FEBS Letters 587 (2013) 1310–1315

FEBS Letters

journal homepage: www.FEBSLetters.org

A transthylakoid proton gradient and inhibitors induce a non-photochemical fluorescence quenching in unicellular algae *Nannochloropsis* sp.

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ARTICLE INFO

Article history:
Received 7 November 2012
Revised 21 December 2012
Accepted 28 December 2012
Available online 6 March 2013

Edited by Richard Cogdell

Keywords:
Non-photochemical fluorescence quenching
Xanthophyll cycle
Transthylakoid proton gradient
Inhibitor
Nannochloropsis sp.

ABSTRACT

Non-photochemical quenching (NPQ) of chlorophyll fluorescence is thought to be an indicator of an essential regulation and photoprotection mechanism against high-light stress in photosynthetic organisms. In this report, special chemicals were used to perturb the kinetics of the ΔpH build-up and the xanthophyll cycle (XC) in *Nannochloropsis* sp. We found that NPQ was stimulated rapidly on exposure to high light and relaxed rapidly in darkness. The ΔpH could be obligatory for NPQ and ΔpH alone was not sufficient to induce NPQ. The XC, being strictly mediated by ΔpH, was also essential for NPQ. The results demonstrate that the mechanism of NPQ in *Nannochloropsis* sp. resembled that of diatoms.

1. Introduction

Photosynthetic organisms live in variable environments and are often exposed to changing light intensities that may vary over several orders of magnitude from limiting to excess conditions rapidly in both the short- (seconds–minutes) and long-term (hours–months) under natural conditions [1]. Over-excitation by surfeit light can produce harmful reactive oxygen intermediates detrimental to pigments, proteins and lipids [2]. Several protective mechanisms can be stimulated when light absorption exceeds its utilization in photosynthesis [3]. The fastest feedback regulatory mechanism is non-photochemical quenching (NPQ), which consists in the thermal dissipation of excess absorbed energy and is activated within seconds upon a change in light intensity [4]. The process of energy dissipation in NPQ is triggered by low pH in the thylakoid lumen and modulated by several factors including zeaxanthin (Zx)/diatoxanthin(Dtx) [5,6], the LHCSR protein [7] and the PsbS protein [1]. As the NPQ process involves the de-excitation of chlorophyll molecules from their exited states, NPQ is usually detected indirectly by analyzing chlorophyll fluorescence [8], rather than directly by monitoring heat emissions [3,9].

Although NPQ has been studied in higher plants, mosses, algae and cyanobacteria, the basic principle of NPQ, the safe dissipation of excessive light irradiation as heat, is identical across all organisms. However, crucial differences exist in its structural mechanisms and regulation [3]. For example, NPQ in diatoms and brown algae is located in fucoxanthin–chlorophyll *a*/*c* antennae [10], which are non-homologous to chlorophyll *a*/*b* antennae of higher plants. In addition to the structural differences, the ability of transthylakoid ΔpH to trigger NPQ also differs. When compared to higher plants, the capacity of ΔpH to induce quenching is decreased in some green algae [11]. On the regulatory level, PsbS is known to be active in NPQ of higher plants; however, another group of light-harvesting proteins from the LHCSR (formerly LI818) family – which are missing in higher plants – are involved in NPQ in green algae [7], brown algae and diatoms [12,13]. Recent studies on the moss *Physcomitrella patens* provided direct evidence that both PsbS and LHCSR are active in NPQ in mosses [14]. Additionally, differences also exist in xanthophyll cycles (XCs): a viola-

Abbreviations: Ax, antheraxanthin; Chl, chlorophyll; DCCD, N,N′-dicyclohexylcarbodiimide; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DC, diadinoxanthin cycle; Ddx, diadinoxanthin; DTT, dithiothreitol; Dtx, diatoxanthin; NPQ, non-photochemical fuorescence quenching; Vx, violaxanthin; XC, xanthophyll cycle; Zx, zeaxanthin; ΔpH, transthylakoid proton gradient

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0014-5793/$36.00
http://dx.doi.org/10.1016/j.febslet.2012.12.031

xanthin (Vx) cycle found in higher plants, green algae and in members of brown algae [6]; and a diadinoxanthin cycle (DC) in diatoms [5]. Furthermore, in cyanobacteria and red algae there is a completely different mechanism of NPQ, triggered/regulated by light-activation of the orange carotenoid protein (OCP), located/operating in the phycobilisomes [15]. NPQ in cryptophytes also represents a novel class of effective and flexible NPQ, in which a direct antennae protonation is involved and there is no light-induced XC [3].

