

Characterization of a Novel Non-Constitutive Photomorphogenic *cop1* Allele¹

Monika Dieterle², Claudia Büche³, Eberhard Schäfer, and Thomas Kretsch*

Alberts-Ludwigs-Universität Freiburg, Institut für Biologie 2/Botanik, Schänzlestrasse 1, 79104 Freiburg, Germany

A specific light program consisting of multiple treatments with alternating red and far-red light pulses was used to isolate mutants in phytochrome A-dependent signal transduction in *Arabidopsis* seedlings. Because of their phenotype, the mutants were called *eid* (empfindlicher im dunkelroten Licht, which means hypersensitive in far-red light). One of the isolated mutants, *eid6*, is a novel recessive allele of the *COP1* gene (constitutive photomorphogenic 1) that carries an amino acid transition in a conserved histidine residue of the RING finger domain. Mutant seedlings exhibited an extreme hypersensitivity towards all tested light qualities, but in contrast to known *cop1* alleles, no constitutive photomorphogenic phenotype was detectable in darkness. Thus, the novel *cop1^{eid6}* allele seems to encode for a protein whose remaining activity is sufficient for the suppression of photomorphogenesis in dark-grown plants. In adult *cop1^{eid6}* plants, the development of the Cop1 phenotype is dominated by phytochrome B. Comparison of the phenotype of the novel *cop1^{eid6}* and the weak *cop1-4* allele under continuous far-red light indicates that the RING finger and coiled-coil domains of COP1 are sufficient for some specific regulatory function in phytochrome A-dependent high irradiance responses.

Light is one of the major environmental factors that controls plant development. To perceive light, plants have evolved a large set of photoreceptors. Among these, the cryptochromes and phototropins are responsible for UV-A/blue light sensing, whereas the phytochromes predominantly regulate responses to red and far-red light (Neff et al., 2000). The phytochromes are dimeric proteins of approximately 125-kD subunits. Each monomer is covalently linked to a phytychromobilin chromophore, a linear tetrapyrrole. Phytochromes can exist in two different forms: the red light-absorbing Pr and the far-red light-absorbing Pfr form. Because the Pfr conformer is the physiologically active form, the photoreceptor can function as a red/far-red photoreversible light switch upon light pulse treatments for several physiological responses (Neff et al., 2000; Møller et al., 2002).

Phytochromes are encoded by a small multigene family of five different genes (*PHYA* to *PHYE*) in *Arabidopsis* (Clack et al., 1994). Phytochrome A

(*phyA*) accumulates to a high level in the dark, and its Pfr form is rapidly degraded (Clough and Vierstra, 1997). The light-labile *phyA* mediates the very low fluence responses that can be induced by extremely low amounts of photons. Furthermore, *phyA* is also responsible for the so-called high irradiance responses (HIRs) that need high fluence rates of continuous far-red light to be fully induced. The light-stable phytochrome B (*phyB*) is the main photoreceptor to sense continuous red light and the classical red/far-red light photoreversible low fluence responses (Whitelam and Devlin, 1997; Neff et al., 2000; Møller et al., 2002).

A very drastic effect of light is seen during seedling development of higher plants. In darkness, seedlings follow a developmental program called skotomorphogenesis. They have a long hypocotyl, a hypocotyl hook, and small pale cotyledons. Upon irradiation, seedlings become de-etiolated. The induction of photomorphogenesis leads to the inhibition of hypocotyl elongation, opening of the hypocotyl hook, and unfolding of the cotyledons.

These drastic changes between skoto- and photomorphogenesis were used to screen for mutants in components of light signal transduction pathways (Hudson, 2000; Neff et al., 2000). The *det* (*de-etiolated*)/*cop* (*constitutive photomorphogenesis*)/*fus* (*fusca*) class (Chory et al., 1989; Deng et al., 1991; Miséra et al., 1994) of mutants was identified because of their de-etiolated seedlings phenotype even in darkness. In the last few years, considerable progress has been made in cloning and characterization of the respective genes. The *det2* mutant is defective in the biosynthesis of brassinosteroids (Li and Chory, 1996). Another group carries mutations in components of

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² Present address: Institut de Biologie Moléculaire des Plantes du Centre National de la Recherche Scientifique, 12 rue de Général Zimmer, 67084 Strasbourg, France.

³ Present address: Deutsches Krebsforschungszentrum, Molekulare Genomanalyse, Im Neuenheimer Feld 580, 69120 Heidelberg, Germany.

* Corresponding author; e-mail thomas.kretsch@biologie.uni-freiburg.de; fax 49-761-203-2791.

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the COP9 multiprotein complex. *COP1*, *COP10*, and *DET1* do not belong to one of these groups (Hardtke and Deng, 2000; Møller et al., 2002).

