Arabidopsis mutants define downstream branches in the phototransduction pathway

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Light regulates the development of *Arabidopsis* seedlings in a variety of ways, including inhibition of hypocotyl growth and promotion of leaf development, chloroplast differentiation, and light-responsive gene expression. Mutations that uncouple most or all of these responses from light control have been described, for example, *det1*, *det2*, and *cop1*. To identify regulatory components that define downstream branches in the light-regulated signal transduction pathway, mutants specifically affected in only one light-regulated response were isolated. A screen was designed to isolate mutants that overexpressed the *CAB* (photosystem II type I chlorophyll *a/b*-binding proteins) genes in the dark, by use of a transgenic line containing a T-DNA construct with two *CAB3* promoter-reporter fusions. Eight mutants that showed aberrant expression of both *CAB3* promoters were isolated and were designated *doc* mutants (for dark overexpression of *CAB*). All of the mutants have normal etiolated morphology in the dark. Genetic and phenotypic analyses indicate that most of the mutations are recessive and define at least three loci (*doc1*, *doc2*, *doc3*). Unlike *det1* and *det2* mutants, which affect the expression of *CAB* and *RBCS* (the small subunit of RuBP carboxylase) to approximately the same extent, all three *doc* mutations are much more specific in derepressing the expression of *CAB*. The phenotypes of *doc* mutants suggest that morphological changes can be genetically separated from changes in *CAB* gene expression. Moreover, the regulation of *CAB* gene expression can be separated further from the regulation of *RBCS* gene expression. Epistasis studies suggest that *DOC1* and *DET3* act downstream from *DET1* on two separate branches in the phototransduction pathway. In contrast, *DOC2* appears to act on a distinct pathway from *DET1*. Mutations in *doc1*, *doc2*, or *doc3* also impair plant growth under short-day conditions.

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Light plays a pivotal role in the development of plants. It regulates germination, leaf development, organelle morphology, photosynthesis, and gene expression (Chory 1991). One of the most important challenges in studying plant development is to understand the light-regulated signal transduction pathways that lead to these developmental responses.

The best-defined molecular event in light-induced responses is the expression of light-regulated genes, such as the genes encoding the chlorophyll *a/b*-binding proteins of photosystem II (*CAB*), the small subunit of RuBP carboxylase (*RBCS*), and chalcone synthase. Analyses of light-regulated expression of these genes have increased our understanding of the light signal transduction pathways. For example, deletion and mutational analyses of the promoters of these genes and in vitro protein-binding assays using promoter fragments have identified several *cis*-acting sequences and *trans*-acting factors with possi-

ble functions in light-regulated transcription (for review, see Gilmartin et al. 1990; Li et al. 1993). Some common features can be found among the different *cis*-acting elements and also among the various *trans*-acting factors. For instance, two or more distinct *cis*-acting elements are required for the expression of a light-inducible promoter (Weisshaar et al. 1991) and the light-regulatory elements are redundant (e.g., Gilmartin et al. 1990). Also, in vitro DNA-binding activities of most nuclear factors (and the mRNA levels of those factors for which a cDNA has been isolated) are not light-regulated and are often found to be present in all tissues (Gilmartin et al. 1992; Perisic and Lam 1992; Schindler et al. 1992). Explanations for this lack of specificity are that post-translational modifications or some other protein factor, for example, a repressor in the dark or an activator in the light, are required for the proper function of these *trans*-acting factors. At present, the biological functions of these nuclear factors are not clear.

Genetics is an alternative tool for studying light-regulated gene expression, with the potential to identify genes on the basis of their functional importance. Mu-

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tants have been isolated that express light-induced genes at high levels in the dark, for example, several of the *det* (de-etiolated, Chory et al. 1989, 1991) and *cop* (constitutively photomorphogenic; Deng et al. 1991; Wei and Deng 1992) mutants of *Arabidopsis thaliana* and the *lip* (light-independent photomorphogenesis) mutant of pea (Frances et al. 1992). However, these mutants were isolated on the basis of their abnormal de-etiolated morphology in the dark, that is, they have opened cotyledons and short hypocotyls. Mutations identified this way are either pleiotropic and affect multiple downstream events or have no effect on light-regulated gene expression at all. For example, the *det1* and *cop1* mutants of *Arabidopsis* not only express light-regulated genes at relatively high levels but also make leaves and chloroplasts in the dark (Chory et al. 1989; Deng et al. 1991). Tissue-specific expression of several light-regulated genes in light-grown *det1* mutants is also altered (Chory and Peto 1990). Thus, DET1 and COP1 most likely regulate early steps in the light-regulated signal transduction pathways, and their effect on photosynthetic gene expression may be indirect. On the other hand, although the *det3* mutant and, to some extent *cop2* and *cop3* mutants have de-etiolated morphology in the dark, they exhibit a wild-type pattern of light-regulated gene expression (Cabrera y Poch et al. 1993; Hou et al. 1993).

We have designed a genetic screen that allows us to isolate mutations that specifically affect the expression of light-regulated genes in the absence of morphological changes. A stably transformed line of *Arabidopsis* was constructed that contained a transgene with two *CAB3* (also called *CAB180*, Leutwiler et al. 1986) promoters fused to two different reporter genes (Chory et al. 1993). Seeds from transgenic plants homozygous for the transgene were mutagenized and used to screen for mutants that expressed both reporter genes at aberrantly high lev-

els in the dark. We report here the characterization of several of the mutants isolated by use of this screen.

