A study on PsbS and its role as a pH sensor

Krishnan, M.

1

Introduction

Solar energy harnessed by plants and algae has great potential to be converted into biofuels for future generations. Understanding the regulatory mechanism of photosynthesis can increase biomass yield and drive the generation of biofuels to its maximum. PsbS, a membrane protein, is an essential component for NPQ and has been studied extensively in this thesis. PsbS is hypothesized to sense pH and to regulate photosynthesis under light stress conditions. The first objective in this thesis is to produce, purify and refold recombinant PsbS with sufficiently high yields for biochemical studies along with optimizing the detergent conditions for maintaining its stability. The second objective is the spectroscopic characterization of PsbS to understand its role as a pH sensor and its molecular mechanism during photoprotection.

1

By the year 2050, the human population is projected to reach 9.1 billion, bringing with it a major energy-related crisis[1]. The shortage of fuels will be one of the major issues to deal with due to the limited availability of non-renewable resources like coal, oil, and natural gas. For more than a century, scientists are exploring the possibility of biofuels generated from plants and plant products to overcome this crisis. Although direct synthesis of biofuels like hydrogen, isoprene, toluene[2] and longer hydrocarbons can be achieved by photosynthetic microorganisms[3], some inhibitory pathways make it complicated to achieve high efficiency of biofuel production[4]. To improve the production of biofuel, an increase in biomass production or genetic engineering of photosynthetic proteins is necessary, but this requires an in-depth knowledge of the photosynthetic mechanism that improves CO_2 assimilation. Currently, many countries are attempting to develop bio-solar fuels, for example, *BioSolar Cells*, a Dutch research program in the field of photosynthesis aimed at the sustainable production of food and renewable energy[5]. Apart from such programs, the emergence of new technologies and advancements in scientific tools enable us today to unravel many photosynthetic mechanisms[6]. These scientific effort may one day enable us to solve food and energy crisis to free the world from hunger and from exploiting our limited resources like petrol and oil.

1.1 Photosynthesis and photoprotection

Oxygenic photosynthesis is the conversion of solar energy to chemical energy while producing oxygen that takes place in microalgae/cyanobacteria and all land plants. When photons of sun rays hit a leaf surface, they are absorbed by an antenna that transfers excitations to the two photosystems, the oxygen-evolving PSII and the ferredoxin-reducing PSI[7]. Both PSII and PSI are important for light driven charge separation and transport of electrons. The splitting of water takes places in PSII which generates electrons that are used in oxygen evolution. The remaining electrons from the PSII are then transported to PSI via plastoquinones, the cytochrome b_6f complex and plastocyanin. The electron acceptor $NADP^+$ is converted to NADPH, which contributes to carbon fixation of solar energy, the prime objective of photosynthesis[8]. The protons evolved from water oxidation also contribute to drive the ATP synthase to produce ATP, another essential energy source.

One way to measure the efficiency of photosynthesis is through *Solar Energy Storage Efficiency*, which is the percentage of solar energy converted into biomass[8]. This conversion efficiency in direct sunlight is less compared to low irradiance conditions[9] due to the *light-saturation effect*[8], depicted in Figure 1.1. This light-saturation effect is driven by a mechanism called *photoprotection*. Photoprotection against sunlight takes place to prevent damage from toxic ROS that are produced when electrons leak out of

the electron transfer chain or when photosynthetic pigments transfer energy to oxygen to produce singlet oxygen.

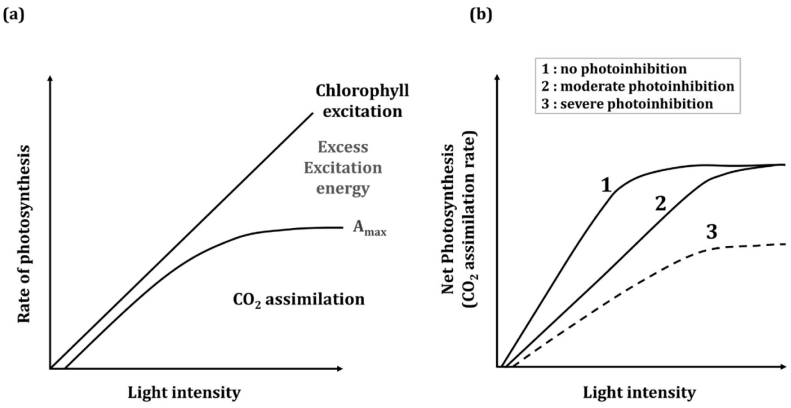

Figure 1.1 (a) Principle of photoprotection showing excess excitation energy that is dissipated as compared to the absorbed light. A_{max}, the maximal light-saturated photosynthetic rate is achieved; (b) Effect of photoinhibition on quantum yield and CO_2 assimilation rate is shown. A_{max} is also affected by other photo-inhibitory conditions; therefore, it is a dotted line. (Adopted from[10])

The photoprotective mechanisms that under high light conditions quench the excitations, which in return dissipate the excess photon energy as heat or fluorescence, are known as NPQ and lead to ~55 % irradiance loss (average in USA)[11]. From the absorbed sunlight, an estimated 8 - 10 % of the solar energy converts into biomass, which is significantly low. As seen in Figure 1.1 a, the A_{max}, which is the light-saturated photosynthetic rate is reached when there is too much light intensity leading to dissipation of excess excitation energy[10]. The quantum yield and CO_2 assimilation rates also drop when severe photoprotection takes place, as shown in Figure 1.1 b. To recapitulate, adjusting NPQ is a key for improving CO_2 assimilation which could assist in efficient biofuel production.

