A Role for Cytokinins in De-Etiolation in Arabidopsis

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When grown in the absence of light, Arabidopsis thaliana de-etiolated (det) mutants develop many of the characteristics of light-grown plants, including the development of leaves and chloroplasts, the inhibition of hypocotyl growth elongation, and elevated expression levels of light-regulated genes. We show here that dark-grown wild-type seedlings exhibit similar phenotypic traits if any one of a variety of cytokinins are present in the growth medium. We further show that the striking phenotype of det mutants is unlikely to be caused by different levels of cytokinins in these mutants. The three major Arabidopsis cytokinins, zeatin, zeatin riboside, and isopentenyladenosine, accumulate to similar levels in wild-type seedlings grown in either the light or the dark. There is no consistently different pattern for the levels of these cytokinins in wild-type versus det1 or det2 mutants. However, det1 and det2 have an altered response to cytokinin in a detached leaf senescence assay and in tissue culture experiments. A model is proposed in which light and cytokinins act independently or sequentially through common signal transduction intermediates such as DET1 and DET2 to control the downstream light-regulated responses.

Light effects on plant growth and development can be observed at almost every stage of the life cycle, from germination to floral induction. In particular, light causes profound changes in the morphology of the developing young dicotyledonous seedling. These changes include production of Chl, differentiation of leaves and cotyledons, inhibition of stem growth elongation, and the development of the photosynthetically competent chloroplast. In the few cases for which there are available data, the molecular basis for light-regulated development involves the modulation of gene expression (for reviews, see Gilmartin et al., 1990; Chory, 1993).

The molecular mechanisms by which light regulates development are largely unknown. Except for the red/far-red absorbing photoreceptor, phytochrome, little is known about the biochemistry of signal perception and transduction (Quail, 1991). A further complication exists in the plethora of literature documenting that light and hormones cause similar effects in developing plants. For instance, control of germination has been shown to involve GAs, cytokinin, and ABA in various species (Moore, 1979; Jacobsen and Chandler, 1987). GAs, auxins, and ethylene are each involved in the control of cell elongation and morphological responses required for seedling emergence from the soil (Evans, 1985; Potts et al., 1985; Abeles et al., 1992; Kieber et al., 1993). Cytokinins promote cotyledon expansion, leaf development, and chloroplast differentiation (Miller, 1956; Stetler and Laetsch, 1965; Huff and Ross, 1975). During later vegetative growth, cytokinins and ethylene control the onset of leaf senescence (Leopold and Kawase, 1964; Gepstein and Thiemann, 1981). Finally, the ratio of cytokinins to auxins is a primary determinant in the control of apical dominance (Moore, 1979). The overlapping roles of light and plant hormones in development raises the interesting question of whether light and hormones act independently to affect developmental responses or whether plant hormones are involved in the sequence of events initiated by physiologically active photoreceptors.

We have taken a genetic approach to the study of how light interacts with endogenous developmental programs in developing young Arabidopsis seedlings. Our studies have identified a phenotypic class of mutants that show many characteristics of light-grown plants even when grown in complete darkness. We have designated these mutants det (for de-etiolated) because they are de-etiolated in the dark, instead of having the usual etiolated seedling morphology. Recessive mutations in any one of four DET genes result in

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dark-grown plants that grow similarly to light-grown wild-type seedlings (Chory et al., 1989, 1991b; Cabrera et al., 1993; H. Cabrera and J. Chory, unpublished data).

We have been studying homozygous mutant det1 and det2 seedlings in the most detail. Dark-grown det1 and det2 plants have short hypocotyls, expanded cotyledons, and developed leaves. Additionally, several light-regulated genes, including cab (codes for the light-harvesting Chl proteins of PSII), rbcS (codes for the small subunit of Ribulose), and chs (codes for chalcone synthase) are expressed at high levels in the dark in these mutants (Chory et al., 1989, 1991b). When grown in the light, det1 and det2 are small and have reduced apical dominance and fertility when compared with wild-type Arabidopsis (Chory and Peto, 1990; Chory et al., 1991b). These data imply that the DET1 and DET2 gene products play a role in light-grown plants, as well as in dark-grown plants. Specifically, light-grown plants appear to require DET1 for spatial repression of light-regulated genes, because cab, chs, and rbcS are ectopically expressed in det1 mutants (Chory and Peto, 1990). In contrast, homozygous recessive det2 mutations affect photoperiodic responses in light-grown plants, including delayed timing of leaf and chloroplast senescence, delayed flowering, and reduced dark adaptation of cab gene expression (Chory et al., 1991b). Therefore, DET2 may play a negative role in the temporal elaboration of light responses during Arabidopsis development. Epistasis analyses between det1 and det2 and det1 or det2 with phytochrome-deficient mutants indicate that DET1 and DET2 act on distinct pathways and are signal transduction elements that act downstream from phytochrome to affect developmental responses (Chory, 1992).

