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The Phenotype of *Arabidopsis thaliana det*1 Mutants Suggests a Role for Cytokinins in Greening

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SUMMARY

When grown in the absence of light, the *det*1 mutants of *Arabidopsis thaliana* develop characteristics of light-grown plants by morphological, cellular, and molecular criteria. Further, in light-grown plants, mutations in the *DET*1 gene affect cell-type-specific expression of light-regulated genes and the chloroplast developmental program. Here we show that the addition of exogenously added cytokinins (either 2-isopentenyl adenine, kinetin, or benzyladenine) to the growth medium of dark-germinated wild-type seedlings results in seedlings that resemble *det*1 mutants, instead of having the normal etiolated morphology. Like *det*1 mutants, these dark-grown seedlings now contain chloroplasts and have high levels of expression of genes that are normally "light"-regulated. These results suggest an important role for cytokinins during greening of *Arabidopsis*, and may implicate abnormal cytokinin levels or an increased sensitivity to cytokinins as explanations for some of the observed phenotypes of *det*1 mutants.

INTRODUCTION

The mechanisms underlying developmental processses in multicellular eukaryotic organisms are largely unknown. In plants, the environmental stimulus of light triggers a profound change in the developmental program of the organism, leading to alterations in gene expression, tissue differentiation, and gross morphology (Dale, 1988, Mullet, 1988). The development of photosynthetically active chloroplasts from the small, undifferentiated proplastids present in meristematic cells (a process called greening) is one dramatic transition that occurs in response to light signals (Leech, 1976, Mullet, 1988, Gruissem, 1989). In addition to light, the development of chloroplasts is also regulated by intrinsic developmental signals that control leaf differentiation. Only particular leaf-cell types, namely the mesophyll cells, house mature chloroplasts, indicating that cell-specific signals are also important determinants of chloroplast biogenesis. Several of the growth regulators have been implicated in greening, including cytokinins and gibberellins (Stetler and Laetsch, 1965, Flores and Tobin, 1986, Mathis, et al., 1989). How light might interact with these hormone signal transduction pathways is not understood.

Aside from the red-light photoreceptor, phytochrome (Colbert, 1988), the molecular biology of light-regulated developmental pathways in higher plants is unknown. In order to better understand the molecular mechanisms which 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 (Chory et al., 1989a, Chory and Peto, 1990). We have currently assigned these mutants to three complementation groups, designated *det*1, *det*2, and *det*3, and have studied alleles of *det*1 in the most detail. Alleles *det*1-1 and *det*1-2 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 *det*1

mutants to those found in light grown wild-type plants, and are 20-100 fold higher than those found in dark-grown wild-type seedlings (Chory et al., 1989a). These results suggest that DET1 may be a master regulatory molecule exerting negative control over the light response.

*det*1 mutants are small, 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. We have recently shown, by histology and RNA analysis, that the role of *DET*1 in light-grown plants is likely to be in regulating the cell-type-specific expression of light-regulated genes and chloroplast development (Chory and Peto, 1990). Using several light-regulated promoters fused to screenable marker genes that were introduced into *det*1 and wild-type plants, we showed that in light-grown *det*1 plants, these promoters are active in cell-types where they are normally silent or expressed at very low levels in wild-type plants. Taken together with the dark-grown *det*1 phenotypes, these results suggest that *DET*1 is involved in the integration of the light and tissue-specific signal transduction pathways that regulate greening in *Arabidopsis*.

When *det*1 tissue was put into culture on synthetic medium containing only auxin, we observed that the calli were green, instead of achlorophyllous like the wild-type, indicating that the undifferentiated calli derived from *det*1 leaves contained chloroplasts. Since the growth regulator, cytokinin, is normally required for greening in calli, this observation implied that the *det*1 mutants had become cytokinin attenuated. Here, we test the possibility that aberrant cytokinin physiology is related to the numerous phenotypes observed in *det*1 seedlings. On the basis of our results with exogenously added cytokinins, we suggest that cytokinins play an important role during greening of *Arabidopsis*, and that abnormally high cytokinin levels or an increased sensitivity to cytokinins may be explanations for some of the observed phenotypes of *det*1 mutants.

