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The role of calcium in blue-light-dependent chloroplast movement in *Lemna trisulca* L.

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Summary

Chloroplast movements are a normal physiological response to changes in light intensity and provide a good model system to analyse the signal transduction pathways following light perception. Blue-light-dependent chloroplast movements were observed in *Lemna trisulca* using confocal optical sectioning and 3-D reconstruction or photometric measurements of leaf transmission. Chloroplasts moved away from strong blue light (SBL) towards the anticlinal walls (profile position), and towards the periclinal walls (face position) under weak blue light (WBL) over about 20–40 min. Cytoplasmic calcium ($[Ca^{2+}]_{cyt}$) forms part of the signalling system in response to SBL as movements were associated with small increases in $[Ca^{2+}]_{cyt}$ and were blocked by antagonists of calcium homeostasis, including EGTA, nifedipine, verapamil, caffeine, thapsigargin, TFP (trifluoperazine), W7 and compound 48/80. Treatments predicted to affect internal Ca^{2+} stores gave the most rapid and pronounced effects. In addition, artificially increasing $[Ca^{2+}]_{cyt}$ in darkness using the Ca^{2+} ionophore A23187 and high external Ca^{2+} (or Sr^{2+}), triggered partial movement of chloroplasts to profile position analogous to a SBL response. These data are all consistent with $[Ca^{2+}]_{cyt}$ acting as a signal in SBL responses; however, the situation is more complex given that both WBL and SBL responses were inhibited to a similar extent by all the Ca^{2+}-signalling antagonists used. As the direction of chloroplast movement in WBL is exactly opposite to that in SBL, we conclude that, whilst proper regulation of $[Ca^{2+}]_{cyt}$ homeostasis is critical for both SBL and WBL responses, additional factors may be required to specify the direction of chloroplast movement.

Introduction

Light is central to plant growth and development as it provides both an energy supply, through photosynthesis, and information on the local environment. With appropriate signal transduction systems, plants interpret and respond to the prevailing light regime and modify their development and physiology accordingly. Useful information may be present in the light intensity, wavelength, polarization angle or duration (Hangarter, 1997; Haupt and Scheuerlein, 1990; Horwitz and Berrocal, 1997; Jenkins *et al.*, 1995; Trewavas and Malho, 1997; Yatsuhashi, 1996; Zurzycki, 1980). Perception of the light signal involves specific photoreceptors that absorb the light, become photochemically changed and are subsequently coupled to the downstream signal transduction system. Although knowledge about the whole transduction chain is still incomplete, calcium has already been implicated as an important component following light absorption by both phytochrome and/or blue-light photoreceptor(s) (Khurana *et al.*, 1998 and references therein).

Calcium is present in all compartments of the cells, but its resting concentration in the cytoplasm ($[Ca^{2+}]_{cyt}$) is very low at about 0.1 μM. The external concentration is of the order of 1 mM, so calcium homeostasis within the cells is maintained by the action of channels, pumps and transporters and by accumulation in intracellular stores or organelles (chloroplasts, mitochondria, vacuoles) (Bush, 1993; Evans *et al.*, 1991; Schroeder and Thuleau, 1991). Changes in $[Ca^{2+}]_{cyt}$ have been reported in response to stimuli such as light, gravity, phytohormones (Hepler and Wayne, 1985) or touch and wind (Trewavas and Knight, 1994). The information from the initial stimulus may be transformed into the magnitude, duration and/or speed of $[Ca^{2+}]_{cyt}$ changes or into the spatial organization of elevated $[Ca^{2+}]_{cyt}$. The $[Ca^{2+}]_{cyt}$ changes are then interpreted by the effector system. The source of calcium involved in signalling may vary, and may include cycling through the plasma membrane or internal stores, such as ER or vacuole (Dawson, 1990). Signalling through calcium may also involve the inositol triphosphate ($InsP_3$) pathway, which can mobilize the vacuolar Ca^{2+} pool in plants by opening channels in the tonoplast (Alexandre and Lassalles, 1992; Allen and Sanders, 1994; Drøbak, 1991; Muir *et al.*, 1997).

Here we have focused on the role of Ca^{2+} in blue-light-dependent chloroplast movement as a model system to dissect out the signalling events underlying a physiologically important blue-light response. In *Lemna,* for example, chloroplasts are distributed randomly around all the cell walls in darkness. In weak blue light (WBL), they move towards the periclinal cell walls perpendicular to the light direction to obtain face position, and in strong blue light (SBL) to the anticlinal walls parallel to the light direction to reach profile position (Zurzycki *et al.*, 1983).

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Responses are rapid, being complete within 20–40 min, are easily observed under the microscope and readily manipulated.

Changes in $[Ca^{2+}]_{cyt}$ have been implicated in the movement of the single chloroplast mediated by phytochrome in the alga *Mougeotia* (Haupt and Schönbohm, 1970; Haupt and Weisenseel, 1976; Haupt, 1982; Wagner and Klein, 1978). In this species, movements can be triggered by artificially increasing plasma membrane calcium fluxes using ionophores (Serlin and Roux, 1984); however, there is also evidence that calcium is released from internal stores (Russ *et al.*, 1991; Schönbohm *et al.*, 1990). In most other cells, these stores are typically the ER or vacuole (Bush, 1995); however, in *Mougeotia*, additional calcium-binding vesicles have been identified (Grolig and Wagner, 1987; Wagner and Rossbacher, 1980) and appear to be required for chloroplast movement (Grolig and Wagner, 1989). There is also evidence of calcium transport across the thylakoid membrane of chloroplasts (Evans *et al.*, 1991; Kreimer *et al.*, 1985) regulated by the Ca^{2+}/H^{+} antiporter (Ettinger *et al.*, 1999), but the importance of this system has not yet been investigated in chloroplast movements. The molecular details of the subsequent signal transduction cascade following increases in $[Ca^{2+}]_{cyt}$ have yet to be determined, except that the final effector system appears to involve a functional actin cytoskeleton (Blatt and Briggs, 1980; Blatt *et al.*, 1980; Kadota and Wada, 1992a; Malec *et al.*, 1996).

The possible connections between chloroplast movement and $[Ca^{2+}]_{cyt}$ in plants that are sensitive to blue light have been investigated in some species. Thus, treatments which change $[Ca^{2+}]_{cyt}$ alter the pattern of chloroplast movement in *Eremosphaera viridis* (Weidinger and Ruppel, 1985), *Lemna trisulca* (Tlałka and Gabryś, 1993) and *Adiantum* cells (Kadota and Wada, 1992b). In the latter case, both phytochrome and blue-light-mediated responses are affected. In *Mougeotia*, increases in calcium triggered by UV/blue light increase the velocity of chloroplast re-orientation in comparison to red light (Russ *et al.*, 1991).

In this paper, we specifically set out to test the extent that $[Ca^{2+}]_{cyt}$ can be considered as part of the signal transduction network controlling blue-light-dependent chloroplast movements in *Lemna trisulca*. Given that the response is freely reversible depending on the light intensity, it was also of great interest to test whether responses to WBL and SBL were pharmacologically distinct. We have adopted the three criteria initially set out by Jaffe (1980) to assess the importance of calcium signalling, namely that (i) the physiological response should be accompanied by a change in calcium, (ii) artificially increasing calcium should stimulate the response, and (iii) blocking the change in calcium should inhibit the response.