Genetic analyses indicated that most of the mutations were recessive and defined a minimum of three loci. Unlike previously isolated mutants, all of the mutants had normal etiolated morphology in the dark. At least two of the mutations specifically affected the accumulation of *CAB*, but not of *RBCS*, mRNAs in the dark. The existence of these mutants indicates that during dark-grown seedling development, *CAB* gene expression can be separated from morphological changes. Moreover, regulation of *CAB* expression can be genetically separated from *RBCS* expression. A pathway positioning these genes in the light-regulated signal transduction chain is proposed based on epistasis studies of these mutants and the previously described *det1* and *det3* mutants.

Results

Isolation of mutants using a dual promoter-reporter transgenic line

We designed a reporter construct to identify mutants in the phototransduction pathway. The T-DNA construct, pOCA107, contained two full-length *CAB3* promoters fused either to the hygromycin phosphotransferase (*hph*) gene or to the *Escherichia coli* β -glucuronidase (*uidA* or GUS) gene (Fig. 1A). We transformed the *Columbia* ecotype of *Arabidopsis* with the construct and generated a number of transgenic lines. One of the resulting transgenic lines, pOCA107-2, was homozygous for a single insertion of the transgene on chromosome II and displayed proper light-regulated and tissue-specific expression of the *CAB3-uidA* transgene (Chory et al. 1993). To isolate mutants that had high *CAB3* promoter activity in the dark, M_2 progeny of ethylmethane sulfonate (EMS) or

Figure 1. Selection of *doc* mutants. (A) Schematic representation of the pOCA107 T-DNA construct. (LB and RB) Left and right borders of the T-DNA, respectively. The kanamycin resistance gene (*nptII*) used for selection is driven by the constitutive cauliflower mosaic virus 35S promoter (35S). One *CAB3* promoter was fused to the hygromycin phosphotransferase (*hph*) gene. The second *CAB3* promoter was fused to the *E. coli* β -glucuronidase (*uidA*) gene. All three reporter genes were fused to the no-palinaline synthase (*nos*) terminator. (B) Hygromycin resistance as measured by hypocotyl length. From the left are shown wild-type, two putative mutants, and the PHG1 (positive control) line. Seedlings were grown for 7 days in the dark on media containing 20 μ g/ml of hygromycin B. (C) The eight *doc* mutants were grown on 25 μ g/ml of hygromycin B for 7 days in the dark. From the left: Wild type, *doc0-6*, *doc1*, *doc3*, *doc2-1*, *doc2-2*, *doc0-11*, *doc0-13*, and *doc0-19*.

γ -ray mutagenized pOCA107-2 seeds were first screened in the dark for hygromycin resistance (i.e., for overexpression of the *CAB3*-*hph* transgene). A similar transgenic line, PHG1, in which the *hph* gene was fused to the phenylalanine ammonia-lyase (PAL) promoter (S. Ohl, B. Kraft, J. Chory, and C.J. Lamb, unpubl.), served as a positive control. Because the PAL promoter was highly expressed in the dark, the PHG1 line was hygromycin resistant when grown in the dark on media containing hygromycin. It had a normal etiolated morphology with a hypocotyl length of ~14 mm (Fig. 1B). In contrast, the unmutagenized pOCA107-2 line had a hypocotyl length of 1 to 4 mm (Fig. 1B). Hypocotyl length was thus used as an indicator for hygromycin resistance.

We selected 375 putative mutants with long hypocotyls on hygromycin media in the dark and allowed them to recover in the light and set seeds. To eliminate mutants that had only *cis*-acting mutations in the *CAB3*-*hph* transgene, progeny from the putative mutants were screened further for the expression of the second transgene, *CAB3*-*uidA*, by assaying GUS activity of pooled dark-grown seedlings. Forty putative mutant lines also had higher specific GUS activities than the wild type. After rescreening for the expression of both reporter genes in the next few generations, eight putative mutant lines consistently showed longer hypocotyls on hygromycin-containing media (Fig. 1C) and had three- to sevenfold higher specific GUS activities (Table 1) than the wild type (pOCA107-2). These mutants most likely contained mutations that acted in *trans* to affect the expression of both *CAB3* promoters in the transgene. The putative mutants were thus designated *doc* for dark overexpression of *CAB*. Several hygromycin-resistant mutants with a de-etiolated morphology (short hypocotyl and opened cotyledon) were also isolated during the screen. Genetic analyses indicated that they were allelic to the *det1* mutant (H. Cabrera y Poch, L. Atlshmiel, and J. Chory, unpubl.).

Genetic analysis of *doc* mutants

To determine the nature of the mutations, putative mutants were backcrossed to either the parent pOCA107-2

line or to the wild-type *Columbia* ecotype. If the mutations were recessive, the F_1 progeny from the backcross should be hygromycin sensitive with short hypocotyls, that is, wild type in appearance. As shown in Table 2, most of the mutant lines arose from single gene recessive mutations, except *doc0-6*. *doc0-6* was a dominant mutation as indicated both by the backcross and by complementation crosses with other mutants (Table 3). We have not finished the studies with *doc0-19*. *doc3* and *doc0-13* were most likely single gene recessive mutations, even though the segregation ratios of the F_2 progeny fitted better with 3:1 instead of the expected 13:3 (see Table 2 footnote). This might indicate that the mutations were linked to the pOCA107-2 transgene or simply that we encountered difficulty when scoring the mutants.

