

Characterization of Tryptophan Oxidation Affecting D1 Degradation by FtsH in the Photosystem II Quality Control of Chloroplasts

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Abstract

Photosynthesis is one of the most important reactions for sustaining our environment. Photosystem II (PSII) is the initial site of photosynthetic electron transfer by water oxidation. Light in excess, however, causes the simultaneous production of reactive oxygen species (ROS), leading to photo-oxidative damage in PSII. To maintain photosynthetic activity, the PSII reaction center protein D1, which is the primary target of unavoidable photo-oxidative damage, is efficiently degraded by FtsH protease. In PSII subunits, photo-oxidative modifications of several amino acids such as Trp have been indeed documented, whereas the linkage between such modifications and D1 degradation remains elusive. Here, we show that an oxidative post-translational modification of Trp residue at the N-terminal tail of D1 is correlated with D1 degradation by FtsH during high-light stress. We revealed that *Arabidopsis* mutant lacking FtsH2 had increased levels of oxidative Trp residues in D1, among which an N-terminal Trp-14 was distinctively localized in the stromal side. Further characterization of Trp-14 using chloroplast transformation in *Chlamydomonas* indicated that substitution of D1 Trp-14 to Phe, mimicking Trp oxidation enhanced FtsH-mediated D1 degradation under high light, although the substitution did not affect protein stability and PSII activity. Molecular dynamics simulation of PSII implies that both Trp-14 oxidation and Phe substitution cause fluctuation of D1 N-terminal tail. Furthermore, Trp-14 to Phe modification appeared to have an additive effect in the interaction between FtsH and PSII core in vivo. Together, our results suggest that the Trp oxidation at its N-terminus of D1 may be one of the key oxidations in the PSII repair, leading to processive degradation by FtsH.

Significance Statement

In photosynthetic organisms, maintenance of photosynthetic light reaction is manifested by so called Photosystem II (PSII) repair system, where the reaction center protein D1 is targeted to photo-oxidative damage and rapidly degraded by the processive protease FtsH. While this system is well known to cope with photoinhibition, the actual oxidation within the D1 polypeptide and its association to degradation remained elusive. Here, we characterized oxidative modification of tryptophan (Trp) residues in the PSII core, and hypothesize that the oxidation of N-terminal Trp is one of the key oxidations in the PSII repair, likely enhancing D1's accessibility to FtsH.

eLife assessment:

This study adds a **fundamental** new perspective to a long-standing question: What controls the repair of photosystem II (PSII), a key process in maintaining and optimizing photosynthesis? The work supports a role for chemical modification in the recognition and subsequent degradation of a key protein subunit of PSII by a bacterial-type protease, suggesting that tryptophan oxidation of components of the photosynthetic apparatus after high light stress plays a critical role in initiating the PSII repair system. The evidence supporting the authors' conclusions is **solid**.

Introduction

Light energy is essential for photosynthesis, which sustains our environment on Earth by generating oxygen and chemical energy for carbon fixation. The first step of photosynthetic electron transfer in the thylakoid membrane occurs at Photosystem II (PSII), where light energy is transferred to P₆₈₀ chlorophyll molecules to drive water oxidation, and electron is transferred to plastoquinone. PSII core complex is formed by two reaction center proteins D1 (PsbA) and D2 (PsbD), along with core antenna CP43 (PsbC) and CP47 (PsbB). However, light energy is known to cause photooxidative damage in PSII, especially reaction center protein D1. Photo-damaged D1 needs to be degraded to replace it with a newly synthesized one in the PSII repair (Lindahl, 2000 [\[1\]](#); Bailey et al., 2002 [\[2\]](#); Silva et al., 2003 [\[3\]](#); Kato et al., 2009 [\[4\]](#)). When photooxidative damage in PSII exceeds the capacity of PSII repair, it ultimately leads to the status called 'photoinhibition' (Aro et al., 1993 [\[5\]](#); Murata et al., 2007 [\[6\]](#)). PSII repair proceeds with the following steps, i) oxidative damage to D1 protein in the PSII complex, ii) migration of photo-damaged PSII laterally from grana stacks to non-appressed regions of thylakoid membranes, iii) detachment of CP43 from photo-damaged PSII allows for access of protease degrading damaged D1, and iv) concomitant D1 synthesis and reassembly of PSII into grana thylakoid. Substantial efforts to understand the mechanisms of PSII repair suggest that reversible phosphorylation of PSII core subunits is involved in fine-tuning the photo-damaged D1 turnover (Baena-González et al., 2002 [\[7\]](#); Fristedt et al., 2009 [\[8\]](#); Kato and Sakamoto, 2014 [\[9\]](#)).

Our previous studies along with those from other groups have shown that the fundamental D1 degradation in PSII repair is performed by FtsH, a membrane-bound ATP-dependent zinc metalloprotease that degrades membrane proteins in a processive manner, although several Deg proteases seem to facilitate the effective degradation by creating additional recognition sites for FtsH (Lindahl, 2000 [\[1\]](#); Bailey et al., 2002 [\[2\]](#); Sakamoto, 2003 [\[10\]](#); Silva et al., 2003 [\[3\]](#); Kato et al., 2009 [\[4\]](#); Kato and Sakamoto, 2014 [\[9\]](#)). These proteins are universally conserved in prokaryotes and eukaryotic organelles (Hanna Janska, Malgorzata Kwasniaka, 2013 [\[11\]](#)). Photosynthetic organisms have hetero-hexameric FtsH complex in the thylakoid membrane, which is composed

of type A and type B subunits (Kato and Sakamoto, 2018 [↗](#); Yi et al., 2022 [↗](#)). In the thylakoid membrane of *Arabidopsis thaliana*, for example, FtsH1 or FtsH5 (type A) and FtsH2 or FtsH8 (type B) form functional FtsH complex (Yu et al., 2004 [↗](#); Yu et al., 2005 [↗](#); Zaltsman et al., 2005 [↗](#)). *Arabidopsis* mutants lacking FtsH5 or FtsH2 are, though viable, highly vulnerable to PSII photodamage under high light and are named *yellow variegated1* (*var1*) and *var2* from the characteristic variegated phenotype (Chen et al., 2000 [↗](#); Takechi et al., 2000 [↗](#); Sakamoto et al., 2002 [↗](#)). This variegated phenotype implies that FtsH is required for proper protein quality control during proplastid-to-chloroplast differentiation in seed plants (Miura et al., 2007 [↗](#); Sakamoto et al., 2009 [↗](#)). Similarly, the FtsH complex in the thylakoid membranes of *Chlamydomonas reinhardtii* consists of FtsH1 (type A) and FtsH2 (type B) (Malnoe et al., 2014 [↗](#)). *Chlamydomonas* studies revealed that FtsH is also involved in the degradation of cytochrome *b₆f* complex and light-harvesting antenna of photosystem I (PSI) (Malnoe et al., 2014 [↗](#); Bujaldon et al., 2017 [↗](#)). Furthermore, recent studies suggest that increased turnover of FtsH is crucial for their function under high-light stress. That is compensated by upregulated *FtsH* gene expression (Wang et al., 2017 [↗](#); Kato et al., 2018 [↗](#)).

In *Escherichia coli*, FtsH was shown to recognize either N- or C-terminal tail of membrane protein substrates to pull their substrates and push them into the protease chamber by ATPase activity (Ito and Akiyama, 2005 [↗](#)). Similarly, the fact that both D1's N-terminal end and the catalytic site of FtsH are exposed to the stromal side implies D1 to be recognized by FtsH from its N-terminus (Umena et al., 2011 [↗](#)). Supporting this, processive degradation of D1 by FtsH was shown to be attenuated by the loss of N-terminal tail of D1 (Komenda et al., 2007 [↗](#); Michoux et al., 2016 [↗](#)). While these observations suggest involvement of the N-terminal region in D1, whether D1 undergoes oxidative modification at its N-terminal region has not been investigated. What is the disorder of photosystems leading to photo-oxidative damage in PSII?

Light energy frequently leads to the generation of reactive oxygen species (ROS) such as singlet oxygen at around PSII (Ohnishi et al., 2005 [↗](#); Tyystjärvi, 2008 [↗](#); Yamamoto et al., 2008 [↗](#)), which may cause oxidative post-translational modification (OPTM) of subunit proteins (Li and Kim, 2022 [↗](#)). It is noteworthy that light-dependent oxidation of amino acids, either in free forms or as peptide residues, has been reported, including thiol-containing (Cys and Met) and aromatic (Tyr, Phe, Trp) amino acids. For example, Cys and Met are prone to oxidation, whereas the oxidized Cys and Met can be reduced enzymatically. In contrast to these reversible OPTMs, OPTM of Trp is irreversible (Rinalducci et al., 2008 [↗](#); Ehrenshaft et al., 2015 [↗](#)). Thus, the only way to remove irreversible oxidized residues is proteolytic degradation, implying that Trp oxidation might trigger D1 degradation, either directly or indirectly in the PSII repair. As summarized in **Fig. 1a** [↗](#), oxidation of Trp side chain results in the formation of oxindolylalanine (OIA), N-formylkynurenine (NFK), and kynurenine (KYN). ROS attacks and opens the pyrrole ring of Trp, and forms a di-oxidized Trp derivative, NFK. Indeed, Trp residues in photosynthetic protein components were shown to be oxidized both in vitro and in vivo (Anderson et al., 2002 [↗](#); Dreaden et al., 2011 [↗](#); Dreaden Kasson et al., 2012 [↗](#)). However, although oxidative modification of D1 and other subunits has been documented previously, how these molecules are recognized and undergo degradation remains elusive. In this study, we investigated whether Trp oxidation in PSII core proteins influences D1 degradation mediated by FtsH. Our integrative approaches to address this question, by mass-spectrometry, site-directed mutagenesis, D1 degradation assay, and simulation model suggest that an N-terminal Trp oxidation is likely to be a key OPTM to trigger D1 degradation in the PSII repair.

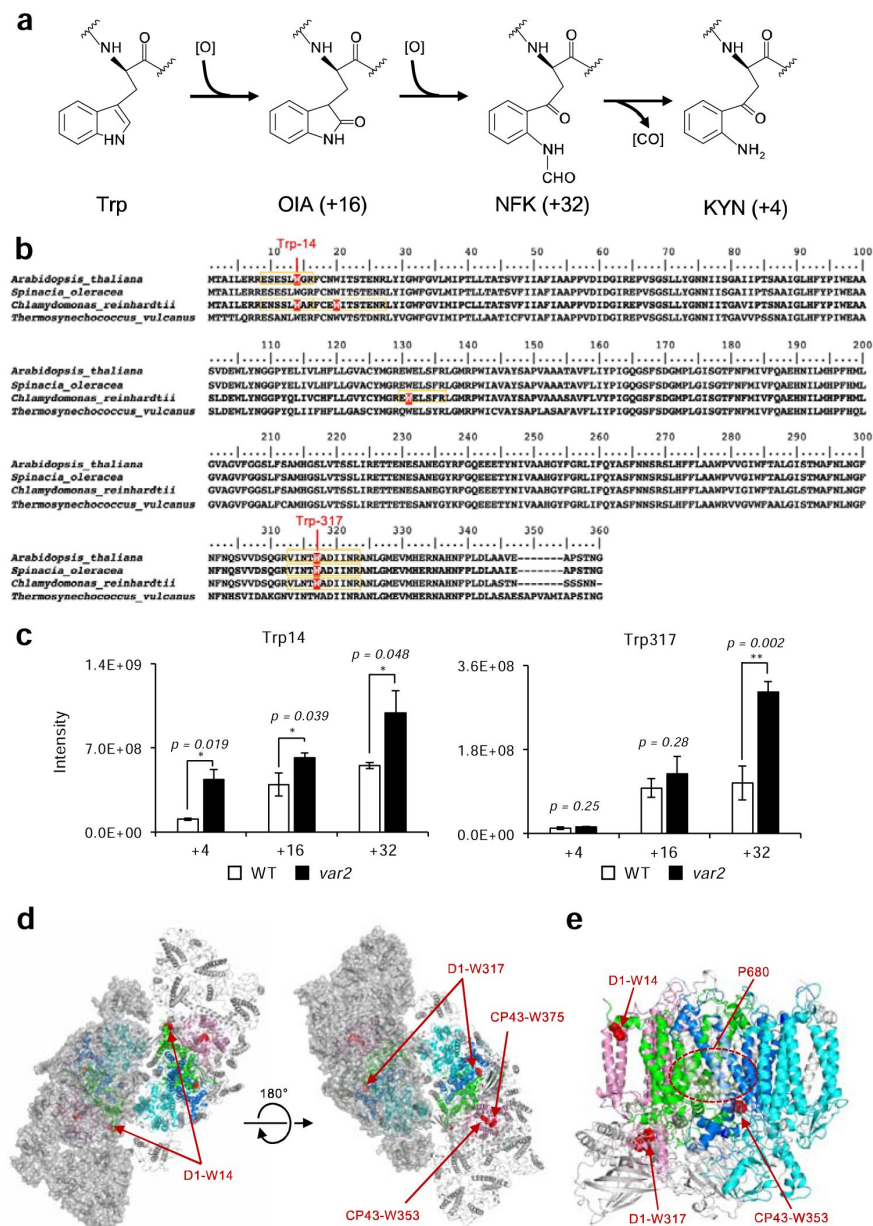


Figure 1.

The Oxidized Trp residues in PSII complex.

a, Trp-oxidation pathway. OIA: oxindolylalanine, NFK: N-formylkynurenine, KYN: Kynurenine. **b**, Multiple alignment of D1 protein from *Arabidopsis*, spinach, *Chlamydomonas*, and *Thermosynechococcus vulcanus*, showing oxidized Trp residues. Orange color boxes indicate the identified peptide by the MS-MS analysis. Oxidized Trp residues are highlighted in red. **c**, Oxidation levels of three oxidative variants of Trp in Trp14 and Trp317 containing peptides in *var2* and WT obtained by label-free MS analysis. The abundance of oxidized variants (+4: KYN, +16: OIA, and +32: NFK) of Trp14 and Trp317 were calculated using the intensity values. Asterisks indicate statistically significant differences between the mean values (* < 0.05, ** < 0.01; Student's t-test). **d-e**, Structural positions of oxidized Trp residues in PSII core proteins. The side chain of oxidized Trp residues are shown with red-colored space-filling model and indicated with arrows. The P680 special chlorophyll pair is indicated with dark-green colored ball-stick model in panel e. PSII dimer (panel d) and monomer (panel e) from *Chlamydomonas reinhardtii* (PDB ID is 6KAC) is shown in cartoon model without cofactors Top view from stromal side or luminal side (**d**) and the side view from the dimer interface (**e**) are shown respectively. The color code of each subunit is, Green, D1; Dark blue, D2; Purple, CP43, Cyan, CP47. Protein structure graphics were generated with PyMOL ver. 2.4.0 software.

Increased OPTM of Trp residues in *Arabidopsis var2* mutant

Previous studies using isolated spinach thylakoid membranes and *Arabidopsis* seedlings revealed several Trp residues oxidized in PSII core proteins, as summarized (Fig. 1b and Supplementary Table S1) (Rinalducci et al., 2008; Dreaden et al., 2011; Dreaden Kasson et al., 2012). Trp-oxidized derivatives, OIA, NFK, and KYN, give the shifts of peptide mass to + 16, + 32, and + 4 Da, respectively (Fig. 1a, Fig. S1 and S2). In this study, we attempted to assess if the oxidation of certain Trp residues is associated with D1 degradation in the PSII repair cycle. To investigate this, comprehensive detection of Trp oxidation within protein extracts in *Arabidopsis* has been established using label-free quantitative mass-spectrometry, as previously reported (Dogra et al., 2019). First, we characterized Trp oxidation from total proteins of *Arabidopsis* wild-type seedlings grown in continuous light (100 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$). Consistent with previous results (Dreaden Kasson et al., 2012; Dogra et al., 2019), two Trp residues in D1, namely Trp-14 and Trp-317, were shown to be oxidized (Fig. S1 and S2). The total sequence coverage obtained by our mass spectrometry for D1 was 26%. Further mass spectrometry in extracts of *Arabidopsis* mutants *var2* lacking FtsH2, which is shown to impair D1 degradation and exhibit substantial accumulation of ROS (Kato et al., 2009), revealed the accumulation of oxidized Trp in the PSII complex. The levels of Trp-oxidized derivatives, OIA, NFK, and KYN in Trp-14 and the level of KYN in Trp-314 were significantly increased in *var2* compared to the wild type, respectively (Fig. 1c). These results prompted us to characterize the role of these Trp oxidations in the PSII repair further.

OPTM of Trp residues in *Chlamydomonas* PSII core proteins

To validate whether Trp oxidation detected in *Arabidopsis* seedlings is also detectable in other model organisms, we investigated extracts from *Chlamydomonas* thylakoid membranes (Table 1 and Supplementary Table S2). Mass spectrometry following trypsin digestion demonstrated that several Trp residues in PSII were oxidized; among the core subunits, D1 had oxidation of four residues, D2 had three, CP43 had five, and CP47 had three (Table 1 and Supplementary Table S2). The position of oxidized Trp residues in their amino acid sequence was shown in Fig. 1b and Fig. S3. Together with previous studies, Trp-14 and Trp-317 in D1, Trp-21 and Trp-328 in D2, Trp-353 and Trp-375 in CP43, and Trp-275 and Trp-302 in CP47 were commonly identified among at least two organisms. Spatial arrangement of these Trp residues that were commonly oxidized in *Arabidopsis* and *Chlamydomonas* was compared within the structure of PSII complex, as shown in Fig. 1d and e (oxidized Trp residues were assigned in the PSII dimer, and its 3D image is shown in Supplementary Movie 1 online). Also, oxidized Trp residues were assigned in the PSII structure from *Thermosynechococcus vulcanus* and were shown in Fig. S4. Intriguingly, most of these oxidized Trp residues are positioned at the luminal side of the PSII core complex, which appeared to surround the $\text{Mn}_4\text{O}_5\text{Ca}$ cluster in the PSII structure model. In contrast, two oxidized Trp residues, Trp-14 in D1 and Trp-21 in D2 close to the N-terminus of the polypeptides, are located on the stromal side. The fact that the oxidized Trp residues are predominantly observed around the $\text{Mn}_4\text{O}_5\text{Ca}$ cluster may reflect photoinhibition of PSII electron donor side and concomitant ROS generation. In contrast, stromal Trp oxidation is novel and localized at the N-terminal alpha-helix, which might suggest its effects in processive D1 degradation.

