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Digestion and bioconversion of microalgal galactolipids by direct action of lipolytic enzymes on *Chlamydomonas reinhardtii* biomass

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Abstract

Microalgae, like *Chlamydomonas reinhardtii*, are rich in polyunsaturated fatty acids (PUFAs) bound to galactolipids. Their bioaccessibility to digestive enzymes in whole cells was tested using guinea pig pancreatic lipase-related protein 2 (GPLRP2) and *Fusarium solani* cutinase, showing high lipolysis levels with total galactolipid fatty acid release of 93% with GPLRP2. These enzymes also converted galactolipid fatty acids into ethyl esters with ethanol, what could facilitate PUFA recovery from microalgal biomass. *In vitro* digestion studies mimicking gastric and intestinal conditions revealed that human pancreatic galactolipases release α-linolenic acid (ALA) as the main free fatty acid (≥35%). NMR analysis indicated PUFA oxidation when microalgal endogenous enzymes were not inactivated before digestion. ALA oxidation into phytoprostanes occurred, especially after lipolysis. Thus, *C. reinhardtii* galactolipids are bioaccessible to digestive enzymes, making them a valuable PUFA source, provided oxidation is controlled.

Keywords: Alcoholysis, *Fusarium solani* cutinase, galactolipase, lipolysis, microalgae, pancreatic lipase related protein 2, NMR spectroscopy.

Abbreviations: ALA, α-linolenic acid; ALAEE, ethyl linolenate; BSSL, bile salt-stimulated lipase; C8-MGDG, monogalactosyl-dioctanoyl glycerol; CRF, chloroplast-rich fractions; DGDG, digalactosyldiacylglycerol; DGG, digalactosyl glycerol; DGTS, betaine lipid 1,2-diacylglyceryl-3-O-4'-(N,N,N-trimethyl)-homoserine; DHA, docosahexaenoic acid; DW, dry weight; EPA, eicosapentaenoic acid; FW, fresh weight; FA, fatty acid; FAEE, fatty acid ethyl ester; FFA, free fatty acid; FsCut, *Fusarium solani* cutinase; GC-FID, gas chromatography coupled to flame ionization detector; GI, gastrointestinal; GPLRP2, guinea pig pancreatic lipase related protein 2; HPJ, Human pancreatic juice; HPL, Human pancreatic lipase; HSQC, heteronuclear single quantum coherence; IS, internal standard; MGDG, monogalactosyl-diacyl glycerol; MGG, monogalactosyl glycerol; MGMG, monogalactosyl-monoacyl glycerol; NaTDC; sodium taurodeoxycholate; NMR, Nuclear Magnetic Resonance; PE, phosphatidylethanolamine; PhytoP, phytoprostane; PLRP2, pancreatic lipase related protein 2; PUFA, polyunsaturated fatty acids; RGE, rabbit gastric extract; ROS, reactive oxygen species; TAG, triacylglycerol; TLC, thin-layer chromatography; TES, N-tris[hydroxymethyl]methyl-2-aminoethane-sulfonic acid.

1. Introduction

Microalgae are microscopic photosynthetic organisms that play a crucial role in Earth's ecosystems, significantly contributing to global oxygen production and carbon fixation. They can produce lipids like triacylglycerols (TAG) as carbon-storage molecules and their potential use as a source of energy has led to tremendous research efforts [1, 2]. Apart from alkanes, TAG are the biomolecules with the highest density of energy storage (9 kcal/g), contained in the three fatty acids (FA) bound to the glycerol backbone. TAG are usually produced by microalgae under stress or dormancy conditions, and these conditions are not compatible with increasing biomass [3]. Under conditions of optimum growth, most of the FA are found in galactolipids that are constitutive bricks of photosynthetic membranes. Galactolipids like monogalactosyldiglycerides (MGDG) and digalactosyldiglycerides (DGDG; Figure 1) are essential constituents of thylakoid membranes, which contribute to photosynthesis by capturing solar energy and converting it into chemical energy [4-6]. Due to their prevalence in the chloroplasts, and their abundance under optimum growth conditions, they represent another attractive source of FA, although they only contain two FA bound to glycerol [7].

Another attractive feature of chloroplast galactolipids is their natural enrichment in long-chain polyunsaturated fatty acids (PUFA), such as α-linolenic acid (ALA; 18:3 n-3), eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3) depending on the microalgal species [8]. These PUFA play a critical role in the plasticity of thylakoid membranes that show a high protein to lipid ratio, as well as in the adaptation of the photosynthetic machinery to varying light conditions [9] [10-13]. These PUFA make galactolipids more attractive for nutritional applications [8].

Apart from lipids, microalgae also present a promising solution to global nutritional demands due to their rich composition in proteins, carbohydrates, vitamins and minerals. With a protein content reaching 40% of dry weight (up to 70% in spirulina), they are valuable for

human and animal nutrition [14]. The microalga *Chlamydomonas reinhardtii*, in particular, is noted for its high-value biomolecules, including polysaccharides, lipids, proteins and pigments, which have significant applications in nutraceuticals and pharmaceuticals [15]. Bioprocessing techniques, such as extraction, microencapsulation, and enzymatic treatments, can enhance the digestibility and bioavailability of these nutrients, making microalgae a potent source of health-promoting compounds. The integration of these techniques can lead to the development of functional and nutritious food products, maximizing the potential of microalgae for human health [16, 17]. Studies have shown that *C. reinhardtii* surpasses popular dietary supplements like *Chlorella* and *Spirulina* in protein content, amino acid profile, and antioxidant activity, indicating its potential as a functional food ingredient [15] . Interestingly, zebrafish fed with *C. reinhardtii* showed a yellow pigmentation due to lutein absorption while β-carotene was converted into vitamin A [18]. Increased levels of ALA (C18:3 n-3) and hexadecatrienoic acid (C16: 4-n-3) were observed in zebrafish tissues, indicating that lipids from *C. reinhardtii*, probably galactolipids, were well digested and their fatty acid absorbed [18].

Exploring the enzymatic digestion and conversion of galactolipids is crucial for defining their potential applications. Besides their nutritional interest, their enzymatic transformation into various esters as emulsifiers and lipid excipients may provide a sustainable alternative to chemical production. Research in this area has focused so far on the identification of enzymes, galactolipases, catalyzing the conversion of galactolipids into free fatty acids and various esters through hydrolysis and alcoholysis reactions [7, 19, 20]. Galactolipases can also catalyse FA exchanges in galactolipids [21]. Among the enzymes showing high galactolipase activity, two notable candidates are Guinea-pig pancreatic lipase-related protein 2 (GPLRP2) and *Fusarium solani* cutinase (FsCut) which have been shown to act directly onto leaves of various plant species [7, 22], as well as on isolated chloroplasts [23, 24]. The hydrolysis of galactolipids by these enzymes involves the cleavage of acyl ester bonds linked to the glycerol backbone,

targeting MGDG and DGDG molecules in photosynthetic membranes. The process results in the release of free fatty acids, with preferential action on the ester bonds at *sn*-1 position, that can be followed by the release of the second acyl chain, either by direct action at the sn-2 position (slow) or after sn-2 to sn-1 acyl chain migration (most common mechanism). Thus, MGDG and DGDG hydrolysis yields two molecules of free fatty acids (FFA), and monogalactosyl glycerol (MGG) and digalactosyl glycerol (DGG), respectively, as ultimate reaction products *in vitro* [24-26]. MGG and DGG have also been detected *in vivo* in rat intestinal contents [27] and these water-soluble galactose-containing compounds can also be generated upon incubation of radiolabeled MGDG and DGDG with human duodenal contents and pancreatic juice [28].

Regarding alcoholysis, the principle remains similar to hydrolysis, with the alcohol acting as the nucleophile instead of water. The same enzymes can convert galactolipid FAs into methyl and ethyl esters in the presence of methanol and ethanol, respectively [7, 20, 22].

The primary objective of this study was to evaluate the capacity of GPLRP2 and FsCut to access to and convert galactolipids from *C. reinhardtii* whole cells into FFA and fatty acid ethyl esters (FAEE). Second, a two-step *in vitro* digestion assay combining gastric and a small intestinal phase, was carried out to evaluate the ability of gastrointestinal enzymes to digest the galactolipids from the whole microalga, having in mind its applications as a food.

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2. Materials and methods

2.1. Reagents

Monogalactosyl dioctanoyl-glycerol (C8-MGDG; M_w=506 g/mole), monogalactosyl distearoyl-glycerol (C18-MGDG; M_w=646 g/mole) and digalactosyl dioctanoyl-glycerol (C8-DGDG; M_w=632,21 g/mole), was synthetised as previously described [29, 30]. Boron trifluoride (BF3; 14% in methanol), Deuterium oxide, Galactose, N-tris[hydroxymethyl]methyl-2-aminoethane-sulfonic acid (TES), Sodium taurodeoxycholate (NaTDC), Sulphuric acid, Thymol and natural long chain MGDG and DGDG from wheat were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Calcium chloride ($CaCl_2$) and sodium chloride (NaCl) were obtained from Euromedex (Souffelweyersheim, France). Acetonitrile, chloroform, ethanol, and methanol were all HPLC grade from Carlo Erba (Peypin, France). Rabbit gastric extracts (RGE) containing gastric lipase were purchased Lipolytech (Marseille, France). Human pancreatic juice (HPJ) was provided by Prof. René Laugier (Gastroenterology department, La Timone Hospital, Assistance Publique-Hôpitaux de Marseille) and was collected as previously described [31].

2.2. Culture and harvesting of C. reinhardtii

The *C. reinhardtii* wild-type strain CC-5101 T222+ mt- with natural cell wall [32] was obtained from the laboratory Photobiologie et Physiologie des Plastes et des Micro-algues (UMR 7141, Institut de Biologie Physico-Chimique (IBPC), Sorbonne University, Paris) and the cell wall-deficient *C. reinhardtii* strain CC-4349 cw15 mt+ was purchased from the *Chlamydomonas* Resource Center (University of Minnesota, Minneapolis, MN, USA). They were maintained in Tris Acetate Phosphate (TAP) medium and grown in photoautotrophic conditions at 23°C in

minimal MOBS culture medium (20 mM MOPS pH 7.4, 0.4 mM KH_2PO_4, 0.6 mM K_2HPO_4, Beijering Salts solution containing 7.5 mM NH_4Cl, 0.4 mM $MgSO_4$ and 0.34 mM $CaCl_2$ and Hutner's trace elements [33]and under continuous photosynthetically available illumination at 110 µmol photon $m^2.s^{-1}$, constantly shaken at 110 rpm. Cultures were bubbled with air enriched in CO_2 at partial pressure of 20 000 ppm using a El-Flow Bronkhorst flowmeter. Cells were harvested during the exponential phase when the OD_{680} value reached 1.66, corresponding to $8.33 \pm 0.5 \times 10^6$ cells/mL and 314 ± 22 mg dry weight (DW)/L. Cells were recovered by centrifugation at 4 ºC, 2000 g for 10 min, and transferred into 15-mL Falcon tubes. To prevent endogenous lipolysis, some cell pellets were blanched at 100 ºC for 5 minutes by dipping the tube with the cell pellet in boiling water. After centrifugation or after blanching, the cell pellets were either used directly for enzymatic digestion or stored at -20 ºC before lyophilization overnight (around 16-18h).