The pleiotropic nature of the det1 and det2 mutations suggested our original proposal that these genes may play a role in central developmental pathways that respond to the light environment (Chory et al., 1989). This notion was further reinforced by an experiment in which we put det1 tissue into culture on synthetic medium containing only auxin. We observed that det1 calli were somewhat green instead of achlorophyllous like the wild type, indicating that the undifferentiated calli contained chloroplasts. Because cytokinin is normally required for greening, this observation suggested that det1 mutants had become cytokinin autonomous. Here, we test the possibility that aberrant cytokinin physiology is related to the numerous phenotypes observed in det1 and det2 seedlings. We show that many of the phenotypes of det1 mutants can be mimicked by the addition of cytokinins to the growth medium; however, det1 and det2 seedlings do not simply contain elevated cytokinin levels. Responses of det1, det2, and wild-type explants to various concentrations of cytokinins suggest that an altered response to cytokinins is partially responsible for the de-etiolated phenotypes observed in det mutants. A model is proposed in which light and cytokinins act in concert to regulate either the pool size or activity of DET1 and DET2, which in turn affects the elaboration of the downstream light-regulated developmental responses.

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions**

All strains of Arabidopsis used in this study were ecotype Columbia. The det1 and det2 mutants have been described previously (Chory et al., 1989, 1991b). Seedlings were grown aseptically on synthetic medium supplemented with 2% Suc and cytokinins as indicated. Seeds were cold treated overnight and then germinated in high photon fluence rate white light at 20°C under a mixture of fluorescent and incandescent lights at an intensity of 350 μE m⁻² s⁻¹. Dark-grown seeds were germinated for 24 h in the light before transfer to total darkness for 7 to 21 d. A green safelight filter was used during all dark manipulations.

In the experiments described in Figure 1, hypocotyl elongation was measured with a ruler after growth for 7 d in the dark. At least 100 seedlings were measured for each sample.

**Gene Expression Studies**

The construction of the transgenic lines containing cab3-GUS and chs-GUS was published elsewhere (Chory and Peto, 1990). GUS assays with protein extracts from dark- and light-grown seedlings were carried out using the fluorometric assay (Jefferson, 1987).

**EM**

Cotyledon tissue from 8-d-old dark-grown seedlings was used as the source of material. Tissues were processed as described by Chory et al. (1991b).

**Cytokinin Extraction and Measurement**

The method of cytokinin measurement was similar to that described by Schreiber (1990) with the following modifications. Tissues were frozen with liquid N2, ground with mortar and pestle, and homogenized in a Polytron with 5 mL g⁻¹ fresh weight of extraction buffer (methanol:H₂O:acetic acid [70:27:3] plus 10 mg L⁻¹ of butylated hydroxytoluene plus 10% PVP g⁻¹ fresh weight). [³²⁵]Z, [³⁶]Z, and [³⁵]Z were added to extracts for recovery estimation. After 24 h of equilibration, the extract was pelleted and the pellet was reextracted. The combined supernatant was dried, and the residue was treated with acid phosphatase. The extract was applied to a 2-mL C₁₈ Megabond elute cartridge and washed with 2 volumes of 0.1 M acetic acid (pH 3.3) with triethanolamine (solvent B) and eluted with 2 volumes of methanol. The sample was dried and chromatographed over a 2-mL DEAE column onto a 1-mL Affigel anti-[³⁵]Z, (diH)[³⁵]Z, and [³⁵]Z antibody column (washed sequentially with 10 mM Bis Tris, pH 6.5 [buffer A], buffer A plus 1 M NaCl, H₂O, and methanol). The methanol eluant was dried, resuspended in solvent B plus 100 ng of kinetin, injected onto a C₁₈ HPLC (0.45-× 25-cm column at 1.5 mL min⁻¹), and eluted with a gradient of solvent A (CH₃CN) and solvent B (0.9-1, 0–9 min; 17.83, 9–40 min; and 100.0, 40–48 min). Under these conditions cytokinins had retention times (min) of 5.3 for [³⁵]Z, 7.6 for Z, 8.5 for (diH)Z, 14.1 for [³⁵]Z, 14.8 for (diH)[³⁵]Z, 17.0 for kinetin, 26.1 for iP, and 29.1 for [³⁵]Z. Endogenous cytokinins (base and nucleoside plus nucleotide) were identified and quantified by diode array detection and corrected for recovery of radiolabeled cytokinins by scintillation counting.
Detached Leaf Senescence Assays

