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Blue-Light-Induced Shrinking of Protoplasts from Maize Coleoptiles and Its Relationship to Coleoptile Growth[1]

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Protoplasts isolated from red-light-grown maize (*Zea mays* L.) coleoptiles shrank transiently upon brief exposure (e.g. 30 s) to blue light under background irradiation with red light. The maximal volume reduction (about 4% at a saturating fluence) occurred about 5 min after blue-light stimulation. The response was prevented by the anion-channel blocker 5-nitro-2-(3-phenylpropylamino)-benzoic acid. Red light and far-red light did not induce any comparable response. Protoplasts of different sizes and those isolated from different coleoptile positions showed similar responses. After treatment with a saturating blue-light pulse, the protoplasts became responsive to a second pulse and gained full responsiveness within 5 min, suggesting that the photoreceptor system involves a dark-reversible component. The response to continuous blue light was also found to be transient. The protoplast volume was reduced during about 6 to 9 min of irradiation and returned within the next 30 min to the control level. The response to continuous blue light was saturated at 30 μmol m^{-2} s^{-1}. However, when the fluence rate was enhanced 10-fold after a period of irradiation at 30 μmol m^{-2} s^{-1}, the protoplasts showed another shrinking response. These and other kinetic results indicate that the photoreceptor system undergoes a photosensory adaptation. Growth in different zones of the coleoptile was inhibited by blue light transiently after pulse stimulation, as well as during continuous stimulation. It was concluded that the observed protoplast shrinking is related to the blue-light-induced inhibition of coleoptile growth.

Plants control their growth and development by responding to their light environment. A group of light responses have been identified that are uniquely sensitive to blue light (and usually also to near-UV light). Phototropism (Iino, 1990) and blue-light-induced growth inhibition (Cosgrove, 1994) are the best-studied examples. Physiological and genetic evidence have indicated that these two types of blue-light responses are mediated by distinct photoreceptor systems (Iino, 1990; Liscum et al., 1992; Cosgrove, 1994; Liscum and Briggs, 1995). Therefore, the photoreceptor and the phototransduction mechanism for a blue-light response must be considered separately in phototropism and blue-light-dependent growth inhibition. Although the chemical nature of any of the blue-light-sensitive photoreceptors long remained unclear, Ahmad and Cashmore (1993) were able to clone a gene for the receptor (or at least one of the receptors) responsible for blue-light-induced growth inhibition of Arabidopsis hypocotyls.

Some promising results have been reported recently concerning reaction elements involved in the transduction of the blue-light signal. In phototropism, phosphorylation of a 120-kD protein was shown to participate close to the photoreception (for review, see Short and Briggs [1994]). The activation of anion channels was shown to be involved in blue-light-dependent growth inhibition (Cho and Spalding, 1996).

The effects of light on protoplasts have been studied extensively. The observed responses include blue-light-induced swelling of guard cell protoplasts (Zeiger and Hepler, 1977) and red-light-induced or phytochrome-mediated swelling of protoplasts isolated from etiolated wheat leaves (Blakeley et al., 1983), oat leaves (Chung et al., 1988), and mung bean hypocotyls (Long et al., 1995). Such responses, induced by direct treatment of protoplasts with light, are useful in analyzing the physiological and kinetic properties of light responses at the cellular level. The anion channel activation shown to be induced by blue light in Arabidopsis hypocotyls in connection with the membrane depolarization response (Spalding and Cosgrove, 1988; Cho and Spalding, 1996) suggests that protoplasts shrink when responding to blue light. This has not yet been demonstrated. In fact, shrinking of protoplasts has not yet been shown to follow any light treatment.

In the present study we investigated the effect of blue light on protoplast volume using protoplasts isolated from maize (*Zea mays* L.) coleoptiles. It was found that these protoplasts indeed shrink in response to blue light. Following this finding, we extended the study to characterize kinetic features of the protoplast response in detail and to resolve the causal relationship of this response to blue-light-induced growth inhibition.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seedlings of maize (*Zea mays* L. cv Royaldent Hit 85; Takii, Kyoto, Japan) were grown in a light-tight room kept at 25°C. The room was equipped with overhead red-light sources, which consisted of red fluorescent tubes (FL20SP, Mitsubishi Electric, Tokyo) and a filter combination of red plate acrylic

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Abbreviation: NPPB, 5-nitro-2-(3-phenylpropylamino)-benzoic acid.

(one layer of no. 102, 3 mm thick; Mitsubishi Rayon, Tokyo) and orange plastic (two layers of Ryutate no. 31; RDS, Tokyo). Plants received red light (2.5–3 μmol m^{-2} s^{-1}) from these sources continuously from the time of sowing. Preparation of protoplasts and growth experiments were carried out under the same temperature and light conditions.

Maize caryopses were rinsed in running tap water for 5 h and cultured on wet paper towels (Kimtowel, Jujo Kimberly, Tokyo) in plastic trays covered with clear polyvinylidene chloride film. To isolate protoplasts, coleoptiles that reached 18 to 22 mm in length were selected 3 d after sowing, and a defined 2-mm zone (e.g. the apical 2 mm) was excised from each coleoptile with a razor blade. For each experiment, zone segments were obtained from about 50 plants over a period of about 15 min and subjected immediately to protoplast preparation.

To investigate the growth of coleoptiles, germinated caryopses prepared as described above were transplanted 2 d after sowing into a pot filled with 1% agar (Iino, 1995). The transplanted seedlings were placed in boxes made of red plate acrylic (one layer, see above). After an additional day of incubation, seedlings selected for uniform, straight coleoptiles (20–22 mm) were used for growth experiments.

Protoplast Preparation

The excised coleoptile segments were sliced with a razor blade and placed into 5 mL of an enzyme solution containing 2% (w/v) cellulase RS (Yakult, Tokyo), 0.2% (w/v) pectolyase Y-23 (Seishin Pharmaceutical, Tokyo), 0.5 M sorbitol, 10 mM KCl, 1 mM $CaCl_2$, 20 mM Glc, and 5 mM Mes/Tris (pH 6.0). Following vacuum infiltration (55 mm Hg for 15 min), the tissues were incubated for 2 h on a rotary shaker (60 rpm). The mixture was next filtered through a nylon mesh to remove tissue debris and centrifuged at 50*g* for 10 min. The pellet was suspended in 2 mL of an incubation medium containing 0.5 M sorbitol, 10 mM KCl, 1 mM $CaCl_2$, 20 mM Glc, and 5 mM Mes/Tris (pH 6.0). It was loaded in a glass tube (diameter: 10 mm) on 2 to 3 mL of a 20% Percoll (Sigma) solution containing other components identical to the incubation medium and centrifuged at 110*g* for 5 min. The protoplasts located at the interface between the Percoll solution and the loaded incubation medium were collected, suspended in 5 mL of the incubation medium, and centrifuged (10 min at 80*g*). The sedimented protoplasts were suspended in a small amount of the incubation medium (about 0.5 mL) to obtain the final preparation (1×10^5 to 5×10^5 protoplasts mL^{-1}).

The osmolality of the incubation medium determined by a vapor pressure osmometer (model 5500, Wescor, Logan, UT) was 569 mΩ. Protoplasts showed more than 95% viability when assayed by the method of Widholm (1972) at 0.02% (w/v) fluorescein diacetate.

Treatment of Protoplasts with Blue Light and Measurement of Protoplast Volume

A microscope system placed in a temperature-controlled dark room was used to treat protoplasts with blue light and to monitor the diameter of individual protoplasts. This system consisted of an inverted microscope (IMT-2, Olympus) equipped with a camera (SC 35, Olympus) and two blue-light sources. The sample stage of the microscope was covered with a sample box ($35 \times 24 \times 21$ cm^3) made of red plate acrylic (one layer). A red interference filter (IF-BPF-640, Vacuum Optics, Tokyo) was placed in the light pass of the microscope light source, and the observation light of the microscope was passed through this interference filter and a red acrylic layer of the sample box. The two blue-light sources, each of which consisted of a projector (Kodak Ektagraphic III with EXR 300-W lamp) and a blue glass filter (no. 5–50, Corning, Corning, NY), were placed on either side of the sample box, with the heads of the lenses inserted into the box through holes. The angle of the light pass from the horizon was 35°. Unless otherwise specified, treatment with blue light was carried out using only one light source. When two sources were used, each source provided one-half of the total fluence rate.

