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## **Using enzymes to harvest fatty acids from galactosyldiacylglycerols, the most abundant lipids in plant biomass**

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†These authors equally contributed to this work; ¶this article is dedicated to our colleague Frédéric Demarne, former R&D Director at Gattefossé SAS, who passed away on April 24<sup>th</sup>, 2023.

## Abstract

Mono- and digalactosyldiacylglycerols (MGDG, DGDG), the main lipids of plant photosynthetic membranes, represent a large but unexploited reservoir of fatty acids on earth. They are dispersed in plant biomass (mg per gram of dry mass) and not as accessible as vegetable oils by simple physical means. The identification and characterization of galactolipid acyl hydrolases, or galactolipases, raises the possibility to use these biocatalysts for the bioconversion of galactolipids. Here, we show that two enzymes of mammalian and microbial origins, pancreatic lipase-related protein 2 from guinea pig (GPLRP2) and cutinase from *Fusarium solani*, have the capacity to directly and fully release the fatty acids of MGDG and DGDG present in various plant leaves and green wastes. This high substrate accessibility to enzymes was further explored by performing alcoholysis reactions *in situ* and showing the conversion of galactolipid fatty acids into fatty acid alkyl esters (FAAE) when the enzyme and leaves were incubated in the presence of 6M methanol or 2.5M ethanol. These findings pave the way to the recovery of fatty acids dispersed in green biomass and the exploitation of an additional and renewable source of fatty acids for oleochemistry and nutrition in a context of competition for vegetable oils.

*Key words:* Biocatalysis; Cutinase; Digalactosyldiacylglycerol; Galactolipase; Galactolipids; Lipase; Monogalactosyldiacylglycerol; Pancreatic lipase-related protein 2;

## 1. Introduction

Fatty acids (FA) are essential molecules in the living world where they are building blocks for lipids involved in energy storage (triacylglycerols) and membrane structure (phospholipids, galactolipids) (**Figure 1**). The hydrocarbon chain of FA confers to lipids the highest energy content (9 kcal/g) among macronutrients and the ability to self-organize in various supramolecular assemblies (oil droplets, mono- and bilayers, micelles). Besides these basic biological functions, FA are major components in a variety of foods, in which they contribute to physical, sensorial and health properties. They have for long been used as a source of light and energy by burning oils and fats and they provide basic compounds for oleochemistry. The increasing demand for biofuel production based on vegetable oils has triggered and diversified their production. This situation leads to a rising competition for vegetable oils that might severely reduce the availability of edible oils for human nutrition and feed, particularly in developing countries where the production of vegetable oils is a main economic resource. A large part of vegetable oil production (208 Mt in 2022; Data from <https://www.fao.org/faostat/en/> ) comes from palm (75.9 Mt), soybean (58.6 Mt), rapeseed (25.2 Mt), and sunflower (20.6 Mt). These oils are used for human food (80 %), animal feeding (6%) and oleochemistry (14%). Regarding this last sector, oils and fats represent the most important renewable feedstock of the chemical industry where they are used for the production of surfactants and cosmetics (56 %), paints and coatings (7.2 %), lubricants (4 %), polymers (15 %), adhesives and others (17.8 %).<sup>1</sup> The increasing demand can hardly be supported without increasing the worldwide surface of oil plant cultures. These plantations have already created a major concern for the preservation of natural resources in countries like Malaysia and Indonesia where palm trees are replacing the rain forest.<sup>2</sup>

This competition and environmental threat trigger the quest for alternative sources of oils and fatty acids. Among these, microalgae are considered as the most productive and

promising living organisms regarding the production of oil as third-generation biofuel, the first and second generation biofuels being those derived from oleaginous crops and lignocellulosic biomass, respectively.<sup>3</sup> The economically-viable development of microalgal oils requires however a serious improvement of the production capacity by metabolic engineering. Indeed, the production of oil reserves by microorganisms is synonymous of stress conditions that are antagonistic to cellular growth and global biomass productivity.<sup>3</sup> So far, the vegetable oils obtained from oleaginous fruit and seeds remain the most abundant sources of FA.

It appears however that the complete biomass of plants may not have been totally explored for harvesting FA. Galactolipids, especially monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), are the main lipids found in the photosynthetic membranes of plant cells (**Figure 1**), in which they account for as much as 70-80% of the total lipids.<sup>4</sup> Based on its high proportion in the thylakoid membranes of chloroplasts and the abundance of plants, it was estimated that MGDG was the most abundant lipid in nature.<sup>5</sup> As plants are by far the most important reservoir of biomass on Earth (**Figure 2**),<sup>6</sup> it is hypothesized that the galactolipids constituting photosynthetic membranes could contain as much as 80% of total biomass FA,<sup>7</sup> and thus, be a much larger reservoir than oils and fats from plant and animal sources.

Despite their abundance in plant biomass, galactolipids have not been exploited as a source of FA so far. Their dispersion in the biomass, in the range of mg per gram of dry mass<sup>8-11</sup> prevents their recovery by simple physical means, whereas the triacylglycerols concentrated in intracellular lipid droplets of oleaginous seeds and fruit can be easily recovered by pressure to give vegetable oils. The recovery of galactolipids by solvent extraction is possible but requires the treatment of large volumes of biomass by organic solvent to reach interesting yields.<sup>8,9</sup> Moreover, galactolipids are polar lipids with elution properties close to those of plant

pigments (chlorophylls, carotenoids) and their separation to homogeneity at a large scale by chromatographic means is not straightforward.<sup>9</sup>

The discovery and the biochemical characterization of enzymes acting on galactolipids opens new tracks for getting access to the FA contained in these lipids. Pancreatic lipase-related protein 2 (PLRP2), an enzyme present in the exocrine pancreatic secretion of mammals, is the main enzyme involved in the digestion of galactolipids in the gastrointestinal tract.<sup>7</sup> Among PLRP2s that were characterized, the guinea pig enzyme (GPLRP2) displays the highest galactolipase activity reported so far.<sup>12</sup> Several microbial lipases have also been tested for their ability to hydrolyse galactolipids.<sup>12-14</sup> Among them, well-known fungal lipases of industrial interest such as *Fusarium solani* cutinase (FsCut) and *Thermomyces lanuginosus* lipase (TLL) were identified.<sup>12</sup> In both 3D structures of FsCut<sup>15</sup> and GPLRP2,<sup>16</sup> the active site can accommodate acylglycerols with large polar heads like DGDG, as shown by molecular docking (**Figure 3**). In addition to hydrolysis reactions, some microbial lipases have already been studied for their ability to catalyze acyl transfer in galactolipids.<sup>17</sup> More information on the structure-function relationships and biological function of these enzymes can be found in a recent review by Sahaka et al.<sup>7</sup>

The use of galactolipases for getting access to the FAs of galactolipids directly from the plant biomass has never been investigated so far. Here we report the release and conversion of galactolipid FA from various plant sources by GPLRP2 and FsCut used as model enzymes with galactolipase activity. Both hydrolysis and alcoholysis reactions were tested *in situ* using whole leaves to produce either free fatty acids (FFA) or fatty acid alkyl esters (FAAE).

## 2. Materials and Methods

### 2.1. Chemicals

Lipid standards,  $\alpha$ -linolenic acid (ALA), methyl linolenate and natural DGDG and MGDG isolated from wheat, were purchased from Sigma Aldrich. Pure galactolipid standards, 1,2-dipalmitoyl-3- $\beta$ -D-galactosyl-*sn*-glycerol (C16-MGDG) and 1,2-dipalmitoyl-3-O-( $\alpha$ -D-galactosyl-1-6)- $\beta$ -D-galactosyl-*sn*-glycerol (C16-DGDG), were synthesized as previously described<sup>18</sup>. All other chemicals are described in Supporting Information.

### 2.2. Production and purification of recombinant enzymes

Recombinant GPLRP2 and FsCut were produced in the yeast *Pichia pastoris* and purified as described in Supplementary Information.

### 2.3. Assay of enzyme activities

Lipase activities of GPLRP2 and FsCut were routinely measured using the pHstat technique and tributyrin as a substrate for monitoring the enzyme production in *Pichia pastoris* culture medium and estimating the enzyme levels in purification fractions (see Supporting Information). The lipase activities were expressed in international lipase unit (1 U = 1  $\mu$ mole of FA released per minute). Specific activities of pure GPLRP2 and FsCut are  $2700 \pm 300$  and  $2596 \pm 96$  U/mg, respectively, under these conditions.<sup>12</sup>

### 2.4. Galactolipid hydrolysis reaction on plant material

Spinach leaves, parsley, leek and romaine lettuce were purchased from a local supermarket. The cyanobacterium *Anabaena* sp. PCC7120 was grown as previously described.

<sup>19</sup> Mature Grass was obtained from a mixture of 70 % w/w *Festuca arundinacea* (Schreb.)

