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COP1b, an isoform of COP1 generated by alternative splicing, has a negative effect on COP1 function in regulating light-dependent seedling development in Arabidopsis

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Abstract COP1 is a negative regulator of *Arabidopsis* light-dependent development. Mutation of the *COP1* locus causes constitutive photomorphogenesis in the dark. Here, we report the identification of an isoform of the COP1 protein, named COP1b, which is generated by alternative splicing. COP1b has a 60-amino acid deletion in the WD-40 repeat domain relative to the full-length COP1. This splicing step is light-independent and takes place mostly in mature seeds and in germinating seedlings. Transgenic Arabidopsis plants that overexpress COP1b show a de-etiolated phenotype in the dark, with a short hypocotyl, open and developed cotyledons. The transgenic seedlings are adult-lethal. These phenotypes closely resemble that of severe *cop-1* mutants, indicating that COP1b has a dominant negative effect on COP1 function.

Key words Photomorphogenesis · COP1 · Alternative splicing · Seedling development · *Arabidopsis thaliana*

Introduction

Light as a signal plays a critical role in regulating plant growth and development. Light signals perceived by specific photoreceptors (phytochromes, blue and UV light receptors) are transduced via largely unknown signaling components to trigger diverse downstream physiological responses, including seed germination, stem elongation, chloroplast and leaf development, floral induction, and expression of many nuclear and chloroplast genes (Kendrick and Kronenberg 1994). In the light, Arabidopsis seedlings have short hypocotyls, no apical hooks, expanded cotyledons with developed chloroplasts, differentiated cell types and show a dramatic induction of the transcription of many genes. In the absence of light, this photomorphogenic pathway of seedling development is repressed. Instead, the seedlings have elongated hypocotyls, apical hooks, and small and unopened cotyledons with etioplasts. The cells are largely undifferentiated, with little or no expression of the light-induced genes.

Genetic analyses of Arabidopsis seedling development have led to the identification of a class of light regulatory loci, mutation of which results in constitutive photomorphogenic responses in the absence of light, with aberrant induction of many photosynthetic genes in the dark and in inappropriate tissues, indicating that the corresponding wild-type loci encode repressors that inhibit photomorphogenesis of Arabidopsis seedlings in the dark (reviewed in Chory 1993; Deng 1994). These loci include the genes of the *De-et*iolated (*DET*), *co*nstitutive *p*hotomorphogenic (*COP*) and embryonic purple color *FUSCA* (*FUS*) series. Because these mutations are recessive, and their effects are pleiotropic, it is thought that the wild-type gene products act negatively in the dark to repress photomorphogenesis, and that the repression is inactivated by light. Epistatic analyses have demonstrated that these loci act downstream of phytochromes and blue-light receptors in the light signaling pathways (Ang and Deng 1994; Miséra et al. 1994).

Sequence analysis has shown that the *COP1* gene contains 13 exons, coding for a 76-kDa protein with a zinc ring finger at the N-terminus followed by a putative coiled-coil structure and a large domain that is homologous to the β subunit of trimeric G-proteins (Gβ) at the C terminus (McNellis et al. 1994a). The Gβ homology domain consists of several WD-40 repeats, which are thought to be involved in protein-protein interactions of many regulatory proteins (Duronio et al. 1992). This

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domain seems to be functionally important, since most of the characterized copy mutations are localized in this region (McNellis et al. 1994a). Moreover, COP1 shares homology with *Drosophila* $TAF_{II}80$, a **T**ATA-box binding protein (TBP) **A**ssociated **F**actor found in the general transcription factor TFIID (Dynlacht et al. 1993); this suggests that COP1 may interact with the transcriptional initiation machinery to repress the transcription of relevant genes.

The transcription of the *COP1* gene itself does not seem to be regulated by light (Wei et al. 1994), suggesting that the effect of light on the inactivation of COP1 function occurs at a post-transcriptional level. Studies using a chimeric fusion protein between COP1 and the reporter protein β-glucuronidase (GUS) showed that the subcellular localization of COP1 in a few cell types is however light regulated (Von Arnim and Deng 1994).

