The Plant Cell, Vol. 5, 329-339, March 1993 © 1993 American Society of Plant Physiologists

A New Class of Arabidopsis Constitutive Photomorphogenic Genes Involved in Regulating Cotyledon Development

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Light signals have profound effects on morphogenesis of hypocotyls and cotyledons of Arabidopsis seedlings, but the mechanisms by which light signals are transduced and integrated to control these processes are poorly understood. We report here the identification of a new class of constitutive photomorphogenic (*cop*) mutants, *cop2*, *cop3*, and *cop4*, in which dark-grown seedlings have open and enlarged cotyledons resembling those of light-grown wild-type seedlings. The epistatic relationships of these three mutations to previously characterized phytochrome-deficient mutations suggest that *COP2*, *COP3*, and *COP4* may act downstream of phytochrome in the light regulatory pathway. Mutations in each of the three loci alleviate the normal inhibition of cell-type differentiation, cell enlargement, and lateral cell division observed in cotyledons of dark-grown wild-type seedlings, but do not affect plastid differentiation. The *cop4* mutation also leads to high-level dark expression of nuclear, but not plastid-encoded, light-inducible genes. We further show that for the nuclear *cab1* gene encoding a chlorophyll *a/b* binding protein of the photosynthetic light-harvesting complex, activation in dark-grown *cop4* mutants is achieved by modulation of promoter activity. Interestingly, *COP4* modulates *cab1* promoter activity through a pathway distinct from that of *COP1* and *COP9*. Furthermore, *cop4* mutants are defective in both root and shoot gravitropic responses, indicating that the *COP4* locus may be involved in both light-signaling and gravity-sensing processes.

INTRODUCTION

As typical dicotyledonous plants, Arabidopsis seedlings are capable of two distinct developmental strategies, skotomorphogenesis in darkness and photomorphogenesis in the light (Mohr and Shropshire, 1983; Kendrick and Kronenberg, 1986; Adamse et al., 1988). The most striking differences between dark-grown and light-grown seedlings are the morphologies of hypocotyls and cotyledons. Dark-grown Arabidopsis seedlings have long hypocotyls consisting mostly of undifferentiated and elongated cells, whereas hypocotyls of light-grown seedlings have much shorter cells with clear cell-type differentiation (Deng et al., 1992). In addition, dark-grown seedlings have small and unopened cotyledons, which are retarded in cell-type differentiation and contain etioplasts. In contrast, light-grown Arabidopsis seedlings have open and enlarged cotyledons with clear cell-type differentiation, such as mature stomatal structures and functional chloroplasts. Finally, there are dramatic differences in the pattern of gene expression between the dark-grown and light-grown plants (Gilmartin et al., 1990; Thompson and White, 1991). At least three photoreceptors, phytochrome, a blue light receptor (also called cryptochrome), and a UV light receptor, are utilized to perceive the light signals and mediate light-regulated processes (Kendrick and Kronenberg, 1986; Gilmartin et al., 1990; Quail, 1991; Young et al., 1992).

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Two genetic approaches have been used to identify genes that play key regulatory roles in light-regulated seedling development. One approach has been to isolate mutants that show dark-grown morphology when germinated in the light (Koornneef et al., 1980; Liscum and Hangarter, 1991; Chory, 1992). These mutants, including six *hy* loci and three *blu* loci, develop long hypocotyls in the light. Mutants in three of these loci (*hy1*, *hy2*, and *hy6*) are deficient in functional phytochrome due to a defect in the biosynthesis of the phytochrome chromophore (Parks and Quail, 1991; Chory, 1992). Mutations at the *hy3* locus cause a specific reduction in type B phytochrome (Somers et al., 1991). *hy4*, *blu1*, *blu2*, and *blu3* affect blue light-specific responses (Koornneef et al., 1980; Liscum and Hangarter, 1991). *hy5* mutants appear to be deficient in both phytochrome and blue light receptor-mediated responses (Koornneef et al., 1980).

A complementary approach has been to isolate mutants that show a light-grown morphology when germinated in the dark. Mutations in four different loci producing dark-grown seedlings with both a short hypocotyl and open and enlarged cotyledons have been described previously (*de*etiolated-1 [*det1*] and *det2*, Chory et al., 1989, 1991; *c*onstitutive *p*hotomorphogenic-1 [*cop1*], Deng et al., 1991; Deng and Quail, 1992; *cop9*, Wei and Deng, 1992). Recently, we have shown that the *COP1* locus encodes a novel protein with both a putative zinc binding motif and a domain homologous to the β subunit of the trimeric

G-protein (Deng et al., 1992). Because all above-mentioned mutants are affected in both hypocotyl and cotyledon development, they may be involved in early steps of the light regulatory pathway. However, no mutant that only affects hypocotyl or cotyledon development in the dark has been described.

To identify regulatory components functioning further downstream in the light regulatory pathway, it would be useful to identify mutations that only affect a subset of photomorphogenic responses. Toward this end, we have initiated a systematic screening for Arabidopsis mutants that, when grown in the dark, have open and enlarged cotyledons with normal hypocotyl development. We report here the identification and characterization of three such mutants that define three new *COP* loci.

RESULTS

Mutant Isolation

Ethylmethane sulfonate–mutagenized M_2 seeds were germinated and screened for dark-grown seedlings with open and enlarged cotyledons, but with a hypocotyl approximately normal in length, as shown in Figure 1. Three such mutants were isolated and designated as *cop2*, *cop3*, and *cop4* for their constitutive photomorphogenic phenotype. Segregation and complementation tests shown in Table 1 demonstrated that they are single gene recessive mutations at three different genetic loci. Light-grown *cop2*, *cop3*, and *cop4* mutants are nearly indistinguishable from the wild type in both seedling and adult stages, with the exception that the adult *cop4* mutants are smaller in size than wild-type plants of the same age (data not shown). Close examination of their dark-grown morphologies revealed that *cop2* and *cop3*, but not *cop4*, have rather long petioles in the base of their cotyledons, as shown in Figure 2.

Table 1. Phenotypic Segregation in the Progeny of Crosses between *cop* Mutants and Wild-Type Plants or between *cop* Mutant Pairs

Parental Genotype	Generation	Number of Seedlings: WT	Number of Seedlings: Mutant	WT/Mutant Ratio
cop2/cop2 × *COP2/COP2*	F_1	15	0	
	F_2	204	62	3.3:1
cop3/cop3 × *COP3/COP3*	F_1	23	0	
	F_2	162	48	3.4:1
cop4/cop4 × *COP4/COP4*	F_1	19	0	
	F_2	198	68	2.9:1
cop3/cop3 × *cop2/cop2*	F_1	26	0	
cop3/cop3 × *cop4/cop4*	F_1	32	0	
cop2/cop2 × *cop4/cop4*	F_1	28	0	

Seedling phenotypes were examined after growth for 6 days in the dark.
WT, wild type.

