Title
Dissipative response to excess light is catalyzed in monomeric and trimeric light harvesting complexes by two independent mechanisms

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Oxygenic photoautotrophs require mechanisms for rapidly matching the level of chlorophyll excited states from light harvesting with the rate of electron transport from water to carbon dioxide. These protective reactions prevent formation of reactive excited states and photooxidation. The fastest response to excess illumination is the so-called non-photochemical quenching which, in higher plants, requires the luminal pH sensor PsbS and other yet unidentified components of the photosystem II antenna. Both trimeric light-harvesting complex II (LHClI I) and monomeric LHC proteins have been indicated as sites of the heat-dissipative reactions. Different mechanisms have been proposed: energy transfer to a lutein quencher in trimers, formation of a zeaxanthin radical cation in monomers. Here, we report on the construction of a mutant lacking all monomeric LHC proteins but retaining LHClI I trimers. Its non-photochemical quenching induction rate was substantially slower with respect to the wild type. A carotenoid radical cation signal was detected in the wild type, although it was lost in the mutant. We conclude that non-photochemical quenching is catalysed by two independent mechanisms, with the fastest activated response catalysed within monomeric LHC proteins depending on both zeaxanthin and lutein and on the formation of a radical cation. Trimeric LHClI I was responsible for the slowly activated quenching component whereas inclusion in supercomplexes was not required. This latter activity does not depend on lutein nor on charge transfer events, whereas zeaxanthin was essential.

Plants and algae use light as an energy source for carbon dioxide (CO2) fixation into sugars. Their photosystems are composed of a core complex, where charge separation events fuel electron transport from H2O to NADP+, and an antenna system, which expands the absorption cross-section. Large antennas favour energy supply in low-light conditions, yet at high-light (HL) intensities they cause excess excitation beyond the maximal capacity for photochemical reactions. Unquenched singlet excited states of chlorophyll (Chl*) undergo intersystem crossing and the resulting photochemical reactions. Unquenched singlet excited states of energy supply in low-light conditions, yet at high-light (HL) intensity expand the absorption cross-section. Large antennas favour P680, have been indicated as the primary target of photoinhibition. Within the photosynthetic machinery, photochemical quenching which, in higher plants, requires the luminal pH sensor PsbS and other yet unidentified components of the photosystem II antenna. Both trimeric light-harvesting complex II (LHClI I) and monomeric LHC proteins have been indicated as sites of the heat-dissipative reactions. Different mechanisms have been proposed: energy transfer to a lutein quencher in trimers, formation of a zeaxanthin radical cation in monomers. Here, we report on the construction of a mutant lacking all monomeric LHC proteins but retaining LHClI I trimers. Its non-photochemical quenching induction rate was substantially slower with respect to the wild type. A carotenoid radical cation signal was detected in the wild type, although it was lost in the mutant. We conclude that non-photochemical quenching is catalysed by two independent mechanisms, with the fastest activated response catalysed within monomeric LHC proteins depending on both zeaxanthin and lutein and on the formation of a radical cation. Trimeric LHClI I was responsible for the slowly activated quenching component whereas inclusion in supercomplexes was not required. This latter activity does not depend on lutein nor on charge transfer events, whereas zeaxanthin was essential.

NPQ can be dissected into a number of kinetic components: qE, qZ, qM and qI. qE (energy quenching) is the dominant NPQ component, which is rapidly induced (within 20–60 s), is reversible in seconds and is triggered by the over-acidification of the thylakoid membrane. Signal transduction of luminal acidification involves PsbS, through protonation of two lumen-exposed glutamate residues. Since PsbS is an atypical LHC protein, not binding pigments, quenching reactions must be located in interacting pigment-binding subunits of PSII, located in grana partitions together with PsbS.