1.2 Non-photochemical quenching

NPQ takes place primarily in the antenna associated with the PSII complex located in the thylakoid membrane of chloroplasts[12]. qE, the major component of NPQ, is defined as the thermal dissipation of excess absorbed light energy. qE has two main requirements, one involves the activation of an enzyme for conversion of violaxanthin to zeaxanthin[13] during the VAZ cycle and the other is the protonation of light stress sensor proteins[14]. The requirements for the fast component of NPQ, qE are explained below:

1.2.1 Role of low lumenal pH

The most important aspect for qE is the lowering of the lumenal pH in the thylakoid membrane, which senses the photosynthetic state during varying light conditions. In low light conditions, the approximate lumenal pH is 7.5 and the photosynthetic antenna is in a light harvesting state. However, in high light, the lumenal pH decreases until the acidification of the internal thylakoid compartments reaches a threshold value triggering the NPQ process[15]. This low lumenal pH is required to trigger several mechanisms involved in NPQ. The change in the pH of the lumen occurs due to the proton influx into the lumen region, the imbalance between protons at the lumen site and available ADP and NADP. The lumenal pH acts as a measure of the photosynthetic stage, that is regulated or de-regulated under excess light conditions[16]. The first step in the mechanism for NPQ in plants is the activation of a membrane protein called PsbS due to acidification of the lumenal pH. This is followed by a slow enzymatic conversion of V to Z and leads to de-excitation of chlorophylls of the PSII antenna[14].

1.2.2 Role of the VAZ cycle

The VAZ cycle is a forward reaction where the di-epoxy xanthophyll V is de-epoxidized to xanthophyll Z, with an intermediate xanthophyll A. It is one of the most important reactions in the induction of NPQ taking place in high light intensities[17,18]. The enzyme that catalyzes this reaction is called VDE, which gets activated and binds to the thylakoid membrane only under conditions where the luminal pH has lowered to ~pH 5.0. After decades of research, the function of Z still remains controversial, with the current hypothesis that Z acts as an allosteric regulator that controls the sensitivity of qE and conformational changes of LHC's during pH lowering[19–21]. Even though the role of Z is not clear, its involvement in NPQ has been explained by models[22].

1.2.3 Role of PsbS

Although the crucial importance of PsbS in NPQ in higher plants has been established[23] for the past 15 years, there is still a great deal of uncertainty about the mechanism by which PsbS is involved in NPQ[24,25]. Studies have proved that PsbS is a prime requirement for excited-state quenching (qE) upon proton accumulation *in vivo*[26,27]. Several mechanisms have been proposed for PsbS. Earlier models placed PsbS as the site of actual quenching for heat dissipation from PSII, but later it was proposed that PsbS has the role of an antenna organizer that senses pH lowering and facilitates structural changes leading to quenching of LHCII[28]. The LHCII associated with PSII emerged as one of the site of NPQ and may undergo aggregation after conversion of V to Z under low pH conditions[14,29,30]. Along with LHCII, the minor antenna proteins like CP29, CP26 and CP24 have also been proposed to be the site of quenching during NPQ[19].

PsbS is one of the major components in triggering photoprotection due to its role in rearrangement and structural changes during NPQ associated with lowering of the lumenal pH[23,31]. In the absence of PsbS, low levels of NPQ have been observed, suggesting that PsbS is essential for effective pH sensing and rearrangement of antenna proteins under high irradiance[27].

1.2.4 Recent models for NPQ

In the early 1990s, the first model for NPQ was proposed[14,19]. The model proposed four states for NPQ. State 1 occurs in low light conditions, where the LHCII is in a non-aggregated state and the excitation energy is used to drive electron transport. The VAZ cycle starts with the presence of V in the VAZ binding pocket in State 1. With high light intensities, state 4 occurs. LHCII is aggregated and excess of excitation energy is present and the VAZ binding pocket contains Z. State 3 consists of NPQ levels lower than state 4 and is found in the thylakoid membrane immediately after high illumination. The VAZ binding pocket still contains V. State 2 is found when the change from high light to low light occurs. LHCII is unprotonated and Z is present in the VAZ binding pocket. However, the exact role of PsbS in NPQ is still unclear. Another model for NPQ suggests the occurrence of two quenching states: Q1 and Q2. This model is based on time-resolved fluorescence measurements of the two states[31]. Q1 occurs during high light conditions and detachment of the LHCII from PSII and its aggregation takes place. Here, PsbS is in the protonated form and heat dissipation of excess light energy takes place. No Z is required during Q1. Here, the fast phase of NPQ occurs within 1-5 mins. Q2, on the other hand, takes place in minor LHCII complexes like CP29, CP26 and CP24, which are still attached to PSII. Conversion of V to Z in Q2 is hypothesized. Here, the slow phase of NPQ takes ~10-15 mins. NPQ in the Q2 state majorly depends on the VAZ cycle.