Compared to higher plants, the understanding of NPQ in various algal groups is much more fragmented or missing completely. This is especially true for the genus of *Nannochloropsis*, which are marine unicellular oleaginous alga [16,17]. Recent studies have reported that marine *Nannochloropsis* sp. are potential microalgae for biofuel production as they can produce lipid contents of 10–60% (by weight) in dry matter and have high biomass productivity [18,19]. *Nannochloropsis* is distinguished from higher plants and other algae by lacking chlorophyll (Chl) *b*, Chl *c* and lutein. However, it can develop high concentrations of a range of pigments such as astaxanthin, canthaxanthin and Zx. Furthermore, as in higher plants, the algal xanthophyll contains Vx, antheraxanthin (Ax) and Zx, involved in the modulation of NPQ by the XC. Although photosynthetic performance of outdoor *Nannochloropsis* mass cultures under a wide range of environmental conditions has been researched previously [20], the mechanism of NPQ in *Nannochloropsis* is still unclear [21]. In this report, specific inhibitors were used to alter the amplitude of the ΔpH and/or Vx de-epoxidation reaction. We analyzed the effects of a transthylakoid proton gradient and inhibitors inducing XC and NPQ in unicellular algae *Nannochloropsis* sp.

2. Materials and methods

2.1. Culture conditions

Nannochloropsis sp. cells were grown photoautotrophically in sterile natural seawater F/2 medium at 20 ± 1 °C. They were illuminated at a light intensity of 40 μmol photons m^{-2} s^{-1} with white fluorescent tubes. The light regime used was a classical 12 h light/12 h dark photoperiod. Cells were harvested during the exponential phase of growth, centrifuged at 4000×*g* for 5 min and resuspended in their culture medium to a final concentration of 10^7 cells ml^{-1}.

2.2. Chl fluorescence yield and NPQ

In vivo chlorophyll fluorescence in *Nannochloropsis* sp. was measured at room temperature (RT) with a Dual-PAM-100 fluorometer (Walz, Effeltrich, Germany), using saturating light at 10000 μmol photons m^{-2} s^{-1} and actinic light of 825 μmol photons m^{-2} s^{-1}. After a dark adaptation of 20 min at RT, cells were subjected to a saturating light pulse and then illuminated with an actinic light of adjustable intensity. The average fluorescence measured during the last 300 ms of the saturating pulse was taken as F_m or F_m'. The parameters F_v/F_m, ΦPSII and NPQ were calculated as $(F_m - F_0)/F_m$, $(F_m' - F_t)/F_m'$ and $(F_m - F_m')/F_m'$, respectively, where F_0 is intrinsic fluorescence of the dark-adapted sample, F_m' is the maximum PSII fluorescence in the light-adapted state and F_m that in the dark-adapted state. This Stern–Volmer expression of NPQ is proportional to the concentration of the quencher state [21,22]. For each experiment, 2 ml of cell suspension was used. Sodium bicarbonate was added at a concentration of 4 mM from a freshly prepared 0.2 M stock water solution to prevent any limitation of the photosynthetic rate by carbon supply. When appropriate, NH_4Cl (ammonium chloride), nigericin, DCCD (*N*,*N*′-dicyclohexyl-carbodi-imide), DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea] or DTT (dithiothreitol) were added at the start of dark incubation to perturb the kinetics of the ΔpH build-up, of the XC and of NPQ.

2.3. Xanthophyll contents

Cells collected from the PAM fluorometer (see below) were frozen in liquid nitrogen. Pigments were extracted in methanol:water (95:5, v/v) using sonication (59 kHz, 250 W, Kudos, Shanghai, China). Pigment analyses were performed by high-performance liquid chromatography (HPLC) following the method of Yao et al. [23]. Pigments were identified by comparison of their chromatographic retention times and absorption spectra with those of pure pigment standards, which were obtained commercially: Chl *a*, Vx, Ax, Zx and lutein from ^{14}C Agency (DHI, Denmark). The detection limit of Chl *a* was 0.02 mg l^{-1} (for a sample volume of 1000 ml and an extraction volume of 1.5 μl) and the difference between two replicate measurements was usually <1%.

