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Mutations in the *DET1* gene affect cell-type-specific expression of light-regulated genes and chloroplast development in *Arabidopsis*

(gene expression/tissue specificity/light regulation)

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ABSTRACT **When grown in the absence of light, the *det1* mutant of *Arabidopsis thaliana* develops characteristics of a light-grown plant by morphological, cellular, and molecular criteria. Here, we show that recessive mutations at the *DET1* locus also result in cell-type inappropriate accumulation of RNAs for light-regulated nuclear and chloroplast genes. *det1* root plastids are differentiated into chloroplasts and are present in very high numbers in root cortex cells in contrast to the few starch-containing amyloplasts normally found in *Arabidopsis* roots. To assay the effect of the *det1* mutation on the expression of photoregulated promoters, we used fusion constructs to stably transform wild-type and *det1* mutants. We show that the three red-light-regulated chlorophyll a/b binding protein promoters are inappropriately expressed in the roots of *det1* seedlings and the blue-light-controlled anthocyanin biosynthetic gene, chalcone synthase, is expressed ectopically in leaf mesophyll cells. These results, together with our previous findings, suggest that the *DET1* gene product is a negatively acting regulatory molecule that is used in common by the light stimulus transduction pathway and by temporal or spatial regulatory signals in plants.**

In developing plants, photosynthetically active chloroplasts are derived from proplastids, the small undifferentiated plastids present in meristematic cells (for reviews, see refs. 1 and 2). In general, chloroplasts are restricted to the leaf and stem tissues of plants and, within leaves, to a certain cell type, the leaf mesophyll cell. Thus, the differentiation of proplastids to chloroplasts is related to leaf development in higher plants and must also involve cell-specific signals. Further, in dicotyledonous plants, cotyledon and leaf development is dependent on light (3). Most chloroplastic proteins are expressed from nuclear genes. Of the nuclear light-regulated genes that have been studied, most information is available on the chlorophyll a/b binding protein genes (*cab*) and the genes for the ribulose bisphosphate carboxylase/oxygenase small subunit (*rbcS*) (refs. 4 and 5 and references therein). The expression of these genes is highly regulated, transcripts being most abundant in leaves and lower or undetectable in other organs. Moreover, the rate of transcription in leaves is at least 20-fold induced by light. Gene transfer experiments have shown that as little as 300–400 base pairs (bp) of upstream DNA are needed for light/dark- and tissue-specific expression of *cab* and *rbcS* genes. The upstream cis-acting regulatory elements contain functionally redundant elements [light regulatory elements (LREs)] that mediate positive and negative regulation by light. It has been impossible to separate the upstream LREs from those that confer tissue-specific gene expression. These data indicate that light- and tissue-specific regulation are mediated through the same or overlapping cis-regulatory elements in many of these genes.

A second group of nuclear light-regulated genes, not involved in chloroplast functions, is expressed in cells that do not contain chloroplasts. These include the anthocyanin biosynthetic genes, the best characterized of which is the gene encoding chalcone synthase (*chs*), the first committed step in the isoflavonoid biosynthetic branch of the phenylpropanoid pathway (6). Depending on the plant species examined, the *chs* gene has been shown to be regulated by either UV, blue, or red light or a combination of the above (7–10). In parsley, *in situ* mRNA hybridization of leaf sections revealed that the *chs* gene is expressed exclusively in the epidermal layer of the leaf but not in either the vascular or mesophyll layers (10). Thus, depending on the gene family examined, *chs* vs. *cab* or *rbcS*, light mediates a developmental pattern of gene expression that is exquisitely defined for each cell type.

