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FtsH-mediated repair of the photosystem II complex in response to light stress

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Abstract

A common feature of light stress in plants, algae, and cyanobacteria is the light-induced damage to the photosystem II complex (PSII), which catalyses the photosynthetic oxidation of water to molecular oxygen. A repair cycle operates to replace damaged subunits within PSII, in particular, the D1 reaction centre polypeptide, by newly synthesized copies. As yet the molecular details of this physiologically important process remain obscure. A key aspect of the process that has attracted much attention is the identity of the protease or proteases involved in D1 degradation. The results are summarized here of recent mutagenesis experiments that were designed to assess the functional importance of the DegP/HtrA and FtsH protease families in the cyanobacterium *Synechocystis* sp. PCC 6803. Based on these results and the analysis of *Arabidopsis* mutants, a general model for PSII repair is suggested in which FtsH complexes alone are able to degrade damaged D1.

Key words: Cyanobacteria, DegP/HtrA proteases, FtsH proteases, light stress, photoinhibition.

Introduction

An inevitable side-reaction of oxygenic photosynthesis is the light-induced formation of reactive oxygen species (ROS) during photosynthetic electron transport (Asada, 1999). Despite the presence of scavenging enzymes, ROS cause damage to a broad spectrum of cellular components including protein, pigment, lipids, and nucleic acid. The chief target in the thylakoid membrane is the photosystem II complex (PSII) which functions as a water:plastoquinone oxidoreductase (Prasil *et al.*, 1992; Aro *et al.*, 1993; Ohad *et al.*, 1994). Damage to PSII is thought to be due mainly to the action of singlet oxygen generated from triplet chlorophyll species formed following charge recombination within PSII. However, the highly oxidizing species generated by PSII to oxidize water can also cause damage. These two processes are sometimes referred to as acceptor- and donor-side mechanisms, respectively (Barber and Andersson, 1992).

Although PSII is composed of over 25 subunits, the reaction centre subunit D1 appears to be the chief site of photodamage in PSII (Kyle *et al.*, 1984; Ohad *et al.*, 1984; reviewed by Aro *et al.*, 1993; Adir *et al.*, 2003). The reason for this is probably due to the fact that D1 plays a key role in binding the CaMn oxygen-evolving complex and the chlorophyll molecules involved in the charge recombination reactions that can form singlet oxygen (Diner and Rappaport, 2002). However, it is increasingly clear that damage is not exclusive to D1 and, depending upon illumination conditions, other PSII subunits are damaged such as D2 and CP43 (Komenda and Masojídek, 1995).

A key feature of D1 that has been recognized since 1974 is its rapid synthesis and degradation in response to light (Bottomley *et al.*, 1974; Eaglesham and Ellis, 1974). This is assumed to reflect a repair cycle that is used to replace a damaged D1 subunit within a damaged PSII complex by a newly synthesized copy (Aro *et al.*, 1993). A remarkable feature of this process is the specificity. Only the damaged subunit is replaced; the rest of the subunits in the complex appear to be recycled. As yet, the molecular details of PSII repair are ill-defined, despite the fact that PSII is considered

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to represent one of the most vulnerable enzymes in plants to light stress, not only because of its relatively high rates of damage but also because the repair process itself is sensitive to ROS (Nishiyama *et al.*, 2001).

So far most of the understanding of the processes involved in PSII repair has been based upon experimental data obtained from higher plants, mainly from experiments conducted *in vitro*. However, a variety of recent work suggests that the cyanobacterium *Synechocystis* sp. PCC 6803 is likely to play an important role in investigating PSII repair, particularly for studies *in vivo*, and that many of the features could be conserved in the chloroplast. Recent advances in determining the structure of PSII from the related cyanobacterium *Thermosynechococcus elongatus* have also strengthened the case for using *Synechocystis* 6803 (Ferreira *et al.*, 2004). In this paper, recent work from our laboratories on the role of FtsH proteases in D1 degradation is summarized and our current working model of PSII repair is described.

