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Is PsbS the site of non-photochemical quenching in photosynthesis?

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Abstract

The PsbS protein of photosystem II functions in the regulation of photosynthetic light harvesting. Along with a low thylakoid lumen pH and the presence of de-epoxidized xanthophylls, PsbS is necessary for photoprotective thermal dissipation (qE) of excess absorbed light energy in plants, measured as non-photochemical quenching of chlorophyll fluorescence. What is known about PsbS in relation to the hypothesis that this protein is the site of qE is reviewed here.

Key words: *Arabidopsis*, chlorophyll fluorescence, light harvesting, mutant, non-photochemical quenching, photosynthesis, PsbS, zeaxanthin.

Introduction

Light is necessary for photosynthesis in plants, but the supply of light in natural environments is not constant. Incident light can vary rapidly due to passing clouds or sunflecks, as well as on a daily or seasonal basis. With increasing light intensity, photosynthetic utilization of absorbed light energy reaches saturation, while light absorption continues to increase. This can result in a mismatch between excitation of photosynthetic pigments and a plant's ability to use the excitation energy for photosynthesis. Under such excess light conditions, how do plants manage to balance the input and utilization of light energy in photosynthesis?

One of the ways in which this balancing act is accomplished is through the regulation of photosynthetic light harvesting. On a time scale of seconds to minutes, non-photochemical quenching (NPQ) processes in photosystem II (PSII) can be induced or disengaged in response to changes in light intensity. The term NPQ reflects the way in which these processes are routinely assayed through measurements of chlorophyll fluorescence (Maxwell and Johnson, 2000; Müller *et al.*, 2001). Under most circumstances, the major component of NPQ is due to a regulatory mechanism, called qE, which results in the thermal dissipation of excess absorbed light energy in the light-harvesting antenna of PSII. qE is induced by a low thylakoid lumen pH (i.e. a high ΔpH) that is generated by photosynthetic electron transport in excess light, so it can be considered as a type of feedback regulation of the light-dependent reactions of photosynthesis (Fig. 1). Because qE involves the de-excitation of singlet excited chlorophyll, it is also sometimes referred to as feedback de-excitation (Külheim *et al.*, 2002).

The low thylakoid lumen pH that induces qE has two roles (Fig. 2). One role is the pH-dependent activation of a lumen-localized violaxanthin de-epoxidase (VDE) enzyme that catalyses the conversion of violaxanthin to zeaxanthin via the intermediate antheraxanthin (Demmig-Adams and Adams, 1996). Zeaxanthin and/or antheraxanthin (xanthophylls with a de-epoxidized 3-hydroxy β-ring end group) are necessary for qE in plants (Demmig-Adams, 1990; Demmig-Adams *et al.*, 1990; Gilmore and Yamamoto, 1993; Niyogi *et al.*, 1998). In limiting light, zeaxanthin epoxidase (ZE) converts zeaxanthin back to violaxanthin. Together, these light intensity-dependent interconversions are known as the xanthophyll cycle (Yamamoto *et al.*, 1999). The second role of low thylakoid lumen pH is in driving protonation of one or more PSII proteins that are involved in qE (Horton and Ruban, 1992). It has been hypothesized that protonation activates a binding site for zeaxanthin in one of the proteins (Gilmore, 1997), and as a result an absorbance change (ΔA_{535}) is detectable in leaves and isolated

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Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; LHC, light-harvesting complex; NPQ, non-photochemical quenching of chlorophyll fluorescence; PSII, photosystem II; qE, pH- and xanthophyll-dependent component of NPQ; QTLs, quantitative trait loci; VDE, violaxanthin de-epoxidase; ZE, zeaxanthin epoxidase.

thylakoids, which might be due to a change in the absorption spectrum of zeaxanthin (Aspinall-O'Dea *et al.*, 2002; Ruban *et al.*, 2002). This alteration of the properties of one or a few zeaxanthin molecules per PSII might allow zeaxanthin to facilitate directly the de-excitation of singlet excited chlorophyll via energy or electron transfer (Ma *et al.*, 2003; Holt *et al.*, 2004).