A 200-μL portion of the freshly prepared protoplast suspension was added to a quartz cuvette (five sides clear, base area 10×10 mm, height 45 mm). The cuvette was covered with a glass coverslip and placed on the sample stage of the microscope system. The protoplasts were incubated under continuous red light (50 μmol m^{-2} s^{-1}) provided by the microscope light source. This incubation was always initiated 2.5 to 3 h after the start of protoplast preparation. Treatment with blue light was initiated 60 to 65 min after the start of incubation. For a scheduled period, which included a period before blue-light treatment, photographs of the protoplasts in a selected field were taken at 1- or 3-min intervals using technical pan film (Kodak). A scale (in 10-μm intervals) was photographed at the end for calibration purposes. During the entire incubation period the temperature on the sample stage was maintained at 25 ± 1°C. The temperature was monitored with a digital multimeter (TR 6846 with TR1101–130 sensor, Advantest, Tokyo).

In some control experiments protoplasts were treated with red or far-red light (in addition to the background red light from the microscope light source), which was obtained by replacing the blue glass filter with a red interference filter (see above) or a far-red filter (Delaglass, 2 mm thick, Asahi Chemical Industry, Tokyo).

The negative protoplast images recorded on film were magnified by about 1500 times their actual size by means of a slide projector. From the first photograph of a time series, protoplasts (usually 10–12) were selected for size, roundness, and clarity of the margin. The selection was otherwise random. Diameters of the same protoplasts in a series of photographs were recorded on paper and determined using a digitizer (KD4030, Graphtec, Tokyo) interfaced with a computer. The volume of each protoplast was calculated from its diameter (d) using the formula, $(4/3)\pi(d/2)^3$.

Treatment of Coleoptiles with Blue Light and Measurement of Coleoptile Growth

The light-treatment box used to treat the coleoptile with blue light and to monitor its growth consisted of a sample compartment equipped with a camera (MF-21 with AF Micro Nikkor 60-mm lens, Nikon) and two air-circulated

light-source compartments located on either side of the sample compartment. The sample compartment had an open top window through which red light was provided. Each light-source compartment accommodated a projector (see above), and the light from the projector was introduced to the sample compartment through a blue glass filter (see above). In some control experiments the far-red filter was used.

For growth measurements the coleoptile of a selected seedling was marked with India ink at 5-mm intervals from the top (Iino, 1996). The seedling was placed in a box (6.5 × 13.5 cm, height 15 cm) made of clear acrylic (no. 001, 2 mm thick, Mitsubishi Rayon) and set in the sample compartment of the light-treatment box. One narrow side of the coleoptile faced the camera, and the broad sides faced the light sources. Treatment with blue light was initiated 1.5 to 2 h after marking. The blue light was given from both sides simultaneously with the same fluence rate. For a scheduled period, which included a period before blue-light treatment, photographs of the coleoptile were taken at 1-min intervals using technical pan film. A ruler (with 1-mm intervals) was photographed at the end for calibration purposes. The length of a zone between two marks was determined from its negative image on film after magnification by about 60 times the actual length with a slide projector. The digitizer was used to measure the length.

Other Details of Light Treatment

Photon fluence rates were controlled with calibrated neutral-density filters (Vacuum Optics). The fluence rates of blue and red light were measured with a quantum photometer (LI-189, Li-Cor, Lincoln, NE). The fluence rate of far-red light was measured with a spectroradiometer (Li-1800, Li-Cor) for the wavelength range between 695 and 800 nm (there was no detectable light below 695 nm; the peak was at 744 nm). Throughout plant culture and experiments, plants and protoplasts were not exposed to any light other than the background red light and the lights for experimental treatments.

RESULTS

Size Distribution of Protoplasts

The volume of the protoplasts prepared using the standard procedure ranged from 5×10^3 to 200×10^3 μm^3, and the size distributions were similar among the preparations obtained from three coleoptile positions: 0 to 2, 5 to 7, and 10 to 12 mm from the top (Fig. 1A). Protoplasts were most abundant between 20×10^3 and 50×10^3 μm^3 (diameter 34–46 μm).

Figure 1B shows the size distribution before the centrifugal purification on a Percoll solution. When the data were compared with those in Figure 1A, we noted that the proportion of large protoplasts was somewhat reduced by this purification, especially in the preparations obtained from the lower coleoptile parts (5–7 and 10–12 mm). On the other hand, the proportion of small protoplasts (smaller than about 30×10^3 μm^3) was reduced in the preparation obtained from the apical part (0–2 mm). However, since the overall distribution pattern was not much affected, it is unlikely that protoplasts of certain cell types were selectively lost by the purification.

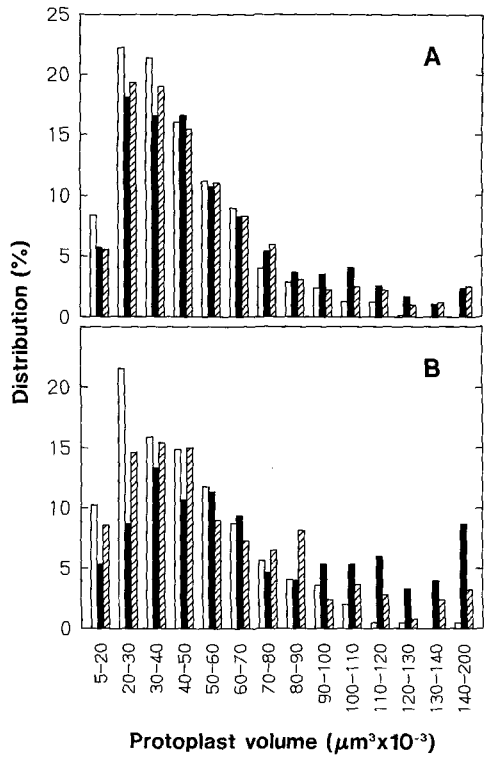

Figure 1. Size distribution of protoplasts of maize coleoptiles. A, Distribution of the final preparation (after centrifugal purification on a Percoll solution), determined from photographs obtained about 10 min after the addition of freshly prepared protoplasts to the measurement cuvette. B, Distribution before the centrifugal purification, determined from photographs obtained similarly after the addition of the enzyme solution containing protoplasts to the measurement cuvette at the end of the 2-h digestion period. Protoplasts were prepared from 2-mm coleoptile zones: 0 to 2 mm (white bars), 5 to 7 mm (black bars), and 10 to 12 mm (hatched bars) from the top. The proportion in each volume range is given as a percentage of the total number of protoplasts (600–700) examined.