RESULTS

Review of the phenotype of *det*1 mutants. Dark-grown dicotyledonous seedlings are developmentally arrested. They have extended hypocotyls, no cotyledon expansion, no true leaves, and are white. A set of well-characterized genes, the so-called light-regulated genes (e.g., *cab*, the chlorophyll a/b binding protein gene, *rbc*S, the small subunit of the RuBP carboxylase, *chs*, the chalcone synthase gene, and a number of chloroplast genes) are not expressed or are expressed at very low levels. In contrast, recessive mutations in the *DET*1 gene affect a wide variety of light-regulated traits, including leaf and chloroplast development, gene expression, anthocyanin accumulation, and germination (Table I) (Chory, et al., 1989a). *det*1 mutants also have an aberrant phenotype when grown in the light (Table I). Light-regulated traits that are affected in light-grown *det*1 mutants include: ectopic development of chloroplasts in root cortex cells, inappropriate expression of nuclear photosynthesis genes in roots, and ectopic expression of the *chs* promoter in leaf mesophyll cells and flowers (Chory and Peto, 1990). In addition, *det*1 mutants lack apical dominance, have green roots, and green in tissue-culture in the absence of exogenously added cytokinins. These characteristics implied to us that *det*1 mutations affected cytokinin physiology, either directly or indirectly. To test this hypothesis, we undertook the experiments described below.

Exogenously applied cytokinins cause morphological changes in wild-type plants. We tested the effects of exogenously supplied cytokinins on dark- and light-grown wild-type plants. Seeds were surface-sterilized, and plated on MS medium containing various concentrations of cytokinin, either in the light or in the dark. The cytokinins tested included: 2-isopentenyl adenine, benzyladenine, and kinetin; the range of concentrations tested were from 0-20 mg/L. The effects of each cytokinin on dark-grown seedlings was examined at 7d and 20d post-germination. Each cytokinin had a similar effect on wild-type morphology, and only the results from the 2-isopentenyl adenine

experiments are presented. 2-isopentenyl adenine had a dramatic effect on hypocotyl elongation of wild-type seedlings (Fig 1). The hypocotyl length was increasingly shorter with increasing concentrations of cytokinin, up to 20 mg/L. At concentrations higher than this (e.g. 30 mg/L), the seedlings did not germinate well. Further, at 7 d, the cotyledons were expanded and open, unlike wild-type seedlings grown in the absence of cytokinin (Fig 2a). By 20 d, the cytokinin-treated seedlings had developed true leaves (Fig 2b). All of these characteristics, i.e., inhibition of hypocotyl elongation, cotyledon expansion, and leaf development, are similar to the *det*1 mutant. Thus, we were able to make an almost perfect phenocopy of *det*1 by the addition of cytokinin to our growth media. Interestingly, when *det*1 seedlings were subjected to the same cytokinin treatments, they were much more sensitive to the concentration of cytokinin in the medium than the wild-type. By concentrations of 10 mg/L, the *det*1 seeds did not germinate well (data not shown).

Exogenously supplied cytokinin also had an effect on the morphology of light-grown wild-type plants. These plants were smaller than wild-type without cytokinin, paler, had increased anthocyanins, and decreased apical dominance. As for dark-grown seedlings, we observed phenotypes that were very similar to *det*1 seedlings. The one noticeable difference is that wild-type seedlings treated with cytokinin did not develop extensive roots, while *det*1 mutants had fairly normal root growth.

