Obscurity of chlorophyll tails - Is chlorophyll with farnesyl tail incorporated into PSII complexes?

André T. Graça | Jenna Lihavainen | Rana Hussein | Wolfgang P. Schröder

1Department of Chemistry, Umeå University, Umeå, Sweden
2Department of Plant Physiology, Umeå Plant Science Centre (UPSC), Umeå University, Umeå, Sweden
3Humboldt-Universität zu Berlin, Department of Biology, Berlin, Germany

Correspondence
Wolfgang P. Schröder,
Email: wolfgang.schroeder@umu.se

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Abstract
Chlorophyll is essential in photosynthesis, converting sunlight into chemical energy in plants, algae, and certain bacteria. Its structure, featuring a porphyrin ring enclosing a central magnesium ion, varies in forms like chlorophyll a, b, c, d, and f, allowing light absorption at a broader spectrum. With a 20-carbon phytyl tail (except for chlorophyll c), chlorophyll is anchored to proteins. Previous findings suggested the presence of chlorophyll with a modified farnesyl tail in thermophilic cyanobacteria Thermosynechococcus vestitus. In our Arabidopsis thaliana PSII cryo-EM map, specific chlorophylls showed incomplete phytyl tails, suggesting potential farnesyl modifications. However, further high-resolution mass spectrometry (HRMS) analysis in A. thaliana and T. vestitus did not confirm the presence of any farnesyl tails. Instead, we propose the truncated tails in PSII models may result from binding pocket flexibility rather than actual modifications.

1 | INTRODUCTION

Chlorophyll is the most abundant pigment in plants and the key pigment in the process of photosynthesis (Björn et al. 2009). It allows plants and other photosynthetic organisms to absorb energy from the sun as they undergo the process of photosynthesis. The chlorophyll molecule contains a central magnesium ion surrounded by a nitrogen-containing structure called chlorin, whose foundation is a porphyrin ring. Chlorins can have different possible side-group modifications that change the absorption properties of the molecule. Those modifications establish the chlorophyll type: a to f. Chlorophyll a (Chl a, Figure 1B) is universal among oxygenic photosynthetic organisms and is found in the reaction center of Photosystem II (PSII, Figure 1A) and other light-harvesting proteins (Grimm et al. 2006).

Connected to the chlorin-ring of most chlorophyll types (except chlorophyll c and some bacteriochlorophylls) is a 20-carbon long phytyl side chain (Figure 1E) (Durrett and Welti 2021). This side chain, made of four isoprenoid building blocks, is known as the chlorophyll tail. During chlorophyll biogenesis, the tail, in its diphosphate and unsaturated form (termed geranylgeranyl pyrophosphate, GGPP, see Figure 1C), is attached to one of chlorophyllide pyrrole rings by the enzyme chlorophyll synthase (ChlG; EC 2.5.1.62) (Solymosi and Mysliwa-Kurdziel 2021). At the last step of chlorophyll synthesis, the enzyme geranylgeranyl reductase (ChlP; EC 1.3.1.83) is responsible for hydrogenating the tail, yielding a fully synthesized chlorophyll a. Chlorophyll’s hydrophobic tail is responsible for anchoring the pigment to photosynthetic proteins in the thylakoid membrane (Durrett and Welti 2021). The need to anchor the pigments to maintain an optimal position for intra-chlorophyll energy transfer (Fiedor et al. 2008) could explain why the phytyl tail is conserved across kingdoms.

Currently, several atomic-resolution structures from cyanobacterial PSII have been solved by X-ray crystallography in the resolution range of 1.85–6.56 Å (Umena et al. 2011; Hellmich et al. 2014; Suga et al. 2015; Young et al. 2016; Sheng et al. 2019; Gisriel et al. 2020, 2023; Hussein et al. 2021; Nagao et al. 2022; Caspy et al. 2023; Simon et al. 2023). Furthermore, with cryo-EM developments in the last
decade, the structure of several PSII complexes that previously resisted crystallization, such as those from *Pisum sativum* (Cao et al. 2018), *Spinacia oleracea* (Wei et al. 2016), and *Arabidopsis thaliana* (Graça et al. 2021), have been resolved at around 3 Å overall resolution or better. These structures are of comparable detail to other existing PSII atomic models from cyanobacteria, diatom, and green algae. Commonly, at resolutions better than 3 Å, detailed information on the structure of chlorophylls, lipids, and other cofactor molecules bound to the proteins can be retrieved from X-ray diffraction (XRD) and cryo-EM maps (Loll et al. 2005, 2007; Mühl and Zouni 2020).

