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1 **Fatty acid photodecarboxylase is an ancient photoenzyme that forms hydrocarbons in the**
2 **thylakoids of algae**

3

4 Solène L.Y. Moulin¹, Audrey Beyly-Adriano¹, Stéphan Cuiné¹, Stéphanie Blangy¹, Bertrand Légeret¹,
5 Magali Floriani², Adrien Burlacot^{1,*}, Damien Sorigué¹, Poutoum-Palakiyem Samire¹, Yonghua Li-
6 Beisson¹, Gilles Peltier¹, Fred Beisson¹

7

8 ¹Aix-Marseille University, CEA, CNRS, Institute of Biosciences and Biotechnologies of Aix-
9 Marseille (BIAM), UMR7265, CEA Cadarache, 13108 Saint-Paul-lez-Durance, France.

10 ²Institut de Radioprotection et de Sûreté Nucléaire (IRSN), PRP-ENV/SRTE/LECO,
11 Cadarache, 13108 Saint-Paul-Lez-Durance, France.

12 *Present address: Howard Hughes Medical Institute, Department of Plant and Microbial
13 Biology, 111 Koshland Hall, University of California, Berkeley, CA 94720-3102 USA

14

15 ***One sentence summary* : Most algal lineages have the ability to produce hydrocarbons in**
16 **thylakoids of the chloroplast.** FAP is present in thylakoids and conserved beyond green algae.

17 ***Short title: FAP location, function and biodiversity***

18 Corresponding author: Fred Beisson

19 CEA Cadarache, France; Email: frederic.beisson@cea.fr

20 Tel: +33442252897 Fax: +3344225626

21

22 Author Contributions:

23 F.B. conceived the original research project; F.B., S.M. and G.P. designed the experiments and
24 analyzed the data; S.M., A.B.-A., S.C., S.B., D.S., B.L., A.B., P.S. performed experiments;
25 M.F. performed the TEM study; S.M. performed phylogenetic analysis; F.B. and S.M. wrote
26 the article with contributions from Y.L.-B. and G.P.

27

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ABSTRACT

Fatty acid photodecarboxylase (FAP) is one of the few enzymes that require light for their catalytic cycle (photoenzymes). FAP was first identified in the microalga *Chlorella variabilis* NC64A, and belongs to an algae-specific subgroup of the glucose-methanol-choline oxidoreductase family. While the FAP from *C. variabilis* and its *Chlamydomonas reinhardtii* homolog CrFAP have demonstrated *in vitro* activities, their activities and physiological functions have not been studied *in vivo*. Furthermore, the conservation of FAP activity beyond green microalgae remains hypothetical. Here, using a *C. reinhardtii* FAP knockout line (*fap*), we showed that CrFAP is responsible for the formation of 7-heptadecene, the only hydrocarbon of this alga. We further showed that CrFAP was predominantly membrane-associated and that >90% of 7-heptadecene was recovered in the thylakoid fraction. In the *fap* mutant, photosynthetic activity was not affected under standard growth conditions, but was reduced after cold acclimation when light intensity varied. A phylogenetic analysis that included sequences from Tara Ocean identified almost 200 putative FAPs and indicated that FAP was acquired early after primary endosymbiosis. Within Bikonta, FAP was retained in secondary photosynthetic endosymbiosis lineages but absent from those that lost the plastid. Characterization of recombinant FAPs from various algal genera (*Nannochloropsis*, *Ectocarpus*, *Galdieria*, *Chondrus*) provided experimental evidence that FAP photochemical activity was present in red and brown algae, and was not limited to unicellular species. These results thus indicate that FAP was conserved during the evolution of most algal lineages where photosynthesis was retained, and suggest that its function is linked to photosynthetic membranes.

61 INTRODUCTION

62 Most organisms have the ability to synthesize highly hydrophobic compounds made only of
63 carbon and hydrogen called hydrocarbons (HCs). Many HCs are isoprenoids but others like *n*-
64 alkanes and their unsaturated analogues (*n*-alkenes) derive from fatty acids (Herman and Zhang,
65 2016). In plants, C29-C35 *n*-alkanes are synthesized in the epidermis from very-long-chain
66 fatty acids, and secreted onto the surface of aerial organs (Lee and Suh, 2013). Plant *n*-alkanes
67 are important for adaptation to the terrestrial environment because they constitute a major part
68 of the wax layer of the extracellular cuticle that prevents the loss of internal water. Occurrence
69 of *n*-alka(e)nes has also been reported in microorganisms such as microalgae (Sorigué et al.,
70 2016) and cyanobacteria (Schirmer et al., 2010). The magnitude and biogeochemical
71 importance of HC production by marine cyanobacteria (and possibly microalgae) is an
72 intriguing question (Lea-Smith et al., 2015; Valentine and Reddy, 2015). Microbial HCs are
73 presumably mostly located in membranes, but their physiological function has not been studied
74 much. While in cyanobacteria *n*-alka(e)nes have been proposed to play roles in cell growth, cell
75 division, photosynthesis and salt tolerance (Berla et al., 2015; Lea-Smith et al., 2016; Yamamori
76 et al., 2018; Knoot and Pakrasi, 2019), in microalgae the biological function of *n*-alka(e)nes is
77 still completely unknown. Besides the elucidation of their biological roles, *n*-alkanes and *n*-
78 alkenes have also attracted attention because of their interest as fuels, cosmetics, lubricants and
79 as synthons in organic chemistry (Jetter and Kunst, 2008; Jiménez-Díaz et al., 2017). Bio-based
80 alka(e)ne production would be highly desirable to replace part of petroleum-derived HCs, and
81 is currently the focus of intense research efforts (Zhou et al., 2018; Liu and Li, 2020).

82 A number of *n*-alka(e)ne-forming enzymes has been identified and characterized in the
83 last decade, and it is now clear that conversion of fatty acids to HCs occurs through a variety
84 of reactions and proteins (Herman and Zhang, 2016). Besides, the biosynthetic enzymes
85 involved in the same types of reactions are not conserved across phylogenetic groups. For

86 instance, it has been shown in bacteria that synthesis of terminal olefins (1-alkenes) occurs
87 through decarboxylation of a saturated long-chain fatty acid, and that this reaction is catalyzed
88 by a cytochrome P450 in *Jeotgalicoccus* spp. (Rude et al., 2011) and by a non-heme iron
89 oxidase in *Pseudomonas* (Rui et al., 2014). In the bacterium *Micrococcus luteus*, yet another
90 pathway has been discovered, which consists of the head-to-head condensation of fatty acids to
91 form very-long-chain *n*-alkenes with internal double bonds (Beller et al., 2010). Cyanobacterial
92 *n*-alka(e)nes are produced by two distinct pathways (Schirmer et al., 2010; Mendez-Perez et al.,
93 2011), which have been shown to be mutually exclusive (Coates et al., 2014). In insects, a
94 cytochrome P450 oxidative decarbonylase acts on an aldehyde to produce cuticular alka(e)nes
95 (Qiu et al., 2012). The plant pathway producing the very-long-chain *n*-alkanes of the cuticular
96 waxes may also involve an aldehyde intermediate, and is known to require the ECERIFERUM
97 (CER) proteins : CER1 and CER3 (Bernard et al., 2012), which are homologous to the oxygen-
98 dependent membrane class of diiron fatty acid desaturases.

99 In microalgae, we have shown that C15-C17 *n*-alka(e)nes occur in *Chlorella variabilis*
100 NC64A (named *C. variabilis* from here on), and that they are synthesized through
101 decarboxylation of long-chain fatty acids (Sorigué et al., 2016). A *C. variabilis* protein with a
102 fatty acid decarboxylase activity was then identified as a photoenzyme (Sorigué et al., 2017), a
103 rare type of enzyme that requires photons at each catalytic cycle (Björn, 2015). The *C. variabilis*
104 protein was thus named fatty acid photodecarboxylase (FAP, E.C. 4.1.1.106). It is one of the
105 few photoenzymes discovered so far, the others being the photosystems, DNA photolyases, and
106 light-dependent protochlorophyllide oxidoreductase. FAP belongs to a family of flavoproteins
107 (Sorigué et al., 2017), the glucose-methanol-choline (GMC) oxidoreductases, which includes a
108 large variety of enzymes present in prokaryotic and eukaryotic organisms (Zámocký et al.,
109 2004). FAP activity thus represents an additional type of chemistry in the GMC oxidoreductase
110 family (Sorigué et al., 2017). Molecular phylogenetic analysis has shown that *C. variabilis* FAP

111 and *Cr*FAP belong to an algal branch of GMC oxidoreductases. However, whether FAP activity
112 is conserved in other algal lineages beyond green algae and whether FAP is indeed responsible
113 for *n*-alka(e)ne formation *in vivo* remain to be demonstrated. Besides, the subcellular location
114 and role of FAP in algal cells have not yet been investigated.

115 In this work, we isolate and characterize in *C. reinhardtii* an insertional mutant deficient
116 in FAP (*fap* mutant strain). We show that FAP is indeed responsible for the formation of 7-
117 heptadecene, the only fatty acid-derived HC present in this alga. In addition, we provide
118 evidence for a thylakoid localization of *C. reinhardtii* FAP and its alkene product. We also
119 show that growth and photosynthesis are not affected in the knockout under laboratory
120 conditions, but photosynthetic efficiency is impacted under cold conditions when light intensity
121 varies. Finally, we build a large molecular phylogeny of GMC oxidoreductases based on TARA
122 Ocean data and identify almost 200 uncharacterized putative FAP sequences across algal
123 lineages. Experimental evidence demonstrates that FAP photochemical activity is conserved in
124 red and brown algae, and, since it is also present in macroalgae, is not limited to unicellular
125 species.

126

127 **RESULTS**

128 **FAP is responsible for alkene synthesis in *C. reinhardtii***

129 *Chlamydomonas reinhardtii* (hereafter named *C. reinhardtii*) has been previously shown to
130 produce 7-heptadecene (C17:1-alkene) from *cis*-vaccenic acid (Sorigué et al., 2016) and to have
131 a FAP homolog that can also perform photodecarboxylation of fatty acids *in vitro* (Sorigué et
132 al., 2017). Although it seemed likely that FAP proteins are indeed responsible for the synthesis
133 of alka(e)nes produced by *C. reinhardtii* and *C. variabilis*, the possibility that the alkenes are
134 formed *in vivo* by another enzyme could not be ruled out. In order to address this issue and
135 investigate the biological role of FAP, a *C. reinhardtii* strain mutated for FAP was isolated from

136 the *Chlamydomonas* Library Project (CLiP) (Li et al., 2016). This strain showed undetectable
137 levels of FAP protein (**Fig. 1**) and was named *fap-1*. The only fatty acid-derived HC in *C.*
138 *reinhardtii*, i.e., 7-heptadecene, could not be detected in *fap-1* knockout or in the two other *fap*
139 mutants isolated (**Fig. 1**). After performing nuclear complementation of *fap-1* using the
140 genomic *FAP* gene expressed under the promotor PsaD, 4 independent transformants with
141 different expression levels of *C. reinhardtii* FAP (*CrFAP*) were isolated (named Cp-1 to -4). In
142 these complemented strains, production of 7-heptadecene was clearly related to FAP amount
143 (**Fig. 1**). These results thus demonstrate that *CrFAP* is responsible for alkene formation *in vivo*
144 in *C. reinhardtii*. Cp-4 was one of the three complemented strains with 7-heptadecene levels
145 similar to the WT strains used for physiological studies (see Material and Methods for the
146 isolation of the WT, *fap* and *Cp* strains).

147

148 **FAP activity is conserved beyond green microalgae**

149 Molecular phylogeny of GMC oxidoreductases has previously shown that *CrFAP* and the FAP
150 from *C. variabilis* (*CvFAP*) are present in an evolutionary branch containing only sequences
151 from algae (Sorigué et al., 2017). The term “algae” is used here in the classical sense of
152 photosynthetic organisms that have chlorophyll *a* as their primary photosynthetic pigment and
153 lack a sterile covering of cells around the reproductive cells (Lee, 2008). To investigate whether
154 FAP activity has been conserved in algal groups other than green algae, genes encoding putative
155 FAPs from selected algal lineages were cloned and expressed in *E. coli*, and the bacterial HC
156 content was analyzed. Considering the basal position of red algae, we decided to explore FAP
157 activity in Rhodophytes, selecting the microalga *Galdieria sulphuraria* and the macroalga
158 *Chondrus crispus*. For algae deriving from secondary endosymbiosis, we also chose the
159 microalga *Nannochloropsis gaditana* and the macroalga *Ectocarpus silicosus*. When cultivated
160 under light, all *E. coli* strains expressing the various FAPs produced a range of *n*-alkanes and

161 *n*-alkenes with different chain lengths (C15 to C17) in various proportions (**Fig. 2A**). The small
162 amount of HCs detected in *E. coli* cells cultivated under dark was not observed in the non-
163 transformed strain or culture medium, and is thus likely to be due to brief illumination required
164 in the cell treatment process. Interestingly, among the FAPs investigated, we observed some
165 profiles of conversion of *E.coli* fatty acids to HCs that are different from that of CvFAP (**Fig.**
166 **2B**). Taken together, these results therefore show that FAP photoenzymatic activity i) is present
167 in red algae, ii) has been conserved in algae with secondary plastids, iii) is not limited to
168 unicellular algae and iv) is likely to be diverse with respect to fatty acid specificity.

169

170 **Identification of a reservoir of putative FAPs**

171 To provide a wider picture of the occurrence and evolution of putative FAP photoenzymes
172 within algal groups and to increase the reservoir of FAPs for future biotechnological purposes,
173 a large phylogenetic analysis of GMC oxidoreductases sequences was conducted. We used
174 GMC oxidoreductases retrieved from public databases, from sequenced algal genomes (Blaby-
175 Haas and Merchant, 2019) and from the Tara Ocean project (de Vargas et al., 2015). Tara data
176 gave a unique opportunity to enlarge the FAP dataset with marine algal species that may not be
177 easy to grow under laboratory conditions and whose genomes have not been sequenced. Protein
178 sequences sharing between 50 to 33% of homology with the sequence of *C. variabilis variabilis*
179 FAP were retrieved using Basic Local Alignment Search Tool (BLAST) searches in algal
180 genomes and in the TARA dataset (**Supplemental Tables S1, S2**). Over 500 putative GMC
181 oxidoreductases were thus identified. Additional putative GMC oxidoreductases from various
182 taxa of the three domains of life were selected from public databases in order to have a
183 representative diversity of the whole GMC oxidoreductase superfamily.

