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Interactions of PsbT with Val247 and Ser254 in the DE-loop of the D2 protein stabilize the quinone–iron electron acceptor complex of Photosystem II

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Abstract

The non-heme iron of Photosystem II (PSII), found between the primary (Q_A) and secondary (Q_B) plastoquinone electron acceptors, is coordinated by histidine residues from the D1 and D2 reaction center proteins and a bicarbonate ion. The D2-Val247 and D2-Ser254 residues of the DE hydrophilic loop of D2 are located in the Q_A -binding region and interact with the PsbT subunit. Substitutions at these residues caused reductions in photoautotrophic growth, increased sensitivity to high light, and weakened bicarbonate binding. In addition, the core antenna CP43 protein accumulated in these mutants and electron transfer from Q_A^- to Q_B was perturbed. In several mutants, bicarbonate addition only partially restored electron transfer, consistent with disruption of a hydrogen-bonding network linking D2-Ser254 to D2-Asn250 and PsbT. These results demonstrate that D2-Val247 and D2-Ser254 stabilize the quinone–iron–bicarbonate acceptor complex of PSII and influence the stable association of CP43 with the photosystem.

Keywords: chlorophyll fluorescence; formate; oxygen evolution; site-directed mutations; *Synechocystis* sp. PCC 6803.

Introduction

Photosystem II (PSII) catalyzes the light-driven oxidation of water and reduction of plastoquinone to plastoquinol in oxygenic photosynthesis (Shen *et al.* 2021, Shevela *et al.* 2023). Cyanobacteria have served as model systems for studying PSII, and several high-resolution structures from different cyanobacteria are available (Umena *et al.*

2011, Kern *et al.* 2018, Gisriel *et al.* 2022, Hussein *et al.* 2024). In cyanobacteria, PSII is a dimeric complex of approximately 700 kDa, with each monomer comprising ~20 subunits. The reaction center proteins D1 and D2 coordinate the majority of the redox-active cofactors, with D1 providing most of the ligands for the Mn_4CaO_5 oxygen-evolving complex (OEC) (Ferreira *et al.* 2004, Shen 2015). The D1–D2 heterodimer is flanked by

Highlights

- Two quinones Q_A and Q_B act in series as electron acceptors in PSII
- The D2 and PsbT proteins of PSII optimize electron transfer between Q_A and Q_B
- D2-Val247 and D2-Ser254 interact with PsbT and, when mutated, inhibit electron transfer

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Abbreviations: Bis-Tris – 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; BG-11 – blue-green 11 medium; BN-PAGE – blue-native polyacrylamide gel electrophoresis; CP43 – 43 kDa chlorophyll *a*-binding core antenna protein; CP47 – 47 kDa chlorophyll *a*-binding core antenna protein; D1 – the *psbA*-encoded reaction center protein subunit; D2 – the *psbD*-encoded reaction center protein subunit; DCBQ – 2,6-dichloro-1,4-benzoquinone; DCMU – 3,4-dichloro-1,1-dimethyl urea; DMBQ – 2,5-dimethyl-1,4-benzoquinone; F – fluorescence level; F_m – maximum fluorescence level; F_o – initial fluorescence level; HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LMM – low-molecular-mass; OEC – oxygen-evolving complex; O, J, I and P – initial fluorescence level, J inflection, I inflection and peak of the fluorescence induction curve; PCC – Pasteur Culture Collection; ROS – reactive oxygen species; S states – the five, S_0 – S_4 , oxidation states of the oxygen-evolving complex following single-turnover flashes applied to dark-adapted cells.

Conflict of interest: The authors declare that they have no conflict of interest.

the chlorophyll *a*-binding core antenna proteins CP43 and CP47, with CP43 additionally contributing to the OEC-binding environment. This CP43-D1-D2-CP47 core is surrounded by 13 low-molecular-mass (LMM) membrane proteins, while three to four extrinsic proteins on the luminal side stabilize the OEC and facilitate substrate water access, proton release, and oxygen release (Shen 2015, Gisriel *et al.* 2022).

Biogenesis of PSII is initiated by the association of preformed D1 and D2 modules that combine to form a reaction center complex (Nickelsen and Rengstl 2013). Subsequent binding of the CP47 module forms the RC47 intermediate, followed by binding of the CP43 module, light-driven assembly of the OEC, and attachment of the extrinsic luminal subunits (Zabret *et al.* 2021, Komenda *et al.* 2024). Each preassembly module and intermediate complex contains a defined subset of LMM subunits and requires dedicated assembly factors that facilitate module or complex formation but are not retained in the mature photosystem (Komenda *et al.* 2024). In the final step, PSII dimerizes and associates with the peripheral phycobilisome antenna system (Domínguez-Martín *et al.* 2022).

Despite its central role in oxygenic photosynthesis, PSII is prone to oxidative damage due to the formation of reactive oxygen species (ROS) during water splitting and electron transfer (Adir *et al.* 2003, Vass 2012, Pospíšil 2016). This vulnerability is exacerbated under excess light, where increased ROS production necessitates continuous PSII turnover and repair, with photoinhibition occurring when damage outpaces repair capacity, resulting in reduced photosynthetic efficiency (Nishiyama *et al.* 2011, Johnson and Pakrasi 2022).

In PSII, absorbed light energy drives electron transfer from water to plastoquinone *via* the P_{680} reaction center chlorophylls (Cardona *et al.* 2012). Photoexcitation of P_{680} generates the excited state P_{680}^* , which transfers an electron to pheophytin, followed by reduction of the primary plastoquinone electron acceptor, Q_A , bound to the D2 protein. The electron is subsequently transferred to the secondary plastoquinone acceptor, Q_B , located on the D1 protein. Q_B undergoes two sequential reduction and protonation steps to form plastoquinol (PQH_2), which then dissociates from the Q_B -binding site and is replaced by an oxidized plastoquinone from the thylakoid membrane pool (Müh *et al.* 2012). The oxidized reaction center is reduced by electrons supplied from the OEC, which advances through a series of S-state transitions (S_0 – S_4), ultimately catalyzing the splitting of two water molecules and the release of molecular oxygen after four charge-separation events (Kok *et al.* 1970, Bhowmick *et al.* 2023, Li *et al.* 2024).

A non-heme iron (NHI) is located between Q_A and Q_B on the electron-acceptor side of PSII. The NHI is not redox-active and is coordinated by four histidine residues, two from D1 (His215 and His272) and two from D2 (His214 and His268), together with a bidentate bicarbonate (HCO_3^-) ligand (Umena *et al.* 2011, Shevela *et al.* 2012, Tamura *et al.* 2021). Bicarbonate binding facilitates efficient electron transfer from Q_A^- to Q_B and stabilizes plastoquinone exchange at the Q_B -binding site (Robinson

et al. 1984). Loss or displacement of bicarbonate disrupts forward electron transfer and impairs PQH_2/PQ turnover, highlighting its essential role in acceptor-side function (Eaton-Rye and Govindjee 1988a,b). In addition to its role in catalysis, bicarbonate binding modulates the redox potential of the Q_A/Q_A^- couple and likely contributes to photoprotection. Under photoinhibitory conditions, prolonged accumulation of Q_A^- can promote dissociation or reorientation of the bicarbonate ligand, inducing a positive shift in the Q_A/Q_A^- redox potential that favors charge recombination with P_{680}^+ and suppresses 1O_2 formation, thereby limiting oxidative damage (Brinkert *et al.* 2016, Kato and Noguchi 2021, Fantuzzi *et al.* 2022, Sugo and Ishikita 2022).

Several polypeptides or structural elements contribute to stabilizing the bicarbonate-NHI-quinone complex of PSII. One such contribution is made by the conserved DE loop of the D2 protein, which links transmembrane helices D and E (Kless *et al.* 1992) (Fig. 1S, *Supplement*). Mutations within this region impair electron transfer from Q_A^- to Q_B and disrupt PSII assembly (Cardona *et al.* 2015, Khaing *et al.* 2022, Zhong *et al.* 2025). Furthermore, the D2 DE loop is positioned at the interface between the reaction center and CP43, placing it in close proximity to the LMM PSII subunit PsbT. The involvement of PsbT in the susceptibility of PSII to light-induced photodamage and in its subsequent repair has been demonstrated in *Chlamydomonas reinhardtii* and in the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803) (Ohnishi and Takahashi 2001, Ohnishi *et al.* 2007, Bentley *et al.* 2008, Fagerlund *et al.* 2020).

Strains of *Synechocystis* 6803 with PsbT inactivated also exhibited reduced rates of photoautotrophic growth and forward electron transfer (Bentley *et al.* 2008, Fagerlund *et al.* 2020). Furthermore, the deletion of PsbT interferes with the binding of PsbM, leading to the destabilization of PSII dimerization in *Nicotiana tabacum*, *Thermosynechococcus elongatus*, *Thermosynechococcus vulcanus*, and *Synechocystis* 6803 (Iwai *et al.* 2004, Bentley *et al.* 2008, Henmi *et al.* 2008, Umate *et al.* 2008). In addition, it has been shown that PsbT stabilizes the DE loop of the D1 protein through an interaction with D1-Phe239. Mutants with substitutions at the D1-Phe239 position showed reduced oxygen evolution, impaired electron transfer, and slower growth under high light (Forsman and Eaton-Rye 2021).

In this study, the roles of D2-Val247 and D2-Ser254 within the DE loop of D2 were investigated through targeted mutations in *Synechocystis* 6803. These residues are adjacent to D2-Met246 and D2-Trp253, which are important for the binding of Q_A (Vermaas *et al.* 1990, Zhong *et al.* 2025). D2-Val247 is involved in hydrophobic interactions with D1-Phe239 (3.9 Å), and PsbT-Ile29 (3.5 Å) (Fig. 1). Similarly, D2-Ser254 likely forms hydrogen bonds with PsbT-Arg24 (2.7 Å) and D2-Asn250 (2.6 Å) (Fig. 1). Disrupting the interactions involving D2-Val247 and D2-Ser254 perturbed the roles of the D2 DE loop in stabilizing the Q_A and bicarbonate binding environments, and in mediating the incorporation and release of CP43 during PSII assembly and repair.

