Stereospecific Analysis and Mass Spectrometry of Triacylglycerols from *Arabidopsis thaliana* (L.) Heynh. Columbia Seed

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ABSTRACT: *Arabidopsis thaliana* continues to be an excellent model organism for studying plant molecular genetics and biochemistry. In particular, the generation and analysis of mutant lines has facilitated the study of fatty acid biosynthesis, lipid bioassembly and the regulation of these processes. In view of its importance in understanding the pathways specific to seed storage lipid biosynthesis, we report here, for the first time, stereospecific and mass spectral analyses of the triacylglycerols present in *A. thaliana* (L.) Heynh. Columbia wild-type seed. The use of NH_4^+–chemical-ionization mass spectrometry/mass spectrometry is described as a powerful technique in analyzing even trace amounts of individual triacylglycerol species.
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KEY WORDS: Acyltransferases, ammonia chemical-ionization–mass spectrometry/mass spectrometry, *Arabidopsis thaliana,* Brassicaceae, eicosenoic acid, mass spectrometry, stereospecific analysis, triacylglycerols.

The fatty acyl composition of seed triacylglycerols (TAGs) determines their physical and chemical properties and, thus, their use in edible oil or industrial applications. Most oilseed crops accumulate a limited range of fatty acids in their seed oil. Just six fatty acids contribute more than 95% to world oil production (1). Many other fatty acids are of considerable interest as renewable feedstocks for chemical industries. These include the very long-chain fatty acids (VLCFAs), C_{20}, C_{22}, and C_{24}, characteristic of the seed oils of a number of species within the Brassicaceae, Tropaeolaceae, and Limnathaceae (2). Seeds of the small diploid, annual, self-compatible crucifer *Arabidopsis thaliana,* like other members of the Brassicaceae, accumulate VLCFAs, predominantly eicosenoic (20:1) rather than erucic (22:1) acid (2–4). The 20:1 is confined exclusively to seed lipids and is not found in leaf or root lipids (4). It is primarily found in the TAG fraction and is largely excluded from polar or membrane lipids. Studies in several laboratories have exploited mutants of *A. thaliana* that are defective in various aspects of lipid metabolism to increase our understanding of lipid biosynthesis in leaves and seeds (2–7). Furthermore, with the development of *Arabidopsis* as a model organism for plant molecular genetics, it has become possible to clone genes in the lipid pathway affected by mutations by using the techniques of T-DNA tagging (8) and chromosome walking from restriction fragment length polymorphism (RFLP) sites (3,9). While most of the work to date has focused on the characterization of *A. thaliana* mutants with lesions associated with leaf membrane lipid biosynthesis, there is promise that this plant will be an equally useful model system in the study of seed storage lipid biosynthesis and its regulation (2–4,6,10). This is precluded, however, by a lack of detailed baseline information about the process of TAG bioassembly in *A. thaliana* seeds, and published schemes are admittedly speculative (6). In this regard, even the most fundamental studies, such as a full stereospecific analysis of the TAG fraction from wild-type *A. thaliana* seed, have not been reported. Without such information, mutations in key Kennedy pathway enzymes will be difficult to identify and characterize during *A. thaliana* mutant analysis. Thus, we report here for the first time detailed stereospecific and mass spectral analyses of the seed TAGs present in *A. thaliana* (L.) Heynh. Columbia wild-type.

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EXPERIMENTAL PROCEDURES

Seed material. Seeds of *A. thaliana* (L.) Heynh. Columbia wild-type were obtained from G. Haughn (Department of Biology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada). Seeds were multiplied for several generations by growing plants under continuous fluorescent illumination (150–200 $\mu E \cdot m^2 \cdot s^{-1}$) at 22°C on Terra-lite Redi-earth (W.R. Grace and Co., Canada Ltd., Ajax, Ontario, Canada). Under these growth conditions, mature seeds were obtained 6–8 wk after sowing and bulked for analysis.