The COP1 protein carries three structural modules: a RING finger motive, a coiled-coil domain, and multiple WD-40 repeats (Deng et al., 1992). COP1 is enriched in the nucleus in the dark and disappears from the nucleus in the light (von Arnim and Deng, 1994). The recessive nature of *cop1* mutants and their light-grown phenotype in darkness suggest that the respective protein functions as a repressor of photomorphogenesis (Deng et al., 1991). RING finger proteins very often act as ubiquitin ligases, which target proteins for proteolysis in the proteasome (Tyers and Jorgensen, 2000). COP1 also functions in the regulation of ubiquitylation and protein stability. COP10, a COP1-interacting protein, shows homologies to ubiquitin-activating E2 proteins (Suzuki et al., 2002). HY5 and HYH, two bZIP transcription factors involved in light signaling, interact with COP1 and are degraded in darkness in a COP1-dependent pathway. HY5 is stabilized upon light treatments, most probably due to a reduced phosphorylation of the protein (Hardtke et al., 2000; Osterlund et al., 2000; Holm et al., 2002). The myb transcription factor LAF1, which is involved in phyA signal transduction, also interacts with the repressor and becomes destabilized by inducible overexpression of COP1 (Seo et al., 2003). Using an in vitro system, Seo et al. (2003) could demonstrate that COP1 catalyzes self-ubiquitylation and the ubiquitylation of LAF1.

To screen for hypersensitive mutants in phyA signaling, a screening program consisting of alternating 20-min red and far-red light pulses was used (Büche et al., 2000; Dieterle et al., 2001). In the wild type, the red light pulse causes a degradation of phyA that abolishes far-red light-induced HIR. *phyB-5* seeds mutagenized with ethyl methylsulfonate were used for screening to exclude effects caused by phyB under the multiple red light treatment. With the screening program, seven different mutant lines were isolated that exhibited a strong photomorphogenic growth under the red/far-red light pulse treatment. The mutants formed six different complementation groups named *eid1* to *eid6* (empfindlicher im dunkel-

roten Licht, which means hypersensitive to far-red light; Büche et al., 2000). Here, we describe the physiological and molecular analysis of *eid6*. As shown below, a mutation in the *COP1* gene was responsible for the observed Eid phenotype. Therefore, the novel allele is called *cop1^{eid6}* throughout the text.

RESULTS

Isolation and Genetic Analysis of the *cop1^{eid6}* Mutant

Seven different mutant lines were isolated that exhibited a strong photomorphogenic growth under a screening program consisting of alternating 20-min red and far-red light pulses (Büche et al., 2000). The screened *cop1^{eid6} phyB-5* mutant was backcrossed to the *phyB-5* background line and to the Landsberg *erecta* (*Ler*) wild type. F₂ seedlings of both crosses exhibited a 3:1 segregation behavior for wild type: mutant phenotype that is characteristic for monogenic recessive mutations (Table I). F₂ plants of the *Ler* backcrosses were used to isolate homozygous *cop1^{eid6}* mutants in a *PHYB* wild-type background.

The *cop1^{eid6} phyB-5* and the *cop1^{eid6}* mutant displayed a very strong phenotype under the screening program (Fig. 1A), whereas they did not exhibit a constitutive photomorphogenic development in darkness (Fig. 1B). *cop1^{eid6}* and *cop1^{eid6} phyB-5* seedlings also showed an enhanced photomorphogenic response under continuous weak and strong red light (Fig. 1, C and D). Weaker effects were observed under strong continuous white and far-red light. Under continuous far-red light, *cop1^{eid6}* exhibited a clearly increased anthocyanin accumulation (Fig. 1, E and F).

eid6 Carries a Mutation in the RING Finger Motif of the *COP1* Gene

We mapped *cop1^{eid6}* using simple sequence length polymorphism and cleaved amplified polymorphic sequences (CAPS) markers (Konieczny and Ausubel, 1993; Bell and Ecker, 1994). No recombinants were observed with the *COP1* CAPS marker after the analyses of 80 F₂ plants homozygous for *cop1^{eid6}*. There-

Table I. Segregation of crosses under different light conditions

Cross	Treatment	F ₁ Phenotype	F ₂ Segregation (Etiolated:De-Etiolated)	Expected Ratio Etiolated:De-Etiolated	χ^2 ($\chi^2_{99\%} = 6.64$)
<i>cop1^{eid6} phyB-5</i> × <i>phyB-5</i>	Darkness	Etiolated	All:0	All:0	–
	Weak red light	Etiolated	488:183	3:1	1.39
<i>phyB-9</i> × <i>cop1^{eid6} phyB-5</i>	Darkness	Etiolated	All:0	All:0	–
	Weak red light	Etiolated	399:129	3:1	0.07
<i>cop1^{eid6} phyB-5</i> × <i>Ler</i>	Darkness	Etiolated	All:0	All:0	–
	Weak red light	Etiolated	807:256	3:1	0.36
<i>cop1^{eid6}</i> × <i>Ler</i>	Darkness	Etiolated	All:0	All:0	–
	Weak red light	Etiolated	145:45	3:1	0.13
<i>cop1^{eid6} phyB-5</i> × <i>cop1-4</i>	Darkness	Etiolated	612:240	3:1	5.53
	Weak red light	De-etiolated	0:368	0:All	–

