Stimulation of the blue light phototropic receptor NPH1 causes a transient increase in cytosolic Ca^{2+}

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Blue light regulates plant growth and development, and three photoreceptors, CRY1, CRY2, and NPH1, have been identified. The transduction pathways of these receptors are poorly understood. Transgenic plants containing aequorin have been used to dissect the involvement of these three receptors in the regulation of intracellular Ca^{2+}. Pulses of blue light induce cytosolic Ca^{2+} transients lasting about 80 s in *Arabidopsis* and tobacco seedlings. Use of organelle-targeted aequorins shows that Ca^{2+} increases are limited to the cytoplasm. Blue light treatment of *cry1*, *cry2*, and *nph1* mutants showed that NPH1, which regulates phototropism, is largely responsible for the Ca^{2+} transient. The spectral response of the Ca^{2+} transient is similar to that of phototropism, supporting NPH1 involvement. Furthermore, known interactions between red and blue light and between successive blue light pulses on phototropic sensitivity are mirrored in the blue light control of cytosolic Ca^{2+} in these seedlings. Our observations raise the possibility that physiological responses regulated by NPH1, such as phototropism, may be transduced through cytosolic Ca^{2+}.

Light is one of the most important signals controlling plant growth and development. Separate photomorphogenic effects of red light (R) and blue light (B) (which also interact with each other) are well established. B signaling controls important plant processes such as phototropism, suppression of stem extension, chloroplast movement, circadian timing, and expression of numerous genes. Three photoreceptors absorbing in the B region of the spectrum have been identified thus far. The *Arabidopsis HY4* gene encodes a protein, CRY1, for cryptochrome (1). *hy4* mutants are impaired in several extension growth responses and in the expression of genes concerned with flavonoid biosynthesis in B (2, 3). CRY1 is thought to possess FAD and pterin chromophores (4, 5). A similar protein, CRY2, controls B-induced cotyledon expansion and is involved in the regulation of flowering (6, 7). A third B photoreceptor, NPH1, was discovered as a result of the isolation of mutants with impaired phototropic sensitivity (8). Although there was some doubt initially as to whether NPH1 was a receptor or a downstream component, more recent evidence shows that heterologously expressed NPH1 has an FMN chromophore and is autophosphorylated as a result of B irradiation (9, 10). Ahmad *et al.* (11) have reported recently that CRY1 and CRY2 may interact with NPH1 in the regulation of phototropism. This interaction implies crosstalk between the signal transduction pathways for these three receptors.

The nature of the B signal transduction pathways remains uncertain, but protein phosphorylation or a kinase cascade is likely for NPH1. Furthermore, the effect of B is to change the redox status of B receptors, and redox processes have been predicted to be involved in B signal transduction (12, 13). However, despite the facts that intracellular Ca^{2+} is involved in more processes in plant cells than any other second messenger (14–16) and that calcium has been implicated in B light signaling (13, 17, 18), the direct involvement of Ca^{2+} in B signal transduction has not yet been established. In an important paper, Lewis *et al.* (12) addressed this question by using *Arabidopsis* seedlings transformed with the Ca^{2+}-sensitive luminescent protein, aequorin (aeq). However, changes in Ca^{2+}-dependent luminescence could not be detected on B irradiation. Lewis *et al.* (12) concluded that either other signal transduction processes such as phosphorylation were the primary pathway or Ca^{2+} changes were so (undetectably) small as to be irrelevant to signal transduction.

Deciphering the path of information flow is essential if the primary transduction pathways and the interactions between B and R signals are to be understood. Transgenic aeq (without modification) is cytoplasmic, and changes in other compartments would not therefore have been detected, for example, by Lewis *et al.* (12). Targeted aeqs have now enabled investigation of Ca^{2+} concentration in different plant cell compartments (19, 20). More recent improvements in technology have greatly extended the flexibility and sensitivity of the aeq method (e.g., ref. 21). The results presented in this paper indicate an involvement of Ca^{2+} in B signal transduction in the cytoplasmic compartment; they identify a B receptor involved and indicate a complex of interactions between R and B. We have detected both adaptation and crosstalk between the B and R signal transduction pathways. These data raise the possibility that cytosolic Ca^{2+} ($[Ca^{2+}]_c$) may regulate tropic bending mediated by NPH1.

Materials and Methods

Light Sources and Filters. For broad bandpass illumination, a cold light source (KL750 from Schott, Wiesbaden, Germany) was used in conjunction with blue filter BG37 and red filter RG610 (both from Schott).

For narrow bandpass illumination, a monochromator was used (PTI, South Brunswick, NJ; model 101, 0.2 m f/4 monochromator with 600 lines per mm, 500-nm blaze-ruled grating) coupled with a 75-W xenon lamp. To block the second-order light from the monochromator, a 450-nm high-pass filter was added when illuminating at wavelengths above 500 nm.

