Evolution, biosynthesis and protective roles of oligogalactolipids: Key molecules for terrestrial photosynthesis?

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A B S T R A C T

Galactolipids (GLs) are the main lipids in chloroplast membranes and by default are also the most abundant polar lipids on earth. GLs with one or two galactose residues, monogalactosyldiacylglycerol (MGDG) and digalactosyl-diacylglycerol (DGDG), are ubiquitous and essential for photosynthesis. GLs with a headgroup formed by three to five galactoses, the so-called oligogalactolipids (OGLs), are only detected in some taxa, organs and environmental conditions. OGLs can be synthesized by two metabolic pathways: successive galactosylation by DGDG synthase (DGD) or transgalactosylation from MGDG by the GL:GL galactosyltransferase (GGGT/SFR2). While the first route appeared early in the evolution (cyanobacteria), the second evolved associated to the process of terrestrialization in the streptophytes. Both routes also differ on the anomeric type of glycosidic linkages formed: α-type in DGD and β-type in GGGT/SFR2. Despite functional differences between both configurations, the anomeric analysis of OGLs allows tracking their biosynthetic origin. While α-OGLs are constitutive and present in some algae and non-vegetative organs of vascular plants, β-OGLs are typically stress-inducible in photosynthetic tissues. Land colonization by plants involved new challenges, such as the risk of dehydration, which required developing biochemical and physiological strategies to stabilize chloroplast membranes and safeguard their functioning. Based on the integrated assessment of data available we propose that the appearance of OGLs was one of those adaptations that simultaneously could have provided advantages against other environmental constraints such as freezing.

1. Introduction

1.1. Chloroplast lipids

Chloroplasts contain three different types of biological membranes: outer and inner envelopes and thylakoids. In quantitative terms chloroplast thylakoids comprise the vast majority of plant cell membranes representing a surface 300–800 times higher than the corresponding leaf area (Antal et al., 2013). Chloroplasts membranes are basically composed by glycolipids and phospholipids (PLs), with the first increasing their abundance from the outer to the inner envelopes, and from them to the thylakoids (LaBrant et al., 2018), where they represent 80–90% of total polar lipids (Rolland et al., 2009).

The lipid composition of thylakoids is quite unique, and it has been preserved relatively stable during the course of the evolution of oxygenic photosynthesis (Boudière et al., 2014). The most abundant lipid in chloroplasts is the galactolipid (GL) monogalactosyldiacylglycerol (MGDG) which represents more than one half of total lipid content and is considered as the most abundant polar lipid in nature (Gounaris and Barber, 1983). The second in abundance is the digalactosyldiacylglycerol (DGDG), followed by sulphoquinovosyldiacylglycerol (SQDG). The remaining fraction is mostly composed by PLs, in particular phosphatidylglycerol (PG) (Wada and Murata, 2007). While the conic-

shaped MGDG is unable to form stable bilayer structures (Garab et al., 2016), the other three, with a cylindrical shape contribute to thylakoid stability. Chloroplasts also contain a high amount of triacylglycerol (TAG), mostly forming lipid droplets or plastoglobules in the stroma (Bréhelin et al., 2007).

The structure of GLs consists on a glycerol esterified with two acyl chains and a polar head formed by one to five galactose residues. Given the extension of thylakoids in plant cells, the replacement of PLs by GLs in those membranes probably represents a mechanism to reduce phosphate demand (LaBrant et al., 2018). In fact the complete replacement of GLs by PLs would represent an increase of P content of more than 30%, being P a nutrient frequently limiting in plants. However the presence of galactose in the head groups not only can be understood as a mechanism to reduce P requirements, but its specific interaction with photosynthetic protein complexes is also a requirement for the full engagement of the photosynthetic apparatus (Hölzl et al., 2006).

Besides constituting the majority of thylakoid lipids, GLs also play a lot of fundamental functions. First, these lipids are essential constituents of multi-protein photosynthetic complexes: MGDG is present in photosystem I (PsI), photosystem II (PSII) and Cytochrome b_6f; while DGDG is part of the PsI and PSII (Quin et al., 2015; Wei et al., 2016). Second, the inverted hexagonal structures (H_{II}) formed by MGDG are essential for the activity of the xanthophyll cycle, as is the only mean for the violaxanthin de-epoxidase (VDE) to reach access to its substrate (violaxanthin) and produce photoprotective zeaxanthin (Z) (Hieber et al., 2004; Jahns et al., 2009). Third, the proportion of bilayer and non-bilayer forming lipids determines chloroplast morphology (Deme et al., 2014; Rocha et al., 2018). Fourth, MGDG to DGDG ratio regulates jasmonic acid production (Li and Yu, 2018).

1.2. Biosynthesis of GLs

GLs were identified for the first time in benzene-extractable lipids of wheat flour (Carter et al., 1956) and the first advances in the elucidation of GLs biosynthesis came in the late 50 s and 60 s when the metabolic route was elucidated by radiolabeling studies (Benson et al., 1958; Ferrari and Benson, 1961). These authors incubated green algae (*Chlorella*) with $^{14}CO_2$ and observed its rapid incorporation in GLs, inferring that the only pathway was through the incorporation of uridine diphosphate galactose (UDP-Gal). This pathway was later confirmed *in vitro* with isolated spinach chloroplasts by the rapid incorporation of UDP-Gal^{14}C through a sequence of successive galactosylations giving rise to GLs with up to four galactose residues (Neufeld and Hall, 1964). A few years later, Ongun and Mudd (1968) proposed that two different enzymes were involved in GLs biosynthesis (one forming MGDG and the other DGDG). Interestingly, in this initial study Neufeld and Hall (1964) observed that only 17% of the terminal galactose was hydrolysed with α-galactosidase, suggesting that the remaining fraction was linked by a β-linkage. However, this fact was overlooked until a decade later, when a second alternative enzyme able to form DGDG in the absence of UDP-Gal, was demonstrated in isolated chloroplasts through a double labelling (^{14}C and ^{3}H) experiment (Van Besouw and Wintermans, 1978). This enzyme with activity galactolipid:galactolipid galactosyltransferase (GGGT) catalyses a transglycosylation from MGDG to either MGDG or DGDG. However, these authors did not study the anomeric configuration of the newly formed GL. In fact, it was more than twenty years later when the β-anomeric configuration of the products of GGGT was confirmed by proton-nuclear magnetic resonance (NMR) (Moellering et al., 2010).

Nowadays it is considered that the bulk of MGDG and DGDG under non-stress conditions is synthesized through the action of the MGDG Synthase (MGD) and DGDG Synthase (DGD) by the successive addition of galactose residues (for recent reviews on GLs biosynthesis see: Benning and Ohta, 2005; Hölz and Dörmann, 2007; Moellering and

Benning, 2011; Boudière et al., 2014; Li and Yu, 2018; Rocha et al., 2018). The first galactose forms a β-glycosidic linkage with diacylglycerol (DAG) giving rise to 1,2-diacyl-3-O-(β-D-galactopyranosyl)-*sn*-glycerol (β-MGDG) (Fig. 1). Successive galactosylation generates 1,2-diacyl-3-O-(α-D-galactopyranosyl-(1-6)-O-β-D-galactopyranosyl)-*sn*-glycerol (αβ-DGDG). Alternatively, GGGT pathway acts on MGDG, converting it into DGDG with concomitant production of DAG, using MGDG as galactosyl donor, instead of UDP-Gal. In contrast with DGD, linkages formed by GGGT are in β conformation, giving rise to 1,2-diacyl-3-O-(β-D-galactopyranosyl-(1–6)-O-β-D-galactopyranosyl)-*sn*-glycerol (ββ-DGDG) (Fig. 1). This enzyme is encoded by one of the genes needed for freezing tolerance, the *SENSITIVE TO FREEZING2* gene (*SFR2*) (Moellering et al., 2010), and is non detectable in the model plant Arabidopsis under non-stress conditions (Moellering and Benning, 2011). Because of the name of the encoding gene, the GGGT enzyme is frequently referred to as SFR2 in the literature. For the sake of simplicity, the term SFR2 is used hereinafter as synonym of GGGT.

Further galactosylation of DGDG is possible by both routes, with concomitant production of the so-called oligogalactolipids (OGLs): trigalactosyldiacylglycerol (TGDG), tetragalactosyldiacylglycerol (TeGDG), and presumably also pentagalactosyldiacylglycerol (PGDG) (Heemskerk et al., 1983). As occurs with DGDG, OGLs synthesized by both routes differ in the type (α or β) of glycosidic linkage formed. Thus, when TGDG is the result of incorporation of UDP-Gal residues by a DGD synthase, the TGDG formed is 1,2-diacyl-3-O-(α-D-galactopyranosyl-(1–6)-α-O-D-galactopyranosyl-(1–6)-O-β-D-galactopyranosyl)-*sn*-glycerol (ααβ-TGDG) (Gent and Gigg, 1975; Kelly et al., 2002) (Fig. 1). On the other hand, when TGDG is formed by transglycosilation by SFR2 the product formed is 1,2-diacyl-3-O-(β-D-galactopyranosyl-(1–6)-β-O-D-galactopyranosyl-(1–6)-O-β-D-galactopyranosyl)-*sn*-glycerol (βββ-TGDG) (Moellering et al., 2010; Xu et al., 2003). The sequential activity of DGD and SFR2 could also lead to 1,2-diacyl-3-O-(β-D-galactopyranosyl-(1–6)-α-O-D-galactopyranosyl-(1–6)-O-β-D-galactopyranosyl)-*sn*-glycerol (βαβ-TGDG) (Kojima et al., 1990; Gasulla et al., 2013) (Fig. 1, see Section 3 for further information about the enzymes and their regulation). This last OGL synthesis pathway, though, has not been confirmed by *in vitro* experiments yet (Roston et al., 2014). Further galactosylation is possible by both routes enlarging the polar head to form TeGDG and PGDG.