The PSII repair cycle in cyanobacteria

Figure 1 presents a hypothetical scheme for PSII repair in cyanobacteria such as *Synechocystis*. This model is based largely on schemes developed for D1 replacement in the chloroplast (Aro *et al.*, 1993). The key aspects are (i) photodamage to PSII so that electron transport is impaired, (ii) induction of a conformational change that signals the need to remove the damaged subunit, (iii) monomerization of PSII and partial disassembly of the PSII complex to allow access to the damaged subunit, (iv) degradation of damaged D1 and synchronized replacement by a newly synthesized subunit (Komenda and Barber, 1995), and (v) rebinding of various extrinsic proteins and the light-driven assembly (termed photoactivation) of the CaMn cluster, which can only occur after C-terminal processing of the D1 subunit.

Probing the role of the DegP/HtrA and FtsH families of protease in D1 degradation *in vivo*

Recently, much interest has focused on the identification of proteases that are involved in the degradation of damaged D1. Based on a variety of studies conducted *in vitro*, a model has been proposed for chloroplasts in which damaged D1 is removed through the action of two proteases (reviewed by Adam and Clarke, 2002). DegP2, which is a member of the DegP/HtrA family of serine proteases, is proposed to perform the primary cleavage event within the Q_B-binding pocket (Haußühl *et al.*, 2001) in a GTP-regulated process (Spetea *et al.*, 1999), after which, the two breakdown products are removed by one or more members of the FtsH protease family (Lindahl *et al.*, 1996, 2000).

