Light perception and the role of the xanthophyll cycle in blue-light-dependent chloroplast movements in *Lemna trisulca* L.

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Summary
In most higher plants, chloroplasts move towards the periclinal cell walls in weak blue light (WBL) to increase light harvesting for photosynthesis, and towards the anticlinal walls as an escape reaction, thus avoiding photo-damage in strong blue light (SBL). The photoreceptor(s) triggering these responses have not yet been identified. In this study, the role of zeaxanthin as a blue-light photoreceptor in chloroplast movements was investigated. Time-lapse 3D confocal imaging in *Lemna trisulca* showed that individual chloroplasts responded to local illumination when one half of the cell was treated with light of different intensity or spectral quality to that received by the other half, or was maintained in darkness. Thus the complete signal perception, transduction and effector system has a high degree of spatial resolution and is consistent with localization of part of the transduction chain in the chloroplasts. Turnover of xanthophylls was determined using HPLC, and a parallel increase was observed between zeaxanthin and chloroplast movements in SBL. Ascorbate stimulated both a transient increase in zeaxanthin levels and chloroplast movement to profile in physiological darkness. Conversely, dithiothreitol blocked zeaxanthin production and responses to SBL and, to a lesser extent, WBL. Norflurazon preferentially inhibited SBL-dependent chloroplast movements. Increases in zeaxanthin were also observed in strong red light (SRL) when no directional chloroplast movements occurred. It thus appears that a combination of zeaxanthin and blue light is required to trigger responses. Blue light can cause cis-trans isomerization of xanthophylls, thus photo-isomerization may be a critical link in the signal transduction pathway.

Introduction
The ability to perceive and respond to varying light intensity and light quality is a critical adaptation that allows plants to link their physiology and development to the local illumination regime. Classically, responses to light have been grouped by the range of wavelengths that initiate the response, and include UV-C (200–280 nm), UV-B (280–320 nm), UV-A (320–390 nm), blue-light responses (390–500 nm) and red/far-red responses (600–750 nm) (Batschauer, 1998; Jenkins et al., 1995; Khurana et al., 1998). Multiple blue/UV photoreceptors are known to be present in plants based on physiological evidence, such as the action spectrum and fluence dependence, and molecular evidence from cloning of several different photoreceptors with distinct functions assayed in corresponding mutants (Ahmad, 1999; Briggs and Liscum, 1997; Cashmore et al., 1999; Horwitz, 1994; Horwitz and Berrocal, 1997; Jenkins, 1997; Jenkins et al., 1995; Lasèe et al., 1999; Niyogi et al., 1998; Parks and Hangarter, 1994). What is not clear at this stage is the extent to which multiple receptors may contribute to parallel or converging transduction pathways that affect the same physiological or molecular response.

A diverse range of physiological and developmental changes are triggered by blue light, including phototropism (Firn, 1994), stomatal opening (Assmann and Shimazaki, 1999; Zeiger, 1994) and chloroplast movement (Haupt and Scheuerlein, 1990; Wada et al., 1993; Yatsuhashi, 1996). Movement of chloroplasts has been proposed as a mechanism to optimize light harvesting for photosynthesis. Movement of chloroplasts towards light is correlated with a range of intensities below the saturation point of photosynthesis, while their escape from light protects chloroplasts from damage by excess radiation (e.g. Park et al., 1996) and is found in the saturation range of photosynthesis (Zurzycki, 1955).

In *Lemna*, chloroplast movements are bidirectional depending on the fluence rate. Chloroplasts migrate from a random arrangement in darkness to face position near the periclinal cell walls under weak blue light (WBL), and to profile position near the anticlinal cell walls under strong blue light (SBL) (Zurzycki et al., 1983). The action spectra for both WBL and SBL responses have two major peaks between 400 and 500 nm, and a UV peak at 340 nm (Zurzycki, 1962). Currently it is not clear whether both responses are triggered by a single photoreceptor, or...
whether separate systems co-operate to switch between WBL- and SBL-induced movements.

The most likely chromophores in the blue-light photoreceptor(s) are flavins, porphyrins or carotenoids such as the xanthophylls (Briggs and Liscum, 1997; Galland and Senger, 1988a; Galland and Senger, 1988b; Presti, 1983). Flavins in solution typically have an absorption spectrum with two broad peaks, one centred around 370 nm and the other around 460 nm. Flavin mononucleotide (F MN) binds non-covalently to the NPH1 apoprotein (phototropin) via two LOV (light, oxygen or voltage) domains, and the fluorescence excitation spectrum of the photoreceptor shows additional fine structure between 400 and 500 nm (Christie et al., 1998; Christie et al., 1999). Carotenoids such as β-carotene and xanthophylls also show a complex fine structure in the 400–500 nm range, but do not show any UV absorption in the all-trans conformation. Recently, however, the cis-xanthophylls have been shown to have an absorption peak in the UV/Vis at 350 nm (Molnár and Szabolics, 1993), similar to that found in many action spectra for blue-light responses (Horwitz and Berrocal, 1997; Karlsson et al., 1992; Presti, 1983; Zurzycki, 1962).

The action spectra in Lemma are almost identical to the absorption spectrum for cis-xanthophylls. This coincidence has implications for the localization of the photoreceptor as the xanthophylls are present within the chloroplasts, whilst flavoproteins are thought to be cytoplasmic or bound to the plasma membrane (Horwitz, 1994; Presti, 1983). In addition, the xanthophyll cycle plays an important role in photoprotection (Demmig-Adams, 1990; Demmig-Adams et al., 1996; Niyogi, 1999; Young, 1991) which would enable close coupling between signal perception, the physiological state of each individual chloroplast and the direction of chloroplast movement.

