

# Cytokinin, Acting through Ethylene, Restores Gravitropism to Arabidopsis Seedlings Grown under Red Light

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Cytokinin replaces light in several aspects of the photomorphogenesis of dicot seedlings. *Arabidopsis thaliana* seedlings grown under red light have been shown to become disoriented, losing the negative hypocotyl gravitropism that has been observed in seedlings grown in darkness or white light. We report here that cytokinin at micromolar concentrations restores gravitropism to seedlings grown under red light. Cytokinin cancels the effect of red light on the gravity-sensing system and at the same time replaces light in the inhibition of hypocotyl elongation. Furthermore, application of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid acts similarly to cytokinin. Cytokinin cannot restore gravitropism under red light to an ethylene-insensitive mutant that is defective at the *EIN2* locus. Stimulation of ethylene production, therefore, can explain the action of cytokinin in restoring negative gravitropism to the hypocotyls of *Arabidopsis* seedlings grown under continuous red light.

Growth and development of dicot seedlings are sensitive to environmental signals such as light and gravity (Pilet et al., 1978; Feldman and Briggs, 1987). Seedlings of *Arabidopsis thaliana* grown in total darkness show the typical features of etiolated dicots: long hypocotyls and small cotyledons. *Arabidopsis* stems exhibit the normal gravitropic response, and this plant has been suggested as a suitable genetic and physiological model system for studying shoot gravitropism (Fukaki et al., 1996a, 1996b). When seedlings are grown under white light, hypocotyl growth is negatively gravitropic; however, when seedlings are grown under continuous red light, the growth direction of the hypocotyls is randomized (Liscum and Hangarter, 1993). This action of light is surprising, since light usually potentiates gravitropic sensitivity (Feldman and Briggs, 1987). Disorientation under red light may therefore provide a way to demonstrate the existence of a new photomorphogenetic response pathway.

Exogenous cytokinins replace light in the induction of several aspects of de-etiolation. In the dark cytokinin can induce expression of genes that are normally induced by light (Flores and Tobin, 1986; Chory et al., 1991). Chloroplast development, which normally depends both on the inductive actions of light and on chlorophyll synthesis, proceeds at least partially in the dark when cytokinins are applied (Kasemir and Mohr, 1982; Chory et al., 1991). Cytokinin also inhibits hypocotyl elongation and acts inde-

pendently and additively with light, in the sense that neither factor can cause further inhibition under conditions in which the other factor is saturating (in cucumber, Cohen et al., 1991; in *Arabidopsis*, Cary et al., 1995; Su and Howell, 1995). Recently, it was shown that cytokinin inhibits the elongation of *Arabidopsis* hypocotyls by increasing ethylene levels (Cary et al., 1995; Su and Howell, 1995). Light acts by a mechanism that does not depend directly on ethylene, as shown also for red-light-grown pea seedlings, in which blue light and ethylene acted independently to inhibit epicotyl growth (Laskowski et al., 1992).

In this study we investigated the relation between red-light-induced randomization of hypocotyl gravitropism and two growth regulators, cytokinin and ethylene. Cytokinin could modulate gravitropism by the same pathway as hypocotyl inhibition (through ethylene) or by another mechanism (as for light). Coupling to ethylene was investigated by increasing ethylene levels through application of the precursor ACC. Furthermore, the ethylene-insensitive mutant *ein2-1* (Guzman and Ecker, 1990) provided a way to test the involvement of ethylene in the action of cytokinin.