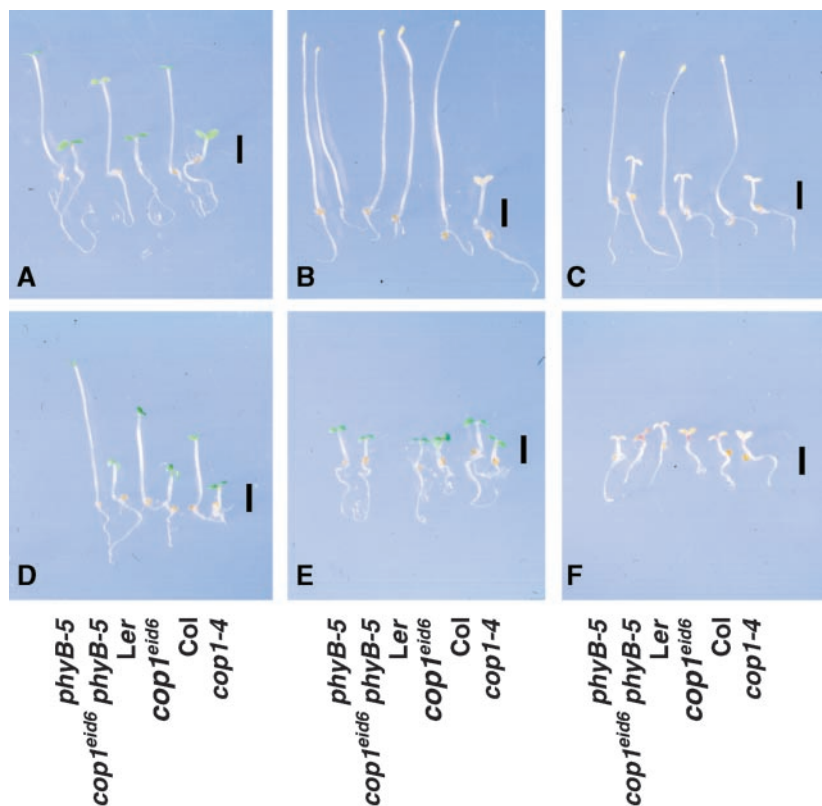


Figure 1. Seedling morphology of *phyB-5*, the screened *cop1^{eid6}* *phyB-5* mutant, *Ler* wild type, *cop1^{eid6}* in *Ler* background, Columbia (*Col*) wild type, and *cop1-4* in *Col* background. Seedlings were grown for 3 d after induction of germination. Bars = 2 mm. A, Seedlings grown under the screening program (alternating pulse treatments of 20 min of red followed by 20 min of far-red light). B, Seedlings grown in continuous darkness. C, Seedlings grown under weak continuous red light ($15 \text{ nmol m}^{-2} \text{ s}^{-1}$). D, Seedlings grown under strong red light ($14.3 \mu\text{mol m}^{-2} \text{ s}^{-1}$). E, Seedlings grown under continuous white light ($15 \mu\text{mol m}^{-2} \text{ s}^{-1}$). F, Seedlings grown under strong far-red light ($14 \mu\text{mol m}^{-2} \text{ s}^{-1}$).

fore, it could not be excluded that *cop1^{eid6}* is a novel *COP1* allele with an unusual, non-constitutive photomorphogenic seedling phenotype.

To test whether *cop1^{eid6}* is a novel *COP1* allele, the mutant was crossed with the weak recessive *cop1-4* allele. The *cop1-4* mutation created a stop codon at position 283 of the *COP1* protein, which should result in the accumulation of a truncated version in mutant plants (McNellis et al., 1994a). Seedlings of the F_1 and F_2 generation of the *cop1^{eid6} cop1-4* crossing displayed a de-etiolated phenotype under weak red light similar to their parental lines (Table I). This result clearly indicates that *cop1^{eid6}* and *cop1-4* belong to the same complementation group because both mutants were recessive under selective light conditions when crossed with their respective wild type. In continuous darkness, seedlings of the F_1 generation remained etiolated, and the F_2 exhibited a 3:1 segregation for etiolated:de-etiolated seedlings. Thus, the constitutive photomorphogenic *cop1-4* phenotype remained recessive in crosses between the weak *cop1* allele and *cop1^{eid6}*.

Sequencing of the *COP1* gene from *cop1^{eid6}* revealed a single C to T base pair substitution at position 247 of the open reading frame. This mutation leads to a replacement of the His at position 69 of the *COP1* protein by an aromatic Tyr residue (Fig. 2). His-69 is one of the highly conserved amino acids in the RING (really interesting new gene) finger motif of the *COP1* protein (von Arnim and Deng, 1993). The

RING finger domain comprises eight metal ligands formed by the consensus motif C3HC4 (Fig. 2). RING finger domains usually bind two zinc ions in a unique cross brace arrangement (Jackson et al., 2000).

