Role of Calcium in Phytochrome-Controlled Nyctinastic Movements of Albizzia lophantha Leaflets

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ABSTRACT

The involvement of Ca2+ on phytochrome-controlled nyctinastic closure in Albizzia lophantha has been studied by testing the effect of the calcium ionophore 6S-[8-[2S*,3S*],8(R*),9S,11a]-5-methyl-amino)-2-[[3,9,11-trimethyl-8-[1-methyl-2-oxo-2-(1H-pyrrol-2-y)]ethyl]-1,7-dioxaaspiro[5.5]-undec-2-yl] methyl]-4-benzoxazolcarboxylic acid (A23187) and the intracellular calcium antagonist 8-(diethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride (TMB-8). An external supply of Ca2+ or calcium ionophore A23187 to the Albizzia leaflets emulates the effect of red light irradiation and counteracts the inhibitory effect of far red light. The intracellular calcium antagonist TMB-8 supplied to Albizzia leaflets inhibits the effect of red light, but had no effect on far red irradiated plants. This suggests a dependence between phytochrome action and intracellular free Ca2+. We suggest that calcium acts as a phytochrome messenger on control of ion fluxes that drive turgor changes in pulvini motor cells.

Albizzia lophantha Benth. leaflets show both nyctinastic and rhythmic movements from a nearly horizontal position (open) during daylight to a vertically folded position (closed) at night. Similar leaflet movements in other related legumes such as Albizzia julibrissin or Samanea saman are controlled by an internal clock that induces a circadian rhythmic oscillation in the leaflets when plants are kept in continuous darkness for several days (19). Both nyctinastic closure (6, 18) and internal clock setting (26) are modulated by phytochrome photoconversion. Turgor changes of pulvini motor cells induced by fluxes of K+ and Cl− drive leaflet movement in these plants (21, 22).

It is well known that phytochrome photoconversion promotes nyctinastic closure of leaflets and resets the internal clock of various systems that have an endogenous circadian rhythm (18, 22, 26); however, the mechanism of phytochrome action is not yet clearly understood. Roux et al. (17) suggest that phytochrome R2 activation can induce an increase in concentration-free Ca2+ in the cytosol and assign the role of a second messenger in triggering R-stimulated responses to this ion. Support for this idea comes from previous research on phytochrome-controlled Ca2+ fluxes in Nitella (30), in isolated mitochondria from oats (17), in Avena coleoptiles (5), and in Onoclea spores (29). Other R-induced physiological responses such as chloroplast rotation in Mougeotia (27) also depend on the presence of Ca2+ in the external medium.

Effects produced by the calcium ionophore A23187 and by calmodulin inhibitors suggest that calcium and calmodulin are involved in nyctinastic closure of Cassia fasciculata (15, 16). However, the relationships between the effects on nyctinastic closure of these two substances and phytochrome photoconversion have not been studied.

According to Satter and Galston (19), phytochrome controls K+ and Cl− fluxes in pulvinar motor cells by changes in intracellular free Ca2+, possibly through a calmodulin-dependent reaction. Calcium can be taken up from the extracellular medium and/or may be released from intracellular stores. In a recent work (10, 11) phosphatidyl inositol turnover has been found to be stimulated by white light in Samanea pulvini, and it has been suggested (1) that it could be involved in calcium release from intracellular stores as in animal tissues.

We have examined the role of calcium as a second phytochrome messenger by testing the effects of the A23187 calcium ionophore (4, 8) and TMB-8 intracellular calcium antagonist (2) on pulvini movements as well as Ca2+ and K+ fluxes during phytochrome-controlled nyctinastic closure of A. lophantha leaflets. If Pfr stimulates nyctinastic closure by increasing intracellular Ca2+ concentration, then any process blocking this increase should inhibit the effects of R and, consequently, any process that artificially increases the concentration of intracellular-free Ca2+ should stimulate leaflet closure. Thus, the activity of the calcium ionophore will emulate the effect of R by increasing free Ca2+ concentration in the cytosol while TMB-8 will inhibit this effect by blocking intracellular calcium. The results presented here show that an external supply of Ca2+ and A23187 mimics the effect of R while the Ca2+ antagonist TMB-8 inhibits the action of Pfr. We suggest that these treatments affect intracellular free Ca2+ which in turn regulates leaflet opening and closing.