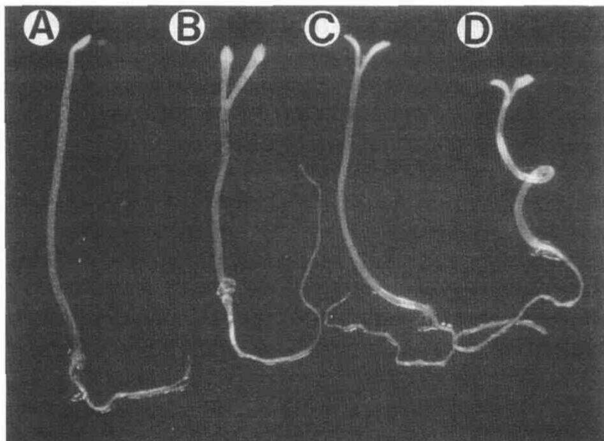

Figure 1. Morphologies of the Dark-Grown Wild-Type and *cop* Mutant Seedlings.

(A) Wild type.
(B) *cop2*.
(C) *cop3*.
(D) *cop4*.
Seedlings were grown in darkness for 6 days and photographed. The hypocotyl curling of the *cop4* mutant seedlings was observed very frequently, possibly due to the defect in gravitropism (see Figure 9).

COP2, *COP3*, and *COP4* May Act Downstream of Phytochrome

To examine the relationship of the new mutants to the phytochrome signaling pathway, we chose to analyze the interaction of the *cop* mutations and the phytochrome deficiency mutations (*hy1* and *hy3*). Double mutant lines homozygous for a *hy1* mutation and each of the three *cop* mutations were constructed, and their dark-grown and light-grown seedling phenotypes are summarized in Table 2. Whereas *hy1* mutants have long hypocotyls in the light and a wild-type phenotype in the dark, *cop2*, *cop3*, and *cop4* mutants all have open and enlarged cotyledons in the dark but a wild-type appearance in the light. Interestingly, the phenotype of each double mutant

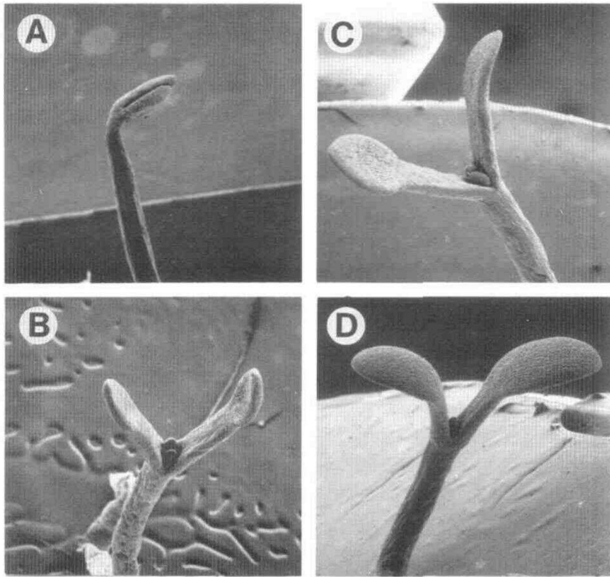

Figure 2. Morphogenetic Comparison of Cotyledon and Apical Hook Development of 6-Day-Old Dark-Grown Seedlings.

(A) Wild type.
(B) *cop2*.
(C) *cop3*.
(D) *cop4*.

The morphology of the upper part of the seedlings was examined by scanning electron microscopy, and the same magnification scale was used for all panels. All three *cop* mutants have open and enlarged cotyledons but no apical hook. The *cop2* and *cop3* mutants also have highly elongated cotyledon petioles.

tant is not a simple combination of the respective parental phenotypes. Instead, double mutant seedlings have the same dark-grown phenotype as the corresponding *cop* mutant. In the light, however, the *cop2*, *cop3*, and *cop4* mutations partially suppress the *hy1* mutation, as determined by their hypocotyl lengths. Among the three *cop* mutations, the *cop2* mutation shows the most complete suppression of the *hy1* mutation. These results suggest that the *cop2*, *cop3*, and *cop4* mutations act downstream of phytochrome during light-regulated seedling development. To further test this hypothesis, double mutant lines homozygous for *cop4* and *hy3*, the latter being deficient specifically in phytochrome B (Somers et al., 1991), were constructed and analyzed. As shown in Table 2, *cop4* is able to suppress the long hypocotyl phenotype of the *hy3* mutants and therefore acts downstream of phytochrome B. Taken together, those results suggest that the *COP2*, *COP3*, and *COP4* loci are involved in the light-regulated seedling development mediated through the phytochrome system.

Cellular Differentiation in *cop2*, *cop3*, and *cop4* Seedlings

To define the effects of the *cop2*, *cop3*, and *cop4* mutations on cellular differentiation during seedling development, hypocotyls and cotyledons of dark-grown mutants were examined by light and scanning electron microscopy (SEM). The SEM examination of the dark-grown *cop2*, *cop3*, and *cop4* hypocotyls showed highly elongated cells without cell-type differentiation (data not shown). This is similar to the phenotype of dark-grown wild-type seedlings and different from the phenotypes of dark-grown *cop1* mutants and light-grown wild-type plants (Deng et al., 1992).

Table 2. Phenotypes of Double Mutant Seedlings Containing Individual *cop* Mutations and the *hy1* or *hy3* Mutation[a]

Arabidopsis Lines	Independent Lines Analyzed[b]	Dark-Grown Phenotype	Light-Grown Hypocotyl Length (mm ± SD)[c]
WT[d] (Landsberg)	1	WT	1.66 (±0.43)
WT (Columbia)	1	WT	1.92 (±0.27)
hy1	1	WT	7.58 (±1.36)
cop2	1	*cop2*	1.15 (±0.38)
cop3	1	*cop3*	1.21 (±0.52)
cop4	1	*cop4*	1.56 (±0.33)
cop2/hy1	3	*cop2*-like	2.39 (±0.60)
cop3/hy1	3	*cop3*-like	4.29 (±0.73)
cop4/hy1	2	*cop4*-like	3.91 (±0.96)
hy3	1	WT	6.50 (±1.39)
cop4/hy3	2	*cop4*-like	2.58 (±1.14)

[a] Seedling phenotypes were examined after growth for 6 days.
[b] Only for the double mutants were two or three independent lines constructed and examined.
[c] Hypocotyl lengths are averages of at least 50 seedlings. In cases where more than one line was analyzed, equal numbers of seedlings (at least 50) from each line were used for hypocotyl measurements. Numbers in parentheses are calculated standard deviations.
[d] WT, wild type.

