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Acquisition of Desiccation Tolerance Unveiled: Polar Lipid Profiles of Streptophyte Algae Offer Insights

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Abstract

Terrestrialization by photosynthetic eukaryotes took place in the two branches of green microalgae: Chlorophyta and Charophyta. Within the latter, the paraphyletic streptophytic algae divide into two clades. These are named Klebsormidiophyceae-Chlorokybophyceae-Mesostigmatophyceae (KCM), which is the oldest, and Zygnematophyceae-Coleochaetophyceae-Charophyceae (ZCC), which contains the closest relatives of vascular plants. Terrestrialization required the emergence of adaptations in response to new challenges, such as irradiance, temperature oscillations and water deprivation. In this study, we evaluated lipid composition in species representative of distinct phylogenetic clusters within Charophyta and Chlorophyta. We aim to study whether the inherent thylakoid lipid composition, as well as its adaptability in response to desiccation, were fundamental factors for the evolutionary history of terrestrial plants. The results showed that the lipid composition was similar to that found in flowering land plants, differing only in betaine lipids. Likewise, the largest constitutive pool of oligogalactolipids (OGL) was found only in the fully desiccation-tolerant species *Klebsormidium nitens*. After desiccation, the content of polar lipids decreased in all species. Conversely, the content of OGL increased, particularly trigalactosyldiacylglycerol and tetragalactosyldiacylglycerol in the ZCC clade. The analysis of the molecular species composition of the newly formed OGL may suggest a different biosynthetic route for the KCM and ZCC clades. We speculate that the appearance of a new OGL synthesis pathway, which eventually arose during the streptophyte evolutionary process, endowed algae with a much more dynamic regulation of thylakoid composition in response to stress, which ultimately contributed to the colonization of terrestrial habitats.

1 | INTRODUCTION

Green algae refer to a varied and paraphyletic group of organisms, primarily found in aquatic habitats but with some species also living in aeroterrestrial environments. The phylogenetic origin of algae dates back to more than 1.5 billion years ago when a symbiotic event in which a photosynthetic prokaryotic organism was engulfed by a non-photosynthetic eukaryotic host cell gave rise to the first photosynthetic eukaryote (Archaeplastida). There are three groups of Archaeplastida: glaucophytes, red algae (rhodophytes) and green algae (chlorophytes)

(Keeling, 2010). Approximately one billion years ago, green algae split into two main lineages: Chlorophyta and Streptophyta (Becker, 2013). Although the former is the group that includes the best-known algal species (*Ulva*, *Chlamydomonas*, *Chlorella*, *Volvox*, *Trebouxia*, etc.), the latter is the group that gave rise to embryophytes (de Vries et al., 2016). Streptophyta includes mostly freshwater algae and is grouped into two clades: the oldest KCM clade, including the classes Klebsormidiophyceae, Chlorokybophyceae and Mesostigmatophyceae, and the most recently evolved ZCC clade, including the classes Zygnematophyceae, Coleochaetophyceae and Charophyceae. The last group includes the closest relatives of embryophytes, the group that succeeded in dominating all terrestrial habitats, later evolving into land plants. The conquest of terrestrial habitats, also known as terrestrialization, brought new challenges for photosynthetic organisms (Lang et al., 2008). The closest relatives of terrestrial plants are supposed to have been empowered by a set of physiological tools, frequently acquired by a process of exaptation (de Vries et al., 2016; de Vries & Archibald, 2018), which allowed the final establishment on dry land. Among the physiological tools, these organisms acquired stronger cell walls to counteract gravity and wind, UV-B absorbing phenylpropanoids (de Vries et al., 2017) or environmentally tuned stress-signalling pathways (de Vries et al., 2018; Fürst-Jansen et al., 2020).

A pivotal strategy in the evolutionary transition from aquatic to terrestrial habitats is likely the acquisition of tolerance to desiccation in vegetative cells. It is interesting in that sense that mainly Zygnematophyceae but also Choleochaetophyceae, Chlorokybophyceae and Klebsormidiophyceae comprise taxa that live in terrestrial habitats that consequently are naturally exposed to stressors such as desiccation (Holzinger & Pichrtová, 2016). This had to be particularly relevant in the initial steps of terrestrialization, given that the first terrestrial organisms lacked anatomical structures to prevent water loss (i.e., they were poikilohydric). A likely outcome thereof is that photosynthetic tissues tolerant to desiccation (i.e., able to equilibrate their water potential to that of the environment but also able to re-establish metabolism upon rehydration) are rather common in evolutionarily ancient groups, including cyanobacteria and algae (Gaff & Oliver, 2013). Several eukaryotic groups have developed the capacity to tolerate dehydration, particularly in intertidal, freshwater and aeroterrestrial habitats. This is the case for certain brown algae (Fernández-Marín et al., 2011), red algae (García-Plazaola et al., 2022), and green algae (Holzinger & Karsten, 2013). Within Chlorophyta, desiccation tolerance has been described in a wide diversity of free-living or symbiotic ulvophytes and trebouxiophytes (i.e., virtually all lichens contain desiccation-tolerant photobionts) (Kranner et al., 2008; Holzinger & Karsten, 2013). In the Streptophyta lineage, the frequency of desiccation-tolerant taxa diminished in parallel to the acquisition of water-loss prevention traits (i.e., homoiohydric organisms) but is present among streptophyte algae (Holzinger & Karsten, 2013; Becker et al., 2020), bryophytes, pteridophytes and even angiosperms (Fernández-Marín et al., 2016). Overall, these mechanisms include a set of anatomical and physiological adaptations to counteract both the mechanical stress related to severe dehydration of cells (changes in volume) and the oxidative processes taking place mainly in the dry state and upon rehydration, reviewed in (Rascio & La Rocca, 2005; Fernández-Marín et al., 2016; Oliver et al., 2020). Importantly, these mechanisms must guarantee the preservation of the integrity of molecules and cell compartments. Hence, in addition to the accumulation of specific osmolytes and the presence of an efficient antioxidant system (Rascio & La Rocca, 2005; Fernández-Marín et al., 2016; Oliver et al., 2020), a specific composition of cell membranes and its modification upon drying seems to play a crucial role (Gasulla et al., 2013; Gasulla et al., 2016; Tshabuse et al., 2018; Gasulla et al., 2019). In that sense, the preservation of chloroplast membranes is particularly relevant. Interestingly, the mechanisms of desiccation tolerance in angiosperms likely originated in streptophyte ancestors (Gaff & Oliver, 2013; Becker et al., 2020).

