Molecular and Global Time-resolved Analysis of a psbS Gene Dosage Effect on pH- and Xanthophyll Cycle-dependent Nonphotochemical Quenching in Photosystem II*

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Photosynthetic light harvesting in plants is regulated by a pH- and xanthophyll-dependent nonphotochemical quenching process (qE) that dissipates excess absorbed light energy and requires the psbS gene product. An Arabidopsis thaliana mutant, npq4-1, lacks qE because of a deletion of the psbS gene, yet it exhibits a semidominant phenotype. Here it is shown that the semidominance is due to a psbS gene dosage effect. Diploid Arabidopsis plants containing two psbS gene copies (wild-type), one psbS gene (npq4-1/NPQ4 heterozygote), and no psbS gene (npq4-1/npq4-1 homozygote) were compared. Heterozygous plants had 56% of the wild-type psbS mRNA level, 58% of the wild-type PsbS protein level, and 60% of the wild-type level of qE. Global analysis of the chlorophyll a fluorescence lifetime distributions revealed three components in wild-type and heterozygous plants, but only a single long lifetime component in npq4-1. The short lifetime distribution associated with qE was inhibited by more than 40% in heterozygous plants compared with the wild type. Thus, the extent of qE measured as either the fractional intensities of the PSII chlorophyll a fluorescence lifetime distributions or steady state intensities was stochiometrically related to the amount of PsbS protein.

Absorption of light in excess of photosynthetic capacity necessitates mechanisms to protect plants from photo-oxidative damage (1). Overexcitation of chlorophyll (Chl) and overreduction of the electron transport chain can result in increased generation of reactive intermediates and harmful byproducts of photosynthesis. For example, when excitation energy in single-excited Chl (1Chl*) cannot be used to drive electron transport, the lifetime of 1Chl* increases, resulting in an increased yield of triplet-excited Chl (3Chl*) via intersystem crossing. 3Chl* can generate singlet O₂ (1O₂*), which can directly damage pigments, proteins, and lipids in the photosynthetic apparatus. To maintain a short lifetime of 1Chl* and minimize photo-oxidative damage, a nonphotochemical quenching process, called qE, is induced in excessive light, resulting in de-excitation of 1Chl* molecules and thermal dissipation of excess absorbed light energy in the light-harvesting antenna of photosystem (PS) II (2, 3). Because it decreases the lifetime of 1Chl*, qE can be measured easily as a decrease in the maximum yield of Chl fluorescence in isolated chloroplast membranes, algal cells, or intact leaves.

qE is induced by a low pH in the thylakoid lumen of chloroplasts during illumination with excessive light (reviewed in Refs. 2–5). Low pH activates the violaxanthin de-epoxidase, which converts violaxanthin, V, into antheraxanthin, A, and then zeaxanthin, Z, as part of a xanthophyll cycle (6–9). Binding of de-epoxidized xanthophylls (A and Z) and protons (H+) to undefined PSII proteins is hypothesized to result in a conformational change that effectively switches a PSII unit into a quenched state in which nonphotochemical de-excitation of 1Chl* is favored (reviewed in Refs. 10 and 11).

The decrease in the maximum yield of Chl fluorescence caused by qE is associated with characteristic changes in Chl fluorescence lifetime distributions that can be described by a three-state model for PSII (12–14). According to this model, without lumen acidification and with PSII traps closed, the main Chl fluorescence lifetime distribution that is broad and centered at 1.7–2.2 ns is referred to as the W state. Thylakoid membrane energization by a ΔpH results in a distinct conversion of the W state distribution to a shorter, usually narrower lifetime, centered at 1.6–1.8 ns and referred to as the X state. The W to X shift is hypothesized to be related to protein conformational changes (15) in the molecular environment of the protein-bound fluorophores, in this case fluorescing Chls (12, 13, 16), caused by protonation of lumen-exposed carboxylate groups of proteins in the PSII inner antenna. The conformational changes are associated with the activation of potential structural interactions between the xanthophylls Z and A and the PSII proteins. The final step in the model is the binding of Z (or A) to convert PSII from the X state to the Y state that exhibits a short lifetime distribution typically at 0.3–0.5 ns (Y state) (12, 13, 17, 18). The W to X to Y state conversion is typically saturated by levels of Z or A above 2–3 molecules per PSII (12, 13, 19, 20). Hence, it was concluded that the energy dissipation in PSII exhibited saturable kinetics and may be defined by a pH-dependent binding isotherm (13). According to this model, and as tested in the experiments below, the energy dissipation mechanism should therefore exhibit limiting (and potentially saturating) characteristics for both the available xanthophyll substrate con-

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‡ The abbreviations used are: Chl, chlorophyll; A, antheraxanthin; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyleurea; DPS, de-epoxidation state; DTT, dithiothreitol; LHC, light-harvesting complex; NPQ, non-photochemical quenching; PS, photosystem; qE, pH- and xanthophyll-dependent component of NPQ; V, violaxanthin; Z, zeaxanthin; HPLC, high performance liquid chromatography.
centration [A+Z] and the xanthophyll binding enzyme concentration.

Characterization of mutants that are defective in qE has helped to define the factors that are required for the photoprotective mechanism (1). Several npq mutants of Arabidopsis and the green alga Chlamydomonas reinhardtii have been identified in forward genetics screens based on video imaging of the Chl fluorescence yield (21–25). Besides providing genetic evidence for the necessary roles of the ΔPH and de-epoxidized xanthophylls in qE (22, 23, 26), mutant analysis has recently identified the PSII subunit PsbS as a critical component of the qE mechanism (21, 27).