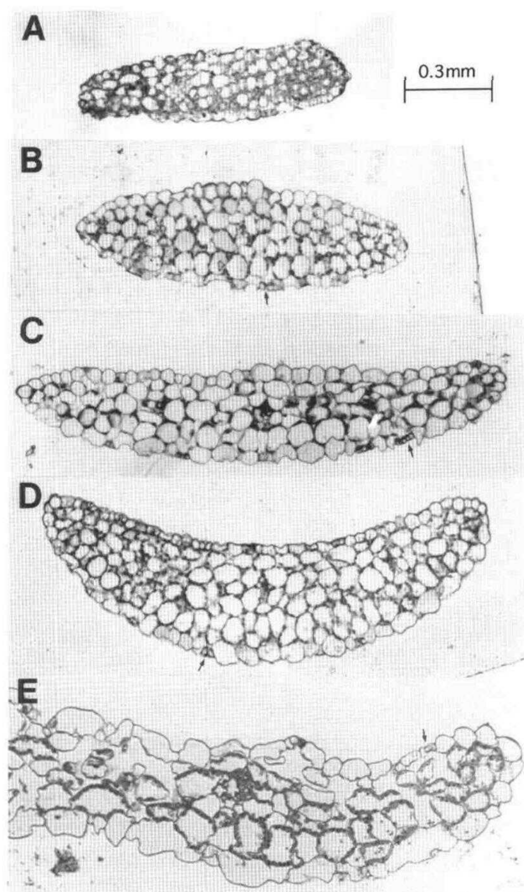

Figure 3. Morphogenetic Comparison of Cotyledon Cross-Sections from 6-Day-Old Wild-Type and Mutant Seedlings.

(A) Dark-grown wild type.
(B) Dark-grown *cop2*.
(C) Dark-grown *cop3*.
(D) Dark-grown *cop4*.
(E) Light-grown wild type.
The same magnification, shown in **(A)**, was used for all panels. Examples of stomatal structures are indicated by arrows in **(B)** to **(E)**.

Figure 3 shows that cell-type differentiation in dark-grown cotyledons of all three *cop* mutants is clearly more advanced than in the wild type. In the mutants (Figures 3B, 3C, and 3D), there is cell layer differentiation, clear cell-type differentiation in the epidermal cell layer, increased cell numbers per layer (due to lateral cell division), and cell enlargement. These advanced differentiation patterns are normally observed only in light-grown wild-type seedlings (Figure 3E) and are absent from dark-grown wild-type seedlings (Figure 3A). To examine whether mutations in the *COP2*, *COP3*, or *COP4* locus also result in mature stomatal structures in dark-grown seedlings as they do in dark-grown *cop1* seedlings or in light-grown wild-type plants, the epidermal surface cells of cotyledons were examined by SEM, as shown in Figure 4. When compared to dark-grown wild-type cotyledons, there are clear cell size differences between guard cells and epidermal cells in all three mutants. However, both dark-grown mutant and wild-type seedlings have similar "immature" stomatal structures. These properties are quite different from those of *cop1* mutants, which have mature stomatal structures with openings between guard cells (Deng et al., 1992).

The *cop2*, *cop3*, and *cop4* Mutations Have No Effect on Plastid Differentiation

In most higher plants, plastids in photosynthetically competent cells differentiate into etioplasts in darkness and chloroplasts in light (Kirk and Tilney-Bassett, 1978). To examine whether plastid differentiation is aberrant in *cop2*, *cop3*, and *cop4*, plastid morphology was examined by transmission electron microscopy. As shown in Figure 5, all mutants have etioplasts with typical prolamellar bodies in their dark-grown cotyledons (Figures 5A to 5C), which are identical to those of dark-grown wild-type plants (Figure 5D) and distinct from chloroplasts of light-grown seedlings (Figure 5E). These results are similar to those observed in *det2* mutants (Chory et al., 1991), but are in contrast to *cop1*, *cop9*, and *det1* mutants, which lack prolamellar bodies and contain parallel and sometimes stacked thylakoid membranes when grown in the dark. Taken together, these results suggest that the cotyledon chloroplast development and cellular differentiation can be uncoupled during light-regulated seedling development.

Effects of the *cop2*, *cop3*, and *cop4* Mutations on the Expression of Light-Regulated Genes

The expression of a variety of plant nuclear-encoded genes and plastid-encoded genes is either positively or negatively regulated by light (Gilmartin et al., 1990; Quail, 1991; Thompson and White, 1991). It seemed likely, therefore, that some or all of these *cop* loci would be involved in the light regulation of gene expression. Figure 6 shows the expression of four representative light-regulated genes (ribulose-1,5-bisphosphate carboxylase small subunit [*rbcS*], the photosynthetic light-harvesting complex chlorophyll *a/b* binding protein [*cab*], and ferredoxin type A [*fedA*] nuclear genes and *psbA*, which is a plastid gene encoding the 32-kD D1 protein of the photosystem II reaction center) in 6-day-old dark-grown and light-grown wild-type and mutant seedlings. The data show that mutations in the *COP2* and *COP3* loci do not have significant effects on the expression of the genes examined, whereas the levels of all three nuclear-encoded light-regulated mRNAs were elevated in the dark-grown *cop4* plants. However, the *cop4* mutation has no effect on the expression of the plastid-encoded *psbA* gene. This is in contrast with the coordinated activation of both nuclear and plastid genes in *cop1*, *cop9*, *det1*, and *det2* mutants

(Chory et al., 1989, 1991; Deng et al., 1991; Wei and Deng, 1992).

