Chlorophylls (Chls) are the most important cofactors for capturing solar energy to drive photosynthetic reactions. Five spectral types of Chls have been identified to date, with Chl \( f \) having the most red-shifted absorption maximum because of a C21-formyl group of Chl \( f \) and to verify whether Chl \( f \) is synthesized from Chl \( a \) in the cyanobacterial species *Halo- micronema hongdechloris*. In the presence of either H\(_2\) or O\(_2\), the origin of oxygen atoms in the newly synthesized chlorophylls was investigated. The pigments were isolated with HPLC, followed by MS analysis. We found that the oxygen atom of the C21-formyl group originates from molecular oxygen and not from H\(_2\)O. Moreover, we examined the kinetics of the labeling of Chla and Chlf from *H. hongdechloris* grown in 50% D\(_2\)O-seawater medium under different light conditions. When cells were shifted from white light D\(_2\)O-seawater medium to far-red light H\(_2\)O-seawater medium, the observed deuterium in Chlf indicated that Chl(id) \( a \) is the precursor of Chlf. Taken together, our results advance our understanding of the biosynthetic pathway of the chlorophylls and the formation of the formyl group in Chlf.
atoms present in the Chl a molecule (C_{55}H_{72}O_5N_4Mg). The biosynthetic pathway of the Chl a molecule and the origin of its five oxygen atoms are well established (6). The four oxygen atoms at the C13= and C17= carboxyl groups in the Chl a molecule are derived from water during ALA formation (6, 15). Thus, these four oxygen atoms originate from water via a hydratase-type reaction mechanism during synthesis of the precursor ALA. The fifth oxygen atom at the C13=1-oxo group of Chl a comes from molecular oxygen (O_2) through an oxygenase-type reaction in all oxygenic photosynthetic organisms (6). Using ^18O-labeling technology, it was further revealed that the sixth oxygen atom at the C7 formyl group of Chl b is derived from O_2 and not from water (12). The sixth oxygen atom of the formyl group at the C3 position in Chl d is also derived from O_2 through an oxygenase-type reaction mechanism, although the enzyme directly involved in the reaction is yet to be characterized (14).

D_2O (2H_2O) has been used extensively in biochemical studies to trace the fate of hydrogen atoms and in mechanistic studies on enzyme reactions, as well as for measuring protein synthesis rates (16–18). However, it has been less widely used in the examination of metabolic pathways because of unpredictable toleration of D levels; some of them cannot survive with high levels of D even after acclimation treatments (19–21). Part of the reason for this is that the d-proton can be toxic to an organism at sufficiently high concentrations, possibly because of the stronger hydrogen bond formed with D, which may result in the slowing down or severely limiting metabolic reactions, and in interactions where hydrogen exchange is part of the reaction or binding mechanisms (20, 22, 23). However, a number of studies have reported that a sizable fraction of D_2O can be tolerated by some microbes (24). D_2O has also been applied to in vivo investigations of the biomembrane structure (25, 26) and in the study of Chl biosynthesis (27, 28).

Because Chl f is a newly discovered chlorophyll, the origin of all of its six oxygen atoms needs to be investigated, especially the one located at the C2=1-formyl group (Fig. 1). Whether the oxygen at the C2=1 formyl group in Chl f is incorporated using the same reaction mechanisms as that of Chl b or Chl d is still under debate. However, Chl f has been proposed to have an analogous chemical mechanism for its formyl group substitution (29). Recently, a single enzyme, Chl f synthase, was reported to be responsible for the synthesis of Chl f under far-red light (RL), although the pathway of Chl f biosynthesis still needs to be experimentally validated (30).

Using ^18O labeling, we explored whether the origin of the oxygen atoms in Chl f was from water (H_2^{18}O) or from oxygen (^18O_2). The kinetics of Chl a and Chl f biosynthesis in response to changed light conditions was investigated using D_2O-seawater in the culture medium. By monitoring the changes in the percentage of deuterated chlorophylls induced by the changed light conditions, we aimed to examine whether Chl f is derived from Chl a under far-red light conditions.

**Results**

**H_2^{18}O experiment**

In the Chls extracted from Halomicronema hongdechloris cells grown in the culture having either H_2^{18}O or ^18O_2, a 2 Da higher mass above the monoisotopic mass will be observed if one ^18O is incorporated. A maximum difference of 10 Da added to the monoisotopic mass is expected for labeled Chl a because it contains five oxygen atoms and a maximum of an
additional 12 Da for labeled Chl $f$ as it contains six oxygen atoms.

