Plant Physiol. (1997) 114: 779–788

Genetic and Developmental Control of Nuclear Accumulation of COP1, a Repressor of Photomorphogenesis in Arabidopsis[1]

Albrecht G. von Arnim[2], Mark T. Osterlund, Shing F. Kwok, and Xing-Wang Deng*

Department of Biology (A.G.v.A., M.T.O., X.-W.D.), and Department of Genetics (S.F.K.), Yale University, New Haven, Connecticut 06520–8104

Using a β-glucuronidase (GUS) reporter-COP1 fusion transgene, it was shown previously that Arabidopsis COP1 acts within the nucleus as a repressor of seedling photomorphogenic development and that light inactivation of COP1 was accompanied by a reduction of COP1 nuclear abundance (A.G. von Arnim, X.-W. Deng [1994] Cell 79: 1035–1045). Here we report that the *GUS-COP1* fusion transgene can completely rescue the defect of *cop1* mutations and thus is fully functional during seedling development. The kinetics of GUS-COP1 relocalization in a *cop1* null mutant background during dark/light transitions imply that the regulation of the functional nuclear COP1 level plays a role in stably maintaining a committed seedling's developmental fate rather than in causing such a commitment. Analysis of GUS-COP1 cellular localization in mutant hypocotyls of all other pleiotropic *COP/DET/FUS* loci revealed that nuclear localization of GUS-COP1 was diminished under both dark and light conditions in all mutants tested, whereas nuclear localization was not affected in the less pleiotropic *cop4* mutant. Using both the brassinosteroid-deficient mutant *det2* and brassinosteroid treatment of wild-type seedlings, we have demonstrated that brassinosteroid does not control the hypocotyl cell elongation through regulating nuclear localization of COP1. The growth regulator cytokinin, which also dramatically reduced hypocotyl cell elongation in the absence of light, did not prevent GUS-COP1 nuclear localization in dark-grown seedlings. Our results suggest that all of the previously characterized pleiotropic *COP/DET/FUS* loci are required for the proper nuclear localization of the COP1 protein in the dark, whereas the less pleiotropic *COP/DET* loci or plant regulators tested are likely to act either downstream of COP1 or by independent pathways.

Ambient light conditions affect plant development throughout all developmental stages (Kendrick and Kronenberg, 1994; Liscum and Hangarter, 1994; Smith, 1995). For instance, seedling development in the angiosperm *Arabidopsis thaliana* depends on light signals to choose between the photomorphogenic and the skotomorphogenic (etiolation) pathway (for review, see von Arnim and Deng [1996]). Seedlings grown under light conditions show open and expanded cotyledons, a short hypocotyl, chloroplast development, and a high expression level for light-inducible genes. Under dark conditions, however, the seedlings exhibit long hypocotyls, closed and undeveloped cotyledons with etioplasts instead of chloroplasts, and low or undetectable levels of light-inducible genes. A complex and still largely unknown signaling network that coordinates seedling development in response to light is being unraveled (for review, see Bowler and Chua [1994]; Millar et al., 1994; McNellis and Deng, 1995; Quail et al., 1995).

During recent years, genetic analysis has demonstrated that a set of regulatory genes functions in darkness to suppress the photomorphogenic pathway, which in turn allows etiolation to occur (Chory et al., 1989, 1991; Deng et al., 1991; Wei and Deng, 1992; Hou et al., 1993; Miséra et al., 1994; Wei et al., 1994b; Kim et al., 1996; Kwok et al., 1996). Among them, 10 pleiotropic de-etiolated (*DET*), constitutively photomorphogenic (*COP*), or fusca (*FUS*) loci have been identified by way of mutations that result in pleiotropic photomorphogenic seedling development in darkness (Chory et al., 1989; Deng et al., 1991; Wei and Deng, 1992; Castle and Meinke, 1994; Miséra et al., 1994; Kwok et al., 1996). This class of pleiotropic *COP/DET/FUS* genes is presumably involved in the primary switch between seedling developmental patterns in response to light. One common feature of the pleiotropic *COP/DET/FUS* genes is that their severe alleles invariably lead to lethality after seedling development (Castle and Meinke, 1994; Miséra et al., 1994; Kwok et al., 1996).

On the other hand, several loci that have mutations that resulted in less pleiotropic photomorphogenic developmental phenotypes in darkness have also been identified (such as *cbb2* [Kauschmann et al., 1996]; *cpd/cbb3* [Kauschmann et al., 1996; Szekeres et al., 1996]; *dwf1*, allelic to *dim/cbb1* [Kauschmann et al., 1996; Takahashi et al., 1995]; *det2* [Chory et al., 1991]; *cop2/amp1/pt-1*, *hls1/cop3* [Chaudhury et al., 1993; Hou et al., 1993; Lehmann et al., 1996]; and *cop4* [Hou et al., 1993]; suppressors of *hy2* or *shy* [Kim et al., 1996]). Their dark-grown seedlings have lost some of the characteristics of the dark developmental path-

[1] This work was supported by National Institutes of Health (NIH) grant no. GM47850 to X.-W.D. and Department of Energy grant no. DE-FG02-96ER20223 to A.G.v.-A. X.-W.D. is a National Science Foundation Presidential Faculty Fellow, and M.T.O. and S.F.K. were NIH predoctoral trainees.

[2] Present address: Department of Botany, HBB 437, The University of Tennessee, Knoxville, TN 37996–1100.

* Corresponding author; e-mail xingwang.deng@yale.edu; fax 1–203–432–3854.

Abbreviations: BR, brassinolide; DAPI, 4,6-diamidino-2-phenylindole.

way and exhibit aspects of photomorphogenic development. It is interesting that phenotypic defects in three of the less pleiotropic genes are rescued by treatment with brassinosteroids, suggesting that this class of plant growth regulators is required for efficient hypocotyl cell elongation during the etiolation response (*det2* [Li et al., 1996]; *cpd/cbb3* and *dwf1/dim/cbb1* [Kauschmann et al., 1996; Szekeres et al., 1996]).

To date, the precise molecular function of the pleiotropic *COP/DET/FUS* gene products remains unknown. At least some of the COP/DET/FUS proteins, such as COP9 and COP11, form part of a light-modulated multisubunit protein complex (Wei et al., 1994a; Chamovitz et al., 1996; Staub et al., 1996). Both COP1 and DET1 were also reported to be nuclear regulators (Pepper et al., 1994; von Arnim and Deng, 1994). Overexpression analysis of COP1 has substantiated the genetic model that it acts as a repressor of photomorphogenic development (McNellis et al., 1994b, 1996). When expressed as a fusion protein with the GUS reporter, COP1 is found in the nuclei of Arabidopsis hypocotyl cells when plants are grown in darkness. In contrast, under light conditions, the hypocotyl cell nuclei lack GUS-COP1 protein. Moreover, the light-dependent subcellular partitioning of COP1 is tissue-type specific: In the cells of the root, light signals do not affect the subcellular localization of GUS-COP1, and the protein remains nuclear at all times (von Arnim and Deng, 1994). Thus, it was proposed that COP1 acts within the nucleus to repress photomorphogenesis, whereas its activity is controlled at least in part by a light-modulated and tissue-specific regulation of its concentration in the nucleus. These studies have not only suggested an intriguing mechanism for the reversible inactivation of the nuclear regulator COP1 by light but have also provided a framework to test the functional relationship between COP1 and other photomorphogenic genes. For example, mutations in genes that are directly involved in properly localizing COP1 to the nucleus would result in a phenotype similar to *cop1* mutations themselves, whereas other genes involved in more downstream or independent pathways may have no effect on COP1 nuclear localization.

