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Rapid Communication

Light-Stimulated Cotyledon Expansion in the *blu3* and *hy4* Mutants of *Arabidopsis thaliana*[1]

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Cotyledon expansion in response to blue light was compared for wild-type *Arabidopsis thaliana* (L.) Heynh. and the mutants *blu3* and *hy4*, which show reduced inhibition of hypocotyl growth in blue light. White, blue, and red light stimulated cotyledon expansion in both intact and excised cotyledons of wild-type seedlings (ecotypes No-0, WS, Co-0, La-*er*). Cotyledons on intact *blu3* and *hy4* seedlings did not grow as well as those on the wild type in response to blue light, but pretreatment of *blu3* seedlings with low fluence rates of red light increased their responsiveness to blue light. Excision of cotyledons alleviated the mutant phenotype so that both mutant and wild-type cotyledons grew equally well in blue light. The loss of the mutant cotyledon phenotype upon excision indicates that the *blu3* and *hy4* lesions affect cotyledon expansion indirectly via a whole-plant response to light. Furthermore, the ability of excised, mutant cotyledons to grow normally in blue light shows that this growth response to blue light is mediated by a photosystem other than the ones impaired by the *blu3* and *hy4* lesions.

Blue light-induced photomorphogenic responses in angiosperms include phototropism, inhibition of hypocotyl and epicotyl elongation, hook unfolding, and cotyledon and leaf expansion. The latter is a complicated process that can be driven through several photoreceptors acting at different sites. For example, in *Phaseolus vulgaris* (L.), full expansion of the primary leaves can be driven by both blue and red light acting photosynthetically and photomorphogenically (Van Volkenburgh and Cleland, 1990; Van Volkenburgh et al., 1990; Blum et al., 1992). Perception of the red light signal is at two sites, the hook and the primary leaves (De Greef et al., 1978).

Cellular pathways mediating responses to specific stimuli can be elucidated by analyzing mutants deficient in these responses. Several *Arabidopsis thaliana* mutants, *blu1*, *blu2*, *blu3* (Liscum and Hangarter, 1991), and *hy4* (Koornneef et al., 1980), have been identified by a screen for altered hypocotyl growth inhibition. These mutants do not show wild-type inhibition of hypocotyl growth in response to blue light, but they display normal response to white and far-red light in the case of the *blu* mutants (Liscum and Hangarter, 1991) and to far-red light in *hy4* (Koornneef et al., 1980). Additionally *blu1*, *blu2*, and *blu3* do not exhibit full cotyledon expansion in blue light when compared to white light (Liscum and Hangarter, 1991). Of the mutants found by Liscum and Hangarter, *blu3* showed the greatest difference between white- and blue-light-induced cotyledon expansion.

To understand the cellular mechanism of blue-light-driven cell growth in cotyledons, their growth response in the mutants *blu3* and *hy4* has been investigated. Realizing that several sites of perception may be involved (Black and Shuttleworth, 1974; De Greef et al., 1978; Oelze-Karow and Mohr, 1988) and that excision sometimes changes responses (Cosgrove, 1981), we also examined whether the mutant phenotype would be expressed when the cotyledons were excised. The effect of a low-fluence pretreatment with red light was also determined.

MATERIALS AND METHODS

Seeds of wild-type *Arabidopsis thaliana* (L.) Heynh., ecotypes Co-O, No-O, and WS-1, were obtained from Dr. R.D. Bradley (University of Washington). Seeds of *blu3-1* were originally obtained from Dr. R. Hangarter (Ohio State University); plants were back-crossed one time and their seeds were used in these experiments. Seeds of La-*er* and *hy4* were obtained from Dr. M. Koornneef (Wageningen Agricultural University, The Netherlands).