Plant Materials and Growth Conditions. Transgenic *Arabidopsis thaliana* that express cytosolic aeq (22) and transgenic *Nicotiana plumbaginifolia* that express cytosolic aeq (23) were used to measure changes in $[Ca^{2+}]_c$. All seedlings used for experiments were grown on half-strength Murashige and Skoog basal salt mixture and 0.8% agar in luminometer cuvettes at 21°C (*Arabidopsis)* or 24°C (*Nicotiana*) with a 16-h photoperiod. The plants were used when 10–16 days old.

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Abbreviations: B, blue light; R, red light; aeq, aequorin; $[Ca^{2+}]_c$, concentration of cytosolic Ca^{2+}; apoaeq, apoaequorin; coel, coelenterazine; *cp* coel, *cp* coelenterazine.

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***cry1, cry2,* and *nph1* Mutants.** *nph1* and *cry2* mutants were crossed with transgenic *Arabidopsis* expressing apoaequorin (apoaeq). The F_1 generation was selected for kanamycin resistance and selfed. The progeny were selected for aeq expression and the mutant phenotype (6, 8). Homozygous F_3 plants were used for $[Ca^{2+}]_c$ measurements. The *cry1* (*hy4-2.23N*) mutant was in the Landsberg ecotype, which necessitated transformation with the apoaeq-expressing construct (22). Wild-type *Arabidopsis* (Landsberg ecotype) were also transformed with the same apoaeq construct to be used as control seedlings.

aeq Reconstitution and $[Ca^{2+}]$ Measurements. Luminescence was measured by using a digital chemiluminometer consisting of an EMI photomultiplier model 9829A with an EMI FACT50 cooling system (Electron Tubes, Middlesex, U. K.). For *in vivo* reconstitution of aeq from expressed apoaeq and coelenterazine (coel), seedlings were germinated as described above. aeq was reconstituted *in vivo* by wetting the plants to ensure even distribution of coel over the seedlings, and three to four 10-μl droplets of 10 μM *cp*-coelenterazine (*cp*-coel) were placed between the cotyledons. aeq was allowed to reconstitute overnight in the dark. Inhibitors were added together with the coel when they were used.

To determine *in vivo* calcium concentrations, the following equation was adapted from Allen *et al.* (24): $L/L_{max} = [(1 + K_R \times [Ca^{2+}])/(1 + K_{TR} + K_R \times [Ca^{2+}])]^3$, where L is the amount of light per second, L_{max} is the total amount of light present in the entire sample over the course of the experiment, $[Ca^{2+}]$ is the calculated Ca^{2+} concentration, K_R is the dissociation constant for the first calcium ion to bind, and K_{TR} is the binding constant of the second calcium ion to bind to aeq.

We had found that $K_R = 26 \times 10^6\ M^{-1}$ and $K_{TR} = 57\ M^{-1}$ for *cp*-coel by fitting the curve of this equation with the relationship between luminescence and calcium concentration for aeq reconstituted with *cp*-coel. The resulting equation is $[Ca^{2+}](nM) = \{[X^{1/3} + (X^{1/3} \times 57) - 1]/(1 - X^{1/3})\}/0.026$, where X is the amount of light per second divided by the total light emitted after that time point until all the aeq was discharged.

All results were analyzed by Student's *t* test, and the responses that were found to be statistically significant are stated.

Light Stimulation and Luminescence Measurement. The luminometer cuvette with the seedlings was placed in the cuvette holder of the luminometer in total darkness avoiding perturbations, which elicit mechanical signaling. The basal luminescence level was measured for several minutes. Then the cuvette holder was turned away from the photomultiplier tube, and the seedlings were illuminated for the time indicated without taking the tube out of its chamber. In this way, the mechanical stimulation and the time required to turn the holder back to the photomultiplier tube and commence measurement were minimal. To calculate $[Ca^{2+}]$ from the luminescence, all of the remaining aeq after the experiment was discharged by a series of cold and touch shocks given by fast injection of ice-cold water on top of the plants. Because of the high affinity (and high sensitivity) of *cp*-aeq for Ca^{2+}, more than 95% of the *cp*-aeq in the seedling is discharged by the first combined shock.