Here we report further analyses of the *GUS-COP1* fusion transgene in Arabidopsis and show that it is fully functional during seedling development, demonstrating the suitability of the fusion protein as an indicator for COP1 localization. By analyzing GUS-COP1 localization in a panel of mutant backgrounds and in the presence of plant growth regulators, we were able to address the genetic and developmental regulation of COP1 nuclear abundance and thus the light control of plant development.

MATERIALS AND METHODS

The transgenic lines harboring *GUS-COP1*, *GUS-NIa*, and *GUS* transgenes have been described (von Arnim and Deng, 1994). Mutant alleles of the genes used in this work are *det1–1* (Chory et al., 1989; Pepper et al., 1994), *cop4–1* (Hou et al., 1993), *det2–1* (Chory et al., 1991; Li et al., 1996), *cop8–1* (Wei et al., 1994b), *cop9–1* (Wei and Deng, 1992), *cop10–1* (Wei et al., 1994b), *cop11–1* (*fus6*, Wei et al., 1994b), *fus4–1*, *fus5–1*, *fus 11–1*, and *fus12–1* (Miséra et al., 1994). Mutant plants carrying the desired transgene were generated by standard genetic crossing. Progeny F_1 plants heterozygous for both the transgene and the mutation were selected based on the transgene selectable marker, kanamycin resistance, as well as on F_2 segregation analysis. Homozygous mutant plants carrying the GUS-fusion transgene were examined in the F_2 or the following F_3 population.

Complementation Analysis

Arabidopsis lines carrying the indicated transgenes were crossed to homozygous *cop1–4* or heterozygous *cop1–5* mutant plants, and F_1 plants heterozygous for both the *cop1*-allele and the transgene were identified. Segregation of the mutation and the transgene were analyzed in the selfed progeny (F_2 population). In the absence of complementation, we expected to observe one-quarter mutant seedlings. In addition, both mutants and wild-type plants should show GUS expression. In the event of complementation, we expected an increase in the ratio of wild-type to mutant plants from 3:1 up to 15:1, and none of the mutants should stain for GUS. To confirm the result, we examined segregation of the *cop1* allele and the transgene in F_3 populations derived by selfing of homozygous mutant F_2 plants that were heterozygous for GUS-COP1 (*cop1/cop1; GUS-COP1/wt*). These F_2 plants lacked copies of the wild-type *COP1* allele and could be identified because the ratio of wild-type to mutant plants reverted to 3:1 in their progeny, with none of the mutants but all of the wild-type plants staining for GUS. Such *cop1/cop1*-mutant F_3 populations were used for the kinetic analysis of GUS-COP1 relocalization.

Histochemical Localization of GUS Activity

Arabidopsis seedlings were harvested and stained in GUS assay buffer as described previously (von Arnim and Deng, 1994), except that the initial fixation step before GUS staining was usually omitted. Omitting the fixation did not alter the pattern of subcellular localization, but it increased the overall speed or intensity of GUS staining. For microscopic observation of GUS-COP1 localization, areas of tissue were chosen that showed intense cytoplasmic inclusion bodies but lacked diffuse blue staining, because the diffuse staining would otherwise have obscured the staining of the nucleus. For quantitative comparisons of nuclear staining intensity, care was taken to compare cells from areas of tissue with equally intense cytoplasmic inclusion bodies. Staining times varied between 3 and 6 h, with photomorphogenic seedlings usually requiring longer staining times than etiolated seedlings. Placing seedlings into the staining solution should interrupt protein-trafficking processes, and any effect of the staining time on GUS-COP1 relocalization can therefore be excluded.

For GUS-NIa and GUS alone, which do not form cytoplasmic inclusion bodies as a reference point, staining was allowed to proceed until the staining intensity in the root tip was approximately equal to that in corresponding GUS-

COP1 transgenic seedlings under the same experimental conditions. Stained seedlings were fixed in 10% acetic acid, 3.7% formaldehyde, and 50% ethanol or as described by von Arnim and Deng (1994) and destained through a graded ethanol series before mounting in water with 1 mg/L DAPI. Samples were examined on a microscope (Axiophot Zeiss) with epifluorescence optics and a DAPI filter set no. 02 (Zeiss). Micrographs were usually taken with a color CCD camera (model 72, Sony, Tokyo, Japan) with a low-light (fluorescence) extension package (Sony) to visualize DAPI staining. In some cases micrographs were taken on 35-mm slide film (400 ASA, Kodak), and slides were scanned using Polaroid (Cambridge, MA) or Nikon slide scanners. Micrograph were assembled using Adobe Photoshop software (Adobe Systems Inc., Mountain View, CA) for the Macintosh computer.

Kinetics of GUS-COP1 Relocalization

Wild-type and *cop1–5* seedlings carrying the *GUS-COP1* transgene were germinated in continuous white light (120 μE m^{-2} s^{-1}) for 5 d. They were kept under light conditions for 5 d or transferred to darkness for 12, 24, and 36 h toward the end of the 5-d period. Seedlings were harvested into wells of coded tissue culture dishes and stained for GUS activity. For each treatment we typically evaluated the percentage of cells showing nuclear staining by examining at least 20 cells in each of at least three individual hypocotyls. Before decoding the information concerning genotype and sample treatment, we graded stained samples according to their increasing nuclear localization of GUS-COP1. We investigated whether one genotype showed consistently more intense staining than the other and found that this was not the case.

Growth Regulator Treatments

BA was added to cooled autoclaved growth medium (Deng et al., 1991) at the indicated concentrations from a stock solution in DMSO. BR was added from a stock solution in 100% ethanol. BR treatment of light-grown seedlings (Fig. 3) was performed on seedlings growing under 30 μE m^{-2} s^{-1} white light. Under these conditions, the GUS-COP1 seedlings are slightly elongated compared with the controls lacking GUS-COP1, indicating that the light intensity used did not completely saturate the inhibition of hypocotyl elongation.

RESULTS

GUS-COP1 Transgene Is Fully Functional during Seedling Development

GUS-COP1 has been used previously to analyze the subcellular localization of COP1 (von Arnim and Deng, 1994). To test whether the GUS-COP1 fusion is a suitable indicator for the subcellular localization of the COP1 protein, the *GUS-COP1* transgene was introduced into both a weak (*cop1–4*) and a null (*cop1–5*) mutant. Subsequently, the segregation ratio of mutant and wild-type-like F_2 progeny was analyzed and compared with the segregation of the *GUS-COP1* transgene. As shown in Table I, the wild-type to mutant ratios in F_2 populations from crosses between *GUS-COP1* transgenic plants and *cop1* mutants approached 15:1, just as expected if *GUS-COP1* can functionally rescue the mutant defects. Most importantly, it was further confirmed by GUS staining that none of the F_2 seedlings showing a *cop1* mutant phenotype contained a *GUS-COP1* transgene and that all *GUS-COP1* transgene-bearing plants showed the wild-type phenotype. As illustrated in Figure 1, seedlings homozygous for the *cop1* mutations but containing the *GUS-COP1* transgene were phenotypically indistinguishable from the wild type. Hence, it is concluded that *GUS-COP1* can functionally substitute for *COP1*. As a control, the *GUS* gene itself did not rescue the *cop1* mutant phenotype. Because the *GUS-COP1* gene could replace the function of the endogenous *COP1* gene, the subcellular localization of GUS-COP1 should be indicative of the endogenous COP1 protein.

