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Light-Stimulated Cotyledon Expansion in *Arabidopsis* Seedlings[1]

The Role of Phytochrome B

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Leaf and cotyledon expansion in dicotyledonous plants is a light-dependent developmental process. The unique role of phytochrome B has been tested by investigating expansion of cotyledons in wild-type and phytochrome-deficient mutants of *Arabidopsis thaliana* (L.) Heynh. A relatively rapid method for measuring cotyledon area was developed to quantify growth in large populations (average $n \geq 100$) of wild-type or mutant seedlings under different light and chemical treatments. Three-day-old wild-type (La-*er*) *Arabidopsis* seedlings, grown in saturating, low-fluence red light (2–4 μmol m^{-2} s^{-1}), showed a >250% increase in cotyledon area after 48 h of bright-red light when compared with the phytochrome mutants *hy1*, *hy2*, and *hy3*. An increase in epidermal cell area was observed in wild-type cotyledons but not in *hy3*, indicating that light-stimulated growth is due in part to cell expansion. The mutant phenotype was rescued by feeding the chromophore precursor biliverdin to the chromophore biosynthesis mutants *hy1* and *hy6*. This treatment did not rescue the *hy3* mutant. Since the *hy3* lesion is specific to phytochrome B, we conclude that this pigment is involved in the enhancement of cotyledon cell expansion in bright-red light.

One dramatic developmental effect of light is to stimulate leaf expansion while simultaneously inhibiting stem elongation, and this is especially evident during de-etiolation of dicotyledonous seedlings. In some species this response is readily observed as light-stimulated cotyledon expansion and is one of a suite of developmental processes mediated by the family of red-light photoreceptors, the phytochromes (Mohr, 1987; Devlin et al., 1992). The growth response of cotyledons along with light-inhibited hypocotyl elongation has been used as a marker to characterize mutants in developmental responses to light (Liscum and Hangarter, 1991; Reed et al., 1993).

The cellular mechanism by which light stimulates cotyledon growth has not been analyzed physiologically to determine the role of phytochrome. On the other hand, physiological characteristics of leaf expansion in response to light have been partially characterized in primary leaves of bean (*Phaseolus vulgaris* L.) (reviewed by Dale, 1988). An action spectrum for this response shows that this is a red-light-driven, high-irradiance response; blue and red light stimulate expansion of excised leaf discs and far-red light inhibits this response (Van Volkenburgh et al., 1990). Light-stimulated growth of green leaf tissue is further enhanced by photosynthesis and therefore involves the activity of the photoreceptor Chl; however, separate, additional roles for both phytochrome and a blue-light receptor have been indicated in regulation of leaf cell expansion in beans (Van Volkenburgh et al., 1990; Blum et al., 1992).

A powerful approach for determining the cellular role of phytochromes is to make use of mutants deficient in phytochrome or regulation of its signal transduction pathway. It has recently become clear that there are multiple phytochrome genes (Sharrock and Quail, 1989; Somers et al., 1991; Furuya, 1993) and that these phytochromes perform different functions (McCormac et al., 1993; Nagatani et al., 1993; Parks and Quail, 1993). A genetic approach to understanding photomorphogenic signal transduction has been employed with the mustard *Arabidopsis thaliana*. One method involves screening for mutants failing to respond to a given light treatment. Mutants have been identified with hypocotyls that fail to show growth-inhibition in either bright red (*hy1*, *hy2*, *hy3*, and *hy6*) (Koornneef et al., 1980; Chory et al., 1989a), far-red (*hy8*, *fre1*, and *fhy2*) (Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993), or blue light (*hy4*, *blu1*, *blu2*, and *blu3*) (Koornneef et al., 1980; Liscum and Hangarter, 1991). This method has also been used to identify plants with altered kinetics of blue-light-mediated phototropism (Khurana and Poff, 1989). A second method involves identification of mutants that no longer need a light cue to elicit a developmental response. This approach led to the identification of *det1* and *det2*; (Chory et al., 1989b, 1991; Chory and Peto, 1990) and *cop1*, *cop2*, *cop3*, *cop4*, and *cop9* (Deng et al., 1991; Wei et al., 1992; Hou et al., 1993).