Leaf senescence assays were performed as described by W. Su and S. Howell (personal communication). Plants were grown on synthetic medium plus agar for approximately 3 weeks (Chory et al., 1989). Whole leaves from wild type, det1, and det2 were then detached with a scalpel and placed into Petri dishes containing several layers of filter paper saturated with water or solutions containing various levels of BA in water. The Petri dishes were incubated in the dark for 7 d, taking care to keep the leaves moist. Specific Chl content was calculated as follows. Two leaves per sample were blotted dry, weighed, and placed in a 1.5-mL Eppendorf tube. The samples were resuspended in 80% acetone, ground with a disposable pestle, and incubated in the dark for 30 min. Total Chl (µg mL⁻¹) was determined according to the equation: 20.2 + 8.02 A₆₆₃. At least 10 leaves per treatment were used.

Tissue Culture Experiments

Wild type, det1, and det2 plants were grown aseptically on minimal salts medium plus Suc for 2 weeks (Chory et al., 1989). At that time, stem, root, and leaf explants were incubated for 4 d on callus induction medium (Valvekens et al., 1988). The explants were then transferred to various media with no hormones or different quantities of kinetin as described in "Results." Organogenesis was scored after 2 weeks of growth.

RESULTS

Exogenously Applied Cytokinins Result in a Wild-Type Phenocopy of det1 Mutants

The striking phenotype of dark-grown det1 seedlings prompted us to examine the role of cytokinins in the deetiolation response in Arabidopsis. Using exogenously added cytokinins, we were able to make a wild-type phenocopy of the det1 mutant. Seeds were surface sterilized and plated in the light or the dark on Murashige-Skoog medium containing various concentrations of cytokinin. Three different cytokinins were tested initially: iP, BA, and kinetin at concentrations from 0 to 95 µmol L⁻¹. The effect on hypocotyl elongation of each of the cytokinins was examined after 7 d of growth in the dark (Fig. 1). The hypocotyl length was increasingly shorter with increasing concentrations of cytokinin, up to 95 µmol of iP L⁻¹. Concentrations higher than this affected the germination rate of the seeds. Upon determining the identity of endogenous Arabidopsis cytokinins to be Z, [9R]Z, and [9R]iP (see below), we repeated the phenocopy experiments with these hormones with similar results (data not shown). Similar concentrations of the auxin, IAA, did not affect the morphology of dark-grown seedlings, suggesting that the differences in hypocotyl morphology are specifically caused by cytokinins (data not shown).

Wild-type seeds germinated in the dark on cytokinin did not have the developmentally arrested phenotype of etiolated seedlings. We examined the morphology of wild-type seedlings germinated in various concentrations of cytokinins after growth in the dark for 6, 12, or 28 d (Fig. 2). Each of the six cytokinins had a similar effect on wild-type morphology, and only the results from the [9R]iP experiments are presented. After 6 d of growth in the dark, the cotyledons were expanded and open, in contrast to seedlings grown in the absence of cytokinins (Fig. 2A). By 12 d, the cytokinin-treated wild-type seedlings had developed true leaves (Fig. 2B), and by 28 d, full rosettes had formed (Fig. 2C). It should be noted that concentrations as low as 100 nmol L⁻¹ of kinetin or iP were sufficient to overcome developmental arrest in the dark. These traits, e.g. inhibition of hypocotyl elongation and promotion of cotyledon expansion and leaf development, are similar to those of dark-grown det1 mutants. Furthermore,
det1 seedlings were more sensitive than the wild type to exogenously added cytokinins. At concentrations of iP as low as 24 µmol L⁻¹, the det1 seeds did not germinate well (data not shown).

Exogenously applied cytokinins also had an effect on the morphology of light-grown wild-type plants. Treated plants were smaller and paler and had increased anthocyanins and decreased apical dominance when compared to wild-type plants grown in the absence of cytokinins (data not shown). As for the dark-grown seedlings, these phenotypes were very similar to det1 seedlings. The one notable difference was that wild-type seedlings treated with cytokinin did not develop extensive roots, whereas untreated det1 mutants had normal root growth.