In this report we show that the activity of COP1 is also regulated at the level of mRNA maturation. We have identified an alternatively spliced mRNA species of *COP1*, which is produced in mature seeds and in germinating seedling cotyledons. The protein product (named COP1b) of this mRNA has a 60-amino acid deletion in the WD-40 repeat domain. Over-expression of COP1b in transgenic Arabidopsis causes a dominant-negative effect, interfering with the ability of COP1 to suppress photomorphogenic development processes in the dark. The physiological function of COP1b in wild-type Arabidopsis is discussed.

Materials and methods

Primers, RNA samples and RT-PCR

Two PCR primer sets were used for RT-PCR in this work. The first primer set (5′-agatctATGGAAGAGATTTCGAC-3′ and 5′-gaattcGAATCACGCAGCGAGT-3′) was used to amplify the full-length *COP1* coding sequence. The second primer set (5′-TGGTAGTGACGACTGC, 5′-TCGTCCATGTCTGGCG), corresponding respectively to sequences in exons 10 and 12 was employed to detect the presence or absence of exon 11 in RNA samples extracted from different Arabidopsis organs.

For RNA extraction, wild-type or transgenic *A. thaliana* (ecotype Columbia 24) plants were grown in soil or in synthetic medium at 22° C in continuous light. RNA samples were prepared using the LiCl precipitation method.

For RT-PCR, 1 µg of total RNA was reverse-transcribed at 37° C for 60 min with 20 Units of SuperScript RT (BRL) using the manufacturer's recommended procedures. The reverse-transcribed products were then ethanol-precipitated and suspended in Taq reaction buffer in the presence of 1 Unit of Taq DNA polymerase (Appligene), 0.2 mM of each of the four deoxynucleotides and 0.1 µg of each primer. The reaction mixtures were heated at 95° C for 5 min and subjected to 40 cycles of 1 min at 94° C, 1 or 2 min at 60° C and 1 min at 72° C. After the reaction, aliquots of each mixture were loaded on agarose gels for electrophoresis.

Plasmids

For cloning the RT-PCR products, 1 µl of the PCR reaction was ligated with the pCR™II cloning vector (Invitrogen) using the supplier's protocol. The cloned inserts were then sequenced using either the manual dideoxy method or by using the ALF automatic sequencing machine (Pharmacia).

The translational fusion cassette GUS-COP1b was constructed by standard procedures and inserted into a plant transformation vector (pBI101, Clontech) to yield the plasmid pBIGB.

Transformation of Arabidopsis plants

Arabidopsis Columbia plants were transformed with pBIGB by either the root infection method or by in planta infiltration (Bechtold et al. 1993).

For GUS staining of seedlings, the samples were incubated in the dark or in the light at 28° C in staining buffer containing X-glucuronic acid (X-gluc) for 2 h before fixation with ethanol. Fluorometric measurements of GUS activity were carried out according to Jefferson et al. (1987).

Results

Identification of an alternatively spliced *COP1* mRNA species

In order to isolate COP1 cDNA, we performed reverse transcriptase-polymerase chain reaction (RT-PCR) assays on mRNAs from 6-day-old *A. thaliana* (Columbia ecotype) seedlings. The primers matched the ends of the translated region of COP1 cDNA (Fig. 1). The amplified products were analyzed by electrophoresis in an agarose