MATERIALS AND METHODS

Plant Growth Conditions

Albizzia lophantha Benth. plants were grown outdoors from seeds in pots containing a mixture of garden earth, peat and perlite (1:1:1, v/v). Plants were watered with tap water on...
alternate days and fed from the second week after sowing with N:P:K (15:15:15, w/w) fortnightly. Prior to use in experiments, 12-month-old plants were kept for at least 7 d in a growth chamber under the following conditions: 16 h light/8 h dark cycle at 24 to 28°C. Illumination was provided by white fluorescent tubes (Osram-L Fluora 65W/77R) giving 254 W·m⁻² at the plant apex.

**Light Treatments**

The red light source was a bank of eight 15 W natural-white fluorescent tubes (Sylvania F15/T8/N) each wrapped in one layer of red filter No. 14 Cinemoid (Rank Strand Electrics Ltd, London) and another layer of primary yellow No. 1 Cinemoid. The FR source was a bank of five 150 W incandescent bulbs (Sylvania BRS) filtered through 10 cm of water, one layer of blue filter No. 20 Cinemoid, and one layer of deep orange filter No. 5A Cinemoid. The fluence rates used were R, 4.10 W·m⁻², and FR, 30.35 W·m⁻². A dim green safelight was used in the dark (15 W daylight fluorescent tube Sylvania covered with three layers of green filter No. 39 Cinemoid).

**Measurement of Leaflet Movement**

Effects of A23187 and TMB-8 on leaflet movement were measured as summarized in Figure 1. In the first one (Fig. 1a), adapted from Satter et al. (21), pairs of leaflets were excised at 2 h of photoperiod just prior to experimental use. They were floated with their adaxial side up on 5 mL control or test solutions in Petri dishes and transferred to darkness after R (15 min) or FR (5 min) irradiation. The second method of measurement (Fig. 1b), was adapted from Roblin and Bonnort (14). Pairs of leaflets were excised at 5 h of photoperiod and 3 h before experimental use. They were floated on 5 mL control or test solutions in Petri dishes for 3 h in a growth chamber, and later they were exposed to R (15 min) or FR (5 min) and transferred to darkness in the middle of the photoperiod. All leaflet pairs for a given experiment were taken from the same leaf, normally the fourth or fifth from the plant apex, excluding the four basal and two apical pairs of leaflets. In each case, leaflet angles were estimated at 30 min intervals during a 3 h dark period by comparison with angles on a chart. Initial angles were measured after cutting. When leaflets were allowed to incubate in control or test solutions, in the growth chamber for 3 h, the angles were again measured before exposure to R or FR. In each experiment, 10 leaflet pairs received the same treatment. The results are expressed as degrees of closure (i.e. the difference between the initial and the final angles). Each experiment was repeated at least three times. Graphs summarize all the repetitions and each point represents the mean value ± SE.

**Analysis of Contents of Potassium and Calcium**

After 3 h of incubation in darkness, control and test solutions were collected in plastic vials in order to analyze potassium and calcium content in the medium. Net efflux of Ca²⁺ or K⁺ is the difference between the ion concentration measured in the control solutions without leaflets and those in which the leaflets were incubated for 3 h.

The measurement of calcium levels was performed by atomic absorption spectrophotometry using a Pye Unicam (Philips) model SP 1900 atomic absorption spectrophotometer, wavelength of 422.7 nm, lamp current of 4 mA, slit width of 0.20 mm, and a calibration scale of 0.0 to 2.0 µg/mL. A sodium solution (3000 g·m⁻³) was added in the proportion 1:1 (v/v) to each solution before measurements were taken to assist the release of Ca²⁺ into the solution. The measurement of potassium levels was performed by atomic emission spectrophotometry using a Pye Unicam (Philips) model SP 1900 atomic absorption spectrophotometer, wavelength of 766.5 nm, slit width of 0.02 mm, and a calibration scale of 0.0 to 10.0 µg/mL. A sodium solution (600 g·m⁻³) was added in the proportion 1:1 (v/v) to each solution before measurements were taken to assist the release of K⁺ into the solution.

**Chemicals**

A23187 and TMB-8 were purchased from Sigma (St. Louis, MO). A23187 stock (10 mM) was dissolved in DMSO, TMB-8 stock (10 mM) was dissolved in ethanol. In the experiments, the final DMSO or ethanol concentrations were never higher than 0.1% (v/v) for the highest product concentrations tested. Control sets received the same final DMSO or ethanol concentrations. These concentrations have no effect on modifying leaflet movement. The pH of the solutions was adjusted to 6.5 with 0.1 N NaOH.

