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The Lhcb protein and xanthophyll composition of the light harvesting antenna controls the ΔpH-dependency of non-photochemical quenching in *Arabidopsis thaliana*

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Abstract **Nonphotochemical quenching (NPQ) is the photoprotective dissipation of energy in photosynthetic membranes. The hypothesis that the ΔpH-dependent component of NPQ (qE) component of non-photochemical quenching is controlled allosterically by the xanthophyll cycle has been tested using *Arabidopsis* mutants with different xanthophyll content and composition of Lhcb proteins. The titration curves of qE against ΔpH were different in chloroplasts containing zeaxanthin or violaxanthin, proving their roles as allosteric activator and inhibitor, respectively. The curves differed in mutants deficient in lutein and specific Lhcb proteins. The results show that qE is determined by xanthophyll occupancy and the structural interactions within the antenna that govern allostericity.**

Keywords: Xanthophyll cycle; Thylakoid membrane; Non-photochemical quenching; Light harvesting complex; Lutein; *Arabidopsis*

1. Introduction

Non-photochemical quenching (NPQ) is the process by which excitation energy is rapidly dissipated in the light harvesting antenna complexes of plant photosystem II (LHCII), providing photoprotection during periods of excess illumination. The main component of NPQ is the ΔpH-dependent component of NPQ (qE). The trigger for the induction of qE is the increased acidification of the thylakoid lumen that results from protonmotive electron transport [1]. Protonation of lumen facing acidic residues of antenna proteins, either PsbS [2] or the minor antenna complexes CP26 and CP29 [3,4], cause conformational changes in the antenna that create a quenching channel through which energy is dissipated [5]. The induction of qE correlates with the extent of de-epoxidation of the xanthophyll cycle carotenoids [6]: in limiting light this pool comprises principally violaxanthin, whereas in excess light up to 90% of this may be de-epoxidised to antheraxanthin and zeaxanthin (zea). Most of the xanthophyll cycle carotenoids are bound by the trimeric antenna complex LHCIIb [7–9] at the peripheral V1 site [10,11], whilst the minor monomeric antenna CP26, CP24 and CP29 each bind 1–2 molecules [10,12], one of which may be at one of the internal L2 site that binds lutein in LHCIIb [13].

The relationship between qE and the xanthophyll cycle is controversial and not yet determined conclusively. Zeaxanthin is proposed to be a direct quencher of chlorophyll excited states because of the strength of the correlation between qE and the de-epoxidation state in leaves [6] and isolated thylakoids [14]. Evidence for the direct quenching model is the detection of carotenoid radical states upon qE formation [15]. Zeaxanthin radical cations are also detected in purified antenna complexes [16,17], although the extent of quenching was much less than needed to account for qE in vivo without further factors being introduced. An alternative explanation of the role of the xanthophyll cycle in qE is that the effect is an indirect one, allosteric regulation of a quenching process that is intrinsic to the antenna complexes [18–20]. Evidence supporting this hypothesis is twofold: firstly, significant amounts of qE form in the absence of zeaxanthin [21–23]; and secondly, isolated antenna complexes readily assume a highly quenched state resembling NPQ that does not depend upon zeaxanthin [19]. The mechanism of quenching in vitro involves energy transfer from chlorophyll a to the S1 state of lutein bound to the L1 site [5], and the conformational change accompanying this quenching has been detected in vivo, indicating that quenching at the this site on the antenna complexes is responsible for qE.

Quenching in the presence and absence of zeaxanthin have different ΔpH-dependency [21,24,25]; titration of qE against pH difference across the thylakoid membrane (ΔpH) in thylakoids prepared from dark-adapted plants containing only violaxanthin gives a sigmoidal curve with an apparent $pK < 5$, whereas the curve for thylakoids from light-treated leaves with a de-epoxidation state of the xanthophyll cycle pool (DES) of about 60% is less sigmoidal and has a $pK > 5$ [21,25]. In leaves, the physiological ΔpH is around 5 [26,27]. Therefore, qE

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Abbreviations: 9aa, 9-aminoacridine; DES, de-epoxidation state of the xanthophyll cycle pool; LHCII, light harvesting complexes of photosystem II; Lhcb, the proteins of LHCII; NPQ, non-photochemical quenching; ΔpH, pH difference across the thylakoid membrane; PSII, photosystem II; q9aa, quenching of 9aa fluorescence; qE, the ΔpH-dependent component of NPQ; WT, wild-type; zea, zeaxanthin

