Photoreversible Calcium Fluxes Induced by Phytochrome in Oat Coleoptile Cells

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ABSTRACT

The chromometallic dye murexide was used to measure photoreversible Ca fluxes in apical tips of etiolated oat coleoptiles and in suspension cultures of protoplasts derived from the coleoptile segments. Phytochrome presence in the protoplasts was indicated by a repeatedly photoreversible ΔA(725 – 900 nm) of >0.001 A centimeters−1, recorded on a dual wavelength spectrophotometer. Concentrations of Ca in the solution bathing the cells were observed to change photoreversibly, red irradiation inducing an increase in the medium Ca concentration and subsequent far-red irradiation inducing a decrease down to near dark control levels. These changes could be measured in media with or without exogenously added Ca. Protoplasts from green primary leaves of oat, which had no spectrophotometrically detectable phytochrome, showed no photoreversible Ca fluxes when measured by the same method. These data imply that red light induces an efflux of Ca from phytochrome-containing cells and that far red light can reverse this change by promoting a Ca reentry into these cells.

All organisms have cellular receptors by which they can sense environmental stimuli and convert these into useful physiological responses. For many different cell types including both procaryotes and eucaryotes, a common early response to the reception of a new sensory input is a change in cytoplasmic Ca levels (9). Specifically in higher plants, changes in cellular Ca content have been correlated with the transduction of a gravity stimulus into the upward growth curvature in hypocotyls (7, 21), and Ca fluxes have been implicated as an early cellular response to the photoactivation of the pigment phytochrome in Nitella (19) and Mougeotia (5).

The important role of Ca fluxes in the mediation of most forms of sensory transduction and the known influence of phytochrome on phototropism (1) and on geotropism (11, 20) make it appear plausible that phytochrome could influence Ca flux in higher plant tissue as it apparently does in Nitella. The experiments reported here were designed to test that idea, using intact apices of etiolated oat coleoptiles and protoplasts derived from them as the experimental material. Cells from this tissue have a high phytochrome titer (2) and have the sensory receptors for both phototropism and geotropism.

To assay for Ca2+ in these experiments we used the metallocromic indicator murexide which undergoes a rapid A change at 540 nm when the concentration of Ca2+ in the solution changes (16). For phytochrome studies, this assay method has an advantage over radiotracer methods in that it permits a continuous monitoring of Ca2+ fluxes into or out of cells within seconds after actinic irradiation. Murexide is not taken up by live cells and does not bind to them (16). Thus these experiments measured changes in the concentration of Ca2+ in the solution surrounding coleoptile cells after the cells were irradiated with R and FR. Under the conditions used here the dye is selectively sensitive to Ca2+ fluxes over those of other cations and can detect changes in the concentration of this cation as small as 1 μM (13).

MATERIALS AND METHODS

Preparation of Coleoptile Tips. Seeds of Avena sativa (L.) var. Garry were sown on water-soaked Kimpak in 15-cm diameter Petri dishes and grown in the dark at 30°C for 4 days. The seedlings were harvested near the base of the coleoptile, sterilized in 70% ethanol for 2 min, then rinsed in sterile distilled H2O. The top 2 cm of the coleoptiles were removed with a razor blade and floated on a sterile solution of buffer A (0.6 M mannitol and 10 mM Pipes, titrated to pH 6.8 with NaOH) until use.

Preparation of Protoplasts. Protoplasts were obtained from oat coleoptile tips, prepared as described above. Three-g lots of the tissue were digested in a 3% enzyme solution containing 2% w/w cellulysin and 1% w/v Macerase (Calbiochem), 0.6 M mannitol, and 10 mM Pipes (pH 5.8). Digestions were 5–7 h in the dark at 30°C with periodic, gentle agitation. Protoplasts were harvested by filtering the digests through silk screening (45 μM) followed by two low speed centrifugation washes with buffer A. Protoplast viability was determined by observing cyclosis and/or Evans blue vital dye exclusion. Protoplast quantitation was performed using a hemocytometer. Typical yields were 2 × 10⁶ protoplasts from 3 g etiolated coleoptile tips and 2 × 10⁷ protoplasts from 3 g greened leaves.