Recently, a specific role was proposed for zeaxanthin as the blue-light photoreceptor both for phototropism in maize (Quinones and Zeiger, 1994) and blue-light-stimulated stomatal opening in Vicia (Srivastava and Zeiger, 1995a; Srivastava and Zeiger, 1995b; Zeiger and Zhu, 1998). In both systems a strong correlation between the response and the level of zeaxanthin was observed and both parameters were sensitive to inhibition by dithiothreitol (DTT), which prevents zeaxanthin synthesis by inhibition of violaxanthin de-epoxidation (Neubauer, 1993; Sarry et al., 1994; Winter and Koenigen, 1989; Yamamoto and Kamite, 1972). This role for zeaxanthin in phototropic responses was questioned by Palmer et al. (1996). They observed phototropic responses in maize seedlings that were deficient in carotenoids, either through genetic lesions in phytoene desaturase, a key enzyme in the synthesis pathway for carotenoids, or after treatment with norflurazon which specifically inhibits this enzyme (Bartels and McCullough, 1972). The different interpretations of the role of zeaxanthin may arise in part from differences in the fluence rates used in each study (Horwitz and Berrocal, 1997) and do not exclude the existence of several co-operating photoreceptors controlling or modulating different phases of the complete phototropic response (Christie et al., 1998; Horwitz and Berrocal, 1997) or functional redundancy, as suggested for cry1 and cry2 (Ahmad, 1999; Ahmad et al., 1998). Equally, however, recent analysis of several blue-light receptor mutants indicates that each photoreceptor appears to activate separate signal transduction pathways (Lasècre et al., 1999).

Given the precedent for blue-light perception by xanthophyll lipids (Quinones and Zeiger, 1994; Zeiger, 1994), the congruence in the action spectrum (Zurzycki, 1962) and the physiological link between xanthophylls, excess light and chloroplast movements, we set out to determine whether the xanthophylls are part of the blue-light-perception system underlying chloroplast movements in higher plants. As a working hypothesis we would expect that (i) individual chloroplasts should respond to their local light regime, i.e. the early stages of signalling should be chloroplast autonomous; (ii) changes in the levels of xanthophyll lipids should correlate with the response; (iii) agonists and antagonists of the xanthophyll cycle should affect chloroplast movements; and (iv) mutants that are defective in xanthophyll cycle turnover should have modified responses. In this paper we present data for the first three criteria that are consistent with a role of xanthophyll lipids in modulating blue-light-dependent chloroplast movements in Lemma.

Results

Chloroplast movement in response to blue and red light

Photometric measurements indicated that there was no net change in the overall tissue transmission (ΔT) when leaves were illuminated only with the low-intensity red measuring beam (660 nm, 0.155 μmol m⁻² s⁻¹) (Figure 1a). Confocal optical sectioning and 3D reconstruction showed that chloroplasts were randomly distributed around the cell periphery and showed only limited, non-directional movements over time (Figure 1b). To allow comparison with results obtained from photometric measurements, an estimate of the predicted amount of light transmission was calculated from the percentage of chloroplast-free area (%CFA). The percentage CFA was an average from three different regions of each leaf excluding idioblasts, and was determined for at least three independent experiments. In darkness the percentage CFA was 16.5 ± 0.6 (mean ± SEM, n = 3) and there was no significant change in percentage CFA (Δ%CFA) with time (Figure 1c), confirming the photometric results for tissue transmission (Figure 1a).

In response to WBL, chloroplasts moved rapidly towards both the inner and outer pericellular wall and reached a
Figure 1. Chloroplast movements in *Lemma* under different light regimes. (a) A photometric trace showing changes in light transmission through dark-adapted leaves illuminated only with the measurement beam (660 nm, 0.155 μmol m⁻² s⁻¹). The trace is an average of four different experiments. (b) Height-coded views showing the position of chloroplasts over time. Bright chloroplasts are closer to the viewer, dark further away. Some slight movements of chloroplasts can be observed between different time points (representative of three experiments). Each projection was calculated from 50 optical sections collected at 1 μm intervals with excitation at 543 nm and emission at >575 nm. Scale bar, 25 μm. (c) Changes in the percentage of chloroplast-free area (Δ%CFA) calculated from the confocal images shown in (b). In the absence of a blue-light stimulus no significant changes in percentage CFA were observed over time from the dark value. Measurements of the Δ%CFA were done from three separate regions of each *Lemma* leaf, avoiding idioblasts, and repeated for three independent experiments. (d) Height-coded views of chloroplast distribution in response to continuous WBL and SBL. Dark-adapted chloroplasts were distributed randomly around all the cell walls. After 30 min of WBL they moved towards the periclinal walls to reach face position. In the following SBL treatment, chloroplasts moved towards the anticlinal cell walls and attained profile position. Scale bar, 10 μm. (e) A photometric trace showing the change in light transmission (ΔT) from the dark value after exposure to WBL and SBL at times indicated by arrows. Response in WBL was connected with the decrease in light transmission (ΔT₀) and in SBL with an increase of transmission (ΔTₙ). (f) Changes in Δ%CFA calculated from confocal images over time during WBL and SBL treatments (arrows). The Δ%CFA decreased after the treatment with WBL and increased in SBL. Each time point is the average Δ%CFA value obtained from four different regions of the leaf, excluding idioblasts, and is representative of three independent experiments. Vertical bars represent SD.

full-face position after about 30 min (Figure 1d). In response to SBL, virtually all of the chloroplasts moved rapidly to the anticlinal walls and reached a full-profile position after 20–30 min (Figure 1d). In 3D views, chloroplasts were occasionally observed to remain near face position (Figure 1d).