Two models for the function of PsbS in NPQ have been proposed, both assigning PsbS as the pH sensor for sensing lumen acidification during high light irradiance. One model proposes that PsbS triggers conformational changes in LHCII leading to aggregation and NPQ, while the other model suggests PsbS to be the quenching site along with its binding to the pigment Z[16]. NPQ mechanism to regulate photosynthesis is quite biodiverse in nature. In plants, PsbS is known to be the only protein for pH sensing while in green algae, another class of proteins called LHCSR are found. In the moss *Physcomitrella patens,* the presence of both PsbS and LHCSR proteins are found to play equal roles in photoprotection[32]. Why plants adopted for PsbS-dependent NPQ, why LHCSR was lost in vascular plants and where did PsbS come from, are some intriguing questions to ask[33]. Is moss the intermediate during the evolution to land and holds answers to how the PsbS gene came into its role as a pH sensor?

From an evolutionary standpoint, photoprotection has evolved to achieve survival under high light-stress conditions over maximum carbon fixation efficiency[8]. An improvement of the efficiency of photosynthesis by reduction of the size of the antenna system and spread of the excitation throughout the canopy has been demonstrated[11]. Other ways, like deletion or inactivation of genes coding for antenna proteins or accessory pigments, have resulted in a balanced dispersion of energy to the two antenna systems[11,34,35]. Overexpression of membrane protein PsbS in Tobacco plants, together with xanthophyll-cycle enzymes, led to an increase in qE along with accelerating NPQ relaxation, which significantly increased the quantum yield of CO_2 assimilation[36]. In conclusion, understanding the mechanism of PsbS is vital for improving biomass production.

1.3 Discovery and mechanism of PsbS

The membrane protein PsbS was first discovered as a component of PSII preparation in 1986[37] and co-precipitated with other subunits[38]. Sequencing of PsbS showed[39] that it is a unique protein containing four transmembrane helices.

After more than 20 years of research, it is clear that PsbS has a central role in sensing the thylakoid luminal pH and activation of a series of complex structural rearrangements[40] that lead to chlorophyll de-excitation in the antenna, which is the basis of NPQ[23,41]. A model of the hypothesized function of PsbS in photoprotection is depicted in Figure 1.2. PsbS belongs to the LHC protein family[42]. It has a molecular weight of 22 kDa and has four transmembrane helices in its structure, unlike all other members of the LHC family.

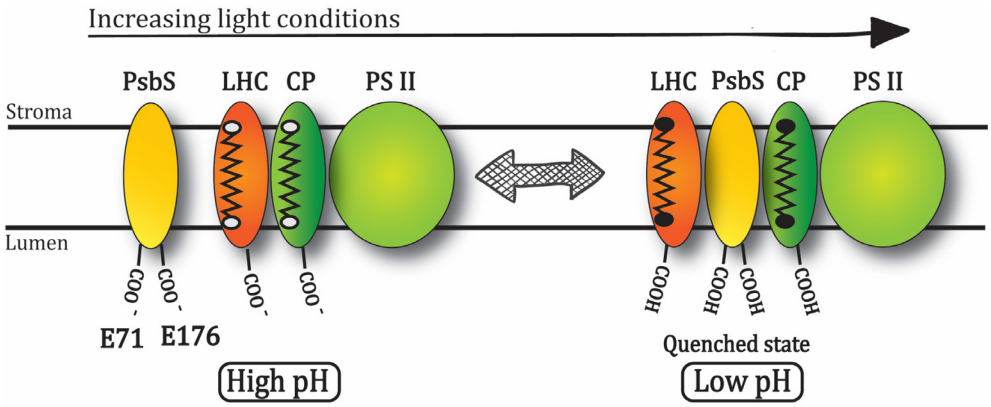

Figure 1.2 Top: The hypothesized role of PsbS during NPQ adopted from Niyogi *et al.*, 2005[13]. V to Z (white to black dots) conversion takes place with protonation of glutamate residues.

A homology structure of PsbS from *Physcomitrella patens* and the crystal structure of PsbS from *Spinacia oleracea* is shown in Figure 1.3 followed by a protein sequence alignment between PsbS from *P. patens, S. oleracea* and *A. thaliana* species. PsbS does not bind specific Chl pigments and acts as a pH sensor to trigger structural changes in PSII-LHCII super-complexes leading to qE and NPQ[24,43]. Recent studies, focusing on PsbS interaction, showed that Patens-PsbS associates with one of the monomeric unit of LHCII trimer[44]. For PsbS of higher plants, enhanced interactions with Lhcb1 antenna complexes were observed under high-light conditions[45] and increased binding of PsbS to minor antenna complexes was observed with the combination of low pH and zeaxanthin[46].

Mutational studies on proton-accepting residues in the lumen region of PsbS, especially on two glutamate residues located at the lumenal loops, have shown to severely influence qE, indicating that they are critical for the function of PsbS[47]. In Patens-PsbS, these two glutamate sites are at Glu-71 and Glu-176 that protrude at the lumenal side of the membrane (Figure 1.3c, red box). Protonation of these two active glutamates is proposed to induce a dimer to monomer transition, which is suggested to be the first step in PsbS activation[28]. The recent crystallography structure, however, shows that PsbS forms a stable dimer under low-pH conditions[48].

