3. METHODS AND PROTOCOLS FOR ARABIDOPSIS LIPID ANALYSES

3.1. Lipid Extraction Methods and Separation

(Katherine M. Schmid[1])

The following sections summarize methods that have been applied specificially to analysis of lipids from Arabidopsis and plant systems with similar challenges. Many other protocols are available, and readers may be especially interested in *The Lipid Library*, available online at http://lipidlibrary.aocs.org.

Although recent advances in nuclear magnetic resonance (NMR)-based techniques have made quantitation of oil in intact Arabidopsis seeds feasible (Jako et al., 2001; Colnago et al., 2007), most lipid analyses require extraction of the desired fractions into organic solvents. Since polarities and solubilities of lipids differ radically, methods of extraction will vary with the goals of the researcher. In all cases, oxidation of unsaturated fatty acids should be minimized by drying and storing samples under nitrogen, including antioxidants such as 25 mg L^{-1} butylated hydroxytoluene (BHT), and avoiding diethyl ether and unstabilized chloroform. Sample containers and equipment should be chosen to prevent introduction of contaminating plasticizers and lubricants (Christie, 1993). Thus, glass or Teflon containers rinsed with high purity solvents should be used rather than plastics, Teflon tape and solvent-washed aluminum foil should replace parafilm and plastic wraps, and stopcock grease and homogenizers with exposed lubricated bearings must be avoided.

If native lipid species are to be characterized, it is critical to immediately denature lipases, esterases, and oxidases, which may even be stimulated by some solvents or by freezing and persist at subzero temperatures (Christie, 1993). Therefore, even quickly frozen tissues will undergo degradation when stored in the freezer. Wounding quickly and strongly activates conversion of Arabidopsis leaf fatty acids to oxophytodienoic acids (Buseman et al., 2006). Immediate brief treatment of plant tissue with boiling isopropanol to inactivate enzymes (Kates and Eberhardt, 1957) is the typical remedy and is highly recommended. To prevent transesterification artifacts, extracts should not be stored in solvents containing primary alcohols such as methanol (Christie, 1993).

Arabidopsis lipids have most often been extracted with chloroform:methanol (2:1; Folch et al., 1957) or (1:2; Bligh and Dyer, 1959) (v/v). The latter permits lower solvent-to-sample ratios, since additional methanol increases the water content at which phase separation and concomitant loss of extraction efficiency occur beyond the 6.54% (w/v) limit of chloroform:methanol (2:1; Schmid 1973). Bligh and Dyer (1959) stipulated three subsequent chloroform washes, bringing the combined extracts to chloroform:methanol (2:1). Both protocols call for induction of phase separation once extraction is complete. Use of a salt solution such as 0.88% (w/v) potassium chloride (KCl) rather than pure water to achieve optimal chloroform:methanol:water (8:4:3 v/v/v) helps keep most acidic lipids protonated, so that they partition into the lower lipid phase (chloroform:methanol:water 86:14:1) rather than being lost with polar contaminants to the upper phase (3:48:47; Folch et al., 1957). Additional washes of the lower phase with salt solution reduce lipid yield compared to washes with salt solution:methanol (1:1 v/v; Christie, 1993). For a variant of the chloroform:methanol method suggested by the Kansas City Lipidomics Research Center (http://www.k-state.edu/lipid/lipidomics/leaf-extraction.html), see the protocol below.

Given the toxicity of chloroform and the advantages of boiling isopropanol pretreatment, some Arabidopsis researchers have adopted extraction with hexane:isopropanol (3:2) as proposed by Hara and Radin (1978). The initial hexane:isopropanol solution yields a relatively uncontaminated extract that can be used for gas chromatography (GC) or thin-layer chromatography (TLC) analysis without partitioning or washing. However, to remove nonlipids, the extract may be partitioned into an upper hexane phase by addition of aqueous sodium sulfate (e.g., ½ volume 6.5% [w/v] Na_2SO_4; (Y.H. Li et al., 2006). Back extraction of the lower phase with hexane:isopropanol 7:2 is necessary to avoid loss of polar lipids. For a sample protocol, see below.

Unfortunately, few direct comparisons of laboratory scale extractions of plant material are available. Fishwick and Wright (1977) found that the procedure of Bligh and Dyer (1959) gave better lipid yields than that of Folch et al. (1957) for spinach leaf, tomato fruit, and potato tuber. A small-scale study by Khor and Chan (1985) suggested that the hexane:isopropanol procedure of Hara and Radin (1978), including a 6.5% Na_2SO_4 wash, extracted neutral lipids from soybean seeds but left behind about 4/5 of phospholipid extractable with chloroform/methanol (2:1). Shäfer (1998), however, obtained better extraction from a mixture of wheat, soybean meal, and barley with hexane:isopropanol (3:2) than with chloroform:methanol (2:1).

In addition to the general techniques above, many specialized applications are available. High-throughput techniques such as screening of total fatty acid profiles by simultaneous extraction and transmethylation of tissue samples in hot acidic

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methanol (Browse et al., 1986a) have been popular in the Arabidopsis community. For complete transesterification of Arabidopsis seeds or other tissues high in triacylglycerols, a cosolvent such as toluene should be included (Y.H. Li et al., 2006).

Finally, some lipids are poorly represented in extracts prepared by standard methods. **Table 1** provides references to approaches for lipid classes requiring special attention.

Techniques for fractionating lipid extracts include TLC, high-performance liquid chromatography (HPLC), and column chromatography (Christie, 2003). Often a gross separation into nonpolar lipids, glycolipids, and phospholipids by silicic acid column chromatography (Rouser et al., 1967) precedes further analysis.

Protocols for general extraction of Arabidopsis lipids.

- For minimum oxidation, all solvents should contain 0.01% BHT.
- Samples should be placed in glass tubes with Teflon-lined screw caps and extracted immediately after harvesting.
- Isopropanol should be preheated prior to step 1 for effective killing of phospholipase D.
- Appropriate internal standard(s) may be added in step 1.
- After extraction is complete, lipid extracts may be concentrated under a stream of nitrogen for TLC and preparation of derivatives.

Choroform/Methanol method	Hexane/Isopropanol method
For soft tissue samples up to 0.5 g fresh weight (approx. 30 mg dry weight)	For up to 1 g fresh weight
1. Cover sample with 3 mL preheated isopropanol. Cap and heat at 75^{o}C for 15 min. Cool to room temperature.	1. Add 8 volumes (v/w) to sample. Cap and heat at 80°C for 5 min. Cool to room temperature.
2. Add 1.5 mL chloroform and 0.6 mL water.	2. Add 12 v/w of hexane.
3. Incubate with shaking for 1 hr.	3. Homogenize (mortar and pestle, polytron, or other suitable equipment).
4. After transferring lipid extract to a fresh tube, reextract tissue with 4 mL chloroform:methanol (2:1 v/v).	4. Briefly centrifuge to facilitate separation of lipid extract from tissue.
5. Repeat step 4 until tissues is white.	5. Transfer the upper phase (hexane:isopropanol-containing lipids) to another tube.
6. To combined lipid extracts from steps 4 and 5, add 1 mL 1M KCl. Vortex, centrifuge, and dicard upper phase.	6. For complete recovery, re-extract the pellet with hexane:isopropanol (7:2) and combine extracts with upper phase from step 5.
7. To lower phase from step 6, add 2 mL water. Vortex, centrifuge, and discard upper phase.	7. If less contamination of the extract is required, partition the hexane:isopropanol with Na_2SO_4 as described by Hara and Radin (1978) or Y.H. Li et al. (2006).

Modified Bligh and Dyer (1959) Protocol for lipid extraction from Arabidopsis leaves.
1. Put frozen plant material (snap-frozen in liquid nitrogen and stored in –80°C, put back in liquid nitrogen prior to extraction) in a glass homogenizer
2. Add 3.75 mL MeOH: $CHCl_3$ (2:1, v/v).
3. Add 1mL 1mM EDTA in 0.15M HAc (acetic acid).
4. Homogenize carefully.
5. Transfer to a fresh glass tube with screw cap (cap with teflon rubber inside).
6. Rinse homogenizer with 1.25 mL $CHCl_3$; transfer to the glass tube.
7. To the glass tube, add 1.25 mL 0.88% (w/v) KCl.
8. Vortex carefully.
9. Centrifuge appr. 3000 rpm for 2 min.
10. Transfer lipid ($CHCl_3$-) phase (lower phase) with a Pasteur pipette and transfer to a fresh glass tube, store in freezer –20°C.

Table 1. Extraction Methods for Acyl Lipids Not Amenable to Standard Chloroform/Methanol Extraction Techniques

Lipid Class	Extraction Solvent or Approach	Reference
Acyl ACP	2.5% trichloroacetic acid; resuspend in buffer and analyze by immunoblotting	Post-Beittenmiller et al., 1991
Acyl coenzyme A	isopropanol:50 mM KH_2PO_4 7.2:glacial acetic acid:50 mg mL^{-1} BSA (2:2:0.05:0.08 v/v/v/v)	Larson and Graham, 2001[a]
Acylcarnitine	acetonitrile:MeOH (4:1 v/v)	Bourdin et al., 2007[a]
Cutin/suberin	hydrogenolysis or transmethylation of solvent-insoluble epidermal residue	Bonaventure et al., 2004b[a]
Lysophospholipids	n-butanol	Bjerve et al., 1974; Seo et al., 2008[a]*
	chloroform:methanol (2:1 v/v) minus partitioning	Bjerve et al., 1974; W. Li et al., 2008[a]
Phosphatidylinositol phosphates	Chloroform:methanol:2.4M HCl:0.4M EDTA 36:36:18:9 (v/v/v/v)	König et al., 2008[a]
Complex sphingolipids	direct hydrolysis in dioxane:10% aqueous $Ba(OH)_2$ (1:1 v/v)	Sperling et al., 1998[a], 2005[a]
	isopropanol:hexane:water (50:20:25 v/v/v)	Markham and Jaworski, 2007[a]

[a]Citation includes application to Arabidopsis.

3.2. Determination of Total Fatty Acid Profiles

(Martine Miquel[2])

In plants, fatty acids are mainly present as esters linked to glycerol, sterols, or waxes (long-chain alcohols) or as amides to sphingolipids, while free (unesterified) fatty acids are minor constituents. The total fatty acid profile of plant tissues other than seeds (see Section **3.4**) or from lipid extracts can be determined by direct transesterification followed by analysis by GC or GC-MS. This process applies to the most commonly found fatty acids ranging from 14 to 24 carbon straight chains with zero to three double bonds. Fatty acids are identified by comparison of retention times (and also slit patterns) to standards. With addition of an internal standard such as heptadecanoic acid (C17:0), which is normally not present in the lipid extracts, the quantity of each fatty acid in the sample analyzed can be determined. A typical GC chromatogram of Arabidopsis leaf is provided in **Figure 13**.

For fresh tissue, for example, from leaf or root, the following acid-based procedure (Browse et al., 1986a) is widely used. This procedure allows methylation of both free fatty acids and transmethylation of *O*-acyl lipids. *O*-acyl lipids and free fatty acids in lipid extracts can be transesterified/methylated using the same protocol. For particular cases (short-chain and unusual fatty acids, free fatty acids, sphingolipids and *N*-acyl lipids, derivatization for GC-MS), protocols are available (Christie, 1993, 2003), as well as beginner's guides on methylation of fatty acids (http://www.lipidlibrary.co.uk/topics/methests/index.htm and mass spectrometry of fatty acids (http://www.lipidlibrary.co.uk/topics/ms_fa_1/index.htm

A direct acid-catalyzed transmethylation protocol.

