Plant lipidomics based on hydrophilic interaction chromatography coupled to ion trap time-of-flight mass spectrometry

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Abstract Plants synthesize a wide range of hydrophobic compounds, generally known as lipids. Here, we report an application of liquid chromatography ion trap time-of-flight mass spectrometry (LC-IT-TOF-MS) for plant lipidomics. Using hydrophilic interaction chromatography (HILIC) for class separation, typical membrane lipids including glycerolipids, steryl glucosides and glucosylceramides, and hydrophobic plant secondary metabolites such as saponins were analyzed simultaneously. By this method, we annotated approximately 100 molecules from Arabidopsis thaliana. To demonstrate the application of this method to biological study, we analyzed Arabidopsis mutant trigalactosyldiacylglycerol3 (tgd3), which has a complex metabolic phenotype including the accumulation of unusual forms of galactolipids. Lipid profiling by LC-MS revealed that tgd3 accumulated an unusual form of digalactosyldiacylglycerol, annotated as Gal(β1→6)βGalDG. The compositional difference between normal and unusual forms of digalactosyldiacylglycerol was detected by this method. In addition, we analyzed well-known Arabidopsis mutants ats1-1, fad6-1, and fad7-2, which are also disrupted in lipid metabolic genes. Untargeted lipidome analysis coupled with multivariate analysis clearly discriminated the mutants and their distinctive metabolites. These results indicated that HILIC-MS is an efficient method for plant lipidomics.

Keywords Hydrophilic interaction chromatography · Ion trap time-of-flight mass spectrometry · Lipidome · Lipidomics

1 Introduction

Recent developments in mass spectrometry make it feasible to analyze metabolome. The metabolome represents the ultimate cell phenotype, which is controlled through the transmission of genetic information. Thus, one must analyze a snapshot of the metabolome to precisely understand cell status. However, plants synthesize a huge array of metabolites that cannot be revealed by any single methodology (Saito and Matsuda 2010). Plant lipidomics based on tandem mass spectrometry using a triple quadrupole mass spectrometer is frequently applied to the analyses of several polar glycerolipids and sphingolipids using highly selective functions including precursor ion and neutral loss scanning or multiple reaction monitoring according to well-characterized mass fragmentation patterns (Brügger et al. 1997; Welti et al. 2003; Welti and Wang 2004; Markham and Jaworski 2007). These approaches have revealed many new insights into plant lipid metabolism and its genetic foundations (Chen et al. 2008; Beaudoin et al. 2009; Jao et al. 2009; Michaelson et al. 2009; Zhang et al. 2009; Peters et al. 2010; Roudier et al. 2010; Xiao et al. 2010). However, plants synthesize lipids that are not yet classified or well characterized by mass spectrometry. Focused metabolomics
approaches cannot address these lipids. An improved analytical method appropriate for a wide range of plant lipid classes and lipidome can facilitate future studies on plant lipid metabolism and our understanding of the diversity of plant metabolome.

In this study, liquid chromatography ion trap time-of-flight mass spectrometry (IT-TOF-MS) was used to analyze the plant lipidome; high resolution MS² was expected to be an efficient method to characterize the complex mixture of plant metabolites (Shibano et al. 2007; Zhou et al. 2009; Aboshi et al. 2010; Liu et al. 2010). The power of MS² for metabolite profiling can be enhanced by combination with chromatographic separation. Since many lipid classes consist of hydrophobic moieties (i.e., fatty acid and ceramide) and polar functional groups (phosphate, glucose, and galactose), normal phase chromatography provides efficient class separation. We employed hydrophilic interaction chromatography (HILIC), which is often used to analyze polar compounds. A similar analytical system was used for targeted profiling of polar glycerolipids of mutant plants (Okazaki et al. 2009). We expanded the coverage of this analytical system to other major membrane lipid classes and lipophilic plant secondary metabolites. In addition, we applied our method to the analysis of the lipidome of several *A. thaliana* mutants.