Dumort., 20 % w/w *Poa pratensis* L. and 10 % w/w *Lolium perenne* L. seeds. It was purchased in rolls ready for use from La Gazonnière (Saint-Laurent-d'Aigouze, France). Leaves of sugar cane (*Saccharum officinarum* L., cultivars B693799 and FR96047) grown in CIRAD experimental greenhouses were kindly supplied by Jean-Heinrich Daugrois (CIRAD, Montpellier, France).

Leaves were blanched for 5 min in boiling distilled water to inactivate endogenous enzymes and avoid partial lipolysis and FA oxidation. After removing the excess of water on 3MM Chr Whatman paper sheet, leaves were finely sliced into 1-2 mm pieces before enzymatic treatments. Leaves were either used directly in hydrolysis reaction or tested after a combination of various treatments including a freezing (-20°C)/thawing step, a grinding step and/or an enzymatic pre-treatment with glycosyl hydrolases to disrupt the cell wall (see Supporting Information and Scheme S1). In all cases, subsequent hydrolysis experiments with GPLRP2 and FsCut were conducted in 2 mL-Eppendorf tubes with 50 mg of plant material in 1 mL of 2 mM Tris-HCl, 150 mM NaCl buffer, pH 8.0. Various amounts of enzyme powders, corresponding to 2.5, 5, 10 and 50 µg of pure GPLRP2 or 10, 50 and 100 µg of pure FsCut, were tested and the mixtures were incubated for 1, 2 or 3 hours at 37°C under stirring at 200 rpm in an orbital shaker. Controls without added enzyme were performed in order to check the inactivation of leaves' endogenous enzymes through the absence of FFA produced by these enzymes. The hydrolysis of cyanobacteria was tested as described for leaves using 50 mg of heat-treated/frozen cell pellet and 50µg of GPLRP2.

### *2.5. Galactolipids alcoholysis reaction on plant material*

The alcoholysis reactions were carried out on the same amounts of leaves (50 mg) dispersed in 1 mM Tris-HCl, 150 mM NaCl buffer, pH 8.0, containing methanol or ethanol (2.5-6 M final concentration), using 250 µg of biocatalyst powder containing either 2% w/w



GPLRP2 (5  $\mu\text{g}$ ) or 3 % w/w FsCut (7.5  $\mu\text{g}$ ). Reactions were performed at  $37\pm 1^\circ\text{C}$  under orbital stirring (250 rpm) for 0.5h to 96h.

## 2.6. Lipid extraction

The whole reaction mixture of leaves treated with enzymes (1 mL) was used for lipid extraction. First, lipolysis was stopped by adding 200  $\mu\text{L}$  of 1 M HCl and 5 mL of a chloroform/methanol mixture (2:1, v/v) in a 15-mL glass tube with a Teflon-lined screw cap. A vigorous mixing was applied before centrifugation for 10 min at 5000 rpm in Sigma 3-18K centrifuge (rotor 19776) for phase separation. The lower organic phase was transferred to a 15-mL test tube and dried over anhydrous magnesium sulfate. Once  $\text{MgSO}_4$  had precipitated, the organic phase was filtered through 0.45 $\mu\text{m}$  Millex-FH PTFE syringe filter (Merck-Millipore) and stored at  $-20^\circ\text{C}$  before lipid analysis by thin-layer chromatography (TLC) and gas chromatography (GC).

## 2.7. Lipid separation and quantitative analysis by TLC

All separations were performed onto TLC silica gel 60 pre-coated plates (Merck, Darmstadt, Germany) using various solvent mixtures for elution and staining reagents depending on the compounds to be quantified (DGDG, MGDG, FFA and FAAE). Galactolipids were specifically revealed using the reaction of galactose with thymol-sulfuric acid reagent.<sup>20</sup> Detailed procedures, including calibrations with reference standards and scanning densitometry for quantification are given in Supporting Information.

## 2.8. GC analysis of fatty acid methyl esters

The FA generated upon galactolipid hydrolysis were derivatized into FA methyl esters (FAME) using borontrifluoride (BF<sub>3</sub>; 14 %)-methanol (86%). FAME were analyzed by GC coupled to flame ionization detector (GC-FID) and identified using a mixture of methyl esters as external standards (see Supporting Information).

## 2.9. Estimation of lipid amounts and reaction rates

All lipid amounts were expressed in mg per g of leaves dry weight (DW). For each experiment and sample, DW was measured after weighting a given amount (50-500 mg) of blanched leaves and placing it in an oven at 100°C overnight. The dried leaves were then cooled down at room temperature in a dessicator before measuring DW. The drying (15 min) and cooling procedures were repeated until two successive weightings gave identical DW ( $\pm 0.5$  mg). The production of FFA and FAAE was expressed in mg per g of leaves DW and mg of enzyme. Initial rates ( $V_0$ ) were estimated from curve-fitting the time-course variations in FFA and FAAE using CurveExpert 1.3 software program (<https://www.curveexpert.net/support/documentation/>) and expressed in mg of product per g of DW per mg of enzyme per hour ( $\text{mg.g DW}^{-1}.\text{mg Enz}^{-1}.\text{h}^{-1}$ ). These rates were also expressed in  $\mu\text{mole}$  of product per minute per mg of enzyme ( $\mu\text{mole. min}^{-1}.\text{mg}^{-1}$ ) in order to compare them with known specific activities of GPLRP2 and FsCut. Since ALA was the most abundant FA in FFA and FAAE, we used its molar mass (278.4 g/mole) and those of methyl (292.5 g/mole) and ethyl (306.48 g/mole) linoleate for the conversion of masses into moles.

### 3. Results and Discussion

#### 3.1. Production of recombinant enzymes

Recombinant GPLRP2 and FsCut were produced in the yeast *Pichia pastoris* using a constitutive expression system under the control of the glyceraldehyde-3-phosphate dehydrogenase promoter and they were secreted into the culture medium thanks to their N-terminal fusion to the *Saccharomyces cerevisiae*  $\alpha$ -factor secretion peptide. The recombinant enzymes were recovered from the culture supernatant and purified by batch adsorption onto cation-exchange SP-Sepharose gel, eluted with NaCl and further purified to homogeneity by anion-exchange chromatography on a Mono Q column (see Figures S1 and S2 in Supporting information). The correct processing of the  $\alpha$ -factor and production of mature proteins were checked by N-terminal sequencing with the obtention of the AEVCYSHLGCFS and QLGRTT sequences for GPLRP2 and FsCut, respectively. Their global masses determined by MALDI-ToF mass spectrometry were  $47827 \pm 59$  Da (+0.3 % versus theoretical mass of mature protein) and  $20857 \pm 4$  Da (-0.4% versus theoretical mass of mature protein), respectively (see Figures S3 and procedures in Supporting information).

#### 3.2. Enzymatic hydrolysis of galactolipids from various plant materials

Prior to testing the enzyme activity on whole plant leaves, we established the best conditions to observe the activity of an exogenous galactolipase added to the leaves and limited as much as possible the interference of endogenous galactolipases known to be present in plants.<sup>21, 22</sup> Indeed, an endogenous lipolysis of galactolipids was found to occur in fresh spinach leaves incubated at 37 °C (Figure S4). Therefore, we implemented a blanching step (5 min in boiling water) to inactivate the endogenous enzymes. Additional treatments were also tested, including

freezing at  $-20^{\circ}\text{C}$ /thawing, grinding and enzymatic pre-treatment with a mix of glycosyl hydrolases to disrupt the plant cell wall. When the leaves were subjected to freezing/thawing and/or grinding before incubation with the enzyme, the galactolipid hydrolysis by GPLRP2 was only slightly increased compared to controls in which lipolysis was already very efficient (Figure S5). The addition of glycosyl hydrolases led to identical results (Figure S6), indicating that the use of these enzymes was not required to allow the accessibility of the enzyme to thylakoid membranes. These trials were repeated with FsCut, leading to identical results (data not shown). In the final protocol, only blanching and cutting the leaves into small pieces were retained for preparation of the plant material submitted to enzyme hydrolysis.

Several plant materials, including leaves from leek, parsley, lettuce, spinach and sugar cane, as well as grass cuts were then incubated in the presence of GPLRP2 (Figure 4). A culture of the cyanobacterium *Anabaena* sp. PCC7120 (freeze-dried pellet) was also included in these trials. In all cases, MGDG and DGDG were largely hydrolyzed and converted into FFA after 1 hour of incubation at  $37^{\circ}\text{C}$  and pH 8 (Figure 4A).

FFA analysis (Figure 4B and Table S1) revealed that ALA was the most abundant FA released from spinach, parsley, lettuce, sugar cane and grass (46 to 62 % of total FA) by GPLRP2. In *Anabaena* and leek, palmitic acid (16:0) was the main FA released (25 to 62 % of total FA).