Aside from the red-light photoreceptor, phytochrome (11), the molecular biology of light-regulated developmental pathways in higher plants is unknown. To better understand the molecular mechanisms that control this process, we isolated mutations that mimic the light signal and induce the light developmental program in darkness. From among a population of mutagenized dark-grown seedlings, we obtained mutants that displayed many phenotypic characteristics of light-grown wild-type plants (12). We have currently assigned these mutants to three complementation groups, designated *det1*, *det2*, and *det3*, and have studied alleles of *det1* in the most detail. Alleles *det1-1* and *det1-2* were shown to be recessive single gene mutations. When grown in the dark, these mutants have the gross morphology of light-grown plants, including the development of chloroplasts and leaf mesophyll tissue. The mRNA levels for several nuclear and chloroplast photogenes are similar in dark-grown *det1* mutants to those found in light-grown wild-type plants, and are 20- to 100-fold higher than those found in dark-grown wild-type seedlings (12). These results suggest that DET1 may be a master regulatory molecule exerting negative control over the light response.

We had previously noted that *det1* mutants are small, are pale-green, and lack apical dominance when grown in the light, implying that DET1 has a function in light-grown plants as well as in dark-grown plants. Here we show, by histology and RNA analysis, that the role of *DET1* in light-grown plants is likely to be in regulating the cell-type-specific expression of light-regulated genes and chloroplast development. Using several light-regulated promoters fused to screenable marker genes that were introduced into *det1* and wild-type plants, we further show that, in light-grown *det1* plants, *cab* and *chs* promoters are active in cell types where they are normally silent or expressed at very low levels in wild-type plants. We

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Abbreviations: Kan, kanamycin; ^{r}, resistant; GUS, β-glucuronidase; CAT, chloramphenicol acetyltransferase.

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extend this analysis to show that the aberrant dark expression of nuclear light-regulated genes in the *det1* mutants is correlated with increased transcription from these promoters. Taken together, these results suggest that *DET1* is involved in the integration of the light- and tissue-specific signal transduction pathways in *Arabidopsis*.

METHODS

Plant Material and Growth Conditions. The *det1-1* mutant allele used in this study has been described (12). This mutant was backcrossed into the wild-type Columbia background four times prior to the experiments described here. For all experiments, *det1* and the Columbia wild type were grown side-by-side under the same light and humidity conditions. For RNA and microscopy experiments that involved root tissue, plants were grown in liquid MS medium for 10 days.

Construction of Chimeric Genes for Transformation. A transcriptional fusion between the *A. thaliana cab3* promoter and the *Escherichia coli* β-glucuronidase (GUS) gene was constructed in the binary vector pBI101.1 (13). The promoter cassette was constructed by making a series of BAL-31 deletions in a *Bam*HI-linearized *cab3* gene (14). The resulting construct contained 950 bp of upstream sequence and has its 3′ end at +50 from the start of transcription (data not shown). The fusion was verified by nucleotide sequence analysis. The *Arabidopsis chs*-GUS-*chs* chimeric transgene used in this study will be described in detail elsewhere (R. L. Feinbaum, G. Storz, and F. M. Ausubel, personal communication). The chimeric gene contains ≈2.0 kilobases (kb) of the *chs* 5′ regulatory region and 0.5 kb of the 3′ untranslated region of the *chs* gene (15). For analysis of the expression of the three individual *A. thaliana cab* genes in the *det1* and wild-type transformants, translational fusions to the *E. coli* chloramphenicol acetyltransferase (CAT) gene were used (16). The resulting constructions in *Agrobacterium* binary vectors, called pGA568 (*cab1*), pGA569 (*cab3*), and pGA570 (*cab2*), have been described (16).

Introduction of Chimeric Transgenes into Wild Type and *det1*. The root transformation protocol of Valvekens *et al.* (17) was used to transform the wild-type and, in some cases (*chs*-GUS, *cab*-CAT), the *det1* mutant. Sterile seedlings for transformation were grown in liquid medium with shaking rather than solid medium as previously described.

The seeds from the primary transformants (T_0) were plated on kanamycin (Kan) (50 μg/ml) and scored for inheritance of the dominant resistance marker. Individual Kan^r seedlings were transferred to pots and seeds were harvested (T_1). We then scored the T_2 progeny from these "selfed" individuals to identify lines that were homozygous for the chimeric transgene. Only plants that were homozygous for the Kan^r marker were analyzed further. For all constructs described above, five independent homozygous lines were analyzed.