PSII is made by a Chl a- and b-carotene-binding dimeric core complex surrounded by an antenna system binding Chl a, b and xanthophylls. Antennas are arranged into an inner layer of monomeric LHC proteins called CP29, CP26 and CP24 (encoded by the Lhcb4, Lhcb5 and Lhcb6 genes, respectively) and an outer layer of trimeric LHClI I subunits made of Lhcb1-Lhcb3 gene products. Together, the PSII core and antenna system form supercomplexes, whose composition undergoes dynamic changes depending on acclimation to light conditions and NPQ activation. Several lines of evidence suggest that the site of quenching is located within the PSII antenna system: (1) lutein (Lut) and zeaxanthin (Zea), ligands of LHC proteins, are essential for NPQ activity; (2) Chl b-less plants lack both LHCs and qE although depletion of PSII core complexes does not affect qE activity; (3) DCCD binding to lumen-exposed protonatable residues of LHC proteins shares spectroscopic features with qE. Nevertheless, identification of PsbS partners in quenching reactions is complex due to the high number of gene products involved: in Arabidopsis, only Lhcb5 and Lhcb6 subunits are encoded by single genes, whereas Lhcb4 and LHClI I are encoded, respectively, by three and nine genes.
Also, open questions include the role of Zea, which enhances the amplitude of quenching reactions20, and the biophysical mechanism(s) by which the quenching reactions are initiated. Proposals include (1) Chl-Chl interactions, yielding into a mixing of charge transfer (CT) states and excitonic states, acting as quenchers21,22; (2) formation of short-living Chl-xanthophyll excited states, which serve as traps for ‘Chl*’23,24; (3) CT events in a Chl a→Zea heterodimer, followed by charge recombination to the ground state25,26. Interaction between Chl a and Zea might be promoted by a conformational change from the interaction of protonated PsbS with CP29.26 or between PsbS and LHCII, forming a Zea–PsbS/LHCII complex at the interface27. It was also shown that ‘Chl*’ quenching can occur by the formation of a transient Chl ‘-Lut’ state28. The large number of models for the NPQ mechanism clearly shows that knowledge is limited: many hypotheses are based on measurements in vitro, which, although mimicking, might not closely reflect in vivo phenomena.

In this work, we isolated and characterized the Arabidopsis koLhcb4.1 koLhcb4.2 koLhcb5 triple mutant (hereafter referred to as NoM), which lacks all monomeric Lhcb5s but retains a full trimeric LHCII complement. Further, we introduced the npq1. lut2 and npq4 mutations, preventing, respectively, Zea and Lut synthesis or PsbS accumulation. Lack of monomeric antenna complexes delayed substantially the onset of quenching reactions and changed the xanthophyll- dependence of the residual NPQ activity, implying the fast- and slow-activated components contributing to NPQ in wild type were catalysed, respectively, by monomeric and trimeric components of the PSII antenna system.

Results
NoM is a triple knock-out mutant of Arabidopsis, lacking all monomeric Lhcb subunits of the PSII. NoM plants were obtained by crossing homozygous transfer DNA (T-DNA) mutants carrying insertions in genes encoding Lhcb4.1, Lhcb4.2 and Lhcb5, as previously described29–31. When grown in a climate chamber for 4 weeks under controlled conditions (150 μmol photons m⁻² s⁻¹, 23°C, 8/16 h light/dark), NoM plants showed a significant growth reduction with respect to wild-type plants (Fig. 1 and Table 1). The possibility that such a phenotype was due to the presence of unrelated mutation(s) was ruled out since parental knock-out (KO) mutants were not affected in their growth29–31. Moreover, the reduced growth was not due to altered development, since leaf formation rates were similar in the two genotypes (Supplementary Fig. 1a).

Dark-adapted NoM plants showed a high Chl fluorescence phenotype: images of minimum Chl fluorescence (F₀) were captured and false-colour images relative to Fᵦ parameter were generated from the fluorescence data. A far higher emission in the mutant suggested that absorbed light energy was not used for photochemistry as efficiently as in the wild type, thus yielding an enhanced excitation level in the antenna (Fig. 1).

The quantum efficiency of PSII photochemistry was assessed by Chl fluorescence analysis in vivo32. The ratio of variable to maximum fluorescence (Fᵥ/Fₘₐₓ), which is the maximal photochemical yield of the PSII RC, was 0.57 ± 0.02 in NoM versus 0.81 ± 0.01 in wild type (Table 1), indicating partial loss of PSII activity. NoM leaves, illuminated at 150 μmol photons m⁻² s⁻¹ for 25 min, showed a significant reduction in both maximal efficiency of PSII photochemistry (Fᵥ'/Fₘₐₓ) and the efficiency of PSII-harvested light for Qₐ reduction (Φₚₛₗ), with respect to wild type. These effects suggest a defective connection of the LHC antenna to the PSII RC, consistent with the increased F₀ level compared to wild type (Table 1 and Fig. 1).

NoM plants grown in control light showed a slight but significant decrease in Chl content per leaf area compared to wild type as well as lower Chl a/b and Chl/Car ratios (Table 1). In dark-adapted plants, the content of xanthophylls (neoxanthin, Vio and Lut) was higher in NoM, whereas the level of β-carotene was unaffected. Synthesis of Zea, induced by exposing plants for 20 min to HL (1,200 μmol photons m⁻² s⁻¹, 23°C), was the same in both genotypes (Supplementary Table 1).