Amongst streptophyte algae, the formation of dormant spores is a common survival strategy for freshwater organisms, whereas true terrestrial groups have developed the ability to tolerate stress in their vegetative state (Holzinger & Pichrtová, 2016). Tolerance to desiccation has been described in vegetative cells of some taxa within Zygnematophyceae (Holzinger & Pichrtová, 2016; Herburger et al., 2019), Klebsormidiophyceae (Holzinger & Pichrtová, 2016) and, to some extent, Coleochaetophyceae (Graham et al., 2012). Within Charophyceae, desiccation tolerance has been described in reproductive cells (oospores) only (Davis, 1972). Desiccation tolerance in vegetative cells of streptophytes is achieved through various strategies to compensate for both mechanical and biochemical consequences of cell dehydration. Several of these strategies include cell aggregation, flexible cell walls, mucilage production, accumulation of osmotically active compounds and chloroplast photoprotection (Holzinger & Pichrtová, 2016). Additionally, these groups exhibit high photophysiological plasticity and accumulate UV-screening compounds to protect themselves from high levels of irradiation (Holzinger & Pichrtová, 2016). The biochemical composition of the cell wall and extracellular matrix and their physical properties are thus relevant in the adaptation to severe cell dehydration. For example, a high homogalacturonan content enhances cell resistance to dehydration-induced stress in Zygnematophyceae (Herburger et al., 2019), while in *Klebsormidium*, flexible cell walls accompany cell shrinkage during water loss, enabling the preservation of cell integrity (Holzinger et al., 2011). Shifts in the amino acid composition of extramembrane regions of plastid and mitochondrial proteins towards more stable proteins may have also represented a biochemical adaptation to desiccation in streptophytes that helped green plants colonize the land (Jobson & Qiu, 2011).

During dehydration, the loss of membrane integrity is one of the main causes of viability loss (Hoekstra et al., 2001). Biological membranes in algae are mainly composed of glycerolipids (glycoglycerolipids and phosphoglycerolipids), as is the case in higher plants (Kobayashi et al., 2016). The most common glycolipids in chloroplasts are galactolipids, which may bind to one galactose, i.e., monogalactosyldiacylglycerol (MGDG), or to two galactose residues, i.e., digalactosyldiacylglycerol (DGDG). The other two major chloroplast lipids are sulfoquinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG). Likewise, some species also contain betaine lipids, a lipid class only present in cryptogamic plants (Kalisch et al., 2016). In particular, these are glycerolipids characterized by a nonphosphorous polar head connected through an ether bond to a diacylglycerol backbone. Among these

glycerolipids, diacylglyceryl-*N*-trimethylhomoserine (DGTS) is the most common and is present in different groups, including Bacteria, Protozoa, Chromista, Fungi and Plantae. It has thus far been found in chlorophytes, streptophytic algae, bryophytes, and ferns but not in flowering plants (Künzler & Eichenberger, 1997; Cañavate et al., 2016). Monoacylglyceryl-*N*-trimethylhomoserine (MGTS)-type lipids have only a monoacylglycerol backbone and are thus more polar. To date, they have been studied more rarely, although they are supposed to be common constituents of the membrane lipid bilayers (Cauchie et al., 2021). In addition, some organisms, particularly when exposed to freezing or desiccation, also contain significant amounts of galactolipids with three or more galactose residues, the so-called oligogalactolipids (OGLs). Given that membrane fusion is one of the main sources of damage in desiccated tissues, this causal relationship of OGLs and stress has led to the proposal that their cylindrical shape with large polar heads would stabilize membranes, acting both directly by maintaining the bilayer structure (Moellering et al., 2010; Gasulla et al., 2013; Gasulla et al., 2019) or indirectly by interacting with late embryogenesis-abundant (LEA) proteins that accumulate in response to desiccation (Hoekstra et al., 2001). OGLs can be produced by the successive addition of galactose units through the action of a DGDG synthase (DGD) (Benning & Ohta, 2005) or by transgalactosylation from MGDG through the action of galactosyltransferase (GGGT/SFR2) (Moellering et al., 2010). DGD supposedly represents the ancestral route of OGL biosynthesis and results in the constitutive accumulation of OGLs, while SFR2 appeared more recently at some point in streptophyte evolution (Gasulla et al., 2019), providing a mechanism for faster membrane remodelling in response to environmental fluctuations (Moellering & Benning, 2011). Interestingly, the very scarce literature available regarding galactolipid composition and its changes under stress in green algae indicates a higher DGDG/MGDG ratio in desiccation-tolerant than in desiccation-sensitive species and an inducible further rise in response to stress, i.e., high irradiance, desiccation or freezing (Montero et al., 2021).

In the present study, we propose that thylakoid glycolipids, specifically OGLs with three or more galactose residues, are essential to enable streptophyte algae to tolerate tissue desiccation by enhancing the stability of bilayer membranes in the absence of water molecules. This adaptation was likely a prerequisite for the process of plant terrestrialization. We speculate that during the evolution of streptophytes, there could have been a shift from constitutive to stress-inducible OGL presence, likely due to the emergence of new metabolic tools. To support this hypothesis, constitutive polar lipid composition was evaluated in cultures of five lineages of streptophytes atop the chlorophyte model species *Chlamydomonas reinhardtii*, which was used as a reference. Despite the critical role played by thylakoid lipid composition in the evolution of streptophyte algae, this aspect has received limited attention in the scientific literature. To the best of our knowledge, this study represents the first comparative investigation of polar lipid profiles across various Charophyta lineages. In fact, the lipidome has only been described in one species of *Klebsormidium* (Hori et al., 2016). Further analysis of the effects of desiccation on these species provides insights into the activation of metabolic responses.

2 | MATERIALS AND METHODS

2.1 | Algae Material and Growth Conditions

The 6 studied species are specified in Figure 1. All the streptophyte algae strains, except for *Chara fragilis*, were cultivated at 75–100 µmol photons m^{-2} s^{-1}, 14/10 h light/dark cycle and 22/20°C temperature in a Sanyo Environmental Test Chamber MLR-350H (Sanyo). *Chlorokybus cerffii* (SAG 34.98), *Coleochaete scutata* (SAG 50.90) and *Zygnema circumcarinatum* (SAG 698-1a) strains were grown in a modified 3 N-BBM medium (Bischoff & Bold, 1963), as per Starr & Zeikus (1993), supplemented with 50 mg L^{-1} of fungicide Nystatin (300 U mL^{-1}, Sigma-Aldrich). *Klebsormidium nitens* was grown with Gamborg's B-5 Basal Medium with minimal organics (Sigma-Aldrich; Gamborg et al. (1968)) supplemented with 50 mg L^{-1} of fungicide Nystatin (300 U mL^{-1}, Sigma-Aldrich) and vitamin B12. *Chara fragilis* was collected from a clean stream located near Villanañe (Basque Country Spain, 42°49' N, 3°04' W, altitude 560 m). The material was identified, gently cleaned and processed in the laboratory for the desiccation experiments. The chlorophyte *Chlamydomonas reinhardtii* WTCW15 was grown in TAP growth liquid media (Gibco™, Thermo Fisher Scientific) at 75-100 µmol photons m^{-2} s^{-1}, a 14/10 h light/dark and 22/20°C temperature cycles with a 150-rpm shaking orbital incubator (Climo-Shaker ISF1-X).