The *cab*-GUS fusion was introduced into the *det1* mutant by crossing with wild-type lines. Kan^r F_1 seedlings were allowed to self, and Kan^r *det1* mutants were screened in the F_2 progeny. As described above for the wild type, lines that were *det1* and homozygous for the transgene were selected from individual F_2 *det1* plants.

Analytical Techniques. RNA techniques, blotting protocols, and DNA probes were as described (12). GUS assays with protein extracts of leaves and stems or roots were carried out using the fluorometric assay described by Jefferson (13). Quantitative CAT assays were performed as described by Seed and Sheen (18) and in some cases were visualized on silica gel TLC plates (19).

The histochemical localization of GUS in transformed plants was performed as described (13). After staining, fixation, and dehydration, the tissue pieces were stored at 4°C in 70% ethanol. For examination of leaf cross sections, the tissue was rehydrated and embedded in 2% agar. Fifty- to 100-μm sections were cut using a Vibrotome.

Light and Electron Microscopy. Root tissue from 10-day-old seedlings grown in continuous light (on liquid synthetic medium supplemented with 1% sucrose) was used as the source of material. Tissues were processed as described (12).

RESULTS

***det1* Mutations Affect the Root Plastid Developmental Program.** The morphology of light-grown *det1* plants suggests that DET1 does not simply have a function in dark-grown plants but also plays a role in light-grown plants. We had previously ruled out the possibility that DET1 functions in the repression of photosynthesis gene expression following light-to-dark transitions (12). Since *det1* roots become green upon exposure to light (noticeable green pigmentation about 1 week after germination, whereas roots from wild-type plants typically do not green, or occasionally green 7–8 weeks after germination), we examined the possibility that DET1 is involved in the tissue specificity of chloroplast development and gene expression.

det1 and wild-type seedlings were grown in liquid medium for 7 days and roots were excised. Longitudinal sections through roots showed that *det1* roots were remarkably different from the wild type (Fig. 1). In contrast to wild-type roots (Fig. 1*A*), *det1* root cells were considerably smaller and contained a large number of plastids (Fig. 1*B*). We examined the ultrastructural characteristics of *det1* and wild-type root plastids (Fig. 2). As for other plants, wild-type *A. thaliana* roots contain amyloplasts (starch-containing plastids) (Fig. 2*A*). In contrast to the wild type, *det1* root plastids had clearly differentiated to chloroplasts (Fig. 2*B*), as evidenced by the larger size of the plastid and the formation of stacked thylakoid membrane structures. These *det1* root chloroplasts looked like "young" leaf chloroplasts with a similar structure to wild-type leaf chloroplasts that had been exposed to 12 hr of light (data not shown).