The kinetics of FFA release upon hydrolysis of spinach leaves (50 mg) by GPLRP2 (50  $\mu\text{g}$ ) or FsCut (100  $\mu\text{g}$ ) were then measured for 3 hours (Figure 5A. Panel A). FFA production reached levels of  $24.4 \pm 0.4$  and  $21.1 \pm 2.3$  mg FFA per gram DW of spinach leaves upon hydrolysis by GPLRP2 and FsCut, respectively. In order to express FFA release in % of the total galactolipid FA present in spinach leaves, the amounts of galactolipids were estimated from TLC analysis. Spinach leaves were found to contain  $25.1 \pm 2.8$  mg MGDG/g DW and

14.9 ± 1.3 mg DGDG/g DW, i.e. a total of 40.0 ± 3.90 mg/g DW. These values were similar to those previously reported for spinach leaves<sup>11</sup> and chloroplasts.<sup>10</sup> From the known FA composition of MGDG and DGDG from spinach leaves (Figure 5B), mean molar masses for FA, MGDG and DGDG were estimated (see Tables S2 and S3) and the amounts of galactolipids in spinach leaves could be converted into mg of FA equivalents. From the value obtained (23.9 ± 2,8 mg FA/g DW), it was deduced that GPLRP2 and FsCut were able to release 101.9 % and 88.3 % of galactolipid FA, respectively, after 3 hours of incubation. These high values are consistent with the kinetics of FFA release showing a saturation profile (Figure 5A) and they demonstrate the high accessibility of membrane galactolipids to lipolytic enzymes. Some preliminary assays using attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) also support the direct action of galactolipases on spinach leaves (see Figure S7 in Supporting Information). However, due to the broad substrate specificity of GPLRP2 and FsCut, some FFA might be released from other polar lipids (OPL), mainly phospholipids, present in spinach leaves in lower amounts than galactolipids (Figure 5B). The main FA released from spinach leaves by GPLRP2 was ALA (61.8 ± 0,7 % of total FFA), as expected from the FA composition of total lipids including MGDG, DGDG and OPL (Figure 5B).

Interestingly, the initial rates of FFA production by GPLRP2 (15.0 ± 10,59 mg FA/g DW/ h) and FsCut (14.8 ± 6.5 mg FA/g DW/ h) were nearly identical. Knowing the mean molar mass of galactolipid FA (269 g/mole), these rates were converted into μmole FA/min (or international units; 0.66 ± 0.04 and 0.40 ± 0.03 μmole FA/min, respectively) and then in specific activities (13.12 ± 0.81 and 4.04 ± 0.25 μmole FA/min/mg of enzyme; Table 1), knowing the respective amounts of enzyme (50 μg for GPLRP2 and 100 μg for FsCut). These values are two orders of magnitude lower than those obtained with the same enzymes acting on purified galactolipids presented in the form of mixed micelles with bile salts.<sup>12</sup> They are however closer to the specific activity of GPLRP2 on the galactolipids from isolated spinach chloroplasts (89

$\pm 7 \mu\text{mole FA}/\text{min}/\text{mg}$  of enzyme.<sup>23</sup> Moreover, they were estimated within a time frame of 1 hour and one can assume that the initial activity was higher.

### *3.3. Enzymatic alcoholysis of galactolipids from spinach leaves*

The ability of GPLRP2 and FsCut to catalyse the alcoholysis of galactolipids directly from the leaves was evaluated in the presence of alcohol (4-6 M MeOH or 2.5 M EtOH), for both enzymes, the concomitant formation of FA alkyl esters (FAAE) and FFA, was observed as illustrated in Figure 6 for FsCut. No alcoholysis was observed in pure alcohol. With GPLRP2, FFA appeared to be produced at a faster initial rate than FAAE in both MeOH and EtOH concentrated solutions (Figure 7A and B; Table 1), but an opposite trend was then observed with the production of esters exceeding that of FFA after 2 hours in the presence of MeOH (Figure 7A) and after 4 hours in the presence of EtOH (Figure 7B). The final proportions of FAME and FAEE reached 64.7 % and 73.9 %, respectively. It is worth noting that the rate of FFA production in 6M MeOH was identical to the rate of hydrolysis in water ( $13.6 \mu\text{mole}\cdot\text{min}^{-1}\cdot\text{mg Enz}^{-1}$ ; Table 1). A faster rate was even recorded in 2.5 M EtOH ( $22.27 \mu\text{mole}\cdot\text{min}^{-1}\cdot\text{mg Enz}^{-1}$ ; Table 1). The presence of alcohols had therefore no inhibitory effect on GPLRP2 under these conditions. Regarding the production of esters by GPLRP2, the faster rate was recorded in 6 M MeOH ( $3.49 \mu\text{mole}\cdot\text{min}^{-1}\cdot\text{mg Enz}^{-1}$ ; Table 1). With FsCut, the reaction rates were slower than with GPLRP2 (Table 1). Moreover FFA, FAME (Figure 7C) and FAEE (Figure 7D) appeared at similar initial rates, revealing a different selectivity of FsCut for the nucleophile compared to GPLRP2. Ester levels exceeded that of FFA after 24 h in the presence of MeOH or EtOH. The final proportions of FAME and FAEE reached 79.6 % and 73.2 %, respectively. Whatever the enzyme and alcohol, it is worth noting that the final levels of FAAE

were close to those expected at the thermodynamic equilibrium between long chain FA and their esters.<sup>24</sup>

### 3.4. Enzymatic conversion of galactolipids from green wastes

Sugar cane leaves and grass cuts were used as models of green wastes from agriculture and horticulture. Both contained MGDG and DGDG in similar amounts to those found in spinach leaves (Table 2). The hydrolysis of sugar cane leaves by GPLRP2 allowed a FA recovery corresponding to 85 % of the FA esterified to galactolipids ( $11.4 \pm 0.9$  mg/g DW). Alcoholysis with aqueous MeOH was less efficient with only 68 % of total FA recovered as FFA and FAME. With grass, the MGDG and DGDG levels were similar to those found in a chloroplast-rich fraction (CRF) of another green waste, pea vine haulm (PVH; Table 2).<sup>10</sup> However, FA recovery upon hydrolysis/alcoholysis by GPLRP2 was much lower than with spinach and sugar cane leaves and it hardly exceeded 20%. We have no explanation at this point for the lower enzyme activity recorded with grass. One hypothesis could be the presence of lipase inhibitory compounds in grass. Nevertheless, these preliminary trials and previous results on the digestion of galactolipids from PVH CRF<sup>10</sup> suggest that galactolipid FA may also be recovered from green wastes using galactolipases.

### 3.5. Enzyme accessibility to galactolipids within plant leaves

It was shown here that two enzymes with galactolipase activity, GPLRP2 and FsCut, could hydrolyse galactolipids in whole plant leaves with a high efficiency and a nearly complete release of FA *in situ* (Figure 5A). Using the same enzymes in the presence of alcohol aqueous solutions (methanol or ethanol) allowed the *in situ* alcoholysis (or transesterification) of galactolipids from the whole leaves. While the initial production of FFA was usually faster than

that of FAAE (Table 1), all reactions finally led to an excess of FAAE versus FFA (64.7 to 79.6 %) that was close to the value expected at the thermodynamic equilibrium in the presence of alcohol in excess. GPLRP2 was the most efficient enzyme to convert galactolipids in FFA and FAAE (Figure 7 and Table 1). Both GPLRP2 and FsCut have the ability to reach the galactolipid substrates within the whole plant cell (Figure 5), which means that these enzymes can cross the plant cell wall and diffuse towards the photosynthetic membranes of the chloroplast. This process has not been deciphered so far but the enzymes might pass through the plasmodesmata, which are microscopic channels in the polysaccharide cell wall of plants and some algae.<sup>25</sup> The larger size of GPLRP2 (47.6 kDa) compared to that of FsCut (20.9 kDa; Figure 3) does not seem to have any impact on the enzyme accessibility to its substrate. As a matter of fact, it has been shown that porcine pancreatic lipase, an enzyme homologous to GPLRP2, could diffuse within almond cells prior to reaching lipid droplets (oleosomes).<sup>26</sup> The action of GPLRP2 on the galactolipid membranes in plant cells is in line with previous observations showing that GPLRP2 can hydrolyse galactolipids within intact chloroplasts<sup>23</sup> and plant biomimetic monolayers composed of galactolipids, phospholipids and sterols.<sup>27</sup>

### *3.6. Enzymatic versus chemical hydrolysis*

Since galactolipid fatty acids could be released from plant biomass by simple acid or alkaline hydrolysis, we searched for data on chemical hydrolysis in the literature to compare them to the current results. Most articles on plant biomass hydrolysis are related to the pretreatment of plant lignocellulosic biomass for the production of sugar monomers from cellulose using glycosyl hydrolases and the subsequent conversion of these sugars into second-generation bioethanol or other chemicals by fermentation.<sup>28</sup> Chemical pretreatment by acid (hydrochloric, phosphoric, sulfuric acid or organic acids) or alkaline (sodium hydroxide, ammonia, sodium carbonate or calcium hydroxide) hydrolysis allows improving the



biodegradability of cellulose by removing hemicellulose and lignin. Indeed, the tight structure of lignin is a barrier that limits the access of cellulases to their substrate.<sup>29</sup> Various plant sources and agricultural residues have been submitted to chemical hydrolysis<sup>30</sup> but there is no report to our knowledge on the fate of galactolipids under these conditions and a possible recovery of the released fatty acids. Therefore, no comparison of processes and reaction rates can be made at this stage. Nevertheless, the enzymatic release of galactolipid fatty acids is specific, performed under mild conditions and does not require neutralisation. Moreover, it seems that galactolipases have a higher accessibility to their substrate than cellulases when considering the complete green plant biomass. It suggests that the use of galactolipases to recover galactolipid fatty acids could be implemented prior to the conversion of the lignocellulosic biomass in the context of biorefinery development.