We also examined plastid morphology in dark-grown wild-type seedlings grown in the absence or presence of cytokinin (45 µmol L⁻¹). Figure 3A shows the typical etioplast structure of dark-grown wild-type A. thaliana seedlings. These etioplasts were small (less than 5 µm in diameter) and irregularly shaped and contained a central paracrystalline assembly of tubules, the prolamellar body. In contrast, plastids found in dark-grown wild-type seedlings grown in the presence of 45 µmol L⁻¹ of iP showed chloroplast development, as evidenced by the lack of prolamellar bodies in the plastids, somewhat larger size and more regular lens shape of the plastid, and formation of some bithylakoid membrane structures (Fig. 3B). These developing chloroplasts appeared similar to the chloroplasts that we previously observed in dark-grown det1 seedlings (Chory et al., 1989).

In dark-grown det1 and det2 seedlings, several light-regulated genes, including cab, chs, and rbcS, are expressed at high levels. Because cytokinin-treated wild-type seedlings had many of the morphological phenotypes of det1 mutants, we were curious to test whether these light-regulated genes were now expressed in the dark in cytokinin-treated wild-type plants. We used previously constructed transgenic lines that contained either the cab3 or chs promoter fused to GUS. Measurements of GUS activity indicated that the chs and cab promoters were 7 to 8 times more active in cytokinin-treated wild-type seedlings than in untreated controls (Table I). The GUS levels observed were similar to those that we previously observed when transgenic det1 mutants were grown in the dark (Chory and Peto, 1990). As a control, the levels of actin mRNA increased only 1.5- to 2-fold after treatment with cytokinins. Thus, by three criteria, morphology, plastid development, and gene expression, cytokinin-treated wild-type seedlings had characteristics similar to those of det1 seedlings (Table II).
Table I. Expression of cab and chs promoters in seedlings treated with cytokinins

Seedlings were grown for either 7 d in the light (light) or in the dark for 10 d (dark).

<table>
<thead>
<tr>
<th>Construct</th>
<th>Wild Type (no cytokinins)</th>
<th>Wild Type (+45 pmol L⁻¹ of iP)</th>
<th>det1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Light²</td>
<td>Dark</td>
<td>Light</td>
</tr>
<tr>
<td>pcab3-GUS</td>
<td>41,000</td>
<td>350</td>
<td>23,000</td>
</tr>
<tr>
<td>pchs-GUS</td>
<td>34,000</td>
<td>2,200</td>
<td>49,500</td>
</tr>
</tbody>
</table>

* The transgenic lines are from Chory and Peto (1990). b GUS units are pmol of 4-methyl umbelliferone min⁻¹ mg⁻¹ of protein. The values presented are averages of three separate experiments.

Table II. Comparison of phenotypes of wild type, wild type plus cytokinin, and det1

<table>
<thead>
<tr>
<th>Phenotypic Trait</th>
<th>Wild Type</th>
<th>Wild Type + iP</th>
<th>det1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dark</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cotyledon development</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Leaf development</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Hypocotyl</td>
<td>Long</td>
<td>Short</td>
<td>Short</td>
</tr>
<tr>
<td>Chloroplast development</td>
<td>No</td>
<td>Partial</td>
<td>Partial</td>
</tr>
<tr>
<td>Activation of light-regulated promoters</td>
<td>Low</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Light</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Height</td>
<td>Tall</td>
<td>Short</td>
<td>Short</td>
</tr>
<tr>
<td>Color</td>
<td>Green</td>
<td>Pale green</td>
<td>Pale green</td>
</tr>
<tr>
<td>Fertility</td>
<td>Normal</td>
<td>Reduced</td>
<td>Reduced</td>
</tr>
<tr>
<td>Green roots</td>
<td>No</td>
<td>No roots</td>
<td>Yes</td>
</tr>
<tr>
<td>Apical dominance</td>
<td>Normal</td>
<td>Reduced</td>
<td>Reduced</td>
</tr>
</tbody>
</table>

Quantitative Cytokinin Analysis in Light- and Dark-Grown Wild-Type and Mutant Arabidopsis

The cytokinin-induced phenocopy of det1 suggests that an increase in available cytokinins in dark-grown seedlings is sufficient to override a light requirement for leaf and chloroplast development and gene expression in Arabidopsis. One way to explain the cytokinin results in terms of the recessive nature of det1 mutations is that the wild-type DET gene products may be involved in the negative regulation of cytokinin synthesis in Arabidopsis. If this were indeed the case, det1 mutants should have elevated levels of active cytokinins. This would also explain the observed increased sensitivity of det1 over the wild type to cytokinin treatments.