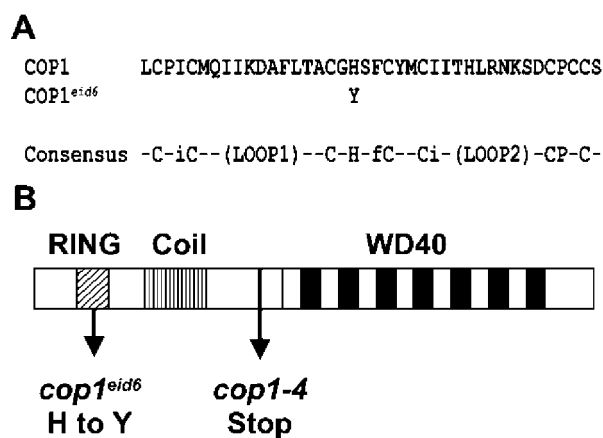


Figure 2. Mutations in the *COP1* protein. A, Amino acid exchange in the *cop1^{eid6}* mutant: Amino acids 51 to 90 of the *COP1* protein are given. The consensus of the RING finger motif is adapted from von Arnim and Deng (1993). Uppercase letters in the RING finger motif represent the eight conserved zinc ligands. Lowercase letters indicate other highly conserved amino acids. B, Schematic overview of the *COP1* protein with the RING finger motif (RING), the coiled-coil domain (Coil), and the WD40 repeats (WD40). The arrows indicate the positions of mutations in *cop1^{eid6}* and *cop1-4*.

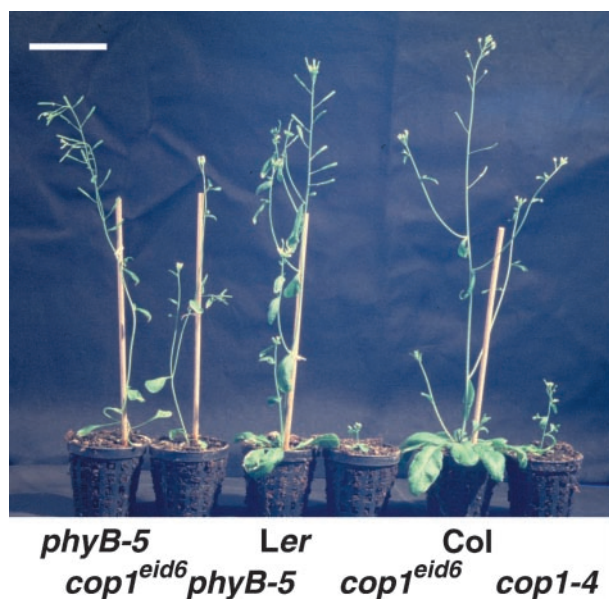


Figure 3. Phenotypes of adult plants. Plants were grown for 35 d under long-day conditions (16 h of light/8 h of dark). Bar = 5 cm. Ler, Ler wild type. Col, Col wild type.

Adult *cop1^{eid6}* Plants Are Dwarf and Exhibit an Early Flowering Phenotype

Mutations in the *COP1* gene are either homozygous lethal or lead to severe phenotypes in adult plants, such as dwarfism and early flowering (Deng and Quail, 1992; McNellis et al., 1994a). The *cop1^{eid6} phyB-5* double mutant exhibited only a weak reduction in size when compared with its *phyB-5* background (Fig. 3). In contrast, adult *cop1^{eid6}* single mutants were clearly smaller compared with wild-type plants and had a decreased vitality. Thus, they closely resembled the phenotype of the *cop1-4* mutant under the same growth conditions (Fig. 3).

Another strong difference between *cop1^{eid6}* mutants and their respective background lines was observed for flower induction. Under short-day conditions (8 h white light: 16 h darkness), *cop1^{eid6} phyB-5* and *cop1^{eid6}* always exhibited an early flowering phenotype (Table II). Under long-day conditions (16 h of white light and 8 h of darkness), no difference in the induction of flowering was observed between *cop1^{eid6} phyB-5* and *phyB-5* mutants, whereas *cop1^{eid6}* flow-

ered earlier when compared with its *Ler* wild-type background. Even though flowering starts much earlier in both *cop1^{eid6}* lines, a weak effect of the applied photoperiod still remains because plants flower a little bit earlier under long-day conditions. *cop1-4* also exhibited an early flowering phenotype under short- and long-day conditions, but no clear difference between the two photoperiods was detectable (Table II).

Anthocyanin Accumulation

Anthocyanins accumulate in Arabidopsis seedlings under continuous far-red and blue light absorbed by phyA and blue light photoreceptors (Kunkel et al., 1996; Neff and Chory, 1998; Poppe et al., 1998). Anthocyanin accumulation was measured spectroscopically after extraction from wild-type, *cop1-4*, *cop1^{eid6}*, and *cop1^{eid6} phyB-5* seedlings grown under different light conditions. No anthocyanin was detectable in dark-grown seedlings (Fig. 4A). All examined *cop1* mutants showed a light-dependent anthocyanin accumulation. Under strong continuous blue light, *cop1^{eid6}* accumulated a more than 5-fold higher anthocyanin level when compared with its *Ler* wild type, whereas *cop1-4* exhibited the same response as seen with the corresponding *Col* background. Surprisingly, anthocyanin accumulation was detected under continuous red light in *cop1^{eid6}* and *cop1-4* seedlings (Fig. 4A), whereas both wild types exhibited no response under this light quality. This red light effect seems to be partially dependent on the presence of phyB because the *cop1^{eid6} phyB-5* double mutant exhibited a reduced anthocyanin level compared with *cop1^{eid6}*.