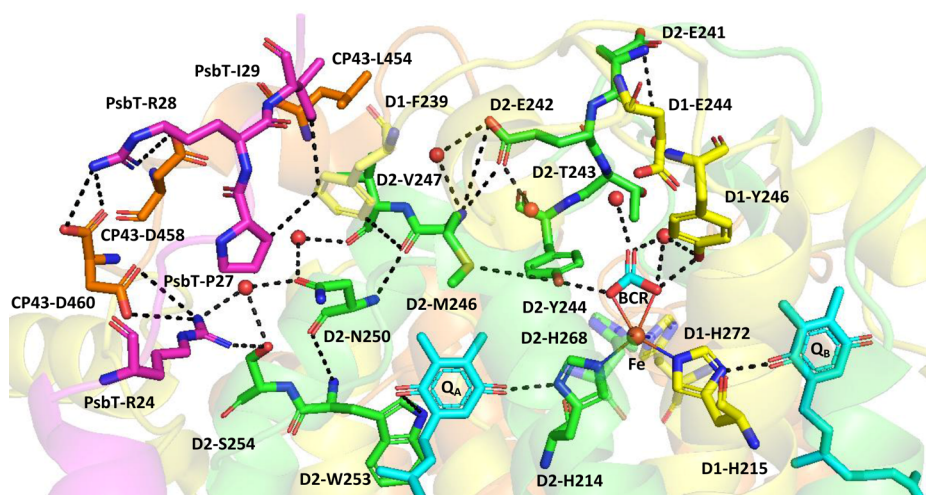


Fig. 1. Diagram of the PSII acceptor side hydrogen-bond network around the non-heme iron and bicarbonate-binding environment. BCR represents bicarbonate, Fe is the non-heme iron, and red spheres indicate water molecules. Q_A and Q_B are shown in light blue. Oxygen atoms are shown in red and nitrogen atoms are shown in blue. D1 residues are in yellow; D2 residues are in green, PsbT residues are in magenta, and CP43 residues are shown in orange. Dashed black lines indicate putative hydrogen bonds. The figure was generated using PyMOL and PDB 7N8O.

Materials and methods

Strains and mutant construction: The glucose-tolerant *Synechocystis* sp. PCC 6803 substrain GT-O1 was used throughout this study (Williams 1988, Morris *et al.* 2014). In *Synechocystis* 6803, the D2 protein is encoded by two genes, *psbDI*, located within the *psbDI:psbC* operon, and *psbDII* (Golden *et al.* 1989). The control strain retained the wild-type *psbDI* sequence, while mutations in *psbDI* were introduced using the *QuikChange II* site-directed mutagenesis kit (Agilent, Santa Clara, CA, USA) with primers listed in Table 1S (Supplement). Modified *psbDI:psbC* operons were introduced into a deletion strain lacking both *psbDII* and the native *psbDI:psbC* operon (Khaing *et al.* 2020). In this system, *psbDII* was replaced with a kanamycin-resistance cassette and the *psbDI:psbC* operon was substituted with a chloramphenicol-resistance cassette. A spectinomycin-resistance cassette was inserted downstream of *psbC* to facilitate selection of successful incorporation of the modified operons. The control strain was constructed in the same way as the mutants and hence contained the spectinomycin-resistance cassette. Genotype and full segregation were confirmed using colony PCR and Sanger sequencing.

Strains were maintained on BG-11 agar plates supplemented with 5 mM glucose, 20 μ M atrazine, 10 mM TES-NaOH (pH 8.2), and 0.3% sodium thiosulfate. Liquid cultures were grown mixotrophically in BG-11 medium containing 5 mM glucose, with appropriate antibiotics: chloramphenicol (15 μ g mL⁻¹), kanamycin (25 μ g mL⁻¹), and spectinomycin (25 μ g mL⁻¹). Cultures were incubated at 30°C under 30 μ mol(photon) m⁻² s⁻¹ provided by Philips HPL-N 250 W metal halide bulbs. Photoautotrophic growth assays were conducted under the same light and temperature conditions but without glucose (Eaton-Rye 2011).

Oxygen evolution and photodamage assays: Oxygen evolution rates were measured using a Clark-type oxygen electrode (Hansatech, King's Lynn, UK) maintained at 30°C. Samples were adjusted to 10 μ g(chlorophyll (Chl) *a*) mL⁻¹, and PSII-specific electron transfer was monitored in the presence of 0.2 mM 2,5-dimethyl-1,4-benzoquinone (DMBQ) supplemented with 1 mM potassium ferricyanide [K₃Fe(CN)₆]. Whole-chain electron transfer was assessed with 15 mM sodium bicarbonate, and 25 mM sodium formate was included when indicated. Saturating illumination was provided by a red light [2,000 μ mol(photon) m⁻² s⁻¹] from an *FLS1* light source (Hansatech, UK) equipped with a 580-nm bandpass sharp-cutoff filter (OG 580, Melles-Griot, Carlsbad, CA, USA).

To evaluate photodamage and PSII repair, samples were exposed to high-light stress [2,000 μ mol(photon) m⁻² s⁻¹] for 45 min using an *Ektalite* slide projector (Kodak, Rochester, NY, USA). Following this treatment, cultures were returned to standard low-light conditions [30 μ mol(photon) m⁻² s⁻¹] for 135 min to allow PSII recovery. Oxygen evolution measurements were taken every 15 min over a 180-min period in the presence of DMBQ with K₃Fe(CN)₆ or sodium bicarbonate to support electron transport throughout the assay.

Low-temperature (77 K) fluorescence emission spectra: Fluorescence emission spectra at 77 K were recorded using a modified MPF-3L fluorescence spectrometer (PerkinElmer, Waltham, MA, USA) equipped with a custom-built Dewar. Samples containing 5 μ g(Chl *a*) mL⁻¹ were snap-frozen, and spectra were collected following excitation at either 440 nm or 580 nm. All spectra were normalized to the PSI emission maximum at 725 nm (Jackson *et al.* 2014).

Measurement of variable chlorophyll *a* fluorescence: Chl *a* fluorescence induction and decay kinetics were

measured using an *FL-3500* fluorometer (*Photon Systems Instruments*, Brno, Czech Republic) at a Chl *a* concentration of 5 $\mu\text{g mL}^{-1}$. For fluorescence induction, cells were dark-adapted for 5 min and then exposed to a constant blue (455 nm) actinic light with superimposed measuring flashes (455 nm), in the presence or absence of 40 μM DCMU. For decay measurements, samples were dark-adapted for 5 min and illuminated with either a single-turnover actinic flash (455 nm) or a train of saturating flashes at 5 Hz (200 ms intervals), with fluorescence monitored using 455 nm measuring flashes. Kinetic analyses followed the procedures of Vass *et al.* (1999). When indicated, samples contained 15 mM sodium bicarbonate or 25 mM sodium formate.

Thermoluminescence measurements: Thermoluminescence (TL) measurements were performed following Cser and Vass (2007). Samples containing 30 μg of Chl *a* were dark-adapted and exposed to a single actinic flash at -10°C , then further cooled to -40°C . TL signals were recorded while heating the samples at $20^{\circ}\text{C min}^{-1}$ up to 80°C . When indicated, 60 μM DCMU was added to the samples in the dark before the actinic flash.

Isolation of thylakoid membranes: Thylakoid membranes were isolated from cultures grown to an OD_{750} of 0.8. Cells were harvested and washed in a buffer containing 1 mM 6-amino caproic acid, 2 mM benzamidine, 20 mM CaCl_2 , 50 mM HEPES-NaOH (pH 7.5), 10 mM MgCl_2 , and 1 mM phenylmethylsulfonyl fluoride. The cells were then resuspended in a disruption buffer, composed of the wash buffer supplemented with 1 M betaine monohydrate and 800 mM sorbitol, and transferred to tubes containing 0.1-mm zirconia beads (*BioSpec Products*, Bartlesville, OK, USA). Mechanical disruption was performed using a bead beater for five cycles (25 s at 4,800 rpm, followed by 5 min on ice per cycle). Cell debris and intact cells were removed by centrifugation at $8,000 \times g$ for 5 min, and thylakoid membranes were collected by centrifugation at $25,000 \times g$ for 1 h at 4°C . Membranes were resuspended in disruption buffer and centrifuged again at $25,000 \times g$ for 25 min at 4°C . The purified thylakoids were snap-frozen in liquid nitrogen and stored at -80°C in 25 mM Bis-Tris-HCl (pH 7.0), 20% glycerol, and 0.25 mg mL^{-1} *Pefabloc* (*Sigma-Aldrich*, St. Louis, MO, USA).

Blue-native polyacrylamide gel electrophoresis and western blotting: Blue-native polyacrylamide gel electrophoresis (BN-PAGE) was performed following Fagerlund *et al.* (2020). Thylakoid samples containing 1.2 μg of Chl *a* were loaded onto 4–16% Bis-Tris gradient gels (*Life Technologies*, Carlsbad, CA, USA) and electrophoresed at 4°C . After separation, proteins were transferred to polyvinylidene difluoride membranes and probed with antibodies specific for D1 (AS05 084), D2 (AS06 146), and CP43 (AS11 1787) (*Agrisera*, Vännäs, Sweden). Anti-rabbit IgG peroxidase (*Sigma-Aldrich*) was used as the secondary antibody, and proteins were visualized using enhanced chemiluminescence.