### 3.7. Conclusion

We have seen that a major limitation to the recovery of galactolipid FA is their dispersion in the plant biomass ( $40.0 \pm 3.90$  mg/g DW in spinach leaves) compared to those of vegetable oils (triacylglycerols) concentrated in specific organelles, the lipid droplets. So far, natural galactolipids have been isolated and recovered using organic solvents<sup>9, 11, 31</sup> including chloroform, methanol and hexane. Such procedures appear viable only for the production of high added-value molecules showing anti-inflammatory,<sup>31</sup> anti-tumor<sup>32</sup> and anti-HIV reverse transcriptase<sup>33</sup> properties. The potential valorisation of galactolipids as a reservoir of FA will require simpler and greener methods to get access to these FA. The demonstration that plant galactolipids can be converted *in situ* into FFA and FAAE using enzymes (Figure 9) paves the way to an easier separation of FA from plant biomass using physical means (molecular distillation, liquid-liquid extraction, phase separation by centrifugation...). Further steps

following the enzymatic bioconversion need however to be implemented before a complete process can be proposed. Nevertheless, one can already envision three main directions for process development: (1) hydrolysis for the production of FFA and eventually its coupling with other FA-modifying enzyme, (2) alcoholysis for the production of various esters and (3) glycerolysis for the production of acylglycerol mixtures (Figure 9). This latter reaction can lead to various emulsifiers and lipid-based formulations that found applications in the solubilization /delivery of lipophilic compounds.<sup>34</sup> Regarding the downstream processing of FFA, various enzymes including P450 monooxygenase and lipoxygenase<sup>35</sup> could be coupled to galactolipases to produce FA derivatives. Nevertheless, after the discovery of FA photodecarboxylase (FAP) that converts FA into hydrocarbons,<sup>36</sup> one of the most attractive challenges is currently the coupling of FAP with lipases to produce hydrocarbons.<sup>37</sup> So far, the proof of concept has been established using either a thioesterase<sup>36</sup> or microbial lipases.<sup>37</sup> Producing FFA with galactolipases could be another way forward.<sup>38</sup>

An interesting feature of galactolipids is their natural enrichment in long chain polyunsaturated FA (PUFA) such as the essential ALA (18:3 n-3) which usually accounts for more than 60% of total FA in galactolipids from plant photosynthetic membranes.<sup>39</sup> This was confirmed here with ALA being the main FA released from spinach, parsley, lettuce, grass and sugar cane leaves (Figures 4B and 5B) and converted into esters in the presence of alcohols (Figure 8). Galactolipids therefore represent an interesting source of FA for applications in health and nutrition, rather than for producing biofuels.

While galactolipids can be recovered from the leaves of various edible plants,<sup>8</sup> it would be more attractive to recover them from non-valORIZED parts of these plants and green wastes. It has already been shown that wastes like PVH can be used to produce CRF as dietary supplements<sup>40</sup> and the galactolipids present in these CRF are well digested by galactolipases.<sup>10</sup> Here we showed that sugar cane leaves, and to a lower extent, grass cuts, can be used for the

production of FFA enriched in ALA (Figure 4) and FAAE (Table 2). With the development of microalgae cultures for production of oil and other compounds, algal cakes resulting from oil extraction and rich in photosynthetic membranes should also be available as a source of galactolipids and additional FAs beyond the algae oil. Whatever the resource, plants or algae, galactolipases could be used for an optimum recovery of the FA present in these biomasses.

### **Data availability**

Data will be made available on request.

### **Conflicts of interest**

The authors declare no known competing financial interests.

**Supporting Information:** Addendum to Materials and Methods (Chemicals; Production and purification of recombinant enzymes; N-terminal sequencing; MALDI-ToF mass spectrometry; Assay of enzyme activity; Galactolipids hydrolysis and alcoholysis reactions on plant material; Pre-treatment of plant leaves with glycosylhydrolases; Lipid extraction; Lipid separation and quantitative analysis by thin-layer chromatography; Gas chromatography analysis of fatty acid methyl esters; Study of spinach leave lipolysis by ATR-FTIR). Addendum to Results (Production of recombinant enzymes; Hydrolysis of galactolipids from spinach leaves); Scheme S1: Various preparations of plant leaves prior to their incubation with galactolipases ; Figures S1: SDS-PAGE (A) and western blot (B) analysis of the production and purification of recombinant GPLRP2 expressed in the yeast *Pichia pastoris*; Figure S2: SDS-PAGE analysis of the production and purification of recombinant *Fusarium solani* cutinase (FsCut) expressed in the yeast *Pichia pastoris*; Figure S3: MALDI-ToF mass spectrometry of recombinant

GPLRP2 (A) and FsCut (B) purified from *Pichia pastoris* culture medium; Figure 4S: Fate of galactolipids in spinach leaves in the absence of heat treatment (blanching); Figure 5S: Effect of grinding and/or freezing/thawing treatments on the lipolysis of galactolipids from blanched spinach leaves (50 mg) incubated in the presence of GPLRP2 (50 $\mu$ g); Figure 6S: TLC analysis of polar (A) and neutral (B) lipids of spinach leaves subjected to enzyme treatments; Figure S7: Monitoring of spinach leaf lipolysis by FsCut using ATR-FTIR spectroscopy; Tables S1: GC analysis of the methyl ester derivatives of FFAs (% w/w) released from various plant leaves and cyanobacteria by GPLRP2; Table S2: Estimation of mean molar masses for the fatty acids of spinach MGDG and DGDG based on their individual fatty acid compositions; Table S3: Mean molar masses of spinach MGDG and DGDG based on their individual fatty acid compositions (Table S1).