The changes in light transmission through the tissue were measured photometrically (Figure 1e) or as changes in percentage CFA for well defined regions of the leaf, excluding idioblasts (Figure 1f). The maximum changes in transmission were ΔT₀ = -4.8 ± 0.3% for WBL and ΔTₙ = 14.1 ± 1.3% for SBL, measured from the dark value (T₀) (n = 6). Similar kinetics but greater overall responses were observed for measurement of percentage CFA from time-lapse 3D images (Figure 1e). The maximum changes in percentage CFA were Δ%CFA₀ = -7.0 ± 2.9% for WBL and Δ%CFAₙ = 30.9 ± 7.0% for SBL (n = 3).

Chloroplasts within one cell can respond to their local light regime

To test whether chloroplasts within a single cell could react independently to different light treatments, *Lemma* leaves were illuminated with abutting light regimes or
adjacent light and physiological darkness. Within each cell, the chloroplasts responded to the local illumination under all combinations tested (Figure 2). Thus chloroplasts in the part of the cell illuminated with SBL responded by moving towards the anticlinal cell walls and reached full-profile position in 40 min, irrespective of whether the rest of the cell was in darkness (Figure 2a,b), strong red light (SRL) (Figure 2c), or weak blue light (data not shown). The only exception was an occasional chloroplast that remained in the centre of the periclinal wall even in SBL (e.g. Figure 2a). In WBL, the chloroplasts started to move within a single cell towards the periclinal walls and obtained full-face

Figure 2. Changes in chloroplast distribution in Lemma cells with abutting illumination regimes. (a) Half the leaf was maintained in darkness whilst the other half was illuminated first with WBL and then with SBL; (b) the order of illumination was reversed; and (c) leaves were exposed to adjacent SRL and SBL followed by WRL and WBL. A significant difference in chloroplast distribution between each pair of regions was observed over time, even within a single cell. Note that there was only limited directional movement induced by WRL or SRL. Each image of chloroplasts was generated from 30 optical sections spaced at 2 μm, collected using a ×60 lens with excitation at 543 nm and emission at >575 nm. Images were displayed as maximum projections after z-attenuation correction. Scale bar, 10 μm.

Figure 3. Content of xanthophyll lipids in Lemma cells in different light conditions.
HPLC traces showing the separation of the xanthophyll lipids in darkness (a) and after 40 min SBL (b), where v = violaxanthin; a = antheraxanthin, z = zeaxanthin and l = lutein. Violaxanthin was the most abundant xanthophyll in dark-adapted leaves. Levels of zeaxanthin and to a lesser extent antheraxanthin increased in SBL at the expense of violaxanthin. Changes in the total xanthophyll pool (v + a + z) over time were calculated and normalized to chlorophyll a content for WBL (c), SBL (d), and SRL (e), respectively. Xanthophylls were followed over 40 min in WBL and 120 min in SBL and SRL. Changes within the xanthophyll pool of violaxanthin (●), antheraxanthin (■) and zeaxanthin (▲) were also calculated from HPLC data and presented as the percentage of the whole pool over time for WBL (f), SBL (g), and SRL (h).

position after 30 min (Figure 2) irrespective of whether the WBL was applied before (Figure 2a) or after (Figure 2b) the SBL treatment. The magnitude of SBL and WBL responses in split-light experiments was comparable to the normal SBL and WBL responses (Δ%CFA_{a} = -6.3 ± 2.0, Δ%CFA_{z} = 27.3 ± 7.0, n = 9).

Only very limited movements were triggered by strong red light (SRL) (Figure 2c). If anything, chloroplasts moved slightly towards face position after 40 min SRL (Δ%CFA = -2.0 ± 3.0, n = 3), even though chloroplasts in the same cell irradiated with SBL moved to profile position. The movement in SRL could have been caused by some light scattering from the irradiated part of the leaf and/or continuation of the random movements observed in darkness.

Levels of xanthophylls change with illumination
To determine whether there was any correlation between the levels of xanthophyll lipids and chloroplast positions, violaxanthin (v), antheraxanthin (a) and zeaxanthin (z) were measured using HPLC (Figure 3a,b). Under the
Table 1. Changes in the content of xanthophylls within the xanthophyll pool after irradiation with different light wavelengths and intensities, and after treatments with inhibitors

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>[(v + a + z)/chl a]</th>
<th>Percentage of xanthophyll pool</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>(%)</td>
<td>violaxanthin v/(v + a + z)</td>
</tr>
<tr>
<td>Darkness</td>
<td>100 (5)</td>
<td>95.9 ± 2.1 (5)</td>
</tr>
<tr>
<td>30 min WBL</td>
<td>110 ± 39 (3)</td>
<td>97.4 ± 0.9 (3)</td>
</tr>
<tr>
<td>30 min SBL</td>
<td>85 ± 41 (5)</td>
<td>24.9 ± 2.8 (5)</td>
</tr>
<tr>
<td>40 min SRL</td>
<td>64 ± 4 (4)</td>
<td>56.5 ± 0.9 (4)</td>
</tr>
<tr>
<td>30 min ascorbate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 min darkness</td>
<td>107 ± 18 (5)</td>
<td>87.1 ± 3.8 (5)</td>
</tr>
<tr>
<td>10 min darkness</td>
<td>108 ± 11 (6)</td>
<td>94.3 ± 2.1 (6)</td>
</tr>
<tr>
<td>3 h 10 mM DTT darkness</td>
<td>118 ± 15 (4)</td>
<td>95.7 ± 1.1 (4)</td>
</tr>
<tr>
<td>40 min SBL</td>
<td>82 ± 2 (4)</td>
<td>94.1 ± 1.0 (4)</td>
</tr>
<tr>
<td>20 h 10 μM norflurazon</td>
<td></td>
<td></td>
</tr>
<tr>
<td>darkness</td>
<td>91 ± 2 (4)</td>
<td>94.8 ± 0.2 (4)</td>
</tr>
<tr>
<td>40 min SBL</td>
<td>93 ± 10 (4)</td>
<td>82.8 ± 0.2 (4)</td>
</tr>
</tbody>
</table>

