Chapter 13

PsbS-Dependent Non-Photochemical Quenching

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Summary

Much of the research on the thermal dissipation of excess absorbed light, measured as non-photochemical quenching (NPQ) of chlorophyll fluorescence, has been focused on a major, rapidly induced and rapidly reversible component of fluorescence quenching termed energy-dependent quenching, feedback de-excitation, or qE. A breakthrough in this field came with the discovery of the involvement of the photosystem II subunit S (PsbS) protein, a thylakoid membrane protein required for qE induction and relaxation. In this chapter, we discuss the history of how PsbS was first identified as a photosystem II subunit, the genetic characterization that defined its important role in qE, and the biochemical work describing how PsbS might regulate photosynthetic light harvesting. We emphasize how mutants affecting PsbS (i.e., *npq4)* have been invaluable tools in defining structural and spectroscopic changes associated with qE, and how these mutants have contributed to our current understanding of the physiological role of the thermal dissipation process accompanied by non-photochemical fluorescence quenching.

I Introduction

Energy-dependent fluorescence quenching or feedback de-excitation (qE) is the fastest component of the non-photochemical quenching (NPQ) of chlorophyll fluorescence, inducing and relaxing within seconds to minutes, and used as a measure of thermal dissipation of singlet-excited chlorophyll *a*, the primary mechanism by which plants cope with excess energy in fluctuating light conditions (Murchie and Niyogi 2011; Jahns and Holzwarth 2012; Ruban et al. 2012). Thermal dissipation of excess absorbed light is thought to limit the production of reactive oxygen species by decreasing the lifetime of singlet-excited chlorophyll (Chl) *a* and reducing the excitation pressure on photosystem (PS) II (Niyogi 1999). The signal that activates thermal dissipation (measured as qE) is a low lumen pH, which occurs when the trans-thylakoid proton gradient is formed faster than it is dissipated by ATP synthase (Kanazawa and Kramer 2002; Takizawa et al. 2008). In this way, thermal dissipation (measured as qE) acts as a feedback mechanism, switching the PS II antenna to a quenching state in response to the (excess) light perceived.

There are at least three requirements for the activation of thermal dissipation (qE) in vascular plants. The first is, as stated above, the trans-thylakoid pH gradient formed in the light (Wraight and Crofts 1970). By preventing formation of, or removing, the pH gradient, using uncouplers, the qE component of NPQ can be abolished (Briantais et al. 1979). Conversely, using chemicals (Takizawa et al. 2008; Johnson and Ruban 2011), or decreas-

Abbreviations: BBY – Berthold-Babcock-Yocum; Co-IP – Co-immunoprecipitation; CP24 – Chlorophyll protein of 24 kDa molecular mass; CP26 – Chlorophyll protein of 24 kDa molecular mass CP29 – Chlorophyll protein of 29 kDa molecular mass; DAD – Diaminodurene; DCCD – Dicyclohexylcarbodiimide; LHC – Light-harvesting complex; NPQ – Non-photochemical quenching of chlorophyll fluorescence; PS – Photosystem; PsbO – Photosystem II subunit O; PsbP – Photosystem II subunit P; PsbS – Photosystem II subunit S; qE – Energy-dependent, rapidly reversible component of NPQ, also termed energy-dependent quenching or feedback de-excitation; QTL – Quantitative trait locus; V – Violaxanthin; VDE – Violaxanthin de-epoxidase; Z – Zeaxanthin

ing CO_2 fixation (Schreiber et al. 1986; Fichtner et al. 1993), in order to increase the pH gradient can increase qE. There is also evidence that qE can be modulated in vivo by fine-tuning the pH gradient via cyclic electron transport (Heber and Walker 1992; Miyake et al. 2005) or changes to the conductivity of ATP synthase (Kanazawa and Kramer 2002; Avenson et al. 2004). The chemical dicyclohexylcarbodiimide (DCCD) that binds to protonatable residues in hydrophobic environments was also shown to inhibit qE (Ruban et al. 1992b). It was later shown that the light-harvesting complex (LHC) proteins to which DCCD binds are CP26 and CP29, suggesting that the low pH can be sensed through lumen-exposed residues, and implicating the latter two proteins in qE (Walters et al. 1994; Pesaresi et al. 1997).

The second requirement for qE in vivo is the xanthophyll (VAZ) cycle, in particular the conversion of the carotenoid violaxanthin (V) to antheraxanthin (A) and zeaxanthin (Z) (Demmig-Adams 1990; Niyogi 1999). V is converted to Z through the action of V de-epoxidase (VDE), a lumenal enzyme of the lipocalin family of proteins (Yamamoto et al. 1999). The first evidence for the role of the VAZ cycle in qE came from the correlation between Z levels and the amount of quenching upon exposure of leaves to high light (Demmig et al. 1987). This relationship was confirmed by the identification of mutants affecting VDE (*npq1*) and Z epoxidase (*npq2*) that also affected NPQ (Niyogi et al. 1998). Despite years of study, the exact role of Z in qE remains controversial, owing largely to the lack of consensus on the molecular mechanism of qE (Holt et al. 2004; Jahns and Holzwarth 2012; Ruban et al. 2012). A direct role in qE has been proposed for Z through either an energy transfer (Owens 1994; Frank et al. 2000; Ma et al. 2003) or charge-transfer (Holt et al. 2005; Ahn et al. 2008; Avenson et al. 2008, 2009) mechanism from Chl to Z (see also Walla and Fleming, Chap. 9). Recently, it has also been shown that this function is not specific to Z, as accumulation of lutein can partially compensate for the qE defect in *npq1* (Li et al. 2009). Alternatively, an indirect role for Z in qE has been put forward, in which Z acts as an allosteric regulator, controlling the sensitivity of qE to the pH gradient and/or conformational changes within the LHCs where qE occurs (Crouchman et al. 2006; Johnson et al. 2008, 2012; Ruban et al. 2012). These roles for Z are not necessarily mutually exclusive, and models have been put forth to explain Z involvement in NPQ by different roles depending on site and type of quenching (Jahns and Holzwarth 2012).