1. Place up to 50 mg tissue in a Teflon-lined screw capped glass tube.
2. Add 1 mL of 2.5 % H_2SO_4 (v/v) in methanol (freshly prepared).
3. Heat at 80°C for 1 h.
4. Cool to room temperature.
5. Add 500 µL of pentane followed by 1.5 mL 0.9% NaCl (w/v) to extract fatty acid methyl esters (FAME).
6. Shake vigorously and then briefly centrifuge to facilitate phase separation.
7. Transfer some of the upper phase (pentane-containing FAME) to an injection vial. Concentrate with stream of nitrogen if necessary for GC sensitivity.
8. Run GC with a flame ionization detector (FID) on a polar column such as Econo-Cap™ EC™-WAX capillary column (15 m long, 0.53 mm i. d., 1.20 µm film, Alltech Associates, Deerfield, Ill.).
9. Typical GC conditions—split or splitless mode injection, injector, and flame ionization detector temperature, 250°C; oven temperature program—160°C for 1 min, 40°C min^{-1} to 190°C, 4°C min^{-1} to 230°C, holding this temperature for 4 min.

Notes:

1. This method works well for Arabidopsis leaves and also roots, although duration of the transmethylation step for the latter should be increased to 1.5 h.
2. Hexane and heptane have been used traditionally for extraction of FAMEs. However, due to known long-term toxicity of hexane, one may consider using pentane, which is less toxic. Should hexane or heptane be used, care should be taken.
3. Care should be taken when applying nitrogen stream to evaporate the solvent, as a considerable amount of C14 (even C16) FAMEs can be lost if the sample is heated or the nitrogen flow too vigorous.

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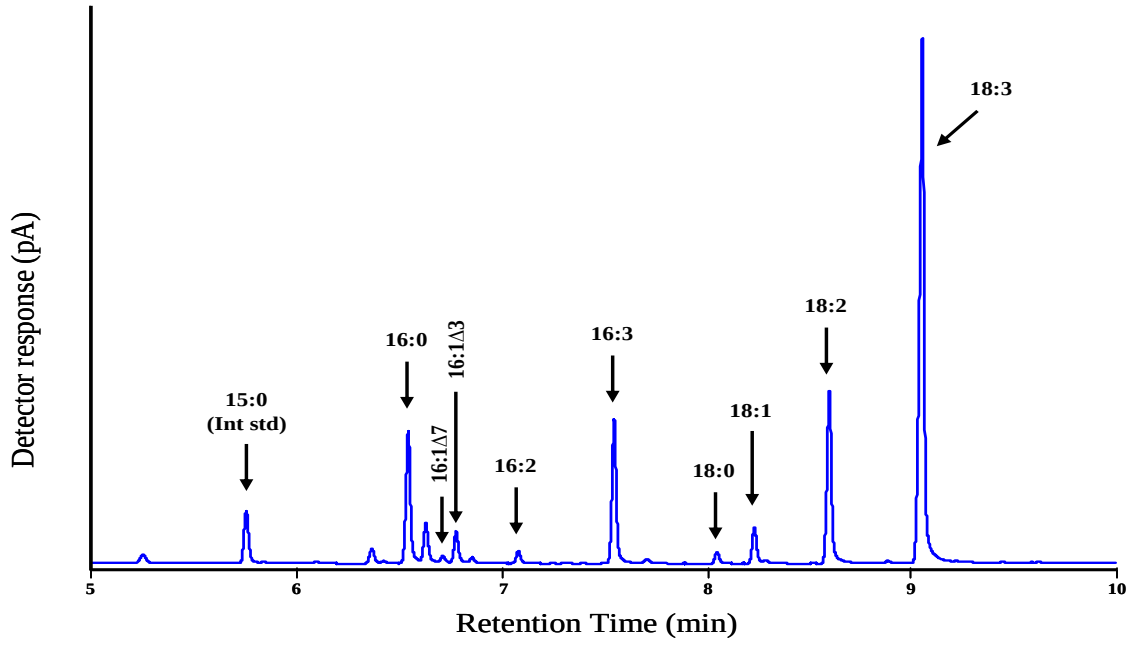

Figure 13. Separation of the Methyl Ester Derivatives of Fatty Acids From Arabidopsis Leaf.
A capillary fused silica column coated with (50% Cyanopropyl)-methylpolysiloxane (DB-23; J&W Scientific) was temperature-programmed from 140°C to 260°C at 10°C min^{-1} with helium as carrier gas. One µL of sample was injected in a 270°C inlet with a 30:1 split ratio. Methyl ester derivatives were detected using a FID at 270°C. (Courtesy of Dr. Imad Ajjawi, Michigan State University, Michigan)

3.3. Glycerolipid Analysis Methods

(Philip D. Bates[3])

Glycerolipid analysis involves separation of individual polar and neutral glycerolipid classes. Separation may be followed by quantitation of acyl groups within the class, stereospecific analysis to determine composition of acyl groups at each position of the glycerol backbone, or characterization of molecular species with specific combinations of acyl groups esterified to a single glycerol backbone.

Accurate analysis of glycerolipids requires care in lipid extraction to minimize oxidation, lipolysis, or transesterification [see Section 3.1]. Although initial separation of the extract may be performed HPLC (Beermann et al., 2003), the ease of use and low cost of TLC have made it a dominant technique for more than 60 years. Once separated, individual lipid classes can be quantified and fatty acid composition determined by direct conversion to fatty acid methyl esters and GC (Wu et al., 1994), or the glycerolipids may be collected for other analytical methods. Many different TLC separation and analysis methods are available for lipids and have been reviewed in depth (Christie, 2003, http://www.lipidlibrary.co.uk/).

3.3.1. Separation of Glycerolipid Classes by TLC

TLC plates are typically made of glass and coated with silica gel. Differences in gel composition and binders may affect lipid migration and downstream analysis (Sowa and Subbaiah, 2004). Comigration of lipid class standards should be used for identification of unknown compounds. Commercial TLC plates can be used directly; however, sometimes it is beneficial to heat TLC plates to ~110°C to drive off any moisture, especially in areas of high humidity. The ability of TLC plates to separate plant lipids can be enhanced by impregnating the plates with salts or compounds that will interact with the lipids. For instance, the silver ions of $AgNO_3$ will slow the migration of double bond–containing lipids, allowing separation of molecular species (Christie, 2003; Bates et al., 2009). To prevent oxidation of fatty acids, both spotting of samples on plates and drying of samples eluted from plates are performed under nitrogen, and 0.01% BHT may be added to samples, TLC solvents, and detection sprays.

Several reagents are available for detection of lipids on TLC plates. Lipids containing double bonds can be stained with iodine vapor by placing a TLC plate for 15 to 60 min together with iodine crystals in a TLC tank. The iodine staining is mostly reversible, but because iodine can destroy double bonds, very light staining is advised if further analysis is required. Alternatively, general lipids can be sprayed lightly with 0.005% primulin in 80% acetone and lipids visualized under UV light. This sensitive, nondestructive stain will not interfere with most downstream analyses. Other nondestructive sprays for lipid detection under UV include 0.01% Rhodamine 6G (w/v) in water and 0.1% (w/v) 2',7'-dichlorofluorescein in 95% methanol. Both solvents and sprays should be handled in a fume hood.

Figure 14 illustrates separation of the major lipid classes found in Arabidopsis and other plants on Partisil K6 silica gel 60 Å TLC plates (Whatman, Maidstone, U.K.). Neutral lipids are separated on TLC plates developed once in hexane/diethyl ether/acetic acid (70/30/1, v/v/v) (**Figure 14A**). Waxes and sterol esters migrate the most quickly, followed by triacylglycerols, free fatty acids, diacylglycerols, and monoacylglycerols, while polar lipids remain at the origin. For polar lipid separation, TLC plates are dipped in a solution of 0.15 M $(NH_4)_2SO_4$ and allowed to air dry. Just before use, the plates are heated to 110° C for at least 3 h. After lipid application, the plates are developed once or twice in acetone:toluene:H_2O (91/30/8, v/v/v) (Khan and Williams, 1977). Typical migration order of the major plant membrane lipids is as follows: total neutral lipids, monogalactosyldiacylglycerol, phosphatidylglycerol, sulfoquinovosyldiacylglycerol, digalactosyldiacylglycerol, phosphatidyinositol and phosphatidylserine, phosphatidylethanolamine, and phosphatidylcholine. See **Figure 14B** for separation of polar lipids after metabolic radiolabeling of soybean embryos or Härtel et al. (2000) for separation of Arabidopsis leaf lipids.

3.3.2. Stereochemical Analysis of Glycerolipids

The acyltransferases of glycerolipid synthesis have different specificities for acyl groups and thus produce lipids with different acyl compositions at each position of the glycerol backbone. Stereospecific phospholipase A_2 will cleave acyl groups from the *sn*-2 position of phospholipids. The free fatty acid and lyso-lipid products can be separated for analysis by TLC. Stereospecific analysis of glycolipids can be done with the TAG lipase from *Rhizopus arrhizus* that will cleave at the *sn*-1 position. There is no enzyme that differentiates the *sn*-1 and *sn*-3 positions of TAG, and thus a regiochemical analysis of *sn*-2 versus *sn*-1/3 is commonly employed with TAG lipase. Complete stereochemical analyses of TAG can be done through multistep procedures that involve partial degradation of TAG, generating a mixture of 1,2-DAG and 2,3-DAG. The mixed DAGs are either separated by chiral chromatography or are chemically converted to phospholipids for stereospecific PLA_2 digestion of *sn*-1,2 species as for natural phospholipids above. Protocols can be found in Christie (2003).

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3.3.3. FAME and Molecular Species Separations

GC and mass spectrometry are the preferred methods of measurement for lipid-derived FAME and individual lipid molecular species, respectively. For detailed coverage of molecular species analysis by mass spectrometry, see Section 3.10. However, TLC separations can be very useful in circumstances such as analysis of radiolabeled lipids from metabolic labeling experiments. For example, molecular species of triacylglycerols and acetylated diacylglycerols may be separated based on their number of double bonds by TLC on $AgNO_3$-impregnated TLC plates developed with a series of chloroform/methanol mixtures (Bates et al., 2009; Figure 14C and Figure 14D). FAME may be separated based on the number of double bonds with $AgNO_3$-TLC (Figure 14E) or based on both number of double bonds and fatty acid chain length with reverse phase TLC plates (Christie, 2003; Marquardt & Wilson, 1998).

