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The causes of altered chlorophyll fluorescence quenching induction in the *Arabidopsis* mutant lacking all minor antenna complexes

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ABSTRACT

Non-photochemical quenching (NPQ) of chlorophyll fluorescence is the process by which excess light energy is harmlessly dissipated within the photosynthetic membrane. The fastest component of NPQ, known as energy-dependent quenching (qE), occurs within minutes, but the site and mechanism of qE remain of great debate. Here, the chlorophyll fluorescence of *Arabidopsis thaliana* wild type (WT) plants was compared to mutants lacking all minor antenna complexes (NoM). Upon illumination, NoM exhibits altered chlorophyll fluorescence quenching induction (i.e. from the dark-adapted state) characterised by three different stages: (i) a fast quenching component, (ii) transient fluorescence recovery and (iii) a second quenching component. The initial fast quenching component originates in light harvesting complex II (LHCII) trimers and is dependent upon PsbS and the formation of a proton gradient across the thylakoid membrane (ΔpH). Transient fluorescence recovery is likely to occur in both WT and NoM plants, but it cannot be overcome in NoM due to impaired ΔpH formation and a reduced zeaxanthin synthesis rate. Moreover, an enhanced fluorescence emission peak at ~679 nm in NoM plants indicates detachment of LHCII trimers from the bulk antenna system, which could also contribute to the transient fluorescence recovery. Finally, the second quenching component is triggered by both ΔpH and PsbS and enhanced by zeaxanthin synthesis. This study indicates that minor antenna complexes are not essential for qE, but reveals their importance in electron stransport, ΔpH formation and zeaxanthin synthesis.

1. Introduction

Photosystem II (PSII) is a large pigment-protein complex responsible for the light-dependent oxidation of water to molecular oxygen in photosynthetic organisms. In plants, PSII is organised in super-complexes containing dimeric cores with associated complexes (CP43/47) and a peripheral antenna system consisting of major light-harvesting complexes (the trimeric LHCII, composed by the polypeptides Lhcb1-3) and minor light-harvesting complexes (the monomeric CP24 (Lhcb6), CP26 (Lhcb5) and CP29 (Lhcb4)) [1–7]. The D1/D2 heterodimer forms the reaction centre of PSII, where charge separation takes place [8]. Because of the high efficiency of the peripheral antenna system at capturing photons, excess light energy absorption can easily oversaturate the electron transport chain capacity. This increases the chances of reactive oxygen species formation leading to damage [9–11]. Plants efficiently deal with the exposure to excess light energy and resulting damage either through dissipative processes that prevent detrimental energy accumulation or through PSII repair pathways [6,7,12].

Non-photochemical quenching (NPQ) of chlorophyll fluorescence is the process by which excess light energy is dissipated in the photosynthetic membrane. NPQ consists of a number of components including qT (state transitions), qZ (zeaxanthin-dependent quenching) and qI (photoinhibitory quenching and other slow components) [6,13–24]. qE, or energy-dependent quenching, is often the major and fastest acting component (within minutes of illumination) and is

Abbreviations: 9-AA, 9-aminoacridine; ΔpH, proton gradient across the thylakoid membrane; AL, actinic light; DAD, diaminodurene; DCCD, *N,N′*-dicyclohexylcarbodiimide; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DES, de-epoxidation state; Fm, maximum fluorescence in the dark with all reaction centres closed; Fm′, maximum fluorescence in the dark-adapted state (Fm) during the course of actinic illumination (Fm′); Fo, minimum fluorescence in the dark with all reaction centres open; Fo′, minimum fluorescence in the dark after illumination; FR, far red (light); Fv, variable fluorescence defined as Fm-Fo; Fv/Fm, maximum quantum yield of photosystem II in the dark; LHC, light harvesting complex; MV, methyl viologen; NoM, no minor antenna complex mutant; NPQ, non-photochemical quenching; PSI, photosystem I; PSII, photosystem II; qE, energy-dependent quenching; qI, photoinhibitory quenching; qT, state transitions; qZ, zeaxanthin-dependent quenching; SP, saturating pulse; WT, wild type

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triggered by the generation of a proton gradient across the thylakoid membrane (ΔpH) [25,26]. The kinetics of qE formation and relaxation have been shown to be slower than that of ΔpH suggesting that conformational changes and rearrangements within PSII are required in order to attain the photoprotective state [21,25,27–29]. This led to the proposal that protons must target a specific site where qE is formed, leading to a conformational change and the formation of the quencher (s) [6,12].