2.3.Production, purification and assay of GPLRP2 and Fusarium solani cutinase

Recombinant GPLRP2 and *Fusarium solani* cutinase (FsCut) were produced in the yeast *Pichia pastoris* and purified as previously described [7]. Stock solutions containing 2.17 mg/L GPLRP2 and 1.92 mg/mL FsCut were prepared and stored at -20°C. Both GPLRP2 and FsCut catalyse the hydrolysis of triacylglycerols (TAG), phospholipids and galactolipids, which generate FFA [19]. The titration with time of these fatty acids allows the determination of the enzyme activity of both lipases. This activity can be expressed in international units: 1 U = 1 µmole of fatty acid released per minute. For routine analysis of GPLRP2 and FsCut samples, either tributyrin or C8-MGDG, a synthetic galactolipid with eight-carbon acyl chains [29], were used as substrates. FFA release was monitored using the pHstat technique (Metrohm Titrando 842 and burette Dosino 800, Herisau, Switzerland), the TIAMO version 1.3 software and 0.1 M NaOH, at a pH endpoint of 7 or 8. The assay solution contained 1 mM Tris, 150 mM NaCl, 1 mM $CaCl_2$, and 0.5 mM sodium taurodeoxycholate (NaTDC). All assays were performed in

triplicate at 37 ºC for 5 minutes. The specific activities of GPLRP2 and FsCut are 2700 ± 300 and 2596 ± 96 U/mg, respectively, on tributyrin and 5420 ± 85 and 984 ± 62 U/mg, respectively, on C8-MGDG[19].

2.4.Hydrolysis reactions.

The hydrolysis of *C. reinhardtii* cells was performed with both GPLRP2 and FsCut. Each hydrolysis reaction was performed using 300-400 mg fresh weight (FW; equivalent to 50-75 mg DW) of *C. reinhardtii* cell pellets. The cell pellet, fresh or after blanching was resuspended in 3 mL of 0.3 mM Tris buffer, pH 8.0, 150 mM NaCl and 2 mM $CaCl_2$. Based on previous studies performed on plant leaves [7], 50 µg of enzyme was added (either 23 µL stock solution of GPLRP2 or 26 µL stock solution of FsCut) and the tubes were incubated at 37 ºC for 3 h, under continuous orbital agitation at around 250 rpm. Control experiments were performed with non-blanched cells with and without adding enzyme. The reactions were stopped after 3 h and a lipid extraction was performed immediately. The addition of chloroform-methanol and 1N HCl for lipid extraction allows stopping the enzyme-catalyzed reactions by killing the enzymes (see section 2.7).

2.5. Alcoholysis reactions.

The ethanolysis reactions were carried out with both enzymes, GPLRP2 and FsCut, with an optimum ethanol concentration of 2.5 M defined in a previous work [7], in 2 mM Tris buffer, pH 8.0, 150 mM NaCl. Absolute ethanol was added to the buffer solution, and 1 mL of this solution was added to 50-75 mg DW of freeze-dried microalgae cells in 15-mL Falcon tubes. The reaction mix was vortexed before adding the enzyme stock solution (50 µg). Reactions

were performed in triplicate at 37 °C with stirring (200 rpm), including a blank reaction without enzyme. For FsCut, the reaction time was 72 h with samples taken at 4, 8, 24, 48, and 72 h. For GPLRP2, the reaction time was 24 h with samples taken at 0.5, 1, 2, 4, 8, and 24 h. All samples underwent immediate lipid extraction for killing the enzymes and stopping the reaction at given times, and the organic fraction was analysed by thin-layer chromatography (TLC).

2.6. In vitro gastrointestinal digestion

The experiment included two phases: a gastric step at pH 5, using RGE as a source of gastric lipase and pepsin [34], and an intestinal step at pH 6, using HPJ and bile salts (NaTDC) solution. The final concentrations were set to 16 μg gastric lipase /mL (equivalent to 20 U/mL) in the gastric step, 250 μg human pancreatic lipase (HPL) /mL (equivalent to 2,000 U/mL) and 4 mM NaTDC in the intestinal step. Besides HPL, HPJ also contains human PLRP2 and bile salt-stimulated lipase (BSSL) that both display galactolipase activities [31]. The assay solution contained 10 mM MES, 150 mM NaCl, with pH adjusted to 5.0 for the gastric phase and 6.0 for the intestinal one, in order to fit with pH values observed in gastric and duodenal contents at half-gastric emptying of test meals [35, 36].

For each digestion assay (performed in triplicate), approximately 250 mg of freeze-dried microalgae were added to a 50-mL Falcon tube and re-suspended in 10 mL of the above-mentioned pH 5.0 assay solution. 1 mL of the suspension was taken for lipid extraction and analysis at time zero. After collecting the t_0 sample, RGE was added, and the tube was incubated for 30 min at 37 °C with gentle stirring (100 rpm). Two 1-mL samples were taken at 15 min and 30 min. 2 mL of pure water was added to gastric samples prior to lipid extraction.

At 30 min, the intestinal phase was started by diluting the remaining gastric phase by half with 7 mL of the prepared HPJ-NaTDC solution and adjusting pH to 6.0. The mixture was vortexed for homogenization and incubated for 60 min at 37 °C with gentle stirring (100 rpm), totalling 90 min of incubation. Five points were taken at 35, 40, 45, 60 and 90 min. Since the reaction mixture was diluted by half, 2-mL samples were taken for lipid extraction at each time point and completed to 3 mL with pure water. All samples underwent immediate lipid extraction for killing the enzymes and stopping the reaction at the selected time points, and the organic phase was analysed by TLC.

2.7. Lipid extraction and analysis by thin-layer chromatography

At each time point of reactions, 1 to 3 mL of the reaction mixture containing *C. reinhardtii* cells was collected and completed with pure MilliQ™ water to 3 mL when required. 3 mL of chloroform-methanol (2:1 v/v) and 500µL 1N HCl were added to stop the reaction and start the extraction procedure. The samples were frozen at -20°C, thawed and vortexed to ensure cell disruption for efficient extraction. Phase separation was performed by centrifugation at 1,000 rpm for 10 min at 4°C (Sigma 3-18K centrifuge) and the lower organic phase was collected with a Pasteur pipette. Extraction was repeated twice and the organic phases were pooled and washed with 3 mL of saturated NaCl solution. After phase separation, the organic phase was dried using $MgSO_4$.

Lipid extracts and reference standards were spotted on silica gel 60 TLC plates (10 X 20 cm, from Merck-Sigma-Aldrich, Saint-Quentin Fallavier, France) using a Linomat IV sample spotter (Camag, Muttenz, Switzerland) equipped with a 100 µL Hamilton syringe. For each plate, four calibration lanes with increasing amounts of standards were spotted, for plotting calibration curves and subsequent quantification of the compounds separated by TLC.

For galactolipid separation, migration was performed using Chloroform:Methanol:Water (47.5:10:1.25 v/v/v) as the eluant for polar lipids. The migration proceeded until about 80% of the plate height, taking around 20 min. After drying for 10 min at room temperature (RT), the plate was dipped into thymol-sulfuric acid reagent for galactolipid staining [26], using a Chromatogram Immersion Device 3 (Camag, Muttenz, Switzerland). It was then dried again and placed in an oven at 100 ºC for 10 min. Afterward, a densitometry analysis of the plate allowed establishing calibration curves for MGDG, DGDG, and ALA used as reference standards and the estimation of their concentrations in the samples. C8-DGDG (0.25 mg/mL), C18-MGDG (0.5 mg/mL) and ALA (0.5 mg/mL) in $CHCl_3$-MeOH 2:1 (v/v) were used as reference standards and calibration curves were established with 10 to 400 ng of MGDG and DGDG.

For FFA and FAEE analysis, migration was performed using Heptane:Diethyl Ether:Formic Acid (55:45:1 v/v/v) as the eluant for neutral lipids. The migration proceeded until about 80% of the plate height, taking around 15 min, followed by drying for 10 min. The plate was then dipped in a staining solution of copper acetate solution and phosphoric acid using a Chromatogram Immersion Device 3. After drying again, it was placed in an oven at 180 ºC for 15 min. The same protocol as for the polar lipids was followed regarding the densitometry analysis, except that ALA (0.5 mg mL^{-1}) and ethyl linolenate (ALAEE; 0.5 mg mL^{-1}) in $CHCl_3$-MeOH 2:1 (v/v) were used as reference standards. Calibration curves were established with 50 to 750 ng of ALA and ALAEE spotted onto the plate.

2.8. Analysis of fatty acid methyl esters by gas chromatography-flame ionization detection (GC-FID)

The fatty acids from *C. reinhardtii* lipids and free fatty acids (FFA) generated upon digestion were derivatized into FAME prior to GC-FID analysis. This was achieved by adding 2 mL of boron trifluoride (14% BF_3 in methanol) to the dried lipid extract, 1 mL heptane as co-solvent and a known amount of pentadecanoic acid (C15:0) as internal standard (IS). The reaction was performed by heating at 105°C for 30 min. After cooling, the FAME were extracted by adding 2 mL of heptane. Samples were shaken, and the upper heptane layer was recovered after phase separation. When required it was concentrated by solvent evaporation under a stream of nitrogen and stored at –20 °C before GC analysis. Samples were analysed by GC using a Thermo Scientific TRACE GC UltraTM Gas Chromatograph equipped with an Agilent Technologies J&W HP-5ms GC Column (5 % phenyl, 95 % dimethylpolysiloxane; 60 m x 0.25 mm ID x 0.25 µm film) and a flame ionization detector (FID). Separation of FAME was achieved by injecting 1 µL of each extract in the split mode (1/80). The carrier gas was hydrogen (1 mL/min). The oven temperature was hold at 100 °C for 2 min and then a temperature gradient was established, ranging from 100 °C to 225 °C at a rate of 10 °C/min, to 240 °C at a rate of 1 °C/min, to 260°C at a rate of 5°C/min and 300°C at a rate of 30°C/min, before a final hold at 300°C for 10 min. The FID detector was set at 280 °C. The identification of fatty acids was based on the elution time of reference standards including FAME 37 from Supelco and *C. reinhardtii* FAME previously produced and characterized in our laboratory.

2.9 Nuclear magnetic resonance (NMR) spectroscopy

^{1}H and ^{13}C NMR spectra were recorded at 298 K, on a Bruker Avance III 600 MHz spectrometer equipped with a TCI cryoprobe, using Topspin 3.2 software. Spectra were recorded on lipid

extracts. A given volume of each extract (500 µL) was evaporated under nitrogen and the dry extract was then re-dissolved in deuterated chloroform (500 µL $CDCl_3$) before NMR analysis. HSQC (heteronuclear single quantum correlation) spectra were recorded to determine proton-carbon single bond correlations. The acquisition times were 106.5 ms in the ^{1}H dimension and 4.2 ms in the ^{13}C dimension. The spectral width was set to 16 ppm in the ^{1}H dimension, centred on water (4.7 ppm) and to 200 ppm in the ^{13}C dimension, centred at 110 ppm. 16 scans were accumulated for a total acquisition time of 1 h and 18 min.

2.10. Determination of selected non-enzymatic oxylipins (ng/g) in lipid extracts

Briefly, 500 µL of each lipid extract in $CHCl_3$/MeOH (2:1) was transferred into a centrifuge tube and brought to a final volume of 3 mL with the same solvent. Then, 4 µL of an Internal Standards (IS) mixture and 1 mL of water were added. The mixture was vortexed for 30 seconds, followed by the addition of 500 µL of phosphate buffer (pH 2). After another 30 seconds of vortexing, 3 mL of cold $CHCl_3$/MeOH (2:1) was added. The mixture was homogenized and centrifuged at 2000 rpm for 5 min at 4°C. The lower organic phase was transferred to a Pyrex® tube and concentrated to dryness under a nitrogen stream. Subsequently, the samples were hydrolysed with 950 µL of KOH (1M in H_2O) in a thermostatic mixer set to 100 rpm, alternating between 57°C for 15 seconds and 50°C for 4 seconds, for a total of 30 min at 40°C. After hydrolysis, 1 mL of formic acid solution (40 mM in H_2O) was added to the mixtures, which were then loaded onto an SPE cartridge (Oasis Max 3CC 60mg, Waters®). The samples were cleaned by sequentially washing with 1.5 mL of 2% (v/v) NH_3, 1.5 mL of MeOH/formic acid (20 mM, 30:70 v/v), 1.5 mL of hexane, and finally 1.5 mL of hexane/acetate (70:30 v/v). After drying, targeted metabolites were eluted with 2 × 750 µl of hexane/ethanol acid (70:29.4:0.6 v/v/v). The eluates were evaporated under a nitrogen stream and reconstituted in 100 µL of H_2O/ACN (83:17 v/v). The mixtures were transferred to a 0.45 µm filtering

Eppendorf® tube and centrifuged at 10,000 rpm for 1 min. The filtrates were then ready for injection.