In all experiments except those with *hy4* (see Fig. 4), seeds were surface sterilized for 20 min in 30% (v/v) commercial bleach, rinsed several times with distilled water, and stratified for 48 h at 5°C either in water or on agar plates. After stratification in water, seeds were germinated and grown on filter paper moistened with 5 mM KCl in Petri plates. This medium proved less optimal for growth than agar plates containing full-strength Murashige and Skoog salts (Sigma), 2% (w/v) Suc, and 0.8% (w/v) agar (Difco). In experiments with *hy4*, seeds were sterilized for 5 min with 70% ethanol, for 10 min with 50% (v/v) bleach, 0.05% (v/v) Tween 80, and then rinsed four times with sterile, distilled water. Seeds were sown on growth media plates (Valvekens et al., 1988) with 0.8% phytagar and without Suc.

White light was provided by halogen projector lamps passed through a fiber-optic cable (see Figs. 1–3) or a 1000-W GE quartz halogen lamp (see Fig. 4). Bright red light was obtained by passing white light through red plexiglass providing equal fluence rates of red and far-red light. Blue light was obtained either by passing white light through Rohm and Haas blue plexiglass No. 2424 and 3 mm of 1.5% (w/v)

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copper sulfate (470-nm peak) (see Fig. 3) or by passing white light through Rohm and Haas blue plexiglass No. 2424 (472-nm peak) (see Fig. 4). Both blue light sources had a half-bandwidth of 100 nm. Fluence rates were measured with a quantum meter (Li-Cor, Lincoln, NE).

Experiments were performed either at room temperature (20–25°C) with excess heat being removed by passing the light through water (see Figs. 1–3), or in a temperature-controlled box kept at 25°C (see Fig. 4). Temperature differences among treatments never exceeded 1.5°C.

Cotyledon diameters were measured at 20× to a resolution of 0.13 mm. Cotyledon areas were measured from traces of camera lucida projections observed at 40 and 100×. Areas were calculated by comparison to a 1-mm^2 standard with a digitizing tablet (Kurta, Phoenix, AZ) using SigmaScan software. In whole-plant experiments, cotyledons were excised or flattened directly onto the agar with a coverslip at the end of the treatment and measured. In experiments with excised cotyledons, after *blu3* cotyledons were excised they were traced and placed on moistened tissue paper with 5 mm of KCl. Cotyledons were retraced after 24 h and percent increase in area was calculated. The *hy4* cotyledons were excised, placed on agar, and measured at the end of the experiment.

RESULTS AND DISCUSSION

Light-stimulated cotyledon expansion was observed in four ecotypes of *A. thaliana* in red, blue, and white light. This response was inducible by growing seedlings first in low-fluence-rate (4 μmol m^{-2} s^{-1}) red light and then transferring them to higher fluence rates. This protocol was developed for studies of light-stimulated leaf expansion (Van Volkenburgh and Cleland, 1979) and is useful for saturating phytochrome responses to low-fluence red light such as cell division and greening of leaves and cotyledons. Cotyledons on intact, wild-type seedlings de-etiolated but remained small (0.6 mm diameter) when plants were grown in low-fluence red light (Fig. 1). Exposure to high fluence rates (100 μmol m^{-2} s^{-1}) of either red or blue light stimulated cotyledon expansion with similar effectiveness. The response to light decreased with age, as shown by the decreasing initial growth rate of 9- and 13-d-old compared to 5-d-old cotyledons (Fig. 1).

The growth response of cotyledons to bright blue light was also observed in excised cotyledons sampled early in development (d 4–6); their ability to grow in response to the light treatment was mostly lost by d 10 (Fig. 2). These experiments were performed with ecotypes No-O (Fig. 2) and Co-O (data not shown) with similar results, although Co-O lost its ability to respond earlier than No-O.

The fluence rate dependence for expansion of excised cotyledons was determined. Equivalent maximal responses were obtained for 50 and 100 μmol m^{-2} s^{-1} blue light; at 25 μmol m^{-2} s^{-1} growth was half maximal (data not shown). All subsequent experiments comparing *blu3* and Co-0 wild type and *hy4* and La-*er* wild type were conducted at 50 μmol m^{-2} s^{-1}. The response to white light at 100 μmol m^{-2} s^{-1} was slightly higher than the response to blue light. The growth response of excised cotyledons to blue light was partially but not completely inhibited by 10^{-5} M DCMU, an inhibitor of PSII (data not shown), as has been reported previously for leaf expansion in bean (Blum et al., 1992).