2. Occurrence of OGLs across photosynthetic life forms

The presence of MGDG and DGDG is essential in photosynthetic life forms, from green non-sulfur bacteria and cyanobacteria to angiosperms. Plant mutants deprived of MGDG/DGDG display serious morphological and functional anomalies that can be lethal (Kobayashi et al., 2007). The synthesis of OGLs (TGDG and TeGDG) is also widespread in photosynthetic organisms (Table 1). They have been found in primitive cyanobacteria and in modern monocots, in unicellular chlorophyte algae and in the leaves of angiosperms (Fig. 2). However, unlike MGDG and DGDG, OGLs are not accumulated by all the photosynthetic organisms and apparently are not needed for growing under non-stress conditions (see Sections 2 and 4).

2.1. Presence of OGLs among cyanobacteria and "algae"

TGDG has been detected in several species of cyanobacteria (Fig. 2), the most primitive oxygenic-photosynthetic organisms. Zepke et al. (1978) observed that *Tolypothrix tenuis* and *Oscillatoria chalybea* accumulated TGDG during growth, but not *Anabaena cylindrica* or *Nostoc calcicola*. More recently, the presence of TGDG has been confirmed in the model strain *Anabaena* (*Nostoc*) sp. PCC 7120 growing under optimal conditions and under cold stress (Awai, 2016).
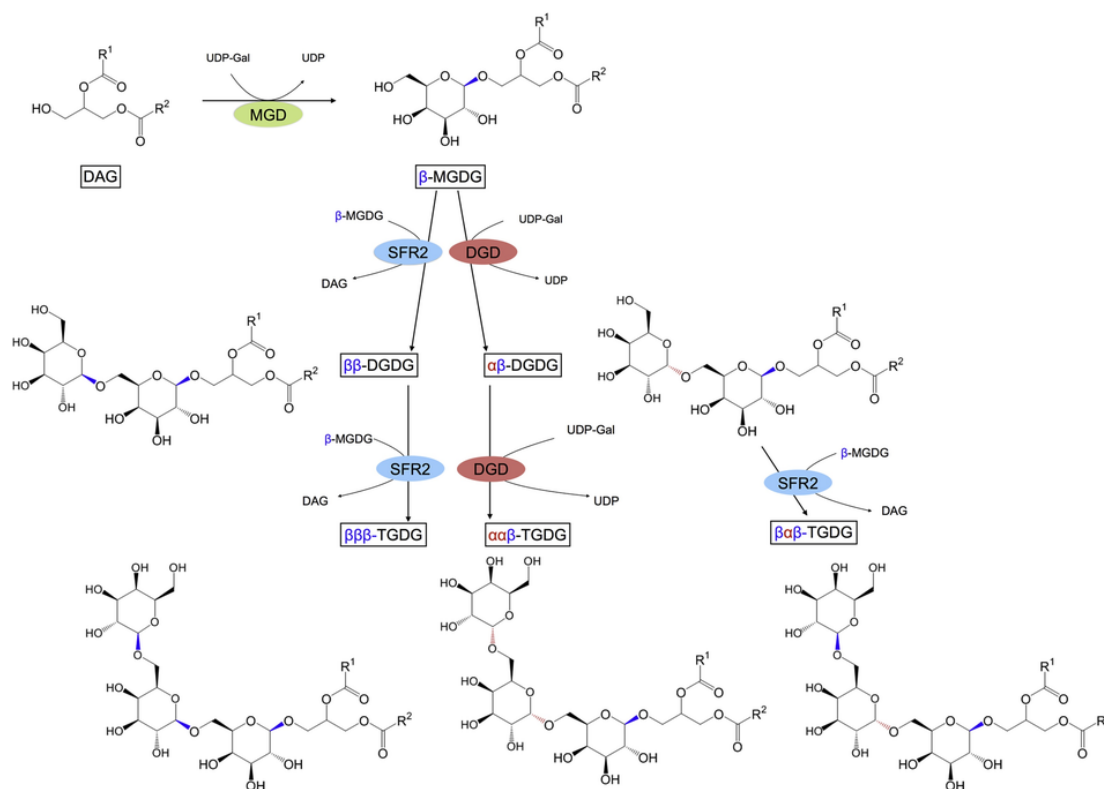
Fig. 1. Schematic view of the two main biosynthetic pathways of OGLs. The enzymes are indicated in color. The synthesis of OGLs starts with the addition of a galactose to DAG through a β-glycosidic linkage. The enzyme MGD mediates this reaction from UDP-Gal producing one molecule of β-MGDG. Two different enzymes can mediate successive galactosylations. The DGD enzyme (in red) adds galactoses through a α-glycosidic linkage, producing DGDG and OGLs, with UDP as by-product. This enzyme is universally present in photosynthetic organisms. The GGGT/SFR2 (in blue) synthesizes DGDG and OGLs using MGDG as substrate and producing DAG as by-product. This enzyme adds galactoses through a β-glycosidic linkage and has only been found in organisms within the Streptophyta. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Within the paraphyletic group of the algae -defined as photosynthetic eukaryotic organisms that lack specialized multicellular reproductive structures- the capacity to synthesize OGLs have been found in several phyla (Fig. 2). Within green algae (Chlorophyta), the synthesis of OGLs was firstly reported when *Chlorella* cells (Trebouxiophyceae) were exposed to radioactive $^{14}CO_2$ and after a short period of time radioactive-TGDG was detected (Benson et al., 1958) (see Section 1.2). More recently, the constitutive accumulation of TGDG and TeGDG has been observed in *Asterochloris erici*, another Treboxuiophyceae alga that establishes a symbiosis with fungi to form lichens (Gasulla et al., 2016). On the contrary, Mendiola-Morgenthaler et al. (1985) were not able to found OGLs in the alga *Chlamydomonas reinhardtii* (Chlorophyceae) by incorporation of UDP-Gal^{14}C, and further mass spectrometric (MS) analysis have not detected them either (Vieler et al., 2007; Yang et al., 2015).

The presence of OGLs is frequent in the superclass Dinoflagellata. Gray et al. (2009b) surveyed the lipid composition of four cold-adapted dinoflagellates and found that TGDG, a lipid previously unreported in dinoflagellates, was a major glycolipid of *Gymnodinium* sp. (Fig. 2). After this finding, the data from a previous study (Gray et al., 2009a) was re-interpreted revealing also the presence of TGDG in some warm-adapted peridinin-containing dinoflagellates. In the case of the genus *Pyrocystis*, dinoflagellates accumulate TGDG constitutively and do not show significant response to growth temperature (Leblond et al., 2010). In contrast, the presence of TGDG has not been detected in Euglenophyta (e.g. *Euglena gracilis*) (Blee and Schantz et al., 1978) (Fig. 2). The presence of TGDG in Bacillariophyceae is not clear. Thus, Vieler et al. (2007) carried out a MS analysis of the lipid composition of the diatom *Cyclotella meneghiniana* and detected a peak that might

be attributed to TGDG, but they did not confirmed it. Further studies are required to determine the presence of OGLs in other species of these phyla and in other groups like the brown or the red algae.

2.2. Occurrence of OGLs within the Streptophyta

Among the clade of the Streptophytes –the monophyletic group that includes the Charophyta algae and Embryophyta– TGDG has been found in high abundance in *Klebsormidium flaccidum*, a charophyta alga, growing under optimal conditions (Hori et al., 2016) (Fig. 2). In bryophytes, TGDG was detected by thin-layer chromatography (TLC) in the gametophytes of four field-collected moss species: *Mnium cuspidatum*, *Mnium medium*, *Hylocomium splendens* and *Pleurozium schreberi* (Gelleerman et al., 1974) (Fig. 2). Among ferns (Pteridophyta), the only available study showed that the chlorophyllous-spores of the fern *Osmunda regalis* contained high levels of TGDG, but its presence was residual in gametophytes (López-Pozo et al., 2018).