184 Molecular phylogenetic analysis identified almost 200 sequences of putative GMC
185 oxidoreductases that grouped with the 5 FAPs that have demonstrated biochemical activity

186 (CvFAP, CrFAP and the four additional FAPs characterized in this study). These sequences
187 constitute a reservoir of putative FAPs. Sequences in this FAP group all belonged to algal
188 species (**Fig. 3, Supplemental Fig. S1**). Logo sequences built with the putative FAPs exhibited
189 highly conserved patterns, some of which were absent in the other GMC oxidoreductases
190 (**Supplemental Fig. S2**). Interestingly, the residues C432 and R451 of CvFAP active site
191 (Sorigué et al., 2017; Heyes et al., 2020) were strictly conserved, and this feature was specific
192 to the FAP group. It is thus likely that all proteins of this group are genuine FAPs.

193 Sequences from plants as well as other streptophytes (including charophytes) did not
194 group with algal FAPs (**Fig. 3, Supplemental Fig. S1**). Absence of FAP in charophytes
195 indicated early loss of FAP function in streptophytes. No putative FAP sequences could be
196 found in cyanobacteria, although this group is highly represented in TARA data (de Vargas et
197 al., 2015). Phylogeny within the FAP branch indicated that red algae (rhodophytes) sequences
198 were the most basal. Interestingly, FAP sequences from secondary endosymbiosis-derived
199 species appeared to be more closely related to FAPs of green algae (chlorophytes) than red
200 algae.

201 Overall, the putative FAPs that could be identified in algae were present in a variety of
202 algal groups, including stramenopiles (heterokonts), haptophytes and dinophytes. Most
203 eukaryotic algae harbored one putative FAP and no other GMC oxidoreductase, but a few algae
204 contained no FAP and/or several non-FAP GMC oxidoreductases (**Fig. 4**). Indeed, no putative
205 FAP could be found in the sequenced algal genomes of the glaucocystophyte *Cyanophora*
206 *paradoxa*; Mamiellophyceae species: *Ostreococcus* spp., *Micromonas* spp. and *Bathycoccus*
207 spp.; and the diatom *Thalassiosira pseudonana*. Conversely, only a few algal sequences could
208 be found in other branches of the GMC oxidoreductase family. Existence in the diatom
209 *Thalassiosira pseudonana* of a GMC oxidoreductase grouping with bacterial choline
210 dehydrogenase was supported by one sequence from the sequenced genome (Tps-GMC) and

211 one sequence from Tara (48230190). A Tara sequence annotated as a *Pelagomonas* protein
212 (5166790) also turned out not to be located on the FAP clade. The cryptophyte *Guillardia theta*
213 contained 3 different GMC oxidoreductases in 3 different branches but none of them grouped
214 with FAPs. *Ulva mutabilis* had 11 predicted GMCs, but only one was in the FAP clade. The ten
215 other members of this multigene family of *Ulva* appeared to form a group close to plant GMC
216 oxidoreductases. Although exceptions similar to these probably exist in algal diversity, the
217 general picture is that most algae have only one GMC oxidoreductase and that it groups with
218 the characterized FAP clade in the phylogenetic tree.

219

220 ***C. reinhardtii* FAP binds membranes, and most of its products are found in the thylakoid**
221 **fraction**

222 *CrFAP* is predicted by PredAlgo (Tardif et al., 2012), a software dedicated to the analysis of
223 subcellular targeting sequences in green algae, to be localized to the chloroplast. Consistently,
224 *CrFAP* is found in a set of 996 proteins proposed to be chloroplastic in *C. reinhardtii*
225 (Terashima et al., 2011). A broader study of putative FAP targeting peptides, also using
226 PredAlgo, indicated that FAPs from various green and red algae were largely predicted to be
227 chloroplastic (**Supplemental Fig. S3**). In algae with secondary plastids (i.e. containing 3 or 4
228 membranes), the presence of a signal peptide was consistent with targeting to the ER or
229 chloroplast ER (CER) membrane. Further analysis performed using ASAFind (Gruber et al.,
230 2015), a prediction tool designed to recognize CER targeting motifs in signal peptides, indicated
231 that such a motif was present in *Ectocarpus silicosus* and *Nannochloropsis gaditana*. Taken
232 together, these results suggest that FAP homologs are very likely to be localized to chloroplasts
233 in green algae, in red algae, and also in at least some of the algae that acquired plastids through
234 secondary endosymbiosis.

235 Since *CrFAP* is a soluble protein (Sorigué *et al.* 2017) and is predicted to be plastidial,
236 we then sought to determine whether it is a stromal protein with no affinity for membranes or
237 if it has some ability to bind thylakoid membranes. Subcellular fractionation of *C. reinhardtii*
238 cells was therefore performed (**Fig. 5A**). Most of the FAP protein was found in the total
239 membrane fraction. Thylakoid-enriched membranes were then isolated from whole cells using
240 a sucrose gradient purification procedure (**Fig. 5A**). Co-purification with thylakoids was
241 performed using the D2 protein (PsbD), a thylakoid membrane protein from the photosystem II
242 (PSII) core complex. The fact that the phosphoribulokinase (PRK), used as a control for stroma,
243 could barely be detected in our thylakoid fraction, indicated the presence of little intact
244 chloroplasts or cells. After the thylakoid purification procedure, the amount of *CrFAP* still
245 bound to this fraction varied between purifications, but the protein was always detectable. A
246 salt wash experiment confirmed that the *CrFAP* remaining in the thylakoid fraction was tightly
247 bound to the thylakoids, and thus not likely to be a contaminating stromal protein. When
248 analyzing the percentage of 7-heptadecene in total fatty acids in whole cells versus thylakoid-
249 enriched membranes, a slight but significant enrichment in alkene was found in the thylakoid
250 fraction (**Fig. 5B**). Most importantly, by using the fatty acid C16:1(3t) as a marker of the
251 thylakoid lipids, it could be estimated that the enrichment in 7-heptadecene corresponds, in fact,
252 to the localization of >90% of this compound to the thylakoid fraction (**Fig. 5C**). Taken
253 together, these results indicated that *CrFAP* is a chloroplast-targeted soluble protein that has
254 the ability to bind membranes (including the thylakoid fraction) and whose *n*-alka(e)nes
255 products are mostly associated to the thylakoid fraction.

256

257 **7-Heptadecene content varies with cell cycle in *C. reinhardtii***

258 The lack of FAP and HCs in chloroplasts of *C. reinhardtii* did not result in any obvious
259 differences in the overall organization of cells or chloroplasts as seen by transmission electron

260 microscopy (TEM) (**Supplemental Fig. S4**). To try to gather clues on FAP function, publicly
261 available FAP transcriptomic data were mined. Transcriptomic data (Zones et al., 2015) shows
262 that FAP has a similar expression pattern as those genes encoding proteins of the photosynthetic
263 apparatus (**Supplemental Fig. S5**). In order to determine whether FAP product varied with
264 time, we monitored total fatty acids and 7-heptadecene content during a day-night cycle in
265 synchronized *C. reinhardtii* cells. While a constant level of 7-heptadecene representing 0.45%
266 of total fatty acids was found most of the time, a substantial peak (0.7%) was observed before
267 the night (after 12 hours of light), and a steep decrease in 7-heptadecene content relative to total
268 fatty acids occurred at the beginning of the night (**Fig. 6A**). This decrease in relative 7-
269 heptadecene content was concomitant with cell division, as evidenced by the drop in total fatty
270 acid content per cell that occurred at that time (**Fig. 6B**). Interestingly, the peak in 7-
271 heptadecene (**Fig. 6A**) was not associated with an increase in total FAP amount between 8h and
272 16h (**Fig. 6C**).

273

274 **Fatty acid and membrane lipid compositions differ in the *fap* mutants**

275 Since 7-heptadecene content varied during cell cycle and may thus play a role in cell division,
276 growth of the WT and *fap* strains were analyzed. Growth at 25°C was compared in
277 photoautotrophic conditions (mineral medium (MM)) and in mixotrophic conditions (Tris-
278 acetate-phosphate medium (TAP)). No difference between WT and *fap* could be detected,
279 neither in growth rates nor in cell volumes under these conditions (**Supplemental Fig. S6**). In
280 addition, no difference between WT and *fap* strains could be observed when cells were grown
281 under various concentrations of sodium chloride (**Supplemental Fig. S7**). Although the lack of
282 HCs had no effect on growth, fatty acid profiling showed some differences in C16:1(9),
283 C18:1(9), and C18:3(9-12-15) (**Supplemental Fig. S8**). Synchronized cells also exhibited no
284 growth differences between WT and *fap* strains, but exhibited differences in the dynamics of

285 some fatty acid species (**Supplemental Fig. S9**). Changes in fatty acid profiles prompted us to
286 perform a lipidomic analysis by UPLC-MS/MS. Interestingly, this analysis revealed that a
287 limited set of lipid molecular species were significantly different between WT and *fap* and that
288 they were all plastidial lipids belonging to the galactolipid classes digalactosyldiacylglycerol
289 (DGDG) and monogalactosyldiacylglycerol (MGDG) (**Fig. 7 and Supplemental Fig. S10**).
290 The decrease in the relative content of these galactolipid species appeared to be fully restored
291 by complementation in the case of DGDG but not of MGDG (**Fig. 7**). Taken together, these
292 results indicate that the lack of 7-heptadecene in the *fap* mutant causes a change in thylakoid
293 lipid composition, which is evidenced by the decrease in the relative content of at least 3
294 galactolipid species belonging to the DGDG class.

295

296 **FAP is not strongly associated to photosynthetic complexes, and lack of HCs has no effect**
297 **on their organization**

298 In cyanobacteria, a role of HCs in photosynthesis has been suggested (Berla et al., 2015) but is
299 controversial (Lea-Smith et al., 2016). In *C. reinhardtii*, there was no difference in the 77K
300 chlorophyll fluorescence spectrum between WT, complementation and *fap* mutant strains,
301 which indicated no major changes in antenna distribution around photosystems (**Supplemental**
302 **Fig. S11A**). No difference in photosynthetic efficiency could be detected among WT,
303 complementation and *fap* strains grown under standard laboratory conditions (**Supplemental**
304 **Fig. S11B**). Membrane inlet mass spectrometry (MIMS) experiments conducted to quantify O₂
305 exchange showed no difference in respiration or photosynthesis rates between the two
306 genotypes (**Supplemental Fig. S11C**). Native electrophoresis of proteins from purified
307 thylakoids and FAP immunodetection revealed that FAP could only be detected at an apparent
308 molecular size of the monomeric FAP (**Fig. 8**), indicating no strong association with proteins

309 of photosynthetic complexes. Besides, no difference in organization of photosynthetic
310 complexes between WT and *fap* could be seen on the native protein electrophoresis.

311

312 **Photosynthesis is affected under light and cold stress in the *fap* mutants**

313 Lack of HCs in the *fap* strain did not cause changes in the photosynthesis activity under standard
314 growth conditions. However, since significant modifications in the composition of membrane
315 lipids could be detected, we explored harsher conditions to further challenge photosynthetic
316 membranes. We chose to investigate chilling temperatures because cold is well-known to affect
317 both membrane physical properties and photosynthesis (Los et al., 2013). Using a
318 multicultivator in turbidostat mode, we first stabilized cultures at 25°C under medium light (200
319 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), finding the apparent electron transfer rate (ETR) showed no difference
320 under this condition (**Fig. 9A**). When cooling down the culture to 15°C and after 3 days of
321 acclimation, both ETR and 77 K chlorophyll fluorescence spectra still showed no differences
322 (**Fig. 9B, Supplemental Fig. S11A**). However, after one day at a lower light intensity (50 μmol
323 $\text{photons m}^{-2}\text{s}^{-1}$, 15°C), while the maximal PSII yield was equal for all the strains (**Fig. 9C**), the
324 ETR was lower for the mutant when measured at high light intensities (**Fig. 9D**). Interestingly,
325 longer acclimation to this condition (3 days) led to the disappearance of this phenotype. This
326 transient phenotype of the *fap* KO was clearly linked to low temperature because shifting
327 cultures at 25°C from medium light to low light did not result in any difference in maximal PSII
328 yield or ETR among the strains (**Fig. 9E**).

329 In order to provide support for a possible link between HCs and cold acclimation, 7-
330 heptadecene content was quantified under various growth temperatures. Relative HC content in
331 WT cells clearly increased under cold conditions (**Fig. 10**). Enrichment of WT membranes in
332 HC was due to a slower decrease in HC synthesis compared to fatty acid synthesis (**Fig. 10B**).
333 As expected, an increase in the relative content of polyunsaturated species occurred upon cold

334 treatment in both WT and *fap* strains (**Supplemental Fig. S12**), but no difference in the
335 dynamics of fatty acid remodeling was observed between the two strains.

336

337 **DISCUSSION**

338 Here, we report the isolation and characterization of an insertional *C. reinhardtii* mutant
339 deficient in FAP, and we perform phylogenetic and functional analyses of algal homologs. We
340 show that FAP and the vast majority of its 7-heptadecene product are associated to thylakoid
341 membranes. It is also shown that a gene encoding for a putative FAP is present in most algal
342 lineages and indeed encodes a functional fatty acid photodecarboxylase in some species of red
343 algae, and of secondary algae, including some macroalgae. By studying a FAP knock-out *C.*
344 *reinhardtii* mutant, we provide evidence that lack of HCs is associated correlated with small
345 changes in galactolipid composition, but has no impact on photosynthesis and growth in *C.*
346 *reinhardtii* under standard culture conditions. However, in the absence of HCs generated by
347 FAP, the photosynthetic activity is transiently affected during cold acclimation when light
348 intensity varies. The possible significance of these results for algal physiology, as well as FAP
349 function and evolution, are discussed below.