Cotyledons of *blu3* did not expand as well as wild-type cotyledons in response to blue light when measured on intact plants grown in continuous light treatments (Liscum and Hangarter, 1991). Replication of those experiments in this laboratory showed that after 5 d in continuous light, cotyledon area for intact, wild-type seedlings in blue light was 90% of the area in white light, whereas *blu3* cotyledons grew 60% in blue light compared to white light (data not shown). When cotyledons were allowed to develop first in low-fluence red light and then were transferred to bright light (as described for Fig. 1), the mutant phenotype was less pronounced (Fig. 3A). Under these conditions, wild-type cotyledons grew 94% in blue light compared to white light, whereas *blu3* cotyledons grew 77% in blue light compared to white light (Fig. 3A).

The mutant phenotype was completely lost when cotyledons were excised. When plants were grown in dim red light for 3 or 4 d, cotyledons excised, and expansion measured after 24 h of light treatment, wild-type and *blu3* cotyledons responded equally well to light, with no significant difference between the blue and white light treatments (Fig. 3B). To determine if this loss of mutant phenotype was due solely to the dim red pretreatment, plants were grown for 4 d in the dark and cotyledons were excised and exposed directly to white and blue light. Again, there was no difference between *blu3* and Co-O with respect to their growth response to blue and white light (data not shown). In these experiments with *blu3*, excised cotyledons (Fig. 3B) grew less than cotyledons on intact seedlings (Fig. 3A); the former were grown on filter paper moistened with 5 mM KCl and the latter were grown on agar. Excision itself did not cause the reduced growth response as shown below for *hy4* (Fig. 4).

The loss of the mutant phenotype in excised cotyledons was also observed in the *hy4* mutants. The expansion of *hy4*

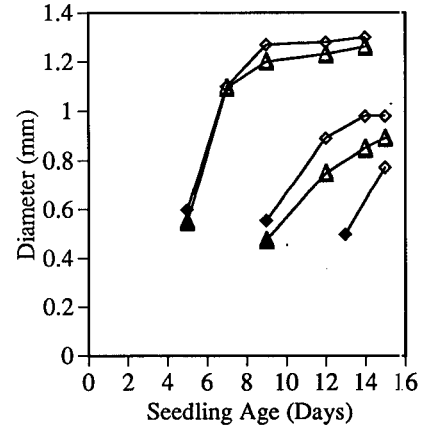

Figure 1. Cotyledon expansion for intact seedlings in response to red or blue light. *A. thaliana* seeds were given a 48-h cold treatment, grown on agar plates in 4 μmol m^{-2} s^{-1} red light for 5, 9, and 13 d (filled symbols), then placed in bright red (◇) or blue light (100 μmol m^{-2} s^{-1}) (△) for various times up to 10 d. Results shown here are for ecotype No-0. Similar results were obtained with ecotype WS.

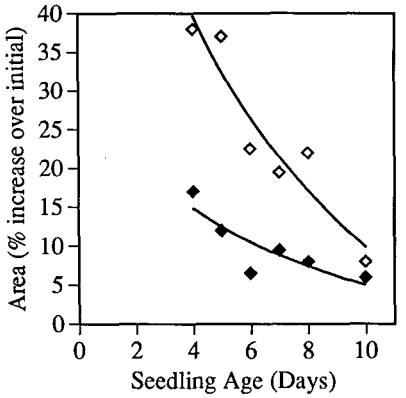

Figure 2. Expansion of excised cotyledons in response to blue light. *A. thaliana* seeds were grown on filter paper moistened with 5 mM KCl in low-fluence red light (4 μmol m^{-2} s^{-1}). On the day indicated, cotyledons were excised, area was determined, and one cotyledon from each pair was placed in 50 μmol m^{-2} s^{-1} blue light (open symbols; $r^2 = 0.89$) or darkness (filled symbols; $r^2 = 0.74$) for 24 h. Final areas were measured and percent increase over initial area was determined. A logarithmic curve was fit to the data. Results shown here are for ecotype No-0. Similar results were obtained with ecotype Co-0.