Based on the analyses of an X-ray map (from *Thermosynechococcus vulcanus* and low-resolution mass spectrometry data (from *Thermosynechococcus elongatus*, now designated *T. vestitus*), an earlier report (Wiwczar et al. 2017) suggested that thermophilic cyanobacteria could integrate chlorophyll *a* with a farnesyl tail (Chl *a*F, see Figure 1D for farnesyl structure) in place of a Chl *a* with a longer phytyl tail. Chl *a*F was suggested to be present in at least one specific position within the PSII reaction center (Wiwczar et al. 2017). This idea is supported by in vitro studies (Rudiger et al. 1980) that suggested that ChlG, besides catalyzing the condensation reaction of geranylgeranyl pyrophosphate with chlorophyllide *a*, can also use farnesyl pyrophosphate as substrate although at lower efficiency. This unique modification of the chlorophyll molecule has only been reported in thermophilic cyanobacteria and heliobacteria with a side group modification ([8-hydroxyethyl]-Chl *a*F) (Mizoguchi et al. 2005). Other studies (Shpilyov et al. 2005; Li et al. 2019) have demonstrated that cyanobacteria with inactivated chlP gene (encoding the geranylgeranyl reductase enzyme) can incorporate Chl *a* molecules with a geranylgeranyl tail into PSII. However, the incorporation of this modification leads to instability and rapid degradation of the photosystems, inhibiting the organism from photoautotrophic growth (Shpilyov et al. 2005; Li et al. 2019).

![Figure 1](https://onlinelibrary.wiley.com/doi/fig/10.1111/ppl.14428)
It was postulated that mesophilic cyanobacteria do not have a specific farnesyl pyrophosphate synthase (FPPS; EC 2.5.1.110) (Wiwczar et al. 2017), but like other cyanobacteria, they possess a poly-prenyl synthase, an enzyme responsible for catalyzing all the prenyl condensations leading to GGPP termed CrtE (Feng et al. 2020; Satta et al. 2022). However, like thermophilic cyanobacteria, plants, and most other photosynthetic organisms have a FPPS, the enzyme responsible for the synthesis of farnesyl chains (Closa et al. 2010; Wichmann et al. 2022). This prompted us to search for support of Chl $a_{\alpha}$ existence in the structural data available for PSII of other photosynthetic organisms. We started by looking at our cryo-EM map of Arabidopsis PSII (EMDB-13078) (Graça et al. 2021). In the corresponding atomic model, we observed that the same Chl $a$ molecule – Chl $a_{504}$ ligated to the CP43/PsbC subunit – has a tail density that cannot be modeled beyond its 15th carbon atom. We also analyzed mesophilic cyanobacterial PSII structures and PDB PSII structures of classes of organisms, which we identified as having a gene coding for FPPS. Furthermore, high-resolution mass spectrometry (HRMS) was performed for pigment extracts purified from PSII complexes from A. thaliana and T. aestivum. Our mass spectrometry analyses do not support the existence of Chl $a_{\alpha}$ in the two organisms. Instead, we suggest that PsbC-CLA504’s appearance as a truncated molecule in different XRD and cryo-EM maps is due to its mobility and the geometry of the pocket where it is found that allows for the added flexibility of the cofactors present in it. Ultimately, a certain degree of flexibility in this pocket is of interest to PSII function as this lipophilic region is suggested to be the pathway for plastoquinone (PQ) / plastoquinol (PQH$_2$) exchange in PSII.

2 | MATERIALS AND METHODS

2.1 | Isolation of photosystem II

Photosystem II complexes have been isolated from Arabidopsis thaliana, as described in (Graça et al. 2021) and T. vulcanus, as described in (Kern et al. 2005).

2.2 | Pigment extraction and analysis of non-acidified samples with UPLC-DAD-HRMS

Acetonitrile, methanol, isopropanol, chloroform, hexane, and formic acid were purchased from Sigma-Aldrich/Merck and were LC grade. Chlorophyll $\alpha$ and $\beta$, pheophytin $\alpha$, violaxanthin, neoxanthin, and antheraxanthin standards, and a mixture of phytoplankton pigments were purchased from DHI Lab products (Denmark). Zeaxanthin, lutein, and beta-carotene were purchased from Extra-synthese (France). DHI pigment mixture was concentrated by drying an aliquot under a stream of nitrogen gas and dissolved in acetonitrile:methanol 7:3 before UPLC-HRMS analysis.