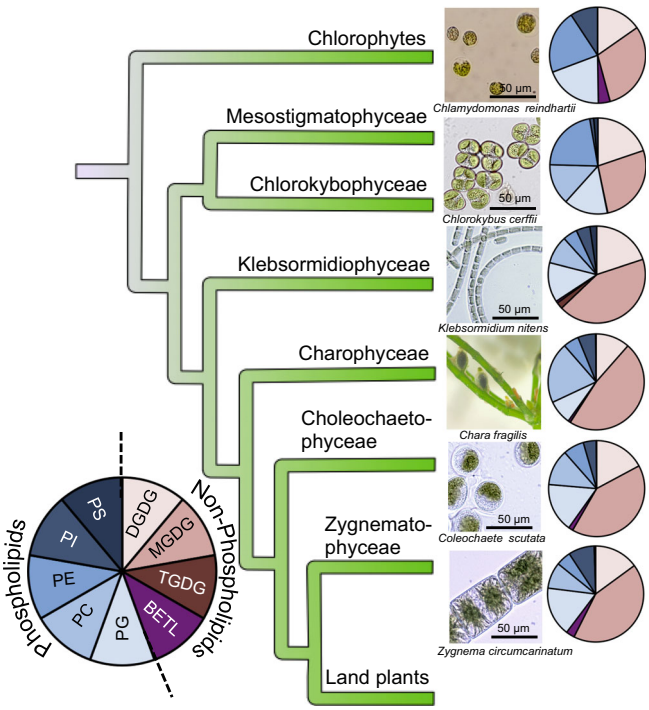

FIGURE 1 Overview of the main evolutionary groups of green algae evaluated, the general aspect of the studied species under the light microscope and their overall lipid composition in the hydrated state. Mesostigmatophyceae and land plants lineages are also shown but were not object of study in this work. Legend to the pie-charts for phospholipids and other lipids is shown at the bottom left of the figure.

2.2 | Desiccation Treatments and Tolerance Tests

Two different desiccation regimes were applied to the algae material for this experiment, as further explained onwards. (1) A severe desiccation was employed to determine the tolerance of the analysed species. The samples were subject to a sequential desiccation treatment at a decreasing relative humidity (RH) of 80%, then 50% and, finally, at 15% using the same desiccant treatments as those explained in López-Pozo et al. (2019). The samples were subject to each relative humidity for 24 h. Finally, the samples were rehydrated with a saturated atmosphere for yet another 24 h. The extent of tolerance to severe desiccation was estimated as the percentage of recovery of the initial maximal photochemical efficiency of PSII (F_v/F_m). (2) A moderate desiccation was employed to obtain the material for the lipid analysis, subjecting the samples to a RH of 80% for 24 h. The rehydration was performed as in the severe desiccation. The objective of this desiccation was to favour the changes in the lipidome with a slower and less aggressive desiccation to prevent generalised metabolic collapse.

2.3 | Chlorophyll a fluorescence

To determine their survival rate, the chlorophyll *a* fluorescence was measured before and after the desiccation treatments using a portable pulse amplitude modulation fluorometer PAM-2500 (Walz, Effeltrich) at a 630 nm wavelength. Prior to the measures, the samples were subjected to 30 min of darkness in order to determine the basal fluorescence (F_0). The maximal fluorescence (F_m) was determined by applying a saturating pulse (8000 µmol photons m^{-2} s^{-1}). The maximal photochemical efficiency of the PSII (F_v/F_m) was calculated as:

$$F_v/F_m = \frac{(F_m - F_0)}{F_m}$$

2.4 | Relative Water content

Relative Water content (RWC) was used to determine the extent of desiccation of the samples. Samples were weighed at the hydrated state (W_H), at the desiccated state (W_{DH}) and at the end of the measurements, after 48-72 h in an oven at 80°C to obtain the dry weight (DW). RWC of the samples was calculated as:

$$RWC\,(\%) = \frac{(W_{DH} - DW)}{(W_H - DW)}$$

2.5 | Quantification of polar lipids by Q-TOF

Polar lipids were analysed from freeze-dried and powdered material. The methodology by Gasulla et al. (2013) was followed for the extraction procedure. Briefly, a first extraction with 1 mL of $CHCl_3$: MeOH (1:2, v/v) was performed, and the organic phase was collected. The extraction was repeated 3-fold with 1 mL of $CHCl_3$: MeOH (2:1, v/v) until the pellet turned white. In some of the samples, an additional extraction was required to obtain a completely white pellet. Pure chloroform and aqueous ammonium acetate 0.3 M were added to the combined organic phases to obtain an organic phase of $CHCl_3$:MeOH: $C_2H_7NO_2$ (2:1:0.75, v/v), which was left overnight at −20°C. Afterwards, the organic phase was harvested and evaporated completely under a stream of N_2. Finally, the lipids were dissolved in 1 mL of $CHCl_3$, transferred to a 2.0 mL clear glass vial and vacuum dried at room temperature (SPD 121P, Speed-Vac, Thermo Scientific Savant).

Quantification of phospholipids and glycolipids was performed by the Kansas Lipidomics Research Center at Kansas State University. Briefly, samples were dissolved in 1 mL of chloroform. Aliquots were mixed with internal standards and solvents, as described in Shiva et al. (2013). Internal standards are indicated in Appendix S1. Lipid measurements were performed using a Sciex 4000 Q TRAP using the acquisition and data processing parameters indicated in Appendix S1. Internal standards were from the same class or for classes without available internal standards: PC was used for diacylglyceryl trimethyl homoserine (DGTS), LysoPC for monoacylglyceryl trimethyl homoserine (MGTS) and DGDG 18:0/18:0 for TGDG and TeGDG. Factors to account for the difference in the response of the instrument to MGDG and DGDG analytes vs their standards were applied.

2.6 | Statistical analysis

To examine the total analysed lipid content among species and with the desiccation treatment, a two-way ANOVA was performed. To determine whether the desiccation treatment altered the lipid profile of each species, a one-way ANOVA was performed for each lipid group. To analyse the response of the different species to the desiccation treatment, a one-way ANOVA was performed. The correlation was calculated as the Pearson coefficient. Levene's test was used to check the homogeneity of variances, and the Kolmogorov–Smirnov test was used to check whether the residuals followed a normal distribution. Duncan post-hoc was used to detect differences among treatments. Unless otherwise stated, $p < 0.05$ was considered significant. Statistical analyses were performed with SPSS v28.0.

3 | RESULTS

3.1 | Constitutive lipid composition

When lipid composition was analysed in the control cultures (Figure 1), MGDG was the main polar lipid in all species, particularly in *C. fragilis*, where it represented almost half of the total lipid pool. Among this pool, chloroplast lipids (galactolipids + betaine lipids + PG) accounted for the highest fraction (60 to 80%). Other minor glycerolipids were present at proportions higher than 1% in *C. reinhardtii*, *C. scutata* and *Z. circumcarinatum* (Appendix S1). The presence of trigalactosyldiacylglycerol (TGDG) was only significant in *K. nitens* and was negligible in other species. The content of tetragalactosyldiacylglycerol (TeGDG) was close to the detection

TABLE 1 Changes induced by moderate desiccation in the double bound index (DBI) of the analysed polar lipids (e.g. the unsaturation index of their fatty acids). DBI was calculated as: (∑[N x mol% lipid]/2, where N is the total number of double bonds in each molecular species of each polar lipid analysed. Data are mean ± SE (n = 5). Letters indicate significant differences between species and treatment ($p < 0.05$). DGDG, digalactosyldiacylglycerol; DGTS, diacylglyceryltrimethylhomoserine; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPG, lysophosphatidylglycerol; MGDG, monogalactosyldiacylglycerol; MGTS, monoacylglyceryltrimethylhomoserine; PA, phospatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; TeGDG, tetragalactosyldiacylglycerol; TGDG, trigalactosyldiacylglycerol