Apart from the structure of the protein PsbS, its evolution and existence in vascular plants are also very intriguing. In *Chlamydomonas reinhardtii*, which is an alga, previously it was believed that the PsbS gene is present but it is not active and NPQ is LHCSR-dependent[49], however, recently PsbS was shown to be transiently expressed and to play a transient role during NPQ in algae[50]. Recent studies showed that in *P. patens*, both PsbS and LHCSR genes are present and active during NPQ[51]. This role is however taken over by PsbS in higher green plants like *S. oleracea* and *A. thaliana*[52]. A reason for this switch is proposed to be the less robust LHCSR-dependent NPQ that was discarded during evolution[32]. Another explanation lies in the function of LHCSR as both a sensor of excess light and quenching site in lower organisms and mosses, that was later replaced by PsbS as sensor and LHCII as a site of quenching in higher plants[33].

The sequence alignment between *P. patens*, *S. oleracea* and *A. thaliana* shows that several regions are conserved for all the three species (Figure 1.3c).

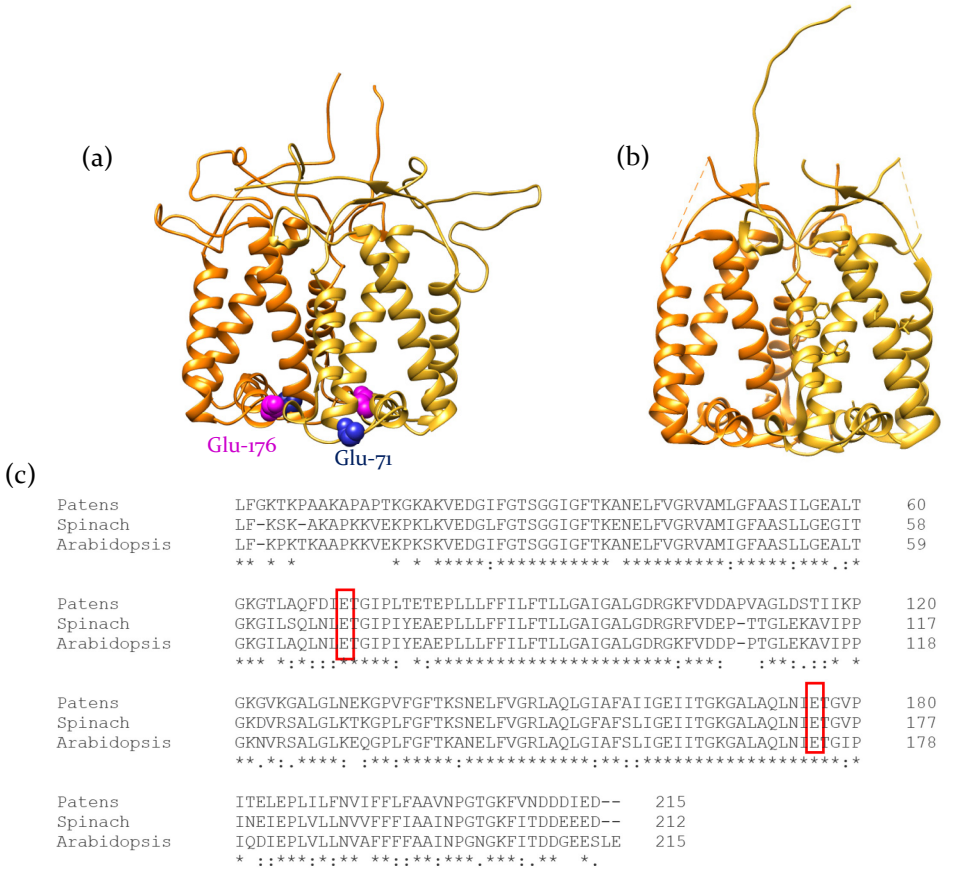

Figure 1.3 A homology model of (a) Patens-PsbS[53] in comparison with (b) Spinach-PsbS, PDB 4RI2 [48]. Two glutamate residues are shown as pink and blue spheres that are hypothesized to be the site of protonation. (c) Protein sequence alignment of *P. patens*, *S. oleracea* and *A. thaliana* using Clustal Omega from EMBL-EBI. In red marked are the two active glutamates.

1.4 Challenges in PsbS structural characterization

In vivo and *in vitro* studies have been carried out on PsbS to understand its underlying mechanism in the regulation of NPQ and its interaction with antenna proteins[43,46,47,54]. It is challenging to determine the molecular function of PsbS, due to its lack of specific-bound pigments, its low production in plants, and the difficulty of PsbS purification. Some studies performed *in vitro* synthesis of PsbS[24,28,43,54] producing detergent-refolded PsbS in moderate quantities, and PsbS has also been refolded in liposomes together with LHCII to study its quenching mechanism *in vitro*[54,55].

However, the yield of PsbS attained is not enough for carrying out spectroscopic studies and there are many challenges to solve.