The maximum incorporation of $^{18}$O atoms can be accounted for by the heaviest mass peak. For Chl $a$ extracted from *H. hongdechloris* grown in $H_2^{18}$O, the heaviest mass peak observed was 892.5 m/z, which was 8 Da heavier than the monoisotopic mass of Chl $a$ (892.5). The results indicate that four oxygen atoms in Chl $a$ are derived from water (Fig. 2A), which is consistent with previous reports (13, 15). Similar to the Chl $a$, the newly synthesized Chl $f$ was 8 Da heavier with a maximum mass of 914.5 m/z, also indicating that four oxygen atoms in Chl $f$ are derived from water (Fig. 2B). The five $^{18}$O-labeled Chls (both Chl $a$ and Chl $f$) were mainly observed after 12 days of incubation in $H_2^{18}$O-seawater medium to a maximum percentage of ~10%, which could be due to $^{18}$O$_2$ produced from $H_2^{18}$O by oxygenic photosynthesis. It was noted that both Chl $a$ and Chl $f$ showed similar profiles of $^{18}$O incorporation, with a maximum labeled percentage of ~80% obtained at 10–12 days and ~10% for five $^{18}$O-labeled chlorophylls at the end of the experimental period (16 days). There were no significant differences observed between Chl $a$ and Chl $f$ for the five $^{18}$O atom incorporations. The $H_2^{18}$O incubation experiment suggested that there was a complete turnover of Chl $a$ at ~10 days, and almost all of the extracted Chl $a$ contained $^{18}$O atoms. However, the newly synthesized Chl $f$ labeling peaked at ~12 days, indicating a two-day lag phase between newly synthesized Chl $a$ and Chl $f$ (Fig. 2). The slight delay in the synthesis of Chl $f$ containing $^{18}$O may have been caused by the fact that Chl $f$ is synthesized from Chl $a$ (Fig. 2).

$^{18}$O$_2$ gas experiment

The maximum mass of newly synthesized Chl $a$ extracted from the *H. hongdechloris* cells grown in the presence of $^{18}$O$_2$ was 893.5 m/z, which was 2 Da heavier than the monoisotopic mass of Chl $a$ (892.5 m/z) (Fig. 3A). The time course $^{18}$O-labeling profile indicated that only one $^{18}$O atom was incorporated even after 14 days of incubation time. No Chl $a$ was detected containing two labeled $^{18}$O, which was consistent with previous reports of only one oxo-group oxygen atom at the C13$^+$ position originating from O$_2$ gas (13). There was no significant increase in the percentage of $^{18}$O-labeled Chl $a$ (Fig. 3A) or $^{18}$O-labeled Chl $f$ (Fig. 3B) observed after 5 days. This may be due to the relatively small volume of gas phase used in the experimental setup (an enclosed 6 ml culture vial containing 4 ml gas phase) and that the $^{18}$O$_2$ will be equilibrated with $^{16}$O$_2$ produced from water by oxygenic photosynthesis activities.

The mass spectrometry analysis of purified Chl $f$ from the $^{18}$O$_2$ experiment showed a similar profile for $^{18}$O labeling, with a slight delay of 2 days for maximum labeling as compared with the $^{18}$O-labeled Chl $a$ (Fig. 3, A and B). However, the maximum mass of the newly synthesized Chl $f$ was 910.5 m/z, which was 4 Da heavier than its monoisotopic peak of 906.5 m/z (Fig. 3B), which suggested that the oxygen atoms at the C2$^+$ formyl group and the oxo-group at C13$^+$ came from oxygen molecules. The total percentage of $^{18}$O-labeled chlorophyll $f$ (including one and two $^{18}$O-labeled chlorophyll $f$) is higher than the total percentage of $^{18}$O-labeled Chl $a$ molecules at the same time points. However, the percentage of doubly $^{18}$O-labeled Chl $f$ is the same or lower than the percentage of $^{18}$O-labeled Chl $a$, whereas the percentage of singly $^{18}$O-labeled Chl $f$ is much higher. No Chl $f$ with three $^{18}$O labels was detected, which is in agreement with the $H_2^{18}$O results that four oxygen atoms of chlorophylls are derived from water molecules and not from oxygen molecules. The population of the one $^{18}$O-labeled Chl $f$ included an $^{18}$O substitution either at the C2$^+$ formyl group or at the C13$^+$ oxo-position. The delay observed in the doubly labeled Chl $f$ was consistent with the profiles of one $^{18}$O labeling in Chl $a$ from the same cultures. The percentage of the doubly $^{18}$O-labeled Chl $f$ (Fig. 3B) is always the same or lower than the percentage of $^{18}$O-labeled Chl $a$ (Fig. 3A), which would be expected if Chl $a$ is the precursor of Chl $f$. Taken together, Fig.
Chlorophyll f traced using isotopes