3. Results

3.1. NPQ kinetics

In order to elucidate the modulate illumination of NPQ in *Nannochloropsis* sp. we measured Chl *a* fluorescence quenching using different intensities of actinic light. The NPQ was activated only by irradiancies >100 μmol photons m^{-2} s^{-1} (Fig. 1), lower irradiances were efficiently utilized in photosynthesis as indicated by the high efficiency of PSII photochemistry (ΦPSII of 0.59–0.70). Increasing the actinic light did not induce a stronger final NPQ for actinic light >825 μmol photons m^{-2} s^{-1} (Fig. 1). Thus actinic light was set to 825 μmol photons m^{-2} s^{-1}, which was shown to be sufficient to ensure maximal NPQ activation for *Nannochloropsis* sp.

3.2. NPQ in Nannochloropsis sp. exhibits fast induction and reversibility

In order to elucidate the basic characteristics of NPQ in *Nannochloropsis* sp. we measured Chl *a* fluorescence quenching using 825 μmol photons m^{-2} s^{-1} actinic light. The decrease in F_m' (Fig. 2) showed that irradiation induced rapid quenching of

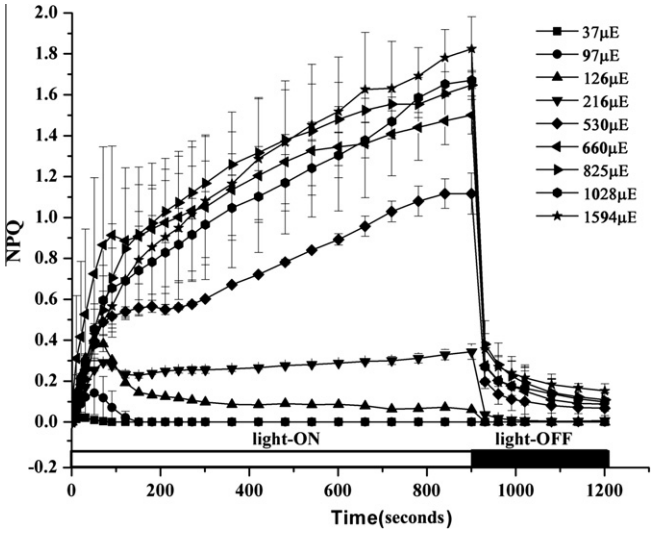

Fig. 1. Non-photochemical quenching (NPQ) kinetics measured using different actinic light intensities. After dark adaptation of 20 min at room temperature, cells were subjected to a saturating light pulse and then illuminated with actinic light of adjustable intensity.