Protoplast Response to a Pulse of Blue Light

Protoplasts were isolated from the apical 2 mm of coleoptiles. The volumes of these protoplasts were monitored before and after treatment with a 30-s pulse of blue light. Only those protoplasts with an initial volume between 50×10^3 and 80×10^3 μm^3 (diameter 46–53 μm) were analyzed. Without blue-light treatment, protoplasts swelled continuously at a rate of about 10% h^{-1}. When treated with a blue-light pulse the protoplasts shrank transiently. Examples of the response, measured at three different blue-light fluences, are shown in Figure 2, A to C. The protoplast volume decreased almost immediately after blue-light stimulation to reach a minimum at 4 to 5 min. The volume then increased toward the level extrapolated from the steady increase taking place before blue-light stimulation. The extent of response depended on the flu-

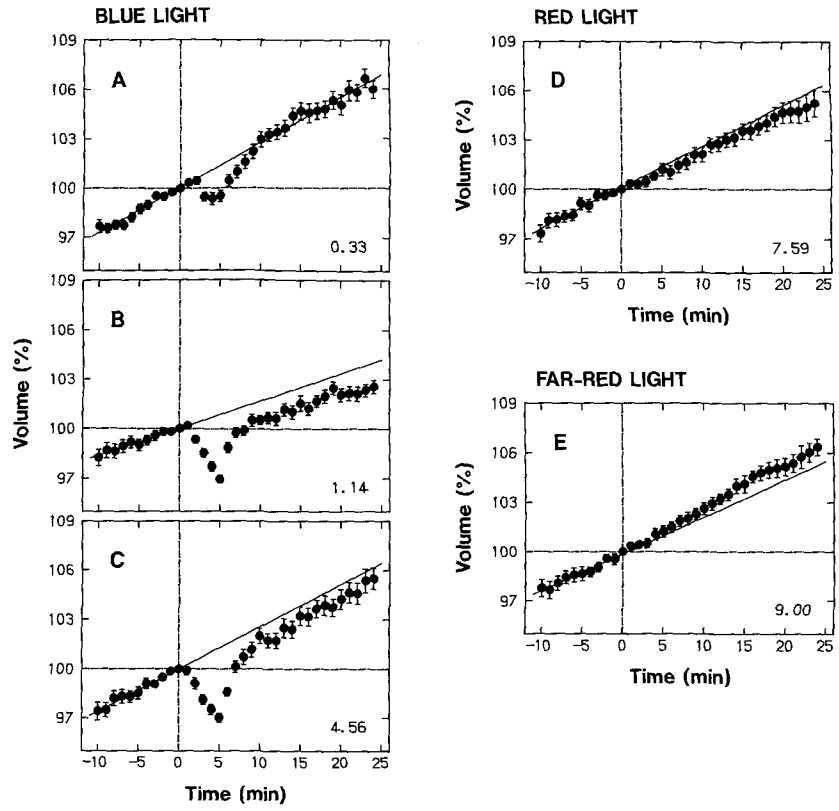

Figure 2. Time courses of protoplast shrinking induced by a blue-light pulse. The volumes of individual protoplasts (50×10^3 to 80×10^3 μm^3), prepared from the apical 2 mm of maize coleoptiles, were monitored before and after the onset at time 0 of 30-s irradiation with blue light (A–C), red light (D), and far-red light (E). The value in the lower right of each graph indicates the total fluence (mmol m^{-2}) applied. The light treatment was given while protoplasts were under background red light (50 μmol m^{-2} s^{-1}). The treatment with far-red light was carried out using two light sources to apply the required high fluence. The relative volume of each protoplast was calculated as a percentage of the volume at time 0. The data are the means ± SE from 20 to 24 protoplasts (two experiments). The solid line represents the linear regression line of the points at and before time 0.

ence, but the response kinetics were similar among the different fluences.

Treatment with a blue-light pulse would affect the level of Pfr. Since we incubated protoplasts under a relatively high fluence rate of red light, a decrease in Pfr was expected to occur only for a short period. To determine whether such an alteration of phytochrome status actually resulted in the observed protoplast response, the effects of red light and far-red light were investigated. As shown in Figure 2, D and E, the protoplasts did not show any apparent response to a high-fluence pulse of either red light (7.6 mmol m^{-2}) or far-red light (9.0 mmol m^{-2}). It was concluded that the shrinkage of protoplasts induced by blue light was not mediated by phytochrome but by a specific blue-light receptor.

The relationship between blue-light fluences and the shrinking response was determined next. The response was quantified by two methods. With one method, the maximal decrement in volume was estimated by subtracting the relative response 5 min after the onset of a 30-s blue-light stimulation from the response at the corresponding time on the linear regression line, which was obtained from the measurements before blue-light stimulation (Fig. 2). With the other method, the volume decrement was integrated over the time after blue-light stimulation. Since the linear regression line from prestimulation measurements could not exactly predict the baseline after the transient response, we used the three measurement points at and before the onset of blue-light stimulation (−2, −1, and 0 min) and the points at 12, 13, and 14 min to estimate the baseline (see the legend to Fig. 3). The differences in the measured volume from the baseline at each 1-min interval after blue-light stimulation were calculated, and the values were summed over the period of 12 min to obtain the integrated response.

As shown in Figure 3, either of the two data sets for maximal response and integrated response indicated a saturation curve. In Figure 3 the two data sets were displayed so that the means of the values at the three highest fluences match. The response showed a major increase from 10^2 to 10^3 μmol m^{-2}.

Protoplast Response to Continuous Blue Light

The protoplasts isolated from the coleoptile tip shrank in response to continuous irradiation with blue light (Fig. 4, A–C), but, again, this response was transient. The minimal volume was achieved about 6 min after the onset of blue-light stimulation, and the protoplasts returned gradually, within the next 30 min, to the levels extrapolated from the measurements before blue-light stimulation. The extent of shrinking depended on the fluence rate, but the overall response kinetics was similar among different fluence rates (Fig. 4, A–C). The protoplasts did not respond to high-fluence-rate red light given in addition to the background red light (Fig. 4D).

The relationship between blue-light fluence rates and the maximal volume decrement achieved during blue-light stimulation was investigated. To determine the maximal decrement, the differences between the measured volume

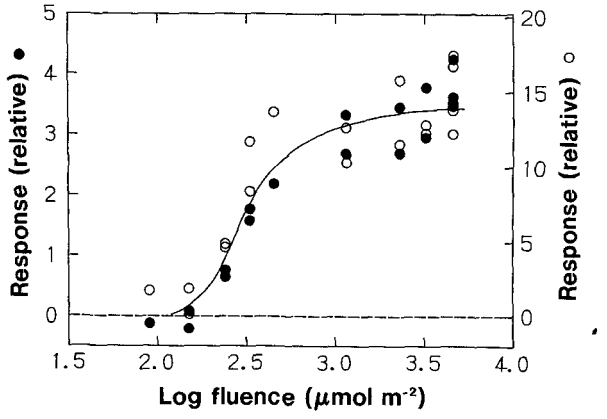

Figure 3. Fluence-response relationship of protoplast shrinking induced by a blue-light pulse. The protoplasts isolated from the apical 2 mm of maize coleoptiles were stimulated with a 30-s pulse of blue light providing the fluences indicated. Experiments were conducted and time courses were analyzed as described in Figure 2. ●, Maximal volume decrement, which was calculated by subtracting the measured relative volume at 5 min from the volume on the extrapolated regression line, obtained from the measurements before blue-light stimulation (Fig. 2). ○, Integrated volume decrement: the measured relative volume was subtracted from the volume on the baseline, obtained by joining the mean of the measurements at −2, −1, and 0 min with the mean of those at 12, 13, and 14 min at each min for a 12-min period from the onset of blue-light stimulation; the calculated values were summed to obtain the integrated decrement. The two sets of data are displayed so that the means of the values at the three highest fluences (log fluences of 3.36, 3.51, and 3.66) match. A line was fitted by eye to all data. Each point is the mean obtained from 10 to 12 protoplasts in a separate experiment.

and the volume on the extrapolated linear regression line were calculated from time-course measurements. In all of the time courses obtained, the largest difference occurred either 6 or 9 min after the onset of blue light, with a small difference between 6 and 9 min. Therefore, the maximal volume decrement was estimated as the mean of the differences at 6 and 9 min. The response to continuous blue light obtained in this way was plotted as a function of fluence rate (Fig. 5). The response became detectable from about 0.01 μmol m^{-2} s^{-1} and was saturated at about 30 μmol m^{-2} s^{-1}. The width of the fluence rate-response curve, from threshold to saturation, was about 4 orders of magnitude.

Response to Blue Light of Protoplasts Obtained from Different Coleoptile Positions

The preceding experiments were conducted using protoplasts isolated from the apical 2 mm of coleoptiles and monitoring only those protoplasts in the range of 50×10^3 to 80×10^3 μm^3. In the next experiments, protoplasts were isolated from different positions of the coleoptile to investigate if the observed response was limited to the tip region. In addition, protoplasts falling into three size ranges were analyzed separately. The coleoptile positions chosen were three 2-mm zones located 0 to 2, 5 to 7, and 10 to 12 mm from the top. The ranges of the initial protoplast volume selected for analyses were 20×10^3 to 40×10^3, 50×10^3 to 75×10^3, and 80×10^3 to 120×10^3 μm^3. The comparisons were made for the response induced by a 30-s blue-light pulse providing a saturating fluence (4.6 mmol m^{-2}).

