570 bp (intron 4). The three underlined sequences represent the three recognizable structural domains of COP1. Starting from the N terminus, these domains are the Zn binding, the coiled-coil, and the G_β-protein homology domains.

Summary of Structural Features of the *COP*1 Gene and Its Encoded Protein

Because the *cop*1 mutant alleles examined in this study were isolated from either the Columbia or Landsberg *erecta* ecotypes, the complete *COP*1 cDNA sequence from the wild-type Landsberg *erecta* ecotype of Arabidopsis was determined and compared with that of the Columbia ecotype, as shown in Figure 4. Only two single base substitutions at third base positions of the codons 216 and 560 were observed, and neither change altered the encoded amino acids. A comparison of cDNA sequences with the corresponding genomic sequences revealed the presence of 12 introns, ranging from 94 bases to 570 bp, all of which are located within the protein coding region of the transcribed sequence. The detailed sequence analysis performed in this study revealed a sequence error in our previously reported COP1 protein sequence from the Columbia ecotype (Deng et al., 1992): an extra base was detected after codon 611 of the previously reported sequence. Therefore, a different reading frame was used for amino acids after codon 611. Thus, the originally predicted C-terminal 47 amino acids (from 612 to 658) were replaced by a different 64 amino acids. The corrected sequence is shown in Figure 4, and it predicts a 675–amino acid protein with a molecular mass of 76.2 kD. The validity of the newly predicted C-terminal COP1 sequence is supported by its high degree of conservation with corresponding regions of COP1 homologs in spinach and rice (M. Matsui, D. Chamovitz, and X.-W. Deng, unpublished data).

Computer analysis of the revised COP1 sequence revealed an expanded domain (234 amino acids, position 386 to 619) homologous to the β subunit of the trimeric G-proteins (Figure 4). This domain now contains four complete WD-40 repeats and two half WD-40 repeats (Figure 5). Secondary structure predictions for the COP1 protein suggested a continuous α-helical structure in an 81–amino acid stretch of the COP1 protein (positions 128 to 209) following the "ring-finger"–type Zn binding domain (Figure 4). This α-helical region is highly charged and contains 37% acidic and basic residues. When compared with known sequences, it shows limited homology (up to 20% identity) with the α-helical tail domain of the myosin heavy chains and with intermediate filament proteins (data not shown). Because both types of proteins contain coiled-coil structures (Cohen and Parry, 1986), this domain of COP1 may also have the potential to form a coiled-coil structure. Indeed, analysis with the COILED-COIL program (Lupas et al., 1991) predicted a high probability for this domain to participate in a coiled-coil structure (M. Matsui and X.-W. Deng, unpublished data).

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    Part A                          Part B
LxGHxxxIxxΦxδ (spacer) ΦΦSGGxDxxΦxIWDδ
F       L                 TAA N   C LFN   WD-40
        V                   S       YY    consensus

                          FATAGVSRCIKVFDF (386-400)
                          EMSTRSKLSCLSWNK (416-430)
HEKRAWSVDFSRTEPSM..LVSGSDDCKVKVWCT (462-493)
MKANICCVKYNPGSSNY..IAVGSADHHIHYYDL (504-536)
FSGHKKAVSYVKFLSNNE.LASASTDSTLRLWDV (545-577)
FRGHTNEKNFVGLTVNSEYLACGSETNEVYVYHK (586-619)

FxGHxxxVxΦVxx             LASGSxDxxΦxΦWDx  COP1 consensus
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Figure 5. The Predicted WD-40 Repeats of COP1 and the Consensus.

The WD-40 consensus shown on the top is based on published G_β-protein homology domain sequences (van der Voorn and Ploegh, 1992; Dynlacht et al., 1993). WD-40 repeats can generally be divided into two subdomains: part A of 13 residues and part B of 15 residues. A spacer of four to six amino acids exists between parts A and B. The WD-40 repeats identified in COP1 are shown below the WD-40 consensus. The amino acid positions comprising each repeat are shown on the right. In COP1, two extra copies of part B were found without the accompanying part A, a common feature of many proteins with G_β-protein homology domains. The WD-40 consensus for COP1 is shown at the bottom. x, any amino acid: δ, noncharged amino acid; Φ, hydrophobic amino acid; all standard amino acids are indicated by the single-letter code. For spacer regions less than six amino acids long, empty positions are indicated by a period.