Cytokinins allow chloroplast development in the absence of light. Since the morphological changes observed after cytokinin treatment were reminescent of *det*1 mutants, we wanted to test if other light-regulated traits were also affected in the cytokinin-treated wild-type seedlings. We used electron microscopy to examine the plastid types of wild-type seedlings either grown in the absence or presence of 2-isopentenyl adenine (20 mg/L). Fig 3A shows the typical etioplast structure of dark-grown wild-type *A. thaliana* seedlings. These etioplasts were small, irregularly shaped, and contained a central paracrystalline assembly of tubules, the prolamellar body. In contrast, plastids in the cytokinin-treated wild-type plants (Fig 3B) showed clear signs of chloroplast development,

as evidenced by the lack of prolamellar bodies in the plastids, the somewhat larger size and more regular lens shape of the plastid, and the formation of some thylakoid membrane structures. These developing chloroplasts appeared similar to the chloroplasts that we previously observed in dark-grown *det*1 seedlings (Chory et al., 1989a).

"Light"-regulated genes are expressed in the dark in the presence of cytokinins. A remarkable trait of *det*1 mutants is that light-regulated gene expression is uncoupled from the presence of a light signal (Table I). Since cytokinin-treated wild-type seedlings had many of the morphological phenotypes of *det*1 mutants, we tested to see if light-regulated genes were now expressed in the dark in cytokinin-treated wild-type seedlings. To do this experiment, we used previously constructed transgenic lines that contained either the *chs* or *cab*3 promoter fused to the *E. coli* B-glucuronidase gene (GUS) (Chory and Peto, 1990). We chose these two promoters because work in our lab (Chory et al., 1989b), and by others (Feinbaum and Ausubel, 1988, Karlin-Neumann et al., 1988) has shown that the *chs* gene is regulated primarily by blue-light signals, while *cab*3 is red-light controlled. Thus, these transgenic lines would allow us to assess two divergently light-regulated genes. Measurements of GUS activity indicated that the *chs* and *cab*3 promoters were at least 25-fold more active in cytokinin-treated dark-grown seedlings than in control seedlings (Table II). The levels observed were similar to those that we observed when transgenic *det*1 mutants were plated in the dark in the absence of cytokinins (Table II). Thus, by a third criterion, gene expression, cytokinin-treated wild-type seedlings had similar characteristics to *det*1 seedlings.

Putative cytokinin-insensitive mutants have greening phenotypes. Since dark-grown wild-type seedlings had such a striking phenotype when grown on cytokinins, we decided to screen for mutants that looked etiolated in the presence of cytokinins. These cytokinin-insensitive mutants might be opposite to *det* mutants, and may aid in the further analysis of greening in *Arabidopsis*. Cytokinin-insensitive mutants were identified in a population of mutagenized M_2 generation seeds that were sown at a density of

approximately 1000 seeds per 100 mm petri dish and allowed to grow in complete darkness in the presence of 20 mg/L 2-isopentenyl adenine. Following 7 days of growth in the dark, the plates were scored for seedlings that appeared etiolated. Out of 60,000 M_2 seeds tested, 16 putative cytokinin-insensitive mutants were identified. Of these, 5 never developed leaves and died; 11 grew to maturity and were re-screened for the cytokinin-insensitive phenotype in the M_3 generation. Three of the 13 tested positive for the cytokinin-insensitive phenotype in the M_3 generation. Interestingly, 2 of these are pale yellow-green, i.e., have a greening phenotype. Further molecular and genetic analysis is being performed on these mutants. It will clearly be of interest to cross these mutants to the *det*1 mutants, and examine the phenotype of the double mutants.

CONCLUSIONS

We have shown that many of the phenotypes of *det*1 mutants can be mimicked by the addition of 2-isopentenyl adenine to the growth medium. The traits we examined included leaf and chloroplast development and gene expression. To our knowledge, this is the first report of de-etiolating a dicotyledonous seedling in the dark in the presence of cytokinins.