After transferring light-grown wild-type plants to darkness, the accumulation of mRNA for light-inducible genes decreases dramatically (Chory et al., 1989; Deng et al., 1991), and the *cop1* and *cop9* mutants are defective in this adaptive response (Deng et al., 1991; Wei and Deng, 1992). To determine whether the *cop4* mutation also affects this response, expression of the three light-regulated nuclear genes was examined after dark adaptation for different periods of time. The results shown in Figure 7 clearly demonstrate that the mRNA levels of all three genes examined decreased dramatically in wild-type plants. Interestingly, different genes seem to follow distinct kinetics of dark-adaptive changes. For example, both *rbcS* and *fedA* mRNA levels reached their lowest levels in less than 24 hr after transfer to darkness, whereas the *cab* mRNA level took more than 36 hr to reach the plateau. In all three genes examined, the dark-adaptive changes of their mRNA levels in the *cop4* mutants followed patterns identical to those in the wild-type plants. Therefore, we concluded that the *cop4* mutation does not affect the dark-adaptive changes of expression of light-regulated genes. This property of the *cop4* mutants is reminiscent of the *det1* mutants (Chory et al., 1989) but not of the *cop1*, *cop9*, and *det2* mutants (Chory et al., 1991; Deng et al., 1991; Wei and Deng, 1992).

A Distinct *cis* Element Outside of a Minimal Light-Responsive Promoter Element Is Required for Modulation of *cab1* Expression by *COP4*

To test whether the high level of mRNA accumulation for light-inducible genes in dark-grown *cop4* seedlings results from transcriptional activation, we analyzed the activity of a representative light-regulated promoter by introducing a full-length *cab1* promoter (−1281 to +67)–β-glucuronidase (*GUS*) reporter gene fusion into the *cop4* mutant. The results in Figure 8A show that both the GUS activity and the *GUS* mRNA levels are significantly elevated in the dark-grown *cop4* mutants when compared to dark-grown wild-type seedlings, but light-grown

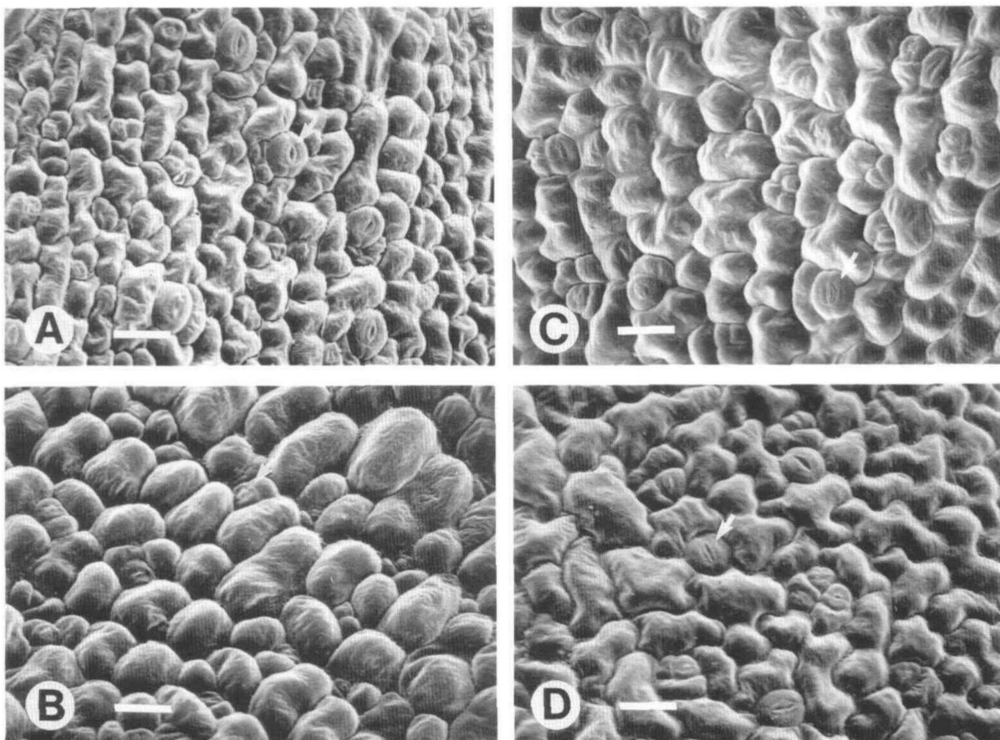

Figure 4. Epidermal Cell Differentiation Patterns in the Cotyledons of Dark-Grown Wild-Type and Mutant Seedlings.

(A) Wild type.
(B) *cop2*.
(C) *cop3*.
(D) *cop4*.
The cotyledons of 6-day-old seedlings were examined using scanning electron microscopy. Representative stomatal structures are indicated by arrows. Bars = 15 μm for all panels.

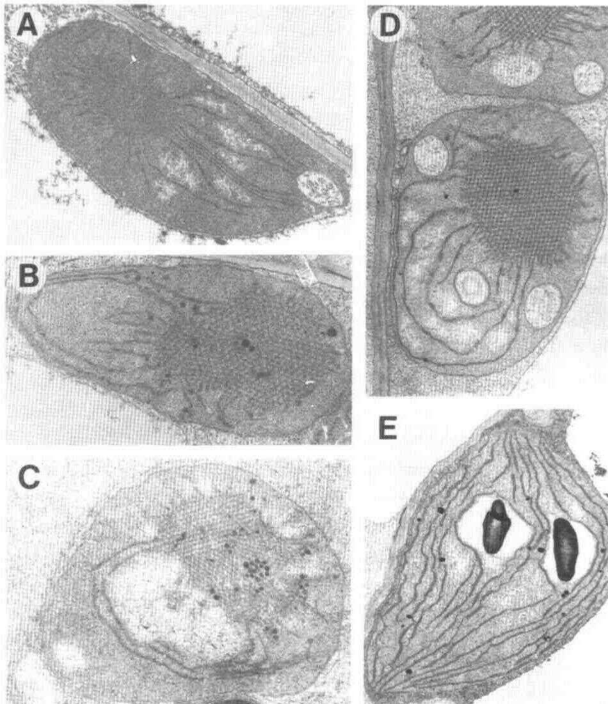

Figure 5. Plastid Morphologies of Wild-Type and Mutant Seedlings.

(A) Dark-grown *cop*2.
(B) Dark-grown *cop*3.
(C) Dark-grown *cop*4.
(D) Dark-grown wild type.
(E) Light-grown wild type.
Plastids in cotyledons of 6-day-old seedlings were examined using transmission electron microscopy.

wild-type and *cop*4 mutant seedlings have similar GUS activities and *GUS* mRNA levels. These results correlate well with the mRNA level of the endogenous *cab* gene and suggest that the *cop*4 mutation leads to activation of the *cab*1 promoter in the dark.