Results

Photoautotrophic growth and PSII assembly:

Substitutions at D2-Val247 and D2-Ser254 impaired photoautotrophic growth and PSII assembly (Fig. 2). The V247A mutant (16 h) grew at a rate comparable to the control (15 h), whereas the V247D (25 h) and V247G (20 h) strains grew at a slower rate (Fig. 2A). Low-temperature (77 K) fluorescence emission spectroscopy was used to assess the relative abundance of PSII and PSI complexes and the efficiency of energy transfer from the phycobilisome antenna into PSII. Upon excitation at 440 nm, the emission spectra displayed peaks at 685 and 695 nm, corresponding to PSII, and a peak at 725 nm arising from PSI (Lamb *et al.* 2018). The emission at 685 nm originates predominantly from CP43 and PSII preassembly intermediates, whereas the 695 nm peak is attributed to CP47 within fully assembled PSII reaction centers (Boehm *et al.* 2011). Upon 440 nm excitation, all D2-Val247 mutants showed reduced emission at 685 and 695 nm, indicative of decreased levels of PSII, with the effect most pronounced in the V247D and V247G mutants (Fig. 2B). Under 580 nm excitation, reduced phycobilisome emission peaks (645 and 665 nm) compared to the control were observed, accompanied by an enhanced 685 nm peak, particularly in the V247D and V247G strains (Fig. 2C). This pattern is consistent with inefficient energy transfer from phycobilisomes to PSII, likely due to reduced numbers of fully assembled reaction centers.

PSII assembly was also independently examined through BN-PAGE, followed by western blotting using PSII-specific antibodies (Fig. 3). Near wild-type levels of PSII monomers and dimers were observed in V247A cells, whereas the V247D and V247G strains accumulated two distinct CP43-containing preassembly complexes, indicative of impaired CP43 incorporation during PSII assembly or turnover (Fig. 3A–D). A mobility shift in these complexes was also detected, suggesting stress-associated changes, potentially linked to carotenoid accumulation (Choo *et al.* 2022).

In contrast to the D2-Val247 substitutions, mutations at D2-Ser254 had a more severe impact on photoautotrophic growth. While the S254A strain showed moderately reduced growth (23 h), the S254H and S254T mutants exhibited prolonged doubling times of 69 h and 50 h, respectively (Fig. 2D). In addition, low-temperature fluorescence emission spectra revealed a corresponding reduction in PSII assembly and impaired energy transfer from the phycobilisome in these mutants. Following 440 nm excitation, the S254H and S254T strains showed a near-complete loss of the 695 nm emission peak (Fig. 2E); and under 580 nm excitation, an increase in the 685 nm peak was observed, indicating uncoupling of energy transfer from the phycobilisomes into PSII (Fig. 2F).

BN-PAGE and western blotting further demonstrated reductions in PSII monomer and dimer levels in the S254H and S254T mutants, together with accumulation of two distinct CP43-containing complexes, pointing to possible defects in both PSII assembly and repair

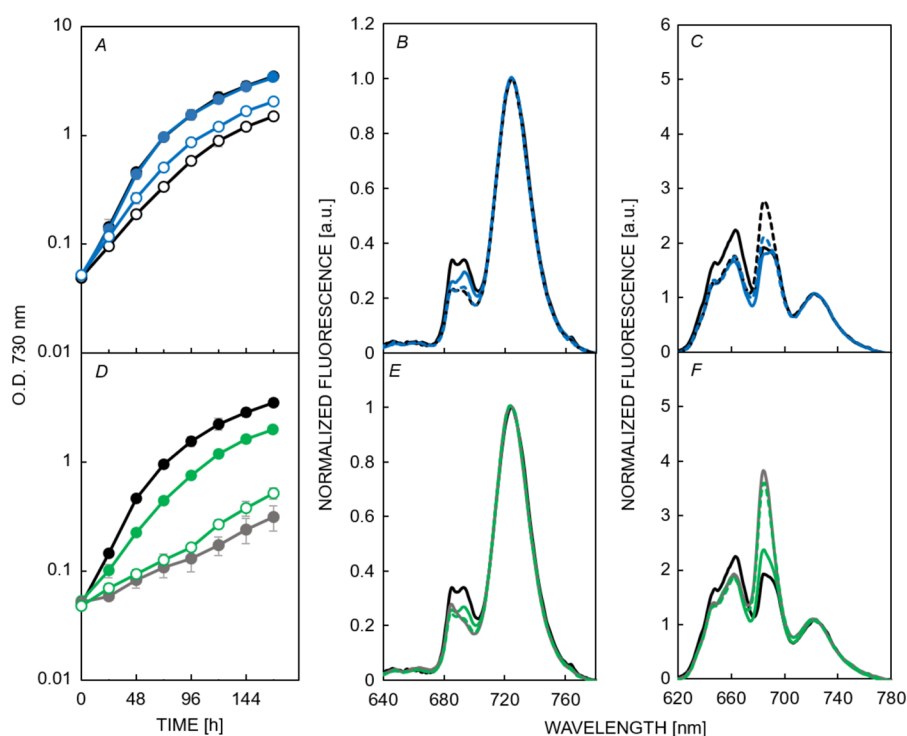


Fig. 2. Photoautotrophic growth and low-temperature (77 K) fluorescence emission spectra. (A) Photoautotrophic growth measured by turbidity at 730 nm. Control (black filled circles), V247A (blue filled circles), V247D (black empty circles), and V247G (blue empty circles). Data are the average of three independent experiments, and the standard error is shown. (B) Low-temperature emission spectra from cells excited at 440 nm. Control (black line), V247A (blue line), V247D (black dashed line), and V247G (blue dashed line). (C) Low-temperature emission spectra from cells excited at 580 nm. Strains are the same as shown in panel B. (D) Photoautotrophic growth measured by turbidity at 730 nm. Control (black filled circles), S254A (green filled circles), S254H (gray filled circles), and S254T (green empty circles). Data are the average of three independent experiments, and the standard error is shown. (E) Low-temperature emission spectra from cells excited at 440 nm. Control (black line), S254A (green line), S254H (gray line), and S254T (green dashed line). (F) Low-temperature emission spectra from cells excited at 580 nm. Strains are the same as shown in panel E. The low-temperature emission spectra are the average of three independent experiments and normalized to the PSI peak at 725 nm.

(Fig. 3E–H). The S254A mutant, however, displayed only minor accumulation of these intermediates and largely retained normal PSII complex levels. Notably, an additional CP43-containing unassembled complex was detected in the S254H strain. As observed for several D2-Val247 mutants, mobility shifts in protein complexes were also detected, consistent with a stress response.

Together, these findings indicate that substitutions at both D2-Val247 and D2-Ser254 impair PSII assembly to varying extents, with the most severe defects seen in the V247D, V247G, S254H, and S254T strains. The accumulation of CP43-containing complexes suggests that these residues play a role in stabilizing CP43 incorporation, and mutations at these positions may therefore compromise both PSII biogenesis and repair.

Steady-state PSII activity in mutants with substitutions at Val247: Variable Chl *a* fluorescence induction measurements were used to assess steady-state electron transfer in the mutants. In the absence of DCMU, the control strain exhibited a characteristic OJIP fluorescence transient, in which the initial rise from

F_0 (O) to the J step reflects photochemical reduction of Q_A , followed by the I phase and a maximum at F_m (P) as downstream electron acceptors become progressively reduced (Fig. 4A). After reaching F_m , fluorescence declined as a consequence of Calvin–Benson–Bassham cycle activation and the engagement of additional quenching processes (Stirbet and Govindjee 2011). Upon addition of DCMU, Chl fluorescence rapidly rose to F_m due to inhibition of forward electron transfer from Q_A^- (Fig. 4B). Under these conditions, the variable fluorescence yield provides an estimate of the relative abundance of active PSII centers (Vass *et al.* 1999).

The V247A strain exhibited a similar fluorescence induction curve to that of control cells, while the V247G mutant showed similar kinetics but with a reduced fluorescence yield. The V247D mutant had the lowest overall fluorescence yield with a minor P peak, consistent with reduced Q_A^- accumulation and fewer assembled PSII centers. Both the V247D and V247G strains also showed a flattened J–I transition before the P peak, suggesting impaired plastoquinone pool filling. Likewise, in the presence of DCMU, the V247A mutant had a fluorescence maximum similar to that of the control, whereas

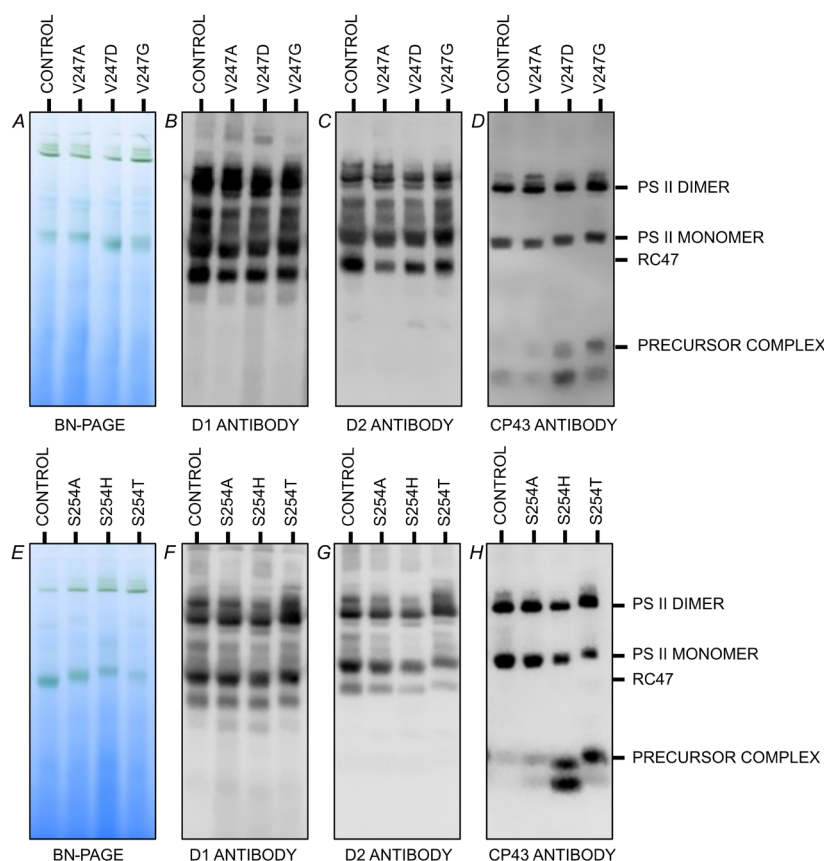


Fig. 3. Blue-native (BN) PAGE followed by western blot analysis. Panels *A–D* are for strains with mutations at Val247, and panels *E–H* are for strains with mutations at Ser254. (*A,E*) BN-PAGE gel. (*B,F*) Western blot with antibody against D1. (*C,G*) Western blot with antibody against D2. (*D,H*) Western blot with antibody against CP43. The strains are labeled above their corresponding lane. RC47 is an assembly intermediate of PSII that lacks bound CP43.

the V247D and V247G strains displayed a markedly lower F_m .