Results

Movement of chloroplasts in *Lemna* was recorded either directly by visualizing chloroplast autofluorescence using confocal microscopy or indirectly by the photometric measurement of changes in light transmission (ΔT) associated with changes in chloroplast position. Collection of confocal serial optical sections free from out-of-focus blur through the cells provided clear images of the three-dimensional (3-D) disposition of chloroplasts throughout the tissue in different light conditions after correction for depth and sample-dependent attenuation (Figure 1a). Representative images from at least 10 separate experiments are shown as height-coded projections, with chloroplasts closest to the viewer coded as white and those furthest away coded as dark. Differences in chloroplast positions between dark, face and profile arrangements were readily apparent in these views. Photometric analysis of light transmission through the tissue provided quantitative measurements of chloroplast movements. Schematic traces from at least six photometry experiments using continuous WBL followed by SBL or pulse blue light (PBL) are presented (Figure 1b,c), together with the parameters used to describe the chloroplast movement. The sign convention we have used relates to the change in the parameter measured, thus $\Delta T_{(+)}$ indicates an increase in light transmission. This convention differs from previous authors where positive values were associated with movement towards the light (Ślesak and Gabryś, 1996; Trojan and Gabryś, 1996). Transmission values are expressed as changes with respect to the dark value. The typical traces shown in Figure 1 have been included in subsequent figures for comparison.

The effects of artificially increasing $[Ca^{2+}]_{cyt}$ on chloroplast movement

There was no net chloroplast movement in physiological darkness when the leaf was only irradiated with the measurement beam (data not shown). Rapidly increasing the external concentration of Ca^{2+} or the calcium analogue Sr^{2+} 10-fold immediately stimulated a small increase in leaf transmission in the absence of a light stimulus (Figure 2a,b, Table 1). The direction of the response was analogous to the movement to profile observed in SBL but not WBL (compare with Figure 1); however, the magnitude was only around 7% (Ca^{2+}) or 15% (Sr^{2+}) of the average $\Delta T_{(+)}$ for the SBL response measured photometrically (Table 1).

As the normal calcium homeostatic mechanisms may have been able to accommodate these changes in external calcium, the calcium ionophore, A23187, was also used to increase the permeability of the membranes to calcium. Addition of Ca^{2+} in the presence of A23187 enhanced both

the rate and magnitude of the Ca^{2+}-dependent response in darkness (Figure 2b). With 10 mM Ca^{2+} and 3.8 μM A23187, the change in transmission was 32% of a full SBL response and was associated with movement of chloroplasts towards the anticlinal walls (Figure 2a).

To determine the extent that $[Ca^{2+}]_{cyt}$ changed during ionophore treatment, we estimated the changes in $[Ca^{2+}]_{cyt}$ using fluorescent techniques. A large number of methods and dyes were tested following the protocols described in (Fricker *et al.*, 1999) but virtually all proved unsuccessful with *Lemna*. These included mechanical scrubbing of the leaf cuticule, enzyme digestion, vacuum infiltration, microinjection or AM-ester (acetoxymethyl ester) loading using a wide range of Ca^{2+} dyes including Fluo-3, calcium green, Fura Red and calcium orange (data not shown). Some cytoplasmic loading was achieved with the calcium-sensitive fluorescent indicator Fluo-3, following treatment with cutinase and low-pH loading. Low-pH loading was used in preference to AM-ester loading, as the residual cutinase remaining after the wash stages was sufficient to hydrolyse the AM-esters. The dye was localized in the cytoplasm and small vesicles initially (Figure 2c) and accumulated in the vacuole after a variable length of time, ranging from 30 min to 24 h. The resting cytoplasmic calcium level was estimated using the Ca^{2+}-sensitive fluorescence indicator, Fluo-3, as 120 ± 50 nM (n = 7) following *in situ* calibration. After treatment with low concentrations (3.8 μM) of A23187, there was a global increase in $[Ca^{2+}]_{cyt}$ to a relatively stable plateau around 300 nM (n = 6) (Figure 2d). *In situ* calibrations required threefold higher concentrations of ionophore and 10 mM Ca^{2+} to drive the fluorescence to F_{max}.

The activity of A23187 is pH-dependent and increases with increasing pH (Hell and Donath, 1990). To test whether the relatively small response observed even in the presence of A23187 was a consequence of the relatively low pH of the medium (pH 6.0), the effects of varying external pH were examined. The magnitude of the transmission response induced by A23187 and Ca^{2+} was similar between pH 4.0 and pH 7.0; however, the rate of movement decreased sharply above pH 6.0 (Table 1).

Interestingly, chloroplasts still responded to continuous WBL after treatment with Ca^{2+}–A23187 or Sr^{2+} (data not

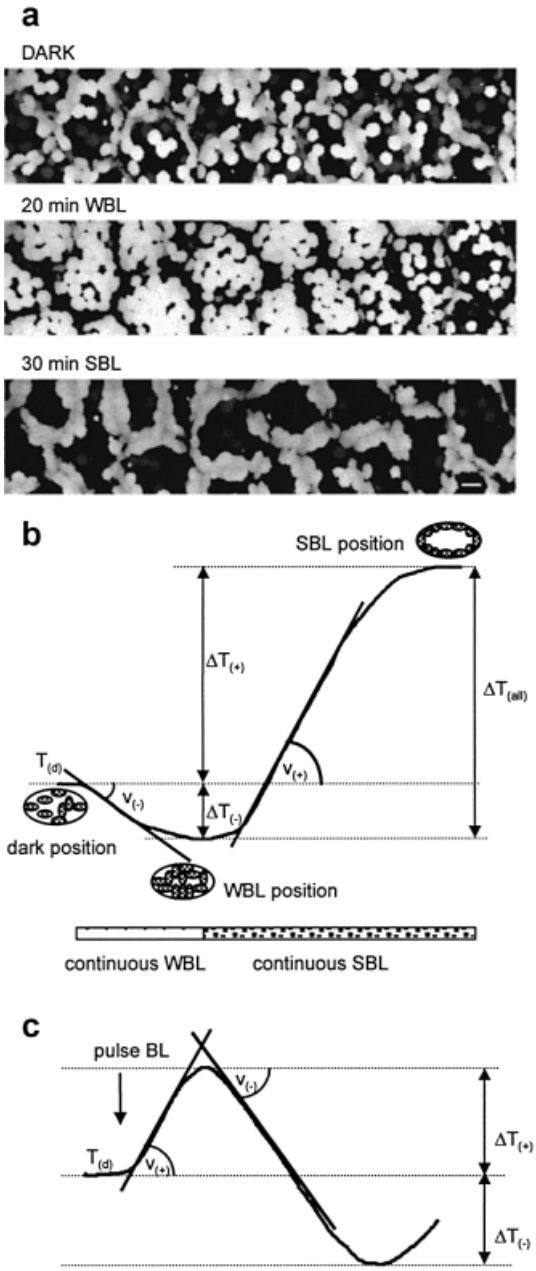


Figure 1. Chloroplast movement in *Lemna* in response to continuous and pulse blue light.