For a case study of the biological application of our method, we analyzed the *Arabidopsis tgd3* mutant (trigalactosyldiacylglycerol3) (Fig. 1) (Lu et al. 2007). The biosynthesis of lipids occurs in the discrete subcellular biogenic membranes of different cellular compartments.

**Fig. 1** Glycerolipid biosynthesis in *A. thaliana* leaf. An abbreviated biosynthetic scheme for the major plant glycerolipids is represented. Breaks in the pathway represent the mutation sites described in case studies 1 and 2. The set of reactions occurring within the chloroplast are termed the “prokaryotic pathway” and those that involve glycerolipid synthesis in the ER and subsequent transfer to the chloroplast constitute the “eukaryotic pathway”. Dashed arrows indicate the enhanced biosynthetic pathway of oligogalactolipids in the *tgd3* mutant. For simplification, another pathway to transport ER-derived glycerolipid to chloroplasts, mediated by lipin-like phosphatases, is omitted (Nakamura et al. 2009). This metabolic pathway is illustrated according to Somerville et al. (Somerville et al. 2000) with modifications. ACP acyl carrier protein, PA phosphatidic acid, DAG diacylglycerol, PhosCho phosphocholine, PhosGly phosphoglycerol, t16:1 hexadec-3-trans-enoic acid, SQ sulfogluconovose, GGGT galactolipid:galactolipid galactosyltransferase, βGalβGal Gal(β1 → 6)βα Gal, βGalβGal Gal(β1 → 6)β Gal, βGalβGal Gal(β1 → 6)βα Gal
Therefore, the intracellular trafficking of lipids is essential for the assembly of membrane systems. In seed plants, the quantitatively largest flux of lipid precursors is between the 2 major sites of glycerolipid assembly, namely the endoplasmic reticulum (ER) and the plastid envelopes (Somer-ville and Browse 1996; Benning 2009). These biogenic membranes cooperate and constitute parallel pathways for thylakoid lipid biosynthesis in the ER and the plastid. Recently, mutants of Arabidopsis lacking ER-derived thylakoid lipid species were isolated and four proteins involved in ER-to-plastid lipid transport were identified (Xu et al. 2003; Xu et al. 2005; Awai et al. 2006; Lu et al. 2007; Xu et al. 2008). These loci were named TRIGALACTOSYLDIACYLGLYCEROL (TGD) because of the accumulation of the new galactoglycerolipid trigalacto- soyldiacylglycerol (TGDG). Based on detailed analyses of the mutants and the respective proteins, it was proposed that TGD 1, 2 and 3 form a complex in the inner chloroplast envelope membrane responsible for delivering ER-derived phosphatidate (PA) to the inside of the inner envelope membrane. PA dephosphorylation gives rise to DAG, which is used for galactolipid biosynthesis in plastid envelope membranes. The fourth protein, TGD4, is involved in lipid transport between the ER and outer chloroplast envelope membrane (Xu et al. 2008). In addition to TGDG, Arabidopsis mutants deficient in TDG1 accumulate an unusual form of digalactosyldiacylglycerol (DGDG), Gal(β1 → 6)/GalDG. NMR analysis of purified DGDG revealed that DGDG in tgd1 was a mixture of Gal(β1 → 6)/GalDG and normal DGDG (Gal(α1 → 6)/GalDG), but the detailed profile of Gal(β1 → 6)/GalDG in tgd mutants remains unclear (Xu et al. 2003). Since all the TGD proteins are involved in the same metabolic function, tgd3 was also expected to accumulate Gal(β1 → 6)/GalDG, although there has been no detailed analysis of intact lipids in tgd3. We analyzed DGDG in tgd3 to evaluate the separation ability and coverage of our method, and generated a detailed profile of two forms of intact DGDG in this mutant.

We also analyzed several well-characterized Arabidopsis mutants (ats1-1, fad6-1, and fad7-2) which were used for studies on the biosynthesis of polyunsaturated fatty acids and plasticid membrane lipids (Fig. 1) (Kunst et al. 1988; Iba et al. 1993; Falcone et al. 1994; Xu et al. 2006), to determine whether untargeted lipidome analysis of these mutants could reproduce the results of these previous reports.

