The Plant Cell, Vol. 3, 445–459, May 1991 © 1991 American Society of Plant Physiologists

Phenotypic and Genetic Analysis of *det2*, a New Mutant That Affects Light-Regulated Seedling Development in *Arabidopsis*

Joanne Chory,[a,1] Punita Nagpal,[a] and Charles A. Peto[b]

[a] Plant Biology Laboratory, The Salk Institute for Biological Studies, P.O. Box 85800, San Diego, California 92186-5800
[b] Laboratory of Neuronal Structure and Function, The Salk Institute for Biological Studies, P.O. Box 85800, San Diego, California 92186-5800

The greening phenotypes produced by recessive mutations in a gene designated *de-etiolated-2* (*DET2*) are described. Recessive mutations in the *DET2* gene uncouple light signals from a number of light-dependent processes. *det2* mutations result in dark-grown *Arabidopsis thaliana* seedlings with many characteristics of light-grown plants, including hypocotyl growth inhibition, cotyledon expansion, primary leaf initiation, anthocyanin accumulation, and derepression of light-regulated gene expression. In contrast to these morphological and gene expression changes, however, the chloroplast development program is not initiated in the dark in *det2* mutants, suggesting that light-regulated gene expression precedes the differentiation of etioplasts to chloroplasts. *det2* mutations thus reveal at least two classes of downstream light-regulated responses that differ in their timing and control mechanisms. Homozygous *det2* mutations also affect photoperiodic responses in light-grown plants, including timing of flowering, dark adaptation of gene expression, and onset of leaf senescence. The phenotype of *det1 det2* double mutants is additive, implying that DET1 and DET2 function in distinct pathways that affect downstream light-regulated genes. Furthermore, these pathways are not utilized solely during early seedling development but must also be required to regulate different aspects of the light developmental program during later stages of vegetative growth.

INTRODUCTION

Seedling development proceeds according to a complex schedule of cell differentiation, cell division, and morphogenesis. This schedule includes the development and differentiation of the leaf meristem, the biogenesis of chloroplasts from undifferentiated plastids, and the regulated expression of both nuclear and chloroplast-encoded genes (Dale, 1988; Mullet, 1988; Steeves and Sussex, 1989). Dicotyledonous plants are unique in that the primary trigger for the regulation of this complex developmental program is an environmental signal, light. Dark-grown (etiolated) dicotyledonous seedlings are developmentally arrested. They have extended hypocotyls, no cotyledon expansion, no leaves, and are white. A set of well-characterized genes, the "light-regulated" photosynthesis genes, is not expressed or is expressed at a very low level (Silverthorne and Tobin, 1987; Gilmartin et al., 1990). The transition from dark to light growth causes profound differences in the morphology of the plant, including rapid inhibition of stem elongation, expansion of cotyledons, development of leaves, and the development of the photosynthetically competent chloroplast, a process called de-etiolation (Thompson, 1988; Gruissem, 1989). During de-etiolation in *Arabidopsis thaliana*, for instance, the light-regulated genes become expressed at 100 to 200 times the levels at which they were expressed in the dark.

The mechanisms by which light exerts its influence on this multifaceted developmental program are unknown. Both red and blue light inhibit hypocotyl elongation and induce expression of genes required for leaf and chloroplast development (Mancinelli and Rabino, 1978; Link, 1988; Thompson, 1988), whereas UV light is the strongest inducer of anthocyanin biosynthetic genes (Lipphardt et al., 1988; Schulze-Lefert et al., 1989; Staiger et al., 1989). It has been inferred that plants use at least three photoreceptors to perceive light, corresponding to red/far-red, blue, and UV wavelengths; however, the only characterized photoreceptor is the red/far-red photoreceptor phytochrome (Colbert, 1988). The number and sequence of events that occur after light excitation of the photoreceptors are not known. For instance, are there parallel pathways that regulate light-mediated seedling development?

[1] To whom correspondence should be addressed.

Is there a central regulatory pathway with shared signal transduction elements that branches out into several pathways, each having an independent effect on a different aspect of light development (i.e., gene expression, leaf development, decrease in the rate of stem elongation, anthocyanin accumulation, etc.)? Is there a linear sequence of events that occurs during seedling development?

Understanding the genetic control of dicotyledonous seedling development in response to light will require identifying genes involved in directing the downstream light-regulated responses. One candidate for such a gene is the *Arabidopsis DET1* (*de-et*iolated-1) gene, which we identified during a search for mutations that induce the light developmental program in darkness (Chory et al., 1989a). *det1* mutants display many phenotypic characteristics of light-grown wild-type plants, even when grown in total darkness. These characteristics include leaf and chloroplast development, anthocyanin accumulation, and the accumulation of mRNAs for several light-regulated nuclear and chloroplast-encoded genes (Chory et al., 1989a). Using chimeric fusion genes that were introduced into wild-type and *det1* lines, we have also shown that in light-grown *det1* plants two divergent light-regulated promoters are active in cell types where they are normally silent or expressed at very low levels in wild-type plants (Chory and Peto, 1990). Taken together, these results suggest a common point, *DET1*, to the light-specific and tissue-specific signal transduction pathways associated with leaf and chloroplast development in *Arabidopsis* (Chory and Peto, 1990).

The identification of mutations at the *DET1* locus has been helpful in defining a potentially important signal transduction pathway involved in light-regulated early seedling development in *Arabidopsis*; however, these mutations have not aided the dissection of the downstream light responses (leaf development, decrease in stem elongation, chloroplast biogenesis, photoregulated gene expression). Furthermore, it is clear that additional mutations will be necessary to define the number and complexity of the pathway(s) involved in light-regulated seedling development. Here we describe the phenotype of mutations in a second gene involved in de-etiolation, *DET2*. Like *det1* mutants, *det2* mutants show a deceleration of hypocotyl elongation, expanded cotyledons, anthocyanin accumulation, and increased accumulation of light-regulated nuclear and chloroplast RNAs in the absence of light. In contrast to *det1*, however, recessive *det2* mutations do not result in the initiation of the chloroplast developmental program. Thus, *det2* mutations have shown that two assayable downstream light-regulated responses (gene expression and leaf development) can be separated from the differentiation of chloroplasts. Moreover, the phenotype of *det1 det2* double mutants is additive, suggesting that two distinct pathways may be involved in the de-etiolation response in *Arabidopsis*.

RESULTS

Isolation and Genetic Characterization of *det2* Mutants

Five recessive alleles of *det2* were isolated during a screen for seedlings that had a de-etiolated morphology after 7 days of growth in total darkness (see Methods). All alleles were assigned to a single locus on chromosome 2 (position 32.9) by mapping and complementation tests, as shown in Table 1 (see also Methods). All five of these lines had the same phenotype after backcrossing (see below).

Three (*det2-1*, *det2-2*, and *det2-3*) of the five lines were backcrossed an additional three times, and the phenotypes were analyzed in detail. Figure 1 shows the dark-grown and light-grown phenotypes of *det2-1* compared with wild type. After 7 days of growth in total darkness, *det2* mutants were short, had thick hypocotyls, accumulated anthocyanins, and had open, expanded cotyledons (Figure 1A). This is in contrast to wild-type dark-grown (etiolated)

Table 1. Results of Crosses with *det2-1* Mutant

Cross	Type	Total	De-etiolated	Etiolated	χ^2 [a]
det1-1/det1-1 × *det2-1/det2-1*		37	0	37	
det2-1/det2-1 × *DET2/DET2*	F_1	55	0	55	
DET2/det2-1 × *DET2/det2-1*	F_2	1349	326	1023	0.50
DET2/det2-1 × *det2-1/det2-1*	Testcross	68	35	33	
DET2/det2-1 × *DET2/det2-2*		302	70	232	0.53
DET2/det2-1 × *DET2/det2-3*		745	187	568	0.02
DET2/det2-1 × *DET2/det2-4*		614	143	471	0.32
DET2/det2-1 × *DET2/det2-5*		462	110	352	0.34

[a] χ^2 calculation for an expected segregation of one de-etiolated to three etiolated.