2.2.1. Occurrence of OGLs within angiosperms

The presence and biosynthetic pathways of OGLs have been more deeply studied in angiosperms than in any other photosynthetic clade. The first evidence of OGLs was obtained by Neufeld and Hall (1964) in chloroplasts isolated from spinach. Later, studies using different approaches (immunological, isotopes…) confirmed the presence of OGLs in spinach (Allen et al., 1966; Ongun and Mudd, 1968; Webster and Chang, 1969). Immuno-technical approaches also confirmed the presence of TGDG in the chloroplast of two other angiosperms, *Urtica dioica* and *Antirrhinum majus* (Radunz, 1976). Additional studies demonstrated that isolated chloroplasts (Cline and Keegstra, 1983; Heemskerk et al., 1990) from several monocots and dicots (Table 1) were able to

Table 1

List of photosynthetic organisms in which the presence (+) or absence (-) of trigalactolipids (TGDG) has been reported employing different biological material (Biol. mat.): vegetative cells (Veg. cell.) of cyanobacteria or algae; gametophytes (Gamet.) from bryophytes or pteridophytes; bud plastids (Bud Pl.), cell plastids (Cell Pl.), chloroplasts (Chloropl.), chromoplasts (Chromopl.), inflorescence meristems (Inflor.), protoplasts (Protopl.), fruits, tubers, seeds, shoots or leaves from angiosperms. In some experiments the TGDG analysis were carried out in organisms growing under favorable conditions (Fav.) but also in response to different kind of stress. Only in a few studies the anomeric configuration of the glycosydic bonds of TGDG has been determined.

Group /Species	Biol. mat.	Fav.	Stress	Bonds	Reference
Cyanobacteria					
Tolypothrix tenuis	Veg.cell	+		ααβ	Zepke et al., 1978
Oscillatoria chalybea	Veg.cell	+			Zepke et al., 1978
Anabaena cylindrica	Veg.cell	–			Zepke et al., 1978
Nostoc calcicola	Veg.cell	–			Zepke et al., 1978
Anabaena sp. PCC 7120	Veg.cell	+	+[c]	ααβ	Awai, 2016
Dinoflagellata					
Pyrocystis lunula UTEX 2166	Veg.cell	+			Leblond et al., 2009
Pyrocystis lunula UTEX 2271	Veg.cell	+			Leblond et al., 2009
Pyrocystis noctiluca PP7	Veg.cell	+			Leblond et al., 2009
Pyrocystis noctiluca Scripps	Veg.cell	+			Leblond et al., 2009
Pyrocystis fusiformis Scripps	Veg.cell	+			Leblond et al., 2009
Pyrocystis fusiformis NOAA	Veg.cell	+			Leblond et al., 2009
Scrippsiella hangoei	Veg.cell	–			Gray et al., 2009b
Woloszynskia halophila	Veg.cell	–			Gray et al., 2009b
Gymnodinium sp. C5	Veg.cell	+			Gray et al., 2009b
Peridinium aciculiferum	Veg.cell	–			Gray et al., 2009b
Borghiella dodgei	Veg.cell	+			Flaim et al., 2012
Diatomea					
Cyclotella meneghiana	Veg.cell	+?			Vieler et al., 2007
Euglenophyta					
Euglena gracilis	Veg.cell	–			Lin and Chang, 1971
Euglena gracilis	Veg.cell	–			Blee and Schantz, 1978
Chlorophyta					
Chlorella sp.	Veg.cell	+			Benson et al., 1958
Chlamydomonas reindhartii	Veg.cell	–			Mendiola-Morgenthaler et al., 1985
Chlamydomonas reindhartii	Veg.cell	–			Vieler et al., 2007

Group /Species	Biol. mat.	Fav.	Stress	Bonds	Reference
Asterochloris erici	Veg.cell	+	+[d]		Gasulla et al., 2016
Charophytes					
Klebsormidium flaccidum	Veg.cell	+			Hori et al., 2016
Bryophytes					
Mnium cuspidatum	Gamet.	+			Gellerman et al. (1975)
Mnium medium	Gamet.	+			Gellerman et al. (1975)
Hylocomium splendens	Gamet.	+			Gellerman et al. (1975)
Pleurozium schreberi	Gamet.	+			Gellerman et al. (1975)
Pteridophytes					
Osmunda regalis	Spores	+			López-Pozo et al., 2018
Osmunda regalis	Gamet.	–			López-Pozo et al., 2018
Angiosperms					
Spinacia oleracea	Chloropl.	+			Neufeld and Hall, 1964
Spinacia oleracea	Chloropl.	+			Ongun and Mudd, 1968
Brassica oleracea	Inflor.	+			Ongun and Mudd, 1968
Solanum tuberosum	Tuber	+		ααβ	Galliard, 1968, 1969
Spinacia oleracea	Leaf		+*		Webster and Chang, 1969
Spinacia oleracea	Chloropl.	+			Poincelot, 1973
Cucurbita maxima	Fruit	+		ααβ	Ito and Fujino, 1975
Urtica dioica	Chloropl.	+		ααβ	Radunz, 1976
Antirrhinum majus	Chloropl.	+			Radunz, 1976
Spinacia oleracea	Chloropl.	+			Radunz, 1976
Glycine hispida	Seed	+			Radunz, 1976
Triticum aestivum	Seed	+		ααβ	Morrison et al., 1978
Oryza sativa	Seed	+		ααβ	Fujino and Miyazawa, 1979
Pisum sativum	Chloropl.	+			Cline and Keegstra, 1983
Brassica oleracea	Bud Pl.	+			Alban et al., 1988
Acer pseudoplatanus	Cell Pl.	+			Alban et al., 1988
Lycopersicon esculentum	Fruit	+	+[se]		Güçlü et al., 1989
Spinacia oleracea	Chloropl.	+			Heemskerk et al., 1990
Avena sativa	Chloropl.	+			Heemskerk et al., 1990
Pisum sativum	Chloropl.	+			Heemskerk et al., 1990
Sinapis alba subsp. alba	Chloropl.	+			Heemskerk et al., 1990
Zea mays	Chloropl.	+			Heemskerk et al., 1990
Nicotiana sylvestris	Chloropl.	+			Heemskerk et al., 1990

Table 1 (*Continued*)

Group /Species	Biol. mat.	Fav.	Stress	Bonds	Reference
Tropaeolum majus	Chloropl.	+			Heemskerk et al., 1990
Narcissus pseudonarcissus	Chromopl.	+			Heemskerk et al., 1990
Vigna angularis	Seed	+		α/βαβ	Kojima et al., 1990
Spinacia oleracea	Leaf	–	+ [O3]		Sakaki et al., 1990a, 1990b
Vicia faba	Protopl.	+			Sakaki et al., 1995
Vicia faba	Leaf	–			Sakaki et al., 1995
Cucurbita pepo	Fruit	+			Sugawara and Miyazawa, 1999
Arabidopsis thaliana tgd2/dgd2	Leaf	+		βββ	Xu et al., 2003
Avena sativa	Seed	+			Moreau et al., 2008,
Avena sativa	Seed	+			Doehlert et al., 2010
Arabidopsis thaliana	Leaf	–	+ [f]	βββ**	Moellering et al., 2010
Craterostigma plantagineum	Leaf	–	+ [d]	βαβ	Gasulla et al., 2013
Lindernia subracemosa	Leaf	–	+ [d]		Gasulla et al., 2013
Lindernia brevidens	Leaf	–	+ [d]		Gasulla et al., 2013
Arabidopsis thaliana	Leaf	–	+ [d]		Gasulla et al., 2013
Arabidopsis thaliana	Leaf	–	+ [f]		Barnes et al., 2016
Arabidopsis thaliana	Shoots	–	+ [ac]		Barnes et al., 2016
Pisum sativum	Leaf	–	+ [ac]		Barnes et al., 2016
Solanum lycopersicum	Leaf	–	+ [d,s]		Wang et al., 2016
Boechera stricta	Leaf	–	+ [f]		Arisz et al., 2018

[c] cold, [se] senescence, [O3] ozone, [f] freezing, [d] desiccation, [ac] acid, [s] salt stress.

* Lipids were isolated from detached leaves and stored at 4 °C and, therefore, stressed. ** Anomeric configuration of TGDG glycosidic bonds inferred from DGDG *in vitro* synthesis by SFR2.

synthesize TGDG and/or TeGDG *in vitro*, suggesting that the plastids of all angiosperms have the capacity to produce OGLs. In spite of the potential capacity, OGLs are hardly synthesized in the leaves of seed-plants growing under optimal conditions, being the proportion of TGDG and TeGDG lower than 0.2% of the total polar lipids (Moellering et al., 2010; Gasulla et al., 2013). However, OGLs can be accumulated in the plastids of leaves in response to different kind of stresses (see Fig. 2 and Section 4).