The red-light mutants *hy1*, *hy2*, and *hy6* have been shown to have severely depressed levels of all phytochromes (Chory et al., 1989a; Parks et al., 1989; Parks and Quail, 1991). The *hy3* lesion affects only levels of phytochrome B (Nagatani et al., 1991; Somers et al., 1991; Reed et al., 1993). Because phytochrome B in particular is considered to be active in de-etiolated tissues, and because cell expansion in leaves and cotyledons is a phytochrome-mediated growth response occurring in such tissues, this study was designed to test the

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involvement of phytochrome B in light-stimulated cotyledon expansion by comparing these mutants in a quantitative analysis of light-stimulated cell expansion.

MATERIALS AND METHODS

Plant Material

All wild-type and mutant *Arabidopsis thaliana* (L.) Heynh. seeds were obtained from the Arabidopsis Stock Center. The Landsberg *erecta* ecotype was used for the *hy1*, *hy2*, and *hy3* experiments (and corresponding wild-type control). The Columbia ecotype was used for experiments with *hy6*. Seeds were sterilized for 5 min with 70% ethanol, for 10 min with 50% bleach, 0.05% (v/v) Tween 80, and then rinsed four times with sterile, distilled water. Seeds were sown onto standard growth media plates (Valvekens et al., 1988) without Suc (0.8% phytagar). For biliverdin rescue experiments, plates were prepared according to Parks et al. (1991) (0.25 mM biliverdin) with 0.8% phytagar. A 48-h cold treatment at 4°C preceded all light-condition protocols.

Light Sources

Dim-red light (2–4 μmol m^{-2} s^{-1}) was supplied by two 40-W, cool-white fluorescent light bulbs wrapped with red plastic (fluence rates at 660:730 nm = 1:1) in a 25°C growth chamber. Bright-red light (50–55 μmol m^{-2} s^{-1}) was supplied by a 1000-W GE quartz halogen lamp filtered though red plexiglass (660:730 nm = 1:1). Bright-light box temperature was maintained at 25°C by containing the entire setup in a 10°C cold room and pulling cool air through the box with a standard computer fan regulated by a 120-V rheostat. Bright-blue light (50–55 μmol m^{-2} s^{-1}) was supplied by the same light and cooling source as bright-red light, except that the filter was Rhomm and Hass No. 2424. Fluence rates were measured with a quantum meter (Li-Cor, Inc., Lincoln, NE).

Cotyledon Area, Length, and Width Determination

After the appropriate light treatment, all plates were placed in the dark at 4°C until they could be measured. No significant growth occurred in controls after 4 d (data not shown). Cotyledon areas, lengths, and widths were measured by flattening seedlings with a coverslip and tracing the outline of cotyledons using a camera lucida. Cotyledon areas, lengths, and widths were calculated by comparing them to a 1-mm^2 standard with a digitizing tablet (Kurta, Phoenix, AZ) using SigmaScan software. Excised cotyledons were removed in dim-red light with a razor blade under a dissecting scope and placed flat on fresh plates of the same growth agar. Area was measured and calculated the same as for intact plants. Error bars represent ±1 SE. All graphs represent data from three independent experiments. Each replicate was performed on a different day.

Epidermal Cell Area Determination

Seeds were planted on growth media plates (Valvekens et al., 1988) without Suc (0.8% phytagar). After the appropriate light treatment, all plates were placed in the dark at 4°C until they could be measured. Patches of epidermal cells on the upper side of cotyledons were measured on lightly vacuum-infiltrated (with 10 mM KCl) excised cotyledons temporarily mounted on standard microscope slides. Patches of epidermal cells were traced with a camera lucida and the number of epidermal cells per patch was determined (minimum $n = 50$; average $n = 125.9$). Epidermal patch area was calculated by comparing to a 1-mm^2 standard using a digitizing tablet (Kurta) with SigmaScan software. Error bars represent ±1 SE. All graphs represent data from three independent experiments. Each replicate was performed on a different day.
day.