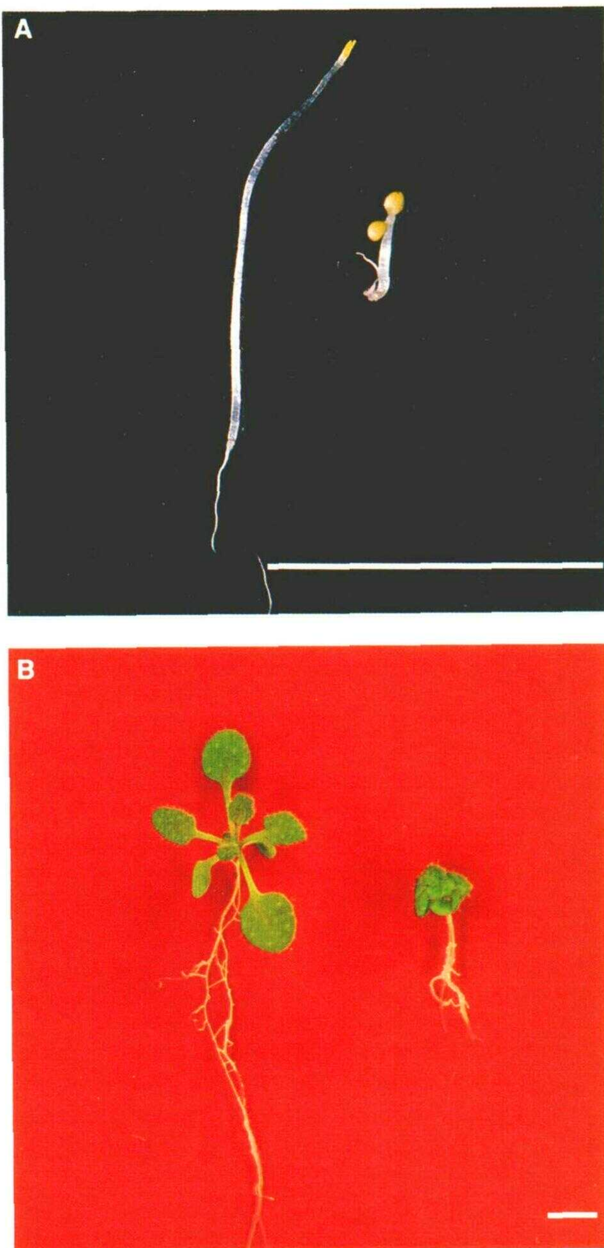


Figure 1. Phenotypes of Dark-Grown and Light-Grown *det2* and Wild-Type *Arabidopsis* Seedlings.

(A) Seven-day-old dark-grown (etiolated) wild type (left) and 7-day-old dark-grown *det2* (right).
(B) Twelve-day-old light-grown wild type (left), and 12-day-old light-grown *det2* (right).
Bar = 1cm.

seedlings, which had long hypocotyls and folded cotyledons and did not accumulate anthocyanins (Figure 1A). If kept in the dark for longer periods of time, *det2* alleles developed primary leaf buds, although no further leaf development was observed (data not shown). Wild-type etiolated seedlings never developed further than shown in Figure 1A.

The *det2* homozygotes also had an abnormal phenotype when grown in the light (Figure 1B). They were smaller and darker green than wild-type plants grown under the same conditions. In addition, *det2* mutants had a prolonged vegetative phase (see below). *det2* mutants made at least twice the number of leaves as wild type grown under the same light regimes (an average of 19 ± 3 leaves/plant, compared with 9 ± 2 leaves/plant for wild type), and had reduced apical dominance (average of five to seven bolts/plant for *det2*, whereas wild type had one to two bolts/plant). Finally, homozygous *det2* alleles had smaller flowers than wild type and all five alleles had severely reduced male fertility. The significance of this increased male sterility is not known.

Nuclear and Chloroplast Light-Regulated Genes Are Constitutively Expressed in the Dark in *det2* Plants in the Absence of Chloroplast Development

We examined the accumulation of mRNAs known to be positively regulated by light in wild-type and *det2* seedlings grown in the light and dark. These included both nuclear and chloroplast genes for chloroplast-localized proteins, as well as a blue-light-regulated nuclear gene encoding chalcone synthase (*chs*) (Feinbaum and Ausubel, 1988), which encodes an anthocyanin biosynthetic enzyme. The mRNAs examined included those corresponding to the nuclear genes for the small subunit of ribulose bisphosphate carboxylase, *rbcS* (Krebbers et al., 1988), the genes for the light-harvesting chlorophyll *a/b*-binding proteins, *cab* (Leutwiler et al., 1986), and four different chloroplast-encoded mRNAs: *psaA-B*, which codes for the 66-kD chlorophyll apoproteins of photosystem I; *psbA*, the 32-kD Q_B-binding protein of photosystem II; *rbcL*, the large subunit of ribulose-bisphosphate carboxylase; and 16S rRNA (Mullet and Klein, 1987). In all of the nuclear and chloroplast photosynthesis genes that we examined, we observed a high level of mRNA accumulation in dark-grown *det2* seedlings. Several are shown in Figure 2. These levels were 10-fold to 20-fold higher than those in dark-grown wild-type seedlings (Figure 2) and were about 10% to 20% of wild-type light-grown levels. In the case of the *chs* gene, the mRNA levels in dark-grown *det2* seedlings were at least 50-fold higher than wild-type etiolated seedlings and were about half of the levels accumulated in wild-type light-grown plants.

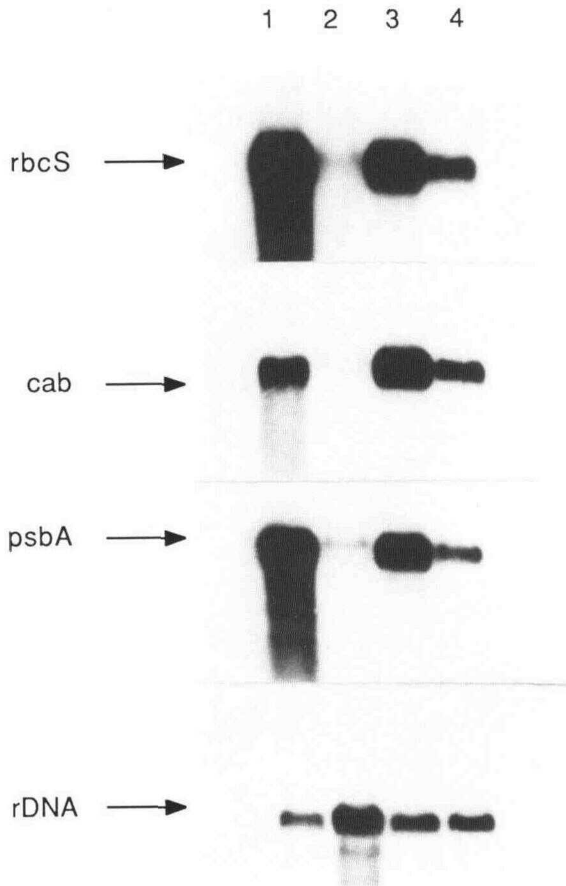


Figure 2. Accumulation of mRNAs for *rbcS* and *cab* (Nuclear Genes) and *psbA* (a Representative Chloroplast Gene) in Light-Grown and Dark-Grown Wild-Type and *det2* Plants.

Lane 1, light-grown wild type; lane 2, dark-grown wild type; lane 3, light-grown *det2*; lane 4, dark-grown *det2*. An *Arabidopsis* rDNA probe was used to normalize the load in the various lanes. Three micrograms of total RNA was loaded per lane. Similar results were observed for other chloroplast-encoded genes (*rbcL*, *psaA-B*) as for *psbA* (data not shown).

We previously showed, for *det1* mutants, that the aberrant dark expression of nuclear light-regulated genes is correlated with increased transcription from these promoters (Chory and Peto, 1990). Similarly, we analyzed the *det2* mutant for aberrant transcriptional regulation using *det2* mutant lines carrying a transgenic *Escherichia coli* β-glucuronidase (GUS) marker gene under the control of the blue-light-regulated *chs* promoter (Feinbaum and Ausubel, 1988; Chory and Peto, 1990) or the phytochrome-regulated *cab3* promoter (Chory and Peto, 1990). The *cab*-GUS and *chs*-GUS fusions were introduced into *det2* mutants by crossing with well-characterized wild-type transgenic lines (see Methods) (Chory and Peto, 1990). Table 2 depicts the average GUS levels observed in wild-type and *det2* transgenic seedlings either grown in the light or germinated and grown in darkness. In dark-grown *det2* seedlings, the levels of GUS activity from the *cab3* promoter were about 10% of the levels in light-grown *det2* seedlings (Table 2). This 10-fold light induction contrasts with the greater than 80-fold level of light induction observed in wild-type plants. In *chs*-GUS transgenic *det2* seedlings, the *chs* promoter drove GUS expression to greater than 5 times the levels in dark-grown wild-type seedlings, which corresponded to approximately one-third of the light-grown *chs*-GUS activity (Table 2). These data indicate that, in addition to the high levels of RNA accumulated, there was also aberrant high constitutive transcription from these promoters in dark-grown *det2* seedlings.

