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Low pH-induced regulation of excitation energy between the two photosystems

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ABSTRACT

Earlier studies have proposed that low pH causes state transitions in spinach thylakoid membranes. Several *Arabidopsis* mutants (*stn7* incapable in phosphorylation of LHC II, *stn8* incapable in phosphorylation of PSII core proteins, *stn7 stn8* double mutant and *npq4* lacking PsbS and hence qE) were used to investigate the mechanisms involved in low pH induced changes in the thylakoid membrane. We propose that protonation of PsbS at low pH is involved in enhancing energy spillover to PS I.

1. Introduction

In oxygenic photosynthesis, a concerted action of Photosystem I and II (PSI and PSII) is required to ensure efficient electron transfer from water to NADP+ in the thylakoid membrane. Under different environmental conditions, plants are capable of regulating the relative reducing abilities of PSII and PSI through several mechanisms such as (i) control of relative excitation of the two photosystems, (ii) regulation of electron transfer rates at Cytochrome b_6f ($Cytb_6f$) complex [1], (iii) manipulating the amount of active/inactive reactions centres of PS II [2], (iv) regulation of electron transport by the lumen pH [3]. A fine tuning and concerted function of all these mechanisms is required to optimize photosynthetic efficiency under changing light intensities, temperature etc. [4].

The light-induced activation of photosynthetic electron transport chain (ETC) results in the translocation of protons across the thylakoid membrane leading to alkalization of the stroma and acidification of lumen [5]. Acidic lumen leads to inhibition of PS II activity probably because of a reversible dissociation of Ca^{2+} from the water splitting enzyme [6]. There are two mechanisms of down-regulation of photosynthetic electron transport resulting from pH dependent regulation of PS II activity. One is deceleration of protolytic steps of PSII turnover caused by acidic lumen and another is enhancement of thermal dissipation of energy in the light harvesting antenna of PS II, measured as Non-Photochemical Quenching (NPQ) of fluorescence [5]. NPQ has been shown to be modulated by several factors including xanthophyll cycle, the PsbS protein and state transitions [7]. A correlation between NPQ and a minor light harvesting complex of PSII (LHCII) protein CP29 has likewise been reported where CP29 plays an important role in regulating the kinetics of PsbS-dependent NPQ formation under fluctuating light conditions [8]. In higher plants, there are two well characterized elements which are associated with qE, the major component of NPQ. First is protonation of the PSII protein PsbS, which if absent, inhibits qE and makes the plant more susceptible to photoinhibition [9] and second is the activation of the xanthophyll cycle leading to an accumulation of Zeaxanthine (Zx).

Acidification of lumen has been shown to induce PsbS- and xanthophyll cycle dependent thermal dissipation of excitation energy [10,11]. Likewise, a reversible dissociation of PsbS dimers into monomers, which is the relevant form of the protein for NPQ, has been shown to occur [10]. Electron microscopic studies have revealed that PsbS protonation regulates the macro-organisation of PS II complexes in the grana membranes of higher plant chloroplasts [12]. A role of PsbS in regulating cyclic electron flow by controlling the tight coupling between $Cytb_6f$ and PSI was proposed [13]. Protonation of PsbS drives concerted conformational changes

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in LHC II leading to qE [14]. Direct interactions of LHCs of PSII with PsbS monomers have recently been shown to regulate NPQ in *Arabidopsis* [15].

Nevertheless, qE has also been observed in intact *Arabidopsis* chloroplasts isolated from plants lacking PsbS [16]. In an another study, the role of low pH was emphasized in driving state transitions [17] deduced from the fact that low luminal pH resulted in distribution of more energy towards PSI. Low pH was also shown to cause reversible structural reorganizations in the thylakoid membrane, affecting the long range order of the protein complexes [18] thereby providing an explanation for pH induced redistribution of excitation energy. Changes in the PSII-LHCII macro-structure are a crucial element in the regulation of qE and have been shown to depend on the formation of ΔpH [19]. However, there are debates still going onto explain the pH-induced changes in terms of so-called "State-transitions". According to classical State transition theory, phosphorylation of LHCII is a prerequisite for its migration from PSII to PSI [20]. Live cell imaging of phosphor-LHCII (P-LHCII) dissociation during state transitions has been reported in *Chlamydomonas reinhardtii* [21]. On the other hand, there are several reports demonstrating no direct correlation between phosphorylation and migration of LHCII [17,22].