Site-directed mutagenesis of Trp residues undergoing OPTM in D1

To test whether any change in the oxidized Trp residues is associated with D1 degradation, we performed site-directed mutagenesis using chloroplast transformation in *Chlamydomonas*, to substitute the corresponding Trp for other amino acids in D1. Based on the mass-spectrometric results, we focused on Trp-14 and Trp-317, each of which was replaced by Ala (non-polar and hydrophobic) or Phe (aromatic and hydrophobic), respectively. The vectors harboring spectinomycin/streptomycin-resistant *aadA* cassette and the mutated *psbA* gene were transformed

into *ΔpsbA* mutant Fud7 (**Fig. 2a**). Transformants were selected on mixotrophic Tris-acetate-phosphate (TAP) plates containing spectinomycin, and their homoplasmy was subsequently confirmed by PCR using specific primers and sequencing. All transformants grew like the control strain on mixotrophic TAP plates (**Fig. 2b**). However, the transformants in which Trp-14 or Trp-317 was substituted to Ala, (W14A, W317A, and W14A/W317A) showed significantly impaired growth on photoautotrophic high salt minimal (HSM) plates. Ala substitution at both Trp-14 and Trp-317 led to decreased photosynthetic activities due to reduced accumulation of D1 and other PSII core proteins (**Fig. 2b - d**), indicating its defect in stability and/or the translation of D1 protein.

In contrast, Phe substitution at the same sites had little effect on their growth under growth light (30 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$). These transformants (W14F, W317F, and W14F/W317F) accumulated PSII core proteins whose amounts were comparable to the control levels. They did not show a substantial change in photosynthetic activities as evidenced by comparable electron transport rates through the PSII complex and oxygen-evolving activity (**Fig. 2c** and **e**). We next examined their photoautotrophic growth under high light (320 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$). Under this condition, however, W14F exhibited significantly impaired growth, and W317F grew slightly slower than control cells. Double mutant W14F/W317F synergistically increased high-light sensitivity but the growth defect appeared to be similar to W14F, suggesting that Phe substitution at Trp-14, but not at Trp-317 had profound effects in the PSII repair cycle (**Fig. 2b** and **Fig. S5**).

Site-directed mutagenesis of Trp residues in CP43

We next examined high-light sensitivity in the CP43 Trp mutants. As an important step in the PSII repair, PSII complex is partially disassembled by CP43 detachment, and this process likely allows FtsH to access photo-damaged D1. Therefore, Trp oxidation in CP43 may play a role in PSII disassembly and D1 degradation concomitantly. To test this, we substituted Trp-353 and Trp-375 for either Ala or Phe as carried out in D1. Transformants were generated by cotransformation of Fud7, using the vector harboring wild-type *psbA* gene and the vector harboring the mutated *psbC* gene. Consequently, we obtained four single mutants (W353A, W353F, W375A, and W375F) and two double mutants (W353A/W375A and W353A/W375F) (**Fig. S6**). Mixotrophic growth on TAP plates was comparable among all transformants and control cells (**Fig. S6**). All the transformants except for W353A/W375A grew normally on the phototrophic condition under growth light condition. Supporting normal growth, immunoblot analysis showed normal accumulation of PSII core proteins, D1 and CP43, in all lines except for the double mutant W353A/W375A. On the other hand, D1 and CP43 were severely reduced in W353A/W375A, indicating that Ala substitution in these residues resulted in highly unstable or impaired PSII complex formation (**Fig. S6**). Further analysis of these mutants under high light showed that the transformants except for W353F did not increase high-light sensitivity on their growth. Only W353F, but not W353FW375F, showed a slight reduction in their growth under high-light conditions. This is consistent with a previous study (Anderson et al., 2002). However, the impaired growth of W353F was not as severe as that of D1 W14F or W14F/W317F, suggesting its effect was limited. At least in our site-directed mutagenesis, Trp oxidation in CP43 appeared to have little impact on the PSII repair.

Substitution of Trp-14 with Phe accelerates D1 degradation

To evaluate whether Trp substitution in D1 affects PSII damage or repair, we next measured the maximum quantum yield of PSII (F_v/F_m) and subsequently monitored D1 levels under growth or high-light conditions. Trp-substituted lines grown in TAP medium under growth light were pre-incubated in the presence or absence of chloramphenicol (CAM), an inhibitor of chloroplast protein synthesis. CAM blocks the PSII repair at the step of D1 synthesis and allows us to evaluate photodamage and D1 degradation. Cells incubated under growth light or high light were subjected to chlorophyll fluorescence measurement and immunoblot analysis. Under growth light condition

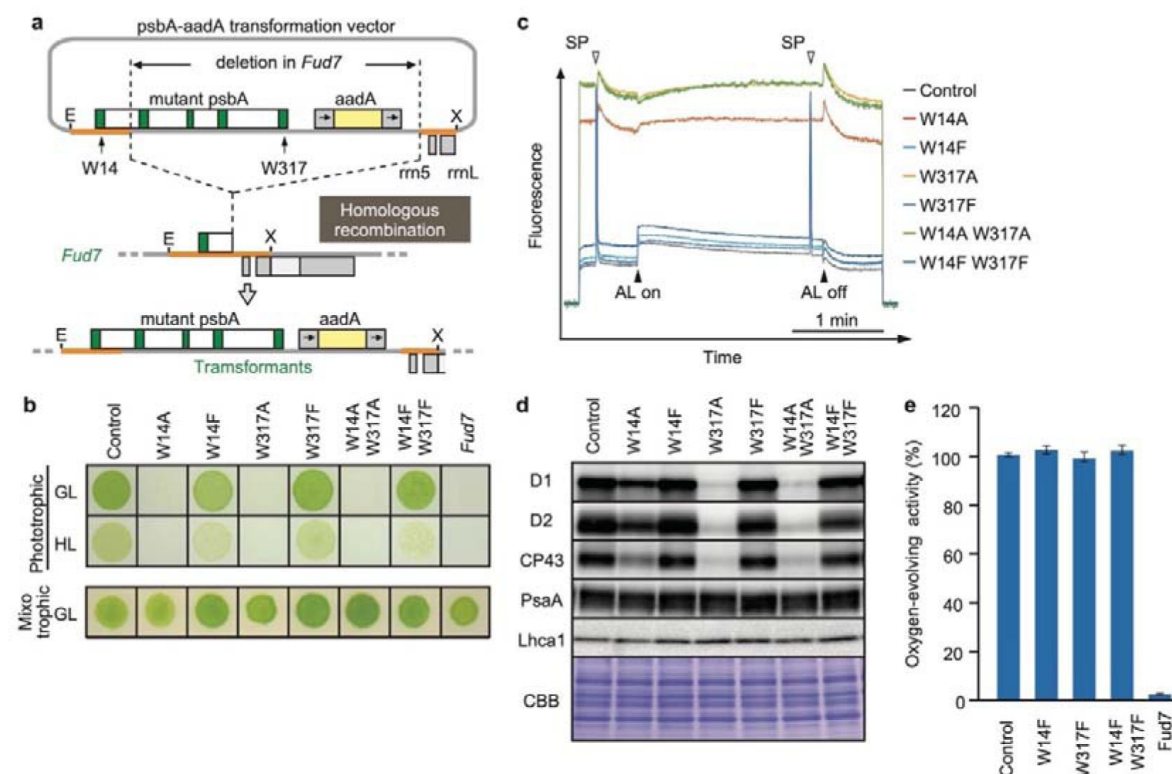


Figure 2.

High-light sensitive phenotype in the *Chlamydomonas* D1 transformants in which Trp-14 and Trp317 were mutated.

a, Schematic drawing of the transforming vector carrying *psbA*, its flanking regions of the chloroplast DNA, and the selectable *aadA* marker cassette. E and X represent restriction sites of *EcoRI* and *XhoI*, respectively. Green boxes represent exons 1–5 of *psbA*. *Fud7* is the *psbA* deletion mutant of *Chlamydomonas*. **b**, Phototrophic growth of Trp-substituted transformants on HSM medium and mixotrophic growth on TAP medium. GL, growth light ($30 \mu\text{mol photons m}^{-2}\text{s}^{-1}$); HL, high light ($320 \mu\text{mol photons m}^{-2}\text{s}^{-1}$). **c**, Chlorophyll fluorescence induction kinetics in Trp-substituted transformants. SP, saturating pulse. AL, actinic light. **d**, Protein accumulation in the transformants. Thylakoid proteins of cells grown in TAP medium under growth-light condition were separated by SDS-PAGE and analyzed by immunoblotting with antibodies against PSII subunits (D1, D2, and CP43), PSI subunits (PsaA), and light-harvesting complex of PSI (Lhca1). **e**, oxygen-evolving activity of the transformants.

and in the absence of CAM, both PSII activity (F_V/F_M values) and D1 levels were comparable among all Trp-substituted lines and the control (**Fig. 3a**). This result was consistent with their photoautotrophic growth under growth light. When CAM was added, D1 levels decreased only slightly during incubation (90 min) in the control. D1 degradation rate was comparable in all Trp-substituted lines and control (**Fig. 3c**), indicating that all Trp-substituted D1 proteins formed stable and functional PSII complex under growth light.

Under high-light condition, however, F_V/F_M values in Trp-substituted lines significantly decreased, compared to that observed in the control even in the absence of CAM (**Fig. 3b**). These vulnerabilities to high light were consistent with their impaired growth under high light (**Fig. 2**). To our surprise, D1 levels in W14F and W14F/W317F concomitantly decreased during high-light incubation (**Fig. 3b**). In contrast, D1 levels in W317F were similar to those in control cells. When the PSII repair engages properly, high-light irradiation does not alter D1 levels because rapid D1 synthesis compensates turnover of photo-damaged D1. Given decreased D1 under high light, W14F was likely to cause faster D1 degradation. To confirm this possibility, D1 degradation in the presence of CAM was measured. PSII activity in all Trp-substituted lines fell at similar rates compared with control cells in the presence of the CAM (**Fig. 3d**), indicating the light-induced damage was at the similar level among all Trp-substituted lines and the control. In contrast, our time course experiment indicated that W14F and W14F/W317F decreased D1 faster than the control and W317F (**Fig. 3d**); the D1 level in W14F and W14F/W317F decreased approximately 60% and 50% of the initial level, respectively, whereas those in control cells and W317F remained 80% (**Fig. 3d**).

Based on these D1 degradation assays, we assumed that D1 degradation by proteolysis was enhanced by W14F substitution, despite the fact that PSII suffered from photodamage equally among other lines and the control during high-light irradiation. To exclude the possibility that W14F decelerates D1 synthesis rather than accelerating degradation, we analyzed protein synthesis in Trp-substituted lines by *in vivo* pulse labeling in the presence of cycloheximide, which prevents the synthesis of the nuclear-encoded proteins. As shown in **Fig. S7**, D1 synthesis was shown to proceed comparably in all lines. Collectively, our findings demonstrated that Trp-14 substitution to Phe enhanced D1 degradation, whereas it affected neither the light-induced damage in PSII, D1 synthesis, nor PSII stability.

Enhanced D1 degradation due to the substitution of Trp-14 is mitigated in the *ftsH* mutant

To address whether the increased D1 degradation in W14F (and W14F/W317F) involved proteolysis by FtsH, these Trp substitutions were introduced into an *ftsH* mutant deficient in thylakoid FtsH activity. In the thylakoid membrane of *Chlamydomonas*, a hetero-oligomeric FtsH complex composed of FtsH1 (type-A) and FtsH2 (type-B) exists, and the *ftsH1-1* mutant, expressing an inactive FtsH1 due to the amino-acid substitution in the ATP-binding domain, has been reported (Malnoe et al., 2014). We performed mating W14F and W14F/W317F transformants (mt+) with *ftsH1-1* (mt-), and the resulting mutants, W14F *ftsH1* and W14F/W317F *ftsH1*, were subjected to D1 degradation assay. The results indicated that as expected, the enhanced D1 degradation observed in W14F and W14F/W317F cells was partially mitigated under *ftsH1-1* background (**Fig. 4**), when CAM was added. These results suggested that FtsH plays a key role in the increased D1 degradation in W14F and W14F W317F.

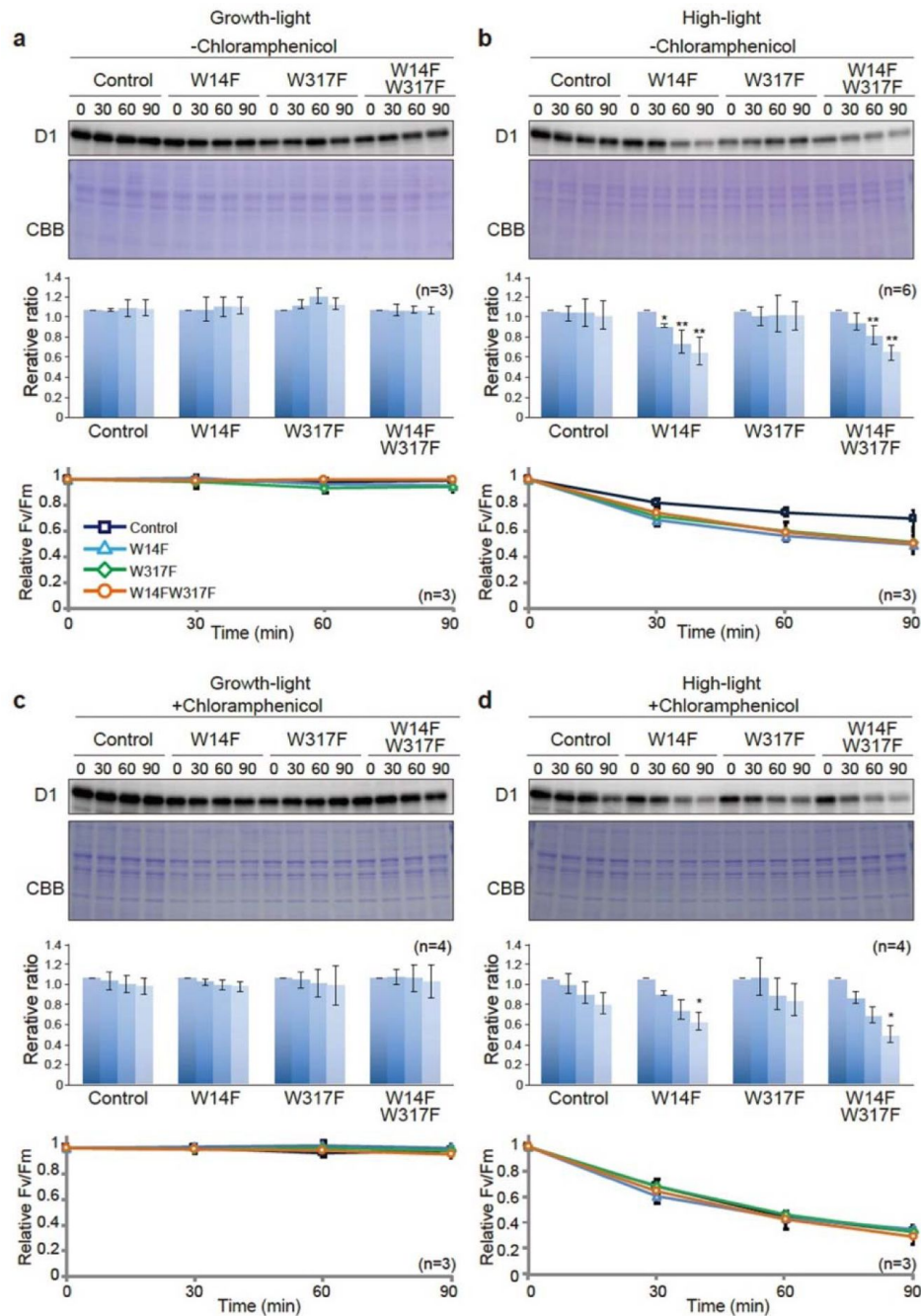


Figure 3.

D1 degradation assay in W14F and W317F transformants demonstrating enhanced D1 degradation under high-light stress.

The transformants were incubated under high-light ($320 \mu\text{mol}\cdot\text{photons m}^{-2}\text{s}^{-1}$) or growth-light ($30 \mu\text{mol}\cdot\text{photons m}^{-2}\text{s}^{-1}$) conditions in the absence or presence of inhibitor of chloroplast protein synthesis, CAM, and subjected to D1 degradation assay. **a**, growth-light in the absence of CAM; **b**, high-light in the absence of CAM; **c**, growth-light in the presence of CAM; **d**, high-light in the presence of CAM. Immunoblot results of D1 in the transformants are shown at the top of each panel. A representative immunoblot using anti-D1 is depicted. Quantified D1 levels using NIH Image program are shown in the middle. Values are means $\pm$ SD. Asterisks indicate statistically significant differences between the mean values (* < 0.05 , ** < 0.01 ; Student's t-test). Time course analysis of maximal photochemical efficiency of PSII, F_v/F_m , are shown at the bottom.