To further examine PSII activity, oxygen evolution was measured. In the presence of the PSII-specific acceptor DMBQ, the rate of oxygen evolution by V247D cells was 60% of the control rate, while V247G exhibited a rate that was 75%. The V247A mutant, however, showed a modest reduction (81% of control) (Fig. 4C) (Table 2S, Supplement). When bicarbonate was supplied, oxygen evolution in the V247A (114%), V247D (115%), and V247G (122%) mutants exceeded the control rate (Fig. 4D, Table 2S). Since the rate-limiting step in whole-chain electron transport is the oxidation of PQH₂ by the cytochrome *b₆* complex, elevated rates of oxygen evolution above that of control cells are not expected when measured with saturating actinic light. This could occur if there was less reduction of the PQ pool via PSI cyclic electron transfer or if there were alternative pathways for PQH₂ oxidation, as both of these scenarios would allow additional electrons from PSII to flow into the PQ pool; however, the explanation for this phenomenon is yet to be established (Lea-Smith *et al.* 2016, Choo *et al.* 2022).

Steady-state PSII activity in mutants with substitutions at Ser254: The S254A strain displayed an OJIP curve similar to the curve obtained with the control (Fig. 5A); however, S254A cells exhibited a decrease in fluorescence yield (~79%) in the presence of DCMU (Fig. 5B). In contrast, the S254H and S254T strains showed a substantial reduction in fluorescence yield under both conditions, suggesting

an increased number of inactive PSII centers in these mutants. PSII-specific oxygen evolution measurements in the presence of DMBQ supported these observations, with reduced rates observed in both the S254A (80%) and S254T (69%) strains, and a more pronounced decline to 51% in the S254H mutant (Fig. 5C). Notably, whole-chain electron transport exhibited enhanced rates of oxygen evolution. Bicarbonate-supported rates of oxygen evolution in S254A and S254T cells were 106 and 115% of the control rate, respectively, while the S254H strain reached 89% (Fig. 5D) (Table 3S, Supplement).

Impaired PSII activity under high light: Sensitivity to high light was assessed to determine the susceptibility of the mutants to photodamage and their capacity for subsequent recovery. Cells were exposed to high light [$2,000 \mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$] for 45 min, followed by recovery under low light [$30 \mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$]. While oxygen evolution in control cells initially dropped, it acclimated during the high-light exposure, and an enhanced rate was observed upon transition to low light (Fig. 6A). All D2-Val247 mutants, however, displayed a heightened sensitivity to high light when PSII activity was measured in the presence of DMBQ. Oxygen evolution in V247A cells dropped to 47% of its initial rate and recovered to 92% in low light. The rate in the V247G mutant declined more severely to 15% but also recovered to 92% of the initial rate in these cells. Additionally, the V247D mutant was particularly light-sensitive, with the rate of oxygen evolution falling to 3% of its initial rate after

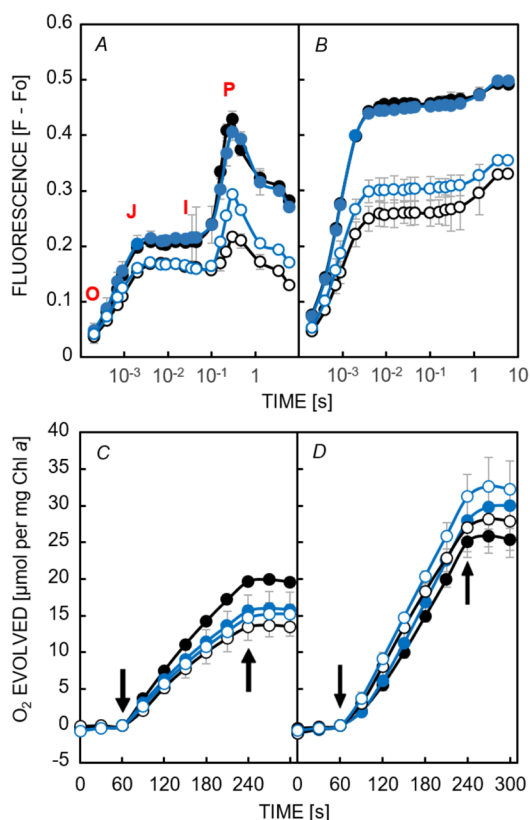


Fig. 4. Photosystem II activity in strains with mutations at Val247. (A) Variable chlorophyll *a* fluorescence in the absence of DCMU. Control (black filled circles), V247A (blue filled circles), V247D (black empty circles), and V247G (blue empty circles). O is the origin, J and I are inflections, and P is the peak of the observed fluorescence in the control. (B) Variable chlorophyll *a* fluorescence in the presence of DCMU. Strains are the same as shown in panel A. (C) Oxygen evolution supported by 200 μM DMBQ and 1 mM $\text{K}_3\text{Fe}(\text{CN})_6$. Arrows indicate light on and off. Strains are the same as shown in panel A. (D) Oxygen evolution supported by 15 mM bicarbonate. Arrows indicate light on and off. Strains are the same as shown in panel A. Data are the average of three biological replicates.

15 min in high light and recovering to only 56% after 135 min in low light (Fig. 6A). When oxygen evolution was assayed in the presence of bicarbonate, the V247D mutant still showed inhibition, dropping to 56% and recovering to 90% of its initial rate, while the V247A and V247G strains resembled the control (Fig. 6B).

Photodamage and recovery assays were also conducted on the strains carrying mutations at D2-Ser254. All D2-Ser254 mutants exhibited heightened light sensitivity, with severity depending on the substitution. When assayed in the presence of DMBQ, the S254A mutant most closely resembled the control, declining to 56% of its initial rate under high light and exhibiting a rate of approximately 94% of its initial rate after 135 min under 30 $\mu\text{mol}(\text{photon})\text{m}^{-2}\text{s}^{-1}$. The S254T mutant dropped to 25% of its initial rate yet recovered to 93% upon return to low light. In contrast, the S254H strain showed the most severe phenotype, with oxygen-evolving activity dropping to 9% under high light

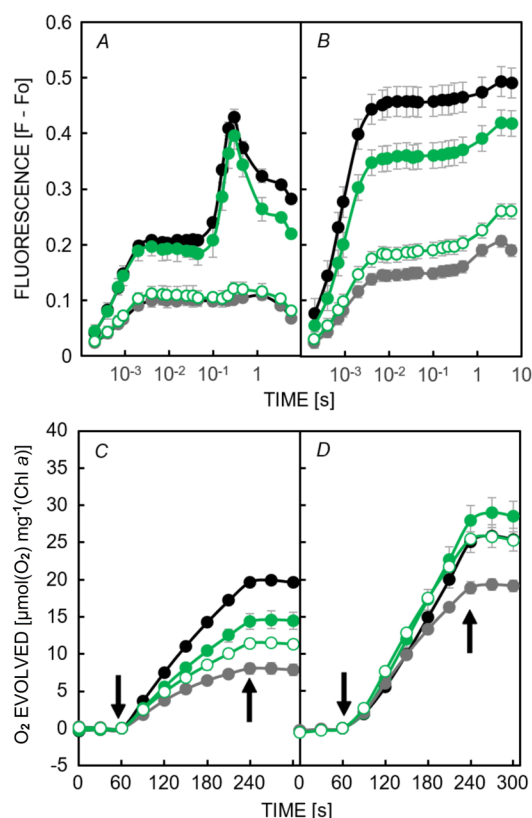


Fig. 5. Photosystem II activity in strains with mutations at Ser254. (A) Variable chlorophyll *a* fluorescence in the absence of DCMU. Control (black filled circles), S254A (green filled circles), S254H (gray filled circles), and S254T (green empty circles). (B) Variable chlorophyll *a* fluorescence in the presence of DCMU. Strains are the same as shown in panel A. (C) Oxygen evolution supported by 200 μM DMBQ and 1 mM $\text{K}_3\text{Fe}(\text{CN})_6$. Arrows indicate light on and off. Strains are the same as shown in panel A. (D) Oxygen evolution supported by 15 mM bicarbonate. Arrows indicate light on and off. Strains are the same as shown in panel A. Data are the average of three biological replicates.

and recovering to only 24% after 135 min (Fig. 6C). When S254H cells were assayed in the presence of bicarbonate, the attenuation of oxygen-evolving activity was less apparent with the activity dropping to 43% in response to the high-light treatment followed by a slight recovery to 61%, while the S254A strain closely resembled the control and the S254T strain showed only a slight reduction in activity under high light (Fig. 6D). Together, these findings indicate that both D2-Val247 and D2-Ser254 substitutions reduce the robustness of PSII to high-light exposure and compromise the cells' ability to undergo repair during a subsequent period of low light. From these data, the most pronounced defects were observed in the V247D and S254H mutants.

Decay of variable Chl *a* fluorescence and thermo-luminescence in mutants with substitutions at Val247: Chl *a* fluorescence decay kinetics after a single actinic flash were examined to investigate electron transfer between Q_A^- and Q_B (Fig. 7A,B and Table 4S, Supplement).

