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NPQ activation reduces chlorophyll triplet state formation in the moss *Physcomitrella patens*

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ABSTRACT

Plants live in variable environments in which light intensity can rapidly change, from limiting to excess conditions. Non-photochemical quenching (NPQ) is a regulatory mechanism which protects plants from oxidative stress by dissipating excess Chl singlet excitation. In this work, the physiological role of NPQ was assessed by monitoring its influence on the population of the direct source of light excess damage, i.e., Chl triplets (^{3}Chl*). ^{3}Chl* formation was evaluated in vivo, with the moss *Physcomitrella patens*, by exploiting the high sensitivity of fluorescence-detected magnetic resonance (FDMR). A dark adapted sample was compared with a pre-illuminated sample in which NPQ was activated, the latter showing a strong reduction in ^{3}Chl* yield. In line with this result, mutants unable to activate NPQ showed only a minor effect in ^{3}Chl* yield upon pre-illumination.The decrease in ^{3}Chl* yield is equally experienced by all the Chl pools associated with PSII, suggesting that NPQ is effective in protecting both the core and the peripheral antenna complexes. Moreover, the FDMR results show that the structural reorganization in the photosynthetic apparatus, required by NPQ, does not lead to the formation of new ^{3}Chl* traps in the LHCs. This work demonstrates that NPQ activation leads to effective photoprotection, promoting a photosystem II state characterized by a reduced probability of ^{3}Chl* formation, due to a decreased singlet excited state population, while maintaining an efficient quenching of the ^{3}Chl* eventually formed by carotenoids.

1. Introduction

Absorption of sunlight for photosynthesis is performed by pigments bound to light-harvesting pigment–protein complexes (LHCs) associated with the reaction centers (RCs) of the photosynthetic apparatus. Light absorption results in singlet state excitation of the pigments, mainly chlorophylls (Chls). In limiting light conditions, all the excitation energy is efficiently transferred to the reaction centers, where it is converted into chemical energy. Large antenna systems are suitable for efficient light capture in limiting light, but they may represent a problem when light is absorbed in excess with respect to the capacity of the photochemical turnover. In these conditions, the excitation energy absorbed by Chls exceeds its use in the photochemistry taking place in the reaction centers, and the ^{1}Chl* population in LHCs increases. As a consequence, the probability of intersystem-crossing (ISC) to the triplet states of Chl (^{3}Chl*), which can produce $^1O_2$, becomes higher [1,2]. A second mechanism of $^1O_2$ production, due to excess light conditions, takes place at the level of reaction center, mainly that of photosystem II (PSII) where, owing to over-reduction of the electron acceptors in the electron transport chain, charge recombination to the triplet state $^3P_{680}$ occurs, with the consequent formation of $^1O_2$ and photo-inhibition of RC [3].

The oxidative stress generated by the absorption of excess light energy led to the evolution of protective mechanisms targeting the agents directly responsible for $^1O_2$ production, i.e., Chls in their excited singlet (^{1}Chl*) or triplet (^{3}Chl*) states [1–5].

The high quantum efficiency of photochemistry in limiting light results in a decrease of fluorescence called photochemical quenching (qP). Plants present a mechanism active in quenching ^{1}Chl* in PSII light-harvesting complexes when light energy is absorbed in excess. This phenomenon, called 'Non-Photochemical Quenching' (NPQ), corresponds to a non-radiative deactivation channel for ^{1}Chl* states [6]. In relation to its time course, most NPQ takes place through a fast component called 'energy-dependent quenching' (qE) or feedback de-excitation, but a slower component is also present, called qI, due, at least in part, to recovery from photo-inhibition [7]. Rapid induction and relaxation of NPQ are required for plants to deal with frequent, rapid changes in the natural light environment [8]. A current model for NPQ activation in plants is related to acidification of the thylakoid lumen under excess light. The low lumenal pH induces

Abbreviations: NPQ, non photochemical quenching; Car, carotenoid; Chl, chlorophyll; ZFS, zero field splitting; ISC, inter system crossing; ODMR, optically detected magnetic resonance; FDMR, fluorescence detected magnetic resonance

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the synthesis of zeaxanthin, via the xanthophyll cycle, and protonation of a PSII protein, PSBS. The latter was suggested to be involved in the light-induced re-organization of the complexes in the membranes, which switch between light-harvesting and photoprotective states [9–11]. Recent results indicate that, owing to their NPQ activation, algae rely on a different LHC-like protein, called LHCSR, as demonstrated by specific knockout (KO) mutants in the green alga *Chlamydomonas reinhardtii* and in the diatom *Phaeodactylum tricornutum* [12,13]. However, LHCSR-dependent NPQ, most probably depends on a different activation mechanism, with the protein itself acting as a quencher [14]. Both PSBS and LHCSR are present in the moss *Physcomitrella patens* and are active in NPQ [15,16].

NPQ acting on ^{1}Chl* is not the only photo-protection mechanism of plants. In fact direct deactivation of ^{3}Chl* and $^1O_2$ is constitutively provided by carotenoids (Cars) closely bound to LHC proteins. ^{3}Chl* states are quenched by carotenoids by a triplet–triplet energy transfer (TTET) mechanism [17]. The ^{3}Car* states thus produced, lying in energy below the $^1O_2$ energy level, decay directly to the ground state by internal conversion. TTET takes place via an exchange mechanism which depends on the overlap of the wavefunctions of donor and acceptor. Chls and Cars must therefore be in close contact for this process to occur, since transfer efficiency decreases exponentially with donor–acceptor distance. Such close distances are maintained in the LHCs by the protein moiety which coordinates the pigments. Although chlorophyll-to-carotenoid triplet transfer has been found to be efficient in several isolated antenna proteins, it is not complete, leaving some unquenched ^{3}Chl* states (18). This incomplete quenching, also proven in intact systems [19,20], confirms that chlorophyll in the antennas are a potential source of $^1O_2$.