The SDS–polyacrylamide gel electrophoresis (SDS–PAGE) of thylakoid membrane polypeptides showed that NoM plants completely lacked Lhcb4 (Fig. 2a), despite still containing the Lhcb4.3 gene, in agreement with previous reports that Lhcb4.3 did not accumulate in mutants deleted in both Lhcb4.1 and Lhcb4.220. The band corresponding to Lhcb6 was also missing, because of destabilization in the absence of its docking site CP2930. The NoM mutant is, thus, lacking all monomeric LHCB proteins of PSII.

The organization of pigment-binding complexes was then analysed by non-denaturing PAGE (Fig. 2b). The photosystem I (PSI) formed a single green band at 650 kDa including the core complex and its antenna moiety. In the case of PSII instead, the component pigment–proteins migrated as multiple bands: namely, the monomeric Lhcb5s, the trimeric LHCII, the Lhcb4–Lhcb6–LHCII-M complex and the PSI core. The upper region of the gel contained undissociated supercomplexes, which formed multiple green bands according to their different LHC complements. The pattern from NoM thylakoids differed in the lack of all PSI supercomplex bands, as well as of the Lhcb4–Lhcb6–LHCII-M complex. The mobility and abundance of PSI-LHCII, PSI core and monomeric Lhcb5s were unaffected, whereas the abundance of the trimeric LHCII was enhanced in NoM with respect to wild type.

Quantification of pigment-binding proteins by immunotitration (Fig. 2c,d) yielded the same PSI/PSII (PsaA/CP47) ratio for wild type and NoM and an increased LHCII/PSII ratio, suggesting the mutant reacted to the lack of monomeric LHCBs by over-accumulating (+60%) the trimeric LHCII antenna.

NPQ of chlorophyll fluorescence. We assessed the ability of wild type and NoM to undergo quenching of Chl fluorescence upon exposure to HL and analysed the known components of the NPQ mechanism including the abundance of the luminal pH sensor PsbS, the extent of thylakoid lumen acidification and the capacity for Zea synthesis. PsbS was present in both genotypes and its abundance with respect to LHCII was similar in NoM versus wild type (Supplementary Fig. 2a). Measuring lumen acidification higher in NoM, whereas the level of β-carotene was unaffected. Synthesis of Zea, induced by exposing plants for 20 min to HL (1,200 μmol photons m⁻² s⁻¹, 23°C), was the same in both genotypes (Supplementary Table 1).

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from the light-induced quenching of 9-aminoacridine showed that mutant and wild-type chloroplasts had the same $\Delta pH$ over a wide range of light intensities (Supplementary Fig. 2b). Consistently, the kinetic of Zea accumulation was the same in both wild-type and NoM leaves, and the same de-epoxidation index was measured over different light intensities (Supplementary Fig. 2c,d). These results are consistent with proton-pumping not being affected in NoM versus wild-type chloroplasts. Thus, changes in quenching activity are expected to reflect altered efficiency of quenching reactions only.

The NPQ activity of the two genotypes is shown in Fig. 3a. Upon exposure of wild-type plants to saturating irradiance (1,200 μmol photons m$^{-2}$ s$^{-1}$, 23 °C), NPQ suddenly reached a value of 1.2 in the first minute, followed by a biphasic rise, faster at 1–4 min and then slower, reaching a maximal value of 2.2 upon 12 min of illumination. NPQ of NoM underwent an initial rise, followed by a transient decrease. Quenching resumed only after 5 min illumination, after which quenching rapidly rose to 90% of wild-type value at 12 min illumination. This is consistent with three phases of quenching overlapping in wild type: phase 1 (P1, 0–1 min), phase 2 (P2, 1–4 min) and phase 3 (P3, 4–12 min), although P2 is missing in NoM. Recovery in the dark was faster and more complete in wild type: phase 1 (P1, 0–1 min) and phase 3 (P3, 12–24 min). These results are consistent with proton-pumping not being affected in NoM versus wild-type chloroplasts. Thus, changes in quenching activity are expected to reflect altered efficiency of quenching reactions only.