The components of the xanthophyll pool were calculated in ng. Total levels of xanthophylls are expressed as the percentage of the pool in darkness, after normalization against chl a content. The proportion of violaxanthin (v), antheraxanthin (a) and zeaxanthin (z) are given as a percentage of the total pool. Each value was calculated as the mean ± SEM with the number of independent experiments given in brackets.

elution conditions used, the three xanthophylls were clearly separated from each other and from other peaks, such as lutein (l) (e.g. Figure 3b). The identity of the peaks was confirmed by comparison with standards. For comparison of total xanthophyll levels between experiments, the sum of the individual xanthophylls was normalized to chlorophyll a content.

In dark-adapted leaves, violaxanthin was the most abundant xanthophyll, with low levels of antheraxanthin and zeaxanthin (Figure 3a, Table 1). In WBL the total level of xanthophylls (v + a + z) decreased slightly after 5 min, but subsequently recovered to 110% of the dark value (Figure 3c). There was relatively little change between the different component lipids within the xanthophyll pool. Antheraxanthin (Figure 3f) increased from 2.4 to 10% of the whole pool after 10 min with a corresponding decrease in violaxanthin; however, values had returned to the initial levels after 40 min.

During irradiation with SBL there was also a slight decrease in the total xanthophyll pool within 10 min, that remained more or less stable over the next 120 min (Figure 3d). Within the xanthophyll pool there was a rapid fourfold decrease in the levels of violaxanthin (Figure 3g) accompanied by a transient increase in the level of antheraxanthin up to about 10% of the whole xanthophyll pool after about 5 min and a rapid, 40-fold increase of zeaxanthin to 70% of the total pool after 40 min (Figure 3g, Table 1). After 120 min these trends reversed, with a decrease in zeaxanthin to 56% and a corresponding increase in violaxanthin.

A small decrease in the total xanthophyll pool was also found after 10 min in SRL (Figure 3e); however, the decline continued and at 40 min values were 64% of the dark values. The pool size recovered after 120 min SRL. Changes in the proportions of each xanthophyll were two to three times smaller over the first 40 min in SRL compared to SBL at the same fluence rate, and took place significantly more slowly (Figure 3h). Thus levels of zeaxanthin reached about 35% of the total xanthophyll pool (Table 1) after 40 min and remained unchanged thereafter up to 120 min (Figure 3h).

Chloroplast movements are affected by agonists and antagonists of the xanthophyll cycle

Ascorbate is a non-specific reducing agent but is also required as a substrate for the de-epoxidation enzyme to convert violaxanthin and antheraxanthin to zeaxanthin. Addition of ascorbate is known to stimulate synthesis of zeaxanthin in isolated chloroplasts (Siefemann-Harms et al., 1980) or leaves (Bilger et al., 1989). Addition of sodium ascorbate (30 mM) in darkness caused movement of chloroplasts towards profile position within 10–15 min (Figure 4a,b). The maximum effect measured photometrically was 33% of a control SBL response (Table 1). Subsequent illumination with WBL reversed the movement (Figure 4a,b) although the final transmission changes were lower than a normal WBL response (Figure 4a, Table 1) and chloroplasts did not quite reach full-face position (Figure
Figure 4. The effect of agonists and antagonists of the xanthophyll cycle on chloroplast movement. Photometric traces (a, c, e) and height-coded images of chloroplast positions (b, d, f) are shown for treatments with sodium ascorbate (a, b), DTT (c, d) and norflurazon (e, f). Sodium ascorbate (30 mM) promoted an increase in light transmission (a) and chloroplast movement to profile position (b) in darkness within 2 min of application. Subsequent responses to WBL (a, b) were slightly lower than controls, whilst responses to SBL followed the normal pattern (b). In contrast to ascorbate, DTT (10 mM) did not induce changes in light transmission in dark, but responses were reduced in subsequent WBL and SBL treatments (c). Incomplete movement and clustering of chloroplasts was apparent in the confocal height-coded views (d). Treatment in norflurazon (10 μM, 20 h) did not influence the position of chloroplasts in darkness, but reduced changes in light transmission in response to WBL and to a greater extent in SBL (e). In confocal images, disruption of chloroplast movements was apparent after pretreatment with norflurazon for even shorter (3 h) periods (f). Chloroplasts formed clusters in SBL and did not respond to the following WBL treatment. Chloroplasts were imaged with excitation at 543 nm and emission at >570 nm, using a ×60 lens. Each image of chloroplasts was generated from 16 optical sections spaced at 3 μm (b), 20 sections spaced at 3 μm (d), or 25 sections spaced at 2 μm (f). Images were displayed as height-coded projections after z-attenuation correction. Scale bar, 25 μm.