The third requirement for qE in plants, and the focus of this chapter, is the thylakoid membrane protein PS II subunit S (PsbS). While this protein has been the topic of a great deal of research over the last 15 years, a surprisingly large amount of uncertainty remains regarding its location within the thylakoid membrane, what other protein(s) it interacts with, and the biochemical mechanism by which it is involved in NPQ. In this chapter, we will describe experiments and proposed models leading to our current understanding of PsbS-dependent qE and discuss what questions remain.

II Discovery of PsbS and Involvement in qE

A Initial Discovery as a Photosystem II Subunit

The PsbS protein was initially identified as a component of PS II-enriched Berthold-Babcock-Yocum (BBY) preparations (Ghanotakis and Yocum 1986) and by co-immunoprecipitation (Co-IP) with the oxygen-evolving complex subunits PsbO and PsbP (Ljungberg et al. 1984, 1986). While these results initially suggested that PsbS might be a component of the oxygen-evolving complex, subsequent experiments showed that isolated PS II core complexes depleted in PsbS did not lose any oxygen-evolving ability (Ghanotakis et al. 1987). Sequencing of the *psbS* cDNA revealed that it encoded a protein with similarity to LHC proteins (Kim et al. 1992; Wedel et al. 1992). PsbS

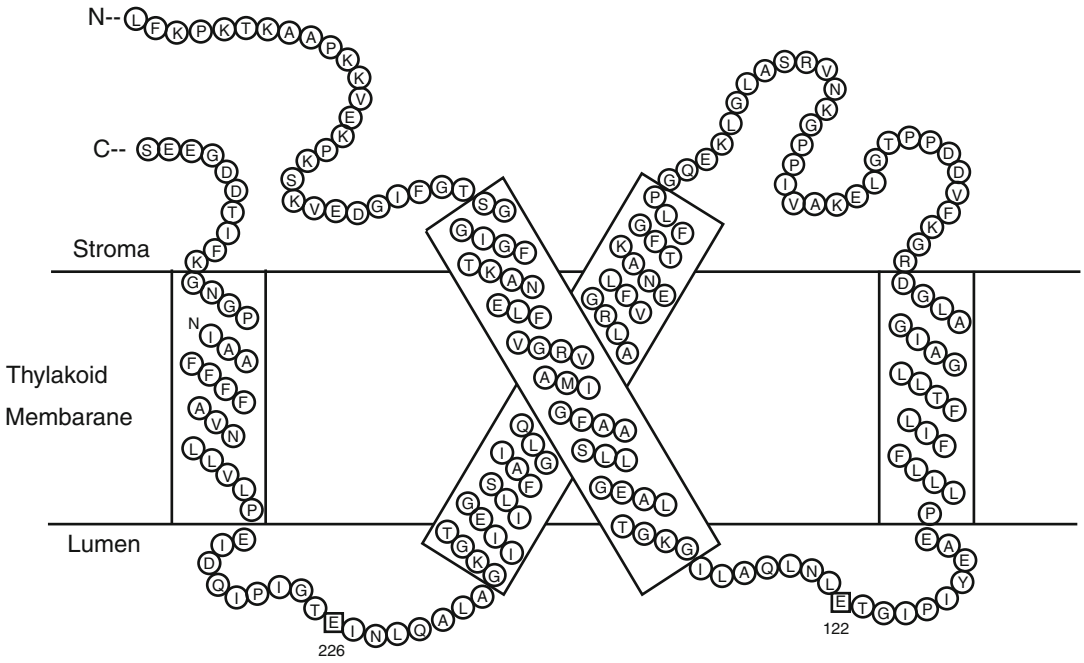

Fig. 13.1 Proposed topology of PsbS in the thylakoid membrane. Two glutamate residues shown to be involved in sensing of lumen pH are marked by *squares.*

is unique, however, as it contains four rather than the typical three transmembrane helices (Fig. 13.1). The similarity between the first/third and second/fourth helices suggested that PsbS likely arose through duplication of a two-helix LHC protein (Kim et al. 1992). Analysis of extant LHC protein sequences indicates that PsbS and typical light-harvesting proteins arose via independent duplication events (Engelken et al. 2010).