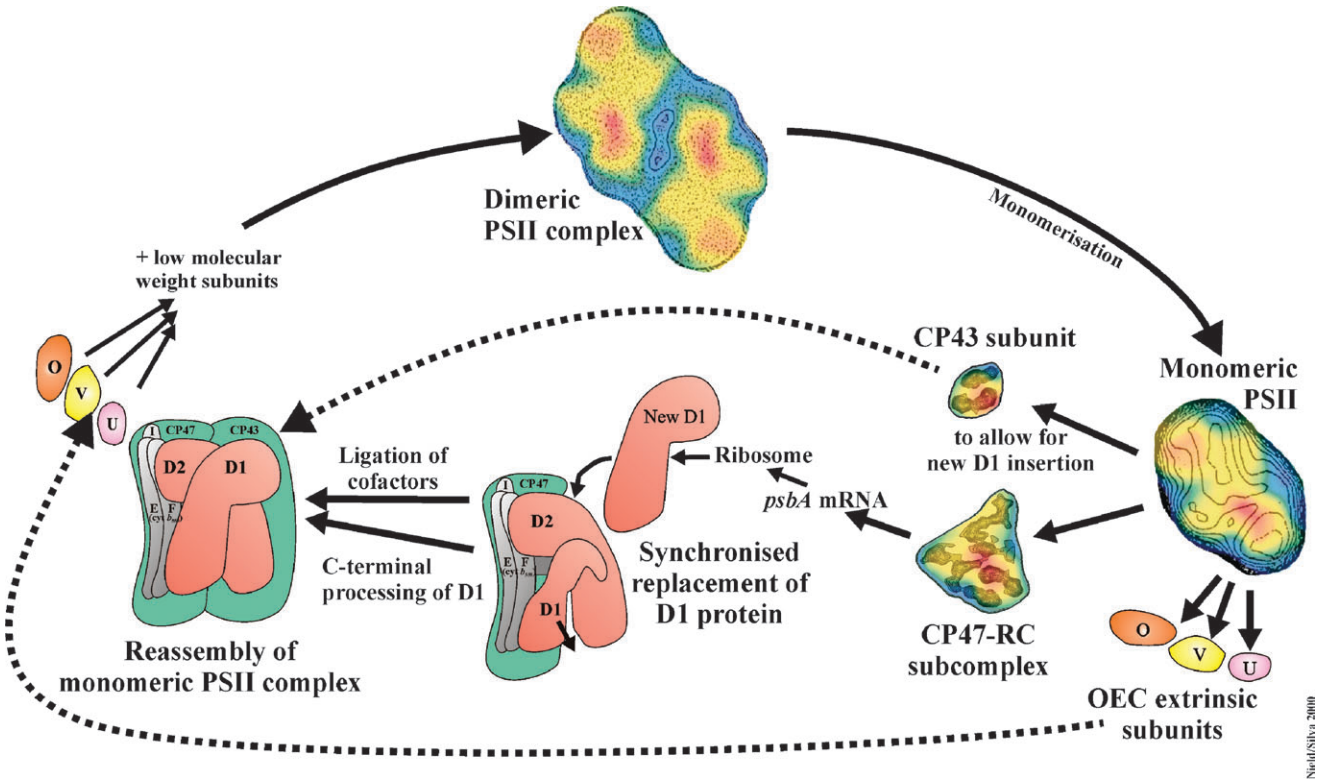

Fig. 1. Model of the PSII repair cycle in *Synechocystis* sp. PCC 6803 (kindly provided by Paulo Silva and Jon Nield). A functional dimeric PSII complex undergoes a series of disassembly steps to allow the synchronized replacement of a damaged D1 subunit by a newly synthesized copy. The PSII complex is then reassembled and the water-oxidizing CaMn cluster photoactivated.

In *Synechocystis* 6803, among the 62 predicted proteases/peptidases (Sokolenko *et al.*, 2002), there are three potential members of the DegP/HtrA family of proteases, termed HtrA (slr1204), HhoA (sll1679), and HhoB (sll 1427). This class of protease possesses the catalytic triad typical of serine proteases and contains PDZ domains involved in binding to the C-terminal region of target proteins. Structurally DegP/HtrA proteases are thought to be hexameric and to form a chamber in which either refolding or proteolysis of protein occurs (reviewed by Clausen *et al.*, 2002). To test the involvement of the DegP/HtrA proteases in PSII repair, a triple mutant has been constructed in which all three genes were insertionally inactivated. Although growth of the mutant is sensitive to high irradiance (Silva *et al.*, 2002), recent pulse-chase analyses and oxygen-evolution assays indicate little effect on PSII repair and D1 turnover (PJ Nixon, unpublished data). These data therefore suggest that these proteases are not crucial for D1 turnover in *Synechocystis* 6803. Given this it is likely that the involvement of DegP2 in PSII repair in chloroplasts might have occurred after the divergence of cyanobacteria and chloroplasts. Importantly, though, PSII repair has not so far been examined in a *degP2* mutant *in vivo*, so it remains unclear how relevant the DegP2 pathway is for D1 cleavage *in planta*.

The FtsH proteases are membrane-bound, and contain an AAA^+ module (ATPase associated with various cellular activities) and a Zn^{2+}-binding site which catalyses proteolysis (Fig. 2A) (Ogura *et al.*, 1991). The C-terminus might also contain a leucine zipper (Shotland *et al.*, 2000). In *Synechocystis* 6803 there are four potential FtsH proteases, two of which are crucial for cell viability (slr1390 and slr1604) and two that are dispensable (slr0228 and sll1463) (Mann *et al.*, 2000). Recent studies on an slr0228 insertion mutant have revealed that this particular member is needed for photoprotection, and is required for normal rates of D1 degradation *in vivo* as assessed in pulse-chase assays (Silva *et al.*, 2003). Importantly, full-length D1 was stabilized in the mutant during light stress and there was no evidence for the accumulation of D1 fragments, which would be expected if the role of FtsH were solely to remove D1 fragments. FtsH (slr0228 and slr1604) were also found at low levels in His-tagged PSII preparations of *Synechocystis* 6803 (Kashino *et al.*, 2002; Silva *et al.*, 2003). Although there are a number of interpretations of these data, the simplest model is one in which FtsH (slr0228) plays a direct role in the early stages of D1 degradation, not just in the removal of breakdown fragments.

In the case of *Arabidopsis thaliana*, nine FtsH homologues are targeted to the chloroplast (Sakamoto *et al.*, 2003). Mutation of FtsH2 (VAR2) and FtsH5 (VAR1) give rise to a yellow variegated phenotype, whereas *ftsH1*, *ftsH6* or *ftsH8* mutants show no visible phenotypic difference to WT (Sakamoto *et al.*, 2003). Importantly both *var1* (*ftsH5*) and *var2* (*ftsH2*) mutants show impaired PSII repair (Bailey *et al.*, 2002; Sakamoto *et al.*, 2002). In the case of *var2*, degradation of damaged D1 was blocked at an early stage, in a similar manner to that observed in the cyanobacterial