H. hongdechloris in D2O-seawater culture media

H. hongdechloris cells were grown in three different concentrations of D2O-seawater culture medium with final D2O concentrations of 30, 50, or 70% under RL conditions to determine the highest concentration of D2O that could be used without having an adverse effect on growth while still providing sufficient labeling of the chlorophylls to easily discriminate labeled from unlabeled chlorophylls. There are 72 hydrogen atoms in Chl a and 70 hydrogen atoms in Chl f. The mass spectral profiles of deuterated Chl a obtained from the different percentages of D2O medium are presented, respectively, in Fig. 4. In 30% D2O medium, the peaks of 892–897 m/z comprised of the standard mass distribution of undeuterated Chl a. (i.e. the envelope of deuterated Chl a showed overlaps with undeuterated (unlabeled) Chl a resulting in one mass peak cluster centered at 899 (m/z) in the 30% D2O media (Fig. 4A, insets)). In contrast the Chl a from 50% D2O and 70% D2O cultures has three distinct mass peak clusters or envelopes. In 50% D2O medium, there are undeuterated Chl a molecules at 892–897 m/z, but two distinct deuterated clusters at 898–913 m/z, centered at 907 m/z, and at 914–932 m/z, centered at 920 m/z (Fig. 4B). In 70% D2O medium, the deuterated clusters were at 902–920 m/z, centered at 912 m/z, and at 921–942 m/z, centered at 931 m/z (Fig. 4C). In contrast the chlorophyllide fragment ions of these chlorophylls only have a single deuterated envelope (Fig. 4). The second higher molecular mass clusters correspond to a 36 atom % incorporation of deuterium from the 50% D2O culture and 51 atom % incorporation of deuterium from the 70% D2O culture. The same atom % deuterium is observed in the chlorophyllide fragment ions of these chlorophylls, indicating that the first cluster consists of either undeuterated chlorophyll macrocycle and deuterated phytyl side chain or deuterated chlorophyll macrocycle and undeuterated phytyl side chain (Fig. 4, B and C). MS/MS of 912 m/z ion of the 70% D2O gave both undeuterated and deuterated mass clusters of the chlorophyllide fragment ion, indicating that this ion is indeed a mixture of undeuterated chlorophyll macrocycle and deuterated phytyl tail, as well as deuterated chlorophyll macrocycle and undeuterated phytyl tail.

Further confirmation of the identities of the different clusters seen in the Chl a from the 50 and 70% D2O cultures was via modeling and MS/MS of the center of each envelope. Fig. 5 shows the MS of Chl a from 50% D2O culture at weeks 1 and 4. In both cases all three clusters are seen, but the quantity of each is different. The modeled intensities shown in Fig. 5C assume one or both parts of the chlorophyll being deuterated at the specified atom %D of the macrocycle ring structure (32 hydrogens) or the phytyl tail (40 hydrogens). In addition MS/MS of the central peak to yield the Chlide a fragment ion confirmed that the first deuterated envelope was a mixture of deuterated and undeuterated macrocycle. The fact that the center of the envelopes does not change significantly between weeks 1 and 4 indicates that the lower than expected atom %D incorporation is likely due to H for D isotope discrimination and maybe also some dilution of the D2O during set up of the experiment.