350

351 ***Cr*FAP localization and regulation in algal cells**

352 Based on the characterization of a *fap* mutant, we first show that *Cr*FAP is responsible for the
353 synthesis of all fatty acid-derived HCs found in *C. reinhardtii* cells (**Fig. 1**). Our result clearly
354 demonstrates that the fatty acid photodecarboxylase activity measured *in vitro* for *Cr*FAP
355 (Sorigué et al., 2017) is not a promiscuous secondary activity and indeed corresponds to a
356 genuine biological activity, namely the light-driven synthesis of 7-heptadecene from *cis*-
357 vaccenic acid (**Fig. 11**). Also, the results from the *fap* knockout line demonstrate that no other
358 enzyme is able to synthesize 7-heptadecene in *C. reinhardtii*. Furthermore, the HC production

359 was found to correspond to the quantity of *CrFAP* present in complementation lines. Thus, FAP
360 is a limiting factor for 7-heptadecene production *in vivo*, but the lipase(s) that may act upstream
361 of FAP to generate the free *cis*-vaccenic acid does not limit the pathway. This conclusion is
362 consistent with a previous observation that 7-heptadecene is increased 8-fold in *C. reinhardtii*
363 when *CvFAP* is overexpressed (Yunus et al., 2018).

364 *CrFAP* is a soluble protein (Sorigué et al. 2017). Using subcellular fractionation of *C.*
365 *reinhardtii* cells and anti-*CrFAP* antibodies, we show here that *CrFAP* is predominantly found
366 in the total membrane fraction (**Fig. 5A**). Based on the predicted plastid localization of *CrFAP*
367 (**Supplemental Fig. S3**) and the fact that the thylakoid fraction harbors >90% of the 7-
368 heptadecene product (**Fig. 5C**), we propose that *in vivo CrFAP* is a soluble chloroplastic protein
369 that can bind to and unbind from thylakoid membranes. The capacity of *CrFAP* to bind to
370 thylakoids is supported by the observation that after the thylakoid purification procedure, a
371 fraction of *CrFAP* is still tightly bound to this fraction (**Fig. 5A**). Plastidial localization may be
372 a general feature of FAPs because presence of plastid transit peptides in FAPs seems to be a
373 general rule in green and red algae (for primary plastids) and is also predicted for some
374 secondary endosymbiosis-derived algae (**Supplemental Fig. S3**).

375 Concerning the regulation of FAP activity in membranes, it is clear that the peak in 7-
376 heptadecene during the cell cycle (**Fig. 6A**) is not due to an increase in the total amount of FAP
377 protein (**Fig. 6C**), and another mode of regulation of FAP activity must exist. Availability of
378 free fatty acid substrates provided by a lipase acting upstream of FAP is a possibility (**Fig. 11**).

379 The peak in *CrFAP* gene expression during the day (**Supplemental Fig. S5**) may correspond
380 to the necessity of a high turnover of the *CrFAP* protein due to photodamage to the protein.
381 This would be consistent with the idea that *C. variabilis* FAP has a radical-based mechanism
382 (Sorigué et al. 2017), which may be the cause of photoinactivation of the protein (Lakavath et
383 al. 2020) and degradation under excessive light (Moulin et al. 2019).

384

385

386 **Role of CrFAP products in membranes**

387 In a work reporting cyanobacterial mutants devoid of fatty acid-derived HCs, it has been
388 suggested that HCs are located in membranes and may play a role in cell division (Lea-Smith
389 et al., 2016). In the proposed cyanobacterial model, integration of HCs into the lipid bilayer
390 would be responsible for membrane flexibility and curvature. HCs may play a similar role in
391 thylakoid membranes of green algae. In light of this connection, it is interesting to note that in
392 *C. reinhardtii*, HC production follows lipid production during cell growth except just before
393 mitosis (**Fig. 6A,B**). The ratio of HCs to FAMES decreased at the beginning of night, when
394 cells are dividing, indicating that the extra amount of HCs synthesized before the night must be
395 somehow lost or metabolized during cell division. Simple mechanisms that could explain HC
396 loss during cell division involve enrichment in HCs at breaking points of plastidial membranes
397 before cell division, exclusion from these membranes during division, and/or loss to the gas
398 phase of the culture due to HC volatility (**Supplemental Fig. 5B**).

399 HCs might thus impact local flexibility of algal plastidial membranes and participate in
400 lipid membrane remodeling during cell division. However, under standard culture conditions,
401 the presence of HCs is apparently not critical for chloroplast structure (**Supplemental Fig. 4**),
402 cell size and cell division rate (**Supplemental Fig. 6**). A role of cyanobacterial HCs in tolerance
403 to salt stress has also been suggested (Yamamori et al., 2018). In *C. reinhardtii*, contrary to
404 what has been shown in cyanobacteria, no difference could be detected in growth under
405 increasing salt concentrations (**Supplemental Fig. 7**). One could thus hypothesize that even if
406 HCs are produced in chloroplasts and accumulate in thylakoids, their function might be
407 different from in cyanobacteria. It is also possible that laboratory culture conditions used for *C.*
408 *reinhardtii* (this study) are far from natural growth conditions where HCs may be necessary.

409 Alternatively, a compensation mechanism for HC loss may operate differently in *C. reinhardtii*
410 and in cyanobacteria. In *C. reinhardtii*, part of this mechanism may involve changes in
411 membrane lipid composition. Interestingly, lipidomic analysis under standard growth
412 conditions unraveled specific changes in DGDG molecular species (**Fig. 7**) but no other
413 significant differences in other class of lipids (**Supplemental Fig. S8**). Taken together, these
414 results suggest that in *C. reinhardtii* HCs play no crucial role in cell division and growth under
415 standard conditions. Cells may adapt to a lack of HCs by some changes in the composition of
416 membranes, which could specifically involve some DGDG galactolipids. Alternatively, or in
417 addition to this proposed effect on properties of the membrane lipid phase, it cannot be ruled
418 out that 7-heptadecene may act locally to disrupt or enhance some specific protein-protein
419 interactions, or may play a yet to be defined role, such as acting as a signaling molecule or its
420 precursor (**Fig. 11**).

421 The fact that FAP gene expression follows that of photosynthesis genes in day-night cycles, the
422 likely localization of FAP in plastids of green and red algae (as well as in some secondary
423 algae), and the localization of part of FAP and almost all its alkene product in *C. reinhardtii*
424 thylakoids, point toward a role of FAPs in the photosynthetic function of algal cells. This idea
425 is strongly reinforced by the conservation of the FAP-encoding gene in many eukaryotic algae
426 but not in non-photosynthetic protists (**Fig. 3 and Fig. 4**) and in *Polytomella*, an algae that has
427 kept some of its plastidial function but lost photosynthesis (Smith and Lee, 2014). As standard
428 culture conditions did not reveal any photosynthesis phenotype in *C. reinhardtii* *fap* mutant
429 (**Supplemental Fig. S11**), more challenging conditions involving colder temperatures and
430 variations in light intensity were tested. These experiments have revealed a difference between
431 WT and *fap* mutant in the photosynthesis activity during acclimation to cold after light intensity
432 varied from medium to low (**Fig. 9**). This difference could be highlighted under the high-light
433 ETR measurement conditions. Interestingly, colder temperatures were associated with

434 membrane enrichment in HCs (**Fig. 10**) and fatty acid profiles followed similar variations with
435 temperature in both WT and *fap* strains (**Supplemental Fig. S12**). Taken together, these
436 observations indicate that adaptations in membrane lipid composition compensate partly for the
437 loss of HCs in standard growth conditions, but not in harsher conditions, such as light intensity
438 variations under cold temperatures.

439 At temperatures lower than 20°C, it is clear that cell division and FAME production slow down
440 (**Fig. 10B**). Interestingly, HC production does not follow the same pattern, and increases with
441 decreasing temperatures (except at 8°C). Enrichment of membranes in HCs with decreasing
442 temperatures is consistent with our proposal that HCs are lost during cell division
443 (**Supplemental Fig. S5**): lower temperature causes reduced division and would hence result in
444 lower HC losses. Whether the sustained production of HC at low temperature is an adaptation
445 mechanism to low temperature or not, remains to be elucidated.

446

447 **Conservation of FAP in algae**

448 According to molecular phylogeny (**Fig. 3, Fig. 4**), FAP proteins appear to be specific to algae
449 and highly conserved in many algae species. A noticeable exception is the Mamiellophyceae
450 class of the green algae. Algae is a common denomination that covers photosynthetic
451 eukaryotes which mainly live in aquatic environments. This polyphyletic group includes
452 organisms derived from a first endosymbiosis, as well as organisms derived from a secondary,
453 or even tertiary, endosymbiosis. However, a functional FAP can be found in Chlorophyta (green
454 algae), Rhodophyta (*Chondrus* and *Galdieria*) and Stramenopiles (in the phaeophyceae
455 *Ectocarpus* and the Eustigmatophyceae *Nannochloropsis*), as demonstrated by heterologous
456 expression in *E. coli* of the corresponding identified FAPs (**Fig. 2**). FAP activity was therefore
457 conserved during secondary endosymbiotic event(s) that gave rise to the red algae lineage.
458 Moreover, FAP activity is not specific to the unicellular state, as FAPs were also functional in

459 the pluricellular algae (macroalgae) *Ectocarpus silicosus* and *Chondrus crispus*. Considering
460 homology of sequences, FAP function is thus expected to be present in most algal phyla,
461 including Haptophyta and Dinophyta. Importantly, some amino acid residues that are likely to
462 be involved in fatty acid substrate stabilization or photocatalysis, such as CvFAP Arg451 or
463 Cys432 (Sorigué et al., 2017; Heyes et al., 2020), are strictly conserved in the 198 putative
464 FAPs (**Supplemental Fig. S2**). This observation reinforces the idea that all the putative FAPs
465 identified in this work have the ability to photo-produce HCs from fatty acids.

466 FAP neofunctionalization from GMC oxidoreductases may have occurred early during
467 evolution of algae, almost concomitantly with the very first endosymbiosis shared by green and
468 red algae. No GMC could be found in Glaucocystophyta (**Fig. 4**), which may indicate that this
469 neofunctionalization event has occurred after separation of this group from red and green algae.
470 However, it should be noted that so far only one complete Glaucocystophyta genome is
471 available. Absence of FAP in charophytes (**Supplemental. Fig. 1**) indicates early loss of FAP
472 function in Streptophyta. Phylogenetic analysis indicates that FAPs from secondary
473 endosymbiosis lineages are more closely related to core Chlorophyta than to Rhodophyta. FAP
474 could thus be one of the genes that was inherited from green algae by horizontal gene transfer
475 (Moustafa et al., 2009).

476 Concerning the conservation of FAP activity, it should be noted that some of the FAPs
477 selected for heterologous expression had fatty acid preference profiles different from that of
478 CvFAP (**Fig. 2B**). For example, while CvFAP converted *E. coli* fatty acids into HCs with a
479 slight preference for C18 over C16 fatty acids, *Chondrus* and *Galdieria* FAPs showed higher
480 preference for C16 over C18, and saturated over unsaturated fatty acids. By contrast,
481 *Ectocarpus* FAP had a strong preference for C16:0 over C16:1 fatty acid, and *Nannochloropsis*
482 *gaditana* FAP preferred unsaturated over saturated chain for both C16 and C18 fatty acids. This
483 indicates that the algal biodiversity is likely to contain FAPs with fatty acid specificities

484 different from the FAPs of *C. variabilis* and *C. reinhardtii*. FAPs with different properties may
485 be useful for a biobased production of specific HCs, for instance shorter chain semi-volatile
486 HCs for liquid fuels (Moulin et al., 2019).

487 In conclusion, the results presented here show that FAP activity is conserved beyond
488 green microalgae, and identify a big reservoir of FAPs that may be useful for biotechnological
489 applications. It also provides some important clues for future studies aiming at unravelling the
490 exact role of the FAP photoenzyme in eukaryotic algae.

491

492 **MATERIALS AND METHODS**

493 **Strains and culture conditions**

494 The *fap* mutant and its corresponding wild type strain of *C. reinhardtii* were ordered from the
495 CLiP library (Li et al., 2016). Upon receipt, strains were plated on Tris-acetate-phosphate
496 (TAP) medium and streaked to allow formation of single colonies. For each strain, after 1-week
497 growth in the dark, three single-clone derived colonies were randomly chosen for
498 characterization. Wild type strains are CC-4533 cw15 mt⁻ for mating type minus and CC-5155
499 cw15 mt⁺ (Jonikas CMJ030 F5 backcross strain) [isolate E8] for mating type plus. Mutant
500 LMJ.RY0402.226794 was used in this study, which is predicted to harbor a first insertional
501 cassette in the coding sequence of Cre12.g514200 which encodes FAP. A second insertion in
502 the line LMJ.RY0402.226794 was predicted in Cre14.g628702. To remove this side mutation,
503 we backcrossed the mutant strain to CC-5155. Analysis of one full tetrad showed 2 progeny
504 strains resistant to paromomycin and which were mutated in *FAP* gene. The region of
505 Cre14.g628702 was amplified by PCR and sequenced for the 4 progeny strains of the tetrad.
506 No insertion was actually found, therefore a potential insertion at this locus was ruled out. Work
507 on mutant strains was conducted on one isolated parental strain with the mutation from
508 LMJ.RY0402.226794, and on two mutants from the backcross with CC-5155 (full tetrad).