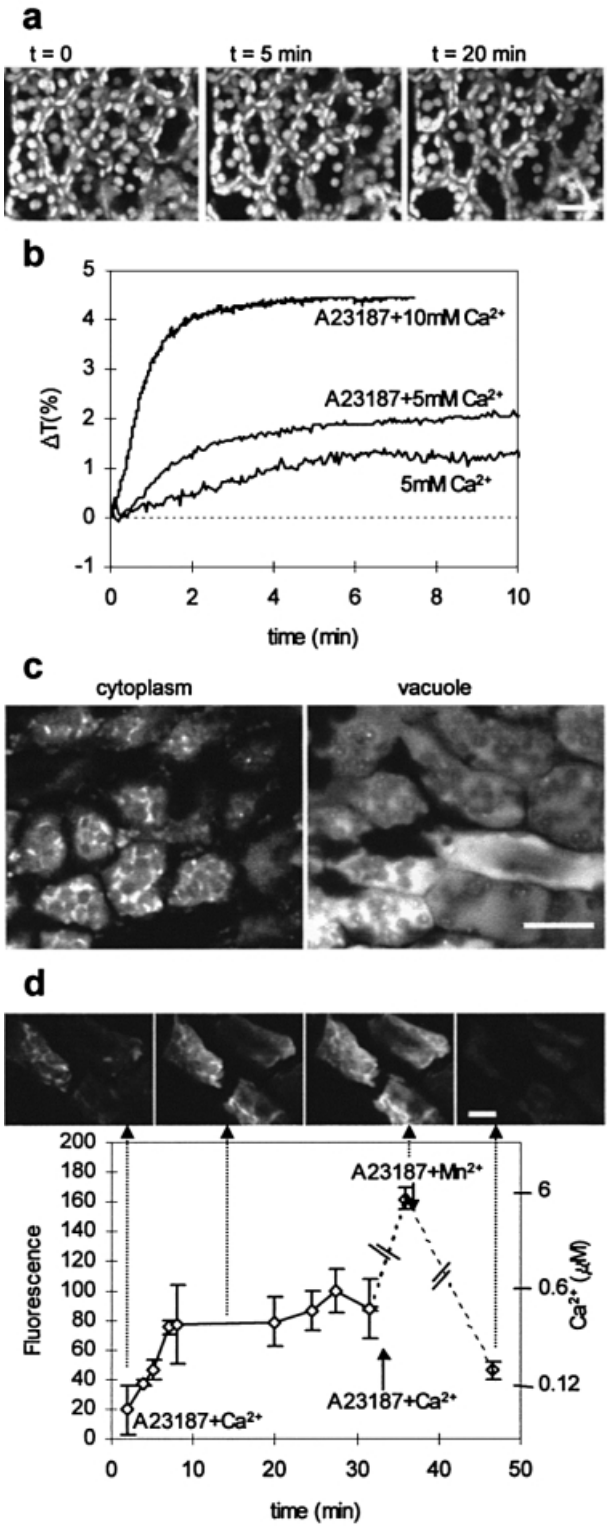
(a) Dark-adapted leaves of *Lemna trisulca* were exposed first to continuous WBL and after completion of this response, to SBL. Chloroplasts were visualized from projections of serial optical sections collected using confocal microscopy. Dark-adapted chloroplasts were distributed randomly around all the cell walls. In WBL they moved towards the periclinal walls and reached the face position. In the subsequent SBL irradiation, chloroplasts re-oriented towards the anticlinal cell walls to reach profile position. Each 3-D image was collected as 50 optical sections spaced at 1 μm, using a ×25 lens, with excitation at 543 nm and emission at >575 nm. Images were displayed as height-coded projections after *z*-attenuation correction. The scale bar represents 10 μm.

(b) Changes in light transmission during the movement were recorded photometrically and the measurement was characterized by the following parameters: $T_{(d)}$, light transmission for the dark-adapted leaf; $\Delta T_{(-)}$, the decrease in transmission from $T_{(d)}$ in response to WBL; $\Delta T_{(+)}$, the increase in transmission in response to SBL; $\Delta T_{(all)}$, the total change in transmission; $v_{(-)}$ and $v_{(+)}$, the maximum rate of transmission changes in WBL and SBL, respectively.

(c) Chloroplast movements in response to a 10 sec blue light pulse were characterized by the following parameters: $T_{(d)}$, the light transmission for the dark adapted leaf; $\Delta T_{(+)}$, the relative increase in transmission from $T_{(d)}$ in the first phase; $\Delta T_{(-)}$, the decrease in the transmission in the second phase; $v_{(+)}$ and $v_{(-)}$, the maximum rate of transmission change for each phase.

shown) giving an immediate decrease in transmission with characteristics similar to a normal WBL response.

As the artificial increase of calcium caused movement of chloroplasts typical for SBL responses (see above), we have tried to establish whether the SBL stimulus itself was able to increase calcium within the cells. In most cases, fluorescence was measured from cytoplasm and vesicles together due to difficulties in separating signals from the



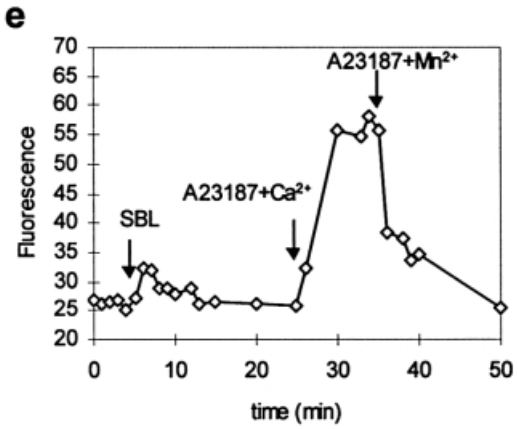


Figure 2. The role of calcium in chloroplast movement in Lemna.

(a) Visualization of chloroplast redistribution over time in a *Lemna* leaf treated with calcium ionophore A23187 and Ca^{2+} in physiological darkness and recorded by confocal microscopy. A dark-adapted *Lemna* leaf ($t=0$) was exposed to 10 mM Ca^{2+} and 3.8 μM A23187 and the position of chloroplasts was detected over time. Two time points ($t=5$ and 20 min) are presented from a series of 5. Chloroplasts positions were imaged from their autofluorescence with excitation using a 543 nm laser which did not induce chloroplast movements. Each image is a height-coded projection of 12 optical sections spaced at 3 μm, collected with a ×60 lens after *z*-attenuation correction. Movements of chloroplasts towards the anticlinal cell walls were observed. The scale bar represents 25 μm.

(b) Responses of dark-adapted chloroplasts to treatment with Ca^{2+} or Ca^{2+} and calcium ionophore A23187. All recordings of the light transmission changes took place in physiological darkness, i.e. only the red light measuring beam, within 1 min of the stimulus application. Representative curves from $n \geqslant 3$ experiments for each treatment are presented.

(c) Fluorescence imaging of Fluo-3 in *Lemna* cells. Cells were loaded with the free acid form of 12 μM Fluo-3 at pH 4.5 following a cutinase pre-treatment. Confocal optical sections of the cells were collected for a single (x,y) plane over time. The dye accumulated in the cytoplasm and small vesicles and no significant fluorescence was detected in the idioblasts containing calcium oxalate. Compartmentalization of Fluo-3 in the vacuoles occurred after 60 min. Strong fluorescence was then observed in the idioblast. Fluo-3 was imaged with laser excitation at 488 nm, using a ×60 lens, and fluorescence was observed at 540 ± 15 nm together with chloroplast autofluorescence at >570 nm. The scale bar represents 25 μm.

(d) Changes in calcium after ionophore treatment measured with Fluo-3. After treatment with 3.8 μM A23187, calcium increased from the resting level around 100 nM to a stable plateau around 400 nM. Calibration of the fluorescence was performed using higher levels of ionophore (10 μM), followed by Mn^{2+} to quench the signal. Each time point is an average of the signals from three different regions of the leaf and is given as the mean ± SEM.

(e) Blue-light response in Fluo-3-loaded cells of *Lemna*. Loading conditions were as in (d). An increase of fluorescence signal was observed in the first 2 min after SBL application, followed by a decrease. *In situ* calibration was attempted with A23187 in the presence of high Ca^{2+} (10 mM) followed by Mn^{2+} (10 mM) to quench the signal. However, in this case, the signal did not appear to reach F_{max}, reflecting the difficulty in calibrating Fluo-3 in *Lemna*.