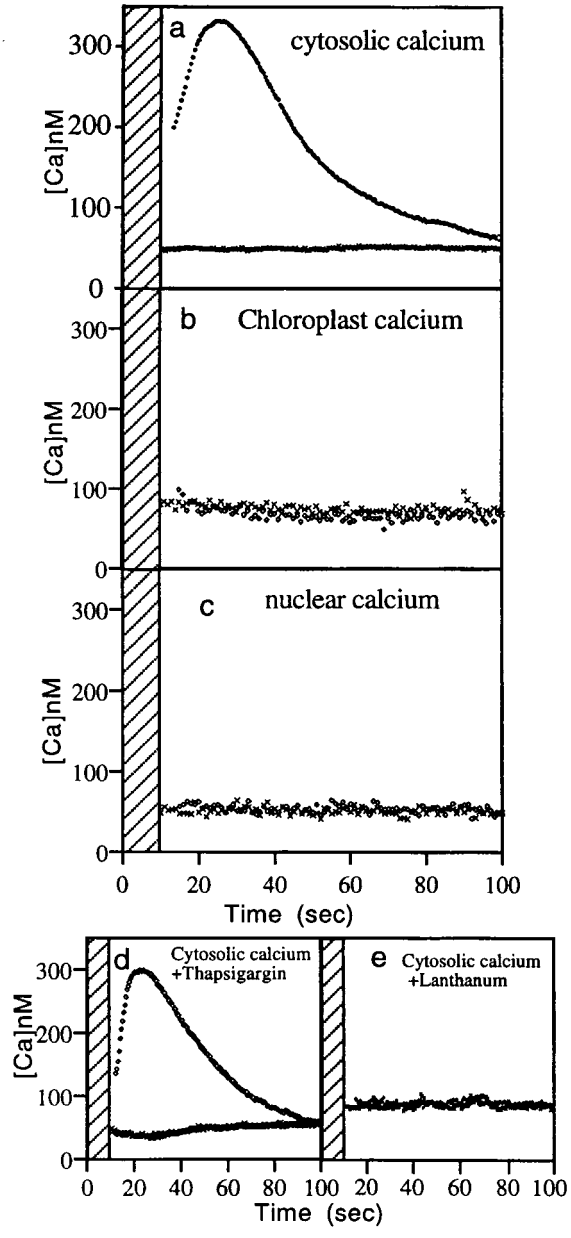

Fig. 1. B-induced changes in cytosolic, chloroplast, and nuclear Ca^{2+}. All seedling batches were exposed to 10 s of B, and chemiluminescence was allowed to decay before $[Ca^{2+}]_c$ measurement commenced. (*a*) The averaged transient of $[Ca^{2+}]_c$ from 50 *A. thaliana* measurements and 50 *N. plumbaginifolia* measurements. Luminescence was measured every 0.2 s for over 3 min, and each point was converted to $[Ca^{2+}]_c$ before averaging. (*b* and *c*) *N. plumbaginifolia* expressing aeq in the chloroplast (n = 20; *b*) and in the nucleus (n = 20; *c*). (*e* and *d*) The effect of 200 μM thapsigargin (*d*) and 3 mM lanthanum chloride (*e*) on the cytosolic response in *Arabidopsis*. ◇, light-treated; ×, dark control (the same plants before stimulation).

Results

B Transiently Increases $[Ca^{2+}]_c$. When 12- to 16-day-old tobacco (*N. plumbaginifolia*) and 10- to 14-day-old *A. thaliana* (Columbia) seedlings containing transgenic aeq were exposed to 10 s of B (fluence rate of 600 $\mu mol \cdot m^{-2} \cdot s^{-1}$), a Ca^{2+} transient was induced, lasting about 80 s (n = 100). Fig. 1*a* shows the averaged transient of equal numbers of measurements (n = 50) of both seedlings. The immediate effect of seedling illumination with B is the production of chemiluminescence, which decays rapidly within 3–5 s, with only slight variation between different seedlings. Because Ca^{2+} (luminescence) measurements can start only when chemiluminescence has decayed sufficiently, most kinetic profiles shown in this paper contain a short period in which no data can be collected, in addition to the illumination period (which is marked by hatched boxes in all figures). With illumination periods longer than 10 s, truncated Ca^{2+} transients were observed, suggesting either that the Ca^{2+} response is saturated or that a refractory period to further B exposure is rapidly induced (data not shown).

Each signal that has been investigated in plant cells seems to induce unique kinetics in $[Ca^{2+}]_c$ (14). Specificity in Ca^{2+} signaling may therefore relate to the $[Ca^{2+}]_c$ kinetics. Most of the Ca^{2+} profiles that we have recorded here suggest that there may be a lag period after B exposure and before Ca^{2+} is increased. Because B illumination and luminescence measurement cannot take place concurrently and because chemiluminescence induces a delay in measurement, precise estimation of putative lag periods is difficult. However, extrapolation from the upward arm of the transient to resting levels of Ca^{2+} and occasional experiments with shorter B exposure times suggest that a lag period from 3–6 s in length may exist. Ca^{2+} transients normally peak at about 300 nM free Ca^{2+}. Use of lower fluence rates ($<$600 $\mu mol \cdot m^{-2} \cdot s^{-1}$) reduces Ca^{2+} peak height, but the transient length remains unchanged at about 80 s.