509 These three strains are thereafter named *fap-1*, *fap-2*, and *fap-3*. Wild type (WT) strains were
510 parental strain CC5155 (WT-1) and single colony-derived lines of background strain CC4533
511 (WT-2, WT-3). The average of the three wild type strains and the average of the three knockout
512 strains are labeled WT and *fap*, respectively. For liquid culture experiments, cells were grown
513 in 24 deep well plates of 25 mL culture, under 100 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ with constant shaking
514 at 25°C. Cells were grown in TAP or minimal medium (MM) (Harris, 1989) for mixotrophic
515 and autotrophic conditions, respectively. Cell growth was followed using a cell counter
516 Multisizer (Beckman Coulter). For the day night cycle experiment, cells were cultivated
517 autotrophically in 1L-photobioreactors in turbidostat mode (Sorigué et al., 2016) ($\text{OD}_{880\text{nm}}$ at
518 0.4) under 16 hours of light (40 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) and 8 hours of dark at 25°C. For
519 photosynthesis analysis, cells were grown autotrophically in 80 mL photobioreactors
520 (multicultivator, Photon Systems Instruments) in turbidostat mode ($\text{OD}_{680\text{nm}}$ at 0.8). Conditions
521 were 25°C, with medium light intensity (200 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) or low light intensity (50
522 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$); or 15°C, with medium or low light intensity. All cultures were performed
523 under ambient air.

524

525 **Complementation of the *fap* mutants**

526 The construct for complementation of the knockout strain for *FAP* gene was performed using
527 pSL-Hyg vector containing an *AphVII* cassette conferring hygromycin resistance. This vector
528 allowing nuclear transformation was kindly provided by Pr. Steven Ball (University of Lille,
529 France). A WT copy of the *FAP* gene was obtained by PCR of WT genomic DNA. It was cloned
530 into TOPO-XL vector. pSL-Hyg vector and *FAP* gene were digested with *EcoRV* and *SpeI* and
531 ligated. Then, the vector was linearized with *PvuI* and was electroporated into the *fap* strains.
532 Level of complementation was verified by immunoblot (to assess quantity of protein) and by
533 transmethylation of whole cells (to assess quantity of HCs). The two *fap* mutants from a full

534 tetrad were complemented. Three complemented strains with HC levels similar to WT were
535 kept for physiological studies (*e.g.* Cp-4 shown in Figure 1).

536

537 **SDS PAGE and Immunodetection**

538 Cells (10–15 mL) were harvested by centrifugation at 3,000g for 2 min. Pellets were then frozen
539 in liquid nitrogen and stored at -80°C until use. Pellets were resuspended in 400 mL 1% (w/v)
540 SDS and then 1.6 mL acetone precooled to -20°C was added. After overnight incubation at -
541 20°C, samples were centrifuged (14,000 rpm, 10 min, 4°C). Supernatant was removed and used
542 for chlorophyll quantification using SAFAS UVmc spectrophotometer (SAFAS). Pellets were
543 resuspended to 1 mg chlorophyll mL⁻¹ in LDS in the presence of NuPAGE reducing agent
544 (ThermoFischer), and loaded on 10% (w/v) PAGE Bis-Tris SDS gel. To load equal protein
545 amounts for immunoblot analysis, protein contents were estimated by Coomassie Brilliant Blue
546 staining of the gel using an Odyssey IR Imager (LICOR). After gel electrophoresis, proteins
547 were transferred to nitrocellulose membranes for 75 min at 10 V using a semi-dry set up.
548 Membranes were blocked in TBST with milk 5% (w/v) overnight at 4°C then incubated at room
549 temperature in the presence of the following antibodies: anti-Cyt f, anti-AtpB, anti-PsaD, anti-
550 PsbA, anti-LHCSR3 (Agrisera), or anti-FAP (see below). After 2 hour incubation, primary
551 antibody was removed by rinsing 3 times in TBST, and a peroxidase-coupled secondary
552 antibody was added for at least 1 h. Luminescence was detected with a Gbox imaging system
553 (Syngene).

554

555 **Production of anti-CrFAP antibodies**

556 A codon-optimized synthetic gene encoding *C. reinhardtii* FAP (Sorigué *et al.* 2017) was
557 cloned into the pLIC7 expression vector, allowing the production of a recombinant FAP fused
558 to TEV-cleavable His-tagged *Escherichia coli* thioredoxin. Production was performed in the *E.*

559 *coli* BL21 Star (DE3) strain initially grown at 37°C in TB medium. Induction was initiated at
560 an OD_{600nm} of 0.8 by adding 0.5 mM isopropyl b-D-thiogalactoside (IPTG), and cultures were
561 then grown at 20°C. Following overnight incubation, cells were centrifuged and protein was
562 purified as described previously (Sorigué *et al.* 2017). Purity of the purified protein was
563 controlled on SDS-PAGE and it was brought to a final concentration of 2 mg mL⁻¹ using an
564 Amicon-Ultra device (Millipore). Polyclonal antibodies against FAP were raised in rabbits
565 (ProteoGenix, Schiltigheim, France).

566

567 **Analysis of hydrocarbons and fatty acids**

568 For quantification of HCs, approximately one hundred million cells were pelleted by
569 centrifugation in glass tubes. Transmethylation was conducted by adding 2 mL of methanol
570 containing 5% (v/v) sulfuric acid to the cell pellet. Internal standards (10 µg of hexadecane and
571 20 µg of triheptadecanoylglycerol) were added for quantification. Reaction was carried out for
572 90 min at 85°C in sealed glass tubes. After cooling down, one mL of 0.9% (w/v) NaCl and 500
573 µL of hexane were added to the samples to allow phase separation and recovery of fatty acid
574 methyl esters (FAMES) and HCs in the hexane phase. Samples were mixed and then centrifuged
575 to allow phase separation. Two µL of the hexane phase was injected in the GC-MS/FID.
576 Analyses were carried out on an Agilent 7890A gas chromatographer coupled to an Agilent
577 5975C mass spectrometer (simple quadrupole). A Zebron 7HG-G007-11 (Phenomenex) polar
578 capillary column (length 30 m, internal diameter 0.25 mm, and film thickness 0.25 mm) was
579 used. Helium carrier gas was at 1 mL min⁻¹. Oven temperature was programmed with an initial
580 2-min hold time at 35°C, a first ramp from 35 to 150°C at 15°C min⁻¹, followed by a 1-min hold
581 time at 170°C then a second ramp from 170 to 240°C at 5°C min⁻¹ and a final 2-min hold time
582 at 240°C. The MS was run in full scan over 40 to 350 amu (electron impact ionization at 70

583 eV), and peaks of FAMES and HCs were quantified based on the FID signal using the internal
584 standards C17:0 FAME and hexadecane, respectively.

585

586 **Chlorophyll fluorescence measurements and MIMS analysis**

587 Chlorophyll fluorescence measurements were performed using a pulse amplitude-modulated
588 fluorimeter (Dual-PAM 100) upon 15-min dark-adaptation under continuous stirring. Detection
589 pulses (10 mmol photons $m^{-2} s^{-1}$ blue light) were supplied at a 100-Hz frequency. Basal
590 fluorescence (F_0) was measured in the dark prior to the first saturating flash. Red saturating
591 flashes (6,000 mmol photons $m^{-2} s^{-1}$, 600 ms) were delivered to measure F_m (in the dark) and
592 F_m' (in the light). PSII maximum yields were calculated as $(F_m - F_0)/F_m$, and PSII yield for
593 each light intensity was measured with a saturating flash after 2 to 3 minutes of illumination
594 and was calculated from $(F_m' - F)/F_m'$. The apparent electron transfer rate (ETR) was calculated
595 as the product of light intensity and PSII yield. MIMS was used to measure gas exchange as
596 described previously (Burlacot et al., 2018) .

597

598 **Transmission electron microscopy**

599 Cells were grown photoautotrophically in photobioreactors under 40 $\mu\text{mol photons } m^{-2} s^{-1}$ in
600 turbidostat ($OD_{880\text{nm}}$ at 0.4). The algal cells were collected by centrifugation (1,500g, 1 min)
601 and were immediately fixed with 2.5% (v/v) glutaraldehyde in 0.1 M, pH 7.4 sodium cacodylate
602 buffer for two days at 4°C. They were then washed by resuspending 5 min in the same buffer
603 for three times. Samples were post-osmicated with 1% (w/v) osmium tetroxyde in cacodylate
604 buffer for 1 h, dehydrated through a graded ethanol series, and finally embedded in monomeric
605 resin Epon 812. All chemicals used for histological preparation were purchased from Electron
606 Microscopy Sciences (Hatfield, USA). Ultra-thin sections for transmission electron microscope
607 (90 nm) were obtained by an ultramicrotome UCT (Leica Microsystems GmbH, Wetzlar,

608 Germany) and mounted on copper grids and examined in a Tecnai G2 Biotwin Electron
609 Microscope (ThermoFisher Scientific FEI, Eindhoven, the Netherlands) using an accelerating
610 voltage of 100 kV and equipped with a CCD camera Megaview III (Olympus Soft imaging
611 Solutions GmbH, Münster, Germany).

612

613 **Isolation of thylakoids and native PAGE**

614 Thylakoids were isolated according to a protocol described previously (Chua *et al.* 1975). All
615 steps were performed on ice or at 4°C with as little light as possible. Briefly, cells were pelleted
616 and resuspended in 8 mL 25 mM HEPES, 5 mM MgCl₂, 0.3 M sucrose, pH 7.5 with a protease
617 inhibitor cocktail for plant cell and tissue extracts (Sigma P9599). Cells were disrupted with
618 French press at a pressure of 6000 Psi. Total membranes were collected by centrifugation
619 (1,000g, 10 min) and washed first in 5 mM HEPES, 10 mM EDTA, 0.3 M sucrose, pH 7.5 and
620 then in 5 mM HEPES, 10 mM EDTA, 1.8 M sucrose, pH 7.5. Sucrose gradient was 0.5 M
621 sucrose (5 mL), 1.3 M sucrose (2 mL), and 1.8 M sucrose, initially containing thylakoids (5
622 mL). After ultracentrifugation (274,000g, 1h), thylakoids were collected at the interface
623 between 0.5 and 1.3 M sucrose. They were washed with 5 mM HEPES, 10 mM EDTA, pH 7.5
624 (with or without 0.5 M NaCl) and resuspended at 1 mg mL⁻¹ chlorophyll for subsequent SDS-
625 PAGE analysis. For non-denaturing conditions, thylakoids were resuspended in NativePAGE
626 sample buffer (Life technologies) at 1 mg mL⁻¹ chlorophyll, then thylakoids were solubilized
627 for 30 min on ice in the same volume of 1% (w/v) n-Dodecyl-alpha-D-Maltoside, 1% (w/v)
628 digitonine (final concentrations : 0.5 mg mL⁻¹ chlorophyll, 0.5% (w/v) n-Dodecyl-alpha-D-
629 Maltoside and 0.5% (w/v) digitonine. For each sample, 20 µL were then loaded with 2 µL of
630 G-250 sample additive (Life technologies) on 4-16% (w/v) NativePAGE gels (Life
631 technologies). Cathode running buffer (Life technologies) was supplemented with 0.02% (w/v)
632 G-250 for two-thirds of the migration, and with 0.002% (w/v) G-250 for the remaining third.

633 Annotation of observed bands was done according to a previous publication (Pagliano et al.,
634 2012). For immunoblot analysis, native gel was incubated in Tris Glycine SDS buffer, 10%
635 (v/v) ethanol for 15 min and transferred onto PVDF membrane using XCell II Blot module
636 (25v, 1h). Immunodetection was performed as described above.

637 Based on C16:1(3t) FAME, we determined a factor of enrichment expected for a
638 compound that would be located exclusively within thylakoids (ratio EF=C16:1 (3t) FAME_{whole}
639 cells /C16:1 (3t) FAME_{thylakoids}). Considering the amount of C17:1 alkene found in thylakoids,
640 we calculated the expected content in whole cells, which equals C17:1 alk_{thylakoids}*ratio EF. This
641 calculated value for thylakoids was divided by the value for whole cells that had been
642 determined experimentally, which gives the proportion of C17:1 alkane that is present in
643 thylakoids.

644

645 **Lipidomic analysis by UPLC-MS/MS**

646 Lipid molecular species analysis was done by Ultra Performance Liquid Chromatography
647 coupled with tandem Mass Spectrometry (UPLC-MS/MS). Lipids were first extracted with a
648 modified hot isopropanol method. Briefly, *C. reinhardtii* cells were harvested by centrifugation
649 at 4000 rpm for 2 min in glass tubes. Pellets were immediately resuspended in 1 mL of hot
650 isopropanol (85°C) containing 0.01% (w/v) butylated hydroxytoluene (BHT). Sealed tubes
651 were heated at 85°C for 10 minutes to inactivate lipases. Internal standards were added. Lipids
652 were then extracted in 3 mL methyl tert-butyl ether(MTBE) with a phase separation with
653 1 mL of water. Organic phase was collected and aqueous phase was washed with an additional
654 mL of MTBE. Organic phases were evaporated under a gentle nitrogen stream and resuspended
655 in 500 µL of a mixture of acetonitrile/isopropanol/ammonium acetate 10 mM (65:30:5, v/v/v).
656 Lipid molecular species were analyzed on an ultimate RS 3000 UPLC system (ThermoFisher,
657 Waltham, USA) connected to a quadrupole-time-of-flight (QTOF) 5600 mass spectrometer(AB

658 Sci ex, Framingham, MA, USA) equipped with a duo-spray ion source operating in positive
659 mode. Lipid extracts were first separated on a Kinetex™ (Kinetex, Atlanta, USA) C182.1×150
660 mm 1.7 μm column (Phenomenex, Torrance, USA). Two solvent mixtures, acetonitrile-water
661 (60:40, v/v) and isopropanol-acetonitrile (90:10, v/v), both containing 10 mM ammonium
662 formate at pH 3.8, were used as eluents A and B, respectively. A 32-min-long binary
663 gradient elution was performed; eluent B was increased from 27 to 97% in 20 min and the
664 mixture was maintained for 5 min; eluent B was then decreased to 27% and the mixture
665 maintained for another 7 min for column re-equilibration. The flow-rate was 0.3 mL min⁻¹ and
666 the column oven temperature was maintained at 45°C. Lipid identification was based on
667 retention time and on mass accuracy peaks from the MS survey scan compared with theoretical
668 masses and fragment ions from MS/MS scan. Relative quantification was achieved with
669 multiquant software (AB Sciex, USA) on the basis of intensity values of extracted masses
670 of the different lipids previously identified. Detector response was normalized by the quantity
671 of FAME previously measured by GC-MS for each sample.