Table 1. Characteristic parameters from photometric measurements of chloroplast movement in response to Ca^{2+}, Sr^{2+}, calcium ionophore, thapsigargin and caffeine treatments

Treatment	Change in transmission from $T_{(d)}$ in response to blue light, ΔT [a]	% of the control ΔT	Rate of transmission changes in response to blue light, v [b]	% of the control v
Chloroplast movement in response to WBL				
Control WBL	–4.8 ± 0.3 (6)	100	–0.32 ± 0.03 (6)	100
Caffeine (0.5–1 mM, 2 min)	–1.3 ± 0.2 (3)	27	–0.15 ± 0.06 (3)	47
Caffeine (0.5–1 mM, 2 h)	0 (3)	0	0 (3)	0
Caffeine (20 mM, 2 min)	0 (3)	0	0 (3)	0
Thapsigargin (0.15 μM, 2 min)	–3.1 ± 0.8 (3)	65	–0.30 ± 0.07 (3)	94
Thapsigargin (15 μM, 2 min)	–2.9 ± 0.7 (3)	60	–0.26 ± 0.09 (3)	81
Chloroplast movement in response to SBL				
Control SBL	14.1 ± 1.3 (6)	100	1.08 ± 0.04 (6)	100
Caffeine (0.5–1 mM, 2 min)	2.6 ± 0.4 (3)	18	0.28 ± 0.08 (3)	26
Caffeine (0.5–1 mM, 2 h)	0 (3)	0	0 (3)	0
Caffeine (20 mM, 2 min)	0 (3)	0	0 (3)	0
Thapsigargin (0.15 μM, 2 min)	11.6 ± 0.5 (3)	82	0.98 ± 0.04 (3)	91
Thapsigargin (15 μM, 2 min)	4.1 ± 1.3 (4)	29	0.45 ± 0.16 (4)	42
Chloroplast movement to profile position in physiological darkness				
Ca^{2+} (5 mM pH 6.0)	1.0 ± 0.2 (3)	7	0.18 ± 0.05 (3)	17
Sr^{2+} (10 mM pH 6.0)	2.1 ± 0.4 (6)	15	0.32 ± 0.08 (6)	30
A23187 (pH 4.6), 5 mM Ca^{2+}	3.2 ± 0.1 (3)	23	0.39 ± 0.08 (3)	36
A23187 (pH 6.0), 5 mM Ca^{2+}	2.7 ± 0.4 (5)	19	0.64 ± 0.14 (5)	59
A23187 (pH 7.4), 5 mM Ca^{2+}	2.1 ± 0.5 (3)	15	0.07 ± 0.02 (3)	6
A23187 (pH 6.0), 10 mM Ca^{2+}	4.5 ± 0.2 (3)	32	1.31 ± 0.18 (3)	121
Chloroplast movement in response to PBL – first phase				
Control	2.5 ± 0.3 (3)	100	1.07 ± 0.35 (3)	100
Thapsigargin (0.15 μM, 2 min)	0.8 ± 0.3 (4)	32	0.28 ± 0.13 (4)	26
Thapsigargin (15 μM, 2 min)	1.0 ± 0.4 (5)	40	0.25 ± 0.09 (5)	23
Chloroplast movement in response to PBL – second phase				
Control	–3.6 ± 0.6 (3)	100	–0.60 ± 0.19 (3)	100
Thapsigargin (0.15 μM, 2 min)	–0.7 ± 0.6 (3)	19	–0.16 ± 0.08 (4)	27
Thapsigargin (15 μM, 2 min)	–1.1 ± 0.9 (3)	31	–0.18 ± 0.05 (4)	30

Each value was calculated as the mean ± SEM, the number of experiments is given in parentheses. For comparison, the parameters for the control experiments are included. Values of the change in transmission and rate are also expressed as percentages of the controls.
[a]Units are % of transmission; [b]Units are % of transmission per min.

individual components. Small increases in Fluo-3 fluorescence were observed within 2–3 min after SBL in 7 out of 10 experiments (Figure 2e). The response was around two- to threefold increase in resting levels and did not appear to be spatially localized in the cytoplasm. The fluorescent Ca^{2+}-imaging techniques used allowed limited measurements of $[Ca^{2+}]_{cyt}$ responses; however, the loading technique itself appeared to interfere with the normal physiology of the cells as in most cases only limited chloroplast movements were observed.

Effect of treatments to reduce the influx of external calcium

Prolonged (18 h) incubation in 1 mM EGTA completely inhibited chloroplast movements in response to both WBL and SBL (Figure 3a). Inhibition was partially reversed after extensive (12–24 h) wash-out of the EGTA solution. Shorter EGTA treatments (0.5–1 h) at lower concentrations (0.5 mM) inhibited SBL responses to a marginally greater extent than WBL responses, although no consistent effects could be observed if the incubation period was less than about 30 min (data not shown).

Partial inhibition of PBL responses was achieved with the calcium-channel blockers nifedipine (30 μM) and verapamil (20 μM) after 1–3 h incubation (Figure 3b). There was an overall trend showing that the longer the incubation time, the greater the inhibition of chloroplast movement observed; however, there was no evidence for a differential effect on the SBL and WBL components of the response with these inhibitors.

Investigation of the role of internal calcium pools

To test whether internal pools of Ca^{2+} might contribute to light responses, the effects of caffeine and thapsigargin

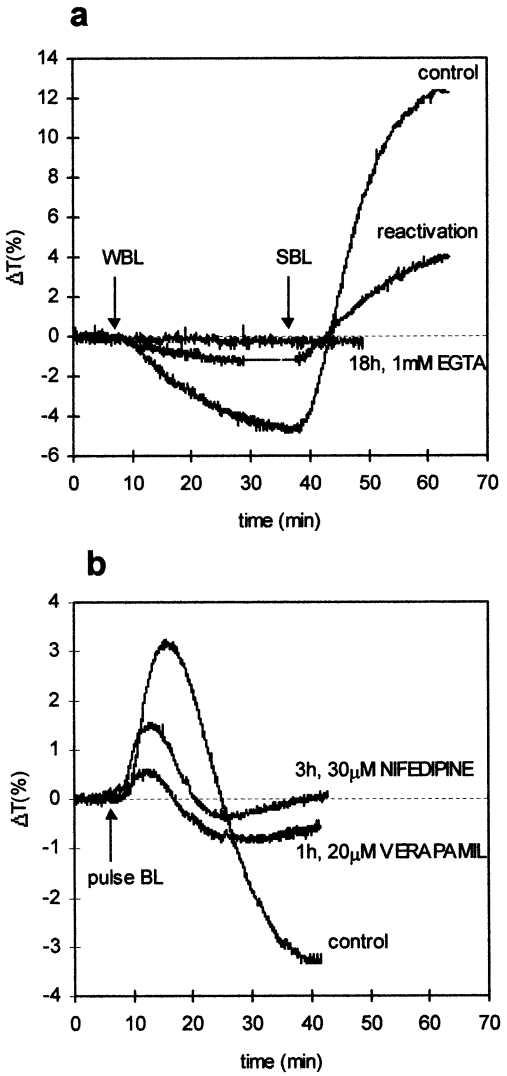


Figure 3. Effects of chelating external Ca^{2+} or blocking Ca^{2+} influx on chloroplast movement.
(a) The response of the dark-adapted chloroplasts to continuous WBL and SBL was blocked in the presence of EGTA (1 mM, 18 h). Partial reactivation was obtained after wash-out in calcium-containing medium for 12 h. The curves were co-aligned after completion of the WBL response for easier comparison of the subsequent SBL treatments (indicated by a dotted line).
(b) The calcium-channel blockers nifedipine (30 μM, 3 h) and verapamil (20 μM, 1 h) caused significant reductions in both phases of the pulse blue-light response. Responses are representative of at least three experiments.

were tested for their ability to disrupt chloroplast movements. Caffeine is known to evoke Ca^{2+} release from intracellular stores in animal cells (Liu and Meissner, 1997) and thapsigargin is reported to be a selective inhibitor of the ER Ca^{2+}-ATPase in plants and animals (Takemura *et al.*, 1989).