## MATERIALS AND METHODS

### Plant Material and Growth Conditions

Seeds of wild-type *Arabidopsis thaliana* (L.) Heynh, ecotype Columbia, and of the mutant *ein2-1* (Guzman and Ecker, 1990; obtained from the *Arabidopsis* Biological Resource Center, The Ohio State University, Columbus) were surface-sterilized for 90 to 120 s in 1% NaOCl with 0.05% (v/v) Tween 20 (Sigma). About 200 seeds were sown per 9-cm plate as described previously (Lifschitz et al., 1990). The seeds were washed thoroughly with sterile, distilled water until no hypochlorite smell was detectable, and they were suspended in 0.15% agar for homogeneous spreading on standard mineral nutrient medium without Suc, to which hormones or effectors were added after autoclaving. The plates were kept at 4°C for 2 d to enhance germination and oriented vertically and incubated for 5 d at 25 to 26°C in a growth chamber or temperature-controlled darkroom. The synthetic cytokinin BA and the ethylene precursor ACC were from Sigma.

### Light Sources and Measurement of Hypocotyl Gravitropism

Continuous light was from two cool-white fluorescent tubes (40  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , Osram, Frankfurt, Germany) and for red light (1.5  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) the light was passed

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through a layer of red and a layer of yellow acrylic plastic (YavinPlast, Haifa, Israel). No detectable blue light was passed by this red filter. At the end of the treatments plates were inserted into a photographic enlarger, and the projected images were analyzed. Growth orientation was measured and expressed in degrees with respect to the vertical ( $90^\circ$ ). If the hypocotyls were curved (Fig. 1), the growth direction was taken as that of the cotyledon end of the hypocotyl. Frequency distributions were generated by grouping the data every  $5^\circ$  (wild type) or  $10^\circ$  (mutant). SDs were calculated from the original (continuous) data sets. The SD of the angles is a good measure of the extent of randomization: higher SD values represent weak gravitropic orientation, whereas lower SD values indicate a stronger gravitropic response.