All analyses were carried out using a micro-LC-MS/MS. Extracts were chromatographed on a HALO C18 analytical column (100 × 0.5 mm, 2.7 µm; Eksigent Technologies, CA, USA) using an Eksigent ekspert MicroLC 200 instrument. Mass spectrometry measurements were conducted on an AB Sciex QTRAP 5500 (Sciex Applied Biosystems). The ionization source was electrospray ionization (ESI) in negative mode. Detection of the fragmentation ion products from each deprotonated molecule $[M-H]^-$ was performed in multiple reaction monitoring mode (MRM). Analyte concentrations were determined using calibration curves based on the area ratio of the analytes to the IS. Data processing was carried out using MultiQuant 3.0 software (Sciex Applied Biosystems).

3. Results and Discussion

3.1. *Hydrolysis of galactolipids from whole algal cells by GPLRP2*

The hydrolysis reaction was monitored by lipid extraction and TLC analysis of galactosylated lipids and FFA after incubating *Chlamydomonas* whole cells with GPLRP2. We initially worked with the cell wall-deficient strain CC-4349 cw15 mt+, commonly available at our laboratory for genetic engineering [37].

Control experiments with blanched cells without addition of GPLRP2 allowed visualizing and estimating MGDG and DGDG contents in *C. reinhardtii* cells (Figure 2A, lane 2; Table 1). When GPLRP2 was added (Figure 2A, lane 1), MGDG and DGDG bands disappeared, while a band corresponding to monogalactosylmonoglyceride (MGMG) was clearly observable. This result indicates that GPLRP2 can directly hydrolyse the galactolipids from *C. reinhardtii* cells without needing any specific pre-treatment beforehand.

The staining with thymol-sulfuric acid makes ALA visible on the TLC plate of polar lipids, with a yellow colour (Figure 2A, lane 1). The reaction responsible for this color is not yet known, but probably results from a reaction with fatty acid double bonds. Lipolysis and production of FFA was confirmed by analysing neutral lipids after staining them with copper acetate and phosphoric acid (Figure 2B, lane 1).

A control with non-blanched cells and no GPLRP2 was performed (Figure 2, lane 3). Interestingly, the MGDG and DGDG bands have disappeared compared to the control with blanched cells (Figure 2A, lane 2), suggesting that all galactolipids were digested by endogenous lipases. However, no fatty acid band stained in yellow can be observed on the TLC plate for polar lipids (Figure 2A, lane 3). On the TLC plate for neutral lipids (Figure 2B, lane 3), two bands corresponding to FFA appear where only a single and strong band appears in the sample of blanched cells incubated with GPLRP2. From these findings and comparison, it can

be hypothesized that the FFA released from non-blanched cells were further modified after lipolysis and probably oxidized by endogenous lipoxygenases and non-enzymatic reactions.

In Figure 2B, lane 3, the two bands migrating like FFA could correspond to non-oxidized fatty acids for the upper one and to oxidized and more polar fatty acids for the lower one.

From the quantitative TLC analysis of galactolipids and FFA released from blanched cells by GPLRP2, the conversion rates for the lipolysis reaction were calculated (Table 1). As the initial substrate, microalgae cells contained approximately 278.5 ± 72.1 µmole/g DW of FA equivalents in MGDG and DGDG. After the lipolysis reaction, FFA represented 258.2 ± 8.1 µmole/g DW, i.e. a lipolysis level of 93%. Taking into account the presence of MGMG as an intermediate lipolysis product (14.7 ± 5.2 µmole/g DW), the recovery of fatty acids initially present in *C. reinhardtii* galactolipids reached 98% (Table 1). These results show the ability of GPLRP2 to directly digest galactolipids from whole *Chlamydomonas* cells and convert them into FFA with a high efficiency.

Since the *C. reinhardtii* strain (CC-4349 cw15 mt+) is characterized by a cell wall produced in minute quantities or absent [38], what might have facilitated the access of GPLRP2 to galactolipids in cell membranes, we repeated the galactolipid hydrolysis experiments using another strain known to have a full natural cell wall (*Chlamydomonas reinhardtii* wild-type strain CC-5101 T222+) [32]. The results obtained in terms of galactolipid hydrolysis (Figure 2C, lane 1) and FFA production (Figure 2D, lane 1) were identical to those obtained with the cell wall-deficient strain with a complete disappearance of MGDG and DGDG. The access of GPLRP2 to *C. reinhardtii* galactolipids and its activity were therefore not impaired by the microalgal cell wall, thus confirming previous results obtained with plant leaves [7]. In this previous work, it was also shown that GPLRP2 can hydrolyse galactolipids in the absence of

blanching (see Supporting Information in [7]), thus ruling out an increased accessibility of GPLRP2 due to cell wall disruption upon blanching.

3.2. NMR spectroscopy analysis of C. reinhardtii lipid extracts

NMR spectroscopy analysis was performed on the lipid extract from blanched cells incubated with GPLRP2 for 3 h at 37°C (spectrum 1 in Figure 3). The spectrum was compared to those obtained from blanched cells without added enzyme (spectrum 2 in Figure 3; blank for lipolysis and oxidation), non-blanched cells incubated without added enzyme for 3 h at 37°C (spectrum 3 in Figure 3) and pure ALA.

Specific areas of the ^{1}H-NMR spectra are displayed in Figure 3. In the first area of the spectra (2.6-1.2ppm), the matching pattern between spectrum 1 and the α-CH2 and β-CH2 of ALA standard spectrum is striking. It also matches with the complex multiplet of allylic protons. This only appears in sample 1 spectrum. Other spectra do not show clear signals for ALA, although the spectra indicate, in each case, a complex mixture of lipids. These results are in agreement with the high level of FFA in sample 1 and the large proportion of ALA in *C. reinhardtii* galactolipids [39].

The absence of clear signals for ALA in the spectrum of non-blanched sample 3 suggests that FFA have been further modified after their release by lipolysis. Several signals indicate an oxidation of fatty acids in this sample. A multiplet at 6.1 ppm indicates dienic proton associated with an hydroxyl group [40]. This could correspond to the formation of 13-(S)-hydroperoxy linolenic acid (Figure 4). An even stronger evidence of fatty acid oxidation in non-blanched sample 3 is the doublet at ~9.5 ppm [40] that correlates with a ^{13}C-NMR signal at 194 ppm in the HSQC spectrum (See Figure S1 in Supplementary Information). These chemical shifts are typical of carbonyl carbon bound to an aldehydic proton. This suggests the conversion of fatty

acids into hydroperoxides and then aldehydes according to the pathway shown in Figure 4. The doublet could be attributed to a conjugated aldehyde that could result from the isomerization of 3-hexenal into 2-hexenal. The other samples do not show such unshielded ^{13}C signals, which indicates the absence of a carbonyl bound to a proton in those samples.

Finally, the three samples also differ in the 3-5 ppm area of their respective spectra (Figure S2). This area is the expected area for carbohydrate protons, proton in the α position of an alcohol function, and is generally crowded if a sugar is present. Interestingly, sample 1 did not display much signal, especially compared to sample 2 (control without lipolysis). In sample 1, this may reflect the extent of galactolipid lipolysis leading to water-soluble end-products like MGG and DGG. Indeed, these products are expected to be present in lipid extracts [26] and the galactose initially present in galactolipids remains in the aqueous phase upon lipid extraction.

Sample 2 show signals that could match galactose whilst sample 3 has a great variety of signals in this area that do not quite match galactose. It is possible that endogenous enzymes acted on galactose and degraded it in galactose-like or sugar-like molecules explaining these changes. The TLC analysis shown in Figure 2 (Panel A) confirms that galactose from galactolipids is mainly revealed in sample 2 with MGDG and DGDG, and to a lower extent in sample 1 with MGMG, but not in sample 3.

The NMR analysis led to some interesting findings regarding the comparisons between the three samples. The TLC and NMR analysis both highlight the same conclusions. The formation of free ALA resulting from galactolipid lipolysis by GPLRP2 in sample 1, as well as the oxidation of FFA in sample 3. This oxidation is probably promoted by endogenous enzymes that are otherwise inactivated by blanching.

3.3. Search for non-enzymatic fatty acid oxidation products in C. reinhardtii lipid extracts

Since galactolipids from *C. reinhardtii* contain large amounts of ALA (Table 2) [39], we also searched for non-enzymatic oxidation products of ALA, the so-called phytoprostanes (PhytoPs) [41]. Reactive oxygen species (ROS) like the free radical $OH^{\bullet}$ can trigger lipid peroxidation by hydrogen abstraction from PUFAs and the addition of molecular oxygen to form a peroxyl radical [42]. The produced intermediates can undergo a nonenzymatic series of cyclization, addition of a second molecular oxygen, and reduction to form isoprostanoids (IsoP) [41], which are more stable than other peroxidation products, such as aldehydes or peroxyl radicals [43]. In addition to free radicals, the non-enzymatic formation of isoprostanoids requires the presence of at least three double bonds in the PUFA, such as in ALA, the precursor of PhytoPs [44].

Six PhytoPs (Figure 5) were identified in lipid extract of *C. reinhardtii* by microLC-MS/MS using a method validated with macroalgae isoprostanoids [45]. The highest levels were observed in non-blanched cells (Figure 5A) with 9-F_{1t}-PhytoP, 9−L_{1t}−PhytoP and 9-*epi*-9-F_{1t}-PhytoP being the most represented PhytoPs. On the contrary, the lowest levels of PhytoPs were observed with blanched cells without added enzyme (Controls; Figure 5A). Blanched cells treated with GPLRP2 showed an increased in PhytoP levels compared to controls, especially, with 9−L_{1t}−PhytoP that exceeded the levels found in non-blanched cells (Figure 5A). These results indicate that non-enzymatic oxidation of ALA preferentially occurs on free ALA generated upon lipolysis by either endogenous lipases (non-blanched cells) or GPLRP2 added to blanched cells. When IsoPs were discovered in humans, it was indicated that the non-enzymatic free radical-catalyzed peroxidation of PUFAs initially occurred on fatty acids esterified to phospholipids before IsoPs were subsequently released presumably by phospholipases [46]. Regarding PhytoPs, it was assumed that they could be produced according to a similar mechanism on esterified fatty acids. Moreover, some oxidized fatty acids such as

10-oxo-11,15-phytodienoic acid (10-OPDA) are known to be esterified to galactolipids in *Arabidopsis thaliana* [47]. These molecules, named Arabidopsides (A to G) and linolipins (A to D), can be derived from both MGDG and DGDG [48]. However, there is still a debate, on whether oxidation preceeds lipolysis or vice versa. Some studies have shown that lipolysis can be anti- or pro-oxidative depending of various factors [49]. Here we show that the lipolysis of *C. reinhardtii* galactolipids is pro-oxidative and triggers the production of PhytoPs.

