The roles of specific xanthophylls in photoprotection

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ABSTRACT Xanthophyll pigments have critical structural and functional roles in the photosynthetic light-harvesting complexes of algae and vascular plants. Genetic dissection of xanthophyll metabolism in the green alga Chlamydomonas reinhardtii revealed functions for specific xanthophylls in the nonradiative dissipation of excess absorbed light energy, measured as nonphotochemical quenching of chlorophyll fluorescence. Mutants with a defect in either the α- or β-branch of carotenoid biosynthesis exhibited less nonphotochemical quenching but were still able to tolerate high light. In contrast, a double mutant that was defective in the synthesis of lutein, loroxanthin (α-carotene branch), zeaxanthin, and antheraxanthin (β-carotene branch) had almost no nonphotochemical quenching and was extremely sensitive to high light. These results strongly suggest that in almost all photosynthetic eukaryotes, the majority of xanthophylls are bound with chlorophyll (Chl) molecules to proteins of integral membrane, light-harvesting complexes (LHCs) (2–5). The LHCs absorb and transfer excitation energy to the photosynthetic reaction centers to drive electron transport; these reactions convert light energy into chemical energy that is used to fix atmospheric CO₂ into sugars. Xanthophylls can function as accessory light-harvesting pigments, as structural entities within the LHC, and as molecules required for the protection of photosynthetic organisms from the poten- tally toxic effects of light.

The importance of carotenoids in photoprotection is evident from the phenotypes of organisms that cannot synthesize carotenoids, either as a consequence of mutations or treatment with herbicides (e.g., norflurazon) that block carotenoid biosynthesis (6–9). There are several mechanisms by which carotenoids function to protect plants against photodamage. Specific xanthophylls are involved in the de-excitation of singlet Chl (¹Chl) that accumulates in the LHC under conditions of excessive illumination (10–14). This de-excitation, measured as nonphotochemical quenching of Chl fluorescence (NPQ), depends on a large transthylakoid proton gradient that be- comes established in excessive light. The development of NPQ correlates with the synthesis of zeaxanthin (Z) and antheraxanthin (A) from violaxanthin (V) via the xanthophyll cycle, which is depicted in Fig. 1. Estimates of excited state energy levels suggest that the lowest singlet state (2¹Ag or S₁) of Z and A can accept excitation energy directly from ¹Chl (15–17); the excited xanthophylls return to ground state by nonradiative heat dissipation. ¹Chl can also enter the triplet state (³Chl) by intersystem crossing, and ³Chl can facilitate the formation of the highly toxic singlet oxygen molecule (¹O₂). The triplet states of xanthophylls can de-excite both ³Chl and ¹O₂ (18). The arrangement of the xanthophyll and Chl molecules in the LHC (19) allows for singlet and triplet energy transfer between these pigments either by a coulomb or a Dexter electron exchange mechanism (20). Finally, xanthophylls may be in- volved in inhibiting lipid peroxidation (21).

To elucidate the roles of specific xanthophylls in photoprotection, we used a video imaging system to isolate mutants of the unicellular green alga Chlamydomonas reinhardtii that were impaired in NPQ and xanthophyll metabolism (22). Analysis of the npq1 mutant, which is unable to convert V to A and Z, demonstrated that operation of the xanthophyll cycle is not required for all pH-dependent NPQ in C. reinhardtii (22). In this report, we provide genetic evidence of a role in NPQ for xanthophylls derived from α-carotene, in addition to the xanthophyll cycle pigments, which are derived from β-carotene (Fig. 1). Characterization of single and double mutants has enabled us to define the specific xanthophylls that are required for protection of the photosynthetic apparatus from photo-oxidative damage in excessive light.