qE is a plant trait of major ecophysiological significance (Demmig-Adams *et al.*, 1999). Plants that experience excess light stress in their environment (i.e. sun plants) generally have higher qE capacities and larger xanthophyll cycle pool sizes (violaxanthin+antheraxanthin+zeaxanthin) than plants, growing in shaded environments (Thayer and Björkman, 1990; Demmig-Adams and Adams, 1992, 1994; Johnson *et al.*, 1993; Demmig-Adams, 1998), and the maximum extent of qE is considered to be an important ecophysiological factor for the adaptation of plants to excessive light. Furthermore, mutants that lack qE (see below) are less resistant to light stress (Graßes *et al.*, 2002; Li *et al.*, 2002*b*) and have decreased ecological fitness in fluctuating light environments (Külheim *et al.*, 2002).

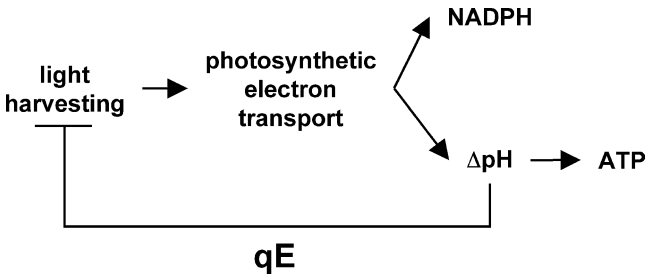

Fig. 1. Diagram depicting feedback regulation of photosynthetic light harvesting (qE) by one of the products of the light reactions of photosynthesis (ΔpH). The ΔpH is used to drive ATP synthesis, and NADPH and ATP are used in CO_2 fixation and other assimilatory reactions. qE down-regulates photosynthetic light harvesting by de-exciting singlet excited chlorophyll in the PSII antenna and thereby dissipating excess absorbed light energy as heat.

The importance of qE as a photosynthetic regulatory process has stimulated tremendous interest in understanding its ecophysiology, genetics, biochemistry, and biophysical mechanism. This paper reviews the role that a specific PSII protein, PsbS, plays in qE.

A genetic approach revealed a role for PsbS

By isolating and characterizing *Arabidopsis thaliana* mutants that lack qE, it was shown that qE requires PsbS, in addition to a low lumen pH and the presence of de-epoxidized xanthophylls like zeaxanthin (Li *et al.*, 2000). qE-deficient mutants were isolated by video imaging of chlorophyll fluorescence quenching during exposure of mutagenized *Arabidopsis* seedlings to excess light (Niyogi *et al.*, 1998; Shikanai *et al.*, 1999; Peterson and Havir, 2000). The *npq1* and *npq2* (*aba1*) mutants were defective in VDE and ZE, respectively (Niyogi *et al.*, 1998), whereas *npq4* mutants lacked qE and ΔA_{535} but had a normal xanthophyll cycle (Li *et al.*, 2000; Peterson and Havir, 2000). The *npq4-1* mutation was mapped to chromosome 1 and ultimately shown to affect the gene encoding PsbS (Li *et al.*, 2000). The *npq4-1* mutant had a complete deletion of the *psbS* gene, so the PsbS protein was missing. Other PSII proteins, however, were present at wild-type levels in the mutant, and light harvesting and photosynthesis appeared to be normal (Li *et al.*, 2000). Characterization of several independently isolated alleles of *npq4* with various lesions in the *psbS* gene (Peterson and Havir, 2001; Graßes *et al.*, 2002; Li *et al.*, 2002*c*) confirmed that PsbS is necessary for qE in *Arabidopsis*.

A working hypothesis for the role of PsbS

The PsbS protein had been identified previously as an integral component of PSII in other plants through biochemical approaches (Ljungberg *et al.*, 1984; Ghanotakis