All of the investigated protoplast groups showed very similar shrinking responses with respect to both kinetics and magnitude (Fig. 6). Since the protoplast volume was expressed as a percentage of the volume at time 0, the results indicate that the relative extent of volume decrease was similar among the different protoplast sizes, although the actual decrease in volume was greater in larger protoplasts. It was noted that the steady increase in relative protoplast volume, which took place before blue-light stimulation, was steeper in smaller protoplasts (see slopes of the regression lines, Fig. 6). This tendency, however, appeared to have no relation to the blue-light-induced shrinking response.

Protoplast Response to Two Pulses of Blue Light

Although the response to a blue-light pulse was saturable (Fig. 3), it was next demonstrated that protoplasts recover responsiveness to blue light after receiving a saturating pulse. The protoplasts isolated from the apical 2 mm of coleoptiles were first treated with a 15-s blue-light pulse (2.3 mmol m^{-2}) and after various time intervals with another identical pulse. An additional shrinking response to a second blue-light pulse could be identified in time courses when sufficient intervals were allowed between the two pulses (Fig. 7, B–D). When the interval was longer than 10 min, a nearly identical response followed each pulse (Fig. 7D). The response to a 15-s pulse (Fig. 7A) was very similar to the response to a 30-s blue-light pulse, i.e. two 15-s pulses without an interval (Fig. 2C), confirming that the 15-s pulse was sufficient to saturate the response to a single pulse.

The response to two pulses was quantified by the integration method used to obtain the data shown in Figure 3 and plotted against the time interval between two pulses (Fig. 8). At time 0, the response to a 15-s pulse is shown in addition to the response to two pulses given without an interval. The data demonstrated that the ability of protoplasts to respond to a second pulse increased sharply within the first few minutes. The response to the second pulse was saturated at about 5 min. At saturation, the response due to two pulses was doubled, indicating that the responsiveness to the second pulse was fully recovered.

Protoplast Response to an Increase in Fluence Rate during Continuous Blue-Light Irradiation

The fluence rate-response curve shown in Figure 5 indicated that the shrinking response of protoplasts to continuous blue light was saturated at a fluence rate of 30 μmol m^{-2} s^{-1}. In the following experiments, protoplasts were first irradiated with 30 μmol m^{-2} s^{-1} blue light and, at 27 min of this irradiation, the fluence rate was enhanced to 300 μmol m^{-2} s^{-1}. The data (Fig. 9A) indicated that the protoplasts showed another transient shrinking response to this 10-fold increase in fluence rate, a response comparable to the preceding one taking place at the lower fluence rate. As shown in Figure 9B, irradiation of protoplasts at

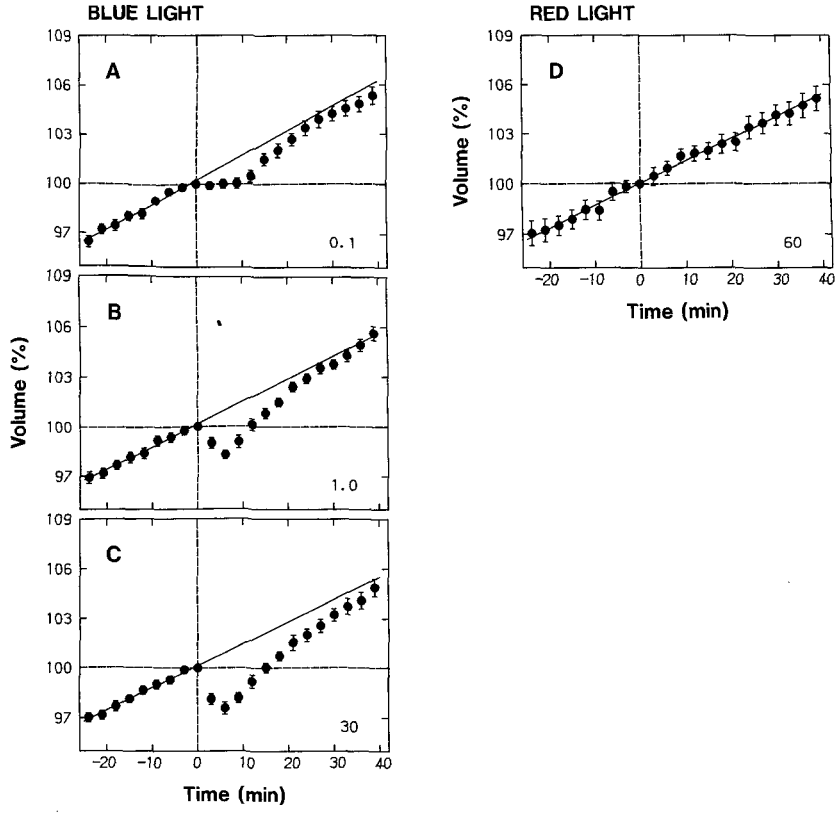

Figure 4. Time courses of protoplast shrinking induced by continuous blue light. The volumes of individual protoplasts were monitored every 3 min for 36 min before (but shown for 24 min) and 39 min after the onset at time 0 of continuous irradiation with blue light (A–C) and red light (D). The value in the lower right of each graph indicates the fluence rate (μmol m^{-2} s^{-1}) used. The data are the means ± SE from 20 to 25 protoplasts (two experiments). The solid line represents the linear regression line of the points at and before time 0, including those not shown. Other details are as described in Figure 2.

300 μmol m^{-2} s^{-1} from the beginning caused a response that was very similar to one obtained at 30 μmol m^{-2} s^{-1}. These results demonstrated that protoplasts became responsive to an enhanced fluence rate after saturation at a lower fluence rate.

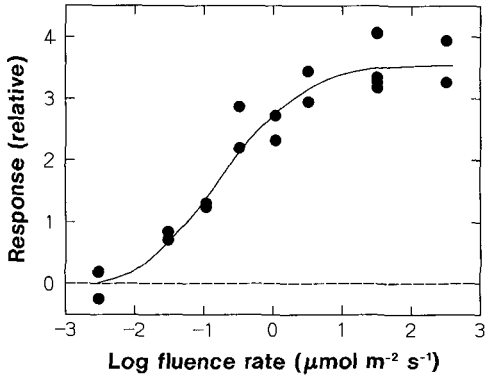

Figure 5. Fluence rate-response relationship of protoplast shrinking induced by continuous blue light. Experiments were conducted and time courses were analyzed as described in Figure 4. The highest fluence rate (300 μmol m^{-2} s^{-1}) was provided using two blue-light sources. The relative response shown is the mean of volume decrements at 6 and 9 min after the onset of blue-light irradiation, calculated as the differences of measured relative volume from the volume at corresponding times on the extrapolated regression line (Fig. 4). Each point is the mean obtained from 10 to 13 protoplasts in a separate experiment.

The Effect of NPPB on Protoplast Response

To find a possible link between the blue-light-induced activation of anion channels (Cho and Spalding, 1996) and the protoplast-shrinking response detected in the present study, the effect of NPPB, an anion channel blocker, on the blue-light-induced protoplast shrinking was investigated. NPPB was added to the protoplast suspension 20 min before treatment with a blue-light pulse (4.6 mmol m^{-2}). The final concentration of NPPB was 20 μM. The total length of preincubation in the measurement cuvette before blue-light treatment was identical to that in the experiments shown in Figure 2. The NPPB addition was achieved by gently mixing an NPPB solution (50 μL), which contained other components identical to the incubation medium, with the protoplast suspension (150 μL), which had been incubated in the cuvette. The original NPPB solution was prepared in ethanol at a concentration of 20 mM; the final ethanol concentration was 0.1% (v/v).