Table III. Cytokinin levels in dark-grown Arabidopsis seedlings

<table>
<thead>
<tr>
<th>Cytokinin</th>
<th>Wild type (7 d)</th>
<th>Det1 (7 d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp 1</td>
<td>Exp 2</td>
</tr>
<tr>
<td>Z</td>
<td>4.3⁶ (0.26)</td>
<td>nd⁷ (nd)</td>
</tr>
<tr>
<td>[9R]Z</td>
<td>6.1 (0.37)</td>
<td>4.6 (0.28)</td>
</tr>
<tr>
<td>[9R]iP</td>
<td>1.8 (0.11)</td>
<td>2.3 (0.14)</td>
</tr>
</tbody>
</table>

a ng g⁻¹ fresh weight. b ng/100 seedlings, fresh weight/seedling computed from a separate experiment. c nd, Not detected. The cytokinin levels were 2.9 times higher in 26-d-old det1 (Table IV). Wild type, det1, and det2 showed similar trends in cytokinin levels, with total levels at d 14 being higher than at d 26. The levels of cytokinins detected in wild-type seedlings at d 14 and d 26 were 64 and 18 nm g⁻¹ fresh weight. For det1 and det2, these values were 50 and 37 and 95 and 25 nm g⁻¹ fresh weight, respectively.

det1 and det2 Have Reduced Leaf Senescence

Cytokinins are well characterized for their role in preventing leaf senescence. When detached leaves are placed in the dark, a rapid loss of Chl occurs. If cytokinins are added to the detached leaves, however, there is protection from senescence. As shown in Figure 4, wild-type Arabidopsis detached leaves contain approximately 10-fold lower levels of Chl g⁻¹ fresh weight after incubation in the dark for 7 d. In contrast, det1 leaves (which are slightly pale at the start of the experiment) have Chl levels that are only 1.3-fold lower than the controls. Likewise, det2 leaves (which are slightly greener at the beginning of the experiment) are approximately 1.25-fold lower than the control leaves. When increasing concentrations of BA are added to the detached leaves during the 7-d incubation, wild-type leaves are partially protected from senescence. With concentrations of 5 μM BA, Chl levels are approximately 50% of the wild-type controls (Fig. 4). det1 shows a slight recovery in the presence of BA. However, det2 leaves show no significant recovery in the presence of BA (Fig. 4). Therefore, we conclude that neither det1 nor det2 mutants senesce as rapidly or to the same extent as wild type. Moreover, neither of the mutants responds significantly to exogenously added BA, indicating that the mutants may
already have saturated a cytokinin response pathway. Thus, increasing the concentration of cytokinin in the medium does not cause any further changes in the mutant leaves.

**det1 and det2 Have an Altered Response to Cytokinins in Tissue Culture Experiments**

*det1* and *det2* mutants show a significant delay in senescence from the wild type, yet they do not have significantly increased levels of cytokinins. Therefore, we further examined whether the mutants might have an enhanced sensitivity to cytokinin in tissue culture experiments. Wild-type, *det1*, and *det2* seedlings were grown aseptically on synthetic medium. After 2 weeks of growth, explants from various tissues were dedifferentiated on callus-induction medium (0.5 mg L\(^{-1}\) of 2,4-D, 0.05 mg L\(^{-1}\) of kinetin) (Valvekens et al., 1988) for 4 d and then transferred to synthetic medium with no hormones or with a lower cytokinin-to-auxin ratio (0.5 mg L\(^{-1}\) of 2,4-D, 0.015 mg L\(^{-1}\) of kinetin). We then examined over time the regeneration capability of the various tissues. Each explant was scored for the number of regenerative organs as well as the type of tissue regenerated (shoot, root, callus). Under these growth conditions, wild-type leaf and root explants predominantly formed roots (Table V). In contrast, *det1* and *det2* root and leaf explants continued to callus and only occasionally made roots at low cytokinin-to-auxin ratios or in the absence of hormones (Table V). A small percentage of *det2* leaf explants (6%) differentiated into shoots. The results of these experiments suggest that *det1* and *det2* mutants have a lower requirement than the wild type for cytokinins to express a typical cytokinin response.