The initial fast phase P1 of NPQ induction is transient, and depends on the trans-thylakoid $\Delta pH$ and PsbS and has been proposed to originate in the PSI core complex. The slower kinetic components, P2 and P3, depend on both lumen acidification, Lut, and Zea accumulation. In order to further evaluate the modulation of kinetics by Zea, we measured NPQ during two consecutive cycles of HL, separated by a dark relaxation. Zea was synthesized during the first light treatment and was present at the onset of the second illumination period owing to the slow kinetic of the Zea-Viola back-reaction. The resulting kinetics showed that preloading with Zea resulted in a much faster rise of quenching, reaching near maximal amplitude in both genotypes already in P1 with no further increase in P2 and P3. A transient decline of quenching in P2 followed by recovery in P3 was still evident in the mutant trace (Fig. 3b). The kinetic of dark relaxation was still faster than in wild type and the difference increased during the second dark period. Since the kinetic of Zea accumulation was the same in both wild-type and NoM leaves (Supplementary Fig. 2c), the differences in quenching kinetics are likely due to differences in the availability of Zea-binding sites. The results of Fig. 3a,b imply that the lack of monomeric LHCs affects NPQ mainly during illumination of dark-adapted leaves. In order to further assess this point, we measured the effect of preloading with Zea on the kinetic of quenching in wild-type and NoM leaves (Supplementary Fig. 2d). NoM leaves showed a faster rise of quenching, reaching near maximal amplitude in both genotypes already in P1 with no further increase in P2 and P3. A transient decline of quenching in P2 followed by recovery in P3 was still evident in the mutant trace (Fig. 3b). The kinetic of dark relaxation was still faster than in wild type and the difference increased during the second dark period. Since the kinetic of Zea accumulation was the same in both wild-type and NoM leaves (Supplementary Fig. 2c), the differences in quenching kinetics are likely due to differences in the availability of Zea-binding sites. The results of Fig. 3a,b imply that the lack of monomeric LHCs affects NPQ mainly during illumination of dark-adapted leaves.

Table 1 | Measurement of Chl and Car content, fresh weight and key photosynthetic parameters on leaves of Arabidopsis wild type and NoM.

<table>
<thead>
<tr>
<th>Chl a/b</th>
<th>Chl/Car</th>
<th>µg Chl per cm$^2$</th>
<th>Fresh weight (µg)</th>
<th>$F_0/Chl$ (a.u.)</th>
<th>$F_v/F_m$</th>
<th>$\Phi_{PSII}$</th>
<th>P700 max (a.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>2.97 ± 0.02*</td>
<td>3.85 ± 0.06*</td>
<td>22.1 ± 0.8</td>
<td>0.47 ± 0.21</td>
<td>63.8 ± 4.7</td>
<td>0.60 ± 0.02*</td>
<td>325 ± 4.7</td>
</tr>
<tr>
<td>NoM</td>
<td>2.72 ± 0.06*</td>
<td>3.70 ± 0.06*</td>
<td>19.3 ± 1.3*</td>
<td>0.14 ± 0.04*</td>
<td>177.7 ± 16.3*</td>
<td>0.37 ± 0.04*</td>
<td>1587 ± 196*</td>
</tr>
</tbody>
</table>

PSII function was determined on plants either dark-adapted ($F_0/Chl$, $F_v/F_m$) or upon illumination at 150 µmol photons m$^{-2}$ s$^{-1}$ for 5 min in the presence of saturating CO$_2$ ($P_{700}$ max), the maximum amount of photo-oxidizable PSI RC ($P_{700}$ max) on dark-adapted leaves. Symbols and error bars show means ± s.d. (n > 5). Values that are significantly different (Student’s t-test, P < 0.05) from the wild type (WT) are marked with an asterisk (*).
measured NPQ during steady-state photosynthesis at different light intensities (Fig. 3c), showing that the overall NPQ activity was indeed significantly lower in NoM versus wild type at light intensities below 1,000 μmol photons m⁻² s⁻¹.

In order to verify in vivo the differential role of Zea and of other known factors determining NPQ activity, further genetic analysis was undertaken. To this aim, Arabidopsis NoM mutants devoid of Zea (NoM npq1), Lut (NoM lut2) or both (NoM npq1 lut2), or lacking the PsbS subunit (NoM npq4) were generated. P1 was maintained in the npq1 and lut2 mutants, whereas P2 was reduced in lut2 and both P2 and P3 were reduced in npq1 with respect to wild type. Contrary to lut2, the NPQ kinetic of NoM lut2 was identical to NoM in all components whereas NoM npq1 retained the P1 component only.

Introducing the npq1 mutation in wild type and NoM had a strong decreasing effect in the NPQ activity of both genotypes (Fig. 4a,b), suggesting Zea-binding to antenna subunits active in NPQ occurred in both genotypes. The effect of introducing the lut2 mutation was, instead, significant in wild type only (Fig. 4a,b), implying Lut-binding sites active in NPQ were missing in NoM. The effect of introducing the double mutation npq1 lut2 or the npq4 mutation caused a full depletion of activity in wild-type and NoM background (Fig. 4a,b).