RESULTS

Cotyledons of Mutants with Altered Phytochrome Pools Fail to Expand in Response to Bright-Red Light

We tested the role of phytochromes in light-stimulated cotyledon expansion by comparing the phytochrome mutants *hy1*, *hy2*, and *hy3* with the wild type after 48 h in continuous dim-red, bright-red, or bright-blue light. To saturate any possible low-fluence component of cotyledon expansion, these plants were germinated and grown for 3 d in dim-red light (2–4 μmol m^{-2} s^{-1}). Figure 1 shows that the cotyledons of wild-type plants expanded >250%, whereas cotyledons of the mutants expanded only slightly after a 48-h exposure to high-fluence red light. All three mutants expanded cotyledons in high-fluence blue light (Fig. 1), although not as much as the wild type. The mutant *hy6* (ecotype CO-0) showed a cotyledon response similar to *hy1*, *hy2*, and *hy3* (data not shown). Since the *hy3* mutation resides in the phytochrome B gene (Reed et al., 1993), these data demonstrate that phytochrome B normally participates in these growth responses.

The growth response of cotyledons to light was reflected by changes in epidermal cell area. The average epidermal cell area was calculated for wild-type and *hy3* cotyledons after

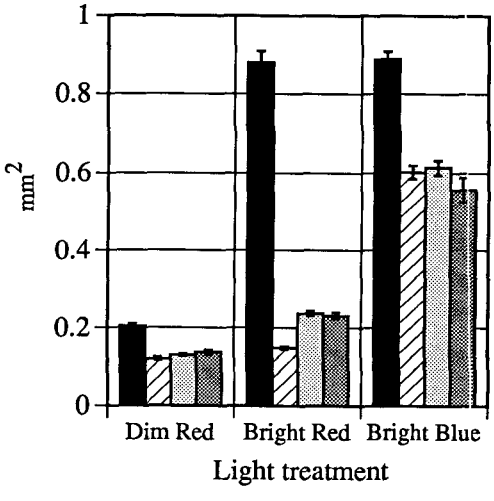

Figure 1. Cotyledon area of intact wild-type (La-*er*) and mutant *Arabidopsis* seedlings after a 48-h light treatment. ■, Wild type (La-*er*); ▨, *hy1*; □, *hy2*; ■, *hy3*. Average number of cotyledons per experiment = 99.6; minimum $n = 58$.

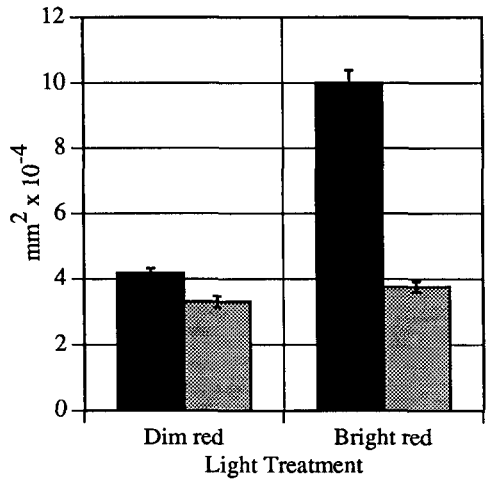

Figure 2. Average epidermal cell area of wild-type (La-*er*) and mutant *Arabidopsis* seedlings after a 48-h light treatment. ■, Wild type (La-*er*); ■, *hy3*. Average number of epidermal patches per experiment = 21.25; minimum n = 16.