Most of the Lethal *cop*1 Mutations Result in Undetectable Levels of COP1 Protein

As a first step toward defining the molecular lesions of the various *cop*1 mutations, we analyzed the accumulation of COP1 protein in homozygous mutant plants with polyclonal antibodies against COP1. Of the 17 available *cop*1 mutant alleles, two of the lethal alleles had very low germination rates, rendering them impractical for protein analysis. Therefore, only 15 alleles were examined, and Table 1 summarizes these results. In wild-type plants, COP1 protein of the predicted size was detected at a low level on protein gel blots, as shown in Figure 6. Many of the *cop*1 mutations have effects on the size and quantity of the COP1 protein present in the plant. The two weak mutants both produced detectable COP1 protein. *cop*1-6 plants produced COP1 protein of wild-type size and quantity, whereas *cop*1-4 plants accumulated a protein of much smaller size (~33 kD) at a reduced level (see Figure 6). Two strong mutants, *cop*1-1 and *cop*1-2, produced a slightly smaller COP1 protein (~74 kD) that accumulated to wild-type levels, whereas *cop*1-3 mutant plants produced a greatly reduced (~5%) quantity of wild-type size protein. Four lethal mutants produced detectable COP1. Both *cop*1-8 and *cop*1-10 plants produced COP1 proteins with an apparent molecular mass of 66 kD and at wild-type levels. *cop*1-9 mutant plants produced a COP1 protein of wild-type size, which accumulated to a level higher than COP1 in wild-type plants. *cop*1-11 plants produced a 70-kD COP1 protein that also accumulated to a level higher than COP1 in wild-type plants. The remaining six lethal mutant alleles, *cop*1-5, *cop*1-7, *cop*1-12, *cop*1-13, *cop*1-14, and *cop*1-15, produced no detectable COP1 protein (much less than 5% of wild-type level). The absence of COP1 protein in those mutants could be caused by the effects of these mutations on gene transcription, transcript processing or stability, or the translatability of the mRNAs or the stability of the proteins. Because all six alleles that did not accumulate COP1 protein belong to the lethal class of mutations, the phenotype of lethal *cop*1 mutants may represent that of null *cop*1 mutations.

The molecular defects of two of the six alleles that did not accumulate COP1 protein were determined and are shown in Figure 7. *cop*1-5 has a large T-DNA insertion in intron 8 just after the first two partial WD-40 repeats. *cop*1-7 has a G-to-T mutation that changes the Glu-416 GAG codon to a UAG stop codon. This removes all of the G_β-protein homology domain except for the first WD-40 repeat. Somehow, the mutations in these alleles either prevent translation of the mRNA or destabilize the translated protein. It is highly likely that the lethal phenotype observed in these two mutants is due to the lack of COP1 protein rather than to the structural alterations.

Clearly, determination of molecular defects in the mutants that still accumulate COP1 protein will be most informative with regard to structure–function relationships. These mutants include all strong and weak mutants and four of the lethal mutants (Table 1). In each of these alleles, the altered COP1 protein structure is likely to be the cause of the phenotypic defect. Thus, these *cop*1 mutant alleles represent a mutagenized series of COP1, all of which lead to the constitutive photomorphogenic phenotype. Therefore, the *COP*1 cDNAs from these selected alleles were cloned and sequenced.

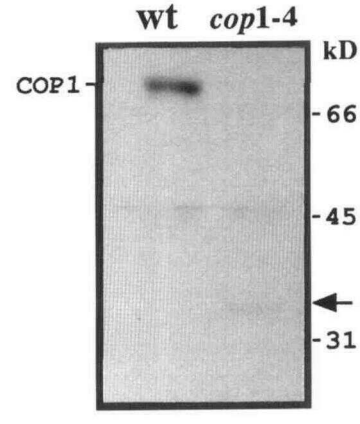

Figure 6. Protein Gel Blot Analysis of the COP1 Protein in Wild-Type and *cop*1-4 Mutant Seedlings.

The COP1 protein at the expected 76-kD position in the wild-type (wt) plants is indicated; the truncated COP1 protein (~33 kD) in *cop*1-4 is marked with an arrow. A weak band at the 46-kD position is present in both wild-type and mutant extracts and is thus likely to be a nonspecific cross-reaction.