In subsequent analysis, the kinetics of deuterated Chl formation was restricted to examining total deuterated compared with undeuterated Chls, with undeuterated chlorophyll being represented by monoisotopic masses in the range 6 mass units higher than the lowest monoisotopic mass and deuterated chlorophylls being >6 mass units above the lowest monoisotopic mass. So undeuterated Chl a was 892–897 m/z, and deuterated Chl a had monoisotopic masses of >897. Using this criterion the rate of deuterated Chl a formation in the cells cultured in 50% D2O and 70% D2O-seawater culture medium was similar (Fig. 6A). 47% of the Chl a molecules from 50% D2O-seawater culture medium were deuterated (labeled) at week 4, which was similar to the 49% of molecules that were deuterated (labeled) from cells grown in 70% D2O-seawater medium. This indicates a similar synthesis and turnover rate for Chl a in both culture conditions. However, the rate of accumulation of deuterated Chl f (Fig. 6B) is lower than the rate of accumulation of Chl a (Fig. 6A). The simplest explanation of this observation is that deuterated Chl f is derived predominantly from the turnover of deuterated Chl a located in the newly synthesized photosynthetic protein complexes. It would be expected that these complexes would contain a higher proportion of unlabeled Chl a than any chlorophylls derived from new synthesis. This is also consistent with the 18O-labeling experiments described above.

Chlorophyll a and f synthesis rates under changing light conditions

Growth in far-red light induces the synthesis of Chl f. Thus, by switching between growth in D2O and/or growth in red or white light and monitoring the changes in the rates of formation of deuterated Chl a and Chl f, we can gain insight into the possible biosynthetic origin of Chl f.

The following D2O-labeling experiments were focused on the dynamic changes of d-labeling ratio of both chlorophylls during changing culture and light conditions.

Treatment 1: white light D2O-labeling experiments—After an initial growth phase of H. hongdechloris cells in 50% D2O-seawater medium under white light (WL)-D2O illumination for 2 weeks, the cells were subjected to three different treatments for another 2 weeks. Chl f was only detected in the cells that were shifted to RL conditions (WR-H2O; Fig. 7A). Because we removed the D2O source at the time when we changed the culture light condition, the deuterated Chl f observed in the WR-H2O treatment at weeks 3 and 4 after incubation will come from either newly synthesized tetrapyrrroles and thus have lower percentage labeling than Chl a, or it will have the same labeling percentage as Chl a if it is derived from Chl a. As shown in Fig. 7A, the changing trends of Chl a and Chl f labeling percentage were the same and decreased at the same rate over time, indicating that Chl f is likely to be synthesized from Chl a rather than completely de novo. That is, if Chl f was made de novo, unlabeled or very limited labeling of Chl f might be expected, which is not consistent with what we have observed.
in Fig. 7. Additionally, the significant decrease in labeling percentages of Chl \(a\) in the WW-H\(_2\)O treatment \((p = 0.0027)\) was the same as that of WR-H\(_2\)O \((p < 0.001)\), except that cells were kept under WL illumination. This indicates a similar rate of synthesis and turnover of Chl \(a\) in the WW-H\(_2\)O treatment and in the WR-H\(_2\)O treatments (Fig. 7A).

**Treatment 2: far-red light D\(_2\)O-labeling experiments**—After an initial growth phase of \(H. hongdechloris\) cells in 50% D\(_2\)O-seawater KES medium under RL illumination for 2 weeks, the cells were subjected to three different treatments for another 2 weeks. Both deuterated Chl \(a\) and Chl \(f\) were present in RL cultures, and the kinetics of d-labeled Chl \(a\) and Chl \(f\) is shown.

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**A. 30% D\(_2\)O culture**

**B. 50% D\(_2\)O culture**

**C. 70% D\(_2\)O culture**

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**Figure 4.** Mass spectrum of deuterated chlorophyll \(a\) extracted from cells grown in 30% (A), 50% (B), and 70% (C) D\(_2\)O-seawater KES medium under far-red light conditions for 4 weeks. The envelope formed by the empty bars represents the simulated mass spectra with the predicted deuterated rate of Chl \(a\). If 14% of the hydrogen atoms of chlorophyllide \(a\) were deuterated, the mass distribution of Chlile \(a\) would have centered at 619 m/z. If 7% of hydrogen atoms of Chl \(a\) were deuterated, the mass distribution of Chl \(a\) would have centered at 899 m/z. The atom %D is increased following the increased D\(_2\)O percentage in the KES medium.
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mass spectrum of chlorophyll a collected from 50% D$_2$O culture at week 1

mass spectrum of chlorophyll a collected from 50% D$_2$O culture at week 4

unlabelled  observed  simulated

Relative Intensity (%) vs. Molecular mass (m/z)

macrocycle 36 atom% D

18 atom% D (macrocycle 36 atom% D or phytyl 36 atom% D)

36 atom% D (macrocycle + phytyl)
Figure 6. The deuterated chlorophyll percentage recorded from *H. hongdechloris* grown under far-red light, but in different concentrations of D$_2$O-seawater in the KES medium. *A*, deuterated percentage of Chl *a*. *B*, deuterated percentage of Chl *f*. The error bars represent standard deviation with *n* = 6.