The Kinetics of GUS-COP1 Repartitioning Are Consistent with a Role in Maintaining the Developmental Program Triggered by Environmental Light Cues

The nuclear localization of GUS-COP1 in darkness and its nuclear depletion in the light are consistent with a role for COP1 as a repressor of photomorphogenesis. To investigate whether GUS-COP1 repartitioning is a cause or an effect of the switch between the etiolation pathway and the photomorphogenic pathway, the kinetics of GUS-COP1 repartitioning during dark to light transitions and vice versa were analyzed in the *cop1–5* null mutant background, i.e. in the absence of any endogenous COP1 protein (Deng et al., 1992; McNellis et al., 1994a). Here the localization of GUS-COP1 should be indicative of all COP1 activity.

As shown in Figure 2, GUS-COP1 staining of the hypocotyl cells in homozygous *cop1–5* mutant seedlings carry-

Table I. *Complementation of the cop1 mutant seedling phenotype by GUS-COP1 transgene*

Cross	Generation	Total	Phenotype		Ratio Wild-Type:Mutant	GUS+ among cop1-Like
			cop1	Wild type		
cop1-4 × *GUS-COP1*	F_2	286	16	270	16.9	0
	F_3[a]	45	8	37	4.6	0
cop1-5 × *GUS-COP1*	F_2	171	8	163	20.4	0
cop1-5 × *GUS*	F_2	559	121	438	3.6	n.d.[b]

[a] F_3 population from an F_2 individual with *cop1/cop1*; transgene/wild-type genotype. [b] n.d., Not determined.

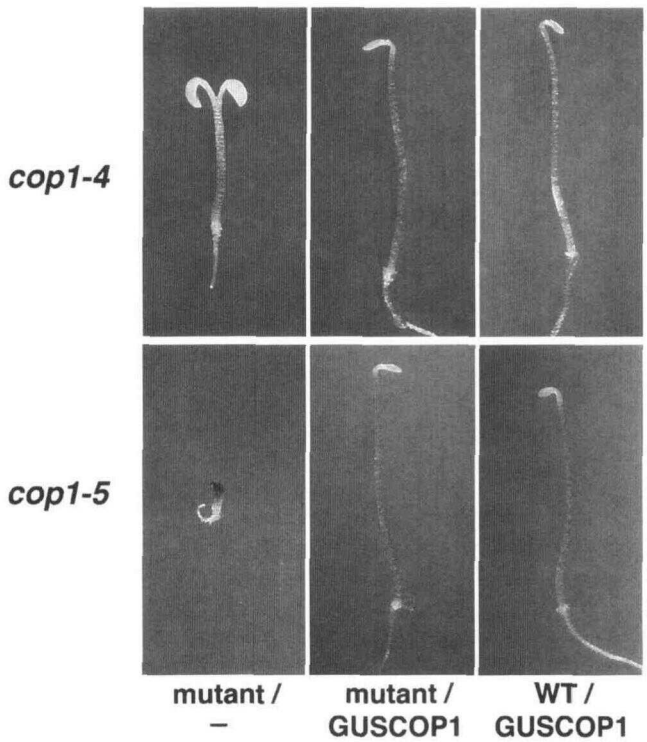

Figure 1. Rescue of *cop1* mutant phenotypes by the *GUS-COP1* transgene. Populations of seedlings derived from selfing of *cop1/cop1; GUS-COP1/+* plants were germinated in darkness. Representative examples of the seedling phenotypes in the absence (left column) and in the presence (center column) of the transgene are shown for the mild *cop1–4* allele (top) and the lethal *cop1–5* (bottom). Wild-type (WT) (*COP1/COP1*) seedlings transgenic for GUS-COP1 are shown for comparison (right column).

ing the *GUS-COP1* transgene was examined after growth in darkness or light and then a shift to light or darkness. Light or dark treatments of 12, 24, or 36 h were carried out toward the end of a 5-d (120 h) growth period. Note that, as described previously (von Arnim and Deng, 1994), cytoplasmic GUS-COP1 accumulated in particulate domains (inclusion bodies) rather than as the soluble protein typical for the unfused GUS enzyme alone. Corresponding inclusion bodies have been visualized by electron microscopy using immunogold labeling with GUS-specific and COP1-specific antibodies and therefore do not reflect an artifact of the histochemical enzyme assay (A. von Arnim, unpublished data).

In the hypocotyl cell nuclei, the modulation of GUS-COP1 staining in response to light treatments was observed in both wild-type and *cop1–5* mutant seedlings. Images for the wild type have been published previously (von Arnim and Deng, 1994) and only the data for the *cop1–5* mutant are shown here (Fig. 2, A–F). Figure 2, A and D, shows the expected nuclear and nonnuclear staining in the dark-grown and light-grown seedlings, respectively. After 12 h of light or dark treatment we observed little change in the intensity of nuclear staining (not shown). A change in the staining pattern was apparent after 24 h of light (Fig. 2B) or darkness (Fig. 2E) and after 36 h (Fig. 2, C and F) of treatment. However, for each treatment there was no difference between the kinetic behaviors of the nuclear level of GUS-COP1 in the wild-type or *cop1–5* null backgrounds. We therefore conclude that the endogenous COP1 protein does not interfere significantly with the nuclear localization of GUS-COP1 and that the kinetics of relocalization for the functional GUS-COP1 protein is a rather slow process. This conclusion implies that the repartitioning of GUS-COP1 in response to light is unlikely to be the cause for the concurrent developmental switch; rather, repartitioning must be a consequence of other, earlier, and as-yet undefined light-triggered events. However, it is possible that light-regulated GUS-COP1 accumulation or depletion within the nucleus plays a role in stabilizing the resulting developmental program under the prevailing light conditions, and other photomorphogenic genes may be involved in this light-regulated subcellular repartitioning process.

GUS-COP1 Nuclear Localization in Darkness Is Defective in All Pleiotropic *cop/det/fus* Mutants

If any of the pleiotropic *COP/DET/FUS* genes (*COP8–COP10, DET1, FUS4–FUS6, FUS11,* and *FUS12,* apart from *COP1*) were required for the nuclear accumulation of COP1, their loss-of-function mutations should prevent the nuclear accumulation of COP1 in the dark, which would result in the observed pleiotropic photomorphogenic phenotype. To test this hypothesis, the *GUS-COP1* transgene was crossed into representative alleles of all available *cop/det/fus* mutant backgrounds other than *cop1*. The effect of the mutations on COP1 nuclear localization was examined by analyzing the subcellular GUS-staining patterns. Similar to the *cop9* mutant (Chamovitz et al., 1996), each of the other eight pleiotropic mutants tested exhibited the same phenotype with respect to GUS-COP1 localization, and the data obtained with dark-grown *cop10, fus12,* and *det1* seedlings are shown as examples in comparison with the wild type (Fig. 2, G–N). It is evident that no nuclear staining for GUS-COP1 was detected in the dark-grown Arabidopsis hypocotyls of any of the mutants analyzed. This absence resembles that of the GUS protein control (data not shown). Therefore, the wild-type gene products of all of the nine pleiotropic *cop/det/fus* genes are required for the nuclear accumulation of the GUS-COP1 protein under dark-grown conditions.