B Involvement in qE Revealed Through a Genetic Screen

More than 15 years after the initial discovery of the PsbS protein, a functional role for PsbS in NPQ (and thus presumably thermal dissipation) was first established through a genetic approach. Using video imaging of chlorophyll fluorescence to screen for qE-deficient mutants, the *npq4* mutant was isolated (Li et al. 2000). Unlike the previously characterized *npq1* and *npq2* mutants, *npq4* plants contained a normal xanthophyll (VAZ) cycle, yet lacked qE (Fig. 13.2) and ΔA_{535} (Li et al. 2000; Peterson and Havir 2000), a spectroscopic signature correlated to qE (Ruban et al. 1993). The *npq4* mutation was mapped to the gene encoding PsbS, and this protein was demonstrated to be necessary for qE in plants (Li et al. 2000). It was also shown that, despite a complete lack of PsbS protein in the *npq4-1* allele, all other PS II subunits accumulated to normal levels, and other photosynthetic properties were unaffected. Later, rice PsbS knockout mutants (Zulfugarov et al. 2007; Ishida et al. 2011; Kasajima et al. 2011) and *Populus* PsbS RNAi lines (Fig. 13.3) were shown to have a similar phenotype as *npq4*, confirming that the function of the protein is conserved.

Since the discovery that PsbS is essential for qE (and presumably thermal dissipation of excess energy) in plants, the *npq4* mutant has been a crucial tool for exploring possible mechanisms involved in qE. While *npq1* mutants have also been used as qE-deficient controls, the role of Z as an antioxidant (Havaux et al. 2007), and the trace amounts of Z remaining in the *npq1* mutant complicates the interpretation of results. Likewise,

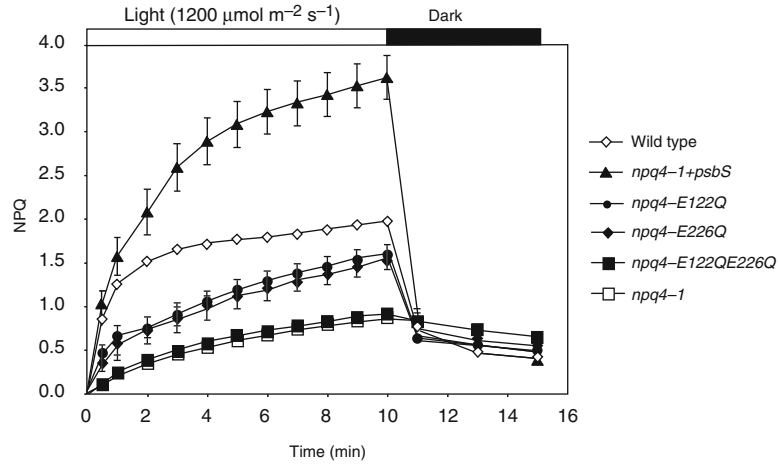

Fig. 13.2 Induction and relaxation of NPQ in mutants affecting PsbS. NPQ was measured during 10 min of illumination with high light (1,200 μmol photons m^{-2} s^{-1}), followed by relaxation in the dark for 5 min. Data are means ± SE (n = 8), from Li et al. (2004).

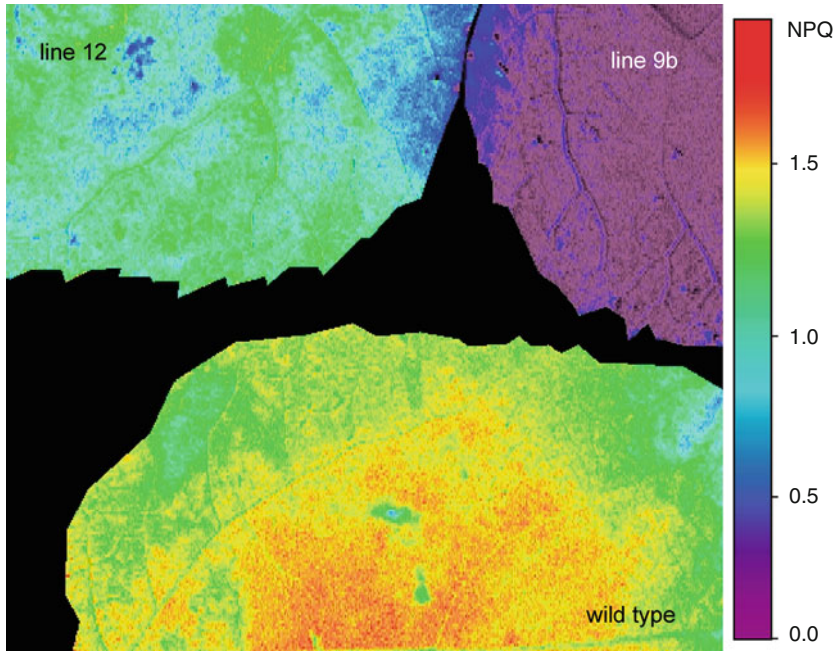

Fig. 13.3 Chlorophyll fluorescence imaging of NPQ in leaves of wild type and two independent PsbS RNAi lines (9b and 12) of *Populus.*

the use of pH uncouplers to prevent qE may also have other effects on the cell. Because PsbS has thus far been implicated solely in NPQ, and specifically qE, with no other effect on light harvesting or photosynthesis, the *npq4* mutant is an ideal control for studies examining the spectroscopic and structural changes associated with qE.

III Biochemical Function of PsbS

A Does PsbS Bind Pigments?