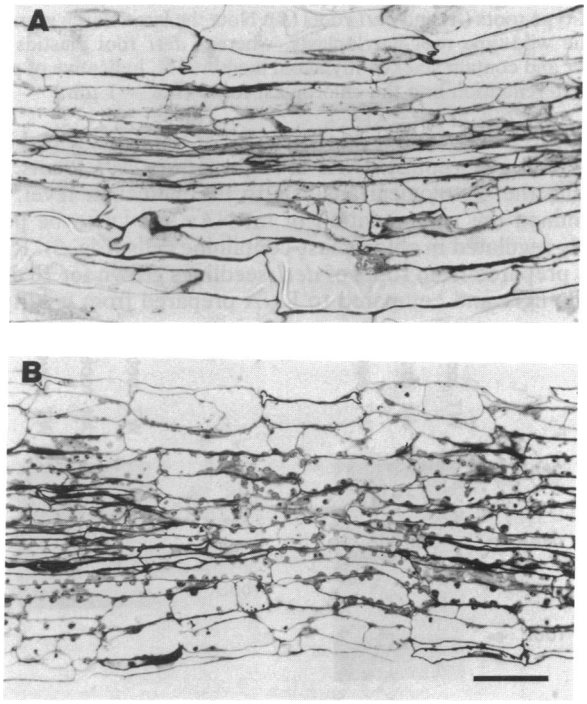

FIG. 1. Light micrographs of 1-μm longitudinal sections from wild-type roots (*A*) and *det1* roots (*B*). Note the large number of plastids that can be seen in the *det1* cortex cells. Wild-type root cells contain very few plastids in comparison. (Bar = 50 μm.)

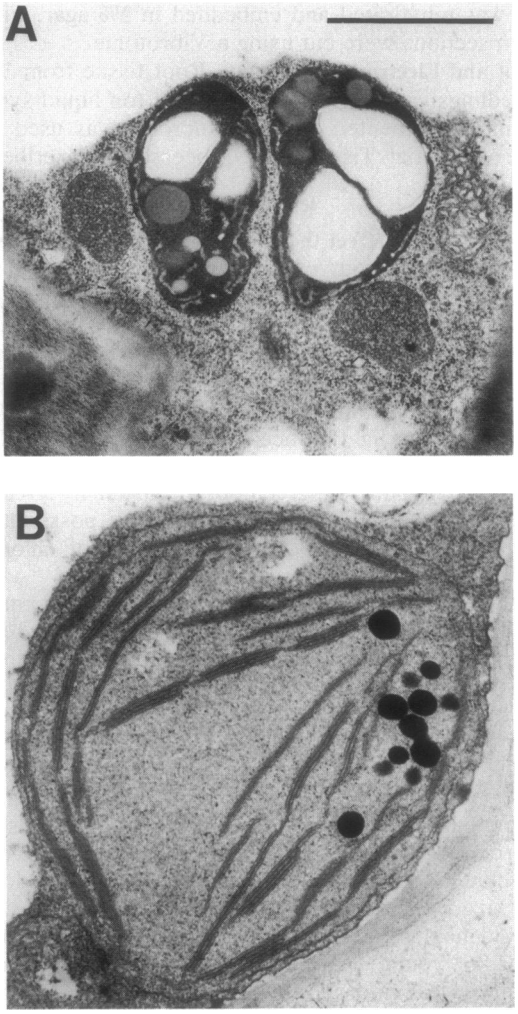

FIG. 2. Electron micrographs of representative plastids from wild-type roots (*A*) and *det1* roots (*B*). Note the large starch granules in the wild-type root amyloplasts, whereas *det1* root plastids are larger and contain stacked thylakoid membranes, indicative of plastid differentiation into the chloroplast form. (Bar = 1 μm.)

Tissue-Inappropriate Accumulation of Light-Regulated Nuclear and Chloroplast RNAs in Light-Grown *det1* Plants. To extend the histological analysis to the molecular level, we examined the accumulation of mRNAs known to be positively regulated in chloroplast-containing cells (Fig. 3). RNA was prepared from roots of *det1* seedlings grown for 10 days in the light and compared to RNA prepared from wild-type roots and leaves. The mRNAs examined included those for the nuclear genes, *cab* (14) and *rbcS* (20), and for the chloroplast genes, *psaA*, *psbA*, and *rbcL* (21). When chloroplast gene expression was examined in wild-type roots, we observed low levels of accumulation for several different genes (Fig. 3*A*). These chloroplast mRNAs accumulated at ≈10-fold higher levels in *det1* roots than in wild-type roots. The RNA levels in *det1* roots were ≈10 times lower than for wild-type leaves (Fig. 3*A*). In wild-type roots, we did not detect RNAs for either the *cab* or *rbcS* gene families (Fig. 3*B*). In contrast, we consistently observed *cab* and *rbcS* mRNAs in the *det1* roots; however, these levels were 10- to 20-fold lower than the levels accumulated in either wild-type or *det1* leaves (Fig. 3*B*).

