Arabidopsis mutants define downstream branches in the phototransduction pathway

Hsou-min Li, Lothar Altschmied, and Joanne Chory

Plant Biology Laboratory, The Salk Institute, San Diego, California 92186-5800 USA

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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muts, 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 detl 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 detl 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 levels 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 detl 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 B-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, M2 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 B-glucuronidase (uidA) gene. All three reporter genes were fused to the nosynthetic control) line. Seedlings were grown for 7 days in the dark on media containing 20 \( \mu \text{g/ml} \) of hygromycin B. (B) The eight doc mutants were grown on 25 \( \mu \text{g/ml} \) of hygromycin B for 7 days in the dark. From the left: Wild type, doc0, 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. Atlantis, 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 F1 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 F2 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 F1 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 Niederzenz ecotypes, and the DNAs from the F2 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.

### Table 1. Specific GUS activities of doc mutants

| Lines   | GUS activity [pmole/mg per min] | Ratio of mutant/wild type |
|---------|--------------------------------|--------------------------|
| pOCA107 | 74.3 ± 0.0                     | 1.0                      |
| doc1    | 500.9 ± 2.5                    | 6.7                      |
| doc2-1  | 526.9 ± 20.0                   | 7.1                      |
| doc2-2  | 226.8 ± 13.3                   | 3.1                      |
| doc3    | 277.0 ± 2.4                    | 3.7                      |
| doc0-6  | 249.7 ± 0.0                    | 3.4                      |
| doc0-11 | 270.5 ± 18.9                   | 3.6                      |
| doc0-13 | 377.0 ± 3.3                    | 5.1                      |
| doc0-19 | 485.2 ± 17.0                   | 6.5                      |

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

### 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-
All conditions are the same as described in Table 2.

Table 2. Results of crosses with doc mutants

| Cross          | F1         | F2              | ratio of wild type/mutant | x^2  |
|----------------|------------|------------------|----------------------------|------|
| Co × doc1      | WT         | 54               | 16*                        | 3.38 | 0.17^b |
| doc2-1 × pOCA107| WT         | 163              | 62                         | 2.63 | 0.78^b |
| pOCA107 × doc2-2| WT         | 447              | 154                        | 2.90 | 0.12^b |
| Co × doc3      | WT         | 184              | 61                         | 3.02 | 0.001^b|
| Co × doc0-6    | mutant^c   | 222              | 406                        | 0.55 |       |
| Co × doc0-11   | N.D.       | 95               | 28                         | 3.39 | 1.3^d |
| Co × doc0-13   | 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 µg/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.

*The mutant was scored with the phenotype of reduced apical dominance.
^The x^2 value is given for the ratio of 3:1 (wild type/mutant).
^The 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.
^The x^2 value is given for the ratio of 13:3 (wild type/mutant).

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 µg) 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.

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 mutations 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.
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;
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 *Lemma*, 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 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 *DO2* and *DO3* respond to some environmental conditions that require the modulation of *CAB*, but not *RBCS*, expression. *DO2* and *DO3* then regulate 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
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-docl 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 docl also has reduced apical dominance as does det1. Because the docl 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 isolation 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 μE/m² 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 M2 seeds from 200 pools were screened for hygromycin resistance by growing the seedlings in the dark on a medium containing 40 μg/ml of hygromycin B for 7.5 days. Seedlings with noticeably longer hypocotyls than the wild type were rescued to the media without hygromycin and transferred to the light. doc1 and doc0-6 were isolated in this screen. An additional 48,000 EMS-mutagenized M2 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 μg/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 M2 seeds on 40 μg/ml of hygromycin B for 7 days in the dark.
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 Niederenz. 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 then outcrossed to a multiply marked Landsberg line (an-er-py-gll-cer4). Mutants in the F2 progeny were identified with 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 double mutants with the det phenotype in the F2 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 F2 generation.

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H M Li, L Altschmied and J Chory

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