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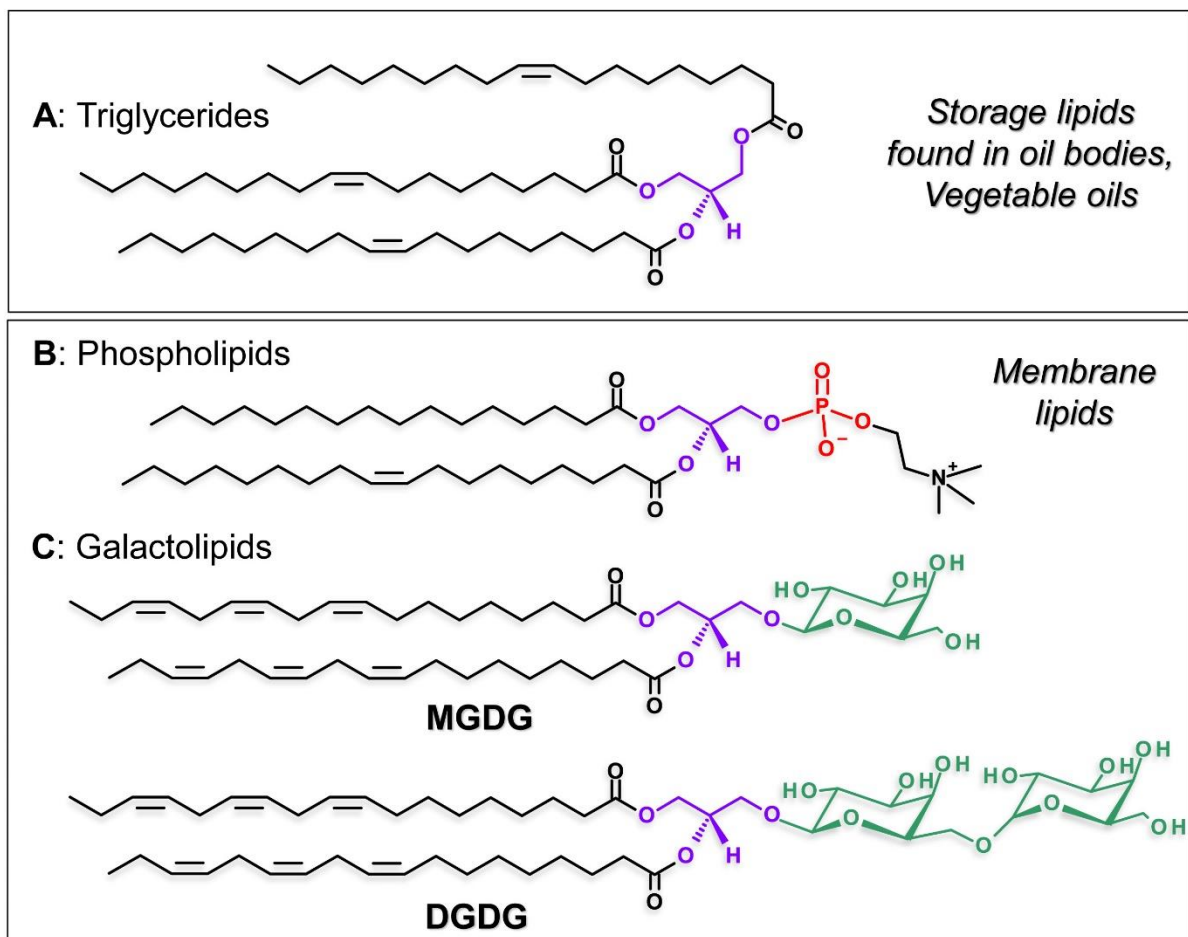
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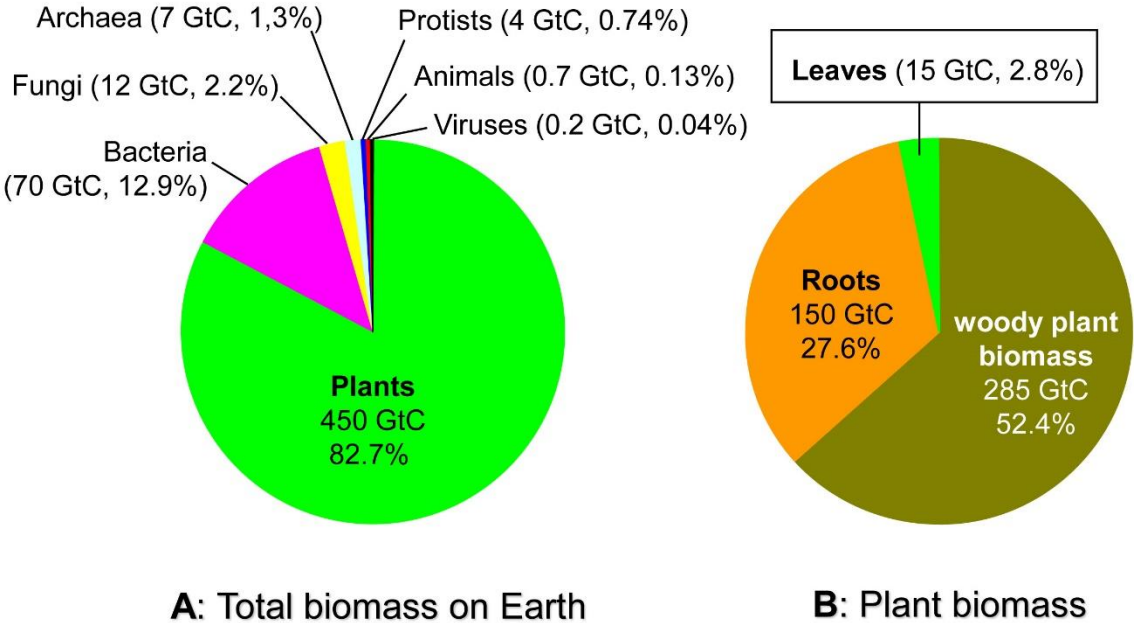
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## Figure legends

**Figure 1.** *Main acylglycerols found in plant biomass.* A: Triacylglycerols, illustrated here by a molecule of triolein, are the main energy storage lipids found in vegetable oils. B: Phospholipids, illustrated by a molecule of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PC), are major components of biological membranes. C: Galactolipids, illustrated by molecules of MGDG and DGDG, are also polar lipids involved in biological membranes in plants and algae, especially within the chloroplasts.

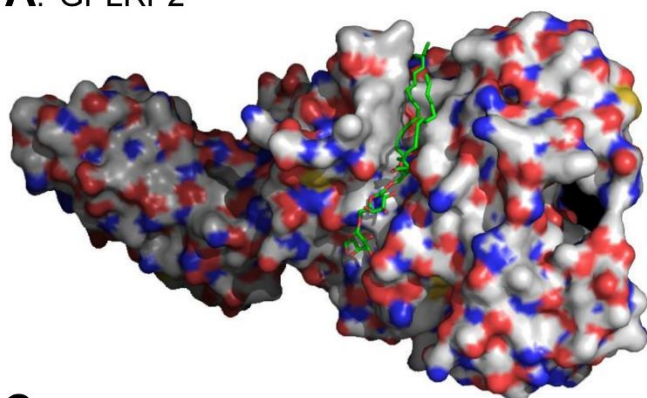


**Figure 2.** Contribution of plant biomass to the overall biomass on Earth. The pie charts in panels A and B were built from the data reported by Bar-on et al.<sup>6</sup> Values are gigatons of carbon (GtC) and percent of the total amount of carbon in biomass. For comparison, the world production of vegetable oils was 208 Mt/year or 0.16 GtC/year in 2022.

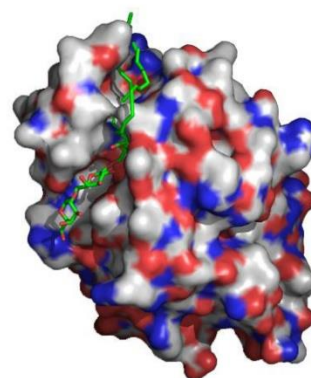


**Figure 3.** *Galactolipases and galactolipid hydrolysis.* X-ray crystallography 3D structures of a chimeric guinea pig pancreatic lipase-related protein 2 (GPLRP2; panel A; PDB ID: 1GPL<sup>16</sup>) and of *Fusarium solani* cutinase (FsCut; Panel B; PDB ID: 1CEX<sup>15</sup>), showing the molecular docking of a 18:3 n-3/18:3 n-3 DGDG molecule (green sticks) in their active site. Molecular docking of DGDG was performed using the Autodock vina program<sup>41</sup> and displayed using PyMOL software program (PyMOL Molecular Graphics System, Version 1.3. Schrödinger, LLC; <http://www.pymol.org/>). Molecular surfaces of GPLRP2 and cutinase are shown with the following color codes: white for C and H atoms; blue for N atoms; red for O atoms and yellow for S atoms). Panel C shows the hydrolysis reaction of a MGDG molecule leading to the generation of two FFA and one molecule of monogalactosylglycerol (MGG).