To determine how many complementation groups these new mutations defined, the mutants were crossed to one another, and the F_1 progeny were tested for resistance to hygromycin. The results indicated that there were two alleles of *doc2*, and *doc1* appeared to complement *doc2-1*, *doc2-2*, and *doc3* (Table 3). We confirmed this by mapping *doc1*, *doc2-1*, and *doc3*. These three mutants were crossed to either *Landsberg* or *Niederzenn* ecotypes, and the DNAs from the F_2 progeny were analyzed using codominant ecotype-specific PCR-based markers (Konieczny and Ausubel 1993). The *DOC1* locus was mapped to the upper portion of chromosome III between the markers *GapC* and *myb4*. *DOC1* was ~3 centimorgans (cM) from *GapC* and 2 cM from *myb4*. The *DOC2* locus was positioned between the markers *DFR* (*tt3*) and *ASA1* on chromosome V, ~34 cM from *DFR*. The *DOC3* locus was mapped to the lower portion of chromosome I between the markers *GapB* and *ADH* (~26 cM from *GapB* and ~31 cM from *ADH*). The dominant mutation, *doc0-6*, was mapped to chromosome II ~23 cM from the pOCA107-2 transgene. Thus, we have identified at least four loci that affected the expression of the two *CAB3* promoters in the transgene, with one allele of *doc1*, *doc3*, and *doc0-6* and two alleles of *doc2*.

doc mutations affect the accumulation of endogenous CAB mRNAs and define a new branch in the signal transduction pathway

Because the mutations in the *doc* mutants increased the expression of both *CAB3* promoters in the transgene, it was likely that the endogenous *CAB* promoters were also affected. Figure 2 shows the result of an RNA gel blot hybridized with a *CAB* gene probe that detects mRNAs from all members in the photosystem II type I *CAB* gene family. All of the mutants, except *doc0-6*, had elevated endogenous *CAB* transcript levels in the dark when compared to the wild type. The extent of derepression varied with each allele. On the basis of three to four separate experiments and densitometer scans, *doc1* had ~6.2-fold elevation of *CAB* mRNAs over the wild type, and *doc2-1* and *doc3* had ~3.5- and 3.2-fold elevation, respectively. The remaining mutants had less than two-

Table 1. Specific GUS activities of *doc* mutants

Lines	GUS activity (pmole/mg per min)	Ratio of mutant/wild type
pOCA 107	74.3 $\pm$ 0.0	1.0
<i>doc1</i>	500.9 $\pm$ 2.5	6.7
<i>doc2-1</i>	526.9 $\pm$ 20.0	7.1
<i>doc2-2</i>	226.8 $\pm$ 13.3	3.1
<i>doc3</i>	277.0 $\pm$ 2.4	3.7
<i>doc0-6</i>	249.7 $\pm$ 0.0	3.4
<i>doc0-11</i>	270.5 $\pm$ 18.9	3.6
<i>doc0-13</i>	377.0 $\pm$ 3.3	5.1
<i>doc0-19</i>	485.2 $\pm$ 17.0	6.5

GUS activity was assayed on seedlings grown for 6 days in the dark.

Table 2. Results of crosses with *doc* mutants

Cross	F ₁	F ₂			
		wild type (hygromycin ^a)	mutant (hygromycin ^a)	ratio of wild type/mutant	χ^2
Co × <i>doc1</i>	WT	54	16 ^a	3.38	0.17 ^b
<i>doc2-1</i> × pOCA107	WT	163	62	2.63	0.78 ^b
pOCA107 × <i>doc2-2</i>	WT	447	154	2.90	0.12 ^b
Co × <i>doc3</i>	WT	184	61	3.02	0.001 ^b
Co × <i>doc0-6</i>	mutant ^c	222	406	0.55	
Co × <i>doc0-11</i>	N.D.	95	28	3.39	1.3 ^d
Co × <i>doc0-13</i>	WT	139	50	2.78	0.21 ^b

Each cross is indicated as female parent × male parent. Seedlings were scored for hygromycin resistance by measuring hypocotyl lengths after growth for 7 days in the dark on media containing 25–40 μ g/ml of hygromycin B. If the mutant phenotype arises from a single gene recessive mutation, the F₁ progeny from the cross should be hygromycin sensitive (short hypocotyls), i.e., wild type (WT). F₂ progeny should show a segregation ratio of 3:1 wild type/mutant if the mutant was crossed to pOCA107, or a ratio of 13:3 wild type/mutant if the mutant was crossed to *Columbia* (Co), as one-fourth of the F₂ mutant seedlings would not have the pOCA107 transgene, which is necessary to confer hygromycin resistance. (N.D.) Not determined.

^aThe mutant was scored with the phenotype of reduced apical dominance.

^bThe χ^2 value is given for the ratio of 3:1 (wild type/mutant).

^cThe F₁ seedlings showed a mixture of hygromycin-sensitive and -resistant plants, indicating that the mutant parent could be heterozygous for the mutation, or the hygromycin-sensitive seedlings resulted from selfing of the *Columbia* parent. The hygromycin-resistant F₁ plants were used to generate the F₂ progeny.

^dThe χ^2 value is given for the ratio of 13:3 (wild type/mutant).

fold increases of *CAB* mRNA over the wild type, and we did not study them further. Surprisingly, *doc0-6* did not have elevated *CAB* mRNA accumulation, despite the observations that the seedlings were hygromycin resistant, had a higher specific GUS activity than the wild type, and the mutation mapped 23 cM away from the transgene.

