Lipidomics of *Thalassiosira pseudonana* as a function of valve SDV synthesis

Philipp Schwarz · Cornelia Herrfurth · Claudia Steinem · Ivo Feussner

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Abstract
Silica polycondensation occurring in diatom organelles called silica deposition vesicles (SDVs) leads to valve and girdle band formation to complete the biosilica cell wall after cell division. As SDVs have as yet not successfully been isolated, the role of SDV membranes in silica biogenesis is still largely unexplored. Here we used the possibility of synchronizing the diatom *Thalassiosira pseudonana* to produce two synchronized cultures in different cell cycle stages that differ in their valve SDV production. Lipid subclass fold changes between valve SDV-enriched samples and cells in the G1 state are interpreted as indications for SDV-relevant membrane lipids. The lipid classes phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidic acid (PA), and lysophosphatidylcholine (LPC) were found to be elevated in valve SDV-enriched cells, also showing accumulation of the very long-chain polyunsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). It is conceivable that PA and LPC are preferentially found in SDV regions with high membrane curvature like the rims of the pancake-shaped valve SDV, while PC and PE molecules are presumably found in the planar regions of the SDVs. In addition, DHA-enriched phospholipids are known to form highly disordered membrane domains, which might be involved in membrane protein localization, possibly used for cytoskeleton-mediated positioning of the SDV.

Keywords Biosilica · Diatoms · Silica deposition vesicle · Silicalemma · Synchronized cultures

Introduction
Diatoms are a species-rich family of single-celled algae that produce silica-based cell walls with highly species-specific morphologies. The silica cell walls of diatoms, also termed frustules, are supposed to be involved in more than mere protection from predators due to mechanic stability (Hamm et al. 2003). The siliceous cell wall may also provide necessary support for the large vacuole (Raven and Waite 2004; Finkel and Kotrc 2010), enhance nutrient uptake (Mitchell et al. 2013), buffer protons for carbon dioxide uptake (Milligan and Morel 2002), facilitate light harvesting (Fuhrmann et al. 2004; Romann et al. 2015), and/or protect the cell against UV radiation (Aguirre et al. 2018).

The frustule is composed of valves and girdle bands and is produced in a compartment called silica deposition vesicle (SDV) (Drum and Pankratz 1964; Stoermer et al. 1965), which is enclosed by the silicalemma (Reimann et al. 1966). Biogenesis of the valves and girdle bands is tightly linked to the cell cycle with valves only being produced during cell division, while girdle bands are made exclusively during interphase. Polycondensation of silicic acid to silica occurs in the acidic environment of the SDV (Vrieling et al. 1999). Inside the SDV, silica formation is templated and catalyzed by an organic matrix composed of long-chain polyamines (LCPAs) and several protein classes (cingulins, silacidins, silaffins, silicanins).
to finally build up the species-specific porous patterned frustule (Sumper 2002, 2004; Vrieling et al. 2002; Kröger and Poulsen 2007; Scheffel et al. 2011; Kotzsch et al. 2017). By the recent identification of the transmembrane protein silicanin-1 and silicalemma-associated proteins (SAP) 1 and 3 that are associated with SDVs and that are involved in biosilica morphogenesis, it was suggested that the silicalemma also directly contributes to biosilica formation in diatoms (Kotzsch et al. 2017; Tesson et al. 2017; Görlich et al. 2019). After cytokinesis, valve SDVs occur in proximity to the cleavage furrow and grow with succeeding valve biosilica production, at first in the x,y-plane parallel to the cell membrane and finally, in z-direction (Pickett-Heaps et al. 1990; Hildebrand et al. 2006). After completion of biosilica formation, the newly formed valves and girdle bands are exocytosed. To prevent the plasma membrane from being altered by the silicalemma, exocytosis is probably followed by a compensatory endocytosis (Kröger and Poulsen 2008).