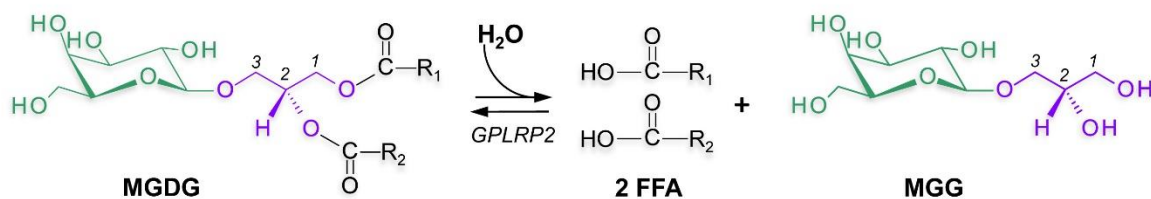
**A:** GPLRP2



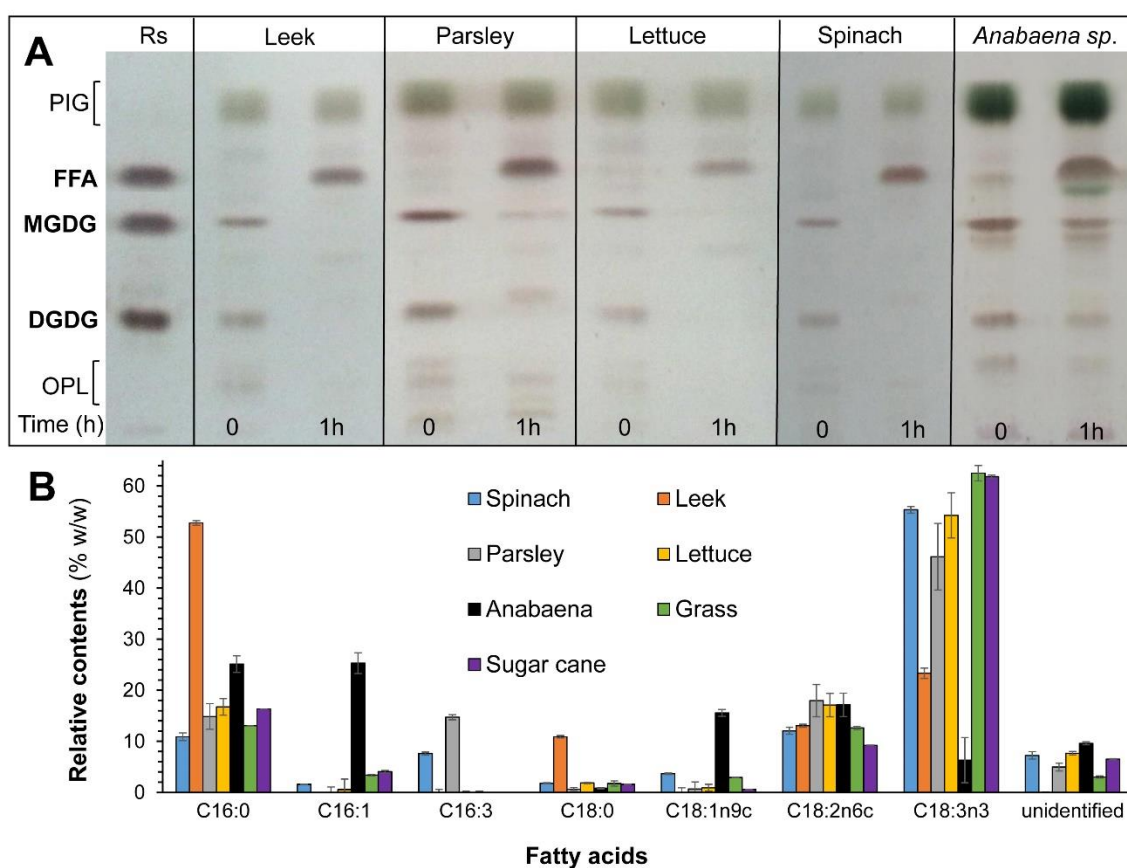
**B:** *Fusarium solani* cutinase



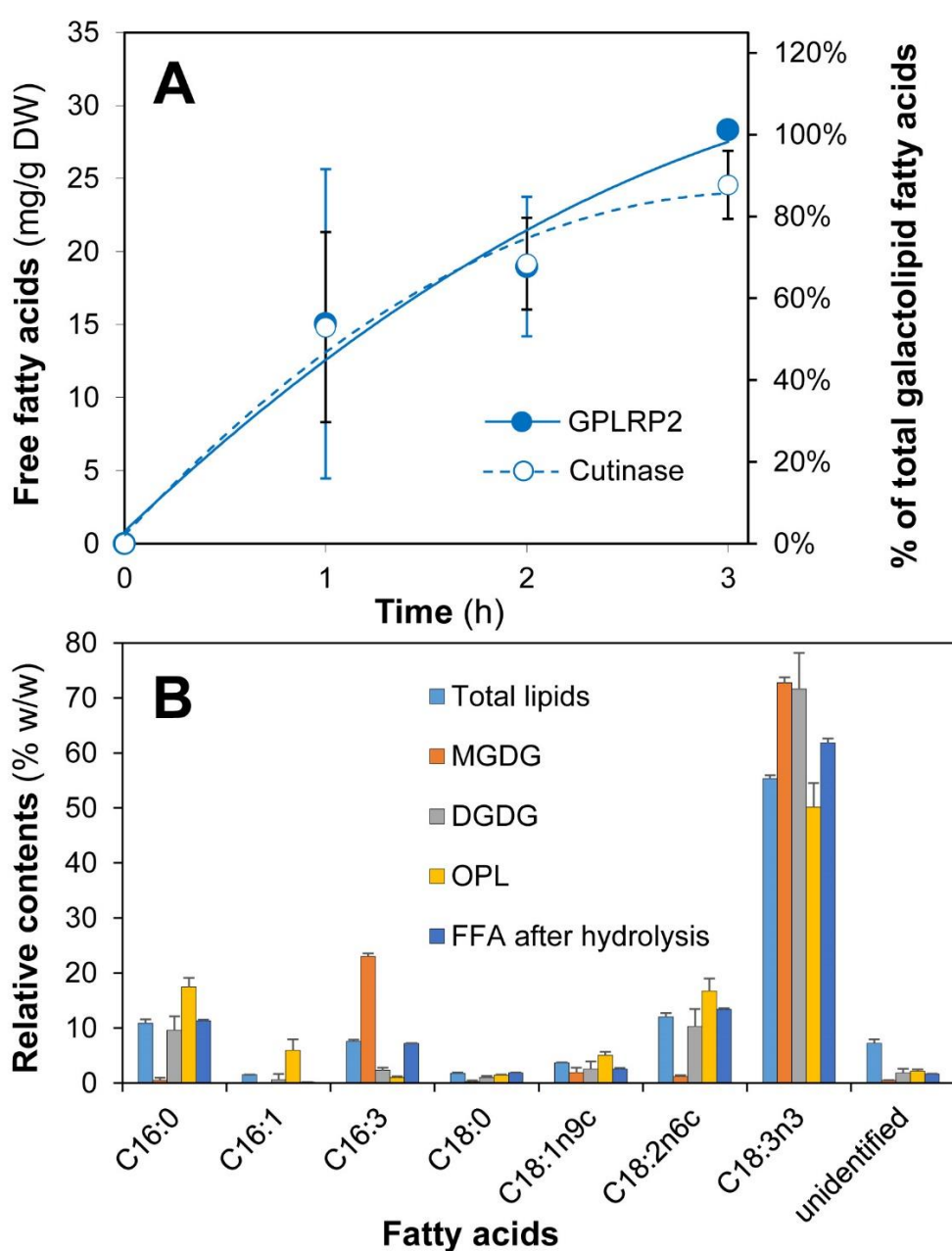
**C**



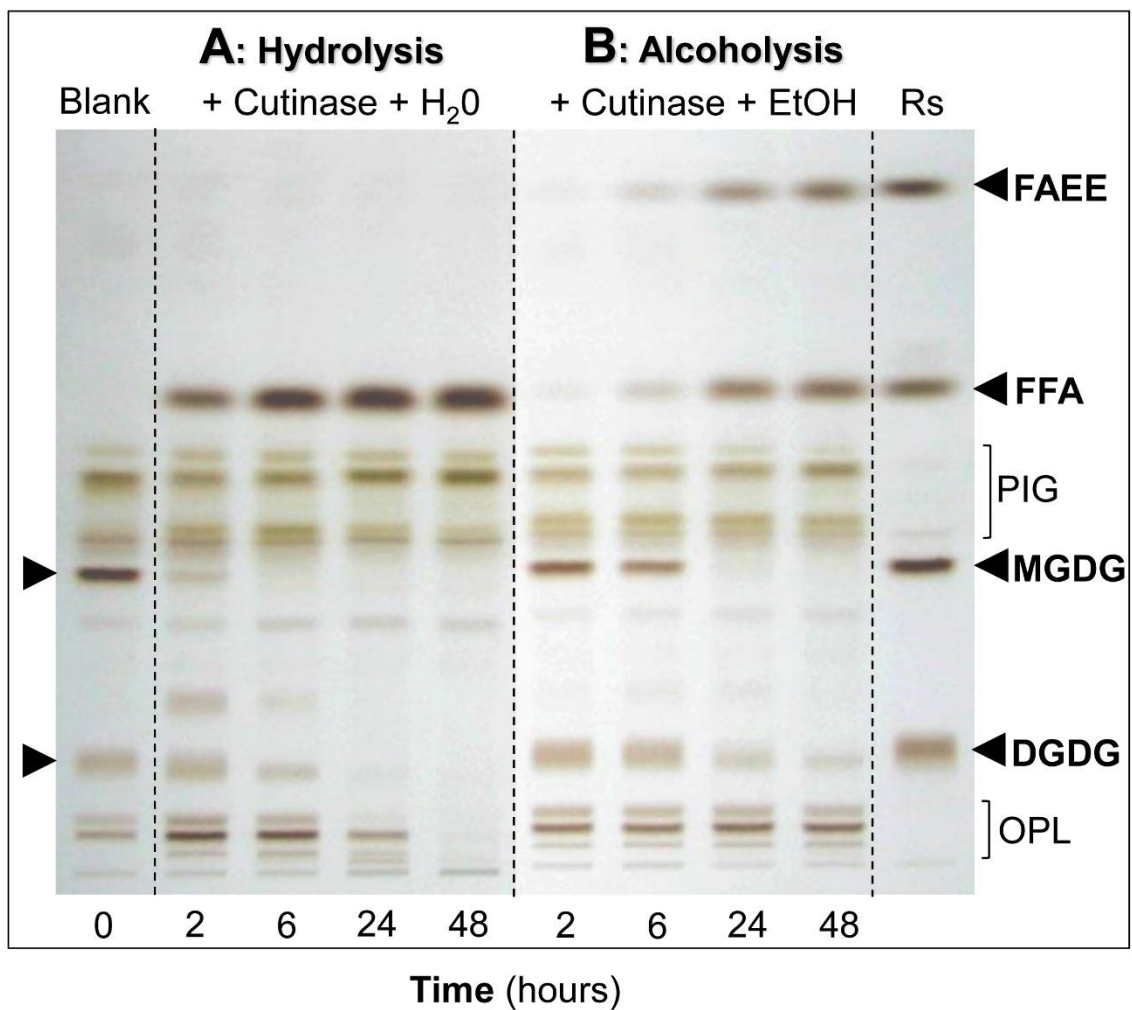
**Figure 4.** *Enzymatic hydrolysis of galactolipid from various plants leaves and cyanobacteria by GPLRP2.* Panel A: TLC analysis of lipid extracts of blanched plant leaves (leek, parsley, romaine lettuce, spinach) and *Anabaena sp.* culture pellet (50 mg each) after their incubation with GPLRP2 (50  $\mu$ g) for 1 hour. Reference standards are  $\alpha$ -linolenic acid for FFA and natural long chain MGDG and DGDG. Bands corresponding to pigments (PIG) and other polar lipids (OPL) are indicated. Panel B: GC analysis of the methyl ester derivatives of FFA (% w/w) released from various plant leaves and cyanobacteria. Values are mean  $\pm$  SD (n=3).