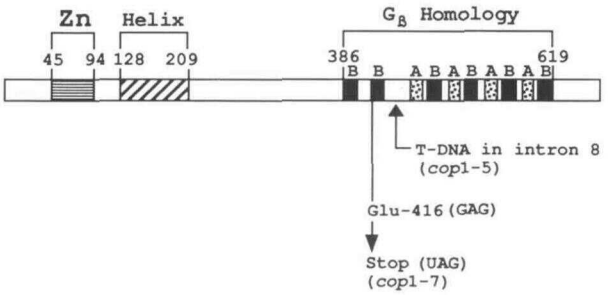

Figure 7. Summary of Molecular Lesions in Two Lethal *cop*1 Alleles, *cop*1-5 and *cop*1-7.

The three structural domains of the COP1 protein, the Zn binding motif, putative coiled-coil region, and the WD-40 repeats (see legend to Figure 5) are diagrammed. The *cop*1-5 mutation was caused by a T-DNA insertion within intron 8, and the *cop*1-7 mutation was a single base substitution that results in a stop codon. Both mutations led to undetectable COP1 protein. A, subdomain part A; B, subdomain part B (see legend to Figure 5).

C-Terminal Region after the G_β-Protein Homology Domain Is Essential for COP1 Function

*cop*1-3, a strong allele, and *cop*1-11, a lethal allele, are both defective in the splicing of intron 12 due to mutations in the splice junction at the 3′ end of the intron (data not shown), and each mutant accumulated two cDNAs, as shown in Figure 8A. In the case of *cop*1-11, the smaller transcript (transcript 1) is more abundant, and it represents an incorrect splicing of intron 12, which resulted in the deletion of the G residue from codon 620 (GAA) in the mRNA. This caused a frameshift that resulted in a truncated protein with five novel amino acids added after codon 619, as shown in Figure 9. Transcript 2 contains an unspliced intron 12, creating a 111-bp insertion after codon 619. This resulted in the addition of 16 novel amino acids after codon 619 (Val-His-Lys-Ser-Glu-Thr-Ser-Ala-Leu-Asn-Phe-Ser-Ile-Phe-His-Ser-[UGA]) before a stop codon was reached. Conceptual translation of both transcripts produces a COP1 protein lacking the final 56 amino acids right after the end of the G_β-protein homology domain. These results correlate well with the protein gel blot results for homozygous *cop*1-11 plants; the data revealed a 70-kD COP1 protein that accumulates to a higher level than COP1 in wild-type plants.

*cop*1-3 also affects splicing at intron 12, and it accumulated two transcripts of similar sizes to those found in *cop*1-11 (Figure 8A). The larger transcript (transcript 2) contains an unspliced intron 12, just like transcript 2 of *cop*1-11, except that in *cop*1-3, this transcript predominates. In contrast to *cop*1-11, however, the less abundant transcript 1 has a wild-type sequence. Thus, the *cop*1-3 mutation only led to a decreased efficiency in the splicing of intron 12. Protein gel blots of *cop*1-3 mutants revealed the presence of only a very low level (5%) of COP1 protein of wild-type size. This protein must be translated from the low-abundance wild-type transcript. The slightly smaller protein expected from the abundant transcript 2 was not detected by protein gel blot analysis (Table 1 and data not shown), suggesting that it is either not translated or unstable in vivo. Therefore, the phenotype of this mutant is due simply to the reduced level of COP1 protein.

Because the large transcripts (transcript 2) in both *cop*1-3 and *cop*1-11 are identical, the smaller COP1 protein observed in *cop*1-11 mutants is likely to be translated only from the smaller transcript (transcript 1), as shown in Figure 9. The lethal phenotype of *cop*1-11 mutants suggests that the C-terminal region after the G_β-protein homology domain is essential for the function of the protein. After examining more than 500 homozygous mutants, a complete cosegregation of the lethal phenotype with the *cop*1-11 mutation was observed. This would rule out the possibility that a second site mutation might be responsible for the observed lethality.