2.2.2. Occurrence of OGLs in reproductive and storage structures

Literature on OGLs from non-photosynthetic tissues is very scarce and most studies are merely reports or methodological improvements rather than biochemical or physiological studies. Thus, the synthesis, roles and distribution of OGLs are rather unknown in tissues/organs such as fruits, seeds, flowers or tubers (within phanerogams) and in spores or rhizomes in other tracheophytes (ferns). Changes in TGDG upon ripening were studied in cherry tomato fruit (*Lycopersicon esculentum*) by Güçlü et al. (1989) (Table 1). In this experiment, TGDG decreased in parallel to MGDG during tomato ripening (e.g. reddening) and increased later upon fruit senescence (Güçlü et al., 1989). TGDG was also found in fruit flesh extract from pumpkin (*Cucurbita pepo*) by

Sugawara and Miyazawa (1999). The same authors highlighted the presence of TGDG also in potato tuber (*Solanum tuberosum*) with not-shown data (Sugawara and Miyazawa, 1999). But the presence of ααβ-TGDG in potato had already been evidenced by Galliard (1968). With regard to plastids from other plant tissues, OGLs were detected in amyloplasts from cultured cells of *Acer pseudoplatanus* and from cauliflower inflorescences (Alban et al., 1988). Besides, plastids isolated from petals of *Narcissus pseudonarcissus* were able to synthesize TGDG when incubated with UDP-Gal (Heemskerk et al., 1990), but no further studies were conducted to elucidate whether TGDG can be naturally synthesized in flowers.

Among seeds, the constitutive accumulation of OGLs has been reported in *Glycine hispida* (Radunz, 1976), *Triticum aestivum* (Morrison et al., 1978), *Oryza sativa* (Fujino and Miyazawa, 1979), *Vigna angularis* (Kojima et al., 1990), and *Avena sativa* (Wang et al., 2016) (Table 1). In oat kernel (*Avena sativa*), TGDG and TeGDG have been quantified as 8% and 0.3% of the total lipid fraction (Moreau et al., 2008) representing TGDG more than 5% of the total GLs fraction (Doehlert et al., 2010). When the anomeric configuration has been determined, TGDG from seeds appears frequently in the ααβ-configuration, e.g. in wheat, rice and *V. angularis* (Morrison et al., 1978; Fujino and Miyazawa, 1979; Kojima et al., 1990).

3. Two pathways for OGLs synthesis

Two types of enzymes are responsible for the synthesis of OGLs: the DGDG synthases, which are common to all photosynthetic organisms, and the SFR2 present only in the Streptophyta (See Section 1.2 and Fig. 2).

3.1. The DGDG synthase pathway

3.1.1. The origin and evolution of the DGDG synthases

Two different DGDG synthases emerged independently during evolution of photosynthetic organisms, DgdA and DGD (Hori et al., 2014) (Fig. 2). Both synthases produce DGDG by linking a galactose moiety to MGDG with an α-glycosidic bond (Sato and Murata, 1982; Kelly and Dörmann, 2002; Awai et al., 2007). The capacity to synthesize ααβ-TGDG has been demonstrated by *in vitro* assays for DGD (Kelly and Dörmann, 2002) but not yet for DgdA. However, based on current knowledge, DgdA is the most plausible candidate for OGLs synthesis in cyanobacteria (see Section 2.1 for synthesis of OGL in cyanobacteria). DgdA is ubiquitous in cyanobacteria and Cyanidiophytina, a basal group of Rhodophyta (Hori et al., 2016). Based on phylogenetic analyses, it has been proposed that the DgdA was originated from a SqdX –an enzyme that transfers sulfoquinovose to DAG– in a green non-sulphur bacterium before the emergence of cyanobacteria (Sato and Awai, 2016). The DgdA gene has been found in the plastid genome of some Rhodophyta (Awai et al., 2007; Sakurai et al., 2007; Sato, 2016) and in Glaucophyta species (Awai, 2015). On the contrary a DGD gene is encoded in the nuclear genome in other Rodophyta species (Bhattacharya et al., 2013), in diatoms (which are supposed to originate from a red algal secondary endosymbiosis) (Sato, 2016) and in the remaining algal groups and land plants. Because all the chloroplasts of Archaeplastida (plants, glaucophytes, green and red algae) are monophyletic, it has been proposed that the two types of DGDG synthases probably coexisted in a common ancestor, and one of them was independently selected early during the evolution of the different lineages (Sato, 2016). DGD genes underwent further functional divergence and led before the emergence of angiosperms into two DGD genes (*DGD1* and *DGD2*) (Hori et al., 2016). Thus, a simple question rises, if all the photosynthetic organisms have DGD synthase genes and synthesize constitutively DGDG (Fig. 1), why TGDG is only constitutively present in some species?
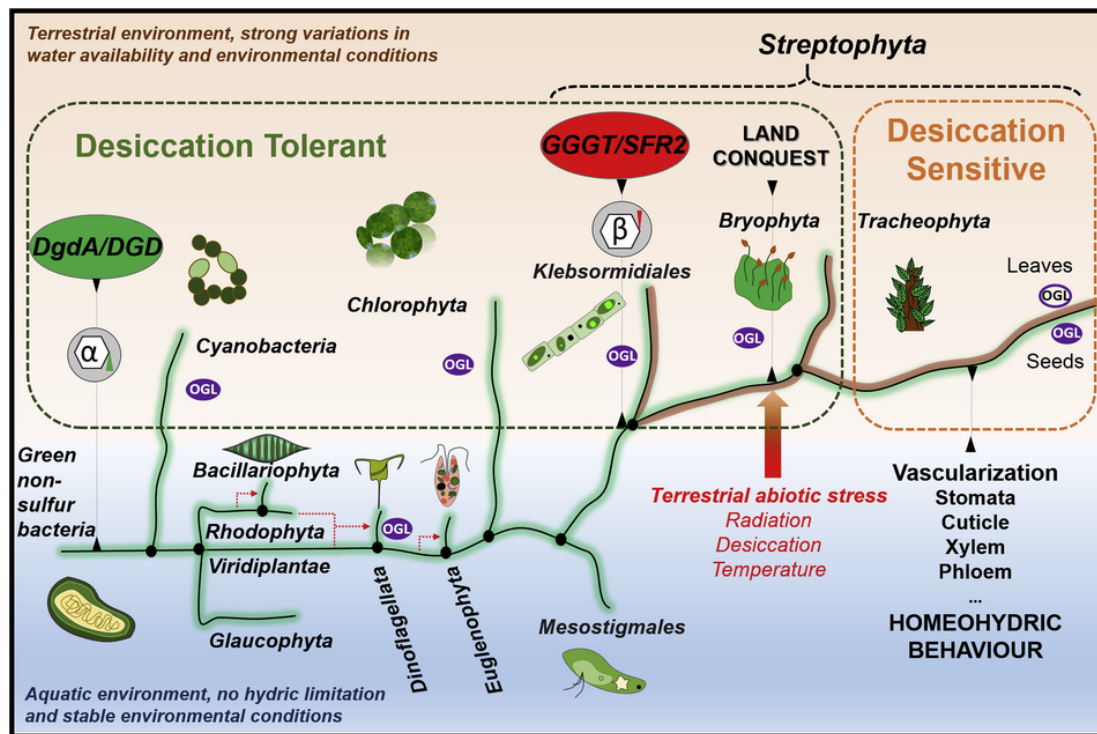
Fig. 2. Plant evolution and OGLs. Representative scheme of the appearance in the evolutionary tree of the two enzymes responsible for the synthesis of oligogalactolipids (OGLs). Only groups of photosynthetic organisms in which OGLs have been studied (Table 1) are represented in the figure. DgdA/DGD enzyme is present in all photosynthetic organisms, from green non-sulfur bacteria to angiosperms (shaded green branches). In cyanobacteria and algae with this enzyme, the synthesis of OGL is constitutive (closed purple circles) in terrestrial species, whereas OGL are not present in aquatic ones (with the exception of Dinoflagellates, see text). GGGT/SFR2 enzyme appeared for the first time in Klebsormidiales (Streptophyta) during land conquest and all photosynthetic organisms after this clade present the enzyme (shaded red branches). Despite the presence of the enzyme, OGL synthesis is still constitutive in Klebsormidiales and in bryophytes (closed purple circles). On the other hand, in tracheophyta, the synthesis of OGL is inducible in leaves (open purple circles), whereas is constitutive in reproductive structures (closed purple circles). Figure also shows the occurrence of DT among photosynthetic organisms. In general DT is widely distributed in less evolved terrestrial groups (dotted green box), becoming a rare feature in Tracheophytes, except in reproductive structures (dotted orange box). Blue background indicates aquatic environment. Brown background indicates terrestrial environment. Dotted red lines represent several secondary endosymbioses. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.1.2. The regulation of the DGDG synthase pathway

The cellular mechanism involved in the regulation of DGD is poorly understood and several hypotheses have been proposed. One possibility is that the activity of DGD is transcriptionally regulated. Thus, plants growing under phosphate deprivation accumulate DGDG in plastidial and extraplastidial membranes (Andersson et al., 2005; Jouhet et al., 2004; Härtel et al., 2000; Holzl et al., 2009; Kelly and Dörmann, 2002; Kelly et al., 2003) as a result of the increase in the expression of *dgd1* and *dgd2* genes (Kelly and Dormann, 2002; Kelly et al., 2003). Likewise, the up-regulation of DGD in leaves of maize during drought-induced senescence is related with an increase in the relative amounts of DGDG and the DGDG:MGDG ratio (Cheng et al., 2018). Radunz (1976) reported the presence of ααβ-TGDG by immunological techniques in the chloroplast of several dicotyledoneous species, which indicates that DGD can produce TGDG under non-stress conditions. Thus, it might be hypothesized that the constitutive synthesis of OGLs observed in some algae and cyanobacteria species -lacking SFR2- could be the result of a higher DGD activity. However, there are no evidences of a higher DGD synthase activity in those species since the ratio DGDG:MGDG is not related with the presence of OGLs (Suppl Table S1). In addition, Holzl et al. (2010) reported that the over-expression of DGD synthases in Arabidopsis did not cause any further DGDG accumulation. Thus, the species-specific levels of OGL in algae/cyanobateria seem to be the result of the coordination of DGD expression and other unknown control mechanisms.

