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Roles of the transthylakoid proton gradient and xanthophyll cycle in the non-photochemical quenching of the green alga *Ulva linza*

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ABSTRACT

Non-photochemical quenching (NPQ) is one of the most important photoprotection mechanisms in photosynthetic organisms when they are exposed to excessive irradiation. The basic principle of NPQ, which is the safe dissipation of excessive absorbed light as heat, is identical in all photoautotrophs. However, crucial differences in its regulation and structural mechanisms exist in different phototrophs. Here, we investigated NPQ in the green alga *Ulva linza* coupled with inhibitors to alter the amplitude of the transthylakoid proton gradient (ΔpH) and/or de-expoxidation of xanthophyll cycle (XC) under high light conditions. The data demonstrates that NPQ started with a rapid initial rise within the first minute of illumination, followed by a decline before a further rise in quenching. During the whole phase, NPQ was triggered and completely controlled by ΔpH, then strengthened and modulated by zeaxanthin. NPQ relaxation was slower in *U. linza* when compared to plants and other green algae, and it may be mainly caused by the slow conversion of zeaxanthin to violaxanthin. NPQ in *U. linza* is controlled to a greater extent by XC, which show more similarities to *Arabidopsis* than to *Chlamydomonas* and may be an adaptive mechanism for its successful colonization of coastal ecosystems.

1. Introduction

Light is essential for photosynthetic eukaryotes to assimilate carbon through photosynthesis. However, when the amount of absorbed light exceeds photosynthetic capacities, reactive O_2 species are generated in the chloroplasts causing photoinhibition that can limit plant photosynthetic activity, growth and productivity (Barber and Andersson, 1992). Photosynthetic organisms have developed diverse photoprotection mechanisms to avoid net photoinhibition: reactive oxygen species (ROS) scavenging systems; thermal dissipation of excess light energy (non-photochemical quenching, NPQ); cyclic electron flow (CEF) around photosystem I (PSI); and the photorespiratory pathway (Takahashi and Badger, 2011). The fastest and most-studied photoprotective mechanism is non-photochemical quenching (NPQ) of excitation energy, which is activated within seconds after changes in light intensity and mostly due to thermal deactivation of excited states of pigments in the antenna of PSII (Horton et al., 1996, 2008; Horton and Ruban, 2005).

NPQ is a very complex and finely feed-back regulated process, and the exact mechanism is different between oxygenic photosynthetic organisms such as that in higher plants and algae. It was best characterized in higher plants (Horton and Ruban, 2005; Horton et al., 2005), in which different NPQ components based on different molecular mechanisms have been defined. The fast component is the energetic or pH-dependent quenching (qE) which is triggered by the proton gradient across the thylakoid membrane (ΔpH), and modulated by several factors including the xanthophyll cycle (XC) (Ruban and Horton, 1999) and the PsbS protein (Li et al., 2004; Johnson and Ruban, 2010). Low pH in the thylakoid lumen activates the lumen-located violaxanthin (Vx) de-epoxidase (VDE), which converts Vx first to antheraxanthin (Ax) and then to zeaxanthin (Zx) through the xanthophyll cycle (Bugos and Yamamoto, 1996). Zeaxanthin-dependent quenching (qZ) is further stimulated under prolonged irradiation which appears to remain active even in the absence of a low lumen pH (Nilkens et al., 2010). Photoinhibitory quenching (qI) is a slow reversible damage to PSII reaction centers after a long-term exposure (hours and days) to excessive irradiation (Aro et al., 1993; Ruban and Horton, 1995; Osmond et al., 1997), and data suggest that qZ might contribute substantially to this process (Dall'Osto et al., 2005). State transition quenching (qT) is a change in the relative antenna sizes of PSII and PSI, due to the reversible phosphorylation and migration of antenna proteins (LHCII) (Allen, 1992).

The basic principle of NPQ in algae is identical to that in higher plants, however, the mechanisms of NPQ show more structural and

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regulatory diversity in algae. NPQ in green algae is essentially controlled by ΔpH and further depends on the action of xanthophylls (Niyogi et al., 1997a,b), but the capacity of ΔpH to induce quenching is decreased in some green algae when compared to higher plants (Finazzi et al., 2006). The maximum NPQ was found to be less dependent on Zx in comparison with higher plants (Niyogi et al., 1998) or even independent of Zx (Bonente et al., 2011). Light harvesting proteins from the LHCSR (formerly LI818) family are essential for NPQ in diatoms and green algae instead of the PSBS protein in higher plants (Bonente et al., 2008; Peers et al., 2009; Zhu et al., 2010). NPQ in diatoms is located in the fucoxanthin–chlorophyll a/c antennae (Lavaud et al., 2002; Gundermann et al., 2008; Miloslavina et al., 2009) which are non-homologous to the chlorophyll a/b antennae of higher plants. And the NPQ is associated with a diadinoxanthin cycle involving diadinoxanthin (DD) and its de-epoxidized form diatoxanthin (DT) (Goss et al., 2006). In addition, in diatoms and brown algae, low lumen pH alone does not induce NPQ in contrast to higher plants, and the role of ΔpH seems to be related only to activation of the de-epoxidase enzyme (Lavaud et al., 2002; Garcia-Mendoza and Colombo-Pallotta, 2007; Garcia-Mendoza and Govindjee, 2011). NPQ in cyanobacteria and red algae is distinct, in which quenching is located in the phycobilisomes instead of PSII and is triggered by light activation of the orange carotenoid protein (OCP) (Rakhimberdieva et al., 2004; Wilson et al., 2006; Kirilovsky, 2007) in an unknown manner (Wilson et al., 2008).