The similarity between PsbS and other members of the LHC superfamily suggested a role of PsbS as a pigment-binding protein. On the other hand, many of the amino acids

that coordinate chlorophylls, and that are conserved in most LHC proteins, are absent in PsbS (Dominici et al. 2002). Early studies, in which PsbS was isolated from solubilized BBY membranes, indicated that the protein bound five chlorophyll molecules (Funk et al. 1994, 1995b). Unlike other LHCs, however, PsbS accumulated in etiolated tissues (Funk et al. 1995a) and chlorophyll-deficient mutants of barley (Dominici et al. 2002), indicating that the protein is stable in the absence of chlorophyll. Subsequently, two research groups purified PsbS from thylakoids by the same method as above, as well as using other techniques, and were unable to observe any chlorophyll binding for PsbS (Aspinall-O'Dea et al. 2002; Dominici et al. 2002). In addition, while all other recombinant LHCs require pigments in order to fold in vitro, recombinant PsbS protein does not exhibit such a requirement (Dominici et al. 2002; Wilk et al. 2013). Binding of Z to purified PsbS in vitro was suggested to cause a red shift in the pigments's absorbance, similar to the ΔA_{535} signature observed in vivo (Aspinall-O'Dea et al. 2002). This result has been questioned, however, as the signal attributed to PsbS-xanthophyll interaction was shown to be the result of Z aggregation (Bonente et al. 2008), and PsbS reconstituted into liposomes along with or without Z did not show any differences (Wilk et al. 2013). Interestingly, mutagenesis of the glutamate-arginine ion pairs, modeled as Z ligands in PsbS (Haripal et al. 2006), resulted in a complete loss of PsbS function without affecting protein stability and accumulation (Schultes and Peterson 2007). There is thus no firm evidence that PsbS is a "typical" pigment-binding protein, although it is possible that PsbS somehow interacts with at least carotenoid molecules to fulfill its function.

B Insights into How PsbS Functions in qE

The amount of PsbS protein present modulates a plant's maximal qE level. This effect was hypothesized based on the semi-dominant nature of *npq4* heterozygous plants obtained from the backcross to wild type; the heterozygotes had an intermediate level of qE relative to the parental strains (Li et al. 2000). In subsequent work the PsbS mRNA and protein levels were shown to correlate to the amount of qE in *npq4* heterozygotes, wild type and homozygous *npq4* plants (Li et al. 2002a). The dosage effect (the amount of PsbS protein determining the amount of qE) was further confirmed by transforming wild-type plants with an additional copy of the *psbS* gene and showing that these plants accumulated more protein and were able to reach NPQ values approximately twice as high as wild type (Li et al. 2002b; Fig. 13.2). These results suggested that differences in qE capacity observed among plant species and in response to high light (Demmig-Adams and Adams 1992; see also Demmig-Adams et al., Chap. 24) might result from changes in PsbS expression. While this has been shown to be the case in certain situations (Demmig-Adams et al. 2006), it is not always the case as discussed below.

1 PsbS as a Lumen pH Sensor

The presence of several conserved acidic residues on each of the two lumen-exposed loops of PsbS (Li et al. 2000) suggested that the protein may be capable of sensing lumenal pH. It has been demonstrated that, just as CP26 and CP29, PsbS is also able to bind DCCD (Dominici et al. 2002), consistent with the hypothesis that protonatable amino acid residues occur in PsbS. Two glutamate residues, one on each lumen-exposed loop of PsbS, were identified by site-directed mutagenesis as being critical for qE function in vivo (Li et al. 2002c, 2004). Mutation of either one of these residues (E122Q or E226Q) reduced qE capacity to approximately one-third of the control level, while a double point mutant expressed wild-type levels of protein but lacked all qE (Fig. 13.2). Finally, DCCD-binding by PsbS was completely abolished in the E122Q/E226Q double mutant, suggesting that the latter two residues are solely responsible for the pH-sensing capability of PsbS (Li et al. 2004).

2 Interactions with Other Photosynthetic Complexes

Interactions between PsbS and other thylakoid proteins have not been resolved completely. As mentioned in the previous section, PsbS was originally found to be associated with the oxygen-evolving complex (Ljungberg et al. 1984, 1986). Co-IP experiments in various crop species, using antibodies raised against PsbS, resulted in the appearance of a band at approximately 42 kDa (Bergantino et al. 2003). This band was determined to be a PsbS dimer based on migration in the gel and the lack of signal when blotted with other PS II subunits of similar sizes. Since formation of this band was pH dependent, it was concluded that PsbS is present as a homodimer in the dark, and that low pH induces monomerization in the light (Bergantino et al. 2003). It would thus be interesting to explore whether or not mutation of the pH-sensing glutamates affects monomer-to-dimer transition. Co-IP was also used to show interaction between PsbS and several photosynthetic complexes, including not only LHCII components and CP29 but also, surprisingly, several PS I, cytochrome b_6f, and ATPase subunits (Teardo et al. 2007). More recently, a direct interaction between PsbS and LHCII was demonstrated when both proteins were reconstituted into proteo-liposomes (Wilk et al. 2013). Cross-linking experiments performed in several laboratories have failed to provide informative results.