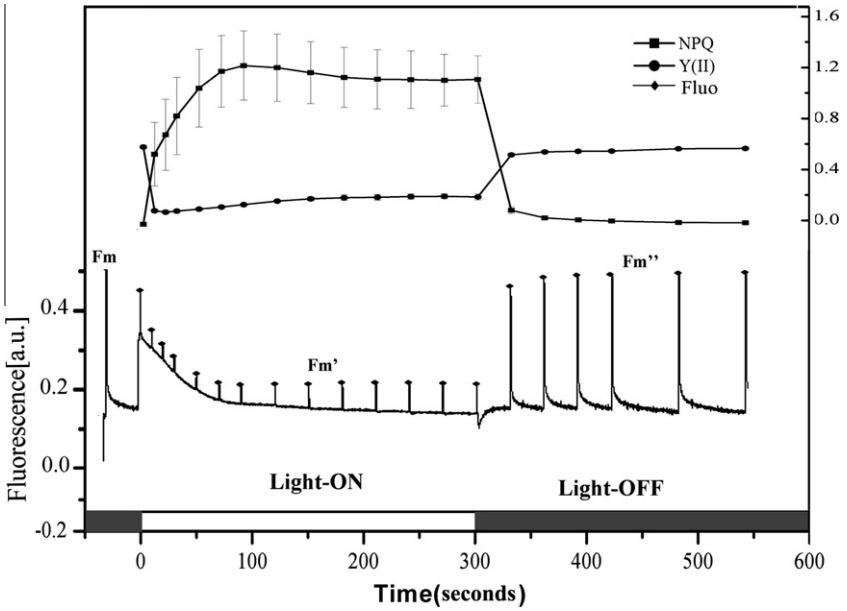

Fig. 2. Chlorophyll *a* fluorescence quenching in *Nannochloropsis* sp. Cells were dark adapted for 20 min before irradiation. NPQ was induced by 300 s of orange actinic light (620 nm, 825 μmol photons m^{-2} s^{-1}; white bar). Fluorescence induction curve (black line) represents a typical curve. The extent of NPQ (squares symbols) was calculated as quenching of maximal fluorescence ($F_m' - F_m$)/F_m' for every saturating flash (n = 3); the maximal fluorescence measured after a light period (F_m'') reflects a fast recovery of F_m quenching.

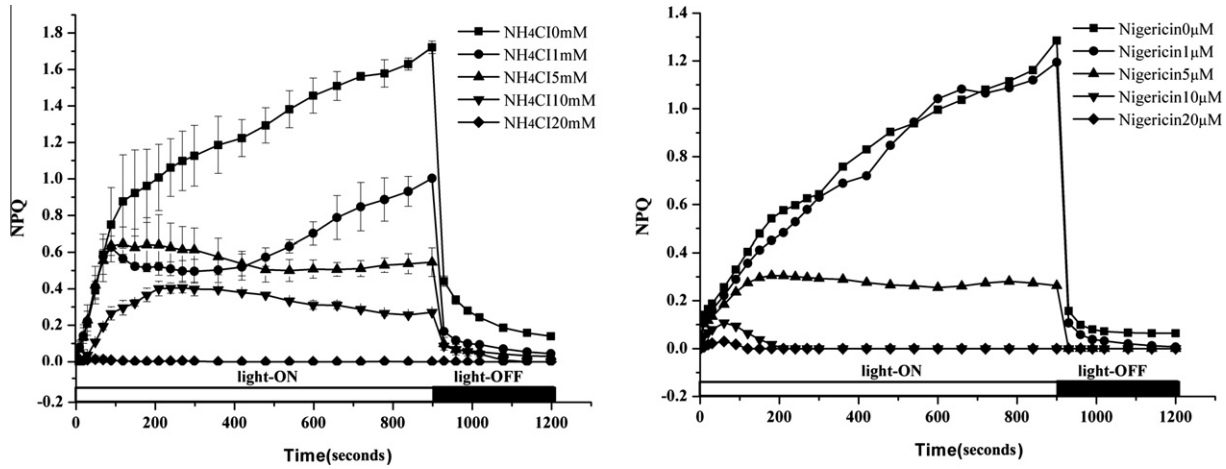

Fig. 3. Effect of increasing concentrations of NH_4Cl and nigericinon NPQ in *Nannochloropsis* sp. Cells were subjected to a saturating light pulse and then illuminated with an actinic light of 825 μmol photons m^{-2} s^{-1} after a dark adaptation of 20 min at room temperature.

maximal fluorescence during the 100 s of irradiation. The calculated photochemical efficiency in the light ΦPSII was around 0.1 (Fig. 2), suggesting the majority of quenching was caused by stimulation of non-photochemical processes and only a minor fraction of the actinic irradiance was used in PSII photochemistry. The recovery kinetics of F_m' (Fig. 2) showed that NPQ was reversed within seconds in darkness. This type of NPQ, characterized by rapid stimulation (in tens of seconds) and fast relaxation, is also well described for other organisms (e.g. *Arabidopsis*, *Rhodomonas salina* and *P. patens*) [3,4,24]. These data show that *Nannochloropsis* sp. could acclimate and modulate photosynthetic capacity in response to environmental conditions.