det2 mutants accumulated high levels of mRNAs for photosynthesis genes in the dark. To determine whether the proteins translated by these RNAs were also accumulated in dark-grown *det2* seedlings, we examined the accumulation of several photosynthetic proteins using antibodies specific for purified proteins or complexes. The same plants that were used for the RNA gel blot analyses were used for these studies. A representative protein gel blot for light-harvesting chlorophyll *a/b* proteins (LHCPII) (which are coded for by the *cab* genes) and the ribulose-bisphosphate carboxylase/oxygenase (Rubisco) (coded for by *rbcS* and *rbcL*) is shown in Figure 3. For Rubisco, aberrant high levels of both the small and large subunits were observed in dark-grown *det1* and *det2* mutants (about 15% of wild-type light levels) (Figure 3B, lanes WT, det1, and det2). This contrasts to dark-grown wild-type seedlings where no Rubisco proteins were observed (Figure 3B, lane et). In the case of LHCPII, no protein accumulated in either dark-grown *det1* or *det2* seedlings, even though high levels of *cab* mRNAs were present in these seedlings. This result was not surprising because neither

Table 2. Light-Dark Expression of *cab3* and *chs* Promoters in Wild-Type and *det2* Seedlings

	GUS Units[a]					
	Wild Type			*det2*		
Construct	Light	Dark	L/D[b]	Light	Dark	L/D[b]
cab3-GUS	16,500	200	82.5	11,900	1050	11.3
chs-GUS	34,600	1700	20.0	29,700	9300	3.2

Plants were grown for 14 days in the light (light) or were germinated in the light overnight and then grown in the dark for 7 days (dark).

[a] GUS units are picomoles of 4-methylumbelliferone per minute per milligram of protein.

[b] Ratio of light versus dark GUS units.

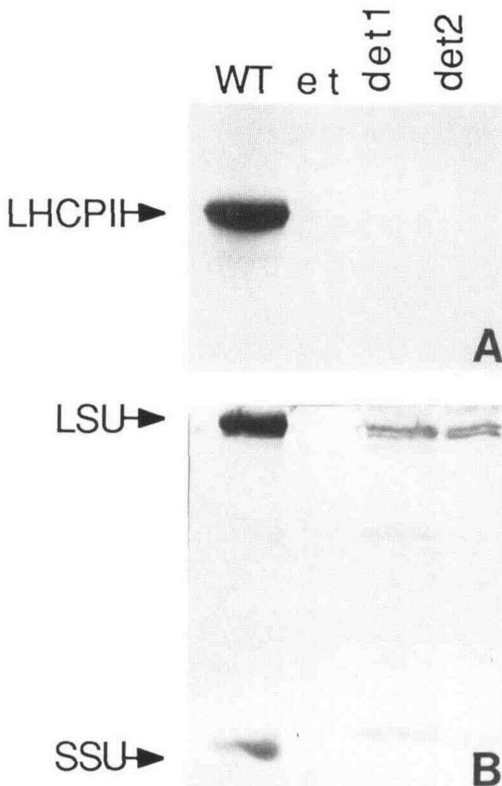


Figure 3. Immunoblot Analyses of the Levels of LHCPII or Rubisco Proteins Accumulated in Dark-Grown *det*1, *det*2, and Wild-Type Seedlings Compared with Light-Grown Wild Type.

Lane WT, wild type grown for 14 days in the light; lane et, wild type grown for 7 days in total darkness (etiolated); lane det1, *det*1 grown for 7 days in total darkness; lane det2, *det*2 grown for 7 days in total darkness. LSU and SSU refer to the large and small subunits of the Rubisco, respectively. Fifty micrograms of protein was loaded per lane.

*det*1 nor *det*2 accumulates chlorophylls when grown in the dark. Previous work from several laboratories has shown that the light-harvesting chlorophyll apoproteins are rapidly turned over in the absence of chlorophylls (Apel, 1979; Bennett, 1981), and we assume this is the basis for the observation here.

We examined plastid morphology in dark-grown *det*2 and wild-type seedlings. Figure 4 shows that the chloroplast developmental program has not been initiated, even though there are cotyledon expansion, aberrant high levels of nuclear and chloroplast gene expression, and accumulation of Rubisco in dark-grown *det*2 mutants. Etiolated wild-type seedlings have a plastid form, the etioplast, that is typified by the structure shown in Figure 4A; these plastid types were characterized by their irregular shape, a large paracrystalline body, the prolamellar body, and the notable absence of thylakoid membranes. *det*2 plastids were also etioplasts (Figure 4B). No chloroplasts were ever observed in dark-grown *det*2 plants.

*det*2 Mutants Have a Phenotype in the Light, Characterized by a Prolonged Juvenile Phase and a Lack of Leaf Senescence

Plants possess distinct juvenile and adult developmental phases. The juvenile or vegetative phase is the period of growth during which no flowering is possible (Hart, 1988). One of the most important factors that affects the transition from vegetative to floral growth is the environmental signal, light; however, other factors such as nutrition, temperature, and unknown endogenous factors are also involved (Hart, 1988). In *Arabidopsis*, the length of the juvenile phase can vary greatly, with flowering possible as early as the cotyledonary stage if the plants are stressed, or delayed for several months if the daylength is short. However, if the growth conditions are well defined, the vegetative growth phase is actually quite predictable.

Wild-type *Arabidopsis* (ecotype Columbia) seedlings, grown in a 22°C growth chamber on a 16-hr day/8-hr night growth regime, germinated in approximately 3 days and produced a rosette of 9 ± 2 leaves. Plants made the transition from the vegetative to floral growth phases approximately 21 days postgermination. This transition was characterized by the rapid growth of the floral blot and was followed by leaf senescence (beginning at about 30 days). During leaf senescence, chloroplast volume and number per mesophyll cell declined, resulting in the concomitant decrease in chlorophyll concentration and causing the plants to yellow. Light-regulated genes coding for photosynthetic functions were expressed at relatively low levels during senescence, as shown in Figure 5, lanes 4 and 5. By the time of seed set (approximately 7 weeks), the rosette leaves were brown and atrophied.

*det*2 mutants, in contrast, had a prolonged vegetative growth phase. Under the same growth conditions just described for wild-type plants, *det*2 mutants produced a rosette of 19 ± 3 leaves, had small leaves that resembled young leaves, and did not flower until 33 days postgermination. Moreover, no visible leaf senescence was observed even after 7 weeks of growth. To assess whether other light-regulated functions associated with greening were affected, we prepared samples for RNA and chloroplast analysis from wild type and *det*2 . Figure 5 shows an autoradiograph of an RNA gel blot of *cab* mRNA accumulation with time. Wild-type leaves showed a dramatic (10-fold) decline of *cab* mRNA by 22 days of growth in light; however, *det*2 plants had high levels of *cab* mRNA even after 45 days of growth. The results were reinforced by measurements of the specific chlorophyll content in these plants with time, as described in Table 3. Together,