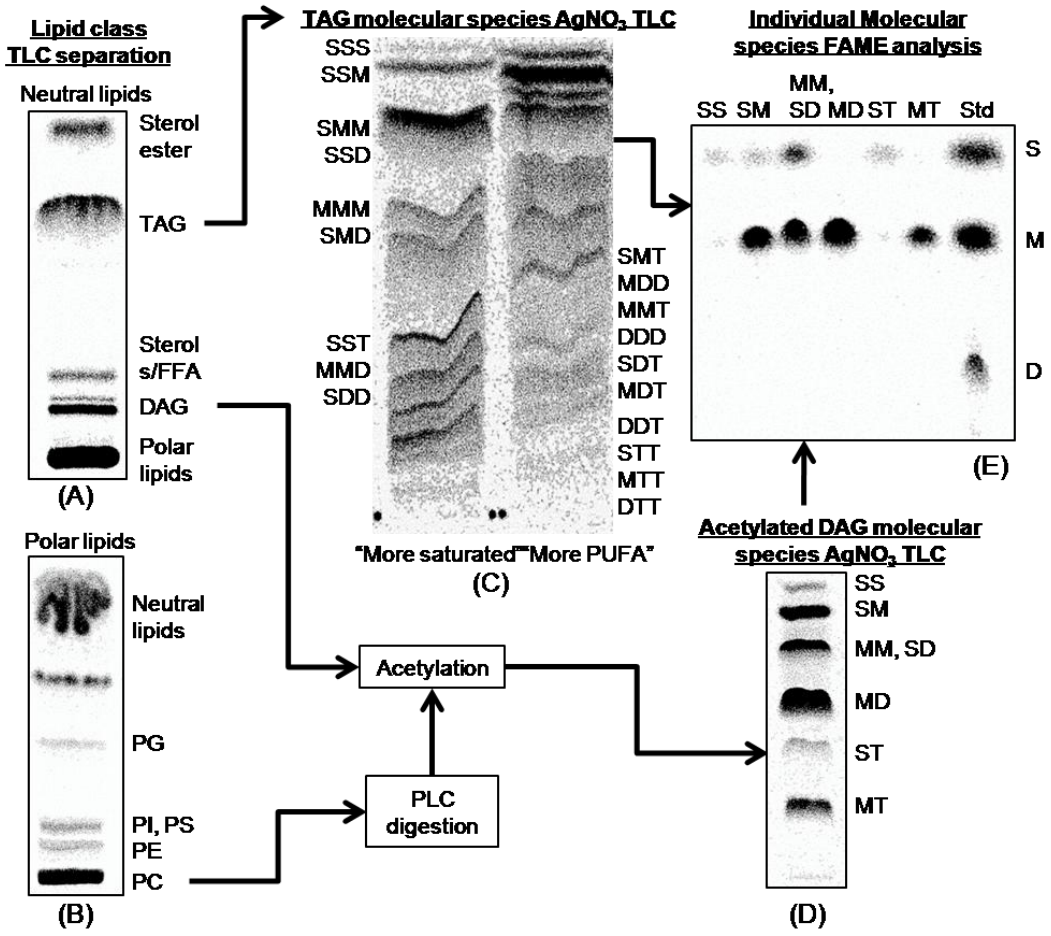

Figure 14. TLC Image Examples and Flow Chart for Analysis of Radiolabeled Glycerolipids From [^{14}C]acetate and [^{14}C]glycerol Labeled Soybean Embryos.

Examples of the major TLC systems used during analysis of radiolabeled glycerolipid classes, molecular species, and FA composition. Molecular species (e.g. SSS, SM, etc.) are represented as a combination of two or three FA, total saturates, S; monoenes (18:1), M; dienes (18:2), D; trienes (18:3), T.

(A) Neutral lipid class TLC, pictured 6 min [^{14}C]acetate labeling.

(B) Polar lipid class TLC, pictured 6 min [^{14}C]acetate labeling.

(C) TAG molecular species $AgNO_3$-TLC, pictured 30 min [^{14}C]glycerol labeling.

(D) Molecular species separation of PC (after phospholipase C digestion) and DAG as 3-acetyl-1,2-diacyl-glycerols, pictured 6 min [^{14}C]acetate labeled PC.

(E) FAME $AgNO_3$-TLC, pictured FAMEs from different 6 min [^{14}C]acetate labeled PC molecular species separated in **D**. See (Bates et al., 2009) for labeling experiment details and TLC protocols.

Abbreviations: DAG, diacylglycerol; FFA, free fatty acid; PG, phosphaglycerol; PI, phosphainostol; PE, phosphoethanolamine; PC, phosphacholine; PLC, phosphalipase C; PS, phosphaserine; PUFA, polyunsaturated fatty acid; TAG, triacylglycerol;

3.4. Seed Oil Quantification

(Yonghua Li-Beisson[4])

Arabidopsis stores over 35% oil in its seeds as energy and carbon reserves. A range of methods has been applied to quantify oil amount in Arabidopsis seeds (**Table 2**). These methods differ in sample size, sensitivity, instrument required, and information provided. Functional genetic screens have increasingly been used as a way to identify genes/proteins involved in storage oil metabolism. To facilitate mutant identification, a reliable and medium- to high-throughput oil quantification method is desirable.

Arabidopsis seeds have a thin seed coat and are tiny (~20 µg per seeds). Each seed has 5 to 8 µg of fatty acids, and over 90% of these fatty acids are stored as triacylglycerols. It has been shown that total TAGs can be quantified based on fatty acid composition compared to internal standard (Y.H. Li et al., 2006). A direct whole seed transmethylation protocol is described below. It has several advantages: (1) It bypasses oil extraction; (2) it requires a GC-FID, which is found in most lipid labs, and GC-FID-based methods offer very high sensitivity for quantifying acyl chains; (3) it provides not only total TAG content but also fatty acid composition; and (4) it is suitable for medium- to high-throughput screening and can be used in functional genetic screens. A typical GC chromatogram of the FAME profile for seeds is shown in **Figure 15**

Table 2. Comparison of Methods Reported for Quantifying Oil Content of Arabidopsis Seeds

Method	Sample Size	Analytic Procedure	Precision	Equipment (Core)	Information Provided
Gravimetric analysis[a]	>100 mg	Destructive Time consuming	Moderate	Analytical balance	Total lipid content (TAGs, phospholipids, etc.)
NMR[a]	>50 mg	Nondestructive Short scans (<1 min)	Moderate	NMR analyzer	Total lipid content
Lipid extraction transmethylation[a]	>100 mg	Destructive Time consuming	High	GC-FID	TAG content Fatty acid composition
Whole seed transmethylation[a]	20 seeds[b]	Destructive Suitable for high-throughput screening	High	GC-FID	TAG content Fatty acid composition
Carbon/Nitrogen ratio[a]	2–4 mg seeds	Destructive Suitable for high-throughput screening	High	Element analyzer	Relative value
TLC[b]	10 mg	Destructive	Low	TLC	TAG content
High temperature GC[b]		Destructive	High	GC-FID	TAG content TAG molecular species
LC-MS/MS (Burgal et al., 2008)	50 seeds	Destructive	High	LC-MS/MS	TAG content TAG molecular species

TAG = triacylglycerol; NMR = nuclear magnetic resonance; GC-FID = gas chromatography flame ionization detector; TLC = thin-layer chromatography; GC = gas chromatography; LC-MS/MS = liquid chromatography tandem mass spectrometry.

[a]For references, please refer to Y.H. Li et al., 2006; new methods available after this publication will be added here.

[b]Please refer to Christie's website http://www.lipidlibrary.co.uk/ for more details.

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A whole seed acid-catalyzed transmethylation protocol.

1. Count 20 seeds and add them to a Teflon-lined screw-capped glass tube.
2. Add 1 mL 5% H_2SO_4 (v/v) in methanol (freshly prepared), 50 µg BHT, 20 µg C17:0 TAG (triheptadecanoin) as internal standard, and 300 µL of toluene as cosolvent.
3. Vortex vigorously for 30 s.
4. Heat at 85º to 90°C for 1.5 h.
5. Cool to room temperature.
6. Add 1.5 mL 0.9% NaCl (w/v) and add 1 mL of hexane to extract FAME.
7. Mix well (vortex) and then centrifuge briefly to facilitate phase separation.
8. Transfer the upper organic phase (hexane-containing FAME) to a new tube.
9. Evaporate the extracts under a stream of nitrogen.
10. Redissolve in 50 µL of hexane (vortex well).
11. Run GC with a flame ionization detector on a polar column–like DB23 (30 m by 0.25 mm i.d., 0.25 µm film; J&W Scientific, Folsom, CA).
12. The GC conditions were as follows: split mode injection (1:40); injector and flame ionization detector temperature, 260°C; oven temperature program 150°C for 3 min, then increasing at 10°C min^{-1} to 240°C and holding this temperature for 5 min.

Notes:

1. This method works well for Arabidopsis seeds because almost all of the fatty acids are esterified to form TAGs, and the tiny seeds can be extracted without first grinding. For larger seeds (such as seeds of *Brassica napus*) or in cases where significant amounts of fatty acids are stored in other forms, TAGs might need to be isolated.
2. Before applying this method to other systems, total oil content and fatty acid distribution need to be verified by other methods.
3. Often researchers use as little as 1 or 2 seeds for the analysis. From our experience, this is sufficient to screen a large number of lines aiming to find changes in fatty acid profile or production of an unusual fatty acid. For quantification purpose, this usually gives very low value. Variations are likely due to seed-to-seed variation and inaccuracy in seed weight determinations.
4. Toluene is added as a cosolvent since neutral lipids such as TAGs and wax esters do not dissolve well in methanol alone.
5. Care should be taken when applying nitrogen stream to evaporate the solvent as a considerable amount of C14 (even C16) FAME can be lost if the nitrogen flow is too vigorous.
6. This method gives information on FAME content and composition; therefore, when reporting oil content, calculation is required: percent oil by weight = 100 (4 total mol FAME/3) + total g FAME)/g tissue, where 4 is the Mr. difference between TAG and three moles of FAME.

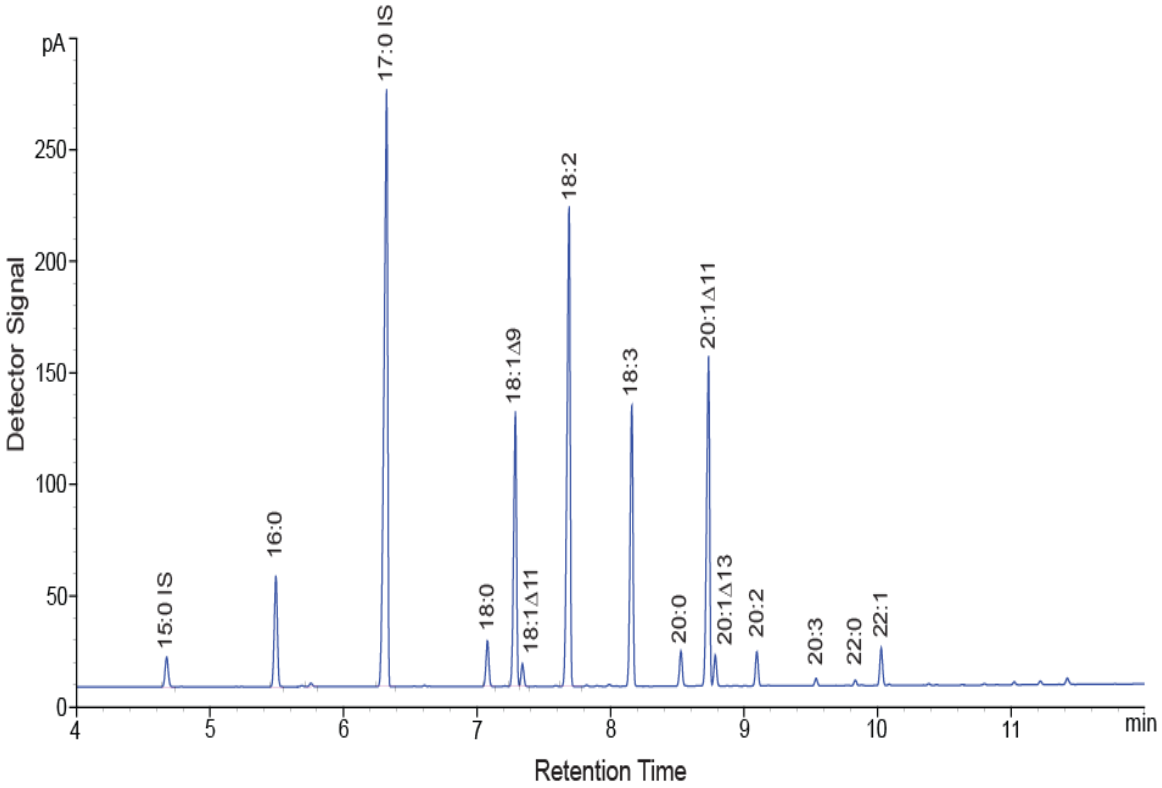

Figure 15. GC-FID Analysis of Fatty Acid Methyl Esters Derived From Neutral Lipids Isolated From Arabidopsis Col-0 Seed.