![Graph](image)

Discussion

Chlorophyll *f* is known as a “red light–induced” chlorophyll (31) and has a unique modification with a formyl group at the C2' position instead of the methyl group of Chl *a* (32). The conversion of the C2'-methyl group of Chl *a* to the C2-formyl group of Chl *f* may have a similar reaction mechanism as that of Chl *b* synthesis, which has a formyl group at the C7 position (29). Using $^{18}$O$_2$ as the isotopic source, two $^{15}$O atoms are observed to be incorporated in the newly synthesized Chl *f*, which was different to only one $^{18}$O detected as labeled in Chl *a* (Fig. 3), indicating that the additional oxygen atom of Chl *f* originates from molecular oxygen. This clearly demonstrates that the oxygen in the formyl group of Chl *f* is derived from molecular oxygen and that the formation of the C2-formyl group of Chl *f* is an oxygenase-type reaction.

The observation in the $^{18}$O$_2$ experiment is that doubly $^{18}$O-labeled Chl *f* is always the same or at a lower percentage than the $^{18}$O-labeled Chl *a* in the same experiment. In addition, the singly $^{18}$O-labeled Chl *f* is much higher than that of Chl *a*. These findings support the conclusion that Chl *f* is derived from Chl *a* that comes from both new synthesis as well as from previously synthesized and hence unlabeled Chl *a*. It also suggests that the substitution of the formyl group at C2' position occurred at a late stage of the chlorophyll biosynthesis pathway.

In our study, *H. hongdechloris* cells were able to grow in 50% D$_2$O-seawater medium without any significant effects on their growth rate, indicating the tolerances of *H. hongdechloris* against the toxicity of D. Our results were similar to the other study where strains of *Escherichia coli*, *Bacillus subtilis*, and *Bacillus thuringiensis* showed no significant change in growth rate when grown in the presence of up to 50% D$_2$O; however, a higher percentage of D$_2$O (>50%) reduced the growth rate of
the most of these organisms (17). Similarly, Lester et al. (33) evaluated a number of bacterial species including E. coli and found that 50% D2O did not inhibit their growth rate. Thus, D2O up to 50% may provide an ideal measure for labeling studies such as the present study in different bacterial communities. A few studies have also extended the scope of growing algae and other microbes in a high concentration of D2O of ~99% by either providing them an extended period of adaptation in D2O or by acclimatizing them through a sequential subculturing into increasing concentrations of deuterium (24, 34, 35). In our study, 50% D2O-seawater was used for H. hongdechloris culture without any prior acclimation. This was necessary to perform the experiment where cells were shifted from H2O-seawater to 50% D2O-seawater medium and vice versa.