In *cop*1 and *cop*9 mutants, the minimal *cab*1 promoter region (−250 to +67) responsible for dark activation coincides with the minimal light-responsive region (Deng et al., 1991; Wei and Deng, 1992). To determine whether the same *cab*1 promoter region is also responsible for dark activation in the *cop*4 mutant, the minimal *cab*1 promoter–*GUS* reporter gene fusion (Deng et al., 1991) was introduced into the *cop*4 mutant, and GUS activity and *GUS* mRNA accumulation in dark-grown and light-grown mutant and wild-type seedlings were compared. As shown in Figure 8B, the activity of the minimal *cab*1 promoter in both wild-type and *cop*4 mutant seedlings, as indicated by both GUS activity and *GUS* mRNA levels, is very low in the dark and elevated to a similarly high level in the light. Because the minimal *cab*1 promoter does not show any elevated activity in dark-grown *cop*4 mutants, we concluded that the activation of the *cab*1 promoter in dark-grown *cop*4 mutants involves a promoter element(s) located outside of the minimal light-responsive promoter region, somewhere between positions −1281 and −250. Therefore, *COP*4 modulates *cab*1 promoter activity through a pathway distinct from the one involving *COP*1 and *COP*9.

Agravitropism of the *cop*4 Mutant

Arabidopsis plants display typical gravitropism, with roots growing toward and shoots growing away from the gravity center (Bullen et al., 1990; Evens, 1991; Okada and Shimura, 1992). We noticed that both the roots and shoots of the *cop*4 mutant seedlings, regardless of light conditions, grew in all directions on agar plates. To test whether the abnormal growth was due to a defect in gravitropism, we examined *cop*4 seedling growth on plates placed in a vertical orientation (Bullen et al., 1990; Okada and Shimura, 1992). Because similar results were

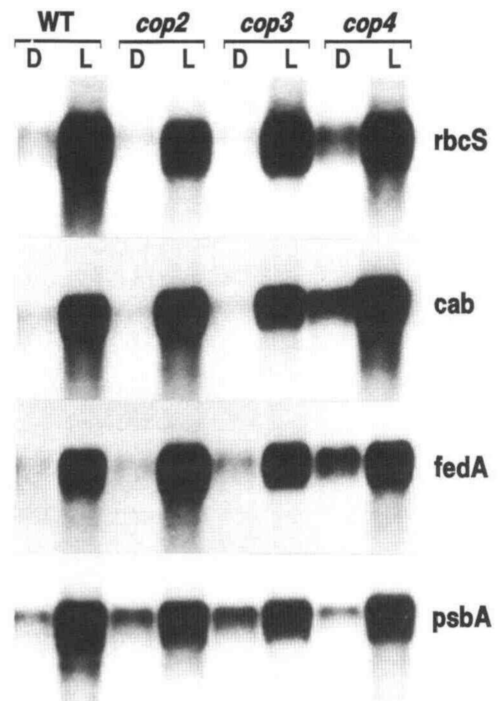

Figure 6. RNA Gel Blot Analysis of Steady State RNA Levels of Nuclear-Encoded and Plastid-Encoded Genes.

RNA levels of 6-day-old dark-grown (D) and light-grown (L) wild-type (WT), *cop*2, *cop*3, and *cop*4 seedlings were analyzed. Equal amounts of total RNA (2 μg for *rbc*S and *cab*; 5 μg for *fed*A and *psb*A) from different plant samples were used, and identical blots were hybridized with ^{32}P-labeled gene-specific probes. The three nuclear genes are as follows: *rbc*S (Krebbers et al., 1988), *cab* (Leutwiler et al., 1986), and *fed*A (Somers et al., 1990). The representative plastid gene is *psb*A (Zurawski et al., 1982). Blots were exposed to x-ray film for different periods of time to obtain suitable exposures for each transcript.

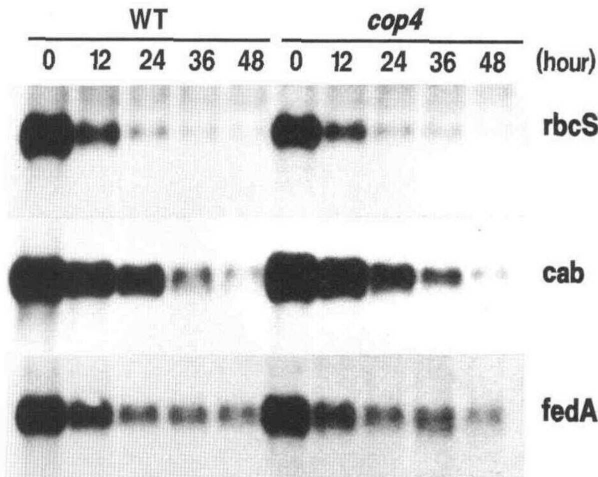

Figure 7. Dark-Adaptive Changes in mRNA Levels of Light-Regulated Genes in Wild-Type and *cop4* Mutant Plants.

Total RNA samples were prepared from continuous light-grown plants (0 hr) and from light-grown plants that had been dark adapted for 12, 24, 36, or 48 hr. Equal amounts of total RNA (2 μg for *rbc*S and *cab*; 5 μg for *fed*A) were used for analysis of mRNA levels. Blots were exposed to x-ray film for different periods of time to obtain suitable exposures for each transcript. WT, wild type.

obtained with both dark-grown and light-grown plants, only the results for light-grown plants are shown in Figure 9. The roots and shoots of wild-type seedlings grew as expected, and after rotating the plate 90° sideways, both the roots and shoots were able to alter their growth directions accordingly (compare top rows, Figures 9A and 9B). In contrast, the directions of *cop4* root and shoot growth were highly variable (middle and bottom rows, Figure 9A) and were not noticeably affected by rotating the plate (middle and bottom rows, Figure 9B). These results clearly indicate that the *cop4* mutants are defective in both root and shoot gravitropic responses.

Characteristics of the *cop4/cop1* Double Mutant

As the first step in investigating the relationship of this new class of *COP* loci to our previously reported *COP1* locus (Deng et al., 1991), the *cop4/cop1-1* double mutant was constructed and examined. Compared to the parental *cop4* and *cop1-1* mutants, *cop4/cop1-1* double mutant seedlings had most of the same morphological characteristics as the *cop1-1* mutant in both darkness and light, as shown in Figure 10 and Table 3. These characteristics included hypocotyl length, cotyledon development, anthocyanin accumulation, and the overall morphology of seedling and adult plants. Due to the fact that both the *cop4* and *cop1-1* mutations lead to constitutive photomorphogenic phenotypes in darkness, we were unable to distinguish whether *cop1* and *cop4* act epistatically or additively during light-regulated seedling morphogenesis. On the other hand, the *cop4/cop1-1* double mutants were as deficient in both root and shoot gravitropisms as the *cop4* mutants (Table 3). This property of the double mutant would be consistent with an additive interaction between the *cop4* and *cop1-1* mutations.