3 Bypassing the Need for PsbS in qE

Treatment of thylakoids with chemicals has been a traditional way of investigating NPQ, and revisiting several of those experiments with mutants has proven informative. A particularly interesting example is the use of diaminodurene (DAD), shown in the 1970s to cause a higher than normal ΔpH by facilitating artificial electron transport around PS I (Wraight and Crofts 1970; Hauska and Prince 1974). Recently, the effects of DAD on isolated chloroplasts from wild type and *npq* mutants were compared (Johnson and Ruban 2011; Johnson et al. 2012). Surprisingly, while control thylakoids behaved as expected, DAD was able to induce qE to an equal and significantly higher level in both wild type and *npq4* (Johnson and Ruban 2011). Similar results were demonstrated for *lut2 npq1* mutants lacking the xanthophylls involved in qE (Johnson et al. 2012). The authors used these results to suggest that the role of PsbS and xanthophylls in qE is to modulate the pKa of lumen-exposed residues involved in qE and allow qE at a physiologically relevant lumen pH in vivo (Johnson and Ruban 2011; Johnson et al. 2012; Ruban et al. 2012).

IV Does PsbS Affect the Organization of Photosynthetic Complexes?

More recently, it has been proposed that the conformational change occurring as a result of PsbS protonation may affect the organization of complexes within the grana membrane. It was shown that the amount of PsbS protein affected Mg^{2+}-induced thylakoid restacking, possibly by altering interactions between PS II and LHCII complexes (Kiss et al. 2008). This effect on thylakoid restacking did not depend on the capability of PsbS to function in qE, but rather solely on PsbS protein level as demonstrated using mutants in the pH-sensing glutamate residues. Evidence for a role in membrane organization also came from sucrose-gradient fractionation of *Arabidopsis* thylakoids and the observation that a band (called band 4), containing CP29, CP24 and LHCII trimers, was present in dark-acclimated samples but disassociated upon high light treatment in wild type (Betterle et al. 2009). In the *npq4* mutant, band 4 remained even after high light treatment and, even more strikingly, dissociation of this complex was reduced in the single glutamate mutants by approximately 50 %. The latter authors concluded that, while dissociation of band 4 was not sufficient for qE, it is possible that this complex stabilizes an unquenched light-harvesting state. They also noted that PsbS is likely to have other roles besides allowing the dissociation

of band 4, as knockouts of CP24 constitutively lacked band 4, yet contained disconnected unquenched LHCs and were able to activate NPQ upon exposure to high light (Kovacs et al. 2006; Betterle et al. 2009).

Electron microscopy has also been used to investigate the location of PsbS and how it affects grana organization. Using single particle analysis of cryo-images, Nield et al. (2000) determined that the electron density of PS II-LHCII super-complexes does not allow for the presence of a protein containing a fourth transmembrane helix, such as PsbS, and that PsbS is more likely associated with LHCII-rich regions of the membrane. NPQ was also correlated to structural changes in thylakoid properties and grana organization using freeze fracture and thin-section electron microscopy, and from these results it was suggested that NPQ requires the dissociation and aggregation of LHCII (Johnson et al. 2011a, b). Subsequent work used the *npq4* mutant and PsbS-overexpressing lines to show that PsbS is able to increase fluidity of the membrane and reduce ordering of PS II complexes (Kereïche et al. 2010; Goral et al. 2012). That PsbS affected the fluidity of the membrane was supported by fluorescence recovery after photobleaching (FRAP) experiments, a technique that uses a strong laser pulse to bleach a region of the thylakoid membrane, and then follows the kinetics of recovery of chlorophyll fluorescence (Goral et al. 2012). Using FRAP it was shown that in the *npq4* mutant, PS II complexes had a reduced mobility within the thylakoid membrane, whereas mobility increased in PsbS-overexpressing lines.

A drawback to most of the techniques used to analyze grana organization is that they are rarely performed on intact leaves, but rather on isolated chloroplasts or grana membranes. The time it takes to prepare the samples is also important given how rapidly qE relaxes. Care must be taken when interpreting results particularly from experiments using detergent-solubilized membranes, since it is unclear what effect various detergents have on interactions between complexes and proteins or on extracting specific proteins from the membrane. For instance, in sucrose gradients PsbS has been found either solely as a monomer (Nield et al. 2000), or in an aggregated form (Caffari et al. 2009), depending on which isomer of dodecyl maltoside was used to solubilize BBY membranes.

V Using Spectroscopic Measurements to Understand the Mechanism of qE

A Multiple Models of NPQ

Spectroscopy is another frequently used tool used to investigate qE. As mentioned previously, qE has been correlated with specific spectroscopic changes, such as the ΔA_{535} absorbance change (Ruban et al. 1993). A dosage-dependent effect of PsbS after illumination with high light has been demonstrated by measuring chlorophyll fluorescence lifetimes of wild type, *npq4* and PsbS overexpressors (Li et al. 2002a, b). While most current research indicates that quenching occurs in the LHCs associated with PS II (LHCII), there is some evidence that PS II reaction centers can also form quenching complexes during photoinhibitory stress (Huner et al. 2006). Higher plants have six LHCII proteins associated with PS II (Lhcb1-6), with Lhcb1-3 forming the major trimeric antenna while Lhcb4-6 exist as monomers (Peter and Thornber 1991; Jansson 1999). The observation that isolated LHCII trimers form aggregates in vitro with spectroscopic features that have been correlated with qE in vivo (Ruban et al. 1992a; Phillip et al. 1996) has led to the proposal of the LHCII aggregation model (Horton et al. 2008; Ruban et al. 2012). In this four-state model, aggregation of LHCII trimers is controlled cooperatively by PsbS protonation and Z formation, which presumably allow for more rapid formation of the quenching state.