The pigment composition of xanthophyll- or PsbS-deficient mutants, in either dark-adapted state or upon 12 min illumination with HL, is shown in Supplementary Table 1. Xanthophyll content was higher in NoM mutants with respect to the corresponding genotypes accumulating monomeric LHCs, and β-carotene was the same. Lut was absent in all the lut2 genotypes, and compensated for by increased Vio. Treatment with HL induced Zea synthesis in npq4 and NoM npq4 plants to the same level as in wild type, whereas lut2 and NoM lut2 accumulated 2.5-fold more Zea. In npq1 and NoM npq1 genotypes, HL did not induce Vio de-epoxidation as in npq1 lut2 or NoM npq1 lut2 genotypes. The double illumination experiment (Fig. 4c,d) confirmed that NPQ in the NoM mutants was fully dependent on Zea although independent from Lut. This was strikingly different from wild type which depended both on Lut and Zea, in agreement with previous reports. In NoM lut2, the second actinic illumination was accompanied by an increase in the amplitude of slowly relaxing quenching (qL), the latter being an indicator of photodamage or sustained down-regulation of PSII. The inability of NoM npq1 plants to undergo qE was maintained even in the second illumination, whereas the increase in max NPQ was mostly due to the higher amplitude of the qL component (Fig. 4d).

From the above results, we conclude that NPQ activity in wild type has three phases: namely, a very fast P1 at the dark-light transition; a second, P2, partially overlapping with P1 due to monomeric LHC proteins, which is absent in NoM; and a more slowly activated P3 involving LHCII, retained in NoM. The P2 depends on both Lut and Zea, whereas the P3 depends on Zea only. All three components were dependent on PsbS.

**Investigations on the mechanism(s) of NPQ.** The mechanistic models proposed for excess energy dissipation include (1) the establishment of LHCII aggregates in vivo, opening a channel for energy transfer from Chls to the Lut S1 state, and (2) the Chl a – Zea CT, which was proposed to occur within monomeric LHCs.

According to the former proposal, the transition into the dissipative state engages a clustering of LHCII proteins into aggregates with low fluorescence yield that can be reproduced in vitro by inducing aggregation of purified LHCII antenna proteins in low detergent and low pH. This can be observed in vivo by 77 K fluorescence emission spectroscopy of leaves as a decrease of the red emission peak concomitant to an increased far-red emission at 727 nm. In order to determine whether the phases of NPQ described above could be attributed to any of the previously proposed mechanisms, we carried out specific assays: first, we performed 77 K Chl fluorescence quenching experiments on wild-type and NoM leaves, either HL-treated using 1,200 μmol photons m⁻² s⁻¹ light, or after recovery of HL-treated leaves in darkness for 10 min. In both wild type and NoM, 77 K emission spectra recorded upon HL treatment showed a lower amplitude of the PSII emissions (685 and 695 nm components) with respect to the dark-recovered sample. Instead, the amplitude of the long wavelength component (727 nm) was significantly enhanced (Supplementary Fig. 4). Light minus dark difference spectra clearly showed the HL-induced increase in the 727 nm emission was absent in npq4 genotypes whereas it was stronger in the case of NoM leaves, suggesting the rate of the process undergoing this red-shifted PSII emission, namely the clustering/aggregation of trimeric LHCII, was associated with the build-up of NPQ and enhanced in NoM with respect to wild type (Fig. 5).

We then proceeded to investigate the relevance of the second quenching mechanism, namely the formation of a CT state between Chl a and Zea, which was first observed by ultra-fast pump-probe experiments on isolated thylakoid membranes. In order to verify whether NoM was competent in the formation of a Zea‘ radical cation, we measured ultrafast transient absorption (TA) kinetics in isolated thylakoids, before and after inducing qE. The samples were excited at 665 nm and individual TA kinetic traces were measured on spinach, Arabidopsis wild-type and NoM thylakoids at 1,030 nm, where carotenoid radical cations have substantial absorption. In both
spinach (Supplementary Fig. 5) and Arabidopsis wild-type thylakoids (Fig. 6, upper panel), the differential optical density ($\Delta$OD) traces under actinic light (red lines) revealed additional rise and decay components versus the traces recorded in the absence of qE (black line), thus showing the characteristic pattern of a $\text{Zea}^+$ radical cation signal. The amplitude of the signal was slightly higher in spinach chloroplasts owing to their higher NPQ activity.