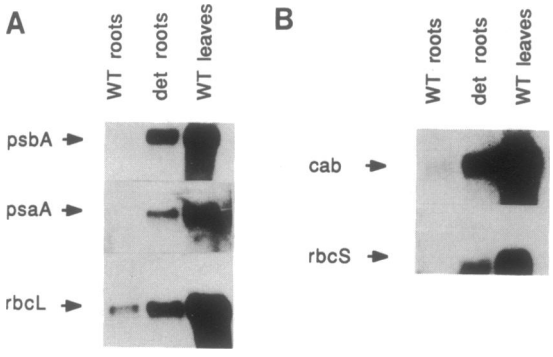

FIG. 3. Accumulation of mRNAs for *psbA*, *psaA*, and *rbcL*, representative chloroplast genes (*A*), and *rbcS* and *cab*, representative nuclear genes (*B*), in wild-type (WT) and *det1* roots compared with wild-type (WT) leaves. Five micrograms of total RNA was loaded per lane.

The *cab* and *chs* Promoters Are Expressed in a Tissue-Inappropriate Manner in *det1* Plants. *A. thaliana* contains four *cab* genes (14), three of which have been cloned (14) and characterized in some detail (16, 22–24). Previous studies using the three cloned *cab* promoters fused to the bacterial CAT gene have indicated that about 200 bp of upstream sequences are sufficient for light- and tissue-specific gene expression in tobacco. Similarly, *chs*, a single-copy gene in *A. thaliana*, is transcriptionally regulated in response to light (15). To assay the expression of these photoregulated promoters, the *cab* and *chs* promoters fused to marker genes were stably introduced into *det1* and wild-type plants.

We initially studied the spatial patterns of expression of the transgenes in *det1* roots. Table 1 shows the expression of the *cab*-CAT transgenes in *det1* roots compared to wild-type roots. Wild-type root extracts contained no CAT activity, whereas *det1* root extracts had activity that was about 10% of the levels of either wild-type or *det1* leaves. This corresponds to the steady-state *cab* RNA levels measured by Northern analysis (Fig. 3). In contrast, the *chs*-GUS chimeric transgene resulted in high and comparable levels of GUS activity in wild-type and *det1* root extracts. By histochemical staining, we observed that *chs* was expressed throughout the entire root of *det1* and wild-type seedlings. In contrast, the spatial expression of *chs* differed in the mutant and wild type when other organs were examined (see below).

The tissue and cell-type specificity of *chs*-GUS expression in wild-type and *det1* leaves was examined by histochemical analysis of GUS activity *in situ*. In wild-type *A. thaliana* leaves, *chs* expression was restricted to the epidermal and vascular layers (Fig. 4*B*), unless the section was near a wound site. As can be seen in Fig. 4*A*, however, *chs* expression was induced at the site where the leaf was excised; in those cells adjacent to the wound, weak *chs*-GUS expression was also observed in the mesophyll cell layers (data not shown). In the *det1* mutant, *chs*-GUS staining was observed in every leaf cell type, including cells of the vascular system, the meso-

Table 1. Tissue-specific expression of *cab* and *chs* promoters in wild-type and *det1* seedlings

	GUS or CAT units*			
	Wild type		*det1*	
Construct	Leaves	Roots	Leaves	Roots
cab1-CAT	1,300	ND	850	70
cab2-CAT	800	ND	150	20
cab3-CAT	1,200	ND	1,300	120
chs-GUS	32,000	11,000	71,000	20,000

Plants were grown on Petri dishes for 10 days in the light. ND, none detected.

*GUS units are pmol of 4-methylumbelliferone per min per mg of protein. CAT units are nmol of chloramphenicol per min per 50 μg of crude protein extract. To minimize variation due to position effects of the inserted transgenes, values are expressed as an average of five independent transformants.