Neutral lipids were isolated from wild type Col-0 seed and transmethylated and the subsequent fatty acid methyl esters separated on a J+W DB-23 (50% cyanopropyl) methylpolysiloxane 30 m column and detected using a flame ionization detector. The column temperature was initially held at 150 °C for 3 min, then increased to 240 °C at rate of 10 °C min^{-1}, and then held at 240°C for 10 min. Tripentadecanoin (15:0) and triheptadecanoin (C17:0) were used as seed lipid extraction and transmethylation internal standards, respectively. IS = internal standard (Courtesy of Dr. Timothy Durrett, Michigan State University)

3.5. TAG Analysis by Liquid Chromatography Mass Spectrometry

(Tony Larson[5])

The yield and fatty acid composition of seed triacylglycerols reflects the combined activities of fatty acid synthesis, desaturation, elongation, and transferase reactions, the latter of which are reversible. TAG analysis is therefore an important tool in evaluating how the process of acyl chain assembly into storage lipids is controlled, which may be especially important in metabolically engineered oil synthesis (Burgal et al., 2008). Three components of TAG analysis are potentially useful measures: (1) absolute quantity (i.e., yield), (2) relative TAG molecular species distribution, and (3) acyl position-specific information for a given TAG species. In the outlined method, absolute quantification is difficult as MS-based methods return biased responses depending on acyl chain length and degree of unsaturation (X.W. Li and Evans, 2005); instead, GC-FID-based FAME analyses of TAG derivatives is recommended. However, seed oil TAGs from Arabidopsis, comprising mixed acyl chains in the narrow 18–20 carbon number and 1–3 double bond range, generally provide a proxy of FAME-calculated yield within an error of ±10%. Less than half of the 80 to 120 resolvable *Arabidopsis* TAG molecular species are chromatographically separated; the remainder is resolved in the MS dimension by nominal mass. Constituent acyl species are then empirically assigned by reconciling the MS2 neutral-loss DAG fragments with the parent ammoniated molecular ion. In some cases, *sn*-2 position can be assigned due to the theoretically favorable loss of acyl chains from *sn*-1,3 positions (represented as more intense *sn*-1,2 and *sn*-2,3 DAG fragments in MS2 spectra). However, full positional assignments are not possible with MSn techniques alone; prior stereospecific digestion techniques are required and are not covered here.

TAGs are extracted by grinding 20 to 200 Arabidopsis seeds in a 1.5 mL microfuge tube with 10 µL 1,1,1^{13}C-triolein (0.5–5 mg mL^{-1} in chloroform; internal standard) + 400 µL hexane/isopropanol (3:2, v/v), snap-freezing in liquid nitrogen, incubating at 4ºC for 60 min, and centrifuging at 14,000 RPM for 5 min, and the supernatant is transferred to a fresh tube. The pellet is washed 3 times with 100 µL hexane:isopropanol and the supernatants pooled, combined with 350 µL 6.7% sodium sulphate (w/v), vortexed and centrifuged for 30 s at 14,000 RPM, and the supernatant dried *in vacuo* in an HPLC vial. The lipid residue is reconstituted in 100 µL chloroform and 10 µL injected on an LCQ-MS (Thermo Finnigan) equipped with a C30 HPLC column (YMC, 250 x 4.6 mm, 5 µm particle size) held at 30°C. A ternary separation gradient is used at 1 mL min^{-1} with solvents containing 0.2% formic acid (v/v): Solvent A is 20 mM ammonium formate in 80% (v/v) methanol, B is methanol, and C is tetrahydrofuran. Gradient is 0 to 5 min isocratic 5% A, 95% B; 5 to 45 min to 5% A, 35% B, 60% C, then isocratic 45-50 min; 10 min re-equilibration time between injections. The column eluent fed unsplit into an atmospheric pressure chemical ionization (APCI) source: vaporizer temperature 350°C; sheath gas (N_2) flow 60 units, aux gas flow 60 units; source current 5 µA; capillary voltage 32 V; capillary temperature 150°C. Full-scan MS data are collected over the range 450 to 1500 m/z, and MS2 fragmentation data are collected in data-dependent mode at 60% normalized collision energy and an isolation width of 4 m/z.

A representative total ion current trace for Col-0 seed extract is shown in **Figure 16A**, with the identification process shown for a dominant TAG species outlined in **Figure 16B**.

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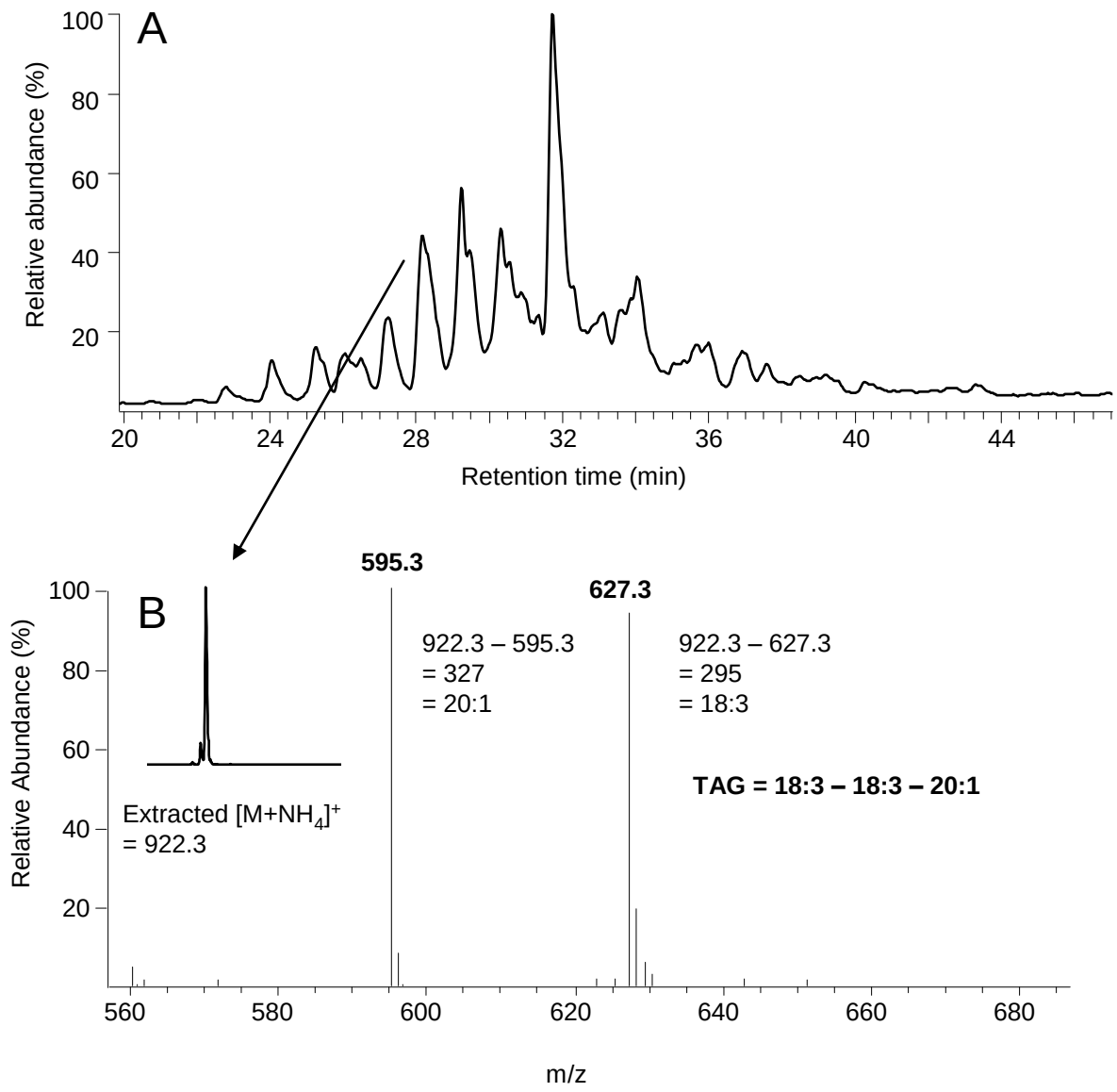

Figure Figure 16. TAG Profile of Col-0 Dry Seed.
The total ion current **(A)** is used for the data-dependent selection of parent ions (inset in **(B)**), which are subjected to MS^2 fragmentation analysis. Up to three daughter diacylglycerol (DAG) fragments are generated per triacylglycerol (TAG); in this example, two are generated because there are only two unique constituent fatty acids. The neutral losses for the observed DAG fragments (after correction for adducts) can be used for a constrained calculation of fatty acid identity **(B)**. The correct stoichometric combination of these fatty acids to give the parent ion is used to calculate the TAG molecular formula.

3.6. Acyl-CoA Analysis by High-Performance Liquid Chromatography

(Tony Larson[6])

Long-chain (C16–18) and very long chain acyl-CoAs (C20–C24) are cytosolic intermediates for glycerolipid synthesis, and their accumulation would suggest a bottleneck or limiting step. Additionally, these acyl-CoAs (and also short-, medium-, and branched-chain variants) are measureable intermediates during peroxisomal β-oxidation in all tissues, especially those tissues undergoing rapid catabolism (e.g., seedlings, senescing leaves). CoA species also include the rapidly turned-over ubiquitous intermediates, acetyl and malonyl CoAs, which, although occasionally seen, are troublesome to recover quantitatively from extracts using the method described below.

The method given is an HPLC-reversed-phase-based separation with an integral washing step to remove interfering components. It is based on established techniques for acyl-CoA extraction from biological samples (Mancha et al., 1975), with extraction optimized to maximize recovery from plant tissues and detection specificity and sensitivity increased to cope with the low inherent concentration of acyl-CoAs in complex plant tissue matrices (Larson and Graham, 2001). Sample extraction combines prepurification with conversion to stable *etheno* fluorescent derivatives that can be readily separated by HPLC and sensitively and quantitatively detected. The acyl chain moiety of novel acyl-CoAs can be structurally determined in preconcentrated extracts using liquid chromatography tandem mass spectrometry (LC-MS/MS) techniques (Ishizaki et al., 2005). Acyl-CoA standards for verification of unknowns can be purchased or synthesized using enzymatic techniques for long or very long chains (Taylor et al., 1990) or chemical synthesis (Kawaguchi et al., 1981) for short or medium chains.