As shown in Figure 10, the shrinking response was inhibited almost totally by the NPPB treatment. The addition of 0.1% ethanol alone did not affect the shrinking response (not shown). The results support the possibility that the blue-light-induced shrinking response is mediated by activation of anion channels.

Effects of Blue Light on Coleoptile Growth

A possible causal relationship between the protoplast shrinking induced by blue light and the growth response to

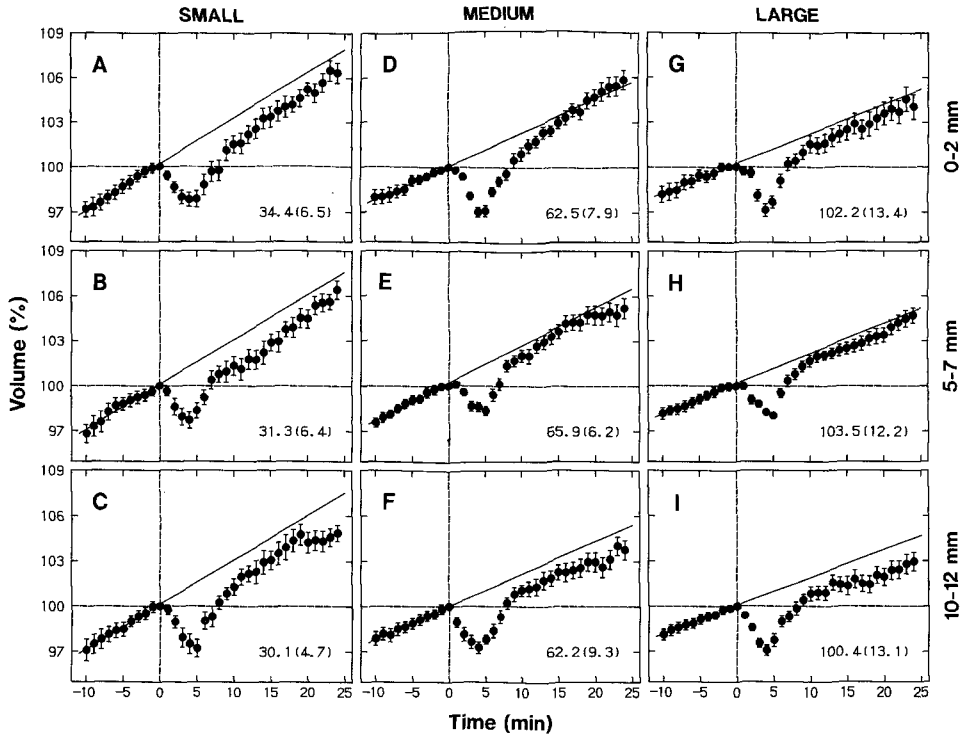

Figure 6. Blue-light-induced shrinking response of protoplasts isolated from different coleoptile positions. Protoplasts were isolated from 2-mm coleoptile zones located 0 to 2 mm (A, D, and G), 5 to 7 mm (B, E, and H), or 10 to 12 mm (C, F, and I) from the top. The initial protoplast volume (the volume at −10 min) fell into the ranges of 20×10^3 to 40×10^3 μm^3 (small; A, B, and C), 50×10^3 to 75×10^3 μm^3 (medium; D, E, and F), or 80×10^3 to 120×10^3 μm^3 (large; G, H, and I), with the mean (10^3 μm^3) and the SD (in parentheses) given in the lower right of each graph. Protoplasts were treated with a 30-s pulse of blue light (152 μmol m^{-2} s^{-1}). Each time course was obtained from 18 to 22 cells (two experiments). Other details are as described in Figure 2.

blue light was investigated. The coleoptiles of intact seedlings, growing under background red light, were treated with a blue-light pulse that provided a fluence (4.5 mmol m^{-2}) sufficient to saturate the protoplast-shrinking response. Growth of three coleoptile zones (initially 5 mm long) was monitored before and after blue-light treatment (Fig. 11, D–F). Growth of these zones was inhibited considerably by the blue-light pulse (compare with Fig. 11, A–C). Growth inhibition occurred immediately after blue-light stimulation, and the response was transient in all zones. It appeared that the inhibition lasted longer in the most apical zone than in the other zones. In all zones the growth rate returned to the prestimulation rate within 5 min.

Additional control measurements were obtained by treating coleoptiles with a high-fluence pulse of far-red light (8.4 mmol m^{-2}). This treatment did not cause any significant growth response (Fig. 11, G–I).

Coleoptiles were next treated with continuous blue light (30 μmol m^{-2} s^{-1}) and, as shown in Figure 12, growth of the three coleoptile zones was inhibited. Inhibition occurred immediately after the onset of blue-light irradiation. However, the growth rate returned to near the prestimulation rate, indicating that the response was transient, even during continuous blue-light irradiation. The growth inhibition appeared to last longer in the most apical zone.

The blue-light-induced growth inhibition shared the following features with the blue-light-induced protoplast shrinking: (a) the response was not limited to the tip of the coleoptile; (b) the response to pulse irradiation was transient, occurring within several minutes after blue-light irradiation; and (c) the response to continuous irradiation was also transient, occurring during the first several minutes of irradiation.

DISCUSSION

Blue-Light-Induced Protoplast Shrinking and Osmoregulation

We have shown here that the protoplasts isolated from maize coleoptiles shrink in response to blue light. The results demonstrate for the first time to our knowledge that shrinking of protoplasts, which reflects enhanced net efflux of osmotic substances, can be induced by blue light. The extent of the volume decrease was small: the maximal volume decrease induced by either pulse stimulation (Fig. 3) or continuous stimulation (Fig. 5) was 3 to 4%. Nevertheless, the response was always detectable and was reproducible with regard to both magnitude and kinetics. Time courses of volume changes plotted for individual protoplasts indicated that most of the protoplasts responded to blue light. This was also reflected in the relatively small SEs of the time-course measurements. Clearly, the shrinking response is not limited to a small fraction of protoplasts. The response must at least be made by the protoplasts of parenchyma cells.

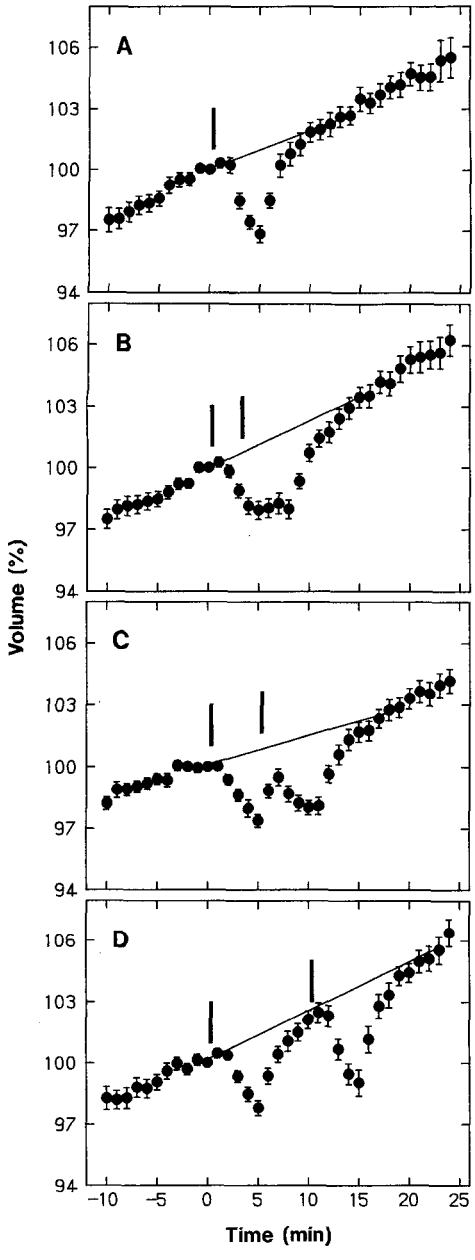

Figure 7. Time courses of protoplast shrinking induced by two blue-light pulses. The volumes of individual protoplasts isolated from the apical 2 mm of maize coleoptiles were monitored before and after the onset at time 0 of a 15-s pulse of blue light (152 μmol m^{-2} s^{-1}). A second 15-s pulse of identical blue light was given after intervals (onset to onset) of 3 min (B), 5 min (C), and 10 min (D); no second pulse was given in A. The solid line (baseline) was generated by joining the mean of the three measurements at and before the onset of the first blue-light pulse (0, −1, and −2 min) with the mean of the measurements at 12, 13, and 14 min after the onset of the first (A) or the second (B–D) blue-light pulse. Each vertical bar indicates the time of blue-light stimulation. The data are the means ± SE obtained from 20 to 24 protoplasts (two experiments). Other details are as described in Figure 2.