**DISCUSSION**

We have shown that many of the dark-grown phenotypes of *det* mutants can be mimicked in wild-type plants by the addition of any one of a number of cytokinins to the growth medium. The phenotypic traits examined included the promotion of cotyledon and leaf development, the inhibition of hypocotyl growth, and the induction of "light-regulated" gene expression. Although other workers have long studied the effects of adding cytokinins to undifferentiated tissue culture cells or to excised cotyledons, to our knowledge this is the first example of de-etiolating a dicotyledonous seedling in the dark in the presence of cytokinins.

**Table IV. Cytokinin levels in light-grown Arabidopsis seedlings**

<table>
<thead>
<tr>
<th>Cytokinin</th>
<th>Wild type, 10 d, green</th>
<th>Wild type, 14 d, green</th>
<th>det1, 14 d, green</th>
<th>det2, 14 d, green</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zeatin</td>
<td>4.0 (11)(^b)</td>
<td>2.2 (6.2)</td>
<td>7.3 (45)</td>
<td>7.4 (46)</td>
</tr>
<tr>
<td>[9R]Z</td>
<td>8.3 (23)</td>
<td>1.4 (3.9)</td>
<td>4.0 (25)</td>
<td>3.5 (22)</td>
</tr>
<tr>
<td>[9R]P</td>
<td>2.8 (7.8)</td>
<td>nd (nd)</td>
<td>9.8 (60)</td>
<td>3.5 (22)</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>Exp 1</th>
<th>Exp 2</th>
<th>Exp 1</th>
<th>Exp 2</th>
<th>Exp 1</th>
<th>Exp 2</th>
<th>Exp 1</th>
<th>Exp 1</th>
<th>Exp 1</th>
<th>Exp 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zeatin</td>
<td>6.6 (30)</td>
<td>1.4 (6.4)</td>
<td>21 (110)</td>
<td>0.7 (46)</td>
<td>nd (nd)</td>
<td>2.9 (170)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[9R]Z</td>
<td>nd</td>
<td>nd</td>
<td>8.4 (39)</td>
<td>nd (nd)</td>
<td>nd (nd)</td>
<td>nd (nd)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[9R]P</td>
<td>7.4 (34)</td>
<td>6.0 (28)</td>
<td>nd (nd)</td>
<td>nd (nd)</td>
<td>2.3 (160)</td>
<td>8.1 (290)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

\(a\) ng g\(^{-1}\) fresh weight. \(b\) ng/100 seedlings, fresh weight/seedling computed from a separate experiment. \(c\) nd, Not detected.

**Table V. Rooting frequency of calli from wild type, det1, and det2 leaf and root explants transferred to a medium without hormones**

At least 50 explants were examined in two separate experiments. The explants were grown for 4 d on synthetic medium plus 0.5 mg L\(^{-1}\) of 2,4-D, 0.05 mg L\(^{-1}\) of kinetin (Valvekens et al., 1988) and then transferred for 2 weeks to Murashige and Skoog plates lacking hormones. Similar results were obtained when the explants were transferred to 0.5 mg L\(^{-1}\) of 2,4-D, 0.015 mg L\(^{-1}\) of kinetin (data not shown). WT, Wild type.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Roots</th>
<th>Calli</th>
<th>Shoots</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT leaves</td>
<td>87</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>WT roots</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>det1 leaves</td>
<td>22</td>
<td>78</td>
<td>0</td>
</tr>
<tr>
<td>det1 roots</td>
<td>15</td>
<td>85</td>
<td>0</td>
</tr>
<tr>
<td>det2 leaves</td>
<td>45</td>
<td>49</td>
<td>6</td>
</tr>
<tr>
<td>det2 roots</td>
<td>23</td>
<td>77</td>
<td>0</td>
</tr>
</tbody>
</table>