	Chlamydomonas reindhartii		*Chlorokybus cerffii*		*Klebsormidium nitens*		*Chara fragilis*		*Coleochaete scutata*		*Zygnema circumcarinatum*	
	Control	**Desiccation**	**Control**	**Desiccation**	**Control**	**Desiccation**	**Control**	**Desiccation**	**Control**	**Desiccation**	**Control**	**Desiccation**
MGDG	262.84 ± 0.61 c	262.10 ± 1.93 c	229.91 ± 4.05 e	228.36 ± 3.37 e	266.88 ± 4.87 c	263.72 ± 3.84 c	288.66 ± 1.11 a	280.66 ± 2.32 b	279.34 ± 1.82 b	279.71 ± 1.97 b	261.72 ± 1.48 c	252.77 ± 1.84 d
DGDG	175.69 ± 1.63 d	183.04 ± 2.05 d	224.56 ± 3.59 c	225.04 ± 3.04 c	256.56 ± 4.37 a	256.82 ± 3.47 a	235.14 ± 2.90 bc	232.00 ± 3.86 c	250.24 ± 1.48 a	250.21 ± 2.84 a	233.62 ± 1.39 bc	241.18 ± 3.17 b
TGDG	227.21 ± 8.18 c	218.76 ± 5.11 c	237.24 ± 6.59 bc	229.33 ± 3.40 c	212.65 ± 1.38 c	232.05 ± 1.80 c	170.91 ± 17.86 d	266.67 ± 25.00 a	266.60 ± 12.57 ab	262.06 ± 2.61 ab	256.80 ± 15.91 c	266.42 ± 2.52 a
TeGDG	254.35 ± 2.50 abc	232.06 ± 10.28 abc	250.00 ± 0.00 abc	224.33 ± 8.75 bc	255.73 ± 4.27 abc	250.23 ± 4.79 abc	249.00 ± 12.05 abc	199.71 ± 18.99 c	264.29 ± 25.00 a	267.74 ± 12.55 ab	233.55 ± 50.00 abc	249.36 ± 10.50 abc
PG	132.36 ± 2.82 c	153.66 ± 0.81 b	132.04 ± 5.74 c	132.18 ± 6.18 c	160.71 ± 2.11 ab	158.65 ± 1.89 ab	167.70 ± 2.69 a	159.37 ± 1.92 ab	154.96 ± 4.41 b	150.91 ± 1.96 b	137.11 ± 1.64 c	136.42 ± 2.44 c
PC	268.98 ± 39.47 a	238.68 ± 46.61 cd	158.40 ± 1.25 d	162.18 ± 1.10 d	173.35 ± 3.93 d	175.61 ± 2.38 d	248.81 ± 2.54 ab	247.29 ± 11.09 bc	195.94 ± 2.61 cd	187.56 ± 1.65 cd	174.85 ± 1.12 d	155.38 ± 2.51 d
PE	172.41 ± 1.39 f	138.61 ± 6.10 h	149.52 ± 1.30 gh	158.43 ± 1.73 fg	232.92 ± 4.97 c	212.81 ± 3.50 d	314.26 ± 1.41 a	296.01 ± 16.55 b	203.54 ± 5.13 de	170.15 ± 6.71 fg	192.77 ± 1.31 e	150.84 ± 3.60 gh
PI	53.42 ± 0.09 g	58.63 ± 0.73 gh	62.75 ± 4.01 f	90.73 ± 2.06 e	128.43 ± 3.05 d	144.48 ± 1.49 c	162.30 ± 2.38 a	156.13 ± 1.83 b	141.57 ± 1.51 c	153.47 ± 0.90 b	131.30 ± 0.58 d	128.69 ± 1.81 d
PS	146.50 ± 11.87 cd	187.92 ± 24.66 ab	122.92 ± 1.19 d	117.35 ± 0.91 d	197.83 ± 0.78 a	198.34 ± 0.36 a	203.12 ± 2.98 a	180.08 ± 16.94 bc	195.42 ± 5.45 a	207.16 ± 3.20 a	178.59 ± 2.05 ab	163.08 ± 3.79 bc
PA	133.09 ± 3.03 de	124.82 ± 3.61 e	131.27 ± 5.93 e	131.96 ± 1.68 e	205.63 ± 13.30 a	179.41 ± 1.37 b	227.64 ± 5.11 a	213.27 ± 3.40 a	99.14 ± 1.18 f	167.30 ± 1.74 bc	163.70 ± 4.01 c	147.63 ± 0.84 d
LPG	86.19 ± 9.81 ab	48.14 ± 2.71 b	100.00 ± 0.00 a	76.95 ± 9.44 ab	101.18 ± 3.70 a	102.30 ± 13.72 a	95.78 ± 27.79 b	42.75 ± 1.91 b	102.58 ± 15.82 a	53.32 ± 11.71 b	68.25 ± 16.43 ab	41.20 ± 13.27 b
LPC	274.16 ± 12.83 a	279.97 ± 10.77 a	76.82 ± 6.54 c	70.15 ± 2.63 c	94.46 ± 15.04 bc	80.62 ± 4.70 bc	84.41 ± 4.16 bc	80.46 ± 7.95 bc	79.47 ± 8.16 bc	72.35 ± 5.50 c	109.13 ± 6.10 b	80.04 ± 9.10 bc
LPE	81.25 ± 13.29 ab	51.33 ± 3.10 b	65.45 ± 2.06 ab	79.39 ± 1.51 ab	98.38 ± 22.80 a	102.06 ± 5.44 a	85.93 ± 18.40 ab	65.34 ± 18.43 ab	49.31 ± 8.55 b	68.87 ± 7.36 ab	104.90 ± 8.97 a	71.95 ± 17.21 ab
MGTS	58.40 ± 5.42 cd	61.91 ± 1.01 cd	35.14 ± 8.31 f	36.64 ± 3.88 f	67.65 ± 3.95 bcd	61.76 ± 6.51 d	59.48 ± 2.74 d	78.17 ± 1.91 b	74.32 ± 1.50 bc	40.07 ± 1.67 ef	109.49 ± 8.30 a	51.31 ± 1.30 de
DGTS	178.77 ± 2.08 b	146.57 ± 1.53 c	115.78 ± 4.75 d	113.63 ± 8.94 d	147.15 ± 5.25 c	140.07 ± 1.98 c	117.49 ± 1.24 d	114.31 ± 1.85 d	201.28 ± 4.53 a	186.58 ± 2.94 b	178.46 ± 1.49 b	143.99 ± 3.66 c
Weighted average	160.86 ± 3.86 f	101.04 ± 2.25 h	156.54 ± 2.73 f	156.06 ± 2.08 f	213.44 ± 2.38 b	180.77 ± 1.95 e	238.37 ± 2.34 a	198.09 ± 2.34 cd	206.39 ± 2.31 bc	166.34 ± 4.86 f	188.97 ± 1.83 de	119.99 ± 1.13 g