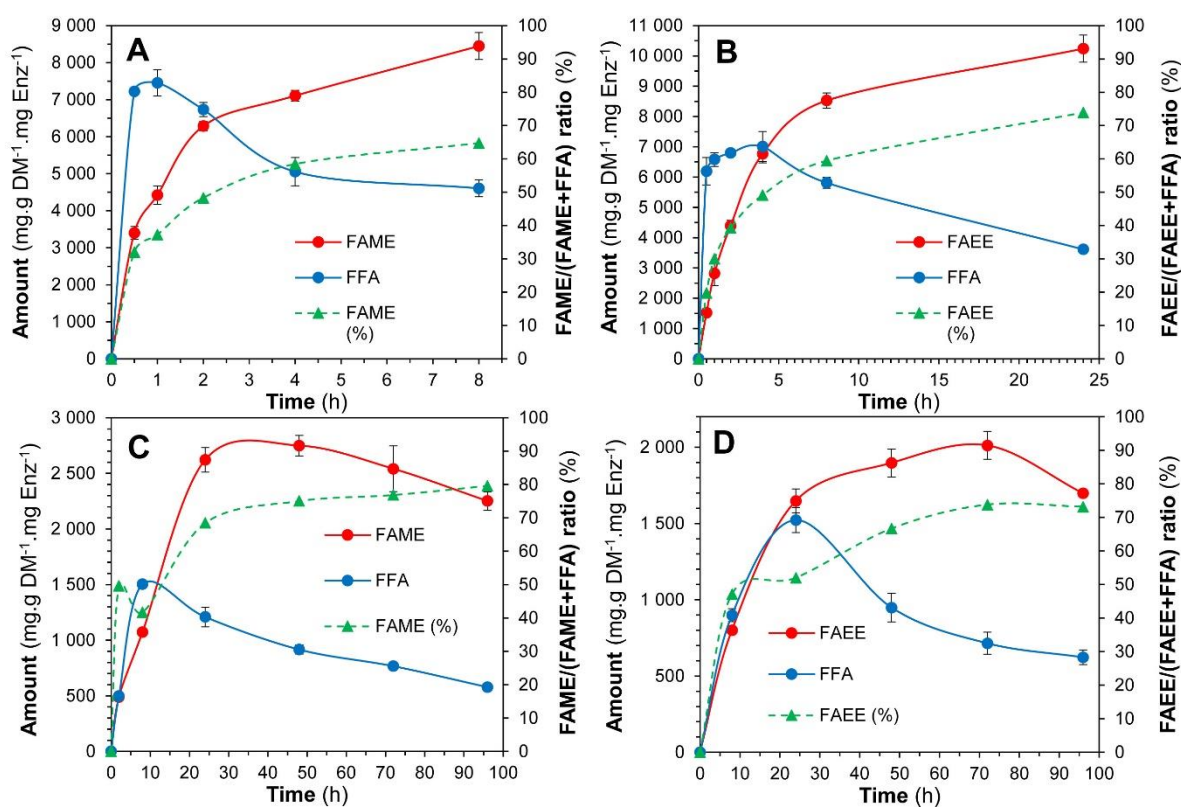
**Figure 5.** Fatty acid release upon hydrolysis of spinach leaves by GPLRP2 and *FsCut*. Panel A: kinetics of FFA production (mg per dry weight (DW) of spinach leaves) upon hydrolysis of 50 mg spinach leaves incubated with 50  $\mu$ g of GPLRP2 or 100  $\mu$ g *FsCut*. FFA release is also expressed in % of the total galactolipid fatty acids present in spinach leaves. Panel B: FA composition (% w/w) of the lipids initially present in spinach leaves (Total lipids, MGDG, DGDG and other polar lipids (OPL)) and FFA recovered after hydrolysis of spinach leaves by GPLRP2. Values are mean  $\pm$  SD (n=3).



**Figure 6:** TLC analysis of the *in situ* hydrolysis and alcoholysis of galactolipids from spinach leaves by *FsCut*. Panel A: time-course analysis of the hydrolysis reaction performed in 2 mM Tris-HCl, 150 mM NaCl buffer, pH 8.0. Panel B: time-course analysis of the alcoholysis reaction performed in the presence of 2.5 M EtOH added to the 2 mM Tris-HCl, 150 mM NaCl buffer, pH 8.0. Reference standards (Rs) for TLC analysis are ethyl linoleate for FA ethyl esters (FAEE),  $\alpha$ -linolenic acid for FFA and natural long chain MGDG and DGDG. The blank lane corresponds to the extract of polar lipids initially present in spinach leaves. Bands corresponding to pigments (PIG) and other polar lipids (OPL) are observed above and below the galactolipid bands.

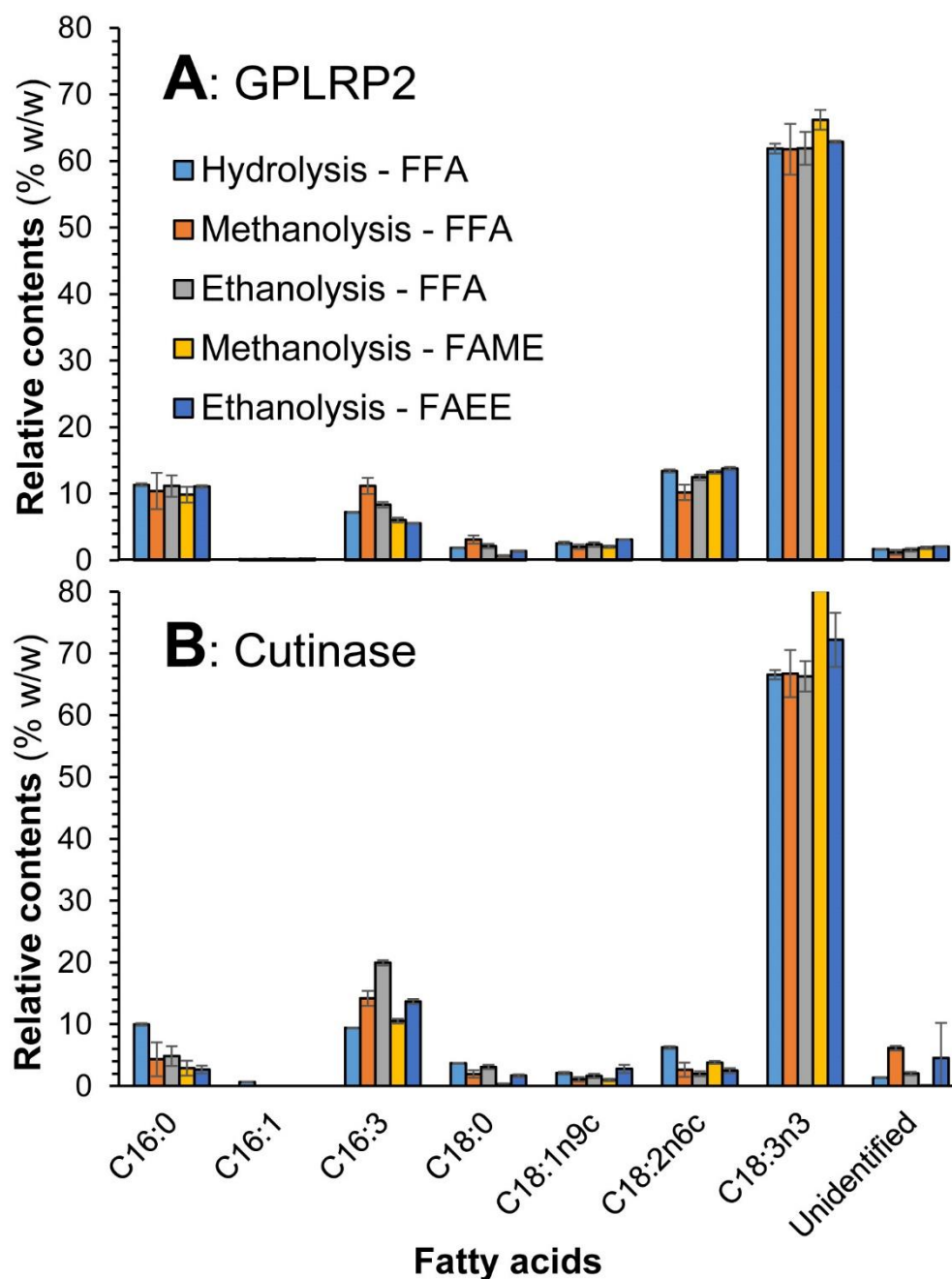


**Figure 7:** kinetics of free fatty acids (FFA), fatty acid methyl esters (FAME) and fatty acid ethyl esters (FAEE) production during the *in situ* methanolysis and ethanolysis of spinach galactolipids by GPLRP2 (Panels A, B) and FsCut (Panels C, D), respectively. Amounts of FFA and esters are expressed in mg per gram of spinach leaves dry weight (DW) per mg of enzyme. For each reaction, 50 mg spinach leaves were incubated with GPLRP2 (250  $\mu$ g powder; 5  $\mu$ g enzyme) or FsCut (250  $\mu$ g powder; 7.5  $\mu$ g enzyme) in 2 mM Tris-HCl, 150 mM NaCl buffer, pH 8.0, containing 6M (A) or 4M (C) methanol or 2.5M ethanol (B, D). Values are mean  $\pm$  SD (n=3).