PsbS is a member of the light-harvesting complex (LHC) protein superfamily (28, 29). PsbS has been reported to bind substoichiometric levels of V (30), although recent attempts to purify or reconstitute the protein with bound pigments were unsuccessful (31). In the npq4-1 mutant of Arabidopsis, the nuclear gene encoding PsbS has been completely deleted, resulting in the absence of PsbS protein and a severe defect in qE (21). In contrast to other mutants in which the accumulation of de-epoxidized xanthophylls is affected, the npq4-1 mutant exhibits xanthophyll cycle conversion and generation of the thylakoid ΔPH that are indistinguishable from that of the wild type. Loss-of-function mutations like the npq4-1 deletion usually behave as recessive mutations in genetic crosses, yet npq4-1 is semidominant: heterozygous F1 plants resulting from a cross between npq4-1 and the wild type have a phenotype that is intermediate between those of the parents. This observation suggested that the dosage of the psbS gene somehow affects the level of qE (21).

In this study, the effect of psbS gene dosage was investigated systematically by comparing psbS mRNA levels, PsbS protein levels, extent of qE, and global analysis of the PSII Chl fluorescence lifetime distributions. Materials in the investigation included the homozygous npq4-1 mutant plants (no psbS gene copies), heterozygous F1 plants (one psbS gene copy), and homozygous wild-type plants (two psbS gene copies). The targeted global analysis employed the measured xanthophyll concentrations, lumen pH, and multifrequency phase and modulation data to simulate the changes in the fractional intensity changes in the PSII components corresponding to the W, X, and Y states (13, 15, 32, 33). The implications of the results for the concentration-dependent function of the PsbS protein in the pH- and xanthophyll-dependent control of qE are discussed.

EXPERIMENTAL PROCEDURES

Plant Materials, Growth Conditions, and Thylakoid Preparation—A. thaliana plants (Col-0 background) were grown in a growth chamber with a short-day photoperiod of 10 h light (150 μmol photons m⁻² s⁻¹, 22–23 °C) and 14 h dark (19–20 °C). Plants between the ages of 5 and 7 weeks, prior to bolting, were used for all experiments except for genomic DNA extraction and crosses. Heterozygous plants (npq4-1/ NPQ4; hereafter referred to as F1) were generated by crossing wild-type plants (NPQ4/NPQ4) with homozygous npq4-1 mutant plants (npq4-1/npq4-1; hereafter referred to as npq4-1). Wild-type plants have two copies of the psbS gene, the F1 plants have only one copy of the psbS gene, and npq4-1/npq4-1 plants do not have any psbS gene.

For RNA extraction and immunoblot analysis, leaves were harvested 3 h into the light period, frozen immediately in liquid nitrogen, and then stored at −80 °C. Chloroplast thylakoid membrane isolations, including grinding, resuspension, and reaction buffers and sample preparation protocols for the lifetime measurements were described previously (13, 14).

Determination of thylakoid membrane energization (Fₐ₋ₐ) was determined for 30 s with the weak 1.6-kHz measuring beam (0.25 μmol of photons m⁻² s⁻¹). A 2-s pulse of high intensity white light (10,000 μmol of photons m⁻² s⁻¹, Walz DT-Cyan filter) was delivered simultaneously with switching the PAM measuring beam from 1.6 to 100 kHz to determine the maximal fluorescence level (Fₐ) with all PSII traps closed. A train of identical high intensity pulses ensued at a rate of 1 pulse every 100 s for the duration of the experiment to determine the fluorescence level above the contribution of thylakoid membrane energization (Fₐ₋ₐ). Immediately following the initial Fₐ and Fₐ₋ₐ determination, the leaf samples were exposed to a continuous white actinic light (750 μmol of photons m⁻² s⁻¹, Walz DT-Cyan filter) for 300 s at a temperature of 25 °C with the PAM measuring beam at 100 kHz. The temperature was attenuated to −2 °C over the next 300 s upon which time actinic light was extinguished, and the PAM measuring beam was switched to 1.6 kHz. The Fₐ₋ₐ was determined every 100 s for 600 s upon which time the temperature was ramped back up to 25 °C over a 300-s interval. The Fₐ₋ₐ (or Fₐ₋ₐ) determinations (depending on the interpretation of dark-sustained thylakoid membrane lumen acidification) continued for an additional 600 s.

Chl Fluorescence Intensity and Lifetime Measurements with Thylakoids—The determination of the PSII Chl fluorescence intensity and lifetimes followed the eight-step protocol defined by Gilmore et al. (13). The only notable exception was that the lifetimes were measured with an ISS K2-004 lifetime spectrofluorimeter (ISS Inc., Urbana, IL) via a quartz fiber optic probe using the emission/excitation wavelength specifications defined by Gilmore & Yamamoto (20). In addition to the reaction buffer, other chemical additions included 7.5 μM total Chl, 50 mM sodium ascorbate, 0.3 mM ATP, and 35 μM methylviologen in 3-mI quartz cuvettes for all samples. When necessary, DCMU was added to 10 μM and nigericin to 2 μM. The thylakoids were treated in the same temperature-controlled cuvette described for the leaf experiments above except that the actinic light intensity was 500 μmol of photons m⁻² s⁻¹ for 10 min at 25 °C followed by addition of diethiothreitol (DTT) to a total concentration of 5 mM and continued actinic illumination for 10 min while the reaction temperature was lowered to 0 °C. The level of violaxanthin de-epoxidation was controlled by addition of a saturating level of DTT (0–1 mM) prior to actinic illumination.