Like all eukaryotes, the diatom cell cycle consists of a G1, S, G2, and M phase, the latter two also combined to G2 + M (Brzezinski et al. 1990). G2 + M includes chloroplast division and translocation, karyokinesis, and cytokinesis (Huysman et al. 2014). Before complete cell division and separation can occur, new silica valves have to be produced in SDVs during the late G2 + M phase. The G1 phase then serves for cell growth with girdle band synthesis in further SDVs and preparation for DNA synthesis in the S phase (Hildebrand et al. 2007). The synchronization of microalgae cultures allows to investigate cell cycle dependent processes. For example, a synchronized culturing method was developed for Thalassiosira pseudonana, which enriches for cells making valves (Hildebrand et al. 2007). In this procedure, cells are starved for silicic acid for 24 h, during which time the majority (80%) of the cells rest in the G1 phase (Hildebrand et al. 2007) or late G2 + M phase (Brzezinski et al. 1990) of the cell cycle. Resting T. pseudonana cells need 1–4 h to reach the next cell cycle stages (mostly S followed by G2 + M) (Hildebrand et al. 2018). The percentage of synchronized cells can be increased by two subsequent silicon starvation/repletion processes and a maximum valve SDV concentration was observed 3 h after silicon repletion (Heintze et al. 2020). Silicon starvation changes the metabolite composition of Navicula pelliculosa, especially an increase in lipids was observed (Coombs et al. 1967). d’Ippolito et al. (2015) found no change in triacylglycerides and glycolipids under silicon depleted conditions but a significant increase in glycerocephospholipid production in Thalassiosira weissflogii. In contrast to T. weissflogii, Cyclotella cryptica showed no such increase. Smith et al. (2016) found for T. pseudonana that cell division arrested under silicon starvation for 24 h, but lipid concentrations increased nearly threefold.

SDVs have as yet not been successfully isolated and separated from chloroplasts, but first proteins from that reside in their membrane have been identified that serve as valuable tools for further functional analysis. However, the lipid composition of the silicalemma is still unknown. In this study, we made use of the synchronization of T. pseudonana cells to elucidate SDV membrane composition by detecting possible changes in lipid composition during the cell cycle to identify valve SDV-relevant lipids. Cell synchronization and harvest were executed according to the protocol of Heintze et al. (2020), who found valve SDV-enriched T. pseudonana cells to be present 3 h after silicon replenishment.

Understanding the role of the silicalemma in the biogenesis of silica will help to find out the molecular mechanisms and prerequisites for the formation of a defined porous structure. Such structures are of great interest for future nanotechnology developments. Applications for biosensing and gene or drug delivery are being investigated, among many other potential uses of diatom frustules or their biomimetics (Kröger and Poulsen 2008; Townley et al. 2008; Gordon et al. 2009; Dolatabadi and De La Guardia 2011; Rabiee et al. 2021).

Materials and methods

Culture of Thalassiosira pseudonana

Cultures of Thalassiosira pseudonana (Tp) CCMP1335 (300 mL each) were grown for five days with 12-h/12-h day/night light cycles (100–250 μmol photons m−2 s−2) in artificial seawater (ASW) to a density of 5 × 105 cells mL−1 (measured with Bio-Rad TC10 Automated Cell Counter). Harvested cells were incubated with ASW (−Si) lacking a silicon source for 24 h before adding Na2SiO3 (final concentration 200 μM) at time t = 0 h. At t = 3 h, half of the hereby synchronized cells were harvested and called Tp-G1. At t = 6 h, the other half was harvested and called Tp-G1. All cells were washed 3 × with water, frozen in liquid nitrogen and lyophilized (Christ Alpha 2–4 LD Plus). The dried cells were stored at −80 °C.

Lipid extraction

For lipid extraction, between 2 and 8 mg of lyophilized cells were used. The extraction was done according to Bligh and Dyer (1959) with some modifications. At first, the cells were extracted with 3 mL chloroform/methanol (1:2, v/v). After centrifugation (450 × g, 4 °C, 10 min), supernatant and pellet were separated and the pellet was reextracted with 3 mL chloroform/methanol (2:1, v/v). After centrifugation both supernatants were combined. A
1.5 mL NaCl solution (0.45% w/v) was used for liquid–liquid extraction, followed by phase separation and addition of Na₂SO₄ to the organic phase. After centrifugation, the supernatant was evaporated under a stream of nitrogen and the dried lipids were dissolved in 800 µL tetrahydrofuran/methanol/water (4:4:1, v/v/v), covered with argon and stored at −20 °C until further use.