MATERIALS AND METHODS

Strains and Growth Conditions. The npq1 mutant was isolated as described (22). The lor1 mutant (originally designated pg-101) (23, 24) was obtained from the Chlamydomonas Genetics Center (Duke University). Although it was first described as lacking only loroxanthin, lor1 also is unable to make α-carotene and lutein and is therefore likely defective in e-cyclase activity. Genetic crosses were performed according to established methods (25). Mutants were backcrossed three times as mt− parents to the standard wild-type strain CC-125 (mt+) to eliminate other mutations that might be present in the different strain backgrounds, including the chloroplast sr-u-2–60 mutation that was in the background of the original npq1 isolate (22).

Cells were grown photoautotrophically in 100 ml minimal (high-salt) medium (25) with shaking in air in sterile beakers at 25°C with a 15 hr light/9 hr dark cycle (22). Viable cell number was determined by using a hemacytometer as described (26). Strain stocks were maintained in very low light (10 μmol photons m⁻²s⁻¹) at 27°C on agar medium containing acetate (Tris–acetate phosphate) (25).

Measurements of Fluorescence and Oxygen Evolution. Cells (30 μg Chl) were deposited on a filter disc, and fluorescence parameters were measured in air (without CO₂ enrichment) by using a modified pulse-amplitude modulation fluorometer system as described (22), except that the modulated measuring beam was provided by an LED with a peak output at 450 nm
Fig. 1. Carotenoid biosynthetic pathway in C. reinhardtii. The defects in xanthophyll metabolism in the npq1 and lor1 mutants are indicated.

RESULTS AND DISCUSSION

A Role in NPQ for Xanthophylls Derived from α-Carotene.

The npq1 mutant is defective in the xanthophyll cycle and could not convert V to A and Z on exposure to excessive light (Figs. 1 and 2) (22). As a consequence, this mutant had a reduced ability to de-excite 1Chl (shown as reduced NPQ in Fig. 3). Nevertheless, excessive light did not impair the growth significantly in photoautotrophic growth in high light (Fig. 4 and Table 1). The C. reinhardtii lor1 mutant exhibited an elevated Chl a/Chl b ratio (Table 2) and decreased amount of neoxanthin (Fig. 2), which reflects a partial defect in assembly or stability of the peripheral LHCCI (23). Similar pigment changes were reported for an analogous mutant of the green alga Scenedesmus obliquus (29). However, it is unlikely that the NPQ defect in lor1 is solely the consequence of altered assembly or stability of the peripheral LHCCI; several observations suggest that lutein has a more direct role in NPQ. Recent data indicate that
the inner rather than the peripheral LHC is the site of xanthophyll-related NPQ (30–35), and there is no evidence of a significant alteration of the inner LHC in the lor1 mutant. The $F_v/F_m$ value of the lor1 mutant (and the lor1 npq1 double mutant) grown in low light was not decreased compared with that of wild-type cells (Table 1), suggesting that the lor1 lesion does not perturb the efficient transfer of absorbed light energy to the photosystem II reaction centers. As shown in Table 1, the $F_v/F_m$ values of low light grown lor1 and npq1 lor1 were significantly greater than that of wild-type C. reinhardtii. Finally, the lutein2 mutant of Arabidopsis thaliana, which is analogous to the lor1 mutant, has a normal Chl $a$/Chl $b$ ratio (36), but is still impaired in NPQ (O.B., B. Pogson, D. DellaPenna, A.R.G., and K.K.N., unpublished data).

Specific Xanthophylls Required for Photoprotection. Analysis of the npq1 lor1 double mutant demonstrated that blocking the synthesis of specific xanthophylls derived from both $\alpha$- and $\beta$-carotene in a cumulative reduction in NPQ. This double mutant was unable to synthesize $\alpha$-carotene, lutein, and loroxanthin or to convert V to A and Z (Figs. 1 and 2). On exposure to high light, this strain displayed almost no reversible NPQ (Fig. 3), suggesting that it is severely impaired in the de-excitation of $1^1$Chl. The reversible NPQ remaining in npq1 lor1 (Fig. 3) probably reflects the low levels of A and Z (Fig. 2 and Table 2) that accumulate as intermediates in the synthesis of V (Fig. 1).