Here we have addressed the questions whether the bulk pH of the thylakoid membrane influences the phosphorylation of LHCII, whether the changes in pH regulate state transitions and what are the probable mechanisms underlying the low pH induced changes in thylakoids? To this end, we have conducted experiments on following mutants of *Arabidopsis*: *stn7* (incapable to phosphorylate LHC II), *stn8* (deficient in phosphorylation of PSII core proteins), *stn7 stn8* double mutant and *npq4* (lacks PsbS and hence the qE mechanism). It is necessary to make it clear that in these experiments, bulk pH of the thylakoid was equilibrated and no pH gradient was formed. The aim of the work was to pin-point the mechanisms caused by low pH.

2. Materials and methods

Wild-type (WT) *Arabidopsis thaliana* (ecotype Columbia) and the various mutants plants were grown in phytotronin a mixture of soil and vermiculite (1:1) under constant moderate light (130 μmol photons m^{-2} s^{-1}), 8 h photoperiod and relative humidity of 60%. Light was provided by OSRAM PowerStar HQIT 400/D Metal Halide Lamps. Mature rosette leaves from 5 to 6 weeks old plants were used for experiments. Thylakoid membranes were isolated from light-adapted plants (four hours after the beginning of light period) as described in [23] and the chlorophyll content was determined according to [24].Treatment with pH 5.5 and 7.5 was given by suspending the thylakoid membranes in a medium containing 0.33 M sucrose, 50 mM HEPES–NaOH (pH 7.5) or MES–NaOH (pH 6.5, 5.5), 1 mM $MgCl_2$ and 1 mM NaCl for 30 min in the dark. Fluorescence emission spectra at 77 K were obtained using a diode array spectrophotometer (S2000; Ocean Optics) equipped with a reflectance probe. To obtain the 77 K fluorescence excitation spectra, the sample were excited with wavelengths from 400 to 540 nm with a 5-nm steps using f/3.4 Monochromator (Applied Photophysics).

3. Results

Thylakoid membranes isolated from leaves of *Arabidopsis* wild type and mutant plants were subjected to measurements of the fluorescence emission spectra at room temperature (RT) (Fig. 1A) and 77 K (Fig 1B). As reported earlier in case of spinach [17], there is a quenching observed in PS II fluorescence at RT and a significant change in the ratio of F730/F685 (PSI/PSII) at 77 K in WT

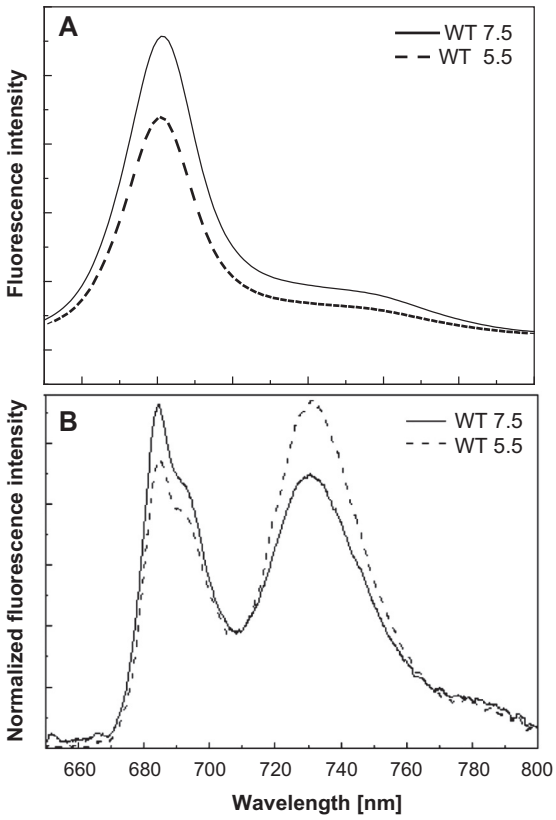

Fig. 1. Fluorescence emission spectra of WT *Arabidopsis* thylakoids at room temperature (RT) and low temperature (LT) 77 K normalized at 710 nm suspended in reaction mixture having buffers of pH 7.5 and 5.5.