**Transesterification**

For gas chromatography with flame ionization detection (GC-FID), fatty acids and acyl residues of lipids were converted to fatty acid methyl esters (FAMEs) according to Miqel and Browse (1992). Forty microliters of lipid extracts dissolved in TMW were evaporated to dryness under a stream of nitrogen and cultivated with methanol/toluene/dimethoxypropane/sulfuric acid (66:33:2:2.5, v/v/v/v) at 80 °C for 1 h after addition of 3 µg glycercyl triheptadecanoate (Merck KGaA, Germany) as internal standard. Adding saturated NaCl solution and n-hexane allowed the FAMEs to be extracted via vigorous mixing. After evaporation of the separated hexane phase under a stream of nitrogen, FAMEs were dissolved in acetonitrile, covered with argon and stored at −20 °C until further use.

**Methylation and acetylation of lipids**

To improve the chromatographic separation and mass spectrometric detection of certain lipid subclasses, lipid extracts were chemically modified. For the LC–MS/MS analysis of phosphatidic acid (PA) and lysophosphatidic acid (LPA), aliquots of the lipid extracts were methylated according to Lee et al. (2013). Eighty microliters of lipid extracts dissolved in TMW were evaporated to dryness under a stream of nitrogen and dissolved in 400 µL methanol. 6.5 µL trimethysilyldiazomethane (2 M in n-hexane; Merck KGaA) were added, followed by 30 min shaking and final addition of 2 µL 1 N acetic acid. The sample was evaporated to dryness under a stream of nitrogen, dissolved in 80 µL TMW, covered with argon and stored at −20 °C until further use. For the LC–MS/MS analysis of free sterols, aliquots of the lipid extracts were acetylated with a modified protocol according to Berdyshhev et al. (2005). Forty microliters of the lipid extracts were evaporated to dryness under a stream of nitrogen and vigorously mixed with 100 µL pyridine and 50 µL acetic anhydride. After incubation at 50 °C for 30 min, the sample was evaporated under a stream of nitrogen and the residues were dissolved in 40 µL TMW, covered with argon and stored at −20 °C until further use.

**LC–MS/MS analysis**

The molecular lipid species were analyzed by an ultra-performance liquid chromatography (UPLC) system coupled with a chip-based nanoelectrospray ionization (nanoESI) source and a triple quadrupole analyzer for tandem mass spectrometry (MS/MS) as described previously (Herrfurth et al. 2021). The analysis of molecular species from 35 different lipid subclasses was performed with lipid subclass-specific parameters as shown in Table S1. For reverse-phase LC separation, an ACQUITY UPLC I-class system (Waters Corp., USA) equipped with an ACQUITY UPLC HSS T3 column (100 mm × 1 mm, 1 µm; Waters Corp) was used. Solvent A was methanol/20 mM ammonium acetate (3:7; v/v) containing 0.1% (v/v) acetic acid, and solvent B was tetrahydrofuran/methanol/20 mM ammonium acetate (6:3:1; v/v/v) containing 0.1% (v/v) acetic acid. All lipid subclasses were separated with linear binary gradients following the same scheme: respective start condition (Table S1) held for 2 min, linear increase to 100% solvent B for 8 min, 100% solvent B held for 2 min, and re-equilibration to start conditions in 4 min. Chip-based nanoESI was achieved with a TriVersa Nanomate (Advion, USA) in the positive or negative ion mode, respectively (Table S1). Lipid molecular species were detected with a 6500 QTRAP tandem mass spectrometer (AB Sciex, USA) by multiple reaction monitoring (MRM) with lipid class-specific parameters (Table S1). For glycerolipid and glycerophospholipid analysis, all MRMs for the distinct lipid subclasses were measured for the putative lipid species having 14:0, 15:0, 16:0, 16:1, 16:2, 16:3, 16:4, 18:0, 18:1, 18:2, 18:3, 18:4, 20:5, 22:6, 24:0, and 24:1 as acyl residues. For sphingolipid analysis, all MRMs for the distinct lipid subclasses were measured for the putative lipid species having 14:0, 15:0, 16:0, 16:1, 16:2, 16:3, 16:4, 18:0, 18:1, 18:2, 18:3, 18:4, 20:5, 22:6, 24:0, and 24:1 as acyl residues. For sterol lipid analysis, all MRMs for the distinct lipid subclasses were measured for the putative lipid species having 14:0, 15:0, 16:0, 16:1, 16:2, 16:3, 16:4, 18:0, 18:1, 18:2, 18:3, 18:4, 20:5, 22:6, 24:0, and 24:1 as LCB residues and chain lengths from C16 to C28 as acyl residues that are saturated or monounsaturated and monoxygenated, monohydoxyated, or dihydroxylated. For sterol lipid analysis, all MRMs for the distinct lipid subclasses were measured for the putative lipid species having brassicasterol/24-methylenecholesterol, campesterol, cholesterol, fucosterol/isofucosterol/stigmasterol, and sitosterol as sterol residue and the fatty acids as acyl residues as described above for the glycerolipid analysis. Peak integration was executed with MultiQuant software (AB Sciex, USA) followed by carbon isotope correction in Microsoft Excel.