The first challenge is to produce recombinant PsbS in high quantity for biochemical and NMR studies. This can be achieved by either cell-free protein expression or overexpression using *E. coli*. Both techniques have great potential in membrane protein production. While cell-free expression allows for easy labelling of isotopic amino acids with minimal scrambling, it is still challenging to produce large amounts of soluble membrane proteins[56]. This makes overexpression using *E. coli* more favorable. Even though this procedure can produce sufficient amounts of proteins, it often leads to the production of inclusion bodies. Inclusion bodies are aggregated protein structures within the bacteria when membrane proteins foreign to *E. coli* system are overexpressed[57]. These inclusion bodies contain the membrane protein in an unfolded form and are very hard to isolate and purify.

The next challenge is to refold the unfolded PsbS into its proper conformation. The refolding of membrane proteins is quite critical to their function. Factors like CMC, aggregation number of detergents, temperature and concentration of PsbS have to be considered for a successful refolding[58–60].

The determination of oligomeric states of PsbS is important as it has been proposed that PsbS activation is associated with dimer to monomer transition upon protonation under high-light conditions[28]. Determining the exact size of PsbS and oligomeric states can be very challenging since the detergent micelles surrounding it will contribute to the total molecular weight of the protein-detergent micelles. Therefore the use of complementary techniques for correct size determination is required[61–63].

The final challenge and major aim of this thesis is to resolve the molecular pH sensing mechanism of PsbS by observing structural changes and dynamics of PsbS. NMR spectroscopy is emerging as a powerful tool[64] to observe the dynamics of a protein in solution, unlike X-ray crystallography that requires crystallization. Resolving the "fingerprint" of a protein can be performed by using 2D ^{1}H-^{15}N HSQC, where each backbone amide bond corresponds to a peak[65]. However, solution-state NMR studies of PsbS are challenging due to the total size of PsbS in detergent micelles being too large, while solid-state NMR requires high quantities of properly folded, membrane reconstituted PsbS, which is challenging to prepare. In this thesis, other spectroscopic techniques like IR and CD spectroscopy have been explored as well to overcome the limitations of NMR spectroscopy.

1.5 Research objectives and dissertation outline

PsbS has been a mysterious protein in photosynthetic research. Its role in NPQ has been hypothesized in various models but the exact mechanism of function is still unclear. How PsbS interacts with other membrane components (like antenna proteins, PSII, zeaxanthin) to induce qE is still unknown as is its molecular pH-response mechanism. Mutation of two key glutamate residues in the lumenal loops of PsbS has shown to reduce NPQ significantly[47]. The exact molecular mechanism of these two glutamate residues in PsbS leading to structural changes have not been determined yet. Several challenges like the proper folding of PsbS, right environmental conditions for NMR or CD spectroscopy, while maintaining the stability of PsbS in different pH and temperature conditions have been overcome in this thesis. Finally, the role of the two glutamate residues in the pH sensing-mechanism of PsbS has been elucidated.

The main research objectives are outlined below:

1. *Can sufficient quantities of recombinant PsbS be produced and purified for spectroscopic studies?*

With the goal to perform structural and biochemical studies on recombinant PsbS, the first step is to produce enough purified PsbS in a proper fold. Two approaches for the production of PsbS were explored: cell-free expression and overexpression by *E. coli*. In Chapter 2, production of PsbS using cell-free expression system is described. After temperature and detergent optimization steps, cell-free production of soluble PsbS was achieved. PsbS was successfully refolded after its production. In Chapter 3, the production of PsbS using overexpression in *E. coli* is documented. Plasmid and temperature optimization led to maximal yields of PsbS of ~120 mg/L. A novel purification protocol was set up, which reduced losses by ~60 %.

2. *What is the optimal environment for PsbS folding and stability?*

In Chapter 3, PsbS was refolded using different detergents and was characterized by CD spectroscopy. Detergents FC-12 and OG proved optimal for the folding and stability of Patens-PsbS.

3. *What is the influence of pH on the oligomeric states of PsbS?*

In Chapter 4, PsbS refolded under high and low pH conditions were analyzed to determine pH-induced changes in oligomerization state using SEC, SDS-gel analysis, DLS and DOSY NMR spectroscopy. The dimer-to-monomer transitions were explored,

which might be the first step in the activation of PsbS. ^{15}N-^{1}H HSQC NMR was performed on PsbS to detect pH-dependent changes in structure or dynamics.

4. *What is the molecular pH response mechanism of PsbS? What is the role of the two active Glu residues in bringing about pH-dependent conformational changes?*

The fold and oligomeric state of selective Glu mutants of PsbS were compared with the WT PsbS by NMR, FTIR and CD spectroscopy, presented in Chapter 5. The roles of Glu-71 and Glu-176 were explored and a model suggesting the structural response of PsbS to low pH was proposed.