The highest level of anthocyanin accumulation was observed in the *cop1^{eid6} phyB-5* double mutant under strong continuous far-red light (Fig. 4B). *Ler*, *Col*, and *cop1-4* mutants reached only about one-half of the level observed for the double mutant (Fig. 4B). To further analyze this effect, fluence rate response curves were measured for anthocyanin accumulation under continuous far-red light. *cop1^{eid6}* clearly exhibited an increased sensitivity, whereas *cop1-4* again behaved very similarly to its wild type. The highest sensitivity was seen for the *cop1^{eid6} phyB-5* double mutant. Thus, phyB seems to reduce the effect of phyA on the regulation of anthocyanin accumulation.

Table II. Flowering time under short- and long-day conditions

Values are means \pm SD.

Plants	Short Day		Long Day	
	Rosette leaf no.	Days until flowering	Rosette leaf no.	Days until flowering
<i>phyB-5</i>	16 \pm 3	54 \pm 3	5 \pm 1	27 \pm 4
<i>cop1^{eid6} phyB-5</i>	7 \pm 1	33 \pm 1	5 \pm 2	28 \pm 4
Ler wild type	46 \pm 5	59 \pm 3	11 \pm 2	32 \pm 6
<i>cop1^{eid6}</i>	8 \pm 1	36 \pm 2	5 \pm 1	33 \pm 4
Col wild type	47 \pm 3	56 \pm 2	13 \pm 2	32 \pm 5
<i>cop1-4</i>	10 \pm 2	35 \pm 2	9 \pm 2	30 \pm 4

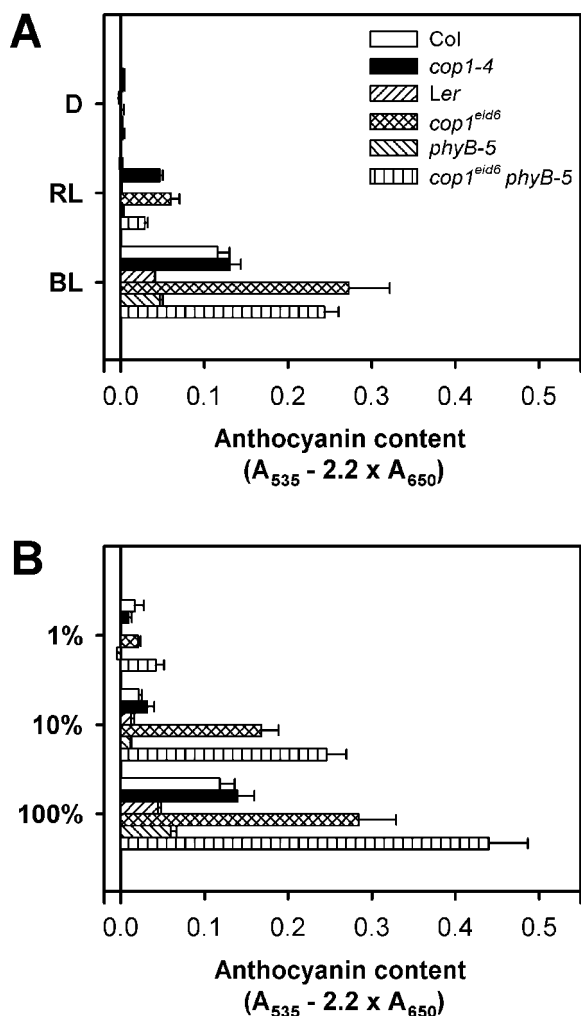


Figure 4. Analysis of anthocyanin accumulation under different light conditions. Seedlings were grown under different light conditions for 3 d after induction of germination before harvesting and anthocyanin extraction. Anthocyanin contents of different single and double mutants and their respective wild types are given. Error bars = SE of the mean. A, Anthocyanin content of seedlings kept in darkness (D) and under strong continuous red (RL; $14.3 \mu\text{mol m}^{-2} \text{s}^{-1}$) or blue light (BL; $38.5 \mu\text{mol m}^{-2} \text{s}^{-1}$). B, Anthocyanin content of seedlings irradiated with different fluence rates of far-red light: 100%, $14 \mu\text{mol m}^{-2} \text{s}^{-1}$; 10%, $1.4 \mu\text{mol m}^{-2} \text{s}^{-1}$; 1%, $0.14 \mu\text{mol m}^{-2} \text{s}^{-1}$.

Most *cop1* alleles also exhibit a *fusca* phenotype (Miséra et al., 1994) that is characterized by a purple seed color caused by an enhanced anthocyanin accumulation in the embryo and the seedling. Both *cop1^{eid6}* and *cop1-4* did not show such a phenotype (data not shown).