The similarity of red-light and cytokinin effects was first noted in 1956 by Miller (Miller, 1956). Since then, work has concentrated on studying the effects of adding cytokinins to undifferentiated tissue culture cells or to excised cotyledons (e.g., Stetler and Laetsch, 1965, Teyssendier de la Serve, et al., 1985). These studies have indicated a role for cytokinins in chloroplast development and expression of genes for chloroplast-destined proteins. More recently, *Lemna gibba* has been used to study cytokinin effects on light-regulated gene expression (Flores and Tobin, 1986). From these latter studies, it was concluded that kinetin regulation of *cab* and *rbc*S mRNA levels was primarily post-transcriptional. In *Arabidopsis*, the increased expression of *cab* and *chs* in the presence of cytokinins is at the transcriptional level, since we show that the promoter is all that is

required for increased GUS activity. Indeed, our studies show that greening in *Arabidopsis* is particularly sensitive to the levels of cytokinin.

The primary mode of action of phytochrome, the blue-light photoreceptor, or cytokinins is currently unknown. Based on their data with red-light and cytokinin treatments, Flores and Tobin (1986) speculated that phytochrome and cytokinins independently change the pool size of a common intermediate, and this intermediate more directly regulates gene expression. It is interesting to speculate that this intermediate is specified by the *DET*1 gene. Since high cytokinin to auxin ratios are known to promote organ (shoots and leaves) formation from callus, it is further tempting to speculate that the cytokinin effects are related to tissue-specificity. In favor of this last argument, it was recently shown that tobacco seedlings, stressed by transformation with *Agrobacterium tumefaciens* overexpressing the T-cytokinin gene, had changes in the tissue-specific control of the levels of several defense-related mRNA species (Memelink et al., 1987). Alternative explanations for the *det*1phenotype that relate to the experiments described here are that the cytokinin levels in *det*1 seedlings are abnormally high or *det*1 mutants have an increased sensitivity to cytokinins.

Clearly, many questions need to be addressed to define the role of cytokinin in the regulation of chloroplast development and gene expression. Quantitative measurements of endogenous cytokinins need to be made in light-grown and etiolated seedlings, and compared with levels in the *det*1 mutant. Molecular clones of the *DET*1 gene will aid in the analysis of its function. Finally, isolation and characterization of additional cytokinin mutants might aid in the elucidation of the mode of action of cytokinin and how cytokinin interacts with the light developmental pathways in plants. All of these experiments are in progress.

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Table I

Summary of Greening Phenotypes in Wild-type and *det*1 Plants

Trait	Dark Grown		Light-Grown	
	*DET*1$^+$	*det*1$^-$	*DET*1$^+$	*det*1$^-$
Leaves	Unexpanded cotyledons	Expanded cotyledons, leaves		
Hypocotyl	Long	Short	Short	Short
Pigments	Absent	Anthocyanins	Green	Pale-green
Germination	20% of light levels	100% of light levels	100%	100%
Plastid Type (Leaf)	Etioplast	Young chloroplast	Chloroplast	Chloroplast
(Root)	n.d.	n.d.	Amyloplast	Chloroplast
Gene Expression*:				
Leaf *chs* (nuclear)	Undetected	100	100	200
cab (nuclear)	1-2	25	100	50
*rbc*S (nuclear)	2-5	80		
Chloroplast genes	1-2	100	100	70
Root *cab*	n.d.	n.d.	0	10
chloroplast genes			1-2	10

*Gene expression values are expressed as a percent of the total RNA accumulated in wild-type plants grown in the light.

Table II

Light-Dark Expression of *cab* and *chs* Promoters in Seedlings Treated with Cytokinins

	Wild-type (no cytokinins)		Wild-type (+20mg/L 2-IP)		*det*1	
Construct	Light	Dark	Light	Dark	Light	Dark
p*cab*3-GUS	6,500	60	5,100	2,100	5,700	3,200
p*chs*-GUS	31,840	1,990	60,000	49,000	71,000	57,000

+GUS units are pmol 4-MU/(min x mg protein). Values are an average of 5 transformants.

Plants were grown for either 10 days in the light (light) or were germinated and grown in the dark for 7 days (dark). The wild-type (no cytokinins) and *det*1 data are from Chory and Peto (1990) and are shown here for comparison.