1

1.6 References

1. FAO's Director-General on How to Feed the world in 2050. *population and development review* **35,** 837–839 (2009).
2. Beller, H. R. *et al.* Discovery of enzymes for toluene synthesis from anoxic microbial communities. *Nature Chemical Biology* **14,** 451–457; 10.1038/s41589-018-0017-4 (2018).
3. Melis, A. Photosynthesis-to-fuels: From sunlight to hydrogen, isoprene, and botryococcene production. *Energy and Environmental Science* **5,** 5531–5539; 10.1039/c1ee02514g (2012).
4. Ghirardi, M. L., Togasaki, R. K. & Seibert, M. Oxygen sensitivity of algal H2-production. *Applied Biochemistry and Biotechnology* **67,** 182; 10.1007/BF02787851 (1997).
5. Robin Purchase, Huib De Vriend, Huub de Groot. *Artificial photosynthesis : for converstion of sunlight to fuel. Published as part of the series "Groene Grondstoffen" ("Green Raw Materials") ,*
6. Govindjee & Krogmann, D. Discoveries in oxygenic photosynthesis (1727-2003): A perspective. *Photosynthesis Research* **80,** 15–58; 10.1023/B:PRES.0000030443.63979.e6 (2004).
7. L.N.M.Duysens, J.Amesz, B.M.Kamp. Two photochemical systems in photosynthesis. *Nature* **190,** 510–511 (1961).
8. Blankenship, R. E. *Molecular Mechanisms of Photosynthesis.* 2nd ed. (Wiley Blackwell, 2014).
9. Melis, A., Neidhardt, J. & Benemann, J. R. Dunaliella salina (Chlorophyta) with small chlorophyll antenna sizes exhibit higher photosynthetic productivities and photon use efficiencies than normally pigmented cells. *Journal of Applied Phycology* **10,** 515–525; 10.1023/A:1008076231267 (1998).
10. Murchie, E. H. & Niyogi, K. K. Manipulation of photoprotection to improve plant photosynthesis. *Plant Physiology* **155,** 86–92; 10.1104/pp.110.168831 (2011).
11. Melis, A. Solar energy conversion efficiencies in photosynthesis: Minimizing the chlorophyll antennae to maximize efficiency. *Plant Science* **177,** 272–280; 10.1016/j.plantsci.2009.06.005 (2009).
12. Kiss, A. Z., Ruban, A. V. & Horton, P. The PsbS protein controls the organization of the photosystem II antenna in higher plant thylakoid membranes. *Journal of Biological Chemistry* **283,** 3972–3978; 10.1074/jbc.M707410200 (2008).
13. Niyogi, K. K., Li, X. P., Rosenberg, V. & Jung, H. S. Is PsbS the site of non-photochemical quenching in photosynthesis? *Journal of Experimental Botany* **56,** 375–382; 10.1093/jxb/eri056 (2005).
14. Horton, P. & Ruban, A. V. Regulation of Photosystem II. *Photosynthesis Research* **34,** 375–385; 10.1007/BF00029812 (1992).

15. Brooks, M. D., Jansson, S., and Niyogi, K. K. . in *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria,* edited by Barbara Demmig-Adams, Gyozo Garab, William Adams III, Govindjee (Springer, Dordrecht2014).

16. Goss, R. & Lepetit, B. Biodiversity of NPQ. *Journal of plant physiology* **172,** 13–32; 10.1016/j.jplph.2014.03.004 (2014).

17. H. Y. Yamamoto, T. O. M. Nakayama, C. O. Chichester. Studies on the light and dark interconversions of leaf Xanthophylls. *Archives of Biochemistry and Biophysics.,* 168–173 (1962).

18. Hager, A. Studies on the light-induced reversible Xanthophyll conversions in Chlorella and spinach leaves. *Planta* **172,** 148–172 (1967).

19. Horton, P., Johnson, M. P., Perez-Bueno, M. L., Kiss, A. Z. & Ruban, A. V. Photosynthetic acclimation: Does the dynamic structure and macro-organisation of photosystem II in higher plant grana membranes regulate light harvesting states? *FEBS Journal* **275,** 1069–1079; 10.1111/j.1742-4658.2008.06263.x (2008).

20. Ruban, A. V., Johnson, M. P. & Duffy, C. D. P. The photoprotective molecular switch in the photosystem II antenna. *Biochimica et Biophysica Acta - Bioenergetics* **1817,** 167–181; 10.1016/j.bbabio.2011.04.007 (2012).

21. Johnson, M. P., Zia, A. & Ruban, A. V. Elevated ΔpH restores rapidly reversible photoprotective energy dissipation in Arabidopsis chloroplasts deficient in lutein and xanthophyll cycle activity. *Planta* **235,** 193–204; 10.1007/s00425-011-1502-0 (2012).

22. Jahns, P. & Holzwarth, A. R. The role of the xanthophyll cycle and of lutein in photoprotection of photosystem II. *Biochimica et biophysica acta* **1817,** 182–193; 10.1016/j.bbabio.2011.04.012 (2012).

23. Li, X. P. *et al.* A pigment-binding protein essential for regulation of photosynthetic light harvesting. *Nature* **403,** 391–395; 10.1038/35000131 (2000).

24. Bonente, G., Howes, B. D., Caffarri, S., Smulevich, G. & Bassi, R. Interactions between the photosystem II subunit PsbS and xanthophylls studied in vivo and in vitro. *Journal of Biological Chemistry* **283,** 8434–8445; 10.1074/jbc.M708291200 (2008).

25. Barbara Demmig-Adams, Gyozo Garab, William Adams III, Govindjee. *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and cyanobacteria. Chapter 13 PsbS-Dependent Non-Photochemical Quenching* (Springer US, 2014).

26. Johnson, M. P. & Ruban, A. V. Arabidopsis plants lacking PsbS protein possess photoprotective energy dissipation. *Plant Journal* **61,** 283–289; 10.1111/j.1365-313X.2009.04051.x (2010).