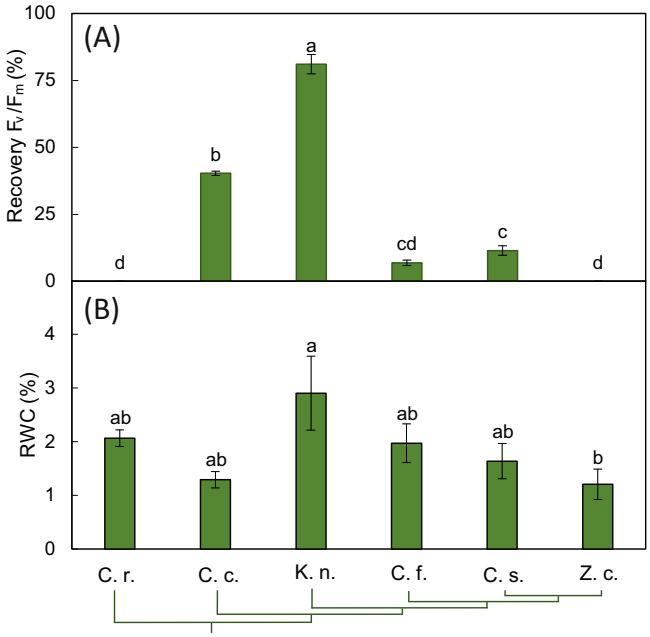

FIGURE 2 Assessment of the tolerance to severe desiccation of the studied species. (A) Estimation of desiccation tolerance through the % of recovery in the maximal photochemical efficiency of PSII after rehydration. (B) Absolute water content reached in the severe desiccation. Bars shows the average ± SE (n = 3). Letters above the bars depict significant differences amongst the species at $p < 0.05$. The species names are abbreviated from left to right: *Chlamydomonas reinhardtii*, *Chlorokybus cerffii*, *Klebsormidium nitens*, *Chara fragilis*, *Coleochaete scutata*, *Zygnema circumcarinatum*.

limits (less than 1‰ of the total polar lipid pool) in all species. Among extraplastidial phospholipids, the dominant forms were phosphatidylcholine (PC) and phosphatidylglycerol (PG), except in *C. cerffii* and *C. reinhardtii*, where phosphatidylethanolamine (PE) was the dominant form.

The most abundant MGDG molecular species in all the taxa were those containing 34 carbons (Table S1, Appendix S1), with 34:6 being the most abundant, except in *C. cerffii* where the main form was 34:4. In DGDG, acyl chains tended to be longer and more saturated. Thus, the majority was 36 carbons in *K. nitens* and *C. cerffii* (36:6, 36:5 and 36:4). With regard to TGDG, both forms of 34 and 36 carbons were equally present.

The highest number of double bonds in the acyl chains was significantly higher in *C. fragilis*, followed by *K. nitens* and *C. scutata* (Table 1). The double bond index (DBI) was higher in galacto- than in phospholipids.

3.2 | Desiccation tolerance tests

After a sequential desiccation treatment at 80, 50 and 15% RH, the relative water content (RWC) decreased to values lower than 3% in all species (Figure 2). The studied species differed significantly in the degree of desiccation tolerance. Thus, while dehydration barely affected F_v/F_m in *K. nitens*, no photochemical activity was measured in *C. reinhardtii* or *Z. circumcarinatum* after desiccation. A residual F_v/F_m was maintained in *C. fragilis* and *C. scutata*, while in *C. cerffii*, F_v/F_m recovered to 40% of the pre-stress values.

3.3 | Effects of desiccation on lipid composition

Desiccation resulted in two general responses that were observed in all species, independent of whether they survived the experimental treatment. The first was a significant decrease in lipid content (except for *C. fragilis*). While the decrease was higher than 40% in all those species that did not tolerate desiccation treatment, in tolerant species, the decrease was only 24 and 10% for *K. nitens* and *C. cerffii*, respectively (Figure S1). The second was a significant decrease in the number of double bonds of the acyl chains, which was more noticeable in those species that did not survive (in *C. cerffii*, the double bond index was maintained after desiccation). This change was mostly due to changes in phospholipids, while the degree of unsaturation remained remarkably stable in galactolipids (Table 1).

The total content of phospholipids decreased in all species (Figure 3). However, this decrease was lower in the case of PI, which even increased significantly in the two species that survived desiccation (*K. nitens* and *C. cerffii*). In contrast, phosphatidic acid (PA) increased significantly (5 to 74-fold) in all species except *C. reinhardtii*, while the other monoacyl forms (lysophospholipids and MGTS) increased in all species except *K. nitens* (Figure 3, Appendix S1). The highest increase in PA and MGTS was observed in *Z. circumcarinatum*.

Among galactolipids, MGDG and DGDG decreased in all species (except DGDG in *K. nitens*, which remained stable; Figure 4). This decrease was more acute in MGDG than in DGDG for all species, leading to a generalized enhancement of the DGDG/MGDG ratio. In contrast, the content of TGDG either remained stable in the species with its highest constitutive content (*K. nitens*) or increased significantly (17 to 70-fold) in *Z. circumcarinatum* and *C. scutata*, respectively, and to a lesser extent in *C. cerffii* (Figure S2). The content of TeGDG increased in almost all species in response to desiccation, particularly in the case of *Z. circumcarinatum* and *C. scutata* (110- and 18-fold, respectively). However, the increase was not significant in *C. reinhardtii* and *K. nitens*.

To test the origin of the newly formed TGDG, its molecular species composition was matched against that of DGDG, MGDG and the sum of DGDG and MGDG in the three species with the largest increase in TGDG (Figure 5). In *C. cerffii* the best adjustment (r^2 0.97) was obtained with DGDG, while in *C. scutata* and *Z. circumcarinatum*, the best match was against the sum of MGDG and DGDG (r^2 0.91 and r^2 0.99, respectively).

4 | DISCUSSION

When analysing a particular potentially useful trait for terrestrialization in streptophyte algae using a neontological approach,