Reagents. All solvents were of high-performance liquid chromatography (HPLC) grade (Omni-Solv) and were obtained from BDH Inc. (Toronto, Ontario, Canada). Silica 60G (Kiesel Gel 60G) was obtained from E. Merck (Darmstadt, Germany), and 0.25 mm silica plates containing 10% boric acid were prepared as described by Christie (11). Commercially prepared Silica 60G plates were purchased from Whatman Inc. (Clifton, NJ). Neutral lipid standards were obtained from Nu-Chek-Prep, Inc. (Elysian, MN), and ethyl magne-

sium bromide was purchased from Sigma (St. Louis, MO). 3,5-Dinitro-phenyl isocyanate was custom-synthesized by Rose Scientific Ltd. (Edmonton, Alberta, Canada).

Isolation and purification of seed TAGs and Grignard-based stereospecific analysis. All methods used to isolate, purify and analyze the TAG fraction from mature *A. thaliana* seed were essentially as described previously for similar analyses of other members of the Brassicaceae (12), and a Grignard-based stereospecific analysis was adapted from the method described by Takagi and Ando (13). The stereospecific analysis was performed on 50 mg of *A. thaliana* seed TAGs, with the Grignard reaction reagents scaled down proportionally. The *sn*-2 and [*sn*-1 + 3] monoacylglycerols (MAGs) were resolved on boric acid thin-layer chromatography plates. The *sn*-1 and *sn*-3 MAGs were separated by conversion to their corresponding di-3,5-dinitrophenylurethane (di-DNPU) derivatives which were resolved by HPLC on two Sumichiral OA-4100 5 μm (4 mm ϕ × 25 cm) columns (YMC Inc., Morris Plains, NJ), linked in series, as described previously (12).

Transmethylation. All fatty acids present in the TAG, *sn*-2 MAG, *sn*-1 or *sn*-3 di-DNPU–MAG and [*sn*-1 + 3] free fatty acid (FFA) fractions were converted to the corresponding fatty acid methyl esters (FAMEs) by 3 N methanolic HCl and analyzed by gas chromatography on a DB-23 column as described previously (14). The total fatty acyl composition of lipids present in intact seeds was obtained by direct transmethylation with methanolic HCl as reported by Kunst *et al.* (2). In all FAME analyses, 17:0 was added as an internal standard.

Direct-probe mass spectrometry (MS). Analyses of intact TAG species by direct-probe electron impact (EI–MS) and ammonia–chemical ionization (NH_4^+–CI–MS) mass spectrometry and MS/MS [collision-induced dissociation (CID) product ion analyses] were performed as described previously (14–16): All analyses were done with a VG 70-250 SEQ hybrid mass spectrometer. TAG samples (1–10 μg) for EI and NH_4^+–CI analyses were introduced *via* the solids probe heated ballistically to 320°C. The source temperature was maintained at 250°C. Magnetic scanned spectra (both EI and NH_4^+–CI) were obtained by scanning from *m/z* 1300 to 130 at a rate of 5 s/decade. CID product ion spectra were obtained by fragmenting the EI molecular ion or the CI $(M + NH_4)^+$ ion and scanning the quadrupole analyzer from *m/z* 100 to 1000 in 7 s. Xenon was used as the collision gas at a pressure of 9×10^{-5} torr. The collision energy was 16 eV (laboratory frame).

RESULTS AND DISCUSSION

An initial fatty acid analysis of the total lipids of *A. thaliana* (L.) Heynh. Columbia wild-type seed showed a composition similar to that reported previously (2) except that small proportions of the VLCFAs 20:2 (11,14-eicosadienoic acid), 22:0 (docosanoic acid), and traces of 24:0 and 24:1 are reported here (Table 1). Overall, this composition was also closely comparable to that recorded in an early analysis of *A. thaliana* Schur (17), except that the lipid content in the present study was about 29% of the dry seed weight. The proportion of 20:1 was about 20%, with linoleic acid (18:2) being the predominant fatty acid present. TAGs comprised 98.6% of the neutral lipid fraction, while diacylglycerols (DAGs), MAGs, and FFA comprised 1.1, 0.12, and 0.14%, respectively.