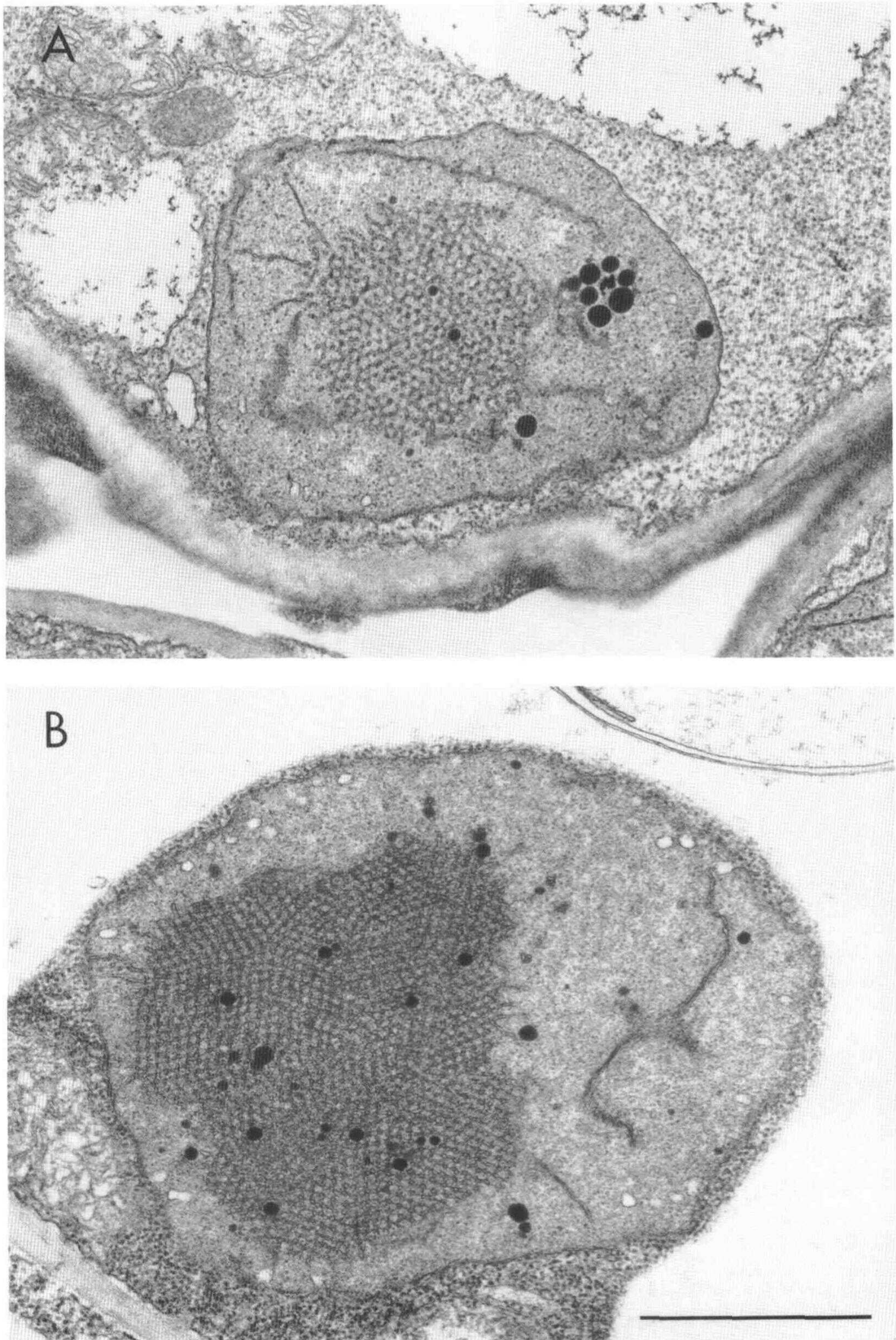


Figure 4. Electron Micrographs of Representative Plastids from Dark-Grown Wild-Type and *det2* Seedlings.

(A) Wild-type etioplast.
(B) *det2* etioplast.
Bar = 1 μm.

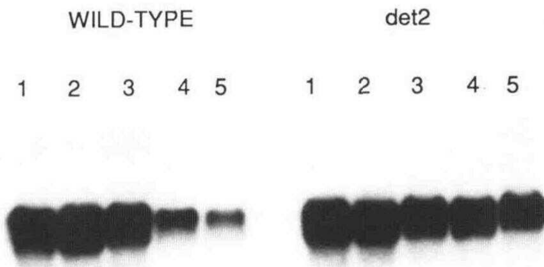


Figure 5. Accumulation of *cab* mRNAs during Development in Wild Type and *det2*.

Lanes 1, 8 days postgermination; lanes 2, 14 days postgermination; lanes 3, 19 days postgermination; lanes 4, 22 days postgermination; lanes 5, 45 days postgermination. Five micrograms of total RNA was loaded per lane.

these results implied that the chloroplasts were still present in high numbers and functionally active in *det2* plants, even after 7 weeks of growth in the light.

We followed the time course of chloroplast development in wild-type versus *det2* plants. Eight-day-old wild-type chloroplasts, shown in Figure 6A, had the typical lens shape and granal stacking of a mature chloroplast. Eight-day-old *det2* chloroplasts (Figure 6E), in contrast, resembled immature leaf chloroplasts, with a smaller, rounder shape and reduced granal stacking. Furthermore, 8-day-old *det2* seedlings had an abnormally high ratio of chlorophyll *a*/*b*, indicating the relatively "young" state of the chloroplasts (Table 3). By day 14, the *det2* chloroplasts were similar morphologically to wild-type chloroplasts and continued to grow and divide through day 22 (Figure 6G). Wild-type chloroplasts looked similar to the 8-day-old chloroplasts until the first flowers were observed (Figure 6D) at approximately day 22. Shortly after that time, visible signs of leaf senescence had occurred, and we did not take any more samples for microscopy. Figure 6I shows a typical *det2* chloroplast after 45 days of growth in the light. In general, the chloroplasts were still intact; however, it appeared that although chloroplast replication events had occurred, the daughter chloroplast remained fused to the mother. Also, in sections, the grana membranes had a novel circular swirling appearance. The membrane stacks were distorted, with many individual stacks curved or bent, instead of oriented somewhat parallel to the outer chloroplast membrane and inclusions. We had not observed this phenomenon previously.

det2 Mutations Affect the Expression of Nuclear Light-Regulated Genes during Light to Dark Transitions

Because the *det2* mutations affected the photoperiodic control of vegetative development, we were interested to see whether the DET2 gene product was also necessary for the proper day–night-regulated expression of photosynthesis genes, such as *cab*. We addressed this question by analyzing RNA from *det2* and wild-type plants grown in continuous light for 3 weeks and then shifted to the dark for 24 hr or 48 hr. It has been shown previously that accumulation of RNA for nuclear genes involved in photosynthesis decreases in the dark (Giuliano et al., 1988; Chory et al., 1989a). When we examined the levels of accumulation of *cab* mRNAs in dark-adapted *det2* mutants, we observed different results than for wild type, as shown in Figure 7. In wild type, the levels of *cab* RNA had decreased by approximately eightfold after 24 hr in the dark and were undetectable by 48 hr; however, in *det2*, *cab* RNA levels had decreased by only about twofold to threefold after 24 hr and 12-fold after 48 hr (Figure 7). Similar results were observed for *rbcS* RNAs (data not shown). These experiments suggest that the DET2 gene product may be involved in a signaling pathway that affects the expression of *cab* and *rbcS* genes during light-dark transitions.

det1 det2 Double Mutant Studies

We generated plant lines homozygous for both *det1* and *det2* mutations to examine the epistatic relations of these two genes and possible phenotypic interactions. The light and dark phenotypes of the double mutants indicate that the combination of two homozygous *det* mutations in a

Table 3. Chlorophyll Content and Chlorophyll *a*/*b* Ratio of Wild Type and *det2* during Development

Days Post-sowing	Chlorophyll Content[a] (μg/seedling)		Chlorophyll *a*/*b* Ratio[a]	
	Wild Type	*det2*	Wild Type	*det2*
8	6.7	3.2	3.6	5.6
14	29.0	17.0	3.0	2.9
17	37.0	27.6	3.2	3.0
22	45.0	39.7	3.1	3.4
28	55.0	53.0	3.1	3.0
45	18.2	57.0	2.2	3.0

[a] Values are an average of three seedlings for two independent experiments. On days 28 and 45, flowering bolts were removed.