G_β-Protein Homology Domain Is Critical for COP1 Function

Three lethal alleles, *cop*1-8, *cop*1-9, *cop*1-10, affect the integrity of the G_β-protein homology domain of the COP1 protein, as shown in Figure 9. The *cop*1-8 mutation resulted in a 180-bp in-frame deletion in the mRNA that corresponds to exon 11. This removed amino acids 523 to 582, leaving the following coding region in-frame. The deletion removed one and one-half of the WD-40 repeats. This correlates with the smaller size of the COP1 protein detected in *cop*1-8 mutants on protein gel

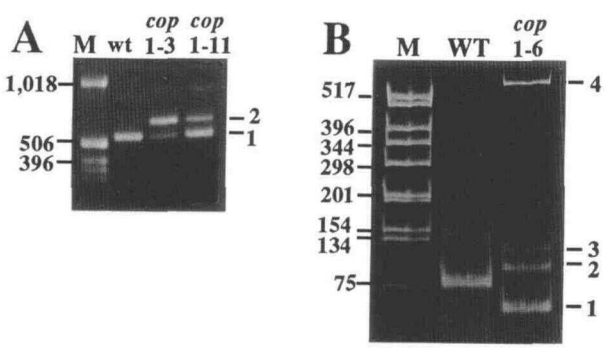

Figure 8. Multiple Splicing Products in *cop*1-3, *cop*1-6, and *cop*1-11 Mutants.

(A) The *cop*1-3 and *cop*1-11 mutations led to a splicing defect at intron 12. Two splicing products of intron 12 (indicated at right) were amplified by PCR using primers corresponding to the adjacent exons, and the products were resolved on a 1% agarose gel. A single spliced product was observed in wild-type (wt) plants. M, molecular size markers in base pairs.

(B) The *cop*1-6 mutation led to four cryptically spliced products (indicated at right) at intron 4. PCR products generated from *cop*1-6 mutant and wild-type (wt) plants using primers corresponding to the adjacent exons were examined on a 10% polyacrylamide gel. Transcript 4 of *cop*1-6 mutants represents an mRNA with intron 4 unspliced. M, molecular size markers in base pairs.

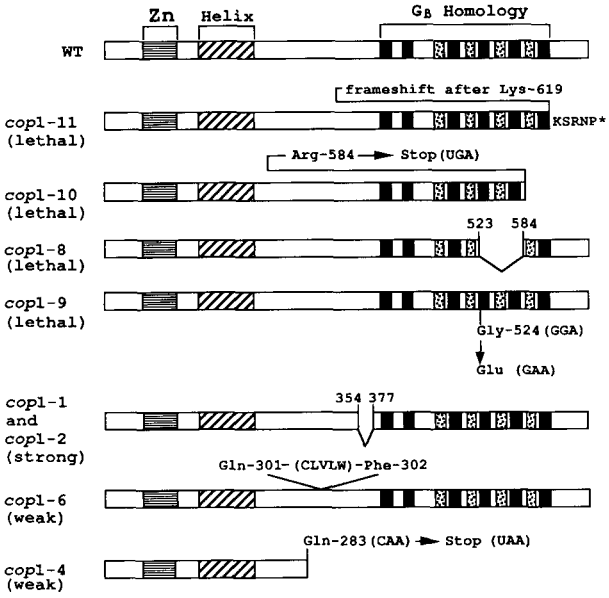

Figure 9. Summary of the Accumulated Mutated Forms of COP1 Proteins in Eight *cop*1 Mutant Alleles.

In the case of an allele that produced multiple transcripts, only the protein form observed by protein gel blot analysis is depicted. Details are described in Results and the legend to Figure 7. wt, wild type.

blots (Table 1). Because the *cop*1-8 mutants expressed the defective COP1 protein at levels comparable to wild-type plants, the alteration of its structure must have disrupted the function of COP1. The *cop*1-9 mutation is a G-to-A point mutation, changing Gly-524 (GGA) to a negatively charged glutamic acid (GAA). The glycine is a highly conserved amino acid in part B of the WD-40 repeat (Figure 5). Protein gel blots of *cop*1-9 mutant protein showed a wild-type–sized COP1 protein accumulating to slightly higher levels than COP1 in wild-type plants (see Table 1). Placing a negatively charged amino acid at a position in which there is usually an uncharged amino acid may disrupt the protein conformation or the interaction of COP1 with other proteins of the light signal transduction pathway. The *cop*1-10 mutation is a single C-to-T base change, changing the Arg-584 codon (CGA) to a stop codon (UGA). This mutation removed the last WD-40 repeat in the G_β-protein domain as well as the C-terminal region. Protein gel blot analysis of *cop*1-10 mutants revealed the presence of a smaller COP1 protein that accumulated to levels similar to COP1 protein in wild-type plants (see Table 1).