Pigments were extracted from the samples by liquid–liquid extraction with chloroform. Ice-cold chloroform was added, and samples were vortexed and centrifuged at 10,000 g for 1 min. Pigment-containing chloroform phase (hypophase) was collected, filtered (Durapure PVDF 0.22 μm, Merck), transferred into an LC insert in an amber vial, and diluted with acetonitrile:methanol (7:3). During the extraction steps, samples were kept in cold and protected from light by working under green light in a cold room. Pigments from the samples were analyzed immediately after extraction with an Agilent Infinity LC system coupled with a diode array detector (DAD) and 6546 QTOF mass spectrometer (Agilent Technologies). Analytes were separated with ACQUITY UPLC BEH C18 (1.7 μm, 2.1 mm, 150 mm, Waters) column at 30°C. The separation of the pigments in the standard mixture and in the samples (2 μL injected) was achieved with mobile phases consisting of water (A) and ACN:MeOH:IPA 80:15:5 (B) using the following gradient: from 85% B to 100% B in 13.0 min and 100% B until 25 min. The flow rate was 0.5 mL min$^{-1}$ until 14.0 min after which it increased to 0.6 mL min$^{-1}$ and switched back to the initial conditions after 25 min, followed by 2.0 min of equilibration. UV–Vis spectra were recorded at 220–640 nm and chlorophyll $\alpha$ and $\beta$ absorption maxima were monitored at 430 and 460 nm, respectively. Electrospray (ESI) and atmospheric pressure chemical (APCI) ionization techniques were used in positive mode. Formic acid (0.01% FA) was added into the mobile phases when using ESI to improve ionization. When using APCI, FA was omitted since it suppressed ionization. The scan range was 125–1500 m/z. In positive mode ESI, the sheath gas temperature was 350°C and flow 11 L min$^{-1}$, the gas temperature was 250°C and flow 8 L min$^{-1}$, the nebulizer was 35 psi, and the capillary voltage was 4000 V. In positive mode APCI, the vaporizer temperature was 350°C, the drying gas temperature 300°C, and the flow 6 L min$^{-1}$, the nebulizer pressure was 45 psi, the capillary voltage 3000 V, and the corona current 4.0 μA. Pigments were annotated based on the standard mixture, m/z, and UV–Vis absorption spectra (Table S1).

2.3 | Pigment extraction and analysis of the acidified and non-acidified samples with UPLC-HRMS

We replicated the experiment of Wiwczar et al. (2017) with the following modifications: extracted pigments were concentrated by flushing with a nitrogen stream (until an approximate volume of 60 μL) and the volume of the concentrated samples was divided into two vials of which one had a few drops of 12 M HCl. The analytes of two differently treated samples were separated by UPLC (Agilent Infinity II 1290) with a POROSHELL 300SB-C8 (5 μm, 1 mm, 75 mm, Agilent) column at 40°C directly coupled to a high-resolution mass spectrometer (Agilent 6230 LC/TOF). For all chromatography runs injected sample volume was 2 μL, and the mobile phases used consisted of water $+0.01\%$ formic acid (A) and acetonitrile $+0.01\%$ formic acid (B). The following gradient was applied: from 5% B to 95% B in 9.0 min and 95% B until 13 min, followed by 1.0 min of equilibration. The flow rate was 0.6 mL min$^{-1}$; Electrospray (ESI) was used in positive mode: sheath gas temperature was 300°C with a flow of 12 L min$^{-1}$, the gas temperature was 250°C and a flow of 10 L min$^{-1}$; the nebulizer pressure was set to 40 psi, and the capillary voltage was set to 4000 V. All settings
were kept constant except for fragmentor voltage which was changed between 200 V and 300 V for each sample type. The scan range was 100–3200 m/z.

2.4 | Bioinformatics

NCBI protein–protein BLAST tool (Altschul et al. 1990) against protein sequence data (blastp) using the protein sequence of FPPS from *A. thaliana* (Text S1) against *T. vestitus*, *Synechocystis sp. CACIAM 05*, *Spinacia oleracea*, and *Pisum sativum*; and genomic sequence data (tblastn) using FPPS protein sequence from *A. thaliana* against the genome of *Chlamydomonas reinhardtii* and *Chaetoceros tenuissimus* strain NIES-3715. A protein annotated as FPPS was found in several mesophilic *Synechococcus* species by wide UniProt search for the gene *ispA* (Table S2).