Unlike the Car/Chl couples belonging to LHCs, the distance between β-carotene and P680 in the RC of PSII is too large to enable direct quenching of the recombination triplet state, ^{3}P680*. Therefore, the primary function of carotenes in the PSII reaction centers must be restricted to direct quenching of $^1O_2$, produced via ^{3}P680* in conditions of photo-inhibition [21].

^{3}Car* and ^{3}Chl* states have been detected in natural photosystems under illumination by means of zero-field optically-detected magnetic resonance (ZF-ODMR) spectroscopy. The technique is unique in allowing the assignment of triplet populations to specific sites, even in intact environments. In particular, fluorescence-detected magnetic resonance (FDMR) has revealed the presence of ^{3}Car* as well as ^{3}Chl* states, belonging specifically to PSI and PSII, not only in isolated pigmen–protein complexes but also in thylakoids and leaves [19,20,22–25]. At least three ^{3}Chl* populations assigned to PSII have been detected and characterized in terms of zero field splitting parameters and microwave-induced fluorescence emission dependence. Since these ^{3}Chl* states have been detected not only in thylakoids but also in intact systems, such as plant leaves and algal cells [19,23], they do not appear to be due to nonspecific ^{3}Chl* belonging to damaged photosystems.

Although ^{1}Chl* quenching has been widely studied in NPQ conditions by detection of fluorescence as an indirect probe, until now ^{3}Chl* states have been monitored only in dark adapted samples, thus in conditions where no NPQ response was induced. Therefore it is not known whether the "quenched" state of the light-harvesting system, which maintains the level of the ^{1}Chl* state population low, is also characterized by a low ^{3}Chl* state yield in *all* the LHCs. In fact the structural rearrangements following the NPQ activation could lead, in principle, to the formation of ^{3}Chl states in new local traps due to modified interactions among pigments inside the complexes and among LHCs. The exact photo-physical origins of the quenchers remain the subject of discussion in the NPQ field. Involvement of: xanthophyll–chlorophyll charge transfer states, excitonic coupling between chlorophylls and xanthophylls, and, recently, formation of coherent chlorophyll–chlorophyll interactions with charge transfer character in quenching have been proposed. Moreover, either aggregation of the light harvesting complexes or detachment of LHC II has been hypothesized (see ref. [26] for a recent review).

At the same time, it is not known if the ^{3}Chl quenching ability by carotenoids, which strictly depends on the molecular requirements of the Chl/Car couples, is affected by the NPQ-induced structural changes and extra mechanisms have been developed to efficiently quench ^{3}Chl* states *after* their formation. In this work, we exploit the sensitivity of FDMR spectroscopy to answer these questions by measuring the various triplet state populations of PSII in frozen "unquenched" (light-harvesting) and "quenched" (photoprotective) states in the moss *P. patens*.

2. Materials and methods

2.1. Plant material

Protonemal tissues of *P. patens*, Gransden WT strain and triple KO mutants lacking psbs, lhcsr1 and lhcsr2, were grown on $PpNH_4$ medium solidified with 0.7% plant agar (Duchefa Biochemie). Plants were propagated in sterile conditions on 9-cm Petri dishes overlaid with cellophane disks (A.A. Packaging Ltd.), as previously described [15].

Plates were placed in a growth chamber in controlled conditions: 24 °C, 16-h light/8-h dark photoperiod, light intensity 40 μmol photons m^{-2} s^{-1}. In all experiments, 7-day-old plants were used. Before all measurements, plates were dark-adapted for 40 min at room temperature.

2.2. NPQ measurements

In vivo chlorophyll fluorescence in *P. patens* was measured at room temperature with a Dual PAM-100 fluorometer (Heinz Walz GmbH, Germany), with saturating light of 6000 μmol photons·$m^{-2}s^{-1}$ and actinic light of 830 μmol photons·$m^{-2}s^{-1}$. The parameters Fv/Fm and NPQ were calculated as (Fm − Fo)/Fm and (Fm − Fm′)/Fm′. Results were in agreement with those previously reported for the same kind of samples and growth conditions [16].

2.3. Sample preparation and treatment

Pieces of tissue were interposed between two plexiglass slides (0.5 × 2 cm) separated by a 1-mm wedge to allow location of the tissue and, after addition of a few drops of buffer, fitted into the helix of the FDMR spectrometer. NPQ was induced by 5 minutes' illumination with actinic light at 800 μmol photons m^{-2} s^{-1}, supplied by a 150-W projector filtered by 5 cm of water. The samples in the helix were directly illuminated at the entrance of the cryostat and, after 5 minutes' NPQ induction, were immediately frozen in the cryostat pre-filled with liquid helium (see Supplementary material for technical details). This procedure ensured that the samples were frozen within 15 s, so that the qE component was only 25–30% relaxed [16].

In the reduced samples, a solution of 30 mM sodium dithionite in $PpNH_4$ buffer was added in drops to the dark-adapted tissue, and incubated for 15 min. Samples were then illuminated at 800 μmol photons m^{-2} s^{-1} at room temperature for 5 min and finally introduced into the cryostat for FDMR measurements. The same procedure had been used before to obtain the double reduction of Q_A in PSII particles and thylakoids. Under illumination at low temperature, formation of the PSII recombination triplet state was consequently induced [27].

2.4. FDMR measurements

Experiments were performed in laboratory-built apparatus, previously described in detail [19]. FDMR is a double resonance technique based on the principle that, when a triplet steady state population is generated by illumination, application of a resonant microwave electromagnetic field between a couple of spin sublevels of the triplet