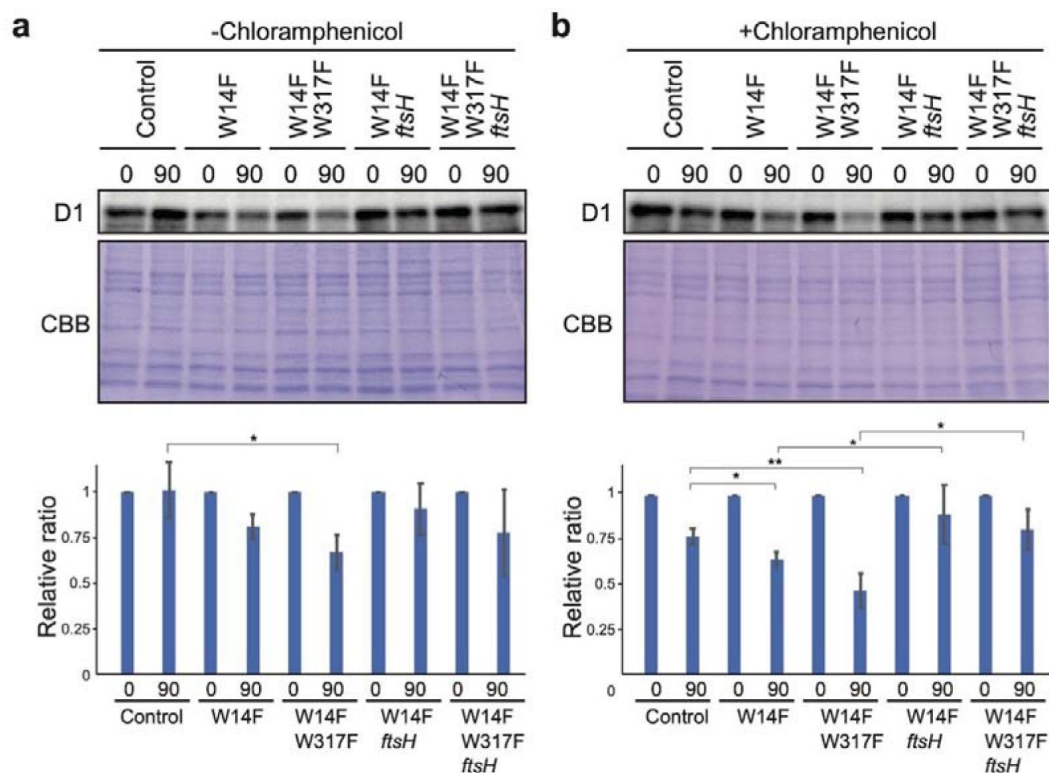


Figure 4.

D1 degradation assay in W14F and W14F/W317F transformants in the *ftsH* mutant background.

Rate of D1 degradation in the W14F *ftsH* and W14F/W317F *ftsH* was investigated as shown in [Fig. 3](#). Cultured cells were incubated under high-light conditions ($320 \mu\text{mol photons m}^{-2}\text{s}^{-1}$) in the absence (a) or presence (b) of CAM. Signals of immunoblots were quantified using NIH Image program. Values are means $\pm$ SD (n = 4). A representative immunoblot using anti-D1 is depicted. Values are means $\pm$ SD. Asterisks indicate statistically significant differences between the mean values (* < 0.05, ** < 0.01; Student's t-test).

Molecular dynamics simulation suggests W14F mimicking Trp-14 oxidation

Although our site-directed mutagenesis in Trp-14 showed its effect in D1 degradation, how Trp oxidation can be structurally correlated with Trp to Phe mutagenesis should be taken into consideration. To investigate this, we employed molecular dynamics (MD) simulation, a powerful tool to simulate movements of amino acids in a protein complex, using the crystal structure of PSII complex from *Thermosynechococcus vulcanus* (Sakashita et al., 2017b; Sakashita et al., 2017a; Kawashima et al., 2018). D1 Trp-14 is located in the first α -helix at the N-terminus and hydrogen-bonded with PsbI Ser-25 (Fig. 5a). It is deduced that this hydrogen bond restricts the conformational change around D1 Trp-14 and limit the fluctuation of D1 N-terminus. The simulation indicated that the hydrogen bond disappeared (Fig. 5b, c) and the structural fluctuation of D1 Trp-14 was increased as compared with WT (Fig. 5d) when Trp-14 is oxidized to NFK or is replaced with Phe residue. The increased fluctuation of the side chain also influences the C β -C β distance between D1 Trp-14 and PsbI Ser-25; the two C β atoms became farther away from each other when D1 Trp-14 is oxidized to NFK (Fig. 5e). Of note, the amino acid substitution on Trp-14 to Phe showed similar trends as those observed when D1-Trp-14 is oxidized to NFK. These results suggest that the structural change of Trp-14 affect the local movement. The increased fluctuation of the first α -helix of D1 would give a chance to recognize the photo-damaged D1 by FtsH protease.

Augmented interaction between D1 and FtsH by substituting Trp-14/317

Presented experimental results collectively raise the possibility that oxidation of Trp-14 is one of the key OPTMs for D1 degradation by FtsH. We raised a possibility that W14F mimics Trp-14 oxidation and shows increased FtsH association with D1. Since quantitative interaction of the protein and the protease remains to be elucidated, we performed differential pull-down assay. To emphasize the effect of the substituted amino acid residues and minimize potential oxidation of other amino acid residues, we decreased light intensity during cell culture and removed oxygen molecule from the buffer solution during the assay. *Chlamydomonas* cells were grown under dim light with gently shaking and were harvested at the mid-log phase. Subsequently, we isolated thylakoid membrane from the gently disrupted cells and performed co-immunoprecipitation in anoxic aqueous solution. Quantification of D1 and D2 levels, normalized by FtsH in the co-immunoprecipitated sample showed that the relative D1 protein amounts was statistically higher in W14F/W317F than the control while D2 was fluctuating (Fig. 6). We concluded that W14F increased affinity between FtsH and the reaction center proteins, which leads to enhanced D1 degradation.

Discussion

Recent progress in mass-spectrometry has advanced our understanding of holistic OPTM in photosynthetic protein complexes. Along this line, we investigated Trp oxidation in PSII in this study, and attempted to address whether any modification in amino acid residues was correlated with the PSII repair. PSII is one of the major sites for ROS generation due to photoinhibition, and oxidized amino acid residues in PSII core proteins have been reported previously (Kale et al., 2017)(Frankel et al., 2012). In general, Met and Cys are sensitive amino acid residues for ROS-mediated oxidation (Rinalducci et al., 2008)(Ehrenshaft et al., 2015). However, those OPTMs can be converted back in reduced forms by methionine sulfoxide reductase and disulfide reductase, respectively. In contrast, Trp oxidation is irreversible, and its replacement requires whole protein degradation and de novo synthesis, implicating Trp suitable for flagging photo-oxidative damaged proteins that undergo degradation in the PSII repair.

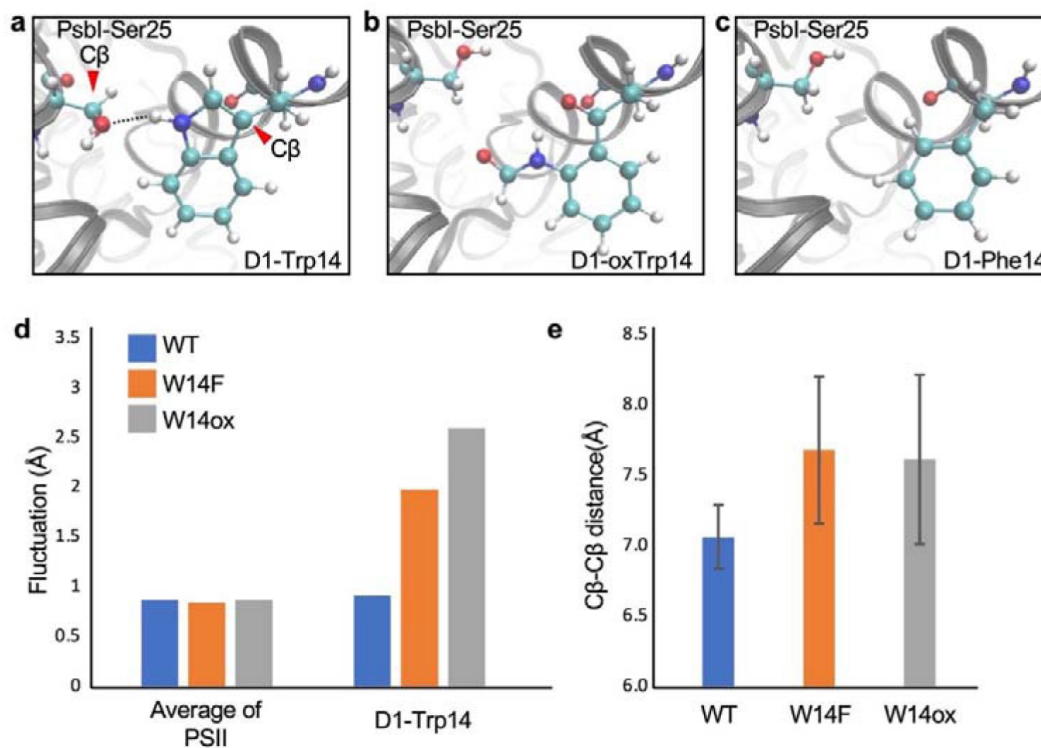


Figure 5.

Snapshots and structural fluctuation of D1-Trp14 in molecular dynamics simulations of PSII.

a, The interaction between D1 Trp-14 and PsbI Ser-25. Dash line indicates the hydrogen bond between the side chains. **b**, Position change of side-chain when D1 Trp-14 is oxidized to N-formylkynurenine. **c**, Position change of side-chain when D1 Trp-14 is substituted to Phe. **d**, The fluctuation of atoms at D1 Trp-14 in the MD simulation. **e**, Averaged C β -C β distance between side chains of D1 Trp-14 and PsbI Ser-25. The error bars represent the standard deviations of the distances. The C β atoms are indicated as red arrowheads in **a**.

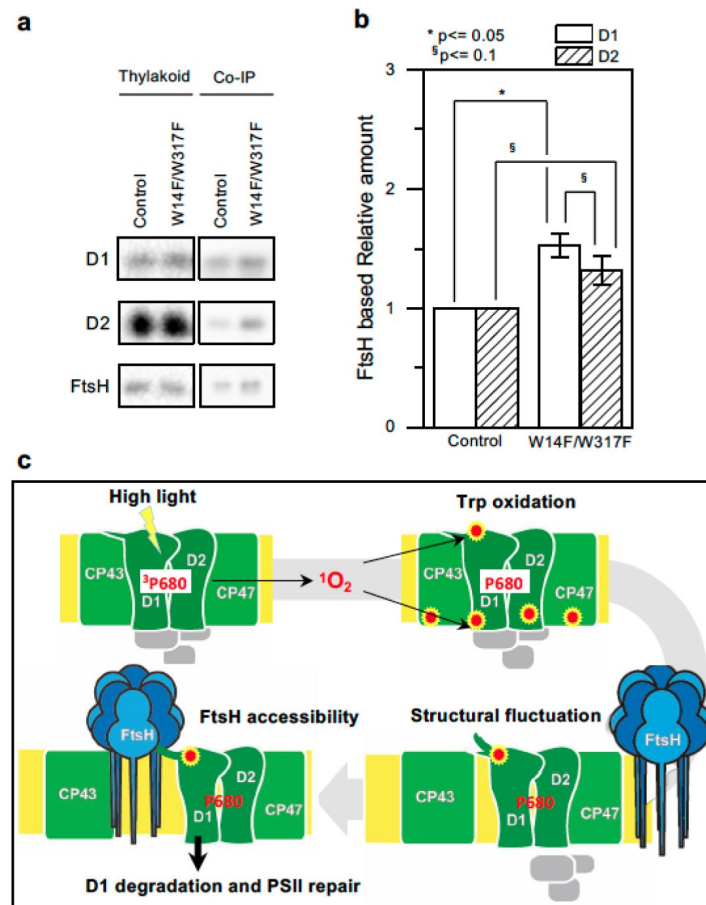


Figure 6.

Augmented affinity of FtsH with D1 by W14F/W317F.

a, Coimmunoprecipitation was performed with anti-FtsH antibody using the thylakoid membrane isolated from control or D1-W14F/W317F. The polypeptides of thylakoid membrane or coimmunoprecipitated samples were separated by SDS-PAGE and detected by immunoblotting with anti-D1, anti-D2, and anti-FtsH antibody. **b**, The immunoblotting signals are quantified and the ratio of D1 or D2 to FtsH are calculated. The averaged value and standard error for three biological replicates are shown. Significant difference was calculated by t-test and 0.1 (§) or 0.05 (*) probability confidence were indicated respectively. **c**, A proposed model of photodamaged D1 recognition, in which Trp oxidation plays a role in recruiting FtsH. FtsH heterocomplexes (blue) and PSII core proteins (green) along with oxygen evolving protein complex (gray) in the thylakoid membrane are schematically shown. Trp-oxidized residues (red) are localized at both lumenal and stromal sides. Trp-14 located at the N-terminus alpha helix enhances association of FtsH, whose catalytic site faces stroma.

Previous studies have reported OPTM of several Trp residues in PSII core proteins in vitro (Dreaden Kasson et al., 2012) (Dreaden et al., 2011) (Anderson et al., 2002). In addition, Dogra et al. confirmed several oxidized Trp residues in PSII core proteins of *Arabidopsis* in vivo (Dogra et al., 2019). Our mass-spectrometry in *Chlamydomonas* further indicated that oxidation in some of the Trp residues was detected among algae and land plants. It is noteworthy that a majority of Trp residues are located on the luminal side (Table 1) in the vicinity of the $\text{Mn}_4\text{O}_5\text{Ca}$ cluster, consistent with the previous observations that amino acid residues around the $\text{Mn}_4\text{O}_5\text{Ca}$ cluster are oxidized at the early stage of photoinhibition (Kale et al., 2017) (Frankel et al., 2012). However, OPTM is not limited to the luminal side but also found in the stromal side, which accounts for photoinhibition at the electron acceptor side (Vass, 2012). Our data indicated the presence of commonly oxidized Trp residues as represented by Trp-14 and Trp-317 in D1 protein. These are in fact Trp residues highly conserved among photosynthetic organisms (Fig. 1). Technically, detection of light-dependent OPTM was considered to be difficult; an extensive OPTM leads to fully inactive PSII, whereas D1 degradation in the PSII repair may diminish OPTM under optimal light conditions. We therefore assumed that OPTM associated with PSII repair might accumulate significantly in the mutant that is defective in FtsH. Supporting this, our quantitative mass spectrometry indicated that *var2* lacking FtsH2 in *Arabidopsis* had increased levels of oxidation in Trp residues, particularly in Trp-14 and Trp-317. Although indirect, these results strongly suggested an interconnection between Trp oxidation and D1 degradation.

OPTM of Trp residues causes irreversible modification and is likely to mark photo-damaged D1 protein as a substrate for degradation. In the PSII repair, a series of events including migration of photo-damaged PSII to non-appressed regions of thylakoid membranes, release of CP43 from the PSII, and recognition of photo-damaged D1 for selective D1 degradation, are essential. In this scenario, FtsH interacts with a partially disassembled PSII complex lacking CP43 protein, called RC47 (Kato and Sakamoto, 2009; Järvi et al., 2015). Close access to the photo-damaged D1, followed by the recognition of its N-terminal region, is concomitantly necessary for FtsH to proceed with processive D1 degradation. Therefore, the OPTM would be involved in the CP43 disassembly or the recognition of damaged D1 protein. A recent study showed that exogenous ROS treatment leads to PSII disassembly supports this model (McKenzie and Puthiyaveetil, 2023). Krynická et al. indicate that the accessibility to PSII core proteins drives selective protein degradation by FtsH in the cyanobacterium *Synechocystis* PCC 6803 (Krynická et al., 2015). This observation suggests that D1 protein in the RC47 complex is promptly degraded even if D1 did not suffer from photodamage. On the other hand, all site-directed mutants mimicking Trp oxidation by Trp to Phe substitution have stable and functional PSII complexes under growth light, suggesting that the OPTM would not induce the disassembly of CP43. We also tested whether Trp oxidation in CP43 affects PSII repair by site-directed mutagenesis. Similarly to the case in D1, none of Trp substitutions at the site of OPTM in CP43 (Trp-353 and Trp-375) affected the D1 degradation. Additionally, the CP43 transformants did not show the increased photosensitivity (Fig. S6). Our results somewhat appear to contradict the previous report in cyanobacterium that the mutants in which Trp-353 (Trp-352 in *Synechocystis* 6803) was substituted to Leu, Cys, or Ala increased photosensitivity under high-light conditions (Anderson et al., 2002). This might be due to the use of extremely high-light irradiation ($5,000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$), under which severe photo-damage in PSII complex was rendered. We consider that irreversible Trp oxidation in CP43, if to be repaired, may require a rapid turnover rate comparable to D1 degradation, which is not the case. Although further study is necessary to elucidate the disassembly mechanisms of CP43 during the PSII repair cycle, Trp oxidation in D1, rather than CP43 disassembly, might be important for the recognition of FtsH.

To examine its effect on D1 degradation, we performed site-directed mutagenesis of the corresponding Trp residues using *Chlamydomonas* chloroplast transformation. While Trp to Ala substitution in these sites (W14A or W317A) appeared to compromise PSII complex formation, Trp to Phe substitution (W14F and W317) gave us a hint in the critical role of OPTM. We showed that

Accession	Protein	Sequence	Range	Modified Trp residue	Oxidation status
DAA00922.1_20	D1	ENSSL(W*)AR	9-16	Trp14	OIA, NFK, KYN
		FC ^{cam} E(W*)ITSTENR	17-27	Trp20	OIA, NFK, KYN
		E(W*)WELSFR	130-136	Trp131	OIA, NFK, KYN
		VLNT(W*)ADIINR	313-323	Trp317	OIA, NFK, KYN
DAA00964.1_63	D2	T(W*)FDDADDWLR	13-23	Trp14	OIA, NFK, KYN
		TWFDDADD(W*)LR	13-23	Trp21	OIA, NFK, KYN
		T(W*)FDDADD(W*)LR	13-23	Trp14, Trp21	OIA, NFK, KYN
		A(W*)MAAQDQPHER	327-338	Trp328	OIA, NFK, KYN
		A(W*)M ^{ox} AAQDQPHER	327-338	Trp328	OIA, NFK, KYN
DAA00966.1_65	CP43	DQETTGF(A(W*))WSGNAR	15-29	Trp23	OIA, NFK, KYN
		DQETTGF(A(W*))SGNAR	15-29	Trp24	OIA, NFK, KYN
		DQETTGF(A(W*))SGNAR	15-29	Trp23, Trp24	OIA, NFK, KYN
		AM ^{ox} YFGGVYDT(W*)APGGGDVR	167-185	Trp177	OIA, NFK, KYN
		GP(W*)LEPLR	351-358	Trp353	OIA, NFK, KYN
		NDIQP(W*)QER	370-378	Trp375	OIA, NFK, KYN
DAA00933.1_31	CP47	YQ(W*)DQGFFQQEIQK	273-286	Trp275	OIA, NFK, KYN
		VQASLAEGASLSDA(W*)SR	288-304	Trp302	OIA, NFK, KYN
		TGAM ^{ox} NSGDGIAVG(W*)LGHASFK	327-347	Trp340	OIA, NFK, KYN

Ccam, Cyc carbamidomethylation; W*, Trp oxidative modifications; Mox, Met oxidation.