Arabidopsis upon incubation of isolated thylakoids in buffer of pH 5.5 (Fig. 1, Table 1). Intriguingly, similar high F730/F685 ratio was recorded also from the *stn7*, *stn8* and *stn7 stn8* double mutant thylakoids at low pH providing evidence that traditional state transitions are not involved in pH-induced changes in the distribution of absorbed excitation energy between the two photosystems (Fig. 2, Table 1). Contrary to WT and the *stn7*, *stn7/8* mutants, no pH induced change in the PSI/PSII ratio was observed in the *npq4* mutants (Fig. 2). This suggested that PsbS dependent NPQ may be involved in causing the pH-induced changes in the fluorescence intensity of the two photosystems which provides some clue about absorption cross section of the photosystems. PsbS protein is known to influence or to be influenced by the pH gradient. A comparison of the PSI/PSII ratio in WT and mutants under different pH conditions is provided in Table 1.

To find out whether the different behavior of the mutants in 77 K fluorescence emission measurements was due to pH-induced changes in the composition or re-organisation of the thylakoid

Table 1
F730/F685 (PSI/PSII) ratios as calculated from the fluorescence emission spectra measured at 77 K.

Type of *Arabidopsis*	F730/F685 pH 7.5	F730/F685 pH 5.5
WT	0.81 ± 0.04	1.21 ± 0.05
stn7	0.78 ± 0.02	1.12 ± 0.02
stn8	0.80 ± 0.03	1.24 ± 0.03
stn7/8	0.81 ± 0.03	1.30 ± 0.06
npq4	0.84 ± 0.01	0.87 ± 0.03
WT + DTT	0.82 ± 0.04	1.25 ± 0.04
WT + DTT + Ascorbate	0.79 ± 0.04	1.20 ± 0.04