The expression of another light-regulated nuclear gene, *RBCS*, was also analyzed (Fig. 2). For most mutants the accumulation of *RBCS* mRNA was not as high as the *CAB* mRNA. In particular, in *doc2* and *doc3* mutants *RBCS* transcript levels were approximately the same as in the wild type (a normalized mutant/wild type ratio of 1.5 ± 0.5 and 1.4 ± 0.2 respectively, on the basis of four experiments). In the *doc1* mutant, *RBCS* mRNA levels

were two- to fivefold higher than the wild-type levels. These results indicated that mutations in *DOC2* and *DOC3* specifically affected the dark expression of *CAB* but not *RBCS* genes. *DOC1* had a greater effect on *CAB* than *RBCS* mRNA accumulation. In contrast to other mutations like *det1* and *det2* (Chory et al. 1989, 1991), which affect the expression of *CAB* and *RBCS* to approximately the same extent, *DOC2* and *DOC3* defined a new branch in the signal transduction pathway that was specific for the regulation of *CAB*. On the basis of the above data, *doc1*, *doc2-1*, and *doc3* were chosen for further analyses because they had the highest elevation of *CAB* transcripts in the dark.

Table 3. Complementation crosses among *doc* mutants

Cross	F ₁	
	mutant (hygromycin ^a)	wild type (hygromycin ^a)
<i>doc2-1</i> × <i>doc1</i>	0	6
<i>doc1</i> × <i>doc3</i>	0	4
<i>doc3</i> × <i>doc1</i>	0	6
<i>doc2-1</i> × <i>doc2-2</i>	32	1
<i>doc1</i> × <i>doc0-13</i>	0	17
<i>doc1</i> × <i>doc2-2</i>	0	4
<i>doc0-19</i> × <i>doc0-13</i>	0	3
<i>doc0-6</i> × <i>doc1</i>	13	0
<i>doc0-6</i> × <i>doc2</i>	4	0
<i>doc0-6</i> × <i>doc3</i>	16	0
<i>doc0-13</i> × <i>doc0-6</i>	24	0

All conditions are the same as described in Table 2.

Figure 2. Accumulation of mRNAs for *CAB* and *RBCS* in dark-grown *doc* mutants as determined by an RNA gel blot. Seedlings were grown for 4 days in the dark on synthetic media. Total RNA (5 μ g) from individual mutants (*top*) was loaded per lane. The *CAB* and *RBCS* probes used detect all members in the two gene families. The RNA load in each lane was normalized by probing for *rRNA*. (WT) Wild type.

CAB1 transcripts are also increased in the *doc* mutants

Arabidopsis has at least five members in the photosystem II type I *CAB* gene family (Leutwiler et al. 1986; McGrath et al. 1992). For the three promoters for which expression has been characterized (*CAB1*, *CAB2*, and *CAB3*, also called *CAB140*, *CAB165*, and *CAB180*, respectively; Leutwiler et al. 1986), it has been shown that the three transcripts are differentially expressed both in the light and in the dark (Sun and Tobin 1990; Brusslan and Tobin 1992). The sequence of the *CAB1* promoter is less homologous to that of the *CAB2* and *CAB3* promoters, which are very similar to each other (Leutwiler et al. 1986). *CAB1* transcripts also constitute the majority of *CAB* messages in wild-type dark-grown seedlings (Brusslan and Tobin 1992). Because our mutants were isolated on the basis of increased hygromycin resistance and GUS activity (Table 1), which were reflections of only *CAB3* promoter activity, and the RNA gel blots as shown in Figure 2 could not distinguish among the various *CAB* transcripts, we sought to investigate whether *CAB1* message levels were also affected in the *doc* mutants. Figure 3 shows the result of an RNase protection experiment with a *CAB1*-specific probe (Brusslan and Tobin 1992). The levels of *CAB1* transcripts in the three *doc* mutants were compared with a ubiquitin gene (*UBQ3*), which was used as a constitutive control (Norris et al. 1993). The result showed that *CAB1* transcripts were elevated in the three *doc* mutants, indicating that the three *doc* mutations derepressed both *CAB3* and *CAB1* expression in the dark.

Phenotypes of *doc* mutants

All of the *doc* mutants had normal etiolated morphology when grown in the dark (Fig. 1C; see also Fig. 5, below), indicating that unlike all of the other photomorphogenic mutants isolated to date, these mutations affected the expression of downstream photosynthetic genes in the absence of obvious morphological changes. The simplest

model to explain our mutants is that they are defective in the normal light/dark expression of *CAB*. Brusslan and Tobin (1992) have shown recently that dark-grown *Arabidopsis* seedlings exhibit a developmental regulation of *CAB* and *RBCS* gene expression that is independent of light. In their studies, the expression of *CAB* mRNA transiently peaks at 3 days postgermination and then gradually drops back to basal level.

We analyzed the timing of *CAB* gene expression in dark-grown *doc* mutants. In the three *doc* mutants, both *CAB* mRNA levels and specific GUS activities from the *CAB3* promoter were elevated to the same extent over the levels in wild-type seedlings of the same age throughout the entire time course studied (2–8 days; data not shown). This indicated that the developmental timing of *CAB* expression was not altered in dark-grown *doc* mutants. Moreover, the level of *CAB* mRNA in light-grown *doc* mutants was not significantly different from the wild type (data not shown). Finally, cell-type-specific expression of *CAB* genes in either light- or dark-grown *doc* mutants, as analyzed by histochemical staining of GUS activity, was not altered (data not shown). These results suggested that mutations in the three *DOC* genes were specific for a pathway(s) that functioned to repress *CAB* gene expression in etiolated seedlings.