3.3. Effect of NH_4Cl and nigericin on NPQ

NPQ is triggered by low lumen pH (ΔpH) in most species, e.g., *Arabidopsis* and *R. salina* [3,24]. The immediate response of NPQ to changes in irradiation is due to ΔpH, as the ΔpH across the thylakoid membrane was rapidly formed in the light and quickly dissipated in darkness [25]. To examine whether activation of NPQ in *Nannochloropsis* sp. was ΔpH dependent, two uncouplers, NH_4Cl and nigericin, which could decrease formed ΔpH during illumination, were added. The observed stimulation of NPQ decreased with increased contents of NH_4Cl and nigericin (Fig. 3). Moreover, NPQ was completely restrained when the concentration of inhibitors increase to a certain degree with 20 mM NH_4Cl or 10 μM nigericin. The results showed that ΔpH was essential for the NPQ process in *Nannochloropsis* sp.

3.4. Effect of DCCD on NPQ

The relationship between ΔpH and NPQ was further investigated by using DCCD to vary the extent of the light-dependent lumen acidification. DCCD is a known inhibitor of ATP synthase [26],

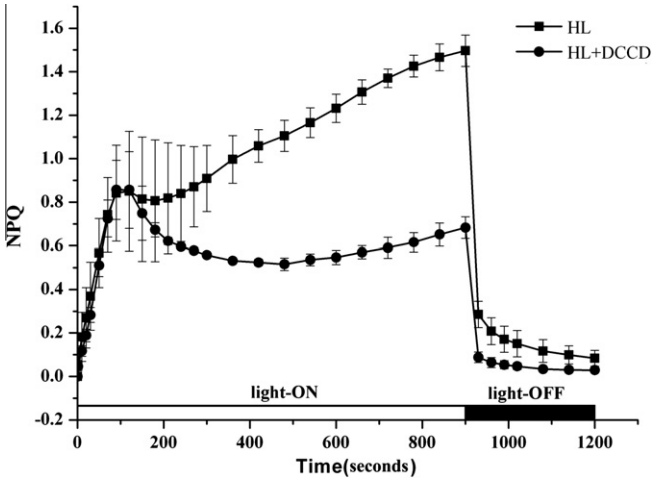

Fig. 4. Effect of 10 min of dark incubation of *Nannochloropsis* sp. cells with 10 μM DCCD on NPQ at 825 μmol photons m^{-2} s^{-1}. DCCD was added at 10 μM at the start of the 10 min dark-adaptation.

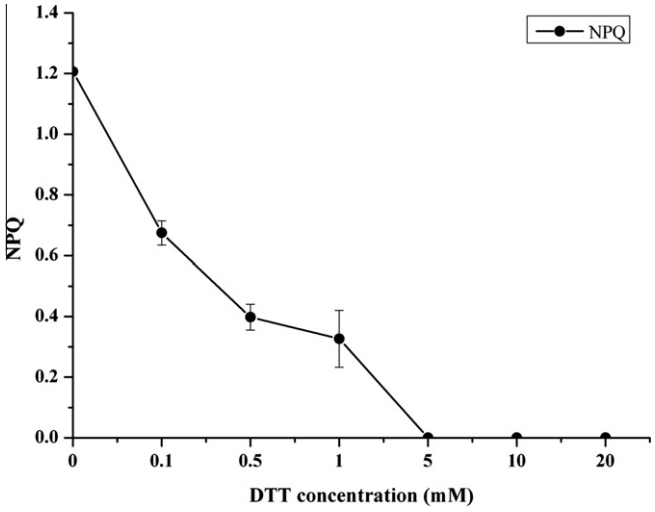

Fig. 5. Effect of increasing concentrations of DTT on NPQ in *Nannochloropsis* sp. Cells were subjected to a saturating light pulse and then illuminated with an actinic light of 825 μmol photons m^{-2} s^{-1} after a dark adaptation of 20 min at room temperature.