3.4. *Hydrolysis of galactolipids from whole algal cells by FsCut*

The digestion of blanched *C. reinhardtii* cells by FsCut followed the same protocol as the one with GPLRP2, using both the cell wall-deficient *C. reinhardtii* strain (CC-4349 cw15 mt+) and the strain with full natural cell wall (*Chlamydomonas reinhardtii* wild-type strain CC-5101 T222+). The hydrolysis of galactolipids (Figure 6A, lane 1 and 6C, lane 2, respectively) and the release of fatty acids (Figure 6B, lane 1 and 6D, lane 2, respectively) were monitored by TLC. The bands corresponding to MGDG and DGDG disappeared or were much reduced compared to the controls without enzyme (Figure 6A, lanes 2 and 6C, lane 1, respectively), while a band corresponding to MGMG was clearly observed when the cell wall-deficient *C. reinhardtii* cells were tested ((Figure 6A, lane 1). With the full cell wall *C. reinhardtii* strain, only a faint MGDG band (or no band at all) was observed (Figure 6C, lane 2). A band corresponding to FFA/ALA was observed on the TLC plates for neutral lipid analysis (Figure 6B, lane 1 and 6D, lane 2, respectively). After the lipolysis reaction, FFA represented 176.7 ± 5.5 µmole/g DW, i.e. a lipolysis level of 63.5%. These results show the ability of FsCut to digest directly galactolipids from whole *C. reinhardtii* cells and convert them into FFA. As seen with GPLRP2, its activity is not impaired by the microalgal cell wall.

3.5. Alcoholysis of galactolipids from whole algal cells by GPLRP2 and FsCut

These experiments were performed with the CC-4349 cw15 mt+ strain only. The ethanolysis reaction was performed in the presence of a large concentration of ethanol (2.5 M) to generate ethyl esters of the galactolipid fatty acids as previously obtained with the same enzymes acting on whole leaves of various plant species [7]. Nevertheless, water was still present in the reaction mixture and some hydrolysis could not be avoided. It was therefore expected to observe both FFA and fatty acid ethyl esters (FAEE) as the main products of the reaction. The reactions were monitored by TLC of both polar (Figure 7A and C) and neutral lipids (Figure 7B and D).

In the presence of GPLRP2, a band corresponding to MGMG was visible after 30 min (Figure 7A) while the MGDG band was already faint. Then the MGMG band intensity decreased with time until it had totally disappeared after 24 h. Bands corresponding to FFA/ALA and FAEE were observed simultaneously (Figure 7B). No DGDG was observed, suggesting that the low levels present in *C. reinhardtii* were rapidly converted into FFA and esters. These results show that GPLRP2 can convert *C. reinhardtii* galactolipids into FAEE directly from the whole cells.

After quantifying the products of the alcoholysis reaction by TLC and densitometry, the mass of FFA and FAEE detected on the plates was converted into molar concentration and their variations plotted as a function of time (Figure 8A). In the presence of GPLRP2, both FFA and FAEE concentrations increased quickly at the beginning of the reaction with FFA levels that were around 2-fold higher than those of FAEE at 30 min. Then, the FAEE to FFA concentration

ratio increased up to 24h, where it reached a value of 1 with a FAEE concentration of 1.92 ± 0.05 mM.

With FsCut, the reaction was slower and monitored for a longer period of 72 h. Bands corresponding to residual MGDG and DGDG were observed until 8 h while MGMG appeared transiently from 4 to 24 hours (Figure 7C). The conversion of galactolipids led to the production of both FFA and FAEE (Figure 7D). Their mass detection was converted into molar concentrations, which were plotted as a function of time (Figure 8B).

Kinetics of FFA and FAEE production by FsCut were slower (Figure 8B) than those observed with GPLRP2 (Figure 8A). Similar levels of FAEE were however reached after 10 h, where the FAEE to FFA ratio reached a value of 0.49. Then, FAEE levels increased up to 72 h, where FAEE concentration was 2.0 ± 0.13 mM and the FAEE to FFA ratio was 0.94.

The choice of 2.5 M for the EtOH concentration and the reaction times were based on previous studies performed with the same enzymes acting on galactolipids from spinach leaves [7]. Globally, the respective behaviours of GPLRP2 and FsCut are the same, the kinetics being faster with GPLRP2, but reaching a lower final FAEE to FFA molar ratio (around 1 versus 2.3 [7]). With spinach leaves galactolipids, a FAEE to FFA molar ratio of 1 was reached after 5 and 25 h for GPLRP2 and FsCut, respectively. Therefore, the conditions for producing FAEE from the galactolipids of whole *C. reinhardtii* cells will have to be optimized. Nevertheless, the proof of concept is made that such a direct galactolipase-catalysed reaction is possible.

Regarding the potential valorisation of FAEE production, one track is that FAEE of galactolipid PUFA might easier be separated from biomass than galactolipids themselves. FAEE of omega-3 FA, usually produced from fish oils and containing DHA and EPA, already find various applications as dietary supplements or FDA-approved drugs [50]. Ethyl linolenate is commonly used in cosmetics as a skin emollient and perfuming agent. It is known for its

antibacterial and anti-inflammatory properties [51, 52], acceleration of wound healing, as an effective and clinically-proven anti-acne agent [53] and possible anti-melanogenesis activity through the inhibition of tyrosinase promoter activity [54, 55]. Recently, it has been shown that microalgal-sourced FAEEs have an antiparasitic effect against *Gyrodactylus turnbulli* infection in fish (guppies and barramundi) and may represent a natural solution for the treatment of fish parasitic diseases [56].

3.6. In vitro digestion of C. reinhardtii under simulated GI tract conditions

An *in vitro* digestion assay was carried out to determine if the gastrointestinal digestive enzymes can release free fatty acids from the galactolipids present in whole *C. reinhardtii* cells (CC-4349 cw15 mt+ strain). The reaction was intended to mimic the conditions encountered during digestion in the GI tract, with a gastric step in acidic conditions, pepsin and gastric lipase provided by RGE, and an intestinal phase with a slightly higher pH and the addition of bile salts along with pancreatic enzymes provided by human pancreatic juice (HPJ). The chosen pH conditions differ from those recommended by the Infogest standard static *in vitro* digestion protocol [57]. Indeed, a pH of 3 as recommended by Infogest corresponds to the late stage of gastric digestion when most of the meal has already been emptied from the stomach, whereas a pH of 5 corresponds to the pH of gastric contents at half-gastric emptying and fits well with the optimum activity of gastric lipase [35, 36]. Similarly, a pH of 6 rather than 7 better fits with the mean pH value of upper intestinal contents during digestion [35, 58, 59]. HPJ provided a final pancreatic lipase concentration of 2000 U/mL as recommended by Infogest [57] and other lipolytic activities, including galactolipase activity displayed by human PLRP2 (HPLRP2) and pancreatic carboxyl ester hydrolase [31]. HPLRP2 is present in the HPJ juice sample we used [60] and its enzyme properties are similar to those of GPLRP2 [61]. It is worth remembering

that classical pancreatic lipase serves as a reference here but is not involved in the digestion of galactolipids [28, 62].

In vitro digestion of galactolipids was monitored by TLC after lipid extraction at various time points (Figure 9). During the gastric phase (0- 30 min), a band corresponding to MGDG was clearly seen (Figure 9A) while DGDG was not detected, probably because of its too low concentration after cells dilution. As expected, the intensity of the MGDG band did not decrease, because gastric lipase provided by RGE does not display galactolipase activity [23].

After the beginning of the intestinal phase (35-90 min), MGDG decreased rapidly and was no more observed after 45 min (Figure 9A and C). A band corresponding to MGMG appeared, reaching a maximum at 60 min (Figure 9A and C). Simultaneously, a band corresponding to FFA was observed using both polar (Figure 9A) and neutral (Figure 9B) lipid staining. After 60 min, at which MGMG was maximum, FFA reached a concentration of 0.2 mM, similar to the initial concentration of MGDG (Figure 9C). One fatty acid out of the two esterified to MGDG was therefore released. Progress in lipolysis was then much slower, which probably reflected the preferential release of the fatty acid at the *sn*-1 position of galactolipids [61]. These results are similar to those previously reported on the *in vitro* digestion of galactolipids from pea chloroplast-rich fractions (CRF) [23]. Then, after 60 min, a slight decrease in MGMG concentration was observed, while FFA still slightly increased. This suggests that more than one acyl chain is released by molecule of MGDG. Additionally, some low levels of FFA might also result from DGDG hydrolysis (not seen on the TLC plates). The duration of the *in vitro* digestion (90 min in total) was chosen from previous studies on meals [63] and emulsions [64], which showed an efficient digestion of TAGs (≥70%). This duration was clearly not long enough to reach a complete digestion of galactolipids. Conditions will have to be adapted for further studies but it can be concluded that galactolipids from whole *C. reinhardtii* cells are bioaccessible to the lipolytic enzymes present in HPJ.

Analysis by GC FID of the FFA released upon *in vitro* digestion allowed comparing them with the FA initially present in *C. reinhardtii*, galactolipids (Figure 10). Overall, the two compositions are similar, except that FFA contain more ALA and less 16:4 n-3. This finding probably reflects the specificity of the galactolipases present in HPJ. Indeed, ALA is preferentially found at the *sn*-1 position in MGDG, while 16:4 n-3 is mainly found at the *sn*-2 position (Table 2) [39]. Again, it seems that fatty acids at position *sn*-1 position are preferentially released in comparison to those at *sn*-2 position, which is a known property of PLRP2 [24, 25, 61]. However, it is worth noting that 16:4 n-3 was found in tissues of zebrafish fed with *C. reinhardtii*[18], suggesting that fish galactolipases have the ability to release also the fatty acid at the *sn*-2 position and thus complete galactolipid digestion.

The detection of 18:4 n-3 (18:4Δ5,9,12,15) could also derive from lipolytic activities on other polar lipids in which this PUFA is present, such as phosphatidylethanolamine (PE) and the betaine lipid 1,2-diacylglyceryl-3-O-4'-(N,N,N-trimethyl)-homoserine (DGTS) [39]. Purified guinea pig and human PLRP2 are known to display a phospholipase A1 activity on PE[65-67], but to our knowledge their activity on DGTS has never been tested.

4. Conclusion

4.1. The enzymatic digestion and bioconversion of C. reinhardtii galactolipids

It was shown that several enzymes with galactolipase activity, GPLRP2, FsCut and galactolipases from HPJ, can directly hydrolyse galactolipids from whole *C. reinhardtii* cells without prior cell disruption. This was achieved with purified enzymes, as well as under conditions mimicking the complex GI tract contents. Galactolipids from *C. reinhardtii*

membranes are therefore bioaccessible to digestive enzymes. This finding explains the bioavailability of *C. reinhardtii* fatty acids observed in zebrafish, with increased levels of ALA and hexadecatrienoic acid (C16: 4-n-3) when the fish diet was enriched with *C. reinhardtii* biomass [18]. In this previous study, C16:4n-3, a known trophic marker of green algae[68], appears to be a marker of *C. reinhardtii* galactolipid digestion and absorption in the fish. It could be a perfect marker for future nutritional studies in humans since we show here that *C. reinhardtii* galactolipids can be digested by human enzymes present in HPJ (Figure 9), presumably human pancreatic lipase-related protein 2 (HPLRP2) and carboxyl ester hydrolase/bile salt-stimulated lipase (CEH/BSSL)[8, 31].

Furthermore, the enzymatic capabilities of galactolipases extend beyond hydrolysis, as they efficiently convert *C. reinhardtii* galactolipid fatty acids into ethyl esters in the presence of ethanol. This may provide an easier route to recover PUFAs from galactolipids dispersed in microalgal biomass, as previously shown with plant leaves[7].