Another model put forth, and in many ways complementary to the aggregation model, proposes the presence of two distinct quenching sites, Q1 and Q2 (Holzwarth et al. 2009; Jahns and Holzwarth 2012). This

model is based on spectroscopic measurements and global target analysis on mutants with varying amounts of PsbS as well as the *npq1* mutant (Holzwarth et al. 2009). Based on this analysis the authors suggested that detached LHCs can aggregate and lead to the formation of Q1, and that this quenching site may in fact be formed upon dissociation of band 4, as described above. The formation of a far-red spectral component assigned to Q1 has been seen during qE formation in both aggregated LHCII (Ruban and Horton 1992; Miloslavina et al. 2008) and intact leaves (Lambrev et al. 2010). Z was shown to enhance this effect, but to not be required, again suggesting an allosteric role (Johnson and Ruban 2009). The Q2 site in the model by Holzwarth et al. (2009) remains associated with PS II and is Z-dependent, but presumably does not require PsbS. More kinetic information will be required to determine whether or not the quenching at this site is, in fact, qE-like, or if it might fit better with one of the slower components of NPQ.

B The Molecular Mechanism

The molecular mechanism responsible for qE (and presumably thermal dissipation of excess absorbed light) is more contentious. One proposed mechanism involves an aggregation-dependent conformational change affecting a lutein located in the L1 site of trimeric LHCII, which positions this lutein molecule as a quencher of Chl through an energy transfer mechanism (Ruban et al. 2007; however, see Demmig-Adams et al., Chap. 24, for relationships between NPQ in nature and foliar zeaxanthin versus lutein levels). Alternatively, it has been suggested that the same conformational change results in quenching through a Chl-Chl charge transfer mechanism (Miloslavina et al. 2008; Müller et al. 2010). In contrast to these scenarios, where Z plays a strictly allosteric role, mechanisms for qE based on direct involvement of Z either through energy or electron transfer from Chl to Z have been proposed (Demmig-Adams 1990; Frank et al. 1994; Dreuw et al. 2003; Holt et al. 2004). Evidence supporting a carotenoid charge-transfer mechanism came from observations of the formation of Z radical cation formed under qE conditions in isolated thylakoids but absent in thylakoids of *npq4* (Holt et al. 2005). While this signal was observed in Lhcb4-6 in vitro (Ahn et al. 2008; Avenson et al. 2008, 2009), no evidence of any radical cation could be found in isolated LHCII trimers (Avenson et al. 2009).

Excitonic coupling between the S1 state of carotenoids and Chl has been observed to be correlated with the amount of quenching and the formation of the red-shifted band in isolated LHCs (Bode et al. 2009; Liao et al. 2010a, b) and in vivo (Bode et al. 2009). This has led to the proposal that, when electronic coupling between the two molecules is sufficient, energy can be trapped in this short-lived state, and further increases in coupling can lead to the charge transfer state (Bode et al. 2009; Liao et al. 2012; see also Walla and Fleming, Chap. 9). Recently, Wilk et al. (2013) reconstituted LHCII along with PsbS and Z in liposomes and showed that the amount of chlorophyll fluorescence quenching observed in these samples was higher relative to liposomes containing LHCII in the absence of PsbS or Z, and the authors were able to correlate this quenching with the electronic coupling measured by two-photon excitation. Importantly, these experiments were performed at LHCII concentrations low enough to avoid aggregation quenching. It remains to be seen whether this phenomenon is specific to LHCII or if the minor complexes are also able to interact with PsbS and Z to form this quenching state, and whether the Z cation radical can be observed in these liposomes.

VI Physiological Function of qE and PsbS

A qE Is Important Under Fluctuating Light Conditions

Despite some remaining uncertainty regarding how PsbS functions at a biochemical level, the *npq4* mutant has been invaluable in studies aimed at defining the physiological function of qE in plants. One important

question in the field is what effect qE has on plant growth and fitness. Using a variety of lines containing various amounts of PsbS, and therefore qE, this question can be addressed directly. There is at least one report suggesting that under continuous high light, *npq4* plants were inhibited in growth relative to wild type (Graßes et al. 2002), while others have reported that growth was similar to wild type (Havaux and Niyogi 1999; Külheim et al. 2002). Experimental variation, such as a difference in the age of plants transferred to high light, may account for this discrepancy. For instance, it has been shown that young but not mature leaves of *npq1* mutants are able to acclimate to high light via other mechanisms, such as increasing the amount of tocopherols (Havaux et al. 2007).

When plants are grown in fluctuating light environments, a more obvious PsbS-dependent growth and fitness phenotype was observed (Külheim et al. 2002; Frenkel et al. 2009; Krah and Logan 2010). One explanation for the more dramatic difference seen under fluctuating versus constant light is that qE might be important to reduce the excitation pressure on PS II and prevent photoinhibition during rapid changes in light intensity. This explanation is supported by experiments showing that exposure of low light-grown plants to higher light intensities resulted in greater excitation pressure in *npq4* mutants than in wild type (Li et al. 2002b; Johnson and Ruban 2010), but lower excitation pressure in PsbS-overexpressing lines than in wild type (Li et al. 2002b).