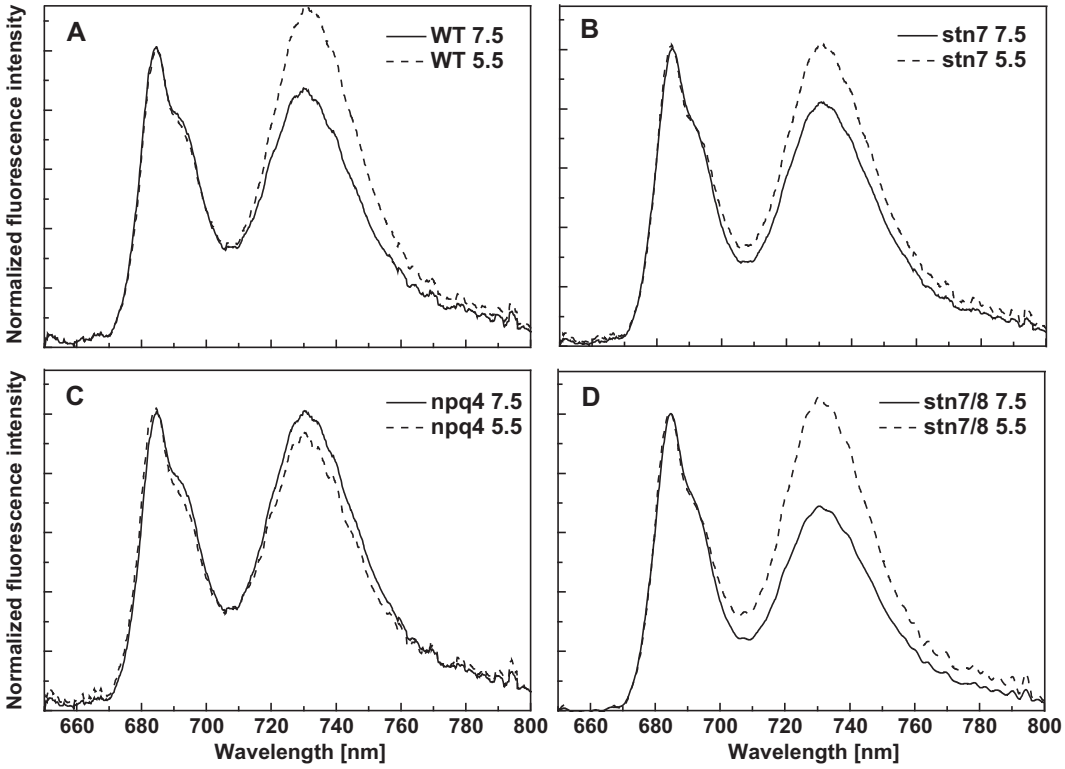

Fig. 2. Fluorescence emission spectra of thylakoid membranes of *WT, stn 7, npq4, stn7 stn8* mutants of *Arabidopsis* treated with pH 7.5 and 5.5.The spectra are normalized at 685 nm.

pigment protein complexes, the WT and all mutant thylakoids were submitted to BN-PAGE. No major pH-induced difference was observed in the thylakoid membrane protein composition of the mutants upon incubation at pH 5.5 and 7.5 (data not shown).

The major component of NPQ, qE, depends on the level of transmembrane proton gradient. After ascertaining with the *stn7* and *stn7 stn8* mutants that state transitions are not involved in pH induced changes in fluorescence intensity of the two photosystems (Fig. 2), we focused on the involvement of other components of qE as probable candidates affected by pH i.e., whether the qE observed as a result of low pH is zeaxanthin dependent/independent and PsbS dependent/independent. For these experiments, the traditional inhibitor of zeaxanthin de-epoxidation (1,4-Dithiothreitol, DTT) was applied. DTT has been used to detect Zx-independent fluorescence quenching in higher plants [25].

The results from WT *Arabidopsis* thylakoid membranes with different treatments are shown in Fig 3. In the presence of DTT there is less quenching of PS II at low pH but increase in fluorescence intensity of PS I remains as much as in control. Since absence of ascorbate does not favour Zea formation, experiment was done in the presence of ascorbate also. However no significant difference in the spectra was observed and the ratio were also almost the same as were obtained without ascorbate (Table 1). This provides evidence that xanthophyll cycle is not responsible for all pH induced effects and the qE observed is independent of the xanthophyll cycle.

Next, despite no pH-induced changes were evident in the composition of the pigment protein complexes, we recorded the fluorescence excitation spectra in order to evaluate whether the change in pH induces differences in absorption wavelengths of PS I and PS II. No significant difference was observed in WT thylakoid membranes treated either with pH 5.5 or 7.5 (Fig. 4). The extent of change in absorption cross section of PSII (Fig. 4A) and PSI (Fig. 4B) both at pH 7.5 and 5.5 was similar. This result provided further

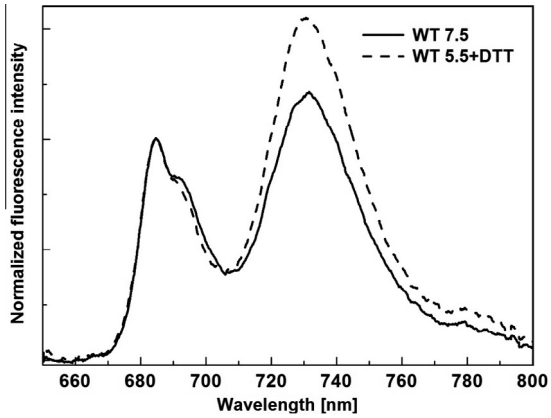

Fig. 3. Fluorescence emission spectra (normalized at 685 nm) of WT thylakoid membranes treated with 5 μM Zx de-epoxidase inhibitor (DTT).

evidence for the fact that no actual migration of LHC II had taken place upon change in pH. After in-depth analysis of the pH effect on the thylakoid membrane , the only possibility that remained to explain the present and previously published results is the occurrence of energy spillover from PS II to PS I under low pH, probably mediated by the PsbS protein.