4b). Chloroplasts obtained full-profile position after subsequent illumination with SBL (Figure 4b).

Ascorbate did not affect the total xanthophyll pool in darkness, but triggered a transient change in the proportion of the component lipids (Table 1). Levels of anteraxanthin and zeaxanthin increased at the expense of violaxanthin within 2 min. After 10 min in darkness, when the transmission values had reached a plateau (Figure 4a), the proportions of violaxanthin, zeaxanthin and anteraxanthin had returned to the dark values (Table 1).

Dithiothreitol (DTT) is also a non-specific reducing agent, but has exactly the opposite effect to ascorbate on the xanthophyll cycle, as it is known to inhibit the de-epoxidase enzyme and prevent formation of zeaxanthin (Yamamoto and Kamite, 1972). Short-term (2 min pre-incubation) exposure to DTT did not affect chloroplast positions in darkness, but reduced chloroplast movements in response to both WBL and SBL (Figure 4c, Table 2). Longer term incubations caused slightly greater disruption of chloroplast behaviour. Thus after 3 h in DTT, confocal imaging revealed that chloroplasts were clustered in SBL rather than reaching a full-profile position along anticlinal walls (compare Figure 4d with Figure 1d). Measurement of the xanthophyll lipids showed that the conversion of violaxanthin to zeaxanthin expected in SBL had effectively stopped under these conditions (Table 1).
Table 2. Effects of agonists and antagonists of the xanthophyll cycle on chloroplast movements

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Change in transmission from Td in response to blue light, ΔT (%)</th>
<th>Percentage of control, ΔT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroplast movement in darkness</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.2 ± 0.3 (4)</td>
<td>—</td>
</tr>
<tr>
<td>AscNa (30 mM, 2 min)</td>
<td>4.6 ± 0.4 (3)</td>
<td>33 of SBL</td>
</tr>
<tr>
<td>DTT (10 mM, 2 min)</td>
<td>0.3 ± 0.4 (4)</td>
<td>—</td>
</tr>
<tr>
<td>AscNa + DTT (2 min)</td>
<td>2.4 ± 0.9 (3)</td>
<td>17 of SBL</td>
</tr>
<tr>
<td>Chloroplast movement in response to WBL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>−4.8 ± 0.3 (6)</td>
<td>100</td>
</tr>
<tr>
<td>AscNa (30 mM, 2 min)</td>
<td>−3.3 ± 0.4 (3)</td>
<td>69</td>
</tr>
<tr>
<td>DTT (10 mM, 2 min)</td>
<td>−2.1 ± 1.1 (3)</td>
<td>44</td>
</tr>
<tr>
<td>AscNa + DTT (2 min)</td>
<td>−0.9 ± 0.4 (3)</td>
<td>19</td>
</tr>
<tr>
<td>Norflurazone (10 μM, 20 h)</td>
<td>−3.8 ± 0.5 (4)</td>
<td>80</td>
</tr>
<tr>
<td>Chloroplast movement in response to SBL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>14.1 ± 1.3 (6)</td>
<td>100</td>
</tr>
<tr>
<td>AscNa (30 mM, 2 min)</td>
<td>18.5 ± 1.2 (3)</td>
<td>134</td>
</tr>
<tr>
<td>DTT (10 mM, 2 min)</td>
<td>7.3 ± 2.5 (3)</td>
<td>52</td>
</tr>
<tr>
<td>AscNa + DTT (2 min)</td>
<td>2.9 ± 0.8 (3)</td>
<td>21</td>
</tr>
<tr>
<td>Norflurazone (10 μM, 20 h)</td>
<td>5.6 ± 1.8 (4)</td>
<td>40</td>
</tr>
</tbody>
</table>

The changes in transmission (ΔT) relative to the control dark value are shown for leaves in darkness, after WBL and SBL, in the presence of sodium ascorbate, DTT and norflurazone. Each value represents the mean ± SEM with the number of experiments given in brackets. For comparison, results are also expressed as a percentage of the normal response for each light treatment.

DTT also reduced the effect of ascorbate on chloroplast movements in darkness, when measured as a change in light transmission (Table 2). Chloroplasts still responded to subsequent illumination with WBL and SBL, but changes were much smaller than in typical controls (Table 2).

Norflurazone, an inhibitor of carotenoid biosynthesis, disrupts chloroplast movements

Norflurazone (SAN 9789) inhibits cis-phytoene desaturase and prevents synthesis of carotenoids but not flavins (Bartels and McCullough, 1972). In Lemna, after 20 h incubation in 10 μM norflurazone in the dark, the total xanthophyll pool had declined by only about 10% (Table 2). In SBL, there was substantially less conversion of violoxanthin to zeaxanthin, which reached only about 15% of the levels normally observed in SBL (Table 2). Under these conditions, whilst chloroplast positions in darkness were not disturbed, subsequent responses to WBL and SBL were reduced by 20 and 60%, respectively (Figure 4e, Table 2). Even after only 3 h treatment with norflurazone in darkness, some disruption to chloroplast movements was observed using confocal imaging (Figure 4f). Thus some chloroplasts reached profile position in SBL, but clusters of chloroplasts also remained on the periclinal cell walls. Interestingly, after exposure to SBL in the presence of norflurazone, chloroplasts failed to move at all in a subsequent WBL treatment (Figure 4f).

Discussion

Is the light-perception system associated with individual chloroplasts?