Our hypothesis that Chl a is the precursor of Chl f was also supported by the D2O-labeling experiments performed under both WL and RL conditions. In the WL-D2O experiment, no Chl f was detected in the first 2 weeks because of being a red light–induced chlorophyll. However, after shifting cells from WL-D2O to WR-H2O for a week, newly synthesized Chl f was as high as ~39% deuterated percentage, even though the cells were transferred from D2O-seawater to H2O-seawater medium after changing the light conditions (Fig. 7A). The deuterated Chl f observed from the week 3 samples in WL-D2O experiments confirms our hypothesis that Chl f is derived from Chl a. Similar to the WL experiment, the percentage of δ-labeling in Chl a and Chl f in RL also suggests that Chl a is the precursor of Chl f. In the RL experiment, a lag phase of incorporation of D in the Chl f was observed after 1 week with only ~1% deuterated Chl f. This was because deuterated Chl a was unavailable at the beginning of the experiment, and hence mostly unlabeled Chl a was metabolized into unlabeled Chl f, resulting into a very small fraction of the deuterated Chl f. After 1 week, newly synthesized deuterated Chl a was metabolized into newly synthesized Chl f, and hence ~10% deuterated labeling was observed in the Chl f. Moreover, a slight increase in the deuterated Chl f at 4 weeks was observed when cells were shifted from culture medium containing D2O at 2 weeks into the medium without D2O (RR-H2O experiment). The observed increase in deuterated Chl f in the RR experiment could be due to the deuterated Chl a metabolizing into Chl f after 1 week of adaptation of the cells into the new medium. Additionally, the delay of 18O-labeling Chl f observed from H218O (37) and 18O2-labeling experiments (Figs. 2 and 3) indicated that Chl f is synthesized from Chl a or at a late stage of the chlorophyll biosynthesis pathway. The current experimental results cannot exclude the possibility that chlorophyllide a may be the immediate precursor of chlorophyllide f instead of Chl a. We know that the last step of chlorophyll biosynthesis is esterification of the chlorophyllide with a phytol-pyrophosphate to produce chlorophylls. The deuterated chlorophylls can originate from the precursors of the magnesium tetrapyrrrole five-membered ring structure (chlorophyllides) and the precursors of the phytol chains. However, we could not distinguish the atom %D in the ring structures from the atom %D in the phytol chains, because of the limitations of mass spectral analysis (> 500 Da) for the small molecular fraction of deuterated phytol chains with a predicted molecular mass of ~278 Da. The deuterated chlorophyll is the sum of the deuterated ring structure (chlorophyllides) and the deuterated phytol chain. The observed atom %D at the chlorophyllide section, which was higher than that of total chlorophyll atom %D from the same samples, could indicate that the deuterated chlorophyllide percentage was the sum of D labeling at the ring structure, with and without the D labeling at the phytol chain, because of two individual biosynthetic pathways. That Chl f is derived from Chl a further supports the Granick hypothesis of forward direction of evolution for the Chl a and heme biosynthesis pathways (36), which states that pathways evolved forward as organisms evolved. However, it is still unclear whether chlorophyllide a or Chl a is the central molecule for Chl f biosynthesis. Moreover, it is also unclear whether the formyl group of Chl f is synthesized through a one-step or two-step reaction from the C21 methyl group of Chl a. Similarly, further work needs to be done to understand whether Chl f degrades via Chl a, similar to Chl b through a hydroxymethyl chlorophyll intermediate (8).

The 18O2 and H218O experiments revealed the origin of all oxygen atoms of Chl f. They also indicate that the extra oxygen at C21 formyl group of Chl f originates through an oxygenase-type of reaction mechanisms. Our D2O and 18O isotope labeling suggests that Chl a is the precursor of Chl f biosynthesis, similar to that of Chl b and Chl d. The results of this study show that D2O can be used as a powerful tool for stable isotope labeling in cyanobacteria to examine biosynthesis pathways. The results reported here have an important implication for understanding the evolution and biosynthesis pathway of chlorophylls and their formyl group incorporations.

Materials and methods

Culture conditions

H. hongdechloris cells were cultured in modified “K” enrichment seawater (KES) medium (37) under either RL with intensity of 10 μE, or under WL at ~20 μE, on a rotating flatbed shaker at ~100 rpm. Isotopic labeling experiments were performed on cultures grown under RL conditions except where detailed light conditions have been indicated. In each experiment, cultures were homogenized and precultured for 4 days prior to subculturing to the specified conditions, because the doubling time of H. hongdechloris is ~3–4 days (38).

Sea salt mixture was bought from a local store (Instant Ocean), and seawater medium was made according to the manufacturer’s instructions. D2O (2H2O) (99.5% H atom in the 2H form) was obtained from the Australian Nuclear Science and Technology Organization. To make the required percentage of D2O-seawater, the sea salt was dissolved in D2O mixed with H2O to the required percentage of D.

H18O experiment

H. hongdechloris cells were harvested from 1 ml of culture with OD700nm = 0.2 and transferred into either 1 ml of H218O (97%, 18O; Sigma–Aldrich) or H218O-modified KES artificial seawater medium. The cells were collected for pigment analysis at 4, 8, 12, and 16 days. The experiment was repeated as above, but cells were sampled at 2, 6, 10, and 14 days. Each sampling point had three technical repeats.
**18O2 experiment**

*H. hongdechloris* cells harvested from 1 ml of the culture with OD707 nm = 0.2 were transferred to 6-ml vacuum vials (Sarstedt). 4 ml of premixed 50% 18O2 and 50% dinitrogen (N2) gas was injected into the vacuum vials before culture inoculation. The controls were set up by injecting 4 ml of premixed 50% 16O2 and 50% dinitrogen (N2) gas. Two biological replicates for each gas treatment including the control were tested and sampled for pigment isolation up to 16 days. Each purified chlorophyll sample was analyzed with three individual replicates for mass spectral analysis. The whole experiment was repeated, and results of the two experiments were combined to verify the experimental data.