Several pieces of evidence indicate that the mutations do not, however, result in a general defect in nuclear uptake. First, as a positive control, we analyzed the ability of each mutation to affect the nuclear localization of the GUS-NIa fusion protein, a constitutively nuclear protein in which nuclear localization in transgenic Arabidopsis was not affected by the presence or absence of light signals (von Arnim and Deng, 1994). The GUS-NIa protein could localize to the nuclei of all photomorphogenic mutant hypocotyls under both light and dark conditions. Second, the root-tip cells of several *cop/det/fus* mutants showed nuclear staining for both GUS-COP1 and GUS-NIa (not

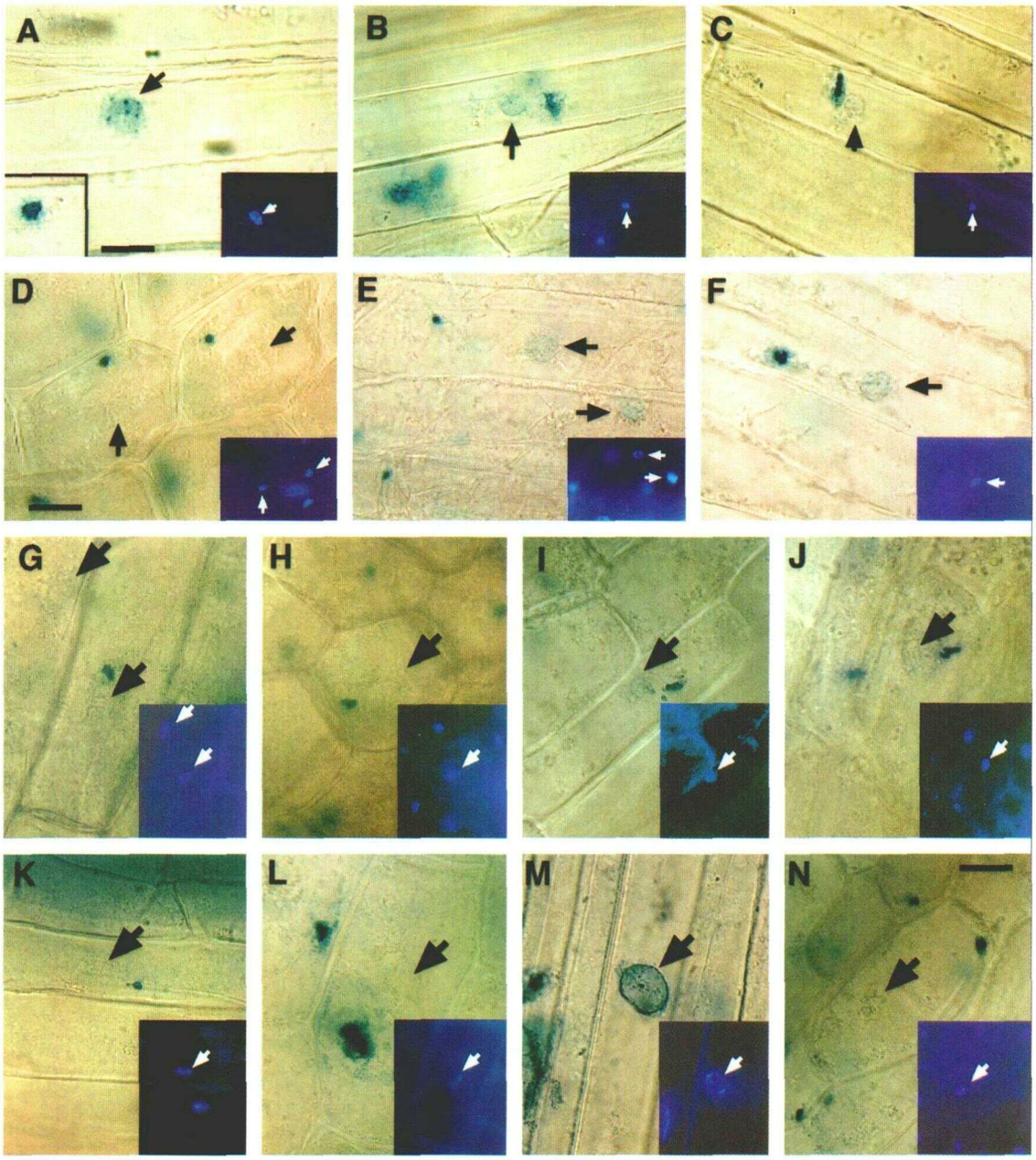

Figure 2. A to F, Kinetics of GUS-COP1 relocalization between cytoplasm and nucleus in *cop1–5* mutant seedlings. Seedlings were stained for GUS activity (brightfield images) and for the position of the nucleus (arrows) using DAPI and epifluorescence illumination (insets). Scale bars = 10 μm. A to C, Dark to light shift: Seedlings were kept in darkness for 5 d (A) or transferred to light for 24 h (B) or 36 h (C) toward the end of 5 d of germination. For the cell shown in A, the cytoplasmic fraction of GUS-COP1 is shown in the left inset. D to F, Light to dark shift: Seedlings were germinated in continuous white light and kept under light conditions for 5 d (D) or transferred to darkness for 24 h (E) or 36 h (F) toward the end of the 5-d period. G to N, Subcellular localization of GUS-COP1 in hypocotyls of severe *cop*, *fus*, and *det* gene mutants. G and H, *cop10*; I and J, *fus12*; K and L, *det1;* and M and N, wild type. Dark-grown (G, I, K, and M) and light-grown seedling hypocotyls (H, J, L, and N) are arranged in pairs.

shown). We therefore conclude that mutations in the nine pleiotropic *cop/det/fus* loci specifically affect, directly or indirectly, the nuclear accumulation of GUS-COP1 in the hypocotyl cells of dark-grown seedlings. It should be noted that none of the mutant phenotypes were detectably affected by the presence of the *GUS-COP1* transgene.

The Less Pleiotropic *cop4* Mutation Has No Effect on Nuclear Accumulation of GUS-COP1

We also tested the effect of less pleiotropic photomorphogenic mutations on the nuclear accumulation of GUS-COP1 using the *cop4* mutant as an example. The dark-grown

cop4–1 mutant exhibits photomorphogenic cotyledon development, including cell differentiation and light-regulated gene expression, but has normal etioplast development (Hou et al., 1993). However, the dark-grown *cop4–1* mutants have a regular long hypocotyl resembling that of the wild type, except for a deficiency in gravitropic orientation (Hou et al., 1993). As shown in Figure 3, C and F, the GUS-COP1 protein localized normally to the hypocotyl cell nuclei of dark-grown and light-grown *cop4* seedlings just as in the wild type. This result indicates that the *COP4* gene product is not required for nuclear accumulation of GUS-COP1 in darkness, which is consistent with the observed normal hypocotyl elongation in *cop4–1* mutant seedlings.