Pigment Measurement—Pigment measurements from leaves were performed by HPLC according to Müller-Moulé et al. (38). For each condition, measurements were done on six individual plants of each
genotype. Thylakoid samples were immediately frozen at 77 K following the experiments and stored at -80 °C until HPLC analysis according to the method of Gilmore and Yamamoto (39).

Global Analysis of Chl Fluorescence Lifetime Data—As outlined in previous studies (12–14, 40), we analyzed the fluorescence lifetime distributions assuming three major distinct PSII Chl fluorescence lifetime states that correspond to the following structural model assumptions. 1) W is a PSII unit containing unprotonated unknown CP protein(s) incapable of binding the Z or A molecules. 2) X is a PSII unit containing the protonated unknown CP protein(s) capable of, but not actively binding a Z or A molecule; and finally 3) Y is a PSII unit with protonated unknown CP protein(s) and with a bound Z or A molecule. In simplest terms conversion of the protonation of the unknown CP protein is defined with a simple Henderson-Hasselbach titration (defined by a $pK_a$ value). Also the concentration dependence of the binding of Z and A to the protonated unknown CP protein is defined as an equilibrium association (defined by a $K_a$ value). In Scheme 1 the total sum of all three PSII states is normalized and defined as $[W] + [X] + [Y] = 1$ where $pK_a = pK_m - \log([W]/[X])$ and $K_a = [X]/[A + Z]$. The following balanced analytical Equations 1–3 defined as,

$$[W] = 1/(1 + 10^{pK_m - pK_a}(1 + K_a[A + Z]))$$  \hspace{1cm} (Eq. 1)

$$[X] = 10^{pK_a - pK_m}(1 + 10^{pK_a - pK_m}(1 + K_a[A + Z]))$$  \hspace{1cm} (Eq. 2)

$$[Y] = K_a[A + Z]10^{pK_a - pK_m}(1 + 10^{pK_a - pK_m}(1 + K_a[A + Z]))$$  \hspace{1cm} (Eq. 3)

were derived from the Scheme 1 with the assistance of Dr. V. P. Shinkarev of the University of Illinois at Urbana-Champaign Center for Biophysics and Computational Biology. Equations 1–3 were used to calculate the $pK_m$, $[W]$, $[X]$, and $[Y]$ given the measured constant level of the ATPase-induced thylakoid lumen pH and the variable ($A + Z/[V + A + Z]$) levels. The integral fractions of the ($A + Z/[V + A + Z]$) parameters were normalized to unity by employing the area form of the Lorentzian distribution equations. The lumen pH was estimated using the 9-aminacridine technique (42), and the levels of ($A + Z/[V + A + Z]$) were varied with DTT concentrations ranging from 0 to 1 mM, essentially as described by Gilmore et al. (13). The measured values for ($A + Z$) and the lumen pH $= 5.18 \pm 0.01$ were fixed. The free parameters included the $pK_m$, $[W]$, and $[X]$ and all the width and lifetime mode parameters of the Lorentzian distributions. The linking scheme assumed all Lorentzian mode and width parameters corresponding to each given PSI1 state represented mean linked values. The heart of the global target scheme reduced to a minimum of nine free parameters, including three modes plus three widths for the entire data set plus two $K_a$ values for the two samples types with measurable (PsbS), namely, the wild type and F1. The $pK_a$ was assumed to be linked between the wild type and F1. In the npq4-1 samples lacking PsbS, the fraction of the ($W$) state was normalized as unity for the final solution since there was little or no observed pH- or xanthophyll-dependent change in the fluorescence lifetime integrals, averages, or intensities. Two other minor modes were also included in the fits for all three materials in addition to the three major modes directly related to the model. One was a narrow distribution ($\approx 50$ ps wide) with the mode and widths parameters linked for all samples and free amplitudes which accounted for rapid decays ($\approx 50$ ps), including energy transfer and exciton equilibration processes. The fast distribution overlapped with the lower lifetime limit of 0 ns and thus possibly accounted for a slight excitation light scatter and/or filter leakage. The second ($\approx 5$ ns) distribution included all free floating parameters and accounted for decay variations attributed to instrumental tuning and decay signals from Chl bands isolated from proteins and other impurities and long-lived background components; the variation in this component was also possibly related to natural variation in the longer lifetime tail of the Lorentzian distributions of the W state attributed to heterogeneity of the Chl protein environment (40).

The global analysis program was written with Visual Basic for Applications 97 for use in Microsoft Windows NT4.0 (32 bit) and Excel 97, and it utilized a Large Scale General Reduced Gradient (Lsgrg) minimization engine developed by Frontline Systems Inc. (Incline Village, NV). The search engine is capable of solving, in Excel 97 with a Pentium III computer with 500 Mb RAM and an 800 MHz processor, all the test problems prescribed by the National Institute of Standards and Technology (Nist) website for Nonlinear Regression (www.itl.nist.gov/div898/strd/nls/nls_info.shtml) with 10 digit precision for the sum of squares parameter. The global reduced chi-square equation (15, 43) was minimized assuming the following uncertainties for the phase angle shift ($\phi$) $\sigma_\phi = 0.025^\circ$ and the demodulation ratio ($M$) $\sigma_M = 0.005$. The final goodness of fit was judged by nonlinear regression of the $\phi$ and $M$ to calculate an $r^2$ regression coefficient and F-test. The standard errors for all global model parameters and regression coefficients were calculated from the diagonal elements of the inverted variance-covariance matrix (44). The randomness and normality of the residual error distribution were further judged by calculation of a second chi square statistic (45) describing the fit of the binned histogram of the residual errors to a Gaussian distribution as described by Gilmore et al. (46).