Of all the mutants, only *doc1* had a clear growth phenotype when grown in the light. Under long-day growth conditions (18 hr light/6 hr dark), mature *doc1* plants had reduced apical dominance (seven to nine inflorescence stems; data not shown) compared with the wild type (one to three inflorescence stems). *doc1* was also shorter than the wild type. In contrast, *doc2* and *doc3* were almost indistinguishable from the wild type when grown under long-day conditions (Fig. 4A). However, when grown under short-day conditions (9 hr light/15 hr dark), all three *doc* mutants were significantly smaller than the wild type and had less than half of the fresh weight of the wild type (Fig. 4B). Thus, mutations that affected transcription of *CAB* promoters in the dark also seemed to have an effect on plant growth when the day length was short.

Figure 3. *CAB1* transcript accumulation in *doc* mutants as determined by RNase protection analysis. Seedlings were grown for 4 days in the dark on synthetic media. Total RNA (3.5 μ g) from individual mutants (top) was loaded per lane. Levels of *UBQ3* RNA were also determined and used to normalize the amount of RNA in each lane. (WT) Wild type.

det-doc double mutant studies

It has been shown that mutations in the *DET1* gene affect several aspects of seedling development in the dark. For example, dark-grown *det1* mutant plants develop as light-grown plants with respect to morphology and the expression of several light-regulated genes (Chory et al. 1989). On the other hand, mutations in the *DET3* gene result in seedlings with a de-etiolated morphology similar to *det1*, but the expression of light-regulated genes is not derepressed, resembling the wild type (Cabrera y Poch et al. 1993). Double mutant studies suggest that *DET3* lies downstream from *DET1* on a branch of the pathway that affects only seedling morphology. We sought to study the epistatic relations and possible interactions of *det1* and *det3* with *doc1* and *doc2* by generating double mutants containing mutations in both groups of genes.

Figure 4. Phenotypes of *doc* mutants when grown in short days. The fresh weights of *doc* mutants were reduced. Plants were grown for 34 days on a synthetic medium in an 18-hr light/6-hr dark long-day cycle (A), or a 9-hr light/15-hr dark short-day cycle (B). At least five plants were measured for each mutant and the wild type (WT).

Seedlings homozygous for both the *det1* and *doc1* mutations (see Materials and methods) had a morphology identical to the *det1* mutant both in the light (data not shown) and in the dark (Fig. 5A). Gene expression changes in the double mutant were analyzed by assaying specific GUS activity of dark-grown doubly mutant seedlings. As shown in Figure 6A, GUS activity of dark-grown *det1-doc1* was comparable to that of the *det1* parent. This indicated that the *det1-doc1* double mutant had the phenotype of *det1* with respect to both *CAB* gene expression and morphological changes, suggesting that

DET1 and DOC1 were on the same signal transduction pathway.

In contrast, seedlings homozygous for *det1* and *doc2* mutations had the additive phenotype of *det1* and *doc2*. Whereas the double mutant had the morphology of *det1* in the dark (data not shown), the specific GUS activity of the dark-grown double mutant was equal to the activity of *det1* plus *doc2* (Fig. 6B). This suggested that the two mutations had independent effects on transcription of the *CAB3* promoter and might act on different signal transduction pathways.

We have also generated the double mutants, *det3-doc1* and *det3-doc2*. Both double mutants had the appearance of *det3* in the dark (*det3-doc2*, Fig. 5B; *det3-doc1*, data not shown). We did not have a *det3* line containing the pOCA107 transgene; however, dark-grown *det3* has *CAB* transcript levels that are similarly low to the wild type (Cabrera y Poch et al. 1993). Therefore, we felt it was valid to compare the specific GUS activity of dark-grown double mutants to that of the wild type. As shown in Figure 6C, GUS activities of the dark-grown double mutants were elevated as in the *doc* mutants, although the degree of elevation was slightly lower than in the *doc* single mutants. These results suggested that the two double mutants had the additive phenotypes of *det3* and the *doc* mutants, suggesting that the *det3* mutation and the two *doc* mutations affected two separate pathways or two different branches of a single pathway.

Discussion

We have identified three new *Arabidopsis* loci (*doc1*, *doc2*, and *doc3*), mutations in which allow derepression of *CAB* expression in dark-grown seedlings in the absence of morphological changes. The phenotypes of these mutants and epistasis studies suggest that these new genes define downstream branches of the light-regulated signal transduction pathways. *doc2* and *doc3* mutations specifically affect the expression of *CAB* genes with little effect on *RBCS* gene expression. Though *CAB* and *RBCS* genes have been shown to be differentially regulated by light fluence (Kaufman et al. 1984), this is the first genetic evidence that the light-regulated expression of *CAB* and *RBCS* genes can be separated. Because the mutations are recessive and affect the expression of multiple genes, these three loci may encode negative regulators or affect the activity of negative factors that repress the expression of *CAB* in the dark.

By a number of criteria, the *doc* mutations are specific for a pathway(s) that functions to repress *CAB* gene expression in etiolated seedlings. First, the levels of *CAB* mRNAs are not derepressed in light-grown *doc* seedlings. In addition, *doc* mutants maintain the normal cell-type-specific expression of *CAB* in both light- and dark-grown seedlings. Finally, the light-independent developmental expression of *CAB* (Brusslan and Tobin 1992) in etiolated seedlings is not altered in *doc* mutants. However, the three *doc* mutations do have some deleterious effects on plants grown in short-day conditions. *doc1*, *doc2-1*, and *doc3* all had severely reduced fresh weights

Figure 5. Dark-grown phenotypes of *det1-doc1* (A) and *det3-doc2* (B) double mutants. Seedlings were grown for 7 days in the dark on synthetic media.

when grown under short-day conditions (Fig. 4). One explanation for this observation is that *doc* mutations result in slower repression of *CAB* gene transcription in the dark. Longer nights would mean more energy is wasted on *CAB* expression in the dark and thus result in impaired growth. An alternative explanation is that the full activation of *CAB* gene expression in the light might require a total inactivation of some repressors that accumulate to down-regulate these genes during the night. Mutations in the *DOC* genes could result in partial or slower inactivation of the repressors in the light. Thus, longer day length might be required for the mutants to

assimilate enough photosynthates to reach wild-type growth levels.