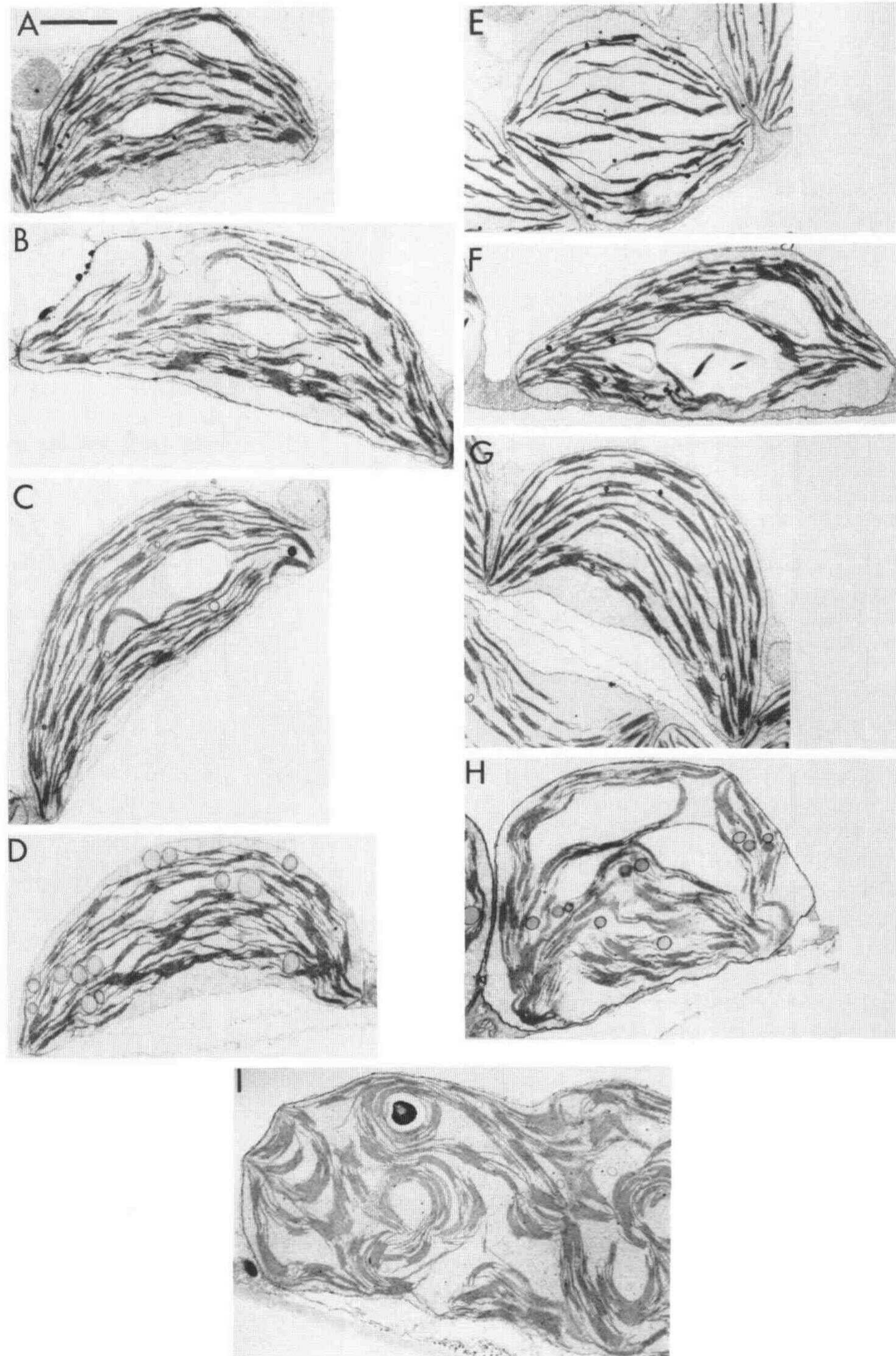


Figure 6. Electron Micrographs of Representative Chloroplasts during Development in Wild Type and *det*2.

(A) Wild type, 8 days postgermination.
(B) Wild type, 14 days postgermination.
(C) Wild type, 19 days postgermination.
(D) Wild type, 22 days postgermination.
(E) *det*2, 8 days postgermination.
(F) *det*2, 14 days postgermination.
(G) *det*2 19 days postgermination.
(H) *det*2, 22 days postgermination.
(I) *det*2, 45 days postgermination.
Bar = 1 μm.

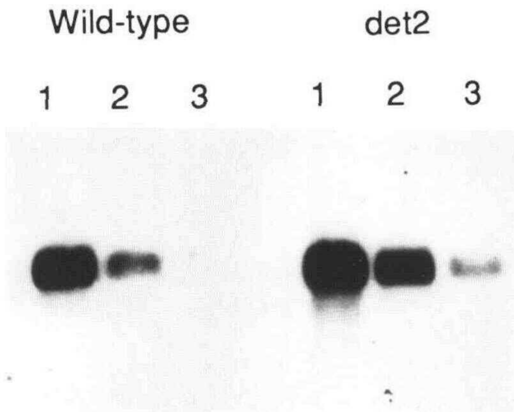


Figure 7. Accumulation of *cab* RNA in Dark-Adapted Wild-Type and *det*2 Plants.

Lanes 1, 15-day-old seedlings grown in continuous light; lanes 2, 15-day-old seedlings grown in continuous light and shifted to total darkness for 24 hr; lanes 3, 15-day-old seedlings grown in continuous light and shifted to total darkness for 48 hr. Two micrograms of RNA was loaded per lane.

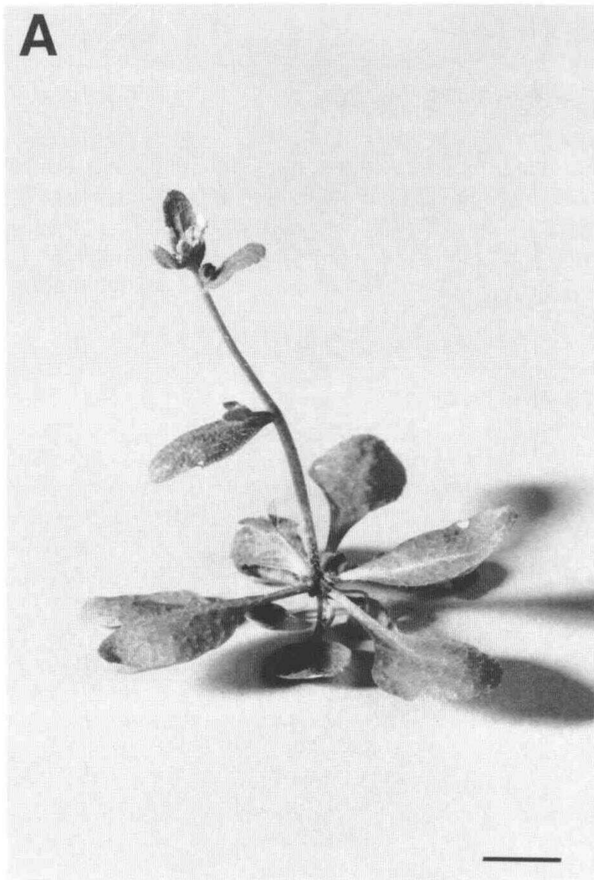
single plant results in an additive interaction. Similar results were observed using two different alleles of *det*2. The light-grown phenotypes for each mutant parent and for the double mutant are shown in Figure 8 and summarized in Table 4. Each individual *det* mutation affected different light-regulated processes in the light, and upon examination of some of these traits, a simple additive phenotype was observed in the double mutants (Table 4). Although the *det*1 *det*2 double mutants make the vegetative to floral transition (Figure 8D), bolts never elongated, and we have been unable to propagate the double mutants by either selfing or crossing with the wild-type or individual mutant parent lines.

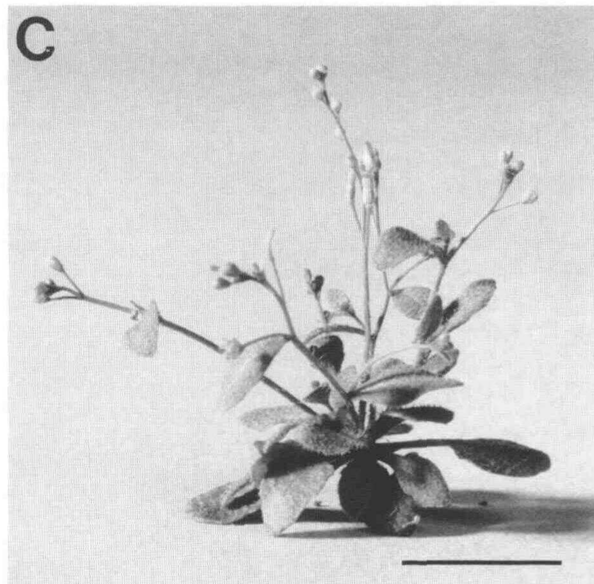
DISCUSSION

We have shown that recessive mutations in the *DET*2 gene uncouple light signals from a number of light-dependent processes. *det*2 mutations result in dark-grown seedlings with many characteristics of light-grown wild-type seedlings, including inhibition of the rate of hypocotyl elongation, cotyledon expansion, development of primary leaf buds, accumulation of anthocyanins, and derepression of light-regulated gene expression. Although *det*2 mutations affect many downstream processes of seedling development that are normally light regulated, one notable exception is the lack of differentiation of etioplasts to chloroplasts. Thus, mutations in the *DET*2 gene have helped in the dissection of some of the downstream light-regulated processes. At the very least, these studies have shown that regulation of genes for chloroplast-destined proteins must precede any morphological differentiation of etioplasts to chloroplasts. This characteristic distinguishes *DET*2 from *DET*1 because in *det*1 mutants, all assayable downstream light-regulated responses are affected, including leaf development, chloroplast development, and gene expression (Chory et al. 1989a).