The results of the detailed Grignard-based stereospecific analysis of TAGs isolated from mature *A. thaliana* seed are presented in Table 2. In Set A, the distribution of all fatty acids at each *sn*-position is presented. This is the traditional method of reporting such data. Only small proportions of 20:1 and other VLCFAs are found at the *sn*-2 position, while 18:2 and 18:3 predominate. This trend is in general agreement with studies of most other members of the Brassicaceae, where VLCFAs are typically found in small proportions at the *sn*-2 position (12). In Table 2, Set B, the stereospecific data are reported as the distribution of each fatty acid across all three *sn* positions. The same general conclusions regarding the presence of only small proportions of most VLCFAs at the *sn*-2 position may be reached from presenting the information in this form. However, the data in Set B more clearly reflect the specificity of the Kennedy pathway acyltransferases and therefore impinge on strategies for modifying seed oil composition by molecular genetic or traditional breeding methods. We believe this form of presenting TAG stereospecific data (Table 2, Set B) to be preferable. From these data, the

TABLE 1
Fatty Acid Composition of Total Lipid Fraction from Seeds of *Arabidopsis thaliana* (L.) Heynh. Columbia Wild-Type[a] and *A. thaliana* Schur[b]

	Fatty acid (wt% of total)											
	16:0	18:0	18:1 *cis* 9	18:1 *cis* 11	18:2	18:3	20:0	20:1	20:2	22:0	22:1	24:0 + 24:1
A. thaliana Heynh. Columbia wild-type	7.6 ± 0.6	3.2 ± 0.04	14.1 ± 0.3	1.4 ± 0.02	28.2 ± 0.1	18.5 ± 0.04	2.2 ± 0.04	20.4 ± 0.1	2.1 ± 0.01	0.4 ± 0.02	2.0 ± 0.02	trace[c]
A. thaliana Schur	6	4	14	nr[d]	27	18	3	22	2	0.3	2	nr[d]

[a]Ten-seed samples were transmethylated; n = 10; ±SE; oil content 28.9% of dry weight. [b]As reported in Reference 17; oil content 43% of dry weight. [c]<0.2% each. [d]nr = Not reported.

TABLE 2
Stereospecific Analysis of Triacylglycerol (TAG) Fraction from Seed of *Arabidopsis thaliana* (L.) Heynh. Columbia Wild-Type

	Fatty acid (mol% of total)										
Fraction	16:0	18:0	18:1 *cis* 9	18:1 *cis* 11	18:2	18:3	20:0	20:1	20:2	22:0	22:1
Set A[a]											
TAG	8.3	3.4	15.0	1.2	28.6	18.5	2.1	19.8	1.2	0.3	1.6
sn-1	11.2	3.6	12.8	1.7	23.3	16.4	2.4	21.7	3.5	0.4	2.7
sn-2	6.4	3.6	17.4	0.9	44.3	22.0	0.9	3.3	0.5	0.4	0.4
sn-3	18.2	9.8	13.9	1.0	6.0	4.8	6.3	34.3	1.5	1.3	2.8
Set B[b]											
sn-1	31.4	21.4	29.1	47.3	31.7	38.0	25.1	36.6	64.3	19.9	46.2
sn-2	17.8	20.9	39.4	24.4	60.1	50.8	9.4	5.6	8.3	19.4	7.0
sn-3	50.9	57.8	31.6	28.2	8.2	11.1	65.5	57.8	27.4	60.6	46.8

[a]Distribution of fatty acids at each *sn*-position. [b]Distribution of each fatty acid across all three *sn*-positions.