The $\alpha$-carotene-derived lutein and loroxanthin and the $\beta$-carotene-derived Z and A function redundantly in protecting C. reinhardtii from photo-oxidative damage. The npq1 and lor1 single mutants grew relatively normally in high light (Fig. 4 and Table 1), although the photosystem II efficiency ($F_v/F_m$) for lor1 appeared to be lower than that of wild-type cells (Table 1). In contrast, the npq1 lor1 double mutant, which attained a similar rate of growth in low light to wild-type cells, grew much more slowly than wild-type cells in high light (Table 1) and rapidly bleached (Fig. 4).

Increases in Z, A, and lutein during growth at elevated light intensities are consistent with a role of these xanthophylls in photoprotection. Wild-type C. reinhardtii grown at 350 $\mu$mol photons $m^{-2}s^{-1}$ had greatly increased levels of Z and A, an increased xanthophyll cycle pool ($V + A + Z$), and elevated lutein relative to Chl $a$ (Table 2). Increases in the xanthophyll cycle pool and lutein in high light occurred even in npq1, despite the inability of this strain to convert V to A and Z. Vascular plants and many algae grown in high light exhibit similar increases in xanthophyll cycle pigments (37–39). The greater accumulation of lutein in wild type and npq1 in high light was accompanied by a lower level of loroxanthin (Table 2). Light intensity-dependent changes in loroxanthin levels have been observed previously for the green alga Scenedesmus obliquus (40). The xanthophyll cycle pool in the lor1 strains was considerably elevated relative to that of wild-type or npq1 cells, even when lor1 was grown in low light (Table 2). This finding suggests that in the lor1 mutant the intermediates normally metabolized by the $\alpha$-carotene biosynthetic pathway are diverted to the $\beta$-carotene branch; in agreement with this interpretation, the sum total of the carotenes and xanthophylls in all of the strains was remarkably similar for each specific growth condition. Together, these results suggest that Z, A, and lutein but not loroxanthin have critical functions in

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**Fig. 3.** Induction of NPQ in C. reinhardtii strains. Cells were grown at an incident PFD of 70 $\mu$mol photons $m^{-2}s^{-1}$ and dark-adapted overnight. Fluorescence was measured before, during, and after exposure to actinic light (1,160 $\mu$mol photons $m^{-2}s^{-1}$) in air, and NPQ was calculated as ($F_{m} - F_0$)/$F_m$. The white bar above the graph indicates the period of illumination with high light (HL); the black bar indicates illumination with weak far red background light.

**Fig. 4.** Growth of C. reinhardtii strains. Cells were streaked on minimal agar medium, incubated overnight at 50 $\mu$mol photons $m^{-2}s^{-1}$, and grown photoautotrophically for 6 days at the indicated PFD.
photoprotection and that the NPQ defect in the lor1 mutant is probably caused by the absence of lutein.

### Multiple Levels of Xanthophyll-Dependent Photoprotection

Z, A, and lutein have 10 or more conjugated double bonds and at least one cyclohexenyl ring with a single oxygen substituent (see Fig. 1). Xanthophylls with these characteristics could alter the fluidity of the photosynthetic membranes (41, 42) and/or the aggregation state of the LHCs within the membranes (43). The lack of an epoxide on at least one cyclohexenyl ring of Z, A, and lutein may facilitate a direct membranes (43). The lack of an epoxide on at least one cyclohexenyl ring of Z, A, and lutein may facilitate a direct photoprotection and that the NPQ defect in the lor1 mutant is probably caused by the absence of lutein.