Small Deletions or Insertions between the Putative Coiled-Coil Region and G_β-Protein Homology Domain Strongly Compromise COP1 Function

The mutations in both *cop*1-1 and *cop*1-2 resulted in identical 66-bp deletions corresponding to exon 6 in the mature mRNA. These deletions removed amino acids 355 to 376, leaving the remaining coding region in frame (Figure 9). The protein gel blot data for *cop*1-1 and *cop*1-2 mutants revealed a COP1 protein that was reduced in size and that accumulated to levels comparable to COP1 in wild-type plants (see Table 1). Presumably, because *cop*1-1 and *cop*1-2 were isolated from different M_1 batches (Deng and Quail, 1992), they represent independent mutational events.

The *cop*1-6 mutation changes the splicing junction "AG" at the 3′ end of intron 4 to "GG" and leads to three principal cryptically spliced mRNA transcripts (transcripts 1 to 3) and a transcript with an unspliced intron 4 (transcript 4), as shown in Figure 8B and Figure 10. Transcript 1 is the most abundant, and it resulted from aberrant splicing at the first downstream AG dinucleotide, which is 16 bases into exon 5. This transcript contains a 16-bp deletion after Glu-301 that changes the reading frame, leading to the addition of 23 novel amino acids before a new stop codon is reached. The second most abundant transcript (transcript 2) and the minor transcript (transcript 3) represent insertions of 15- or 37-bp segments of intron 4 between exon 4 and exon 5. Conceptually, transcript 2 will produce a protein with five novel amino acids (Cys-Leu-Val-Leu-Trp) inserted in frame between Glu-301 and Phe-302 of the wild-type protein (Figure 9). Due to a frame shift, transcript 3 will lead to a truncated protein with 14 novel amino acids added after Glu-301 before reaching a new stop codon (Arg-Leu-Pro-Asn-Ser-Val-Lys-Val-Ser-Cys-Leu-Val-Val-Gln-[UGA]). If translated, transcript 4 would produce a truncated protein with 20 novel amino acids added after Glu-301 before reaching a new stop codon. However, the only COP1 protein detected in *cop*1-6 mutants is of wild-type size and abundance. This protein must be translated from transcript 2. Because no truncated COP1 protein was detected in *cop*1-6 mutants, transcripts 1, 3, and 4 may not be translated efficiently or their protein products may be unstable. This data, together with the fact that the *cop*1-6 mutation is strictly recessive, led us to conclude that the five–amino acid insertion in the COP1 protein is responsible for the reduced activity of COP1 in *cop*1-6 plants.

*cop*1-4 Mutation Results in a Severely Truncated COP1 Protein

The weak *cop*1-4 allele represents a C-to-T mutation that changes the Gln-283 CAA codon to a UAA stop codon. This mutation resulted in a truncated COP1 protein containing only the N-terminal 282 amino acids (Figure 9). Protein gel blot analysis of *cop*1-4 mutant plants confirmed the presence of a 33-kD COP1 protein accumulating to a low level, as shown in Figure 6. No wild-type size COP1 protein has been observed in our protein gel blot analysis of *cop*1-4 mutants, suggesting that translation through the newly created stop codon, if any, is negligible. It is highly unlikely that an undetectable amount of wild-type COP1 protein in *cop*1-4 mutants might be the cause of a weak phenotype, because a reduced but clearly detectable amount of wild-type COP1 protein in *cop*1-3 resulted in

a strong mutant phenotype. Therefore, we concluded that the truncated COP1 protein in *cop*1-4 mutants retains some residual function.

DISCUSSION

In this report, we describe our initial efforts to analyze the functional importance of the different structural domains of the COP1 protein by random in vivo mutagenesis. The results from these studies shed new light on both the biological function of the *COP*1 locus and the functional roles of the various COP1 structural domains.