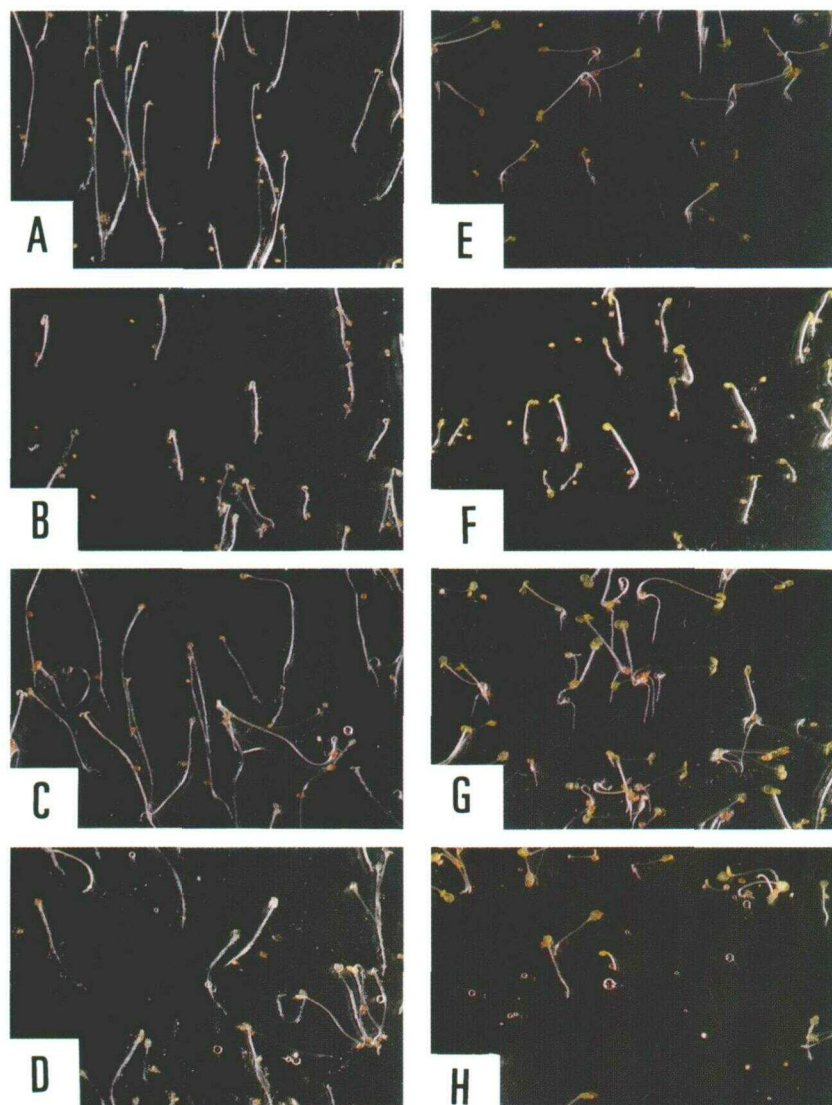
### RESULTS AND DISCUSSION

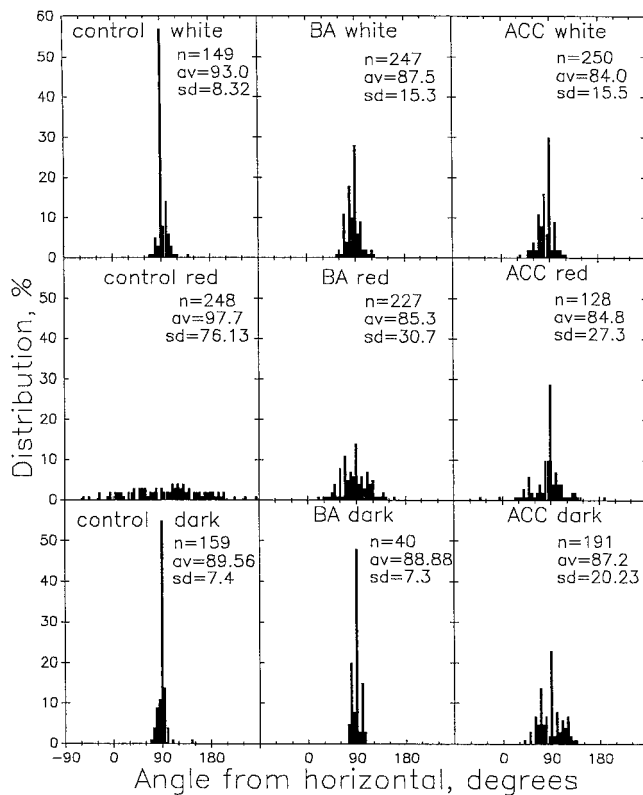
The negative gravitropism of hypocotyls of wild-type *A. thaliana* is evident in dark-grown seedlings (Fig. 1A). In red light, on the other hand, there is a striking randomization

of the angle of growth (Fig. 1E). This randomization effect is mediated by phytochrome (Liscum and Hangarter, 1993). Under white light gravitropism is normal; phototropism or other effects of blue light apparently counteract the disorientation that occurs in red light (Liscum and Hangarter, 1993). Because cytokinins are known to mimic the effect of light, inhibiting hypocotyl elongation (Chory et al., 1991; Cohen et al., 1991; Cary et al., 1995; Su and Howell, 1995), we tested whether the synthetic cytokinin BA would modulate the randomization of gravitropism under red light.

In preliminary dose-response experiments (data not shown; see also Cary et al., 1995) we found that  $4.4 \mu\text{M}$  BA was the minimum concentration that was required to obtain a maximal effect on both hypocotyl growth inhibition and the gravitropic effects described here. Addition of  $4.4 \mu\text{M}$  BA to the medium inhibited hypocotyl elongation in the dark (Fig. 1B) but had no significant effect on gravitropism (Fig. 2). In red light, surprisingly, BA had a strong effect on the seedling orientation, reducing the SD of the

**Figure 1.** Orientation of hypocotyl growth of wild-type and ethylene-insensitive seedlings of *A. thaliana*. Seedlings were grown on vertically oriented plates (see "Materials and Methods") for 6 d in total darkness (A–D) and continuous red light (E–H). A, B, E, and F, Wild type; C, D, G, and H, ethylene-insensitive mutant *ein-2-1*; A, E, C, and G, Without BA; B, F, D, and H,  $4.4 \mu\text{M}$  BA.