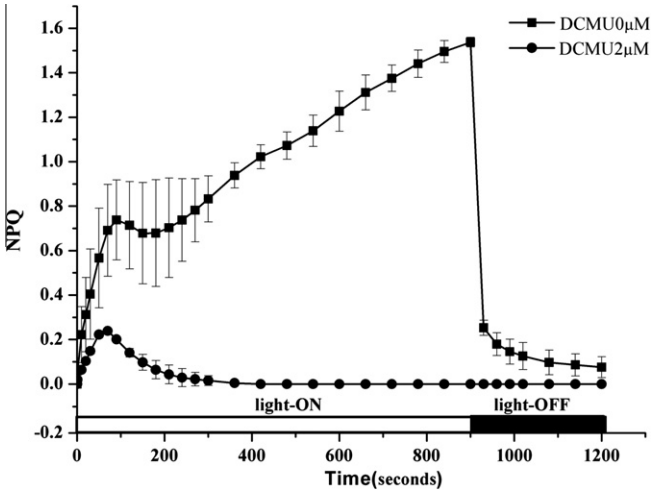

Fig. 6. Effect of increasing concentrations of DCMU on NPQ in *Nannochloropsis* sp. Cells were subjected to a saturating light pulse and then illuminated with an actinic light of 825 μmol photons m^{-2} s^{-1} after a dark adaptation of 20 min at room temperature. Data are presented as mean ± standard deviation of three independent experiments.

Table 1
Relative pigment content in cells of *Nannochloropsis* sp. after 15 min excessive irradiation.

	Vx/Chl *a*	Ax/Chl *a*	Zx/Chl *a*	(Vx + Ax + Zx)/ Chl *a*	(Ax + Zx)/ (Vx + Ax + Zx)
Common light	1.543	0.034	0.037	0.071	0.044
High light	1.941	0.141	0.090	2.172	0.107
NH_4Cl	2.841	0.118	0.077	0.194	0.064
Nigericin	1.716	0.041	0.055	0.095	0.053
DCMU	1.198	0.0148	0.017	0.032	0.026
DTT	1.998	0.0293	0.029	0.058	0.028

blocking the proton conductive channel and thus the reflux of lumenal protons. Its presence should therefore increase the ΔpH formed during illumination. At the same time, DCCD binding to carboxy amino residues located in the hydrophobic domains of light-harvesting antenna can reverse acid-induced fluorescence quenching [27]. For example, in *Chlamydomonas reinhardtii*, NPQ was strongly inhibited by DCCD in high light [28]. In *Nannochloropsis*, NPQ also decreased under higher illumination (825 μmol photons m^{-2} s^{-1}) in the presence of 10 μM DCCD (Fig. 4). This result showed that *Nannochloropsis* had analogous ΔpH-sensing protein and sites to *C. reinhardtii*.

3.5. Effect of DTT on NPQ

DTT is a well-known inhibitor of de-epoxidase and does not affect ΔpH, and has been used to detect Zx-independent fluorescence quenching in higher plants [22]. To elucidate the importance of Zx synthesis through the XC for NPQ formation, *Nannochloropsis* sp. cells were subjected to different DTT concentrations to inhibit Vx de-epoxidase. NPQ decreased as DTT concentrations increased under a saturating light pulse (Fig. 5), indicating a Zx-dependent NPQ in *Nannochloropsis* sp. This is the same as in diatoms [21], where DTT inhibits the accumulation of de-epoxidized xanthophylls with totally suppressing NPQ. However, this differs from results with higher plants [22,29] and green algae [30] in which NPQ was not totally suppressed.

3.6. Effect of DCMU on NPQ

We used the inhibitor DCMU to block electron flow at specific sites of the electron transport chain, thereby blocking the establishment of a proton gradient, to explore how functional components of photosynthetic apparatus contribute to lumen acidification. NPQ was almost completely restrained by 2 μM DCMU (Fig. 6). The inhibitory effect of DCMU on NPQ has been also observed in the diatom *Phaeodactylum tricornutum* [31], in intertidal macroalga *Ulva fasciata* [32] and in the higher plant *Arabidopsis* [33]. Inhibition of PSII by 2 μM DCMU almost completely removed NPQ. This demonstrated that the dependency of NPQ on lumen acidification was related to PSII activity and NPQ was induced only under conditions where electron transport is saturated, in order to avoid undesirable dissipation of excitation energy under light-limiting conditions.

3.7. XC is involved in NPQ in Nannochloropsis sp.

We explored possible changes in pigment composition following short-term light stress to ascertain if *Nannochloropsis* sp. was

able to transform xanthophylls under conditions of excessive irradiation. Carotenoids involved in light-induced XCs (e.g., Ax and Zx) in *Nannochloropsis* sp. cells were detected (Table 1) without lutein. We found lower relative contents of Ax and Zx in treatments of adding inhibitors in comparison to intact cells after 15 min of excessive irradiation. This is similar to the situation in higher plants and diatoms where the VC or DC is activated by high light [21,34]. These data indicated that the XC was involved in NPQ in *Nannochloropsis* sp. – consistent with the recently published results concerning other algae.