2 Materials and methods

2.1 Chemicals

1,2-Dioctanoyl-sn-glycero-3-phosphocholine (10:0/10:0 PC) and 30% ammonium hydroxide (A.C.S. reagent) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other phospholipid standards (PC, PE, PG, PA, and PI) and a soybean glucosylerceramide [comprised primarily (>98%) of ceramide with 4,8-sphingadiene and α-hydroxypalmitic acid] were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Steryl glucoside and tomatine were obtained from Larodan Fine Chemicals (Malmö, Sweden) and Tokyo Chemical Industry (Tokyo, Japan). Solvents for LC-MS and other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan) unless stated otherwise.

2.2 Plant materials

Seeds of A. thaliana mutants (SALK_040335 for tgd3, CS200 for ats1-1, CS207 for fad6-1, and CS8042 for fad7-2) were obtained from the ABRC (Ohio, USA). The T-DNA insertion site in tgd3 was confirmed by sequencing of PCR fragments at the left border of the T-DNA amplified using LBA1 (5’-TGGTTCACGTTAGGCGCATCG-3’) and gene-specific primer (5’-TGCTTCAGTGTAGTCATGTAAGATGT-3’). Arabidopsis plants were grown on agar-solidified Murashige-Skoog medium containing 1% (w/v) sucrose and MS vitamin at 22°C under a 16-h-light/8-h-dark cycle for 18 days. Plant tissues were harvested 6 h after the onset of the light phase, frozen in liquid nitrogen, and stored at −80°C until lipid extraction. Oat seeds (Avena strigosa cv. Hayoats) were purchased from Snow Brand Seed (Sapporo, Japan), and rice seeds (Oryza sativa L. cv. Nipponbare) were the kind gift of Prof. Masahiro Yano at the National Institute of Agrobiological Sciences. Caryopses of rice and oat were incubated for 5 days under the same growth conditions used for A. thaliana.

2.3 Sample preparation

Total lipids were extracted according to the method of Bligh and Dyer (Bligh and Dyer 1959) with slight modifications. The frozen plant material was milled on a Mixer Mill MM300 (Retsch, Haan, Germany) for 2 min at 15 Hz with a zirconium bead. The milling process was carried out under cryogenic conditions (the grinding jars were cooled with liquid nitrogen before each milling process to ensure a low milling temperature to keep the plants frozen). The frozen powdered plant material was kept in liquid nitrogen and a 20-fold volume of extraction solvent (CHCl3−MeOH−H2O = 50:100:31.45, v/v/v) was added with 1 μM 10:0/10:0 PC as the internal standard, followed by vigorous stirring on a vortex mixer. After dark incubation for 5 min at room temperature, the homogenate was centrifuged at 3,000 × g for 3 min at room temperature. Two hundred microliters of supernatant was mixed with CHCl3 and H2O (52.6 μl each), stirred on a vortex mixer, and incubated on
for 15 min in darkness. The mixture was centrifuged at 1,000×g for 3 min, and 85 μl of the lower layer was collected. The organic phase was dried by a centrifugal concentrator at room temperature and the residue was dissolved in 81 μl of CHCl₃ for LC-MS analysis.

2.4 Mass spectrometry and data analysis

Mass spectra were recorded on an LCMS-IT-TOF mass spectrometer combined with an LC-20AD HPLC system (Shimadzu Corporation, Kyoto, Japan) using an Atlantis HILIC silica column (average particle size, 3 μm; 2.1 mm i.d., 100 mm long; Waters, MA, USA) as reported previously with some alterations (Okazaki et al. 2009). A two-solvent system was used to generate the mobile phase: solvent A, methanol–water (95:5, v/v) containing 0.2% ammonium formate (pH 5.9); solvent B, acetonitrile–methanol–water (95:2:3, v/v/v) containing 0.2% ammonium formate (pH 5.9). The pH of both solvents A and B was adjusted by adding 30% NH₄OH to the mixtures of solvents containing 0.2% (v/v) formic acid. The gradient conditions for lipid separation were as follows: 100% solvent B (0–3.33 min), 100–65% solvent B (3.33–10 min), 65–30% (10–11.33 min), 30% solvent B (11.33–14.66 min), 100% solvent B (14.66–40 min). The flow rate was held at 0.2 ml min⁻¹ increased to 0.4 ml min⁻¹ at 14.66 min after gradient initiation, maintained for 13.33 min, and then decreased to 0.2 ml min⁻¹. Total elution time was 40 min. The column temperature was 25°C.