*det*2 mutants also have an aberrant phenotype when grown in the light; therefore, it is likely that DET2 plays some role in light-grown plants. The most striking characteristics of the phenotype of light-grown *det*2 plants are reduced leaf growth, a prolonged vegetative phase, reduced male fertility, and a lack of leaf senescence, even after flowering. The leaves of *det*2 mutants remain green after seed set, and we never observed drastic diminishing of chloroplast numbers or breakdown of granal membranes over a 7-week growth period. This prolonged juvenile phase was characterized by high levels of *cab* mRNA accumulation throughout vegetative growth, which is atypical of wild-type plants. Normally, *cab* mRNA levels are highest during early seedling growth and diminish during later stages of vegetative growth (e.g., much higher levels at 7 days versus 3 weeks in *Arabidopsis*). Heterochronic mutations (*Teopod*) affecting maize shoot development have been described (Poethig, 1988). These three semidominant mutations have a profound effect on both vegetative and reproductive development. Although it is difficult to compare maize to *Arabidopsis* directly, because of morphological differences, certain characteristics of the *Tp* mutants and the *det*2 mutants are similar. For instance, the *Tp* mutants have an increased number of vegetative phytomers, an increased number of phytomers producing ears, a decrease in the size of leaves and internodes, and a decrease in the size of the ear and tassel (Poethig, 1988). Plants homozygous for the *det*2 mutation bear similar phenotypic characteristics. There are, however, several notable differences between the *Tp* mutants and *det*2. For instance, *Tp* mutations are semidominant and probably represent gain-of-function mutations, whereas all five *det*2 alleles are completely recessive and have the same phenotype, implying that each allele may represent the null state of the gene. Also, the *Tp* mutants have a homeotic transformation of reproductive structures into vegetative ones, and we never observed any signs of organ conversion in *det*2 mutants.

The timing of flowering was also delayed in *det*2 mutants (by at least 10 days versus the wild type), and thus *det*2 could be classified as a "late-flowering" mutant. Although the molecular mechanisms involved in floral induction are unknown, physiological studies implicate roles for light (both photoperiod and quality), temperature, and assimilate availability in the timing of floral induction (e.g., Martinez-Zapater and Somerville, 1990). In *Arabidopsis*, several late-flowering mutants have been identified (Koornneef et al., 1983) and a subset of these has been further characterized





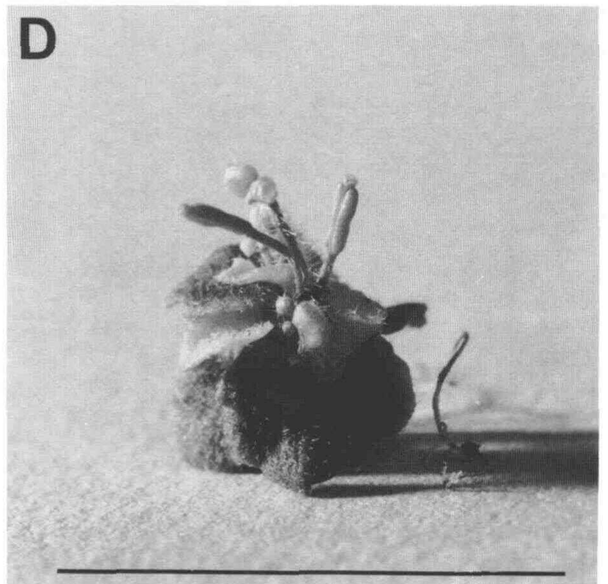


Figure 8. Photographs of Wild Type, Single Mutants, and Double Mutant.

(A) Twenty six-day-old wild-type plant.
(B) Fifty-day-old *det*2-1 plant.
(C) Thirty-five-day-old *det*1-1 plant.
(D) Forty two-day-old *det*1-1 *det*2-1 double mutant.
*det*1 *det*2-2 double mutants had the same phenotype as the seedling shown in **(D)**. Bar = 1 cm.

Table 4. Summary of *det*1 *det*2 Double Mutant Phenotype

	Phenotype				
	Dark	Light			
Genotype		Roots	No. Leaves	Pigmentation	Days to Flower
Wild type	Etiolated	White	9	Green	21
*det*1	De-etiolated	Green	10	Pale green	21
*det*2	De-etiolated	White	19	Dark green	33
*det*1 *det*2	De-etiolated	Green	12	Green	28

(Martinez-Zapater and Somerville, 1990). Studies have not been performed on the greening responses of these mutants. It would be interesting to test whether the prolonged juvenile phase of these mutants is marked by persistent *cab* gene expression and chloroplast development, as reported here for the *det*2 mutants. A third characteristic of the light-grown phenotype of *det*2 mutants is the slower kinetics of the decline of *cab* and *rbc*S mRNA after the transfer of light-grown plants to darkness for 1 day to 2 days. Although the implications of these results are currently unclear, more detailed experiments should be performed to evaluate this phenomenon.

Homozygous *det*2 mutations are pleiotropic, affecting the switch between etiolation and de-etiolation, as well as affecting photoperiodic responses, including timing of flowering, dark adaptation of *cab* gene expression, and delay of leaf senescence. Given the recessive nature of the mutations and the phenotype of the mutants, one prediction is that DET2 is a component in a signal transduction pathway whose activity is high during etiolation, low during early seedling development (de-etiolation), and relatively high during later vegetative growth. Thus, loss-of-function mutations in *DET*2 result in de-etiolated seedlings in the dark and a prolonged vegetative growth in the light, and DET2 may be a molecular switch involved in the decision-making process between etiolation and de-etiolation in *Arabidopsis*. The DET2 switch would be used throughout development to allow plants to progress into an adult phase when chloroplast replication and maintenance are no longer crucial. A second alternative is that *det*2 mutants define a developmental pathway whose activity is modulated by light. A possible candidate would be a cytokinin-controlled pathway involved in the regulation of leaf development and senescence.

*DET*2 is the second genetic locus we have described that appears to be involved in a signal transduction pathway that regulates the etiolation–de-etiolation response. *det*2 mutations share some similarities with *det*1 mutations, yet there is a distinct set of light-regulated processes that are affected differently in *det*1 versus *det*2 mutants. The summary of the dark-grown and light-grown phenotypes of *det*1 and *det*2 mutants is shown in Table 5. Both *DET*1 and *DET*2 appear to be involved in light signal transmission because mutations at either locus uncouple a number of light-dependent processes from the light signal. Mutations in the *DET*1 gene result in derepression of the entire light developmental program, including leaf and chloroplast development and gene expression (Table 5) (Chory et al., 1989a). Furthermore, *det*1 mutants are defective in the proper integration of temporal or spatial regulatory signals because we observed a loss of tissue-specific expression of the chloroplast developmental program and the light-regulated gene expression program, including aberrant expression of *cab* promoters in roots

Table 5. Comparison of *det*1 and *det*2 Mutants

	Wild Type	*det*1	*det*2
Dark phenotype			
Morphology			
Leaves	Unexpanded cotyledons	Expanded cotyledons and leaves	Expanded cotyledons (primary leaf buds)
Hypocotyl	Long	Short	Short
Pigments	Absent	Anthocyanins	Anthocyanins
Chloroplasts	Undifferentiated	Some differentiation	Undifferentiated
Gene expression (% of wild-type levels in the light)			
Nuclear	1–2	25–100	10–30
Chloroplast	1–2	100	10
Light phenotype			
Morphology			
No. of leaves	9 ± 2	10 ± 2	19 ± 3
Bolt no.	2–4	5–7	5–7
Days to flower	21	21	33
Tissue-specific gene expression affected	No	Yes	No
Light-dark regulation affected	No	No	Yes

and the *chs* promoter in leaf mesophyll cells and flowers (Chory and Peto, 1990). Thus, DET1 may be involved in the integration of light with tissue-specific signals that affect greening in *Arabidopsis*.

In general, *det*2 mutations are less severe than *det*1 mutations. *det*2 mutations affect some of the downstream light-regulated processes, including morphology (cotyledon expansion, hypocotyl growth deceleration, and leaf development) and gene expression. However, we saw no effect on the etioplast to chloroplast transition in dark-grown seedlings. Furthermore, unlike *det*1 mutants, *det*2 mutants never make rosettes in the dark; only primary leaf buds were observed. In addition, although light-regulated genes were expressed at 10-fold to 20-fold higher levels in dark-grown *det*2 mutants than wild-type levels in the dark, these levels were still about 10-fold lower than light-grown plants and fivefold to 10-fold lower than *det*1 plants grown in the dark. We recently proposed that the extent of light-regulated gene expression was dependent on the amount of leaf development (Chory et al., 1989a). The characterization of gene expression patterns in dark-grown *det*2 mutant seedlings reinforces this hypothesis. The extent of leaf development is light-grown wild-type plants > dark-grown *det*1 plants > dark-grown *det*2 plants > wild-type etiolated seedlings. When the levels of light-regulated gene expression were measured for the above, we obtained the same relationship described for leaf development. Furthermore, based on the phenotype of *det*2 mutants, chloroplast development does not appear to be required for derepression of the expression of nuclear and chloroplast-encoded photosynthesis genes. Interestingly, both nuclear and chloroplast mRNAs accumulated to about the same levels in dark-grown *det*2 seedlings, indicating that the nuclear and chloroplast compartments were coordinately affected by *det*2 mutations. Nuclear and chloroplast compartments were similarly affected by *det*1 mutations as well (Chory et al., 1989a).