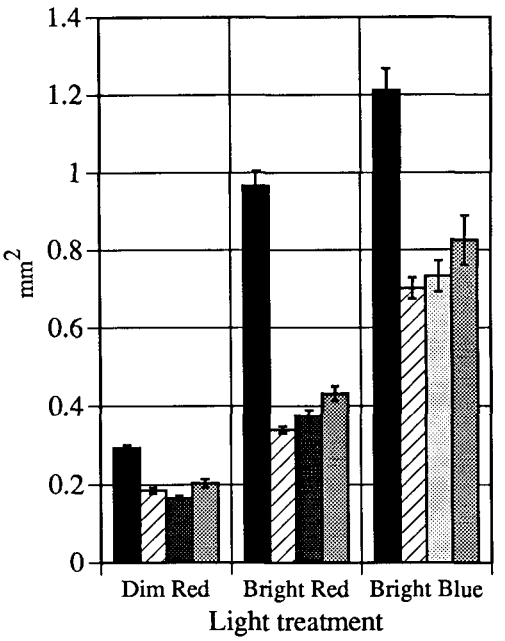

Figure 3. Excised cotyledon area of wild-type (La-*er*) and mutant *Arabidopsis* after a 48-h light treatment. ■, Wild type (La-*er*); ▨, *hy1*; □, *hy2*; ■, *hy3*. Average number of cotyledons per experiment = 67.7; minimum n = 28.

48 h in dim-red or bright-red light (Fig. 2). The epidermal cells of wild-type cotyledons in bright-red light increased about 150% in surface area when compared with the dim-red control. This stimulation in cell size did not occur for the *hy3* mutant. This result implies that one of the roles of phytochrome B is to regulate both light-stimulated cell expansion and division in cotyledons.

To address the directionality of growth in these cotyledons, lengths and widths were compared between wild type and *hy3* in dim-red and bright-red light (Table I). It is clear that the bright-red light-stimulated growth in the presence of functional phytochrome B occurs in both directions. However, the lower length-to-width ratios for wild type compared with *hy3* in both light conditions suggest that phytochrome B effects lateral growth more than longitudinal growth of expanding cotyledons.

The Mutant Phenotype Is Present in Excised Cotyledons

To test the tissue-specific role of phytochrome in these mutants, cotyledons were excised from plants grown for 3 d in dim-red light and placed directly on the agar growth medium. Figure 3 shows that after 48 h, excised cotyledons of the phytochrome mutants *hy1*, *hy2*, and *hy3* maintain their phenotype by failing to expand as much as the wild type in response to bright-red light. Excised cotyledons show greater growth compared with cotyledons on intact plants (Fig. 1). This growth is probably due to greater availability of water as a result of removal of the hypocotyl and placement of cotyledons directly on the water source.

These results are in sharp contrast to the cotyledon phenotype of the blue light mutants *hy4* and *blu3-1*. Cotyledons of these mutants lose their diminished growth response to blue light when they are removed from the rest of the seedling prior to exposure (D.E. Blum, M. Neff, and E. Van Volkenburgh, unpublished results).

Table I. *Directionality of cotyledon growth*

Mean length and width measurements are of wild-type and *hy3* cotyledons from Figure 1 in dim-red and bright-red light. All measurements are in mm. Minimum n = 71; average n = 102.8. SE is included in parentheses.

	Dim-Red	Bright-Red
Wild-type length	0.592 (0.009)	1.082 (0.016)
Wild-type width (w)	0.467 (0.008)	1.032 (0.019)
hy3 length (l)	0.562 (0.012)	0.665 (0.011)
hy3 width	0.358 (0.008)	0.467 (0.01)
Wild-type l:w	1.288 (0.016)	1.061 (0.009)
hy3 l:w	1.595 (0.03)	1.445 (0.021)

Biliverdin Will Restore the Cotyledon Expansion Phenotype in *hy1* and *hy6*

The long-hypocotyl phenotype of *hy1* can be rescued by feeding the plants the chromophore precursor biliverdin (Parks and Quail, 1991). This same treatment did not affect the long-hypocotyl phenotype of *hy3*. Figure 4 shows that the cotyledon phenotype of *hy1* in bright-red light was rescued by providing biliverdin to intact plants through the agar. This chemical treatment did not rescue the mutant response of *hy3*, supporting the hypothesis that phytochrome B mediates this response. The putative chromophore biosynthesis mutant *hy2* showed only partial rescue to biliverdin (data not shown), which is consistent with the results of previous experiments (Parks and Quail, 1991).