When etiolated seedlings are transferred to the light, a number of developmental and gene expression changes occur. The expression of light-regulated genes, for instance, is induced by ~30- to 50-fold over the basal dark levels (Chory et al. 1989). Numerous positively acting *cis* regulatory elements have been identified in the promoters of light-regulated genes, such as *CAB* and *RBCS* (for review, see Gilmartin et al. 1990; Li et al. 1993). These light-regulatory elements are redundant; with the exception of the G-box element (Donald and Cashmore 1990;

Figure 6. Specific GUS activities of dark-grown *det1-doc1* (A), *det1-doc2* (B), *det3-doc1* and *det3-doc2* (C) double mutants. Seedlings were grown for 6 days in the dark. For several samples the standard errors (on the basis of at least four data points from two independent experiments) are too small to be seen in the scale plotted.

S. Kay, pers. comm.), mutation of an individual element results in no or small changes in promoter activity (three- to eightfold decreases where quantitative data are available; Castresana et al. 1988; Gidoni et al. 1989; Schulze-Lefert et al. 1989). In contrast, there are only a limited number of studies that address negatively acting *cis*-acting elements in these promoters. For instance, a mutation in an upstream negative regulatory element in the *Nicotiana plumbaginifolia* *CABE* promoter results in a fourfold increase of expression of this promoter (Castresana et al. 1988). A mutation in a negative regulatory element in the *Arabidopsis* *CAB2* promoter causes three- to fourfold increases in *CAB2* expression in etiolated seedlings (S. Kay, pers. comm.). In *Lemna*, a number of genes have been identified that are negatively regulated by light. Deletion of the *cis* sequence responsible for increased expression in the dark in one of the promoters results in a 3.5-fold decrease in accumulation of the mRNA (Okubara et al. 1993). The level of derepression of the *CAB3* promoter in the *doc* mutants may reflect the somewhat modular nature of *CAB* promoters. We observed a derepression of three- to sevenfold in *CAB* promoter activity and mRNA accumulation in the three *doc* mutants described here. This is directly correlated with the levels of derepression associated with *CAB* promoters when *cis*-acting negative regulatory elements are individually mutated. Experiments are in progress to attempt to correlate a specific *doc* mutation with a defined *cis*-acting regulatory element. Further genetic and biochemical studies may help to elucidate whether the *DOC* gene products act additively or synergistically to affect the activity of the *CAB* promoters.

CAB and *RBCS* represent the two best-studied light-regulated genes in plants. Proteins encoded by these two gene families are central elements in photosynthesis. Expression of both gene families is under phytochrome and blue light control (Thompson and White 1991), and similar promoter sequences have been identified in both promoters (Gilmartin et al. 1990). These data imply that expression of these two genes may be coordinately regulated by light. However, there is also indirect evidence suggesting that these two genes are differentially regulated. For example, the expression of *CAB* genes is more sensitive to photo-oxidative damage (Susek and Chory 1992). High levels of cytosolic carbohydrates result in a much greater suppression of *RBCS* gene expression than of *CAB* gene expression (Krapp et al. 1993). *CAB* is induced by very low fluences of light, whereas *RBCS* requires higher fluences (Kaufman et al. 1984). *doc2* and *doc3* derepress the expression of *CAB* in the dark with little or no effect on the expression of *RBCS*. It is reasonable to postulate that *CAB* and *RBCS* genes have different requirements for and sensitivities to light stimulation and metabolic balance in plants, considering their roles in light-harvesting and carbon fixation, respectively. Thus, separate mechanisms may have evolved to repress *CAB* and *RBCS* expression in the dark. It is possible that *DOC2* and *DOC3* respond to some environmental conditions that require the modulation of *CAB*, but not *RBCS*, expression. *DOC2* and *DOC3* then regu-

late the activities of *CAB* promoters by interacting with unique sequences in the promoters or by modifying the activities of some *CAB*-specific transcription factors. Alternatively, it is possible that the *doc2* and *doc3* mutations are leaky alleles of a group of transcription factors with different affinities for the *CAB* and *RBCS* promoters.

It has been shown that the three *CAB* promoters of *Arabidopsis* (*CAB1*, *CAB2*, and *CAB3*) are differentially regulated (Karlin-Neumann et al. 1988; Sun and Tobin 1990). In the *Columbia* ecotype, *CAB1* is the major transcript in the dark and the *CAB1* promoter is also much more responsive to red light stimulation than the other two *CAB* promoters (Karlin-Neumann et al. 1988; Brusslan and Tobin 1992). In vitro binding assays also indicate that some protein factors that bind to the *CAB1* promoter do not bind to the *CAB2* promoter (S. Kay, pers. comm.). However, both *CAB1* and *CAB3* expression are derepressed by the mutations in the *doc* mutants that we have analyzed. This indicates that the three *CAB* promoters still share some common regulators in the dark. The three *DOC* loci may encode proteins that act directly on the three promoters, or they may encode proteins that modify the activities of transcription factors that regulate the expression of some or all *CAB* promoters. Interestingly, although *doc3* has the lowest level of *CAB3* promoter activity, it has the highest level of *CAB1* transcripts of the three *doc* mutants. This could indicate that different regulatory factors may have variable affinities for different promoters. Modulation of the availability of these factors, for example, by temporal expression or post-translational protein modifications, may result in differential expression of the various *CAB* promoters.