**Figure 4. Results of detached leaf senescence assays.** On the left is shown specific Chl content for wild-type, *det1*, and *det2* control leaves immediately after detachment from the plant. The next four sets of data indicate specific Chl content after 8 d in the dark in the absence (0 \(\mu\)M) or presence of increasing concentrations of BA. The data are shown as averages plus so. *det1* and *det2* show delayed leaf senescence and do not respond significantly to exogenously added cytokinin.
Several laboratories have previously shown that cytokinins increase the accumulation of proteins encoded by cab and rbcS (Flerabend and deBoer, 1978; Teysseindier de la Serve et al., 1985). More recently, Lemna gibba and cucumber have also been used to study cytokinin effects on light-regulated gene expression (Flores and Tobin, 1986, 1988; Cotton et al., 1990). From the Lemna studies, it was concluded that kinetin regulation of cab and rbcS mRNA levels was primarily post-transcriptional. In cucumber, the level of phyA mRNA, a gene whose expression is negatively regulated by light, was determined in etiolated seedlings treated with BA. These authors showed that addition of exogenous BA to etiolated seedlings resulted in a significant decrease in phyA mRNA accumulation. This decrease was of the same order of magnitude as that caused by a red-light pulse, suggesting that BA can completely substitute for phytochrome in down-regulating the accumulation of phyA mRNA (Cotton et al., 1990). In Arabidopsis, the increased accumulation of cab and chs must be at least partially due to increases in transcription rates, because we have shown that the promoter is all that is required for increased activity of either the chloramphenicol acetyl transferase (data not shown) or the GUS marker genes.

The cytokinin phenocopy results imply that an increase in available cytokinins in dark-grown seedlings is sufficient to override a light requirement for leaf and chloroplast development and gene expression in Arabidopsis. There are several ways to explain the cytokinin results in terms of the recessive nature of det mutations. First, DET1 and DET2 may play a negative role in a cytokinin signal transduction pathway that is coupled to photoperception. A second alternative is that DET1 and DET2 conjugate cytokinins, making them inaccessible, or that they modify active cytokinins, creating an inactive form (e.g., cytokinin glucosides). Third, DET1 and DET2 may be involved in the negative regulation of cytokinin synthesis in Arabidopsis.

Quantitative cytokinin analysis in Arabidopsis seedlings indicates that Arabidopsis contains relatively low levels of the cytokinins Z, [9R]Z, and [9R]iP. If det1 was a cytokinin biosynthesis overproduction mutant, the levels of cytokinins in dark-grown det1 would be expected to be consistently higher than in wild-type etiolated seedlings. For instance, in the moss, Physcomitrella patens, cytokinin levels are up to 100-fold higher than wild type in overproducing mutant lines (Wang et al., 1981a, 1981b). However, cytokinin levels did not show a consistent difference between dark-grown det1 and wild-type Arabidopsis. Light-grown wild-type, det1, and det2 mutants also had no consistent difference in cytokinin levels. Although we did make several measurements in light-grown plants, we did not measure kinetics of accumulation of cytokinins throughout the entire developmental time or immediately after transfer of seedlings from dark to light growth conditions. There are reports that cytokinin levels increase transiently after red-light pulses in Scots pine seeds and in etiolated squash seedlings (Qamaruddin and Tillberg, 1989; Kuraishi et al., 1991). Therefore, it is possible that there could be a significant difference in cytokinin levels in the det mutants versus the wild type if measurements were made during the proper “developmental window.”

A second caveat is that the cytokinin levels measured are averages for the entire plant. Because the phenotype of det mutants is expressed in many organs of the mature plant, including roots, leaves, and stems, an increase in cytokinin levels might be expected to occur in several tissues simultaneously. Our experiments do not eliminate the possibility that cytokinins are redistributed in response to light, even though the absolute levels remain the same. The phenotype of dark-grown det mutants might be explained by redistribution of a cytokinin in the absence of light signals. However, complete cytokinin measurements during development and among various tissues would be extremely difficult given the small size of Arabidopsis seedlings and the low endogenous levels of cytokinins. Indeed, tens of thousands of mutant plants were needed to perform the cytokinin quantitation presented here.

We believe that the phenocopy results are significant, even though extremely high quantities of iP were required to see the most dramatic phenotypic differences. Our most striking phenocopy results occurred in the range of 20 to 50 μmol L⁻¹ of iP, which is about 1000-fold higher than the endogenous levels. However, iP is not a very active cytokinin, and release from developmental arrest occurred at concentrations of iP as low as 10 nmol L⁻¹. Moreover, we do not know the amount of exogenously supplied cytokinin actually taken up into dark-grown wild-type seedlings.