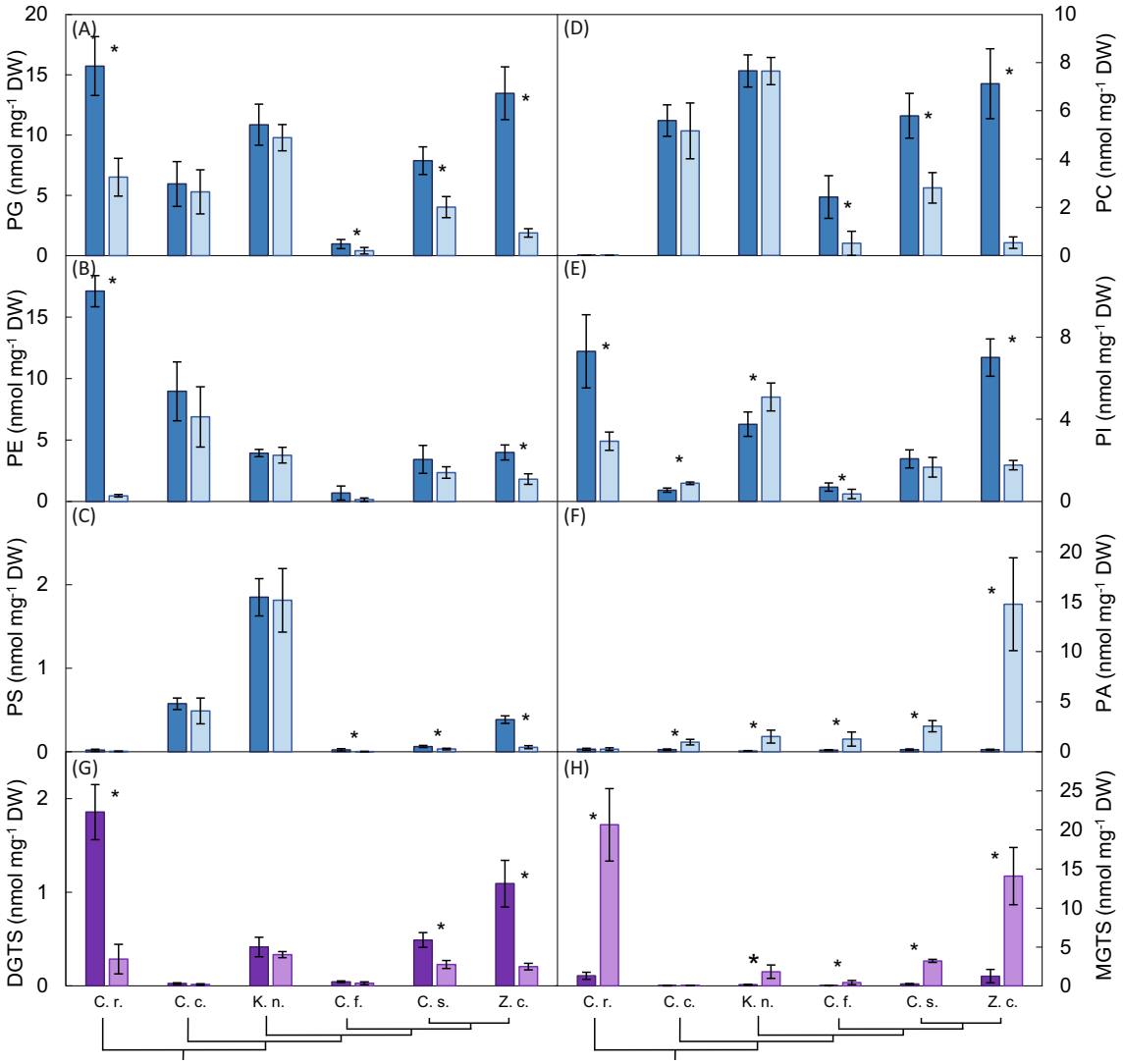

FIGURE 3 Changes induced by moderate desiccation in phospholipid and betaine lipid content. (A) Phosphatidylglycerol (PG). (B) Phosphatidylethanolamine (PE). (C) Phosphatidylserine (PS). (D) Phosphatidylcholine (PC). (E) Phosphatidylinositol (PI). (F) Phospatidic acid (PA). (G) Diacyglyceryl trimethyl homoserine (DGTS). (H) Monoacyglyceryl trimethyl homoserine (MGTS). Dark bars depict control samples and light bars depict dehydrated samples. Data are average ± SE of n = 5. Asterisks depict significant differences between control and desiccated samples within each species at $p < 0.05$. The species names are abbreviated from left to right as in Figure 2.

representatives of multiple lineages must be used to discern whether a trait was present in the early colonizer or evolved afterwards. The use of extant species to infer evolutionary trends has some obvious limitations, but in the case of streptophyte algae, it has been used to characterize various aspects such as the evolution of photoprotection mechanisms (Gerotto & Morosinotto, 2013) or the evolutionary appearance of stress signalling routes (de Vries et al., 2018). Considering that it has been hypothesized that the capacity of biological membranes to mitigate the detrimental consequences of desiccation was a pivotal factor in the process of terrestrialization (de Vries & Archibald, 2018), in the present study, we aimed to characterize evolutionary trends in lipid composition among streptophyte algae with a particular focus on galactolipids.

The lipid composition of the analysed streptophyte algae was similar to that of land plants, as observed in previous studies (Kumari et al., 2013; Khozin-Goldberg, 2016), and the presence of betaine lipids was the main difference in composition. The main polar lipids in land plants are MGDG, followed by DGDG, accounting for up to 40–50% and 20%, respectively (Thompson, 1996; Harwood, 1998). These proportions were also observable in almost all of the studied species, but since sulfoquinovosyl diacylglycerol (SQDG) was not analysed, the real proportions may be different. The phospholipid composition was similar to that of land plants (Harwood, 1998; Reszczyńska & Hanaka, 2020). For example, PA was present in minor amounts, as is usual in land plants (Munnik, 2001). PC and PG were also the most abundant phospholipids, as in land plants, but their proportion was lower in more evolutionary distant species, such *C. cerffii* and *C. reinhardtii*. Indeed, the most basal streptophyte alga, *C. cerffii*, presented the most different overall lipid composition, having some similarities with the

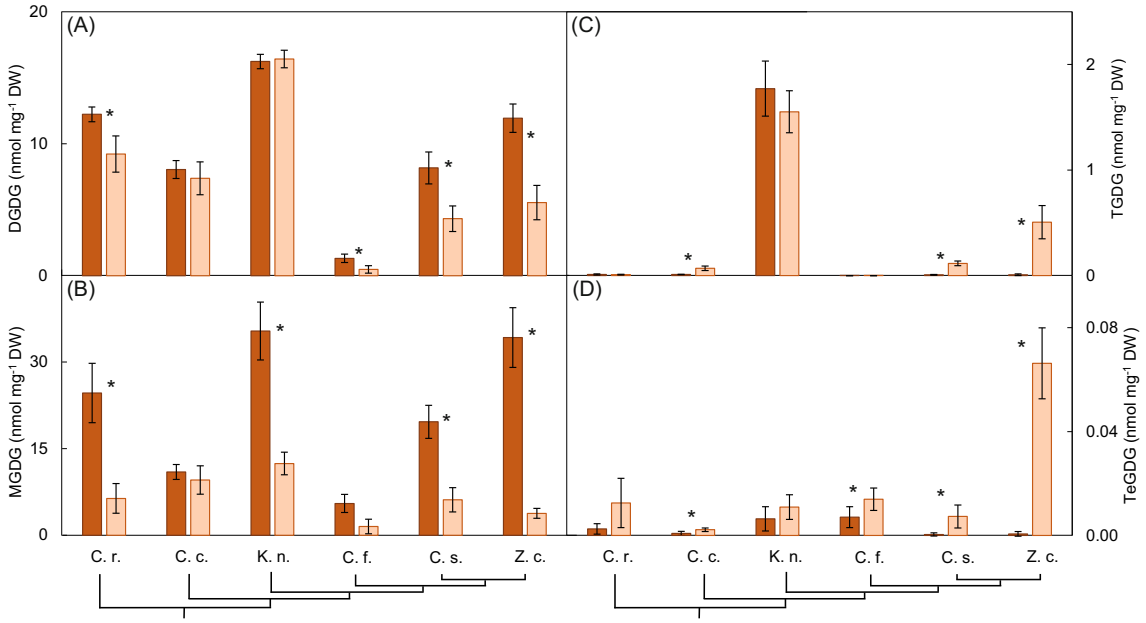

FIGURE 4 Changes induced by moderate desiccation in galactolipids. (A) Digalactosyldiacylglycerol. (B) Monogalactosyldiacylgycerol. (C) Trigalactosyldiacylglycerol. (D) Tetragalactosyldiacylglicerol. Dark bars depict control samples and light bars depict dehydrated samples. Data are average ± SE of n = 5. Asterisks depict significant differences between control and desiccated samples within each species at $p < 0.05$. The species names are abbreviated from left to right as in Figure 2.