doc0-6 is an unusual dominant mutation, mapping 23 cM from the transgenes. This mutation affects the expression of the reporter genes but not the endogenous *CAB* genes. This suggests that the *doc0-6* mutation may have some distal *cis* effect on the expression of the transgenes. This is different from a recently reported *Arabidopsis* mutation that resulted in lower *CAB* mRNA levels (Brusslan et al. 1993). This mutation mapped to the transgene itself, causing a distal effect on the expression of the endogenous *CAB* genes. However, we cannot exclude the possibility that the *doc0-6* mutation has some small but specific effect on the expression of endogenous *CAB2* and/or *CAB3* promoters. Because the *CAB1* transcript is the major transcript in the dark (Brusslan and Tobin 1992), a small increase in *CAB2* or *CAB3* transcripts may not be detected by RNA gel blots.

On the basis of our epistasis studies, we propose a model that incorporates the *DOC* gene products into the proposed phototransduction pathway (Fig. 7). Our previous studies have indicated that *DET1* and *DET2* act downstream from both phytochrome and a blue-light photoreceptor and most likely on separate branches of the signal transduction pathways (Chory 1992). Moreover, *det1* is epistatic to *det3*, suggesting that *DET1* acts upstream from *DET3*, whereas the *det2-det3* double mutant shows the phenotype of both parents (Cabrera y

Figure 7. Model for the hierarchical relationships among *DET* and *DOC* gene products in the light-regulated signal transduction pathway. *DET1* is downstream from the red and blue light photoreceptors and negatively controls multiple branches in the pathway hereafter as indicated (Chory 1992). *DET3* lies on a branch of the pathway that leads to changes in morphology (Cabrera y Poch et al. 1993), and the *DOC1* gene product lies on the *CAB* gene expression branch of the same pathway and may also have a small effect on the expression of *RBCS* genes. *DOC2* appears to specifically repress the expression of *CAB* but not *RBCS*. Its effect on the repression of *CAB* mRNA accumulation is independent of *DET1*; thus, it may lie on a separate signal transduction pathway from *DET1*. The model is formal and makes no prediction as to the precise molecular nature of the proposed interactions among genes or gene products. (→) Positive regulation; (⊣) negative regulation. The broken line indicates that the *doc1* mutation has a smaller effect on *RBCS* gene expression than on *CAB* gene expression.

Poch et al. 1993). Therefore, it is hypothesized that *DET2* acts on a separate pathway from *DET1* and *DET3* (Cabrera y Poch et al. 1993). In the studies presented here, the *det1–doc1* double mutant has the morphology and gene expression pattern of *det1*, suggesting that *DOC1* and *DET1* are in the same signal transduction pathway. This is consistent with the observation that light-grown *doc1* also has reduced apical dominance as does *det1*. Because the *doc1* mutant displays only a subset of the phenotypes of the *det1* mutant, we hypothesize that *DOC1* is downstream from *DET1*. In contrast, *doc2* and *det1* appear to have independent effects on the expression of *CAB* because in the *det1–doc2* double mutant, *CAB3* promoter activity is derepressed to a level that is equal to the addition of the two parental mutants. This suggests that *DOC2* is not directly downstream from *DET1*. It is possible that *DOC2* is under the control of another upstream regulator like *DET2* in the light-regulated signal transduction pathways. Alternatively, the regulation of the *CAB* promoters by *DOC2* may respond to signals other than light, for example, the level of carbon source in the growth media (Brusslan and Tobin 1992; Sheen 1990) or localized hormone concentrations (Chory et al. 1994). Finally, the *det3–doc1* and *det3–doc2* double mutant each have the nearly additive phenotypes of the two parents, suggesting that *DET3* and these two *DOC* gene products act on different branches of the pathways. However, the double mutant phenotypes are not perfectly additive: The GUS activities of the double mutants are slightly reduced when compared with the parental *doc* mutants. It is possible that the *det3* mutation may have some negative effect on *CAB* gene expression in the dark.

The *doc* mutants reported here have helped define two branches in the light-regulated signal transduction pathway. The three *DOC* gene products separated the pathway of *CAB* expression from the pathway of hypocotyl growth and leaf expansion and also divided the pathway of *CAB* expression from *RBCS* expression. With the iso-

lation of these mutants, a more complete picture of the light-regulated signal transduction pathways is beginning to emerge. In the future, consolidating genetic with molecular and biochemical approaches will help in revealing the molecular mechanisms controlling light-regulated gene expression in seedling development.

Materials and methods

Plant materials and growth conditions

In all of the experiments described here, the wild type is the unmutagenized pOCA107-2 line in the *Columbia* background. Plants grown in the light on a synthetic medium (Murashige and Skoog salt mix, Gamborg's vitamin mix, and 2% sucrose) were maintained in growth chambers with a temperature of 21°C and a light intensity of 350 $\mu\text{E}/\text{m}^2$ per sec. Plants grown in the greenhouse on soil were maintained as described (Somerville and Ogren 1982). For dark-grown seedlings, seeds plated on the synthetic medium were cold-treated for 48 hr in the dark and then transferred to the light for 24 hr. The end of this 24-hr light treatment was considered to be the beginning of the dark-growth period. Plates were then wrapped in two layers of aluminum foil and kept in a dark growth chamber in a dark room for the amount of time indicated in each experiment.