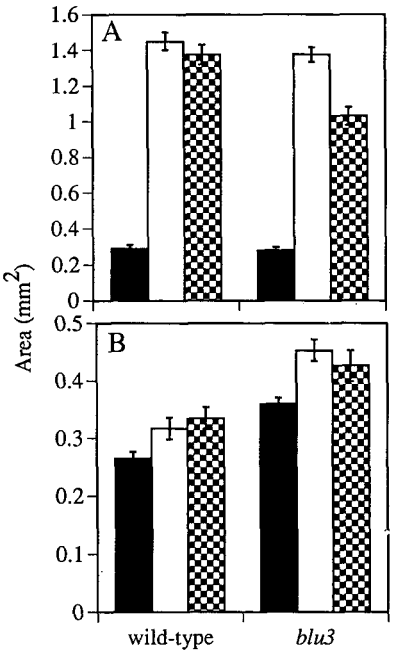

Figure 3. Expansion of intact and excised Co-0 and *blu3* cotyledons. For intact cotyledons (A), seedlings were grown on agar for 4 d in 4 μmol m^{-2} s^{-1} red light and then exposed to 50 μmol m^{-2} s^{-1} white (open bars) or blue (checked bars) light, or dark (filled bars) for 24 h. $n \geq 34$, SE values are indicated. For excised cotyledons (B), seedlings were grown on moistened (5 mM KCl) filter paper for either 3 or 4 d in 4 μmol m^{-2} s^{-1} red light, cotyledons were excised and placed on moistened (5 mM KCl) filter paper in 50 μmol m^{-2} s^{-1} white or blue light, or in the dark for 24 h. $n = 12$, SE values are indicated. Initial cotyledon areas were 0.20 mm^2 for Co-0 and 0.27 mm^2 for *blu3*.

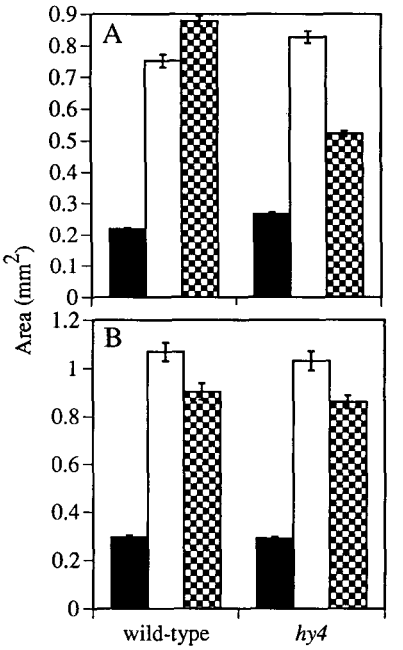

Figure 4. Expansion of intact and excised wild-type (La-*er*) and *hy4* cotyledons. For intact cotyledons (A), seedlings were grown on agar for 3 d in 4 μmol m^{-2} s^{-1} red light, then placed in 50 μmol m^{-2} s^{-1} red (open bars), blue (checked bars), or kept in 4 μmol m^{-2} s^{-1} red (filled bars) light for 48 h. For excised cotyledons (B), seedlings were grown as above except that cotyledons were excised and placed on the same agar in the appropriate light treatment for 48 h. $n \geq 114$, SE values are indicated.

cotyledons in high-fluence red and blue light was compared to cotyledons of the wild type (La-*er*) (Fig. 4). In intact wild-type plants, blue light induced 117% of the expansion observed in high-fluence red light (Fig. 4A). In intact *hy4* plants, bright blue light induced 63% of the red light response, demonstrating a deficient cotyledon expansion in response to blue light (Fig. 4A). Excised cotyledons of both the wild type and *hy4* gave equal response to blue light, 84% of the red-light-induced expansion (Fig. 4B). In these experiments, excised cotyledons grew as well as intact cotyledons.