In Arabidopsis, the TGD (TRIGALACTOSYLDIACYLGLYCEROL) enzyme is a protein involved in the transport of lipid precursors from the endoplasmic reticulum to the chloroplast. It was called with this name because Arabidopsis *tgd* mutants accumulate TGDG as consequence of the activation of the SFR2 enzyme (Awai et al., 2006; Lu et al., 2007; Xu et al., 2003, 2008, 2010). On the contrary, *Chlamydomonas tgd2* knock-out mutants do not synthesize TGDG (Warakanont et al., 2015), which suggests that DGD is not involved in the constitutive accumulation of OGLs in photosynthetic organisms lacking SFR2.

Another possibility is that the DGD is continuously producing OGLs while enzymes involved in their degradation are controlling the levels of OGLs in each species. Some candidates might be the galactolipases, enzymes that can hydrolyse GLs releasing free fatty acids and lyso-galactolipids. The activity and the gene expression of these hydrolytic enzymes are enhanced in vascular plants in response to drought stress, being potentially involved in MGDG degradation (Ferrari-Iliou et al., 1994; Gasulla et al., 2013; Matos et al., 2001; Torres-Franklin et al., 2007). Nevertheless, the role of galactolipases in the hydrolysis and control of OGLs levels is unknown.

In dinoflagellates, the fatty acid composition of TGDG is mostly 18:1/18:1, 18:1/16:0 and 18:1/14:1, while the major form of MGDG and DGDG is 18:5/18:5 (Gray et al., 2009a, b). Consequently, Gray et al. (2009a) proposed that TGDG could be produced by a very different biosynthetic pathway from that of MGDG and DGDG. In this sense, in the lichen-forming Chlorophyta *Asterochloris erici*, 34:1 and 34:2 TGDG are the most abundant forms, a fatty acid composition that is found neither in MGDG nor in DGDG (Gasulla et al., 2016). Up to the date, there are not genomic or physiological evidences of other DGDG/TGDG synthesis pathways in algae/cyanobacteria. However, the capacity of DgdA/DGD to produce OGLs has not yet been demonstrated *in vivo*,

and therefore, the possibility of the existence of an unknown enzyme that also synthetizes OGLs cannot be discarded.

3.2. The SFR2 enzymatic pathway

3.2.1. The origin and evolution of SFR2

Using the genomic data available, Fourrier et *al.* (2008) observed that the *SFR2* gene was present in bryophytes as well as in vascular plants. They suggested that this enzyme appeared in the emergence of land plants 400 million years ago (Mya), and therefore, it had a role in terrestrial life adaptation. More recently, Hori et al. (2016) have identified a *SFR2* homolog in *Klebsormidium flaccidum*, a charophyte alga that is an intermediate organism between green algae and land plants (Fig. 2). This result supports the hypothesis of Fourrier et al. (2008), but brings forward the date of SFR2 origin to 500–700 Mya, when the earlier Streptophytes conquered the land.

Phylogenetic analyses indicate that SFR2 is a member of the glycosil hydrolase family 1 (GH1) (Thorbly et al., 2004), identified in a wide variety of organisms, from bacteria to animals. This family includes structurally related enzymes that catalyze the hydrolysis of glycosidic bonds between two sugars, or between a sugar and a non-carbohydrate moiety (Henrissat, 1991). A characteristic of GH1 enzymes is that they conserve the anomeric configuration of the sugar at carbon 1 (Xu et al., 2004), being the SFR2 among the β-glucosydases (Thorbly et al., 2004; Moellering et al., 2010). However, several phylogenetic studies have demonstrated that SFR2 forms an independent clade, and that this enzyme is different from the remaining β-glucosydases (Thorbly et al., 2004; Fourrier et al., 2008; Hori et al., 2016). In addition, SFR2 has a predominant transferase rather than hydrolase activity, although its catalytic site is highly conserved with that of GH1 (Roston et al., 2014). The authors of this last study proposed that evolutionary pressure changed SFR2 from a hydrolase to a transferase by modifications external to the active site. The changes in the external residues could block the entrance of water to the catalytic site, avoiding the hydrolysis of the glycosyl-enzyme intermediate, and consequently the sugar is transferred to an alternate nucleophile when it enters in the reaction core (Roston et al., 2014).

3.2.2. The regulation of the SFR2 enzymatic pathway

The optimal conditions for the transferase activity of SFR2 were also assayed *in vitro* by Roston et al. (2014), who determined that the highest activity was reached at 24 °C, with a pH optimum of 7.5, and it was dependent of the presence of divalent cations, getting the strongest activation by Mg^{2+}. Within Streptophyta, the SFR2 protein is present in the outer envelope membrane of chloroplasts in any kind of tissue under all conditions (Thorlby et al., 2004; Barners et al., 2016). However, in plants growing under favourable conditions the levels of OGLs are very low, barely detected by TLC or MS techniques and the synthesis of OGLs is only activated in response to different stresses including freezing, wounding, drought, desiccation and salinity stress (see Section 4). The protein and mRNA levels of SFR2 did not change in response to cold (Thorlby et al., 2004; Barnes et al., 2016) or salt stress (Wang et al., 2016), indicating that its activity is non-transcriptionally regulated. Finally the activation of SFR2 is not triggered by interactions with other proteins but by changes in cytosolic pH and Mg^{2+} concentration in plant leaves and roots (Roston et al., 2014, Barners et al., 2016; Wang et al., 2016).

Two main studies (Barnels et al., 2016, Wang et al., 2016) have demonstrated the existence of a common regulation pathway of SFR2 in response to different kind of osmotic stress. Freezing, drought and salt stress are conceptually closely related since all of them result in a decrease of water availability for plant cells (Andrews, 1996; Verslues et al., 2006). The vacuoles are the main reservoir of Mg^{2+} in plant cells and control the ion homeostasis in the cytosol and chloroplast

(Marschner, 1995). While the concentration of Mg^{2+} in the cytosol and chloroplast ranges from 2 to 10 mM (Leigh and Wyn Jones, 1986) –actually, the concentration of free Mg^{2+} might be 10-fold lower (Yazaki et al., 1988)– in the vacuoles the levels of Mg^{2+} vary between 3 mM to more than 120 mM (Shaul, 2002). On the other hand, the cytoplasm is less acidic (pH 7.3–7.6) than the vacuole (pH 4.5–5.9) and the extracellular spaces (pH 5.5) (Kurdjian and Guern, 1989). Thus, as Wang et al. (2016) suggested, independently of the osmotic stress origin, the rupture of membranes during cell dehydration can cause the leakage of small ions, like Mg^{2+} and protons, triggering the activation of SFR2, at the outer chloroplast membrane.

4. Adaptive meaning of OGLs and their response to stress

Sixty years after the discovery of OGLs in plants, the evolutionary origin and functional role of these lipids is still a subject of debate (see Section 5). Several evidences lead to the conclusion that OGLs must have a physiological role related to adaptive responses to environmental stresses like freezing (Moellering et al., 2011; Arisz et al., 2018), desiccation (Gasulla et al., 2013) or osmotic stress (Wang et al., 2016). According to literature, the synthesis of OGLs can be constitutive or inducible. In vascular plants, the capacity to synthetize OGLs is ubiquitous but inducible in vegetative organs (leaves and probably roots), where the accumulation of significant amounts of OGLs is triggered by stress. In the rest of photosynthetic organisms, the capacity to synthesize OGLs in their vegetative cells seems to be constitutive and species-specific (Leblond, 2009; Gasulla et al., 2016; Awai, 2016, see Table S1).

4.1. Constitutive presence of OGLs

Among the primitive cyanobacteria, TGDG has been found to be accumulated in the vegetative cells of *Tolypothrix tenuis* (Zepke et al., 1978) and *Anabaena* sp. PCC 7120 (Awai, 2016), two strong desiccation tolerant (DT) species (Silva et al., 2007; Singh et al., 2013), and in *Oscillatoria chalybea* (Zepke et al., 1978) a filamentous cyanobacteria that grows in soil crusts (Vinoth et al., 2017) and small ponds (Stoyanov et al., 2016) and that therefore, is supposed to withstand desiccation. On the contrary, the vegetative cells of *Anabaena cylindrica* and *Nostoc calcicola* -two cyanobacteria species in which Zepke et al. (1978) did not detected TGDG-, lose rapidly their viability after complete dehydration (Yamamoto, 1975; Agrawal and Singh, 2002). However, these species can produce akinetes that are resting spores tolerant to extreme physical conditions such as, heat, UV or desiccation. It could be interesting to determine whether TGDG is also accumulated in these resistance structures.