Table 1.

Trp oxidation in *Chlamydomonas* PSII core proteins

W14F, but not W317F, caused higher photo-sensitivity with the rapid decrease of D1 under high-light irradiation (**Fig. 2**). Given that W14F affected neither D1 synthesis (**Fig. S7**), stability of PSII complex formation, nor PSII activity under non-photoinhibitory conditions, it was concluded that the mutation results in enhanced D1 degradation. In our D1 degradation assay of wild type, generally, D1 turnover is too fast to detect unless inhibitor of chloroplast protein synthesis (CAM) is added. In sharp contrast, W14F proceeds with rapid D1 degradation even without CAM. Reportedly, numerous amino acid substitutions have been introduced in D1, which may or may not compromise PSII activity. To our knowledge, however, mutations that accelerate D1 degradation have not been found except for W14F in this study. We thus consider that Trp-14 is particularly important, at least for FtsH to recognize photodamaged D1 as described below.

Because FtsH-mediated D1 degradation is crucial for the PSII repair cycle, recognition of photodamaged D1 by FtsH protease is a critical step. Following observations suggest that Trp-14 oxidation is one of the key OPTMs for degrading photodamaged D1. First, enhanced D1 degradation in W14F well fits the notion that PTM in the N-terminus of D1 is important to execute processive degradation by FtsH, as proposed previously. For example, the lack of an N-terminal helix attenuates proper D1 degradation (Komenda et al., 2007)(Michoux et al., 2016). The excision of N-terminal Met by organellar Met aminopeptidase and prokaryotic-like peptide deformylase was shown to be required for FtsH-mediated D1 degradation (Adam et al., 2011). Phosphorylation of the D1 N-terminus was also shown to affect proteolysis and contribute to the spatiotemporal regulation of D1 degradation pathway (Koivuniemi et al., 1995)(Rintamäki et al., 1996)(Kato and Sakamoto, 2014). Together with these, it is possible that Trp-14 oxidation is likely to play a role in ‘photodamage-dependent’ degradation, although we cannot rule out the possibility that other OPTMs may have additive effects. Second, our MD simulation strongly suggests that W14F is similar to Trp-14 oxidation W14* (modified as NFK) in terms of allowing a regional conformational change around the N-terminal helix, thereby increasing fluctuation of the side chain. This fluctuation appears to be manifested by losing hydrogen bonding with Ser-25 of PsbI, a short peptide localized close to D1 and CP43 in the PSII core complex. Although further studies are needed, our simulation is consistent with our notion that Trp-14 is a target of OPTM that alters subtle but critical structural change at the N-terminus of D1.

Based on these observations, we propose a working model of ‘photodamaged D1 recognition’ in which Trp oxidation plays a role in processive degradation by FtsH (**Fig. 6c**). As a consequence of photoinhibition, ROS is produced around PSII and leads to OPTM of numerous residues. Among these, Trp-14 and Trp-317 are prone to oxidation likely due to their relative positions in PSII. While oxidation takes place in both, Trp-14 causes a conformational change at the N-terminus, which triggers enhanced access of FtsH for subsequent processive degradation. Supporting this, we observed augmented association between D1 and FtsH in W14F/W317F (**Fig. 6**). It is unlikely, however, that Trp-14 oxidation alone is sufficient to drive degradation of photodamaged D1, because a stepwise dissociation of PSII core complexes is prerequisite. It is also possible that other oxidative modifications are synergistically involved in D1 degradation. A recent study using *Thermosynechococcus vulcanus* suggests that an oxidative modification of Phe at the DE-loop of the D1 protein disrupts the interaction between the PsbT and D1 (Forsman and Eaton-Rye, 2021). The stroma-exposed DE-loop of D1 is one of the possible cleavage sites by Deg protease, which facilitates the effective D1 degradation by FtsH in photoinhibitory conditions (Kato et al., 2012). Although W14 oxidation would be a key signal for D1 degradation by FtsH, other oxidative modifications in D1 could serve as a general degradation signal. Further study to directly monitor the oxidized D1 captured by FtsH will address these questions.

OPTM of Trp residues has been observed in various proteins (Kasson and Barry, 2012). For example, Trp oxidation has been identified in ATP synthase alpha subunit in mitochondria, one of the target proteins for oxidative stress in the mitochondrial inner membrane (Rexroth et al., 2012). In this case, the oxidation is not random but selectively targets specific Trp. The oxidized ATP synthase might be degraded by mitochondrial FtsH homologs, m-AAA and i-AAA proteases,

which have an essential role in the quality control of aberrant proteins in mitochondrial membranes. Furthermore, a previous study in chloroplasts suggests the Trp oxidation in the stress responses related to singlet oxygen; Dogra et al. (2019) [report](#) specific oxidation of Trp in EXECUTER1 (EX1), a sensor protein of singlet oxygen in plastid signaling. The oxidation of specific Trp residue is required for ROS signaling mediated by light-dependent EX1 degradation by FtsH (Dogra et al., 2019 [report](#)). Together with our results, these reports imply a general mechanism between oxidized modification of target protein and substrate recognition by FtsH. Future proteomic approaches for investigating OPTM will reveal the general substrate recognition mechanisms by FtsH in the thylakoid membranes.

Methods

Detection of Trp oxidation in *Arabidopsis*

Chloroplasts were isolated from 3-week-old plants of WT and *var2* (SAIL_253_A03) grown under continuous light (80 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ at $20 \pm 2^\circ\text{C}$) conditions. The collected rosette leaves were homogenated in chloroplast isolation buffer [50 mM Hepes-KOH pH 8, 5 mM MgCl_2 , 5 mM EDTA pH 8, 5 mM EGTA pH 8, 10 mM NaHCO_3 , and 0.33 M D-sorbitol, supplemented with SIGMAFAST™ Protease Inhibitor (1 tablet per 100 ml)]. The homogenate was filtered through four layers of Miracloth and centrifuged at $400 \times g$ for 8 min at 4°C . The pellets were suspended in isolation buffer and loaded onto a two-step Percoll gradient (40:80%) solution to separate intact and broken chloroplasts. The intact chloroplasts enriched between the two Percoll steps were carefully collected and washed twice with HS buffer (50 mM Hepes-KOH pH 8, and 0.33 M D-sorbitol). Chloroplasts corresponding to equal amounts of chlorophyll were lysed, and the proteins extracted using 6 M guanidine hydrochloride buffer (guanidine hydrochloride dissolved in 100 mM Tris, pH 8.5). The lysed samples were sonicated in an ice bath for 1 min with a pulse of 3 s ‘on’ and 5 s ‘off’, followed by heating at 95°C for 5 min, and then centrifugation at 21 000 g for 30 min at 4°C . Total protein content was estimated using a Pierce™ BCA protein assay kit (ThermoFisher Scientific).

Mass spectrometric analysis for protein identification and PTM analysis was done according to our previous study (Dogra et al., 2019 [report](#)). For MS analysis, equal amounts of total protein (2 $\mu\text{g } \mu\text{L}^{-1}$) from three independent biological samples were denatured using 10 mM DTT at 56°C for 30 min followed by alkylation in 50 mM iodoacetamide at room temperature for 40 min in the dark. Reduced-alkylated proteins were then desalted a Nanosep membrane (Pall Corporation, MWCO 10K) in 200 μL of 100 mM NH_4HCO_3 buffer, followed by digestion in buffer containing 40 ng/ μL trypsin in 100 mM NH_4HCO_3 (corresponding to the enzyme-to-protein ratio of 1:50) at 37°C for 20 h. The digested peptides were dried and resuspended in 0.1% (v/v) formic acid solution. Digested peptides were separated using nanoAcquity Ultra Performance LC (Waters, Milford, MA, USA) and analyzed by using Q Exactive Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) as described in our previous study (Dogra et al., 2019 [report](#)). The mass spectra were submitted to the Mascot Server (version 2.5.1, Matrix Science, London, UK) for peptide identification and scanned against the *Arabidopsis* protein sequences (downloaded from TAIR; <http://www.arabidopsis.org/> [report](#)). Database searches were carried out with peptide mass tolerance of 20 ppm, fragment mass tolerance of 0.02 Da, and a maximum of two missed cleavages. Carbamidomethylation of Cys was set as a fixed modification, while oxidations of Met and Trp were defined as variable modifications. The significance threshold for search results was set at a *P*-value of 0.05 and an Ions score cut-off of 15. For quantification, raw MS data files were processed and analyzed using MaxQuant software (version 1.5.8.3) with a label-free quantitation (LFQ) algorithm. Parent ion and MS2 spectra were searched against the *Arabidopsis* protein sequences. The precursor ion tolerance was set at 7 ppm with an allowed fragment mass deviation of 20 ppm. Carbamidomethylation of Cys was set as a fixed modification, while oxidations of Met and Trp were defined as variable modifications. Peptides with a minimum of six amino acids and a maximum of two missed

cleavages were allowed. False discovery rate (FDR) was set to 0.01 for both peptide and protein identification. The absolute intensity values were used to calculate the abundance of oxidized peptides. Label-free quantitation of oxidized peptides using mass spectrometry were performed according to previously described method (Luber et al., 2010; Schwahnhauser et al., 2011; Duan et al., 2019).

Strains and generation of chloroplast transformants in *Chlamydomonas*

The *psbA* deletion mutant of the green alga *Chlamydomonas reinhardtii*, Fud7 (Bennoun et al., 1986) was used for chloroplast transformation in this study. The vector, which lacks large portion of *psbA* gene (Takahashi et al., 1996), was used for plasmid construction. Details in plasmid construction performed in this study were described as separate Supplementary Materials and Methods. To obtain *psbA* mutants, each *psbA* transformation vector was biolistically delivered into chloroplast of the Fud7 mutant using a particle gun (IDERA GIE-III, TANAKA Co. Ltd., Sapporo, Japan). Chloroplast transformants were selected by at least four rounds of single colony purification on TAP agar plates containing spectinomycin ($150 \mu\text{g mL}^{-1}$) as described previously (Takahashi et al., 1996). The CP43 mutants were generated according to Kuroda et al. (2021) (Kuroda et al., 2021). The *psbD* gene in the Fud7 was disrupted and we obtained Fud7- ΔpsbD mutant as a recipient for CP43 mutagenesis. The DNA delivery methods are the same with *psbA* mutagenesis experiment.

Detection of Trp oxidation in *Chlamydomonas*

Cultivation of the algae was carried out under constant light ($50 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ or $500 \mu\text{mol photons m}^{-2}\text{s}^{-1}$) in TAP medium for 24h. Cells were harvested by centrifugation ($2500 \times g$ for 5 min at room temperature), frozen in liquid nitrogen and stored at -80°C until further use. For protein extraction, lysis buffer (100 mM Tris/HCl pH 8.5, 2% (w/v) SDS, 1mM PMSF, 1 mM benzamidine) was added to frozen cell pellets and incubated for 10 min at 65°C and 1000 rpm in a Thermomixer (Eppendorf, Germany). The lysate was cleared by centrifugation ($18,000 \times g$ for 10 min at 25°C) and the protein content of the supernatant was determined using the Pierce BCA protein assay kit (Thermo Fisher Scientific). Reduction, alkylation and tryptic digestion ($50 \mu\text{g}$ of protein per sample) was performed in centrifugal filters (Amicon Ultra-0.5, 30 kDa cut-off, Merck Millipore) according to the FASP protocol (Wiśniewski et al., 2009). Peptides ($5 \mu\text{g}$ per sample) were desalted using self-packed C18-StageTips as previously described (Kulak et al., 2014), followed by vacuum centrifugation until dry. Prior to LC-MS/MS analysis peptide samples were resuspended in 2% (v/v) acetonitrile/0.05% (v/v) trifluoroacetic acid at a concentration at a concentration of $1 \mu\text{g}/\mu\text{L}$. LC-MS/MS analysis was carried out using an Ultimate 3000 nanoLC (Thermo Fisher Scientific) coupled to an Q Exactive Plus mass spectrometer (Thermo Fisher Scientific) via a nanospray interface. Samples ($1 \mu\text{L}$) were loaded on a trap column (C18, Acclaim PepMap 100, $300 \mu\text{M} \times 5 \text{ mm}$, $5 \mu\text{m}$ particle size, $100 \text{ \AA}$ pore size; Thermo Scientific) at a flow rate of $10 \mu\text{L}/\text{min}$ for 3 min using 2% (v/v) acetonitrile/0.05% (v/v) trifluoroacetic acid in ultrapure water. Subsequently, peptide separation was performed on a reversed phase column (C18, Acclaim Pepmap C18, $75 \mu\text{m} \times 50 \text{ cm}$, $2 \mu\text{m}$ particle size, $100 \text{ \AA}$ pore size, Thermo Fisher Scientific) at a flow rate of $250 \text{ nL}/\text{min}$ using the eluents 0.1% (v/v) formic acid in ultrapure water (A) and 80% (v/v) acetonitrile/0.1 % (v/v) formic acid in ultrapure water (B). The following gradient was applied: 2.5-5% B over 10 min, 5-22% B over 90 min, 22-30% B over 70 min, 30-99%B over 10 min, 99 % B for 20 min.

MS full scans (m/z 350-1600) were acquired in positive ion mode at a resolution of 70,000 (FWHM, at m/z 200) with internal lock mass calibration on m/z 445.120025. The AGC target was set to $3e6$ and the maximum injection time to 50 ms. For MS2, the 12 most intense ions with charge states 2-4 were fragmented by higher-energy c-trap dissociation (HCD) at 27% normalized collision energy. AGC target value was set to $5e4$, minimum AGC target to $5.5e2$, maximum injection time to 55 ms and precursor isolation window to 1.5 m/z .

Peptide and protein identification were carried out in Proteome Discoverer 2.4 (Thermo Fisher Scientific) using the MSFragger node (MSFragger 3.0) (Kong et al., 2017) with default parameters for closed searches (precursor mass tolerance: 50 ppm, precursor true tolerance: 20 ppm, fragment mass tolerance: 20 ppm, maximum missed cleavages: 1). Spectra were searched against a concatenated sequence database containing nucleus-encoded proteins (www.phytozome.org (<http://www.phytozome.org/>), assembly version 5.0, annotation version 5.6), supplemented with proteins encoded in the chloroplast (NCBI BK000554.2) and mitochondria (NCBI NC_001638.1), as well as common contaminants (cRAP, www.thegpm.org/crap/ (<http://www.thegpm.org/crap/>)). Carbamidomethylation was set as static modification. The following variable modifications were defined: N-acetylation of protein N-termini, oxidation of methionine, and various products of tryptophan oxidation (kynurenine (+3.995 Da), hydroxytryptophan (+15.995 Da), hydroxykynurenine (+19.990 Da), N-formylkynurenine (+31.990 Da), dihydroxy-N-formylkynurenine (+63.980 Da). Peptide-spectrum-matches (PSMs) were filtered using the Percolator node to satisfy a false discovery rate (FDR) of 0.01. Subsequently, identifications were filtered to achieve a peptide and protein level FDR of 0.01.

Growth test

Cells were grown in TAP liquid medium without shaking at 23–24°C under the light-dark synchronized condition (10 hours light at 50 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ or less and 14 hours darkness). Subsequently the cells were harvested by centrifugation at 2000 $\times$ g for 10 min at 25°C and were suspended in TP (Tris Phosphate) medium for washing. After finishing the washing process, the cell concentration was adjusted at 25 ng Chl μL^{-1} with TP medium. The liquid culture was spotted on solid medium at 100 ng Chlorophylls/spot. When we evaluate the cellular growth rate in the liquid culture, the cells grown under 30 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ in TAP medium were suspended in the TP medium at 0.1 of OD750 and were incubated under 30 or 350 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$.

Measurement of photosynthetic activity

Chlorophyll fluorescence induction kinetics of *Chlamydomonas* transformants were measured using a pulse amplitude-modulated fluorometer (Dual-PAM-100; Heinz Walz GmbH). Before measurements, cultured cells were maintained in the dark for 5 min to oxidize the plastoquinone pool fully. Initial fluorescence yield of PSII (F_0) and maximal fluorescence yield of PSII (F_M) were measured. Maximal PSII quantum yield (F_V/F_M) was determined as $F_V/F_M = (F_M - F_0)/F_M$. Light-induced oxygen-evolving activity of cells was measured using a Clark-type O_2 electrode (Oxytherm OXYT1; Hansatech Instruments). Briefly, cells were grown in TAP culture under 5 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ to reach 5–10 $\mu\text{g Chl mL}^{-1}$. O_2 -evolving activity of cells (5 $\mu\text{g Chl mL}^{-1}$) in the presence of 0.3 mM 2,6-dichloro-1,4-benzoquinone was measured using a Clarke-type O_2 electrode with an actinic light at 7,800 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ at 25°C as described (Kuroda et al., 2014).

Immunoblotting

Total proteins were solubilized in SDS-PAGE sample (125 mM Tris-HCl, pH 6.8, 2% [w/v] SDS, 100 mM dithiothreitol, 10% [v/v] Glycerol, 0.05% [w/v] BPB) buffer at 96°C for 1 min, and then were loaded based on equal chlorophyll. The proteins were electrophoretically transferred onto polyvinylidene difluoride membrane (Atto Corp.) after SDS-PAGE. The membranes were incubated with specific polyclonal antibodies: anti-D1 (raised against N-terminus, dilution 1:5,000) (Kato et al., 2012), anti-D2 (AS06 146, Agrisera; dilution, 1:5,000), anti-CP43 (AS11 1787, Agrisera; dilution, 1:5,000), anti-PsaA (a gift from Kevin Redding, Arizona State University, dilution 1:5000), and anti-Lhca1, dilution 1:5000 (Ozawa et al., 2018). The signals were visualized by using a Luminata Forte Western HRP Substrate (Merck Millipore) with Molecular Imager ChemiDoc XRS+ imaging system (Bio Rad Laboratories, Inc., USA). Signal intensities were quantified using NIH Image.