Spectrophotometric Measurements. (a) Measurement of Ca flux. The samples to be measured (coleoptile tips or protoplasts) were placed in a 1-ml cuvette containing 25 μM murexide in buffer A and between 0 and 2.5 mM CaCl₂ as given in the figure legends for each experiment. Changes in the Ca concentration of the solution bathing the protoplasts were monitored by measuring changes in the A of the dye at 540 nm with a Perkin-Elmer 556 dual wavelength spectrophotometer, essentially as described by Ohnishi (13). This instrument accurately measured changes as little as 0.0002 A, equivalent to that produced by a change of 2–3 μM in Ca2+ concentration. The reference beam was set at 507 nm, where there is no change in A as the dye complexes with Ca. For the protoplast experiments, a 25 μM murexide solution containing 7% (w/v) Ficoll (Sigma) in buffer A, was placed in the bottom cm of the cuvette. Then the protoplasts, usually about 2 × 10⁶ suspended in 0.5 ml of 25 μM murexide solution in buffer A, were added to the cuvette and allowed to settle onto the Ficoll pad.

Abbreviations: R: red light; FR far red light; Pipes: piperazine-N,N'-bis(2-ethanesulfonic acid).

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concentrating just below where the measuring beam of the instrument passed through the cuvette. With this arrangement, the measuring beams never passed through the cells and so would not detect the minor changes in $A_{540} - A_{507}$ nm that the cellular phytochrome would have responded to R and FR. The system was considered fully equilibrated when the $A_{540} - A_{507}$ nm remained constant for several min, and this usually occurred within 30 min after the cells were introduced into the cuvette. All solutions for the experiments were equilibrated at room temperature, which was maintained at a constant value $\pm 1.0$°C throughout the experiment. The range of temperatures for all the experiments described was 18-21. Continuous measurements of the cuvette solution temperature with a thermistor indicated that this did not vary more than 1°C during the experiments, including the irradiation periods.

(b) Phytochrome measurements. Estimates of the phytochrome content of protoplasts were obtained spectrophotometrically using both a Ratiospect and a Perkin-Elmer 556. The Ratiospect measurements, $\Delta(A,4)$, were made through a 5-cm path length cuvette, as described in (6), and the Perkin-Elmer measurements ($\Delta(A,725)$ nm) were made through a 1-cm path length with the measuring beam set at 725 nm and the reference beam set at $800$ nm.

Irradiations. Except where noted the samples were irradiated in the cuvette used for the spectrophotometric measurements. In Figure 1, the cuvette was removed from the Perkin/Elmer to a Ratiospect where the coleoptiles were given saturating doses of R($\lambda_{max} = 660$ nm) or FR($\lambda_{max} = 730$ nm) through the heat and interference filters of the Ratiospect, or white light (WL) from overhead fluorescent bulbs (40-w Westinghouse cool-white). The irradiance of R was about 2 $\mu$W cm$^{-2}$, of FR was 2.5 $\mu$W cm$^{-2}$, and of WL was 13 $\mu$W cm$^{-2}$. In Figures 2, 3, and 4 the samples were irradiated from above while in the cell holder of the Perkin-Elmer, with the sample chamber open and the voltage to the photomultiplier tube shut off. The R source for these irradiations was a Westinghouse 8-w warm light fluorescent bulb wrapped in two layers of red cellophane, which transmitted $>95\%$ of its light between 580 and 700 nm, with a peak at 620 nm, and produced an irradiance on the cells of 14 $\mu$W cm$^{-2}$. The FR source was light from a 250-w FR heat lamp filtered through a 3-cm-deep ice bath and CBS 750 far red filter (Carolina Biological Supply), which transmitted virtually no light below 700 nm. It produced an irradiance of 26 $\mu$W cm$^{-2}$. The above irradiance measurements were made with a Yellow Springs Radiometer. The sample used in Figure 3B was preirradiated with R before being placed in the spectrophotometer with a total dose between 580 and 700 nm of 4 nE cm$^{-2}$, as measured with a quantum photometer (Li-Cor LI-185, Lambda Instruments Corporation).

RESULTS

Detection of Photoreversible Phytochrome in Oat Coleoptile Protoplasts. As indicated in Table I, the level of phytochrome in etiolated oat coleoptile protoplast preparations was sufficient to be measured spectrophotometrically. Protoplasts obtained from the etiolated primary leaf of oat coleoptiles and protoplasts from the mesophyll cells of greened oat leaves had much lower levels of phytochrome, below the detection limits of the spectrophotometer.