Caffeine did not affect leaf transmission or 3-D chloroplast positions in darkness, but caused a rapid dose- and time-dependent inhibition of blue-light responses (Figure 4a,c). Use of 20 mM caffeine completely and rapidly blocked all responses. The estimated concentration for 50% inhibition (0.5–1 mM) was similar for WBL, SBL and the two phases of the PBL response in short-term (2 min pre-incubation) exposures. Over a longer period of pre-treatment, the extent of inhibition increased for each concentration. Thus, for example, 0.5 mM caffeine caused complete inhibition of PBL responses after 3 h incubation (Figure 4c).

Thapsigargin also dramatically reduced blue-light responses in a dose- and time-dependent manner (Figure 4b,d). Short (2 min) incubations in 15 μM thapsigargin reduced both WBL and SBL responses by 40% and 70%, respectively, whilst longer incubations almost completely abolished the responses (Figure 4d). A normal response to PBL returned after 12 h wash-out of the inhibitor (data not shown).

Interestingly, in 9 out of 17 experiments with thapsigargin, a small increase in light transmission occurred immediately after placing the leaf in the measurement control red light, irrespective of the previous length of exposure to thapsigargin. The value of ΔT observed was around 10% of a full SBL response (data not shown).

Effects of calmodulin inhibitors on chloroplast movement

The influence of the calmodulin inhibitors W7, compound 48/80 and TFP on chloroplast movements were tested at a range of concentrations and incubation periods. The overall trend was that the longer the incubation time, the greater the inhibition of the response observed for all three compounds. However, their relative concentration dependence differed markedly: TFP was the most active (data not shown), W7 marginally less so (Figure 5a), whilst compound 48/80 required about 10-fold higher concentrations and 10–20-fold longer incubation times to exert the same effect (Figure 5b). Thus, chloroplast responses to PBL were already affected after 5 min in TFP (data not shown) or 10 min in W7 (Figure 5a) and completely abolished after 15 min or 1 h, respectively. In contrast, complete inhibition of blue-light responses required 24 h treatment with compound 48/80 at 100 μg ml^{-1} (Figure 5b).

Discussion

Are changes in Ca^{2+} associated with chloroplast movements?

For Ca^{2+} to be considered as a signal intermediate, a transient or sustained increase in internal calcium concentration would be expected upon stimulation. A small transient was observed during SBL responses when measured with Fluo-3. The absolute magnitude of the calcium response reported is subject to the usual range of

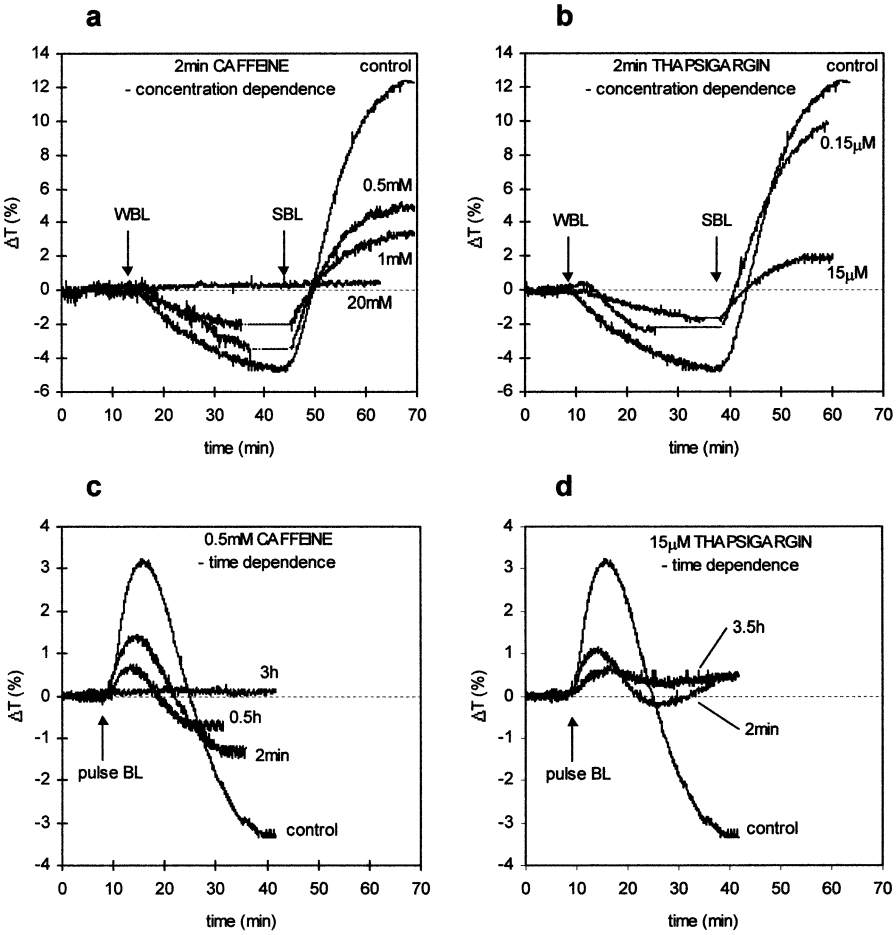


Figure 4. The effects of caffeine and thapsigargin on chloroplast movement.
Caffeine and thapsigargin inhibited all blue-light responses in a concentration- (a and b, respectively) or time- (c and d, respectively) dependent manner. All measurements were carried out after 2 min incubation of *Lemna* leaves before the start of experiments and the inhibitor was present during the experiment. In (a and b), the curves were co-aligned after completion of the WBL response for easier comparison of the subsequent SBL treatments (indicated by a dotted line).

caveats about the accuracy of measurements made with single-wavelength Ca^{2+} dyes and the validity of *in situ* calibrations; however, the large dynamic range of Fluo-3 makes it very useful to detect transient responses (Fricker *et al.*, 1999). The *in situ* calibrations clearly show that if $[Ca^{2+}]_{cyt}$ had increased to micromolar levels, even over limited regions of the cell, a much more substantial change in signal would have been observed. The first sampling point was taken within seconds of applying the stimulus, whilst the transient was recorded after 2–3 min. It is possible that larger, more rapid transients occurred within the sampling intervals; however, it is unlikely that such transients would have been missed in all experiments. One possibility we cannot exclude is that changes in $[Ca^{2+}]_{cyt}$ were highly spatially restricted to the region of the cell close to chloroplasts and could have been masked by the chloroplast autofluorescence signal. Most of the time course measurements followed a single optical section near the top (*x,y*) plane of the cell. A limited number of time-lapse 3-D images were collected and no obvious 'hot-spots' of $[Ca^{2+}]_{cyt}$ were found. From these data we conclude that small transient increases in $[Ca^{2+}]_{cyt}$ may be associated with responses to SBL, but substantial or sustained changes in $[Ca^{2+}]_{cyt}$ do not normally occur.

Artificially elevating $[Ca^{2+}]_{cyt}$ stimulates limited chloroplast movements in one direction

Although only very limited changes in $[Ca^{2+}]_{cyt}$ were detected during SBL responses, all treatments predicted to increase the plasma membrane influx of Ca^{2+} or the calcium analogue Sr^{2+} (e.g. Bauer *et al.*, 1998) promoted slight chloroplast movement towards the anticlinal walls in the absence of a light stimulus. Although the direction of movement mimicked SBL responses, WBL was able to antagonize Ca^{2+}–A23187-induced movements and completely reversed the direction of movement (data not shown). This suggests that the cells remained viable during the ionophore treatments and could overcome the additional Ca^{2+} load.