2.5 | Model analysis and preparation of figures

Molecular graphics and analyses were performed with UCSF ChimeraX (version 1.7) (Pettersen et al. 2020). The map densities for the illustrated chlorophyll molecules were represented by displaying all densities within 2.5 Å of the chlorophyll atoms. It is noteworthy that during this study, the authors did not refine or modify the atomic models in any way.

3 | RESULTS

3.1 | The cryo-EM maps from *Arabidopsis thaliana* and other land plants show poor densities for chlorophyll tails beyond the first two isoprene units

The quality of *A. thaliana* electrostatic potential map (EMDB ID: 13078; PDB ID: 7OUI) empowers an clear fitting of most ligands of the intricate reaction center cofactors network, including the hydrophobic phytyl tails of chlorophylls. In *Arabidopsis* PSI, we have identified chlorophylls with tail densities appearing to be truncated tails (e.g., Chl a 410 ligated to D1 and Chl a 504 ligated to CP43) which are not located at the surface of PSI. At the first glance, they look properly shielded from the solvent environment that surrounds the complex, and the areas where they are located have an overall low atomic displacement parameter (ADP, also known as the Debye-Waller factor, temperature factor, or...
b-factors) (Carugo 2018). Our published structural data from Arabidopsis (PDB 7OU) (Graça et al. 2021), also initially led us to consider that Chl ω 504 ligated to CP43 (abbreviated in atomic model nomenclature to PsbC-CLA504) might resemble a chlorophyll with a farnesyl tail reported in the thermophilic cyanobacteria (Figure 2). Subsequently we sought to determine whether other structures of PSII from other land plants (Streptophyta class) could support this finding.

In early atomic models of PSII from Spinach (PDB ID: 3JC; EMDB ID: 6617) and Pea (PDB ID: 5XNM, EMDB ID: 6742) land plants, the fitting of a full chlorophyll at this position is not representative of the cryo-EM density. Furthermore, we analyzed the 3JC and 5XNM PDB models together with our published structure and their corresponding cryo-EM maps, and we found chlorophyll molecules that were fully modeled despite a lack of continuous density that supports the modeling of a full phytol tail (Figure 2).

Our preliminary observation of the densities of PsbC-CLA504 could imply the existence of chlorophylls with a farnesyl tail across land plant species. This previous lack of reporting Chl αF in land plant species may stem from the lower resolution in the core region of the previous cryo-EM maps and the assumption that chlorophylls should have a full-length phytol tail (biased ligand fitting).

Further analyses of the cryo-EM maps of different land plants’ PSII revealed different conformations of the non-polar chains of the phosphatidylglycerol (PG) lipid LHG410 adjacent to chlorophyll PsbC-CLA504 (see Text S1). In the observed conformations, the ends of the lipid tails partially exchange location, where either of the two tails extends with a continuously resolved density towards the PsbC-CLA504 tail (Figures S1 and S2).

3.2 | Liquid chromatography and Mass Spectrometry analyses of pigments from A. thaliana and T. vestitus PSII did not detect Chl αF

To verify different species of chlorophylls present in PSII from Arabidopsis thaliana, we performed pigment extraction from isolated PSII complexes (Graça et al. 2021) and analyzed them with two different mass spectrometry setups. In addition, we analyzed pigment extract from T. vestitus PSII core complexes (Kern et al. 2005).

First, we analyzed the extracted pigments with ultra-high-performance liquid chromatography coupled with a diode array detector and high-resolution mass spectrometer (UPLC-DAD-HRMS) using electrospray (ESI) (Banerjee and Mazumdar 2012) and atmospheric pressure chemical (APCI) (Gates 2021) ionization techniques in positive mode and annotated the compounds based on commercial standards.

The pigments in A. thaliana samples included Chl α, Chl b, neoxanthin, violaxanthin, zeaxanthin, lutein, beta-carotene, and pheophytin a (Figure 3A). The pigments in PSII samples isolated from T. vestitus included Chl α, beta-carotene, pheophytin a, cryptoxanthin, and zeaxanthin (Figure 3B).