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would only partially form in the absence of zeaxanthin, but would progressively increase as the p*K* shifts with zeaxanthin accumulation, explaining the two kinetic components of the induction of NPQ in dark-adapted leaves [28,29]. The change in sigmoidicity and shift in p*K* is the same as found for allosterically regulated enzyme catalysed reactions, suggesting that zeaxanthin and violaxanthin are positive and negative allosteric effectors of qE, respectively [18,30,31]. However, there are important limitations to this data: thus, dark-adapted leaves are never completely devoid of de-epoxidised xanthophylls and the light-treatment needed for de-epoxidation undoubtedly induces many other changes to the thylakoid membrane. Moreover, an alternative interpretation of this data is the presence of two entirely distinct qE processes that have intrinsically different pH-dependency.

In order to further explore this model for the regulation of qE by the xanthophyll cycle, qE/ΔpH titrations were carried out using the *npq1* and *npq2* mutants in which the DES of xanthophyll cycle is unambiguously 0% and 100% respectively, without any requirement for pre-treatment of plants. Furthermore, we also used lutein-deficient mutants and lines with altered composition of the proteins of LHCII (Lhcb) proteins to test the proposition that the features of the titration curve are dependent upon the protein and pigment composition of the antenna as suggested by Horton et al. [32].

2. Materials and methods

Arabidopsis thaliana cv *Columbia* and mutant and transgenic lines derived from it were grown for 8–9 weeks in Conviron plant growth rooms with a 10-h photoperiod at a light intensity of 120 μmol/m^2/s^1 and a day/night temperature of 22/15 °C. The mutants were *npq1* and *npq2* [22], *lut1* and *lut2* [33], *lut2npq2* [23], *koLhcb6* [34] and *asLhcb2* [35]. Intact chloroplasts were isolated from either dark-adapted or light-treated plants [21] as described by Crouchman et al. [36] and osmotically shocked in 30 mM $MgCl_2$ before addition of 2× strength medium, with 1 μM 9-aa and 20 μg chl/ml. Simultaneous measurement of chlorophyll fluorescence and 9-aa fluorescence was carried out as previously described [21] at 20 °C using an actinic light intensity of 500 μmol/m^2/s. The samples were illuminated for 3 min to reach a steady state NPQ, and small aliquots of nigericin were then added to induce decrease in 9-aminoacridine (9aa) quenching and qE component of NPQ. Titrations lasted ≈7 min. No change in DES occurs during this treatment [21,36]. Lumen pH was estimated from the values of 9aa quenching, as described by Briantais et al. [1], assuming the internal volume of thylakoids is 56 μl/mg Chl [25]. Data analysis used a SigmaPlot software curve-fitting procedure (SPSS, Chicago, IL). The composition of carotenoids in chloroplast samples was determined by HPLC [37].

3. Results

3.1. Validation of the control of the ΔpH-dependency of qE by the xanthophyll cycle carotenoids

Fig. 1 shows typical qE vs ΔpH titration curves (q9aa, the quenching of 9-aa fluorescence being a linear indicator of lumen proton concentration [1]) obtained by progressive addition of nigericin to illuminated chloroplasts prepared from light-treated and dark-adapted wild-type (WT) *Arabidopsis* plants (Fig. 1A). The results are broadly similar to those published for spinach chloroplasts [21,24,25]. Hence the light-treatment, which in this case increased the DES from 3% in the dark to 43% (Table 1), shifted the titration curve to lower q9aa values. The shift was ≈30%, with an estimated p*K* shift of 0.23 pH units (Table 1). Both curves could be fitted to a Hill equation, and the sigmoidicity parameters obtained from the curve fits show a significant decrease from dark-adapted to light-treated.

A titration curve was obtained for chloroplasts prepared from the *npq1* mutant, which contains only violaxanthin. This curve was shifted by 0.1 pH unit compared the dark-adapted wild-type (Fig. 1B), and was also slightly more sigmoidal (Table 1). The titration curve for the *npq2* mutant, which has a xanthophyll cycle pool comprising only zeaxanthin was shifted remarkably to lower q9aa (Fig. 1B). The shift in p*K* from *npq1* to *npq2* was 1 pH unit. The titration curve for *npq2* chloroplasts was hyperbolic.