**Growth in D2O**

Different percentages of D2O-seawater KES medium were used to test the tolerances of *H. hongdechloris* cell culture to the presence of D2O, and the potential toxicities of D2O of the cultures. *H. hongdechloris* cells were cultured in three different concentrations of D2O-seawater as 30, 50, and 70% for 4 weeks. The deuterated percentage of isolated chlorophylls compared with undeuterated chlorophyll remaining was indicative of the toxicity of D2O to the culture. We use 24-well culture plates for examining the tolerance of the cells to the presence of D2O and for the following pulse-chase experiments. Each well contains 2 ml of *H. hongdechloris* cell culture with a Qy value of 0.6 at 665 nm (in 100% methanol) at inoculation time. The samples are collected randomly from individual well following the designed experiments.

**Pulse-chase of D2O and H2O**

The 50% D2O-seawater treatment was selected as the optimal D2O concentration in terms of growth rate and in being able to easily distinguish labeled from unlabeled Chls, and this treatment was used in all further labeling experiments. The experimental setup relied on the previously reported observations that *H. hongdechloris* cells growing under WL produce mainly Chl *a* and no Chl *f*, whereas when *H. hongdechloris* is grown under RL light conditions, Chl *f* accumulates to ~15% of the Chl *a* concentration (31, 39). In switching cultures from WL to RL, Chl *f* becomes detectable within 2 days, and after switching from RL to WL, Chl *f* starts disappearing within 2 days (38). Comparing the labeling patterns of Chl *a* and Chl *f* during a switch of light conditions and at the same time transferring the cultures from D2O to H2O would provide data to determine whether Chl *f* is synthesized from precursors like Chl *a* already present in the culture or from *de novo* synthesis. The cultures were maintained under RL or WL for a minimum of 3 weeks before commencement of the experiments. Two different starting conditions were used in these experiments (Fig. 8): 1) WL-D2O cell cultures were grown under WL in 50% D2O-seawater KES medium for 2 weeks, and 2) RL-D2O cultures were grown under RL in 50% D2O-seawater KES medium for 2 weeks.

**Treatment 1 experiment**

WL-D2O cells (Fig. 8A) were equally divided into three sub-treatments for another 2 weeks. For condition 1 (WL-D2O), the cells were continuously cultured under the same WL conditions in 50% D2O-seawater KES medium. For condition 2 (WW-H2O), the cells were rinsed with H2O-seawater and cultured in H2O-seawater KES medium but remained under WL conditions. For condition 3 (WR-H2O), the cells were rinsed with H2O-seawater and cultured in H2O-seawater KES medium under RL condition. Cells from each treatment were sampled at weekly intervals for 4 weeks for pigment extraction and further HPLC and mass spectral analysis.

**Treatment 2 experiment**

RL-D2O cells (Fig. 8B), were equally divided into three sub-treatments for another 2 weeks. For condition 1 (RL-D2O), the cells were continuously cultured under the same RL conditions in 50% D2O-seawater KES medium. For condition 2 (RW-H2O), RL-D2O cells were rinsed with H2O-seawater and cultured in H2O-seawater KES medium under WL condition. For condition 3 (RR-H2O), the RL-D2O cells were rinsed with H2O-seawater and cultured in H2O-seawater KES medium but remained under RL conditions. Cells from each treatment were sampled at weekly intervals for 4 weeks for pigment extraction and further HPLC and mass spectral analysis.

**Pigment extraction and chlorophyll purification**

All pigment extractions and purifications were conducted under dim green light and at 4 °C where possible. The cells were harvested by centrifugation at 13,000 rpm for 5 min, and pigments were extracted using prechilled 100% methanol. The extracted methanolic samples were immediately used for HPLC purification as described previously (40). Chl *f* and Chl *a* were separated using a C18 reverse phase column (Kinetex, 100 × 4.6 mm; Phenomenex, Lane Cove, Australia) on a Shimadzu HPLC (model 10A series) using 100% methanol as the mobile phase at a flow rate of 1 ml/min. Purified Chl *a* and Chl *f* were collected at their retention times and immediately dried.
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by vacuum centrifugation. Dried Chl a and Chl f were stored in the dark at −80 °C for further mass spectral analysis.