The *Arabidopsis* and tobacco seedlings contain transgenic aeq which is 95–98% cytoplasmic in location (22), with the residue probably in the nucleus. Thus, part of the B-induced Ca^{2+} signal is most definitely cytoplasmic. However, we have constructed two lines of tobacco seedlings, one of which contains $>$99% aeq targeted to the chloroplast (19), whereas the other expresses a fusion protein with the oocyte nuclear protein, nucleoplasmin, in which $>$85% of the aeq is nuclear in location (25). Fig. 1 *b* and *c* shows the effects of B on the Ca^{2+} in these two compartments ($n = 10$ for both compartments). Neither compartment responds directly to B, and the Ca^{2+} signal in Fig. 1*a* is therefore cytoplasmic. Fig. 1*c* suggests that, if Ca^{2+} controls B-induced gene expression, changes in nuclear Ca^{2+} are not involved.

Confirmation of the absence of a nuclear contribution to the Ca^{2+} signal observed in Fig. 1*a* was obtained by using thapsigargin. This inhibitor of endoplasmic reticulum and nuclear envelope Ca^{2+} reuptake substantially enhances the peak size of nuclear Ca^{2+} signals induced by cold and mechanical signals in young tobacco seedlings (25). Fig. 1*d* shows that thapsigargin pretreatment at a concentration that modifies nuclear signals does not modify B-induced Ca^{2+} kinetics. Pretreatment with 3 mM La^{3+} (a Ca^{2+} channel blocker) on the other hand, completely blocks the B-induced Ca^{2+} transient (Fig. 1*e*). These data are consistent with a requirement for uptake of Ca^{2+} at some stage in the B-induced Ca^{2+} signaling in the cytoplasm. Pretreatment with La^{3+} was also observed to increase the resting dark level of Ca^{2+} from 50 nM to 100 nM (Fig. 1*e*). Pretreatment with BAPTA [1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate] and nifedipine also prevented B-induced Ca^{2+} responses (data not shown) consistent again with a cytoplasmic location of the Ca^{2+} signal.

NPH1 Signal Transduction Involves Cytoplasmic Ca^{2+}. We obtained mutant lines of the B receptors CRY1, CRY2, and NPH1 in *Arabidopsis* and transformed them with aeq (*cry1*) or crossed them (*cry2* and *nph1*) into aeq-containing *Arabidopsis* (see *Materials and Methods*). Seedlings of these three mutant lines were exposed to 10 s of B. The averaged Ca^{2+} transients ($n = 10$) are shown in Fig. 2 along with controls in Columbia (*cry2* and *nph1*) and Landsberg (*cry1*). Landsberg consistently had B-induced Ca^{2+} kinetics that differed from those of Columbia (peak height of only 150 nM), although it is not clear without further investigation whether this difference represents an innate difference in responsiveness to B. Mutations in *CRY1* or *CRY2* have little if any effect. However, the mutation in *NPH1* results in a substantial change in Ca^{2+} kinetics. The chemiluminescence was higher relative to the aeq luminescence, thus prohibiting Ca^{2+} measurements for 10 s after illumination. A pronounced Ca^{2+} peak was never observed, and the Ca^{2+} elevation was inhibited by at least 50%; however, elevation lasted for many minutes, decaying only slowly to resting levels. These data suggest that the Ca^{2+} transients shown in Figs. 1 and 2 *a* and *b* are largely regulated by NPH1.

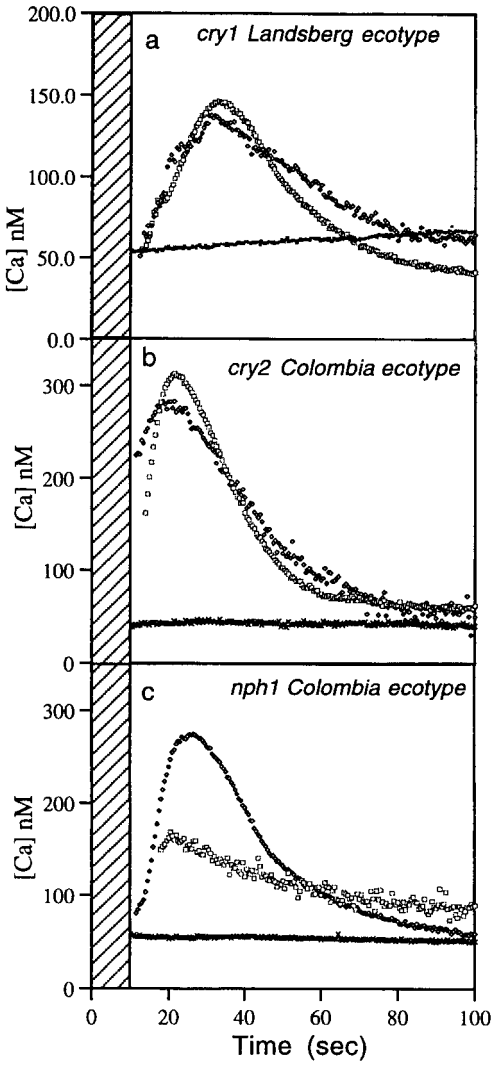

Fig. 2. B-induced changes in $[Ca^{2+}]_c$ in *Arabidopsis* photoreceptor mutants. *cry1*, *cry2*, and *nph1* mutants expressing aeq were illuminated with B for 10 s, and $[Ca^{2+}]_c$ was measured as described in the legend to Fig. 1. The *hy4* mutant and the control wild type used in this experiment are of the Landsberg ecotype. All other *Arabidopsis* plants used were of the Colombia ecotype. $n = 10$ in both panels; □, mutants; ◆, wild type; ×, control (unstimulated mutant).