Peak picking and peak alignment were performed by Profiling Solution (version 1.0.76.0) (Shimadzu Corporation, Kyoto, Japan). The Profiling Solution parameters were as follows: ion m/z tolerance, 20 mDa; Ion RT tolerance, 0.1 min; Ion intensity threshold, 2e4; detect isomer valley, 20%; allow some ion without isotope peak, ON; time range for processing, 0–20 min. The data matrix containing 534 ions was exported from Profiling Solution and normalized based on the intensity of [M – HCO₂]⁻ of 10:0/10:0 PC. Peak height of individual galactolipid molecules were calculated based on the m/z values of [M – HCO₂]⁻. Because lipid species with the same polar head eluted at almost the same retention time, corrections for overlap of isotopic variants in higher-mass lipids were applied. Annotation was based on theoretical m/z values of each possible glycerolipid species in plants and retention time of authentic compounds with same polar head using an in-house Perl script. After this process, data was pareto-scaled and subjected to PCA and OPLS-DA using SIMCA-P (version 11.0.0.0, Umetrics, Umeå, Sweden). LC-MS data and metadata used in case study 2 are available as Supplementary Material 1.

3 Results and discussion

3.1 HILIC-MS can be used to analyze various membrane lipids and lipophilic secondary metabolites

Crude lipid extracts of leaves and roots of the model plant A. thaliana was analyzed by HILIC coupled to IT-TOF-MS. As reported previously, typical membrane glycerolipids including MGDG, DGDG, SQDG, PC, PE, PG, and PI were detected in leaves and roots based on the authentic compounds (Fig. 2) (Okazaki et al. 2009). Similar lipid profiles were observed in the leaves of rice (Oryza sativa), an important cereal plant (Supplementary Fig. 1). Since HILIC is a version of normal phase chromatography, lipid molecules with the same polar head group had similar retention times (Malavolta et al. 2004; Houjou et al. 2005; Taguchi et al. 2007). With these properties, HILIC is similar to silica gel chromatography. However, the chromatography solvents were different: in our method, we used a mixture of methanol, acetonitrile, and water, while ordinary silica gel chromatography for plant lipid analysis is often performed using chloroform (Sugawara and Miyazawa 1999; Malavolta et al. 2004; Pacetti et al. 2007;
Yunoki et al. 2009; Rocha et al. 2010). Thus, lipid analysis by HILIC can reduce toxic halogenated solvent waste and is better suited for electrospray ionization, the most normal ionization mode of LC-MS.