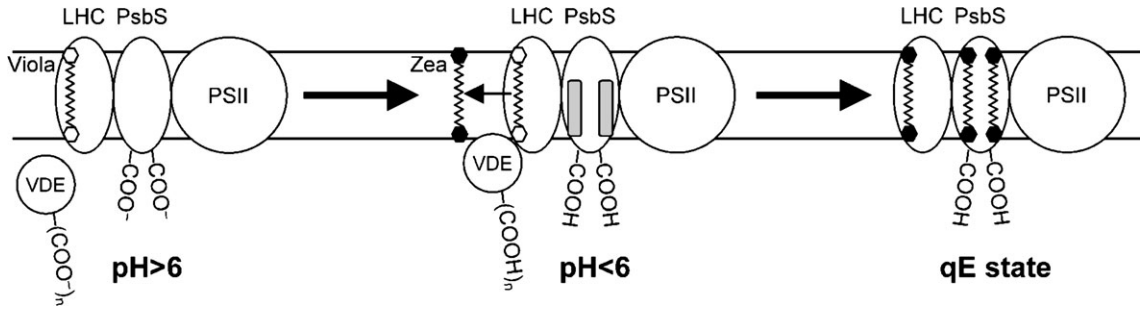

Fig. 2. Schematic model for qE in plants. (Left) In limiting light, the steady-state thylakoid lumen pH is greater than 6 (Kramer *et al.*, 1999). Violaxanthin (Viola) is bound mainly to the V1 site in LHCII and the L2 site in other LHC proteins (such as CP29 and CP26) (Caffarri *et al.*, 2001; Morosinotto *et al.*, 2002). For simplicity, other pigments (chlorophylls and other carotenoids) are not shown, and only one Viola and one LHC protein are shown per PSII. The various components are not drawn to scale. (Middle) In excess light, the thylakoid lumen pH drops below 6, driving protonation of carboxylate side chains in VDE and PsbS. Protonation of VDE activates the enzyme and allows for its association with the membrane (Hager and Holocher, 1994), where it converts multiple Viola molecules to zeaxanthin (Zea). Protonation of glutamate residues E122 and E226 in PsbS activates symmetrical binding sites for xanthophylls with a de-epoxidized β-ring endgroup (i.e. zeaxanthin). (Right) Zea binding to protonated sites in PsbS results in the qE state in which singlet chlorophyll de-excitation is facilitated. Other Zea molecules bind to sites in LHCII and other LHC proteins.

et al., 1987), but its function was uncertain. Cloning and sequencing of the spinach *psbS* gene (Kim *et al.*, 1992; Wedel *et al.*, 1992) had revealed similarity to chlorophyll- and xanthophyll-binding proteins that are members of the light-harvesting complex (LHC) protein superfamily (Green and Pichersky, 1994; Jansson, 1999), and pigment binding by isolated PsbS had been reported (Funk *et al.*, 1994, 1995*b*). This information, coupled with the *npq4* mutant characterization, led to the hypothesis that PsbS might be the site of qE in PSII (Li *et al.*, 2000). Based on this working hypothesis, several predictions could be made.

If PsbS is the site of qE, then (i) other PSII antenna proteins should not be required for qE, except perhaps for the pigment–protein complex from which excitation energy is transferred to the quenching site in PsbS. (ii) PsbS should bind both zeaxanthin and chlorophyll, the pigments that are necessary for qE. (iii) Protonation of PsbS at low thylakoid lumen pH should be necessary for qE. (iv) More quenching sites would mean more quenching, so qE capacity should depend to some extent on the amount of PsbS. In the following sections, the experiments that have been performed to test these predictions will be considered.

Where is PsbS?

PsbS was discovered more than 20 years ago as a 22 kDa protein in isolated PSII preparations (Berthold *et al.*, 1981), but its exact location within PSII is still unknown. In order for PsbS to function as the site of qE, the presumed pigments bound to PsbS would have to be coupled excitationally to one or more chlorophylls in the PSII light-harvesting antenna system. Early biochemical studies showed that PsbS could be co-immunoprecipitated with the (non-pigmented) 33 kDa and 23 kDa subunits of the oxygen-evolving complex in PSII (Ljungberg *et al.*, 1984), suggesting a close association with the PSII reaction centre core. However, PsbS is still present in etiolated plants (Funk *et al.*, 1995*a*) and in mutants that lack PSII (Dominici *et al.*, 2002), indicating that it might be a more peripheral subunit of PSII. Selective extraction of PsbS was shown to disrupt interaction between the peripheral LHCII and the reaction centre, consistent with a location at the interface between these subcomplexes (Kim *et al.*, 1994). A similar conclusion was reached in some studies of PSII supercomplexes, in which PsbS was found to be more closely associated with the reaction centre than with an LHCII fraction (Dominici *et al.*, 2002; Thidholm *et al.*, 2002). Homodimers of PsbS have been described recently, and a dimer-to-monomer transition seems to be triggered by low pH or high light (Bergantino *et al.*, 2003). The dimeric form of PsbS was shown to cofractionate more with the PSII reaction centre, whereas the monomer was also associated with an LHC fraction, and LHC proteins could be co-immunoprecipitated with PsbS (Bergantino *et al.*, 2003). Investigation of qE in chlorophyll *b*-less mutants and antisense plants that lack various LHC proteins (Lokstein *et al.*, 1993; Andersson *et al.*, 2001, 2003), however, have shown that no single LHC protein seems to be necessary for qE (unlike the situation in algae such as *Chlamydomonas*), which casts some doubt on the functional significance of an association between PsbS and a specific LHC protein. Considering all of these results, it seems likely that PsbS is located somewhere between the PSII reaction centre core and the peripheral LHCII (Fig. 2), with the functional association possibly occurring between PsbS and the PSII core antenna.