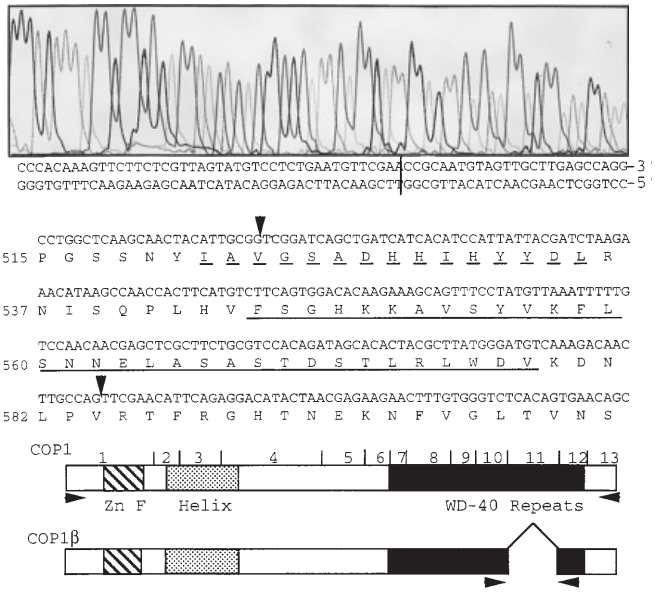

Fig. 1 Identification of an alternatively spliced COP1 mRNA species. In the *upper panel*, a portion of an automated sequencing profile of the 1.8-kb COP1 RT-PCR fragment is shown. The *vertical line* indicates the junction between exons 10 and 12. Exon 11 is missing. The *middle panel* shows sequences flanking exon 11 of COP1. The *arrowheads* indicate the boundaries of exon 11 with exons 10 and 12. One half and one full WD-40 repeat encoded by exon 11 are *underlined* by the *dashed* and *solid lines*, respectively. Schematic diagrams of COP1 and COP1b proteins are shown in the *lower panel*. The positions of the exons are indicated *above* COP1. The *chevrons* represent the primers used in the RT-PCR reactions

gel. Two discrete bands of 1.8 and 2.0 kb were identified (not shown). Both DNA fragments were cloned and sequenced. The 2.0-kb fragment, as expected, corresponded to the published COP1 cDNA (McNellis et al. 1994a), while the 1.8-kb fragment contained the same nucleotide sequence as COP1, except that 180 nucleotides were missing near the 3′ end (Fig. 1). This missing region corresponds exactly to exon 11, the excision of which does not disturb the reading frame through to exon 13 (Fig. 1). This indicates that the 1.8-kb fragment was generated from an alternatively spliced COP1 mRNA species. The 60-amino acid sequence encoded by exon 11 contain about 1.5 WD-40 repeats in the C-terminal domain (McNellis et al. 1994a) (Fig. 1). The protein product of the alternatively spliced mRNA is named here COP1b. Interestingly, the COP1b mRNA is identical to the transcript of the mutant allele *cop1-8*, which was initially identified as an embryonic purple color mutant *fusca* (Miséra et al. 1994; McNellis et al. 1994a).

Developmental regulation of the alternative splicing of exon 11

To determine whether the alternative splicing of exon 11 is regulated at different stages of Arabidopsis development, we analyzed RNA samples extracted from a variety of organs, including hypocotyls, cotyledons, leaves, stems, roots, flowers, siliques and mature seeds by RT-PCR assays. These assays were performed using a second set of primers (Fig. 1) corresponding to sequences in exons 10 and 12. The amplified product of COP1 mRNA should give rise to a DNA fragment of 467 bp, whereas the mRNA lacking exon 11 should produce a fragment of 287 bp. As shown in Fig. 2, the larger fragment was produced from all the RNA samples, confirming the constitutive transcription pattern of the *COP1* gene. In contrast, the smaller fragment was amplified only from RNA samples extracted from mature seeds and cotyledons or from whole young seedlings. No differences were observed between RNA samples extracted from either light- or dark-grown seedlings (Fig. 2). These results indicate that alternative splicing of exon 11 is developmentally regulated and light independent.