27. Johnson, M. P. & Ruban, A. V. Restoration of rapidly reversible photoprotective energy dissipation in the absence of PsbS protein by enhanced by pH. *Journal of Biological Chemistry* **286,** 19973–19981; 10.1074/jbc.M111.237255 (2011).

28. Bergantino, E. *et al.* Light- and pH-dependent structural changes in the PsbS subunit of photosystem II. *Proceedings of the National Academy of Sciences of the United States of America* **100,** 15265–15270; 10.1073/pnas.2533072100 (2003).

29. Ruban, A. V., Phillip, D., Young, A. J. & Horton, P. Carotenoid-dependent oligomerization of the major chlorophyll a/b light harvesting complex of photosystem II of plants. *Biochemistry* **36,** 7855–7859; 10.1021/bi9630725 (1997).

30. Phillip, D., Ruban, A. V., Horton, P., Asato, A. & Young, A. J. Quenching of chlorophyll fluorescence in the major light-harvesting complex of photosystem II: a systematic study of the effect of carotenoid structure. *Proceedings of the National Academy of Sciences* **93,** 1492–1497; 10.1073/pnas.93.4.1492 (1996).

31. Holzwarth, A. R., Miloslavina, Y., Nilkens, M. & Jahns, P. Identification of two quenching sites active in the regulation of photosynthetic light-harvesting studied by time-resolved fluorescence. *Chemical Physics Letters* **483,** 262–267; 10.1016/j.cplett.2009.10.085 (2009).

32. Gerotto, C., Alboresi, A., Giacometti, G. M., Bassi, R. & Morosinotto, T. Role of PSBS and LHCSR in Physcomitrella patens acclimation to high light and low temperature. *Plant, Cell and Environment* **34,** 922–932; 10.1111/j.1365-3040.2011.02294.x (2011).

33. Niyogi, K. K. & Truong, T. B. Evolution of flexible non-photochemical quenching mechanisms that regulate light harvesting in oxygenic photosynthesis. *Current Opinion in Plant Biology* **16,** 307–314; 10.1016/j.pbi.2013.03.011 (2013).

34. Nakajima, Y. & Ueda, R. Improvement of photosynthesis in dense microalgal suspension by reduction of light harvesting pigments. *Journal of Applied Phycology* **9,** 503–510; 10.1023/A:1007920025419 (1997).

35. Blankenship, R. E. & Chen, M. Spectral expansion and antenna reduction can enhance photosynthesis for energy production. *Current Opinion in Chemical Biology* **17,** 457–461; 10.1016/j.cbpa.2013.03.031 (2013).

36. Kromdijk, J., Głowacka, K., Leonelli, L., Gabilly, S. T., Iwai, M., Niyogi, K., Long, S. P. Improving photosynthesis and crop productivity by accelerating recovery from photoprotection. *Science* **354,** 857–860 (2016).

37. Ghanotakis, D. F. & Yocum, C. F. Purification and properties of an oxygen-evolving reaction center complex from photosystem II membranes. *FEBS Letters* **197,** 244–248; 10.1016/0014-5793(86)80335-0 (1986).

38. Ljungberg, U., Akerlund Hans-Erik & Andersson Bertil. Isolation and characterization of the 10-kDa and 22-kDa polypeptides of higher plant photosystem 2. *European Journal of Biochemistry* **158,** 477–482; 10.1111/j.1432-1033.1986.tb09779.x (1986).

39. Kim, S. *et al.* Characterization of a spinach psbS cDNA encoding the 22 kDa protein of photosystem II. *FEBS Letters* **314,** 67–71 (1992).

40. Kereïche, S., Kiss, A. Z., Kouřil, R., Boekema, E. J. & Horton, P. The PsbS protein controls the macro-organisation of photosystem II complexes in the grana membranes of higher plant chloroplasts. *FEBS Letters* **584,** 759–764; 10.1016/j.febslet.2009.12.031 (2010).

41. Li, X. P. *et al.* Regulation of photosynthetic light harvesting involves intrathylakoid lumen pH sensing by the PsbS protein. *Journal of Biological Chemistry* **279,** 22866–22874; 10.1074/jbc.M402461200 (2004).

42. Funk, C. *et al.* The PS II-S protein of higher plants : a new type of pigment binding protein. *Biochemistry* **34,** 11133–11141 (1995).

43. Dominici, P. *et al.* Biochemical properties of the PsbS subunit of photosystem II either purified from chloroplast or recombinant. *Journal of Biological Chemistry* **277,** 22750–22758; 10.1074/jbc.M200604200 (2002).

44. Gerotto, C., Franchin, C., Arrigoni, G. & Morosinotto, T. In vivo Identification of Photosystem II Light Harvesting Complexes Interacting with Photosystem II subunit S. *Plant Physiology* **168,** 1747–1761; 10.1104/pp.15.00361 (2015).

45. Correa-Galvis Viviana & Gereon Poschmann , Michael Melzer, Kai Stühler and Peter Jahns. PsbS interactions involved in the activation of energy dissipation in Arabidopsis. *Nature Plants* **2,** 15225–15232; 10.1038/nplants.2015.225 (2016).