Electron microscopic studies of plant PSII–LHCII supercomplexes have revealed the positions of most peripheral antenna and core subunits of PSII (Hankamer *et al.*, 2001; Yakushevska *et al.*, 2003), but this approach has not thus far been successful in revealing the specific location of PsbS. It turns out that PsbS was not present in these supercomplexes (Nield *et al.*, 2000), because it was removed by the β-dodecylmaltoside detergent that was used to solubilize the supercomplexes (Harrer *et al.*, 1998; Nield *et al.*, 2000). It was recently found that extraction of PSII particles with α-dodecylmaltoside results in the retention of PsbS in supercomplexes (Dominici *et al.*, 2002), so there is hope that a home for PsbS will be found soon.

Does PsbS actually bind pigments?

Although the initial biochemical studies of PsbS did not provide any hint of pigment binding (Ljungberg *et al.*, 1986; Bowlby and Yocum, 1993), the finding that PsbS is a member of the LHC protein superfamily suggested this as a strong possibility (Kim *et al.*, 1992; Wedel *et al.*, 1992). However, PsbS is considered to be a distant relative of the well-known chlorophyll- and xanthophyll-binding members of this superfamily (Green and Durnford, 1996; Jansson, 1999), and it differs from all others in having four transmembrane domains instead of the usual three (Kim *et al.*, 1994) (Fig. 3). Inspection of the predicted amino acid sequence of PsbS shows little conservation of the residues that provide binding sites for chlorophylls in the LHC proteins that are known to function in light harvesting (Kühlbrandt *et al.*, 1994; Bassi *et al.*, 1999; Croce *et al.*, 1999; Liu *et al.*, 2004). The only ligands that appear to be conserved in LHCII and PsbS are the two charge-compensated glutamates (Funk *et al.*, 1995*b*) (Fig. 3) that also have a critical role in the proper folding and stability of LHCII (Bassi *et al.*, 1999; Croce *et al.*, 1999). Furthermore, unlike most other LHC proteins, PsbS is stable in the absence of chlorophyll *in vivo* (Funk *et al.*, 1995*a*).

Efforts to isolate PsbS with bound pigments have met with mixed success. Funk *et al.* (Funk *et al.*, 1994, 1995*b*) were the first to publish evidence supporting pigment binding by isolated PsbS, and they called the protein CP22 (for chlorophyll-binding protein of 22 kDa). More