Thylakoids from NoM instead, despite showing NPQ activity, although slightly lower than in wild type, displayed the same kinetics at 1,030 nm (Fig. 6, lower panel) within the experimental error. We conclude that near infrared absorption changes, detected in wild-type thylakoids and reflecting a CT event, can be correlated with the NPQ component associated with monomeric LHCs only. Instead, no TA signals that can be associated with CT could be detected in genotypes retaining LHCII as the only antenna, despite the fact that the LHCII content was increased by 60% in the NoM versus wild-type thylakoids.

Discussion

All oxygenic photoautotrophs have mechanisms for regulating the efficiency by which the excited states from absorbed light are transferred to RC for photochemical reactions. Eukaryotic algae and land plants evolved feedback-regulated systems in which thylakoid luminal pH signals excess irradiation. Transduction of the low luminal pH into activation of NPQ reactions requires PsbS in land plants. Pigment interactions, either Chl/Chl pairs or Chls/carotenoid pairs, have been proposed to be essential elements of quenching reactions for Chl excited states, thus implying that quenching mechanisms, elicited by the pigment-less subunit PsbS, must occur in interacting pigment-binding subunits of the PSII antenna system. Instead, the organization of PSII–LHCII supercomplexes does not appear to be relevant for NPQ activity since PSII RC level can be reduced by pharmacological treatments or low temperature, leading to the formation of LHCII-only membranes with enhanced NPQ. The difficulty with identification of quenching sites among LHC gene products is redundancy, since members of the PSII antenna system in Arabidopsis are encoded by 14 homologous genes. Reverse genetics has contributed to featuring properties of gene products involved, by showing that quenching reactions are prevented by lack of both Lut and Zea or their ligand LHC proteins. In Lhcb subgroups, down-regulation of Lhcb1 and monomeric LHCs, but not of Lhcb2 or Lhcb3, affected quenching, thus inciting a lively debate on the mechanisms and localization of quenching reactions in either the monomeric or the trimeric antenna proteins.

Results obtained in the present study from the functional characterization of the NoM mutant and by further introducing mutations in this background suggest a series of conclusions.

LHC monomers modulate the middle P2 phase of energy dissipation, namely in the first minutes of transition from darkness to strong illumination (Fig. 3a,b). Slower onset of quenching was previously reported in single KO genotypes, although the effect was weaker. This evidence indicates that monomeric LHCs collectively contribute to the early phase of the quenching response.

LHCII trimers also participate in quenching but their response rate is slower. Indeed, illumination in the NoM genotype leads to

![Figure 4](image-url)
Figure 5 | Spectral changes associated with the formation of NPQ in wild-type, npq4 and NoM genotypes. The 77 K fluorescence emission spectra were recorded from Arabidopsis wild-type (WT), npq4, NoM and NoM npq4 leaves, either illuminated for 12 min with white actinic light (1,200 μmol photons m⁻² s⁻¹, RT) or kept for 10 min in darkness upon illumination, to promote NPQ relaxation. Figure displays comparison of light-minus-dark red fluorescence difference spectra for Arabidopsis wild type versus npq4 (upper panel) and NoM versus NoM npq4 (lower panel) plants. Error bars represent the s.d. values at 686, 694 and 727 nm, corresponding to eight leaves measured individually for each genotype (for details see ref. 37 and Supplementary Fig. 4).

The nature of the quenching reactions is different in monomers versus trimers: the faster-activated P2 in monomers requires both the same quenching amplitude as wild type only after ~10 min of light. This implies that trimeric and monomeric LHCs synergistically contribute to energy dissipation under the fast-changing conditions caused by variable shading under canopies. When considering the relative abundance of trimeric LHCII versus monomeric complexes in wild type and the further 60% increase in trimeric LHCII observed in NoM (Fig. 2d), the specific quenching activity of monomeric LHCs appears far higher than that of trimeric LHCII. The lack of monomeric LHCs leads to dissociation of PSII supercomplexes, confirming that LHCII trimers could be the site of the quenching irrespective of their involvement in PSII supercomplexes.

the nature of the quenching reactions is different in monomers versus trimers: the faster-activated P2 in monomers requires both Lut and Zea, whereas the slower-activated P3 in LHCII is dependent on Zea only (Fig. 4b, d), as shown by the phenotype of the NoM npq4 mutant, hardly distinguishable from the null activity of NoM npq4 and NoM npq1 lut2 (Fig. 4b). This indicates that Lut bound to the LHC monomers is responsible for the changes in NPQ observed in the NoM mutant, so that deletion of Lut gave rise to no further changes. This is further supported by the results of TA measurements of carotenoid radical cation in thylakoids of wild type versus NoM, showing that appearance of the radical cation is associated with the monomeric LHCs (Fig. 6). These findings are fully consistent with previous data on purified LHC proteins showing generation of carotenoid radical cation in LHC monomers and not in LHCII trimers. Moreover, our results show Vio and Lut can substitute for each other in the same quenching site(s) within LHCII, whereas Vio in monomers functions as an inhibitor for quenching. This notion is consistent with evidence that a higher amount of Lut in monomeric LHCs enhances both carotenoid radical cation formation and amplitude of qE.