We performed UPLC-DAD-HRMS-ESI/APCI analyses on three separate occasions, and each analysis included three replicate samples of A. thaliana and T. vestitus. We specifically scanned the data for Chl αF [M + H]⁺/m/z 819.43 ± 0.2 (C₇₉H₈₀N₄O₄Mg), Chl αF with oxidized tail m/z 815.39 ± 0.2 (C₇₉H₈₀N₄O₄Mg) (Table S3), the possible demetallated pheophytin forms [M + H₂-Mg]⁺, and the common adducts [M + Na]+ and [M + K]+ of these molecular species. We did not detect mass spectra that could indicate the presence of a Chl αF in the pigment extracts of A. thaliana nor T. vestitus in the collected HRMS data.

Another set of UPLC-HRMS-ESI analyses was performed with samples that were treated with HCl along with non-treated control samples. HCl acidification demetallizes (Gates 2021), i.e. removes the magnesium from the chlorophyll complexes. We scanned the data for [M + H]⁺ of the oxidized (m/z 793.43 ± 0.05) and reduced (m/z 797.46 ± 0.05) forms of demetallated Chl αF without finding a correspondence. Instead, we spotted a peak with m/z 793.6 (Figure S3), as detected by Wiwczar et al. (2017). However, this peak was present independently of the sample treatment. Since MS data is of high-resolution, we can confidently say that this m/z (a difference of 167.10 mDa or 210.61 ppm) and mass spectra do not support the detected peak would correspond to a chlorophyll-like molecule.

3.3 | Farnesyl pyrophosphate synthase exists in multiple photosynthetic organisms and the observed length of PsbC-CLA504’s tail is independent of its presence

Farnesyl pyrophosphate was suggested to be absent or present in very low amounts in mesophilic cyanobacteria (Wiwczar et al. 2017), presumably because cyanobacteria only have a polyisoprenyl transferase, CrtE, that efficiently synthesizes GGPP while producing GPP and FPP in very low quantities (Pattanaik and Lindberg 2015); however, we found this was not the case for all cyanobacteria (see Table S2 and S4). In this report, we have investigated the genome of organisms from the genera Thermosynechococcus, Synechocystis, Synechococcus, Chaetoceros, Chlamydomonas, Dunaliella, Pismum, Spinacia, Arabidopsis. We have identified at least one gene encoding a possible farnesyl pyrophosphate synthase protein in eight different genera (Table S4).

For the abovementioned genera, we analyzed ten PSII atomic models (with overall resolution equal to or better than 3.2 Å, see Table S5) of ten different species (Wei et al. 2016; Cao et al. 2018; Sheng et al. 2019; Graça et al. 2021; Hussein et al. 2021; Gisriel et al. 2022, 2023; Nagao et al. 2022; Caspy et al. 2023) and their respective cryo-EM / XRD maps. The analysis focused on the densities and the modeled parts of Chl α at the same position as PsbC-504 (nomenclature varies, in some atomic models it corresponds to PsbC-505) in A. thaliana (Figure 4). Like land plant PSII cryo-EM maps, the cryo-EM and X-ray maps of the green algae C. reinhardtii (PDB 6KAC, EMDB – 9955) and D. salina (PDB 7P10, EMDB – 13429) show a chlorophyll density covering the extension of a C15 tail.

In the cryo-EM map of a mesophilic cyanobacterium Synechocystis sp. PCC 6803 (PDB 7N8O, EMDB-24239) PsbC-504 displays a density...
of a C_{20} chlorophyll tail, while in the analyzed X-ray maps of thermo-
mophilic cyanobacteria PSII (PDB: 7RF1, 5V2C) (Figure 4, Figure S4), the

corresponding chlorophyll at the same position does not show a density
for more than a C_{15} long tail. These observations are consistent with