Campbell and Town (1991) measured cytokinin levels in wild-type and radiation-induced tumors of Arabidopsis thaliana, ecotype Landsberg, using immunoaffinity columns and HPLC. The 8-d-old, light-grown wild type was observed to have [9R]Z and [9R]iP at 0.5 to 4.2 and 1.5 to 2.9 ng g⁻¹ fresh weight, respectively, levels similar to 10-d-old, light-grown wild-type Columbia seedlings used in our studies. The authors concluded that the γ-radiation-induced tumors (including a shooty tumor) had cytokinin levels similar to control tissues. Conversely, crown gall shooty tumors had 100 to 1000 times higher levels of cytokinins than wild-type tissues. Clearly, det mutants are not simply cytokinin overproducers as are the Agrobacterium-transformed Arabidopsis shooty tumors of Campbell and Town. The possible biological significance of the relatively small cytokinin changes observed in det mutants is unclear.

Recent studies involving recombinant Agrobacterium rhizogenes rolC protein suggest that the rolC gene product transforms plants by releasing cytokinins from inactive N-glucoside conjugates (Estruch et al., 1991). By corollary, results of the present study infer the presence of an activity or activities that modifies active cytokinins such that they are inaccessible to the plant. In our studies we examined tissue for the presence of cytokinin N-glucosides; however, significant levels of N-glucosides were not observed. Cytokinin O-glucosides were not measured in this study. The current quantitative cytokinin analysis provides no evidence for the hypothesis that DET1 or DET2 modifies active cytokinins into inactive forms. Thus, a regulatory role for DET1 or DET2 as an “anti-rolC” activity is not likely.

We favor a model in which light and cytokinins act independently or sequentially through common signal transduction intermediates, such as DET1 and DET2 to control the downstream light-regulated responses (Fig. 5, A and B). The primary mode of action of phytochrome, the blue-light or UV photoreceptors, or cytokinins is currently unknown.
Plant hormones are involved in the sequence of events independently to affect developmental responses or whether DET1 and DETZ to induce enhanced sensitivity to existing consistent with the interpretation that light may act through intermediate, and this intermediate more directly regulates Flores and Tobin (1986) proposed that phytochrome and gene expression. These intermediates could be specified by cytokinin levels (Trewavas, 1983). Equally plausible is a model in which light or cytokinins act to decrease the activity of DET1 or DET2, which then permits the elaboration of the downstream light-regulated gene expression and morphological responses. Finally, it is possible that DET1 or DET2 act to link photoreceptors to a cytokinin action pathway (Fig. 5C).

The question of whether light and cytokinins act independently to affect developmental responses or whether plant hormones are involved in the sequence of events initiated by physiologically active photoreceptors is still an open one. For instance, in the Lemma studies, exogenously added cytokinin resulted in increased accumulation of cab and rbcS mRNAs to approximately the same levels as when short red-light treatments were applied. However, it was concluded that red light does not act by a cytokinin-mediated mechanism on cab and rbcS expression, because red light or cytokinin was each more effective in the presence of the other than when administered alone (Flores and Tobin, 1986). This result is in agreement with studies designed to measure increases in Chl accumulation in greening mustard cotyledons leading to Chl accumulation. In contrast, in cucumber, phyA mRNA levels are down-regulated to similar levels by either light or BA (Cotton et al., 1990). Our studies do not directly address this question; however, the observation that cytokinin can completely override the necessity for light to induce leaf and chloroplast development and gene expression suggests that these responses are not additive in Arabidopsis.

High cytokinin-to-auxin ratios are known to promote greening and shoot formation from callus tissue in many plants. det1 calli appear to be more sensitive to low ratios of cytokinins to auxin, because they have increased specific Chl content over wild type on low concentrations of cytokinin (data not shown). Even more striking was the lack of root formation in det1 and det2 seedlings transferred to a minimal salts medium after callus induction. This result implies that calli derived from det1 and det2 explants have a lower requirement for cytokinins to express typical cytokinin responses. Alternatively, the mutants may have lower cytokinin catabolism, and the cytokinins are more persistent after transfer of the explants to medium containing no hormones. Thus, det mutants may be opposite to recently isolated cytokinin-resistant mutants (ckr) that have a reduced response to cytokinins (Su and Howell, 1992). In contrast to det2 mutants, the ckr mutants are pale and have longer than normal primary roots. Moreover, auxin-resistant mutants of Arabidopsis (axr) have been described that have decreased responses to auxin (Estelle and Somerville, 1987). When double mutants were made between det1 and axr1, or det2 and axr1, we observed a much more severe phenotype than for either mutant alone (J. Reed and J. Chory, unpublished data). It will be informative to make the det1-ckr and det2-ckr double mutants as well.

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