The plasma membrane is depolarized by blue-light stimulation in hypocotyls of cucumber and Arabidopsis (Spalding and Cosgrove, 1988, 1992; Cho and Spalding, 1996) and in pulvini of *Phaseolus vulgaris* (Nishizaki, 1994). Cho and Spalding (1996) demonstrated, using a patch-clump technique, that the blue-light-dependent plasma membrane depolarization in Arabidopsis hypocotyls results from activation of anion channels. Since the plasma membrane of a living cell maintains an inside-negative membrane potential, it is predicted that activation of anion channels by blue light results in a net efflux of anions (mainly Cl^-), causing a reduction in cell osmolality or a shrinking of protoplasts. Our results indicate that just such a reduction of cell osmolality can follow blue-light stimulation. Furthermore, we were able to show that the anion channel blocker NPPB effectively inhibits the shrinking response of maize protoplasts at a concentration (20 μM) that abolished the anion channel activity (Cho and Spalding, 1996). It was suggested that the blue-light-induced shrinking of coleoptile protoplasts results from activation of plasma membrane anion channels.

In guard cell protoplasts blue light induces swelling of protoplasts (Zeiger and Hepler, 1977; Amodeo et al., 1992) and hyperpolarization of the plasma membrane (Assmann et al., 1985). These responses contrast with the responses found in hypocotyls and coleoptiles. In guard cells, the electrogenic H^+ pump of the plasma membrane is activated by blue light (Assmann et al., 1985; Shimazaki et al., 1986). The transduction mechanism of the blue-light-induced membrane depolarization and protoplast shrinkage, which involves the activation of anion channels, appears to

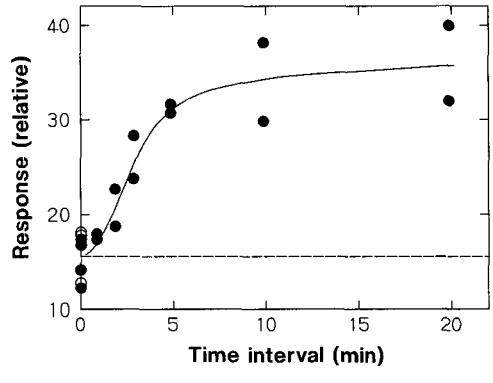

Figure 8. Protoplast shrinking induced by a second blue-light pulse as a function of the time after the first pulse. Experiments were conducted and time-course data were analyzed as described in Figure 7. The measured relative volume was subtracted from the volume at the baseline (Fig. 7) at each minute for the period from the onset of the first pulse to 12 min after the second pulse (or after the first pulse when treated with only one pulse); the calculated values were summed to obtain the relative response. The response was plotted against the time between the end of the first pulse and the onset of the second pulse. Open and closed circles at time 0 represent the responses to a single 15-s pulse and two successive 15-s pulses (i.e. a 30-s pulse), respectively. Each point is the mean obtained from 10 to 12 protoplasts in a separate experiment. The dashed line represents the mean of all points at time 0. Other details are as described in Figure 7.

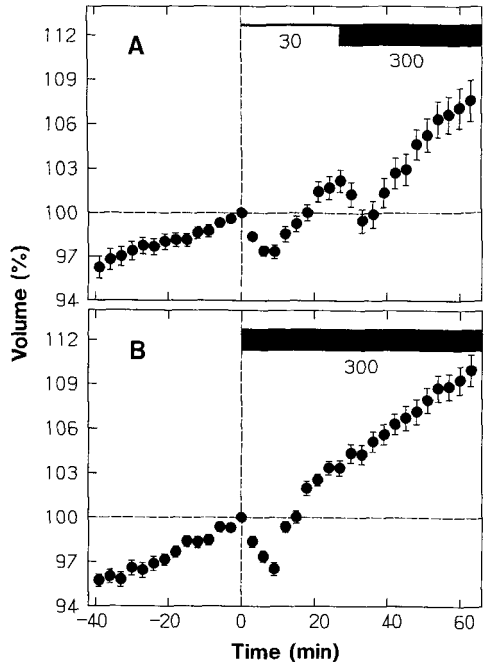

Figure 9. Protoplast shrinking induced by an increase in fluence rate during continuous blue-light irradiation. The volumes of individual protoplasts were monitored every 3 min before and after the onset at time 0 of continuous blue light. A, Treated at 30 μmol m^{-2} s^{-1} for the first 27 min and then at 300 μmol m^{-2} s^{-1} for the remaining period. B, Treated at 300 μmol m^{-2} s^{-1} throughout. The blue light at either fluence rate was provided using two light sources. The data shown the means ± SE from 20 to 25 protoplasts (two experiments). Other details are as described in Figure 4.

be distinct from the one responsible for guard cell responses.

Blue-Light-Induced Protoplast Shrinking and Growth Inhibition

In hypocotyls blue-light-induced depolarization of plasma membranes has been considered to be related to blue-light-induced growth inhibition. This view was initially based on a comparison of response kinetics (Spalding and Cosgrove, 1988). Cho and Spalding (1996) showed recently that NPPB inhibits not only blue-light-induced depolarization but also growth of Arabidopsis hypocotyls. The results, substantiating the link between depolarization response and growth inhibition, indicate that activation of anion channels mediates a blue-light-dependent growth inhibition in hypocotyls.

Although growth inhibition by blue light is commonly observed in hypocotyls (Cosgrove, 1994) and epicotyls (Laskowski and Briggs, 1989; Warpeha and Kaufman, 1989), the reported effects of blue light on coleoptile growth have been controversial. Thornton and Thimann (1967) showed that growth of oat coleoptiles was rapidly inhibited following the onset of blue-light irradiation and that this inhibition ceased soon after its termination. A study by Macleod et al. (1985) had similar results. A detailed study by Gordon and Dobra (1972), however, indicated that growth of oat coleoptiles was stimulated by a pulse of blue light and that growth inhibition became detectable only when the fluence was enhanced. The growth stimulation progressed rapidly, whereas the inhibition began after a lag time longer than 10 min. These growth responses in oats were all detected in dark-adapted plants, so the contribution of phytochrome could not be ruled out (Iino, 1990). In red-light-grown maize, no clear growth inhibition by a pulse of blue light was observed (Iino and Briggs, 1984). It has been demonstrated here, however, that blue light can inhibit growth of red-light-grown maize coleoptiles. The response was transient after pulse stimulation (Fig. 11), as well as during continuous stimulation (Fig. 12). The transient nature of the response explains why it was difficult to detect a blue-light-induced growth inhibition in red-light-grown maize coleoptiles.

Phototropism, another blue-light-dependent response, has been studied extensively in coleoptiles (for review, see Iino [1990]). In coleoptiles phototropic growth redistribution can be demonstrated in the absence of blue-light-dependent growth inhibition (Iino and Briggs, 1984). It is a general conclusion that phototropism and blue-light-dependent growth inhibition are mediated by distinct photoreceptor systems (Iino, 1990). This conclusion has been substantiated by recent genetic studies in which mutants of Arabidopsis were used (Liscum et al., 1992; Liscum and Briggs, 1995). As indicated in "Results," the blue-light-induced shrinking response of protoplasts shared some features with the blue-light-induced growth inhibition. We believe that the protoplast response is related to blue-light-induced growth inhibition rather than to phototropism. The result most critical to this conclusion was that the shrinking response was not limited to protoplasts isolated from the coleoptile tip (Fig. 6). The photosensing for phototropism takes place predominantly in the apical 5-mm region of maize coleoptiles (Iino, 1995). Furthermore, the effective range of blue-light fluences for the protoplast-shrinking response (Fig. 3) is much higher than the fluence range for the major pulse-induced phototropism, the first pulse-induced positive phototropism (Iino, 1987, 1990).