**RESULTS**

**Molecular Analysis of the psbS Gene Dosage Effect**—To investigate the effect of psbS gene dosage at a molecular level, the amounts of psbS DNA and mRNA and PsbS protein were assayed in wild-type, F1, and npq4-1 plants that contain two copies, one copy, and no copies of the psbS gene, respectively. Fig. 1A shows, as expected, that the copy number of the psbS gene in F1 plants was half of that in wild-type plants, and the psbS gene was completely absent in npq4-1 plants. The steady-state level of psbS mRNA in F1 plants, relative to a 5 S rRNA loading control, was $\approx 56\%$ of the wild-type level, and npq4-1 plants did not have any detectable psbS mRNA (Fig. 1B). Immunoblot analysis with a PsbS-specific antibody showed that F1 plants had $\approx 58\%$ of the wild-type level of PsbS protein, normalized to the amount of the PSII reaction center subunit D1 (Fig. 1C).

**Light-induced Changes in Chl Fluorescence Intensity and Pigment Composition**—Chl fluorescence parameters were measured in intact leaves of the three Arabidopsis genotypes during and after illumination with high light. Fig. 2 shows that NPQ was induced rapidly in wild-type and F1 plants, reaching a total extent of 1.9 and 1.3, respectively. During the subsequent dark period, most of the NPQ relaxed along with dissipation of the thylakoid $\Delta$pH, demonstrating that qE is the major component of NPQ in wild-type and F1 plants. In contrast, NPQ was induced slowly in npq4-1 plants to a total extent of only $0.6$, and most of this NPQ failed to relax during the subsequent period in the dark, consistent with the known qE defect of this mutant (21). If we consider NPQ that relaxes within the first 3.5 min in the dark as qE, then F1 plants exhibited $60\%$ of the wild-type level of qE, whereas npq4-1 had only $6\%$.

The differences in qE in wild-type, F1, and npq4-1 plants were not related to any differences in Chl or carotenoid pigment composition. Table I shows that there were no major differences in the levels of xanthophyll cycle pigments in dark-adapted wild-type, F1, and npq4-1 plants, and the high light treatment induced nearly identical changes in the xanthophyll cycle pigments in the three genotypes. There were also no differences in Chl a/b ratio, total Chl, neoxanthin, lutein, or $\beta$-carotene in the three genotypes before or after exposure to high light (data not shown).

Chl fluorescence parameters in leaves and thylakoids of the three genotypes were compared to ensure that the responses of thylakoids used in the lifetime analysis were similar to those of leaves. Fig. 3 shows that dark-adapted leaves and thylakoids exhibited similarly high values of PSII quantum efficiency, as measured by $F_v/F_m$. Following induction of NPQ by illumination with high light and lowering of the temperature, the maximal decrease in the $F_v/F_m$ level ($F_m/F_m$ in the light) in leaves and thylakoids was essentially the same. Consistent with the

![Scheme 1](image-url)
results shown in Fig. 1, wild-type leaves and thylakoids showed the greatest decline in Fm/Fm in the light, whereas the decrease was intermediate in the F1 and nearly completely inhibited in the npq4-1 samples. Lowering the temperature allowed for a sustained decrease in Fm/Fm (and NPQ) in darkness (Fig. 3, Fm/Fm in dark + ΔpH), consistent with the data of Gilmore & Björkman (47). Again in parallel with the maximal level of the fluorescence decrease in the light, the wild type exhibited the largest sustained decrease in Fm/Fm in the dark + ΔpH, with the F1 being intermediate. After reversal of the sustained NPQ by warming the leaves (Fig. 3, panel A) or adding nigericin to the thylakoids (Fig. 3, panel B), the final Fm recovered to ~75–80% of the initial Fm value. For leaves of the three genotypes, the differences in the final Fm were insignificant. For the thylakoids the differences were insignificant for the wild type and F1, whereas the final Fm of npq4-1 thylakoids was slightly lower.

Global Analysis of Chl Fluorescence Lifetimes in Thylakoids—Fluorescence lifetime distributions were determined for wild-type, F1, and npq4-1 thylakoid samples corresponding to the initial Fm and final Fm conditions described above. The lifetime distribution profiles of the three samples were very similar to each other at the initial Fm and at the final Fm (data not shown). The mean lifetimes were 2.59 ± 0.16 ns and 2.27 ± 0.12 ns at the initial and final Fm conditions, respectively. The lower fluorescence lifetime at the final Fm was attributable to mild photoinhibitory damage to the PSII reaction center during the illumination protocol (17). Therefore, subsequent lifetime measurements, which were performed under dark quenching conditions after the illumination period, were compared with lifetimes measured in the final Fm condition.