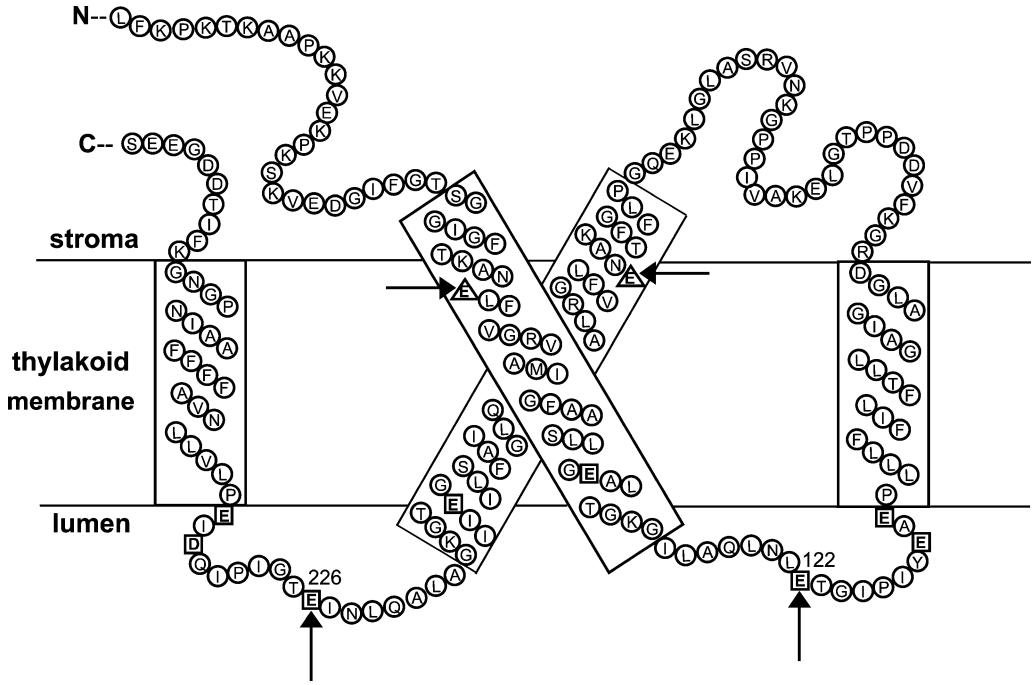

Fig. 3. Topological model of *Arabidopsis* PsbS. Triangles and horizontal arrows denote positions of two highly conserved, charge-compensated glutamates that serve as ligands to bound chlorophylls in LHCII (Kühlbrandt *et al.*, 1994). Squares denote the positions of eight acidic amino acid residues (seven glutamates and one aspartate in *Arabidopsis*) located at or near the lumen side of the protein that are conserved in all known PsbS sequences. The two glutamates that are necessary for qE and DCCD binding are numbered and marked by vertical arrows. Numbering is relative to the predicted initiator methionine of the PsbS precursor protein (prior to import into chloroplasts). Modified from Li *et al.* (2002*c*).

recently, Dominici *et al.* (2002) were unable to demonstrate a stable association of pigments with isolated PsbS, and their attempts to reconstitute recombinant PsbS with pigments *in vitro* were unsuccessful. Aspinall-O'Dea *et al.* (2002) also found no pigment binding that could withstand purification of PsbS, but they were able to provide evidence for an interaction between PsbS and zeaxanthin *in vitro*. This interaction resulted in a change in the absorption spectrum of zeaxanthin that reconstituted the ΔA_{535} that is associated with qE in leaves and thylakoids (Aspinall-O'Dea *et al.*, 2002). Overexpression of PsbS in tobacco was shown to increase the extent of violaxanthin de-epoxidation under relatively low light conditions *in vivo*, and it was suggested that this result could be explained by zeaxanthin binding to PsbS, which would sequester zeaxanthin and prevent feedback inhibition of de-epoxidation by VDE (Hieber *et al.*, 2004). Ultrafast transient absorption studies of qE in isolated thylakoids revealed the PsbS-dependent presence of singlet excited zeaxanthin following the excitation of chlorophyll (Ma *et al.*, 2003). Assuming that the excited zeaxanthin is bound to PsbS, this result implies that there must be chlorophyll in very close proximity to the zeaxanthin, either bound to PsbS itself or on the periphery of a closely associated protein.

The bottom line from the work to date seems to be that, if PsbS does indeed bind pigments *in vivo*, then the nature of this binding interaction must differ substantially from that in other LHC proteins. There is now some evidence for zeaxanthin binding by PsbS (Aspinall-O'Dea *et al.*, 2002), which is consistent with the hypothesis that PsbS is the site of qE, but it is also consistent with other, more complicated hypotheses in which zeaxanthin has an indirect, allosteric role in qE (Horton *et al.*, 2000; Aspinall-O'Dea *et al.*, 2002). Unfortunately, the results showing a lack of chlorophyll binding, because they are negative results, neither support nor rule out the hypothesis.