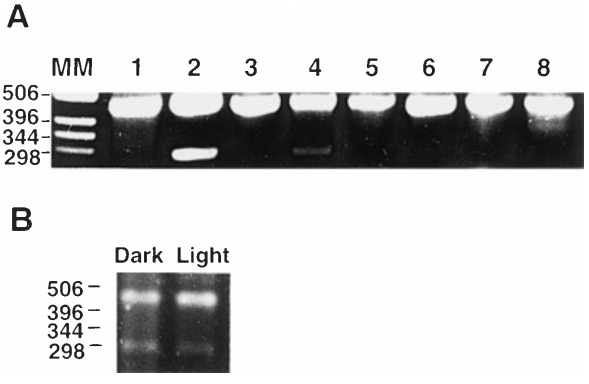

Fig. 2A, B Organ specificity (**A**) and light independence (**B**) of COP1b mRNA expression. RNA samples (1 μg) from rosette leaves (lane 1), mature seeds (2), hypocotyls (3), cotyledons (4), siliques (5), stems (6), roots (7) and flower buds (8) from 6-day-old whole seedlings grown in the dark or in the light (**B**) were amplified by RT-PCR using exon 10- and 12-based primers (see Fig. 1). The RT-PCR products were resolved on a 1.5% agarose gel. A band of 467 bases was amplified from the COP1 mRNA and a band of 287 bases was amplified from the COPb mRNA. MM, molecular size markers

Over-expression of COP1b in transgenic Arabidopsis produces constitutive photomorphogenic phenotypes in the dark

In order to study the function of COP1b, a CaMV 35S promoter-controlled translational fusion between the COP1b cDNA and the β-glucuronidase (GUS) coding region was introduced into *A. thaliana*. Since fusion with GUS does not seem to affect the function of COP1 (McNellis et al. 1994b), the GUS-COP1b fusion can be used to identify by GUS staining transformants that over-express COP1b. To generate GUS-COP1b-expressing lines, two transformation methods were used. Using the in vitro root infection method, many kanamycin-resistant calli showing high GUS activities were obtained (data not shown). During regeneration, most of the shoots appeared to be abnormally purple-colored (Fig. 3F). The majority of the regenerated plants died before maturation, while transgenic plants transformed with a control plasmid (pBI101) appeared normal (data not shown). Most surviving plants were sterile, but a few produced seeds. These results suggest that the over-expression of COP1b severely affects the regeneration of fertile mature plants from transformed calli. In order to overcome this difficulty, we used the in planta infiltration method (Bechtold et al. 1993), which allows direct production of transformed seeds. About 50 Arabidopsis (Columbia ecotype) plants were infiltrated and cultivated to maturation. Seeds from these plants were germinated on a germination medium in the dark without antibiotics. In a population of about 2000–3000 seedlings, about 40 showed a pronounced de-etiolated phenotype: short (or no) hypocotyl, open and developed cotyledons (Fig. 3A, B). To confirm that the de-etiolated seedlings expressed the fusion protein, about a quarter of them were stained with X-gluc for the detection of GUS activity. All these seedlings turned dark blue within 2 h at 28° C, indicating that the GUS-COP1b fusion was expressed in these de-etiolated plants (Fig. 3D, E). In contrast, no staining was detected among the etiolated normal plants (not shown).

In order to determine whether these de-etiolated phenotypes were produced as a result of a co-suppression event, we analysed the COP1 mRNAs in transgenic seedlings by RT-PCR. As shown in Fig. 4, both the full-length COP1 and the over-expressed COP1b mRNAs were present in the transgenic seedlings. These data