3.2 Annotation of nonglycerolipid component in *A. thaliana*

As shown in Fig. 2, there are many signals not attributable to typical polar glycerolipids. To determine what other lipids kinds of can be analyzed by this method, commercially available compounds were subjected to LC-MS, and steryl glucoside (SG) and glucosylceramide (GlcCer) were newly found to show adequate retention on HILIC. These lipids are major nonglycerolipid components of the cellular membrane. To analyze the diverse molecular species belonging to these lipid classes, their mass spectra were analyzed. Since the authentic SG compound was derived from soybean, not chemically synthesized, it contained several SG species that also could be detected in *A. thaliana*. Based on their mass spectra, the SG species in *A. thaliana* were annotated as glucosides of sitosterol, campesterol, and stigmasterol (Table 1). To analyze the diverse molecular species belonging to these lipid classes, their mass spectra were analyzed. Since the authentic SG compound was derived from soybean, not chemically synthesized, it contained several SG species that also could be detected in *A. thaliana*. Based on their mass spectra, the SG species in *A. thaliana* were annotated as glucosides of sitosterol, campesterol, and stigmasterol (Table 1). To analyze the diverse molecular species belonging to these lipid classes, their mass spectra were analyzed. Since the authentic SG compound was derived from soybean, not chemically synthesized, it contained several SG species that also could be detected in *A. thaliana*. Based on their mass spectra, the SG species in *A. thaliana* were annotated as glucosides of sitosterol, campesterol, and stigmasterol (Table 1). To analyze the diverse molecular species belonging to these lipid classes, their mass spectra were analyzed. Since the authentic SG compound was derived from soybean, not chemically synthesized, it contained several SG species that also could be detected in *A. thaliana*. Based on their mass spectra, the SG species in *A. thaliana* were annotated as glucosides of sitosterol, campesterol, and stigmasterol (Table 1). To analyze the diverse molecular species belonging to these lipid classes, their mass spectra were analyzed. Since the authentic SG compound was derived from soybean, not chemically synthesized, it contained several SG species that also could be detected in *A. thaliana*. Based on their mass spectra, the SG species in *A. thaliana* were annotated as glucosides of sitosterol, campesterol, and stigmasterol (Table 1). To analyze the diverse molecular species belonging to these lipid classes, their mass spectra were analyzed. Since the authentic SG compound was derived from soybean, not chemically synthesized, it contained several SG species that also could be detected in *A. thaliana*. Based on their mass spectra, the SG species in *A. thaliana* were annotated as glucosides of sitosterol, campesterol, and stigmasterol (Table 1).

![Relative Intensity (%)](attachment:image)

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**Fig. 3** Analysis of GlcCer by IT-TOF-MS. **a** Positive MS and **b** MS/MS of glucosylceramide consisting of 4,8-sphingadiene and α-hydroxypalmitic acid are displayed (precursor ion, [M + H]+).

**Table 1** Steryl glucosides and glucosylceramides detected in *A. thaliana*

| Annotation                  | *t*<sub>R</sub> (min) | MS (<m/z>)                           | MS/MS (<m/z>)                        |
|-----------------------------|----------------------|--------------------------------------|--------------------------------------|
| Sitosteryl glucoside        | 3.9                  | 621.433<sup>a</sup>, 611.404<sup>b</sup> |                                       |
| Stigmasteryl glucoside      | 3.9                  | 619.415<sup>a</sup>, 609.304<sup>b</sup> |                                       |
| Campesteryl glucoside       | 4.0                  | 607.416<sup>a</sup>                  |                                       |
| d18:1h16:0 GlcCer           | 7.1, 7.5             | 716.565<sup>c</sup>, 554.518<sup>d</sup> | 272.257<sup>e</sup>, 264.269<sup>f</sup> |
| t18:1h16:0 GlcCer           | 8.5, 8.9             | 732.360<sup>c</sup>, 570.509<sup>d</sup> | 272.256<sup>e</sup>, 280.254<sup>i</sup>, 262.243<sup>g</sup> |
| t18:1h22:0 GlcCer           | 8.1, 8.3             | 816.651<sup>c</sup>, 654.607<sup>d</sup> | 262.245<sup>g</sup>                  |
| t18:1h24:1 GlcCer           | 7.9, 8.2             | 842.667<sup>c</sup>, 680.623<sup>d</sup> | 262.242<sup>g</sup>                  |
| t18:1h24:0 GlcCer           | 7.9, 8.1             | 844.687<sup>c</sup>, 682.637<sup>d</sup> | 262.243<sup>g</sup>                  |
| t18:1h26:1 GlcCer           | 8.0                  | 870.695<sup>c</sup>                  |                                       |
| t18:1h26:0 GlcCer           | 7.8                  | 872.711<sup>c</sup>, 710.667<sup>d</sup> | 262.246<sup>g</sup>                  |