Fresh or frozen tissue samples (2–20 mg) are transferred to a microfuge tube and internal standards (10 µL each of 0.2 µM isovaleroyl and heptadecanoyl CoAs) added, followed by 200 µL freshly made ice-cold extraction buffer (200:200:5:8 [v/v] isopropanol:50mM phosphate buffer pH 7.2: acetic acid: 50 mg mL^{-1} BSA). It is important not to exceed 20 mg plant material, or recoveries will be greatly compromised. The samples are ground and lipids removed by 3 x 200 µL washes with water-saturated petroleum ether. Saturated ammonium sulphate (5 µL) is added to each sample, followed by 600 µL 2:1 [v/v] methanol:chloroform. Samples are vortexed and left at room temperature (RT) to precipitate for 20 min before centrifuging at 14,000 RPM for 2 min. The supernatant is transferred to HPLC vials and dried *in vacuo*. Derivatizing reagent (0.5 M chloroacetaldehyde, 0.5% [v/v] SDS, 150 mM citrate buffer pH 4.0; stored at RT for up to 3 months) is added to each vial (40 µL), and the sealed vials are heated at 85°C for 20 min. The derivatized samples (stable for at least a week at RT) are injected (20 µL) for HPLC analysis. The HPLC is equipped with a Luna C18(2) column (Phenomenex, 150 x 2.0 mm, 5 µm particle size) held at 40°C. A quaternary separation gradient is used with solvents: A, 1% acetic acid; B, 90% acetonitrile 1% acetic acid; C, 0.25% triethylamine; D, 90% acetonitrile. The run gradient is as follows: 0–5 min, 0.75 mL min^{-1} A:B (90:10) – A:B (20:80); 5–5.1 min, A:B (20:80) – A:C(20:80); 5.1–7 min, A:C (20:80) – C:D(97:3); 7–10 min, C:D(97:3) – C:D(95:5); 10–10.1 min, flow rate reduced to 0.2 mL min^{-1}; 10.1–50 min, C:D (95:5) – C:D (55:45); 50–50.1 min, C:D(55:45) – D; 50.1–52 min, D; 52–52.1 min, flow rate increased to 0.2 mL min^{-1}; 52.1–57 min D; 57–57.1 min, D – A:B (90:10); 57.1–60 min, A:B (10:90). The eluent is sent to a fluorescent detector with excitation set to 230 nm and emission to 420 nm. Peak area is directly proportional to molar quantities, and concentrations can be determined by reference to the internal standards. An example trace for Col-0 seedling extracts is shown in **Figure 17**.

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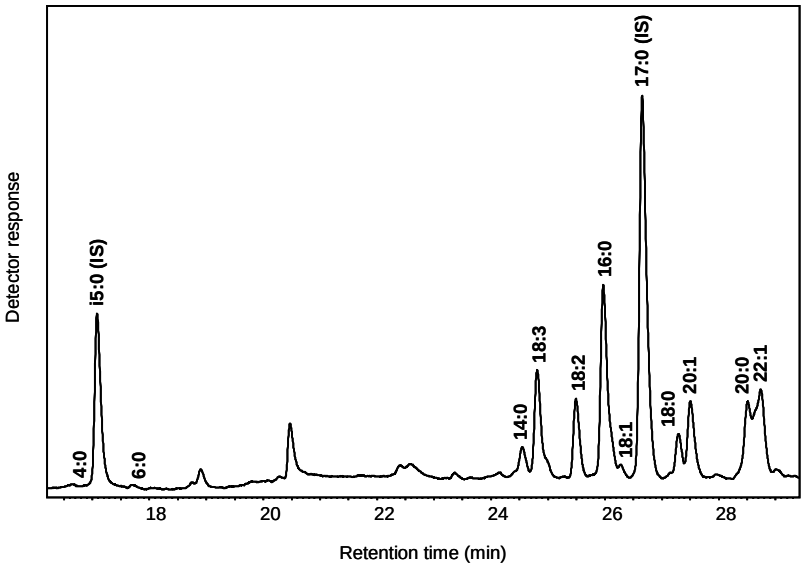

Figure 17. High-Performance Liquid Chromatography Trace of Acyl-CoA From a Col-0 Seedling Extract.
The extract was made as described from 30 seedlings (grown on media plates) harvested at 5 days after imbibition. This developmental stage encompasses both rapid storage lipid breakdown and de novo lipid biosynthesis associated with seedling establishment. Peak areas and heights are directly proportional to the absolute amounts of the indicated acyl-CoAs. IS = internal standard.

3.7. Sphingolipid Analyses

(Jonathan E. Markham[7])

Sphingolipids have unique chemistry that has both advantages and drawbacks for the lipid biochemist. Due to their unique long-chain base component, total sphingolipid content can be quantified from intact, dry tissue by hydrolysis, derivitization, HPLC separation, and fluorescence detection (Markham et al., 2006). A similar technique can be used to release the 2-hydroxy fatty acids (which are almost exclusively found in sphingolipids) to obtain information about the sphingolipid LCB and fatty acid content, although information about which LCBs are found in different classes of sphingolipid and with what fatty acids is not obtained.

Analysis of intact lipids is more challenging due to the highly glycosylated nature of the complex sphingolipids, which means they are largely insoluble in pure solvent and hence not easily extracted by the usual methods to extract lipids (Markham et al., 2006). For this reason, many analyses of sphingolipids from plants have focused on simpler sphingolipids such as ceramide or glucosylceramide (Ohnishi and Fujino, 1981; Imai et al., 1995, 2000). Analysis of complex glycosphingolipids requires more extensive purification and techniques of carbohydrate analysis to uncover the absolute structure of the complex headgroup (Kaul and Lester, 1975; Markham et al., 2006). Intact sphingolipids from Arabidopsis can be extracted and quantified by LC-MS/MS, albeit with some limitations (Markham and Jaworski, 2007), but nonetheless this remains the only viable option for extensive analysis of intact sphingolipids from Arabidopsis.

This protocol is very simple and a quick way to check for changes in sphingolipid metabolism in Arabidopsis due to genetic mutation or environmental challenge. It is robust, very sensitive, quantitative, and free from most artifacts generated by other hydrolysis conditions. In the absence of a HPLC fitted with a fluorimeter, LCBs can be analyzed by making dinitrophenyl derivatives of the LCBs and analyzing by HPLC with a UV detector (Sperling et al., 1998), converting the LCBs to aldehydes and analysis by GC-FID/GC-MS (Bonaventure et al., 2003), or making N-acetyl, O-trimethylsilane-derivatives and also detecting by GC-FID/GC-MS.

Protocol: Hydrolysis and quantification of LCBs from sphingolipids of Arabidopsis.

Materials: Internal standard D-erytho-C20-sphingosine (d20:1, Matreya) dissolved in methanol at 0.1 nmol μL^{-1}; 10% $Ba(OH)_2$ (dissolve 10 g of $Ba(OH)_2 \cdot 8H_2O$ [Sigma] in 96 mL of water with heating and stirring, warm before use); dioxane (HPLC grade), 2% Ammonium sulphate, OPA reagent (dissolve 5mg of O-phthaldialdehyde in 100 µL of methanol, add 5 µL of mercaptoethanol, 6.6 mL of water and 3.3 mL of 3% Boric acid pH 10.5); OPA diluent (combine 10 mL of water, 50 µL of 1M $KHPO_4$ pH7 and 60 mL of methanol).

1. Freeze dry sample. Grind tissue to powder.
    a. Alternatively, dry a lipid extract under nitrogen and proceed to step 3.
2. Weigh approximately 10 mg of tissue into a screw-cap glass tube and record weight.
3. Add 10 µL of d20:1 standard, 1 mL of dioxane, and 1 mL of 10% $Ba(OH)_2$.
4. Screw cap onto tube as tightly as possible.
5. Place tube in 110ºC heat block. Hydrolysis reaction is essentially complete after 8 h.
6. Add 2 mL 2% ammonium sulfate and 2 mL diethylether. Vortex.
7. Spin at 500 g for 10 min. Remove upper phase to a new tube and dry under nitrogen.
8. Add 100 µL of methanol and 50 µL of OPA reagent. Allow to derivatize at room temperature for 20 min.
9. Add 350 µL of OPA diluent.
10. Spin at 500 g for 10 min. Transfer sample to labeled autosampler vial and seal.
11. Run on HPLC as soon as possible. Store samples in dark at 4°C if there is any delay.
12. Quantify the resulting peaks by comparison to the internal standard.

Note: HPLC conditions: Column—Agilent XDB-C18 4.6 x 250mm with guard column; Buffer A 5 mM K_2HPO_4 pH 7; Buffer B Methanol Flow Rate 1.5 mL min^{-1}

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HPLC gradient:

Time	Percent B	Duration
0 min	80%	7 min
7 min	90%	8 min
15 min	90%	10 min
25 min	100%	5 min
30 min	100%	3 min
33 min	80%	1 min
34 min	80%	2 min

Fluorescence detector: Excitation 340 nm Detection 455 nm
Notes: The major peak in Arabidopsis is t18:1$^{\Delta 8E}$ that elutes around 17–18 min. Artifacts generated by the hydrolysis include the partial hydrolysis of the glycosidic bond resulting in Glc-t18:1 peaks and the formation of 1,4-*anhydro* derivatives from the phosphorylated sphingolipids.

3.8. Lipid Polyester Analysis

(Fred Beisson[8])

A protocol for the routine quantitative analysis of ester-linked monomers of Arabidopsis cutin and suberin is described below. Major steps are delipidation of tissues, chemical depolymerization of the residue, and extraction of released monomers in an organic phase and analysis of monomers by gas chromatography after derivatization of their hydroxyl groups. Various protocols using different delipidation, depolymerization, monomer extraction, and derivatization methods have been described (Bonaventure et al., 2004b**;** Franke et al., 2005; Molina et al., 2006). The protocol reported here is mostly adapted from the Bonaventure and Molina references. It can be performed on all Arabidopsis organs and uses minimal amounts of biological material and solvent.

I. Delipidation of tissues.

All solvent extraction steps include 0.01% (w/v) butylated hydroxytoluene (BHT) added from a 5% (w/v) stock solution in methanol. Unless indicated otherwise, each extraction is performed at room temperature by vortexing for 1 h (use a multitube vortexer, glass tubes with Teflon-lined screw caps, and 4 to 10 mL of solvent).

1. Fill preweighed tubes with isopropanol and heat up (85°C). Immerse Arabidopsis tissues in boiling isopropanol (10 min at 85°C). Leaves need to be cut in small pieces, and stems need to be cut in 1–2 cm bits and longitudinally in two halves. Examples of amounts of starting material per tube: 20–100 mg mature seeds, 100–300 mg fresh weight (fw) leaves or stems, 10–20 flowers, 50–200 mg fw secondary roots.
2. Cool down and extract by vortexing for 1 h. Alternatively, shake on a rocking agitator or a rotating wheel for at least 2 h and up to overnight.
3. Discard solvent. Most chlorophyll should have been extracted at this step. Extract again with isopropanol.
4. Discard solvent and extract with chloroform/methanol (2:1, v/v).
5. Discard solvent and extract with chloroform/methanol (1:2, v/v).
6. Discard solvent and extract with methanol.
7. Discard solvent and dry under a gentle stream of nitrogen gas. Dry in vacuum dessicator under reduced pressure until constant weight is achieved (at least 24 h). It is very important to remove as much water as possible.

II. Depolymerization of residue: Base- or acid-catalyzed transmethylation.

Weigh tubes to determine amount of dry residue after delipidation (there should be 10–50 mg depending on tissue). Transmethylations can be done by either acid or base catalysis.

Base catalysis

1. In each tube, add 2 ml of freshly made reaction medium and 5–10 µg of methyl heptadecanoate and ω-pentadecalactone as internal standards. To make 20 mL of reaction medium: In 12 mL methanol, add 3 mL methyl acetate and 5 ml 25% sodium methoxide in methanol (Sigma).
2. Heat the mixture at 60°C for 2 h.
3. Cool, add 4 ml dichloromethane, 0.5 mL glacial acetic acid to neutralize to pH 4–5, and 1 mL 0.9% NaCl (w/v) Tris 100 mM pH 8.0. Shake well and phase separate by centrifugation for 2 min at 1500 g. Check pH of upper phase with pH indicator paper.
4. Collect the lower organic phase, wash it with 2 mL 0.9% NaCl (w/v), and dry over anhydrous sodium sulfate. Evaporate to dryness under a gentle stream of nitrogen gas (heat up tubes at 35°C max).