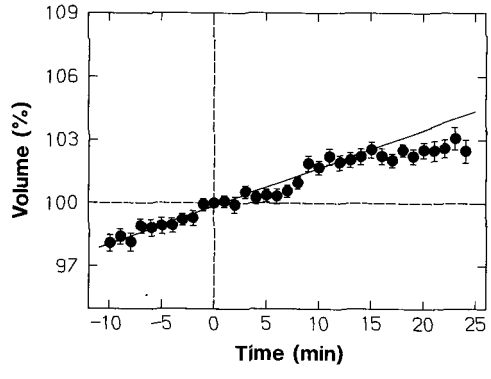

Figure 10. The effect of NPPB on blue-light-induced protoplast shrinking. The protoplasts isolated from the apical 2 mm of maize coleoptiles were stimulated with a 30-s pulse of blue light (152 μmol m^{-2} s^{-1}) at time 0. NPPB (20 μM) was added 20 min before the blue-light treatment. The data are the means ± SE from 21 protoplasts (two experiments). Other details are as described in Figure 2.

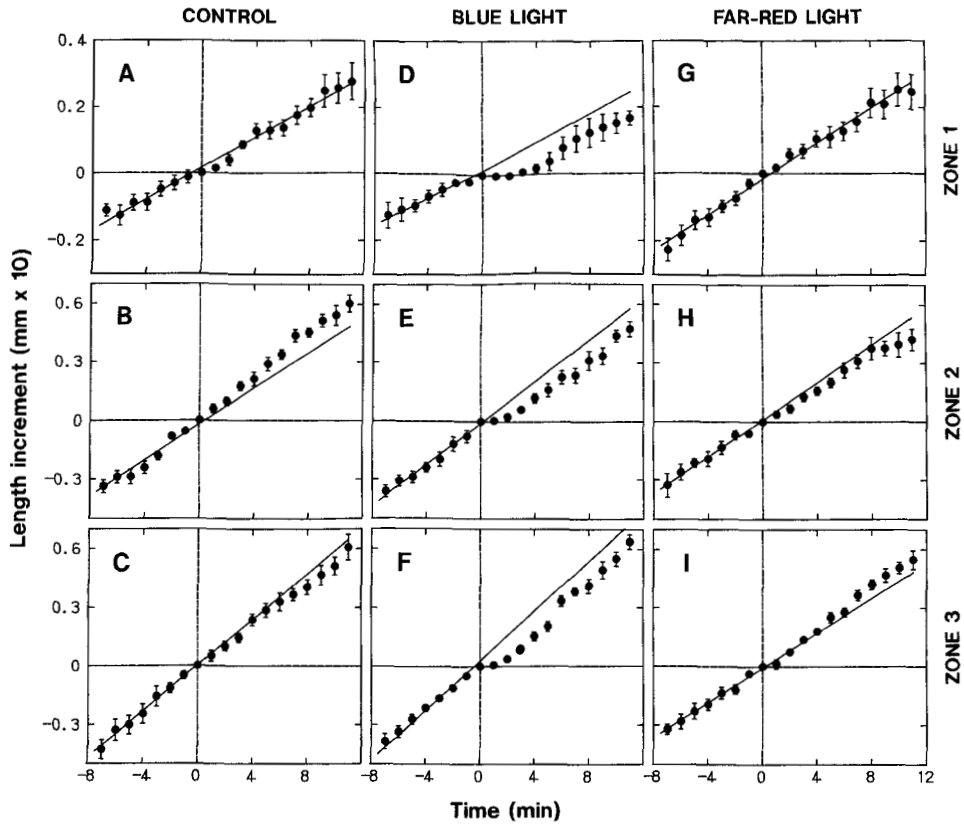

Figure 11. Inhibition of coleoptile growth induced by a blue-light pulse. The coleoptile (20–22 mm) of an intact seedling grown in red light (3 μmol m^{-2} s^{-1}) was marked at 5-mm intervals from the top and allowed to stand for 1.5 to 2 h before the length of each zone was monitored. Zones 1 to 3 were numbered from the top. Left (A–C), Nontreated controls; center (D–F), treated with blue light for 30 s from two opposite sides of the coleoptile (total fluence: 4.5 mmol m^{-2}); right (G–I), treated similarly with far-red light for 30 s (8.4 mmol m^{-2}). The zone length shown is the difference from the length at time 0, the onset of light treatment. Before light treatment, the zone length was monitored for 12 min, but not all points are shown. The data in each graph are the means ± SE obtained from nine plants. The solid line represents the linear regression line of all data at and before time 0.

The results summarized here indicate that the blue-light-dependent growth inhibition found in hypocotyls and coleoptiles is mediated by a common transduction mechanism that includes activation of anion channels and is reflected in plasma membrane depolarization and protoplast shrinkage. A common photoreceptor may also account for the growth responses in hypocotyls and coleoptiles. It is a possibility that this photoreceptor is the product of the gene *CRY1*, identified as responsible for the *hy4* mutation of Arabidopsis (Ahmad and Cashmore, 1993; Lin et al., 1995).

Kinetic Properties of the Blue-Light-Induced Protoplast Shrinking

The blue-light-induced response of maize protoplasts showed the following kinetic features: (a) the response can follow a pulse of blue light (Fig. 2), (b) the pulse-induced response is saturable (Fig. 3), and (c) protoplasts treated with a saturating pulse recover responsiveness to a second pulse (Fig. 8). These features, also shown in other blue-light responses (Briggs, 1960; Iino et al., 1985; Iino, 1987; Iino et al., 1988), suggest the participation of a dark-reversible component in the photoreceptor system (see also Iino [1990]). The dark-reversion kinetics resolved with the two-pulse protocol for the protoplast response is, however, different from the kinetics in other blue-light responses studied. Phototropism of maize coleoptiles (Iino, 1987) and blue-light-dependent stomatal response show a half-time of about 10 min, whereas the blue-light-induced protoplast shrinking shows a half-time of about 3 min (Fig. 8). The rate of the dark reaction appears to be much faster in the protoplast response than in the other blue-light responses.

It has become apparent that continuous blue-light stimulation leads only to a short-lived protoplast response (Fig. 4), as was also the case in the growth response (Fig. 12). These results suggest the participation of photosensory adaptation such as that considered for phototropism of maize coleoptiles (Iino, 1987, 1990): during continuous stimulation at a high fluence rate, the light-responding system becomes less sensitive to light so that the system can exhibit a further response effectively at an enhanced fluence rate. More direct evidence for this was provided by the results shown in Figure 9, which indicated that the