B PsbS Prevents Photoinhibition

Using the *npq4* mutant, qE was shown to prevent high light-induced photoinhibition measured as a persistent decrease in variable fluorescence (Graßes et al. 2002; Li et al. 2002b; Takahashi et al. 2009; for a discussion on photoinhibition, see Adams et al., Chap. 23). While such a role for qE had previously been demonstrated in *npq1* plants (Havaux and Niyogi 1999; Havaux et al. 2000), the additional function of Z in the prevention of lipid peroxidation makes it difficult to isolate the qE-dependent effects (Havaux et al. 2007). How qE protects PS II from photoinhibition remains unresolved, due to conflicting results from leaves treated with a chloroplast protein translation inhibitor and exposed to high light. Graßes et al. (2002) reported that the increased photoinhibition observed in *npq4* was not attributable to increased degradation of D1 protein, but rather to a downregulation of PS II activity by another mechanism(s). In another study, Takahashi et al. (2009) compared wild type to *npq4* and found that the amount of photoinhibition was the same in the absence and presence of the inhibitor, leading to the conclusion that the additional photoinhibition in qE-deficient plants is caused by a lower rate of de novo chloroplast protein synthesis. Most recently, Roach and Krieger-Liszkay (2012) repeated the latter experiment and found that the difference may depend on the light intensity used for high light treatment. It should also be noted that the three groups all used a different inhibitor in their experiments. A more extensive investigation of the effect of a lack of qE (and presumably thermal dissipation) on photoinhibition using a combination of light intensities and chemical inhibitors will be important in future work.

Several groups have previously observed that the amount of photoinhibition cannot be entirely explained by damaged PS II and have proposed that other slowly relaxing NPQ pathways could be responsible (Walters and Horton 1993; Adams et al. 2006; see also Adams et al., Chap. 23, and Demmig-Adams et al. Chap. 24). The results, mentioned above, might suggest that, in some situations, the rapidly reversible form of thermal dissipation indicated by qE may protect plants as well as prevent an activation of slowly relaxing NPQ pathways that might decrease photosynthesis in fluctuating light. An increase in another form of quenching with slower kinetics could explain the decreased photochemical efficiency and the small, yet significantly higher, amount of NPQ that is

observed in *npq4* compared to wild type after high light treatment. Alternatively, the remaining NPQ in high-light-exposed *npq4* plants may have the same mechanism as qE, but form more slowly in the absence of PsbS (Johnson and Ruban 2010; see also Demmig-Adams et al., Chap. 24).

The observation that overexpression of PsbS can lead to increased levels of qE in *Arabidopsis* and prevent photoinhibition raises several questions. What is the optimal amount of qE (and NPQ)? Why do some plants not maximize their qE capacity and does this imply that there are drawbacks of having too much qE (see also Demmig-Adams et al. 2013 for a discussion of trade-offs)? And finally, can altered levels of PsbS be used to optimize crop productivity?

1 Natural Systems Control qE Through PsbS

It has been observed that different species are capable of different levels of qE depending largely on whether they were grown in full sun or in the shade (Demmig-Adams and Adams 1994; see also Demmig-Adams et al., Chap. 24). Variations in qE capacity that depend on growth light conditions have also been documented within a species, and it has been shown that shade or low-light-grown plants had less rapidly reversible NPQ and higher levels of photoinhibition when compared to sun or high-light-grown plants (Demmig-Adams et al. 1998; Mishra et al. 2012; see also Demmig-Adams et al., Chap. 24). With the discovery that PsbS is critical for rapidly reversible thermal dissipation (qE), this analysis was extended to show that the amount of PsbS could be altered depending on growth conditions and affect the capacity of qE (Demmig-Adams and Adams 2006; Demmig-Adams et al. 2006). Quantitative trait locus (QTL) mapping identified PsbS expression level as a determinant of qE capacity in rice cultivars (Kasajima et al. 2011).

However, it should be emphasized that PsbS may not be the only factor contributing to the qE and that plants may adapt in other ways. For example, comparison of *Arabidopsis* ecotypes that exhibited different levels of qE revealed equal levels of PsbS (Jung and Niyogi 2009), despite the earlier observations of a PsbS dosage effect on qE (Li et al. 2002a, b). QTL mapping with two of the ecotypes suggested that the genes responsible had not been identified previously by genetic approaches using induced mutants (Jung and Niyogi 2009). Moreover, when qE levels were compared in a set of *Arabidopsis* ecotypes, grown under lab and field conditions, there was no correlation between PsbS and qE levels, and in the field most lines had qE levels similar to a PsbS-overexpressing line (Mishra et al. 2012). It cannot be excluded that possible differences in electron transport capacity among ecotypes contributed to different qE levels. Nevertheless, it appears that qE level is determined not only by a plant's genetic makeup but can also be adjusted dynamically based on environmental conditions, and that factors other than PsbS level may affect qE level