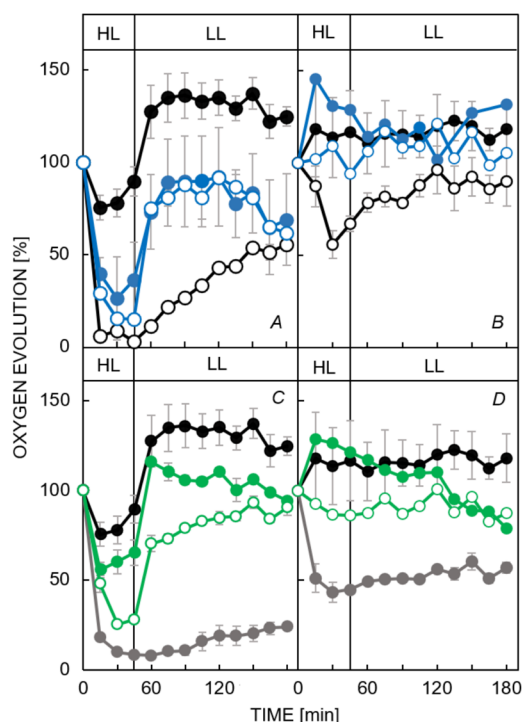


Fig. 6. Sensitivity of oxygen evolution to high light (or photodamage) in control and mutant cells. (A) Photodamage in the presence of DMBQ and $K_3Fe(CN)_6$. Control (black filled circles), V247A (blue filled circles), V247D (black empty circles), and V247G (blue empty circles). (B) Photodamage in the presence of HCO_3^- . Symbols as in panel A. Note that the data for V247A are from a single experiment. (C) Photodamage in the presence of DMBQ and $K_3Fe(CN)_6$. Control (black filled circles), S254A (green filled circles), S254H (gray filled circles), and S254T (green empty circles). (D) Photodamage in the presence of HCO_3^- . Symbols as in panel C. In all panels, high light (HL) was $2,000 \mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$ (for 45 min) and low light (LL) $30 \mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$ (for 135 min). Error bars represent the standard error from three independent experiments. The control data are repeated in panels C and D to facilitate comparison between the different mutants.

In the absence of DCMU, the control strain exhibited three distinct decay phases: a fast component ($t_{1/2} = 289 \mu\text{s}$) with an amplitude of 67%, corresponding to Q_A^- oxidation by prebound Q_B or Q_B^- ; an intermediate component ($t_{1/2} = 3 \text{ ms}$, 25% amplitude) reflecting Q_B binding and subsequent Q_A^- oxidation; and a slow component ($t_{1/2} = 10 \text{ s}$, 8% amplitude) arising from the back reaction between Q_A^- and the S_2 state of the OEC (Vass *et al.* 1999).

The V247A mutant displayed kinetics similar to the control, indicating minimal perturbation of Q_A^- to Q_B electron transfer. By contrast, the V247G mutant exhibited an increased contribution of the intermediate phase (30%), consistent with alterations in the Q_B pocket. This strain also displayed an increase in amplitude (10%) and a faster decay ($t_{1/2} = 3.6 \text{ s}$) of the slow phase, suggesting a shift in equilibrium toward the $Q_A^-Q_B$ state. The V247D mutant showed a pronounced slowing of the fast phase ($t_{1/2} = 446 \mu\text{s}$) and more than a twofold increase in the amplitude of the slow phase (18%), indicative of

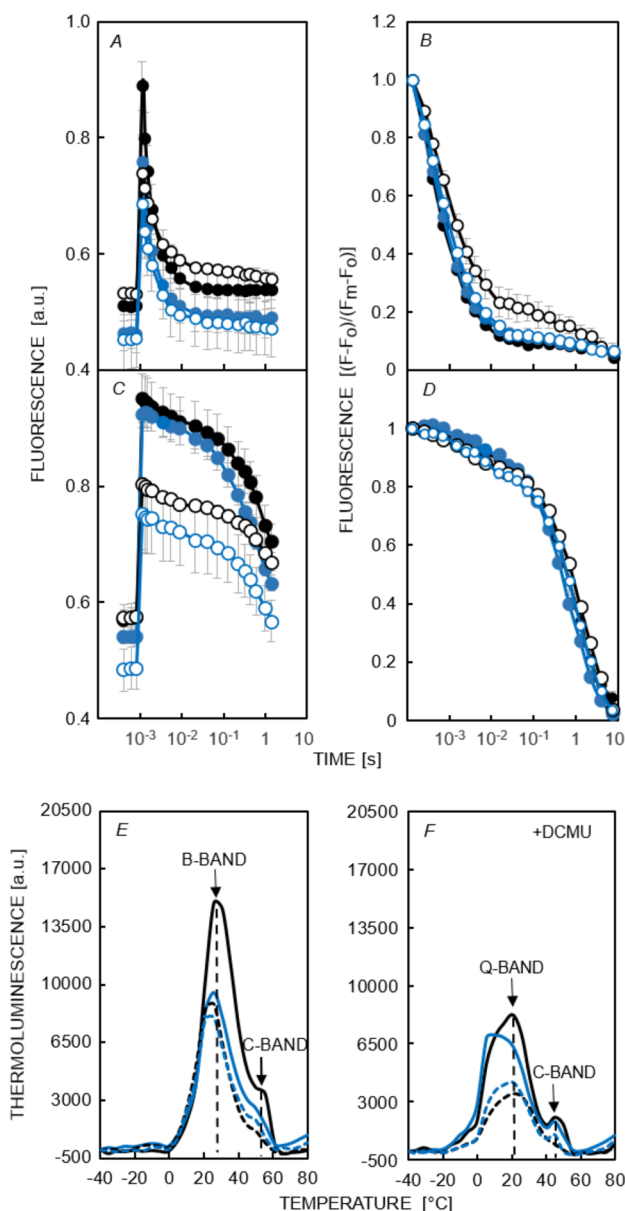


Fig. 7. Chlorophyll *a* fluorescence decay after a single-turnover actinic flash and thermoluminescence emission in the strains with mutations at Val247. (A) Fluorescence decay after three measurements determining the initial (or F_0) fluorescence. Control (black filled circles), V247A (blue filled circles), V247D (black empty circles), and V247G (blue empty circles). (B) Fluorescence decays from panel A normalized to the maximum fluorescence (F_m), where F is the fluorescence at each time point after the actinic flash. (C) Fluorescence decay after three measurements determining F_0 in the presence of DCMU. Strains and symbols are the same as in panel A. (D) Fluorescence decays from panel C normalized to F_m . In panels A–D, data are the average of three independent experiments. (E) Thermoluminescence emission in the absence of DCMU. Control (black line), V247A (blue line), V247D (black dashed line), and V247G (blue dashed line). (F) Thermoluminescence emission in the presence of DCMU. Strains are the same as in panel E. In panels E and F, the data are the average of three independent biological replicates.

impaired electron transfer and stabilization of charge-recombination pathways. In the presence of DCMU, all D2-Val247 strains demonstrated decay kinetics similar to the control but with a slightly accelerated slow phase in the V247A strain (Fig. 7C,D and Table 4S).

Thermoluminescence analysis provided further evidence for altered electron-acceptor-side function in the D2-Val247 mutants. The V247A (26°C), V247D (24°C), and V247G (25°C) strains all exhibited a lower-temperature B-band ($S_2Q_B^-/S_3Q_B^-$ recombination) compared with the control (29°C), reflecting a more negative redox potential of the Q_B/Q_B^- couple and consequent destabilization (Fig. 7E; Fig. 2S, Supplement; Table 5S, Supplement). In the presence of DCMU, the V247D (21°C) and V247G (19°C) mutants displayed Q-band peaks ($S_2Q_A^-$ recombination) similar to the control (19°C). By contrast, the V247A strain exhibited a lower-temperature Q-band at 16°C, pointing to a negative shift in the Q_A/Q_A^- redox potential (Fig. 7F).

Decay of variable Chl *a* fluorescence and thermoluminescence in mutants with substitutions at Ser254:

Chl *a* fluorescence decay kinetics were analyzed to investigate the effects of substitutions at D2-Ser254 on electron transfer (Table 6S, Supplement). In the fast phase, all three mutants showed slower decay compared with the control: S254A ($t_{1/2} = 351 \mu s$), S254H ($t_{1/2} = 337 \mu s$), and S254T ($t_{1/2} = 360 \mu s$). The S254H mutant exhibited an increased contribution of the slow phase (amp = 14% compared to control at 8%) (Fig. 8A,B and Table 6S). The slow phase in the S254A strain, however, resembled the control, but in the S254T cells the slow phase was accelerated ($t_{1/2}$ 3.4 s compared to control 9.8 s), indicative of a shift in the equilibrium for the sharing of an electron between Q_A and Q_B towards Q_A^- . In the presence of DCMU, the S254A mutant again resembled the control. However, the amplitude of the millisecond component was increased in the S254H (26%) and S254T (23%) mutants, consistent with an altered recombination pathway between Q_A^- and Yz^+ (i.e., the oxidized form of the redox-active tyrosine (D1-Tyr161 or Yz^+) found between the OEC and P_{680}), potentially due to a misassembled OEC (Vass *et al.* 1999) (Fig. 8C,D and Table 6S).