*det*1 and *det*2 mutations appear to affect a different subset of light-regulated functions in light-grown plants (see Table 5). Therefore, perhaps it is not surprising that *det*1 *det*2 double mutants have a much more severe phenotype than either single mutation alone. Although we do not have direct proof that the *det*1 and *det*2 alleles are null, the nearly additive interaction observed between the *det*1 and *det*2 mutations suggests an absence of interaction between the two gene products. Therefore, it seems likely that either *DET*1 and *DET*2 do not lie within a common signal transduction pathway or they act in separate branches of a common signal transduction pathway. Moreover, we have evidence that both *det*1 and *det*2 mutations are epistatic to several different *hy* mutations that result in phytochrome deficiency in *Arabidopsis* (J. Chory, unpublished results), and the most probable order of gene action would place both DET1 and DET2 downstream of phytochrome activation. Thus, the identification of *det*1 and *det*2 mutants has allowed us to propose that at least two distinct pathways are involved in the etiolation–de-etiolation response in *Arabidopsis*. Furthermore, these pathways are not utilized solely during early seedling development but must also be required to regulate different aspects of the light developmental program during vegetative development. We are currently in the process of cloning *DET*1 and *DET*2 so that the molecular mechanisms of light-mediated seedling development can be unraveled.

METHODS

Plant Material, Growth Conditions, and Genetic Methods

Two *det* mutants were isolated originally after ethyl methane sulfonate mutagenesis of wild-type seeds from the Columbia ecotype (Chory et al., 1989a). We mutagenized an additional 20,000 M_1 seeds and isolated three additional independent *det*2 alleles (Table 1). Each mutant was backcrossed into the wild-type Columbia background before complementation analysis was performed; three alleles were backcrossed an additional three times before the physiological studies. All five of these lines have the same phenotype after backcrossing (see Results). In all the experiments described here, the wild type was the Columbia line. Genetic nomenclature is based on the Third International *Arabidopsis* Meeting (East Lansing, MI, 1987). If not specified, it is assumed that the mutant allele number is 1 (e.g. *det*2-1 is referred to as *det*2).

Each mutant phenotype segregated as a single recessive nuclear mutation in genetic crosses (see Table 1 for data on *det*2-1). Because of the almost complete male sterility of the *det*2 mutant lines, it was necessary to maintain these lines as heterozygotes. F_1 plants were generated after crossing the original de-etiolated mutant (M_2) with wild-type pollen. The seeds were collected from each F_1 plant and used as a source of segregating homozygous mutant plants. The mutants were identified by germination on synthetic medium plus sucrose in the dark and scoring the de-etiolated phenotype in the F_2 generation (see Table 1). For complementation studies, the recovery of de-etiolated plants among the progeny of a cross between two heterozygotes at a ratio of one de-etiolated to three etiolated (wild type) indicated noncomplementation (Table 1). These results indicated that all five mutations lie in the same complementation group.

To obtain the chromosomal location of the *det*2 mutation, an ecotype Landsberg marker line that contained one visible marker on chromosomes 1 (*an*), 3 (*gl*-1), 4 (*cer*-2), and 5 (*ms*-1), and two markers on chromosome 2 (*er*, position 2.15.9 and *py*, position 2.22) was used (see Koornneef, 1990, for map positions). The *ms*-1 marker was maintained in the heterozygous state (+/*ms*-1). Pollen from the Landsberg tester strain was crossed to *det*2-1, and *det*2 F_2 plants were chosen and scored for segregation of the visible markers. Additional markers on chromosome 2 used for linkage analysis were *hy*1 (position 2.16.9) (Koornneef, 1990), *hy*6 (position 2.21.9) (Chory et al., 1989b; J. Chory and L. Altschmied, unpublished data), and a previously mapped kan^r transgene from a transformation experiment (position 2.13.9) (L. Altschmied and J. Chory, unpublished data). *det*2 was mapped to chromosome 2, position 32.9 (data not shown). We have not resolved the map position of *det*2 relative to the *as* marker on

chromosome 2, which maps to position 33. Using restriction fragment length polymorphism probes from chromosome 2, we have confirmed that *det2* is tightly linked to *as* (data not shown; Nam et al., 1989).

Plants were grown at 20°C under a mixture of fluorescent and incandescent lights at an intensity of 300 $\mu E/m^2/sec$. Methods for the growth of plants in pots, seed harvesting, and cross-pollination have been described (Somerville and Ogren, 1982). For all the experiments on light-grown plants, *det2-1*, *det2-2*, *det2-3*, and wild type were grown side-by-side under the same light and humidity conditions for the times indicated in the text. Dark-grown seeds were germinated for 36 hr in the light on synthetic medium plus sucrose and then transferred to total darkness for 7 days, as previously described (Chory et al., 1989a). Germination of seedlings by this protocol gave the same results as when the seedlings were germinated directly in the dark with gibberellic acid in the medium (data not shown).

RNA Gel Blots

RNA was isolated from small amounts of tissue by using the acid guanidium method of Chomczynski and Sacchi (1987). RNA (2 μg to 5 μg) was separated in formaldehyde-containing agarose gels (Ausubel et al., 1987) and blotted onto Nytran (Schleicher & Schuell) using the manufacturer's recommendations. After transfer to the nylon membrane, the RNA was cross-linked to the membrane by UV irradiation (Stratalinker). ^{32}P-labeled DNA probes (5×10^8 cpm/μg) were generated by random primer extension (Feinberg and Vogelstein, 1983). The filters were hybridized (5×10^6 cpm/mL) using the conditions of Church and Gilbert (1984). Filters were washed three times at 60°C in 0.2 × SSC, 1.0% SDS for 20 min each, and exposed to preflashed Kodak XAR-5 film at −70°C with an intensifying screen. To normalize for RNA loading, filters were stripped and rehybridized with an rDNA probe. Autoradiograms for different exposure times were scanned with a densitometer. Relative amounts of mRNAs were determined by peak-area measurements, and relative mRNA levels reported are an average of two separate hybridizations. The DNA probes for nuclear and chloroplast genes used in these studies were as published elsewhere (Chory et al., 1989a).

Analysis of Chimeric Transgenes in Wild Type and *det2*

The *cab*-GUS and *chs*-GUS fusions were transformed into wild-type Columbia as described elsewhere (Chory and Peto, 1990). For the experiments described here, the *cab*-GUS and *chs*-GUS fusions were introduced into the *det2* mutant by crossing with wild-type transgenic lines. Kan^r F_1 seedlings were allowed to self-pollinate, and kan^r *det2* mutants were screened in the F_2 progeny and used for subsequent analysis.

Electrophoresis and Immunological Detection of Proteins

Seven-day-old dark-grown and 20-day-old light-grown *Arabidopsis* seedlings were quickly frozen and ground with a mortar and pestle in liquid nitrogen. The samples were then transferred to a lyophilization apparatus without thawing. The lyophilized tissue was extracted into SDS extraction buffer (0.5 mL of buffer/30 mg of powder), heated to 70°C for 10 min, and electrophoresed in a 12% SDS-polyacrylamide gel (Laemmli, 1970). The amount of protein loaded was 50 μg per lane. Electrophoretic transfer of protein to nitrocellulose, reaction with antisera, and anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad Immunoblot kit) were performed as described by the manufacturer. The relative levels of specific proteins were quantified by scanning photographic negatives with a densitometer. The antibody probes used in these studies were rabbit anti-LHCPII (pea), obtained from Dr. J. Bennett (Bennett et al., 1984) and rabbit anti-Rubisco (tobacco large subunit, pea small subunit), obtained from Dr. S. Rodermel (Rodermel et al., 1988).

Electron Microscopy

For dark-grown wild type and *det2*, cotyledon tissue from 7-day-old seedlings grown in absolute darkness [on solidified synthetic Murashige and Skoog (1962) medium supplemented with 1% sucrose] was used as the source of material. For light-grown seedlings, leaf samples were harvested at 8 days, 14 days, 17 days, and 22 days from both wild type and *det2* that had been grown side-by-side under the same light and humidity conditions. For *det2*, an additional time point after 45 days in the light was also collected. Tissues were fixed with glutaraldehyde followed by osmium tetroxide, dehydrated, and embedded in Spurr resin.