4.2. The non-enzymatic oxidation of C. reinhardtii PUFAs and potential applications of phytoprostanes

The comparison of blanched, non-blanched and enzyme-treated blanched cells showcased the importance of the blanching procedure to avoid degradation of galactolipids and their PUFAs by endogenous enzymes and non-enzymatic oxidation. Analysis of lipid extract by NMR spectroscopy, corroborating the results obtained by TLC, allowed detecting PUFA oxidation products in extracts from non-blanched cells (Figure 3), suggesting the action of endogenous oxygenases such as lipoxygenases and α-dioxygenases (Figure 4). Phytoprostanes resulting from the non-enzymatic oxidation of ALA were found at higher levels in non-blanched cells than in blanched cells. However, their levels also increased when blanched cells were treated with a galactolipase (GPLRP2). Thus, the non-enzymatic conversion of ALA into

PhytoPs preferentially occurs when lipolysis and release of free ALA occurs first. These findings highlight the risk of PUFA release upon lipolysis and further oxidation in the microalgal biomass. They complete other observations showing high levels of FFA in commercial biomasses of *Chlorella sp*. (up to 17.9% of total lipids)[69] and during wet storage of microalgae [70, 71]. In this work, we have mostly tried to protect the microalgal biomass from endogeneous enzymatic reactions involving lipases and oxygenases, in order to ensure the recovery and bioavailability of non-degraded essential PUFAs such as ALA. The observation that the production level of PhytoPs from *C. reinhardtii* depends on enzymatic lipolysis contributes, however, to a better knowledge on PhytoPs production in microalgae. Recent studies suggest that PhytoPs may have several potential applications per se. They naturally have a potential role in the defense against herbivores and fungal diseases caused by necrotrophic pathogens [48, 72]. They are already used as biomarkers of oxidative stress in plants [73, 74]. Moreover, these isoprostanoids/oxilipins have been seen to exert immunomodulatory and anti-inflammatory activities *in vitro* [75], as well as pro-apoptotic and neuroprotective effects [76]. For instance, PhytoPs obtained from the edible red algae *Gracilaria longissimi* have been shown to display anti-inflammatory effects in endothelial cells [75].

4.3. Impact of the cell wall on the accessibility and digestibility of C. reinhardtii galactolipids

It is to be noted from the comparison of *C. reinhardtii* wild-type strain with its natural cell wall and a cell-wall deficient strain lacking the extracellular matrix made of carbohydrates and glycoproteins, that the presence of the cell wall does not affect the access of the lipases (GPLRP2 or FsCut) to the galactolipids and the release of FFA. However, healthy human gut microbiome possesses cellulolytic activities that should be active concomitantly to HPJ lipolytic enzymes during digestion [77].

4.4. Using C. reinhardtii as a novel food

C. reinhardtii is generally recognized as safe (GRAS) for human consumption in the US by FDA[78] [79]. It is listed as a novel food in the EU Novel Food status Catalogue (https://ec.europa.eu/food/food-feed-portal/screen/novel-food-catalogue/) but a pre-market authorisation is still required before it can be placed as food on the EU market. Assuming a correct handling of its biomass, it is an interesting source of PUFAs, especially the omega-3 ALA, via the digestion of galactolipids. No need here to optimize the culture and growth conditions of microalgae or modify them by genetic engineering to enrich their contents in PUFA-rich oil (TAGs) since PUFAs are constitutive of their main membrane lipids. Moreover, microalgae usually accumulate oil under stress conditions, resulting in a reduced growth, while they continuously produce galactolipids for the biogenesis of photosynthetic membranes. However, the digestion of galactolipids has been long ignored compare to that of TAGs. By showing the digestibility of *C. reinhardtii* galactolipids, one can promote the use of this microalga as food. In the facts, humans and animals are already eating algae and here we only confirm that they can digest their main membrane lipids and benefit from their PUFAs. We identified, however, one mammalian enzyme, PLRP2, involved in this process. We hope that these findings can promote the consumption of edible algal biomass, including that of macroalgae, which are easily accessible from the sea and do not require specific cultivation.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Author contribution

Beatriz Fachini and Cassy Gérard were involved in acquisition, analysis and interpretation of data, in drafting the article and revising it critically for important intellectual content. Achille Marchand and Olivier Bornet were involved in acquisition, analysis and interpretation of NMR data, and revising the article critically for important intellectual content. Valérie Gros, Claire Vigor, Camille Oger, Guillaume Reversat and Thierry Durand were involved in acquisition and interpretation of Phytoprostane analysis, and revising the article critically for important intellectual content. Goetz Parsiegla was involved in revising the article critically for important intellectual content. Brigitte Gontero was involved in revising the article critically for important intellectual content. Frédéric Carrière and Hélène Launay were involved in the conception and design of the study, in analysis and interpretation of data, in drafting the article and revising it critically for important intellectual content. All authors were involved in the final approval of the version to be submitted.

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Figure legends

Figure 1. *Organization of C. reinhardtii cell and main galactolipids found in thylakoid membranes*. C, chloroplast; DGDG, digalactosyldiacylglycerol; ER, endoplasmic reticulum; LD, lipid droplet; MGDG, monogalactosyldiacylglycerol; N, nucleus;

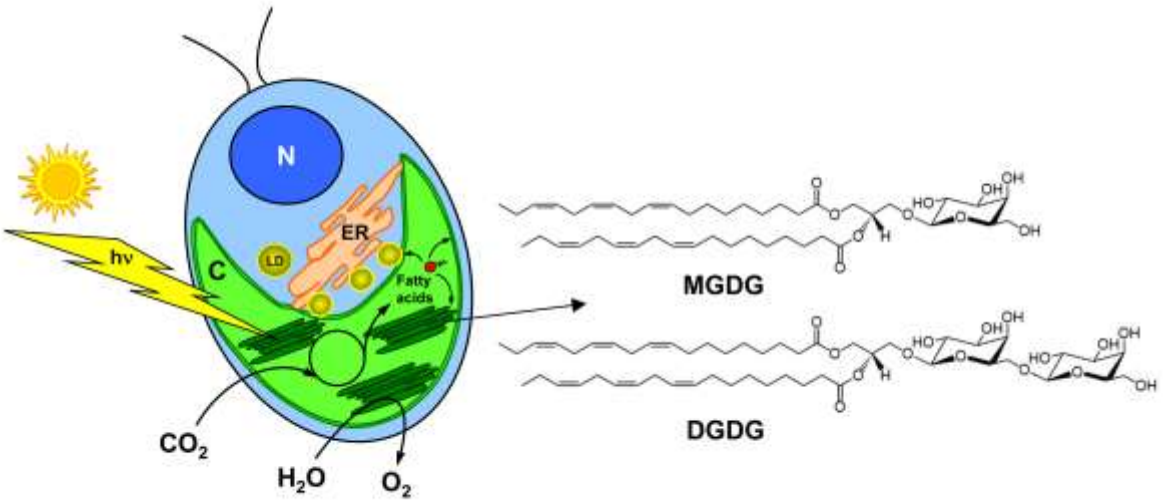


Figure 2. *Direct action of GPLRP2 on whole C. reinhardtii cells*. Panels A and C: TLC analysis of polar lipids (thymol-sulfuric acid staining) of cell wall deficient (A) and full cell wall (C) *C. reinhardtii* strains. In panel A, reference standards (Rs) are α-linolenic acid (ALA), monogalactosyldistearoylglycerol (C18-MGDG), digalactosyldioctanoyl glycerol (C8-DGDG). Lane 1 (two replicates) shows the lipids extracted after the action of GPLRP2 (50 µg enzyme; 3 h at 37°C) on blanched *C. reinhardtii* cells (300-400 mg FW), with one band on the top corresponding to free fatty acids and a band corresponding to monogalactosylmonoacylglycerol (MGMG). Lane 2 (two replicates) is the control showing the lipids extracted from blanched *C. reinhardtii* cells in the absence of added enzyme. Two bands corresponding to MGDG and DGDG are clearly observed. Lane 3 (two replicates) shows the lipid extracted from non-blanched *C. reinhardtii* cells incubated at 37°C for 3 h. In panel C, reference standards (Rs) are α-linolenic acid (ALA) and natural long chain MGDG and DGDG isolated from wheat. Lane 1 (two replicates) shows the lipids extracted after the action of GPLRP2. Lane 2 (two replicates) is the control showing the lipids extracted from blanched C. reinhardtii cells in the absence of added enzyme. Panels B and D: TLC analysis of neutral lipids (phosphoric acid-copper acetate staining) of cell wall deficient (B) and full cell wall (D) *C. reinhardtii* strains. In panel B, reference standard (Rs) is ALA. Lanes 1,2 and 3 correspond to the same samples as in panel A. Lane 1 (two repeats) clearly shows a band corresponding to FFA released from the blanched *C. reinhardtii* cells by GPLRP2. In non-blanched cells (lane 3), two distinct bands of lower intensities are observed in the range of fatty acid elution. An additional band compared to controls (lane 2) and GPLRP2-treated cells (lane 1) appears in the lower part of the lane, which corresponds to an unknown compound with high polarity. In panel D, reference standards (Rs) are oleic acid (OA), diolein (DO) and monoolein (MO). Lanes 1,2 and 3 correspond to the same samples as in panel C. See The original plates used for preparing panels C and D in Figure S3 of Supplementary Information.