Thermoluminescence analysis further highlighted the impact of these substitutions. The S254A mutant exhibited a higher-temperature B-band (31°C) relative to the control (29°C), consistent with a stabilized Q_B^- at the Q_B -binding site. In contrast, the S254H (25°C) and S254T (24°C) mutants displayed lower-temperature B-bands, reflecting destabilization of Q_B^- and a more negative redox midpoint potential for the Q_B/Q_B^- couple (Fig. 8E; Fig. 3S, Supplement; Table 5S). In the presence of DCMU, two mutants exhibited downshifts in the Q-band ($S_2Q_A^-$ recombination), with peaks at 14°C (S254A), and 17°C (S254T), consistent with a more negative redox midpoint potential for the Q_A/Q_A^- couple than in the control in which the Q band peaks at 19°C (Fig. 8F). These findings are consistent with the fluorescence decay assays, which show accelerated decay kinetics in the mutants in the presence of DCMU (Table 6S). In the case

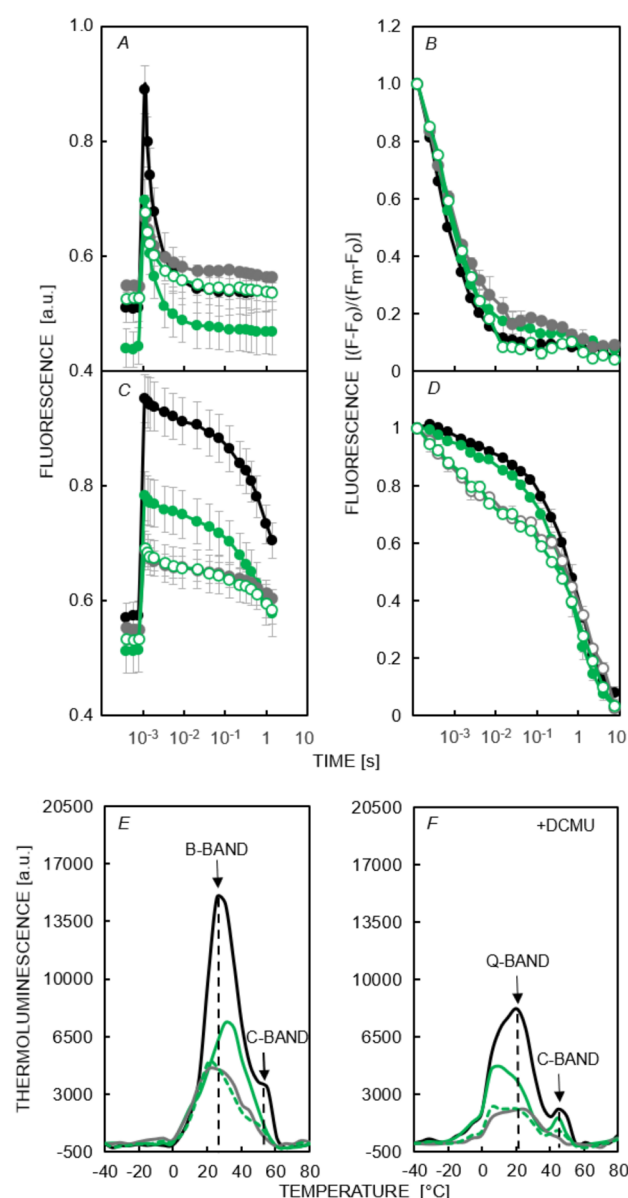


Fig. 8. Chlorophyll *a* fluorescence decay after a single-turnover actinic flash and thermoluminescence emission in the strains with mutations at Ser254. (A) Fluorescence decay after three measurements determining the initial (or F_0) fluorescence. Control (black filled circles), S254A (green filled circles), S254H (gray filled circles), and S254T (green empty circles). (B) Fluorescence decays from panel A normalized to the maximum fluorescence (F_m), where F is the fluorescence at each time point after the actinic flash. (C) Fluorescence decay after three measurements determining F_0 in the presence of DCMU. Strains and symbols are the same as in panel A. (D) Fluorescence decays from panel C normalized to F_m . In panels A–D, data are the average of three independent experiments. (E) Thermoluminescence emission in the absence of DCMU. Control (black line), S254A (green line), S254H (gray line), and S254T (green dashed line). (F) Thermoluminescence emission in the presence of DCMU. Strains are the same as in panel E. In panels E and F, the data are the average of three independent biological replicates.

of the S254H mutant, the peak position of the Q band was somewhat upshifted (to 20°C), which indicated a more

positive redox potential for the Q_A/Q_A^- couple in agreement with the slower fluorescence relaxation in the presence of DCMU (Table 6S). The C-band in the presence of DCMU [$Y_D^+Q_A^-$ recombination (Y_D is Tyr-160 in the D2 protein)] remained similar to the control (48°C) in the S254A (46°C) and S254T (47°C) mutants but exhibited a downshift in the S254H mutant (41°C). However, the explanation for the downshift of the C-band in the S254H cells is unclear.

PSII activity in the presence of bicarbonate and formate in mutants with substitutions at Val247: The D2 DE loop plays a central role in coordinating the bicarbonate ligand bound to the non-heme iron of PSII (Fig. 1). Loss of bicarbonate disrupts forward electron transfer from Q_A^- to Q_B , and this is reversible upon bicarbonate repletion (Govindjee *et al.* 1976, Eaton-Rye and Govindjee 1988a,b). Conversely, sodium formate (HCO_2^-) can competitively displace bicarbonate, impairing Q_A^- to Q_B electron transfer (Robinson *et al.* 1984, Govindjee *et al.* 1991, Sedoud *et al.* 2011, Forsman *et al.* 2019, Forsman and Eaton-Rye 2020).

The structural stability of the D2 DE loop is likely reinforced by its interactions with the small membrane protein PsbT. PsbT possibly forms hydrogen bonds between PsbT-Arg24 and D2-Ser254, while PsbT-Ile29 participates in hydrophobic interactions with D2-Val247

and D1-Phe239 (Fig. 1). These interactions would appear to tether the DE loop to the surrounding protein environment, indirectly stabilizing the bicarbonate-binding site and maintaining the conformation required for efficient electron transfer from Q_A^- to Q_B . Mutations at D2-Val247 and D2-Ser254 are therefore predicted not only to perturb the binding environment of bicarbonate but also to destabilize the PsbT–DE loop interface. This dual effect could impair both forward electron transfer and the protonation of Q_B . To assess these consequences experimentally, Chl *a* fluorescence decay was measured following the separate and combined addition of bicarbonate and formate in the mutants with substitutions at D2-Val247 and D2-Ser254.

Among the V247A, V247D, and V247G strains, the decay of variable fluorescence, following a single-turnover actinic flash, was most impacted in V247D cells, and this was partially recovered upon addition of bicarbonate (Fig. 9A–D and Table 4S). Furthermore, all D2-Val247 mutants displayed sensitivity to the addition of formate. Under formate treatment, the V247A mutant showed a slower fast phase ($t_{1/2}$ 477 μ s [amp 56%], compared to 408 μ s [amp 66%] in the control), and an accelerated slow phase ($t_{1/2}$ 2.6 s [amp. 17%], compared to $t_{1/2}$ 6.6 s [amp 11%] in the control). The V247D and

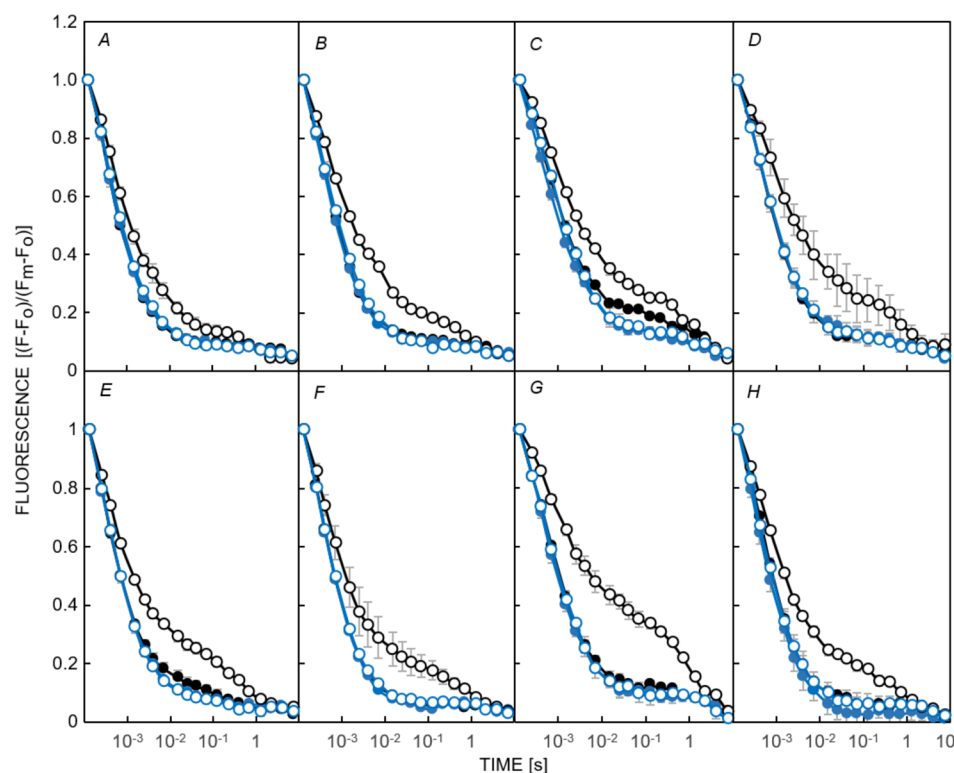


Fig. 9. Chlorophyll *a* fluorescence decay in the presence of bicarbonate, formate, or bicarbonate and formate, in strains with mutations at D2-Val247. (A) Fluorescence decay after one actinic flash in control cells. (B) Fluorescence decay after one actinic flash in V247A cells. (C) Fluorescence decay after one actinic flash in V247D cells. (D) Fluorescence decay after one actinic flash in V247G cells. (E) Fluorescence decay after five actinic flashes given at 5 Hz in control cells. (F) Fluorescence decay after five actinic flashes given at 5 Hz in V247A cells. (G) Fluorescence decay after five actinic flashes given at 5 Hz in V247D cells. (H) Fluorescence decay after five actinic flashes given at 5 Hz in V247G cells. Symbols: No treatment (black filled circles), 15 mM bicarbonate (blue filled circles), 25 mM formate (black empty circles), and 15 mM bicarbonate and 25 mM formate (blue empty circles). All data shown are the average of three independent experiments.

V247G strains exhibited slower fast-phase decays (566 μ s [61%] and 526 μ s [53%], respectively) and a substantial increase in the contribution of the slow phase (28% and 20%, respectively). These formate-induced effects were largely reversed upon addition of bicarbonate (Fig. 9B–D and Table 4S).