**GC-FID analysis**

FAMEs were detected by gas chromatography with flame ionization detection (GC-FID) according to Lang et al. (2011) and Steudel et al. (2016). The system was composed of an Agilent 6890 chromatograph (Agilent Technologies,
Germany) with a DB-23 column (30 m × 0.25 mm, 0.25 μm coating thickness; J&W Scientific, Agilent Technologies, Germany). Helium was used as a carrier gas with a flow rate of 1 mL min⁻¹. The temperature gradient was 150 °C for 1 min, 150–200 °C at 8 K min⁻¹, 200–250 °C at 5 K min⁻¹, and 250 °C for 6 min. “F.A.M.E. Mix C4-C24” (Merck KGaA) was used as external standard. ChemStation software (Agilent) was used for peak area determination. Peaks were assigned to fatty acids via retention time and comparison with the external standard.

**Thin layer chromatography**

A thin layer chromatography (TLC) silica gel 60 plate (20 × 20 cm; Merck KGaA) with a concentration zone of 2.5 × 20 cm was impregnated with 0.15 M (NH₄)₂SO₄ solution and dried on air for at least 2 d. Prior to use the plate was activated by heating to 120 °C for 2.5 h. Lipid extract samples solved in TMW were evaporated to dryness in nitrogen stream, dissolved in 150 µL chloroform/methanol (2:1, v/v), vigorously mixed and centrifuged (450 × g, 20 °C, 20 s). The samples were loaded onto the activated TLC plate in 3-cm broad stripes at the start line. Left and right from Tp-div and Tp-G1 samples standard lipid mixtures (Merck KGaA) were added as reference. The separation was achieved in a TLC glass chamber with acetone/toluene/water (91:30:8, v/v/v) for about 1.5 h. Afterwards, the plate was dried on air, the reference lanes were cut off and dipped into 0.4 M CuSO₄ in 6.8% (v/v) H₃PO₄. The reference staining was achieved by heating the plate parts to 180 °C. The Tp lipid samples were stained by spraying with 0.05% primuline in 80% acetone. The bands were visualized under UV radiation and marked with a pencil. The primuline-stained spots were scratched off, incubated in methanol/toluene/dimethoxypropane/sulfuric acid (66:33:2:2.5, v/v/v/v) over night, transesterified and used for GC-FID analysis.

**Statistical analysis**

Statistical analysis was performed with a two-tailed t-test with unequal variances in Microsoft Excel with a significance level of 0.05 (*) and 0.005 (**).

**Results**

Our analysis was performed from two sample groups of synchronized *Thalassiosira pseudonana* (Tp) cells, called Tp-div and Tp-G1. These groups are defined by different stages in Tp cell cycle and differ in the abundance of valve SDVs. Sample group Tp-div was harvested at 3 h after silicon replenishment when valve SDV enrichment was observed. The second sample group (Tp-G1) was harvested at 6 h after silicon replenishment, when no valve SDVs were observed. Our initial approach to isolate pure SDVs failed due to remaining chloroplast contaminations.

**Total fatty acid composition**

First, fatty acid (FA) residues as major lipid components were determined via gas chromatography with flame ionization detection (GC-FID) to define all possible FA-lipid headgroup combinations, which were then analyzed by our lipidomics workflow by using liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) (Herrfurth et al. 2021).