*COP*1 Is an Essential Plant Gene

The 17 recessive *cop*1 mutant alleles that were isolated fall into three phenotypic classes that, in order of severity, are lethal, strong, and weak. Among these, 12 alleles belong to the lethal mutant class. These lethal mutations result not only in constitutive photomorphogenesis, but also in lethality. The lack of COP1 protein in most of the lethal *cop*1 mutants suggests that they are likely to be the complete loss-of-function (null) mutations. The adult lethality of the putative null *cop*1 alleles has two interesting implications regarding the function of COP1 in wild-type plants. First, COP1 not only serves as a light-inactivated repressor of photomorphogenesis, but it is also essential for normal plant development in the light. This could be explained if COP1 plays a dual role both in light signal transduction and in an essential adult plant developmental process or if light signal transduction processes mediated by COP1 are essential for adult development. Second, except for a high level of anthocyanin accumulation, COP1 appears not to influence Arabidopsis early embryogenesis because neither embryo lethality nor abnormal patterns of morphology were observed for all the lethal alleles (Miséra, 1993).

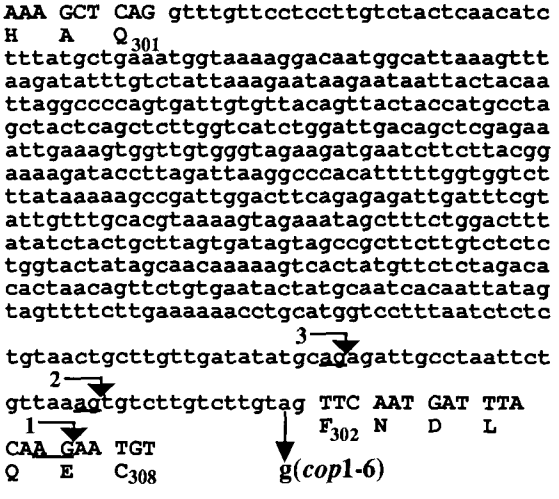

Figure 10. Sequence of Intron 4 of the *COP*1 Gene and the Locations of the Three Cryptic Splice Sites Utilized in *cop*1-6 Mutant Plants.

The intron sequence (570 bp) is shown in lowercase letters and the flanking exon sequences are shown in uppercase letters. The specific genomic mutation (a-to-g substitution at the 3′ end of the intron) in the *cop*1-6 mutant is indicated. The three cryptic splicing sites, each preceded by an "AG" dinucleotide, are marked by arrows.

COP1 also Plays an Essential Role in the Ability of Light-Grown Plants to Respond to a Changing Light Environment

Previously, we have shown that *cop*1 mutations lead to a defect in the ability of light-grown plants to adapt to a dark environment (Deng et al., 1991). In this study, we used physiological experiments on the weak and strong alleles to show that mutations in the *COP*1 locus lead to deficiencies in the shade avoidance (Figure 3A) and end-of-day far-red responses (Figure 3B) and the photoperiodic control of floral induction (Table 2). These observations suggested that *COP*1 plays a role in the ability of light-grown plants to respond to light quality signals and to daylength. Strikingly, *cop*1-6 mutants are able to flower according to a long-day schedule even in total darkness, thus completing most of the life cycle without any light stimulus. However, it is clear that COP1 is not involved in all light-regulated processes, because at least two responses to light, namely the control of seed germination by phytochrome (Deng et al., 1991) and the phototropic response (T.W. McNellis and X.-W. Deng, unpublished data), are unaffected by *cop*1 mutations.

COP1 Structural Domains and Their Functional Roles

COP1 has three structurally recognizable domains: the Zn binding domain, a potential coiled-coil region, and the G_β-protein homology domain, which contains four complete and two partial WD-40 repeats (Figures 4 and 5). It has been demonstrated that each Zn binding domain can bind two Zn atoms, but with drastically different affinities (von Arnim and Deng, 1993). To determine the functional importance of the different structural domains, we conducted a direct in vivo mutagenesis study of the COP1 protein. Through molecular characterization of the accumulated mutant forms of COP1 protein in nine ethyl methansulfonate–induced *cop*1 mutant alleles and by physiological characterization of their phenotypes, several conclusions can be made regarding the functional roles of the COP1 protein motifs. First, the C-terminal 56 amino acids after the G_β-protein homology domain play an essential role for COP1 function. This was demonstrated by the *cop*1-11 mutation, which deletes the C-terminal region and leads to a lethal phenotype. Second, as expected, the integrity of the G_β-protein domain is critical for COP1 function. This was