**RESULTS**

**Effect of Phytochrome**

Data in Figures 2, 4, 5, 6 and 7 show that leaflets irradiated with a 15 min pulse of R both close to a greater extent and more quickly than leaflets irradiated with a 5 min pulse of FR when moved from light to darkness. The effect is R/FR photoreversible proving that phytochrome photoconversion to Pfř is involved in the control of nyctinastic closure as in other plants (15). However, the rate of nyctinastic closure induced by Pfř is not constant throughout the photoperiod. Under the same training schedule of 16 h light and 8 h darkness, the effect of R irradiation is stronger if the light
pulse preceding the change from light to darkness is applied at 8 h after light onset than at 2 h, as can be seen comparing Figures 4 and 5. These different effects are due to the interaction of circadian rhythm and phytochrome action. If R is applied near the light onset, when the leaflets begin to open, the closure induced by Pfr is smaller than if R is applied in the middle of the photoperiod when leaflets are in a completely open position.

**Effect of Ca\(^{2+}\)**

The extent of nyctinastic closure is greater if *Albizia lophantha* leaflets are incubated in a solution containing Ca\(^{2+}\) when they are moved from light to darkness. This increased closure is proportional to the Ca\(^{2+}\) concentration. Figure 2 shows that this increase, produced by externally supplied Ca\(^{2+}\), also occurs if leaflets are irradiated with R or FR pulses of light. The addition of Ca\(^{2+}\) to the R-irradiated leaflets induces a consistent increase of nyctinastic closure up to maximum possible levels. The addition of Ca\(^{2+}\) to leaflets irradiated with FR completely overrides the inhibitory effect caused by FR irradiation if the concentration of external Ca\(^{2+}\) is at least 10 mM.

Figure 3 shows that the effect of external Ca\(^{2+}\) is exactly the same if it is supplied as nitrate or chloride. However, if Na\(^{+}\), K\(^{+}\), or Mg\(^{2+}\) salts are supplied in an incubation solution, no additional effect of nyctinastic closure occurs. These data suggest that Ca\(^{2+}\) is the main ion involved in triggering the turgor change mechanisms of the pulvinular motor cells, but they also suggest that the effect of calcium is independent of the state of phytochrome since the supply of Ca\(^{2+}\) mimics the effect of R in both R and FR irradiated leaflets.

**Effect of A23187**

The calcium ionophore A23187 supplied to pulvini before R or FR irradiation increases the extent of closure of irradiated leaflets. This increased closure depends on the ionophore concentration (i.e., it increases when concentration rises from 1–10 mM) and also on the global effect of R. This effect is even more pronounced if the ionophore is supplied at the beginning of the photoperiod (Fig. 4) when the effect of phytochrome photoconversion is at a low level and is not strong enough to achieve a complete closure of the leaflets. However, this effect is not apparent when R effect is maximum and causes the leaflets to completely close in the middle of the photoperiod (Fig. 5). On the other hand, the promotion of closure of FR-irradiated leaflets due to A23187 is evident after 30 min of darkness or after 2 h of darkness depending on whether experiments were carried out at the beginning or in the middle of the photoperiod (c.f. Figs. 4 and 5).

Table I shows the influence of A23187 on calcium concentration in the external medium. It is conspicuous that the content of calcium in A23187 solutions is lower than in the control solution (0 μM A23187). This effect is independent of phytochrome photoactivation.

A23187, however, does not drastically alter K\(^{+}\) flux (Table II). K\(^{+}\) efflux from control material does not differ from samples which were kept in A23187 when the ionophore is applied immediately before R or FR irradiation. K\(^{+}\) concentration of the control solution is slightly lower than A23187.
Figure 4. Effect of the Ca\textsuperscript{2+} ionophore A23187 on the phytochrome-controlled nyctinastic closure of A. lophantha at 2 h of photoperiod. Leaflet pairs floated on A23187 0 μM (——), 1 μM (-----), or 10 μM (.....). Each point represents the mean value of three experiments. Vertical bars represent SE. R, Red irradiated leaflets; FR, far red irradiated leaflets. See Figure 1 for other symbols.

Figure 5. Effect of Ca\textsuperscript{2+} ionophore A23187 on the phytochrome-controlled nyctinastic closure of A. lophantha at 8 h of photoperiod. Leaflet pairs incubated on A23187 0 μM (——) or 10 μM (.....). Each point represents the mean value of three experiments. Vertical bars represent SE. R, Red irradiated leaflets; FR, far red irradiated leaflets. See Figure 1 for other symbols.

Table I. Effect of A23187 on Ca\textsuperscript{2+} Concentration in the External Medium

Leaflets were floated in A23187 solutions immediately (a) or 3 h prior (b) to red or far red irradiation and then moved to darkness. Light-dark transition at 2 h (a) or 8 h (b) of photoperiod. Solutions were collected after 3 h of darkness and analyzed for Ca\textsuperscript{2+} by atomic absorption spectrophotometry. The Ca\textsuperscript{2+} flux was calculated from the difference of Ca\textsuperscript{2+} content between solutions with and without leaflets. +, Efflux; −, influx. Data represent mean values ± SE (n = 3).