Fluorescence lifetime distributions were determined under sustained quenching conditions (Fm/Fm in dark + ΔpH in Fig. 3B) in the presence of constant thylakoid lumen acidification and varying violaxanthin de-epoxidation. Fig. 4 shows the lifetime distribution profiles for thylakoids of the wild type, F1, and npq4-1 in varying states of quenched fluorescence, and Table II presents the modes and widths of the lifetime distributions. The different curves in Fig. 4 were collected for each genotype by varying the de-epoxidation state ([A + Z]/[V + A + Z]) from ~0.05 to 0.5. The lifetime distribution profiles for the wild type and F1 showed lifetimes corresponding to the W (unquenched), X (lumen acidified only), and Y (fully quenched) states (Fig. 4). The resolved distributions show that the W state had the longest lifetime mode and a broader width than the narrower, slightly shorter lived X state. The Y state was also considerably broader than the X state, especially when plotted on this lifetime-weighted scale, which emphasizes the amplitudes of longer lifetime components.

In contrast to the wild type and F1, the npq4-1 samples exhibited only the W state lifetime distribution (Fig. 4). Importantly, the global-linking scheme for the npq4-1 samples did not exclude the possibility for lifetime mode or fraction changes due to other pH-sensitive PSII antenna proteins besides PsbS, including those known to bind DCCD like CP26 and CP29. The analysis did not resolve a significant fractional intensity change in the npq4-1 samples under conditions of lumen acidification, as the total average fraction of the W state was 68.1 ± 1.9% in the lumen-acidified state and 70.0 ± 2.2% in the after or final Fm state. There were no significant differences in the average lifetimes in npq4-1 thylakoids between the lumen-acidified and final Fm conditions. With respect to the two other components of the model fit that were included for all samples but not shown in Fig. 4, the integral fraction (average lifetime) of the fastest component was 21.5 ± 1.3% (0.01 ± 0.00 ns), and the longer more variable component was 9.0 ± 4.7% (0.66 ± 0.17 ns) inclusive of all 21 samples from all three materials. Neither of these minor components exhibited any significant correlative trends with [A + Z].

The maximal xanthophyll-dependent conversions of W to X and X to Y were inhibited by about 50% in the F1 compared with the wild type. Fig. 5 shows the values of the integral fractions for all three PSII states as a function of de-epoxidation state. The sum of the integral fractions was automatically calculated for each run, including those known to bind DCCD like CP26 and CP29. The analysis did not resolve a significant fractional intensity change in the npq4-1 samples under conditions of lumen acidification, as the total average fraction of the W state was 68.1 ± 1.9% in the lumen-acidified state and 70.0 ± 2.2% in the after or final Fm state. There were no significant differences in the average lifetimes in npq4-1 thylakoids between the lumen-acidified and final Fm conditions. With respect to the two other components of the model fit that were included for all samples but not shown in Fig. 4, the integral fraction (average lifetime) of the fastest component was 21.5 ± 1.3% (0.01 ± 0.00 ns), and the longer more variable component was 9.0 ± 4.7% (0.66 ± 0.17 ns) inclusive of all 21 samples from all three materials. Neither of these minor components exhibited any significant correlative trends with [A + Z].

The maximal xanthophyll-dependent conversions of W to X and X to Y were inhibited by about 50% in the F1 compared with the wild type. Fig. 5 shows the values of the integral fractions for all three PSII states as a function of de-epoxidation state. The sum of the integral fractions was automatically normalized to unity based on the average W state value in the npq4-1 samples, which did not exhibit [A + Z]-dependent changes, because this W state value should include and account for any pH-dependent changes not associated with the PsbS...
Pigments were analyzed by HPLC after exposing leaves to 1000 μmol photons m$^{-2}$s$^{-1}$ high light (HL) for 6 min. Six leaf discs were sampled from six different plants and measured separately. The pigment content is expressed as mmol/mol Chl a, except for [A + Z]/[V + A + Z], which is dimensionless. The data represent means ± S.E. (n = 6).

| Pigment     | Before HL |  | After HL |  |
|-------------|-----------|---|----------|---|
|             | Wild type | F1  | npq4-1   | Wild type | F1  | npq4-1 |
| V           | 48 ± 1    | 47 ± 1 | 50 ± 1  | 26 ± 1 | 30 ± 1  | 27 ± 1  |
| A           | 0 ± 0     | 1 ± 1  | 1 ± 1  | 8 ± 0  | 9 ± 0   | 8 ± 0  |
| Z           | 0 ± 0     | 0 ± 0  | 0 ± 0  | 16 ± 1 | 14 ± 1  | 15 ± 2  |
| V + A + Z   | 48 ± 1    | 48 ± 2 | 51 ± 2  | 50 ± 1 | 53 ± 2  | 50 ± 2  |
| [A + Z]/[V + A + Z] | 0.010 ± 0.010 | 0.010 ± 0.010 | 0.020 ± 0.013 | 0.488 ± 0.019 | 0.432 ± 0.012 | 0.459 ± 0.038 |

The calculated three-state model fit parameters showed that the $K_a$ for the F1 was ~40% of that observed in the wild type. The $K_a$ was 5.588 ± 0.610 in the wild type and 2.270 ± 0.253 in the F1. The $pK_a$ was determined to be ~5.2 and closely coincident to the measured ATPase lumen pH of 5.18. It is clear the ATPase lumen pH was subsaturating under these reaction conditions based on several comparisons with light-saturating conditions in this and other studies (see Fig. 3) (13, 14, 40).