4. Discussion

The dissipation of excess energy as heat is one of the most important photoprotection mechanisms of photosynthetic organisms [35–37]. We demonstrated that efficient NPQ (Fig. 1) operated in *Nannochloropsis* sp. It would be very interesting to compare the NPQ investigated in the present study with the NPQ in the rest of the plant kingdom. *Nannochloropsis* sp. represents a unique evolutionary link within the heterokont line of eukaryotes. It belongs to Eustigmatophyceae, which, together with Phaeophyceae (brown algae) and Bacillariophyta (diatoms) are sister groups in the Stramenopiles. Its lack of Chl *b* and *c* differs from brown algae and diatoms, which contain Chl *a*/*c* antennae but lack phycobiliproteins; and also differs from Rhopdophyta (red algae), which lack Chl *c* but contain phycobilisomes.

The NPQ dependency on lumen acidification has been demonstrated in diatoms and brown algae, green algae and higher plants [1]. In *Nannochloropsis* sp., both ΔpH and XC were crucial for NPQ (Figs. 3 and 5) and ΔpH itself could not induce NPQ without the XC (Fig. 5). In diatoms and brown algae, although low lumenal pH is crucial for the formation of Dtx or Zx (and thus of NPQ induction), high NPQ can be maintained even in the absence of a transmembrane proton gradient [38], once Dtx/Zx has been formed [5] and lumen acidification alone is not sufficient to induce NPQ [21]. In red algae, the quenching located in the phycobilisomes is triggered by light-activation of OCP [1]. In green algae, NPQ is essentially controlled by ΔpH and further depends on the action of xanthophylls and the maximal NPQ was found to be clearly less dependent on Zx compared with higher plants [29], or even independent of Zx [28]. Additionally, the relaxation of NPQ in green algae is strictly dependent on the removal of ΔpH, while in higher plants, only the qE component of NPQ is fully deactivated by collapse of ΔpH, while Zx-dependent NPQ processes appear to remain active even in the absence of a low lumen pH. Therefore, NPQ and its controlling factors in *Nannochloropsis* sp. resemble those in diatoms.

Furthermore, under high light conditions, the addition of DCCD inhibited NPQ of *Nannochloropsis* sp. as previously reported in green algae and in higher plants [28,39], where DCCD binds to the proton-binding sites (carboxy amino residues in the hydrophobic domains) of the antenna protein and LHCSR/PsbS (as a sensor of lumen pH) antenna polypeptides, thus inhibiting NPQ [37,40]. Two putative violaxanthin–chlorophyll proteins (VCPs) have been identified with high similarity to LHCSR proteins in green algae and LHCX proteins in diatoms [41]. NPQ in *Nannochloropsis* sp. was suppressed under high light conditions with DCCD, due to DCCD binding to sites of ΔpH sensors with competition. The results demonstrated there was an analogous sensor of lumen pH in *Nannochloropsis* sp.

In conclusion, we propose that, in *Nannochloropsis* sp., NPQ was rapidly stimulated on exposure to saturated actinic light and rapidly relaxed in darkness in tens of seconds. It was fully mediated by ΔpH and it alone was not sufficient to induce the dissipative function of NPQ. XC, which was controlled by ΔpH, was also essential for NPQ. As a *Nannochloropsis* genome sequence has been published, in which two LHCSR-like proteins were observed, and the NPQ was suppressed under high light with DCCD, we confirmed our assumption that there may be a LHCSR protein acting as a sensor of ΔpH, which is crucial for the induction of NPQ. Further study is required to explicitly identify these proteins.

Acknowledgments

This work was supported by National Natural Science Foundation of China (31200187, 41176153), Hi-Tech Research and Development Program (863) of China (2012AA052103), Shandong Science and Technology plan project (2011GHY11528), the Specialized Fund for the Basic Research Operating expenses Program (20603022012004), Natural Science Foundation of Shandong Province (2009ZRA02075), Qingdao Municipal Science and Technology plan project (11-3-1-5-hy), National Marine Public Welfare Research Project (200805069).

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