<table>
<thead>
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<th>Treatment</th>
<th>External A23187</th>
<th>Ca\textsuperscript{2+} Concentration</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td>R</td>
</tr>
<tr>
<td>a</td>
<td>0</td>
<td>+ 24.97 ± 0.83</td>
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<tr>
<td></td>
<td>1</td>
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<tr>
<td></td>
<td>10</td>
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<tr>
<td>b</td>
<td>0</td>
<td>+ 18.27 ± 1.83</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>+ 14.42 ± 1.59</td>
</tr>
</tbody>
</table>

Table II. Effect of A23187 on K\textsuperscript{+} Concentration in the External Medium

Leaflets were floated in A23187 solutions immediately (a) or 3 h prior (b) to red or far red irradiation and then moved to darkness. Light-dark transition at 2 h (a) or 8 h (b) of photoperiod. Solutions were collected after 3 h of darkness and analyzed for K\textsuperscript{+} by atomic emission spectrophotometry. The K\textsuperscript{+} flux was calculated from the difference of K\textsuperscript{+} content between solutions with and without leaflets. +, Efflux; −, influx. Data represent mean values ± SE (n = 3).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>External A23187</th>
<th>K\textsuperscript{+} Concentration</th>
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</thead>
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<td></td>
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<td></td>
<td>10</td>
<td>+ 83.26 ± 2.08</td>
</tr>
</tbody>
</table>

solutions when the chemical is applied 3 h before R or FR irradiations.

Effect of TMB-8

Figure 6 compares the extent of closure of R-, and FR-irradiated leaflets floated on control (0 μM TMB-8) and TMB-8 (10 and 100 μM) solutions. Externally applied TMB-8 had no effect on FR-irradiated leaflet movement but diminished the extent of closure of R-irradiated leaflets. An inhibitor effect is apparent from the first 30 min following R to the end of the experiment. The TMB-8 inhibitor effect does not increase when concentration raises from 10 to 100 μM. Both concentrations show a similar effect and mimic FR irradiation.

Figure 7 shows that the results were quite similar when the experiment was performed at 8 h of photoperiod, when a preincubation time of 3 h before irradiation of samples was allowed for. At this time of the photoperiod the effect of phytochrome is maximum and the inhibition of the closure

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induced by TMB-8 does not completely override the effect of R irradiation.

Externally applied TMB-8 slightly increases K⁺ (Table III) and Ca²⁺ (Table IV) efflux. This effect is independent of phytochrome photoactivation.

**DISCUSSION**

The effect of R and FR irradiation on nyctinastic closure of *Albizia julibrissin* leaflets clearly indicates that it is controlled by phytochrome photoconversion as in *Albizia julibrissin* and in other related plants (6, 19). *Albizia* leaflets also move with circadian rhythmicity and the phytochrome effect interacts with this rhythmic movement. This movement is controlled by an internal clock as in *Samanea saman* (26) and *Robinia pseudoacacia* (our unpublished data), and the immediate effect of phytochrome on closure depends on the phase of the rhythm at which the R pulse is applied. Thus, if R is applied at the beginning of the opening movement (i.e., 2 h after light onset) the effect on nyctinastic closure is very small compared with the effect produced if R irradiation is applied when the leaflets are completely open in the middle of the photoperiod. These data seem to be in disagreement with the results in *A. julibrissin* reported by Hillman and Koukkari (6) who found a maximum phytochrome effect 2 h after light onset. However, in their experiment plants were trained in a 12 h light/12 h dark schedule, whereas in our experiments the training schedule was 16 h light and 8 h of darkness. Thus, differences in the maximum effect of phytochrome could be due to a difference of rhythm phase, i.e. photoperiod will not start in the same phase of the rhythm in the two experiments.