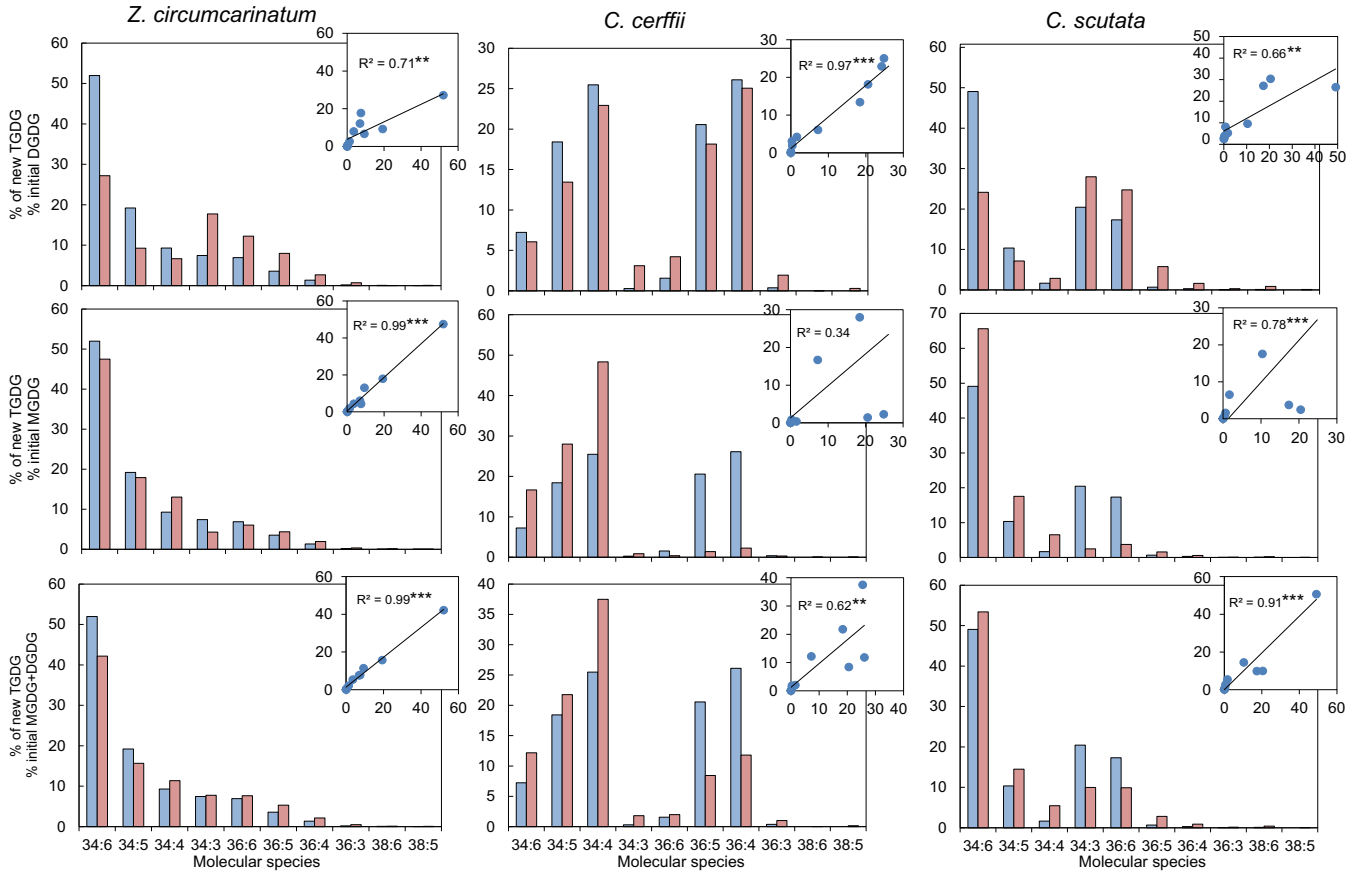

FIGURE 5 Tracing the origin of TGDG synthesised during mild desiccation. The molecular species composition in % of new TGDG (blue) and of initial (pink) DGDG (upper row of panels), MGDG (middle row of panels) or MGDG+DGDG (lower row of panels) are depicted with separate bars. Insets depict the correlation between both percentages per panel. Asterisks depict significant correlation, ** $p < 0.01$, *** $p < 0.001$.

chlorophyte *C. reinhardtii*, such as a lower proportion of MGDG and a higher phospholipid composition, among others. In addition, as reported by other authors (Giroud et al., 1988; Sato, 1988; Riekhof et al., 2005), PC was not found in *C. reinhardtii* and was replaced by higher levels of DGTS. In fact, the DGTS content was the highest among the analysed algae (1.86 nmol g^{-1} DW).

The molecular species of these lipids most commonly found in land plants are typically 34:*x* and 36:*x* (Maatta et al., 2012; Gasulla et al., 2013). Overall, for the analysed streptophyte algae, the main molecular species also belonged to these two types, with the molecular species composed of 34:*x* and 36:*x* being the only ones with content higher than 0.3 nmol mg^{-1} DW. Even in the case of phosphatidylserine (PS), where molecular species with larger fatty acids are generally found (40:*x* and 42:*x*), the molecular species were also similar to those that can be found in land plants (Gasulla et al., 2013). Nonetheless, the number of instaurations was different between species and polar lipids, calculated in this work as DBI (Table 1). The main differences in DBI resided between the types of lipids rather than being linked to the algal species. GLs had the highest DBI values and, therefore, contained the most unsaturated fatty acids. Indeed, MGDG usually has higher amounts of polyunsaturated fatty acids than other polar lipids, such as PG and SQDG, which generally have more saturated fatty acids (Harwood, 1998). However, the lowest DBI was present on the betaine lipid MGTS and in lyso-phospholipids. Small differences between species could only be noticed in phospholipids, where *C. reinhardtii* had a slightly lower DBI index than the streptophyte algae.

During moderate dehydration, the total content of analysed lipids diminished significantly in all species, except for *C. cerffii* (Figure S1). A diminution of polar lipid content during desiccation in land plants has been previously observed (Gasulla et al., 2013). Although the total content of phospholipids decreased in all species, this decrease was lower in the case of PI. This phospholipid, which has a cylindrical shape and a highly polar head, increased even during moderate dehydration in the two species that survived desiccation: *K. nitens* and *C. cerffii*. An increase in PI induced by dehydration has also been reported in desiccation-tolerant tracheophytes (Gasulla et al., 2013). Thus, based on the shape of the PI molecule and its consequent physicochemical properties, a role of PI in extraplastidial membranes analogous to that of OGL in chloroplast membranes has been suggested (Gasulla et al., 2013). The substantial decrease (40%) in total lipid content, together with the large increase in PA and lysolipids (such as MGTS), suggests the activation of a process of lipid cleavage (Giossi et al., 2021) and cellular degradation.