Figure 1 shows the absolute FA amounts of the two sample groups normalized to the initial sample dry weight. Sixteen different FAs with even-numbered chain lengths between 14 and 24 carbon atoms and with different degrees of unsaturation were determined. A single odd-numbered FA (15:0) was detected in addition. Based on its retention time, a methyl-branched structure can be excluded for this odd-numbered FA. The most prominent FAs were 16:1 (n-7), 20:5 (n-3), 16:0, 18:4 (n-3), 14:0, and 22:6 (n-3) sorted in descending order. Overall, the averaged total FA content is 180 ± 13 μmol g⁻¹ d.w. (49 ± 3 mg g⁻¹ d.w.) for Tp-div and 161 ± 5 μmol g⁻¹ d.w. (44 ± 1 mg g⁻¹ d.w.) for Tp-G1, respectively. The total content differences were statistically not significant (p = 0.110, two-tailed Student’s t-test with unequal variances). Considering all FAs with at least 1% abundance, the molar FA amounts as well as the relative FA profiles did not differ significantly between the two sample types (n = 3 for each) except 22:6 (n-3) with p = 0.009.
Although these results indicate that Tp cells do not significantly change their FA composition and amount during their cell cycle from SDV formation to the G1 interphase, there is a tendency towards a slightly higher amount of total FA content at a stage where SDVs are enriched.

**Lipid subclass-specific molecular species profiles**

To determine the absolute abundance ratios of the different subclasses of the glycolipids and glycerophospholipids to each other, the lipid subclasses were separated by thin layer chromatography (TLC) and afterwards quantified with GC-FID analysis with respect to the FA content (Fig. S1, S2 and Table S2). By this way, MGDG, SQDG, PG, DGDG, and PC were identified to be the five major polar lipid subclasses of both sample groups of *T. pseudonana*.

Using our lipidomics workflow, the total lipid extracts were analyzed for the exact molecular lipid composition. For this purpose, all GC-FID-identified FAs were combinatorically assembled together with known polar head groups and lipid backbones that are classified in literature as relevant in diatoms (Hunter et al. 2018). This led to the formation of the mass transition lists for 35 individual lipid subclasses belonging to the lipid categories glycerolipids (betaine lipids: diacylglyceryl carboxyhydroxymethylcholine (DGCC); glycolipids: monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG), and the monoacylglycerolipids MGMG, DGMG, SQMG; neutral lipids: diacylglycerol (DG) and triacylglycerol (TG)), glycerophospholipids (phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidic acid (PA), and the monoacylglycerophospholipids: diacylglyceryl phosphaethanolamine (PEC), diacylglyceryl phosphoserine (PEC), diacylglyceryl phosphocholine (PC), phosphatidic acid (PA), and the monoacylglycerophospholipids: diacylglyceryl phosphatidylcholine (DGPC), diacylglyceryl phosphatidylethanolamine (DGPE), diacylglyceryl phosphatidylserine (DGPS), and diacylglyceryl phosphatidic acid (DGPA)), sphingolipids (sphingoid base (SPB); ceramide (Cer); glycosphingolipids: monohexosyl ceramide (HexCer), dihexosyl ceramide (Hex2Cer); inositolphosphoceramide (IPC); glycosylinositolphosphoceramides: glucuronosylinositolphosphoceramide (GlcA-IPC), hexosyl-GlcA-IPC (Hex-GlcA-IPC), N-acetylhexosaminyl-GlcA-IPC (HexNAc-GlcA-IPC), hexosyl-hexosyl-GlcA-IPC (Hex-Hex-GlcA-IPC), and N-acetylhexosaminyl-hexosyl-GlcA-IPC (HexNAc-Hex-GlcA-IPC)), and sterol lipids (sterylglycoside (SG), acylsterylglycoside (ASG), sterol ester (SE), and free sterol (FS)) (Table S1). To improve the chromatographic separation and mass spectrometric detection, phosphate groups of phosphatidic acid and lysophosphatic acid were methylated before analysis and the hydroxyl group of free steryl was acylated.

**Glycerolipids and glycerophospholipids**

Figure 2 provides an overview about the molecular species profiles of all detected diacylglycerolipid subclasses. PC contained a relatively high proportion of molecular species with 20:5, at least at one sn position (16:0_20:5, 16:1_20:5, 18:4_20:5, 20:5_20:5, and 20:5_22:6). The most common molecular species of PE and PA were 20:5_22:6, and both glycerophospholipid subclasses had significant relative amounts of 20:5_20:5. In contrast, PG, PI, and DG contained shorter fatty acid residues on average since they harbored relatively high amounts of 14:0_16:1, 16:0_16:1, and 16:1_16:1. The most common molecular species of PE and PA was 20:5_22:6, and both glycerophospholipid subclasses had significant relative amounts of 20:5_20:5. In contrast, PG, PI, and DG contained shorter fatty acid residues on average since they harbored relatively high amounts of 14:0_16:1, 16:0_16:1, and 16:1_16:1 and 18:0_18:0 could be found exclusively in PI.