Acid catalysis

1. In each tube, add 2 mL freshly made reaction medium, 0.2 mL toluene as a cosolvent, and 5–10 µg of methyl heptadecanoate and ω-pentadecalactone as internal standards. To make 20 mL of reaction medium: In 19 mL methanol, add 1 mL concentrated sulfuric acid. Alternatively, 3N methanolic hydrochloride can be used.
2. Heat the mixture at 80°C for 2 h.
3. Cool. Add 4 mL dichloromethane and 1 mL of buffer 0.9% NaCl (w/v) Tris 100 mM pH 8.0. Shake well and phase separate by centrifugation for 2 min at 1500 g.
4. Collect the lower organic phase, wash it with 2 mL of buffer, and dry it over anhydrous sodium sulphate. Evaporate to dryness under a gentle stream of nitrogen gas (heat up tubes at 35°C max).

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III. Derivatization of monomers and GC-(MS) analysis.

1. Hydroxyl residues can be acetylated or sylilated.

   Acetylation: Add 100 µL of anhydrous pyridine and 100 µL of acetic anhydride. Heat up at 60°C for 2 h. Evaporate solvent under nitrogen stream.

   Sylilation: Add 100 µL of anhydrous pyridine and 100 µL of BSTFA [N,O-bis(trimethylsilyl)-trifluoroacetamide]. Heat up at 110°C for 10 min.

2. Redissolve monomers in 30–200 µL of heptane:toluene (1:1, v/v) and run samples on GC-(MS). GC-FID conditions: HP-5 capillary column (30 m, 0.32 mm ID, 0.25 µm film thickness) with helium carrier gas at 2 ml min^{-1} and oven temperature programmed from 140°C to 310°C at 3°C min^{-1}) and then held for 10 min at 310°C. Samples are injected in split mode (30:1 ratio, 310°C injector temperature) and peaks quantified on the basis of their FID ion current. For GC-MS, the same column is used with He carrier gas at 2 mL min^{-1} and oven temperature programmed from 110°C to 300°C at 10°C min^{-1}. Splitless injection is used and the mass spectrometer run in scan mode over 40–500 amu (electron impact ionization) with peaks quantified on the basis of their total ion current.

Additional tips and cautionary notes

1. Grinding tissues

For seeds, it is necessary to grind material in liquid nitrogen with mortar and pestle before solvent extractions (Molina et al., 2006). To ensure optimal delipidation of seeds, additional extraction/centrifugation steps should be performed after the final methanol step: methanol (30 min), water (30 min), 2 M NaCl (1 h), water (30 min), methanol (30 min), chloroform/methanol 1:2 (v/v) (1h to overnight), chloroform/methanol 2:1 (v/v) (1h to overnight), and methanol (1h). To improve delipidation steps, tissues such as stems and leaves can also be ground using a Polytron (following immersion in hot isopropanol and cooling down). Recovery of ground material after each extraction can be performed using centrifugation or filtration through several layers of filter paper. One will have to be careful to minimize loss of material at each extraction step, however. Grinding and centrifugation or filtration is thus easier for experiments aiming at quantifying polyester monomer on a dry residue weight basis than those requiring quantification on a leaf/stem surface area basis.

2. Quantification

To express polyester loads as micrograms per unit surface area, scan stems and leaves after harvest. To express polyester loads in ng per seed, weigh 200 seeds.

3. Depolymerization

The acid-catalyzed depolymerization procedure is a bit faster because it does not require acidification prior to monomer extraction. One has to be aware that acid catalysis will release substantial amounts of 2-OH fatty acids that are not O-acylated to polyesters and might come from residual sphingolipids (Molina et al., 2006).

4. Monomer extraction

To extract fatty acid methyl esters, it is important to use dichloromethane (and not hexane) because polyhydroxy fatty acids are very poorly extracted in hexane.

5. Derivatization

Acetylation is less sensitive to water than sylilation. However, it is recommended to perform both methods because a few monomers that do not separate well with one derivatization will separate better with the other one. Ideally, this should be done on two samples of the same monomer extract. Alternatively, the acetyl or trimethylsilyl group from an already derivatized sample can be derivatized again with the other agent by doing a short acid-catalyzed transmethylation (5 min), re-extracting, drying, and derivatizing.

6. Drying organic phase

The drying organic phase can also be achieved by using 2,2-dimethoxypropane (2,2 DMP). This is done by reducing the volume of the organic phase to ~1 mL by evaporating with N_2, adding 1.5–2 volumes of 2,2 Dimethoxypropane (DMP), incubating at 50 60°C for ~ 5–15 min, and evaporating under nitrogen gas to complete dryness (Kosma et al., 2009).

3.9. Analysis of Cuticular Waxes

(Owen Rowland[9])

Cuticular wax is the mixture of compounds removed from plant surfaces by brief immersion in an organic solvent of low polarity. The resulting extract (wax) is typically a mixture of saturated hydrocarbon backbones that may carry an oxygen-containing functional group (e.g., mixture of alkanes, aldehydes, primary and secondary alcohols, ketones, and alkyl esters). Each lipid class is present as a homologous series (e.g., C24:0, C26:0, C28:0, and C30:0 primary alcohols), or one chain length may predominate. In addition to straight-chain aliphatics, cuticular wax may also contain secondary metabolites such as triterpenoids and phenylpropanoids. A detailed discussion of the composition of plant cuticular waxes and methods used for chemical analysis can be found in Jetter et al. (2006).

3.9.1. Wax extraction. After 4 to 7 weeks of plant growth, aerial organs (e.g., 5 cm length of stem or 3–4 rosette leaves) are submerged twice for 30 s each in chloroform at room temperature. Fresh, healthy tissue samples should be used that are free of surface lesions to prevent contamination with internal lipids. A volume required to cover the tissues completely is sufficient. A 13 x 100 mm glass tube holding 10 mL of chloroform is convenient for dipping stems. *n*-Hexane can also be used as the solvent for extraction, but it has a very low polarity, and larger volumes may be required to exhaustively extract the more polar wax constituents. It is important that all vessels are prerinsed thoroughly with solvent to prevent contamination of samples. An internal standard to determine wax quantities is added immediately before or after dipping of organs in solvent. In each tube, 1 µg of n-Tetracosane (C24 alkane) is typically added as the internal standard because it is chemically similar to the common wax constituents but runs at a distinct retention time. The samples are then completely evaporated under a gentle stream of nitrogen and derivatized with bis-*N,O*-(trimethylsilyl)trifluoroacetamide (BSTFA) to transform all hydroxyl- and carboxyl-containing compounds into the corresponding trimethylsilyl derivatives. Derivatization conditions vary, but heating samples resuspended in 50 µL of BSTFA with 1% trimethylchlorosilane (available in 1 mL ampules from Pierce or Sigma-Aldrich) at 80ºC for 60 min or 10 µL of BSTFA mixed with 10 µL of pyridine at 70ºC for 60 min are typical (Rowland et al., 2006; Greer et al., 2007).

3.9.2. Gas chromatography. Derivatized samples are injected onto a capillary GC column with helium or hydrogen as carrier gas. A typical column for wax analysis: 15—30 meter, 0.32 mm i.d., *df* = 1 µm HP-1 column (Agilent, or equivalent column from another supplier). A typical GC method: oven temperature set at 50ºC for 2 min, raised by 40ºC min^{-1} to 200ºC, held for 2 min at 200ºC, raised by 3ºC min^{-1} to 320ºC, and held for 30 min at 320ºC (Wen and Jetter, 2009). After separation by GC, quantitative analysis of individual wax components is usually done with a FID, as it is highly sensitive and has a broad range of proportionality. Absolute values in units of wax mass per surface area are determined by comparison with the known internal standard and measured surface areas. The surface areas of leaves can be conveniently measured using microscope imaging software (e.g., Zeiss Axiovision) and stem surface areas either by microscope imaging or by using a caliper. Values are sometimes reported as units of wax mass per dry or fresh tissue weight, but surface area is more typical and generally preferred. Identification of individual wax components is done by GC-MS in comparison with published MS libraries or authentic standards (many wax constituents are represented in MS libraries).

3.9.3. Thin layer chromatography. TLC is a convenient and rapid way to analyze general alterations of wax compound classes between WT, mutant, and transgenic plants (Greer et al., 2007). Total wax mixtures, extracted as above, of approximately 2 mg are readily separated on silica gel with a mobile phase of $CHCl_3$:ethanol 99:1. The separated fractions are sprayed with 0.01% primuline in acetone:H_2O (4:1) and then visualized under UV light. Individual compound classes can then be scraped from the TLC plate, eluted with $CHCl_3$, filtered, concentrated in a stream of N_2, and then analyzed by GC-MS to identify all homologues and/or isomers of, for example, alkyl esters, secondary alcohols, and ketones (Rowland et al., 2006; Wen and Jetter, 2009).

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3.10. Lipidomics

(Ruth Welti[10])

Lipidomics typically describes the use of electrospray ionization (ESI) triple quadrupole mass spectrometry (MS/MS) to profile lipid molecular species. Quantitative information on numerous individual lipid species is acquired directly from organic extracts **[see Section 3.1]** of plant material, typically without chemical modification. Lipidomics is rapid in comparison to "traditional" lipid analysis and requires relatively small amounts of material (i.e., 0.1 mg of leaf dry weight). Comparison of the lipid profiles of WT plants with those of plants that have been subjected to forward- or reverse-genetic manipulation, in parallel with developmental and physiological phenotyping, can aid in characterization of the roles of the manipulated genes and enzymes (e.g., Welti et al., 2002; Nandi et al., 2003; Cruz-Ramírez, 2006; Devaiah et al., 2006; M.Y. Li et al., 2006b; Welti et al., 2007; Chen et al., 2008; W. Li et al., 2008; Maeda et al., 2008). Association of lipid and genetic alterations can provide clues as to the physiological substrates and products of the altered gene products (enzymes; e.g., Welti et al., 2002).

Lipid extracts can be introduced directly to a mass spectrometer (direct-infusion ESI-MS/MS) or through a liquid chromatography column (LC-MS/MS). Thus far, phospholipids and galactolipids in Arabidopsis have been analyzed primarily by direct-infusion ESI-MS/MS, sphingolipids **[see Section 3.7]**and acyl-CoAs **[see Section 3.6]** have been analyzed primarily by LC ESI-MS/MS (Larson and Graham, 2001; Markham and Jaworski, 2007), and triacylglycerols **[see Section 3.5]** have been analyzed by several mass-spectrometry based approaches. Direct-infusion ESI-MS/MS for analysis of complex lipids is described in this section.