clearly demonstrated by the fact that a single amino acid substitution (*cop*1-9), an internal in-frame deletion (*cop*1-8), and a C-terminal deletion (*cop*1-10) of the G_β-protein domain all led to a lethal phenotype. Third, the region between the helical domain and the G_β-protein domain is also important for COP1 function. It is possible that the spacing between the potential coiled-coil and the G_β-protein domains is critical for the COP1 conformation or activity. This was supported by the fact that both small in-frame deletions (*cop*1-1 and *cop*1-2) and a short in-frame polypeptide insertion (*cop*1-6) seriously compromised COP1 activity. It should be noted, however, that a given mutation in the COP1 protein can disrupt its function in several possible ways, such as altering the protein conformation: that is, the protein's ability to interact with other partners or a specific biochemical activity. At this stage, our results do not allow us to differentiate between those possibilities.

Finally, the N-terminal domains of COP1 retain significant function on their own. The *cop*1-4 mutation creates a stop codon that deletes everything but the first 282 amino acids of the protein. This resulted in the loss of the entire G_β-protein homology domain as well as much of the protein immediately upstream of the G_β-protein domain. Nevertheless, the *cop*1-4 mutation led to a weak phenotype. We have shown that in the *cop*1-3 allele, a low but clearly detectable level of wild-type COP1 protein will still lead to a strong phenotype. Therefore, it is highly unlikely that the weak phenotype of the *cop*1-4 mutant is due to an undetectable level of full-length COP1 protein made by translational read-through of the stop codon. This result contrasts with some lethal mutations, which show that either minor changes in the G_β-protein domain (*cop*1-8 and *cop*1-9) or much shorter deletions of the C terminus (*cop*1-10 and *cop*1-11) are extremely deleterious to COP1 function. The weak phenotype caused by the recessive *cop*1-4 mutation implies that it is better for COP1 not to have a G_β-protein domain at all than to have a defective one. There are at least two possible explanations for this observation. First, the N-terminal portion of COP1 alone retains some function, whereas a defective G_β-protein domain disrupts the function of the N-terminal portion, possibly due to unfavorable conformation. Alternatively, some other protein, such as a different G_β-domain–containing protein in the plant cell, may be able to take the place of the G_β-protein homology domain. This substitution could not take place in the mutants with defective G_β-protein domains due to steric hindrance.

It is interesting to note that we have not yet identified any mutations specifically affecting the N-terminal half, including the Zn binding motif and the potential coiled-coil domain. There are several possible reasons for this. First, because mutations specifically altering the protein structure of the N terminus, but still retaining a normal C-terminal portion are expected to occur at low frequency, it may simply be necessary to screen more mutants to recover such lesions. Second, mutations in the N-terminal region may cause dominant lethal phenotypes. Therefore, we would never recover these mutations in our screen. Third, mutations in the N-terminal region may result in a completely different phenotype and thus would not be recovered by our phenotypic screen. The latter two possibilities can be addressed in the future by creating desired N-terminal mutations in the COP1 protein in vitro and introducing them back into COP1-deficient plants.

METHODS

Plant Materials and Growth Conditions

Plant germination and growth conditions were exactly as described previously by Hou et al. (1993). *cop*1-1, *cop*1-2, *cop*1-3, *cop*1-4, *cop*1-6, and *cop*1-7 are in the Columbia background, *cop*1-5 is in the Wassilewskija background, and *cop*1-8 through *cop*1-17 are in the Landsberg *erecta* background. The *cop*1-6 mutation was originally identified as a *brachytic* (short-internode) mutant by G.P. Redei (University of Missouri–Columbia). For the end-of-day far-red experiments, seedlings were grown for 48 hr in continuous white light and then transferred to a 10-hr light/14-hr dark cycle with a 15-min pulse of far-red light at 20 μmol m^{-2} sec^{-1} at the end of each day for 4 days; hypocotyl measurements were then taken. Control plants were grown under an identical light regimen but without the far-red pulse at the end of each day. Far-red light was provided by far-red–enhanced tubes (F15T8/232 fluorescent tubes; GTE Sylvania, Danvers, MA) wrapped with one layer each of two types of colored plastic filters (Lee Filters HT120 and 106; LEE Colortran Inc., Totona, NJ). For the shade avoidance response experiments, plants were grown for 48 hr in continuous white light and then transferred to a far-red–enhanced chamber with white light provided at 40 μmol m^{-2} sec^{-1}, supplemented with far-red light at 15 μmol m^{-2} sec^{-1}. Plants were grown for 4 days under continuous illumination; hypocotyl measurements were then taken. Control plants were grown in 40 μmol m^{-2} sec^{-1} white light without far-red supplement.