Although the appearance of carotenoid radical cation in wild-type thylakoids under quenching conditions suggests that this mechanism is responsible for quenching in LHC monomers, it can be asked which kind of quenching reaction occurs in trimeric LHCII, leading to sustained fluorescence quenching in vivo (Fig. 4b). Previous work suggested LHCII undergoes quenching by clustering in the thylakoid membrane, leading to red-shifted 400 ps emission which enhances the amplitude of the 727-nm peak in 77 K emission spectra. In Fig. 5, wild type and NoM show an HL-induced 727 nm emission change (ΔF_{727 nm}). This is PsbS-dependent since it is greatly reduced in npq4 and NoM npq4. The signal is enhanced in NoM leaves, in which LHCII content is 60% higher with respect to wild type, implying the ΔF_{727 nm} is related to the abundance of trimeric LHCII. These data are consistent with this type of LHCII-dependent quenching being active in both wild type and NoM, being the only one in the latter genotype. However, we show that quenching in NoM is fully dependent on Zea (Fig. 4) at variance with previous reports. Also, NPQ in NoM is not affected by the lut2 mutation, suggesting the processes underlying quenching by LHCII in vivo differ in some respect to those induced by aggregation in vitro, where Lut was shown to act as a quencher. Our results are consistent with the proposal that quenching sites...
might be formed by the interaction of PsbS with the major LHCIi complex, with the involvement of a Zea molecule. However, the molecular details of PsbS-mediated LHCCI quenching have yet to be determined in future work. Genetic dissection of the interactions between components contributing to LHCCI quenching (Fig. 4) allows for at least two hypotheses. First, (1) PsbS and Zea binding to LHCCI induces a conformational change in the latter which opens a channel for energy transfer from Chl to the S1 state of a Chl-Car heterodimer. Second, a Zea molecule and a peripheral Chl of LHCCI\textsuperscript{19,20}, leading to a quenching interaction. Although our results point to monomeric LHCCs as modulators of the quenching response, it might be asked whether this is consistent with localization of LHCCI monomers in-between the PSI core complex and trimeric LHCCI. Indeed, topological analysis of changes induced in PSI antenna organization upon activation of NPQ has shown that protonated PsbS causes dissociation of CP24 and the LHCCI-M trimer from the C252M2 supercomplex, thus making CP29 accessible to possible interactors activated by lumen acidification\textsuperscript{14}, including PsbS itself. The two moieties of the PSI antenna system produced by the dissociation event are, thus, proposed to become sites for the fast-activated and slow-activated components of NPQ identified by the NoM mutant in the present report.

Illumination of dark-adapted NoM leaves with saturating light resulted in the fast development of a quenching during the first minute (P1), which transiently reversed in the following 3–4 min (P2); then, quenching resumed and rose to the same amplitude of wild type (P3). P1 is partially affected by either Zea or Lut depletion, and it disappears completely in plants devoid of PsbS (Fig. 4). The fluorescence dynamic of NoM leaves is consistent with generation of transient RC quenching, documented both in vitro\textsuperscript{76} and in vivo\textsuperscript{77} within the first 1–2 min of illumination. It was detected in wild-type leaves at saturating irradiance, whereas it was rapidly converted into a large, antenna-type quenching process at saturating light\textsuperscript{77}. In NoM genetic background, the RC quenching becomes evident even upon sustained illumination, due to the slower activation of qE. Clearly, PsbS is involved in generation of the PSII core complex, with the involvement of a Zea molecule. However, the knock-out lines mentioned in the article were obtained from the Arabidopsis Genome Initiative or GenBank/EMBL databases under accession numbers At5g01530 (Lhcb4.1), At3g08940 (Lhcb4.2), At4g10340 (Lhcb5), At1g15820 (Lhcb6), At3g53600 (Lhcb7), At1g15820 (Lhcb6), At1g68550 (violaanthian de-epoxide), At1g44755 (PsbS) and At1g57030 (lycopene-cyclase). The knock-out lines mentioned in the article were obtained from the NASC under the stock numbers N374676 (koLhcb4.1), N877954 (koLhcb4.2), N514669 (koLhcb5), N577953 (koLhcb6) and N500181 (ltu2).