suggestions and remodeling of PsbC-CLA504 in 3WU2/5V2C by

Wiwcza et al. (2017); however, the cryo-EM map (EM-28539) of a more

recent structure of mesophilic cyanobacterium Synechococcus sp. PCC

7335 (PDB 8EQM) published by Gisriel et al. (Gisriel et al. 2023) only

FIGURE 3 Overview of pigment profiles of isolated PSII complexes.
Pigments were extracted from PSII samples isolated from Arabidopsis thaliana (A) and Thermosynechococcus vestitus (B) and analyzed with
UPLC-MS/QTOF/DAD together with a commercial phytoplankton pigment standard mixture (DHI Lab products) (C). UPLC-DAD chromatograms
at 430 nm are presented (Au = arbitrary units). The details of metabolite annotation are in Table S1. Abbreviations: beta-Car = beta-carotene,
Chl a = chlorophyll a, Chl a’ = chlorophyll a’, Chl b = chlorophyll b, Cry = cryptoxanthin, DV-Chl a = divinyl chlorophyll a, DV-Chl b = divinyl
chlorophyll b, Lut = lutein, Neo = neoxanthin, Pheo a = pheophytin a, Vio = violaxanthin, Zea = zeaxanthin.
shows very low density for Chl \( \text{a} \) PsbC-504, suggesting that the density of this chlorophyll tail is dependent on its flexibility.

Like Synechocystis sp. PCC 6803, diatom Chaetoceros gracilis map (PDB 7VD5, EM-31905) shows full density for a complete chlorophyll tail. This observation makes mesophilic cyanobacterium Synechocystis sp. PCC 6803 and diatom Chaetoceros gracilis PSII maps are the only ones displaying an unequivocal density for a complete C20 chlorophyll tail.

In summary, only two of the published high-resolution X-ray and cryo-EM data of PSII show a phytol tail for PsbC-CLA504, including a diatom from the Chaetoceros genus whose genome possibly encodes an FPPS enzyme. Also, some mesophilic Synechococcus species contain a gene, ispA, possibly encoding an FPPS enzyme (see Table S2), contradicting earlier conclusions/assumptions (Ohto et al. 1999; Wiwczar et al. 2017). One of the published mesophilic cyanobacteria PSII structures shows only a density for the first isoprene unit of Chl \( \text{a} \) PsbC-504's tail. These observations indicate that there is another factor responsible for the diversity of observed densities of PSII's Chl \( \text{a} \) PsbC-504, independently of the possible existence of an FPPS enzyme.

### 3.4 PsbC-CLA504 binding pocket is not equally shielded in all PSII structures

To address the question, ‘Why do only diatom and mesophilic cyanobacteria maps display density for the complete phytol tail?’, we looked in-depth at PsbC-CLA504 binding pocket of PSII atomic models from thermophilic cyanobacteria and land plants.
It is important to understand the context of the PsbC-CLA504 binding pocket. Present across all classes of organisms, PsbJ, PsbK, and PsbZ are small transmembrane subunits in the outskirts of PSII that laterally shield the binding pocket of PsbC-CLA504 from the solvent area (Figure 5A-D). PSII from cyanobacteria and diatoms has an additional transmembrane subunit next to PsbJ named Psb30 (Figure 5A, B, and D). Between PsbJ and PsbE, a small opening connects the PsbC-CLA504 pocket with the environment, which is common to all organisms and serves as the diffusion pathway of Qb.

In the land plant PSII, the extrinsic subunit PsbP partially shields this pocket from the lumen (Figure 5C); however, in cyanobacteria and diatoms, PsbV occupies the PsbP area (Figure 5A-C). Diatom’s PsbQ’ protein sequence is longer than CyanoQ and, in complex with PSII, completes the shield that isolates the PsbC-CLA504 binding pocket from the lumen (Figure 5D). When comparing the lumen exposed protein surface near the PsbC-CLA504 binding site, the only difference found between PSII from mesophilic and thermophilic cyanobacteria was the presence of Ca\(^{2+}\) binding between PsbK and Psb30 subunits in mesophilic cyanobacterium PSII (Gisriel et al. 2022). The binding of the calcium ion is accompanied by a slight change of conformation and prolongation of the resolved N-terminal of Psb30. Consequently, it offers slightly better shielding in 7N8O (mesophilic) compared to 5V2C (thermophilic) cyanobacterial PSII.