Hypocotyl Inhibition in *cop1^{eid6}* Is Extraordinarily Hypersensitive toward Light

To further test the influence of light on the *cop1^{eid6}* mutant, fluence rate response curves for the inhibition of hypocotyl elongation were determined under continuous red and far-red light (Fig. 5A). Both the

cop1^{eid6} mutant and the *cop1^{eid6} phyB-5* double mutant showed a maximum hypocotyl inhibition even at the lowest fluence rates of red light used in the experiments. Thus, *cop1^{eid6}* was at least 4 orders of magnitude more sensitive compared with its Ler wild type. The hypocotyls of *cop1-4* remained short even in darkness. Nevertheless, high fluence rates of red light were still able to cause a further reduction of hypocotyl length. Interestingly, further reduction of hypocotyl elongation started at about the same fluence rate as seen for the wild-type background. Thus, the red light HIR seems to be unaltered in *cop1-4*.

In continuous far-red light, *cop1^{eid6}* exhibited a clear log linear relationship between the photon fluence rate and the inhibition of hypocotyl elongation over 5 orders of magnitude (Fig. 5B). The *cop1^{eid6}* mutants

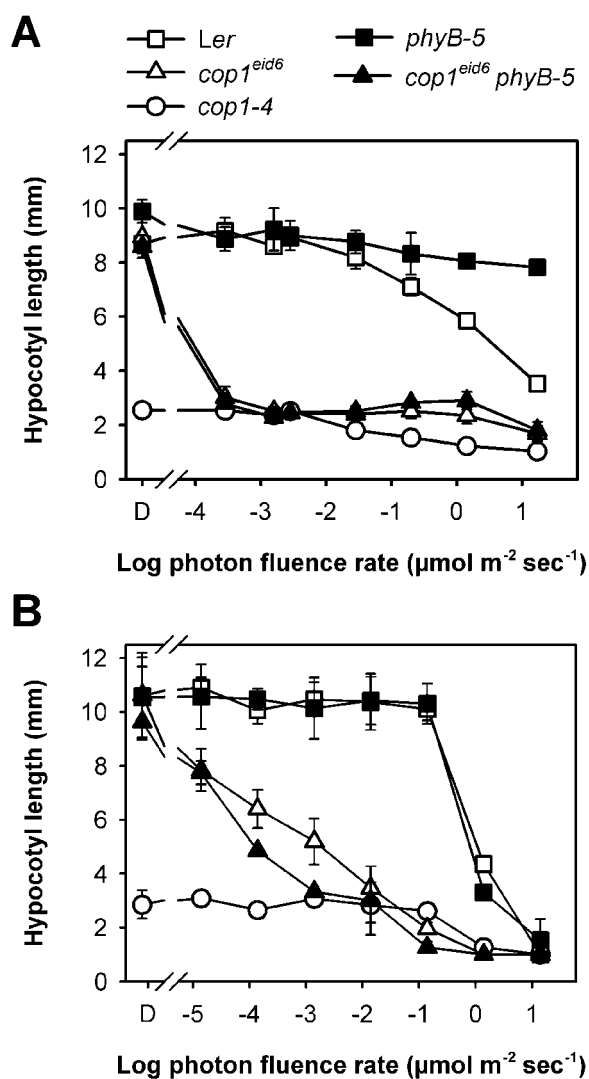


Figure 5. Inhibition of hypocotyl elongation under different light conditions. Seedlings were irradiated at different fluence rates for 3 d after induction of germination. Error bars = SE of the mean. A, Hypocotyl elongation under continuous red light. B, Hypocotyl elongation under continuous far-red light.

were about 10,000 times more sensitive toward continuous far-red light compared with their corresponding background lines. Again, *cop1-4* seedlings were short in darkness. A further inhibition of hypocotyl elongation was observed starting at about the same fluence rate that can induce an HIR in the respective wild type.

DISCUSSION

In this study, we describe the identification and characterization of *cop1^{eid6}*, a mutant that exhibits an extremely increased light sensitivity. The mutated gene was identified by mapping analyses, allelism test after crossing with the *cop1-4* mutant, and sequencing of the respective gene. These data clearly indicate that the phenotype of the mutant is caused by a point mutation in the *COP1* gene. In contrast to all other known *cop1* alleles, the *cop1^{eid6}* mutation does not lead to a constitutive photomorphogenic phenotype. Thus, the induction of photomorphogenesis in the *cop1^{eid6}* mutant remains strictly light dependent.

The lack of a dark phenotype indicates that *cop1^{eid6}* encodes a partially active COP1 protein. The activity of the mutated protein is obviously sufficient for the suppression of a photomorphogenic seedling development in darkness, but even minimal inputs from the photoreceptor systems can overcome the remaining COP1 activity. The remaining activity in darkness would also explain the partial dependence of flower induction on the photoperiod in *cop1^{eid6}* because its reduced activity might still be able to inhibit flower induction under long-night (short-day) conditions.