**DISCUSSION**

*psbS* Gene Dosage Determines Levels of *psbS* mRNA, *PsbS* Protein, qE, and Chl Fluorescence Lifetimes—The genetic semi-
TABLE II
Global fluorescence lifetime model parameters

| Lifetime distribution state | Lorentzian mode | Lorentzian width |
|-----------------------------|-----------------|-----------------|
| W                           | ns              | 0.36 ± 0.02     |
| X                           | 1.74 ± 0.01     | 0.08 ± 0.03     |
| Y                           | 1.60 ± 0.03     | 0.08 ± 0.03     |

dominance of the npq4-1 deletion mutation in Arabidopsis suggested the occurrence of a psbS gene dosage effect on qE (21). Heterozygous npq4-1/npq4 F1 plants that contain only one copy of the psbS gene had 56% of wild-type mRNAs and 58% of wild-type PsbS protein (Fig. 1). This result indicates that psbS gene transcription limits expression of PsbS protein in F1 plants, and thus the endogenous psbS gene copy number can determine the amount of PsbS protein that accumulates in thylakoid membranes.

The lower PsbS protein level in F1 plants resulted in a corresponding decrease in qE to a value that was ~60% of the wild-type level in both leaves and thylakoids (Fig. 2 and data not shown). This finding demonstrates that the amount of the PsbS protein can be a determinant of qE capacity. The decrease in qE, based on measurements of Chl fluorescence yield, was accompanied by a decrease in the pH- and xanthophyll-dependent fractional intensities of the PSI Chl fluorescence lifetime distributions in thylakoids of the F1 plants (Fig. 4).

The lower level of functional PsbS in F1 plants and the absence of PsbS in npq4-1 raise questions about the location and stoichiometry of PsbS in PSII. PsbS was originally identified as a 22-kDa intrinsic subunit of the PSII oxygen-evolving complex in spinach (28, 29, 48), where it was suggested to be situated between the reaction center core and the peripheral light-harvesting antenna (49). However, the exact functional location of PsbS in PSII has been disputed, because it did not appear to be present in dimeric PSII supercomplexes studied by electron microscopy (50, 51). By using different detergent conditions, however, two recent studies have demonstrated the association of PsbS with both monomeric and dimeric PSII supercomplexes (31, 52). These experiments also showed that PsbS appears to be more strongly associated with the PSII core fraction or inner antenna (CP29/CP26) than with the peripheral LHC fraction. Nevertheless, it remains that no one has reported pH- and xanthophyll-dependent qE in PSII reaction centers, BBY particles, or the dimeric supercomplexes. This leads one to suspect that perhaps the detergent treatments required for isolating all these PSII preparations may interfere directly with the location, structural conformation, and function of the PsbS protein.

The previously reported average stoichiometry of the PsbS protein in spinach thylakoids is ~2 PsbS per PSII reaction center (30). However, the functional stoichiometry in terms of limiting and saturating levels of PsbS remains undetermined. Regarding the limiting levels of PsbS in this study, one could reason that if there are normally two PsbS bound per PSII in the wild type, then it is possible for either or both of the binding sites to be unoccupied in the F1 Arabidopsis. Further, it is also likely, as suggested by overexpression of PsbS, that the wild-type samples themselves exhibit a considerable limitation for PsbS on a per PSII unit basis.

PsbS Expression and Variations in qE Capacity—If the level of PsbS protein can be a determinant of qE capacity, as we have shown, then it seems possible that previously observed natural variations in qE capacity are partly related to differences in PsbS expression. There is considerable variation in the extent of qE and $\frac{[A+Z]}{[V+A+Z]}$ exhibited by different plant species (53–56). Within a species, alterations in qE capacity can be related to environmental changes. For example, plants grown in high light generally have a higher maximum extent of qE than plants grown in low light (54, 55). It would be interesting to examine PsbS expression under such conditions in several different species. Protein immunoblot experiments with wild-type Arabidopsis plants show that the level of PsbS protein is indeed responsive to changes in light intensity.

Both the PsbS protein amount and the concentration of de-epoxidized xanthophyll pigments can affect the level of qE. In contrast to PsbS, which appears to function specifically in qE (21), xanthophyll pigments are known to have more than one function. They have structural and protective (as quenchers of triplet Chl and singlet oxygen) function in LHC proteins; they are involved in qE, and they also function as antioxidants in the thylakoid membrane (57, 58). It is clear that de-epoxidized xanthophylls often exist at excess levels that saturate the qE capacity (13), meaning that a significant pool could be available to serve as antioxidants or radical scavengers. Therefore, controlling the level of PsbS in addition to xanthophylls might

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2 X.-P. Li and K. K. Niyogi, unpublished results.

3 P. Müller-Moule and K. K. Niyogi, unpublished results.
have evolved to achieve an efficient, synergistic way of regulating qE capacity.