The ability of excised, mutant cotyledons to grow in response to blue light demonstrates that a photosystem distinct from those associated with HY4 and BLU3 exists in cotyledons and can mediate light-stimulated cotyledon expansion. This is supported by the recent report that the *hy4* mutation is in the sequence coding for the blue light receptor itself (Ahmad and Cashmore, 1993). Despite the presumed loss of the HY4 photoreceptor, excised cotyledons of *hy4* were able to grow normally in response to blue light. It is possible that this response is photosynthetic, although the response in wild type was not eliminated by the PSII inhibitor DCMU. It has been proposed that a nonphotosynthetic mechanism drives blue-light-stimulated leaf expansion in bean (Van Volkenburgh and Cleland, 1990).

A simple explanation for the results is that separate photosystems influence growth of hypocotyl and cotyledon, and that the interaction of hypocotyl with cotyledon shown in

both the *blu3* and *hy4* mutants is a consequence of substrate limitation. The failure of *blu3* and *hy4* cotyledons to expand fully in blue light when on intact seedlings may have been caused by competition for substrates between the growing organs. Continued hypocotyl elongation in the light would retain sink strength in this organ and reduce substrate availability to the cotyledons. However, supplying 2% Suc to the growth medium for *blu3* seedlings did not alleviate the mutant phenotype (Fig. 3A). Another possibility is that water transport to the cotyledons was limited by elongating hypocotyls. Placement of excised cotyledons directly onto a source of water eliminated the difference between wild type and mutants both in water availability and the growth response to blue light.

On the other hand, the observation that pretreatment with dim red light increased the sensitivity of the blue light photosystem in *blu3* seedlings suggests interaction of two or more photosystems. Integrated responses of disjunct photosystems have been described previously for seedlings of several species. For dark-grown *Phaseolus vulgaris*, maximum expansion of primary leaves in white light is attained only when light is perceived at the hook (De Greef et al., 1978). White light shown directly on the leaves resulted in greening but little expansion; additional light shined on the hook region resulted in full expansion of the primary leaves. The red-light-induced growth stimulation of etiolated oat coleoptiles depended on two sites of perception (Mandoli and Briggs, 1982). A light signal perceived at the hook of *Sinapis* seedlings may be translated to the cotyledons, where a "potential capacity to phosphorylate" is detected only if the hypocotyl hook is connected to the cotyledons; the cotyledons themselves are unable to respond to the light (Oelze-Karow and Mohr, 1988). The detection of a light signal may also be communicated from cotyledon to hypocotyl. Perception of red light in the cotyledon inhibits hypocotyl growth of *Cucumis sativus* (Black and Shuttleworth, 1974). Although we have shown that mutant cotyledons can respond normally to blue light when excised, we cannot rule out the possibility that more than one photosystem interact to regulate growth of intact seedlings.

The finding that the mutant phenotype is lost in excised cotyledons of *blu3* and *hy4* demonstrates that these lesions, which result in less light-stimulated cotyledon expansion in intact plants, are not organ autonomous. These results are in contrast to results obtained with the phytochrome mutants of *A. thaliana*, *hy1*, *hy2*, *hy3*, and *hy6*, which retain their phenotype in excised cotyledons (Neff and Van Volkenburgh, 1994). Even in excised material the light-induced expansion of leaves and cotyledons is a complex process driven by different light qualities and fluence rates, working through both photosynthetic and photomorphogenic pigments (Dale, 1988; Van Volkenburgh and Cleland, 1990; Van Volkenburgh et al., 1990; Blum et al., 1992). In whole plants, explanation of phenotypes with cellular models is hindered by the addition of several sites of photoperception and the complication of source/sink relations.

NOTE ADDED IN PROOF

Cotyledon growth assays of *blu3* using the same methods used in experiments with *hy4* (Fig. 4), but comparing blue and white light, corroborated the findings in Figure 3 and show that the mutant phenotype of *blu3* is not observed in excised cotyledons after 48 h.

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