3.2. Alteration in the lutein content affects the ΔpH-dependency of qE

Two *lut* mutants [33], both completely deficient in lutein were investigated: in the *lut2* mutant there is an increased content of violaxanthin; and in the *lut1* mutant, lutein is replaced in part by violaxanthin but also by zeinoxanthin [33,38]. Both mutants contain some antheraxanthin and zeaxanthin even in dark-adapted samples. In chloroplasts isolated from dark-adapted *lut2* no reversible NPQ was inducible. In light-treated *lut2* leaves the de-epoxidation state was increased, and qE was detected in the isolated chloroplasts (Fig. 1C). The titration curve resembled that from the light-treated wild-type, with an estimated p*K* value of 5.3 (Table 1). A sample of the *lut2npq2* mutant was also tested. This mutant has zeaxanthin as its only xanthophyll. The data points obtained from chloroplasts from this mutant were similar to those from *npq2*, consistent with the constitutive presence of zeaxanthin in both these mutants, and with the ability of de-epoxidation to fully "activate" this mutant. The *lut1* mutant behaved differently from *lut2* (Fig. 1D). Dark *lut1* chloroplasts in contrast to those from *lut2* showed an inducible qE; its p*K* was lower than that of either the dark-adapted wild-type or *npq1* chloroplasts (Table 1). For chloroplasts isolated from light-treated *lut1* the p*K* was lower than the corresponding wild-type and the sigmoidicity was much larger.

3.3. The composition of Lhcb proteins also affects the ΔpH-dependency of qE

Titrations were also carried out on thylakoids from *Arabidopsis* plants with altered levels of Lhcb proteins [34,35]: the antisense plants in which Lhcb1 and Lhcb2 are completely absent (*asLhcb2*); and the knockout mutant in which the Lhcb6 protein is missing (*koLhcb6*). The titration curve of chloroplasts from dark-adapted leaves of *asLhcb2* was more sigmoidal than in the wild-type and also shifted to higher q9aa (Fig. 1E). The estimated p*K* was less than either the wild-type or *npq1* (Table 1). Illumination of the leaves prior to chloroplast isolation caused a shift in the titration of about 0.15 pH units, and a similar DES as for the wild-type. The titration curve of chloroplasts from dark-adapted *koLhcb6* plants was also more sigmoidal than that of the wild-type and its titration curve was shifted to slightly higher q9aa values (Fig. 1F). Chloroplasts from light-treated leaves had a higher DES than those of the wild-type (nearly 55%), but showed a much smaller light-dependent shift in the titration curve.