**FIGURE 5**  Protein distribution around the PsbC-CLA504 cavity from four selected species. The cavity is shown from the lumen side (tilted view). Left side of the panel displays two examples – thermophilic cyanobacterium (A) and land plant (C) structures – where the density of PsbC-CLA504 was observed up to the 15th carbon atom. Right side of the panel shows the only two structures – mesophilic cyanobacterium (B) and diatom (D) – where a full phytyl tail density at the same position was observed. The proteins are represented using a protein surface style, while all the cofactors associated with the depicted subunits are represented with stick representation and colored by heteroatom apart from PsbC-CLA504 which is represented with a ball and stick style in yellow and marked by a yellow arrow.
structure. From all PSII atomic models containing Psb30, this Ca$^{2+}$ binding has been observed exclusively in the 7N8O/EMDB-24239 Synecochystis sp. PCC 6803 structure. However, the density for the entire tail of PsbC-CLA504 in 7VD5 (Chaetoceros gracilis) is better defined and less noisy than 7N8O (Synecochystis sp. PCC 6803, mesophilic cyanobacterium), as PsbQ$^*$ completely shields the binding pocket from the lumen. In land plant PSII structures, the higher flexibility of PsbC-CLA504 could be explained by the fact that PsbP alone cannot completely shield this area from the lumen. In diatom’s PSII, PsbQ and PsbV complement each other, providing a better seal of this pocket (Figure 5D). The complete enclosure of this region also relates to a higher stability or lower mobility of the subunits surrounding the PsbC-CLA504 pockets by increased interactions between them.

The structure of mesophilic cyanobacterium Synecochoccus sp. PCC 7335 PSII acclimated to far-red light (Gisriel et al. 2023), 8EQM PDB model, on a per subunit basis, is the least complete core PSII structure among all the structures examined in this study. Compared to 7N8O (also mesophilic cyanobacterium), this complex lacks the PsbJ, PsbR, PsbZ, Psb30, and CyanQ subunits. The missing subunits expose the PsbC-CLA504 pocket to the exterior of the complex, increasing the flexibility of the ligands in this area, and is the reason behind the near absence of densities to the tail of PsbC-CLA504.

The abovementioned structural differences correlate well with the variations in the densities of PsbC-CLA504 observed in cryo-EM and X-ray maps of PSII complexes, elucidating why this specific chlorophyll appears more stable in particular cryo-EM maps compared to others.

4 | DISCUSSION

The discovery of chlorophyll $a$ esterified to a farnesyl tail pigment in green sulfur bacteria (Vogl et al. 2012) and the integration of farnesol into bacteriochlorophyll $g$ from heliobacteria (Mizoguchi et al. 2005) reinforce the potential presence of such chlorophyll variations across different photosynthetic species like plants, cyanobacteria, or other photosynthetic organisms. Building on a prior report concluding the presence of Chl $a_F$ in thermophilic cyanobacteria, initial hypotheses attributed that such chlorophyll characteristics like the special chlorophyll PsbC-CLA504 proposed by Wiwczar et al. 2017 may also be present in land plants. The initial investigations of the density supporting the modeled Chl molecules revealed that such truncated molecules could occur in species other than thermophilic cyanobacteria. However, our HRMS analysis, accompanied by further structural analyses of this chlorophyll in various structures of PSII and its surrounding region, revealed that most of them did not conclusively support the presence of Chl $a_F$ or any other variants of chlorophyll with a truncated tail.

Our HRMS-based experiments were conducted to verify if Chl $a_F$ molecules are present in PSII complexes in A. thaliana. The analysis detected no mass peaks alluding to a chlorophyll with a farnesyl tail in A. thaliana. Surprisingly, the data did not confirm the earlier results suggesting that T. vestitus has chlorophyll molecule with a farnesyl tail. We would like to emphasize that despite our efforts, including experiments with different ionization sources (ESI and APCI) and fragmentor voltage, which could influence in-source fragmentation (Gathungu et al. 2018; Sun et al. 2022), we did not detect the expected molecular ions or adducts that would correspond to the demetallated or intact Chl $a_F$ species. The results of our MS experiments could not identify any chlorophylls with truncated tails in PSII from A. thaliana and T. vestitus, suggesting that their incorporation into PSII is unlikely.

Our structural analysis showed several indications that could make the phytyl tail of the PsbC-CLA504 mobile. Hence, it could explain the incomplete density that does not cover the entire phytol tail of PsbC-CLA504 in some structures. The varying conformations of the non-polar chains of the PG lipid PsbD-LHG410 adjacent to chlorophyll PsbC-CLA504 highlight their mobility and the flexible nature of this region. Even for mesophilic cyanobacterium (7N8O/EMDB-24239) and diatom (7VD5/EM-31905), where PSII structures show the density of the full phytol tail, the density of this phytol tail is less defined, and the ADP values are higher at the last isoprene unit than at its chlorin ring or the beginning of its tail (Figure S5).