The NPH1 photoreceptor was first detected in *Arabidopsis* seedlings with altered phototropic responses, and CRY1 was first detected in seedlings with B-insensitive hypocotyl elongation. Because there are some differences between action spectra for these physiological responses (26), we used a monochromator to obtain information on the relative effectiveness of different wavelengths in the B-induced Ca^{2+} response. The monochromator was set at a 20-nM slit opening, because narrower bandwidths reduced the peak size of the Ca^{2+} elevation and made accurate quantification more difficult. To ensure that seedlings received identical fluences at the different wavelengths, the time period of exposure was adjusted slightly.

Fig. 3 shows the total Ca^{2+} increase (i.e., the area under the transient) plotted against different wavelengths of B from 420–500 nm. This rough action spectrum contains two peaks at 440 nm and 470 nm, although a more detailed analysis at a 5-nm half bandwidth might move these peak values slightly. Action spectra for phototropism, stomatal aperture regulation, and inhibition of hypocotyl elongation are included for comparison (27–29). The

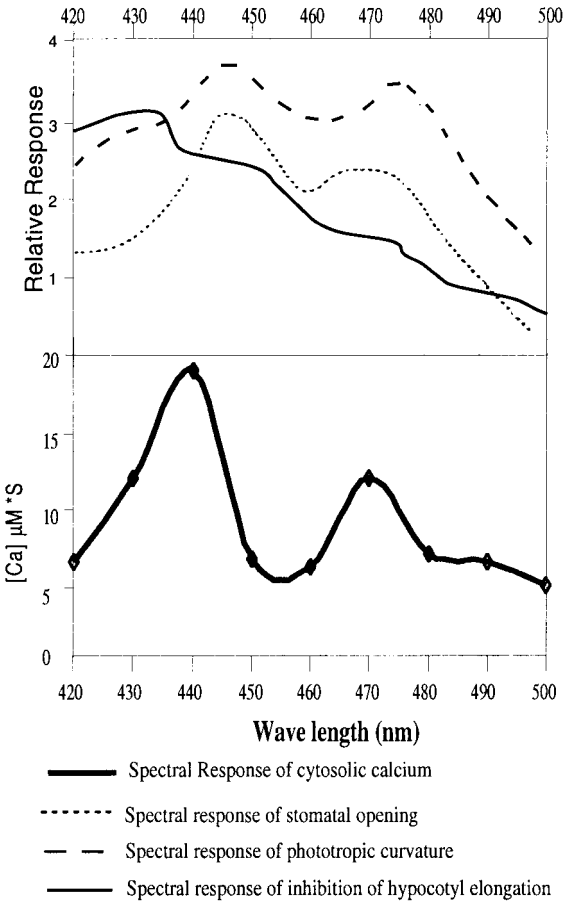

Fig. 3. Spectral response of B-induced $[Ca^{2+}]_c$. *Arabidopsis* seedlings were illuminated with a monochromator (described in *Materials and Methods*) with the slit set to a 20-nm opening. The response was measured at 10-nm steps. The illumination time was calculated to give the same fluence of light at each wavelength (65 mmol·m^{-2}) and changed accordingly from 9.5 to 16 s across the visible spectrum. After the illumination, $[Ca^{2+}]_c$ was measured, and the total increase in Ca^{2+} ([Ca] μM *S, where *S = seconds) was calculated by subtracting the resting Ca^{2+} levels from each measurement and integrating the area under the transient (n = 2). The spectral responses of stomatal opening (25), phototropic curvature (26), and inhibition of hypocotyl elongation by high energy illumination (24) were redrawn for comparison.

first two responses have peaks at 445 and 470–475 nm, which is within experimental error for our estimates of Ca^{2+} change. The action spectrum that we have included for hypocotyl elongation (primarily CRY1) is for strong irradiance (27), equivalent to the fluences we used. The data in Fig. 3 support an involvement of NPH1 in Ca^{2+} regulation, although we cannot exclude interaction with CRY1 and CRY2.