Additional species with the identical saturated FA at both sn positions of the diacylglycerol molecules also existed prominently in SQDG (14:0_14:0 and 16:0_16:0) and PS (15:0_15:0). However, unlike here, these FA combinations never exceeded 5 mol% in the other lipid subclasses. Among glycolipid subclasses, MGDG had a widely distributed molecular species profile with relatively high amounts of 16:2, 16:3, and 16:4, whereas DGDG contained more prominently 16:2_20:5. In summary, the LC–MS/MS analysis revealed that all ten lipid subclasses
had a specific profile at the molecular species levels. On the level of the two different stages in Tp cell cycle, the lipid molecular species profiles exhibited some differences between Tp-div and Tp-G1. For example, the relative levels of PA(20:5_22:6), DG(16:1_16:1), SQDG(14:0_14:0), and PC(20:5_20:5) significantly increased, whereas the relative levels of SQDG(16:0_16:0) and PG(16:0_20:5) decreased in Tp-div in contrast to Tp-G1. The occurrence of the betaine lipid DGCC was also investigated but not a single molecular species of this lipid subclass could be detected.

To further elucidate the developmental-specific differences between the two Tp cultures, FA profiles were calculated using the LC–MS/MS molecular species data. For this purpose, the LC–MS/MS peak areas of the FA-specific mass transitions were summed for each lipid subclass. An absolute comparison between different lipid head groups is not meaningful because they are ionized to different extents during electrospray ionization in the separation process of liquid chromatography, resulting in divergent peak area scales.

The FA profiles for glycerolipids (including TG) and glycerophospholipids resulting from the LC–MS/MS data, C-16 FAs, and the polyunsaturated FAs 18:4, 20:5, and 22:6 are in general the most prominent FAs in all these lipid subclasses (Fig. 3). Analogous to the molecular species profiles, FA profiles of PG, PI, DG, and TG were similar to each other (especially considering 14:0, 16:0, and 16:1). Similarly, PC and PA (and to a lesser extent PE) contained high amounts of the long- and very long-chain (VLC)-polyunsaturated FAs (PUFAs) 18:4, 20:5, and 22:6, in addition to 14:0, 16:0, and 16:1. In contrast, PS, MGDG, DGDG, and SQDG had quite different FA profiles among each other. Here, either 15:0, 16:3, 16:2, and 20:5 or 14:0 and 16:0 dominated in the lipid subclass-specific FA profiles. The less abundant FAs 24:0 and 24:1 were detected exclusively in TG at very low levels. The FAs 18:2 and 18:3 were found only as minor components of the diacylglycerolipid subclasses. Overall, all these lipid subclass-specific profiles obtained from the LC–MS/MS data agreed well with the overall FA profile resulting from the GC analysis. In particular, the TG- and PC-specific profiles showed many similarities with the overall FA profile. In most lipid subclasses, the FA profiles were unchanged in both cell cycle phases. Only SQDG showed a significant relative increase of 14:0 and a relative decrease of 16:0 in Tp-div compared with Tp-G1.

The FA profiles of the monoacylglycerolipids (Fig. 4), constructed from the LC–MS/MS data, followed the trend already shown for the diacyl- and triacylglycerolipids. The profile of LPA was similar to both LPC and LPE. The FA profile of DGMG showed only small amounts of 16:2 and 20:5. For LPS, 18:0 was the only FA detected, although 15:0 would also have been expected according to the LC–MS/MS data of PS.
PUFAs 16:3, 16:4, 18:4, 20:5, and 22:6 (Fig. S7). 16:3 showed a significant relative decrease, whereas 20:5 had a relative increase by about a factor of two for Tp-div compared to Tp-G1. SG and ASG were also analyzed but no single molecular species could be detected for these lipid subclasses. Sphingolipids have also been studied corresponding to Table S1 and molecular species were detected in the following sphingolipid subclasses: Cer, HexCer, Hex2Cer, and Hex-GlcA-IPC (Figs. S8-S11). In all these sphingolipid subclasses, the identified SPB residues harbored 1–3 double bonds and were dihydroxylated (18:1;O2, 18:2;O2, 18:3;O2) and the amide-linked fatty acids were 16:0, 24:0, 24:1, 26:0, or 26:1. Among the molecular species detected, the most prominent ceramide was Cer(18:3;O2/24:1) with about 50 mol% (Tp-div: 55 ± 0.5 mol%, Tp-G1: 46 ± 2 mol%). For HexCer, the molecular species HexCer(18:3;O2/24:1) directly followed this trend with more than 50 mol%, but without a significant difference between Tp-div and Tp-G1. Analogous to this, Hex2Cer(18:3;O2/24:1) and Hex-GlcA-IPC(18:3;O2/24:1) contributed with 50 mol% to Hex2Cer, and about 40 mol% to Hex-GlcA-IPC, respectively, also with no sample type differences.

