

Analytical Platform 7 (Lipidomics) was conducted by the Welte group at the Kansas Lipidomics Research Center, Kansas State University. Immediately upon harvesting, plant material was immersed in 3 ml preheated isopropanol containing 0.01% BHT, and maintained at 75 °C for 15 min. Samples were stored at –20 °C, and were shipped from Iowa State University to Kansas State University on dry ice, using overnight courier services. To each sample, 1.5 ml chloroform and 0.6 ml water were added. The tubes were shaken for 1 h, followed by removal of the extract. The tissues were re-extracted with chloroform/methanol (2:1) with 0.01% BHT 4 additional times with at least 1 h agitation each time. The remaining plant tissue was heated overnight at 105 °C and weighed. The weights of these dried, extracted tissues are designated the “dry weights” (actually dry weight minus lipid). The combined extracts were washed once with 1 ml 1 M KCl and once with 2 ml water. The solvent was evaporated under nitrogen, and the lipid extract was dissolved in 1 ml chloroform. An automated electrospray ionization-tandem mass spectrometry approach was used, and data acquisition and analysis and acyl group identification were carried out as described previously (Devaiah et al., 2006) with modifications. The samples were dissolved in 1 ml chloroform. An aliquot of 5 to 300 µl of extract in chloroform was used. Precise amounts of internal standards, obtained and quantified as previously described (Welte et al., 2002), were added in the following quantities (with some small variation in amounts in different batches of internal standards): 0.66 nmol di14:0-PC, 0.66 nmol di24:1-PC, 0.66 nmol 13:0-lysoPC, 0.66 nmol 19:0-lysoPC, 0.36 nmol di14:0-PE, 0.36 nmol di24:1-PE, 0.36 nmol 14:0-lysoPE, 0.36 nmol 18:0-lysoPE, 0.36 nmol di14:0-PG, 0.36 nmol di24:1-PG, 0.36 nmol 14:0-lysoPG, 0.36 nmol 18:0-lysoPG, 0.36 nmol di14:0-PA, 0.36 nmol di20:0(phytanoyl)-PA, 0.24 nmol di14:0-PS, 0.24 nmol di20:0(phytanoyl)-PS, 0.20 nmol 16:0-18:0-PI, 0.16 nmol di18:0-PI, 2.01 nmol 16:0-18:0-MGDG, 0.39 nmol di18:0-MGDG, 0.49 nmol 16:0-18:0-DGDG, and 0.71 nmol di18:0-DGDG. The sample and internal standard mixture was combined with solvents, such that the ratio of chloroform/methanol/300 mM ammonium acetate in water was 300/665/35, and the final volume was 1.4 ml.

Unfractionated lipid extracts were introduced by continuous infusion into the ESI source on a triple quadrupole MS/MS (API 4000, Applied Biosystems, Foster City, CA). Samples were introduced using an autosampler (LC Mini PAL, CTC Analytics AG, Zwingen, Switzerland) fitted with the required injection loop for the acquisition time and presented to the ESI needle at 30 μ l/min. Sequential precursor and neutral loss scans of the extracts produce a series of spectra with each spectrum revealing a set of lipid species containing a common head group fragment. Lipid species were detected with the following scans: PC and lysoPC, $[M + H]^+$ ions in positive ion mode with Precursor of 184.1 (Pre 184.1); PE and lysoPE, $[M + H]^+$ ions in positive ion mode with Neutral Loss of 141.0 (NL 141.0); PG, $[M - H]^-$ in negative mode with Pre 152.9 or $[M + NH_4]^+$ in positive ion mode with NL 189.0 for PG; lysoPG, $[M - H]^-$ in negative mode with Pre 152.9; PI, $[M - H]^-$ in negative mode with Pre 241.0 or $[M + NH_4]^+$ in positive ion mode with NL 277.0; PS, $[M - H]^-$ in negative mode with NL 87.0 or $[M + NH_4]^+$ in positive ion mode with NL 185.0; PA, $[M - H]^-$ in negative mode with NL 152.9 or $[M + NH_4]^+$ in positive ion mode with NL 115.0; MGDG, $[M + NH_4]^+$ in positive ion mode with NL 179.1; and DGDG, $[M + NH_4]^+$ in positive ion mode with NL 341.1. The scan speed was 50 or 100 u per sec. The collision gas pressure was set at 2 (arbitrary units). The collision energies, with nitrogen in the collision cell, were +28 V for PE, +40 V for PC, -58 V or +20 or +25 V for PI, -57 V or +20 or +25 V for PA and PG, -34 V or +20 or +25 V for PS, +21 V for MGDG, and +24 V for DGDG. Declustering potentials were +100 V for PE and PC, -100 V or +100 V for PA and PG, PI, and PS, and +90 V for MGDG and DGDG. Entrance potentials were +15 V for PE, +14 V for PC, -10 V or +14 V for PI, PA, PG, and PS, and +10 V for MGDG and DGDG. Exit potentials were +11 V for PE, +14 V for PC, -15 V or +14 V for PI, -14 V or +14 V for PA and PG, -13 V or +14 V for PS, and +23 V for MGDG and DGDG. The mass analyzers were adjusted to a resolution of 0.7 u full width at half height. For each spectrum, 9 to 150 continuum scans were averaged in multiple channel analyzer (MCA) mode. The source temperature (heated nebulizer) was 100 °C, the interface heater was on, +5.5 kV or -4.5 kV were applied to the electrospray capillary, the curtain gas

was set at 20 (arbitrary units), and the two ion source gases were set at 45 (arbitrary units).

The background of each spectrum was subtracted, the data were smoothed, and peak areas integrated using a custom script and Applied Biosystems Analyst software. The lipids in each class were quantified in comparison to the two internal standards of that class. The first and typically every 11th set of mass spectra were acquired on the internal standard mixture only. Peaks corresponding to the target lipids in these spectra were identified and molar amounts calculated in comparison to the internal standards on the same lipid class. To correct for chemical or instrumental noise in the samples, the molar amount of each lipid metabolite detected in the “internal standards only” spectra was subtracted from the molar amount of each metabolite calculated in each set of sample spectra. The data from each “internal standards only” set of spectra was used to correct the data from the following 10 samples.

Data in which < 0.002 nmol (< 2 pmol) of a lipid metabolite were detected were removed from the data set. Finally, the data were corrected for the fraction of the sample analyzed and normalized to the sample “dry weights” to produce data in the units nmol/mg.

Devaiah SP, Roth MR, Baughman E, Li M, Tamura P, Jeannotte R, Welti R, Wang X (2006) Quantitative profiling of polar glycerolipid species from organs of wild-type Arabidopsis and a phospholipase D α 1 knockout mutant. *Phytochemistry* **67**: 1907-1924

Welti R, Li W, Li M, Sang Y, Biesiada H, Zhou HE, Rajashekar CB, Williams TD, Wang X (2002) Profiling membrane lipids in plant stress responses.

Role of phospholipase D α in freezing-induced lipid changes in Arabidopsis. *J Biol Chem* **277**: 31994-32002