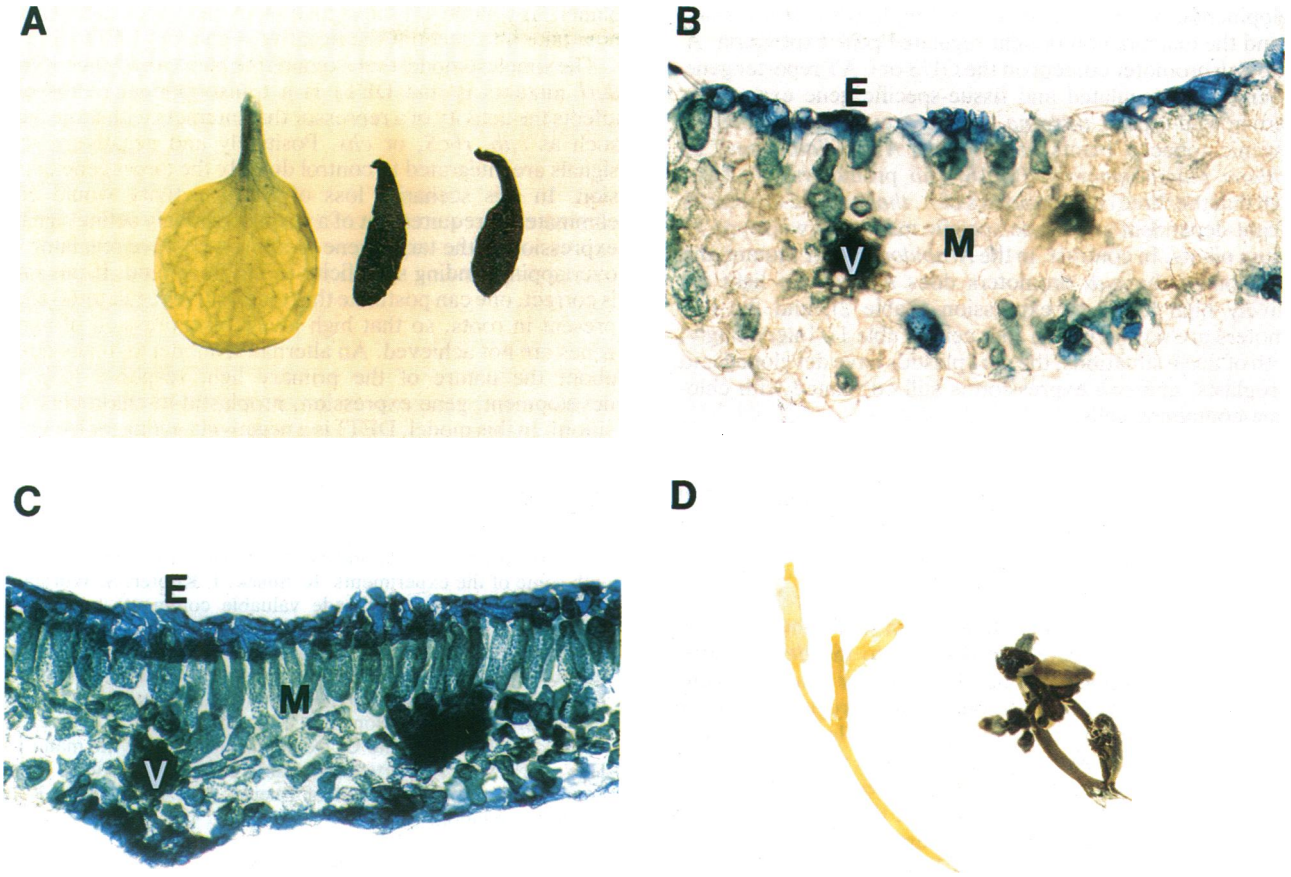

FIG. 4. Histochemical localization of GUS in *chs*-GUS-transformed wild-type and *det1* plants. The green-blue precipitate represents regions of high GUS activity. (*A*) Bright-field image of wild-type leaf (left) and *det1* leaves (two leaves on right) after staining with Glucuronide. (*B*) Bright-field micrograph of transverse section through wild-type leaf. (*C*) Bright-field micrograph of transverse section through *det1* leaf. (*D*) Bright-field image of wild-type flowers (left) and *det1* flowers (right) after staining with Glucuronide. In *B* and *C*, V = vascular tissue; E = epidermis; M = mesophyll cell layers.