We further examined the expression of light-regulated genes in the *cop4/cop1-1* double mutant. Figure 11 shows a comparison of expression patterns of two light-regulated genes (*cab* and *rbc*S) in the *cop4/cop1-1* double mutants with those of the wild type and *cop4* and *cop1-1* mutants. It is clear that both the *cop1-1* and *cop4* mutations lead to partial activation of the two genes examined (Figures 6 and 11, and Deng et al., 1991). Because different promoter elements (at least for *cab1*) are responsible for dark activation in *cop4* and *cop1* mutants (Figure 8), an additive effect of the *cop4* and *cop1-1* mutations would result in a greater activation of these genes in the dark-grown double mutants than in either of the parental mutants. On the contrary, the results in Figure 11 show that the dark expression levels of two light-regulated genes in the double mutant are either similar to or lower than the levels in the *cop1-1* mutant. For *cab* in particular, the mRNA level of the dark-grown double mutant is almost as low as that of wild-type plants. These results are inconsistent with an additive effect of the *cop1-1*

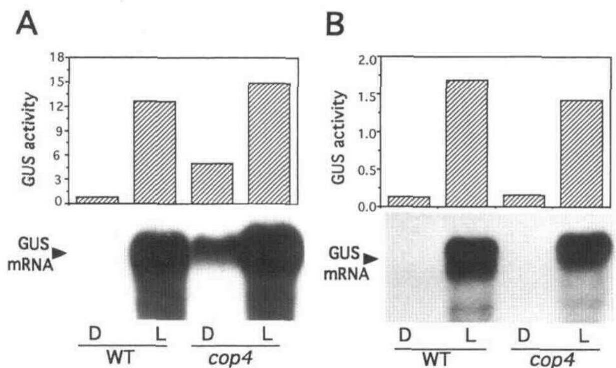

Figure 8. Modulation of *cab1* Promoter Activity by Light and by the *cop4* Mutation.

(A) Regulation of full-length Arabidopsis *cab1* promoter (−1281 to +67) activity.

(B) Regulation of activity of a deleted version of the Arabidopsis *cab1* promoter (−250 to +67).

The promoter–*GUS* fusion constructs were stably introduced into wild-type (WT) plants and the *cop4* mutant, and the promoter activities in the dark (D) and light (L) were analyzed by determining both GUS activity (histograms; measured in micromoles of 4-methylumbelliferone per hour per milligram of protein) and *GUS* mRNA levels. GUS activities are the average of three independent measurements, and the variation among them was less than 5%. For *GUS* mRNA analysis, 4 μg of total RNA from different samples was used in each lane in **(A)**, and 15 μg of total RNA was used for lanes in **(B)**. The GUS activity and *GUS* mRNA levels in light-grown plants for the short *cab1* promoter–*GUS* fusion **(B)** were reduced ~10-fold compared to that of the long *cab1* promoter–*GUS* fusion **(A)**.

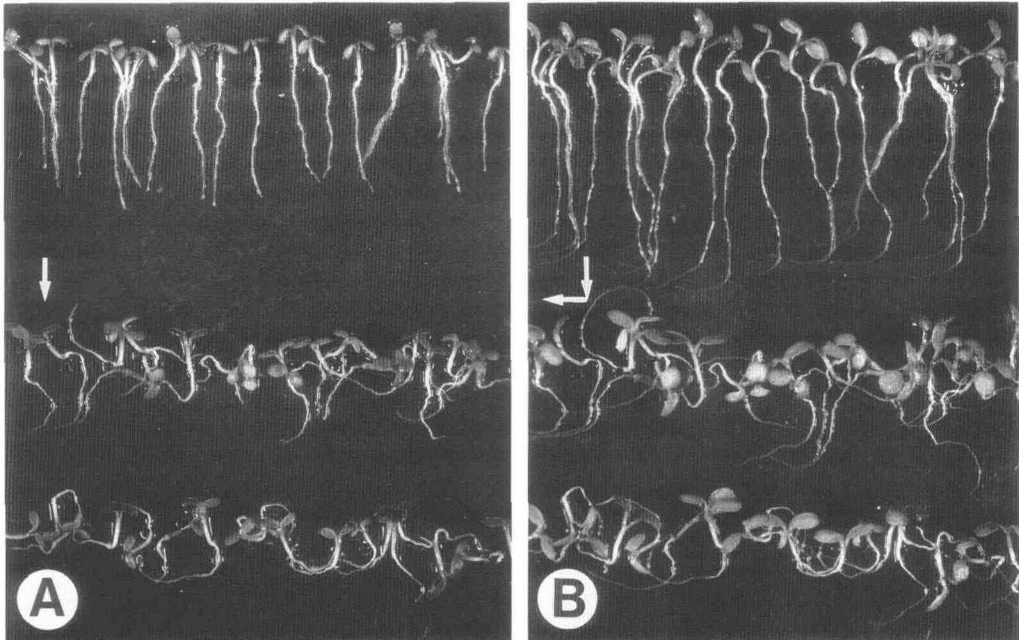

Figure 9. Comparison of Gravitropic Responses of Wild-Type and *cop4* Mutant Seedlings.

(A) The wild-type (top row) and *cop4* mutant seedlings (middle and bottom rows) were grown for 4 days on a vertical plate in the light.
(B) The same plate shown in **(A)** was rotated 90° sideways and incubated for an additional 24 hr.
The directions of gravity are indicated by arrows.

and *cop4* mutations, at least in regard to the regulation of *cab* gene expression. Therefore, it implies that *COP1* and *COP4* interact with each other directly or indirectly in the regulation of *cab* promoter activity.

DISCUSSION

We report here the characterization of a new class of Arabidopsis constitutive photomorphogenic loci, *COP2*, *COP3*, and *COP4*. Mutations in these loci result in dark-grown seedlings with open and enlarged cotyledons and without apical hooks. The epistatic interactions between the new *cop* mutations and the phytochrome-deficient mutations suggest that these newly identified genes are indeed involved in light-regulated seedling development. The ability to isolate mutations that primarily affect the cotyledon but not hypocotyl development suggests that light-regulated development of the cotyledon and hypocotyl are separable processes. Although some regulatory genes, such as *cop1* (Deng et al., 1991), *cop9* (Wei and Deng, 1992), *det1* (Chory et al., 1989), and *det2* (Chory et al., 1991), control both light-regulated hypocotyl and cotyledon development, there are also sets of regulatory genes that primarily regulate the development of either the cotyledon (such as *COP2*, *COP3*, and *COP4*) or hypocotyl (Y. Hou, A. G. von Arnim, and X.-W. Deng, unpublished results). Our results therefore support the hypothesis that photomorphogenic genes act in a hierarchical fashion. More specifically, we propose that a set of regulatory genes (upstream) controls multiple aspects of light-regulated development, whereas other sets (downstream) control more specific light-regulated processes and are possibly subject to control by upstream genes.