Individual chloroplasts in Lemna were able to sense and respond to highly localized illumination and were capable of moving when their neighbours were stationary or even moving in the opposite direction. It was even possible for initially touching chloroplasts to achieve full-face and full-profile positions in adjacent areas of the same cell. The complete perception, transduction and effector system must have sufficient spatial resolution to achieve this level of discrimination. One attractive hypothesis is that part of the perception system is associated with each individual chloroplast. This could be readily achieved if the xanthophylls were the blue-light photoreceptors, as they are located within the thylakoids (Eskling et al., 1997) and chloroplast outer envelope (Costes et al., 1979; Siefermann-Harms, 1985). In contrast, flavoprotein receptors are thought to be localized in the plasma membrane or cytoplasm (Horwitz, 1994; Presti, 1983).
The system operating in *Lemma* appears to be different from that in the fern *Adiantum capillus-veneris*. In *Adiantum*, microbeam irradiation at a high fluence rate not only triggered movement of chloroplasts out of the illuminated area, but also caused movement of distant chloroplasts towards the illuminated region (Kagawa and Wada, 1999). Thus in *Adiantum* part of the signal can be transferred from the irradiated area to the cell periphery, unlike the localized responses observed here for *Lemma*.

The xanthophyll cycle operates in *Lemma* under SBL and SRL

The xanthophyll lipids can act as accessory pigments for photosynthesis (Horwitz, 1994; Lichtenthaler, 1987); however, their major role in higher plants is in photoprotection (Demmig-Adams, 1990; Mathews-Roth, 1997; Niyogi, 1999; Young, 1991). In the xanthophyll cycle, zeaxanthin is formed at high light intensities through de-epoxidation of violaxanthin via antheraxanthin in an enzymatic reaction catalysed by a de-epoxidase. The de-epoxidase functions in the thylakoid lumen at a low pH, 5.2, requires ascorbate and can be inhibited by DTT. The reverse reaction is catalysed by an epoxidase in the stroma and occurs in low light or in darkness in the presence of O₂ and NADPH as co-substrates (Hager and Holocher, 1994). Thus dark-adapted leaves are usually rich in violaxanthin, while zeaxanthin accumulates in leaves exposed to high light (Demmig *et al.*, 1987; Sarry *et al.*, 1994; Siefermann-Harms *et al.*, 1980). The time required for de-epoxidation of violaxanthin to zeaxanthin in high light is of the order of only a few minutes, while epoxidation in low light is always slower.

The levels of xanthophyll lipids in *Lemma* followed the turnover pattern predicted from operation of the xanthophyll cycle under low and high fluence rates of blue and red light. Conversion of violaxanthin to zeaxanthin took place to a greater extent and more rapidly in SBL compared to SRL and was inhibited by DTT and transiently stimulated by ascorbate in darkness. We conclude that the xanthophyll cycle is triggered under SBL in the chloroplasts as a photoprotective mechanism. Chloroplast movement from face to profile reduced the surface area for light absorption and reduced excess light capture. This movement could be responsible for the subsequent reduction in zeaxanthin levels after 120 min SBL.

Do levels of zeaxanthin correlate with chloroplast movements?

Although a functional xanthophyll cycle is operating in *Lemma*, the degree of correlation between zeaxanthin acting as a putative blue-light photoreceptor and chloroplast movements is less clear. In both phototropism and stomatal movements, the magnitude of the response correlated well with the level of zeaxanthin that was already present in the tissues (Quinones and Zeiger, 1994; Srivastava and Zeiger, 1995a). At first sight there appears to be a similar strong correlation between the level of zeaxanthin and the change in transmission during the time course of chloroplast movements (compare Figures 1 and 3). We believe this correlation needs to be treated with caution for three reasons. Firstly, the maximum rate of net chloroplast movement was triggered at the start of the SBL treatment when there was little zeaxanthin formed, and conversely the highest levels of zeaxanthin actually correlated with the point when the chloroplasts had reached their full-profile position and stopped moving. Secondly, increases in zeaxanthin were found in SRL, albeit to a lesser extent than in SBL, but no chloroplast movement to profile position was noted. Thirdly, substantial chloroplast movements to face position were observed in WBL when the absolute changes in levels of zeaxanthin were tiny. Taken together, these data indicate that the absolute level of zeaxanthin *per se* does not appear to correlate with the rate or direction of chloroplast movements.

One possible explanation for the lack of a direct link is that the carotenoids exhibit blue-light-dependent conversion between different isomers. Thus violaxanthin appears to exist in its 15-cis form when bound to LHCI (Gruszeczki *et al.*, 1997). In this form its absorption spectrum is almost identical to the action spectra for chloroplast movements in *Lemma*, including a peak in the UV and multiple peaks between 400 and 500 nm (Molnár and Szabolcs, 1993; Zurzycki, 1962). Bound violaxanthin undergoes a cis–trans isomerization following a triplet–triplet excitation energy transfer from chlorophyll if there is excess light absorption by the photosynthetic apparatus (Gruszeczki *et al.*, 1997), and detaches from LHCI. The released all-trans violaxanthin is a specific substrate for de-epoxidation in the xanthophyll cycle which operates in the lipid phase of the thylakoids. A similar cis–trans photo-isomerization of the polyene chain of retinal in rhodopsin forms the basis of photoperception in vertebrates (Spudich *et al.*, 1986), and Zeiger and Zhu (1998) have suggested that the photo-isomerization event may be the key step in xanthophyll-based blue-light perception.