The phytochrome mutant *hy6* is also rescued by biliverdin (data not shown). This mutant was isolated from ecotype Co-0 (Chory et al., 1989a) instead of from the La-*er* background

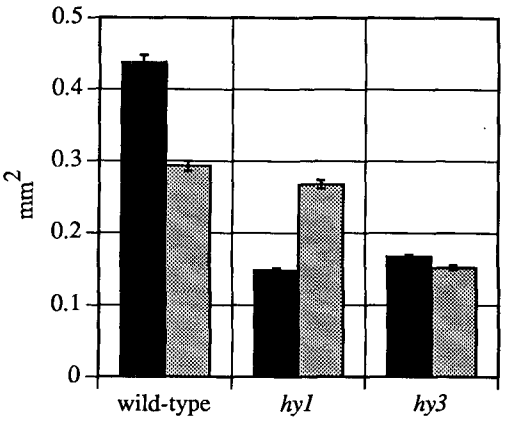

Figure 4. Cotyledon area of intact wild-type (La-*er*) and mutant *Arabidopsis* seedlings in bright-red light. ■, Without biliverdin; ■, with biliverdin. Average number of cotyledons per experiment = 150.2; minimum $n = 122$.

of *hy1*, *hy2*, and *hy3*. Although the general trend is the same as in *hy1* (Fig. 4), *hy6* grows better than its wild-type control under all light conditions when it is provided biliverdin. Furthermore, wild-type (Co-0) cotyledons show no significant depression of growth in the presence of biliverdin (data not shown).

In all of the biliverdin-rescue experiments, cotyledons showed less overall growth than in experiments with standard growth media (Valvekens et al., 1988). This is most likely due to the difference in pH between the two experimental conditions. To keep biliverdin soluble, the agar must be buffered to pH 7.4. Control plates without biliverdin were also buffered at pH 7.4 in these experiments. As we have observed, this pH is not ideal for seedling growth, since it results in 58% shorter roots and hypocotyls when compared with standard growth media (pH 5.7).

DISCUSSION

In cotyledons and leaves of dicotyledons, light induces cells to expand and divide. Cell growth in these tissues can be stimulated by both red and blue light, presumably acting via separate photoreceptor pathways (Blum et al., 1992). The growth response of cotyledons with genetically lower levels of phytochromes was severely reduced compared with the wild type in bright-red light, indicating a requirement for phytochromes in cotyledon expansion under these conditions. Our growth protocol was designed to couple the expansion of cotyledons with the expansion of individual cells in these tissues (Fig. 2), allowing us to address how bright-red light via phytochrome induces individual cells to grow. These cotyledons maintain their growth response to light when they are removed from the rest of the seedling (Fig. 3), demonstrating that the photoreceptor pathway resides within the growing cotyledon cells, and that the phenotype is not a secondary response to growth inhibition at the hypocotyl. This phenomenon is essential if future studies involving isolated cotyledon cells are to be carried out.

All of the mutants examined in this study had altered activity of phytochrome B. There are two classes of *Arabidopsis* mutants with altered phytochrome activity. Three mutants (*hy1*, *hy2*, and *hy6*) appear to affect chromophore biosynthesis; they lack spectrophotometrically detectable phytochrome, yet monoclonal, western analysis shows near wild-type levels of the phytochrome A apoprotein in these plants (Chory et al., 1989a; Parks et al., 1989; Parks and Quail, 1991). Presumably they have depressed levels of all phytochromes. These mutants are clearly leaky since they have been shown to exhibit some phytochrome control of seed germination (Cone and Kendrick, 1985), greening (Lifschitz et al., 1990), and end-of-day far-red responses (Goto et al., 1991). The other class of mutants affects specific phytochrome genes either structurally or in their regulation. Three groups of mutants are in this class—*hy8* (Parks and Quail, 1993), *fre1* (Nagatani et al., 1993), and *fhy2* (Whitelam et al., 1993), which probably affect the PHYA gene product and the phytochrome B mutant *hy3* (Koornneef et al., 1980). The latter mutant has wild-type levels of spectrophotometrically detectable phytochrome, yet western analysis failed to detect the phytochrome B apoprotein (Nagatani et al., 1991; Somers et al., 1991). Sequence analysis of multiple alleles of *hy3* have clearly demonstrated that this mutation resides in the locus that encodes the phytochrome B apoprotein (Reed et al., 1993).