D1 degradation assay

Cells were grown in TAP liquid medium at 22°C under continuous light-condition ($30 \mu\text{mol photons m}^{-2}\text{s}^{-1}$). Cultured cells were harvested by centrifugation at $600 \times g$ for 5 min. The cell pellets were resuspended in a new TAP liquid medium as a final concentration of $0.5 \mu\text{g Chl mL}^{-1}$. Then, the cells were preincubated in the presence or absence of chloramphenicol ($100 \mu\text{g mL}^{-1}$) in the dark for 30 min. Subsequently, the cells were incubated under high-light or growth light conditions (350 or $30 \mu\text{mol photons m}^{-2}\text{s}^{-1}$) with stirring. Cells in $400 \mu\text{l}$ culture were collected at each time points (30, 60, 90 min) by centrifugation, and the resulting cell pellet was resuspended in $100 \mu\text{l}$ of SDS-PAGE sample buffer.

Pulse labeling of chloroplastic proteins

Cells grown in pre-culture medium (TAP media with less sulfur) were harvested by centrifugation at $600 \times g$ for 5 min and were washed by TAP media containing no sulfur. After centrifugation, the cells were resuspended to $25 \mu\text{g Chl mL}^{-1}$ in TAP media containing no sulfur and incubated for 2h. Subsequently, sulfur-starved cells were labeled with $5 \mu\text{Ci mL}^{-1} \text{ } ^{35}\text{S}[\text{Na}_2\text{SO}_4]$ (American Radiolabeled Chemicals) in the light at $50 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ in the presence of $10 \mu\text{g mL}^{-1}$ cycloheximide. At each time point (1, 2, 4 min), cell samples were collected and immediately frozen in liquid nitrogen.

Thylakoid membrane isolation and the following co-immunoprecipitation in anoxic aqueous solution

Cells grown in TAP medium under $5 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ were harvested by centrifugation at $2,000 \times g$ for 10 min at 25°C. All buffers were incubated at 25°C for 60 minutes in the presence of 100 mM glucose, 40 U/mL glucose oxidase, and 50 U/mL catalase to remove oxygen before chilling. Cells were suspended in suspension buffer (10 mM HEPES-KOH pH 8.0), broken by double passage through an airbrush at a pressure of 0.2 MPa (0.2 mm aperture airbrush). The broken materials were suspended in high sucrose concentration solution (1.8 M sucrose, 10 mM HEPES-KOH pH 8.0), and then a low sucrose concentration solution (1.0 M sucrose, 10 mM HEPES-KOH pH 8.0) and suspension buffer were layered in this order. Thylakoid membrane was floated at the interface between high sucrose concentration solution and low sucrose concentration solution after centrifugation (at $20,000 \times g$, for 60 min, at 25°C). The recovered thylakoid membrane was suspended in the suspension buffer.

The anti-VAR2 antibody (Sakamoto, 2003 [DOI](#)) was conjugated with magnetic beads (Magnosphere™, MS 160/Tosyl, JSR life sciences, Japan) by the presence of the fully chemically synthesized polymer (Blockmaster™ CE210, JSR life sciences, Japan) according to the instruction manual. The conjugated and blocked magnetic beads were suspended in the suspension buffer (10 mM HEPES-KOH pH 8.0) after washing TBS-T. Prior to the incubation with solubilized thylakoid membrane, the magnetic beads were resuspended in the suspension buffer of which oxygen was removed enzymatically by incubating at 25°C for 60 minutes in the presence of 100 mM glucose, 40 U/mL glucose oxidase, and 50 U/mL catalase.

Thylakoid membrane was solubilized sequentially; thylakoid membrane (1.0 mg Chlorophyll/mL) was incubated with 1.0% (w/v) glyco-diosgenin (GDN) and subsequently n-dodecyl- α -maltoside was added at 1.0% (w/v), and finally the mixture was diluted at twice volume with suspension buffer. The solubilized material was incubated with FtsH conjugated magnetic beads for 60 minutes at 4°C after removal of debris by centrifugation (at $20,000 \times g$, for 1 min, at 4°C). The beads were washed 6 times with suspension buffer containing 0.02% (w/v) GDN and were incubated with elution buffer (125 mM Tris-HCl pH 6.8, 2% (w/v) Lithium Dodecyl sulfate, 0.1% (w/v) Sodium Dodecyl sulfate, and 25% (w/v) glycerol) for 60 minutes on ice. The eluted sample was directly

loaded on individual sample slot on SDS-PAGE to separate polypeptides. All buffers except elution buffer were incubated at 25°C for 60 minutes in the presence of 100 mM glucose, 40 U/mL glucose oxidase, and 50 U/mL catalase to remove oxygen before chilling.

Molecular dynamics simulations of D1 N-term in PSII complex

The MD simulations for PSII were performed using the X-ray crystal structure determined at 1.9-Å resolution (PDB: 3ARC)(Umena et al., 2011 [DOI](#)) and based on the same procedure described previously (Sakashita et al., 2017b [DOI](#))(Sakashita et al., 2017a [DOI](#))(Kawashima et al., 2018 [DOI](#)), except for the following points. To investigate the structural fluctuation of the N terminal region of the D1 subunit, we restructured the N-terminal region between D1-Met1 and D1-Ser10 that was lacking in the crystal structure, using MOE program (2018). After structural optimization with positional restraints on heavy atoms of the PSII assembly, the system was heated from 0.001 to 300 K over 5.0 ps, with a 0.05-fs time step. The positional restraints on heavy atoms were gradually released over 16.5 ns. After an equilibrating MD run for 40 ns, a production run was conducted over 495 ns with an MD time step of 1.5 fs. The SHAKE algorithm was used for hydrogen constraints (Ryckaert et al., 1977 [DOI](#)). The structure of the D1-W14F mutant was modeled from the crystal structure of WT. The MD simulations were based on the AMBER-ff14SB force field for protein residues and lipids (Maier et al., 2015 [DOI](#)). The water molecules were described by TIP3P model (Jorgensen et al., 1983 [DOI](#)). For NFK, we employed the generalized Amber force field (GAFF) parameter set (Wang et al., 2004 [DOI](#)). The atomic partial charges of NFK were determined by fitting the electrostatic potential by using the RESP procedure (Bayly et al., 1993 [DOI](#)) (for calculated charges, see **Fig. S8** [DOI](#)). The electronic wave functions were calculated after geometry optimization with the density functional theory of the B3LYP/6-31G** level by using JAGUAR (2013). MD Simulations were conducted using the MD engine NAMD (Phillips et al., 2005 [DOI](#)). The atomic fluctuation was calculated as the root mean square fluctuation (RMSF) of heavy atoms from the averaged structure of PSII over the whole MD trajectory.

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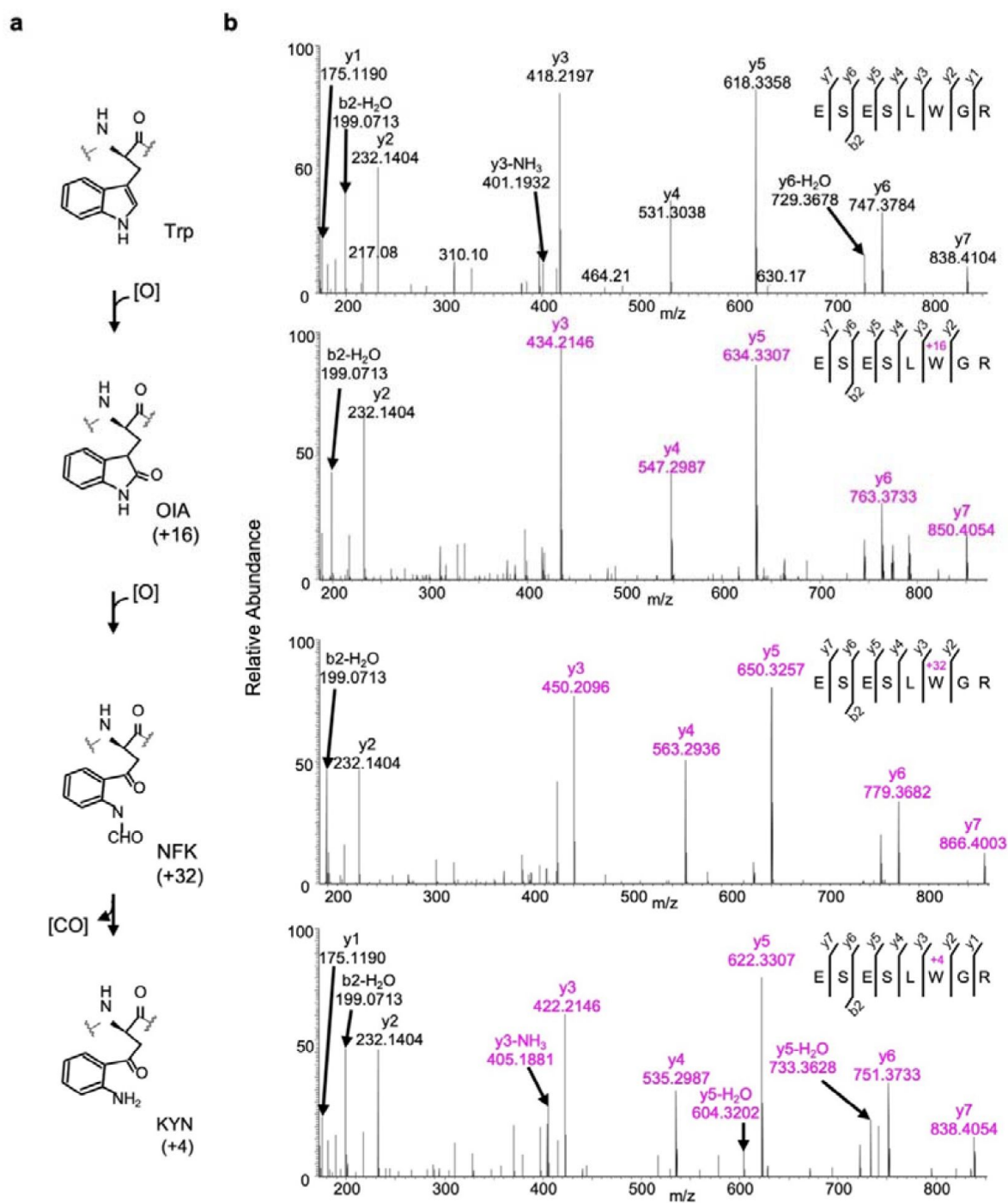
Additional information

Supplementary Table S1. Modification reported in Trp in PSII core proteins.

Supplementary Table S2. Trp oxidation in Chlamydomonas PSII core proteins.

Supplementary Materials and Methods.

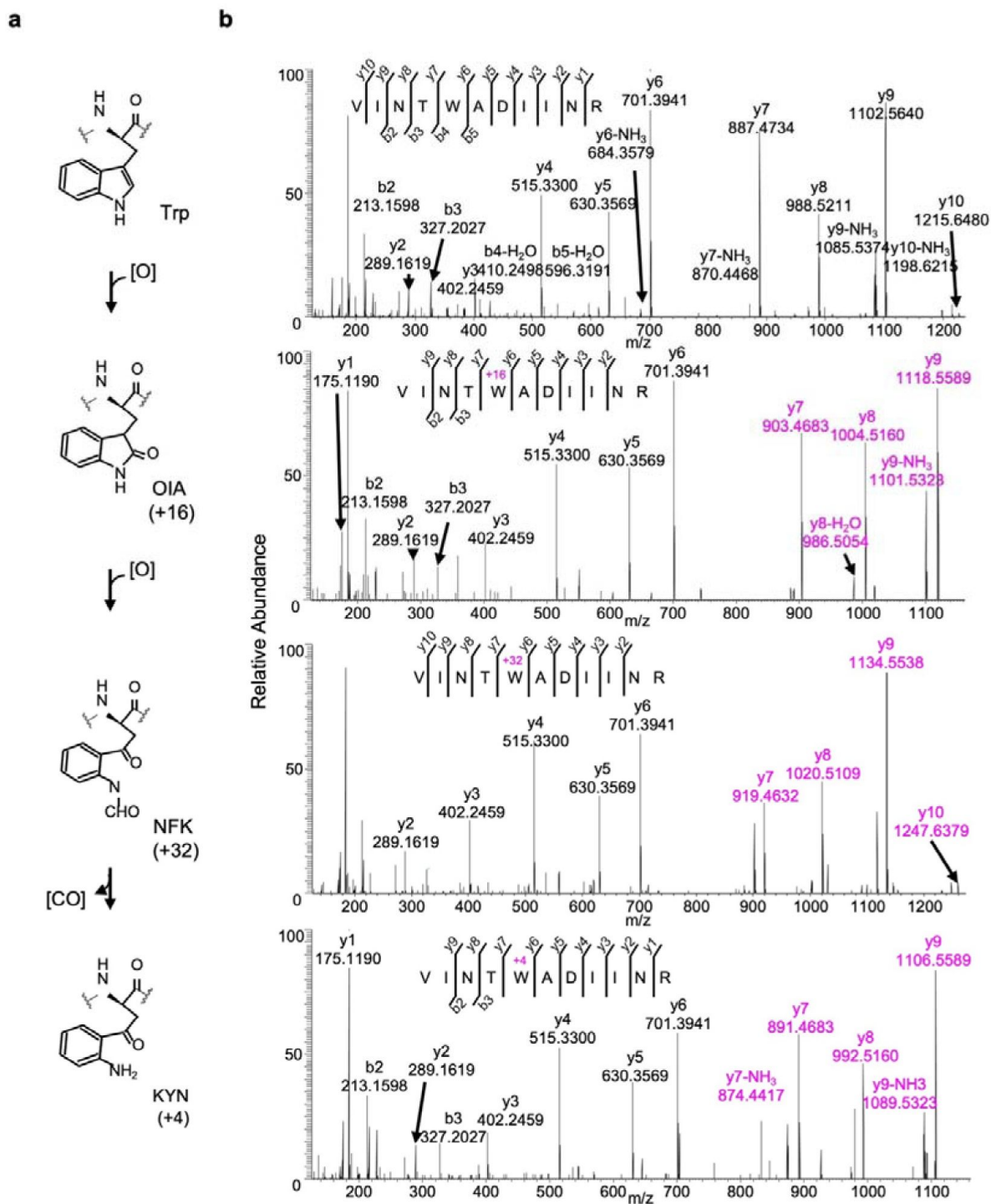
Correspondence and requests for materials should be addressed to W.S.



Supplementary Figure S1

Detected oxidative modifications at Trp14 of D1 in *Arabidopsis thaliana*.

a Trp-oxidation pathway. b Mass spectra of Trp14 carrying peptide ⁹ESEL(W)GR¹⁶ of D1 protein in *var2*. This oxidation led to the formation of oxindolylalanine (OIA), N-formylkynurenine (NFK), and kynurenine (KYN) with ₋16, ₋32, and ₋4 mass shifts, respectively.

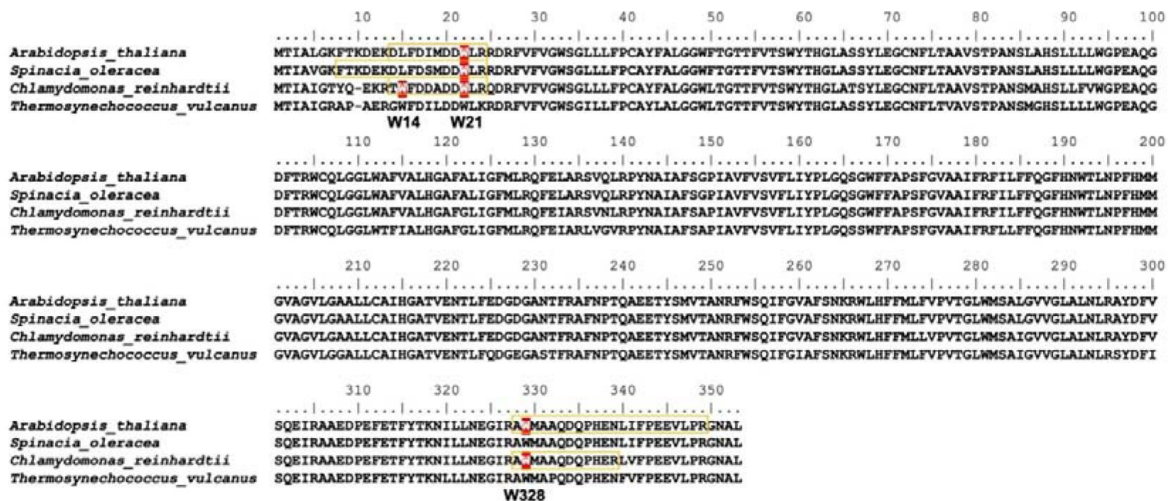


Supplementary Figure S2.

Detected oxidative modifications at Trp317 of D1 in *Arabidopsis thaliana*.

a Trp-oxidation pathway. b Mass spectra of Trp317 carrying peptide $^{313}\text{VINT(W)ADIINR}^{323}$ of D1 protein in *var2*. This oxidation led to the formation of oxindolylalanine (OIA), N-formylkynurenine (NFK), and kynurenine (KYN) with +16, +32, and +4 mass shifts, respectively.

a



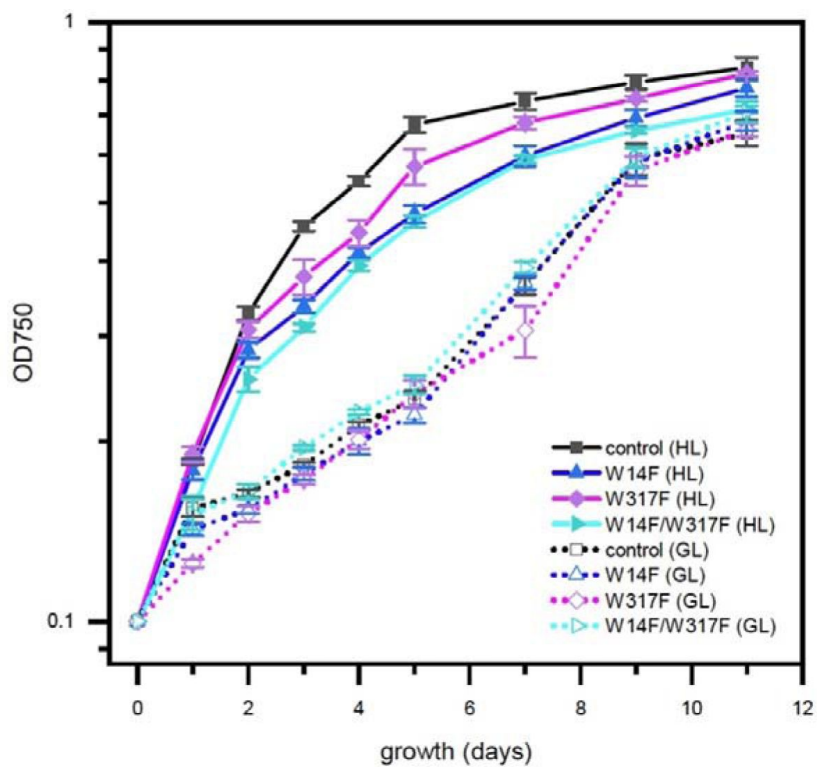
b



Supplementary Figure S3.

