High-throughput lipid profiling of plant tissues using SWATH acquisition and MS-DIAL

Using the SCIEX TripleTOF 6600 system

Cheka Kehelpannala[1], Berit Ebert[1], Hiroshi Tsugawa[2,3,4], Brad Patterson[5], Ute Roessner[1], and Thusi Rupasinghe[1,5]

[1]School of BioSciences, The University of Melbourne, Melbourne, Australia; [2]RIKEN Center for Sustainable Resource Science, Japan; [3]RIKEN Center for Integrative Medical Sciences, Japan; [4]Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology, Japan; [5]SCIEX, Australia

Lipidomics is a growing field of research focused on the global characterization and quantification of lipids within biological matrices such as biofluids, cells, whole organs and tissues. More and more, mass spectrometry (MS) based lipid profiling has become a common technique to track the changes of lipid levels in the field of plant lipidomics. However, there are several challenges associated with MS detection and identification of plant lipids due to their highly complex nature. Here, a robust pipeline for untargeted lipidomics is presented, from extraction to analysis of lipid profiles from *Arabidopsis thaliana,* including rosettes of 14- and 24-day-old plants and samples of main inflorescences from 40-day-old plants. Plant rosette and flower tissues were extracted then analyzed using SWATH acquisition, a data independent acquisition strategy where both MS and MS/MS spectra are acquired on all detectable species. Data analysis was performed using MS-DIAL (mass spectrometry-data independent analysis).

Using this workflow, 779 molecular lipid species were characterized out of 3642 spectral matches confirmed by MS/MS and 6263 unknown features detected in the two plant tissues. The aligned data for all 12310 features with m/z and retention

time showed a clear differentiation of different aged rosettes and flowers as visualized in principal component analysis (PCA) and heat map (Figure 1).

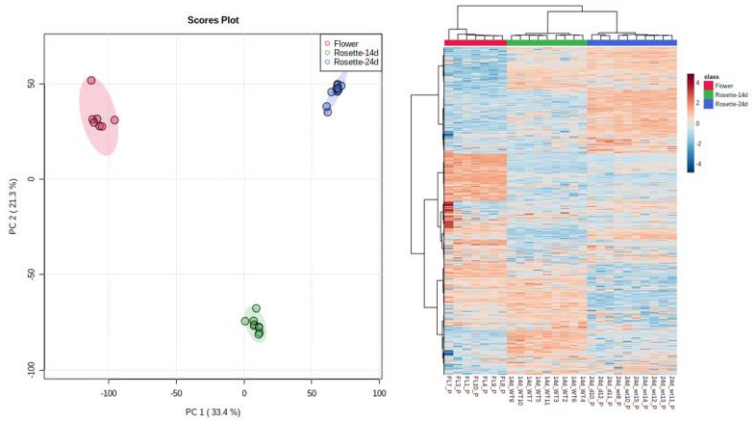

Figure 1. PCA and heat map of all 12310 features detected with MS-DIAL in plant tissues. The PCA score plot and heat map show the clear differentiation of lipid profiles from different tissues.

Untargeted plant lipidomics with MS-DIAL data processing

- Single analysis to provide comprehensive coverage of plant lipids using SWATH acquisition
- 30 min method required to collect a comprehensive lipid map
- Simple data processing workflow and data visualization for lipidomics using MS-DIAL
- Using a data independent method (SWATH acquisition) allows for the collection of MS/MS spectra for all MS features, which are used to comprehend the profile of many lipid species regardless of relative abundance

Methods

Plant growth and sample collection: *Arabidopsis thaliana* (L.) seeds were placed on 42 mm Jiffy-7 pellets (Garden City Plastics, Australia) and vernalized at 4˚C for three days. Following the cold treatment, trays were placed in a growth chamber under a 16 h light/8 h dark regime at 22°C and 50% relative humidity with a daytime light intensity of 100-120 microeinsteins (µE) at the plant level. 14-day and 24-day-old rosettes and all parts of fully open flowers were harvested in liquid nitrogen and stored at -80˚C until lipid extraction.