R and B Pretreatments Modify Subsequent Effects of B on Ca^{2+}. Prior brief exposure of many seedlings (e.g., tobacco) to R induces a substantial reduction in sensitivity to phototropically active B. To gain the same phototropic curvature, higher B fluences have to be used (30–32). The surprising exception to this rule is *Arabidopsis*; R does not modify sensitivity to phototropically active B, but R pretreatment can increase the final phototropic curvature achieved (33). Consequently, we have measured the response of $[Ca^{2+}]_c$ to combinations of B and R illumination.

Tobacco seedlings were irradiated by R for 10 s, followed 5 min later by 10 s of B (Fig. 4 *a–c*). Strong inhibition of the B-induced Ca^{2+} response was observed. Illumination with R on its own has no effect on $[Ca^{2+}]_c$ under these conditions (Fig. 4*b*).

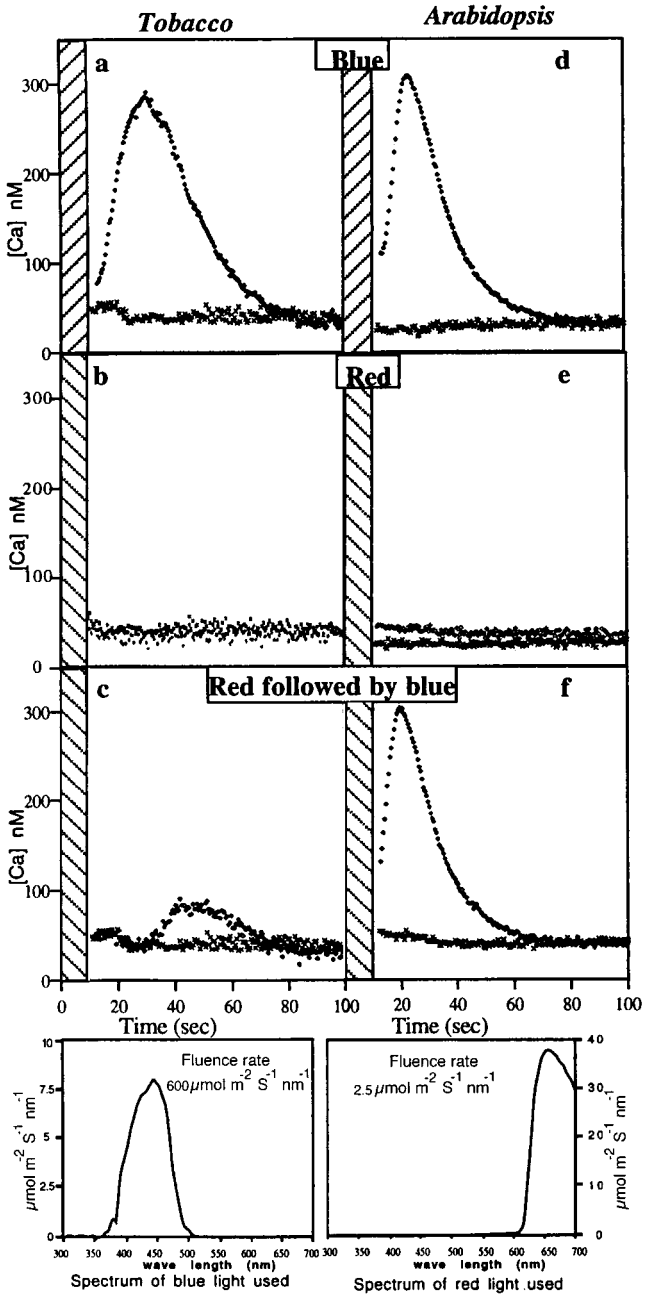

Fig. 4. Interaction between B and R in inducing a $[Ca^{2+}]_c$ in *N. plumbaginifolia* (*a–c*) and *A. thaliana* (*d–f*). Averaged $[Ca^{2+}]_c$ transients (n = 10) are shown in response to 10 s of B (*a* and *d*), 10 s of R (*b* and *e*), and 10 s of B given 5 min after 10 s of R (*c* and *f*). ◆, light-treated; ×, dark control (average of all the plants before stimulation). (*Bottom*) The spectra of the light sources.

Crosstalk between R and B transduction pathways regulating $[Ca^{2+}]_c$ is directly implied.

In *Arabidopsis*, however, prior treatment with R has no effect on the subsequent B-induced $[Ca^{2+}]_c$ response (Fig. 4 *d–f*). These data correlate well with the reported insensitivity of *Arabidopsis* to prior R when treated with a subsequent phototropically active B pulse. Again R on its own had no effect on $[Ca^{2+}]_c$ in *Arabidopsis* seedlings grown under these conditions. These data again suggest the likely involvement of NPH1 in controlling $[Ca^{2+}]_c$.