Mass spectrometry

The purified chlorophylls were mixed with 4 μl of the matrix tert-thiophene (Sigma) (10 mg/ml in acetone) and placed on the sample holder plate. For 18O2 and H218O experiments, pigments were analyzed by MALDI-MS on a Voyager-DE STR (Applied Biosystems) as described before (14). For D2O experiments, mass spectral analyses were performed using an Applied Biosystems Q-STAR Pulsar with a MALDI source (Mass Spectrometry Core Facility, University of Sydney). Data analysis was performed using Data Explorer and Analyst TF 1.7.1 software for the MALDI-MS performed on the Voyager-DE STR and Q-STAR, respectively.

Calculation of isotopic-labeled chlorophylls

Chl a (C₅₅H₇₂N₄O₁₅Mg) contains five oxygen atoms and 72 hydrogen atoms and has average molecular mass and monoisotopic mass values of 893.5426 and 892.5353 Da, respectively; Chl f (C₅₂H₇₀N₄O₁₄Mg) has six oxygen atoms and 70 hydrogen atoms and has average molecular mass and monoisotopic mass values of 907.5219 and 906.5146 Da, respectively. Non-labeled Chl a has seven mass peaks of 892.5 to 898.5 with the relative ion intensities of 100, 75, 42, 15.6, 3.9, 0.7, and 0.1 associated with the natural abundance of isotopic carbon, oxygen, hydrogen, and magnesium. Non-labeled Chl f has seven mass peaks of 906.5 to 912.5 with the relative intensities of 100, 75, 42.2, 15.8, 4, 0.7, and 0.1. The relative concentrations of each isotopic 18O incorporated into the Chls were calculated as described before (10, 14). The values obtained in the control experiment were deducted from the calculated values of the labeled 18O isopomers to avoid any noise and artifacts obtained in the mass spectrum.

Calculation of isotopic-labeled chlorophylls

Chl a molecules ionized by MALDI-TOF analysis have two major ion peak regions at m/z 892.5–898.5 and m/z 614.2–620.5, which correspond to M⁺ masses of chlorophyll a and chlorophyllide a, respectively. Similarly Chl f molecules ionized during MALDI-TOF analysis have two major ion peaks region at m/z 906.5–912.5 and m/z 628.2–634.5, which correspond to M⁺ masses of chlorophyll f and chlorophyllide f, respectively. MS data from both chlorophyll and chlorophyllide ions were calculated using the matrix developed previously in both H218O- and 18O-labeling experiments to calculate the abundance of 18O containing chlorophylls (10, 14).

With 50% D2O in the medium, all hydrogen atoms in the chlorophylls could be potentially labeled. The labeling results in an envelope of mass peaks well separated from the unlabeled chlorophyllous masses. Unless stated otherwise, the calculation of the ratio of deuterated to undeuterated chlorophylls/chlorophyllides was performed by the sum of peak heights of the first six monoisotopic masses as representing undeuterated chlorophyll (unlabeled chlorophylls), whereas the sum of peak intensities of the monoisotopic masses greater than six represented the deuterated chlorophyll (labeled chlorophylls). The peak heights were only summed for peaks with masses ±0.02 mass units from the expected calculated monoisotopic masses and also only with a signal to noise ratio of >3:1.

The extent of deuterated atom percentage (%D per molecule) in 50% D2O-seawater KES medium was calculated by the simulation described previously (41) and available online at https://www.ncbi.nlm.nih.gov/CBBResearch/Yu/midas/index.html.3 The basic assumption of the program is that the amount of deuterium atoms available for each position in Chl a and Chl f is equal. The expected profile of labeled peaks with different percentages of D out of 72 H atoms in Chl a or 70 H atoms in Chl f were thus calculated and compared with the experimental data to determine the extent of labeling.

Author contributions—H. G. performed the experiments. P. C. L. contributed to the 18O-labeling. H. G., R. D. W., and M. C. designed the experiments, analyzed the data, and wrote the manuscript.

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Chlorophyll f traced using isotopes