672

673 **Phylogenetic analysis and logo sequences**

674 The CvFAP protein sequence was used as bait and blasted against different databases using
675 tBLASTn or BLASTp (including NCBI, Phytozome, Fernbase, and Unigene TARA ocean
676 databases). Sequences from the BLAST were pooled with reference sequences from a previous
677 tree of the GMC oxidoreductase superfamily (Zámocký et al., 2004). Alignment of sequences
678 was done with Muscle algorithm (Edgar, 2004) and viewed with Seaview software (Gouy et
679 al., 2010). Selection of conserved sites was done with Gblock. A set of 226 conserved positions
680 were used for tree construction using maximum likelihood algorithm (PhyML, with LG matrix)
681 with 100 replicates for bootstrap analysis. Annotation of the tree was done using annotation
682 data provided by TARA. FAP Logo sequence was based on 35 sequences including at least one

683 sequence of each taxa from the phylogeny. GMC logo sequence was based on sequences of
684 non-FAP GMC oxidoreductases. Alignment of sequences was done using Muscle
685 algorithm and viewed with Seaview software. Construction of Logo sequences was done using
686 WebLogo (<https://weblogo.berkeley.edu/logo.cgi>).

687

688 **Heterologous expression of FAPs in *E.coli***

689 Coding sequences of putative FAPs from *Galdieria sulphuraria* (**Supplemental Fig. S13**) and
690 *Nannochloropsis gaditana* (**Supplemental Fig. S14**) were directly amplified from cDNAs
691 obtained by reverse transcription of total RNAs. The FAP homologs from *Galdieria*, *Chondrus*
692 and *Ectocarpus* were codon-optimized and synthesized, then cloned into pLIC07 as described
693 before for CvFAP and CrFAP (Sorigué *et al.* 2017). Potential transit peptides were removed for
694 better expression in *E. coli*, and N-terminal sequences were as follows:
695 GFDRSREFDYVIVGGG for *Galdieria*; SSEAATTYDYIIVGGG for *Chondrus*;
696 LQSVSMKAPAAVASSTYDYIIVGGG for *Nannochloropsis*; SMSVAEEGHKFIIVGGG for
697 *Ectocarpus*. *E. coli* were cultivated in Terrific broth medium at 37°C until OD_{600nm} reached 0.8.
698 Expression was then induced with 0.5 mM IPTG and cultures transferred at 22°C under 100
699 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$.

700

701 **77K fluorescence emission spectra**

702 Low temperature spectra were measured on whole cells at 77K using a SAFAS Xenius optical
703 fiber fluorescence spectrophotometer (Dang *et al.*, 2014). 200 μL of light-adapted cell
704 suspension was frozen in a liquid nitrogen bath cryostat. The excitation wavelength used was
705 440 nm, and detection wavelength ranged from 600 to 800 nm with a 5 nm split. Fluorescence
706 emission spectra were all normalized to the 686 nm signal.

707

708 **Accession numbers**

709 FAP is referenced under the enzyme classification number E.C. 4.1.1.106. Accession numbers
710 of FAP genes expressed in this study are XP_005842992 (*Chlorella variabilis* NC64A),
711 XP_001703004 (*Chlamydomonas reinhardtii*), CBJ25560 (*Ectocarpus silicosus*) and
712 XP_005714951 (*Chondrus crispus*).

713

714 **SUPPLEMENTAL DATA**

715 **Supplemental Figure S1. Phylogenetic tree of GMC oxidoreductase superfamily.**

716 **Supplemental Figure S2. Logo sequences for FAPs and other GMC oxidoreductases.**

717 **Supplemental Figure S3 .FAP is predicted to be localized in plastids in many algae**

718 **Supplemental Figure S4.Ultrastructure of *C. reinhardtii* wild type and *fap* strains.**

719 **Supplemental Figure S5. FAP gene expression during day-night cycle and hypothetical**
720 **mechanism that may explain HC loss.**

721 **Supplemental Figure S6. Growth curves and cell volume of wild type and *fap* strains.**

722 **Supplemental Figure S7. Growth of wild type and *fap* strains using various concentrations**
723 **of salt.**

724 **Supplemental Figure S8. Fatty acid profile in mixotrophic conditions.**

725 **Supplemental Figure S9. Variation in the proportion of each fatty acid in total fatty acids**
726 **during cell cycle.**

727 **Supplemental Figure S10. Profiles of major glycerolipid species in WT, *fap* and**
728 **complementation strains.**

729 **Supplemental Figure S11. Photosynthetic activity in *fap* and WT strains.**

730 **Supplemental Figure S12. Fatty acid acclimation to cold conditions.**

731 **Supplemental Figure S13. Sequence of *Galdieria sulphuraria* FAP deduced from the cDNA**
732 **cloned.**

733 **Supplemental Figure S14. Sequence of *Nannochloropsis gaditana* FAP deduced from the**
734 **cDNA cloned.**

735 **Supplemental Table S1. Multiple alignment of FAP sequences used for the phylogenetic**
736 **analysis (text file).**

737 **Supplemental Table S2. List of GMC oxidoreductases used for the phylogenetic analysis.**

738

739 **ACKNOWLEDGEMENTS**

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742 providing access to TARA sequences and to Dr. Florence Corellou, Dr. Eric Maréchal and Prof.
743 Stefano Caffarri for useful discussions. Help of Dr. Philippe Ortet and Emmanuelle Billon with
744 analysis of TARA sequences is also acknowledged.

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FIGURE LEGENDS

Figure 1. FAP level corresponds to the amount of 7-heptadecene in *C. reinhardtii* strains mutated in FAP. **A**, CrFAP gene structure and site of cassette insertion in *fap* knockout strain. Yellow boxes: exons; grey boxes: untranslated regions; red arrow: antibiotic resistance cassette. **B**, relative content of total fatty-acid derived HCs measured on whole cells; the only fatty acid-derived HC of *C. reinhardtii* is 7-heptadecene (abbreviated as C17:1 alk). Values are mean \pm SD of n=3 independent experiments for each strain. nd: not detected. **C**, Immunoblot of total protein extract probed with anti-FAP antibody. **D**, Loading control of the immunoblot (Ponceau staining). B, C and D, WT: wild type strains; *fap*: FAP knockout strains; Cp 1 to 4: strains complemented with *FAP* gene (*fap-1* background);. In panels C and D, each track corresponds to the genotype situated just above in panel B.

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Figure 2. Hydrocarbons produced in *E.coli* strains expressing various microalgal FAPs. HC content was analysed by GC- MS after transmethylation of whole cells. Data are means \pm SD of n=3 independent cultures. Cv, *Chlorella variabilis* NC64A; Chcr, *Chondrus crispus*; Esi, *Ectocarpus silicosus*; Gsu, *Galdieria sulphuraria*; Nga, *Nannochloropsis gaditana*.; n.d., not detected. **A**, Amounts of hydrocarbon produced in *E. coli* strains cultivated under light or dark. Empty vector strain was cultivated under light. Blank corresponds to culture medium alone. **B**, Relative conversion of *E. coli* major fatty acids to corresponding HCs. Data represent the amount of a HC produced under light in a FAP-expressing strain divided by the amount of the fatty acid precursor and by the total amount of HCs. *E. coli* strains were cultivated under light or dark. HC content was analysed by GC-MS after transmethylation of whole cells. Data are means \pm SD of n=3 independent cultures. Cv, *Chlorella variabilis* NC64A; Chcr, *Chondrus crispus*; Esi, *Ectocarpus silicosus*; Gsu, *Galdieria*

771 *sulphuraria*; Nga, *Nannochloropsis gaditana*.; n.d., not detected. **A**, Amounts of HCs produced.
772 Empty vector strain was cultivated under light. Blank corresponds to culture medium alone. **B**,
773 Relative conversion of *E. coli* major fatty acids to corresponding HCs. Data represent the
774 amount of a HC produced under light in a FAP-expressing strain expressing divided by the
775 amount of the corresponding fatty acid precursor and by the total amount of HCs.

776

777 **Figure 3. Identification of a set of putative FAPs across algal groups.** **A**, Simplified circular
778 tree of GMC oxidoreductases showing the number of putative FAP sequences found in each
779 group of algae. All 198 identified putative FAPs belong to algae and have been found in
780 TARA data (161 FAPs) or in sequenced algal genomes (37 FAPs). The tree was built using
781 maximum likelihood algorithm using GMC oxidoreductases from various kingdoms.
782 Annotations are focused on the branch of putative FAPs; other branches are other GMC
783 oxidoreductases. Branches have been collapsed; full tree is available in Figure S1. **B**, Names
784 of algae species with at least one putative FAP. For most algal groups, the number of species
785 listed in B is lower than that indicated in A because many species from TARA data have no
786 annotation down to the species level. When the biochemical activity of the FAP homolog is
787 demonstrated (Sorigué et al., 2017 or this study), species names are indicated in bold.

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789

790 **Figure 4. Overview of the number of FAP homologs and other GMC oxidoreductases**
791 **identified in eukaryotic algae and other bikonts.** In most groups, there is one FAP and no
792 other GMC oxidoreductase. Remarkable species whose number of FAP or other GMC
793 oxidoreductases depart from this rule are listed individually. Hyphens indicate that no protein
794 could be identified by BLAST searches. A widely accepted phylogeny of the Bikonta is used.
795 The dashed line connects the tree of the Bikonta to the rest of the phylogenetic tree of the

796 Eukaryotes. Photosynthetic groups or species are colored. Rounds correspond to
797 endosymbiosis: blue round with one black circle, primary endosymbiosis; green and red rounds
798 with two black circles, secondary endosymbiosis; red round with three black circles is for
799 tertiary endosymbiosis in some (not all) Dinophyta; red or green rounds indicate red or green
800 plastid origin, respectively; n: nucleomorph; x: secondary plastid loss.

801

802 **Figure 5. FAP and its 7-heptadecene product are present in a thylakoid membrane-**
803 **enriched fraction of *C. reinhardtii*.** A, Western blot of protein extracts from whole WT cells
804 before (total) and after centrifugation (supernatant and pellet), and from a thylakoid membrane-
805 enriched fraction without (thyl.) or with (thyl. + NaCl) an additional wash with 0.5 M NaCl.
806 PsbD and PRK are thylakoid and stromal proteins, respectively. Proteins were loaded on a
807 constant chlorophyll basis. B, Relative content in 7-heptadecene and C16:1(3t) fatty acid in
808 whole cells and in the thylakoid fraction (thyl.). The C16:1(3t) fatty acid is almost exclusively
809 present in thylakoids and shown for comparison. Values are mean \pm SD of n=4 independent
810 experiments. (*) denote p value < 0.05 in 2-sided Student t-test. C, Estimation of the proportion
811 of total 7-heptadecene present in thylakoids (in green) and elsewhere in the cell (in yellow).
812 Proportion of 7-heptadecene in thylakoids was estimated using C16:1(3t) fatty acid as a
813 thylakoid marker (see Material and Methods for calculation).

814

815 **Figure 6. Variation of 7-heptadecene compared to total fatty acids during *C. reinhardtii***
816 **cell cycle.** A, 7-Heptadecene content of cells expressed as a percent of total FAMES. Values
817 are mean \pm SD (n=3 biological replicates). B, Total fatty acid and 7-heptadecene content per
818 million cells during cell cycle. Total fatty acids were analyzed as FAMES by GC-MS. A and B,
819 Data are mean \pm SD of n=3 independent cultures. The orange dashed line indicates the end of

820 day time and the beginning of night time (gray shading). **C**, Immunodetection of FAP during
821 day time. The large Rubisco subunit (RBCL) was used as a loading control.

822

823 **Figure 7. Identification of lipid molecular species significantly different between *C.***
824 ***reinhardtii* WT and *fap* strains.** Relative abundance of glycerolipids was measured by LC-
825 MS/MS analysis of total lipid extracts of whole cells. Only glycerolipid molecular species
826 showing significant differences between WT and *fap* strains are shown here (See supplemental
827 figure S12 for complete results). WT, *fap* and Cp: wild type, FAP knockout and
828 complementation strains respectively. Lipid extracts from the three strains were loaded on a
829 constant total fatty acid basis. Data are mean \pm SD of n=9 independent cultures (using three
830 different strains for each of the three genotypes). MGDG34:4-1 is one of the two species of
831 MGDG 34:1 species (which differ by 18:3 fatty acid isomers). Stars indicate significant
832 differences according to a Mann-Whitney U-test at p<0.05 (*) or p<0.01 (**). Cells were grown
833 in TAP medium, under 80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ in Erlenmeyers.

834

835 **Figure 8. Thylakoid purification and immunoblot analysis. A**, Purification of *C. reinhardtii*
836 thylakoids using a sucrose density gradient. The thylakoid fraction collected is indicated by an
837 arrow. **B**, Blue native polyacrylamide gel of solubilized proteins (0.5% digitonine, 0.5% α -
838 DM) and corresponding immunodetection. WT: Wild type strain, *fap*: FAP knock out strain. *
839 indicates FAP band. ~~A second, dimensional SDS PAGE and immunodetection (rightmost blot)~~
840 ~~did not reveal an association of FAP with higher molecular weight complexes that would have~~
841 ~~been masked in the 1st dimension BN PAGE.~~

842

843 **Figure 9. Photosynthetic acclimation to cold conditions in *C. reinhardtii* WT and *fap***
844 **strains.** Apparent electron transfer rate (ETR) at various light intensities for cells grown in

845 photoautotrophic conditions at 25°C (A) and 15°C after 3 days acclimation (B). PSII yield (C)
846 and ETR (D) at 15°C after exposure to lower light for one day (50 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$). E, ETR
847 at 25°C after transition to lower light for one day (50 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$). WT, *fap* and Cp:
848 wild type, FAP knockout and complementation strains respectively. Data are mean \pm SD of n=3
849 independent cultures (corresponding to three different strains for each genotype).