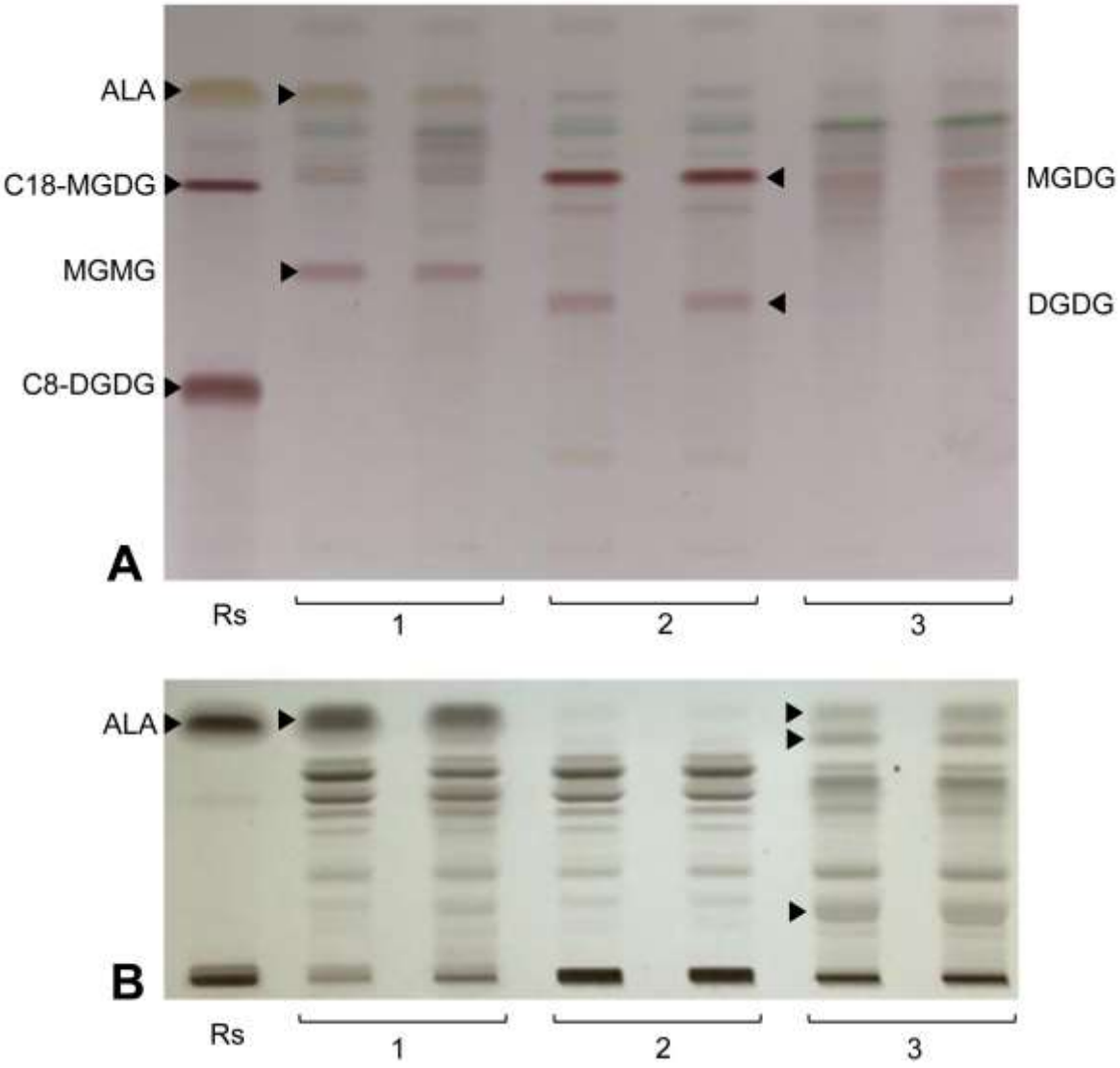

ALA
C18-MGDG
MGMG
C8-DGDG
MGDG
DGDG
A
Rs
1
2
3
ALA
B
Rs
1
2
3

Figure 3. *^{1}H-NMR spectra of C. reinhardtii lipid extracts*. Spectra were acquired for sample 1 (blanched *C. reinhardtii* cells treated with GPLRP2), 2 (blanched *C. reinhardtii* cells without added enzyme), 3 (non-blanched *C. reinhardtii* cells) and for α-linolenic acid (ALA) reference standard. Only the areas of interest are showed. Chemical shifts of fatty acid oxidation products are observed in spectrum 3. The doublet associated with aldehyde is quite characteristic as it is in an area free of signal in the others samples. The aldehyde signal was confirmed using HSQC which gave a carbon signal at 194 ppm.

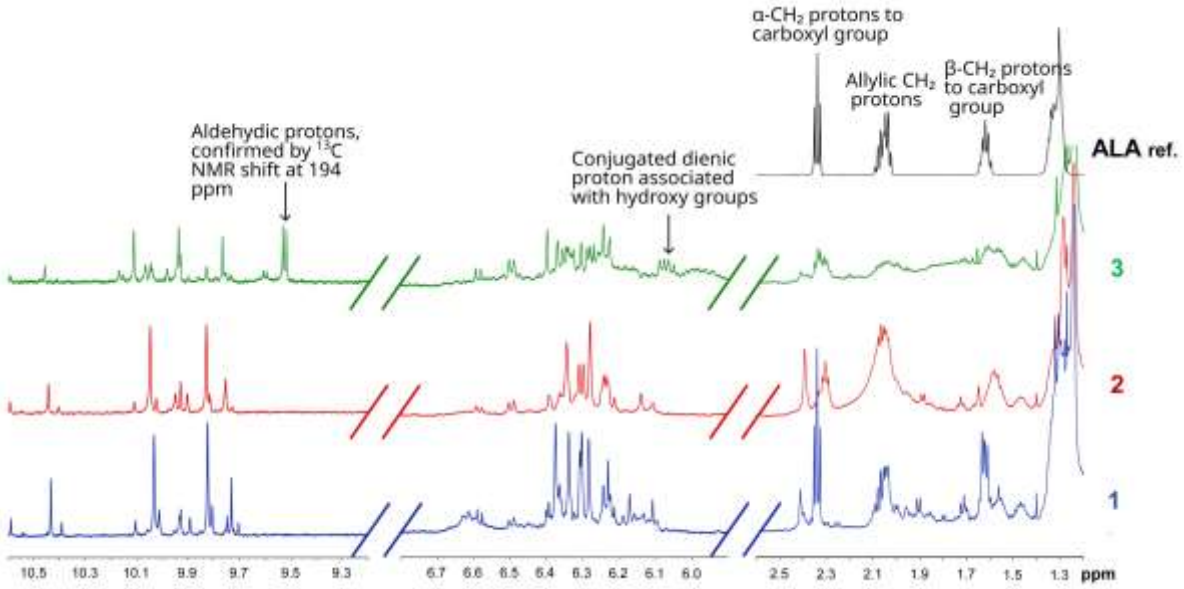


Figure 4. *Oxidation pathway of α-linolenic acid (ALA) released from galactolipids.* Only steps relevant for the interpretation of NMR spectra in Figure 3 are presented. Free ALA results from the enzymatic hydrolysis of MGDG by a galactolipase. It can be further oxidized by lipoxygenase that gives 13-(S)-hydroperoxy linolenic acid, this latter being splitted into 12-oxo-dodecenoic acid and 3-hexenal by hydroperoxide lyase. Finally, 3-hexenal can undergo isomerization to 2-hexenal with its double bond conjugated to the aldehyde group.

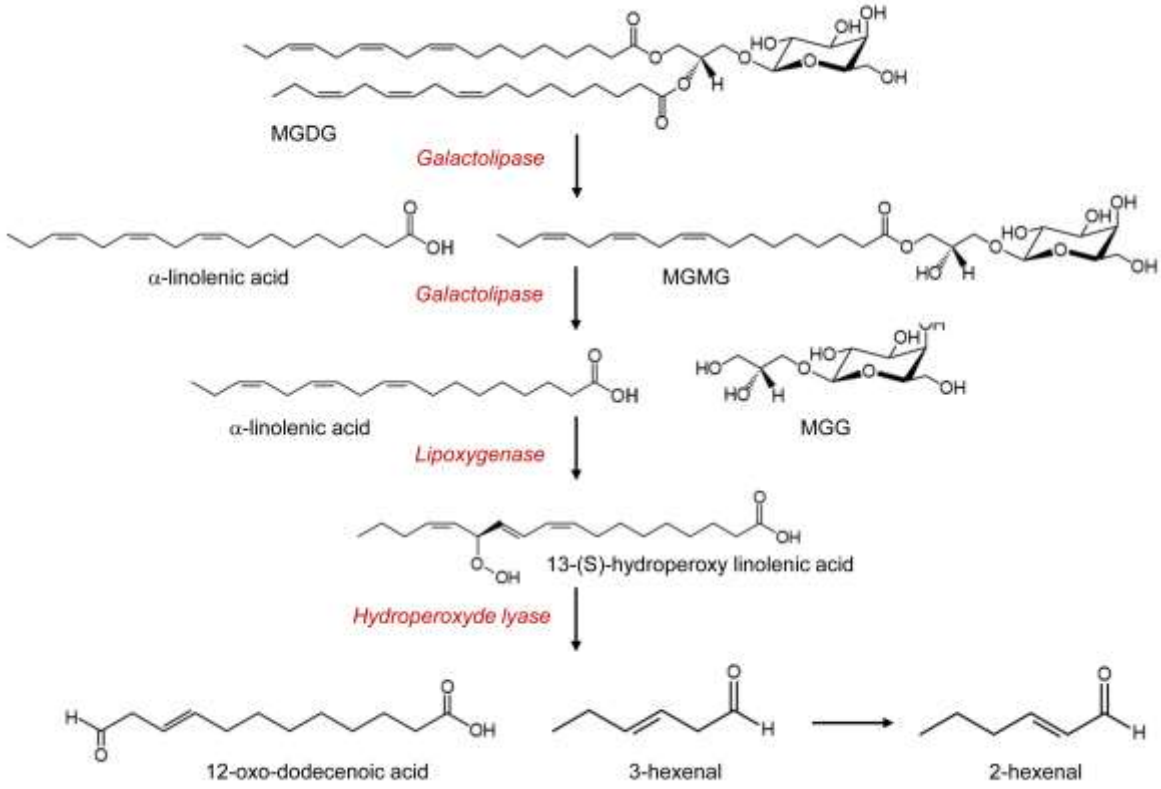


Figure 5. *Identification and quantification of phytoprostanes produced from C. reinhardtii fatty acids.* Panel A: Amounts of phytoprostanes produced from blanched cells (control), blanched cells treated with GPLRP2 and non-blanched cells, all being incubated for 3 h at 37°C. Panel B: Chemical structures of the six identified phytoprostanes resulting from non-enzymatic oxidation of α-linolenic acid.

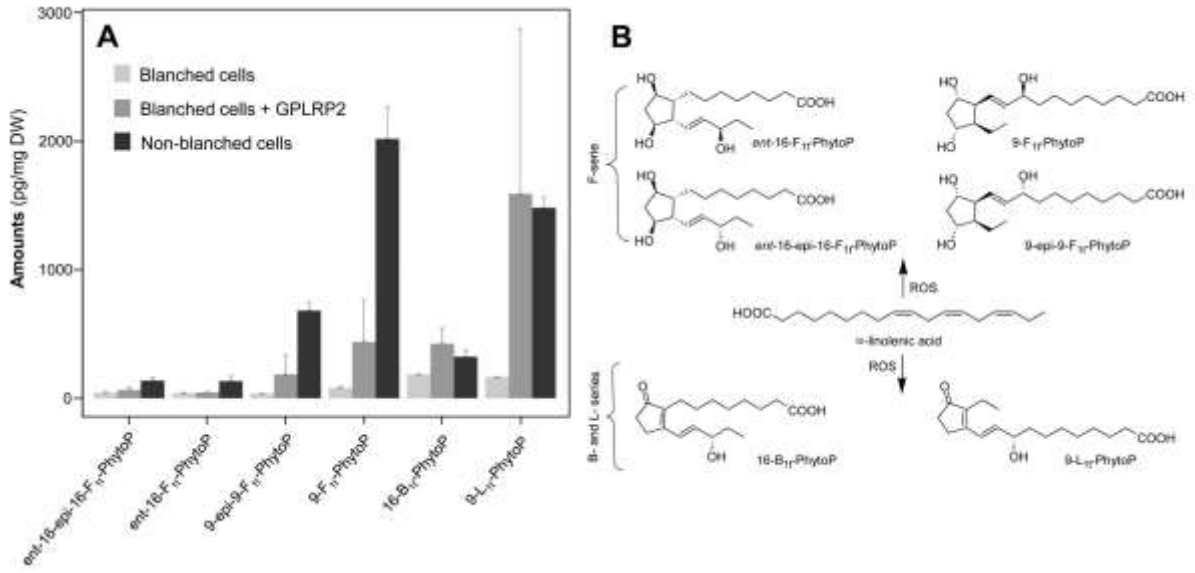


Figure 6. *Direct action of FsCut on whole C. reinhardtii cells*. Panels A and C: TLC analysis of polar lipids (thymol-sulfuric acid staining) of cell wall deficient (A) and full cell wall (C) *C. reinhardtii* strains. In panel A, reference standards (Rs) are α-linolenic acid (ALA), monogalactosyldistearoylglycerol (C18-MGDG), digalactosyldioctanoyl glycerol (C8-DGDG). Lane 1 (three replicates) shows the lipids extracted after the action of FsCut (50 µg enzyme; 3 hours at 37°C) on blanched *C. reinhardtii* cells (300-400 mg FW), with one band on the top corresponding to free fatty acids and a band corresponding to monogalactosylmonoacylglycerol (MGMG). Lane 2 (two replicates) is the control showing the lipids extracted from blanched *C. reinhardtii* cells in the absence of added enzyme. Two bands corresponding to MGDG and DGDG are observed. In panel C, reference standards (Rs) are α-linolenic acid (ALA) and natural long chain MGDG and DGDG isolated from wheat. Lane 1 (two replicates) is the control showing the lipids extracted from blanched C. reinhardtii cells in the absence of added enzyme. Lane 2 (two replicates) shows the lipids extracted after the action of FsCut. Panels B and D: TLC analysis of neutral lipids (phosphoric acid-copper acetate staining) of cell wall deficient (B) and full cell wall (D) *C. reinhardtii* strains. In panel B, reference standard (Rs) is ALA. Lanes 1 and 2 correspond to the same samples as in panel A. Lane 1 (three replicates) clearly shows a band corresponding to free fatty acids released from the blanched *C. reinhardtii* cells by FsCut. In panel D, reference standards (Rs) are oleic acid (OA), diolein (DO) and monoolein (MO). Lanes 1 and 2 correspond to the same samples as in panel C. See The original plates used for preparing panels C and D in Figure S3 of Supplementary Information.