Measurements of $[Ca^{2+}]_{cyt}$ during treatments with A23187 confirmed that $[Ca^{2+}]_{cyt}$ had increased to a plateau that was maintained for tens of minutes. Despite this, the maximum amount of chloroplast movement observed was only 32% of a full SBL response, although the apparent

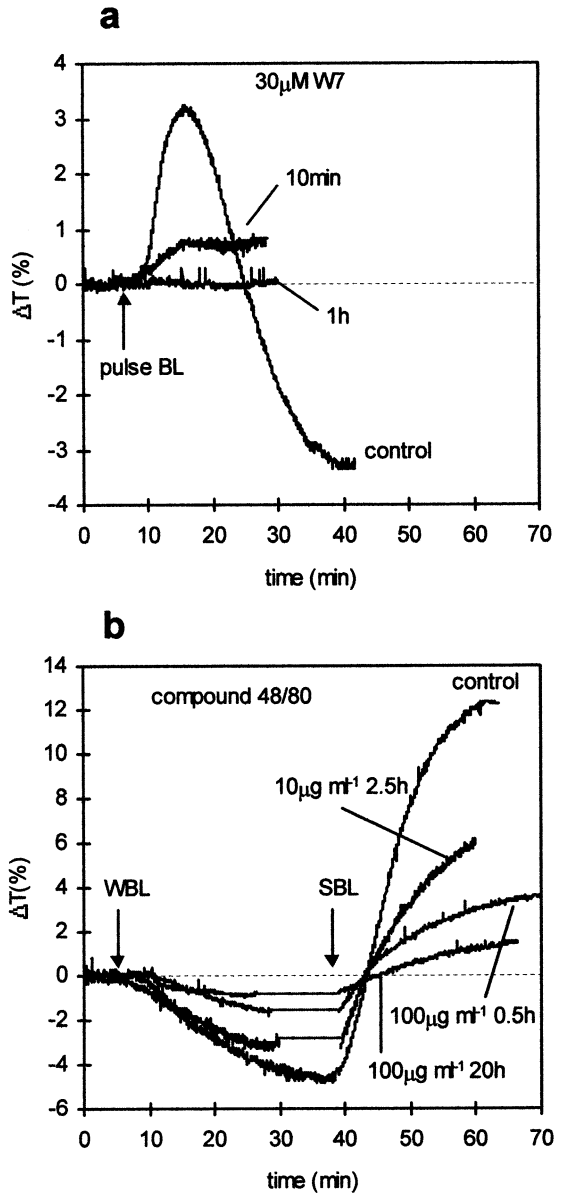


Figure 5. The effects of calmodulin inhibitors on chloroplast movements. Chloroplast movements in response to PBL or WBL and SBL were blocked by W7 (a) or compound 48/80 (b). Dark-adapted *Lemna* leaves were pre-incubated in darkness at a range of concentrations and times, before exposure to light. Selected examples are plotted to show the range of responses obtained; each curve is representative of at least three experiments.

speed of the response was faster (Table 1). Similarly, it has been found that the velocity of chloroplast re-orientation in *Mougeotia* increased with $[Ca^{2+}]_{cyt}$ following treatments with A23187 or the calcium-channel agonist Bay-K8644 (Russ *et al.*, 1991).

As Ca^{2+}–A23187 was effective in triggering chloroplast rotation in *Mougeotia* in darkness (Serlin and Roux, 1984) and increased the velocity of the rotation in red light (Russ *et al.*, 1991) and induced chloroplast movement in *Eremosphaera viridis* (Weidinger and Ruppel, 1985), we tested whether the limited movement initiated in *Lemna* reflected the difficulty in artificially increasing $[Ca^{2+}]_{cyt}$ in this system. The effects of changing external pH in combination with Ca^{2+}–A23187 treatment were investigated, as A23187 is reported to be less effective at acidic pH values (Hell and Donath, 1990). However, the most rapid responses were still found at lower pH values. We can suggest two possible explanations for these data: either protonation of negatively charged groups in the wall polymers at lower pH values increased the rate of diffusion of Ca^{2+} to the cells to a greater extent than it reduced the effectiveness of A23187, or that increased pH values did indeed result in larger increases in $[Ca^{2+}]_{cyt}$ but these caused such a severe disruption in cellular metabolism that the responses were slowed. A similar slowing of chloroplast responses to SBL in very high calcium was observed previously in *Lemna* by Zurzycka and Zurzycki (1951, 1957). Thus, it is possible that chloroplast movements may be induced only if calcium is provided within a certain optimal range. This is reminiscent of the limited concentration window in which $[Ca^{2+}]_{cyt}$ affects chromosome movements in mitosis (Zhang *et al.*, 1990).

In the mitotic system, calcium was shown to depolymerize microtubules (Zhang *et al.*, 1990). Levels of net immunolabelling of cortical microtubules decreased in high-irradiance blue light in *Mougeotia* (Al-Rawass *et al.*, 1997) and disruption of microtubules facilitated the re-orientation of the single chloroplast in the alga (Serlin and Ferrell, 1989). Thus, it is possible that calcium ions may affect the speed of chloroplast movement indirectly through action on the microtubule cytoskeleton.

There is only a limited requirement for an external source of Ca^{2+} for chloroplast movements

Treatments that were predicted to reduce the plasma membrane influx of Ca^{2+} reduced responses to both WBL and SBL, and the sensitivity of the two responses only differed slightly with respect to the concentration and duration or inhibitor required to affect the response. Previous work (Tlałka and Gabryś, 1993) has suggested that long incubation periods in EGTA disturbed calcium homeostasis and completely inhibited chloroplast movements. In this study, shorter (0.5 h) incubations in EGTA were found to affect SBL responses first, but blocked both in longer incubations. The effects of EGTA were to some extent reversible.

Both verapamil and nifedipine block calcium channels, but their action is different. Verapamil blocks the activity of slow Ca^{2+} channels from the cytoplasmic side of the channel. Before it can act, it has to get into the cytoplasm through the open channel, so its activity depends on the membrane potential and concentration (Fleckenstein,

1985). The influence of the L-type calcium-channel blocker (nifedipine) is not connected directly with changes in membrane potential. Nifedipine blocks Ca^{2+} channels in the region of the lipid phase of the membrane and its activity is enhanced by the lipophilic properties of the environment (Fleckenstein, 1985). In spite of these differences, both calcium-channel blockers also partially reduced chloroplast movements; however, relatively high concentrations and long incubation periods were required. On the one hand, partial inhibition of the movement may suggest a role for calcium influx in chloroplast movement. On the other hand, however, depending on the rate of cycling between internal Ca^{2+} pools and external Ca^{2+}, the length of exposure to achieve an effect may have also affected the state of the internal pools. Alternatively, these inhibitors may affect Ca^{2+} release from internal pools in plant cells. These data are consistent with a role of calcium in the regulation of chloroplast movement, but do not unequivocally point to external Ca^{2+} as the primary source, even in the SBL response.

Internal stores of Ca^{2+} may be critical for chloroplast movements

All treatments designed to manipulate internal stores of calcium significantly reduced WBL, SBL and PBL responses of chloroplasts, but did not appear to affect chloroplast positions in darkness. There was no indication that responses to WBL and SBL were pharmacologically distinct despite using a wide range of agonists and antagonists across a broad concentration range and differing exposure times.