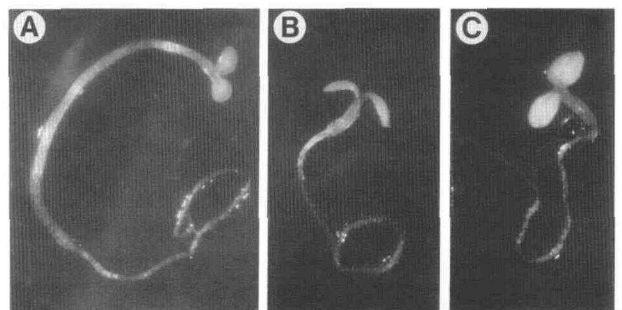

Figure 10. Morphology of *cop4/cop1-1* Double Mutants.

(A) Dark-grown *cop4*.
(B) Dark-grown *cop1-1*.
(C) Dark-grown *cop1-1/cop4* double mutant.
The seedlings were grown in darkness for 6 days before photography.

Table 3. Summary of Phenotypic Characteristics of *cop4*, *cop1-1*, and *cop4/cop1-1* Double Mutants

Characteristics	*cop4*	*cop1-1*	*cop4/cop1-1*
Dark-grown seedlings			
Hypocotyl length	Long	Short	Short
Open and enlarged cotyledon	Yes	Yes	Yes
High anthocyanin content	No	Yes	Yes
Agravitropism	Yes	No	Yes
Partial activation of *cab* gene expression	Yes	Yes	Almost none
Partial activation of *rbcS* gene expression	Yes	Yes	Yes
Light-grown seedlings			
Hypocotyl length	Normal	Very short	Very short
High anthocyanin content	No	Yes	Yes
Agravitropism	Yes	No	Yes

The properties of these new *cop* mutants, together with those of the *cop1* (Deng et al., 1991), *cop9* (Wei and Deng, 1992), *det1* (Chory et al., 1989), and *det2* (Chory et al., 1991) mutants, illustrate several interesting aspects of light-regulated seedling development. First, the enlargement of cotyledons is due to both cotyledon cell enlargement and lateral cell division (Figures 3 and 4). Second, cellular and plastid differentiation in the cotyledon are separable processes. This has been demonstrated by the presence of typical etioplasts in dark-grown *cop2*, *cop3*, *cop4* (Figure 5), and *det2* (Chory et al., 1991) mutants. Third, the absence of altered expression of light-regulated genes in dark-grown *cop2* and *cop3* seedlings (Figure 6) suggests that the expression of these genes is unnecessary for morphogenetic changes in Arabidopsis seedlings.

The *cop4* mutation seems to have different effects on the expression of light-regulated genes that are encoded by the nuclear and plastid genomes. For nuclear genes, such as *rbcS*, *cab*, and *fedA*, the *cop4* mutation leads to partial activation in the dark. This result closely resembles that of the *det2* mutant (Chory et al., 1991), but not that of *cop1*, *cop9*, or *det1* mutants (Chory et al., 1989; Deng et al., 1991; Wei and Deng, 1992). However, the *cop4* mutation does not cause any detectable increase of the mRNA level of *psbA*, a plastid-encoded light-regulated gene. This property of *cop4* differentiates it from *cop1*, *cop9*, *det1*, and *det2* and implies that the light regulation of expression of nuclear and plastid genes can be uncoupled during light-regulated seedling development.

Similar to the *cop1* and *cop9* mutations (Deng et al., 1991; Wei and Deng, 1992), the *cop4* mutation activates *cab1* gene expression in darkness through modulation of its promoter activity. However, different elements of the *cab1* promoter are involved. In *cop1* and *cop9*, a minimal light-responsive *cab1* promoter (−250 to +67) is sufficient to mediate elevated expression in the dark-grown mutant plants (Deng et al., 1991; Wei and Deng, 1992), whereas in *cop4*, the same *cab1* promoter fails to elevate the expression of the *GUS* reporter gene in the dark (Figure 8). Clearly, another *cis* element that is present in the full-length *cab1* promoter but absent in the minimal promoter is required for dark activation of the *cab1* promoter in the *cop4* mutants. Because the minimal *cab1 promoter* is able to properly respond to light signals, it will be interesting to know the specific role of *COP4* in the light-regulated expression of *cab1*. It is unlikely that the *cis* element located between −1281 to −250 is sufficient to mediate the response to the *cop4* mutation, because that would lead to an additive effect on the expression of *cab1* in the *cop4/cop1-1* double mutant, in contrast to the result shown in Figure 11. Therefore, both an upstream *cis* element (between −1281 to −250) and an element located within the minimal promoter are likely required for the responsiveness to the *cop4* mutation in the dark.

The fact that *cop4* mutants are also defective in both root and shoot gravitropism suggests that the *COP4* locus plays a role in the signaling process that controls gravitropism. Previous physiological studies have implied that light signals play some role in the gravitropic response of higher plants (Bullen et al., 1990; Evens, 1991; Okada and Shimura, 1992). The apparent involvement of the *COP4* gene product in both gravitropism and photomorphogenesis indicates that the two processes may share a common regulatory component, which would allow cross-talk. However, the cross-talk between these two signaling pathways appears to be strictly limited, because *cop4* is the only photomorphogenic mutant identified thus far that has a detectable effect on gravitropism.

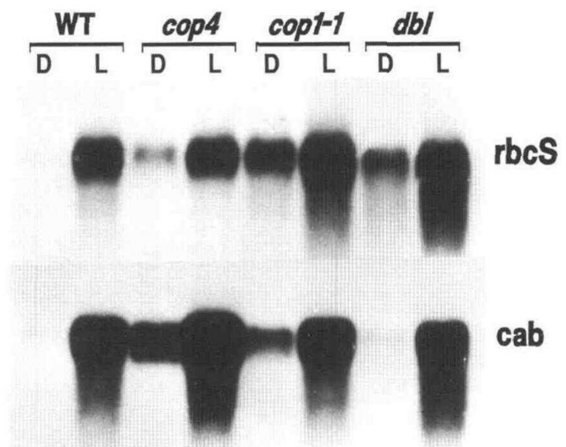

Figure 11. Comparison of Light Regulation of Steady State mRNA Levels of Two Nuclear-Encoded Genes in the Wild Type and *cop* Mutants.