Very long-chain polyunsaturated fatty acids and their saturation levels

FA categorization allows highlighting characteristics like FA chain length or degree of (un)saturation. The VLC-PUFAs eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6) are omega-3 FAs, which current literature often deals with, since EPA and DHA are interesting for food and feed applications (Swanson et al. 2012; Khozin-Goldsberg et al. 2016). Figure 5 displays the relative amount of EPA + DHA in total and with respect to each detected lipid subclass. The overall percentage (“total”) independent of the lipid species was 23.9 ± 1.7 mol% for Tp-div and 24.4 ± 2.0 mol% for Tp-G1, i.e., no significant difference between the two sample types were found. More than 30% of PC-, PE-, PA-, DGDG-, LPC-, LPE-, and LPC-bound fatty acids were EPA and DHA. The largest amount was given for PE with more than 80 mol%. For PS and PI, as well as for their monoacyl lipid species, almost no VLC-PUFAs were found. Also, for SQDG, MGMG, DGMG, and SQMG, the percentage is below 10 mol%. Even though the total percentage of VLC-PUFAs was the same for Tp-div and Tp-G1, the individual lipid subclasses showed significant differences ∆. For PC (∆ = + 6.5 mol%), PE (∆ = + 4.6 mol%), PA (∆ = + 10.3 mol%) and the monoacyl lipid subclasses LPC (∆ = + 12.6 mol%) and LPE (∆ = + 13.4 mol%) as well as for SE (∆ = + 11.5 mol%), an increase in VLC-PUFAs is observed for Tp-div.
compared to Tp-G1, whereas DG (∆ = −4.8 mol%), TG (∆ = −3.0 mol%) and PG (∆ = −4.0 mol%) showed a statistically significant decrease.

A ternary diagram is provided in Fig. 6, which allows gaining an overview of FA distribution in Tp apart a detailed FA profile but taking the saturation levels of the different lipid species-bound FAs into account. The (un)saturation of FAs influences membrane properties like its fluidity. Figure 6 shows that the saturation level of the fatty acids of the total lipidome is rather equally distributed (29% saturated (s), 29% monounsaturated (m), 42% polyunsaturated (p)) and shows no significant difference between Tp-div and Tp-G1. Considering lipid subclass-assigned FAs, saturated and PUFAs distributed over the whole range from nearly 0 to 100%. Monounsaturated FAs did not exceed the 50% level independent of the lipid species. The neutral glycolipid species DG (23% s, 42% m, 35% p) and TG (31% s, 40% m, 29% p) showed FA distributions similar to the total cell. However, for PC, PE, PA, MGDG, DGDG, and SE, the FA composition was strongly shifted towards larger amounts of PUFAs (>68% p). In contrast, for PI (46% s, 45% m, 9% p) and PG (37% s, 44% m, 19% p), low PUFA percentages were found, while the SQDG (71% s, 9% m, 20% p) and PS (97% s, 3% m, 0% p) species were largely saturated.

Sample type-specific distribution of the lipid subclasses (fold change)

All MRM signals in UPLC-nanoESI-MS/MS analysis were merged per lipid subclass. When all sample type-assigned values were summed up to 100% (Fig. S12), Tp-div and Tp-G1 could be compared considering the relative contribution of each lipid subclass to the whole of all detected lipid subclasses. The resulting fold changes (Tp-div/Tp-G1) are displayed for each lipid species in Fig. 7. The three glycerolphospholipid subclasses PC, PE, and PA were significantly increased with a factor of 1.5 ± 0.1, 1.6 ± 0.1, and 1.7 ± 0.4, respectively. LPC was the only monoacyl lipid subclass with a large significant fold change of 3.3 ± 0.6, while LPG significantly decreased by a factor of 0.4 ± 0.1. The glycolipids MGDG (0.8 ± 0.1), DGDG (0.66 ± 0.04), and SQDG (0.40 ± 0.04), as well as the monoacyl lipid subclasses MCMG (0.6 ± 0.1, p = 0.079, not significant), DGMG (0.5 ± 0.1), and SQMG (0.2 ± 0.1) showed decreases in Tp-div compared to Tp-G1. In case of non-glycerolipids, only SE and Cer showed both statistically significant decreases to 0.5 ± 0.1, whereas the fold changes for FS were in the same range (0.4 ± 0.2), but not significant (p = 0.056).