phyll tissue, and epidermal and guard cells of the outer layers (Fig. 4*C*). Wounding had no effect on the levels of expression (Fig. 4*A*). Therefore, the higher GUS activities observed in *det1* leaves may be due to expression in all cells of the leaf, as opposed to higher levels of expression of the *chs* promoter in just the epidermal cell layer. A loss of cell-type-specific gene expression was also observed in *det1* flowers (Fig. 4*D*). *chs* is generally expressed only in the pollen of *A. thaliana* flowers; however, *det1* flowers showed high levels of expression in the sepals, ovary, anthers, and, in some cases, petals (Fig. 4*D*). Therefore, *chs* is aberrantly expressed in several different organs in *det1* plants.

Aberrant Light–Dark Regulation of *cab* and *chs* Promoters in *det1* Seedlings. Table 2 depicts the average GUS and CAT levels observed in wild-type and *det1* transgenic seedlings either grown in the light or germinated and grown in total darkness (etiolated). Similar results were observed for all three *cab* promoters and for the *chs* promoter. For each promoter, the levels of transgene expression were approximately the same for light-grown *det1* and wild-type plants. In dark-grown *det1* plants, the levels of CAT or GUS activity were about half of the levels observed in light-grown plants. This contrasts with the >20-fold level of light induction observed in wild-type plants. The ratio of light-to-dark expression is higher for the *cab*-GUS fusion than the *cab*-CAT fusions due to higher basal levels of *cab*-CAT expression in the dark. This was presumably due to the high stability of the CAT enzyme since CAT mRNA levels were undetectable in etiolated wild-type seedlings (data not shown). These data indicate that, in addition to the spatial-inappropriate expression of the *cab* and *chs* promoters, there was also aberrant high constitutive transcription from these promoters in dark-grown *det1* seedlings.

DISCUSSION

We have shown that recessive mutations in the *det1* gene result in a loss of light- and tissue-specific regulation of the chloroplast

Table 2. Light–dark expression of *cab* and *chs* promoters in wild-type and *det1* seedlings

	GUS or CAT units*					
	Wild type			*det1*		
Construct	Light	Dark	L/D†	Light	Dark	L/D†
cab3-GUS	6,500	60	105	5,700	3,200	1.7
cab1-CAT	1,300	100	13	1,100	850	1.3
cab2-CAT	800	50	16	230	150	1.5
cab3-CAT	1,200	60	20	2,300	1,300	1.7
chs-GUS	31,840	1990	16	71,000	57,000	1.2

Plants either were grown for 10 days in the light (light) or were germinated and grown in the dark for 7 days (dark). Values are an average of five transformants.

*GUS units are pmol of 4-methylumbelliferone per min per mg of protein. CAT units are nmol of chloramphenicol per min per 50 μg of crude protein.

†Ratio of expression of the light vs. dark.

developmental program, as evidenced by ultrastructural analysis and the examination of light-regulated gene expression. A 950-bp *cab* promoter confers on the GUS or CAT reporter gene a specific light-regulated and tissue-specific gene expression pattern in wild-type *A. thaliana*. As was previously observed in transgenic tobacco plants using either *A. thaliana*, pea, or *Nicotiana plumbaginofolia* (5, 16) *cab* promoters, we have shown that the three *cab* promoters of *A. thaliana* are expressed in a light-dependent and tissue-specific manner in wild-type *A. thaliana* plants. In contrast, in the *Arabidopsis det1* mutant, the expression of the *cab* promoters does not require light for relatively high levels of expression (Table 2), and the *cab* promoters are now active in root cells (Table 1). Interestingly, in both of these situations, the *det1* plastids have developed into chloroplasts, and *cab* expression is still correlated with chloroplast-containing cells.