PsbS as a sensor of lumen pH

It was suggested early on that one or more carboxylate side chains in PSII proteins might bind protons at low lumen pH and thereby trigger qE (Horton and Ruban, 1992). This idea was supported by the inhibition of qE by *N*,*N*′-dicyclohexylcarbodiimide (DCCD) (Ruban *et al.*, 1992), which binds to proton-active residues in hydrophobic environments. DCCD was shown to bind to LHC proteins, such as CP29 and CP26 (Walters *et al.*, 1996; Pesaresi *et al.*, 1997), which had been suspected to be involved in qE (Horton and Ruban, 1992; Bassi *et al.*, 1993; Jahns and Schweig, 1995), but antisense experiments showed that CP29 and CP26 are unlikely to be sites of qE (Andersson *et al.*, 2001).

After the involvement of PsbS in qE was discovered, it was shown that DCCD binds to PsbS as well (Dominici *et al.*, 2002), and sequence analysis showed that PsbS has eight conserved acidic amino acid residues (glutamate and aspartate) located at or near the lumen side of the protein

that are candidate proton- and/or DCCD-binding sites (Fig. 3) (Li *et al.*, 2002*c*). These eight residues are arranged as four symmetrical pairs, and they are conserved in all known PsbS sequences, including the recently identified sequences from the green algae *C. reinhardtii* and *Volvox carteri* (Anwaruzzaman *et al.*, 2004). Although many *npq4* point mutant alleles had been isolated following chemical mutagenesis in *Arabidopsis*, none of these mutations affected a potential proton-binding site. Therefore, a site-directed mutagenesis approach was used to test the role of the lumenal acidic amino acid residues in PsbS (Li *et al.*, 2002*c*). Each of seven glutamates and one aspartate in *Arabidopsis* PsbS was changed (both individually and as symmetrical pairs) by mutagenesis *in vitro* to glutamine or asparagine, respectively. The site-directed mutants were transformed into the *npq4-1* mutant that lacks the wild-type *psbS* gene, and the function of each mutant was tested *in vivo*. One pair of glutamates (E122 and E226; Fig. 3) was shown to be necessary for qE, ΔA_{535}, and DCCD binding, strongly suggesting that protonation of these residues in excess light is necessary for qE and that PsbS serves as a sensor of lumen pH (Li *et al.*, 2004).

PsbS and qE capacity

If PsbS is the site of qE, then the level of qE should be related to the amount of PsbS per PSII. If there are more quenching sites, then there should be more quenching (up to a limit, of course). Molecular and genetic analysis of the *npq4-1* mutant showed that there is indeed a *psbS* gene dosage effect on qE. Heterozygous *npq4-1/NPQ4* plants have half the number of *psbS* genes as the wild type, and they have a correspondingly lower level of *psbS* mRNA, PsbS protein, and qE (Li *et al.*, 2002*a*). Increasing PsbS expression in transgenic plants confers a higher qE capacity (Li *et al.*, 2002*b*; Hieber *et al.*, 2004), with saturation occurring in *Arabidopsis* at ~5 times the wild-type level of PsbS on a per PSII basis (Fig. 4). This saturation indicates that there is a maximum number of functional binding sites for PsbS per PSII. Thus, the PsbS protein level can be a determinant of qE capacity, and PsbS expression seems to limit qE in wild-type *Arabidopsis* and tobacco (Li *et al.*, 2002*b*; Hieber *et al.*, 2004). Alterations in PsbS level have been reported as explanations for lower qE in LHCII-deficient plants (Andersson *et al.*, 2003) and higher qE in PsaD-deficient plants (Haldrup *et al.*, 2003).

Does the stoichiometry of PsbS vary naturally in plants? The stoichiometry of PsbS has been reported to be two copies of PsbS per PSII in wild-type spinach thylakoids (Funk *et al.*, 1995*b*), but the *Arabidopsis* mutants and transgenics show that the stoichiometry can vary widely, from zero (in the *npq4-1* mutant) to many times the wild-type value (Fig. 4). The stoichiometry in low-light-grown, wild-type *Arabidopsis* plants has not yet been determined. It seems plausible that variations in PsbS expression might explain at least some cases of environmental (i.e. sun versus shade) and species-dependent variation in qE capacity (Johnson *et al.*, 1993; Demmig-Adams and Adams, 1994; Demmig-Adams, 1998).