Furthermore, in silico experiments, such as coarse-grained molecular dynamic simulations of PSII, have identified a high degree of flexibility around the binding pocket of PsbC-CLA504 (van Eerden et al. 2017), including the PsbJ subunit with elevated mobility. This region of PSII, including the small opening to the environment between subunits PsbJ and PsbE, has been proposed as the diffusion pathway for the mobile quinone Q$_B$ (Loll et al. 2005) and PsbJ is known to have a role in regulating electron flow in PSII (Ohad et al. 2004). Thus, it is unsurprising that this lipophilic pocket needs to be mobile for optimized exchange between PQ$_H^-$ and the PQ pool. The degree of mobility of the PsbJ subunit may differ between organisms of different phyla, depending on the arrangement of the extrinsic lumenal subunits, which may affect the stability of PsbC-CLA504. Further, new all-atom molecular dynamic simulations for all the PSII structures, with a focus on the PsbC-CLA504 pocket, can provide additional insight into the flexibility of this specific chlorophyll in addition to all the cofactors and protein subunits composing this cavity.

These overall findings confirm the flexibility of the phytol tail in this area and suggest that flexibility is the only reason beyond the incomplete density of PsbC-CLA504 phytol tail in most PSII structures. In contrast, the presence of the entire density observed for molecules like PsbC-CLA504 in one mesophilic cyanobacteria and one diatom PSII structure, may be attributed to the shielding effect provided by the surrounding proteins. This protein shield may contribute to stabilizing PsbC-CLA504, reducing its mobility within these structures compared to the others.

Moreover, our HRMS data has implications beyond chlorophyll PsbC-CLA504. Globally, our analysis suggests that the obscured densities of other chlorophyll phytol tails, in XRD and cryo-EM maps, imply chlorophyll molecules whose tail has a higher degree of flexibility.

Hypothetically, a farnesyl tail could influence the regulation and turnover rates of chlorophyll molecules in the cell. Shorter tails might
enable faster degradation and replacement of chlorophyll, which could be advantageous in environments requiring rapid turnover of PSII. However, this would affect the overall chlorophyll pool rather than specifically chlorophylls like Chl a at position PsbC-CLA504. More importantly, if Chl a molecules had a farnesyl tail instead of a phytyl tail, their anchoring strength would be reduced, leading to less efficient energy transfer. This assumes that evolution has optimized chlorophyll positioning for optimal energy transfer.

In conclusion, although bacteriochlorophyll g may be considered a hypothetical ancestor of Chl a in oxygenic photosynthesis, current evidence does not conclusively support its presence in A. thaliana or T. vestitus. Instead, our investigations indicate the exclusive existence of Chl a with only a full phytyl tail in these organisms.

AUTHOR CONTRIBUTIONS
A.T.G. and W.P.S. designed the research; A.T.G performed PSII extraction from A. thaliana and the structural and the bioinformatics data analyses; R.H. performed PSII extraction from T. vestitus and contributed to its structural analysis; A.T.G. conducted the first set of pigment extraction and UPLC-HRMS data collection; A.T.G. and J.L. performed the pigment extractions and UPLC-DAD-HRMS data collection; A.T.G. and J.L. analyzed the MS data; A.T.G. and W.P.S. wrote the paper with input from the other authors.

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DATA AVAILABILITY STATEMENT
The metabolomics data that support the findings of this study are openly available in MetaboLights (Yureken et al. 2024) at www.ebi.ac.uk/metabolights/MTBLS10489, reference number MTBLS10489.

The structural data that supported the findings were derived from the following resources available in the public domain: https://doi.org/10.2210/pdb7RF1/pdb; https://doi.org/10.2210/pdb5V2C/pdb; https://doi.org/10.2210/pdb7NBO/pdb; https://doi.org/10.2210/pdb8EQM/pdb; https://doi.org/10.2210/pdb7VD5/pdb; https://doi.org/10.2210/pdb6KAC/pdb; https://doi.org/10.2210/pdb7P1O/pdb; https://doi.org/10.2210/pdb5XNM/pdb; https://doi.org/10.2210/pdb3JCU/pdb; https://doi.org/10.2210/pdb7OU/pdb;

ORCID
André T. Graça https://orcid.org/0000-0002-5502-1744
Jenna LiHAVAIEN https://orcid.org/0000-0001-7979-8876
Rana Hussein https://orcid.org/0000-0001-6292-2375
Wolfgang P. Schröder https://orcid.org/0000-0001-9831-1533

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