Cytokinin Inhibits Hypocotyl Elongation but Does Not Prevent GUS-COP1 Nuclear Accumulation in Dark-Grown Arabidopsis Seedlings

Since all pleiotropic *cop/det/fus* mutants that lacked nuclear staining of GUS-COP1 in the dark-grown hypocotyl cells also exhibited a severe inhibition of hypocotyl cell elongation, it was necessary to test whether the lack of nuclear accumulation is not a consequence of the inhibition of hypocotyl cell elongation. To address this, we examined the effect of cytokinin, which severely inhibits hypocotyl cell elongation, on the nuclear accumulation of GUS-COP1 in the hypocotyl cells of dark-grown Arabidopsis seed-

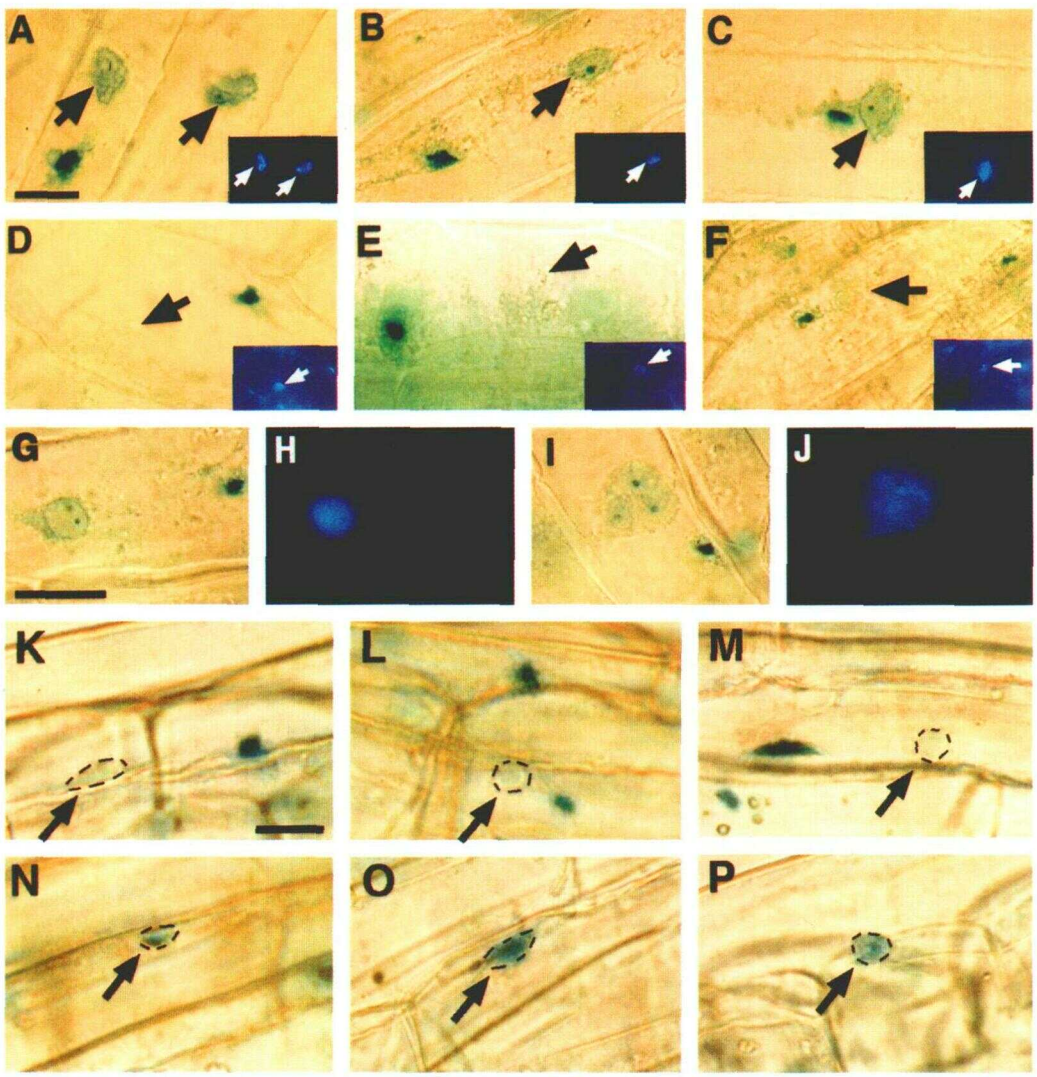

Figure 3. A to F, Subcellular localization of GUS-COP1 in dark-grown (top row) and light-grown (second row) seedlings of the mild photomorphogenic mutants *det2* (B and E) and *cop4* (C and F). The wild type is shown in A and D for comparison. For details, see legend to Figure 2. G to J, Cytokinin treatment of dark-grown wild-type seedlings. Subcellular localization of GUS-COP1 in the presence (G and H) and absence (I and J) of 0.03 mM cytokinin. H and J show nuclear DAPI stains of the cells in G and I, respectively. K to P, BR treatment of seedlings grown for 5 d under constant white light: Subcellular localization of GUS-COP1 (K–N) and GUS-NIa (O and P) in untreated (K and O) or BR-treated (L, M, N, and P) seedlings. K, GUS-COP1, no BR; L, GUS-COP1, 10 nM BR; M, GUS-COP1, 1 μM BR; N, GUS-COP1, 1 μM BR; nuclear staining as seen in N is rare and occurs in approximately 2% of the hypocotyl cells; O, GUS-NIa, no BR; P, GUS-NIa, 100 nM BR. Nuclei are indicated by arrows and dashed oval regions.

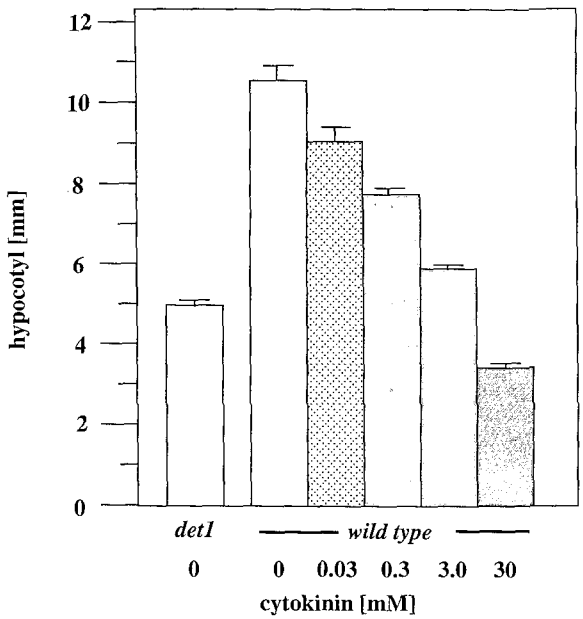

Figure 4. GUS-COP1 transgenic seedlings were grown on increasing concentrations of BA, and hypocotyl lengths were measured 4 d after germination. The hypocotyl length of *det1* mutant seedlings is shown for comparison. SEs are indicated by bars.

lings. Wild-type Arabidopsis seedlings carrying the *GUS-COP1* transgene were germinated in darkness on increasing concentrations of the cytokinin BA. Consistent with data previously reported (Chory et al., 1994; Su and Howell, 1995), increasing the cytokinin concentration resulted in dramatically shortened hypocotyls (Fig. 4) and a decrease in hypocotyl cell length (not shown). Note that the hypocotyl of *det1–1* mutant seedlings, which lacks visible nuclear GUS-COP1 (Fig. 2), was longer than the hypocotyl of wild-type plants treated with 30 μM cytokinin. However, as shown in Figure 3G, GUS-COP1 was retained in the hypocotyl cell nuclei even under the highest concentration of cytokinin used (30 μM). A similar efficiency of nuclear accumulation was observed for the GUS-NIa control protein (not shown). These data suggest that inhibition of hypocotyl elongation does not itself detectably affect GUS-COP1 nuclear localization in the dark.