Photoreversible Ca Fluxes in Etiolated Oat Coleoptile Tips and Protoplasts. Figures 1 and 2 illustrate typical results obtained when etiolated oat coleoptile tips and protoplasts were irradiated with R and FR in a solution containing murexide. When irradiated with R, the solution showed an increased Ca$^{2+}$ concentration (i.e. a decreased $A_{540} - A_{507}$ nm), indicating an efflux and or a dissociation of Ca$^{2+}$ from the tips and protoplasts. This change was reversed by FR, which promoted a decrease in the Ca$^{2+}$ concentration of the medium that was approximately equal to the R-promoted increase. In the experiments shown in Figure 2, when the unirradiated protoplasts were transferred from the Ca-free medium of buffer A into the measuring medium containing 2.5 mM Ca$^{2+}$, there was an apparent uptake of Ca$^{2+}$ by the protoplasts, indicated by a steady increase in the $A_{540} - A_{507}$ nm, which continued for about 0.5 (data not shown). The light treatments were initiated only after this uptake ceased (i.e., when the $A_{540} - A_{507}$ nm became zero.

Protoplasts isolated from greened oat leaf tissue did not produce
were preincubated with EDTA, then irradiated with 2 min of R followed by 2 min of FR (same sources and intensities as for Fig. 2). They were then washed twice by centrifugation (about 100 g for 5 min) followed by resuspension in buffer A plus 25 mM murexide, and loaded onto a 1-ml cuvette for spectrophotometric monitoring as described under "Materials and Methods." Arrow indicates time initial equilibration was reached. B: Sample monitored in this experiment contained about 10⁷ protoplasts suspended in the same medium as used for Figure 3A. Prior to being monitored in the spectrophotometer, these cells were first incubated in buffer A plus 2.5 mM CaCl₂ for 0.5 h at 19 °C, then pelleted and resuspended in 2 ml buffer A plus 0.1 mM EDTA. They were then irradiated with R and FR, pelleted, and resuspended in buffer A plus murexide as were the cells in Figure 3A, then irradiated again with R and loaded into a cuvette for monitoring. Arrow indicates time initial equilibration was reached.

any change in the Ca²⁺ concentration of the medium in response to R or FR irradiations (Fig. 4), a result consistent with the data in Table I, which showed that these protoplasts did not have detectable levels of phytochrome.

Effects of Removing Ca from Measuring Medium. In the aforementioned experiments, the cells were allowed to come to equilibrium with Ca²⁺ in the measuring medium, apparently by taking up Ca²⁺ from the medium, before the irradiations were begun. In another group of experiments, etiolated oat coleoptile protoplasts were preincubated in media containing 0.1 mM EDTA and irradiated with R while in this solution to promote the removal of external soluble Ca²⁺ from the protoplasts. They were then irradiated with FR and transferred to buffer A, with no added Ca²⁺, for flux measurements in the spectrophotometer. R and FR irradiations of these protoplasts had no effects on the solution Ca²⁺ concentration (Fig. 3A). These results suggested that protoplasts must have a prior incubation in a Ca-containing buffer and/or must be in a Ca-containing measuring medium in order to give the phytochrome response indicated in Figures 1 and 2. To clarify this point, another group of experiments was done in which the protoplasts were equilibrated with buffer A containing 2.5 mM Ca²⁺ and then subjected to the same sequence of treatments to remove external soluble Ca²⁺ as the protoplasts used for Figure 3A. When the response of these cells to actinic light in a Ca-free medium was tested, the results of Figure 3B were obtained: R induced an increased Ca²⁺ concentration in the medium and FR reversed this effect, just as in Figures 1 and 2.

The cells used in Figure 3B were exposed to actinic light to convert Pr to Pfr before the data shown were recorded. Consequently these cells showed no response to the initial R irradiation. Once the phytochrome was converted to Pr by FR, however, they did show a Ca²⁺ efflux in response to the next R. Due to the additional processing steps required, fewer cells were available for this experiment in comparison with the experiment shown in Figure 2. Thus the Ca flux changes in Figure 3B are smaller than those recorded in Figure 2.