850

851 **Figure 10. Hydrocarbon production in *C. reinhardtii* WT cells cultivated at low**
852 **temperatures. A, HC content in cells as percentage of total FAMES. B, Dilution rate of culture**
853 **medium and production of FAMES and HCs. For A and B, Cells were grown in TAP medium**
854 **in photobioreactors in turbidostat mode under 50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. HC and fatty acid**
855 **content were analyzed by GC-MS after transmethylation. Data are mean \pm SD of n=3**
856 **independent cultures.**

857

858 **Figure 11. Proposed pathway for hydrocarbon formation from fatty acids in *C. reinhardtii***
859 **and putative roles. The only fatty acid-derived HC in *C. reinhardtii* (7-heptadecene) is**
860 **generated from *cis*-vaccenic acid by FAP only when cells are exposed to blue light. The fatty**
861 **acid precursor must be released from a thylakoid lipid by an unknown lipase. The 7-**
862 **heptadecene FAP product may play several roles in thylakoid membranes depending on**
863 **temperature and light conditions.**

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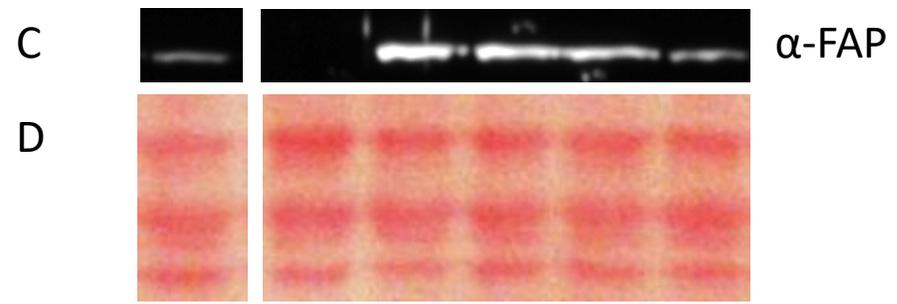
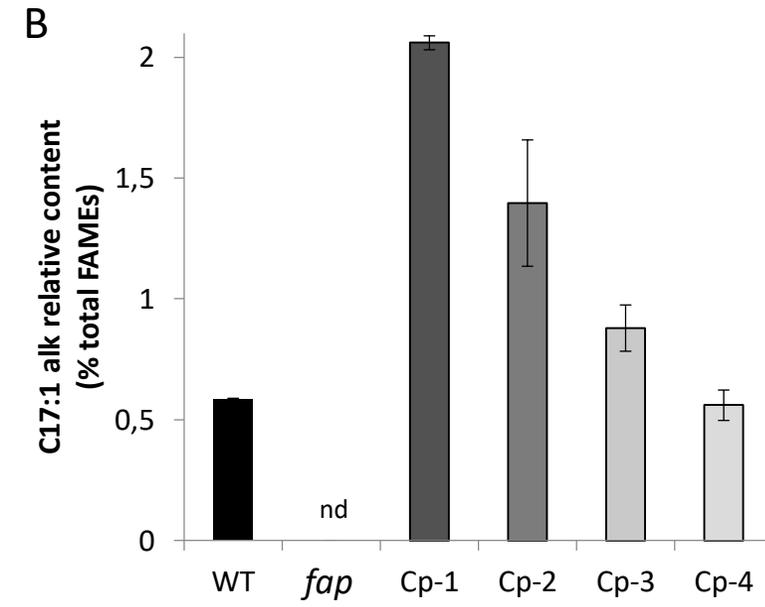
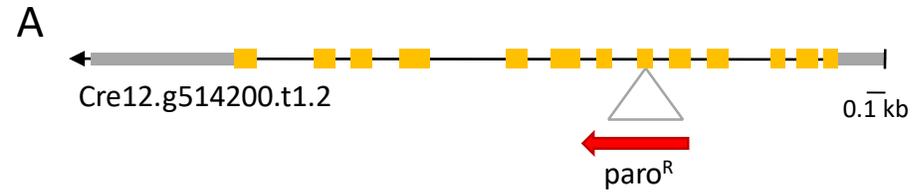
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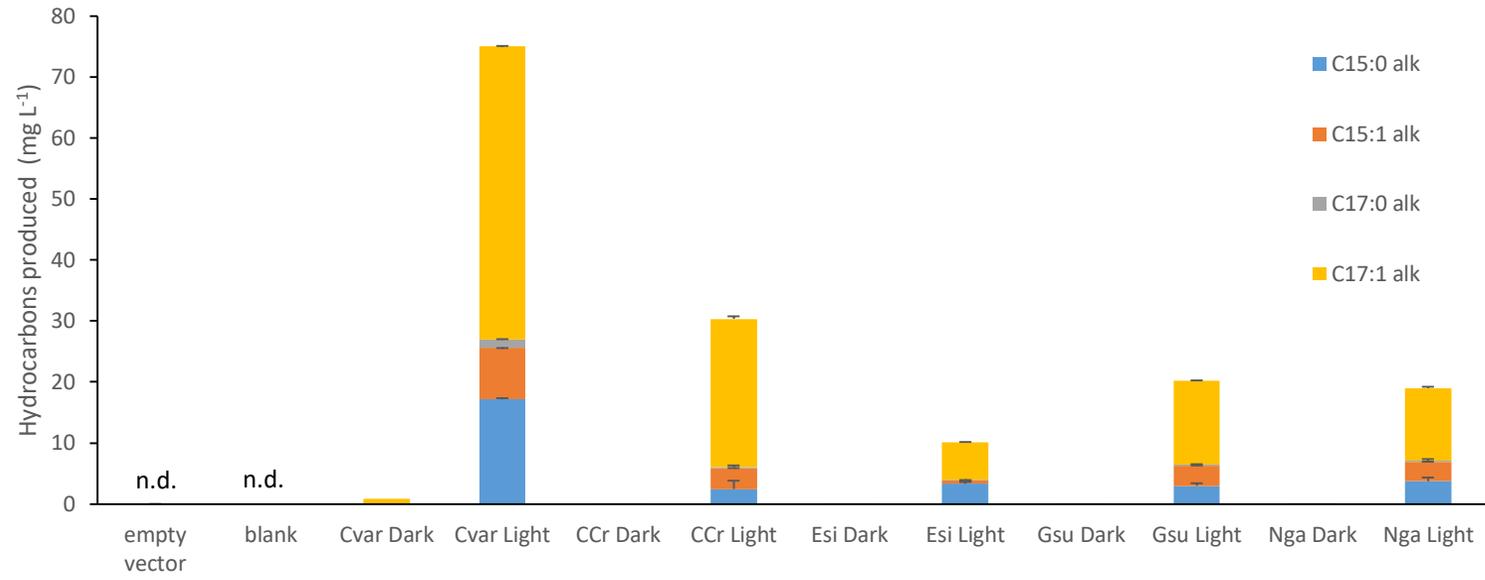
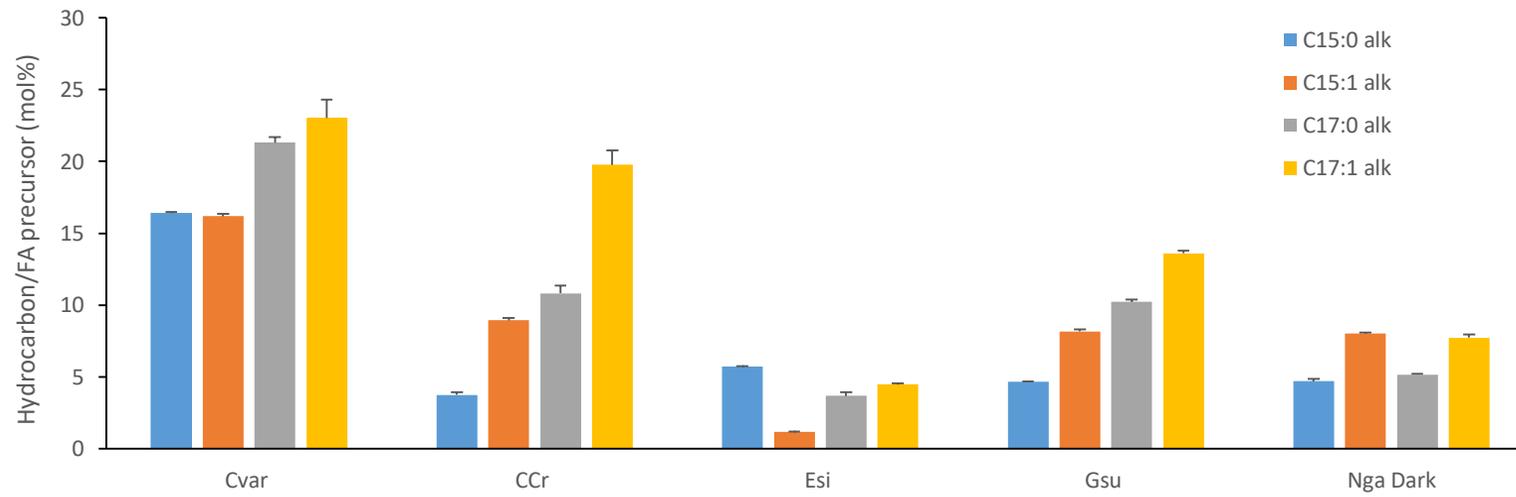
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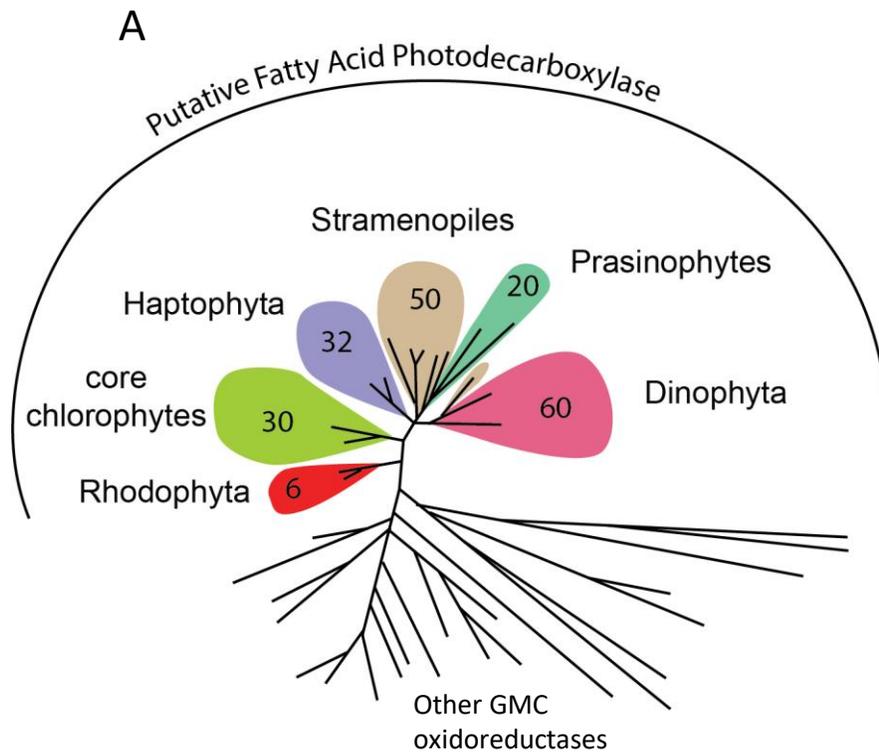
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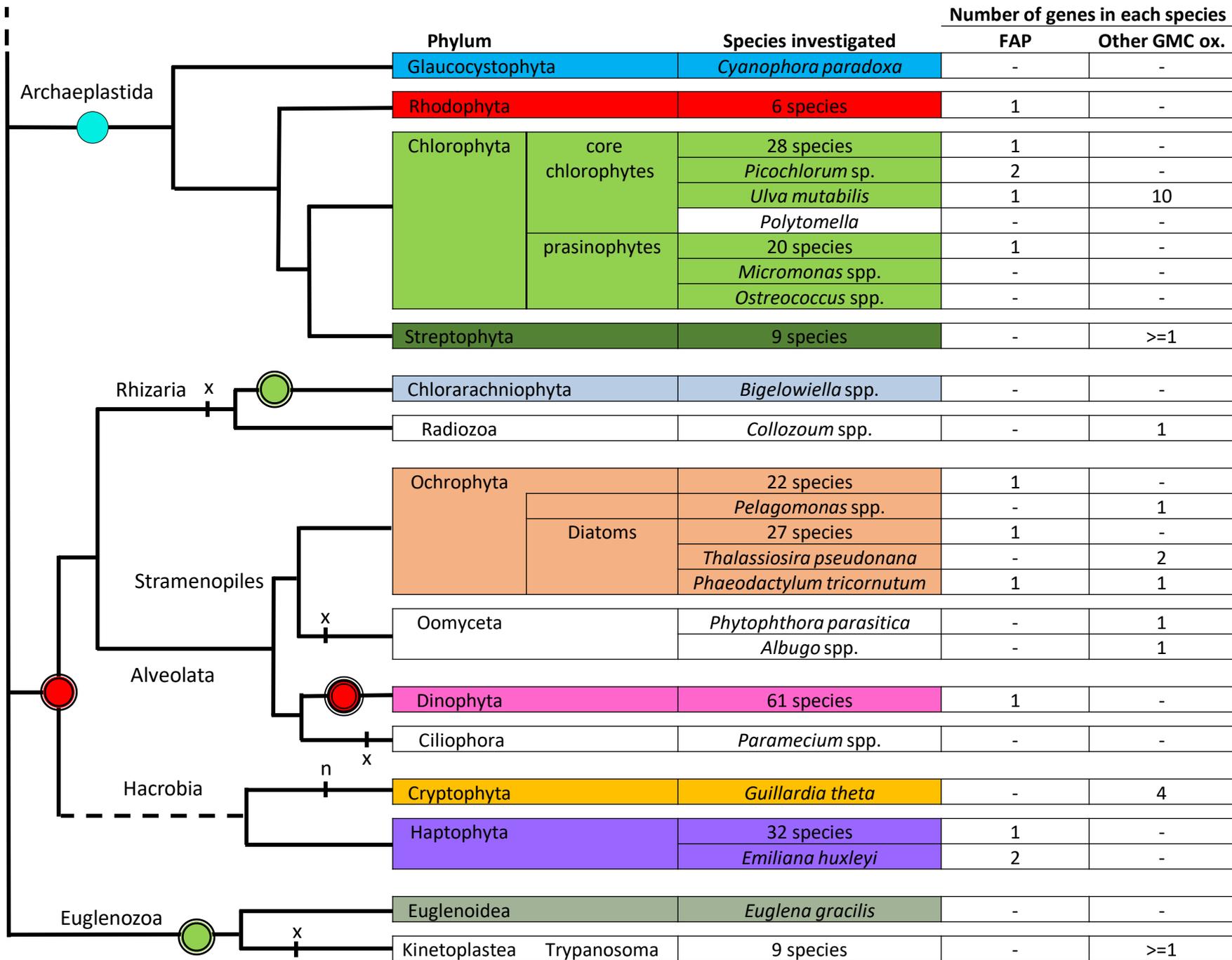
A**B**