The Inhibition of Hypocotyl Elongation Caused by a Defect in the Brassinosteroid Biosynthetic Pathway Does Not Prevent Nuclear Accumulation of GUS-COP1

Arabidopsis *det2* mutants show a reduced hypocotyl cell elongation, similar to *det1* (Fig. 4), as well as a moderate derepression of light-inducible gene expression (Chory et al., 1991). Recently, the molecular cloning of *DET2* has suggested that it encodes a reductase in the BR biosynthetic pathway, implying that brassinosteroids play an important role in light-regulated development of higher plants (Li et al., 1996). To test whether BR can achieve this role by regulating COP1 localization, nuclear GUS-COP1 staining was first examined in wild-type seedlings under light conditions with increasing levels of BR. BR increased the hypocotyl length (Fig. 5) and decreased the cotyledon size (not shown) of 5-d-old light-grown seedlings, suggesting that BR indeed counteracts two typical photomorphogenic growth responses (von Arnim and Deng, 1996). A limited number of hypocotyl cells (approximately 2%) from the seedlings elongated by BR treatment revealed a nuclear GUS-COP1 level that was clearly increased (Fig. 3N) in comparison with the untreated controls (Fig. 3K). However, the majority of hypocotyl cells still exhibited no nuclear staining (Fig. 3, L and M), similar to the untreated wild-type seedlings. Such a result is typical for a minimal increase in nuclear staining, close to the threshold of detection. We also observed this result at early times (12–24 h) after light to dark shifts of GUS-COP1 transgenic seedlings. We interpret this result as an indication that nuclear GUS-COP1 accumulation is stimulated slightly in hypocotyl cells under the influence of BR.

To further examine the extent to which brassinosteroid may stimulate the nuclear accumulation of GUS-COP1, we introduced GUS-COP1 into the severe allele *det2–1*, which is thought to carry a null mutation for BR biosynthesis (Li et al., 1996), and analyzed its nuclear localization pattern. Despite the partial de-etiolation in *det2*, GUS-COP1 remained localized to the nuclei in the dark-grown *det2* hypocotyl cells (Fig. 3, B and E), and the intensity of nuclear staining appeared only slightly reduced as compared with that seen in dark-grown wild-type seedlings (Fig. 3, A and D). To properly interpret the level of nuclear staining, it is illustrative to compare the hypocotyl lengths of dark-grown *det1* and *det2* seedlings alongside their levels of nuclear GUS-COP1 protein. Although *det1* and *det2* seedlings have, in fact, hypocotyls of comparable lengths, the nuclear level of GUS-COP1 is dramatically lower in *det1* (Fig. 2, K and L) than that in *det2* seedlings (Fig. 3, B and E). Therefore, the role of DET1 in securing the nuclear accumulation of GUS-COP1 must be far more significant than

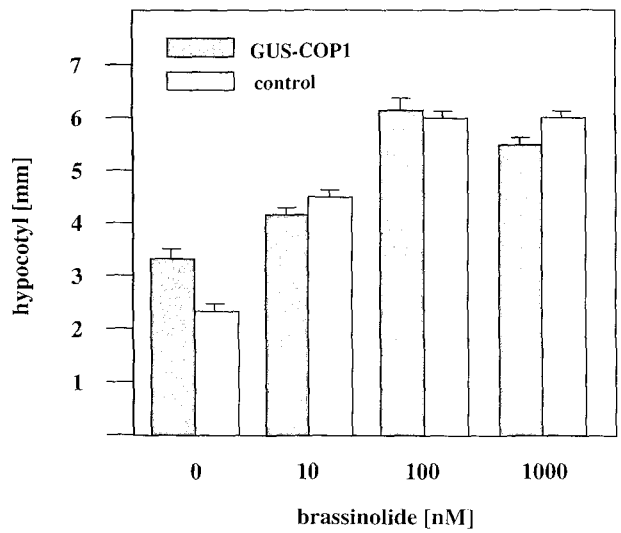

Figure 5. Hypocotyl lengths of *GUS-COP1* transgenic seedlings, and *GUS-NIa* transgenic seedlings as controls, grown for 5 d under constant white light (30 μE m^{-2} s^{-1}) on increasing concentrations of BR. SEs are indicated by bars.

the role of DET2. These distinct effects of *det1* and *det2* mutations on the nuclear localization of GUS-COP1 are consistent with the notion that these two genes act in separate pathways, as reasoned previously (Li et al., 1996). The partial reduction in nuclear staining intensity seen in dark-grown *det2* mutant seedlings is consistent with a partial response to BR.

DISCUSSION

Implications of Temporal Patterns of GUS-COP1 Relocalization

We have previously demonstrated that the activity of the Arabidopsis repressor of photomorphogenesis COP1 may be regulated by a light-mediated nucleocytoplasmic partitioning process (von Arnim and Deng, 1994). Here we analyzed the kinetics of relocalization of COP1 nucleocytoplasmic partitioning during light/dark transitions to clarify the role of this process in the light control of Arabidopsis seedling development. To this end, the GUS-COP1 transgene was first crossed into the *cop1–5* null mutant background, which lacks detectable COP1 protein (Deng et al., 1992; McNellis et al., 1994a). This was possible because the *GUS-COP1* gene completely reverted the lethal *cop1–5* mutant phenotype to wild type. After re-examining the kinetics of GUS-COP1 relocalization in response to shifts from darkness to light and vice versa in the absence of endogenous COP1 protein, we reaffirmed that relocalization of GUS-COP1 proceeds relatively slowly, especially when compared with many known light-inducible developmental and physiological responses (Cosgrove, 1994; Adamska, 1995; Terzaghi and Cashmore, 1995). It is therefore likely that the switch from the etiolation pathway toward the photomorphogenic pathway and vice versa may be triggered by some rapid signaling event(s) other than the subcellular localization of COP1, and the light-regulated nuclear depletion of COP1 may serve to maintain the resulting developmental program. It is unlikely that our analysis severely underestimated the speed of relocalization of the endogenous COP1 protein, which might be expected if the cellular relocalization machinery became saturated by an excess of GUS-COP1 protein. Although GUS-COP1 is overexpressed approximately 10-fold compared with endogenous COP1 (von Arnim and Deng, 1994), the majority of this protein is in the insoluble cytoplasmic "inclusion body" and therefore should not affect the relocalization machinery, if the latter was indeed saturable. Although we cannot rule out that the larger GUS-COP1 fusion protein might have more difficulty interacting with the cellular machinery, the complete phenotypic rescue of GUS-COP1 to the *cop1–5* null mutation would argue against it.

The slow kinetics of relocalization of the GUS-COP1 protein is in line with the assumption that, under photoperiods experienced by *A. thaliana* during the growing season in its natural, temperate zone environment, 8 to 14 h of darkness during the night should be insufficient to reverse the commitment of a light-grown seedling from the photomorphogenic toward the etiolated pathway. Rather, once photomorphogenesis has been initiated, a minute level of nuclear COP1, close to the detection limit imposed by the sensitivity of the GUS reporter, may be all that is required to carry out the functions of COP1 during light-grown development (Deng et al., 1992; Miséra et al., 1994; von Arnim and Deng, 1994). A low level of nuclear COP1 would reflect the commitment to a photomorphogenic program under regular short- or long-day photoperiods, contrasting with the undetectable nuclear COP1 level under high intensity, continuous white light. In fact, such a low level of nuclear GUS-COP1 protein is observed when Arabidopsis seedlings are grown under a day/night cycle (von Arnim and Deng, 1994). The low level of nuclear COP1 may be sufficient to carry out the functions of the COP1 protein during normal light development (Deng et al., 1992) and throughout the vegetative stage of development (Miséra et al., 1994), whereas a higher level of nuclear COP1 is required for full repression of the photomorphogenic program in dark-grown seedlings. The need for a full reversion to the dark developmental pathway, although possible under conditions such as the sudden covering up of a seedling by falling leaves or debris, is probably rarely encountered. To convince the seedling that such a condition has arrived, light deprivation for longer than 14 h may be needed to accumulate a high level of nuclear COP1. In conclusion, our data fit the interpretation that the level and kinetics of nuclear GUS-COP1 are designed to retain the developmental commitment of the Arabidopsis seedling to a particular developmental pathway, and the relatively slow kinetics of relocalization may act as a built-in dampening factor to avoid rapid shifts between the seedling's overall developmental pattern during the frequent fluctuations in ambient light levels over the day/night cycle.