Lipid extraction: The plant material was homogenized by cryo-milling (Precellys 24, Bertin Technologies) with 400 µL of 2-propanol containing 0.01% butylated hydroxy toluene (BHT) for two consecutive 45 sec intervals with a 30 sec pause in between at 6100 rpm and a temperature of 10˚C. Next, samples were incubated at 75˚C for 15 min under constant shaking at 15,700 g. They were then cooled to room temperature and 1.2 mL of a mixture of chloroform/methanol/water (30:41.5:3.5, v/v/v) was added to each sample. The samples were incubated at 25˚C for 24 h under constant shaking at 300 rpm. Finally, the supernatant was separated, and dried in a vacuum concentrator. The dried lipid extracts were re-suspended in 200 µl of butanol/methanol (1:1, v/v) with 10 mM ammonium acetate and subjected to LC-MS analysis as reported.[1,2]

LC-MS/MS analysis: The lipid extracts were placed in the autosampler set to 12 ˚C then 8 µL of each was injected onto a Phenomenex Kinetex C18 Column (2.6 µm, 100 Å, 100 x 2.1 mm) set to 55°C. Separation was performed using a SCIEX ExionLC system. Elution was performed using the gradient and solvents described in Table 1.

Mass spectrometry: Lipids were analyzed using a SCIEX TripleTOF 6600 system equipped with a DuoSpray Turbo V ion source. Electrospray ionization (ESI) probe was used for ionization of the lipid samples and the atmospheric pressure chemical ionization (APCI) probe was used to connect the automated calibrant delivery system (CDS) and calibrate the instrument every five samples (APCI calibration solution). Analysis was performed using SWATH acquisition in both positive and negative ion ionization modes. The MS parameters used are described in Table 2.

Data processing: The raw SWATH acquisition data were processed using the open-source software MS-DIAL version 4.60. The parameters were: MS1 tolerance = 0.01 Da, MS/MS tolerance = 0.05 Da, Retention time = 0.1-28 min, MS1 mass range = 300-1700 Da and minimum peak height = 1000 amplitude. The peaks were aligned to a quality control sample with a retention time tolerance of 0.05 min and MS1 tolerance of 0.015 Da. All other parameters were kept at the default values for conventional LC-MS or data dependent MS/MS data processing.

Table 1. LC gradient. The flow rate was set to 0.26 mL/min.

Time (min)	%B
0	*32*
1.5	*32*
4.0	*45*
5.0	*52*
8.0	*58*
11.0	*66*
14.0	*70*
18.0	*75*
21.0	*97*
25.0	*97*
25.1	*32*
30.0	*32*

Mobile phase A: 60% acetonitrile with 10 mM ammonium acetate in water
Mobile phase B: 90% isopropanol with 10 mM ammonium acetate in acetonitrile

Table 2. Mass spectrometry conditions

Parameter	Setting
Source Temperature	*250 ºC*
Curtain gas	*35 psi*
Gas 1	*25 psi*
Gas 2	*25 psi*
Declustering potential	*80 V*
Ionization voltage	*5500 V*
TOF MS	*100-1700 m/z for 50 msec*
TOF MS/MS	*100-1700 m/z for 10 msec*
Q1 mass range for SWATH acquisition	*300-1700 m/z*
Q1 window size	*15 Da*
Collision energy	*45 ± 15 V*

Lipidome profiling

SWATH acquisition data collected in both positive and negative mode was processed using MS-DIAL. Lipids were annotated using the MS-DIAL internal lipid database with MS1 accurate mass tolerance of 0.01 Da and MS2 accurate mass tolerance of 0.05 Da.[3,4] Only the lipids showing MS/MS spectral similarity to the reference spectra in the MS-DIAL internal database and eluting at the predicted retention times were used for fold-change analysis. The number of identified lipid species across the different lipid classes was 779, covering 16 lipid classes out of a total of 12309 features (Table 3). The MS-DIAL graphical user interface provides the number of peak spots that were identified using either reference matched or suggested as shown in the Figure 2.

Statistical analysis

MS-DIAL outputs consisting of peak areas of the identified lipids were analyzed using Microsoft Excel. The peak areas of annotated lipids were normalized to the median, log transformed, auto-scaled and statistically analyzed using the freely available online software, MetaboAnalyst.[6] Using MetaboAnalyst, various analyses including one-way ANOVA, PCA, and heat maps, were conducted on the lipids identified in the different tissues, to test for significant changes and adjusted p-values were obtained with Benjamini-Hochberg false discovery rate (FDR) correction.[7]

Table 3. Lipid species identified using SWATH acquisition data from MS-DIAL. The SWATH acquisition data analysis by the MS-DIAL program identified a total of 779 unique lipids in positive mode.