Positions of oxidized Trp residues in the identified peptide of PSII core complex by the MS-MS analysis.

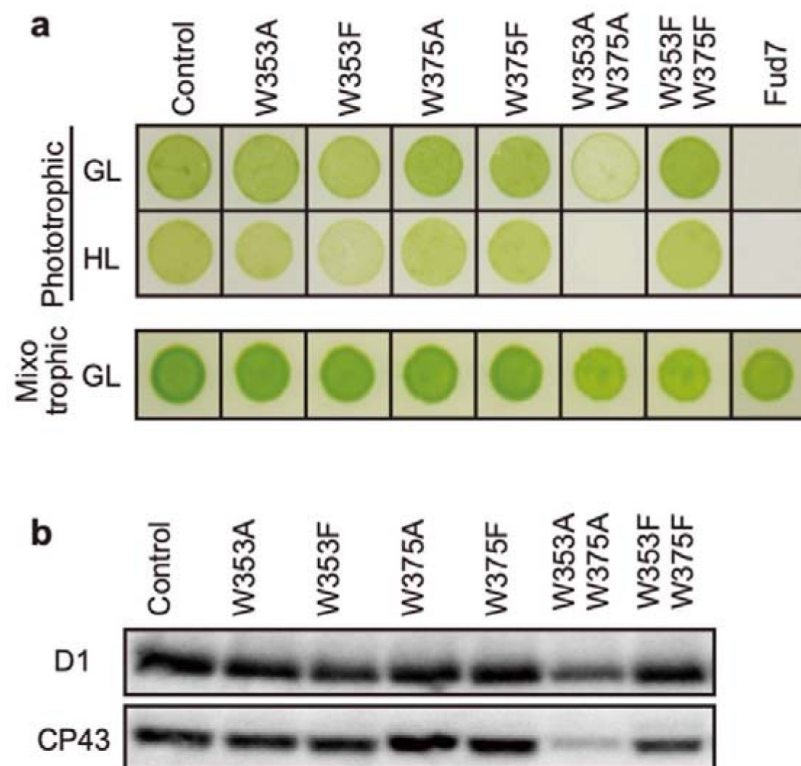
The oxidized Trp residues in D2 (a), CP43(b), and CP47 (c) were highlighted. Orange color boxes indicate the identified peptide by the MS-MS analysis. Oxidized Trp residues are highlighted in red.



Supplementary Figure S5.

High-light sensitive growth phenotype in the *Chlamydomonas* D1 transformants in which Trp-14 and Trp317 were mutated.

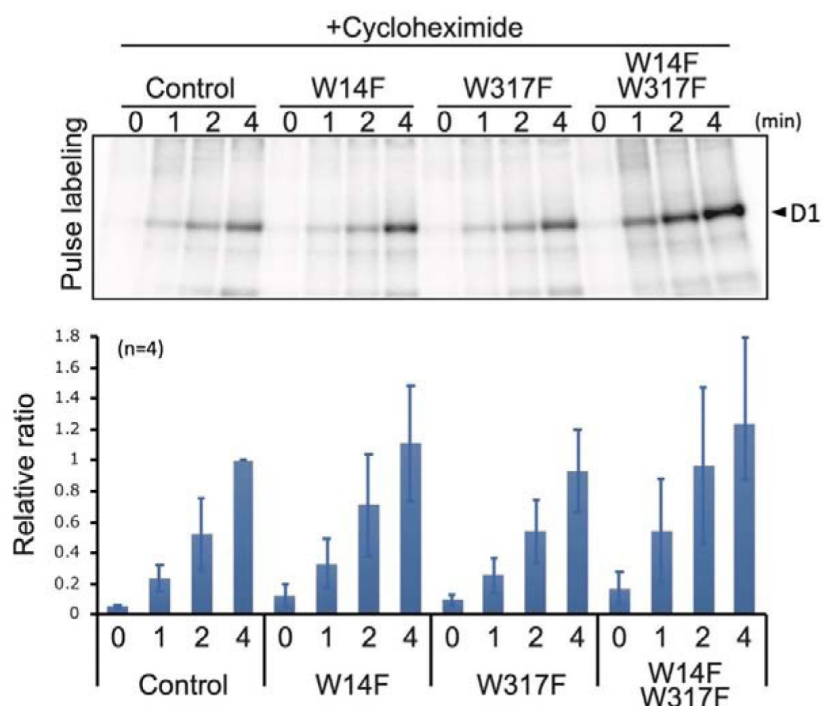
Phototrophic growth of Trp-substituted transformants on HSM medium. GL, growth light ($30 \mu\text{mol_photons m}^{-2}\text{s}^{-1}$); HL, high light ($320 \mu\text{mol_photons m}^{-2}\text{s}^{-1}$).



Supplementary Figure S6.

Characterization of *Chlamydomonas* CP43 transformants in which Trp-353 and Trp-375 were mutated.

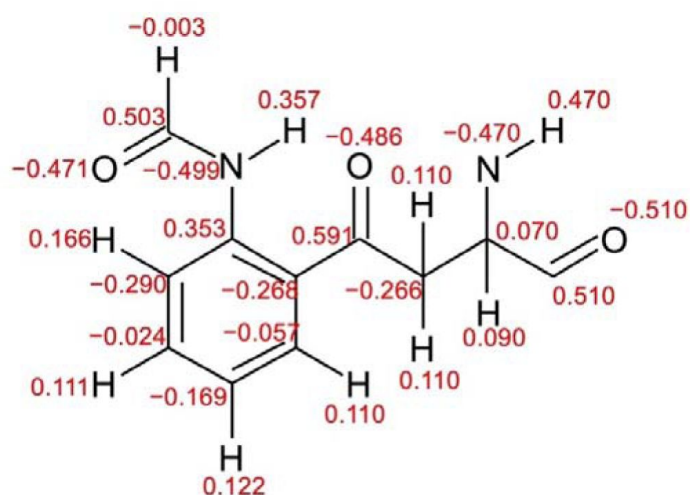
a, Phototrophic growth on HSM medium and mixotrophic growth on TAP medium at growth light (GL) at $30 \mu\text{mol m}^{-2}\text{s}^{-1}$ or high light, (HL) at $320 \mu\text{mol m}^{-2}\text{s}^{-1}$. b, Protein accumulation in the transformants. Thylakoid proteins of cells grown in TAP medium under growth light condition were separated by SDS-PAGE and analyzed by immunoblotting with antibodies against PSII subunits (D1 and CP43).



Supplementary Figure S7.

Protein synthesis in the transformants studied by in vivo protein labeling.

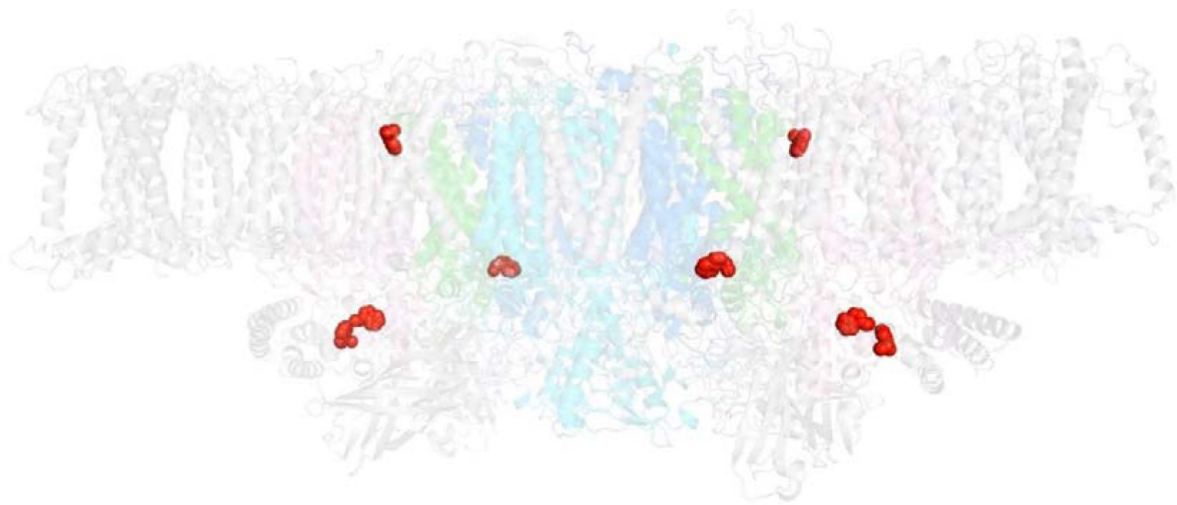
Cells were radio-labeled in vivo with ^{35}S , in the presence of cycloheximide for 1, 2, and 4 min. Total proteins were separated by SDS-PAGE. The bands corresponding to D1 is indicated by arrowheads. Quantified newly synthesized D1 levels using the Image J program are shown in bottom panels. To normalize values from four independent experiments, the ratio of control at 4 min was adjusted as 1, and the relative ratios are indicated. Values are means $\pm$ SD.



Supplementary Figure S8.

Atomic partial charges of NFK for MD simulations.

Red values represent atomic partial charges calculated by using the RESP procedure.



Supplementary Movie 1.

Oxidized Trp residues assigned in the PSII dimer (Movie is available online).

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Reviewer #1 (Public Review):

This manuscript tried to answer a long-standing question in an important research topic. I read it with great interest. The quality of the science is high, and the text is clearly written. The conclusion is exciting. However, I feel that the phenotype of the transgenic line may be explained by an alternative idea. At least, the results should be more carefully discussed.

Specific comments:

1. Stability or activity (Fv/Fm) was not affected in PSII with the W14F mutation in D1. If W14F really represents the status of PSII with oxidized D1, what is the reason for the degradation of almost normal D1?
2. To focus on the PSII in which W14 is oxidized, this research depends on the W14F mutant lines. It is critical how exactly the W-to-F substitution mimics the oxidized W. The authors tried to show it in Figure 5. Because of the technical difficulty, it may be unfair to request more evidence. But the paper would be more convincing with the results directly monitoring the oxidized D1 to be recognized by FtsH.

3. Figure 3. If the F14 mimics the oxidized W14 and is sensed by FtsH, I would expect the degradation of D1 even under the growth light. The actual result suggests that W14F mutation partially modifies the structure of D1 under high light and this structural modification of D1 is sensed by FtsH. Namely, high light may induce another event which is recognized by FtsH. The W14F is just an enhancer.

Reviewer #2 (Public Review):

In their manuscript, Kato et al investigate a key aspect of membrane protein quality control in plant photosynthesis. They study the turnover of plant photosystem II (PSII), a hetero-oligomeric membrane protein complex that undertakes the crucial light-driven water oxidation reaction in photosynthesis. The formidable water oxidation reaction makes PSII prone to photooxidative damage. PSII repair cycle is a protein repair pathway that replaces the photodamaged reaction center protein D1 with a new copy. The manuscript addresses an important question in PSII repair cycle - how is the damaged D1 protein recognized and selectively degraded by the membrane-bound ATP-dependent zinc metalloprotease FtsH in a processive manner? The authors show that oxidative post-translational modification (OPTM) of the D1 N-terminus is likely critical for the proper recognition and degradation of the damaged D1 by FtsH. Authors use a wide range of approaches and techniques to test their hypothesis that the singlet oxygen (1O_2)-mediated oxidation of tryptophan 14 (W14) residue of D1 to N-formylkynurenine (NFK) facilitates the selective degradation of damaged D1. Overall, the authors propose an interesting new hypothesis for D1 degradation and their hypothesis is supported by most of the experimental data provided. The study certainly addresses an elusive aspect of PSII turnover and the data provided go some way in explaining the light-induced D1 turnover. However, some of the data are correlative and do not provide mechanistic insight. A rigorous demonstration of OPTM as a marker for D1 degradation is yet to be made in my opinion. Some strengths and weaknesses of the study are summarized below:

Strengths:

1. In support of their hypothesis, the authors find that FtsH mutants of *Arabidopsis* have increased OPTM, especially the formation of NFK at multiple Trp residues of D1 including the W14; a site-directed mutation of W14 to phenylalanine (W14F), mimicking NFK, results in accelerated D1 degradation in *Chlamydomonas*; accelerated D1 degradation of W14F mutant is mitigated in an *ftsH1* mutant background of *Chlamydomonas*; and that the W14F mutation augmented the interaction between FtsH and the D1 substrate.
2. Authors raise an intriguing possibility that the OPTM disrupts the hydrogen bonding between W14 residue of D1 and the serine 25 (S25) of PsbI. According to the authors, this leads to an increased fluctuation of the D1 N-terminal tail, and as a consequence, recognition and binding of the photodamaged D1 by the protease. This is an interesting hypothesis and the authors provide some molecular dynamics simulation data in support of this. If this hypothesis is further supported, it represents a significant advancement.
3. The interdisciplinary experimental approach is certainly a strength of the study. The authors have successfully combined mass spectrometric analysis with several biochemical assays and molecular dynamics simulation. These, together with the generation of transplastomic algal cell lines, have enabled a clear test of the role of Trp oxidation in selective D1 degradation.
4. Trp oxidative modification as a degradation signal has precedent in chloroplasts. The authors cite the case of 1O_2 sensor protein EXECUTER 1 (EX1), whose degradation by FtsH2, the same protease that degrades D1, requires prior oxidation of a Trp residue. The earlier observation of an attenuated degradation of a truncated D1 protein lacking the N-terminal

tail is also consistent with authors' suggestion of the importance of the D1 N-terminus recognition by FtsH. It is also noteworthy that in light of the current study, D1 phosphorylation is unlikely to be a marker for degradation as posited by earlier studies.

Weaknesses:

1. The study lacks some data that would have made the conclusions more rigorous and convincing. It is unclear why the level of Trp oxidation was not analyzed in the *Chlamydomonas ftsH* 1-1 mutant as done for the var 2 mutant. Increased oxidation of W14 OPTM in *Chlamydomonas ftsH* 1-1 is a key prediction of the hypothesis. It is also unclear to me what is the rationale for showing D1-FtsH interaction data only for the double mutant but not for the single mutant (W14F). Why is the FtsH pulldown of D2 not statistically significant (p value = {less than or equal to}0.1). Wouldn't one expect FtsH pulls down the RC47 complex containing D1, D2, and RC47. Probing the RC47 level would have been useful in settling this. A key proposition of the authors' is that the hydrogen bonding between D1 W14 and S25 of PsbI is disrupted by the oxidative modification of W14. Can this hypothesis be further tested by replacing the S25 of PsbI with Ala, for example?
2. Although most of the work described is in vivo analysis, which is desirable, some in vitro degradation assays would have strengthened the conclusions. An in vitro degradation assay using the recombinant FtsH and a synthetic peptide encompassing D1 N-terminus with and without OPTM will test the enhanced D1 degradation that the authors predict. This will also help to discern the possibility that whether CP43 detachment alone is sufficient for D1 degradation as suggested for cyanobacteria.
3. The rationale for analyzing a single oxidative modification (W14) as a D1 degradation signal is unclear. D1 N-terminus is modified at multiple sites. Please see McKenzie and Puthiyaveetil, bioRxiv May 04 2023. Also, why is modification by only 1O₂ considered while superoxide and hydroxide radicals can equally damage D1?
4. The D1 degradation assay seems not repeatable for the W14F mutant. High light minus CAM results in Fig. 3 shows a statistically significant decrease in D1 levels for W14F at multiple time points but the same assay in Fig. 4a does not produce a statistically significant decrease at 90 min of incubation. Why is this? Accelerated D1 degradation in the Phe mutant under high light is key evidence that the authors cite in support of their hypothesis.
5. The description of results at times is not nuanced enough, for e.g. lines 116-117 state "The oxidation levels in Trp-14 and Trp-314 increased 1.8-fold and 1.4-fold in var2 compared to the wild type, respectively (Fig. 1c)" while an inspection of the figure reveals that modification at W314 is significant only for NFK and not for KYN and OIA. Likewise, the authors write that CP43 mutant W353F has no growth phenotype under high light but Figure S6 reveals otherwise. The slow growth of this mutant is in line with the earlier observation made by Anderson et al., 2002. In lines 162-163, the authors talk about unchanged electron transport in some site-directed mutants and cite Fig. 2c but this figure only shows chl fluorescence trace and nothing else.
6. The authors rightly discuss an alternate hypothesis that the simple disassembly of the monomeric core into RC47 and CP43 alone may be sufficient for selective D1 degradation as in cyanobacteria. This hypothesis cannot yet be ruled out completely given the lack of some in vitro degradation data as mentioned in point 2. Oxidative protein modification indeed drives the disassembly of the monomeric core (McKenzie and Puthiyaveetil, bioRxiv May 04 2023).

Reviewer #3 (Public Review):

Light energy drives photosynthesis. However, excessive light can damage (i.e., photo-damage) and thus inactivate the photosynthetic process. A major target site of photo-damage is photosystem II (PSII). In particular, one component of PSII, the reaction center protein, D1, is very susceptible to photo-damage, however, this protein is maintained efficiently by an elaborate multi-step PSII-D1 turnover/repair cycle. Two proteases, FtsH and Deg, are known to contribute to this process, respectively, by efficient degradation of photo-damaged D1 protein processively and endoproteolytically. In this manuscript, Kato et al., propose an additional step (an early step) in the D1 degradation/repair pathway. They propose that "Tryptophan oxidation" at the N-terminus of D1 may be one of the key oxidations in the PSII repair, leading to processive degradation of D1 by FtsH. Both, their data and arguments are very compelling.

