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A chloroplast "wake up" mechanism: Illumination with weak light activates the photosynthetic antenna function in dark-adapted plants

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ABSTRACT

The efficient and fluent operation of photosynthesis in plants relies on activity of pigment-protein complexes called antenna, absorbing light and transferring excitations toward the reaction centers. Here we show, based on the results of the fluorescence lifetime imaging analyses of single chloroplasts, that pigment-protein complexes, in dark-adapted plants, are not able to act effectively as photosynthetic antennas, due to pronounced, adverse excitation quenching. It appeared that the antenna function could be activated by a short (on a minute timescale) illumination with light of relatively low intensity, substantially below the photosynthesis saturation threshold. The low-light-induced activation of the antenna function was attributed to phosphorylation of the major accessory light-harvesting complex LHCII, based on the fact that such a mechanism was not observed in the *stn7 Arabidopsis thaliana* mutant, with impaired LHCII phosphorylation. It is proposed that the protein phosphorylation-controlled change in the LHCII clustering ability provides mechanistic background for this regulatory process.

1. Introduction

Life on Earth is powered by the energy of light reaching our planet from the Sun but the conversion of energy of electromagnetic radiation to the forms which can be directly used to drive biochemical reactions is realized via the process of photosynthesis. The primary photochemical reactions take place in the photosynthetic reaction centers but their fluent and efficient operation is maintained by the activity of numerous, specialized pigment-protein complexes called antennas, absorbing light quanta and transferring electronic excitations towards the reaction centers. Light-harvesting pigment-protein complex II (LHCII) is the major antenna protein in plants and the most abundant membrane protein in the biosphere (Barros and Kuhlbrandt, 2009). Owing to its relatively high concentration in chloroplasts, the complex has a significant influence on structural and dynamic properties of the thylakoid membranes (Ruban and Johnson, 2015). Exceptionally high protein concentration in the thylakoids of plants, ranging up to 80 % of the surface of the lipid-protein membranes of grana structures, is referred to as a "molecular crowding" (Kirchhoff, 2008). One can expect that such a tight packing of pigment-protein complexes facilitate long-range excitation energy transfer, which is critically dependent on a donor-acceptor distance (Andrews and Demidov, 1999; Förster, 1959). On the other hand, photosynthetic antenna complexes and in particular LHCII, present an exceptionally strong tendency to form clusters in the lipid phase, leading to excitation quenching manifested by shortening of lifetimes of their excited states (Gruszecki et al., 1997; Moya et al., 2001; Natali et al., 2016). Obviously, a process of energy dissipation decreases the efficiency of excitation transfer and reduces a performance of the photosynthetic antenna function. This process seems to be particularly unfavorable under low light conditions, at which the photosynthetic apparatus essentially functions as a quantum

Abbreviations: LHCII, Light-harvesting complex II; Chl *a*, chlorophyll *a*; FLIM, fluorescence lifetime imaging microscopy.

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counter, employing every single light quantum to drive electric charge separation. In the present work, we address the problem of this apparent paradox in experiments carried out on single chloroplasts with the application of the fluorescence lifetime imaging microscopy technique.

2. Materials and methods

2.1. Plant material

Arabidopsis thaliana (L.) plants were grown under controlled conditions in a climate chamber with photosynthetic photon flux density (PPFD) of 120 μmol photons $m^{-2}s^{-1}$, 60% humidity and a 22/18 °C day/night temperature. The photoperiod was 8 h light and 16 h dark. Ecotype Columbia was used in all experiments. Wild-type Columbia (Col) and *stn7* mutant (SALK 073254) used in the experiments were obtained from the Salk Institute. The *stn7* mutant was characterized in (Bellafiore et al., 2005). *Spinacia oleracea* (L.) fresh leaves were purchased from the local market. The leaves were kept at 4 °C for 24 h in the dark in order to facilitate decomposition of starch granules before LHCII isolation and measurements.

2.2. LHCII isolation

Spinach leaves were dark-adapted for 24 h or illuminated with white light (intensity of 100 μmol photons $m^{-2}s^{-1}$) for 2 h. From dark adapted leaves LHCII was isolated according to (Krupa et al., 1987) with slight modifications (Gruszecki et al., 2009). Directly after illumination, phosphorylated LHCII was isolated from leaves as described previously (Janik et al., 2013). The purity of preparations was controlled using HPLC and electrophoretic methods (Gruszecki et al., 2009). The chlorophyll concentration was determined in 80% acetone, according to (Lichtenthaler, 1987). The Chl *a*/Chl *b* molar ratio in the preparations was approximately 1.33. The level of LHCII phosphorylation in preparations obtained from different light regimes was determined using SDS-PAGE electrophoresis and immunoblot analysis methods as described previously (Janik et al., 2013).