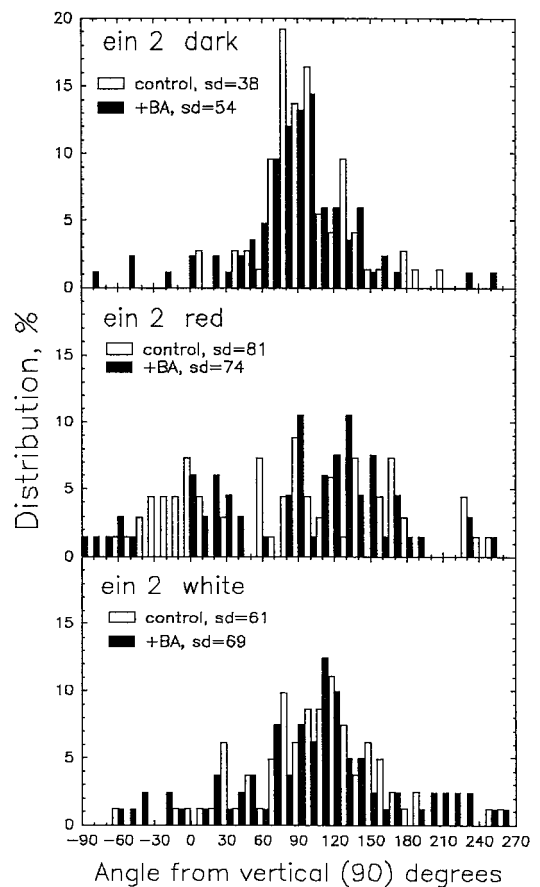


**Figure 2.** Frequency histograms of hypocotyl growth angles of wild-type seedlings. Seedlings were grown as described in Figure 1, in white light, red light, or total darkness, with or without 4.4  $\mu\text{M}$  BA or 1 mM ACC. Frequency distributions of angles from the vertical (90°) are shown for each treatment. A narrow distribution represents a strong negative gravitropic response of the hypocotyl. The number of seedlings, mean (av), and sd are indicated. Each set represents two to three plates; data were consistent and were pooled.

hypocotyl angles from 76 to 30° (Figs. 1F and 2). In other words, BA reduced the randomization that occurs in continuous red light, restoring gravitropism. The restoration of gravitropism by BA is immediately evident upon inspection of a population of red-light-grown seedlings (compare E and F in Fig. 1). In white light the distribution of hypocotyl growth angles is sharply focused, as in the dark (Fig. 2), and BA caused a slight but significant ( $F$  test,  $P = 0.01$ ) randomization.

Cytokinins stimulate ethylene production (Mattoo and White, 1991). Recently, hypocotyl growth inhibition by BA was shown to be ethylene-mediated (Cary et al., 1995). Other effects of cytokinins are not necessarily ethylene-mediated, and in fact cytokinin often acts contrary to ethylene, such as in cotyledon opening and expansion and leaf senescence (Gepstein and Thimann, 1981). Therefore, we used several approaches to test whether the restoration of gravitropism acts also through ethylene. At 1 mM the immediate precursor of ethylene, ACC, acted in a similar manner to BA, restoring gravitropism under red light (Fig. 2). The sd value for ACC was 27°, just slightly less than that for BA, which was 30°. In the dark or under white light, ACC caused a slight but significant randomization (Fig. 2).