Six-day-old wild-type (WT) and *cop4*, *cop1-1*, and *cop4/cop1-1* double (dbl) mutant seedlings grown in the light (L) and dark (D) were used for RNA extraction. Equal amounts of total RNA (1 μg for *rbcS*; 2 μg for *cab*) were used, and blots were hybridized with ^{32}P-labeled gene-specific probes.

METHODS

Plant Materials and Growth Conditions

The wild type and constitutive photomorphogenic (cop) mutants used in this study are in the Columbia ecotype, and the *hy* mutants are in the Landsberg ecotype of *Arabidopsis thaliana.* Production of ethylmethane sulfonate–mutagenized Arabidopsis M_2 seeds and screening for mutants have been described previously (Deng et al., 1991). For germination, seeds were surface sterilized for ~15 min in 30% bleach (Clorox), rinsed at least five times, and plated in Petri plates (150 × 25 mm) containing growth medium (Valvekens et al., 1988). After cold treatment at 4°C for 2 to 4 days in the dark, the plates were incubated in a growth chamber at 22°C in complete darkness or in a cycle of 16 hr light/8 hr dark. For the dark-adaptation experiment, plants were grown in continuous light (24 hr/day) for 3 weeks and then transferred to complete darkness for various amounts of time before harvest. Plants in Petri dishes were either harvested for experiments or transferred to soil to grow to maturity for genetic manipulations or seed set. The light source for Arabidopsis plant growth was a combination of fluorescent and incandescent lights, ranging from 100 to 300 $\mu E\ m^{-2}\ sec^{-1}$.

Construction of Double Mutants

To generate Arabidopsis strains homozygous for two different mutations, we crossed plants homozygous for each mutation and selfed the resulting F_1 progeny. Among the F_2 population from each cross, 10 to 30 plants that were homozygous for one mutation were identified and their seeds were harvested individually. Among these plants, the individuals that were heterozygous for the second mutation were identified by examining phenotypic segregation in F_3 progeny. The plants with a new phenotype among the progeny of these identified F_2 plants were considered as homozygous for both parental mutations. These double mutants were selected and selfed to produce individual double mutant lines. Although the *cop* and *hy* mutants were isolated from Columbia and Landsberg ecotypes, respectively, there are no detectable morphological differences in dark-grown and light-grown seedlings.

Light and Electron Microscopy

The fixation, embedding, sectioning, and examination of Arabidopsis seedlings for light and transmission electron microscopy were performed according to a published procedure of Deng et al. (1991). Unless specified otherwise, seedlings were grown on Petri plates in the dark or light for 6 days.

For scanning electron microscopy (SEM), whole seedlings were fixed in 5% acetic acid, 4% formaldehyde, and 50% ethanol at room temperature for at least 2 hr and dehydrated in a graded ethanol series. Dehydrated material was critical point dried in liquid carbon dioxide. Individual seedlings were mounted on scanning electron microscope stubs, sputter coated with gold palladium, and examined under a scanning electron microscope.

RNA Analysis

Arabidopsis plants were harvested at specific developmental stages and frozen in liquid nitrogen immediately. Dark-grown and dark-adapted plants were harvested under dim green safelights. Isolation of total RNA, electrophoresis and blotting of RNA, and radioactive labeling of DNA probes were as previously described (Deng et al., 1991). Filter hybridizations were performed at 65°C in a solution containing 7% SDS, 500 mM NaH_2PO_4, pH 7.2, 1% BSA, and 1 mM EDTA. The DNA probes used are as follows: a 0.55-kb DNA fragment (generated by polymerase chain reaction) corresponding to the entire open reading frame of the Arabidopsis small subunit of ribulose-1,5-bisphosphate carboxylase *rbc*S gene (Krebbers et al., 1988); a 0.5-kb BamHI-SstI DNA fragment corresponding to the coding region of the Arabidopsis chlorophyll *a*/*b* (*cab*3) gene (Leutwiler et al., 1986); a 1.5-kb HincII-EcoRI DNA fragment containing the Arabidopsis ferredoxin type A (*fed*A) gene (Somers et al., 1990); a 1.2-kb BglII-XbaI DNA fragment containing most of the coding region of a spinach chloroplast plastid (*psb*A) gene (Zurawski et al., 1982); and a 2-kb HindIII fragment containing the entire β-glucuronidase (*GUS*) coding sequence used for the *cab*1 promoter–*GUS* constructs (Deng et al., 1991). For all probes, ~200 ng of each purified DNA fragment was labeled to high specific activity (~2 to 3 × 10^5 cpm/ng DNA) by random oligomer priming, denatured by boiling for 5 min, and used for hybridization.

Promoter-*GUS* Reporter Gene Fusions and GUS Assay

The full-length *cab*1 promoter was a fragment from −1281 to +67 bp from the Arabidopsis *cab*1 gene (Ha and An, 1988; Karlin-Neumann et al., 1988), and a deletion version (from −250 to +67 bp) was used as the minimal Arabidopsis *cab*1 promoter. The position of the transcription initiation site is designated +1. The construction of promoter–*GUS* reporter gene fusions and transformation into wild-type Arabidopsis (ecotype NO-0) were described previously (Deng et al., 1991). Two representative lines for each promoter–*GUS* fusion construct that produced the expression pattern expected of the endogenous genes were chosen for crosses with *cop*4 plants. *cop*4 plants carrying promoter–*GUS* reporter constructs were identified from the F_2 progeny of the crosses. These *cop*4 plants were allowed to set seed, which were used to grow seedlings for GUS activity or *GUS* mRNA analyses.

GUS enzyme activity in transgenic Arabidopsis seedlings was quantified according to a previously published procedure (Jefferson, 1987; Deng et al., 1991) by measuring the fluorescence of 4-methylumbelliferone produced by GUS cleavage of 4-methylumbelliferyl β-D-glucuronide. Protein concentrations were determined by the Bio-Rad protein assay according to the manufacturer's suggested procedure.

ACKNOWLEDGMENTS

We wish to acknowledge the support of Dr. Peter H. Quail during the initial phase of this work. We thank Barry Piekos of the Biology Department electron microscope laboratory, Yale University, for technical assistance with the microscopy analysis, and Drs. David B. Stern and Ning Wei for their critical comments on the manuscript. This work was supported by U.S. Department of Agriculture Grant No. 92-37301-7706 (to X.W.D.). A.G.v.A. is a recipient of a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft.

Received January 4, 1993; accepted January 25, 1993.

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