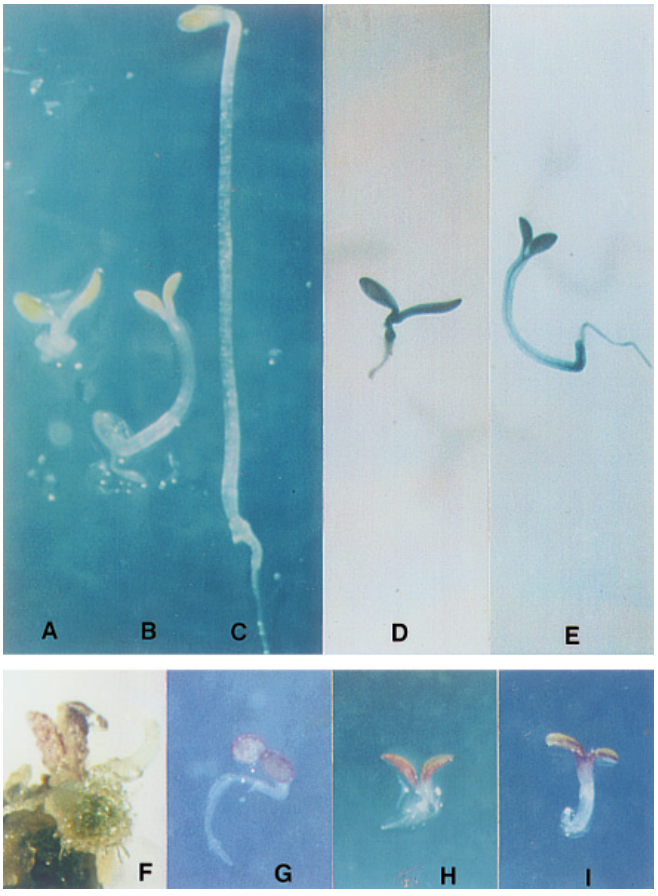

Fig. 3A–I Transgenic Arabidopsis plants overexpressing the GUS-COP1b fusion show a de-etiolated phenotype in the dark and accumulate in the light. *Upper panel* Comparison between two transgenic plants (**A**, **B**) and a wild-type plant (**C**) grown in the dark. Strong GUS staining of plant A and plant B is shown in **D** and **E**, respectively. *Lower panel* Accumulation of anthocyanin in regenerating transgenic shoots (**F**) and in the GUS-COP1b-overexpressing de-etiolated seedlings (G–I) 24 h after being transferred to the light

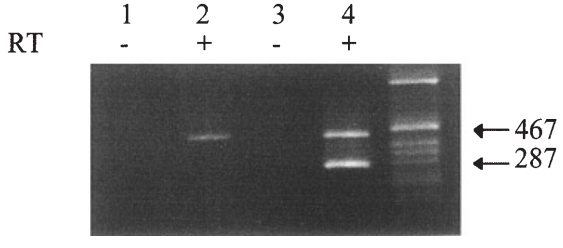

Fig. 4 Detection of both the full-length COP1 and the over-expressed COP1b mRNA in transgenic Arabidopsis plants showing de-etiolated phenotypes in the dark. Total RNAs extracted from wild-type Arabidopsis leaves (lanes 1 and 2) or pooled pBIGUS-COP1b transgenic Arabidopsis seedlings (lanes 3 and 4) were analysed by RT-PCR with the same primers as used in Fig. 2. In lanes 1 and 3, no reverse transcriptase was added to the reactions

indicate that the de-etiolated phenotype observed in the dark-grown transgenic plants was caused by the over-expression of the GUS-COP1b fusion, and is therefore unlikely to result from a co-suppression event. With regard to hypocotyl length, these transgenic plants are

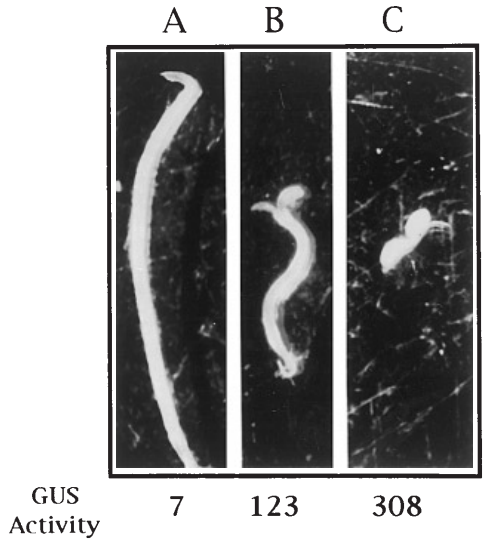

Fig. 5A–C The degree of dark de-etiolation of the transgenic plants is related to the expression level of the GUS-COP1b fusion protein. Wild-type (**A**) and transgenic seedlings with severe (no hypocotyl, **C**) or moderate (short hypocotyl, **B**) de-etiolated phenotypes were pooled, respectively. GUS activities of pooled samples were determined by fluorometric measurements

quite heterogenous (Figs. 3 and 5). This may be due to different expression levels of the GUS-COP1b fusion.