Superscripts indicate the assignment of each ion: a, b, c, d, e, f, and g are corresponding to [M + HCO<sub>3</sub>]<sup>+</sup>, [M + Cl]<sup>+</sup>, [M + H]<sup>+</sup>, [M + H−glucose]<sup>+</sup>, [hydroxyfatty acid amide + H]<sup>+</sup>, [LCB + H−2H<sub>2</sub>O]<sup>+</sup>, [LCB + H−3H<sub>2</sub>O]<sup>+</sup>. LCB and fatty acid nomenclature are as follows, d/t (di/trihydroxy) 18 (18 carbon chain):1 (one desaturation) followed by fatty acid; c/h (non-hydroxy/hydroxy) 12 (12 carbon chain):0 (no desaturation).
compounds against pathogens (Papadopoulou et al. 1999; Friedman 2002), and similar compounds are widely distributed throughout the plant kingdom (Connolly and Hill 2010). Since MS/MS of these compounds were in accordance with their chemical structures (Supplementary Fig. 2), similar compounds could be analyzed by this method. These data indicated that our method could be applied to the analyses of a wide range of plant lipids including glycerolipids, sterol glucosides, glucosyleramides, and lipidic secondary metabolites. In the negative ion mode, we detected approximately 500 lipid signals from Arabidopsis leaves and annotated 96 molecules as shown in Supplementary Table 1. The relative standard deviation of retention time of internal standard (10:0/10:0 PC) was 6.6% (n = 16), and the averaged m/z value of [MH]+ of internal standard was 566.378 ± 1.6 mDa (calcld for C_{25}H_{50}N_{2}O_{3}P: 566.3822) for the dataset shown in Supplemental Table 1. The root-extracted lipids observed at t_R 4 ~ 6 min and t_R 9 ~ 10 min remain unidentified. However, a significant qualitative difference between the lipid profiles of leaves and roots was revealed.

3.3 Applications of HILIC-MS for biological studies—case study 1

We analyzed digalactosyldiacylglycerol (DGDG) in the Arabidopsis tgd3 (trigalactosyldiacylglycerol3) mutant, which cannot transport phosphatidic acid from the endoplasmic membrane (ER) to the plastid (Lu et al. 2007) (Fig. 1). DGDG is a major constituent of the plastid membrane. The diacylglycerol moiety of galactoglycerolipids is synthesized de novo in the plastid or assembled at the ER and must be imported into the plastid (Roughan and Slack 1982). The head group of DGDG is characterized by a terminal z-galactose moiety (1 → 6) linked to the inner β-galactose residue (Gal(z1 → 6)βGalDG) (Benning and Ohta 2005). However, the tgd1 mutant of A. thaliana and some plant species (e.g., Adzuki bean) contain an additional form of DGDG with the second galactose in the β-anomeric configuration (Gal(β1 → 6)βGalDG) (Kojima et al. 1990; Xu et al. 2003; Kelly and Dormann 2004; Benning and Ohta 2005; Hölz and Dörmann 2007). A mixture of these forms of DGDG was observed in an Arabidopsis tgd1 mutant based on the signals of anomeric protons in the 1H NMR spectra of purified DGDG (Xu et al. 2003), but the detailed composition of DGDG in tgd mutants remained unclear. Thus, we evaluated the separation ability and coverage of our method by applying it to the analysis of these two forms of DGDG in a tgd3 mutant. As reported previously (Lu et al. 2007), an accumulation of trigalactosyldiacylglycerol (TGDG) was observed (Supplementary Fig. 3). Thus, we used this experimental material to analyze this unusual form of DGDG. When the composition of DGDG in tgd3 was investigated based on XICs, 2 peaks were observed for the mass-to-charge ratio of a particular DGDG molecule (34:6 DGDG) (Fig. 5). Since the former-eluting isomer was detected only in the tgd3 mutant, this peak was annotated as Gal(β1 → 6)βGalDG. MS/MS of 34:6 Gal(β1 → 6)βGalDG and 34:6 Gal(z1 → 6)βGalDG were quite similar (Supplementary Fig. 4), which supported their annotations. Based on these results, the profile of 2 forms of DGDG in tgd3 was analyzed (Fig. 6). The major molecular species of Gal(β1 → 6)βGalDG was determined to be 34:6, while the major molecules of Gal(z1 → 6)βGalDG were 34:3 and 36:6 Gal(z1 → 6)βGalDG.