Mutagenesis and mutant isolation

pOCA107-2 seeds were mutagenized with EMS (Chory et al. 1989) or γ -ray (30 krad). A total of 100,000 EMS-mutagenized M_2 seeds from 200 pools were screened for hygromycin resistance by growing the seedlings in the dark on a medium containing 40 $\mu\text{g}/\text{ml}$ of hygromycin B for 7.5 days. Seedlings with noticeably longer hypocotyls than the wild type were rescued to media without hygromycin and transferred to the light. *doc1* and *doc0-6* were isolated in this screen. An additional 48,000 EMS-mutagenized M_2 seeds from pools that did not give rise to any surviving seedlings in the first screen were screened again in the same growth condition but using only 20 $\mu\text{g}/\text{ml}$ of hygromycin B. *doc2-1* and *doc3* were isolated in this second screen. A third screen was done with 50,000 γ -ray mutagenized M_2 seeds on 40 $\mu\text{g}/\text{ml}$ of hygromycin B for 7 days in the dark.

doc2-2, *doc0-11*, *doc0-13*, *doc0-19* were isolated from this third screen. All mutant alleles were initially given the designation *doc0-n*. Individual loci were later numbered *doci1*, *doci2*, *doci3*, etc., as they were mapped. All of the analyses with the three *doc* mutants presented in this study were performed with mutant lines that have been backcrossed to the wild type and then repeatedly selected for the mutant phenotype (hygromycin resistant and high GUS activity in the dark).

Northern hybridizations, RNase protection, and GUS assays

RNA was isolated using the acid guanidium thiocyanate-phenol extraction method as described (Chomczynski and Sacchi 1987). RNA was separated on formaldehyde-containing agarose gels as described in Ausubel et al. (1987), except 0.22 M formaldehyde was used both in the gel and in the running buffer. RNA was then blotted onto Nytran nylon membranes (Schleicher & Schuell) and immobilized to the membrane by UV cross-linking and baking. The membrane was prehybridized for 0.5 hr at 60°C in a solution containing 1% BSA, 1 mM EDTA, 0.5 M NaHPO₄ and 7% SDS. Hybridization with specific probes was done in the same solution at 60°C for 16 hr. The membrane was then washed three times at 65°C with 0.1×SSC and 0.5% SDS. The membrane was exposed to X-ray film with an intensifying screen. Quantification of samples was done by scanning the autoradiographs with an LKB UltroScan XL densitometer. The probe for *CAB* mRNAs was generated from a plasmid containing the *CAB2* genomic DNA (Leutwiler et al. 1986) and the probes for *RBCS* mRNA and rRNA were generated from plasmids as described elsewhere (Chory et al. 1989).

RNase protection experiments were done as described by Brusslan and Tobin (1992). Specific GUS activities of seedlings were analyzed with protein extracts from whole seedlings using the fluorometric assay as described by Jefferson (1987).

Mapping of the three *doc* loci

Mapping was done using the codominant ecotype-specific PCR-based markers as described (Konieczny and Ausubel 1993). *doc1* was outcrossed to the ecotype *Niederzenz*. DNA from 154 individual F₂ plants (308 chromatids) that have the phenotype of *doc1* (reduced apical dominance) were tested. From these samples, *doc1* was found to be ~36 cM from the marker *GapA* on chromosome III. *doc1* was then crossed to the *hy2* mutant in the *Landsberg erecta* ecotype. From 48 F₂ DNA samples (96 chromatids), *doc1* was mapped to ~3 cM from the marker *GapC* and 2 cM from an RFLP marker in the *myb4* gene on chromosome III. *doc2* and *doc3* were both outcrossed to a multiply marked *Landsberg* line (*an-er-py-gli1-cer4*). Mutants in the F₂ progeny were identified with the phenotype of hygromycin resistance. Linkage of *doc2* with the *DFR* (*tt4*) marker on chromosome V was based on the analysis of 252 F₂ chromatids and linkage of *doc3* with *ADH* and *GapB* on chromosome I was based on the analysis of 166 F₂ chromatids. Location of *doc0-6* was calculated based on its linkage to the *pOCA107* transgene. *doc0-6* was crossed to the *Columbia* ecotype. The F₂ progeny showed a ratio of 2:1 of mutant/wild type, indicating a linkage with the transgene (a ratio of 9:7 is expected if the mutation is independent of the transgene, and a ratio of 3:1 is expected if the mutation is in the transgene).

Construction of double mutants

Double mutants were constructed by manual cross-pollination, by use of plants homozygous for the desired individual single mutation. The resulting F₁ plants were allowed to self pollinate.

In the F₂ generation, because no new phenotype was observed, mutants with phenotypes of each parent (*det* or *doc*) were isolated and their seeds harvested individually. The seeds from these plants were planted and scored for phenotypic segregation in the F₃ generation. Mutants that are heterozygous for the other mutation should segregate out 1 of 4 double mutants in the F₃ seedlings, and these double mutants could be identified if the phenotype of the double mutant is different from the original F₂ mutant plant. For all of the *doc* mutants isolated from the F₂ generation, more than half of them segregated out 1 of 4 seedlings with the *det* phenotype in the F₃ generation. These *det* seedlings from the F₂ *doc* plants were thus considered double mutants for the respective *det* and *doc* mutations. All the *det* plants isolated from the F₂ generation never segregated any plants with a new phenotype in the F₃ generation.

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Arabidopsis mutants define downstream branches in the phototransduction pathway.

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