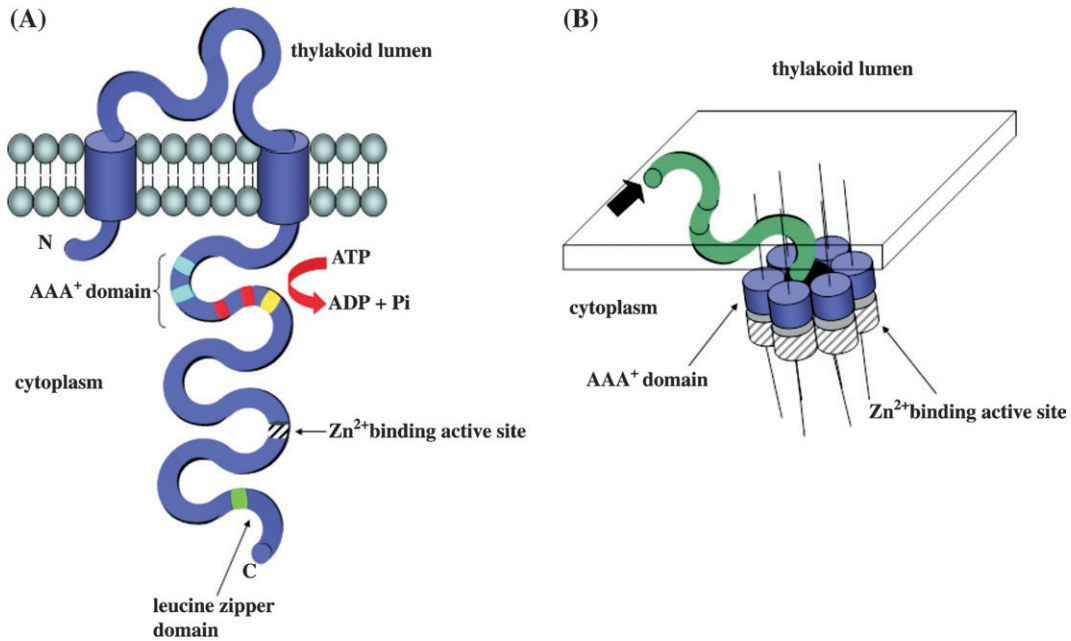

Fig. 2. Structure of the FtsH protease. (A) Schematic representation of a *Synechocystis* thylakoid FtsH protease subunit showing the two transmembrane helices, the ATPase domain (AAA^+) containing various conserved sequence elements, the Zn^{2+}-binding site, and a possible leucine zipper. (B) Cartoon suggesting how a membrane subunit such as D1 (arrowed) might be translocated through a central pore within the hexameric FtsH holoenzyme and subsequently degraded. For clarity only the ATP-ase and the Zn^{2+}-binding site are indicated for each FtsH subunit.

ftsH (slr0228) mutant (Bailey *et al.*, 2002). It therefore seems that the role of FtsH in PSII repair and D1 turnover might be conserved in both cyanobacteria and higher plants.

Model for FtsH-mediated D1 degradation

Based on what is known about the mechanism of FtsH proteases, particularly in *E. coli* (Akiyama and Ito, 2003) and mitochondria (Langer, 2000), a general model for D1 degradation in cyanobacteria and chloroplasts has been suggested (Silva *et al.*, 2003). It is proposed that FtsH (either homo- or hetero-oligomeric) forms a hexameric ring in the membrane. Damaged D1 is then translocated through a central pore in an ATP-driven process and subsequently degraded at the Zn^{2+}-centre (Fig. 2B). The key feature of FtsH-mediated proteolysis of membrane proteins in *E. coli* is that it is a highly processive reaction (Akiyama, 2002) and can occur from either the N- or C-terminus (Chiba *et al.*, 2002), or possibly from the ends generated after an FtsH-mediated endoproteolytic cleavage event (Shotland *et al.*, 2000). Given that the Zn^{2+}-binding site is likely to be located on the stromal side of the membrane (Lindahl *et al.*, 1996), proteolysis would be initiated from stromally exposed regions of D1 such as the N-terminus or after cleavage in the Q_B-binding pocket. Proteolysis from the N-terminus is particularly attractive, as this would allow potential synchronization between the co-translational insertion of a newly synthesized D1 subunit into PSII and the removal of the damaged subunit. It would also explain why N-terminal phosphorylation of D1 and other PSII core subunits might control their degradation in the chloroplast (Koivuniemi *et al.*, 1995).