Among unicellular eukaryotes, some species do not accumulate TGDG. This is the case of *Euglena gracilis* (Euglenophyta), a fresh-water unicellular flagellate that does not contain OGLs (Blee and Schantz, 1978) and in which either vegetative or resistance cells are not DT (Malik, 1993; Strauch et al., 2017; Wieners et al., 2018). In Chlorophyta, two Trebouxiophyceae species have been found to accumulate OGLs in vegetative cells: *Asterochloris erici* (Gasulla et al., 2013), a lichen symbiotic algae strongly DT (Gasulla et al., 2009), and an undetermined *Chlorella* sp. (Benson et al., 1958). *Chlorella* is a cosmopolitan genus living in both aquatic and terrestrial habitats, and many species can withstand desiccation (Gray et al., 2007; Lüttge and Büdel, 2010). On the contrary, the model alga *Chlamydomonas reinhardtii*, a chlorophyta that grows in temperate soils in North America and Japan, does not accumulate OGLs (Vieler et al., 2007; Mendiola-Morgenthaler et al., 1985) and their vegetative cells are desiccation sensitive (DS) (Lewin, 1951; Harris, 1989). In the case of *Cyclotella meneghiniana* the presence OGLs has been suggested but not confirmed (Vieler et al., 2007). This is a common planktonic diatom that grows in streams and

rivers (Stevenson, 1996; Shafik et al., 1997) but it has also been found in soil crusts of entrance caves growing together with desiccation-tolerant algae (Poulíčková and Hašler, 2007).

The relationship between DT and OGLs is not so clear in dinoflagellates. Gray et al. (2009a, b) suggested that TGDG could provide an advantage for cold adaptation, since the cold-adapted *Gymnodinium* sp. C5 accumulated nearly four times more than warm-adapted species. However, this hypothesis is not consistent with their results since not all cold-adapted species accumulated OGL while some warm-adapted species did it (Gray et al., 2009a, b). The possibility that the high TGDG levels in *Gymnoidinium* could be involved in desiccation adaptation cannot be discarded since some *Gymnodinium* species inhabit intertidal zones of sandy shores (Zubizarreta, 2005). On the contrary, universal presence of TGDG in *Pyrocystis* sp. cannot be directly related with DT since these algae are part of the epipelagic plankton in marine waters. Thus, further studies are needed to determine the biological/ecological factors that could trigger the synthesis of OGL in dinoflagellates, like the formation of dormant (and potentially DT) cysts (Delwiche, 2007).

Within the charophyte algae, TGDG has been detected in *Klebsormidium flaccidum* (Hori et al., 2014), which is an intermediate organism between green algae and land plants (McCourt et al., 2004; Delwiche and Cooper, 2015). Curiously, Hori et al. (2016) found that the genome of *K. flaccidum* contains a *SFR2*-like gene but TGDG synthesis is constitutive, with TGDG accounting for 3.12 mol% of all membrane lipids under optimal growth conditions. It is not possible to conclude whether the TGDG was synthesized via the SFR2 pathway or via DGD, since the anomeric configuration of the glycosydic bonds was not determined (see Section 1 and Fig. 1). How to explain then the constitutively high level of OGLs in *K. flaccidum* despite the presence of SFR2 enzyme in this species? The key in the answer to this question could be based on the strategic evolutionary position of Klebsormidiaceae in the conquest of land from fresh-water environments. Klebsormidiophyceae diverged after the Mesostigmatophyceae class (Leliaert et al., 2011). While this latter group uncovers only unicellular freshwater flagellate algae, the first comprises both aquatic and terrestrial pluricellular filamentous species. In fact, the genus *Klebsormidium* is one of the most abundant and diverse microautotrophs in various terrestrial and aerophytic habitats in temperate zones (Ettl and Gärtner, 1995; Lokhorst, 1996; John, 2002, 2003) and several species are tolerant to desiccation and/or to freezing (Elster et al., 2008; Holzinger and Karsten, 2013; Karsten and Holzinger, 2014; Donner et al., 2017). Thus, it is likely that a primitive ancestor of the current *Klebsormidium* species developed strategies to respond quickly to rapid environmental changes that can undergo in aeroterrestrial habitats. Interestingly, in that sense, it has been recently postulated that rehydration rather than temperature acts as driver of photosynthetic adaptations within the Klebsormidiophyceae class (Pierangelini et al., 2018). Thus, presumably, the action of the enzyme SFR2 and, in addition, the constitutive presence of OGLs, would have provided an evolutionary advantage with respect to desiccation in the terrestrial environment to this basal group of Streptophytes. This hypothesis is reinforced by the fact that several seed-plants (recent streptophytes) are able to synthesize $\alpha\alpha\beta$-TGDG (thus, through DGD enzyme) (Table 1) despite having the SFR2 enzyme (see Section 3.2).

Overall, the ecology of the algae employed in the OGLs studies is very diverse since they have different lifestyles, from aquatic to aeroterrestrials, from benthic to planktonic, in free-life or in symbiotic associations, and can be found in temperate or cold regions. However, interestingly, a common ecological feature, frequent among those algae that accumulate OGLs constitutively, is that they cope with cyclic desiccation periods in their natural habitats.

Regarding bryophytes, the most basal group within Embryophytes, available data is very scarce, making extremely speculative to assign a

constitutive or inducible character to the presence of OGLs. When Gellerman et al. (1974) analysed gametophytes of several field-collected mosses, they found that all them contained TGDG. This finding could be interpreted as a constitutive character. Nevertheless, this cannot be directly concluded from the experimental design (lacking a dehardening treatment), since mosses in the field could have been exposed to natural stress conditions (particularly desiccation) able to trigger synthesis of OGLs. The relevance of dehardening in bryophytes concerning interpretation of desiccation-induced responses has been recently reviewed in Stark (2017). In fact, considering that *sfr2* gene has been sequenced in several liverworts and mosses, it is very reasonable to expect a role for the SFR2 in the inducible synthesis of OGLs in bryophytes.

Pteridophytes (ferns) are the oldest evolutionary group included within the tracheophytes (vascular plants), having the ability to regulate their water content (homeohydric organisms). This is the case for the dominant generation, the sporophyte. Nevertheless, dispersive structures (the spores) and the resultant gametophyte generation are typically poikilohydric. The lipid composition has been analysed in the spores and gametophytes (López-Pozo et al., 2019) of the fern *Osmunda regalis*. This fern produces chlorophyllous spores able to cope with desiccation (López-Pozo et al., 2019). Interestingly these spores contained high amounts of OGLs (TGDG and TeGDG) that decreased rapidly in the subsequent developmental stage: the well-developed gametophyte, which is DS. More studies are needed to determine the OGL origin of the spores and the possibility of the inducible SFR2 activity in the fronds in response to dehydration.

Among "seed-plants" the capacity to synthesize constitutively OGLs is restricted to non-photosynthetic plastids of seeds, fruits or tubers (see Table 1). All these organs play storage functions having the ability to resist unfavourable conditions such as drought or adverse temperatures. Several studies have shown that the third galactose of TGDG is in the alpha configuration (see Table 1), indicating that OGLs are synthesized via the DGD pathway in these organs. Probably, the synthesis of OGL is programmed and contributes to confer a permanent cell tolerance to osmotic stresses like desiccation or freezing. On the contrary, the photosynthetic organs synthesize only very little amounts OGL constitutively when plants are growing under favourable conditions. Under the light of those evidences, for the case of homeohydric species, it is reasonably to hypothesise that (i) the constant presence of protective mechanisms against dehydration, like OGLs, are unnecessary in leaves and (ii) the induction of such mechanisms only upon detection of an osmotic stress (eg. SFR2 activity) could have been ecologically and evolutionary more advantageous.

4.2. Inducible synthesis of OGLs

The inducible synthesis of OGLs in photosynthetic tissues of angiosperms is activated in response to different stresses such as ozone (Sakaki et al., 1990), wounding (Vu et al., 2014, 2015), freezing (Moellering et al., 2010; Barnes et al., 2016; Arisz et al., 2018), drought (Wang et al., 2016), desiccation (Gasulla et al., 2013) or salinity (Wang et al., 2016). As specified in Section 3.2.2, the disruption of membranes upon different kinds of stress could lead to the leakage of small ions, like Mg^{2+} and protons into the cytoplasm triggering the activation of SFR2. Interestingly, in that sense, is the fact that the universal plant stress hormone ABA, which is typically involved in stress signalling upon drought and desiccation, but also in O_3-related plant responses (Zhang et al., 2019), can induce cytoplasm acidification (Beffagna et al., 1997). Consequently, a cell-homeostasis disruption orchestrated by a complex interplay of different actors (hormones and other signalling molecules, membrane, ions, pH, etc.) could be the base for a common activation SFR2 mechanism in response to different stress factors.