The direct-infusion ESI-MS/MS approach most applied to plant polar lipids (Welti and Wang, 2004) utilizes a series of "precursor" and "neutral loss" scans (based on Brügger et al., 1997). This method takes advantage of the formation of common fragments from related complex lipids upon collision-induced dissociation (CID) in a triple quadrupole mass spectrometer. Among polar lipids, the CID fragment is typically a head group fragment common to all members of a lipid class. For example, phosphatidylcholine molecular species, which vary in fatty acid composition, produce a common phosphocholine fragment. If the common fragment is charged, a scan for the precursors of the fragment (a precursor scan) yields a spectrum (plot of signal vs. *m/z* or mass/charge ratio, where *z* is typically = 1) in which there are signals at *m/z* corresponding to the masses of intact lipid molecular species ions containing the fragment (Welti and Wang, 2004). If the common fragment is uncharged, then a neutral loss scan provides the spectrum of the molecular species that contain the fragment. A complete lipid profile is obtained by sequentially carrying out characteristic precursor and neutral loss scans for each lipid group or class. Essentially, these scans allow one to look at the molecular species within one class or group of lipids at a time, while the extract is continuously infused into the mass spectrometer. Scans for analysis of many complex plant lipid classes are shown in **Table 3**.

Table 3. Precursor and Neutral Loss Scans Utilizing Characteristic Fragments Generated by Electrospray Ionization **for Analysis of Polar Complex Lipids From Arabidopsis**

Lipids Analyzed	Polarity	Ion Analyzed	Scan Mode	References
Phospholipids				
phosphatidylcholines	+	$[M + H]^+$	Precursors of *m/z* 184	Brügger et al., 1997
phosphatidylethanolamines	+	$[M + H]^+$	Neutral loss of 141	Brügger et al., 1997
phosphatidylserines	+	$[M + H]^+$	Neutral loss of 185	Brügger et al., 1997
phosphatidylglycerols	+	$[M + NH_4]^+$	Neutral loss of 189	Taguchi et al., 2005
phosphatidylinositols	+	$[M + NH_4]^+$	Neutral loss of 277	Taguchi et al., 2005
phosphatidic acids	+	$[M + NH_4]^+$	Neutral loss of 115	

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Sphingolipids				
glycosylinositolphosphoceramides	+	$[M + NH]^+$	Neutral loss of 615 or 179	
hexosylceramides	+	$[M + H]^+$	Neutral loss of 162	
Galactolipids				
sulfoquinovosyldiacylglycerols	–	$[M - H]^-$	Precursors of *m/z* 225	Gage et al., 1992; Welti et al., 2003
monogalactosyldiacylglycerols	+	$[M + NH_4]^+$	Neutral loss of 179	
digalactosyldiacylglycerols	+	$[M + NH_4]^+$	Neutral loss of 341	Moreau et al., 2008
trigalactosyldiacylglycerols	+	$[M + NH_4]^+$	Neutral loss of 503	Moreau et al., 2008
tetragalactosyldiacylglycerols	+	$[M + NH_4]^+$	Neutral loss of 665	Moreau et al., 2008

These scans are recommended for analysis of lipids in chloroform:methanol:300 mM ammonium acetate in water (300:665:35, v/v/v).

The precursor and neutral loss scans thus provide, for each lipid molecular species, the mass and signal of the intact ion, along with the mass of one molecular fragment that allows the lipid to be classified into a class or group. Given the classification, the mass can be interpreted as the total number of carbons and double bonds in the component acyl (or sphingosine/fatty amide) chains. To obtain more complete characterization of the complex lipid molecule, further analysis, such as mass spectral product ion analysis to identify individual fatty acyl components, can be performed. Devaiah et al. (2006) utilized product ion analysis to characterize many Arabidopsis glycerolipid molecular species in terms of fatty acyl composition.

For each detected lipid molecular species, the signal size allows quantification. To achieve accurate quantification by the direct-infusion ESI-MS/MS approach, a large number of internal standard compounds, optimally at least two non-naturally-occurring compounds for each class or group, are required (e.g., Welti et al., 2002; Devaiah et al., 2006). Alternatively, relative quantification among samples can be achieved by comparing mass spectral responses to an arbitrary standard compound detected with the same polarity (i.e., positive or negative mode) and scanning mode (i.e., precursor or neutral loss scanning) as the lipids of interest (e.g., detection of oxylipin-containing complex lipids in Maeda et al., 2008).

Challenges remaining in lipidomics include:

1. Standardizing lipidomics methodology and making the technology widely accessible.
2. Establishment of analyses for additional groups of lipids.
3. Development of a standardized and available data processing system for interpreting mass spectral data to produce lipid profiles.
4. Development of a web-accessible lipid profile database that facilitates integration with genomic, gene expression, proteomic, and other metabolomic data.

3.11. Strategies for Imaging in Plant Lipid Biology

(Allan DeBono and Lacey Samuels*[11])

3.11.1. Background

The goal of the microscopy of lipids is to visualize these hydrophobic compounds in their cellular context, with minimal rearrangements. When planning a microscopy approach, it is useful to think about what level of detailed cell structure is required. For high-resolution information on cellular organelles, transmission electron microscopy must be used, but TEM requires that cells be fixed and cut into thin sections, presenting a static view. Scanning electron microscopy (SEM) is useful for directly visualizing detailed surface structures such as the cuticle. For dynamic processes in live cells, light microscopy is the only choice as live cells fare poorly in the high-vacuum conditions of conventional electron microscopes. However, the resolution of the light microscope is limiting, so organelle identification is often done by correlating "puncta" with markers of known subcellular compartments.

In fluorescence microscopy, a sample containing fluorescent molecules, such as a dye or a fluorescent protein, is excited with energy of a given wavelength from a light source (e.g., a mercury lamp). Lower energy, longer wavelength light is emitted, collected through specific filters, and detected by eye or with a camera. Confocal laser scanning microscopy (CLSM) follows the same excitation and emission principles of fluorescence microscopy but provides improved imaging due to removal of out-of-focus fluorescence. The source of excitation is a laser that scans across the sample. The emission is collected through a pinhole of adjustable size that filters out-of-focus light. This allows for shallow depth of focus, which is exploited to generate a series of optical sections in the z-axis, which can be reassembled into a three-dimensional data set (Nikon Microscopy U website.)

In the study of plant lipids, CLSM is used both to detect fluorochrome dyes and to localize enzymes and other gene products related to lipid metabolism in the cell using fluorescent protein fusions. Storage lipids, such as triacylglycerols in oil bodies, can be imaged in live cells using Nile Red staining and fluorescence microscopy (e.g., Schmidt and Herman, 2008; Quettier and Eastmond, 2009). The plasma membrane can be stained using the amphipathic fluorescent styryl dye, FM4-64, as it partitions into the plasma membrane of live cells (Bolte et al., 2004). Endocytosis of the plasma membrane then can be followed over time as the bilayer is internalized and recycled (Zheng et al., 2005; Dettmer et al., 2006; DeBono et al., 2009). FM1-43, a closely related styryl dye, has also been used to label the plasma membrane, endocytic pathway, and even secretory vesicles (Okamoto et al., 2008; Bove et al., 2008).

The following list of protocols is not comprehensive; rather, it represents the experimental approaches that we have found most robust and reliable. For specialized applications, classical histochemistry stains or other fluorescent probes may be more appropriate, and a search of the literature to find how others have approached imaging lipids in the same system is always the best preliminary step.

3.11.2. Protocols

3.11.2.1. Nile Red, a General Lipophilic Stain

Nile Red is a polycyclic lipid stain that fluoresces intensely in a hydrophobic environment but not in aqueous media (Fowler and Greenspan, 1985). Nile Red has sensitivity to the hydrophobic environment, exhibiting red emission in the presence of polar lipids to more yellow emission in the presence of esterified cholesterol and triacylglycerols (Diaz et al., 2008). Nile Red has been used to stain sites of lipid accumulation in plants (Pighin et al., 2004; Schmidt and Herman, 2008; Dietrich et al., 2009) and alterations of surface lipids (Y.H. Li et al., 2007a; **Figure 18**).

Protocol:

1. A stock solution of Nile Red, also called Nile Blue A Oxazone (Sigma #72485), is dissolved in 100% DMSO to 1 mg/mL and should be stored protected from light at –20^{o}C.
2. Working solutions range from 1–5 µg/mL, diluted with water or buffer.
3. For tissues with cuticle, cut small segments to allow the stain to penetrate. A leaf disc or 5 mm longitudinal segment from a stem or seedling root will provide abundant imaging material. For elongated cell types, avoid transverse sections to prevent cell rupture and think about the cell geometry if the tissue is dissected. The tissue can be rinsed to remove excess dye.
4. Generally, Nile Red is excited with 488 nm or 543 nm laser lines and collected with a 560–615 nm filter. For special applications, Nile Red yellow emission, for nonpolar lipids, can be observed with 460 nm excitation and 535 nm emission; the red emission of Nile Red, for polar lipids, can be observed with 540 nm excitation and a 590 nm long pass emission filter (Diaz et al., 2008).

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3.11.2.2. FM4-64 Staining of the Plasma Membrane and the Endocytic Pathway

FM4-64, which fluoresces in the red range, is a useful counterstain to demonstrate plasma membrane localization in plants expressing green or yellow fluorescent proteins (GFP, YFP). FM4-64 has several properties that have contributed to its widespread use: It is not toxic to cells at the working concentrations; it fluoresces intensely only in a lipidic environment or when bound in membranes, reducing background; and it is soluble in water (http://www.invitrogen.com; Bolte et al., 2004). FM1-43 is the green fluorescent equivalent of the red FM4-64 (both spectra can be viewed at the Invitrogen Spectral Viewer).

Protocol:

1. Stock solutions of FM4-64 and FM1-43 (Invitrogen #T-3166 and #T-3163) are prepared at 10 mM in 100% DMSO and stored in aliquots, protected from light, at –20°C.
2. Typical applications of FM4-64 use a working concentration of 4–10 µM for roots (Dettmer et al., 2006), leaves (Zheng et al., 2005), and stems (DeBono et al., 2009). Typical working concentrations of FM1-43 are 160 nM to 2 µM for pollen (Bove et al., 2008; Sousa et al., 2008) and roots (Okamoto et al., 2008).
3. Dissect tissue to allow dye penetration but minimize cell damage (**Figure 19**) and immerse in dye at room temperature.
4. The plasma membranes of cells from Arabidopsis stems and leaves can be observed after 10 min, while roots require shorter incubation times (less than 60 sec). Endocytosis of FM4-64 typically occurs in 20–30 minutes (**Figure 20**). Timing must be determined empirically for each tissue.
5. FM4-64 can be excited with the 488 nm laser lines and detected with a 560 nm long pass filter. Alternatively, the 543 or 561 nm laser lines can be used for excitation and emission detected with a 584–664 nm band pass filter. Although this means collecting on the shoulder of the FM4-64 emission peak, with the intensity of FM4-64 label, it is often adequate and can be used to limit chloroplast autofluorescence. FM1-43 can also be excited with the 488 nm laser line and detected with a 500–600 nm filter.

3.11.2.3. Scanning Electron Microscopy

Since the surface structures of the cuticle are sensitive to fixatives and dehydrating agents (Reed, 1982), the undisturbed cuticle is best viewed without conventional SEM preparation (Neinhuis and Barthlott, 1997). Samples can be air dried directly on the SEM stub (Jackson, 2002) or frozen and viewed with cryo-SEM (Pighin et al., 2004; Radboud University Nijmegen).

3.11.2.4. Transmission Electron Microscopy

TEM allows high-resolution imaging of lipidic cell structure, including membranes and oil bodies. However, lipids are poorly cross-linked by the first aldehyde fixation step in conventional chemical electron microscopy sample preparation (Hopwood, 1972). This can cause membrane and organelle rearrangements, so cryofixation, such as high-pressure freezing and freeze substitution, is recommended for all lipid-rich systems (Bird et al., 2007; Schmidt and Herman, 2008). However, even in cryofixed cells, lipids are probably extracted during room temperature embedding. For example, we have observed that the ultrastructure of the cuticle of Arabidopsis stems was identical in samples that had been dipped in hexane to remove soluble waxes compared to controls without dipping (Samuels lab, unpublished data). This suggests that the cuticle viewed in the TEM is primarily cutin and cutan components. With these caveats in mind, TEM can still provide useful information about membrane structure, oil body size and distribution, and cutin organization.