Discussion

From our results, we conclude that valve SDV-enriched T. pseudonana cells (Tp-div) and cells in following G1 phase (Tp-G1) differ neither in their total FA composition nor in their total lipid amounts (Fig. 1). The most abundant 16:1, 16:0, and 20:5 FAs were also reported by Tonon et al. (2005) and Smith et al. (2016). However, 14:0 was found with significant lower amounts than reported by others (Volkman et al. 1989; Zhukova 2004; Cook and Hildebrand 2016; Sabia et al. 2018). However, for the different lipid subclasses analyzed with UPLC-nanoESI-MS/MS, the FA composition differed between Tp-div and Tp-G1. The simultaneous
decrease in glycolipids and increase of PC, PE, PA, and LPC contributions to the lipidome in Tp-div was the most significant observed change in lipid composition (Fig. 7). Additionally, PC, PE, PA, and LPC showed an increased amount of VLC-PUFAs (EPA and DHA) compensated by the corresponding decrease in DG, TG, and PG, so that EPA and DHA amounts remained rather constant in total (Fig. 5). The observed 23.9 ± 1.7 mol% (Tp-div) and 24.4 ± 2.0 mol% (Tp-G1, Fig. 5) confirm the results of Tonon et al. (2005), who found 22.8 ± 0.5 mol% and of Volkman et al. (1989), who reported 23.2 wt%. Cook and Hildebrand (2016), however, found only about 15 wt% of EPA + DHA after 24 h silicate starvation, which might be explained by different growing conditions like constant illumination instead of day/night cycles.

Molecular lipid species profiles resulting from UPLC-nanoESI-MS/MS agree with those reported by Hunter et al. (2018) for PC, PE, and PG, but not for MGDG, DGDG, and SQDG. Under phosphate starvation, Hunter et al. (2018) detected the betaine lipid DGCC, but under phosphate-repleted conditions DGCC was not detectable. These findings fit well to the absence of DGCC in our analysis, since phosphate was always present in sufficient amounts. The substitution of PC with DGCC under phosphate stress is a known reaction of diatoms (van Mooy et al. 2009). For SQDG, the lipid profile was dominated by SQDG14:0:14:0 as the most frequent molecular species, different from the report of Hunter et al. (2018). Of note, SQDG experienced a lipid molecular species change from Tp-div to Tp-G1, which suggests that the SQDG lipid composition changes during cell cycle or is a result of silicon starvation leading to higher chloroplast pigmentation and an increased non-photochemical quenching (Smith et al. 2016). DG and TG lipid and FA profiles agree well with those of Hunter et al. (2018) and Tonon et al. (2002). 18:3:0/24:1 was the most prominent LCB-FA combination in Cer, HexCer, Hex2Cer (all also detected by Hunter et al. 2018), and Hex-GlcA-IPC. FS and SE showed a very frequent molecular species with a molecular mass of 398 g mol⁻¹. 24-methylcholesta-5,24(24’)-dien-3β-ol as possible sterol was reported by Volkman and Hallegaeff (1988) and Rampen et al. (2010) with 75% and 85%, respectively. Véron et al. (1998) also detected brassicasterol (24-methylcholesta-5,22-dien-3β-ol) in small amounts, which would also comply with the observed corresponding molecular mass. Next to the 24-methylenecholesterol isomer, we identified campesterol according to the molecular mass. Next to the 24-methylenecholesterol isomer, we identified campesterol according to the molecular mass. Next to the 24-methylenecholesterol isomer, we identified campesterol according to the molecular mass. Next to the 24-methylenecholesterol isomer, we identified campesterol according to the molecular mass.
Data availability The datasets generated and analyzed during the current study are available from the corresponding authors in reasonable request.

Declarations

Competing interests The authors declare no competing interests.

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