The D1 protein repair/degradation pathway in its simplest form can be defined essentially by five steps: (1) migration of damaged PSII core complex to the stroma thylakoid, (2) partial PSII disassembly of the PSII core monomer, (3) access of protease degrading damaged D1, (4) concomitant D1 synthesis, and (5) reassembly of PSII into grana thylakoid. An enormous amount of work has already been done to define and characterize these various steps. Kato et al., in this manuscript, are proposing a very early yet novel critical step in D1 protein turnover in which Tryptophan(Trp) oxidation in PSII core proteins influences D1 degradation mediated by FtsH.

Using a variety of approaches, such as mass-spectrometry (Table 1), site-directed mutagenesis (Figures 2-4), D1 degradation assays (Figures 3, and 4), and simulation modeling (Figure 5), Kato et al., provide both strong evidence and reasonable arguments that an N-terminal Trp oxidation may be likely to be a 'key' oxidative post-translational modification (OPTM) that is involved in triggering D1 degradation and thus activating the PSII repair pathway. Consequently, from their accumulated data, the authors propose a scenario in which the unraveling of the N-terminal of the D1 protein facilitated by Trp oxidation plays a critical 'recognition' role in alerting the plant that the D1 protein is photo-damaged and thus to kick start the processive degradation pathway initiated possibly by FtsH. Coincidentally, Forsman and Eaton-Rye (Biochemistry 2021, 60, 1, 53-63), while working with the thermophilic cyanobacterium, *Thermosynechococcus vulcanus*, showed that when the N-terminal DE-loop of the D1 protein is photo-damaged a disruption of the interaction between the PsbT subunit and D1 occurs which may serve as a signal for PSII to undergo repair following photodamage. While the activation of the processive degradation pathways in *Chlamydomonas* versus *Thermosynechococcus vulcanus* have significant mechanistic differences, it's interesting to note and speculate that the stability of the N-terminal of their respective D1 proteins seems to play a critical role in 'signaling' the PSII repair system to be activated and initiate repair. But it's complicated. For instance, significant Trp oxidation also occurs on the lumen side of other PSII subunits which may also play a significant role in activating the repair processes as well. Indeed, Kato et al., (Photosynthesis Research volume 126, pages 409-416 (2015)) proposed a two-step model whereby the primary event is disruption of a Mn-cluster in PSII on the lumen side. A secondary event is damage to D1 caused by energy that is absorbed by chlorophyll. But models adapt, change, and get updated. And the data provided by Kato et al., in this manuscript, gives us a unique glimpse/snapshot into the importance of the stability of the N-terminal during photo-damage and its role in D1-turnover. For instance, the author's use site-directed mutagenesis of Trp residues undergoing OPTM in the D1 protein coupled with their D1 degradation assays (Figure 3 and 4), provides evidence that Trp oxidation (in particular the oxidation of Trp14) in coordination with FtsH results in the degradation of D1 protein. Indeed, their D1 degradation assays coupled with the use of a *ftsH* mutant provide further significant support that Trp14 oxidation and FtsH activity are strongly linked. But for FtsH to degrade D1 protein it needs to gain access to photo-damaged D1. FtsH access to D1 is achieved by having CP43 partially dissociate from the PSII complex. Hence, the authors also addressed

the possibility that Trp oxidation may also play a role in CP43 disassembly from the PSII complex thereby giving FtsH access to D1. Using a site-directed mutagenesis approach, they showed that Trp oxidation in CP43 appeared to have little impact on the PSII repair (Supplemental Figure S6). This result shows that D1-Trp14 oxidation appears to be playing a role in D1 turnover that occurs after CP43 disassembly from the PSII complex. Alternatively, the authors cannot exclude the possibility that D1-Trp14 oxidation in some way facilitates CP43 dissociation. Further investigation is needed on this point. However, D1-Trp14 oxidation is causing an internal disruption of the D1 protein possibly at the N-terminus of the protein. Consequently, the role of Trp14 oxidation in disrupting the stability of the N-terminal domain of the D1 protein was analyzed computationally. Using a molecular dynamics approach (Figure 5), the authors attempted to create a mechanistic model to explain why when D1 protein Trp14 undergoes oxidation the N-terminal domain of D1 protein becomes unraveled. Specifically, the authors propose that the interaction between D1 protein Trp14 with PsbI Ser25 becomes disrupted upon oxidation of Trp14. Consequently, the authors concluded from their molecular dynamics simulation analysis that "the increased fluctuation of the first α -helix of D1 would give a chance to recognize the photo-damaged D1 by FtsH protease". Hence, the author's experimental and computational approaches employed here develop a compelling early-stage repair model that integrates 1) Trp14 oxidation, 2) FtsH activation and 3) D1- turnover being initiated at its N-terminal domain. However, a word of caution should be emphasized here. This model is just a snapshot of the very early stages of the D1 protein turnover process. The data presented here gives us just a small glimpse into the unique relationship between Trp oxidation of the D1 protein which may trigger significant N-terminal structural changes of the D1 protein that both signals and provides an opportunity for FtsH to begin protease digestion of the D1 protein. However, the authors go to great lengths in their discussion section to not overstate solely the role of Trp14 oxidation in the complicated process of D1 turnover. The authors certainly recognize that there are a lot of moving parts involved in D1 turnover. And while Trp14 oxidation is the major focus of this paper, the authors show in Supplemental Fig S4 the structural positions of various additional oxidized Trp residues in the *Thermosynechococcus vulcanus* PSII core proteins. Indeed, this figure shows that the majority of oxidized Trps are located on the luminal side of PSII complex clustered around the oxygen-evolving complex. So, while oxidized Trp14 may be involved in the early stages of D1 turnover certainly oxidized Trps on the lumen side are also more than likely playing a role in D1 turnover as well. To untangle this complex process will require additional research.

Nevertheless, identifying and characterizing the role of oxidative modification of tryptophan (Trp) residues, in particular, Trp14, in the PSII core provides another critical step in an already intricate multi-step process of D1 protein turnover during photo-damage.

Author Response

The following is the authors' response to the original reviews.

Reviewer #1 (Public Review):

This manuscript tried to answer a long-standing question in an important research topic. I read it with great interest. The quality of the science is high, and the text is clearly written. The conclusion is exciting. However, I feel that the phenotype of the transgenic line may be explained by an alternative idea. At least, the results should be more carefully discussed.

We thank the reviewer #1 for his/her comments that helped to improve the manuscript. We have incorporated changes to reflect the suggestions provided by the reviewer. Here is a point-by-point response to the reviewer's specific and other minor comments.

Specific comments:

1. *Stability or activity (Fv/Fm) was not affected in PSII with the W14F mutation in D1. If W14F really represents the status of PSII with oxidized D1, what is the reason for the degradation of almost normal D1?*

In this study, we used W14F mutation to mimic Trp-14 oxidation. The W14F mutant did not affect the stability and photosynthetic activity under normal growth conditions. However, the W14F mutant showed increased D1 degradation and reduced Fv/Fm values under high light. These results suggested that the W14F mutant has almost normal D1 protein stability under growth light conditions, as pointed out by the reviewer.

However, it should be noted that D1 protein in the W14F strain rapidly degraded under high light. In the discussion part, we mentioned the possibility that other OPTMs may have additive effects on D1 degradation. Synergistic effects such as different amino acid oxidations may cause D1 degradation, and among those oxidative damages, W14 oxidation would be a key signal for D1 degradation by FtsH.

1. *To focus on the PSII in which W14 is oxidized, this research depends on the W14F mutant lines. It is critical how exactly the W-to-F substitution mimics the oxidized W. The authors tried to show it in Figure 5. Because of the technical difficulty, it may be unfair to request more evidence. But the paper would be more convincing with the results directly monitoring the oxidized D1 to be recognized by FtsH.*

We agree that confirming the direct interaction of oxidized D1 protein with FtsH provides more robust evidence. However, since FtsH progressively degrades the trapped substrate, it would be quite a challenging attempt to capture that moment. There are also technical limitations to obtaining sufficient substrate using Co-IP to compare its oxidation state. We included your suggested point in the discussion part. Thank you for your valuable suggestion.

1. *Figure 3. If the F14 mimics the oxidized W14 and is sensed by FtsH, I would expect the degradation of D1 even under the growth light. The actual result suggests that W14F mutation partially modifies the structure of D1 under high light and this structural modification of D1 is sensed by FtsH. Namely, high light may induce another event which is recognized by FtsH. The W14F is just an enhancer.*

Our results indicated that W14 oxidation is one of the keys to D1 degradation. On the other hand, we agree with the possibility that the reviewer points out. There is the possibility that factors other than W14 may act synergistically to promote D1 degradation. High light triggered more D1 degradation in W14F, suggesting that unknown factor(s) may be required for D1 degradation, e.g., oxidative modification at other sites and/or conformational changes of PSII under the high light. However, the current data that we have cannot reveal. We have incorporated the reviewer's comment and discussed it in the discussion part.

Reviewer #2 (Public Review):

In their manuscript, Kato et al investigate a key aspect of membrane protein quality control in plant photosynthesis. They study the turnover of plant photosystem II (PSII), a

hetero-oligomeric membrane protein complex that undertakes the crucial light-driven water oxidation reaction in photosynthesis. The formidable water oxidation reaction makes PSII prone to photooxidative damage. PSII repair cycle is a protein repair pathway that replaces the photodamaged reaction center protein D1 with a new copy. The manuscript addresses an important question in PSII repair cycle - how is the damaged D1 protein recognized and selectively degraded by the membrane-bound ATP-dependent zinc metalloprotease FtsH in a processive manner? The authors show that oxidative post-translational modification (OPTM) of the D1 N-terminus is likely critical for the proper recognition and degradation of the damaged D1 by FtsH. Authors use a wide range of approaches and techniques to test their hypothesis that the singlet oxygen (1O_2)-mediated oxidation of tryptophan 14 (W14) residue of D1 to N-formylkynurenine (NFK) facilitates the selective degradation of damaged D1. Overall, the authors propose an interesting new hypothesis for D1 degradation and their hypothesis is supported by most of the experimental data provided. The study certainly addresses an elusive aspect of PSII turnover and the data provided go some way in explaining the light-induced D1 turnover. However, some of the data are correlative and do not provide mechanistic insight. A rigorous demonstration of OPTM as a marker for D1 degradation is yet to be made in my opinion. Some strengths and weaknesses of the study are summarized below:

We thank reviewer #2 for his/her comments that helped to improve the manuscript. We have incorporated changes to reflect the suggestions pointed out as weaknesses by reviewer #2. Other minor comments were also answered in a point-by-point response.

Strengths:

- 1. In support of their hypothesis, the authors find that FtsH mutants of Arabidopsis have increased OPTM, especially the formation of NFK at multiple Trp residues of D1 including the W14; a site-directed mutation of W14 to phenylalanine (W14F), mimicking NFK, results in accelerated D1 degradation in Chlamydomonas; accelerated D1 degradation of W14F mutant is mitigated in an ftsH1 mutant background of Chlamydomonas; and that the W14F mutation augmented the interaction between FtsH and the D1 substrate.*
- 1. Authors raise an intriguing possibility that the OPTM disrupts the hydrogen bonding between W14 residue of D1 and the serine 25 (S25) of PsbI. According to the authors, this leads to an increased fluctuation of the D1 N-terminal tail, and as a consequence, recognition and binding of the photodamaged D1 by the protease. This is an interesting hypothesis and the authors provide some molecular dynamics simulation data in support of this. If this hypothesis is further supported, it represents a significant advancement.*
- 1. The interdisciplinary experimental approach is certainly a strength of the study. The authors have successfully combined mass spectrometric analysis with several biochemical assays and molecular dynamics simulation. These, together with the generation of transplastomic algal cell lines, have enabled a clear test of the role of Trp oxidation in selective D1 degradation.*

1. *Trp oxidative modification as a degradation signal has precedent in chloroplasts. The authors cite the case of 1O2 sensor protein EXECUTER 1 (EX1), whose degradation by FtsH2, the same protease that degrades D1, requires prior oxidation of a Trp residue. The earlier observation of an attenuated degradation of a truncated D1 protein lacking the N-terminal tail is also consistent with authors' suggestion of the importance of the D1 N-terminus recognition by FtsH. It is also noteworthy that in light of the current study, D1 phosphorylation is unlikely to be a marker for degradation as posited by earlier studies.*

Weaknesses:

1. *The study lacks some data that would have made the conclusions more rigorous and convincing. It is unclear why the level of Trp oxidation was not analyzed in the Chlamydomonas ftsH 1-1 mutant as done for the var 2 mutant. Increased oxidation of W14 OPTM in Chlamydomonas ftsH 1-1 is a key prediction of the hypothesis.*

We thank the reviewer for this valuable comment. We agree with the reviewer that the analysis of oxidized Trp level will reinforce the importance of Trp oxidation in the N-terminal of D1. In our preliminary experiment, we observed a trend toward increase of the kynurenine in Trp-14 in Chlamydomonas ftsH1-1 strain. However, we found large errors, and we could not conclude that this trend is significant. A possible reason for the large error was that the signal intensity of oxidized Trp was insufficient for quantification in a series of Chlamydomonas experiment. In addition, the fact that the amount of D1 in each culture was not stable also might be one reason. On the other hand, we keep note of a previous result that more fragmentation of D1 protein was observed in the Chlamydomonas ftsH1-1 mutant compared to that in Arabidopsis (Malnoë et al., Plant Cell 2014). This result suggests that an alternative D1 degradation pathway involving other proteases is more active in the Chlamydomonas ftsH1-1 mutant than in Arabidopsis var2 mutant. Furthermore, the Chlamydomonas ftsH1-1 mutant, caused by an amino acid substitution, still has a significant FtsH1/FtsH2 heterohexamer, and the level of FtsH1 and FtsH2 proteins increases significantly under high light irradiation. This is a significant difference from the Arabidopsis var2 mutant lacking FtsH2 subunit and showed reduced protein accumulation. These factors may explain to the lower detection levels of oxidized Trp in Chlamydomonas. We believe that improved sensitivity for detection of oxidized Trp peptides and more sophisticated experimental systems could solve this issue in the future.

It is also unclear to me what is the rationale for showing D1-FtsH interaction data only for the double mutant but not for the single mutant (W14F).

We thank the reviewer for the comment. As suggested by the reviewer, the analysis of the mutant crossing ftsH and W14F single mutation will provide more convincing evidence. Fig.3 showed that the photosensitivity in both W14F and W14FW317F was caused by the enhanced D1 degradation observed, which was due to the W14F mutation. Therefore, we crossed the ftsH mutant with W14FW317F, which has a more severe phenotype, to confirm whether FtsH is involved in this D1 degradation.

Why is the FtsH pulldown of D2 not statistically significant (p value = {less than or equal to}0.1). Wouldn't one expect FtsH pulls down the RC47 complex containing D1, D2, and RC47. Probing the RC47 level would have been useful in settling this.

For the immunoblot result of D2 and its statistical analysis, we answered in the following comment; No.2 in the reviewer's comment in Recommendations For The Authors.

We agree with the reviewer's suggestion that further immunoblot analysis for CP47 protein would help our understanding of FtsH and RC47 interaction. Indeed, we attempted the immunoblot analysis of CP47 after the FtsH Co-IP experiment. However, the detection of CP43 protein was not sensitive enough. This reason may be due to the lower titer of the CP47 antibody compared to the D1 and D2 antibodies.

A key proposition of the authors' is that the hydrogen bonding between D1 W14 and S25 of PsbI is disrupted by the oxidative modification of W14. Can this hypothesis be further tested by replacing the S25 of PsbI with Ala, for example?

It is an interesting question whether amino acid substitution in PsbI-S25 affects the stability of D1-N-term and its degradation by FtsH. We would like to analyze the possibility in the future. We thank the reviewer for this helpful suggestion.

1. Although most of the work described is in vivo analysis, which is desirable, some in vitro degradation assays would have strengthened the conclusions. An in vitro degradation assay using the recombinant FtsH and a synthetic peptide encompassing D1 N-terminus with and without OPTM will test the enhanced D1 degradation that the authors predict. This will also help to discern the possibility that whether CP43 detachment alone is sufficient for D1 degradation as suggested for cyanobacteria.

In vitro experimental systems are interesting. However, FtsH is known to function as a hexamer, which has not yet been successfully reconstituted in vitro. Therefore, it would not be easy to perform an in vitro experimental system using the N-terminal synthetic peptide of D1 as a substrate. Thank you for your valuable suggestions.

1. The rationale for analyzing a single oxidative modification (W14) as a D1 degradation signal is unclear. D1 N-terminus is modified at multiple sites. Please see McKenzie and Puthiyaveetil, bioRxiv May 04 2023. Also, why is modification by only 1O2 considered while superoxide and hydroxide radicals can equally damage D1?

We agree with the possibility that oxidative modifications in other amino acids are also involved in the D1 degradation, as pointed out by the reviewer. We also thank the reviewer for pointing us to the interesting article of McKenzie and Puthiyaveetil et al. that showed additional oxidations occurred in the D1-Nterminus, which we had yet to be aware of when we submitted our manuscript. It will be interesting to see how these amino acid oxidations work with W14 oxidation on D1 degradation in the future. The oxidation of Trp by 1O2 can serve as a substrate for FtsH, as in the case of EX1, so we focused on the analysis of Trp oxidation. Single oxygen is believed to be the potential reactive species of Trp oxidation. However, the detected oxidative modifications in this study were not exactly sure depended on singlet oxygen. Thus, we changed several sentences that mention tryptophan oxidation by single oxygen.

1. The D1 degradation assay seems not repeatable for the W14F mutant. High light minus CAM results in Fig. 3 shows a statistically significant decrease in D1 levels for W14F at multiple time points but the same assay in Fig. 4a does not produce a statistically significant decrease at 90 min of incubation. Why is this? Accelerated D1 degradation in the Phe mutant under high light is key evidence that the authors cite in support of their hypothesis.

In Fig. 4a, the p-value comparing the D1 level at 90 min between control and W14F was 0.1075. This value is slightly larger than 0.1. The result that one of the control experiments

showed a decrease in D1 level relative to 0 h might cause this value. Given that the D1 level of the remaining three of the four replicates was unchanged in the control experiments, it can be considered an outlier. We believe the results do not affect our hypothesis that the earlier D1 degradation is occurred in W14F.