Genetic Interactions Regulating the Nuclear Accumulation of COP1

Mutations in at least nine Arabidopsis genes result in a phenotype that is either indistinguishable or closely related to that of *cop1* mutants. These genes have recently been designated as the pleiotropic *COP/DET/FUS* loci (Kwok et al., 1996), and their similar phenotypes are thought to indicate that the corresponding wild-type gene products interact closely in the repression of photomorphogenesis. We have therefore analyzed the requirement for each of eight of these nine loci on the nuclear localization of GUS-COP1 in Arabidopsis hypocotyl cells and found that each of them is required for the nuclear accumulation of GUS-COP1. An identical result was recently described for the ninth gene of the group, *COP9* (Chamovitz et al., 1996). In contrast to the pleiotropic *COP/DET/FUS* loci, mutations in a less pleiotropic gene, *COP4* (Hou et al., 1993), did not reveal an involvement in regulating the nuclear localization of COP1. This result is consistent with the placement of COP4 function in a pathway downstream from or independent of COP1.

Our result reconfirms the close mutual interaction of the 10 *COP/DET/FUS* gene products. It remains to be shown whether the function of the nine gene products is to retain GUS-COP1 in the nucleus after it has been imported, or

whether their function is to mediate nuclear import of GUS-COP1. The nuclear localization of DET1 (Pepper et al., 1994), COP9, (Chamovitz et al., 1996), and COP11/FUS6 (Staub et al., 1996) is consistent with either of these functions, retention in the nucleus or assistance in nuclear uptake. The protein complex formation among at least two of the COP/DET/FUS gene products, namely COP9 and COP11/FUS6, and possibly involving COP8 (Wei et al., 1994a; Chamovitz et al., 1996; Staub et al., 1996), is also consistent with our data. Indeed, if the COP9 complex is essential, one would predict that *cop9* and *cop11* mutations as well as *cop8* have identical effects on GUS-COP1 localization, as has been observed. Additional molecular analysis is required to understand the role of other COP/DET/FUS gene products in retaining COP1 in the nucleus.

Effects of Plant Hormones and Growth Regulators

Apart from the effects of photomorphogenic mutations, a number of plant growth regulators and plant hormones have been shown to affect seedling development in ways that are reminiscent of the effects of light or dark signals (Chory et al., 1994; Su and Howell, 1995; Weatherwax et al., 1996). However, only very recently has it been realized that brassinosteroids can act as growth regulators to contribute to the skotomorphogenic development seen in dark-grown seedlings (Mandava, 1988; Kauschmann et al., 1996; Li et al., 1996; Szekeres et al., 1996). Their contribution is particularly intriguing because the application of brassinosteroids can partially ameliorate some aspects of the mutant phenotypes, *cop1*, for example, of the *COP/DET/FUS* class (Szekeres et al., 1996). Other growth regulators, such as cytokinins and auxins, have so far proven unable to rescue at least the *cop1* mutant phenotype (Deng et al., 1991; Deng and Quail, 1992).

To test the hypothesis that brassinosteroids may mediate hypocotyl cell elongation by increasing the nuclear accumulation of GUS-COP1, we have made use of a mutation that is thought to disrupt the biosynthesis of brassinosteroids in Arabidopsis, namely *det2* (Li et al., 1996). Apart from reduced fertility and slower senescence, *det2* mutants show a mild defect in photomorphogenesis, having partially lost many morphological attributes of the etiolated seedling but retaining the arrest in chloroplast development from the wild-type phenotype (Chory et al., 1991). The DET2 gene product has similarity to mammalian steroid reductases and, importantly, the end product of the DET2-mediated biosynthetic pathway completely rescues the mutant phenotype of *det2* but not of *det1* (Li et al., 1996). It is thought that a functional brassinosteroid synthesis pathway is essential but not sufficient for the full complement of etiolated phenotypes under dark conditions. However, even in the absence of a functional DET2 product, GUS-COP1 protein was found in the nucleus of dark-grown hypocotyl cells (Fig. 3). Clearly, signaling by brassinosteroids is not essential for the nuclear accumulation of COP1. However, a quantitative contribution of brassinosteroid/DET2 signaling could not be excluded. Indeed, such a contribution was suggested by the observed stimulation of GUS-COP1 localization to the nucleus that was caused by treatment of light-grown seedlings with BR (Fig. 3).

While brassinosteroids can mediate cell elongation responses in the context of etiolation, cytokinins are thought to initiate many of the developmental responses that are also controlled by light signals. For example, treatment of dark-grown seedlings with the cytokinin BA or other cytokinins results in opening of the apical hook, partial unfolding and expansion of cotyledons, plastid development, derepression of light-inducible genes, and strong inhibition of hypocotyl cell elongation (Chory et al., 1994; Su and Howell, 1995). Two explanations for the role of cytokinin during seedling development have been put forward. On one hand, cytokinins may be part of the light signal transduction pathway (Chory et al., 1994), and on the other, they may act independently of light on the same morphogenic responses (Su and Howell, 1995). Unfortunately, viable mutations known to specifically reduce the concentration of cytokinins are not available in Arabidopsis. We have therefore examined the possible contribution of cytokinins to the exclusion of GUS-COP1 from the nuclear compartment by growing seedlings in darkness on increasing concentrations of the cytokinin BA.

Although this treatment resulted in a severe reduction in hypocotyl length, GUS-COP1 nuclear localization was not abolished. This was particularly significant in comparison to the complete absence of GUS-COP1 nuclear localization in the *det1* mutant, in which hypocotyl cell length is similar to that after cytokinin treatment. Cytokinin may therefore act downstream or independently of GUS-COP1 localization when mimicking photomorphogenic responses in dark-grown seedlings. This conclusion is consistent with results obtained in a quantitative analysis of the interaction between cytokinin and light treatments on hypocotyl length in Arabidopsis (Su and Howell, 1995).

ACKNOWLEDGMENTS

We are grateful for the mutant lines *det1* and *det2* from Dr. J. Chory and for the mutant lines *fus4*, *fus5*, *fus11*, and *fus12* from Dr. S. Miséra. In addition, the generous supply of BR from Dr. S. Fujioka is acknowledged. We thank L.H. Ang for assistance with a number of the genetic crosses.

Received November 25, 1996; accepted March 28, 1997.
Copyright Clearance Center: 0032-0889/97/114/0779/10.