PsbS and Formation of the Quenching Complex—Although it is clear that PsbS is necessary for qE in Arabidopsis, a critical step in understanding the mechanism of qE is to understand the specific role of PsbS in formation of a quenching complex. It was shown previously that PsbS is also necessary for a high-light-induced leaf absorption change (ΔA535) (21) that is invariably associated with qE. The ΔA535 has been suggested to monitor a pH- and xanthophyll-dependent conformational change in PSII that represents formation of the quenching complex (59). Using resonance Raman spectroscopy, the ΔA535 in Arabidopsis leaves was recently shown to arise from a 22-nm red shift in the electronic absorption of approximately two Z molecules per PSII + PSI (60). The lack of ΔA535 in npq4-1 suggests that PsbS is a pH-sensing trigger involved in formation of the quenching complex and/or a Z-binding component of the quenching complex itself in the PSII antenna (21). The global Chl fluorescence lifetime analysis revealed no significant pH- or xanthophyll-dependent changes in the W state fraction in npq4-1 thylakoids (Fig. 4), confirming that formation of the quenching complex (Y state) requires PsbS. There was a slight (15%) change in the lifetime mode of the W state in npq4-1, suggesting some minor influence of other pH-sensitive proteins in PSII. Nevertheless, the lifetime data for npq4-1 demonstrate that the pH-dependent formation of the X state does not occur in the absence of PsbS, and the F1 data clearly indicate that the concentration of PsbS limits the pH- and xanthophyll-dependent conversion of W to X (Fig. 4). The most straightforward interpretation of these results is that protonation of PsbS itself induces the fractional shift in Chl fluorescence lifetimes from the W state to the X state, and we hypothesize that PsbS is a key component of the unknown CP complex in the three-state model of qE (13). This notion is supported by site-directed mutagenesis of candidate proton-binding sites in PsbS (61). Following protonation of PsbS and conversion of the W state to the X state, binding of one or two de-epoxidized xanthophylls per PSII is required for formation of the Y state complex at the expense of the X state. Future experiments will examine whether the xanthophylls involved in qE are bound directly to PsbS and/or another component of PsbS protein.

The possible involvement of other specific pigment-binding proteins in the formation of a PsbS-dependent quenching complex in PS II and in the energy dissipation mechanism remains unclear. The quenching complex must either contain or interact exquisitely with other Chl-binding antenna proteins of PSII. The minor Chl proteins CP29 and CP26 were suggested early on as sites of qE based on preferential binding of xanthophyll cycle pigments in these protein complexes (62) and the binding of dicyclohexylcarbodiimide, an inhibitor of qE (63, 64). Chlorophyll fluorescence lifetime measurements on CP29 (65) and CP26 (66) showed that binding of zeaxanthin induces the formation of a shorter lifetime component in vitro. Depletion of CP26 and, to some extent CP29, was suggested to explain the qE defect of psbZ (ycf9) knockout mutants in tobacco (67). However, experiments with antisense Arabidopsis plants that specifically lack CP29 or CP26 have demonstrated that these proteins are not necessary for qE in vivo (68). It is possible that protonation of these proteins is involved in the slight shift in the lifetime mode of the W state in npq4-1 thylakoids. In Chlamydomonas, molecular genetic analysis of the npq5 mutant, which has a phenotype similar to that of Arabidopsis npq4-1, has established a role for a major LHC protein of PSII, Lhcbm1, in qE (69). However, vascular plant (barley and Arabidopsis) mutants that lack Chl b and the major LHC proteins of PSII retain the short lifetime distributions characteristic of qE (13, 40). It is likely that there are interesting differences between organisms in the specific antenna components that are involved in qE.

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REFERENCES

1. Niyogi, K. K. (1999) Annu. Rev. Plant Physiol. Plant Mol. Biol. 50, 333–359
2. Björkman, O., and Demmig-Adams, B. (1994) in Biophysics of Photosynthesis (Schulte, E.-D., and kaldew, M. M., eds), pp. 17–47, Springer, Berlin
3. Govindjee (1995) Aust. J. Plant Physiol. 22, 131–160
4. Horton, P., Ruban, A. V., and Walters, R. G. (1996) Annu. Rev. Plant Biol. Plant Mol. Biol. 47, 655–684
5. Müller, P., Li, X.-P., and Niyogi, K. K. (2001) Plant Physiol. 125, 1558–1566
6. Eskling, M., Arvidsson, P.-O., and Åkerlund, H.-E. (1997) Physiol. Plant. 100, 806–816
7. Hager, A. (1969) Planta 89, 224–243
8. Yamamoto, H. Y., Nakayama, T. O. M., and Chichester, C. O. (1962) Arch. Biochem. Biophys. 97, 168–173
9. Yamamoto, H. Y. (1979) Pure Appl. Chem. 51, 639–648
10. Demmig-Adams, B., Gilmore, A. M., and Adams, W. W., III. (1996) FASEB J. 10, 403–412
11. Gilmore, A. M. (1997) Physiol. Plant. 99, 197–209
12. Gilmore, A. M., Hazlett, T. L., and Govindjee. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 2273–2277
13. Gilmore, A. M., Shinkarev, V. P., Hazlett, T. L., and Govindjee. (1998) Biochemistry 37, 13582–13593
14. Gilmore, A. M. (2001) Photosynth. Res. 67, 89–101
15. Aucula, J. R., Gratton, E., and Prendergast, F. G. (1987) Biophys. J. 51, 587–596
16. Richter, M., Goss, R., Wagner, B., and Holzwarth, A. R. (1999) Biochemistry 38, 12718–12720
17. Bukhlov, N. G., Kopecky, J., Puhr, E. K., Klughammer, C., and Heber, U. (2001) Planta 212, 739–748
18. Gilmore, A. M., and Yamamoto, H. Y. (2001) Photochem. Photobiol. 74, 189–202
19. Li, X.-P., Björkman, O., Shih, C., Grossman, A. R., Rosengquist, M., Jansson, S., and Niyogi, K. K. (2000) Nature 403, 391–395
20. Gilmore, K. K., Björkman, O., and Grossman, A. R. (1997) Plant Cell 9, 1369–1380
21. Gilmore, K. K., Grossman, A. R., and Björkman, O. (1998) Plant Cell 10, 1121–1134
22. Peterson, R. B., and Havir, E. A. (2000) Planta 210, 205–214
23. Shikanai, T., Muneoka, Y., Shimizu, K., Endo, T., and Hashimoto, T. (1999) Plant and Cell Physiology 40, 1134–1142
24. Muneoka, Y., Takeda, S., Hara, K., Jahn, P., Hashimoto, T., and Shikanai, T. (2001) Planta 209, 351–359
25. Peterson, R. B., and Havir, E. A. (2001) Planta 214, 142–152
26. Kim, S., Sandusky, P., Bowly, N. R., Aerssens, R., Green, B. B., Vlahakis, S., Youn, C. F., and Pichersky, E. (1999) FEBS Lett. 412, 67–71
27. Wedel, N., Klein, R., Ljungberg, U., Andersson, B., and Herrmann, R. G. (1992) FEBS Lett. 314, 61–66
28. Funk, C., Schröder, W. P., Napiwotzki, A., Tjus, S. E., Renger, G., and Andersson, B. (1995) Biochemistry 34, 11133–11141
29. Dominici, P., Caffarri, S., Armanante, F., Ceoldo, S., Crimi, M., and Bassi, R. (2002) J. Biol. Chem. 277, 22750–22758
30. Beechem, J. M., Conklin, P. L., and Niyogi, K. K. (1999) in Concepts in photobiology: Photosynthesis and Photomorphogenesis (Singhal, G. S., Renger, G., Seopory, S. K., Irgang, K.-D., and Govindjee, eds), pp. 513–548, Narosa Publishing House, New Delhi, India
31. Schuldiner, S., Rottenberg, H., and Avron, M. (1972) Eur. J. Biochem. 25, 64–70
32. Bevington, P. R. (1969) Data reduction and error analysis for the physical sciences, McGraw-Hill, Inc., New York
33. Draper, N. R., and Smith, H. (1988) Applied regression analysis, 3rd Ed., John Wiley and Sons, Inc., New York
34. Struene, M. and Johnson, M. L. (1992) Methods Enzymol. 210, 87–105
35. Gilmore, A. M., Itoh, S., and Govindjee. (2000) Phil. Trans. R. Soc. Lond. B