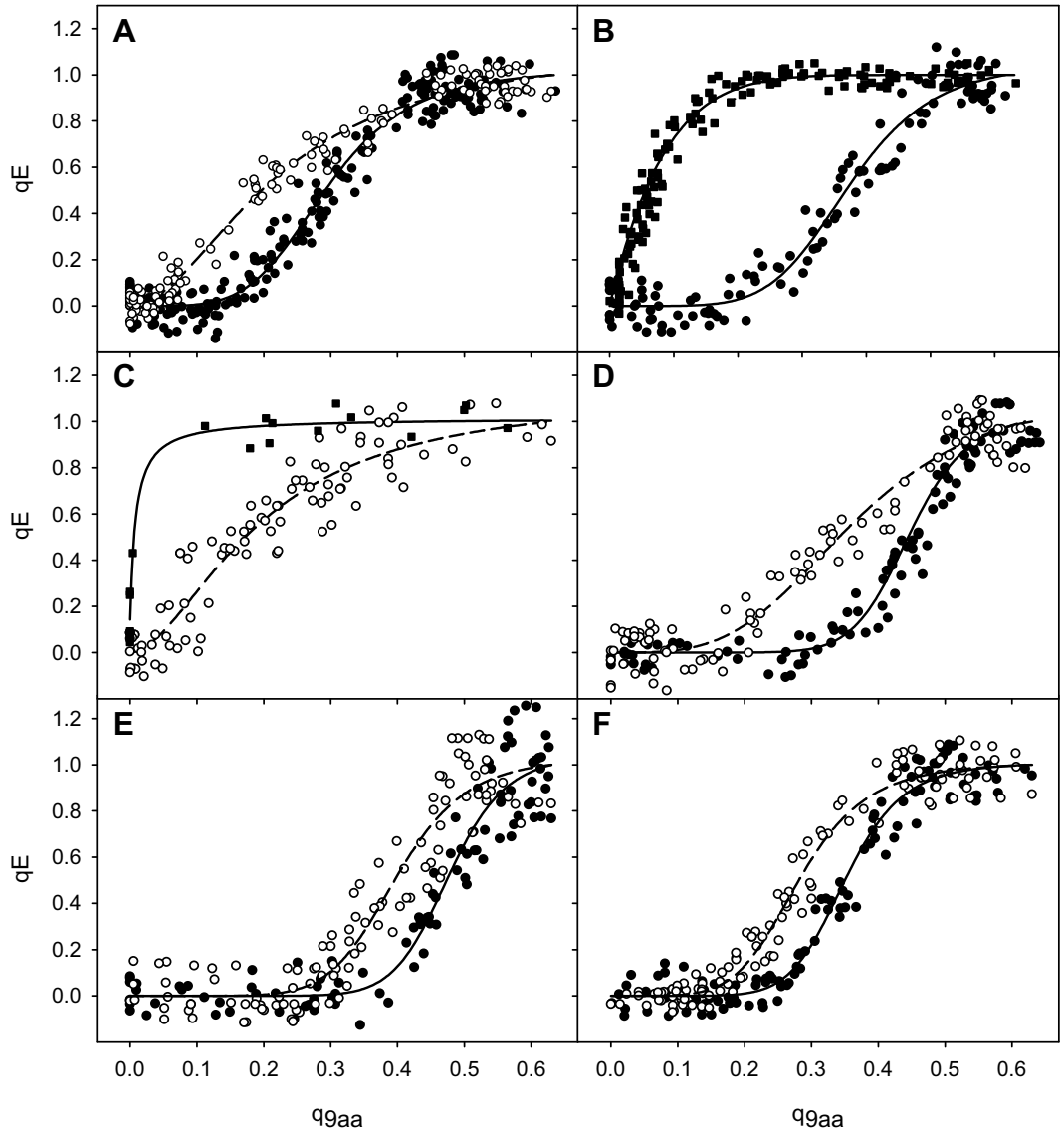

Fig. 1. qE vs. ΔpH titrations of chloroplasts isolated from plants of A, wild-type; B, *npq1* (circles), *npq2* (squares); C, *lut2* (circles), *lut2npq2* (squares); D, *lut1*; E, *asLhcb2*; F, *koLhcb6*. Open symbols and dashed lines, chloroplasts from light adapted plants; closed symbols and solid lines, chloroplasts from dark-adapted plants. q9aa was calculated as (unquenched − quenched)/unquenched fluorescence values of 9aa [1] and qE in the form (nigericin-relaxed − quenched)/quenched for the progressive increase in chlorophyll fluorescence level with each nigericin addition. Data were obtained from 8 to 12 replicate titrations on three to five separate chloroplast preparations (except for *lut2npq2*, which was from one preparation), and normalised to qE = 1 for curve-fitting. Lines are the fitted Hill equations, R^2 values were 0.97, 0.98 (A), 0.95. 0.95 (B), 0.90, 0.97 (C), 0.96, 0.95 (D), 0.92, 0.90 (E), 0.97 and 0.97 (F).

4. Discussion

4.1. The npq1 and npq2 mutants define the limits of allosteric regulation of qE by the xanthophyll cycle

Evidence for the hypothesis that the xanthophyll cycle carotenoids are allosteric regulators of qE [18–20,30,31] is the effect of light-treatment on the p*K* and sigmoidicity of qE of the ΔpH/qE titration curve [21,24,25], attributed to the light-dependent de-epoxidation of violaxanthin. This observation was repeated here using chloroplasts prepared from *Arabidopsis* plants. Moreover, for *npq1*, the ΔpH requirement and sigmoidicity was even greater than for the dark-adapted wild-type, giving the true null point for "zero DES". In *npq2* the full extent of the shift in ΔpH requirement and the absence of sigmoidicity were observed at "100% DES". Since no treatments were applied to the plants prior to chloroplast isolation, these results add very strong support to the proposed role of the xanthophyll cycle carotenoids as allosteric modulators of qE.