Agonists and antagonists of the xanthophyll cycle affect chloroplast movements

Although the absolute levels of zeaxanthin do not follow the model proposed by Quinones and Zeiger (1994), the effects of agonists and antagonists of the xanthophyll cycle support a role for xanthophylls in chloroplast movements. DTT has been extensively used in isolated chlor-
o plast (Sieffermann-Harms et al., 1980) and intact leaves (Bilger et al., 1989) to inhibit the de-epoxidase enzyme and prevent formation of zeaxanthin (Yamamoto and Kamite, 1972). A parallel reduction in blue-light-dependent responses has been interpreted as a causal relationship between the two processes. DTT also caused partial inhibition of both WBL and SBL responses in this study, although unlike blue-light responses in stomatal guard cells (Srivastava and Zeiger, 1995b), it was not possible to achieve complete inhibition of chloroplast movement, even at very high concentrations. DTT is a general reducing agent and cannot be considered as a specific inhibitor of the xanthophyll cycle in vivo, particularly at the millimolar concentrations that have to be used (e.g. Neubauer, 1993), nevertheless it is reported to have little or no effect on photosynthesis under similar conditions (Bilger et al., 1989).

In this study we also tested the effects of ascorbate, which is another general reducing agent but has exactly the opposite effect to DTT on turnover of the xanthophyll cycle. Ascorbate caused a transient increase in turnover of the xanthophyll pool and stimulated chloroplast movement within a similar time frame. Thus the effects of DTT and ascorbate, taken together, are consistent with a role of the xanthophyll cycle in chloroplast movement and provide an internal control for non-specific effects of reducing agents. Equally, however, redox events are well established in control of chloroplast enzyme activities and gene expression (Allen et al., 1995; Escoubas et al., 1995; Ruelland and Migniac-Mislow, 1999), and have also been implicated in other blue-light responses (Jenkins et al., 1995).

Although more specific chemical inhibitors of the epoxidase and de-epoxidase are not available, the absolute level of the xanthophyll lipids can be reduced by inhibition of their synthesis by the herbicide norflurazon which blocks cis-phytoene desaturase (Bartels and McCullough, 1972). Importantly, norflurazon does not affect synthesis of flavins in other systems and thus it should not affect blue-light responses linked to CRY1, CRY2 or NPH1. In this study, norflurazon had relatively little effect on total levels of xanthophyll lipids after 20 h in darkness in Lemna trisulca, but preferentially reduced chloroplast movements in response to SBL. In previous reports for Lemna gibba, norflurazon triggered changes in the levels of carotenoids over several days upon illumination (Wejnàr, 1987; Weinjñar and Appenroth, 1990; Weinjñar and Horn, 1989; Weinjñar and Jungnickel, 1989). We conclude that either the carotenoid pool is relatively stable in darkness in L. trisulca, or norflurazon did not fully inhibit phytoene desaturase in this system. Despite the limited effect on total xanthophyll pool size, norflurazon blocked turnover within the xanthophyll pool in response to SBL and preferentially inhibited chloroplast movements in response to SBL. Thus blue-light-dependent chloroplast movements may be particularly sensitive to de novo synthesis of carotenoids, or norflurazon may have other targets in addition to cis-phytoene desaturase.

Conclusion

In this paper we present data for Lemna that are consistent with a role for the xanthophyll lipids in modulating blue-light-dependent chloroplast movements. Equally, however, the evidence does not entirely match expectations based on the model proposed for zeaxanthin-dependent phototropic movements (Quinones and Zeiger, 1994) or blue-light-dependent stomatal movements (Zeiger and Zhu, 1998), and does not point to control of chloroplast movements exclusively by a xanthophyll-based pathway.

Most experimental manipulations of xanthophyll turnover had the greatest effect on responses to SBL, consistent with the role of the xanthophyll cycle in photoprotection. As none of the treatments used completely abolished SBL responses, it seems likely that a second blue-light-dependent pathway participates in SBL responses. We also infer from the minimal effects of the experimental treatments on WBL responses that a different photoreceptor and transduction system is required to respond to low fluence-rate blue light.

We can only speculate on the link between the xanthophyll cycle and chloroplast movement. The xanthophyll cycle is already tightly coupled to the physiological status of each individual chloroplast, and the extent of turnover in the cycle provides a continuous indication of the excess energy absorbed over that which can be utilized. The specific trigger for chloroplast movement may also involve photo-isomerization between cis and trans forms of zeaxanthin, as suggested for blue-light-dependent stomatal movements (Zeiger and Zhu, 1998).

Experimental procedures

Plant material

Lemna trisulca L. was obtained from the collection of the Jagiellonian University (Kraków) and grown according to Tlalka and Gabryś (1993). Young side leaves were equilibrated in darkness for at least 12 h in a buffer containing 1 mM CaCl2, 10 mM Tris–HCl pH 6.8, adjusted with 0.01 M NaOH (standard incubation buffer).

Chemicals

HPLC standards of violaxanthin and zeaxanthin were obtained from VKI Water Quality Institute (Hørsholm, Denmark). All other chemicals were obtained from Sigma (Poole, Dorset, UK). Norflurazon was a gift from Sandoz Agrochemicals (Ipswich, UK).

Photometric measurement of chloroplast movements

Chloroplast movements were measured photometrically 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 660 nm (28 mW m⁻² or 0.155 μmol m⁻² s⁻¹) which does not trigger any responses and is therefore termed physiological darkness (Walczak and Gabryś, 1980). Dark-adapted *Lemma* leaves were mounted in a chamber under red safe light (15 W bulb, Kodak safe-light filter no. 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 \( \lambda_{\text{max}} \) 450-40 nm), and applied either as continuous WBL of 0.5 mW m⁻² (1.88 μmol m⁻² s⁻¹) or SBL of 20 W m⁻² (75.2 μmol m⁻² s⁻¹). Combinations of neutral-density filters were used to ensure appropriate values of the fluorescence rate and intensities were measured using a calibrated fast silicon photodiode BPW 20 (Telefunken, Heilbronn, Germany).