As a special type of harmful algal bloom, green tides have been increasing in severity and geographic range and are now of growing concern globally (Ye et al., 2011). During the past 7 years, the world's largest green tides have occurred successively in the Yellow Sea. The great majority of green tides consist of members of just one genus: *Ulva* (Zhang et al., 2011). The light irradiance varies more dramatically for algae than for land plants because of the morning and evening tides (Zhang et al., 2012). Moreover, when a green tide occurs, the algae float on the sea surface and are exposed to the sunlight all day long. Thus, *Ulva* must be well adapted to changes in light irradiance which has a xanthophyll cycle similar to higher land plants and has a high NPQ capacity (Franklin et al., 1994; Bischof et al., 2002). In the present study we choose *Ulva linza* as a model to characterize the mechanisms involved in the high NPQ in *Ulva*. Specific inhibitors were used to alter the amplitude of ΔpH and/or the xanthophyll cycle, and coupled NPQ. Their effect on the relationship between ΔpH, zeaxanthin and NPQ was studied.

2. Material and methods

2.1. Sampling and culture conditions

Samples of *Ulva linza* were collected in May 2011 from the intertidal zone (35°35′N, 119°30′E) of Zhanqiao Wharf, Qingdao, China. In the laboratory, the intact samples were washed several times with sterile seawater, sterilized with 1% sodium hypochlorite for 2 min, and then rinsed with autoclaved seawater. The sterilized material was then placed into an aquarium containing enriched seawater (500 μM $NaNO_3$ and 50 μM NaH_2PO_4), and maintained at 15 °C under a cycle comprising 12 of light alternating with 12 h of darkness. The light intensity was 100 μmol m^{-2} s^{-1} provided by cool-white fluorescent tubes. The functions of ΔpH and the XC were characterized by using the inhibitors NH_4Cl, nigericin and DTT. Inhibitors of NH_4Cl and nigericin are known as the uncouplers of the ΔpH, while DTT was used as a specific inhibitor of VDE. When appropriate, NH_4Cl (ammonium chloride), nigericin, or dithiothreitol (DTT) were added at the start of dark incubation to perturb the kinetics of the ΔpH build-up, the xanthophyll cycle and NPQ.

2.2. Chl fluorescence yield and NPQ measurement

In vivo chlorophyll fluorescence measurements of *Ulva linza* cells were performed at room temperature with dual-wavelength pulse amplitude modulated fluorescence monitoring system (Dual-PAM, Heinz Walz, Germany). All of the samples were taken from the aquarium in the morning. The minimum fluorescence yield (F_0) was determined using 30 min dark-adapted samples and the maximum fluorescence yield (Fm) obtained by applying saturating pulses (10,000 μmol m^{-2} s^{-1} for 300 ms). NPQ kinetics in control light was measured at different actinic light intensities: 55, 128, 218, 837 and 1954 μmol m^{-2} s^{-1}. For samples cultured under different inhibitor conditions, actinic light was used at 1954 μmol m^{-2} s^{-1} after the dark adaptation. The energy dissipation that was not used for PSII photochemistry was determined based on the fluorescence quenching effect using the ratio NPQ = (Fm−Fm')/Fm' (Bilger and Björkman 1990). The effective PSII quantum yield was calculated as follows: Y(II) = (Fm'−Ft)/Fm'. All experiments were conducted in triplicate. For NPQ kinetics, to minimize the contribution of the saturating light flashes (which are required to determine the NPQ parameter) to NPQ formation, the flashes were separated at 10 s intervals for the first 30 s of induction, 30 s intervals between 30 s and 300 s, and 60 s intervals from 300 s to 600 s.

2.3. Pigment contents analysis

To extract pigments, samples were treated under different conditions which were repeated three times. Samples of repeated conditions were all mixed together then were pulverized in liquid nitrogen. Pigment contents were extracted in 3 ml cold 85% (V/V) acetone, and then centrifuged at 12000 *g* for 2 min. The supernatant was passed through a 0.2 μm syringe filter into an amber HPLC vial. Pigments were analysed following Thayer and Björkman (1990) with minor modifications using a HP1100 Liquid Chromatograph equipped with a diode array detector (Agilent Technology, Palo Alto, CA, USA). An Agilent Technology non-endcapped Zorbax ODS column (4.5 × 250 mm, 5 mm particle size) was used in the separation, preceded by a C18 Adsorbosphere guard column (Alltech Associates, Inc., Deerfield, IL USA). The concentrations of XC pigments were determined using reversed phase HPLC as described by Zapata et al. (2000) and spectrophotometrically detected by their absorbance at 450 nm. Pigment quantification was performed using calibration curves calculated from HPLC separations using purified pigment standards. The de-epoxidation state of the violaxanthin cycle was calculated as (Z + A)/(V + A + Z). For samples treated with different inhibitor conditions, actinic light was used at 1954 μmol m^{-2} s^{-1} after the dark adaptation, and the pigment quantification was conducted.

2.4. Statistical analysis

The data were analysed with SPSS 17.0 (SPSS, Chicago, IL, USA). Statistical significance was tested using a one-way analysis of variance (ANOVA). All values were expressed as the mean ± SD. Graphs were plotted with OriginPro 8.0 (OriginLab Corporation, Northampton, MA, USA).

3. Results

3.1. NPQ phenotypes in Ulva linza

In order to elucidate the basic characteristics of NPQ in *Ulva linza*, we measured chlorophyll quenching indicated of thermal energy dissipation in different light intensities. The results of NPQ