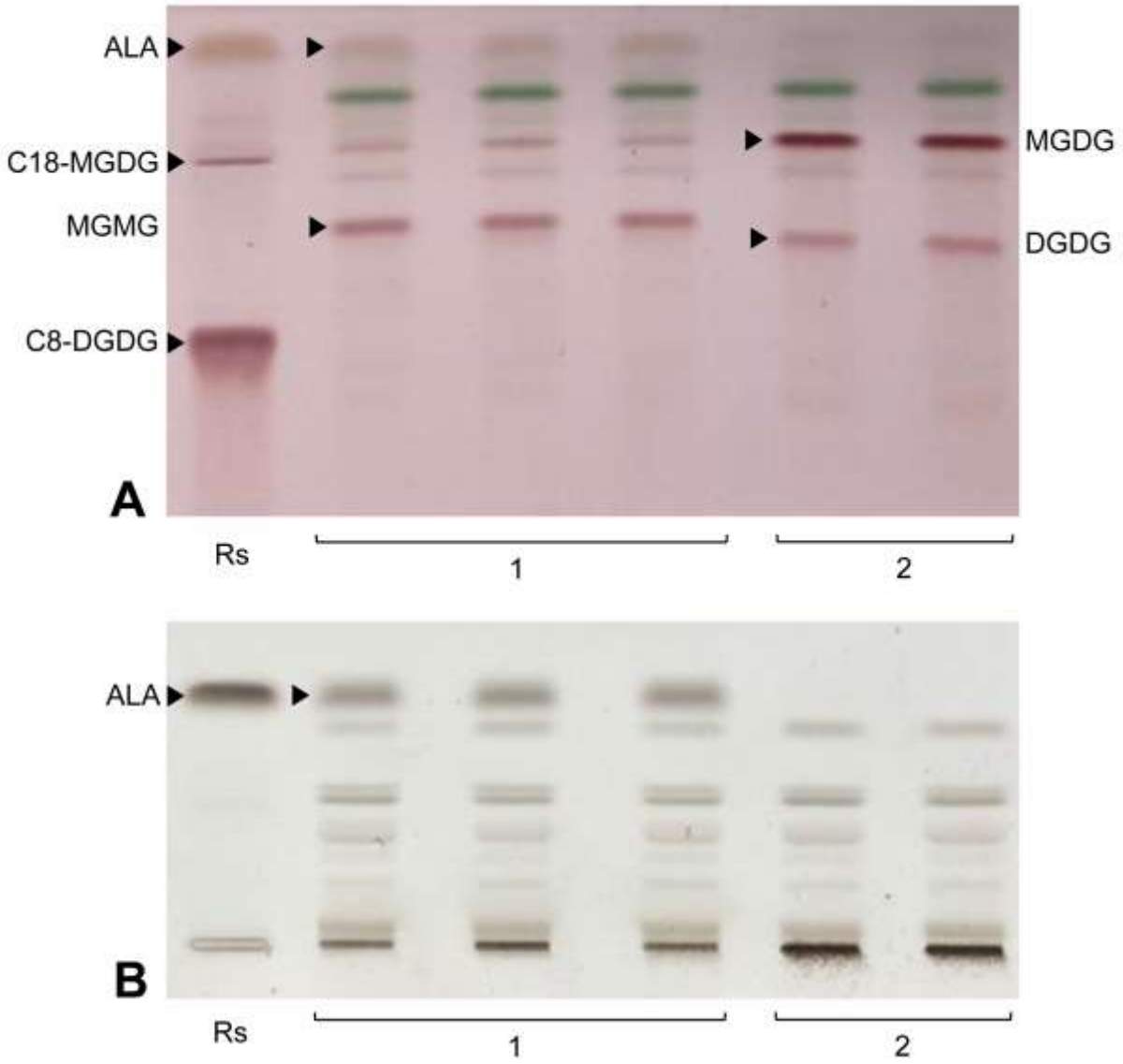

ALA
C18-MGDG
MGMG
C8-DGDG
MGDG
DGDG
A
Rs
1
2
ALA
B
Rs
1
2

Figure 7. *In situ alcoholysis of galactolipids from* whole *C. reinhardtii cells by GPLRP2 and FsCut.* Panel A: TLC analysis of polar lipids upon reaction with GPLRP2 stained with thymol-sulfuric acid. Reference standards (Rs) are α-linolenic acid (ALA), monogalactosyldistearoylglycerol (C18-MGDG), digalactosyldioctanoyl glycerol (C8-DGDG). Lanes show the lipids extracted upon the action of GPLRP2 at various times from 0.5 to 24 hours in the presence of ethanol. Bands corresponding to FFA and MGMG are observed. Panel B: TLC analysis of neutral lipids upon reaction with GPLRP2 stained with phosphoric acid-copper acetate. Reference standard (Rs) are ALA and α-linolenic acid ethyl ester (ALAEE). Each lane corresponds to the same samples as in panel A. Bands corresponding to FFA and fatty acid ethyl esters (FAEE) are observed. Panel C: TLC analysis of polar lipids upon reaction with FsCut stained with thymol-sulfuric acid. Same Rs as in panel A. Lanes show the lipids extracted after action of FsCut at various times from 4 to 72 hours in the presence of ethanol. Bands corresponding to ALA and MGMG are observed. Panel D: TLC analysis of neutral lipids upon reaction with FsCut stained with phosphoric acid-copper acetate. Same Rs as in panel B. Each lane corresponds to the same samples as in panel C. Bands corresponding to FFA and FAEE are observed. All experiments were performed with 50 µg enzyme and 50-75 mg DW of *C. reinhardtii* biomass.

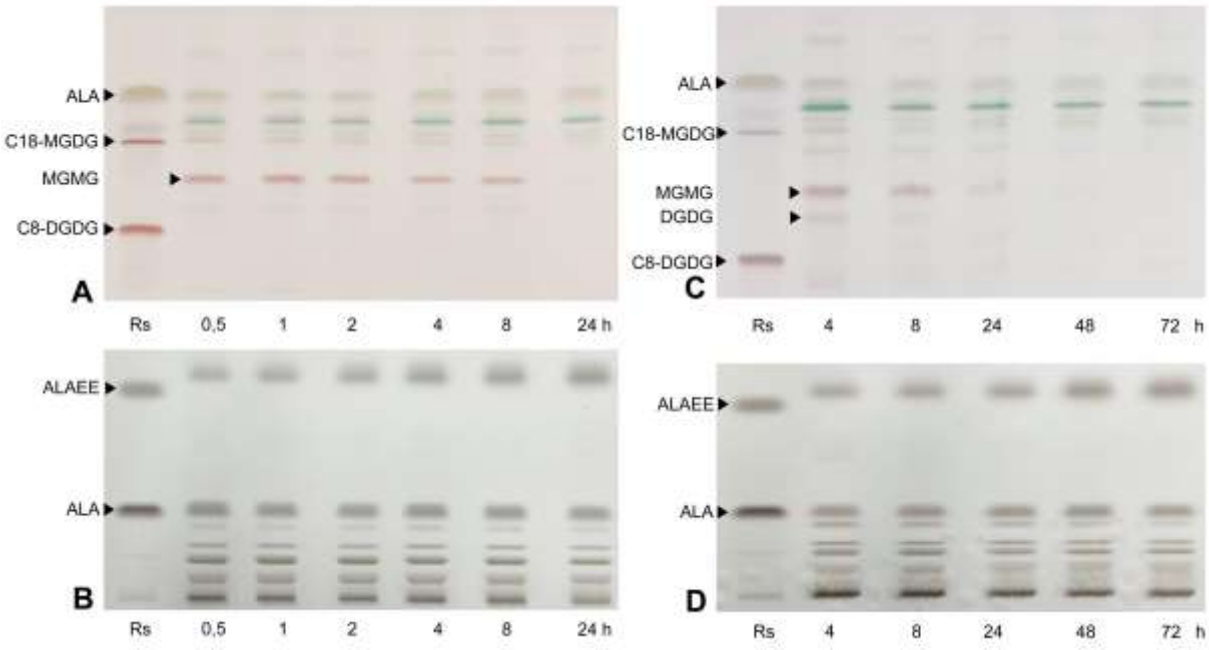

ALA
C18-MGDG
MGMG
C8-DGDG
A
Rs 0,5 1 2 4 8 24 h
ALA
C18-MGDG
MGMG
DGDG
C8-DGDG
C
Rs 4 8 24 48 72 h
ALAEE
ALA
B
Rs 0,5 1 2 4 8 24 h
ALAEE
ALA
D
Rs 4 8 24 48 72 h

Figure 8. *Kinetics of free fatty acids (FFA) and fatty acid ethyl esters (FAEE) production during the in situ alcoholysis of galactolipids from C. reinhardtii cells by GPLRP2 (A) and FsCut (B).* Panel A: Variation with time in FFA and FAEE concentrations during alcoholysis of *C. reinhardtii* cells by GPLRP2. Panel B: Variation with time in FFA and FAEE concentrations during alcoholysis of *C. reinhardtii* cells by FsCut. Values are means ±SD (n=3).