To investigate this hypothesis, the genetic basis for natural variation in qE capacity in *Arabidopsis* accessions (often referred to as 'ecotypes') has started to be examined. A survey of the qE capacity was conducted in more than 50 accessions, and it was found that there is substantial intra-species variation for qE in *Arabidopsis* (Fig. 5). However, selected high and low qE accessions appeared to have the

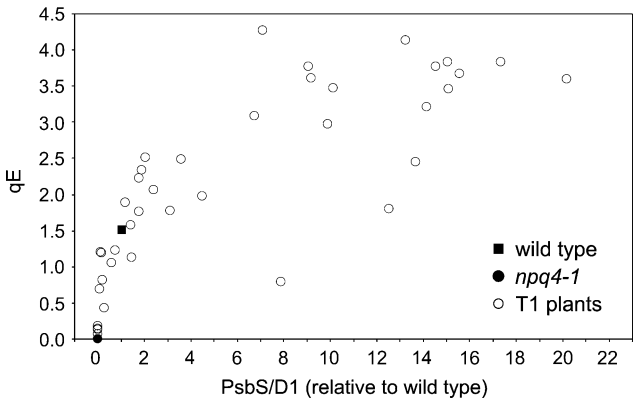

Fig. 4. Relationship between PsbS protein level and qE. The *npq4-1* mutant was transformed with the wild-type *Arabidopsis psbS* gene under the control of its own promoter (Li *et al.*, 2000). T_1 transformants were selected on agar medium containing gentamycin, and the level of NPQ in the transformants was initially assessed using chlorophyll fluorescence video imaging. T1 seedlings exhibiting a range of NPQ values were transferred to soil and grown to maturity. NPQ was then measured using a commercial fluorometer (FMS2; Hansatech, King's Lynn, UK) after illumination at 1200 μmol photons m^{-2} s^{-1} for 10 min. qE of each plant was calculated as (NPQ in T_1 plant)–(NPQ in *npq4-1*). The PsbS protein level in each T_1 plant was determined by immunoblotting and normalized to the level of the PSII reaction centre protein D1 (Li *et al.*, 2002*b*). The qE values of 39 independent T_1 plants are shown with the open circles.

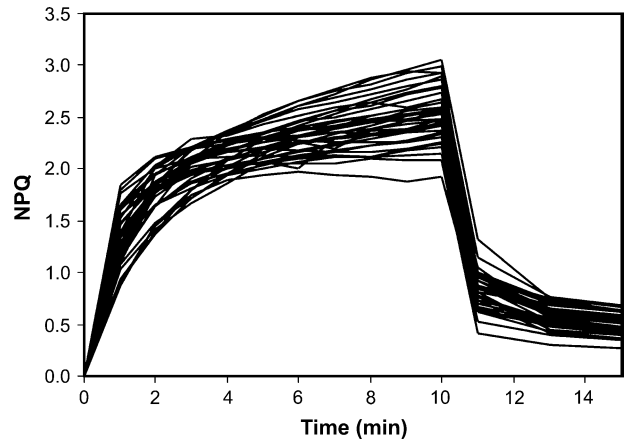

Fig. 5. Natural variation of qE in 38 *Arabidopsis* accessions. Accessions were grown under identical low light conditions (100 μmol photons m^{-2} s^{-1}), and NPQ induction was measured during illumination with high light (1500 μmol photons m^{-2} s^{-1}) for 10 min followed by a relaxation period of 5 min in darkness.

same level of PsbS expression (H-S Jung and KK Niyogi, unpublished results). Analysis of F_2 plants resulting from a cross between a high qE accession (Sf-2) and a low qE accession (Col-0) showed continuous variation of the qE phenotype, indicating that qE capacity in these accessions is a quantitative genetic trait that is controlled by multiple genes. Mapping of quantitative trait loci (QTLs) uncovered two major QTLs that are responsible for much of the variation in qE, but neither of these QTLs mapped to the position of the *psbS* gene, indicating that the naturally occurring variation in qE between these two accessions is not attributable to PsbS (H-S Jung and KK Niyogi, unpublished results). The extent to which this conclusion can be generalized to other *Arabidopsis* accessions and to other species remains to be determined.