Lipid class	# of lipids identified
Acylated sterol glucoside (ASG)	*2*
Ceramide (Cer)	*78*
Diacylglycerol (DG)	*93*
Triacylglycerol (TG)	*223*
LysoPC (LPC)	*16*
LysoPE (LPE)	*3*
Phosphatidylcholine (PC)	*116*
Phosphatidylethanolamine (PE)	*106*
Phosphatidylglycerol (PG)	*31*
Phosphatidylinositol (PI)	*36*
Phosphatidylserine (PS)	*14*
Digalactosyldiacylglycerol (DGDG)	*56*
Monogalactosyldiacylglycerol (MGDG)	*42*
Hex ceramides (HexCer)	*53*
Sulfoquinovosyldiacylglycerol (SQDG)	*14*
Sterol Ester (SE)	*2*

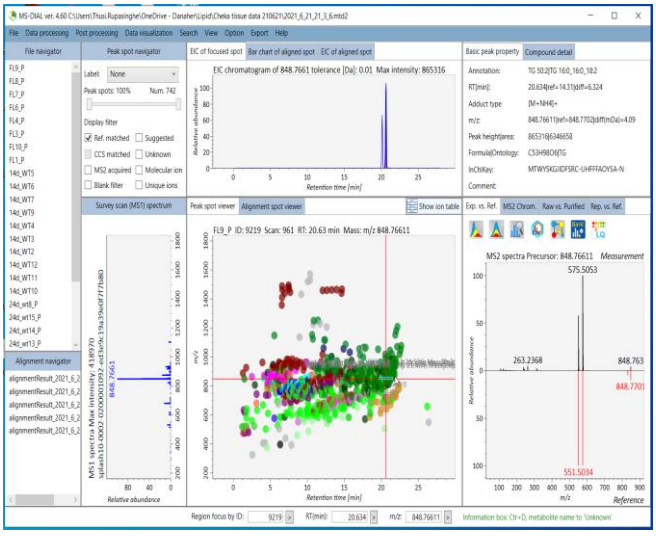

Figure 2. MS-DIAL user interface for data processing. MS-DIAL navigation page provides alignment and identification of the results. By selecting and double clicking, the peak spot in the MS/MS spectrum (shown in black) is shown with the reference spectrum (shown in red).

Lipid results

Results from the one-way ANOVA and post-hoc tests with an adjustable p-value (FDR) of 0.05 showed that 259 features were identified as significantly different between the three different sample types. The untargeted lipid profiles of three tissue samples created using SWATH acquisition and MS-DIAL data processing reveals developmental lipid changes in the different aged rosettes and tissue-type specific differences between flower and rosette tissues. According to the heatmap analysis of the lipid profiles, the two sets of rosette tissue samples (14-day and 24-day-old) show a more similar lipid profile when compared to the profile of flowers (Figure 1, right). The PCA clearly shows significant differences between the lipid profile of the tissues (Figure 1, left).

The box plot analysis for lipids species is showing the abundance of individual lipid species in each tissue, supporting the notion that differences between lipid profiles can clearly be demonstrated by the untargeted lipidomic workflow (Figure 3).