4.2.1. Inducible synthesis of OGLs by ozone

The first evidences of the connection between OGLs and environmental stress were obtained by Sakaki et al. (1990) who treated spinach leaves with ^{14}C-acetate with the aim to confirm that the TAG produced in these treated leaves came mainly from species of MGDG via the GGGT pathway. After ozone fumigation, radioactivity in MGDG decreased, whereas in TAG, DAG and OGLs increased. The proportion of 16:3 content in TAG confirmed its MGDG origin (Sakaki et al., 1985, 1990). Thus, the results confirmed that the GGGT activity was induced in response to ozone in spinach leaves. SFR2 does not respond directly to oxidative stress (Barners et al., 2016), however ozone could affect SFR2 activity through changes in cytosolic pH and/or Mg^{2+}, as consequence of membrane damage.

4.2.2. Inducible synthesis of OGLs by wounding

Recent works have also evidenced that an increase in OGLs can be activated by disruption of cell membranes during wounding (Vu et al., 2014, 2015). *in vitro* experiments, where Arabidopsis leaves were subjected to a mid-vein pressure wounding, have shown significant rise of TeGDG 45 min after the stress and further increase of both TGDG and TeGDG six hour after the treatment (Vu et al., 2015). This took place in parallel to TAG increase upon other lipid rearrangement in the cells.

4.2.3. Inducible synthesis of OGLs by freezing

Most of the information currently available on the stress-induced regulation of OGLs synthesis refers to the effects of freezing on SFR2 activity. In fact *SFR2* gene was identified as one of the genes needed for freezing tolerance (see Section 1.2). Moellering et al. (2010) exposed *A. thaliana* plants to cold acclimation and to freezing and no OGLs were synthesized during cold acclimation in both WT plants and *sfr2* mutants. Nevertheless, WT (but not *sfr2* mutants) accumulated OGLs after the onset of freezing stress. TAG levels were increased upon freezing only in WT plants, whereas the *sfr2* mutants half reduced the amounts of TAG. The NMR spectra of DGDG synthesized *in vitro* by SFR2 showed that the linkages were all in the β-anomeric configuration (ββ-DGDG), confirming that β-glycosidase, and not DGD, was the enzyme responsible of OGLs synthesis in WT in response to freezing (Moellering et al., 2010; see Fig. 1). The concentration of SFR2 does not change upon freezing stress but is rather post-translationally activated by pH and Mg^{2+} (Barnes et al., 2016). In agreement with the results obtained in *A. thaliana*, freezing but not cold-acclimation induced a significant accumulation of TGDG and TeGDG in *Boechera stricta* (a perennial species close relative to Arabidopsis) (Arisz et al., 2018). Based on the concomitant decrease in MGDG, the authors postulated that an enzymatic activity SFR2-like is responsible of the OGLs increase (Arisz et al., 2018).

4.2.4. Inducible synthesis of OGLs by drought and salinity

Wang et al. (2016) found a SFR2 in tomato (*Sl*SFR2) with GGGT activity comparable to its *Arabidopsis* ortholog (*At*SFR2) and obtained evidences for its role in drought and salinity tolerance. The response of WT tomato leaves to drought and salt stress was characterised by an increase in OGLs whereas mutants depleted in *Sl*SFR2 did not synthesize them. Under these stress conditions only WT tomato plants were able to grow. Thus, Wang et al. (2016) concluded that both enzymes may act differently in their species of origin e.g. enhancing freezing tolerance in the freezing-tolerant species *A. thaliana*, but enhancing tolerance to salinity and/or drought in the freezing-sensitive *Solanum lycopersicum*. Despite of it, *At*SFR2 and *Sl*SFR2 seem to act similarly at the biochemical level, e.g: once the stress appears. Interestingly, *Sl*SFR2 exhibited about 2-fold higher activity than *At*SFR2. The similarity of sequences between *Sl*SFR2 and AtSFR2 was only 72% (Wang et al., 2016)

and this could allow enough diversity to explain the difference in the activity of the enzymes.

4.2.5. Inducible synthesis of OGLs by desiccation

Finally, the induction of OGLs synthesis has been evidenced in response to desiccation. Gasulla et al. (2013) carried out a lipidomic comparative approach using plants differing in desiccation tolerance (DT) to determine the protective role of lipids in dried cells. The results of the analysis showed that the synthesis of OGLs by severe dehydration seems to be universal in seed plants, although the levels of OGLs were higher in DT plants (*Craterostigma plantagineum* and *Lindernia brevidens*) than in DS species (*L. subracemosa* and *A. thaliana*). The TGDG produced in *C. plantagineum* was βαβ-TGDG indicating that SFR2 was responsible for the addition of a third galactose to a αβ-DGDG and therefore for the decrease of the MGDG and the increase of OGL upon desiccation (Gasulla et al., 2013).

5. Desiccation tolerance and chloroplast membranes: changes in OGLs and beyond

Desiccation tolerance refers to the capability of some organisms (or organs) to withstand severe intracellular dehydration, equivalent to water potentials of −50 MPa or even lower (Hoekstra et al., 2001; Gaff and Oliver, 2013). The DT in reproductive structures is relatively frequent among many taxa of photosynthetic organisms while the DT in photosynthetic tissues (vegetative cells in the case of unicellular organisms) is much more restricted among taxa, and progressively less frequent in phylogenetically recent groups (e.g. tracheophytes) (Gaff and Oliver, 2013). This has been interpreted as a loss of ecological relevance of DT in photosynthetic tissues (but not in reproductive structures) along with the development of homeohydric characters (vascular system, cuticles, stomata, etc.) during evolution of streptophytes (Oliver et al., 2000). Thus, DT-mechanisms have traditionally been considered as predominantly constitutive in poikilohydric organisms, while predominantly inducible in DT-tracheophtyes (Oliver and Bewley, 1997), with nuances (Proctor and Tuba, 2002). DT of photosynthetic cells has been reported in Cyanophyta, Bacillariophyta, Phaeophyta, Rhodophyta, Chlorophyta and Streptophyta and has still been pretty understudied in some other groups such as the dinoflagellates (Fernández-Marín et al., 2016; Gaff and Oliver, 2013) (Fig. 2).

Although physiological and cellular processes that contribute to the DT of photosynthetic tissues have been more deeply studied in species within Streptophyta (including seed-plants) and Chlorophyta, general response seems to be similar across organisms. Thus, both mechanical strains (e.g. protein denaturalization and membrane fusion) and oxidative stress represent the major cell risks upon desiccation and rehydration cycles being the oxidative pressure exacerbated in chlorophyll containing tissues (Hoekstra et al., 2001; Sahsah et al., 1998; Fernández-Marín et al., 2016). Considering that a dynamic plastid adjustment became particularly relevant upon land colonization by plants and that most evidences available so far relate inducible synthesis of OGLs with SFR2 activity upon either osmotic or oxidative stress (See Section 4) we hypothesise that SFR2 likely evolved as an adaptation to DT and terrestrial-life challenges. Reversible restructuration of the plastids plus an efficient set of energy dissipation and antioxidant systems are crucial to preserve the integrity of the whole cell upon desiccation (Verhoeven et al., 2018). Overall changes in ultrastructure during drying of DT-chloroplasts (desiccoplasts) consist of: sphere-shaped chloroplasts (Fernández-Marín et al., 2016; Proctor et al., 2007), increased number of plastoglobules (Fernández-Marín et al., 2013; Holzinger et al., 2011), alterations in the pattern of thylakoid stacking (Charuvi et al., 2019; Fernández-Marín et al., 2013), and accumulation of unknown electrodense substances in the lumen (Fernández-Marín et al., 2016; Georgieva et al., 2010). Very recently, the formation of new type of

vesicles in the outer envelope of the chloroplast has been reported during drying of the angiosperm *Craterostigma pumilum* and related to a senescence-like process (Charuvi et al., 2019). Overall these changes highlight how the remodelling of plastid membranes plays a key role on the preservation of functional chloroplasts during drying. The synthesis of OGLs in particular and/or the activation of SFR2 in general terms (e.g. leading to a decrease in MGDG proportion) likely play key roles in the safe preservation of plastids at extremely low water contents.

5.1. What is the "raison d'être" for OGLs in desiccoplasts?

As stated in preceding sections there is no doubt that the presence of OGLs in photosynthetic organisms is linked to the occurrence of environmental stresses, in particular desiccation (Section 4.2). Biological logic would suggest an adaptive meaning for such relationship between OGLs and stress, but experimental evidences are completely missing. On the one hand, OGLs could be simply a by-product of the enhanced synthesis of DGDG that forms part of the stress-induced lipid remodelling of chloroplasts membranes. If this is the case, one might wonder why two different enzymatic systems (DGD and SFR2) obstinately produce OGLs, even when from $\beta\beta$-DGDG the reversible reaction back to MGDG could be expected, rather than the further SFR2 activity towards $\beta\beta\beta$-TGDG synthesis (Roston et al., 2014). On the other hand, OGLs could play a genuine protective role. In the absence of any direct mechanistic evidence, at least two specific functions for OGLs can be proposed. First, the accumulation of OGLs could contribute to the preservation of chloroplast membranes since the large polar-heads prevent fusion of membranes during cellular dehydration and also because OGLs enhance the ratio of bilayer against non-bilayer forming lipids (Gasulla et al., 2013; López-Pozo et al., 2019). Second, the large galactosyl polar-heads could specifically interact with late embryogenesis abundant proteins (LEA proteins), which accumulate in desiccoplasts (Hoekstra et al., 2001) contributing to membrane stabilization (Navarro-Retamal et al., 2018).