Debate over the site of qE began in the 1980s with two opposing proposals: (i) the reaction centre is the site of quenching via recombination between $P680^+$ and the acceptor side of PSII [30–32] or (ii) the PSII antenna system (minor or major LHCII complexes) is the site of quenching [29,33]. Spectroscopic studies of qE led to developing the 'LHCII aggregation model' [26,33], which proposes that the quencher resides in the LHCII antenna. Four different structural/functional states of the LHCII antenna are defined [6,33]. Each state corresponds to a different amount of NPQ, proportional to the degree of aggregation and the presence of either violaxanthin or zeaxanthin. Such states and their associated conformational changes are consistent with multiple physiological, spectroscopic and biochemical observations [6]. Although it is now widely accepted that qE takes place in the PSII antenna system [6,34], the specific site of quenching within the antenna is still disputed [6,34]. Furthermore, the mechanism of quenching, which is predicted to occur via chlorophyll-chlorophyll and/or chlorophyll-carotenoid interactions [35–39], is not fully understood.

Demmig-Adams et al. [40–42] were the first group to propose that the carotenoid zeaxanthin may be responsible for quenching based on the correlation between zeaxanthin formation (via violaxanthin de-epoxidation through the intermediate antheraxanthin) and increased ΔpH [43]. However, it was later shown by Horton and co-workers [44,45] that zeaxanthin is not a necessary requirement for qE if the pH of the lumen is lower than 4.5–5.0. Zeaxanthin is therefore considered to act as an allosteric regulator of qE formation and relaxation by affecting the structural reorganisation of the antenna, thus altering the affinity of antenna for protons [6,12,26,42,44–48]. Similarly, it was later found that the protein PSII subunit S (PsbS) is also involved in qE via allosteric regulation and promotion of thylakoid membrane dynamics [6,49–55].

The discovery of a 'proton short circuit', in which protons produced by water oxidation are funnelled from the lumenal side into the reduced bound quinone at the stromal side [56,57,59], and the resulting inhibition of qE brought about by the covalent modification by binding of the reagent *N,N′*-dicyclohexylcarbodiimide (DCCD) [60–62], showed the importance of proton translocation for qE. The minor antenna complexes, particularly CP26 and CP29, were found to form stronger quenching states than the major complexes, which could be more readily reversed by DCCD [47,62,63], further indicating a role of the minor antenna in quenching. CP26 was proposed to either be the primary site for qE formation, via conformational changes in its lumen-facing domain, or to function as a proton channel into the qE locus, found elsewhere in the PSII antenna [6,60,61].

More recent developments in the genetic manipulation of the PSII antenna have shed light upon the role of different LHCII antenna complexes for protection of the photosynthetic membrane, as well as PSII supercomplex organization and function. For example, the role of minor antenna complexes in mediating the binding of the LHCII trimers to the reaction centres of PSII [1,4,64–69]. Nevertheless, several works (reviewed in [6,12]) indicate that no single LHCII complex acts as the sole site of qE and that quenching can, in principle, occur in any of them. Recently, a triple mutant of *Arabidopsis thaliana* lacking all monomeric antenna complexes (NoM) was created [69]. By comparing NoM to WT, as well as mutants depleted in zeaxanthin (*npq1*), lutein (*lut2*) and PsbS (*npq4*), Dall'Osto et al. [70] concluded that the overall qE formation occurs via three independent stages. Firstly, an initial and rapid quenching that takes place in the PSII reaction centre complexes; a middle component, originating from monomeric minor antenna complexes (and therefore absent in NoM), which is dependent on the presence of zeaxanthin and lutein radical cations; and a third, slow component, occurring in trimeric LHCII, which relies entirely upon zeaxanthin synthesis. In the presence of zeaxanthin (i.e. during the second cycle of illumination) the kinetics of NPQ induction in NoM was found to be much faster, with an amplitude similar to that of WT [70]. However, the origins and components of quenching are still greatly debated and the NPQ process has been only partly investigated in this key NoM mutant [69,70].