It has been proposed that qE in the absence of zeaxanthin occurs by a different mechanism than in its presence, including quenching in the reaction centre of photosystem II (PSII) [39,40]. However, the presence of two separate mechanisms for qE is difficult to reconcile with the very similar properties of quenching with and without zeaxanthin; for example, both are sensitive to the same effectors [41], both are diminished in the absence of PsbS [36] and both are reduced in the lutein-deficient mutants [23]. Here, the differences in ΔpH-dependency of qE in mutants lacking lutein and Lhcb proteins are

Table 1
Parameters for isolated chloroplasts from wild-type and mutant plants

Material	Dark/light	DES	Titration parameters		
			Hill coefficients	$q9aa_{50\%}$	Estimated p*K*
WT	Dark	3.04 ± 0.26	4.67	0.292	5.03
WT	Light	43.22 ± 1.92	1.90	0.197	5.26
npq1	Dark	0 ± 0.00	5.70	0.368	4.88
npq2	Dark	100 ± 0.00	1.00	0.056	5.87
lut1	Dark	16.81 ± 0.86	10.29	0.444	4.74
lut1	Light	35.80 ± 1.17	3.55	0.341	4.93
lut2	Dark	15.20 ± 0.49	–	–	—
lut2	Light	35.52 ± 1.52	1.78	0.172	5.33
lut2npq2	Dark	100 ± 0.00	1.00	0.008	6.72
asLhcb2	Dark	3.81 ± 0.29	11.17	0.474	4.69
asLhcb2	Light	40.79 ± 2.56	7.62	0.396	4.83
koLhcb6	Dark	4.06 ± 0.64	8.15	0.350	4.92
koLhcb6	Light	54.73 ± 2.19	5.71	0.281	5.05

Plant materials were either dark-adapted (dark) or light-treated (light) before chloroplast isolation, to induce different de-epoxidation states (DES) defined as ([zeaxanthin] + 1/2[antheraxanthin])/([zeaxanthin] + [antheraxanthin] + [violaxanthin]). Data are means of 3–5 replicates ±S.E.M. Titration parameters were obtained from the data in Fig. 1. $q9aa_{50\%}$ is the fraction of quenching of 9aa fluorescence giving 50% qE. Hill coefficients were derived from the fits to the data in Fig. 1. The p*K* was estimated from q9aa was described in Section 2.

similarly difficult to explain by effects on the PSII reaction centre, but are entirely consistent with modulation of a single quenching mechanism in the antenna. Furthermore, the presence of two quenching mechanisms, each with a different pH dependency, one taking over from the other upon de-epoxidation, would not give rise to the shifts in the titration curve observed here and previously [21,24,25,41]; rather, there would be a mixture of two components, one as seen in *npq1* and the other as seen in *npq2*. Clearly, this is not what has been observed.

4.2. The absence of Lutein affects the ΔpH-dependency of qE

Lutein is important in NPQ [23,33,38] and it is suggested that lutein bound to the L1 site of LHCII is the quencher responsible for qE [5]. The lower reported maximum amplitudes of NPQ in the absence of lutein [33,38] may then be explained by a weaker quencher bound to the L1 site. Whilst alterations in the qE titrations in the mutants could arise from the presence of an alternative weaker quencher, requiring more complexes to be in the quenched state for the same amount of NPQ, lutein deficiency may also indirectly alter the regulatory features of qE. In the absence of light-induced zeaxanthin accumulation, the thylakoids of *lut2* did not form any qE, indicating complete lack of response to ΔpH within the range achievable by light-induced proton transfer. Thus, we propose that there was a putative extreme shift in the titration curve to a ΔpH range above that achievable – this situation is perhaps similar to that found in chloroplasts inhibited with antimycin [25] or in the presence of reduced concentration of Mg ions [41]. The PSII antenna of the *lut2* mutant is less stable, and LHCII trimers are not found following detergent solubilisation of thylakoid membranes [42]. Therefore, it is suggested that this shift results from altered subunit interactions in the PSII antenna. It is significant that in the presence of zeaxanthin, either induced by light or in the double *lut2npq2* mutant, the titration is shifted towards lower ΔpH, as in wild-type and *npq2* plants, respectively. In the *lut1* mutant, lutein is replaced not only by violaxanthin but also by zeinoxanthin [33,38], suggesting that binding of this carotenoid to LHCII in *lut1* gives rise to interactions that are more like "wild-type" than in the case of *lut2*. Titratable qE was found in dark-adapted *lut1* chloroplasts, but its ΔpH requirement was greater than in the wild-type. As for the *lut2* mutant this behaviour can be explained by altered interactions in the PSII antenna in this mutant, which not only shifts the titration curve but also increases its sigmoidicity. Such effects are predicted by the allosteric model of Monod et al. [43], where altered subunit interaction and/or stabilities of different conformations increase the allostericity constant.