### Table 1. Growth and photosynthesis characteristics of *C. reinhardtii* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth PFD, μmol photons m⁻²s⁻¹</th>
<th>Chl doubling time, hr (n)</th>
<th>Fv/Fm (n)</th>
<th>O₂ evolution, μmol O₂ cell⁻¹hr⁻¹ (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>70</td>
<td>20 ± 0 (4)</td>
<td>0.763 ± 0.011 (6)</td>
<td>1.21 ± 0.06 (2)</td>
</tr>
<tr>
<td>npq1</td>
<td>70</td>
<td>19 ± 2 (4)</td>
<td>0.771 ± 0.009 (6)</td>
<td>0.99 ± 0.07 (2)</td>
</tr>
<tr>
<td>lor1</td>
<td>70</td>
<td>23 ± 1 (4)</td>
<td>0.793 ± 0.010 (6)</td>
<td>0.96 ± 0.01 (2)</td>
</tr>
<tr>
<td>npq1 lor1</td>
<td>70</td>
<td>21 ± 1 (4)</td>
<td>0.802 ± 0.004 (6)</td>
<td>1.14 ± 0.16 (2)</td>
</tr>
<tr>
<td>Wild type</td>
<td>350</td>
<td>28 ± 2 (4)</td>
<td>0.769 ± 0.009 (3)</td>
<td>0.90 ± 0.23 (2)</td>
</tr>
<tr>
<td>npq1</td>
<td>350</td>
<td>29 ± 3 (4)</td>
<td>0.761 ± 0.004 (3)</td>
<td>0.92 ± 0.06 (2)</td>
</tr>
<tr>
<td>lor1</td>
<td>350</td>
<td>34 ± 4 (4)</td>
<td>0.718 ± 0.066 (3)</td>
<td>0.36 ± 0.17 (2)</td>
</tr>
<tr>
<td>npq1 lor1</td>
<td>350</td>
<td>67 ± 16 (4)</td>
<td>0.477 ± 0.050 (3)</td>
<td>0.09 ± 0.00 (2)</td>
</tr>
</tbody>
</table>

Each value is the mean ± SD (or for n = 2, mean ± difference from the mean). The maximum rate of O₂ evolution was determined at light and CO₂ saturation as described.

### Table 2. Pigment characteristics of *C. reinhardtii* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth PFD, μmol photons m⁻²s⁻¹</th>
<th>Chl a/Chl b (n)</th>
<th>(A+Z)/(V+A+Z) (n)</th>
<th>(V+A+Z)/Chl a, mmol/mol (n)</th>
<th>Lutein/Chl a, mmol/mol (n)</th>
<th>Loroxanthin/Chl a, mmol/mol (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>70</td>
<td>2.64 ± 0.04 (6)</td>
<td>0.066 ± 0.020 (2)</td>
<td>72.1 ± 5.4 (2)</td>
<td>115.2 ± 6.0 (2)</td>
<td>58.9 ± 3.1 (2)</td>
</tr>
<tr>
<td>npq1</td>
<td>70</td>
<td>2.64 ± 0.03 (6)</td>
<td>0.000 ± 0.000 (2)</td>
<td>73.6 ± 5.1 (2)</td>
<td>113.1 ± 6.3 (2)</td>
<td>57.7 ± 2.8 (2)</td>
</tr>
<tr>
<td>lor1</td>
<td>70</td>
<td>4.02 ± 0.10 (6)</td>
<td>0.213 ± 0.004 (2)</td>
<td>221.5 ± 12.1 (2)</td>
<td>0.0 ± 0.0 (2)</td>
<td>0.0 ± 0.0 (2)</td>
</tr>
<tr>
<td>npq1 lor1</td>
<td>70</td>
<td>4.04 ± 0.07 (6)</td>
<td>0.108 ± 0.001 (2)</td>
<td>219.1 ± 15.1 (2)</td>
<td>0.0 ± 0.0 (2)</td>
<td>0.0 ± 0.0 (2)</td>
</tr>
<tr>
<td>Wild type</td>
<td>350</td>
<td>2.87 ± 0.05 (3)</td>
<td>0.598 ± 0.035 (2)</td>
<td>114.3 ± 1.9 (2)</td>
<td>209.8 ± 1.2 (2)</td>
<td>20.2 ± 1.7 (2)</td>
</tr>
<tr>
<td>npq1</td>
<td>350</td>
<td>2.90 ± 0.02 (3)</td>
<td>0.001 ± 0.001 (2)</td>
<td>125.3 ± 12.1 (2)</td>
<td>215.3 ± 20.4 (2)</td>
<td>21.3 ± 0.1 (2)</td>
</tr>
<tr>
<td>lor1</td>
<td>350</td>
<td>4.17 ± 0.11 (3)</td>
<td>0.696 ± 0.075 (2)</td>
<td>320.8 ± 22.3 (2)</td>
<td>0.0 ± 0.0 (2)</td>
<td>0.0 ± 0.0 (2)</td>
</tr>
<tr>
<td>npq1 lor1</td>
<td>350</td>
<td>4.70 ± 0.29 (3)</td>
<td>0.195 ± 0.001 (2)</td>
<td>440.0 ± 46.8 (2)</td>
<td>0.0 ± 0.0 (2)</td>
<td>0.0 ± 0.0 (2)</td>
</tr>
</tbody>
</table>