Cotyledons of *hy1*, *hy2*, and *hy3* fail to expand significantly after 48 h of growth in bright-red light (Fig. 1). That they are still competent to grow in response to bright light is evident from their expansion in bright-blue light. The possibility that some interaction occurs between responses to red and blue light is suggested by the incomplete growth response of the phytochrome-deficient mutants in bright-blue light compared with the wild type (Fig. 1). The chromophore biosynthesis mutants *hy1* and *hy6* can be rescued by feeding them biliverdin (Fig. 4, data not shown). As a control, the biliverdin treatment will not rescue *hy3* (Fig. 4). It is clear that under our growth conditions the presence of phytochrome B is required for expansion of cotyledons in response to bright-red light.

Previous studies of the *hy3* mutant have concentrated on the failure of inhibition of cell elongation in the hypocotyl in bright light (Koornneef et al., 1980; Parks and Quail, 1993; Reed et al., 1993), as well as seed germination (Cone and Kendrick, 1985), greening (Lifschitz et al., 1990), shade avoidance (Whitelam and Smith, 1991), and end-of-day far-red responses (Goto et al., 1991). Recent studies have also shown that elongation of many tissue types is affected throughout the development of this mutant (Reed et al., 1993). Photographs of cotyledons on intact *hy3* seedlings have been published that suggest that phytochrome B is involved in light-driven cotyledon expansion (Parks and Quail, 1993). A quantitative study of cotyledon expansion utilizing the *ein* mutant of *Brassica rapa* (Devlin et al., 1992) shows a red-light phenotype similar to *hy3*. However, it has not been established that this allele is definitely at the phytochrome B locus. Furthermore, *B. rapa* lacks chromophore biosynthesis mutants that can be rescued by feeding biliverdin. Cotyledons of the *lh* mutant of cucumber fail to expand in red light, and this may be due to reduced levels of phytochrome B (Adamse et al., 1987).

Data quantitating changes in the shape of *Arabidopsis* true leaves (Reed et al., 1993) implicate phytochrome B as participating in the control of leaf elongation. The overexpression of phytochrome B in *Arabidopsis* seedlings conferred a greater sensitivity to red light than the wild type. This treatment also resulted in wider petioles (McCormac et al., 1993). The results presented here indicate the possibility that leaf shape in *hy3* mutants is elongated because growth of veinal tissue dominates due to the inhibition of interveinal cell expansion. For wild-type leaves, phytochrome B may promote nonveinal cell growth as it does cotyledon cell expansion, thus producing wider and therefore rounder leaves.

Cell expansion in plants is accomplished by coordinately regulated loosening of the cell wall and turgor maintenance (Cosgrove, 1986; Dale, 1988). Both of these cellular processes are correlated with the activity of the plasma membrane-bound proton ATPase, acidification of the cell wall, and solute accumulation. Phytochrome has been implicated as a regulator of ion fluxes across the plasma membrane of etiolated leaf cells (Roux, 1986). Red light causes protoplasts to swell (Blakely et al., 1983; Bossen et al., 1988; Zhou et al., 1990), to take up external calcium (Bossen et al., 1988; Shacklock et al., 1992), and to phosphorylate soluble proteins (Fallon et al., 1993). These responses are photoreversible by far-red light, and may involve activity of one or more G-proteins (Bossen et al., 1990). Expansion of greened bean leaf cells is stimulated by red light and inhibited by far-red light, and these wavelengths of light have the expected stimulatory and inhibitory effects, respectively, on proton excretion from epidermal cells of growing pea leaves (M. Staal, unpublished results). It is predicted that, as part of its signal transduction activities, phytochrome interacts with the plasma membrane in some manner to enhance ion flux through specific ion channels and the proton pump in growing leaf cells.

Results from this study demonstrate that phytochrome B is necessary for light-stimulated cell expansion in cotyledons of *Arabidopsis*. The interaction of this molecule with candidates for signal transduction intermediates leading to cellular processes required for growth can now be probed genetically and physiologically using the *hy3* and other mutants of *Arabidopsis*.

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