It is noteworthy that though *det1* roots contain chloroplasts and accumulate light-regulated mRNAs, the chloroplasts look like young chloroplasts, and the levels of accumulation of the RNAs are not nearly as high in *det1* roots (Table 1 and Fig. 3) as they are in wild-type leaves (Table 1) or even in leaves from dark-grown *det1* seedlings (12). The levels of RNA accumulated from *cab* genes could be correlated with the levels of GUS or CAT activity from the chimeric transgene constructs that were introduced into the *det1* mutant. Thus, leaf cells appear to be required for maximal gene activity and there may be additional positive regulators of *cab* gene transcription that are present only in leaf cells. Since *det1* mutants make leaves in the dark unlike wild-type plants, these factors may be mesophyll-cell-specific transcription factors. However, it is equally plausible that they are present in wild-type etiolated seedlings but are unable to act unless the wild-type DET1 gene product becomes inactivated.

The single-copy *chs* gene of *A. thaliana* is regulated by blue light, with little or no observable expression in etiolated seedlings (R. Feinbaum, G. Storz, and F. Ausubel, personal communication). In the experiments described here, we show that the 2.0-kb segment of upstream DNA sequences confers light- and tissue-specific expression on the GUS reporter gene. These results are similar to those observed for the parsley *chs* promoter (10). As with the *cab* promoters, we observed a loss of the light requirement and cell-type-specific expression pattern of the *chs*-GUS transgene in *det1* mutant seedlings.

Regulation of *cab* and *rbcS* gene expression has been intensively studied for the last several years. Analysis of promoter deletion mutants and chimeric promoter constructs in transgenic tobacco plants has shown that the light-regulated expression of photosynthesis genes is mediated by a complex array of cis-acting DNA elements. In the pea *rbcS-3A* gene, there is a sequence with homology to the simian virus 40 core enhancer region that confers positive and negative regulation on reporter genes (25, 26). In the pea *cabAB80* promoter, there is a 247-bp element that enhances expression in leaves in the light and silences expression of the nopaline synthase gene in the roots of transgenic tobacco plants (5). These promoters and several others have been shown to be expressed exclusively in chloroplast-containing cells (27). Though it is possible to generate promoter mutants that have lower levels of gene activity, it has been impossible to separate the upstream light regulatory sequences from those that confer tissue specificity. Likewise, the *chs* promoters of bean and parsley have been shown to contain positively acting light regulatory elements as well as silencing activities (8, 28–30). Sequence elements involved in environmental stimulation of *chs* gene activity appear to overlap those required for tissue-specific gene expression (J. Kooter and C. Lamb, personal communication). Thus, numerous studies on the cis-acting regulatory sequences of these genes have implied that light- and tissue-specific gene regulation may be coupled in plants. Any model for the regulation of these gene families must now take into account the negative regulator, *DET1*.

The simplest model that explains the pleiotropic phenotype of *det1* mutants is that DET1 is a transcriptional repressor or affects the activity of a repressor that interacts with target genes such as *cab*, *rbcS*, or *chs*. Positively and negatively acting signals are integrated to control directly the target gene expression. In this scenario, loss of *DET1* activity would either eliminate the requirement of a strong positively acting signal for expression of the target gene or allow a positive regulator with overlapping binding specificity to DET1 to bind. If this model is correct, one can postulate that the positive regulator(s) are not present in roots, so that high levels of expression of nuclear genes are not achieved. An alternative model assumes nothing about the nature of the primary light response (e.g., leaf development, gene expression, proplastid-to-chloroplast transition). In this model, DET1 is a negatively acting molecule near the top of a regulatory cascade that is used in common by the light stimulus transduction pathway and by temporal or spatial regulatory signals.

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