Electron Microscopy

The first pair of foliage leaves from plants that had been grown for 15 days were used for plastid observations. Plant materials were fixed with 2% glutaraldehyde in 20 mM cacodylate buffer, pH 7.2, and 1% osmium tetroxide in 20 mM cacodylate buffer, pH 7.2, dehydrated in a graded ethanol series, and embedded in Spurr's resin. Sections were made using an ultramicrotome (model MT2B; RMC Inc., Tucson, AZ). Thin sections (0.1 to 0.2 μm) were poststained with 5% uranyl acetate and counterstained with 3% lead citrate. Observations of plastids in these sections were made with a transmission electron microscope.

Protein Analysis

Seedlings were homogenized in extraction buffer (50 mM Tris-Cl, pH 7.5, 2.5 mM EDTA, 400 mM sucrose, 10% glycerol, 1 mM phenylmethanesulfonyl fluoride, 1 mM benzamidine, 10 μg/mL phosphoramidon, 10 μg/mL leupeptin). Ten micrograms of soluble protein was electrophoresed on 10% SDS–polyacrylamide gels, protein gel blotted to polyvinylidene difluoride membranes, and probed with affinity-purified rabbit polyclonal antibodies against the N-terminal 288 amino acids of the COP1 protein produced in *Escherichia coli* (von Arnim and Deng, 1993). Bound antibody was detected with alkaline phosphatase–coupled secondary antibodies, nitro blue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate as substrates. Alternatively, COP1 proteins were immunoprecipitated from the crude extract, in the presence of 0.1%

SDS, 0.5% deoxycholate, and 1% Nonidet-40 (Sigma), using COP1-specific antibodies and protein A–Sepharose (Pharmacia). The immunoprecipitate from ~200 μg of plant protein was electrophoresed, protein gel blotted, and probed as described above.

Sequence Analysis

Total RNA was isolated from 1- to 2-week-old wild-type or homozygous mutant seedlings (1 to 3 g fresh tissue) according to our previously published procedure (Deng et al., 1991). The total RNA was used directly for cDNA synthesis. First-strand cDNA synthesis was performed in reverse transcription buffer (50 mM Tris-Cl, pH 8.3; 75 mM KCl; 3 mM MgCl; 10 mM DTT; 500 μM dATP, dCTP, dGTP, and dTTP; 50 μg/mL oligo[dT] primer) using 20 to 30 μg of total RNA as template and 50 units of murine leukemia virus reverse transcriptase (New England Biolabs, Beverly, MA). First-strand cDNA was used as the template in polymerase chain reactions (PCRs) to amplify the *COP*1 cDNA. The full-length *COP*1 cDNA was amplified in two overlapping fragments (1.0 and 1.3 kb each) using 30 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 3 min with Vent DNA polymerase (New England Biolabs). The products were cloned using the TA cloning system (Invitrogen, San Diego, CA), and the clones were sequenced using the Sequenase kit (U.S. Biochemicals) according to the manufacturer's instructions. Mutations identified were confirmed by direct sequencing of PCR products spanning the region of interest (Chamovitz et al., 1993).

Analysis of Multiple Transcripts

In mutants in which multiple transcripts were implied by sequence analysis (such as *cop*1-3, *cop*1-6, and *cop*1-11), the regions of interest were amplified by performing PCR amplification with specific primers surrounding the affected exon/exon junction, using total first-strand cDNA as template. The PCR conditions were as described above, except that the extension time at 72°C was 1 min. The PCR reaction products were separated on a 1% agarose gel (*cop*1-3, *cop*1-11) or a 10% acrylamide gel (*cop*1-6). Individual PCR products were purified and reamplified by PCR for direct sequencing.

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