Pigment determination by HPLC was performed on samples of exponentially growing cells that were taken during the middle of the light period. Each value is the mean ± SD (or for n = 2, mean ± difference from the mean).
xanthophyll population that can assemble with the LHC monomers (e.g., two luteins, one lutein and one V, or one lutein and one Z) (30). Because V is de-epoxidated efficiently in the lor1 mutant (Fig. 2), the V that assembles with the LHC must be accessible, either directly while still bound to the LHC polypeptides, or on exchange with a pool of free pigment, to the de-epoxidase that catalyzes the conversion of V to Z. This binding site would position Z (or lutein) in close proximity to the Chl a molecules that assemble with the LHC polypeptides and allow for the direct transfer of excitation energy from Chl to Z (or lutein).

Xanthophyll-dependent de-excitation of Chl also depends on acidification of the thylakoid lumen and/or localized thylakoid membrane domains (10–12, 14). Protonation of the LHC, probably specific polypeptides of the inner LHC (29–34), may promote a conformation change that brings the lutein closer to the Chl molecules or by changing the local electric field, which could provide sufficient spectral overlap between the lutein and proximal Chl molecules or alter the dipole strength of the lutein S\textsubscript{1} to S\textsubscript{0} transition (15).

This model accommodates roles in NPQ for both α- and β-carotene-derived xanthophylls and explains both in vivo and in vitro results, some of which have suggested that all NPQ depends on Z and A. From experiments with isolated thylakoid membranes, Gilmore and Yamamoto (51) derived the linear equation NPQ = x\textsubscript{1} [H\textsuperscript{+}] [Z + A] + x\textsubscript{2} [H\textsuperscript{+}] + c. This equation includes the term x\textsubscript{1} [H\textsuperscript{+}] that relates NPQ to the proton concentration and is independent of [Z] and [A]. This term may reflect the contribution of the invariant α-carotene-derived xanthophylls such as lutein. Therefore, we suggest that the equation be modified to NPQ = x\textsubscript{1} [H\textsuperscript{+}] [Z + A] + x\textsubscript{2} [H\textsuperscript{+}] [L] + c, where [L] is the lutein concentration and x\textsubscript{1}/x\textsubscript{2} = x\textsubscript{3}. Assuming that x\textsubscript{3} is relatively small compared with x\textsubscript{1}, which is consistent with lutein being a weaker quencher than Z, lutein-dependent NPQ would be most apparent at a high pH\textsuperscript{+}. These conditions activate the conversion of V to Z; the latter is a more effective acceptor of excitation energy from Chl, thereby allowing dynamic increases in the extent of photoprotection in excessive light. However, the results also emphasize that the xanthophylls can interact with Chl, Chl, and O\textsubscript{2}, proffering several lines of defense against photodamage.

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