Because there is no visible phenotype in darkness, the *cop1^{eid6}* mutant is a good tool to study the interaction between COP1 and the different photoreceptors, which might function upstream of the protein. To date, this kind of analysis has been very difficult because known *cop1* mutants are either lethal (*cop1-5*), show no light response (*cop1-1*), or show just a weak response (*cop1-4*) that is difficult to measure (McNellis et al., 1994a). With the *cop1^{eid6}* mutant, we were able to examine the COP1-dependent light regulation in the presence or absence of phyB under different light qualities. The clear difference in the adult phenotype of *cop1^{eid6}* and *cop1^{eid6} phyB-5* plants and the increased anthocyanin accumulation under continuous blue and red light confirm former data by McNellis et al. (1994b) and Ang and Deng (1994), which demonstrated that COP1 functions downstream of phyB and blue light photoreceptors. The strong phyB effect in adult *cop1^{eid6}* plants is most probably due to a generally increased competence toward signals from this photoreceptor at elder stages of Arabidopsis development. Although adult *phyB* null mutants exhibit drastic changes in their morphology and in flowering time under normal light/dark conditions, all other photoreceptor mu-

tants show more subtle changes, which only become detectable under specific light treatments (Whitelam and Devlin, 1997; Neff et al., 2000; Lin, 2002).

In most Arabidopsis ecotypes, light-dependent anthocyanin accumulation can only be induced by blue or UV light and by a phyA-dependent far-red light response (Kunkel et al., 1996; Neff and Chory, 1998; Poppe et al., 1998). In contrast, both *cop1* alleles exhibited a red light-inducible accumulation of anthocyanin, which was absent in the respective wild types. The results for the *cop1^{eid6} phyB-5* double mutant further indicate that this altered response is partially dependent on the presence of phyB. Therefore, the lack of a red light-induced anthocyanin accumulation in wild-type seedlings seems to be caused by an active, COP1-dependent suppression of signals and not by a general lack of competence toward input signals from phyB or other red light-sensing phytochromes.

The fluence rate response curves for hypocotyl elongation and anthocyanin accumulation under continuous far-red light clearly indicate that the *cop1^{eid6}* allele alters both phyA-dependent HIRs. In *cop1-4*, far-red light-dependent anthocyanin accumulation remains unaltered, and the onset of further reduction of hypocotyl elongation starts at about the same fluence rates as seen with the wild type. Thus, the truncated COP1-4 protein, which carries functional RING finger and coiled-coil domains, seems to be sufficient for a normal function in phyA-dependent HIR. Hoecker and Quail (2001) demonstrated that SPA1, an inhibitor of phyA-specific light responses (Hoecker et al., 1998, 1999), interacts with the coiled-coil domain of COP1. The truncated coiled-coil domain of SPA1 can also enhance the ubiquitin ligase activity of COP1 toward LAF1 (Seo et al., 2003). Because SPA1 also carries WD40 repeats, it is worthwhile to speculate that these repeats might replace the missing WD40 repeats of COP1 and that SPA1 is responsible for the phyA-specific reactions of a COP1-SPA1 ubiquitin ligase complex.

The *cop1^{eid6}* mutation might cause a more general effect compared with *cop1-4* because it most probably alters the interaction with E2-ubiquitin conjugates in all COP1-dependent light reactions. The *cop1^{eid6}* mutation results in an exchange of a conserved His by a Tyr residue in the RING finger domain of COP1. Together with its zinc cofactor, the RING finger domain is responsible for the interaction with the E2-ubiquitin conjugates, which are used to transfer the ubiquitin moiety to target proteins (Jackson et al., 2000; Tyers and Jorgensen, 2000). COP1 is able to catalyze self-ubiquitylation, and the ubiquitylation of LAF1 and ubiquitin ligase activity was abolished by mutations in two conserved Cys residues of the RING finger domain (Seo et al., 2003). His-69 mutated in *cop1^{eid6}* is an important residue in the zinc-binding pocket of COP1. Its replacement by a Tyr residue might result in a partially active ubiquitin

ligase because the aromatic ring of this amino acid might still bind to the cofactor, although with reduced affinity. This partial activity is probably sufficient for the degradation of target proteins in darkness but might be too low to compensate for higher protein levels resulting from light-induced gene expression or for reduced affinities of COP1 substrates caused by light-dependent modifications. Alternatively, the mutation might lead to an increased auto-ubiquitylation rate, which should result in a more rapid turnover in the light.

The amino acid transition in the *cop1^{eid6}* RING finger domain might also change the subcellular localization of the protein because the altered region overlaps with the cytoplasmic localization signal and is close to a subnuclear localization motif (Stacey and von Arnim, 1999; Stacey et al., 1999). Thus, the mutated protein might be excluded more rapidly from its nuclear targets under light irradiation. The mutation might also alter the interaction with target proteins or with the phyB and cryptochrome photoreceptors, although it seems less plausible because these interactions are mainly restricted to the coiled-coil domain and the WD40 repeats (Hardtke et al., 2000; Osterlund et al., 2000; Hoecker and Quail, 2001; Wang et al., 2001; Yang et al., 2001; Holm et al., 2002).