In order to confirm this model it will probably be necessary to develop an *in vitro* assay for studying FtsH-mediated degradation of D1, probably along the lines of one recently developed for *E. coli* FtsH, which required the use of a membrane-based system rather than detergent-solubilized enzyme and substrate (Akiyama and Itoh, 2003).

What is the signal that triggers D1 degradation *in vivo*?

Studies in *E. coli* have highlighted three factors that are important for FtsH-mediated degradation of membrane proteins: (i) the exposed N-terminus of the target protein must be of sufficient length (greater than 20 residues) to engage FtsH; the precise sequence being less important (Chiba *et al.*, 2000), (ii) the target protein must be in a relatively unfolded or destabilized state as FtsH is a weak unfoldase (Herman *et al.*, 2003), and (iii) FtsH must contain transmembrane helices, possibly to interact with the target membrane protein (Akiyama and Ito, 2001).

Exposure of PSII to light is known to damage pigments and to oxidize protein side-chains (Sharma *et al.*, 1997), both of which will act to destabilize the PSII structure. As mentioned earlier, the involvement of D1 in binding many of the key co-factors in PSII means that D1 is potentially more liable to damage and destabilization. This, in combination with an accessible N-terminus, would be the trigger for FtsH-mediated proteolysis to proceed. Initial binding of FtsH to PSII could be through the interaction of transmembrane helices or even through sequences on the lumenal side of the membrane. In this respect, Bailey and colleagues (Bailey *et al.*, 2002) have highlighted an 81-amino-acid sequence of FtsH that is predicted to lie in the lumen of the chloroplast. This region is highly conserved in oxygenic photosynthetic organisms, perhaps because it is involved in recognizing sequences in PSII.

One attractive feature of this model is that FtsH would act to remove destabilized D1, and potentially other PSII subunits, no matter where the damage had occurred. Thus a precise triggering event need not commit D1 to degradation; rather it could be the accumulation of many destabilizing events. In principle, it should be possible to isolate D1 mutants that have slower rates of D1 degradation because of enhanced stabilization of structure or perturbed interaction with FtsH. Interestingly mutants in both the Q_B-binding pocket, at D1-Ser264 and D1-Ala263 (Dalla Chiesa *et al.*, 1997), and at D1-His92 on the lumenal side of the membrane, slow D1 degradation (Lupínková and Komenda, 2004). What is clear is that the so-called 'PEST'-like sequence located close to the Q_B-binding pocket of D1, and suggested to be important for D1 degradation (Greenberg *et al.*, 1987), is not required for D1 turnover (Nixon *et al.*, 1995).

The oligomeric structure of FtsH and its possible interaction with prohibitins

Given the importance of FtsH (slr0228) for the removal of damaged D1 in *Synechocystis* 6803, future studies will aim to clarify the location and oligomeric organization of slr0228 in the membrane. In the case of *A. thaliana*, initial indications are that VAR2 might exist both as a homocomplex and a heterocomplex with VAR1 (Sakamoto *et al.*, 2003). Rodermel and co-workers have also shown that overexpression of FtsH8 can rescue the *ftsH2* (*var2*) mutation, so it seems likely that the chloroplast FtsH subunits could assemble to give rise to a heterogeneous population of hexameric complexes, each with a potentially different activity (Yu *et al.*, 2004).

Of particular interest will be the identification of possible interacting partners of FtsH (slr0228). In *E. coli* it is known that FtsH forms a supercomplex with the HflKC complex (Saikawa *et al.*, 2004), and in yeast mitochondria with a large heterooligomeric prohibitin complex (Steglich *et al.*, 1999). Both HflKC and prohibitin are members of the SPFH (stomatin, prohibitin, flotillin, HflKC) superfamily of proteins (Tavernarakis *et al.*, 1999). One role of the prohibitin

complex in yeast mitochondria is to stabilize newly synthesized membrane subunits (Nijtmans *et al.*, 1999). Consequently, members of the SPFH family might be involved in PSII repair, possibly either to stabilize newly synthesized D1 prior to insertion into a PSII complex in a post-translational step, or to stabilize other PSII subunits to prevent unwanted FtsH mediated proteolysis (Silva and Nixon, 2001). In this regard, it is interesting to note that there are a number of prohibitin and stomatin homologues predicted from analysis of the *Synechocystis* genome sequence.