Prior exposure of a pulse of phototropically active B to seedlings renders phototropism refractory to successive pulses of

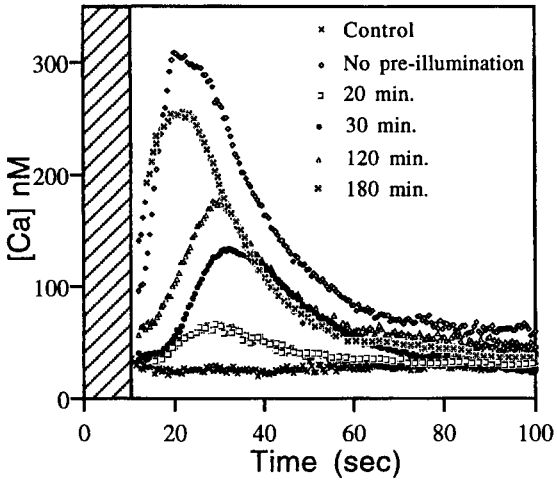

Fig. 5. Desensitization and recovery of B-induced $[Ca^{2+}]_c$ response. *Arabidopsis* seedlings were illuminated with B for 10 s at time zero. The seedlings were then irradiated again with 10 s of B after 20, 30, 120, and 180 min, and the $[Ca^{2+}]_c$ was recorded. The response to the second illumination is shown. ×, dark control.

B (32–34). Recovery from this refractory period can take an hour even with weak B. Consequently, we exposed *Arabidopsis* seedlings first to a pulse of B, measured the change in $[Ca^{2+}]_c$ (data not shown for this experiment), then, after varying times, subjected the seedlings to another B pulse, and recorded the $[Ca^{2+}]_c$ (Fig. 5). Within the first 20 min after the initial B pulse, the seedlings are refractory to further B stimulation. A recovery commences, and within 3–4 h, $[Ca^{2+}]_c$ responsiveness is restored.

The kinetics of the $[Ca^{2+}]_c$ transient change during recovery, with a pronounced lag period detectable for 2 h, which then slowly disappears. Janoudi and Poff (33, 34) reported phototropic response recovery times of up to 60 min by using fluences from 0.2 to 100 $\mu mol \cdot m^{-2}$ in etiolated seedlings. Much higher fluences have been used here with green seedlings. Even after 1 h, a substantial Ca^{2+} signal can be induced by B, and this signal may be sufficient to permit phototropism and any other NPH1 responses to occur.

Discussion

The results described herein clearly show a relationship between B signaling and cytoplasmic Ca^{2+}. There are direct indications that the NPH1 photoreceptor is involved in controlling $[Ca^{2+}]_c$. *nph1* seedlings have markedly altered Ca^{2+} kinetics compared with those of the wild type, but *cry1* and *cry2* do not (Fig. 2). NPH1 was identified originally by using a phototropically insensitive mutant (8). The B induction of Ca^{2+} parallels the action spectrum for phototropism (and stomatal aperture control). Furthermore, interactions between R and B and the effects of successive B pulses on Ca^{2+} mimic surprisingly well the interactions between these two light qualities on phototropic sensitivity reported by others (33, 35). However, Ahmad *et al.* (11) have shown that CRY1 and CRY2 interact with NPH1 in the control of phototropism. Although *cry1* and *cry2* do not affect Ca^{2+} kinetics directly, the use of inhibitors has suggested an involvement of Ca^{2+} in CRY signal transduction (3, 12). These data again emphasize that, in signal transduction, we deal with interactions that construct a network between the constituents (36, 37). Crosstalk between particular transduction pathways is commonly observed but rarely defined.

The interactions between R and B described here might enable crosstalk to be better understood. Because NPH1 seems to be a protein kinase localized in the plasma membrane (9), phosphorylation of a putative plasma membrane Ca^{2+} channel causing it to open is probably the simplest hypothesis to explain the increase in $[Ca^{2+}]_c$. Because recent evidence indicates that phytochrome also has a protein kinase activity (38), interaction between prior R and B in *Arabidopsis* (Fig. 4) might then be explained as resulting from direct regulation of the protein kinase activity of NPH1 by phosphorylation catalyzed by phytochrome or by a protein kinase cascade. Our work implies that, in tobacco, $[Ca^{2+}]_c$ is involved in the crosstalk between the photoreceptors. Successive B pulses make NPH1 refractory to further stimulation by B. A mechanism involving autophosphorylation might then also be involved, a phenomenon already described for this receptor (39). Now that mutants lacking in phytochromes A and B are available, it will be possible to investigate double mutants of these in combination with the B mutants and aeq to uncover which phytochrome modifies B $[Ca^{2+}]_c$ sensitivity.