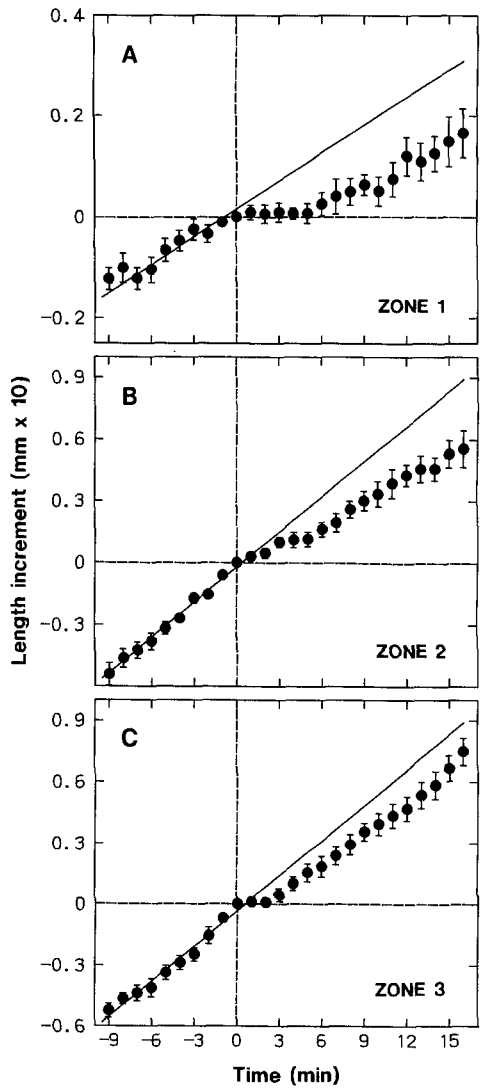

Figure 12. Inhibition of coleoptile growth induced by continuous blue light. Elongation of coleoptile zones 1 to 3 was monitored before and after the onset at time 0 of continuous blue light (30 μmol m^{-2} s^{-1}). The solid line represents the linear regression line of the data at and before time 0 (not all points are shown). Other details are as described in Figure 11.

protoplasts treated initially with a saturating fluence rate of blue light can show an additional response to a 10-fold increase in the fluence rate. Other results suggesting the participation of photosensory adaptation include: (a) that the width of the effective fluence-rate range for the response induced by extended stimulation (Fig. 5) is much wider than the effective fluence range for the pulse-induced response (Fig. 3), (b) that the sharp increase in the response to the second pulse did not appear to begin immediately after the first saturating pulse (Fig. 8), and (c) that the fluence rate-response curve calculated based on the data shown in Figures 3 and 8 (for the principles, see Iino et al. [1988]) occurred in a fluence-rate range lower than the range for the measured response shown in Figure 5 (not shown).

Spalding and Cosgrove (1988) showed that the membrane depolarization induced by extended blue-light stimulation in cucumber hypocotyls is short-lived; the membrane potential returned to the initial level within a few minutes of irradiation. The response was more transient than the shrinking response of protoplasts (Fig. 4). In cucumber hypocotyls, however, growth remained suppressed during at least 10 min of blue-light irradiation (see also Cosgrove [1994]). The transient growth inhibition in maize coleoptiles was detected well within this period (Fig. 12). Other reported data have not indicated the occurrence of a transient growth inhibition during continuous or prolonged blue-light stimulation (Cosgrove, 1981; Laskowski and Briggs, 1989). Further study is necessary to clarify whether the photosensory adaptation, which has become apparent in maize coleoptiles, is a common feature of blue-light-induced growth inhibition in different plant organs.

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LITERATURE CITED

Ahmad M, Cashmore AR (1993) *HY4* gene of *A. thaliana* encodes a protein with characteristics of a blue-light photoreceptor. Nature **366:** 162–166

Amodeo G, Srivastava A, Zeiger E (1992) Vanadate inhibits blue light-stimulated swelling of *Vicia* guard cell protoplasts. Plant Physiol **100:** 1567–1570

Assmann SM, Simoncini L, Schroeder J (1985) Blue light activates electrogenic ion pumping in guard cell protoplasts of *Vicia faba*. Nature **318:** 285–287

Blakeley SD, Thomas B, Hall JL, Vince-Prue D (1983) Regulation of swelling of etiolated-wheat-leaf protoplasts by phytochrome and gibberellic acid. Planta **158:** 416–421

Briggs WR (1960) Light dosage and phototropic responses of corn and oat coleoptiles. Plant Physiol **35:** 951–962

Cho MH, Spalding EP (1996) An anion channel in *Arabidopsis* hypocotyls activated by blue light. Proc Natl Acad Sci USA **93:** 8134–8138

Chung CH, Lim SU, Song PS, Berlin JD, Goodin JR (1988) Swelling of etiolated oat protoplasts induced by phytochrome, cAMP and gibberellic acid: a kinetic study. Plant Cell Physiol **29:** 855–860

Cosgrove DJ (1981) Rapid suppression of growth by blue light. Occurrence, time course, and general characteristics. Plant Physiol **67:** 584–590

Cosgrove DJ (1994) Photomodulation of growth. *In* RE Kendrick, GHM Kronenberg, eds, Photomophogenesis in plants. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 631–658

Gordon SA, Dobra WA (1972) Elongation responses of the oat shoot to blue light, as measured by capacitance auxanometry. Plant Physiol **50:** 738–742

Iino M (1987) Kinetic modelling of phototropism in maize coleoptiles. Planta **171:** 110–126

Iino M (1990) Phototropism: mechanisms and ecological implications. Plant Cell Environ **13:** 633–650

Iino M (1995) Gravitropism and phototropism of maize coleoptiles: evaluation of the Cholodny-Went theory through effects of

auxin application and decapitation. Plant Cell Physiol **36:** 361–367

Iino M (1996) Short-term stimulation of growth induced by the apical application of IAA to intact maize coleoptiles. Plant Cell Physiol **37:** 27–33

Iino M, Briggs WR (1984) Growth distribution during first positive phototropic curvature of maize coleoptiles. Plant Cell Environ **7:** 97–104

Iino M, Nakagawa Y, Wada M (1988) Blue light-regulation of cell division in *Adiantum* protonemata: kinetic properties of photosystem. Plant Cell Environ **11:** 555–561

Iino M, Ogawa T, Zeiger E (1985) Kinetic properties of the blue-light response of stomata. Proc Natl Acad Sci USA **82:** 8019–8023

Laskowski MJ, Briggs WR (1989) Regulation of pea epicotyl elongation by blue light. Plant Physiol **89:** 293–298

Lin C, Robertson DE, Ahmad M, Raibekas AA, Jorns MS, Dutton PL, Cashmore AR (1995) Association of flavin adenine dinucleotide with the *Arabidopsis* blue light receptor CRY1. Science **269:** 968–970

Liscum E, Briggs WR (1995) Mutation in the *NPH1* locus of *Arabidopsis* disrupt the perception of phototropic stimuli. Plant Cell **7:** 473–485

Liscum E, Young JC, Poff KL, Hangarter RP (1992) Genetic separation of phototropism and blue light inhibition of stem elongation. Plant Physiol **100:** 267–271

Long C, Wang X, Pan R (1995) The role of calcium ions in red light-induced swelling of mung bean protoplasts. Chinese Sci Bull **40:** 248–250

Macleod K, Digby J, Firn RD (1985) Evidence inconsistent with Blaauw model of phototropism. J Exp Bot **36:** 312–319

Nishizaki Y (1994) Vanadate and dicyclohexlcarbodiimide inhibit the blue light-induced depolarization of the membrane in pulvinar motor cells of *Phaseolus*. Plant Cell Physiol **35:** 841–844

Shimazaki K, Iino M, Zeiger E (1986) Blue light-dependent proton extrusion by guard-cell protoplast of *Vicia faba*. Nature **319:** 324–326

Short TW, Briggs WR (1994) The transduction of blue light signals in higher plants. Annu Rev Plant Physiol Plant Mol Biol **45:** 143–171

Spalding EP, Cosgrove DJ (1988) Large plasma-membrane depolarization precedes rapid blue-light-induced growth inhibition in cucumber. Planta **178:** 407–410

Spalding EP, Cosgrove DJ (1992) Mechanism of blue-light-induced plasma-membrane depolarization in etiolated cucumber hypocotyls. Planta **188:** 199–205

Thornton RM, Thimann KV (1967) Transient effects of light on auxin transport in the *Avena* coleoptile. Plant Physiol **111:** 433–440

Warpeha KMF, Kaufman LS (1989) Blue-light regulation of epicotyl elongation in *Pisum sativum*. Plant Physiol **89:** 544–548

Widholm JM (1972) The use of fluorescein diacetate and phenosafaranine for determining viability of cultured plant cells. Stain Technol **47:** 189–194

Zeiger E, Hepler PK (1977) Light and stomatal function: blue light stimulates swelling of guard cell protoplasts. Science **196:** 887–889