LITERATURE CITED

Adamska I (1995) Regulation of early light-inducible protein gene expression by blue and red light in etiolated seedlings involves nuclear and plastid factors. Plant Physiol **107:** 1167–1175

Bowler C, Chua N (1994) Emerging themes of plant signal transduction. Plant Cell **6:** 1529–1541

Castle L, Meinke D (1994) A *FUSCA* gene of *Arabidopsis* encodes a novel protein essential for plant development. Plant Cell **6:** 25–41

Chamovitz DA, Wei N, Osterlund MT, von Arnim AG, Staub, JM, Matsui M, Deng X-W (1996) The COP9 complex, a novel multisubunit nuclear regulator involved in light control of a plant developmental switch. Cell **86:** 115–121

Chaudhury AM, Letham S, Craig S, Dennis ES (1993) *amp1*, a mutant with high cytokinin levels and altered embryonic pat-

tern, faster vegetative growth, constitutive photomorphogenesis, and precocious flowering. Plant J **4:** 907–916

Chory J, Nagpal P, Peto CA (1991) Phenotypic and genetic analysis of *det2*, a new mutant that affects light-regulated seedling development in *Arabidopsis*. Plant Cell **3:** 445–449

Chory J, Peto C, Feinbaum R, Pratt L, Ausubel F (1989) *Arabidopsis thaliana* mutant that develops as a light grown plant in the absence of light. Cell **58:** 991–999

Chory J, Reinecke D, Sim, S., Washburn T, Brenner M (1994) A role for cytokinins in de-etiolation in Arabidopsis. *det* mutants have an altered response to cytokinins. Plant Physiol **104:** 339–347

Cosgrove D (1994) Photomodulation of growth. *In* RE Kendrick, GHM Kronenberg, eds, Photomorphogenesis in Plants. Kluwer, Dordrecht, The Netherlands, pp 631–658

Deng X-W, Caspar T, Quail PH (1991) *cop1*: a regulatory locus involved in light-controlled development and gene expression in *Arabidopsis*. Genes Dev **5:** 1172–1182

Deng X-W, Matsui M, Wei N, Wagner D, Chu AM, Feldmann KA, Quail PH (1992) COP1, an *Arabidopsis* regulatory gene, encodes a novel protein with both a Zn-binding motif and a Gβ homologous domain. Cell **71:** 791–801

Deng X-W, Quail PH (1992) Genetic and phenotypic characterization of *cop1* mutants of *Arabidopsis thaliana*. Plant J **2:** 83–95

Hou Y, von Arnim AG, Deng X-W (1993) A new class of *Arabidopsis* constitutive photomorphogenic genes involved in regulating cotyledon development. Plant Cell **5:** 329–339

Kauschmann A, Jessop A, Koncz C, Szekeres M, Willmitzer L, Altmann T (1996) Genetic evidence for an essential role of brassinosteroids in plant development. Plant J **9:** 701–713

Kendrick RE, Kronenberg GHM (1994) Photomorphogenesis in Plants. Kluwer, Dordrecht, The Netherlands

Kim BC, Soh MS, Kang BJ, Furuya M, Nam HG (1996) Two dominant photomorphogenic mutations of *Arabidopsis thaliana* identified as suppressor mutations of *hy2*. Plant J **9:** 441–456

Kwok SF, Piekos B, Miséra M, Deng X-W (1996) A complement of ten essential and pleiotropic Arabidopsis *COP/DET/FUS* genes is necessary for repression of photomorphogenesis in darkness. Plant Physiol **110:** 731–742

Lehmann A, Black R, Ecker J (1996) *Hookless1*, an ethylene response gene, is required for differential cell elongation in the *Arabidopsis* hypocotyl. Cell **85:** 183–194

Li J, Nagpal P, Vitart V, McMorris TC, Chory J (1996) A role for brassinosteroids in light-dependent development in *Arabidopsis*. Science **272:** 398–401

Liscum E, Hangarter RP (1994) Mutational analysis of blue-light sensing in *Arabidopsis*. Plant Cell Environ **17:** 639–648

Mandava BN (1988) Plant growth-promoting brassinosteroids. Annu Rev Plant Physiol Plant Mol Biol **39:** 23–52

McNellis TW, Deng X-W (1995) Light control of seedling morphogenetic pattern. Plant Cell **7:** 1749–1761

McNellis TW, Torii KU, Deng X-W (1996) Expression of an N-terminal fragment of COP1 confers a dominant-negative effect on light-regulated seedling development in Arabidopsis. Plant Cell **8:** 1491–1503

McNellis TW, von Arnim AG, Araki T, Komeda Y, Miséra S, Deng X-W (1994a) Genetic and molecular analysis of an allelic series of *cop1* mutants suggests functional roles for the multiple protein domains. Plant Cell **6:** 487–500

McNellis TW, von Arnim AG, Deng X-W (1994b) Overexpression of *Arabidopsis* COP1 results in partial suppression of light-mediated development: evidence for a light-inactivable repressor of photomorphogenesis. Plant Cell **6:** 1391–1400

Millar AJ, McGrath RB, Chua, N-H (1994) Phytochrome phototransduction pathways. Annu Rev Genet **28:** 325–349

Miséra S, Müller AJ, Weiland-Heidecker U, Jürgens G (1994) The *FUSCA* genes of *Arabidopsis*: negative regulators of light responses. Mol Gen Genet **244:** 242–252

Pepper A, Delaney T, Washburn T, Poole D, Chory J (1994) *DET1*, a negative regulator of light-mediated development and gene expression in *Arabidopsis*, encodes a novel nuclear-localized protein. Cell **78:** 109–116

Quail PH, Boylan MT, Parks BM, Short TW, XuY, Wagner D (1995) Phytochromes: photosensory perception and signal transduction. Science **268:** 675–680

Smith H (1995) Physiological and ecological function within the phytochrome family. Annu Rev Plant Physiol Plant Mol Biol **46:** 289–315

Staub JM, Wei N, Deng X-W (1996) Evidence for COP11/FUS6 as a component of the nuclear localized COP9 complex in *Arabidopsis*. Plant Cell **8:** 2047–2056

Su W, Howell SH (1995) The effects of cytokinin and light on hypocotyl elongation in Arabidopsis seedlings are independent and additive. Plant Physiol **108:** 1423–1430

Szekeres M, Nemeth K, Koncz-Kalman Z, Mathur J, Kauschmann A, Altmann T, Redei GP, Nagy F, Schell J, Koncz C (1996) Brassinosteroids rescue the deficiency of CYP90, a cytochrome P450, controlling cell elongation and de-etiolation in *Arabidopsis*. Cell **85:** 171–182

Takahashi T, Gasch A, Nishizawa N, Chua N-H (1995) The *DIMINUTO* gene of *Arabidopsis* is involved in regulating cell elongation. Genes Dev **9:** 97–107

Terzaghi WB, Cashmore AR (1995) Light-regulated transcription. Annu Rev Plant Physiol Plant Mol Biol **46:** 445–474

von Arnim AG, Deng X-W (1994) Light inactivation of *Arabidopsis* photomorphogenic repressor COP1 involves a cell type specific modulation of its nucleocytoplasmic partitioning. Cell **79:** 1035–1045

von Arnim AG, Deng X-W (1996) Light control of seedling development. Annu Rev Plant Physiol Plant Mol Biol **47:** 215–243

Weatherwax SC, Ong MS, Degenhardt J, Bray EA, Tobin EM (1996) The interaction of light and abscisic acid in the regulation of plant gene expression. Plant Physiol **111:** 363–370

Wei N, Chamovitz DA, Deng X-W (1994a) *Arabidopsis* COP9 is a component of a novel signaling complex mediating light control of plant development. Cell **78:** 117–124

Wei N, Deng X-W (1992) COP9: a new genetic locus involved in light-regulated development and gene expression in *Arabidopsis*. Plant Cell **4:** 1507–1518

Wei N, Kwok SF, von Arnim AG, Lee A, McNellis TW, Piekos B, Deng X-W (1994b) *Arabidopsis COP8, COP10* and *COP11* genes are involved in repression of photomorphogenic developmental pathway in darkness. Plant Cell **6:** 629–643