If BA indeed acts by increasing ethylene levels, the hormone should be ineffective in an ethylene-insensitive mutant. To develop a genetic test for this hypothesis, we first characterized hypocotyl gravitropism in the ethylene-insensitive mutant *ein2-1*. Mutants at the *EIN2* locus are drastically defective in ethylene responses (Guzman and Ecker, 1990). In the dark, in which wild-type seedlings grow vertically (Fig. 1A), the mutant shows a less oriented growth pattern (Fig. 1C). In total darkness or continuous white light, in which the wild-type distribution is sharply focused around the vertical, the mutant is more random (Fig. 3), and under red light the mutant and wild type are virtually indistinguishable (Fig. 1, E and G). The sds of the distributions of growth angles, 76 and 81°, are similar to each other (no significant difference,  $F$  test,  $P > 0.05$ ). The action of cytokinin is significantly reduced in the mutant (Fig. 1, C, D, G, and H), and inhibition of hypocotyl growth is less (Fig. 1, A and D; Cary et al., 1995). Furthermore, restoration of gravitropic orientation by cytokinin under red light is virtually absent in the mutant (Figs. 1H and 3). Consistent



**Figure 3.** Frequency histograms of hypocotyl growth angles of the ethylene-insensitive mutant *ein2-1*. Seedlings of the mutant were grown as described in Figure 1, in total darkness and continuous white or red light, with or without 4.4  $\mu\text{M}$  BA. sds are given as a measure of the width of the distribution of angles from the vertical. Each set represents at least 60 seedlings from two to three plates; results from different plates were consistent and were pooled.

with the insensitivity to ethylene in *ein2* seedlings, the ethylene precursor ACC could not restore negative gravitropism to *ein2-1* seedlings that were grown under red light ( $sd = 109^\circ$ , 31 seedlings). These results provide genetic evidence that BA acts via ethylene to restore negative gravitropism under red light in the wild type.

Independently of cytokinin, *ein2-1* is clearly defective in negative gravitropism of the hypocotyls (Figs. 1, C and G, and 3). This provides support for the view that a low level of ethylene may be required for the efficient functioning of the gravitropic sensory apparatus (Zobel, 1973; Harrison and Pickard, 1986; Philosoph-Hadas et al., 1996). Root gravitropism is only slightly affected by the *ein2-1* mutation, although a separate ethylene-related signaling pathway, defined by *EIR1*, is involved downstream of *EIN2* (Roman et al., 1995). High levels of ethylene induce the triple response in seedlings, which tends to randomize gravitropism. In some sets of seedlings treated with ACC, the triple response became evident by the end of the growth period, with curving of the upper portion of the hypocotyl. Both ACC and BA produced a small but significant randomization in the dark or white light (Fig. 2); this may be attributed to the ethylene triple response. Under red light triple-response randomization is superimposed on the orienting action of BA (or ACC), explaining why full orientation is never reached (Fig. 2).

Based on the data presented here, we propose that red light acts to randomize hypocotyl gravitropism by decreasing the ethylene level (or responsiveness to ethylene). Continuous white light decreases ethylene levels in oat leaves (Gepstein and Thimann, 1980). Light may decrease ethylene levels by promoting ACC malonylation, and there is evidence from red light/far-red light photoreversibility that phytochrome is the photoreceptor (Jiao et al., 1987). In *Arabidopsis* seedlings, on the other hand, growth in (white) light strongly increased ethylene levels, as compared with dark-grown seedlings (Guzman and Ecker, 1990). The model predicts that continuous red light should decrease ethylene levels (at least in the hypocotyl) in contrast to the effect of white light. Red light may alter ethylene levels, responsiveness to ethylene, or both. Thus, phytochrome and ethylene could interact through metabolic control of ethylene biosynthesis or perhaps through receptor or transducer cross-talk between the phytochrome and the ethylene pathways. In this first model, cytokinin would increase ethylene levels high enough to overcome the decrease that is caused by red light. An alternative model would be that ethylene directly increases the sensitivity of a gravitropic transducer molecule and that red light randomizes gravitropism by a mechanism that is completely independent of ethylene levels. The direct effect of ethylene on gravitropism would overcome the inhibition by red light. In this second model, red light would be acting as a dominant signal, disorienting growth. If the second model is correct, ethylene (or cytokinin) may not be the only factor that can overcome the disorienting effect of red light.

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