Time-lapse confocal imaging of chloroplast movements

The 3D positions of chloroplasts in darkness and different light regimes were imaged by their autofluorescence with a modified BioRad MRC600 CLSM (Fricker and White, 1992) attached to a Nikon Diaphot inverted microscope using a Zeiss × 25 0.8 NA multi-immersion lens or Nikon × 60 1.4 NA oil-immersion lens. *Lemma* leaves were mounted on a No. 1.5 cover slip using Dow Corning 355 contact adhesive (Dow Corning Europe Inc, Brussels, Belgium) in a perfusion chamber. Chloroplast autofluorescence was normally excited using a 543 nm laser (1.3 mW, Gre-Ne, Spindler and Hoyer, Milton Keynes, UK). Occasionally the 514 nm of an Argon-ion laser (25 mW, argon-ion, ILT Ltd, Salt Lake City, UT, USA) was used with emission at 575 nm.

Images were collected over a variable rectangular area with an \((x, y)\) pixel spacing between 0.22 and 0.32 μm. Each optical section was averaged over two or three frames and sampling was repeated with a focus motor increment of 1-3 μm throughout the whole leaf. Sampling was repeated at time intervals equal to or longer than 3 min. When required, blue light (450 ± 30 nm) was provided using the microscope transmission lamp with IR/BG38 plus blue acetate (#383 Sapphire blue, Rosco, London, UK) and neutral density filters to give intensities of 20 W m⁻² (75.2 μmol m⁻² s⁻¹) for SBL and 0.6 W m⁻² (2.35 μmol m⁻² s⁻¹) for WBL.

Image correction and analysis

Images were corrected for depth- and sample-dependent attenuation using the protocols developed by Fricker et al. (1997) and White et al. (1996). In brief, the loss of signal associated with increasing penetration into the tissue was estimated from \((x, z)\) images through a uniformly distributed sea of rhodamine B (50 μM) infiltrated overnight into leaves that had previously been fixed and permeabilized in 20 μM m-maleimidoobenzoic acid N-hydroxysuccinimide ester (MBS), 5 mM EGTA, 5 mM MgSO₄, 0.01% (v/v) nonindet P40, 10% (v/v) DMSO, 100 mM mannitol, 10 mM HEPES pH adjusted to pH 6.8 with NaOH, for 1 h. The resulting attenuation profile was fitted using an exponential function. The estimated equation was: \( I = I_0 e^{-0.0128 z} \) for the <25 lens and \( I = I_0 e^{-0.0542 z} \) 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.

Xanthophyll cycle in chloroplast movements

Images are presented as maximum projections (White et al., 1995) of 15-40 optical sections or as height-coded projections in which chloroplasts nearest the viewer are coded as bright and those furthest away are coded as dark. These projections retain information on the \((x, y)\) and \(z\) positions of the chloroplasts in a single 2D image (White, 1995).

To allow statistical comparison between photometric and confocal treatments, the percentage chloroplast-free area (%CFA) was determined from projections of each 3D confocal image after attenuation correction. Chloroplasts were segmented using an intensity threshold set at 50% of the average autofluorescence intensity of the chloroplast above background. This comparison was based on the assumption that large gaps between chloroplasts detected by confocal microscopy correspond to high values of light transmission measured by the photometry system. Each measurement was repeated for at least three different areas of the leaf, excluding idioblasts.

Xanthophyll extraction and HPLC analysis

For each measurement, six *Lemma* leaves were thoroughly ground in a chilled mortar for approx. 2 min in an ice-cold mixture of 0.5 ml acetone and 1 ml hexane. The extract was centrifuged at 5000 g for 2 min (4°C) to remove debris, and the organic phase was carefully collected. The extract was kept on ice and evaporated under \( N_2 \). Samples were kept in a nitrogen atmosphere. The dried residue was dissolved in 100 μl methanol and directly injected to the HPLC (Beckman System Gold, Beckman Instruments, Inc., Fullerton, CA, USA). All the manipulations were performed either in darkness or in safe green light. A reversed-phase C-18 column (6 mm × 25 cm, 5 μm particle size, Hichrom, Reading, UK) was used with a stepwise gradient from A, 76% acetone in water containing 2 mM HEPES pH 7.0, to B, 100% acetone, at a flow rate of 1.2 ml min⁻¹. During the first 9 min only solvent A was pumped, a 2.5 min gradient from A to B followed, and the column was flushed with 100% B for an additional 10 min. Pigments were detected at 440 nm.

Each measurement was performed in triplicate using individually grown *L. trisulca* leaves. Levels of zeaxanthin and violaxanthin were calibrated against standards prepared and run under the same conditions. Levels of antheraxanthin were estimated from its absorption coefficient in comparison to the two other standards. Recovery was estimated as 92.5% from a single extraction by mixing leaf tissue with known amounts of internal standard. The ratio of the different xanthophylls was not altered although the recovery increased in subsequent extractions. Total levels of xanthophylls were normalized in each run against levels of chlorophyll a.

Software

All photometric data were analysed and graphically presented using Microsoft EXCEL. Confocal data were collected and processed using COMOS and MPL (Bio-Rad Microscience Ltd, Herts, UK), CONFOCAL ASSISTANT (I.C. Brebje, University of Minnesota) and Adobe PHOTOSHOP.

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