4.3. Protein composition of the PSII antenna also controls allostericity

Titration of qE in the dark-adapted thylakoids prepared from plants in which the Lhcb1 and Lhcb2 proteins are completely absent showed a shift to higher ΔpH requirement, an increased sigmoidicity, and a reduced light-dependent shift, compared to the wild-type. Again, such effects could arise from altered subunit interactions. Even though the *asLhcb2* plants contain an apparently "wild-type" macro-organisation [44], the trimers in these plants are composed of the normally monomeric Lhcb5 protein (CP26) and may have different properties. The titration of the dark-adapted *koLhcb6* was only slightly shifted compared to wild-type, but its sigmoidicity was greatly enhanced, similar to that found in the *lut1* and *asLhcb2* plants. However, the main feature of *koLhcb6* was the negligible shift upon light-treatment, despite a de-epoxidation state that was even greater than in the wild-type. The composition and macro-organisation of the thylakoids are different in this mutant compared to the wild-type [34], perhaps explaining the altered ΔpH-dependency of qE.

4.4. The ΔpH-dependency of qE explains the kinetics of NPQ induction

The kinetics of NPQ formation in wild-type plants and the differences found in each of the xanthophyll and Lhcb mutants can be explained by the titration curves presented here. At the first illumination there were two kinetic components of NPQ formation in wild-type leaves [22]: a rapid component activated by PsbS and ΔpH [36]; and an additional slower component resulting from the de-epoxidation of violaxanthin to zeaxanthin within the PSII antenna [29,45]. The proportion of qE that is rapidly induced will depend upon the lumen

pH [26,27] and the p*K* of the dark-adapted chloroplast. The second phase then will represent the effect of the DES-dependent shift in the titration to higher p*K*. For *Arabidopsis*, the estimated p*K* of the dark-adapted chloroplast is <5, in agreement with the 20–30% qE formed in the first phase [22] and consistent with the amplitude of NPQ in the *npq1* mutant, the extreme state of zero de-epoxidation [22]. In contrast, *in npq2*, the ΔpH profile predicts 100% of qE would form rapidly in one phase – exactly as observed [22]. In *lut2* the rapid zeaxanthin-independent phase appears to be absent leaving just the slow phase [38], consistent with the absence of qE in the thylakoids from dark-adapted plants. The kinetics of *lut1* are reported in the same paper to be rather intermediate between the wild-type and *lut2*, again as predicted from the titration curves. The absence of specific Lhcb proteins also affects NPQ kinetics. The slower NPQ formation in *asLhcb2* [35] is consistent with a lower amplitude of the fast phase of qE formation, which is predicted by the shift in the ΔpH titration curve. In *koLhcb6* plants, most of NPQ forms rapidly and the slow phase is greatly reduced [34], consistent with the very small shift in the titration curve upon de-epoxidation.

4.5. Concluding remarks

We have validated the assertion that qE be considered to arise from the multi-subunit PSII antenna 'enzyme' in which the substrate is protons and the product is heat [30,31]. L1 is proposed to be the unique 'active site', while V1 is an allosteric site, peripheral to the active site and binding ligands (xanthophyll cycle carotenoids) that affect the enzyme's affinity for the substrate (protons). Recently, it was suggested that NPQ arises within a locus in the PSII macrostructure comprised of PsbS, the minor and the major trimeric LHCII [32]. This locus would provide the subunit interactions and/or multiplicity of proton binding sites needed to explain the sigmoidicity of the titration curves. Specification of the p*K* and extent sigmoidicity by the xanthophyll and protein content of this locus fine-tunes of this regulatory mechanism and gives rise to the physiological optimization of NPQ [32].

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