46. Sacharz, J., Giovagnetti, V., Ungerer, P., Mastroianni, G. & Ruban, A. V. The xanthophyll cycle affects reversible interactions between PsbS and light-harvesting complex II to control non-photochemical quenching. *Nature Plants* **3**; 10.1038/nplants.2016.225 (2017).

47. Li, X. P., Phippard, A., Pasari, J. & Niyogi, K. K. Structure-function analysis of photosystem II subunit S (PsbS) in vivo. *Functional Plant Biology* **29,** 1131–1139; 10.1071/FP02065 (2002).

48. Fan, M. *et al.* Crystal structures of the PsbS protein essential for photoprotection in plants. *Nature structural and molecular biology* **22,** 729–735; 10.1038/nsmb.3068 (2015).

49. Bonente, G. *et al.* The occurrence of the psbs gene product in chlamydomonas reinhardtii and in other photosynthetic organisms and its correlation with energy quenching. *Photochemistry and Photobiology* **84,** 1359–1370; 10.1111/j.1751-1097.2008.00456.x (2008).

50. Tibiletti, T., Auroy, P., Peltier, G. & Caffarri, S. Chlamydomonas reinhardtii PsbS Protein Is Functional and Accumulates Rapidly and Transiently under High Light. *Plant Physiology* **171,** 2717–2730; 10.1104/pp.16.00572 (2016).

51. Alboresi, A., Gerotto, C., Giacometti, G. M., Bassi, R. & Morosinotto, T. Physcomitrella patens mutants affected on heat dissipation clarify the evolution of photoprotection mechanisms upon land colonization. *Proceedings of the National Academy of Sciences* **107,** 11128–11133; 10.1073/pnas.1002873107 (2010).

52. Roach, T. & Krieger-Liszkay, A. The role of the PsbS protein in the protection of photosystems i and II against high light in Arabidopsis thaliana. *Biochimica et Biophysica Acta - Bioenergetics* **1817,** 2158–2165; 10.1016/j.bbabio.2012.09.011 (2012).

53. Krishnan, M., Moolenaar, G. F., Gupta, Karthick Babu Sai Sankar, Goosen, N. & Pandit, A. Large-scale in vitro production, refolding and dimerization of PsbS in different microenvironments. *Scientific Reports* **7,** 1–11; 10.1038/s41598-017-15068-3 (2017).

54. Wilk, L., Grunwald, M., Liao, P.-N., Walla, P. J. & Kühlbrandt, W. Direct interaction of the major light-harvesting complex II and PsbS in nonphotochemical quenching. *Proceedings of the National Academy of Sciences of the United States of America* **110,** 5452–5456; 10.1073/pnas.1205561110 (2013).

55. Liu, C. *et al.* Simultaneous refolding of denatured PsbS and reconstitution with LHCII into liposomes of thylakoid lipids. *Photosynthesis Research* **127,** 109–116; 10.1007/s11120-015-0176-z (2016).

56. Shimono, K., Shirouzu, M. & Yokoyama, S. Production of Membrane Proteins Using Cell-free Protein Synthesis. *Seibutsu Butsuri* **51,** 128–129; 10.2142/biophys.51.128 (2011).

57. Singh, S. M. & Panda, A. K. Solubilization and refolding of bacterial inclusion body proteins. *Journal of bioscience and bioengineering* **99,** 303–310; 10.1263/jbb.99.303 (2005).

58. Garavito, R. M. & Ferguson-Miller, S. Detergents as Tools in Membrane Biochemistry. *Journal of Biological Chemistry* **276,** 32403–32406; 10.1074/jbc.R100031200 (2001).

59. Oliver, R. C. *et al.* Dependence of Micelle Size and Shape on Detergent Alkyl Chain Length and Head Group. *PLoS ONE* **8**; 10.1371/journal.pone.0062488 (2013).

60. Tzitzilonis, C., Eichmann, C., Maslennikov, I., Choe, S. & Riek, R. Detergent/Nanodisc Screening for High-Resolution NMR Studies of an Integral Membrane Protein Containing a Cytoplasmic Domain. *PLoS ONE* **8,** 2–9; 10.1371/journal.pone.0054378 (2013).

61. Chaptal, V. *et al.* Quantification of Detergents Complexed with Membrane Proteins. *Scientific Reports* **7,** 41751; 10.1038/srep41751 (2017).

62. Bowman, G. R. *et al.* Oligomerization and higher-order assembly contribute to sub-cellular localization of a bacterial scaffold. *Molecular microbiology* **90,** 776–795; 10.1111/mmi.12398 (2013).

63. Das, S., Stivison, E., Folta-Stogniew, E. & Oliver, D. Reexamination of the role of the amino terminus of SecA in promoting its dimerization and functional state. *Journal of bacteriology* **190,** 7302–7307; 10.1128/JB.00593-08 (2008).

64. Kwan, A. H., Mobli, M., Gooley, P. R., King, G. F. & MacKay, J. P. Macromolecular NMR spectroscopy for the non-spectroscopist. *FEBS Journal* **278,** 687–703; 10.1111/j.1742-4658.2011.08004.x (2011).

65. Bodenhausen, G. & Ruben, D. J. Natural abundance nitrogen-15 NMR by enhanced heteronuclear spectroscopy (1980).

Incubate for 4 hours
Cell Extract
in vitro
Protein Production