4. Discussion

Despite extensive study, the pH induced changes in isolated thylakoid membranes have remained elusive. Here we report similar pH induced effects in the kinase mutants *stn7* and *stn7 stn8* as in WT, which severely question the role of state transitions in pH-dependent modifications of the absorption cross section of the two

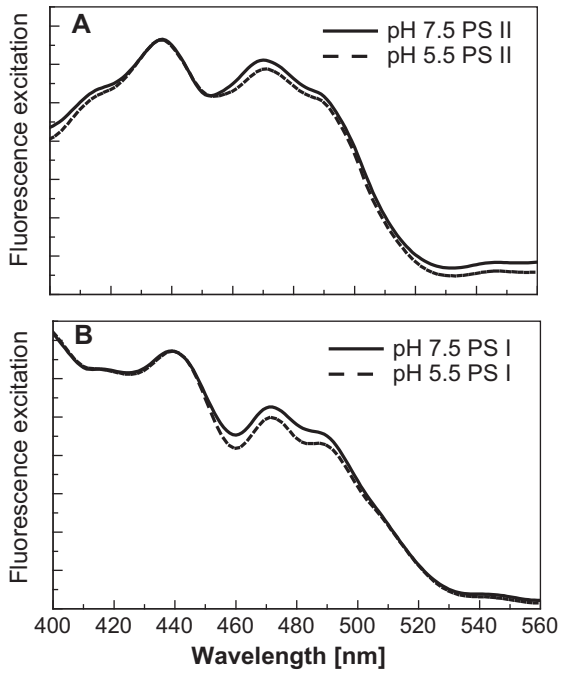

Fig. 4. The 77 K fluorescence excitation spectra of (A) PSII (685 nm) and (B) PSI (730 nm) from the WT thylakoid membranes treated with pH 7.5 and 5.5. The spectra have been normalised to the excitation by Chl *a* at 440 nm.

photosystems. This raises several questions and provides evidence that state transitions (at least the traditional ones in which phosphorylation of LHC II is essential for LHC II migration) are not responsible for pH induced changes in the 77 K fluorescence emission spectra. In search for other mechanisms responsible for the pH induced changes in 77 K fluorescence emission spectra, an important observation was made with the *npq4* mutants. Indeed, it showed an absence of pH induced changes in the absorption cross section of PSI and PSII, suggesting that the PsbS protein is essential in mediating the pH induced changes resulting in an increase in the PSI/PSII ratio. It is probable that structural reorganisations at low pH might be occurring in *npq4* mutant as well, but in the absence of PsbS, quenching of fluorescence is not observed.

The observed change in the PSI/PSII (F730/F685) ratio does not only result from quenching of PS II fluorescence but is also contributed by a significant increase in the intensity of PS I fluorescence at 77 K. Earlier works have identified the pH dependent quenching of PS II fluorescence but none of them observed the change in PSI intensity is response to pH. In one report, change in the ratio (F730/F685) was interpreted as a result of pH change in causing state transitions [17]. The results presented here contradict this report. Since, at pH 5.5, the electron transport through PS II is inhibited, the supply of more electrons to PS I is possible only if PSI is provided by more energy. pH-dependent modulation of the antenna system affects similarly the cross-section of the antennae of both photosystems (Fig. 4) i.e. no antenna migration is involved. Considering the change in the pH-induced F730/F685 ratio in various mutants and WT, the only explanation that remains is an enhanced energy spill-over from PSII to PSI at low pH. Our results with *npq4* mutants provide evidence that the pH effect is dependent on the PsbS protein and independent of zeaxanthin.

PsbS protein is a pre-requisite to enhance PSI excitation relative to PSII excitation under HL (high light) [26] whereas under LL (low light) the phosphorylation of LHCII keeps the excitation balance between PSII and PSI. Indeed, upon transfer of leaves to HL, the phosphorylation of LHCII is no more needed but the PSBS protein instead takes care of optimal distribution of excitation energy to both photosystems [27]. It seems that when both the LHCII phosphorylation and the protonation of the PsbS protein are available and functional when needed, the excitation balance between PSII and PSI is possible to be maintained despite of changes in light intensity. PSI excitation does not increase remarkably under HL [28]. It is conceivable that *in vivo* the protonation and phosphorylation-dependent mechanisms are antagonistic and the excitation balance between PSII and PSI is always maintained whatever changes take place in the light intensity hitting the leaf. On the contrary, by changing pH *in vitro*, the phosphorylation-dependent mechanism can be overruled leading to increased excitation of PSI. Similarly by using traditional state transition conditions, it is possible to rule out the pH dependent mechanism that functions *in vivo* at high light. Thus, the *in vitro* manipulations of the thylakoid properties are important tools to uncover the regulatory mechanisms that govern the function of the thylakoid membrane *in vivo*.