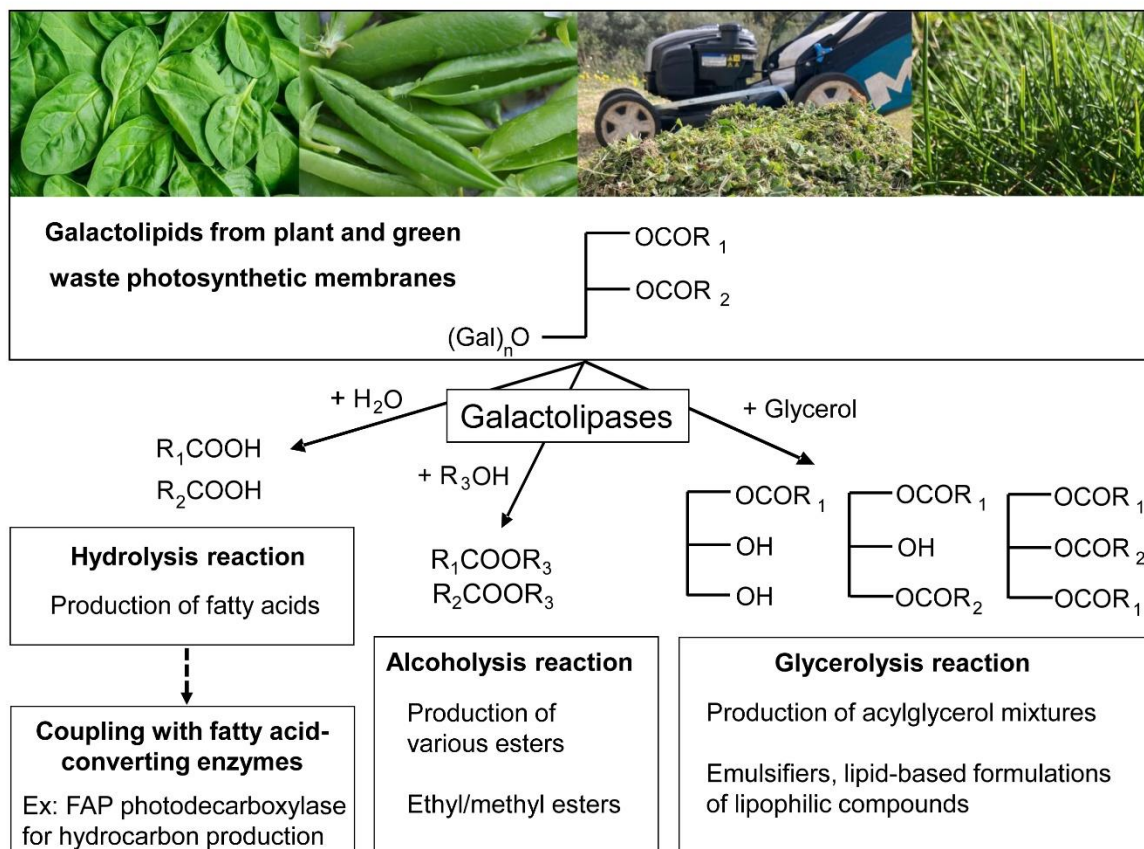




**Figure 8:** Fatty acid composition (% w/w) of the free fatty acids (FFA), fatty acid methyl esters (FAME) and fatty acid ethyl esters (FAEE) formed during the *in situ* methanolysis and ethanolysis of spinach galactolipids by GPLRP2 (Panel A) and FsCut (Panel B). For the sake of comparison, the FA composition (% w/w) of the FFA released during the hydrolysis reaction with the same enzymes are shown in panels A and B, respectively.



**Figure 9:** Various applications of the *in situ* conversion of galactolipids from plant leaves and green waste photosynthetic membranes. Besides the proof of concept made here with the *in situ* hydrolysis and alcoholysis of galactolipids, one could also envision the glycerolysis reaction for the production of partial glyceride mixtures and the coupling of the enzymatic hydrolysis with other FA-converting enzymes.



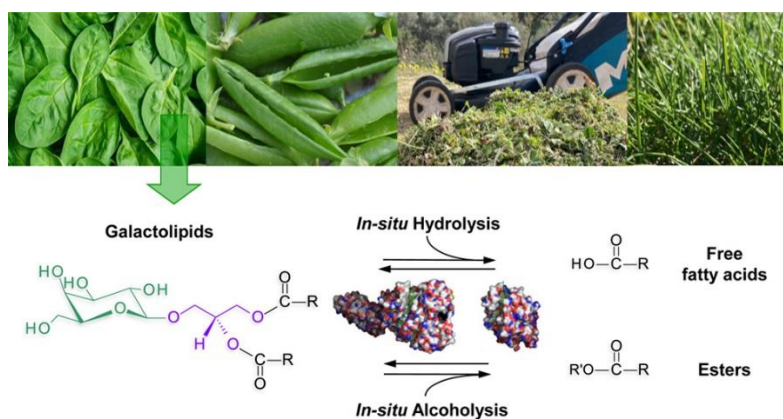
**Table 1:** Rates of FFA and FAAE production upon hydrolysis and alcoholysis of spinach leaves by GPLRP2 and FsCut

<i>Enzyme</i>	<i>Solvent</i>	<i>Initial rate</i> ( $\mu\text{mole}\cdot\text{min}^{-1}\cdot\text{mg}\cdot\text{Enz}^{-1}$ )		<i>Initial rate ratio</i>	<i>Final</i> [FAAE]/[FAAE+FFA] ratio (%)
		<i>FAAE</i>	<i>FFA</i>		
GPLRP2	Water		13.12 ± 0.81		
	6M Methanol	3.49	13.60	0.25	64.7
	2.5 M Ethanol	1.07	22.27	0.05	73.9
FsCut	Water		4.04 ± 0.25		
	4 M Methanol	0.06	0.22	0.27	79.6
	2.5 M Ethanol	0.04	0.04	1	73.2

**Table 2:** Lipid contents of spinach, sugar cane and grass before and after incubation with GPLRP2. Spinach leaves were incubated with GPLRP2 either for 3 h (hydrolysis in water) or 8 h (alcoholysis in 6M MeOH). Sugar cane leaves and grass were incubated for 24 h with GPLRP2. The percents in brackets correspond to the recovery yield of FA versus their contents in galactolipids. \*Data from<sup>10</sup>.

Plant material	Total galactolipids (mg.g DW <sup>-1</sup> )	MGDG (mg.g DW <sup>-1</sup> )	DGDG (mg.g DW <sup>-1</sup> )	FA equiv. (mg.g DW <sup>-1</sup> )	Solvent	After incubation with GPLRP2	
						FAA (mg.g DW <sup>-1</sup> )	FAME (mg.g DW <sup>-1</sup> )
Spinach leaves	40.0 ± 3.90	25.1 ± 2.8	14.9 ± 1.3	23.9 ± 2.8	water	24.4 ± 0.4 (102 %)	
					6M MeOH	18.64 ± 0.13 (78 %)	6.83 ± 0.53 (28 %)
Sugar cane leaves	23.0 ± 1.9	13.1 ± 1.2	9.9 ± 0.7	13.4 ± 1.1	water	11.4 ± 0.9 (85 %)	
					6M MeOH	3.9 ± 0.2 (29 %)	5.2 ± 0.5 (39 %)
Grass	32.6 ± 1.2	19.1 ± 0.7	13.5 ± 0.5	19.0 ± 0.7	water	3.8 ± 0.2 (20 %)	
					6M MeOH	1.8 ± 0.3 (9.5 %)	2.7 ± 0.4 (14.2 %)
Pea vine haulm CRF*	32.1 ± 3.0	18.9 ± 2.0	13.2 ± 1.0	18.6 ± 1.7			
Spinach CRF*	54.4 ± 2.2	28.6 ± 1.1	25.8 ± 1.1	31.5 ± 1.3			

## TOC/Abstract graphic



**Synopsis.** Galactolipids from plant leaves and green waste constitute an underexplored reservoir of renewable FA. The use of enzymes makes them accessible for oleochemistry and nutrition.