The normal form of DGDG, Gal(z1 → 6)βGalDG, is synthesized from MGDG by UDP-galactose-dependent DGD1/2 enzymes (Dörmann et al. 1999; Kelly and Dörmann 2002), whereas Gal(β1 → 6)βGalDG is synthesized from two molecules of MGDG by galactolipid:galactolipid
galactosyltransferase (GGGT) (Fig. 1) (Benning and Ohta 2005). GGGT is also involved in the synthesis of oligo-galactolipids including TGDG, and enhanced accumulation of unusual forms of galactolipids in tgd mutants is due to increased GGGT activity (Xu et al. 2003; Awai et al. 2006; Lu et al. 2007; Xu et al. 2008). Recently, the gene encoding GGGT was identified as SENSITIVE TO FREEZING 2 (SFR2), a gene essential for freeze tolerance in Arabidopsis (Moellering et al. 2010). The molecular profile of Gal(β1 → 6)βGalDG was more similar to that of MGDG than Gal(α1 → 6)βGalDG (Fig. 6). This result is expected, given their biosynthesis, but the molecular profile of Gal(β1 → 6)βGalDG does not exactly reflect that of MGDG; the minor molecular species found in MGDG (34:1 ~ 34:4 species) which were increased in the tgd3 mutant were not detected in Gal(β1 → 6)βGalDG. These data suggest that the GGGT enzyme may have greater affinity for MGDG consisting of polyunsaturated fatty acids or that the substrate-supplying mechanisms for GGGT are biased to polyunsaturated molecules. There is also the possibility that Gal(β1 → 6)βGalDG may be a better substrate than MGDG for plastidic fatty acid desaturases. Increased fatty acid unsaturation has long been correlated with enhanced freezing tolerance or adaptations to growth at low above-freezing temperatures (Steponkus 1984; Uemura et al. 1995). Recently, remodeling of the membrane lipid profile by GGGT was indicated to be important for freezing tolerance in Arabidopsis (Moellering et al. 2010). Thus, the increased fatty acid unsaturation in Gal(β1 → 6)βGalDG may be expected for the lipid class synthesized by GGGT, which is required for freezing tolerance.