Interpretation of these experiments is based on the pharmacology of the agonists and antagonists in animal systems. For example, caffeine is known to evoke Ca^{2+} release from intracellular stores in both animal (Liu and Meissner, 1997) and plant systems (Bauer *et al.*, 1997). Thus a transient increase in $[Ca^{2+}]_{cyt}$ would be anticipated immediately after addition of caffeine; however, subsequent responses involving internal pools would be inhibited as the Ca^{2+} level within the pools would be depleted. The simplest interpretation of the inhibition of WBL, SBL and PBL responses observed in this study with caffeine is that the initial caffeine-induced Ca^{2+} release in darkness was sufficiently slow to affect $[Ca^{2+}]_{cyt}$ only marginally, but the depleted pools were no longer able to participate in chloroplast movements. As both WBL and SBL were affected in parallel across a wide range of caffeine concentrations and incubation times, these data suggest that intracellular stores play a significant role in both types of chloroplast movement but do not appear to determine the direction of movement.

Results with thapsigargin lend some support to this conclusion. Thapsigargin is a relatively selective inhibitor of the ER Ca^{2+}-ATPase in animal cells (Takemura *et al.*, 1989) and may reduce Ca^{2+} levels in intracellular pools in longer-term incubations. Equally, the initial effects of thapsigargin may be to transiently increase $[Ca^{2+}]_{cyt}$ if cycling across the ER is interrupted before other Ca^{2+} sequestration systems can respond. The results in this study with thapsigargin also underline the importance of intracellular Ca^{2+} stores in blue-light responses, but do not provide any clues as to how the direction of movement is controlled.

Interestingly, in about 50% of cases, a small effect of thapsigargin was seen in darkness immediately after assembling the leaf in the measurement system. In all cases, an increase in transmission was observed, although the subsequent response to WBL, SBL or PBL was always attenuated. One possible explanation for these data is that the mechanical stimuli associated with mounting the leaf were sufficient to trigger $[Ca^{2+}]$ transients as reported for other systems (Trewavas and Knight, 1994), which were accentuated in the presence of thapsigargin to block re-uptake into the intracellular stores.

Calmodulin inhibitors block chloroplast movement

Three different classes of nominal calmodulin inhibitors, namely TFP, W7 and compound 48/80, were used to test whether calmodulin was important in blue-light responses. All three inhibitors blocked both WBL and SBL responses, consistent with the view that calmodulin is an integral part of the signalling system; however, as relatively high concentrations and long exposure times were required, particularly in the case of compound 48/80, we believe alternative hypotheses are possible. In *in vitro* assays, the IC_{50} values for inhibition of calmodulin-dependent reactions are typically 6–50 μM, 23–67 μM and 0.85–10 μg ml^{-1} for TFP, W7 and compound 48/80, respectively (Jacoby and Rudich, 1987; Roufogalis, 1985). These inhibitors also affect Ca^{2+}-dependent protein kinases (CDPK) which have a regulatory domain similar to calmodulin (Abo-El-Saad and Wu, 1995; Harmon *et al.*, 1987), at concentrations higher than about 100 μM. In addition, the consequences of their action may not be restricted to the immediate target as, for example, these inhibitors are also known to cause a transient elevation of internal calcium (Gilroy *et al.*, 1987; Schlatterer and Schaloske, 1996), possibly by preventing Ca-calmodulin activation of the plasma membrane Ca^{2+}-ATPase.

The concentrations used here were initially chosen to give 50–100% inhibition of calmodulin-dependent processes based on results with other *in vivo* systems (e.g. Jacoby and Rudich, 1987; Muto and Hirosawa, 1987). Within minutes of application, both W7 and TFP caused substantial inhibition of chloroplast movement at 30 and 50 μM, respectively. In contrast, there was relatively little effect of compound 48/80 at 10 μg ml^{-1} even after 2.5 h

treatment, despite expectations that compound 48/80 is a more potent inhibitor of calmodulin (Gietzen *et al.*, 1983; Gietzen, 1983). Strong inhibition of chloroplast movements required 100 μg ml^{-1} of compound 48/80 for long periods. One explanation for this discrepancy is that compound 48/80 does not penetrate intact *Lemna* leaves as well as TFP or W7. An alternative hypothesis is that the inhibitors are not affecting calmodulin *per se*, but another target, such as a kinase or phosphatase, that shows a differential response to the three inhibitors. These experiments do not unequivocally suggest that calmodulin is involved in reorientation of chloroplasts; however, whatever the target of the inhibitors, both WBL and SBL responses were affected to the same degree. As with the calcium antagonists, the calmodulin inhibitors were effective but did not pharmacologically separate the two processes.

Conclusion

Cytoplasmic calcium ions were found to be important for both weak and strong blue-light-induced chloroplast movements in *Lemna*. The 'rules' set out originally by Jaffe (1980) were fulfilled for SBL responses as small increases in $[Ca^{2+}]_{cyt}$ were detected in SBL, artificially increasing $[Ca^{2+}]_{cyt}$ in darkness triggered partial movement to profile position, and antagonists of $[Ca^{2+}]_{cyt}$ homeostasis inhibited chloroplast movement in response to SBL.

The situation is more complex, however, as WBL responses were also affected by the Ca^{2+} signalling antagonists. Thus, concentrations and incubation periods for a wide range of different compounds that were sufficient to inhibit 50% or more of the SBL response also inhibited the WBL response to a similar extent when measured either as the final magnitude of the transmission change (Figure 6a) or as the rate of the response (Figure 6b). We conclude that although proper regulation of $[Ca^{2+}]_{cyt}$ homeostasis is critical for both SBL and WBL responses to take place, additional factors may be required to specify the direction of chloroplast movement.

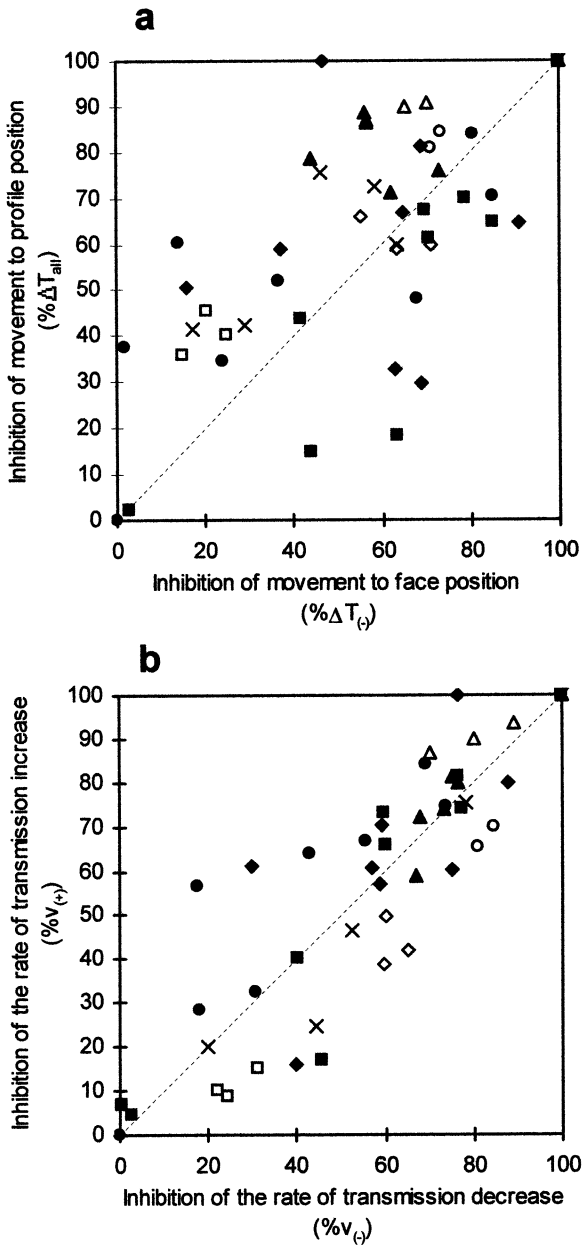


Figure 6. The role of Ca^{2+} in SBL and WBL responses cannot be distinguished pharmacologically.
The extent (a) or rate (b) of inhibition of chloroplast movement to profile position (SBL or the first phase of PBL) was plotted against the extent of inhibition of movement to face position (WBL or the second phase of PBL). Markers represent the following compounds: ◆, 15 μM thapsigargin; ■, 0.15 μM thapsigargin; ▲, caffeine; ●, EGTA; ×, compound 48/80; ○, W7; ◇, verapamil; □, nifedipine; △, TFP. In each case, the symbols refer to a range of concentrations and/or incubation times for each inhibitor.