2 Too Much of a Good Thing

If qE protects leaves from photoinhibition, why do plants often not increase their expression levels of PsbS to the point that saturates qE? PsbS-overexpressing lines indeed had larger rosettes than wild-type plants when grown under a light regime with a single daily sunfleck, suggesting that increasing qE capacity is beneficial under such conditions (Logan et al. 2008). However, the latter conditions are unlikely to be representative of the more common conditions – with frequent, rapid fluctuations in light intensity – plants experience in nature. Field experiments with PsbS-overexpressing lines grown alongside wild-type plants revealed no significant difference in seed production between the two groups (Frenkel et al. 2009). It has thus been proposed that NPQ capacity is optimized in nature and reflects a balance between the gains from an increased protection from photoinhibition versus potential

losses to photosynthesis that might occur during the induction of NPQ. This possibility was examined by measuring photosynthetic assimilation in transgenic rice with varying levels of PsbS, and it was shown that PsbS-overexpressing lines had lower assimilation rates when measured during fluctuating illumination (Hubbart et al. 2012). The possible interplay between NPQ and other stress responses has also led to the suggestion that, when a plant contains more PsbS than necessary, other stress response pathways could become compromised (Frenkel et al. 2009). In particular, both herbivores (Johansson Jänkänpää et al. 2013) and pathogens (Göhre et al. 2012) appear to be able to discriminate between plants with different PsbS levels, perhaps due to changes in gene expression and chloroplast metabolism as a consequence of increased ROS production in plants lacking PsbS (Roach and Krieger-Liszkay 2012; see also Demmig-Adams et al. 2013). Finally, it has also been reported that overexpression of PsbS can inhibit cyclic electron flow and lead to photoinhibition of PS I (Roach and Krieger-Liszkay 2012). This last result is particularly interesting in view of potential interactions between PsbS and PS I subunits (Teardo et al. 2007) (see above). Further research will be necessary to clarify both positive and negative potential effects of PsbS overexpression.

VII Evolutionary Aspects of PsbS

From an evolutionary perspective, it appears as though PsbS-mediated capacity to rapidly modulate NPQ arose before, or at the latest around, the time when plants started to inhabit the terrestrial ecosystem. This latter ability is likely to have been a crucial innovation, as it appears to be widely conserved in land plants. Genes encoding PsbS have been found in the genomes of sequenced green algae, strongly suggesting that PsbS evolved in the common ancestor of green algae and plants (Koziol et al. 2007; see also Morosinotto and Bassi, other Chap. 14). However, there is presently no experimental evidence for PsbS-dependent NPQ in any green alga (Bonente et al. 2008; Niyogi and Truong 2013). Instead, green algae, such as *Chlamydomonas reinhardtii*, use a different LHC-related protein, called LHCSR, for qE (Peers et al. 2009; see also Morosinotto and Bassi, Chap. 14). Both LHCSR- and PsbS-dependent qE have been shown conclusively to co-exist in the moss *Physcomitrella patens* (Alboresi et al. 2010; Gerotto et al. 2012), but LHCSR was presumably subsequently lost during the early evolution of vascular plants, leaving only PsbS-dependent qE (Niyogi and Truong 2013). In the moss, the induction kinetics of PsbS-dependent qE appear to be faster than the LHCSR-dependent qE, which might explain why LHCSR was lost while PsbS was retained (Gerotto et al. 2012).

Although PsbS is not the only mechanism plants possess to modify NPQ capacity, the rapid kinetics of PsbS-dependent qE make the latter an attractive component of plant defense against photo-oxidative stress, and natural selection has presumably maintained this trait in all vascular plant lineages investigated so far. Since the capacity of the PsbS-mediated system is not constant, PsbS level, and hence qE capacity, may be under selection by the plant's natural environment. Enhanced PsbS levels clearly enhance qE capacity in *Arabidopsis* that, however, possesses only the relatively low maximal qE capacity typically seen in rapidly growing annual species compared with other species (especially evergreens) adapted to more extreme environments (see also Demmig-Adams et al., Chap. 24). The level of PsbS in a plant is, of course, selected by evolution to maximize fitness, and less-than-maximal PsbS might be explained by compromised defense against biotic attack, losses to photosynthetic efficiency during the induction or relaxation of NPQ, or something else.

VIII Conclusions

Several important questions remain to be answered concerning the role of PsbS in

qE. Plants with varying amounts of PsbS, in several species beyond *Arabidopsis* and rice, will be needed to elucidate all aspects of the effect of PsbS-dependent NPQ on plant fitness and productivity. Furthermore, sequencing the genomes and characterizing more species between *Chlamydomonas reinhardtii* and vascular plants may help understand what drove the switch from LHCSR-dependent to PsbS-dependent qE, and what function PsbS has where it does not appear to be involved in NPQ.

Conflicting reports on pigment binding, localization, and interaction with other components have led to many different proposals over the years as to how PsbS might be involved in the mechanism of qE. Despite these different views, consensus is forming around the idea that, (1) a conformational change within PsbS occurs upon protonation, which (2) promotes dissociation of a subset of LHCs from the PS II core, and (3) leads to the formation of the qE quenching site. The connection between each of these steps, however, remains poorly defined. The location of PsbS within the grana membrane and/or PS II super-complex is an important piece of information that remains elusive despite several attempts by many labs. In order to help define the molecular trigger for qE, it would also be useful to have a structure of PsbS and, if possible, determine what changes occur in the protein upon protonation. Advances in X-ray crystallography and solid-state nuclear magnetic resonance techniques could lead to exciting results in this area.

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