3.4 Applications of HILIC-MS for biological studies—case study 2

Lipid profiles of well characterized Arabidopsis mutants ats1-1, fad6-1, and fad7-2 were analyzed to examine whether untargeted lipidome analysis of these mutants could produce results consistent with those obtained by conventional analytical method which require preparufication procedure of each lipid class and chemical derivatization. The ats1-1 mutant lacks most of its plastidic acyl-ACP:glycerol-3-phosphate acyltransferase activity (Fig. 1) and is blocked in the plastid galactoglycerolipid biosynthesis pathway (Kunst et al. 1988; Xu et al. 2006). Thus, the amount of galactolipids containing ER-derived diacylglycerol moiety increases to maintain plastidic membrane function. ER-derived glycerolipids have an 18-carbon fatty acid at sn-2 of glycerol, while glycerolipids synthesized de novo in plastids have a 16-carbon fatty acid at sn-2 (Heinz and Roughan 1983; Browse et al. 1986). Thus, the ratio of 16-carbon fatty acids to 18-carbon fatty acids in plastid glycerolipids is reduced by disruption of
ATS1. The fad6-1 mutant lacks the chloroplast ω-6 fatty acid desaturase responsible for synthesis of 16:2 and 18:2 fatty acids in galactolipids, SQDG, and PG (Falcone et al. 1994), while fad7-2 is disrupted for the chloroplast ω-3 fatty acid desaturase responsible for synthesis of 16:3 and 18:3 fatty acids in galactolipids, SQDG, and PG (Iba et al. 1993) (Fig. 1). First, HILIC-MS data of leaf lipids recorded in the negative ion mode were automatically extracted to yield an array of intensities of analytical signals (ions of specific m/z value eluting at a specific time). This processing step generated lists of peak heights for 534 peaks annotated with specific LC retention times and masses (Supplementary Table 1). Listed data were then subjected to principal component analysis (PCA). The PCA model can provide an overview of all observations or samples in the data table by projecting and clustering each sample and highlighting the holistic difference of the complex metabolic state in each sample. The PCA scatter plot showed that the samples were clustered with respect to their corresponding genotypes (Fig. 7), suggesting that each mutant has a distinct metabolite profile. To identify the metabolites contributing to the separation “discriminative metabolites” between the mutant profiles and those of the wild type, for example, orthogonal projection to a latent structure-discriminant analysis (OPLS-DA) model for wild type and the ats1-1 mutant was examined. Figure 8a shows the OPLS score scatter plot of wild type and ats1-1 with one predictive component. The OPLS loading S-plot, a plot of the covariance versus the correlation in conjunction with the variable trend plots allows easier visualization of the data (Fig. 8b). The variables that changed most and strongly contributed to the class separation are plotted at the top or bottom of the “S” shape plot, and those that do not vary significantly are plotted in the middle (Wiklund et al. 2008). The variables circled in Fig. 8b are listed in Tables 2 and 3. The result in Table 2 showed an increase of glycoglycerolipids with two 18-carbon fatty acids in the ats1-1 mutant in comparison to the wild type. In addition, glycerolipids with 18-carbon fatty acid and 16-carbon fatty acid were reduced in the mutant (Table 3). These results were consistent with previous reports (Kunst et al. 1988; Xu et al. 2006). Lipidome data of fad6-1 and fad7-2 were also analyzed by the same procedure, and increased saturations of chloroplast glycerolipids (MGDG, DGDG, SQDG, and PG) were observed (Supplementary Figs. 5 and 6, and Supplementary Tables 2–5), also in accordance with previous reports (Iba et al. 1993; Falcone et al. 1994). These data indicate that untargeted lipidome analysis by our method can be used for detailed analysis of metabolite phenotypes.

4 Conclusions

We applied liquid chromatography ion trap time-of-flight mass spectrometry (LC-IT-TOF-MS) for plant lipidome

![Fig. 7 Principal component analysis of ats1-1, fad6-1, fad7-2, and wild-type lipidome data. Principal component PC1 and PC2 represent the first 2 principal components accounting for 58% of the variance. Each plot represents an independent plant.](image-url)

![Fig. 8 OPLS-DA of ats1-1 and wild-type lipidome data. a Score scatter plot (R2Y = 0.993, Q2 = 0.988, R2X = 0.737). The confidence intervals correspond to the 2 or 3 sigma limits, i.e., 2 or 3 vector standard deviations are displayed. b S-plot. The variables that changed most and strongly contributed to the class separation are circled. Details of variables circled in the upper right and lower left are shown in Tables 2 and 3.](image-url)
analysis. Based on various authentic compounds and biological sample, our method was confirmed to be applicable to simultaneous analysis of a wide range of plant lipids containing typical membrane lipids and lipophilic secondary metabolites. Then, we applied our method for the analysis of Arabidopsis mutant tgd3, and revealed an accumulation of an unusual form of DGDG (Gal(b1→6)GalDG) and compositional differences between intact forms of Gal(b1→6)GalDG, normal form of DGDG (Gal(z1→6)GalDG), and MGDG which is the precursor of Gal(b1→6)GalDG. Furthermore, we analyzed well-known Arabidopsis mutants that are disrupted in lipid biosynthetic genes to examine whether untargeted lipidome analysis could be utilized for evaluation of these mutant metabolic phenotypes. The method coupled with multivariate analysis could clearly discriminate the mutants and yielded results consistent with those from previous reports. These results indicated that lipid analysis using HILIC-MS was efficient for plant lipidomics.

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