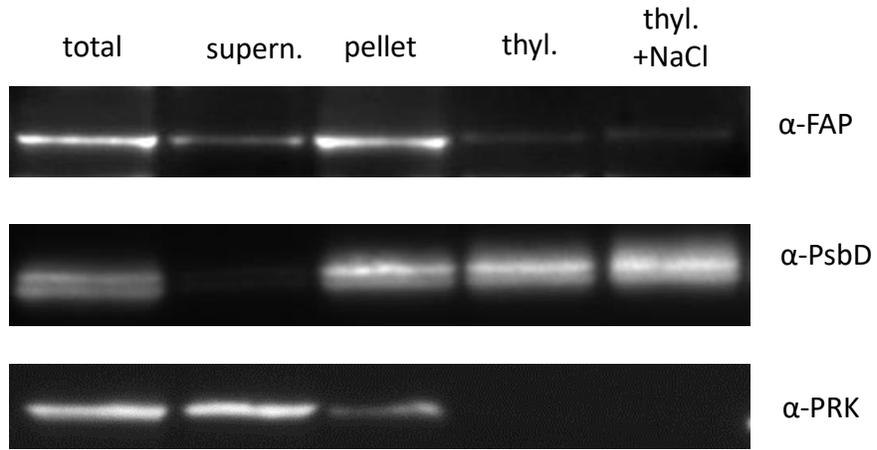
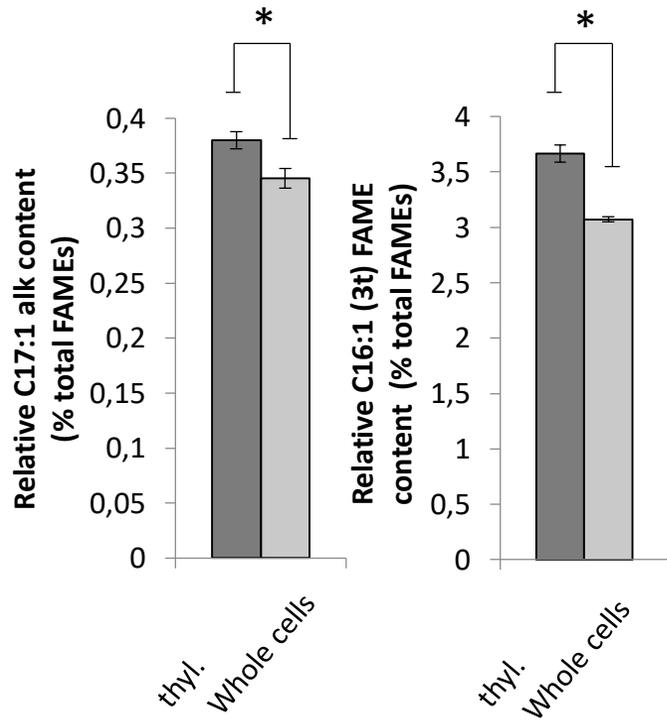
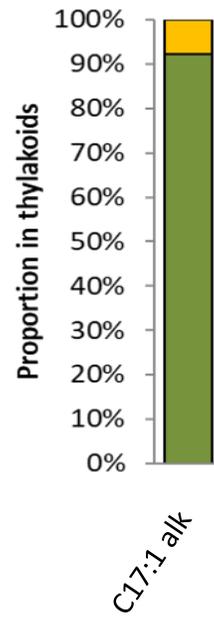


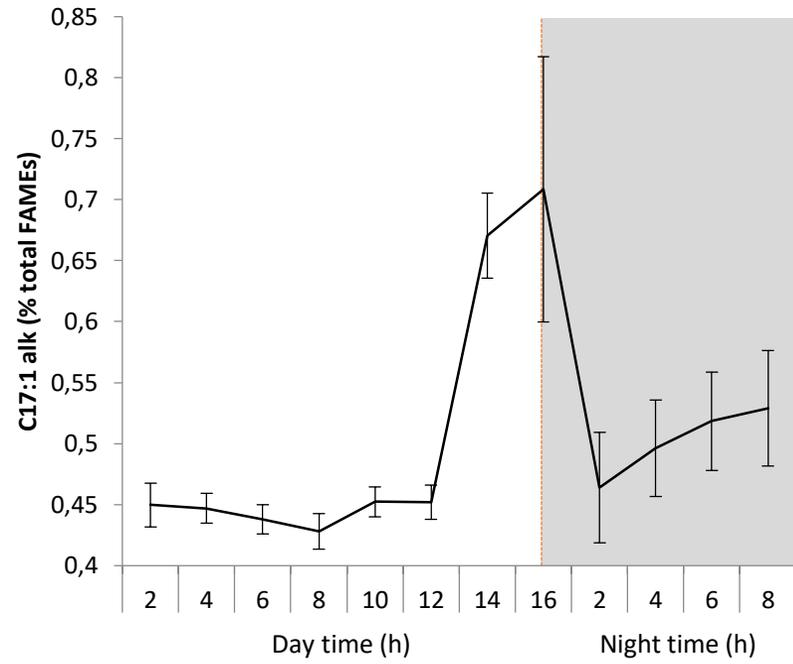
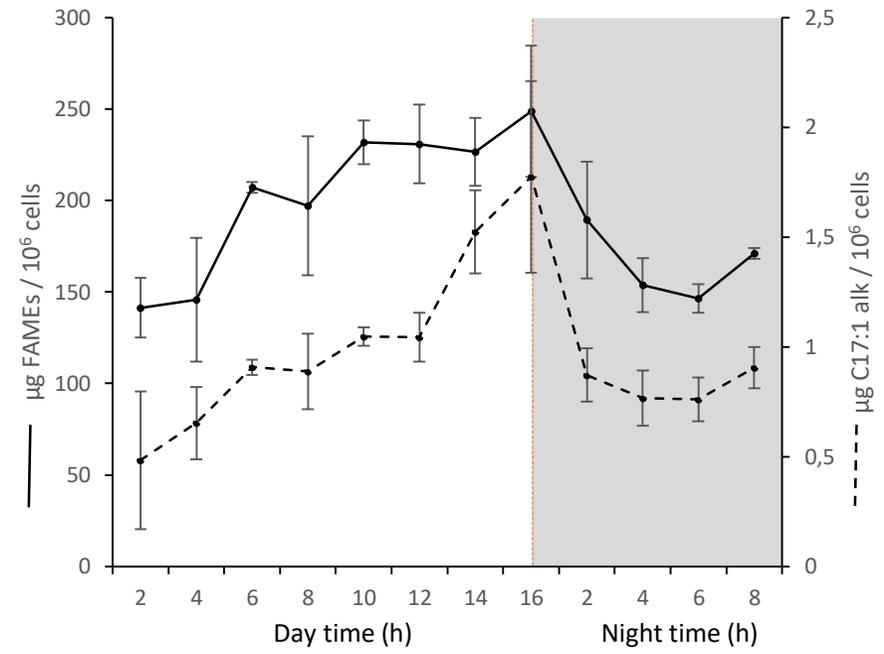
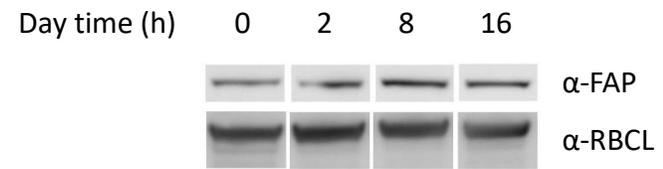
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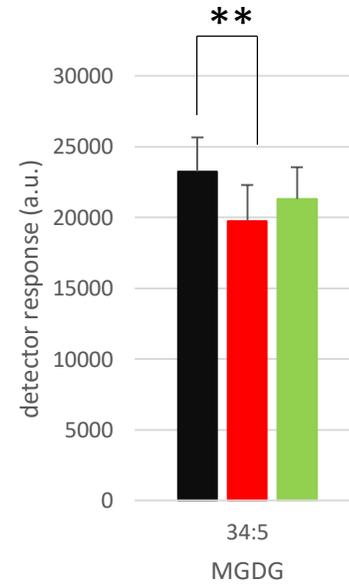
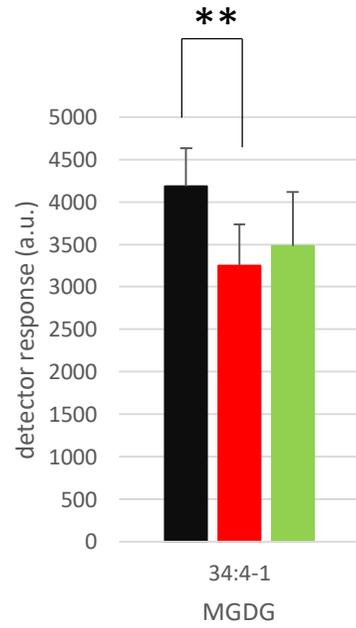
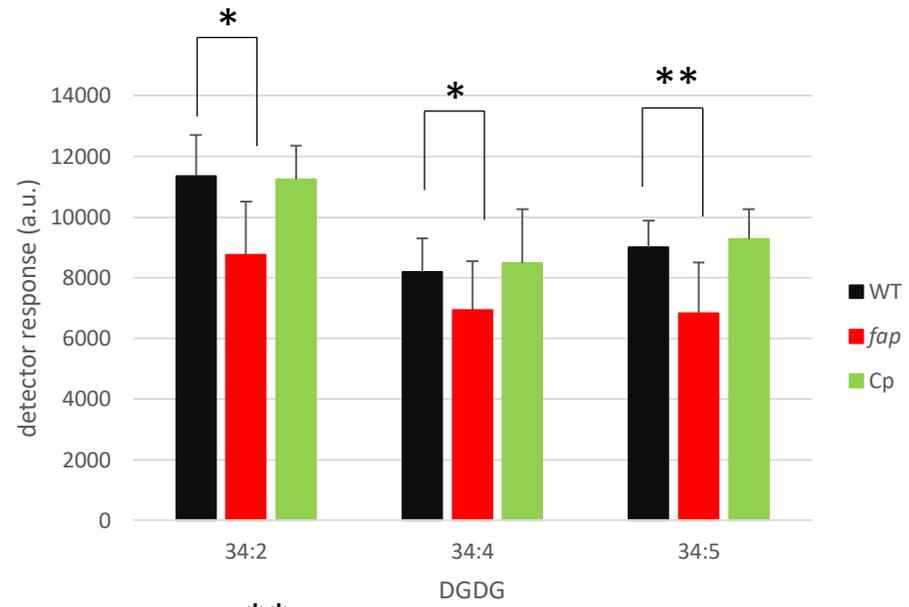
Phylum	Species (abbreviation in full tree and name)
Rhodophyta	<i>Grc</i> <i>Gracilariopsis chorda</i> <i>Chcr</i> <i>Chondrus crispus</i> <i>Pye</i> <i>Pyropia yezoensis</i> <i>Pum</i> <i>Porphyra umbilicalis</i> <i>Gsu</i> <i>Galdieria sulphuraria</i> <i>Cme</i> <i>Cyanidioschyzon merolae</i>
Chlorophyta core chlorophytes	<i>Chles</i> <i>Chlamydomonas eustigma</i> <i>Chlch</i> <i>Chlamydomonas chlamydogama</i> <i>Vc</i> <i>Volvox carteri</i> f. <i>nagariensis</i> <i>Gpe</i> <i>Gonium pectorale</i> <i>Cr</i> <i>Chlamydomonas reinhardtii</i> <i>Chlle</i> <i>Chlamydomonas leiostraca</i> <i>Dus</i> <i>Dunaliella salina</i> <i>Chl68</i> <i>Chlamydomonas</i> sp. <i>Cso</i> <i>Chlorella sorokiniana</i> <i>Mic</i> <i>Micractinium conductrix</i> <i>Cv</i> <i>Chlorella variabilis NC64A</i> <i>Bbr</i> <i>Botryococcus braunii</i> <i>Cs</i> <i>Coccomyxa subellipsoidea</i> C-169 <i>Lobi</i> <i>Lobosphaera incisa</i> <i>Ras</i> <i>Raphidocelis subcapitata</i> <i>Chrzo</i> <i>Chromochloris zofingiensis</i> <i>Ulmu</i> <i>Ulva mutabilis</i> <i>Picsa</i> <i>Picocystis salinarum</i> <i>Pic</i> <i>Picochlorum</i> sp. <i>Picoc</i> <i>Picochlorum oculata</i> <i>Picok</i> <i>Picochlorum oklahomensis</i>
Haptophyta	<i>Chry</i> <i>Chrysochromulina</i> <i>Ehu</i> <i>Emiliania huxleyi</i>
Heterokontophyta (Stramenopiles)	Bacillariophyceae <i>Psm</i> <i>Pseudo-nitzschia multistriata</i> <i>Ptr</i> <i>Phaeodactylum tricorutum</i> <i>Frc</i> <i>Fragilariopsis cylindrus</i> 116393708 <i>Thalassiothrix antarctica</i> 116110556 <i>Corethron pennatum</i>
	Eustigmatophyceae <i>Nga</i> <i>Nannochloropsis gaditana</i> <i>Nsa</i> <i>Nannochloropsis salina</i>
	Phaeophyceae <i>Esi</i> <i>Ectocarpus siliculosus</i>
	Pelagophyceae <i>Aa</i> <i>Aureococcus anophagefferens</i>
Dinophyta	52837172 <i>Neoceratium fusus</i> 97429747 <i>Eterocapsa</i> sp.
Chromerida	<i>Chve</i> <i>Chromera velia</i>