5.2. Protection of desiccoplasts mediated by SFR2 activity

Additionally, SFR2 activity may directly contribute with other protective processes in desiccoplasts, mainly by regulating the content of MGDG and DGDG. MGDG are the only non-bilayer forming lipids in thylakoid membranes (their presence favours the formation of reversal-hexagonal phases H_{II}), and their proportion (ratio of non-bilayer and bilayer forming lipids) together with the protein content and composition greatly determines membrane properties (Holz and Dörmann, 2007) (See Section 1.1). Thus, MGDG are indispensable for the membrane fluidity needed for photochemical activity and they directly interact with photosynthetic protein complexes (Jordan et al., 2001; Loll et al., 2007). In that sense, it has been demonstrated that the lack of MGDG and DGDG provokes a decrease on chlorophyll content and photosynthetic efficiency (Jarvis et al., 2000; Dörmann et al. (1995); Hartel et al., 1997; Reifarth et al., 1997; Steffen et al., 2005). Nevertheless, under stress (mostly osmotic stress) the presence of MGDG favours the formation of H_{II} which can lead to membrane fusion and irreversible damage to membranes that must be prevented. Thus, overall, it is expected that the MGDG will be dismantled once the stress appears, on the one hand, in order to reduce photosynthesis and thus oxidative damage and, on the other hand, to diminish the risk of membrane fusion. Accordingly, lower MGDG/DGDG ratio is related to stress conditions (Torres-Franklin et al., 2007; Gasulla et al., 2013). In DT plants in particular, the decrease in MGDG upon severe dehydration occurs in parallel to a rise in TGDG and TeGDG (Gasulla et al., 2013). In the same sense, the reverse process has been observed upon rehydration (López-Pozo et al., 2019). Although both enzymes DGD and SFR2

could contribute to increase DGDG content, SFR2 activity may represent and advantage because reduction in MGDG and increase in DGDG would take place in the same single enzymatic reaction (Fig. 1).

In addition, it has been suggested that the elimination of MGDG by SFR2 may also help in diminishing the total membrane surface upon desiccation. This may contribute to the safe biophysical readjustment of membranes during the reduction in cell volume associated with dehydration (Moellering et al., 2011). The by-products of SFR2 enzymatic reaction (e.g. DAG which is rapidly converted to TAG) could also be a secondary role for SFR2. Upon stress DAG would lead to the accumulation of membrane degradation residues into energy storage molecules (TAG) that could be of use after stress. In that sense, accumulation of TAG has been found during drying in DT plants (Gasulla et al., 2013) and its consumption upon rehydration was observed in DT-fern spores (López-Pozo et al., 2018). Alternatively, DAG could be used in the phospholipid metabolism as has been suggested in Vu et al. (2015).

5.3. Indirect roles of SFR2 activity in desiccoplasts

Not only GLs, but also all major components of chloroplast membranes such as macromolecular protein complexes and carotenoids, are coordinately altered/rearranged upon drying of DT-chloroplasts. In that sense, the SFR2 activity likely plays indirect additional roles in plastid protection. Dehydration induces the synthesis of Z in chloroplasts of DT-organisms of a broad representation of phylogenetic groups including bryophytes, pteridophytes, angiosperms, chlorophytic algae and brown macroalgae (Augusti et al., 2001; Calatayud et al., 1997; Fernández-Marín et al., 2009, 2010, 2011a, 2013; Kranner et al., 2002, 2003; Xie et al., 2013). This response is shared against freezing stress, at least in angiosperms (Fernández-Marín et al., 2018; Verhoeven et al., 2018). The increase in Z induced by desiccation is mediated by VDE activity (Fernández-Marín et al., 2009). In parallel to this generalised response, drying induces a decrease in the relative amount of MGDG (Gasulla et al., 2016, 2013; Navari-Izzo et al., 1995). An apparent contradiction in the co-occurrence of both processes rises in the fact that MGDG is required for VDE operation in the thylakoid (Garab et al., 2016; Schaller et al., 2010). However, the presence of MGDG-enriched domains with active VDE has already been proved in angiosperms (Goss et al., 2017). Thus, the occurrence of highly enriched MGDG domains within desiccating thylakoids could be a plausible conciliation of both processes (decrease in MGDG and increase in Z) involved in thylakoid rearrangement upon dehydration of DT-organisms. In agreement with this, freeze-fracture cryo-SEM analyses have revealed the parallel formation of (i) H_{II} lipid domains and (ii) ordered arrays of PSII core complexes in thylakoid membranes when the DT-plant *Craterostigma pumilum* is desiccated at relative water contents below 30% (Charuvi et al., 2015). These observations demonstrate that the formation of H_{II} regions in the thylakoids is possible and actually does occur under severe dehydration of DT-chloroplasts. These data also evidence the close interaction between the composition/proportion of GLs and the content/arrangement of other thylakoid components (proteins) as an integrative response to stress. In particular, the formation of H_{II} phases could be, on the one hand the consequence of the decrease in protein concentration (e.g. LHCII complexes), since the presence of LHCII prevents H_{II} formation (Simidjiev et al., 2000), and on the other hand the cause of ordered PSII complexes arrays, since H_{II} establishment can change the lateral membrane pressure altering the microenvironment of the transmembrane proteins (Kirchhoff et al., 2007).

6. Overall view

The first fossil evidences of life on the Earth are the stromatolites formed by cyanobacteria that are about 3.4–3.7 billion years old

(Nutman et al., 2016). Archean stromatolites inhabited intertidal zones where they underwent cyclic desiccation periods (Martin et al., 1980; White, 1984). Therefore, cyanobacteria can be considered the first photosynthetic organisms that successfully survive to direct exposition to the primitive earth atmosphere. Consequently, cyanobacteria had to develop molecular mechanisms to protect all the cellular structures from nearly absolute dehydration during air-drying, among others, cyanobacteria acquired the capacity to produce OGLs (Fig. 2). This protection mechanism was inherited by algae after the symbiotic event in which a heterotrophic host cell captured an ancestral cyanobacterium (Archibald, 2009; Keeling, 2010) giving rise to all groups of photosynthetic eukaryotes. However the dry land remained devoid of life during three billion years, until a monophyletic group of streptophytic algae initiated land colonization (Fig. 2). In this regard, Becker (2013) proposed that "*the Precambrian glaciation event that occurred around 650 Mya, the so-called Gaskier glaciation or snowball earth (a period when the earth was almost completely covered by ice), might have been the trigger for the colonization of the terrestrial habitats by streptophyte algae. Under such drier and colder conditions, freshwater bodies might have dried, forcing organisms to adapt to the hostile terrestrial environment*".

The conquering of land by plants implied the challenge of facing various severe environmental stresses (extreme temperatures, UV radiation, desiccation, freezing,..) (de Vries and Archibald, 2018). More importantly, terrestrial habitats compared to water bodies are much more unstable and unpredictable, and organisms have to adapt to much faster changes than in the highly buffered ocean (Fig. 2). This requires precise environmental sensors and efficient signalling routes. Some of those preadaptations have been identified and proposed (for a recent review see deVries et al., 2016) such as cell walls with xyloglucans, presence of symbiotic genes, UVB protection and repair mechanism or fast photoprotection responses. Consequently, the toolkit of the ancestor streptophyte that initiated land colonization was probably equipped with a huge set of exaptations, including the ability to respond dynamically to fast environmental fluctuations. An example of such evolutionary trend is the transition from a rigid LHCSR-based photoprotective energy dissipation to a more flexible PSBS-based mechanism in Streptophytes, ending with a complete loss of LHCSR in tracheophytes (Gerotto and Morosinotto, 2013). Likewise the appearance of SFR2 likely empowered Streptophytes with the ability to quickly respond to environmental fluctuations through an adaptive chloroplast lipid remodelling. Moreover, the potential cross-tolerance to desiccation and freezing provided by this mechanism was probably a selective advantage in aeroterrestrial habitats. Simultaneously, the constitutive synthesis of OGLs was maintained in reproductive structures, such as spores and seeds, adapted for dispersal and for survival under unfavourable conditions until germination.

This evolutionary proposal fits with a model in which the presence of OGLs is constitutive in poikilohydric DT species and consequently is product of a DGDG synthase activity, while in homoiohydric vascular plants OGLs are a response to stress synthesized by inducible SFR2 activity (Fig. 2). The scarce information available supports this general pattern, but a much higher effort has to be done, particularly on the study of the anomeric configuration of the glycosidic linkage, to confirm or discard it. Despite the biosynthetic machinery employed by plants and algae, the question of whether OGLs are the end-product of the route, and consequently have an adaptive meaning, or they are just a by-product of DGDG synthesis, remains open. Nowadays, correlative data support a role for OGLs on stress tolerance, but the mechanistic evidences are weak. Furthermore, in case of being a genuine protective mechanism, the payback of such protection in terms of photosynthetic efficiency still needs to be elucidated.

Author statement

All authors contributed equally to this manuscript

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.envexpbot.2019.05.003.

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