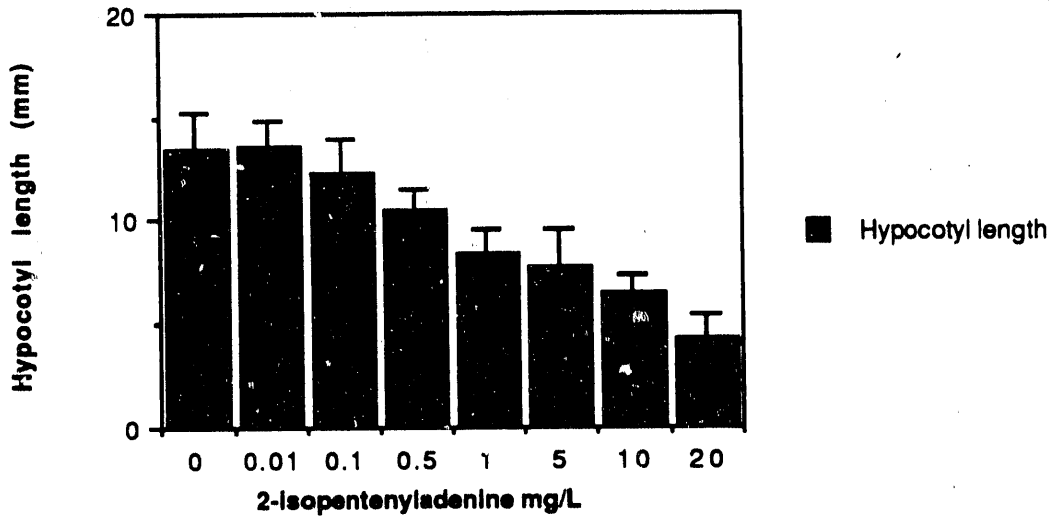
FIGURE LEGENDS

Figure 1: Effect of 2-isopentenyl adenine on hypocotyl length of dark-grown wild-type *Arabidopsis* seedlings. Seedlings were grown for 7 days in the dark on MS medium, 2% sucrose, supplemented with increasing concentrations of 2-isopentenyl adenine as indicated. At least 100 hypocotyls per sample were measured after growth in the dark.

Figure 2: Morphology of wild-type seedlings after growth in the dark on 2-isopentenyl adenine. Panel A: Seven-day old seedlings after growth in the presence or absence of cytokinins. Panel B: Twenty-day old seedlings after growth in the presence or absence of cytokinins. Seedlings were grown in the dark on MS medium supplemented with 2% sucrose and 20mg/L 2-isopertenyl adenine.

Figure 3: Electron micrographs of representative plastids from dark-grown wild-type grown in the absence (A) or the presence (B) of cytokinins. Note the large prolamellar body in (A), while in (B) the plastid is enlarged, has a lens-shape, and contains thylakoid membranes. Bar=1μm.

Effect of 2-isopentenyl adenine on hypocotyl length of dark-grown seedlings



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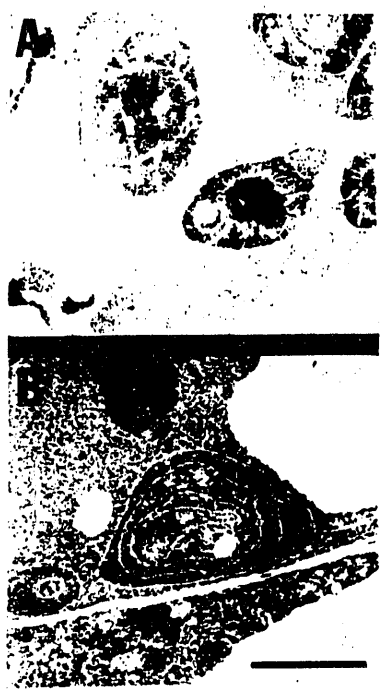
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