In addition to the increase in MGTS, other marked changes upon moderate desiccation included the diminution in DGTS. These are betaine lipids that are rarely studied in streptophytes and are even more rarely studied in response to stress. Among chlorophytes, DGTS has previously been found within Trebouxiophyceae and Chlorodendrophyceae (Cañavate et al., 2016), whereas a phylotranscriptomics analysis indicates that streptophytes are also able to synthesize it (Goh et al., 2019). Interestingly, while most of the chlorophytes and streptophytes studied from a transcriptomic perspective have sequences that imply DGTS synthesis, it is absent in most of the rhodophytes studied so far (Goh et al., 2019). The same trend of higher DGTS presence and content in green vs red algae lineages (which preferentially contain PC) was already pointed out previously based on TLC analyses of betaine lipids (Künzler & Eichenberger, 1997). A division between red and green algae has thus been proposed, with green adopting DGTS and red algae adopting PC (Goh et al., 2019). Therefore, DGTS can substitute for PC in environments that are limited in phosphorus and probably represent an adaptation for that particular purpose (Goh et al., 2019). Our results show different levels among species, but there is not a clear pattern to distinguish Chlorophyta vs Charophyta lineages. Although phylogenetically distant, interesting and recent data have been obtained in relation to the potential functions of DGTS in *Nannochloropsis*, a genus of microalgae that belongs to the stramenopiles, also referred to as heterokonta (Phylum: Gyrista, Class: Eustigmatophyceae) (Murakami et al., 2018). Via the employment of mutants, the authors demonstrated pivotal roles for DGTS in adaptation to phosphorus-deficient conditions and to low temperatures (Murakami et al., 2018). Furthermore, DGTS decreased significantly in the summer period in a seasonal study with horsetail *Equisetum variegatum* (Nokhsorov et al., 2021), further indicating a dynamic remodelling of thylakoids in response to abiotic factors that include this betaine lipid. In response to desiccation, our data also evidence a decrease in DGTS and in the galactolipid MGDG, which are both conical (e.g., DGTS is a structural analogue of PC) and thus nonbilayer-forming lipids (Yu et al., 2021). This may indicate a trend toward reducing the fluidity of the membranes as mechanisms of thylakoid integrity preservation. The decrease in thylakoid fluidity could also be enhanced by the decrease observed in the DBI index during desiccation in almost all the species, which indicates lower polyunsaturated fatty acid content and can thus lead to lower membrane fluidity (Ernst et al., 2016). Nevertheless, it is worth mentioning that the OGLs maintained a similar DBI index, and the changes were mainly due to the phospholipids (Table 1). Based on the different distributions of polar lipids in cell membranes (Kumari et al., 2013), the diminution of DBI only in phospholipids may show a different regulation of membrane fluidity depending on the specific fatty acid unsaturations of GLs and phospholipids.

Among the six species studied, the only one that was constitutively fully desiccation-tolerant was *K. nitens*, while *C. cerffii* survived desiccation with a worse fitness. It should be noted that the experiments to desiccate the algae were carried out on material that had not undergone prior dehydration. In fact, previous studies with *Z. circumcarinatum* showed that its tolerance to desiccation varies with growth conditions as a consequence of the expression of protection genes (Rippin et al., 2017). Therefore, the findings only show how the algae constitutively respond to desiccation and do not demonstrate their ability to adapt to and cope with this condition over time. The remarkable tolerance to desiccation of *K. nitens* and other species belonging to the Klebsormidiaceae has been widely described in a number of previous studies (Holzinger et al., 2011; Herburger & Holzinger, 2015; Pierangelini et al., 2017; Pierangelini et al., 2019; Rippin et al., 2019). This is in agreement with the fact that *Klebsormidium* is a cosmopolitan genus widely distributed in terrestrial and freshwater habitats ranging from polar

tundra (Rippin et al., 2019) to tropical deserts (Karsten et al., 2016). This species was also the only algae constitutively accumulating substantial amounts of TGDG (3% of the total galactolipid pool). These amounts have previously been described in the same species, where they represented 5% of the galactolipid pool (Hori et al., 2016). Additionally, relatively high values of TGDG and TeGDG have been previously found in the green algae *Asterochloris erici* (Gasulla et al., 2016).

Regardless of the survival of algae during desiccation, there was a significant increase in TGDG content in three of the studied species: *C. cerffii*, *Z. circumcarinatum* and *C. scutata*. This is a remarkable finding since, thus far, a rise in the TGDG content induced by desiccation has only been found in tracheophytes (Gasulla et al., 2013). This suggests that during the desiccation process, the metabolism was active enough to induce a certain level of lipid remodelling. Furthermore, TeGDG, which was detected in minimal quantities across all species investigated, also increased in response to desiccation in the six species studied, suggesting that this is a common stress response. Additionally, the analysis of the molecular species composition of newly formed TGDG could be consistent with the hypothesis that suggests a different origin for *C. cerffii* and those species in the clade ZCC. This clade includes the closest relatives of embryophytes (*Z. circumcarinatum* and *C. scutata*) (de Vries et al., 2016). In *C. cerffii* it was likely formed by the addition of one galactose residue to DGDG, while in the other two, both MGDG and DGDG contributed equally to TGDG formation (Figure 5). Currently, the most primitive photosynthetic organism in which the *sfr2* gene has been identified is *Klebsormidium flaccidum* (Hori et al., 2016), a charophyte alga evolutionarily placed between the oldest *C. cerffii* and the more recently evolved *Z. circumcarinatum* and *C. scutata*. Therefore, it could be reasonable to think that the synthesis of TGDG in response to desiccation is mediated by the DGD enzyme in *C. cerffii*, although the mode of regulation is still unknown (Gasulla et al., 2019), whereas SFR2 would be involved in *Z. circumcarinatum* and *C. scutata*. Beyond the ability to activate the synthesis of OGLs in response to water stress, desiccation tolerance in photosynthetic poikilohydric organisms, from cyanobacteria to bryophytes, has been associated with the constitutive accumulation of OGLs in vegetative tissues (Gasulla et al., 2019), which is in agreement with the highest desiccation tolerance observed in *K. nitens*. Moreover, the inducible synthesis of OGLs triggered by osmotic stress, a common response in tracheophytes, increases resistance not only to dehydration but also to other abiotic stresses, such as freezing or salinity stress (Gasulla et al., 2019). Hence, the appearance of the *sfr2* gene at some point in the streptophytic lineage allowed rapid thylakoid remodelling in response to critical changes in water availability. The significant enhancement of TGDG concentration by one or two orders of magnitude in response to desiccation stress may provide further evidence for the appearance and role of an inducible SFR2 during streptophyte evolution. The faster and more plastic response to environmental stressors is considered one of the exaptations that allowed embryophytes to succeed in the conquest of land habitats (de Vries et al., 2018). This may indicate that the appearance of the *sfr2* gene, and therefore, the capacity to induce the synthesis of OGLs, could be one of the exaptations that streptophyte algae developed during terrestrialization and that contributed to the conquest of land.

AUTHOR CONTRIBUTIONS

JIGP and BFM conceived the idea. MIA conducted the measurements and analyses with the supervision of JIGP and BFM. MAG helped with the measurements and obtained the light microscope images. JIGP, MIA and BFM drafted the manuscript. JMA conducted the statistical analyses and contributed to algae culture. JMA and FG contributed to data discussion and interpretation. All authors contributed to the final version of the manuscript.

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DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article and the raw data obtained by the instrument are openly available in Mendeley Data at https://doi.org/10.17632/3tnycnmy4r

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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