A parallel control experiment was run in which about 10⁷

FIG. 3. Photo-reversible Ca fluxes in the absence of exogenous Ca²⁺. A: Sample monitored in this experiment contained about 10⁷ protoplasts suspended in the same medium used for Figure 2, except without Ca²⁺. The irradiations in the spectrophotometer were as described for Figure 2. Prior to being monitored in the spectrophotometer, the cells were suspended in 2 ml buffer A (0.6 m mannitol, 10 mM Pipes [pH 6.8]) plus 0.1 mM EDTA, then irradiated with 2 min of R followed by 2 min of FR (same sources and intensities as for Fig. 2). They were then washed twice by centrifugation (about 100 g for 5 min) followed by resuspension in buffer A plus 25 mM murexide, and loaded onto a 1-ml cuvette for spectrophotometric monitoring as described under "Materials and Methods." Arrow indicates time initial equilibration was reached. B: Sample monitored in this experiment contained about 10⁷ protoplasts suspended in the same medium as used for Figure 3A. Prior to being monitored in the spectrophotometer, these cells were first incubated in buffer A plus 2.5 mM CaCl₂ for 0.5 h at 19 °C, then pelleted and resuspended in 2 ml buffer A plus 0.1 mM EDTA. They were then irradiated with R and FR, pelleted, and resuspended in buffer A plus murexide as were the cells in Figure 3A; then irradiated again with R and loaded into a cuvette for monitoring. Arrow indicates time initial equilibration was reached.

FIG. 4. Ca fluxes in greened oat leaf mesophyll protoplasts. The sample monitored in this experiment contained about 2 × 10⁷ protoplasts in a buffer containing 0.6 m mannitol, 10 mM Pipes, 2.5 mM CaCl₂, and 25 mM murexide (pH 6.8). The experimental procedure followed was identical to that of Figure 2.

Table I. Detection of Phytochrome in Oat Protoplasts

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Protoplast Source*</th>
<th>Protoplast Cells Showing Cyclosis</th>
<th>Sample Photo-reversibility</th>
<th>number/ml</th>
<th>% ΔA (725 nm - 800 nm) cm⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>coleoptile tip</td>
<td>2 × 10⁷</td>
<td>&gt;90</td>
<td>0.0015</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>primary leaf</td>
<td>3 × 10⁷</td>
<td>&gt;90</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>coleoptile tip</td>
<td>2 × 10⁷</td>
<td>&lt;10</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>greened leaf</td>
<td>2 × 10⁷</td>
<td>&gt;90</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
</tbody>
</table>

* Except in experiment 4, all protoplasts were isolated from dark-grown oat tissue.

For experiment 3, protoplasts were severely plasmolyzed in 1 m mannitol prior to monitoring for cyclosis or phytochrome.
protoplasts from the same harvest as used for Figure 3B were
 treated identically to those in Figure 3B except that they were not
 preirradiated with R. In data not shown here, these cells showed
 an efflux of Ca2+ (viz. ∆A (540 – 507 nm) = −0.002) in response
 to the first R irradiation in the spectrophotometer, which was fully
 reversible by FR.

 DISCUSSION

 Assays of Ca2+ flux by indirect spectrophotometric methods,
such as with the dye murexide, require careful controls, especially
when the changes being measured are small. The A changes
measured in Figures 1–3 were well above the accuracy limits of
the spectrophotometer, and the corresponding changes in Ca2+
concentration (15-45 μM) inferred from the A changes are well
within the documented accuracy range of the dye method (13).
Neither the temperature nor the pH of the medium changed
during the R/FR irradiations; nor can the dye A changes be
ascribed to spectrophotometer drift or photomultiplier tube
artifact, since neither photoytichrome-depleted cells nor Ca-depleted
cells produced any change in A 540 – 507 nm in response to R/
FR (Figs. 3A and 4), indicating a fairly stable base line unaffected
by irradiations. In this regard, Figure 3B has a double control
built in: R does not induce any Ca2+ efflux in cells that already
have their phytochrome in the Pfr form (first R irradiation), and
FR does not induce a Ca2+ influx when there is little or no free
Ca2+ in the medium (first FR irradiation). Further validating the
results is the observation that the quantity of Ca2+ flux detected
was roughly proportional to the quantity of cells concentrated on
the Ficoll pad in the cuvette. For example, there were about 50%
fewer cells used for the data shown in Figure 3B compared to the
number used for Figure 2 and the Ca2+ flux measured is propor-
tionately less.