Experimental procedures

Plant material

Lemna trisulca L. was obtained from the collection of the Jagiellonian University (Kraków) and grown according to Tlałka and Gabryś (1993). Young side fronds were equilibrated in darkness for at least 12 h in a buffer containing 1 mM $CaCl_2$, 10 mM Tris–HCl, pH 6.8, adjusted with 0.01 M NaOH (standard incubation buffer).

Chemicals

All chemicals were obtained from Sigma (Poole, Dorset). Concentrations and incubation times of the agonists and inhibitors used are given in the figure legends. Stock solutions (1 mg ml^{-1}) were made up in Milli-Q water (Compound 48/80, W7), ethanol (thapsigargin, nifedipine, verapamil) or DMSO (A23187) and stored at –20°C.

Photometric measurement of chloroplast movements

Chloroplast movements were measured according to Walczak and Gabryś (1980) using a double-beam photometer. In this system, chloroplast movements are detected as changes in light transmission through the leaf tissue at a wavelength that does not trigger any responses (termed physiological darkness). Dark-adapted *Lemna* leaves were mounted in a chamber with access to allow exchange of both solution and gaseous phase. Inhibitors were added either immediately before measurements or leaves were pre-incubated in darkness for varying time periods. In both cases, inhibitors were present during the measurements. All manipulations were carried out in a darkened room in safe red light provided by photographic lamps (15 W bulb, Kodak safe light filter no. 1).

Relative transmission changes through the leaf were recorded in physiological darkness or following stimulation with continuous or pulse blue light (see Figure 1). The irradiation beam was provided by a halogen lamp (100 W, 12 V) and a set of Schott AG filters (Jena, Germany): GG13 + BG12 + BG23 (transmission λ_{max} 450 nm, half-band width 80 nm), and applied either as continuous weak blue light (WBL) of 0.5 mW m^{-2} (1.88 μmol m^{-2} s^{-1}), strong blue light (SBL) of 20 W m^{-2} (75.2 μmol m^{-2} s^{-1}) or a 10 s pulse of blue light (PBL) at 30 W m^{-2} (112.8 μmol m^{-2} s^{-1}). Combinations of neutral density filters were used to ensure appropriate values of the fluence rate. The measuring beam at λ 660 nm and 28 mW m^{-2} (0.155 μmol m^{-2} s^{-1}) intensity had no influence on movement.

To compare the influence of inhibitors on the WBL and SBL responses, the inhibition of the SBL response (or the first phase of the PBL) was plotted as a function of the inhibition of the WBL (or the second phase of the PBL). Inhibition was defined as 0% for the transmission or velocity values equal to the control values and 100% for the complete abolition of the light response.

Confocal measurements of chloroplast movements

The three-dimensional positions of chloroplasts were imaged by their autofluorescence with a modified BioRad MRC600 CLSM (Fricker and White, 1992; Fricker *et al.*, 1994) attached to a Nikon Diaphot inverted microscope using a Zeiss ×25 0.8 N.A. multi-immersion lens or a Nikon ×60 1.4 N.A. oil immersion lens. Chloroplast autofluorescence was excited using a 543 nm laser (1.3 mW, Gre-Ne, Spindler and Hoyer) or occasionally at 514 nm (25 mW, argon-ion, ILT Ltd) with emission at > 575 nm. Images were collected over a variable rectangular area with a pixel spacing of 0.316 μm (×25 lens, zoom 2) or 0.219 μm (×60 lens, zoom 1.2). In some cases, full-frame images were collected and zoom varied between 1 and 2.5. Each optical section was averaged over 2–3 frames and sampling repeated with a focus motor increment of 1–3 μm through the whole leaf. Sampling was repeated over time at intervals equal to or longer than 3 min.

All images were corrected for depth and sample-dependent attenuation using the protocols developed by (White, 1995). In brief, the loss of signal associated with increasing penetration into the tissue was estimated from a uniformly distributed sea of rhodamine infiltrated into the tissue after fixation and permeabilization. The resulting attenuation profile was fitted using an exponential function. The estimated equation was: $I_z = I_0 e^{-0.0128z}$ for the ×25 lens and $I_z = I_0 e^{-0.0542z}$ for the ×60 lens, where z represents depth in μm. Inversion of this function was used to generate an attenuation correction factor for each optical section in the series.

Confocal measurement of cytoplasmic calcium levels

Cytoplasmic calcium levels were measured using the single-wavelength calcium indicator, Fluo-3, and confocal optical sectioning. For AM-ester loading, Fluo-3 AM was diluted immediately prior to use with standard buffer. For low-pH loading (Bush and Jones, 1988), Fluo-3 was diluted with low-pH buffer (10 mM KCl, 10 mM glutaric acid, pH 4.5) to a final concentration of 12 μM. Tissues were incubated at varying concentrations from 1 to 100 μM and for varying times between 0.1 and 24 h. In some cases, tissue was pre-treated with cutinase (Baker *et al.*, 1981; Hepler, 1985) to facilitate loading of the calcium dye Fluo-3 according to Fricker *et al.* (1994).

Fluo-3 was imaged with excitation at 488 nm (25 mW, argon-ion, ILT Ltd) and emission 540 ± 15 nm. Typically, single optical sections were averaged over 3–8 frames and sampling repeated at 1–5 min intervals. To follow changes in Ca^{2+} over time, the average intensity from several user-defined regions was measured on successive images in the time series. The average background was subtracted, and values were calibrated *in situ* at the end of the experiment.

In situ calibration was performed using 10 μM A23187 in buffer containing 10 mM Ca^{2+}, 1 mM KCl, 10 mM Tris–HCl, pH 7.6. Quenching of Fluo-3 fluorescence was obtained by replacing the external calcium with 10 mM Mn^{2+}. The cytoplasmic free calcium ($[Ca^{2+}]_{cyt}$) was calculated according to Minta *et al.* (1989) using the equation: $[Ca^{2+}]_{cyt} = K_d (F - F_{min})/(F_{max} - F)$, where $K_d = 400$ nM (Gehring *et al.*, 1990; Kao *et al.*, 1989; Minta *et al.*, 1989), F_{min} = Ca^{2+}-free fluorescence intensity, F_{max} = Ca^{2+}-saturated fluorescence intensity. The relationships between F_{min}, F_{max} and F_{Mn2+} were defined for the confocal system using *in vitro* calibration buffers containing 10 mM Ca^{2+}, 10 mM Mn^{2+} or 10 mM EGTA and 13 μM free dye, and were $F_{Mn2+} = 7.5 \times F_{min}$, $F_{max} = 30 \times F_{min}$, with the same instrument settings as in the *in vivo* conditions.

Image collection and processing were performed using COMOS™, MPL™, ThruView Plus™ and LaserSharp™ software (BioRad Microsciences Ltd) running on a PC workstation. Graphical analysis was performed using Excel™. Image montages were created using Confocal Assistant™ (© Todd Brelje) and Photoshop™.

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