To confirm this hypothesis, transgenic plants with severe de-etiolated phenotypes (no hypocotyl; Fig. 5C) and plants with hypocotyls that were shorter than those of wild-type plants (Fig. 5B) were pooled separately for protein extraction. GUS activities from those samples were measured by fluorometry. About 2.5 times more GUS activity was observed in plants without hypocotyls than in those with short hypocotyls (Fig. 5), showing that the degree of de-etiolation in the dark of the transgenic plants is related to the expression level of the transgene. This reinforces our conclusion that the de-etiolated phenotypes in the transgenic plants were provoked by the over-expression of COP1b. The remaining de-etiolated transgenic plants were then grown under normal conditions (16 h light/8 h dark). After 24 h under these conditions, all these plants became purple-colored (Fig. 3G–I), while the normal, etiolated plants turned green (not shown). The de-etiolated plants stopped developing at the cotyledonous stage, without any leaf initiation. Later, these plants died. These phenotypes in the COP1b-overexpressing plants are very similar to that of the *cop1* lethal mutants, indicating that the over-expression of COP1b severely impairs the function of *COP1*, resulting in suppression of photomorphogenic development in the dark.

Discussion

The transcription of the *COP1* gene does not seem to be regulated at different developmental stages nor by different light regimes (Wei et al. 1994). It would be

interesting to find out the mechanism by which the COP1 activity is regulated. The finding that the subcellular translocation of COP1 is regulated by light (Von Arnim and Deng 1994) may represent one mode of regulation of COP1 function. The results presented in this paper have revealed that *COP1* gene expression itself is post-transcriptionally regulated by alternative splicing. Although the spliced mRNA species represents only a portion of the total *COP1* mRNA (Fig. 2), the protein product of this mRNA species (COP1b) can impair the function of full-length COP1, thus suppressing photomorphogenic development in the dark. This suggests that the amount of COP1b mRNA normally produced may, at least partially, regulate COP1 function. In addition, it cannot be excluded at this stage that the alternative splicing event might be restricted only to certain cell types and might consequently increase the COP1b/COP1 ratio in that cell type.

The photomorphogenic phenotypes observed in dark-grown COP1b-overexpressing transgenic plants very much resemble the phenotypes of the adult-lethal *cop1* mutations (McNellis et al. 1994a), suggesting that COP1b may function as an antagonist of COP1. Interestingly, the COP1b mRNA actually corresponds to the mRNA species of the mutant allele *cop1-8* (McNellis et al. 1994a). *cop1-8* was initially identified as an embryo-defective mutant (*fus1*) (Miséra et al. 1994; McNellis et al. 1994a). In *fus1*, as in the other *fusca* mutants, one characteristic phenotype is the high level of anthocyanin accumulation in both the embryo and the seedling; other photomorphogenic traits are also expressed in the dark (Miséra et al. 1994). Based on the observation of the adult-lethal phenotype, it has been postulated that *cop1-8* was a null mutation (McNellis et al. 1994a). However, one cannot rule out the possibility that the product of *cop1-8*, clearly identified as a 66-kDa protein in the mutant (McNellis et al. 1994a), actually plays a role in the cell. The results of our COP1b overexpression experiments provide evidence that COP1b has a function, strongly arguing against the notion that *cop1-8* is a null mutation.

The negative-dominant effects of COP1b on COP1 function in transgenic plants could arise in two possible ways. COP1b may interact directly with COP1 and sequester it in the cytoplasm. It has been shown using two-hybrid assays that an N-terminal fragment (N282) of COP1 can interact with itself and, less efficiently, with the full-length COP1 in yeast (McNellis et al. 1996). Having only the 60-amino acid deletion in the WD-40 repeat domain, COP1b could also compete with COP1 for partners and sequester them in the cytoplasm, since it appears that COP1 interacts with other cellular proteins (Matsui et al. 1995). Since the COP1b-overexpressing transgenic plants were adult-lethal in the light, one cannot rule out the possibility that COP1b might function independently in the presence of the full-length COP1 and might indeed act through a distinct mechanism.

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