2.3. Preparation of lipid-LHCII membranes

MGDG and DGDG (from Lipid Products, UK) were mixed in chloroform:methanol (2:1, v:v) solution in a molar ratio of 2:1. Next, the mixture was dried under a stream of nitrogen to a thin film. In order to remove traces of organic solvents, which may possibly remain in dry lipid films, the samples were placed in vacuum (less than 10^{-5} bar) for 30 min. The LHCII complexes were suspended in tricine buffer (20 mM Tricine, 10 mM KCl, pH 7.6) containing 0.1% DM. For incorporation of LHCII complexes into lipid membranes, the LHCII samples were transferred to glass tubes containing the deposited film and subjected to mild sonication using an ultrasonic bath for 30 min. The molar ratio of LHCII:lipids was 1:200. Next, DM was removed from the suspension by incubation with Bio-beads adsorbent (from Bio-Rad) at 4 °C for 12 h. The lipid-LHCII membranes were separated from the detergent-free suspension by centrifugation at 15000xg for 5 min. The pellet was resuspended in tricine buffer. Next, lipid-LHCII multibilayers were formed by a self-organization on nonfluorescent glass slides by evaporation of the water suspension. A structural characterization of lipid-LHCII multibilayers prepared according to a similar procedure is described elsewhere (Gruszecki et al., 2015; Janik et al., 2013).

2.4. Preparation of epidermis

In order to obtain intact cells with chloroplasts, the bottom epidermis from *Arabidopsis thaliana* and *Spinacia oleracea* leaves was prepared by rupture. Subsequent samples were transferred into buffer (20 mM Tricine, 0.4 M Sorbitol, pH 7.8), placed between two non-fluorescent glass slides (Menzel-Glaser) and subjected to measurements.

2.5. FLIM (fluorescence lifetime imaging microscopy)

Time resolved imaging was performed on a confocal MicroTime 200 (PicoQuant, GmbH, Germany) system coupled to an OLYMPUS IX71 microscope. A 470 nm pulsed laser, with repetition adjusted in the range from 0.2 to 40 MHz was used as an excitation source. The full width at half height (FWHM) of the pulse response function was 68 ps (measured by PicoQuant, Inc.). The time resolution was better than 16 ps. More details are presented in Supplementary material. The laser beam was focused on samples with the use of a 60x water immersed objective (NA 1.2, OLYMPUS). In order to select a single focal plane and reduce excitation light a pinhole diameter of 50 μm was used, thereby restricting the confocal volume to x = y = 280 nm and z = 1009 nm. Scattered light was removed by using a ZT 473RDCXT dichroic filter and a 690/70 band pass filter (Chroma Technology Corp.). Fluorescence photons were collected with a single photon sensitive avalanche photodiode (APD) with processing accomplished by the HydraHarp400 time-correlated single photon counting (TCSPC) mode. Laser minimum power was adjusted to 0.66×10^{-10} W, at 1 MHz repetition frequency, in the case of experiments with chloroplasts of *S. oleracea* and to 0.80×10^{-10} W, at 1 MHz repetition frequency, in the case of experiments with chloroplasts of *A. thaliana*. Decay data analysis was performed using the SymPhoTime 64 software package. In order to avoid abrupt fluorescence intensity changes related to Kautsky effect, before recording of the first images, at the lowest repetition frequency, each chloroplast was pre-scanned for 2 min. The following scans at each laser repetition frequency were realized within 3.5 min. Each chloroplast was scanned for several times, each scan with increased repetition frequency. Laser repetition frequency is related linearly to a photon flux density and can be expressed as equivalent of a photon flux density of sunlight absorbed by chloroplasts. In general, it was ensured that difference between photon flux densities of subsequent scans was lower than 10 μmol photons $m^{-2}s^{-1}$. Gradual increase in scanning light intensity, with such a small increment, resulted in the relatively quick adaptation of a chloroplast, manifested by uniform distribution of fluorescence lifetimes over the entire object scanned. All fluorescence photons collected during each chloroplast imaging, were analyzed in terms of a multi-component exponential decay. A set of fluorescence lifetime parameters representing the best fit and characteristic to a chloroplast imaged with the application of a certain light intensity, was used as an initial parameter set in analysis of the same chloroplast imaged with increased laser repetition frequency. FLIM imaging, at different laser pulse repetition rates, was performed for more than 20 chloroplasts, from different leaves, and despite certain varieties in individual samples gave similar results.

3. Results

Fig. 1 presents a chloroplast of *Spinacia oleracea* imaged with the application of fluorescence lifetime imaging microscopy (FLIM), with a laser operating at different repetition frequencies. An increase in repetition frequency results in a linear rise in photon flux density directed to a sample. As can be seen, the increase in the photon flux density results in the alterations in an average chlorophyll *a* (Chl *a*) fluorescence lifetime. A detailed analysis of average fluorescence lifetime in this particular chloroplast is presented in Fig. 2 and fluorescence lifetime components are presented