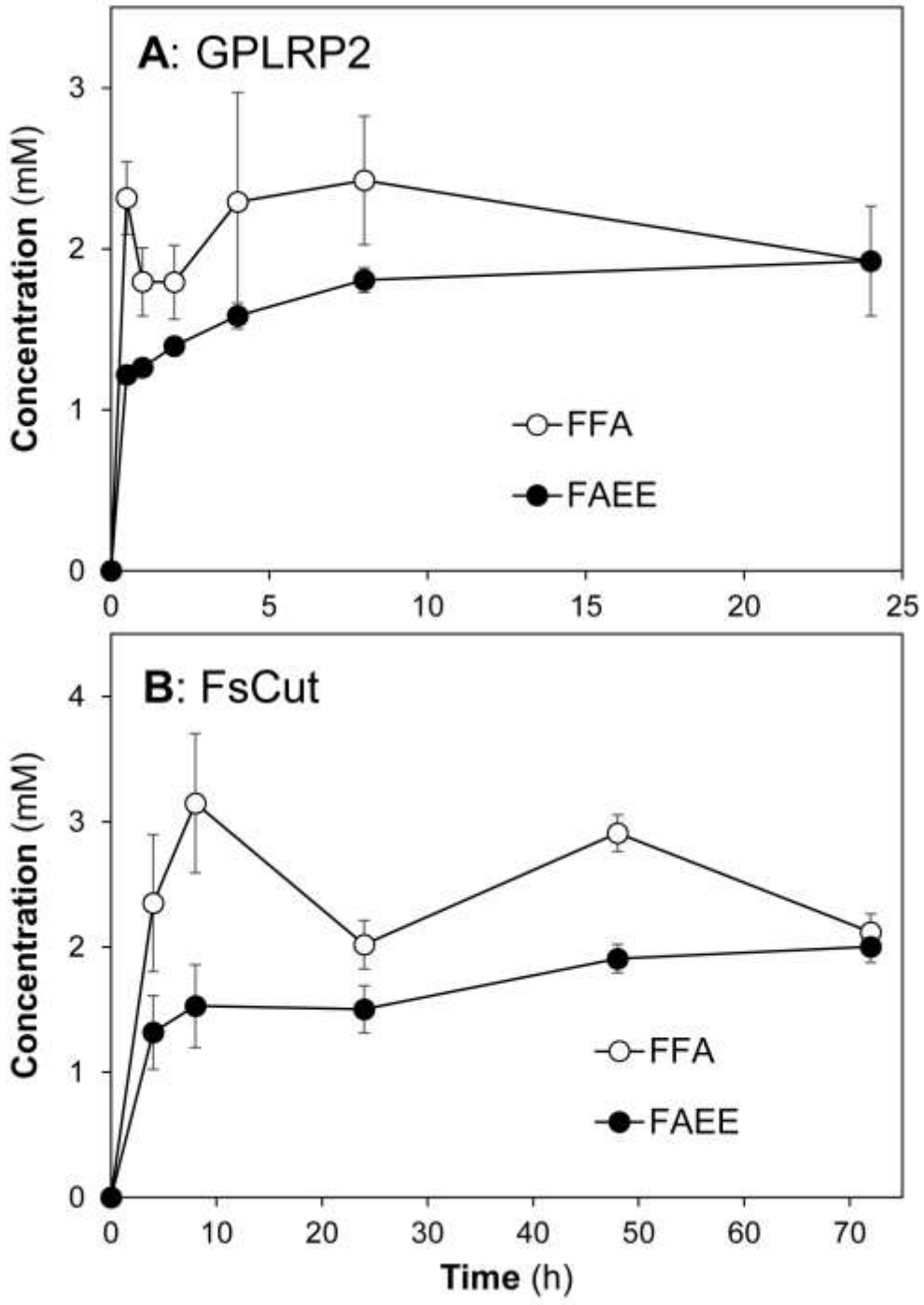


Figure 9. *Two-step in vitro digestion of C. reinhardtii cells*. Panel A: TLC analysis of polar lipids stained with thymol-sulfuric acid. Reference standards (Rs) are α-linolenic acid (ALA), monogalactosyldistearoylglycerol (C18-MGDG), digalactosyldioctanoyl glycerol (C8-DGDG). The three lanes corresponding to time points times 0, 15 and 30 min show the lipid extracts obtained during the gastric phase. The lanes corresponding to time points times 35, 40, 45, 60 and 90 min show the lipid extracts obtained during the intestinal phase. Panel B: TLC analysis of neutral lipids stained with phosphoric acid-copper acetate. Reference standard (Rs) is ALA. Lanes correspond to the same samples as in panel A. They clearly show bands corresponding to FFA after the start of the intestinal phase. Panel C: Time-course variations in the concentration of residual MGDG, MGMG and FFA during the *in vitro* digestion of *C. reinhardtii* cell. Residual MGDG, intermediate monogalactosylmonoacylglycerol (MGMG) and FFA were quantified from the TLC analysis shown in panels A and B from lipid extracts obtained during the gastric (0-30 min) and the intestinal (30-90 min) phases. Values are means ±SD (n=3).

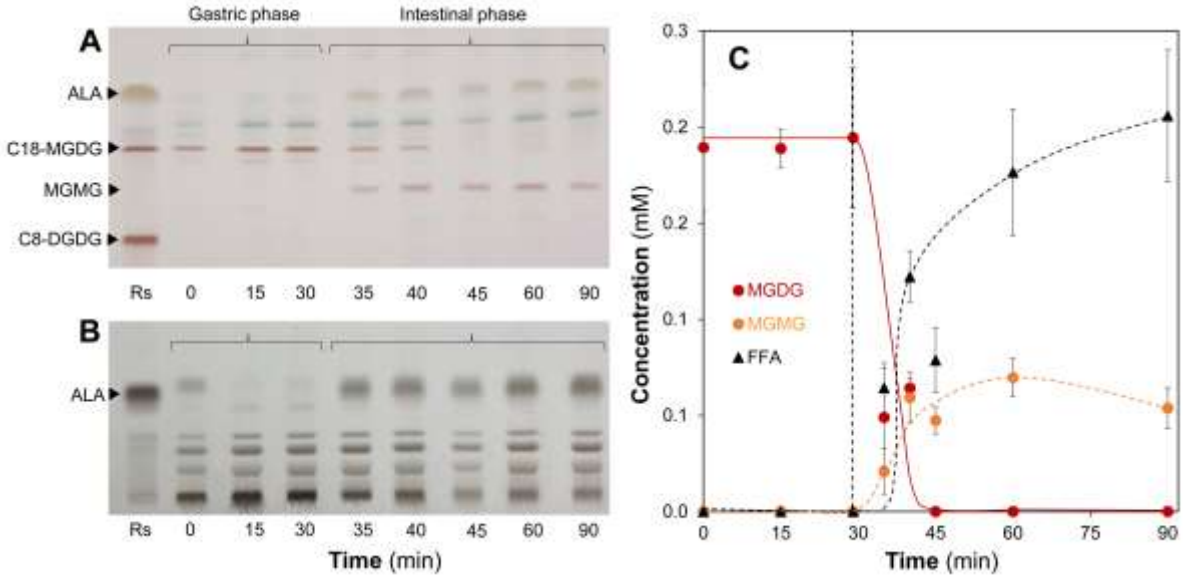


Figure 10. Comparison of fatty acid compositions (FAME) of *C. reinhardtii* total lipids and free fatty acids released upon *in vitro* digestion of algal cells. The main fatty acid released upon digestion, α-linolenic acid (ALA or 18:3D9, 12, 15), is indicated by an arrow. Fatty acids were derived into FAME using MeOH-BF3 and were separated by GC-FID. NI, non-identified. Values are means ±SD (n=3).

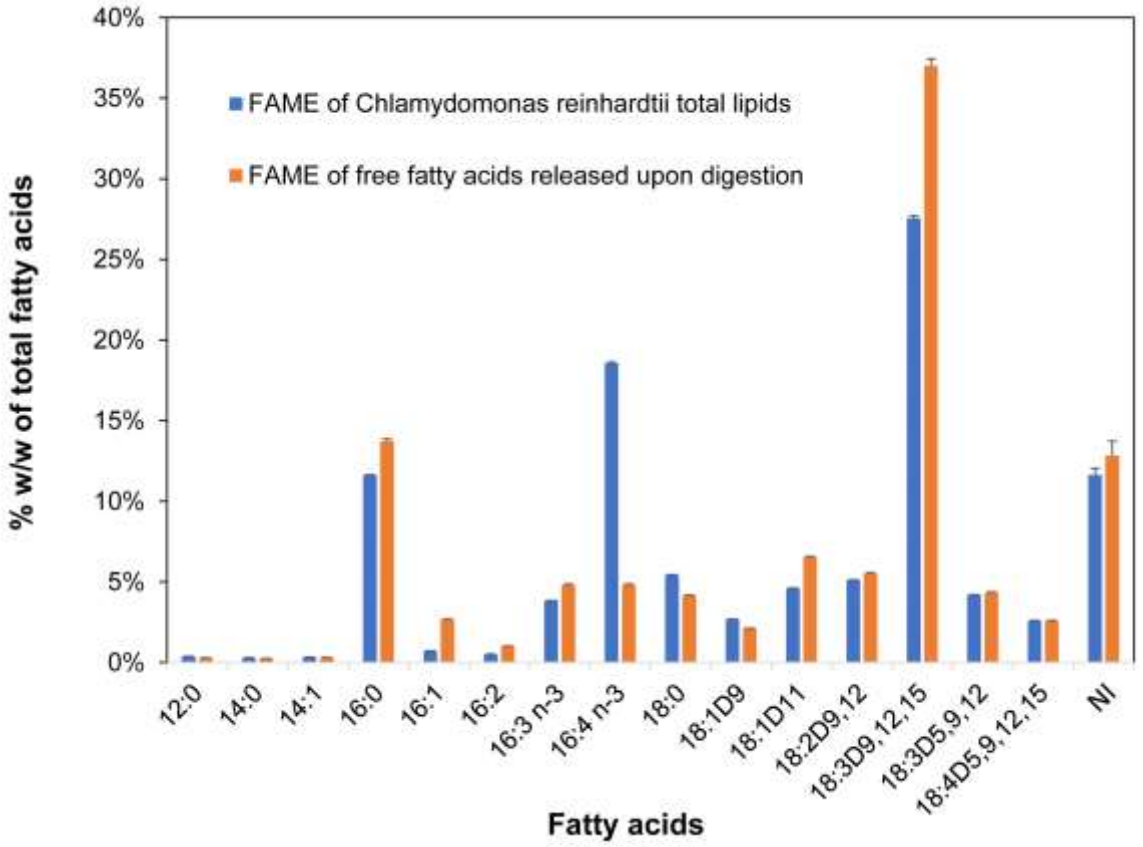


Table 1: *Main galactolipid composition of C. reinhardtii cells and fatty acid recovery upon in situ lipolysis by GPLRP2*. The mass contents expressed in mg per g of cell dry weight (DW) were estimated by TLC analysis. They were converted in µmoles/g DW using the average molar masses of MGDG (737.84 g/mole), DGDG (919.94 g/mole), MGMG (486.16 g/mole) and FFA (269.68 g/mole) estimated from the fatty acid composition of *Chlamydomonas reinhardtii* lipids.

Lipid class		**Contents**		**FA recovery**
		(mg/g DW)	(µmole/g DW)	(% vs total galactolipid FA)
Initial substrate	MGDG	102.4 ± 26.4	138.8 ± 35.8	
	DGDG	0.3 ± 0.2	0.4 ± 0.2	
	FA equivalents		278.5 ± 72.1	
Lipolysis products	FFA	69.6 ± 2.2	258.2 ± 8.1	93 %
	MGMG	7.2 ± 2.5	14.7 ± 5.2	
	FA equivalents		272.9 ± 13.3	98 %

Table 2: *Main molecular species of MGDG and DGDG in C. reihhardtii cells and fatty acid distribution at the sn-1 and sn-2 position of the glycerol backbone*. The relative abundance (%) in the various MGDG and DGDG molecular species in wild-type *C. reinhardtii* strain 137C are from [39]. These data allowed estimating the distribution of fatty acids (FA) at the *sn*-1 and *sn*-2 positions in MGDG and DGDG.

Molecular species	MGDG	DGDG
18:3α/16:4	67	6
18:2/16:4	10	0.4
18:3α/18:3	5	1
18:3α/16:3	4	18
18:1/16:4	3	
18:2/16:3	2	
18:3α/16:0		32
18:1/16:3	2	0.5
18:3α/16:1	1	3
18:3α/16:2	1	3
18:2/16:1	1	
18:2/16:3	0.8	4
18:2/16:2	0.8	2
18:2/16:1	0.4	1
18:2/16:0		14
18:1/16:2	0.7	0.3
18:1/16:1	0.6	0.7
18:1/16:0	0.5	14
18:0/16:0		0.2
18:0/16:4	0.2	

Fatty acid	FA at *sn*-1 in MGDG	FA at *sn*-2 in MGDG	FA at *sn*-1 in DGDG	FA at *sn*-2 in DGDG
16:0	0	0.5	0	60.2
16:1	0	3	0	4.7
16:2	0	2.5	0	5.3
16:3	0	8.8	0	23.3
16:4	0	80.2	0	6.4
18:0	0.2	0	0.2	0
18:1	6.8	0	15.5	0
18:2	15	0	21.4	0
18:3α (ALA)	78	5	63.8	1