The aim of this study was to investigate (i) the site responsible for the fast quenching in NoM; (ii) the origin and components contributing to the transient fluorescence recovery during illumination; and (iii) the levels and kinetics of ΔpH and zeaxanthin formation in chloroplasts and leaves isolated from NoM plants.

2. Materials and methods

2.1. Plant growth

Arabidopsis thaliana wild-type (WT) Col-0, *npq1* (unable to de-epoxidise violaxanthin into zeaxanthin) and NoM (unable to synthesise minor antenna polypeptides) were used in this work [69–71]. Seeds were sterilised in 50% ethanol and 0.1% Triton-X 100 and stored for 72 h at 4 °C prior to sowing. Plants were grown on a 6:6:1 ratio of John Innes No. 3 soil, Levington M3 potting compost and perlite (Scotts UK, Ipswich, UK). All seedlings were grown under 100 μmol $m^{-2} s^{-1}$ for 1 week before being transferred to 190 μmol $m^{-2} s^{-1}$, with a 10 h photoperiod at 22 °C. Water was added to the trays three times a week. Chlorophyll fluorescence and biochemical measurements were made on plants that were between 40 and 60 days old and showed no sign of inflorescence. For plants treated with the chloroplast protein synthesis inhibitor lincomycin, treatment started at the early rosette stage and was delivered three times per week in irrigation water (0.2–0.6 g l^{-1}; Sigma Aldrich, Munich, Germany) until Fv/Fm measured on leaves was in the range of 0.15–0.35 (see [72] for more details).

2.2. Fluorescence measurements

All chlorophyll fluorescence measurements were carried out on a Dual-PAM-100 fluorimeter (Walz, Effeltrich, Germany). NPQ induction measurements were made on intact leaves. Actinic light (AL) was provided by arrays of 635 nm light-emitting diodes. Plants were dark adapted for 40 min prior to measurement. The measuring light intensity was 5 μmol photons $m^{-2} s^{-1}$, or 3 μmol photons $m^{-2} s^{-1}$ for plants treated with lincomycin. The maximum fluorescence in the dark-adapted state (Fm), during AL (Fm′), and in the subsequent dark relaxation periods (Fm″) was determined using a 0.6 s saturating pulse (SP) of 4000 μmol photons $m^{-2} s^{-1}$ (see arrows in Fig. 1). The average values of PSII maximum quantum yield in dark-adapted leaves (Fv/Fm, where Fv = Fm − Fo) were 0.84 ± 0.01 and 0.53 ± 0.01 for WT and NoM, respectively. Plants were dark adapted for 40 min prior to recording the kinetics of NPQ formation and relaxation during two phases of 5 min of light (both at 1382 μmol photons $m^{-2} s^{-1}$) and 5 min of darkness. A SP was applied every minute throughout each cycle in order to assess NPQ, where NPQ = [(Fm − Fm′) / Fm′]. For NoM and WT plants, a manual SP was also applied approximately 30 s after illumination, corresponding to the maximum quenching of chlorophyll fluorescence in NoM (arrow 2, Fig. 1B).

Fo' was determined in NoM during the first 4 min of illumination. A SP was used to measure Fm in dark-adapted leaves. Far red (FR) light was used (grey bar, Fig. 3) prior to AL (1382 μmol photons $m^{-2} s^{-1}$). During illumination, AL was switched off and FR light was manually applied every 10 s to assess Fo′.

Fast fluorescence induction kinetics were measured according to [73]. A custom-made trigger file providing AL of 12 μmol photons $m^{-2} s^{-1}$ was used. After a brief FR light exposure, leaves were