Chl *a* fluorescence decay measurements following five actinic flashes at 5 Hz were also carried out to investigate the impact on turnover at the Q_B -binding site. The resulting impairment was again most pronounced in the V247D mutant, and the impaired decays were again reversed by bicarbonate addition (Fig. 9E–H). The effect of formate and bicarbonate on oxygen evolution supported by DMBQ was also examined. In the presence of bicarbonate, the V247D and V247G strains showed rates of oxygen evolution comparable to the control at 106 and 98%, respectively, whereas V247A (116%) exceeded the control (Fig. 10A). Formate treatment alone reduced oxygen evolution in all strains. The V247A mutant was reduced to 70%, V247G cells to 21%, and the V247D strain showed no detectable oxygen evolution (Fig. 10B and Table 2S). The addition of bicarbonate effectively reversed the formate-induced inhibition, consistent with bound bicarbonate being able to restore PSII activity (Fig. 10C and Table 2S).

PSII activity in the presence of bicarbonate and formate in mutants with substitutions at Ser254: In the S254A and S254H strains, formate treatment slowed the intermediate phase ($t_{1/2}$ 14.8 ms in both mutants) compared to 4.1 and 5.7 ms in untreated S254A and S254H cells, respectively, but with little change in amplitude (Fig. 11A–C and Table 6S). This effect on the intermediate component was less pronounced in the S254T strain, although the slow phase in this mutant, both in the absence and presence of formate, was, when compared with S254A and S254H cells, the most impacted (Fig. 11D and Table 6S). The impairments introduced upon formate addition were, however, largely reversed when bicarbonate was added along with formate (Fig. 11A–D and Table 6S). When subjected to five actinic flashes at 5 Hz, minimal changes were observed under all treatments in the S254A and S254T mutants; however, the S254H strain was particularly perturbed, suggesting slower turnover of

Q_B and Q_BH_2 at the Q_B -binding site in the S254H strain (Fig. 11E–H).

The effects of bicarbonate and formate on oxygen evolution supported by DMBQ were also examined. In the presence of DMBQ and bicarbonate, the S254A (102%) and S254T (91%) mutants displayed rates of oxygen evolution similar to control cells, while S254H cells showed an improved rate relative to when just DMBQ was present (Table 3S). In the presence of formate and DMBQ, oxygen evolution in S254A cells was slightly higher than the control (107%), while the S254T mutant was reduced (77%), and the S254H strain exhibited no detectable oxygen evolution (Fig. 12A,B). The inhibitory effects of formate were, however, largely reversed by bicarbonate, with oxygen evolution restored in all strains when DMBQ, bicarbonate, and formate were present (Fig. 12C and Table 3S). These results indicate that the S254H and S254T strains are particularly sensitive to formate, suggesting disruption of the bicarbonate-binding site that may facilitate bicarbonate dissociation.

Discussion

Structural roles of D2-Val247 and D2-Ser254: Structural data for PSII indicate the DE loop of D2 stabilizes interactions between D1, D2, PsbT, and CP43 (Figs. 1 and 3) (Umena *et al.* 2011, Gisriel *et al.* 2022). Residues within this region are highly conserved across oxygenic phototrophs, reflecting important roles in PSII assembly and function, where they contribute to the electron-acceptor side environment (Khaing *et al.* 2022, Zhong *et al.* 2025). Both D2-Val247 and D2-Ser254 participate in these interactions, but in distinct ways. Substitutions at these positions, which likely disrupt the connections between the D2 DE loop and PsbT (Fig. 1), impair the Q_A -Fe- Q_B electron acceptor complex and may, albeit indirectly, alter the binding environment of the associated bicarbonate that is bound to the NHI.

D2-Val247 contributes primarily through hydrophobic interactions with PsbT. Positioned against D1-Phe239 and PsbT-Ile29, D2-Val247 provides a potential interaction that stabilizes the orientation of PsbT relative to the DE loop of D2 (Fig. 1). This hydrophobic cluster appears

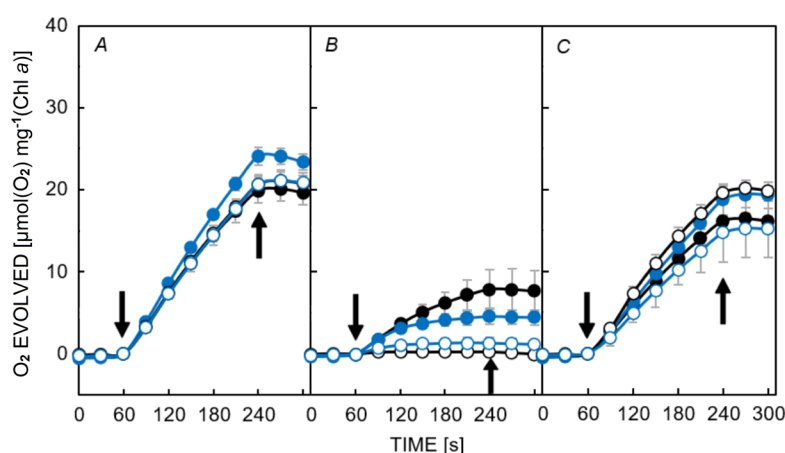


Fig. 10. Oxygen evolution in the presence of bicarbonate, formate, or bicarbonate and formate, in strains with mutations at D2-Val247. (A) Oxygen evolution in the presence of 15 mM bicarbonate and 200 μ M DMBQ. Control (black filled circles), V247A (blue filled circles), V247D (black empty circles), and V247G (blue empty circles). (B) Oxygen evolution in the presence of 25 mM formate and 200 μ M DMBQ. Strain identification as in panel A. (C) Oxygen evolution in the presence of 15 mM bicarbonate, 25 mM formate, and 200 μ M DMBQ. Strain identification as in panel A. In all assays, 1 mM $K_3Fe(CN)_6$ was added together with DMBQ. Arrows indicate actinic light on and off. All data are the average of three independent experiments.

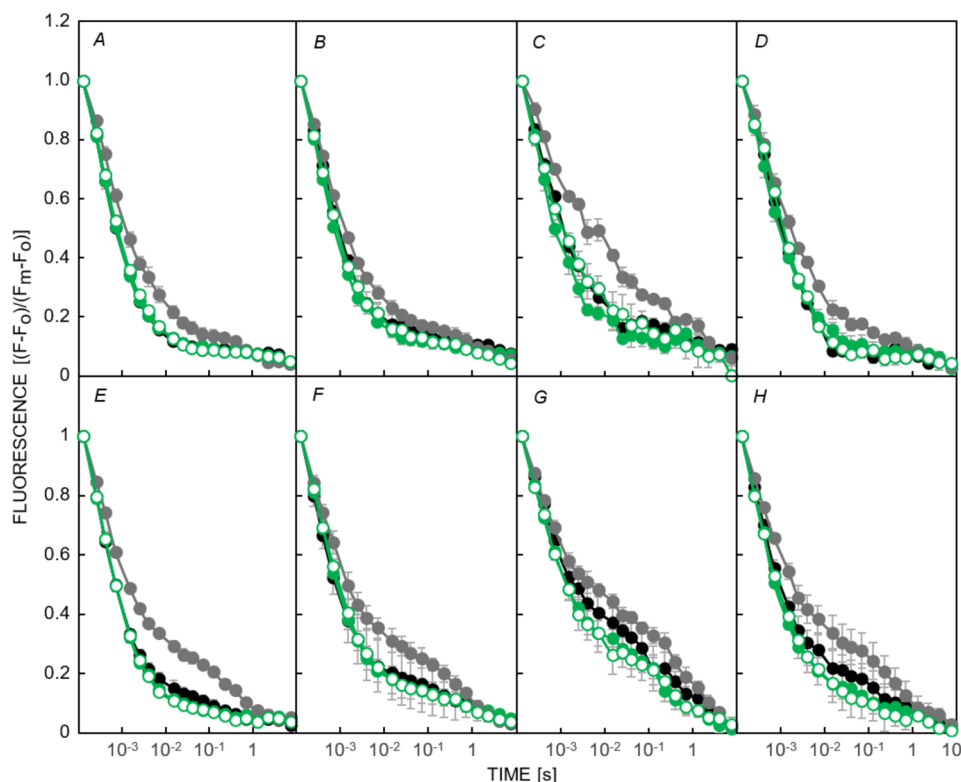


Fig. 11. Chlorophyll *a* fluorescence decay in the presence of bicarbonate, formate, or bicarbonate and formate, in strains with mutations at D2-Ser254. (A) Fluorescence decay after one actinic flash in control cells. (B) Fluorescence decay after one actinic flash in S254A cells. (C) Fluorescence decay after one actinic flash in S254H cells. (D) Fluorescence decay after one actinic flash in S254T cells. (E) Fluorescence decay after five actinic flashes given at 5 Hz in control cells. (F) Fluorescence decay after five actinic flashes given at 5 Hz in S254A cells. (G) Fluorescence decay after five actinic flashes given at 5 Hz in S254H cells. (H) Fluorescence decay after five actinic flashes given at 5 Hz in S254T cells. Symbols: No treatment (*black filled circles*), 15 mM bicarbonate (*green filled circles*), 25 mM formate (*gray filled circles*), and 15 mM bicarbonate and 25 mM formate (*green empty circles*). All data shown are the average of three independent experiments.