MATERIALS AND METHODS

Plant Material, Growth Conditions, Light Sources, and Screening Program

The following ecotypes and mutants were used: Col, *Ler* (both obtained from Lehle Seeds, Tucson, AZ), *phyB-5*, *phyB-9* (Reed et al., 1993), and *cop1-4* (McNellis et al., 1994a).

For seedling analysis, seeds were sown on four layers of water-soaked filter paper in clear plastic boxes. A 48-h cold treatment at 4°C in darkness was followed by induction of germination for 2 h in white light and 22 h in darkness at 25°C. Afterward, the boxes were placed in the different light conditions for an additional 72 h. The used light sources and the performance of the screening are described by Büche et al. (2000).

Genetic Analysis

To obtain *cop1^{eid6}* single mutants, *cop1^{eid6} phyB-5* was crossed with *Ler* wild type, and F₁ plants were allowed to self-fertilize. F₂ plants were analyzed using a CAPS marker (Konieczny and Ausubel, 1993). Genomic DNA was analyzed for the presence (*phyB-5*) or absence (*PHYB*) of a BsaBI restriction site in a *PHYB*-specific PCR product obtained by using the primers 5'-CGTGACGCGCTGCTGGAATTGTT-3' and 5'-TCCATTGATGCAGCTCCGGA-3'. Homozygous *PHYB* plants were self-pollinated, and the phenotype of the F₃ generation was analyzed in weak red light (15 nmol m⁻² s⁻¹) and under the screening program to isolate homozygous *PHYB cop1^{eid6}* lines. A derived CAPS (Neff et al., 1998) marker was developed to detect the *cop1^{eid6}* mutation. PCR was performed with the primers 5'-ATTAAGATGCTTCCCTACGCTTGTTGTT-3' and 5'-AAAGCTAAGGACCAAACACAAATTACGAGT-3. This primer combination creates an AhdI restriction enzyme digestion site in the wild-type *COP1* gene but not in *cop1^{eid6}*. The *COP1* dCAPS marker was used to confirm cosegregation between the *Eid6* phenotype and the mutation in the respective *COP1* allele.

Mapping and Sequencing

The *cop1^{eid6} phyB-5* mutant (*Ler*) was crossed with *phyB-9* (Col) for mapping analysis. Seedlings of the F₂ generation that showed a de-etiolated

phenotype in weak red light (15 nmol m⁻² s⁻¹) were transferred to soil. DNA was extracted from leaf tissue of individual F₂ progenies according to Sawa et al. (1997). Mapping was performed using simple sequence length polymorphism markers and CAPS markers (Konieczny and Ausubel, 1993; Bell and Ecker, 1994). The phenotype of F₃ seed batches was tested to confirm homozygosity of the individual F₂ plants used for mapping. In total, 90 F₂ plants were analyzed.

The coding region of the *COP1* gene was amplified from genomic DNA as two fragments using the following pairs of oligonucleotides: 5'-CAA-AAACCAAATCACAATCGAAGAAATC-3' with 5'-ACCGTACCGAAGAGAAGTCAAAAACCTT-3' (5'-fragment) and 5'-CGTGATGATGAGC-TGTTGCCACTGCTG-3' with 5'-CATGACCGATTACATCACCGCAT-TTTGAT-3' (3'-fragment). The fragments were subcloned into pBluescript KS vector (Stratagene, Amsterdam) using several internal restriction enzyme digestion sites. Sequencing was carried out at the DNA sequencing facility at the University of Freiburg (Germany) with the T₃ and T₇ sequencing primers.

Measurement of Hypocotyl Elongation and Determination of Anthocyanin Accumulation

Hypocotyl lengths were determined manually. Experiments were repeated at least three times independently. Means of three times (20 seedlings for each experiment) are shown.

For anthocyanin extraction, 60 seedlings were transferred to extraction buffer (17% [v/v] 1-propanol and 1% [v/v] concentrated HCl). The samples were boiled in a water bath for 1 min and cooled down on ice. After shaking overnight at 8°C in darkness, samples were centrifuged at 10,000g for 10 min. Anthocyanin content was determined spectroscopically as described by Schmidt and Mohr (1981). Data represent the mean of at least three independent experiments.

Detection of Flowering Time

Seeds were sown on soil and incubated for 2 d in darkness at 4°C. Afterward, they were transferred to climate chambers with either short-day (25°C, 8 h of white light and 16 h of darkness) or long-day (25°C, 16 h of white light and 8 h of darkness) conditions. Flowering time was recorded as the number of days and rosette leaves from the time when the seeds were transferred to light until the opening of the first flower bud. The experiment was repeated two to three times using six to 15 plants from each genotype in each experiment.

Distribution of Materials

Upon request, seeds of mutants will be made available in a timely manner for noncommercial research purposes. No restrictions or conditions will be placed on the use of any materials described in this paper that would limit their use in noncommercial research purposes.

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