We propose that low lumenal pH causes protonation of PsbS [10], in turn regulates the spillover of energy between the photosystems. Intriguingly, most of the low pH induced changes in the thylakoid membrane resemble those caused by high light conditions. Under low light intensities, phosphorylation of LHCII (state transitions) regulates energy distribution whereas at HL conditions, NPQ is the major regulatory mechanism [26,27]. Even the structural reorganizations induced by low pH [18] resembled that under HL conditions (data not shown). It seems that quenching of PS II takes place in HL, probably making PS I a better trap of energy. The same phenomenon seems to work at low pH where quenching of PS II is also observed with more energy going to PS I. The low pH-induced energy spillover phenomenon reported here might simulate the mechanism of regulation in intact leaves when the proton gradient plays a crucial role. Thus when thylakoids are suspended in low pH conditions in darkness, it induces some changes that reduce the excitation of PSII to further guarantee the acidification of lumen. At the same time, the excitation energy is transferred to PSI that can remain functional by maintaining relatively stable photo-oxidized state. Low pH induced changes *in vitro* seem to resemble HL-induced changes *in vivo* where the *npq4* mutant lacking PsbS was found to maintain excitation balance between PS II and PS I under low light. These observations provide strong evidence that the PsbS protein is involved in regulation of not only the quenching of excess energy but also affects the excitation balance between the two photosystems [29].

To summarize, our results show that the increase in 77 K fluorescence of PSI and quenching of PSII fluorescence upon exposure of isolated thylakoid to low pH is not caused by state transitions as evident from the observation that similar change was observed also in the stn7 kinase mutants. On the contrary, the pH induced change in the PSI/PSII ratio was found to be absent in *npq4* mutants, providing evidence that PsbS dependent NPQ is involved in regulating energy distribution between the two photosystems. Zeaxanthin is likely not involved in qE changes induced by pH. No major change by different pH treatments occurred in the thylakoid membrane protein composition of WT or any of the mutants. As to the 77 K fluorescence excitation spectra, the change in pH was shown to cause similar changes in the absorption cross section of both the photosystems. This indicates that pH does not affect the attachment of the LHC system with the photosystems as such, but simply enhances the spillover of energy between the two photosystems. Low pH may lead to isolation of LHCII system from PSII and PSI and concomitantly enhanced direct interaction between PSII and PSI (spillover). Earlier reports have suggested different roles of zeaxanthin and PsbS in qE [30]. A scheme is presented to summarize the results obtained and induced by low pH conditions (Fig. 5). In high light conditions, PsbS and zeaxanthine dependent qE takes place while low pH conditions (in darkness) induce

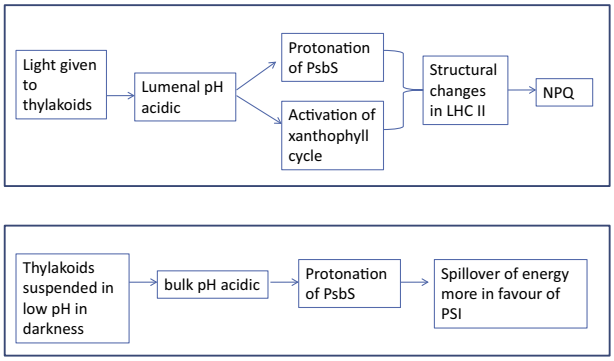

Fig. 5. A scheme comparing the mechanism of low pH induced changes in darkness with high light effects in the thylakoid membranes.

protonation of PsbS solely leading to spillover of excitation energy towards PSI. This is in contention with earlier observations that both PsbS and Zea are required to observe complete NPQ [5]. The possibility of role of alkaline stromal pH under high light conditions, and not during equilibration with pH, cannot be ruled out.

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