PsbS in algae

Is PsbS a key player in qE in other photosynthetic organisms? Besides angiosperms like *Arabidopsis* and tobacco, genes encoding PsbS have been identified in a moss (*Physcomitrella patens*) and in two green algae (*C. reinhardtii* and *V. carteri*) (Anwaruzzaman *et al.*, 2004), but the function of these genes in qE has not yet been tested. *C. reinhardtii* is an interesting case, because qE-deficient mutants have been isolated (Niyogi *et al.*, 1997), but so far no mutants have been shown to affect PsbS. In fact, the most extensively studied *C. reinhardtii* mutant, *npq5*, turned out to be defective in an LHCII gene called *Lhcbm1* (Elrad *et al.*, 2002). This finding raises intriguing questions about the possible relationship between LHCII and PsbS in this alga, and this can be resolved by studying PsbS-deficient mutants generated through reverse genetics.

Although nearly all photosynthetic eukaryotes have qE, it is becoming clear from genome sequencing projects that not all have PsbS. For example, diatoms exhibit robust qE that depends on a low thylakoid lumen pH and the presence of a de-epoxidized xanthophyll (diatoxanthin instead of zeaxanthin and antheraxanthin), but the first completely sequenced genome of a diatom, *Thalassiosira pseudonana* (http://genome.jgi-psf.org/thaps1/thaps1.home.html), lacks a *psbS* gene. There are, however, many genes encoding other members of the LHC protein superfamily, so it is possible that another member of the family performs the function of PsbS in diatoms. As genome sequence information becomes available for other diverse photosynthetic eukaryotes, a challenge will be to identify the proteins that play the role that PsbS has in plants. It is likely that investigation of qE in algae will provide interesting insights into the evolution of function in the LHC protein superfamily.

Conclusion

The discovery that PsbS is necessary for qE in *Arabidopsis* was an important breakthrough in the study of qE (Li *et al.*, 2000), but it certainly did not mean that the problem of understanding qE was solved. On the contrary, the mechanism of qE still remains one of the last major unresolved mysteries in photosynthesis.

A simple hypothesis has been proposed that PsbS is the site of qE in plants (Li *et al.*, 2000). Several experimental tests of the hypothesis have now been conducted, and at present the hypothesis remains viable, although more complicated scenarios are also consistent with the available data. Previously hypothesized sites of qE, such as LHCII, CP29, and CP26, look less promising in the light of recent antisense experiments (Andersson *et al.*, 2001, 2003). On the other hand, the amount of the PsbS protein in thylakoids has been shown to be a determinant of qE capacity (Fig. 4) (Li *et al.*, 2002*b*; Hieber *et al.*, 2004), and two lumen-facing glutamate residues in PsbS (Fig. 3) have been identified as proton-binding sites that are probably involved in sensing lumen pH and turning qE on and off (Li *et al.*, 2002*c*, 2004). Evidence for zeaxanthin binding by PsbS *in vitro* has been reported (Aspinall-O'Dea *et al.*, 2002), and a follow-up of these results is eagerly anticipated. Ultrafast PsbS-dependent excitation of zeaxanthin following laser excitation of chlorophyll has been demonstrated (Ma *et al.*, 2003). This places strict constraints on the distance between the nearest chlorophyll and the excited zeaxanthin, which is assumed to reside in PsbS, but chlorophyll binding to PsbS remains to be unequivocally demonstrated. It is possible that the coupled chlorophyll might be located on the periphery of PsbS, perhaps at the interface between PsbS and PSII, which might explain the difficulty in isolating PsbS with bound chlorophyll.

The next major breakthrough in understanding the role of PsbS in qE will probably depend on biochemical reconstitution of qE in a much simpler system than isolated thylakoid membranes, the simplest system to date. Indeed, a holy grail of qE research is the isolation of a complex containing PsbS, zeaxanthin, and chlorophyll that exhibits pH- and zeaxanthin-dependent de-excitation of singlet excited chlorophyll (qE). In conjunction with methodological advances in spectroscopy and structural biology, it will then be possible to obtain a full picture of the mechanism of qE.

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