Analytical Techniques

Chlorophyll determinations were performed on leaves and stems from wild-type and *det2* plants harvested after 8 days, 14 days, 17 days, 22 days, and 45 days of growth in the light and immediately frozen in liquid nitrogen. The frozen tissue was later ground in liquid nitrogen in a mortar and pestle, and chlorophyll was extracted repeatedly into 80% acetone in the dark until the pellet appeared colorless. Chlorophyll *a* and *b* contents were calculated using MacKinney's specific absorption coefficients (MacKinney, 1941), in which chlorophyll $a = 12.7\ (A_{663}) - 2.69\ (A_{645})$ and chlorophyll $b = 22.9(A_{645}) - 4.48(A_{663})$. The total specific chlorophyll content is expressed as micrograms of chlorophyll per seedling. GUS assays with protein extracts of leaves and stems were carried out using the fluorometric assay described by Jefferson (1987). Protein determinations were made with a Bio-Rad protein assay kit.

ACKNOWLEDGMENTS

J.C. thanks Nathan Aguilar for assistance with some experiments. We thank Stephen Worland, Ron Susek, Terry Delaney, and Alan Pepper for comments on the manuscript, and Paul Sawchenko for generous use of electron microscope facilities. This research was supported by grants from the National Science Foundation and the Department of Energy (to J.C.) and by The Samuel Roberts Noble Foundation. Marc Lieberman of The Salk Institute for Biological Studies prepared the cover photograph and Figures 1 and 8.

Received January 22, 1991; accepted March 6, 1991.

REFERENCES

Apel, K. (1979). Phytochrome induced appearance of mRNA activity for the apoprotein of the light-harvesting chlorophyll *a/b* protein of barley. Eur. J. Biochem. **97,** 183–188.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1987). Current Protocols in Molecular Biology. (New York: Green Publishing Assoc./Wiley Interscience).

Bennett, J. (1981). Biosynthesis of the light-harvesting chlorophyll *a/b* protein. Polypeptide turnover in darkness. Eur. J. Biochem. **118,** 61–70.

Bennett, J., Jenkins, G.I., and Hartley, M.R. (1984). Differential regulation of the accumulation of the light-harvesting chlorophyll *a/b* complex and ribulose bisphosphate carboxylase/oxygenase in greening pea leaves. J. Cell. Biochem. **25,** 1–13.

Chomczynski, P., and Sacchi, N. (1987). Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform-extraction. Anal. Biochem. **162,** 156–159.

Chory, J., and Peto, C. (1990). Mutations in the *DET*1 gene affect cell-type-specific expression of light-regulated genes and chloroplast development in *Arabidopsis*. Proc. Natl. Acad. Sci. USA **87,** 8776–8780.

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

Chory, J., Peto, C.A., Ashbaugh, M., Saganich, R., Pratt, L., and Ausubel, F. (1989b). Different roles for phytochrome in etiolated and green plants deduced from characterization of *Arabidopsis thaliana* mutants. Plant Cell **1,** 867–880.

Church, G.M., and Gilbert, W. (1984). Genomic sequencing. Proc. Natl. Acad. Sci. USA **81,** 1991–1995.

Colbert, J.T. (1988). Molecular biology of phytochrome. Plant Cell Environ. **11,** 305–318.

Dale, J.E. (1988). The control of leaf expansion. Annu. Rev. Plant. Physiol. Plant Mol. Biol. **39,** 267–295.

Feinbaum, R., and Ausubel, F.M. (1988). Transcriptional regulation of the *Arabidopsis thaliana* chalcone synthase gene. Mol. Cell. Biol. **8,** 1985–1992.

Feinberg, A.P., and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132,** 6–13.

Gilmartin, P. M., Sarokin, L., Memelink, J., and Chua, N.-H. (1990). Molecular light switches for plant genes. Plant Cell **2,** 369–378.

Giuliano, G., Pichersky, E., Malik, V.S., Timko, M.P., Scolnik, P.A., and Cashmore, A.R. (1988). An evolutionarily conserved protein binding sequence upstream of a plant light-regulated gene. Proc. Natl. Acad. Sci. USA **85,** 7089–7093.

Gruissem, W. (1989). Chloroplast gene expression: How plants turn their plastids on. Cell **56,** 161–170.

Hart, J.W. (1988). Topics in Plant Physiology. (London: Unwin Hyman Ltd.).

Jefferson, R.A. (1987). Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol. Biol. Rep. **5,** 387–405.

Koornneef, M. (1990). Linkage map of *Arabidopsis thaliana*. In Genetic Maps: Locus Maps of Complex Genomes, 5th ed., S.J. O'Brien, ed (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp 6.94–6.97.

Koornneef, M., van Eden, J., Hanhard, C.J., Stam, P., Braaksma, F.K., and Feenstra, W.J. (1983). Linkage map of *Arabidopsis thaliana*. J. Hered. **74,** 265–272.

Krebbers, E., Seurinck, J., Herdies, L., Cashmore, A.R., and Timko, M.P. (1988). Four genes in two diverged subfamilies encode the ribulose-1,5-bisphosphate carboxylase small subunit polypeptides of *Arabidopsis thaliana*. Plant Mol. Biol. **11,** 745–759.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227,** 680–685.

Leutwiler, L.S., Meyerowitz, E.M., and Tobin, E.M. (1986). Structure and expression of three light-harvesting chlorophyll *a/b* binding protein genes in *Arabidopsis thaliana*. Nucl. Acids Res. **14,** 4051–4064.

Link, G. (1988). Photocontrol of plastid gene expression. Plant Cell Environ. **11,** 329–338.

Lipphardt, S., Brettschneider, R., Kreuzaler, F., Schell, J., and Dangl, J.L. (1988). UV-inducible transient expression in parsley protoplasts identifies regulatory *cis*-elements of a chimeric *Antirrhinum majus* chalcone synthase gene. EMBO J. **7,** 4027–4033.

MacKinney, G. (1941). Absorption of light by chlorophyll solutions. J. Biol. Chem. **140,** 315–322.

Mancinelli, A.L., and Rabino, I. (1978). The high irradiance responses of plant photomorphogenesis. Bot. Rev. **44,** 129–180.

Martinez-Zapater, J.M., and Somerville, C.R. (1990). Effect of light quality and vernalization on late flowering mutants of *Arabidopsis thaliana*. Plant Physiol. **92,** 770–776.

Mullet, J.E. (1988). Chloroplast development and gene expression. Annu. Rev. Plant Physiol. Plant Mol. Biol. **39,** 475–502.

Mullet, J.E., and Klein, R.R. (1987). Transcription and RNA stability are important determinants of higher plant chloroplast RNA levels. EMBO J. **6,** 1571–1579.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. **15,** 493–497.

Nam, H.-G., Giraudat, J., den Boer, B., Moonan, F., Loos, W.D.B., Hauge, B.M., and Goodman, H.M. (1989). Restriction fragment length polymorphism linkage map of *Arabidopsis thaliana*. Plant Cell **1,** 699–705.

Poethig, R.S. (1988). Heterochronic mutations affecting shoot development in maize. Genetics **119,** 959–973.

Rodermel, S.R., Abbot, M.S., and Bogorad, L. (1988). Nuclear-organelle interactions: Nuclear antisense gene inhibits ribulose bisphosphate carboxylase enzyme levels in transformed tobacco plants. Cell **55,** 673–681.

Schulze-Lefert, P., Dangl, J.L., Becker-André, M., Hahlbrock, K., and Schulz, W. (1989). Inducible in vivo DNA footprints

define sequences necessary for UV light activation of a parsley chalcone synthase gene. EMBO J. **8,** 651–656.

Silverthorne, J., and Tobin, E.M. (1987). Phytochrome regulation of nuclear gene expression. BioEssays **7,** 18–22.

Somerville, C.R., and Ogren, W.L. (1982). Isolation of photorespiration mutants in *Arabidopsis thaliana*. In Methods in Chloroplast Molecular Biology, M. Edelman, R.B. Hallick, and N.-H. Chua, eds (New York: Elsevier Biomedical Press), pp. 129–139.

Staiger, D., Kaulen, H., and Schell, J. (1989). A CACGTG motif of the *Antirrhinum majus* chalcone syntase promoter is recognized by an evolutionarily conserved nuclear protein. Proc. Natl. Acad. Sci. USA **86,** 6930–6934.

Steeves, T.A., and Sussex, I.M. (1989). Patterns in Plant Development, 2nd ed. (Cambridge: Cambridge University Press), pp. 100–123.

Thompson, W.F. (1988). Photoregulation: Diverse gene responses in greening seedlings. Plant Cell Environ. **11,** 319–328.