1. *The description of results at times is not nuanced enough, for e.g. lines 116-117 state "The oxidation levels in Trp-14 and Trp-314 increased 1.8-fold and 1.4-fold in var2 compared to the wild type, respectively (Fig. 1c)" while an inspection of the figure reveals that modification at W314 is significant only for NFK and not for KYN and OIA.*

In this sentence, we described the result that is compared with the oxidized peptide levels calculated from all Trp-oxidized derivatives. However, as pointed out by the reviewer, it was not correct to explain the result of Fig.1C. We corrected the sentence following the reviewer's suggestion as below; "The levels of Trp-oxidized derivatives, OIA, NFK, and KYN in Trp-14 and the level of KYN in Trp-314 were significantly increased in var2 compared to the wild type, respectively (Fig. 1c). "

Likewise, the authors write that CP43 mutant W353F has no growth phenotype under high light but Figure S6 reveals otherwise. The slow growth of this mutant is in line with the earlier observation made by Anderson et al., 2002.

As pointed out by the reviewer, the growth of W353F seems to be a little slow under HL. We have changed our description of the result part. However, we still conclude that CP43 had little impact on the PSII repair, because the impaired growth in W353F is not as severe as those in W14F and W14F/W317F under HL

In lines 162-163, the authors talk about unchanged electron transport in some site-directed mutants and cite Fig. 2c but this figure only shows chl fluorescence trace and nothing else.

We agreed with the reviewer's suggestion and changed the sentence. In this study, we did not perform detailed photosynthetic analysis. Based on the analysis of phototrophic growth, oxygen-evolving activity, and Chl fluorescence, we concluded that overall photosynthetic activity was not a significant difference in the mutants.

1. *The authors rightly discuss an alternate hypothesis that the simple disassembly of the monomeric core into RC47 and CP43 alone may be sufficient for selective D1 degradation as in cyanobacteria. This hypothesis cannot yet be ruled out completely given the lack of some in vitro degradation data as mentioned in point 2. Oxidative protein modification indeed drives the disassembly of the monomeric core (Mckenzie and Puthiyaveetil, bioRxiv May 04 2023).*

Thanks for your suggestion. We added a discussion of PSII disassembly by ROS-induced oxidation to the discussion part, and the reference is added.

Reviewer #3 (Public Review):

Light energy drives photosynthesis. However, excessive light can damage (i.e., photo-damage) and thus inactivate the photosynthetic process. A major target site of photo-damage is photosystem II (PSII). In particular, one component of PSII, the reaction center protein, D1, is very susceptible to photo-damage, however, this protein is maintained efficiently by an elaborate multi-step PSII-D1 turnover/repair cycle. Two proteases, FtsH and Deg, are known to contribute to this process, respectively, by efficient degradation of

photo-damaged D1 protein processively and endoproteolytically. In this manuscript, Kato et al., propose an additional step (an early step) in the D1 degradation/repair pathway. They propose that "Tryptophan oxidation" at the N-terminus of D1 may be one of the key oxidations in the PSII repair, leading to processive degradation of D1 by FtsH. Both, their data and arguments are very compelling.

The D1 protein repair/degradation pathway in its simplest form can be defined essentially by five steps: (1) migration of damaged PSII core complex to the stroma thylakoid, (2) partial PSII disassembly of the PSII core monomer, (3) access of protease degrading damaged D1, (4) concomitant D1 synthesis, and (5) reassembly of PSII into grana thylakoid. An enormous amount of work has already been done to define and characterize these various steps. Kato et al., in this manuscript, are proposing a very early yet novel critical step in D1 protein turnover in which Tryptophan(Trp) oxidation in PSII core proteins influences D1 degradation mediated by FtsH.

Using a variety of approaches, such as mass-spectrometry (Table 1), site-directed mutagenesis (Figures 2-4), D1 degradation assays (Figures 3, and 4), and simulation modeling (Figure 5), Kato et al., provide both strong evidence and reasonable arguments that an N-terminal Trp oxidation may be likely to be a 'key' oxidative post-translational modification (OPTM) that is involved in triggering D1 degradation and thus activating the PSII repair pathway. Consequently, from their accumulated data, the authors propose a scenario in which the unraveling of the N-terminal of the D1 protein facilitated by Trp oxidation plays a critical 'recognition' role in alerting the plant that the D1 protein is photo-damaged and thus to kick start the processive degradation pathway initiated possibly by FtsH. Coincidentally, Forsman and Eaton-Rye (Biochemistry 2021, 60, 1, 53-63), while working with the thermophilic cyanobacterium, *Thermosynechococcus vulcanus*, showed that when the N-terminal DE-loop of the D1 protein is photo-damaged that occurs which may serve as a signal for PSII to undergo repair following photodamage. While the activation of the processive degradation pathways in *Chlamydomonas* versus *Thermosynechococcus vulcanus* have significant mechanistic differences, it's interesting to note and speculate that the stability of the N-terminal of their respective D1 proteins seems to play a critical role in 'signaling' the PSII repair system to be activated and initiate repair. But it's complicated. For instance, significant Trp oxidation also occurs on the lumen side of other PSII subunits which may also play a significant role in activating the repair processes as well. Indeed, Kato et al., (Photosynthesis Research volume 126, pages 409-416 (2015)) proposed a two-step model whereby the primary event is disruption of a Mn-cluster in PSII on the lumen side.

A secondary event is damage to D1 caused by energy that is absorbed by chlorophyll. But models adapt, change, and get updated. And the data provided by Kato et al., in this manuscript, gives us a unique glimpse/snapshot into the importance of the stability of the N-terminal during photo-damage and its role in D1-turnover. For instance, the author's use site-directed mutagenesis of Trp residues undergoing OPTM in the D1 protein coupled with their D1 degradation assays (Figure 3 and 4), provides evidence that Trp oxidation (in particular the oxidation of Trp14) in coordination with FtsH results in the degradation of D1 protein. Indeed, their D1 degradation assays coupled with the use of a *ftsH* mutant provide further significant support that Trp14 oxidation and FtsH activity are strongly linked. But for FtsH to degrade D1 protein it needs to gain access to photo-damaged D1. FtsH access to D1 is achieved by having CP43 partially dissociate from the PSII complex. Hence, the authors also addressed the possibility that Trp oxidation may also play a role in CP43 disassembly from the PSII complex thereby giving FtsH access to D1. Using a site-directed mutagenesis approach, they showed that Trp oxidation in CP43 appeared to have little impact on the PSII repair (Supplemental Figure S6). This result shows that D1-Trp14 oxidation appears to be playing a role in D1 turnover that occurs after CP43 disassembly from the PSII complex. Alternatively, the

authors cannot exclude the possibility that D1-Trp14 oxidation in some way facilitates CP43 dissociation. Further investigation is needed on this point. However, D1-Trp14 oxidation is causing an internal disruption of the D1 protein possibly at the N-terminus of the protein. Consequently, the role of Trp14 oxidation in disrupting the stability of the N-terminal domain of the D1 protein was analyzed computationally. Using a molecular dynamics approach (Figure 5), the authors attempted to create a mechanistic model to explain why when D1 protein Trp14 undergoes oxidation the N-terminal domain of D1 protein becomes unraveled. Specifically, the authors propose that the interaction between D1 protein Trp14 with PsbI Ser25 becomes disrupted upon oxidation of Trp14. Consequently, the authors concluded from their molecular dynamics simulation analysis that "the increased fluctuation of the first α -helix of D1 would give a chance to recognize the photo-damaged D1 by FtsH protease". Hence, the author's experimental and computational approaches employed here develop a compelling early-stage repair model that integrates 1) Trp14 oxidation, 2) FtsH activation and 3) D1- turnover being initiated at its N-terminal domain. However, a word of caution should be emphasized here. This model is just a snapshot of the very early stages of the D1 protein turnover process. The data presented here gives us just a small glimpse into the unique relationship between Trp oxidation of the D1 protein which may trigger significant N-terminal structural changes of the D1 protein that both signals and provides an opportunity for FtsH to begin protease digestion of the D1 protein.

However, the authors go to great lengths in their discussion section to not overstate solely the role of Trp14 oxidation in the complicated process of D1 turnover. The authors certainly recognize that there are a lot of moving parts involved in D1 turnover. And while Trp14 oxidation is the major focus of this paper, the authors show in Supplemental Fig S4 the structural positions of various additional oxidized Trp residues in the *Thermosynechococcus vulcanus* PSII core proteins. Indeed, this figure shows that the majority of oxidized Trps are located on the luminal side of PSII complex clustered around the oxygen-evolving complex. So, while oxidized Trp14 may be involved in the early stages of D1 turnover certainly oxidized Trps on the lumen side are also more than likely playing a role in D1 turnover as well. To untangle this complex process will require additional research.

Nevertheless, identifying and characterizing the role of oxidative modification of tryptophan (Trp) residues, in particular, Trp14, in the PSII core provides another critical step in an already intricate multi-step process of D1 protein turnover during photo-damage.

We thank reviewer #3 for all the helpful comments and their supportive review of the manuscript.

We thank the reviewer for raising this interesting study that ROS might disrupt the interaction between the PsbT and D1 in *Thermosynechococcus vulcanus*. The stroma-exposed DE-loop of D1 is one of the possible cleavage sites by Deg protease. Because the D1 cleavage by Deg facilitates the effective D1 degradation by FtsH under high-light conditions, it is interesting to elucidate Deg and FtsH cooperative D1 degradation further. We added this discussion in the manuscript. Other minor comments were also answered in a point-by-point response.

Reviewer #1 (Recommendations For The Authors):

Other minor points

1. L227. How do you eliminate the possibility of reduced stability under high light?

D1 synthesis under HL as pointed out by the reviewer was not tested in this study. Therefore, we can not rule out the possibility of a reduced D1 synthesis rate under HL in the mutant. However, the rate of D1 turnover (coordinated degradation and synthesis) is increased under HL. Since the pulse-labeling experiment is affected by D1 degradation as well as D1 synthesis, even if there is a difference in the rate of D1 synthesis under HL, we can not clearly distinguish whether the cause of reduced labeling is the increased D1 degradation seen in the W14F mutant or the delay in D1 synthesis. We thank the reviewer for this valuable comment.

1. Ls25-26. *It would be quite rare that P680 directly absorbs light energy.*

We changed the sentence.

1. L28. *intrinsic antenna? Is this commonly used? core antenna?*

Corrected to “core antenna”

1. Ls4143. *Because the process is described as step iii), it is curious to mention it again as other critical steps.*

We removed the sentence.

1. L75. *Is it correct? Do you mean damage is caused by inhibition?*

We changed the sentence to “...the disorder of photosynthesis...”

1. Figure 1c. *+4, +16 and +32 should be explained in the legend.*

We added the explanation in the legend.

1. *Supplementary Figures S1 and S2. Title. Is it true that oxidation depends on singlet oxygen? This is a question. If it is not experimentally proved, modify the expression.*

In general, singlet oxygen (1O_2) is believed to contribute in vivo oxidation of Trp. However, as suggested, these detected oxidative modifications were not exactly sure depends on singlet oxygen. Thus, we changed the title of Fig S1 and S2.

1. Figure 3. *Correct errors in + or - in the Figure.*

Corrected

1. L328. *Cys > Cys.*

Corrected

Reviewer #2 (Recommendations For The Authors):

1. *A few suggestions on typos and style:*

- *Lines 2-3, please rephrase the sentence. The meaning is unclear.*

rephased the sentence to “Photosynthesis is one of the most ...”

- *Lines 28-29, "Despite its orchestrated coordination...". Tautology.*

We changed the sentence.

- *Line 31, "...one, known as the PSII repair...". Please rewrite.*

We followed the reviewer suggestion and changed the sentence to “...synthesized one in the PSII repair.”

- *Line 49, "Their family proteins...". Rephrase.*

Rephrased the words.

- *Lines 64-66, please rewrite. I am not sure what the authors imply here. Are they talking about FtsH turnover or regulation of FtsH at the protein or gene level?*

FtsH itself is also degraded under high-light stress. To compensate for this, ftsH gene expression is upregulated and contributes to the proper FtsH level in thylakoid membranes. We rewrote the sentence as follows “increased turnover of FtsH is crucial for their function under high-light stress. That is compensated by upregulated FtsH gene expression”.

- *Line 68, "...to dislocate their substrates..."*

We changed the sentence to “to pull their substrates and push them into the protease chamber by ATPase activity”

- *Line 86, N-formylkymurenine => N-formylkynurenine*

Corrected

- *Lines 111-112, "Consistent with previous results...". Please specify which studies are being referred to and cite them if relevant.*

We added references.

- *Line 114, "...in extracts Arabidopsis..." => "...in extracts of Arabidopsis..."*

Corrected

- *Line 171, "influences in high-light sensitivity." Please rephrase.*

We rephrased the sentence.

- *Line 192, Fv/Fm. "v" and "m" should be subscripts.*

Corrected

- *Line 210, "...encounters...". Unclear meaning.*

We rephrased the sentence.

- *Line 358, hyphen usage. "fine-tuned". This sentence should be rewritten to make the role of phosphorylation clear. "Fine-tuning" is vague.*

We changed the sentence to "...spatiotemporal regulation of D1 degradation"

- *Fig. 6 legend, luminal => lumenal*

Changed to luminal

1. *The statistical notation used for some results is confusing. In Fig. 6b, "*" stands for $p = \{less\ than\ or\ equal\ to\}0.1$ while in fig. 4 it denotes $p = \{less\ than\ or\ equal\ to\}0.05$. If this is not a typo, this usage deviates from the standard one. How is a D2 change in Fig. 6b significant given its p value of $\{less\ than\ or\ equal\ to\}0.1$? The Fig. 6b key for D2 does not correspond with the histogram pattern.*

Thank you for your comments and suggestions. The asterisk in the Figure 6b is not a typo. We revised p value sign for less than 0.05 with a single asterisk to avoid confusion. While the case of p value in less than 0.1, we applied section sign "\$" instead of the single asterisk sign to avoid confusion. Generally accepted p value to indicate statistically difference is less than 0.05. We found that D1 was $p = 0.03322$ and D2 was $p = 0.07418$. As we suspect these p value differences, the results for D2 protein detection were somewhat fluctuating while not in D1 protein detection as you commented. Still the reason of the fluctuating result of D2 signal intensity is not clear yet, we found the p value was between 0.05 and 0.10. We also rewrite the description in the corresponding result part.

1. *There are no error bars in Fig. 5d while the error bars in Fig. 5e show that there are no significant differences between C β distances of W14F and W14ox with WT contrary to the authors' assertion in the text (lines 254-255).*

The reason that there are no error bars in Fig. 5d. is because the fluctuation value in Fig. 5d was calculated from the entire trajectory (i.e., all snapshots) of the MD simulation. In contrast, the C β -C β distance value can be obtained at each individual snapshot of the simulation. Thus, Fig. 5e shows the averaged distances with the standard deviations (the error bars) over all these snapshots. To prevent any confusion for the reader, we have explicitly described "averaged C β -C β distance" and added an explanation of the error bars in the caption of Fig. 5e. It is important to note that our focus in the text (lines 254-255) was not on comparing the C β -C β distance of W14F with that of W14ox but the distance of W14F or W14ox with that of WT.

1. *Figure 3 legends and figure labels do not correspond. Fig. 3b should be labeled as High light - Chloramphenicol and likewise, fig 3c should read growth light + Chloramphenicol to be consistent with the legend.*

Corrected

1. *How are OPTM levels of D1 Trp residues normalized? Is it against unmodified peptides or total proteins?*

Oxidation levels of three oxidative variants of Trp in Trp14 and Trp317 containing peptides were obtained by label-free MS analysis. Fig.1 shows the intensity values of oxidized variants of Trp14 and Trp317. In this analysis, the levels of unoxidized peptides were not significantly changed between var2 and WT.

1. Fig. 1a cartoon might need work. It looks like the oxygen atom in OIA is misplaced.

Corrected

Reviewer #3 (Recommendations For The Authors):

In regard to Table 1, the sequence of the mass spectra fragment listed for Trp14 (i.e., ENSSL(W)AR) in Table 1 is different from the sequence of the mass spectra fragment of Trp14 shown in Supplemental Figure S1 (i.e., ESESLWGR). Likewise, the sequence of the mass spectra fragment listed for Trp317 (i.e., VLNT(W)ADIINR) in Table 1 is different from the sequence of the mass spectra fragment of Trp14 shown in Supplemental Figure S2 (i.e., VINTWADIINR). This discrepancy, I think can be simply explained.

Table 1 shows the newly detected peptide of Trp oxidation in PSII core protein in Chlamydomonas. On the other hand, Figures S1 and S2 are the results of MS analysis used for the level of Trp oxidation analysis in Arabidopsis var2 mutant, as shown in Fig. 1C. To avoid confusion, we added in the supplemental figure title that it was detected in Arabidopsis.

Labeling: In Figure 3, the figure legend states that b, high-light in the absence of CAM; but panel b, shows +CAM conditions. I think this labeling is incorrect and needs to be - CAM. Likewise, the figure legend states that c, growth-light in the presence of CAM. I think this labeling is incorrect and needs to be +CAM.

Corrected

This reviewer has a few comments/suggestions on the presentation of the sequence alignments showing the various positions of oxidized Trps within the D1(Figure 1), D2 and CP43 (Supplemental Figure S3) and CP47 (Supplemental Figure S3):

The authors should consider highlighting in red all the various Trps shown in Table 1 with the corresponding alignments shown in Figure 1 for D1 protein and corresponding alignments in Supplemental Figure S3 (for D2 and CP43) and Supplemental Figure S3 continued (For CP47). Highlighting the locations of oxidized Trps across various species is very informative but as presented here the red labeling somewhat is haphazard, confusing and thus these figures lose some of their impact factor. For instance, in Supplementary Fig. S4, the reader can visualize the structural positions of oxidized Trp residues in the Thermosynechococcus vulcanus PSII core proteins. When one then looks at the various alignments presented by the authors, one can see that other species have a similar arrangement of oxidized Trp residues as well. Consequently, when you now collectively look at the data presented in Table 1, Figure 1, Supplemental Figure S3 and Supplemental Figure S4, a picture emerges that illustrates how common the phenomenon of overall Trp oxidation is and more specifically how oxidized Trp14 across species is playing a similar role in possibly activating D1 turnover. I think these Figures, if presented in a more comprehensive and unified fashion, will really add to the paper.

Thank you for your suggestion. In this study, we tried to show the identified oxidized Trp by the MS-MS analysis, the residue conservation in the sequences, and its position in the

structure. Since we have to show a lot of information, combining them into one figure is difficult. We hope you understand the reason for this.