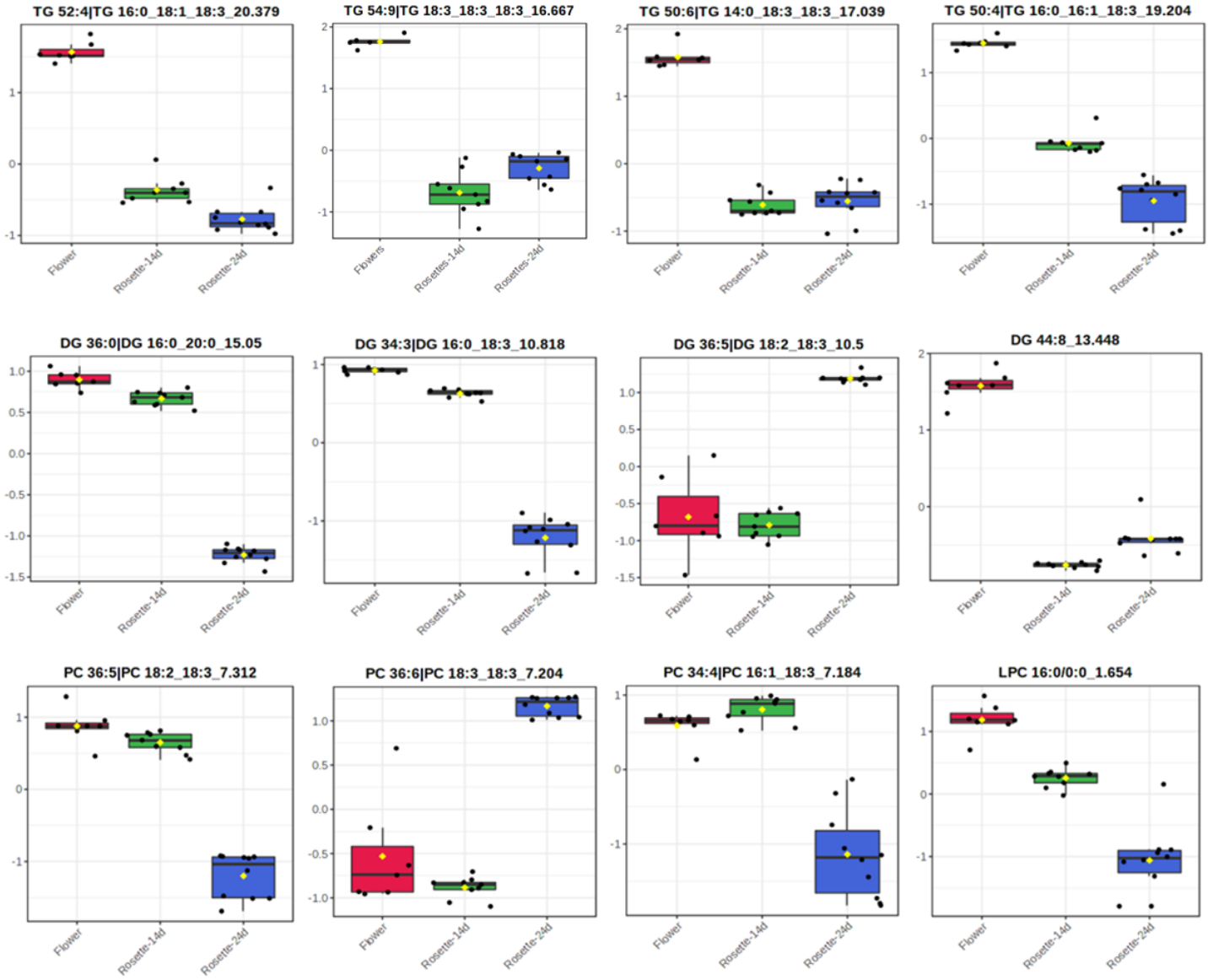

Figure 3. Box plots showing significantly different lipid species. Differences in lipid abundance across the different sample types were determined using one-way ANOVA with adjusted p-value (FDR) as 0.05. Box plots for selected lipid species identified and quantified from the SWATH acquisition data are shown for the flowers (red), the 14-day rosettes (green) and the 24-day rosettes (blue).

Conclusions

The untargeted plant lipidomics workflow was successfully developed using SWATH acquisition on the SCIEX TripleTOF 6600 system, MS-DIAL for data processing, followed by MetaboAnalyst software for statistical analysis. The total number of annotated lipids species, which were based on accurate mass, fragmentation pattern and retention time, was 779 lipids, from 16 lipid classes found in only from positive mode data.

- The untargeted lipid profiling using SWATH acquisition provides comprehensive MS and MS/MS data for lipid identification and quantification in plant tissues
- MS-DIAL data processing provides simple data processing platform with lipid identification using *in silico* predicted MS libraries in both positive and negative modes
- MetaboAnalyst software provides in-depth statistical analysis including PCA, heat maps, dendrograms and box plots for accurate results visualization.

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