4 X.-P. Li, A. M. Gilmore, and K. K. Niyogi, unpublished results.
PsbS and Nonphotochemical Quenching

355, 1371–1384

47. Gilmore, A. M., and Björkman, O. (1995) Planta 197, 646–654

48. Berthold, D. A., Babcock, G. T., and Yocum, C. F. (1981) FEBS Lett. 134, 231–234

49. Kim, S., Pichersky, E., and Yocum, C. F. (1994) Biochim. Biophys. Acta 1188, 339–348

50. Beekema, E. J., van Breemen, J. F. L., van Roon, H., and Dekker, J. P. (2000) Biochemistry 39, 12907–12915

51. Nield, J., Funk, C., and Barber, J. (2000) Phil. Trans. R. Soc. Lond. B 355, 1337–1344

52. Thidholm, E., Lindstrom, V., Tissier, C., Robinson, C., Schröder, W. P., and Funk, C. (2002) FEBS Lett. 513, 217–222

53. Demmig-Adams, B., and Adams, W. W., III. (1994) Aust. J. Plant Physiol. 21, 575–588

54. Demmig-Adams, B., and Adams, W. W., III. (1996) Planta 198, 460–470

55. Johnson, G. N., Young, A. J., Scholes, J. D., and Horton, P. (1993) Plant Cell Environ. 16, 673–679

56. Havaux, M. (1998) Trends Plant Sci. 3, 147–151

57. Havaux, M., and Niyogi, K. K. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 8762–8767

58. Elrad, D., Niyogi, K. K., and Grossman, A. R. (2002) Plant Cell 14, 1801–1816

59. Bilger, W., and Björkman, O. (1990) Photosynth. Res. 25, 173–185

60. Ruban, A. V., Pascal, A. A., Robert, B., and Horton, P. (2002) J. Biol. Chem. 277, 7785–7789

61. Li, X.-P., Phippard, A., Pasari, J., and Niyogi, K. K. (2002) Funct. Plant Biol., in press

62. Bassi, R., Pineau, B., Dainese, P., and Marquardt, J. (1993) Eur. J. Biochem. 212, 297–303

63. Pesaresi, P., Sandona, D., Giuffra, E., and Bassi, R. (1997) FEBS Lett. 402, 151–156

64. Walters, R. G., Ruban, A. V., and Horton, P. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 14204–14209

65. Crimi, M., Dorr, D., Boisinger, C. S., Giuffra, E., Holzwarth, A. R., and Bassi, R. (2001) Eur. J. Biochem. 268, 260–267

66. Frank, H. A., Das, S. K., Bautista, J. A., Bruce, D., Vasil'ev, S., Crimi, M., Croce, R., and Bassi, R. (2001) Biochemistry 40, 1220–1225

67. Swiatek, M., Kuras, R., Sokolenko, A., Higgs, D., Olive, J., Cinque, G., Muller, B., Eichacker, L. A., Stern, D. B., Bassi, R., Herrmann, H. G., and Wollman, F.-A. (2001) Plant Cell 13, 1347–1367

68. Andersson, J., Walters, R. G., Horton, P., and Jansson, S. (2001) Plant Cell 13, 1193–1204

69. Elrad, D., Niyogi, K. K., and Grossman, A. R. (2002) Plant Cell 14, 1801–1816
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