**Methods**

**Plant material and growth conditions.** Wild-type plants of *Arabidopsis thaliana* (Col-0) and mutants koLhcb4.1, koLhcb4.2, koLhcb5 and koLhcb6 were obtained as previously described\textsuperscript{78}. Multiple mutants koLhcb4.1 koLhcb4.2 koLhcb5 (NoM) was isolated as previously described\textsuperscript{21}. Multiple mutants NoM npq1, NoM npq4, NoM lut2 and NoM npq1 lut2 were obtained by crossing single mutants and selecting progeny by either immunoblotting or HPLC. Plants were grown in a phytotron for 6 weeks at 150 µmol photons m$^{-2}$ s$^{-1}$, 23°C, 70% humidity, 8/16 h of day/night.

**Membrane isolation.** Chloroplasts and stacked thylakoid membranes were isolated as previously described\textsuperscript{79}.

**Pigment analysis.** To measure zeaxanthin accumulation, detached leaves floating on water were exposed to 1,200 µmol photons m$^{-2}$ s$^{-1}$ at room temperature (RT, 22°C).

**Results**

**Spectroscopy.** Absorption measurements were performed at RT using an SLM Amino DW-2000 spectrophotometer. 700 nm absorption changes of leaves were sampled by weak monochromatic flashes (10-nm bandwidth, 705 nm) provided by light-emitting diodes (TJS10; Biologic Science Instruments).

**Gel electrophoresis and immunoblotting.** SDS–PAGE analysis was performed using the Tris–Tricine buffer system\textsuperscript{80}. Non-denaturing Deriphat-PAGE was performed as described in\textsuperscript{30}. For immunotitration, thylakoid samples were loaded for each sample and electrophoresed on nitrocellulose membranes, then proteins were detected with alkaline phosphatase-conjugated antibody.

**Analysis of Chl fluorescence.** PSI function during photosynthesis was measured through Chl fluorescence on whole leaves at RT with a PAM 101 fluorimeter (Heinz–Walz). Chl fluorescence parameters were calculated according to\textsuperscript{32}. Colour video images of F$_{v}$/F$_{m}$ (minimal fluorescence from dark-adapted leaves) were obtained with a FluorCam FC 800-C (PSI, Brno, Czech Republic).

**Measurement of ΔpH.** The kinetics of ΔpH formation across the thylakoid membrane were measured in chloroplast suspension using the method of 9-aminoacridine fluorescence quenching, as previously described\textsuperscript{81}.

**Near infrared TA spectroscopy.** Time-resolved experiments to detect the generation of the Car radical cation were performed in a standard pump-probe setup. A KGW amplified laser system (Pharos, Light Conversion) provided the probe wavelength at 1,030 nm and pumped a lab-built non-colinear optical parametric amplifier. The latter provided pump pulses at 665 nm, which were focused to a 220 µm spot at the sample, whereas the probe spot was kept twice smaller. Measured cross-correlation between pump and probe pulses was ~200 fs. A 20 nl per pulse excitation energy and 200 kHz repetition rate were used for the spinach experiments, whereas 40 nl per pulse and 20 kHz were used for the Arabidopsis experiments. Double-frequency lock-in detection was used to reject high scattering from the sample. Optical choppers operating at 179 and 399 Hz were placed in the pump and probe beams, and TA signal was measured at the sum and difference frequencies\textsuperscript{82}. Thylakoid samples with optical density of 0.6–0.8 at the excitation wavelength were kept in the 1 mm path length fused silica optical cell, which was constantly moved in the raster pattern during the experiments. Freshly prepared and activated samples were never measured for longer than 1 h to prevent accumulation of the inactive thylakoids.

**Data availability.** Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under accession numbers At5g01530 (Lhcb4.1), At3g08940 (Lhcb4.2), At4g10340 (Lhcb5), At1g15820 (Lhcb6). Chl fluorescence parameters were calculated according to\textsuperscript{32}. Colour video images of F$_{v}$/F$_{m}$ (minimal fluorescence from dark-adapted leaves) were obtained with a FluorCam FC 800-C (PSI, Brno, Czech Republic).

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**References**


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Author contributions
R.B., K.K.N. and G.R.F. conceived the work and designed the experiments. L.D., S.C. and M.B. performed all the experiments for the isolation of mutants, and their physiological and biochemical characterization. D.Z. coordinated and performed the transient absorption spectroscopy experiments. D.P. and K.Z. contributed to the time resolved analysis experiments. All of the authors contributed to writing the manuscript. All of the authors discussed the results and commented on the manuscript.

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The authors declare no competing financial interests.