There is considerable evidence that B signal transduction involves changes in redox processes. The chromophore of NPH1 is FMN, and B irradiation modifies its redox state; the LOV domains of NPH1 detect this change and activate the protein kinase activity. Because we have already shown that the redox state can control Ca^{2+} (40), our data are consistent with a redox pathway for NPH1 in controlling Ca^{2+} as well. Another alternative is that redox changes may modify plasma membrane potential and that voltage gated channels then open, inducing Ca^{2+} transients. One well characterized aspect of B signal transduction is alterations in membrane potential (41). However, if the calcium transient is induced by changes in plasma membrane potential, we are unable to explain why *cry1* and *cry2* apparently do not modify Ca^{2+}, although double mutants with *nph1* might clarify or even change this situation. Clearly, there has to be some attempt to place these events in kinetic order such that the probable course of information flow can be better understood. It is certainly feasible that the short lag period in the B-induced Ca^{2+} response reported herein might represent the time required to achieve the appropriate redox balance.

There is a considerable amount of data suggesting the involvement of Ca^{2+} in R signaling, including a paper showing a transient increase of Ca^{2+} concentration in response to R (42). These data do not contradict our results (Fig. 4 *b* and *e*). In ref. 42, protoplasts were used to measure the Ca^{2+} response, which was found to be an increase in $[Ca^{2+}]$ followed by a decrease below resting levels. Because the cells did not respond synchronously (42), such a response is impossible to detect in a whole organism.

We were unable to detect changes in nuclear Ca^{2+} as a result of B. This result is significant for understanding the regulation of genes such as *CHS* whose expression is induced by B and for which pharmacological data suggest an involvement of Ca^{2+} (13, 17, 43). A transduction pathway involving $[Ca^{2+}]_c$ in the cytoplasm is therefore likely (13). Studies with *Arabidopsis* cells indicate that La^{3+} has little inhibitory effect on *CHS* induction by B (17), whereas La^{3+} strongly inhibits the Ca^{2+} transient reported herein (Fig. 1*e*). Moreover, the B induction of *CHS* is impaired in *cry1* (3), whereas the Ca^{2+} transient is unaffected (Fig. 2). These observations indicate that the Ca^{2+} transient observed in whole seedlings is unrelated to the *CHS* expression response. Because the whole seedling Ca^{2+} response is the average from a large population of different cell types, to investigate the possible involvement of Ca^{2+} in the B induction of *CHS* further, we need to focus measurements on the specific cells that are engaged in the response. We cannot exclude the possibility that CRY1 or CRY2 may regulate $[Ca^{2+}]_c$ in particular tissues.

An important model that might help to explain the involvement of Ca^{2+} in B signal transduction concerns the movement of COP1. On illumination with B, COP1 moves to the cytoplasm,

and in darkness, COP1 moves back to the nucleus (44). COP1 has a nuclear localizing domain and a cytoplasm retention signal that regulates its localization (45). However, the route into the nucleus involves transit through nuclear pores, and recent data from Clapham (46, 47) indicate that Ca^{2+} regulates pore activity, although the mechanism is not understood. Future studies with a eq targeted to the nuclear pore and into the nuclear envelope cisterna are needed to investigate the intriguing possibility that B affects gene expression by controlling the traffic through the nuclear pore by localized changes in $[Ca^{2+}]$.

One surprising feature is that, in establishing the spectral response (Fig. 3), we were able to use the integrated Ca^{2+} transient plotted against the wavelength of excitation. Good correlation with the expected action spectrum was achieved. There may therefore be a direct (quantal) relationship between B illumination and Ca^{2+} channel activity. A single activated NPH1 might cause the opening of a discrete number of calcium channels. Equally likely, the relationship might instead reflect individual cell $[Ca^{2+}]_c$ responses combined with a threshold. We do not know how many cells respond to differing B fluence rates. Each cell may have a discrete threshold of B-induced processes. When this threshold is exceeded, a full blown $[Ca^{2+}]_c$ transient is achieved. If the threshold is variable for each individual cell and distributed on a population basis, the two possibilities for signaling cannot be distinguished. A possible resolution of these alternatives would be found by imaging the luminescence intensity at an individual cell level as fluence rate is changed. Currently, this resolution poses a challenging technical problem, given the amount of light involved, but it should be solved soon with the use of synthetic coels.

An earlier attempt to show a B effect on $[Ca^{2+}]_c$ by using aeq transformation failed (12). However, subsequent measurements with the moss *Physcomitrella* transformed with aeq showed that B effects could be detected easily (48). There is little doubt in comparing the results described herein with those previously reported (12) that sensitivity in detection has been the key. The relationship between aeq luminescence and Ca^{2+} is not linear. At low Ca^{2+} levels (about 300 nM, as is induced by B), it is advisable to use alternative coels to reconstitute aeq to improve detection. For this study, we used *cp*-coel, which necessitated the construction of new calibration curves but enabled us to detect 100 times more light than was previously possible. We have also greatly improved the aeq technology for plants, thus ensuring that a single *Arabidopsis* seedling can be seen in darkness by the naked eye when discharged with cold shock. Routinely upwards of 100,000,000 total detectable photons from a single seedling can be achieved, which will soon enable single-cell imaging.

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