Another key question that has been addressed is whether FtsH (slr0228) functions solely in PSII repair or whether it has a general role in the removal of damaged or unassembled proteins from the thylakoid membrane. Recent experiments have been performed to look at protein turnover in PSII mutants unable to assemble a functional PSII complex because synthesis of a key PSII subunit is blocked. In all cases degradation of the remaining PSII subunits, which usually occurs rapidly in the presence of FtsH (slr0228) is reduced dramatically in its absence (PJ Nixon, unpublished data). These data therefore provide evidence in favour of a general role for FtsH (slr0228) in the removal of unassembled and/or damaged proteins from the membrane.

Where does PSII repair occur in cyanobacteria?

Although functional PSII complexes are definitely found in the thylakoid membrane of cyanobacteria, there is still uncertainty about the location of the sites of synthesis, assembly and repair. In chloroplasts, repair of PSII is thought to take place in the stromal lamellae (Aro *et al.*, 1993). For *Synechocystis* 6803, immunochemical data have indicated that D1, D2, cytochrome *b*-559, and the PsbO subunit can be found in the cytoplasmic membrane (Smith and Howe, 1993; Zak *et al.*, 2001). It has therefore been suggested that the cytoplasmic membrane might be the site of synthesis of the minimal PSII reaction centre complex, consisting of D1, D2, and cytochrome *b*-559, which then migrates into the thylakoid membrane to be assembled into the holoenzyme (Smith and Howe, 1993; Zak *et al.*, 2001). However, pulse–chase experiments have not yet been conducted to confirm that these subunits are first incorporated into the cytoplasmic membrane. This means that it is still possible that the PSII subunits found in the cytoplasmic membrane could be in the process of being degraded rather than assembled (Smith and Howe, 1993). Another complication could be that the cytoplasmic membrane fraction used in the localization studies might contain other types of membrane system, distinct to the thylakoid and cytoplasmic membrane. Since it is still unclear how proteins and lipids move between the cytoplasmic and thylakoid membrane systems, it is possible that there might be an interconnecting membrane system in which assembly and repair occurs, that co-purifies with the cytoplasmic membrane fraction. Alternatively, if there is no physical connection between the thylakoid and cytoplasmic membranes, a vesicular transport system must be invoked (Westphal *et al.*, 2001).

Are ROS involved in D1 degradation *in vivo*?

A wide variety of studies conducted *in vitro* have led to speculation that ROS are potential candidates for cleaving D1 *in vivo* (Mishra *et al.*, 1994; Miyao *et al.*, 1995). In such a scenario, the primary cleavage event in the Q_B-binding pocket would be mediated by ROS. However, one of the most dramatic observations following high-light treatment of both the *ftsH* (slr0228) mutant and *var2* mutant of *A. thaliana* is the persistence of full-length D1 in the thylakoid membrane, despite extensive inactivation of the PSII complex, and under conditions where D1 is degraded in the WT controls. In the authors' opinion this is convincing evidence against an obligatory role for ROS in the direct cleavage of D1 during PSII repair *in vivo*.

In agreement with earlier conclusions (Komenda and Masojídek, 1995; Lupínková and Komenda, 2004), it is felt that D1 degradation *in vivo* should be considered to occur in two modes depending on the severity of the irradiation and the capacity for repair: (i) under 'normal' conditions a totally enzymatic process (mediated by FtsH) performs the selective replacement of damaged D1, without producing detectable breakdown products, and (ii) ROS-mediated non-enzymatic reactions occur under more 'extreme' conditions when the 'normal' enzymatic removal of damaged subunits cannot keep pace with the rate of damage by ROS. Under these latter circumstances the resulting breakdown fragments and aggregates that are observed might resemble the products studied *in vitro*. Consequently, caution is urged when linking breakdown fragments generated *in vitro* (or under extreme conditions *in vivo*) to the 'normal' enzymatic process of D1 turnover.

However, it is probable that under the more 'extreme' conditions of photodamage, oxidized forms of D1 and the other PSII subunits, as well as their adducts, can be removed from the cell by additional proteases not used in the 'normal' enzymatic process. These could include the DegP/HtrA proteases and other uncharacterized soluble proteases (Yamamoto, 2001; Mizusawa *et al.*, 2003). However, this route for PSII repair is likely to be less specific and slower than the FtsH-mediated pathway.

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