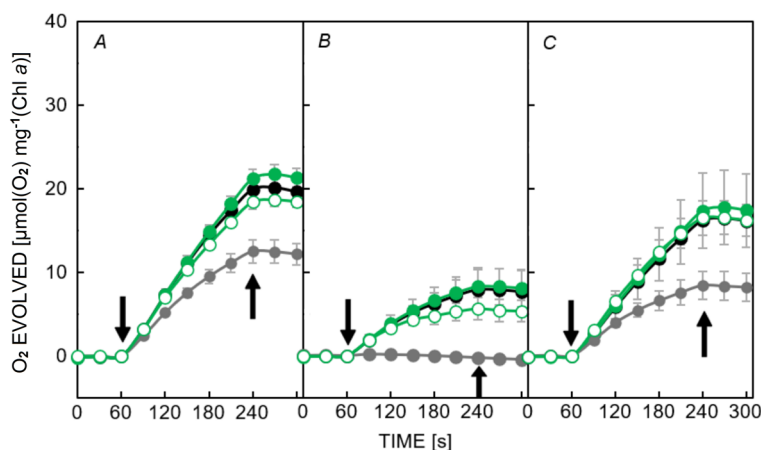


Fig. 12. Oxygen evolution in the presence of bicarbonate, formate, or bicarbonate and formate, in strains with mutations at D2-Ser254. (A) Oxygen evolution in the presence of 15 mM bicarbonate and 200 μ M DMBQ. Control (*black filled circles*), S254A (*green filled circles*), S254H (*gray filled circles*), and S254T (*green empty circles*). (B) Oxygen evolution in the presence of 25 mM formate and 200 μ M DMBQ. Strain identification as in panel A. (C) Oxygen evolution in the presence of 15 mM bicarbonate, 25 mM formate, and 200 μ M DMBQ. Strain identification as in panel A. In all assays, 1 mM $K_3Fe(CN)_6$ was added together with DMBQ. Arrows indicate actinic light on and off. All data are the average of three independent experiments.

to strengthen the interface between the DE loop and the PsbT protein, which in turn may contribute to the correct positioning of CP43. Notably, the C-terminus of the CP43 protein interacts with the DE loop of D2 and PsbT. Loss of these interactions in the D2-Val247 mutant strains appears to weaken the structural framework needed for CP43 incorporation or stabilization. While V247A cells were similar to the control, substitution of D2-Val247 to Gly appeared to weaken the hydrophobic interaction,

while the introduction of a negative charge in the V247D mutant further destabilized the interface, leading to reduced assembly of functional PSII, and the accumulation of CP43-containing complexes (Fig. 3D and Fig. 4B). Because D1-Phe239 itself interacts with both PsbT-Ile29 and PsbT-Pro27, mutations at D2-Val247 may also indirectly perturb the D1 DE loop, destabilizing its interaction with PsbT and further compounding the structural defects previously seen in the D1-Phe239

mutants. Mutations targeting D1-Phe239 have been hypothesized to contribute to the signal for PSII to undergo repair following photodamage (Forsman and Eaton-Rye 2021).

D2-Ser254, by contrast, contributes to a hydrogen-bonding and electrostatic network that is proposed to stabilize the interaction of PsbT with the DE loop. This residue is positioned to seemingly form hydrogen bonds with PsbT-Arg24 and with D2-Asn250, forming a polar anchoring point distinct from the hydrophobic cluster around D2-Val247. Through these contacts, D2-Ser254 may help to stabilize PsbT and indirectly link the DE loop to CP43 *via* the terminal Asp460 residue of CP43, which interacts with Arg24 and Arg28 of PsbT (Khaing *et al.* 2020). Mutations at D2-Ser254 likely disrupted these interactions, leading to destabilization of the DE loop and altered PSII assembly. The S254H and S254T mutants showed the most pronounced phenotypes, with a clear reduction in active PSII centers and increased accumulation of CP43-containing complexes (*cf.* Fig. 3E–H and Fig. 5A,B). These observations indicate that hydrogen bonding at this site is particularly sensitive to perturbation (Fig. 2D–F, Fig. 3H, Fig. 5). These results are consistent with a model in which the D2-Val247–PsbT-Ile29 and D2-Ser254–PsbT-Arg24 pairings, and their putative interactions with CP43 (Fig. 1), may act together to stabilize the PsbT and DE loop interaction contributing to the integrity of the NHI and bicarbonate-binding region. It is also of note that the accumulation of unassembled complexes or modules containing CP43 has been seen in other mutants with introduced substitutions in the DE loop of D2 (Khaing *et al.* 2022, Khaing and Eaton-Rye 2023, Zhong *et al.* 2025).

Effects on electron transfer and bicarbonate binding: Despite being >10 Å from the NHI, mutations at both D2-Val247 and D2-Ser254 exhibited an altered sensitivity towards formate, implicating indirect disruption of the bicarbonate-binding environment.

Mutations targeting D2-Val247 primarily affected Q_B binding and Q_A^- to Q_B electron transfer. The V247D and V247G strains displayed an increased contribution of slow and intermediate fluorescence decay phases and a more negative Q_B/Q_B^- midpoint potential (Fig. 7 and Tables 4S and 5S). In agreement with this effect, the peak position of the TLB-band was shifted to lower temperatures (Fig. 7, Table 5S). In addition, oxygen evolution was abolished in the V247D mutant in the presence of formate, while bicarbonate addition partially restored activity in all mutants (Figs. 9 and 10). These results indicate that destabilization of hydrophobic packing in the DE loop perturbs the conformational environment of the NHI, potentially reducing bicarbonate affinity but allowing functional rescue by bicarbonate supplementation.

Mutations introduced at the D2-Ser254 position caused more severe effects on Q_A^- to Q_B electron transfer than substitutions at D2-Val247. The S254H and S254T strains lacked normal OJIP traces, exhibited slowed fluorescence decay, and the S254T and S254A mutants showed a negative shift in the Q_A/Q_A^- midpoint potential

(Tables 5S and 6S, and Figs. 5 and 8). The S254H strain, however, showed an apparent increase in the Q_A/Q_A^- midpoint potential. In addition, the amplitude of the fast ms component of the fluorescence decay in the presence of DCMU was increased in the S254H and S254T strains, suggesting electron transfer from S_1 to Y_Z^+ was impaired in these strains (Fig. 8D and Table 6S). Bicarbonate addition improved electron transfer in the S254H mutant but not in the S254T strain (Table 6S), suggesting that disruption of the hydrogen bonds at this position through the introduction of histidine propagated into the Q_A -binding pocket and perturbed the bicarbonate-binding environment; notably, D2-Ser254 is positioned 2.6 Å from D2-Asn250, and substitutions at D2-Asn250 have previously been shown to alter Q_A binding, supporting a functional coupling between this hydrogen-bonding network and the stability of the quinone–iron electron acceptor complex (Zhong *et al.* 2025). Furthermore, all three mutants with substitutions at D2-Ser254 were sensitive to the addition of formate, supporting the possibility of weakened bicarbonate affinity in these strains (Figs. 11 and 12, and Tables 3S and 6S).

Susceptibility of PSII to high light in mutants with substitutions at D2-Val247 and D2-Ser254: Exposure to high light places substantial oxidative stress on PSII, increasing charge recombination, generating ROS, and destabilizing cofactors associated with the Q_A -Fe- Q_B environment (Vass 2012, Brinkert *et al.* 2016). Substitutions at either D2-Val247 or D2-Ser254 produced mutants that were susceptible to photodamage (Fig. 6). These results again suggest the proximity of these residues to the C-terminal region of PsbT is important for the stability of the electron acceptor side of PSII.

The involvement of the PsbT protein in the repair mechanism of PSII, especially during high-light-induced photodamage, has been demonstrated (Ohnishi and Takahashi 2001, Ohnishi *et al.* 2007, Bentley *et al.* 2008, Fagerlund *et al.* 2020, Forsman *et al.* 2020). As noted above, in addition to the interaction of the DE loop of D2 with PsbT, the DE loop of D1 has been shown to interact with PsbT, and light-induced damage frequently targets the D1 DE loop, and similar oxidative damage occurs to the D2 protein, but at a slower rate (Ohad *et al.* 1984, Greenberg *et al.* 1987, Jansen *et al.* 1999, Forsman and Eaton-Rye 2021). The combined interactions of the DE loops of D1 and D2 with the PsbT protein may therefore act as a structural feature for the sustained operation of the electron-acceptor side of PSII.

Substitutions at D2-Val247 are expected to disrupt the hydrophobic interactions with PsbT-Ile29 (3.5 Å) and D1-Phe239 (3.9 Å) (Fig. 1) (Forsman and Eaton-Rye 2021). The resulting destabilization may therefore perturb both the D1 and D2 DE loop interactions with PsbT, thereby disrupting the bicarbonate-binding environment and, potentially, the conformational stability necessary for maintaining CP43 interactions under high light. As a result, bicarbonate may become more prone to dissociation during stress, leading to increased sensitivity to the addition of formate and diminished recovery following high-light exposure (Figs. 6A, 9A–D, 10).

By contrast, mutations at D2-Ser254 primarily disrupted the hydrogen-bonding network in the vicinity of the Q_A -binding pocket (Fig. 1). The alanine and threonine substitutions, which were associated with a more negative Q_A/Q_A^- redox potential, would likely increase charge recombination and resulting ROS formation (Vass 2012). In addition, as noted above, D2-Ser254 is positioned such that it may hydrogen bond to PsbT-Arg24 (2.7 Å), and disruption of this interaction would likely destabilize the D2 DE loop. Moreover, the apparent higher ratio of PSII dimers to monomers in the S254H and S254T mutants may arise from altered binding affinity of CP43 (e.g., Fig. 3H). This may be a contributing factor to the sensitivity to high light due to inhibited repair (Fig. 6C,D). The presence of histidine in the S254H mutant may also be altering the Q_A -binding pocket such that Q_A may dissociate under high light, resulting in the inability of S254H cells to recover from photodamage (Fig. 6C,D).

Conclusion: The Val247 and Ser254 residues of the DE loop of the D2 protein participate in interactions with the C-terminal region of the PsbT subunit of PSII. These interactions, coupled with a similar interaction between PsbT and Phe239 of the DE loop of the D1 protein, appear to stabilize the quinone-iron electron-acceptor complex. Collectively, these interactions likely modify the binding of bicarbonate to the NHI and hence play a key role in optimizing the assembly and operation of the electron acceptor side of PSII.

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