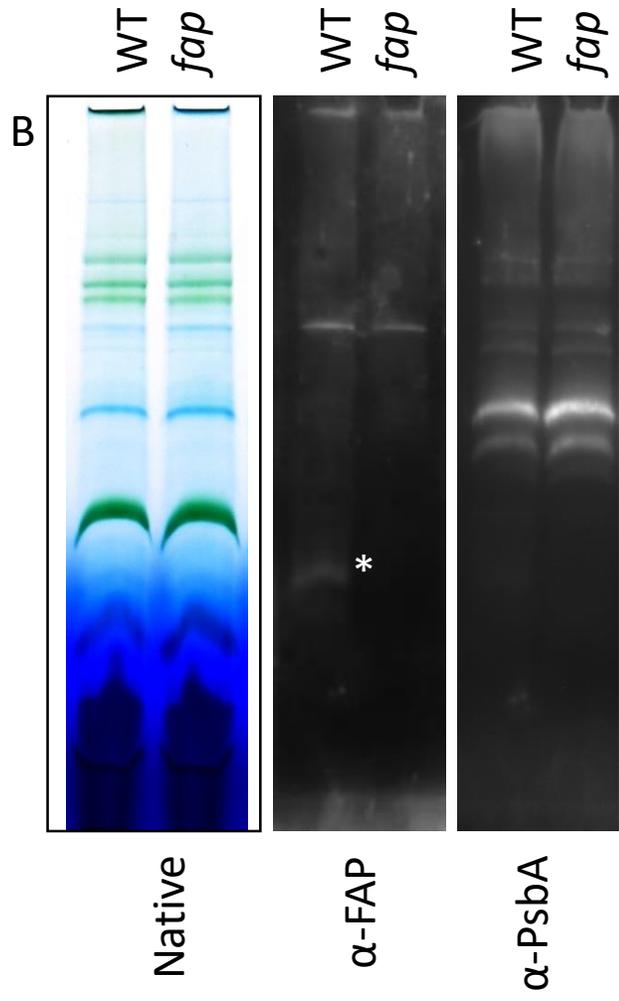
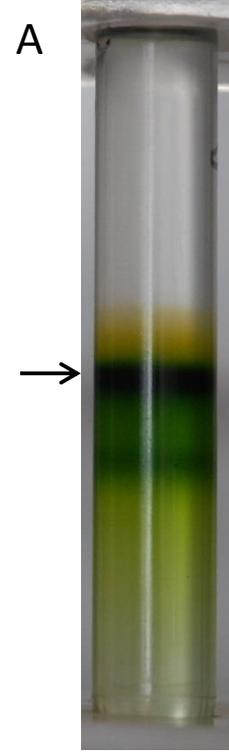
BIKONTA

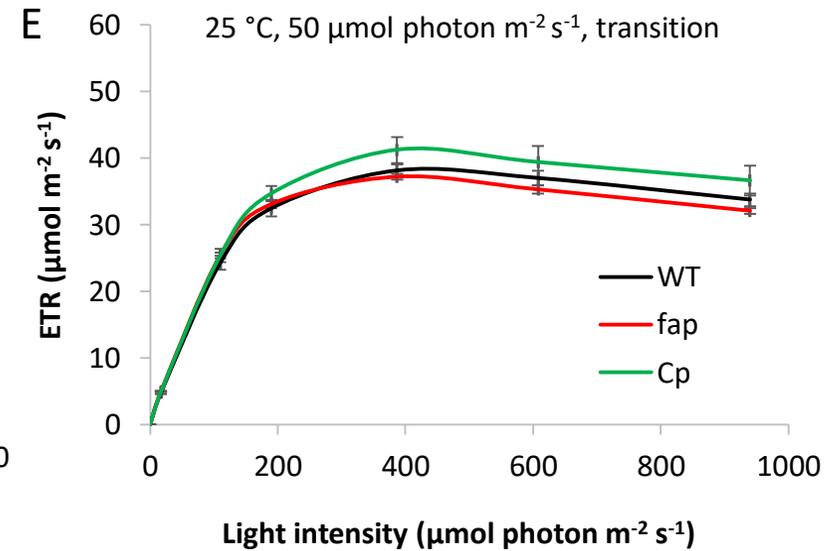
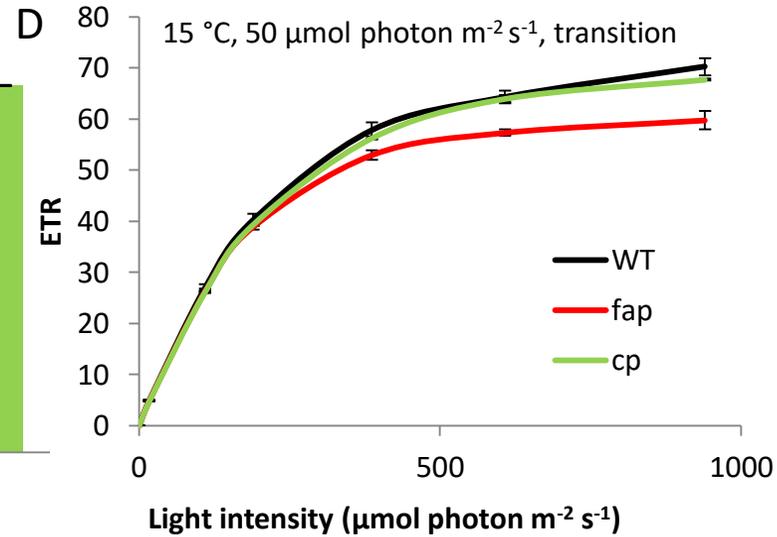
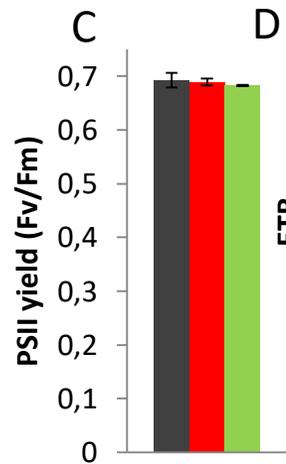
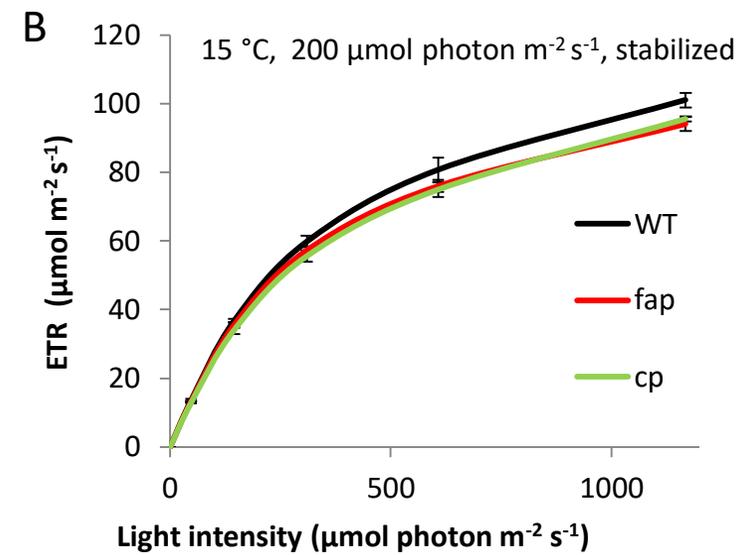
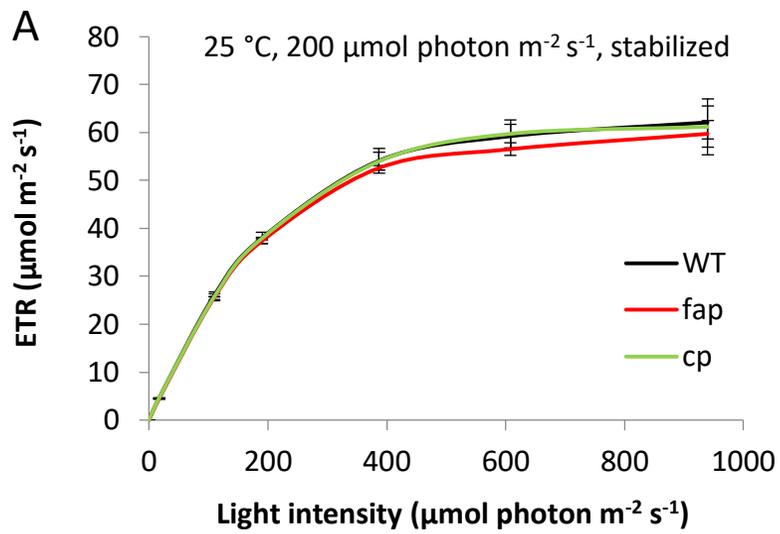


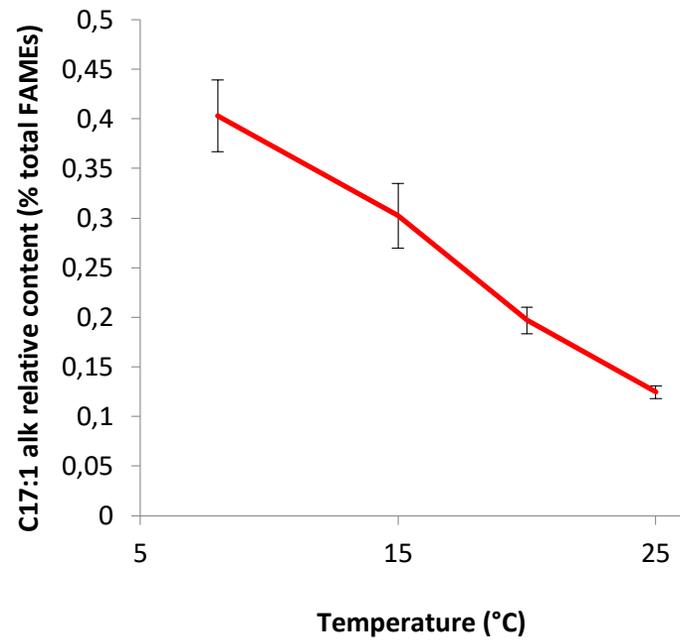
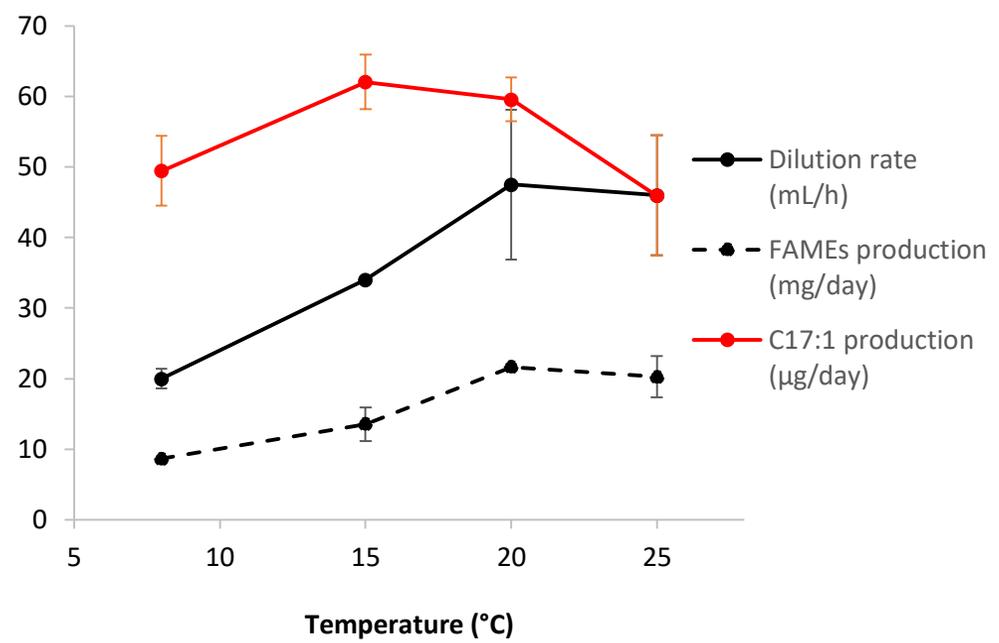
A**B****C**

A**B****C**



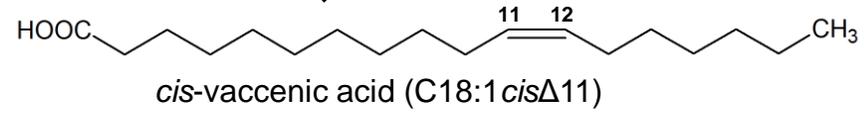




A**B**

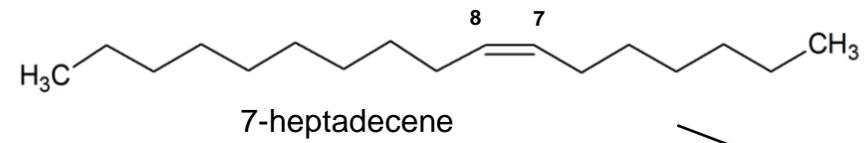
cis-vaccenic acid-containing membrane lipid (thylakoids)

Putative lipase



Blue light

Fatty acid photodecarboxylase (FAP)



Other?

Membrane fluidity, curvature?

Cell signaling?

Direct interaction with specific proteins?