 The photoreversible changes in Ca2+ concentration that are
observed in Figure 1 could have resulted from Ca2+ transport by the
tissue and/or from the binding and release of Ca2+ from the
coleoptile surfaces. The same two explanations are valid options
for the data in Figure 2, although one might expect that the
removal of the cell walls from the coleoptile cells would reduce
their Ca-binding capacity. As a test of which of these two expla-
nations was more probable, we repeated the experiment in a Ca2+-
free solution, using protoplasts that had been washed free of
externally bound Ca2+. We irradiated the cells with R while they
were in the wash medium, a treatment which, according to the
data in Figures 1 and 2, should release any bound Ca2+, if binding
and release of Ca2+ were the phytochrome-controlled events. The
washed cells showed no response to R/FR light (Fig. 3A). These
results were consistent with a requirement either for the presence
of Ca2+ in the medium during the irradiations or for a prein-
cubation in Ca2+ prior to the irradiations to obtain the photorever-
sible fluxes. To distinguish between these two options, the exper-
iment in Figure 3A was repeated, this time using protoplasts that
were incubated in 2.5 mM Ca2+ and then washed free of externally
bound Ca2+ as in Figure 3A. These cells gave photoreversible
changes in the Ca concentration of the medium, once the phyto-
chrome in them was converted to Pr (Fig. 3B). In the absence of
any exogenous Ca2+, the most likely source of Ca2+ for the R-
induced efflux was the cytosol of the protoplasts. Thus the most
likely explanation for the results of Figure 3B (and Figs. 1 and 2)
is that phytochrome can modulate, directly or indirectly, the
transport of Ca2+ across the plasma membrane of the protoplasts.

 We have no direct information on why the preincubation in
Ca2+ is required. But, as noted in the results, freshly isolated oat
protoplasts take up Ca2+ from the medium when they are first
exposed to R. Given the fact that plants regulate the intracellu-
lar levels of Ca2+ (12), one might infer from this observed
uptake that the protoplasts have some metabolic requirement to
increase their endogenous concentration of Ca2+. If this is so, then,
from Figure 3B, one could argue this same requirement critically
affects the ability of Pfr to promote a Ca2+ efflux.

 Phytochrome has previously been shown to influence the flux of
H+ and K+ in higher plants (3, 4, 14); and the addition of Ca2+
to this list might at first seem to be unrelated to these earlier
reports. But K+-H+ exchange mechanisms are well documented to
be an important function of plant cell membranes (8, 10), and
Rubinstein et al. (15) have shown that Ca ions can influence the
medium acidification by oat coleoptiles. Although their work
specifically studied the effect of externally applied 1 mM Ca2+ on
the rate of medium acidification, one might expect that a change
in the external concentration of Ca surrounding coleoptile cells,
such as reported here, could also influence the proton extrusion
rate and any K+ uptake linked with this extrusion.

 The initial inspiration for these experiments was the observation
of Weisenseel and Ruppert (19) that phytochrome and Ca ions
were involved in the light induced depolarization in Nuitella.
The same laboratory has more recently reported that Pfr promoted
an uptake of 45Ca by Mougeotia following a 3- to 10-min dark
incubation in a Ca-free medium (5). One could attribute the
differences in the direction of Pfr promoted Ca2+ flux in their
results compared to that reported here to differences in the cell
material used (green algae versus etiolated oat coleoptile tips or
protoplasts). However, the timing and conditions for the two sets
of experiments were significantly different, and these differences
also could have influenced the observed direction of the photore-
versible Ca2+ fluxes in the two cell types. To resolve this question
we are testing the feasibility of measuring Ca2+ fluxes in Mougeotia
cells with murexide and of measuring 45Ca fluxes in oat coleoptile
protoplasts.

 Weisenseel and Ruppert argue cogently for the potential signifi-
cance of Ca as a second messenger in phytochrome-controlled
morphogenesis. We offer only one further observation here. If Pfr
does indeed promote an efflux of Ca, and this efflux raises the
concentration of Ca2+ in the cell wall, then these results relate
importantly to the recent report by Tepper and Cleland (18) that
Ca2+ inhibits the acid-induced cell wall loosening of auxin in oat
coleoptiles. Taken together these data would predict that, in
certain tissues at least, Pfr would act antagonistically to auxin.
Reports that suggest this conclusion have appeared frequently in
the literature (cf. review in ref. 17). Though most of these reports
develop the idea that the antagonism derives from a Pfr-induced
decrease in auxin content, many of the data are equally consistent
with the hypothesis that Pfr blocks some physiological effect of
auxin, e.g. cell wall loosening. Confirmation of this hypothesis
would have important consequences for the understanding of
phytochrome-mediated growth responses, such as phototropism
and geotropism, that may also involve the action of auxin.

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