An external supply of 10 mm Ca²⁺ solution mimics the effect of R light and if it is supplied to R-irradiated leaflets produces an additional closure effect to that caused by R irradiation. The Ca²⁺ effect depends on the concentration and for concentrations ≥10 mm is stronger on FR- than R-irradi-
Table IV. Effect of TMB-8 on Ca\textsuperscript{2+} Concentration in the External Medium

<table>
<thead>
<tr>
<th>Treatment</th>
<th>External TMB-8</th>
<th>Ca\textsuperscript{2+} concentration</th>
<th>R</th>
<th>FR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µM</td>
<td>µM</td>
<td></td>
<td>SE</td>
</tr>
<tr>
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<td>+ 11.98 ± 1.02</td>
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</tr>
<tr>
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<td>1</td>
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</tr>
<tr>
<td></td>
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<td>+ 14.47 ± 0.81</td>
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</tr>
<tr>
<td>b</td>
<td>0</td>
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<td>+ 26.43 ± 2.72</td>
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</tr>
<tr>
<td></td>
<td>1</td>
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<td>+ 31.40 ± 4.13</td>
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<tr>
<td></td>
<td>10</td>
<td>+ 29.48 ± 2.31</td>
<td>+ 34.02 ± 0.57</td>
<td></td>
</tr>
</tbody>
</table>

Leaflets were floated in TMB-8 solutions immediately (a) or 3 h prior (b) to red or far red irradiation and then moved to darkness. Light-dark transition at 2 h (a) or 8 h (b) of photoperiod. Solutions were collected after 3 h of darkness and analyzed for Ca\textsuperscript{2+} by atomic absorption spectrophotometry. The Ca\textsuperscript{2+} flux was calculated from the difference of Ca\textsuperscript{2+} content between solutions with and without leaflets. +, Efflux; −, influx. Data represent mean values ± SE (n = 3).

account as a longer incubation time could produce side effects, such as disruption of Ca\textsuperscript{2+} homeostasis, which would affect the movement of FR irradiated leaflets (Fig. 5).

A23187 is a Ca\textsuperscript{2+}/H\textsuperscript{+} exchanger (13) and some effects attributed to an increase in cytosolic free Ca\textsuperscript{2+} could be due to pH changes. The activity of a H\textsuperscript{+} pump has been correlated with K\textsuperscript{+} fluxes and turgor changes in *Samanea* (7), but the relationship between changes in the H\textsuperscript{+} extrusion rate and cytoplasmic pH is unknown. However, this possible side effect of the ionophore does not seem to be relevant in *A. lopanthana* since H\textsuperscript{+} extrusion caused by fuscoaicin is related to an inhibition of closure (our unpublished data) as occurs in *Mimosa pudica* (12).

On the other hand, the intracellular Ca\textsuperscript{2+} antagonist TMB-8 counteracts the effect of Pfr by decreasing the extent of leaflet closure (Figs. 6 and 7). However, the effects of TMB-8 are not independent of phytochrome photoconversion and the Ca\textsuperscript{2+} antagonist has no effect on FR irradiated leaflets; thus, Pfr is necessary to show the effect of TMB-8. Furthermore, its effect is maximum when nyctinastic closure and the Pfr effect are also maximum at 8 h after light onset, but then TMB-8 does not completely inhibit the Pfr effect an R irradiated leaflets incubated in TMB-8 close 10% more than those irradiated with FR light.

Phytochrome photoconversion controls the uptake of free Ca\textsuperscript{2+} from the apoplasm according to the model suggested by Satter and Galston (19), but this Pfr-mediated Ca\textsuperscript{2+} transport across the plasmalemma has not been proved up to now. Our data clearly suggest that Pfr uses Ca\textsuperscript{2+} to promote closure but whether or not Ca\textsuperscript{2+} is mobilized from intracellular stores is not clear. A net difference in external Ca\textsuperscript{2+} uptake between R- and FR-irradiated leaflets that were only incubated in water and used as controls is not apparent. However, TMB-8 increases the concentration of Ca\textsuperscript{2+} in the external medium which suggests that the Ca\textsuperscript{2+} antagonist may inhibit extracellular Ca\textsuperscript{2+} uptake (Table IV). Schumaker and Sze (23) found that TMB-8 calcium antagonist blocks the inositol 1,4,5 triphosphate induced Ca\textsuperscript{2+} release from tonoplastic vesicles of oat roots, and more recently Morse et al. (10, 11) found that Ca\textsuperscript{2+} must be released from symplast pools by inositol phospholipid turnover in *Samanea* pulvini since white light increases this turnover. Thus, mobilization of Ca\textsuperscript{2+} could be used to trigger K\textsuperscript{+} fluxes in *Albizia* pulvinal motor cells and this mobilization may be regulated by Pfr which can release Ca\textsuperscript{2+} from symplast pools.

However, externally applied Ca\textsuperscript{2+} promotes pulvinal closure and mimics the effect of phytochrome photoconversion. A possible explanation for this is that Pfr promotes Ca\textsuperscript{2+} transport across cellular membranes and that under certain conditions the Ca\textsuperscript{2+} mobilized by Pfr could come from the extracellular medium. This uptake or utilization can be blocked by TMB-8.

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LITERATURE CITED