Chemical Fixation Protocol:

1. Dissect plant part of interest into pieces no bigger than 2–3 mm. Immersion of the sample in fixative (2% glutaraldehyde in 50 mM PIPES buffer, pH 7.0) at room temperature for 2 h is usually adequate.
2. Wash 3 x in 50 mM PIPES buffer.
3. Postfix in 2% OsO_4 in 50 mM PIPES buffer for 1 h. Samples will turn black.
4. Dehydrate in an ethanol series, 20 min for each step (30% ethanol, 50% ethanol, 70% ethanol, 95% ethanol, 100% ethanol twice).
5. Mix the liquid plastic resin (Spurr's epoxy resin) with the ethanol to gradually infiltrate the resin into the cells. Infiltrate using resin mixed with solvent of increasing concentration series (10% resin, 25% resin, 50% resin, 75% resin, 100% resin). For each mix, let cells incubate for 2 h minimum, overnight maximum.
6. Put samples into BEEM® polyethylene embedding capsules capsules with fresh resin and bake overnight at 60°C.
7. Cut into 0.5 µm sections and stain with 1% toluidine blue in 1% sodium borate to check the morphology of fixed cells with light microscopy. Choose the best blocks and cut 70 nm sections, stain with 2%

aqueous uranyl acetate 15 min and Reynold's lead citrate for 5 min, then view with a transmission electron microscope.

Cryofixation Protocol: For detailed information on sample preparation, download the Practical Methods Manual to High-Pressure Freezing (HPF) by Mary Morphew from the Boulder Lab for 3-D Electron Microscopy of Cells.

1. Prepare HPF according to manufacturer's directions and do test runs.
2. With extra fine razor blades, cut small blocks of tissue under a pool of extracellular cryoprotectant, such as sucrose at a nonplasmolyzing concentration. Transfer cells to sample carriers and freeze without delay. The sample preparation and handling is critical; if samples are anoxic, crushed, or air-dried (mild drying leads to plasmolysis), then the best freezing will be for naught.
3. Following freezing, transfer samples to cryovials with freeze substitution medium (2% OsO_4 in anhydrous acetone with 8% dimethoxypropane).
4. Substitute at –80^{o}C for 3 to 5 days using an automated freeze substitution system or an acetone/dry ice slush in a styrofoam chest, which equilibrates at –80^{o}C.
5. Gradually warm samples to room temperature, ramping the temperature up over an 8-hour period, allowing the OsO_4 to react with the stabilized cell structures.
6. Rinse in clean acetone several times and remove the HPF carriers.
7. Slowly infiltrate with Spurr's resin. Add 1 drop of resin to 1 mL of acetone and mix well, then incubate the samples with the resin and acetone mixture for 5–10 min. Continue to increase the amount of resin by 1 drop/mL until you have added up to 10%–25% resin, then leave overnight.
8. Incubate in fresh 25% resin, 2–3 h; 50% resin, 2–3 h; 75% resin, 2–3 h, 100% resin 2–3 h twice or overnight with an extra 1 h change in the morning.
9. Incubate at 60^{o}C to polymerize the resin.
10. Sectioning, staining, and TEM then follow the conventional protocol above.

3.11.3. Comments

The light and electron microscopy techniques above remain popular approaches, but often simple, classical lipid stains, which are used with bright field microscopy, are the most appropriate technique to identify lipid-rich structures such as the cuticle, suberized endodermis, or periderm (Brundrett et al., 1991; Shen et al., 2003; Y.H. Li et al., 2007b). For practical methods in classical histochemical techniques, see Harris et al. (1994).

Despite the problem that structures in the submicrometer range are difficult to resolve, light microscopy continues to be an important tool for studying plant lipids, especially in conjunction with molecular biology and mutant analyses. The localization of proteins of interest using fluorescent protein fusions provides useful information but requires careful experimental design.

Before beginning a molecular biology protocol to fuse a gene of interest to GFP, the following considerations can make the difference between success and disaster. First, consider predicted targeting sites and topology for the protein, and plan the site of fusion to minimize the probablility that the fluorescent protein tag will be cleaved off the mature protein during targeting. Check the autofluorescence of the tissue where the protein will be expressed, and choose a protein that does not fluoresce in that range. Finally, your work will have greater credibility if you can demonstrate that the GFP-fusion protein is functional in vivo by complementing a mutant phenotype. GFP research has evolved from the older and now obsolete variants of green fluorescent protein to fluorescent proteins with increased brightness, pH stability, and monomerization (e.g., eYFP to CitrineYFP and mRFP to mCherry; Shaner et al., 2007). If your protein of interest is secreted to the acidic cell wall or vacuole, then older GFP variants will be quenched by the low pH. New variants of YFP, Venus, and Citrine have improved pH and chloride sensitivity (Griesbeck et al., 2001; Nagai et al., 2002). After selection of transgenic lines, it is important to screen 10 to 12 lines and select those with appropriate fluorescence levels.

Although live plant cell imaging preparation is relatively straightforward, there are simple provisions that can be made to improve image quality. In addition to differences in anatomical structure, airspaces contribute to the differences in uptake of dyes between leaves and stems versus roots. To overcome the air spaces and the cuticle in such tissues, we have found that a brief centrifugation (30 s) at 500*g* after incubation in a given dye will improve staining, reduce the time required before imaging, and most importantly permit the tissue to be excited using lower, less damaging laser intensity.

The appropriate amount of laser power is the absolute minimum that can excite the fluor without generating autofluorescence in an untreated, nonfluorescent protein control. Too much laser intensity is toxic and will alter membrane morphology and/or cause vesiculation.

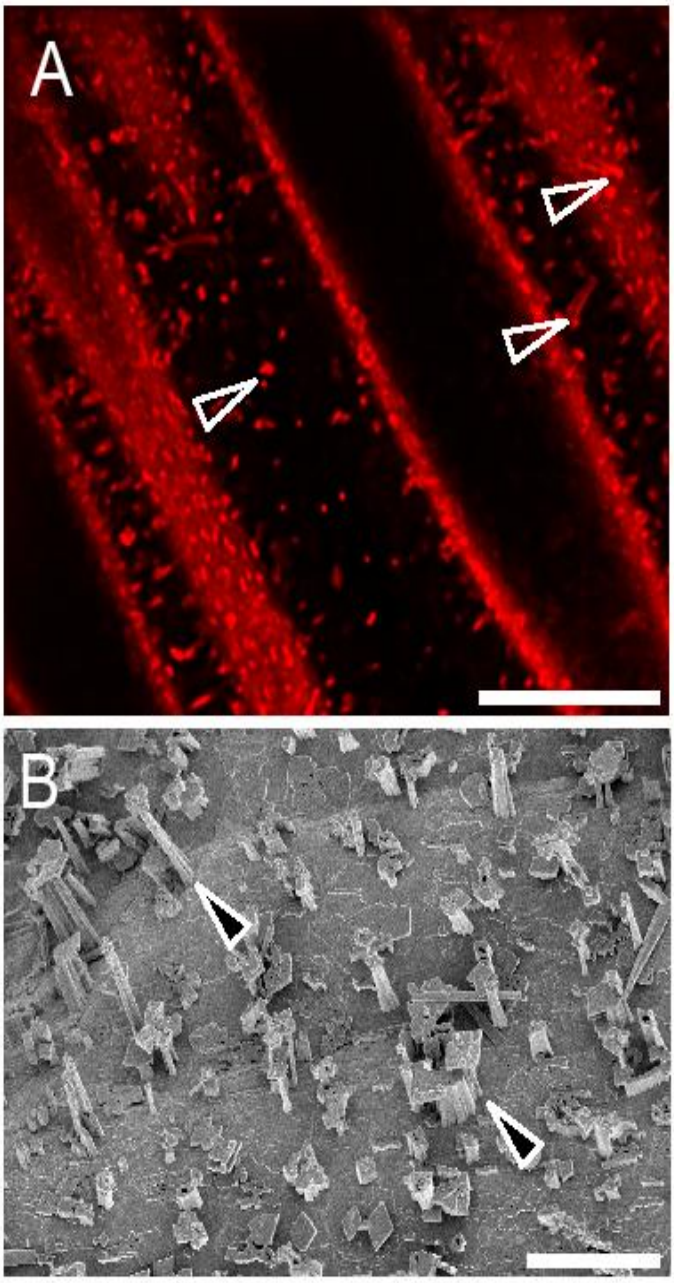

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Figure 18. Wax Crystals of Arabidopsis Stems Viewed by Confocal Microscopy and Cryo-Scanning Electron Microscopy.
(A) Nile Red and confocal microscopy of the Arabidopsis stem epidermis surface with wax crystal structures.
(B) CryoSEM of Arabidopsis stem epidermis surface with wax crystal structures (closed arrowhead).
Bars = 8 μm **(A)** and 5 μm **(B)**.

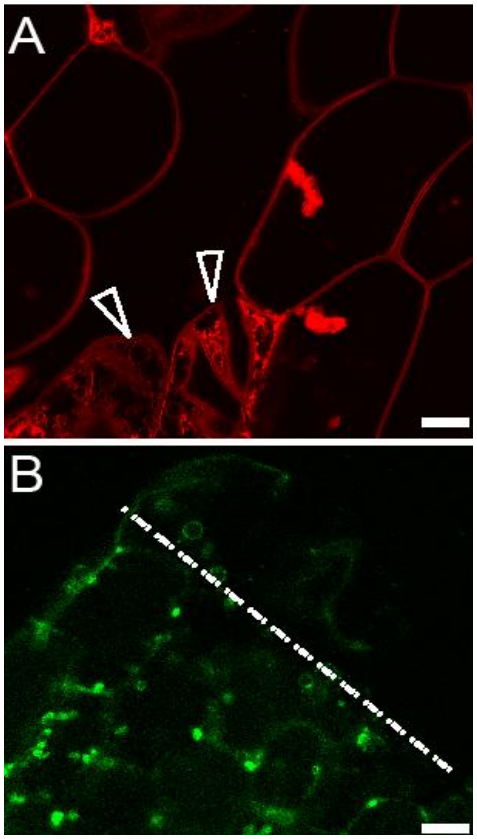

Figure 19. Arabidopsis Hypocotyl Cells Damaged During Handling Prior to Live Imaging.
(A) Hypocotyl cells stained with FM4-64 are vesiculated after crushing (arrowheads).
(B) Yellow fluorescent protein tagged glycosylphosphatidyl-inositol (GPI)-anchored lipid transfer protein (YFP-LTPG) is plasma membrane localized in undamaged cells. In contrast, due to damage during sample dissection, cells along the incision (dotted line) contain numerous large vesicles. Bars = 14 μm.

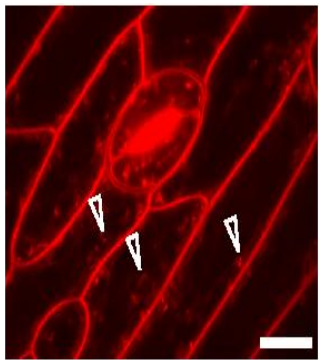

Figure 20. Arabidopsis Stem Epidermal Cells Stained With FM4-64.
Prolonged exposure to FM4-64 stains endocytic compartments (arrowheads) in addition to the plasma membrane. Bar = 9 μm.