acyltransferases appear to be relatively specific in wild-type *A. thaliana*. There is a general preference for placing VLCFAs in the *sn*-3 and *sn*-1 positions with little at the *sn*-2 position. The polyunsaturated fatty acids, 18:2 and 18:3, are preferentially inserted at the *sn*-2 position. The interesting exceptions to these trends are the presence of a substantial proportion (20%) of 22:0 at the *sn*-2 position and the insertion of the majority of 20:2 (63%) at the *sn*-1 position. The latter finding is quite different from the normal distribution of 18:2 in other members of the Brassicaceae (12) or the relatively nonspecific distribution of 5,13-docosadienoic acid (22:2) in meadowfoam (*Limnanthes* spp.) (18). The small amount of erucic acid present in *A. thaliana* is found in almost equal proportions at the *sn*-1 and *sn*-3 positions, with only 6–7% in the *sn*-2 position, as is the case for most, but not all, members of the Brassicaceae studied thus far (12). The relatively nonspecific distribution of oleic acid (18:1) in *A. thaliana* has also been observed in TAGs from certain lines of *Brassica oleracea* and in nasturtium (*Tropaeolum majus*) (12).

The precision and reliability of the method have been well documented (13). However, we are aware that the total composition of 16:0 and 18:0 computed from the proportions at each position are different than the values obtained from a direct analysis of the TAGs. We have no clear explanation for this discrepancy. Some bias is inevitably introduced by omitting from the calculations those fatty acids present in minor proportions (14:0; 24:0; 24:1). Nevertheless, our conclusions with regard to the distribution of the VLCFAs remain valid.

The intact TAGs from *A. thaliana* (L.) Heynh. Columbia were analyzed by direct-probe EI–MS and NH_4^+–CI–MS and displayed major clusters in the molecular ion region characteristic of those reported previously for TAGs of other members of the Brassicaceae rich in VLCFAs (14). However, due to the high level of polyunsaturated fatty acids in TAGs of *A. thaliana* (dienes + trienes = 48.3%; *cf.* Table 2), problems were encountered during EI–MS/MS in the interpretation of the CID product spectra and assignment of product ions, a phenomenon reported previously in analyses of other seed oils rich in polyunsaturated fatty acids (16). In particular, it was noted that under EI conditions, the CID product ions $[M - RCOO]^+$ and $[RCO + 74]^+$ appeared to rearrange so as to reduce one double bond in an acyl group, leading to misinterpretation of the MS data. However, when the analyses were performed under NH_4^+–CI conditions, there was no reduction of double bonds during the formation of the CID product ions, making the technique suitable for the characterization of the specific acyl groups present in each TAG (16). Thus, NH_4^+–CI–MS and the corresponding CID product ion spectra were used in the present study to facilitate structural assignments.

While MS did not give useful stereochemical data, the specific acyl groups present in each molecular species could be determined. The putative *sn*-1/*sn*-2/*sn*-3 acyl assignments are the most probable distribution based on the stereospecific analyses reported in Table 2. The most prominent $[M + 18]^+ = (M + NH_4)^+$ cluster observed was for monoeicosenoyl TAGs ($[M + 18]^+ = 925–933$) containing two C_{18} groups with various degrees of unsaturation. The most intense molecular adduct ion (100% relative intensity) was for 18:2/18:2/20:1 TAG at $[M + 18]^+ = 927$. Clusters representing $C_{20}C_{18}C_{20} + C_{18}C_{18}C_{22}$ TAGs ($[M + 18]^+ = 951–959$) and $C_{20}C_{18}C_{22} + C_{18}C_{18}C_{24}$ TAGs ($[M + 18]^+ = 979–985$) were also present. There was no evidence of trieicosenoin ($[M + 18]^+ = 987$). TAGs containing three C_{18} moieties with differing degrees of unsaturation and traces of a $C_{16}C_{18}C_{20}$ TAG were observed in a cluster at $[M + 18]^+ = 893–907$, while $C_{18}C_{18}C_{16}$ TAGs were found at $[M + 18]^+ = 871–877$. These major TAG cluster assignments were confirmed by MS/MS CID product ion analyses, in which characteristic $[M - RCOOH]^+$ (m/z = 570–700) and $[RCO + 74]^+$ (m/z = 300–500) fragments were identified. Although several acyl compositions were theoretically possible when examining only the molecular ion, MS/MS gave a distinct advantage in that it allowed confirmation of the more probable TAG species. For example, the $[M + 18]^+ = 927$ peak could have been any one of the following TAG species: 18:1/18:3/20:1; 18:2/18:2/20:1; 18:2/18:3/20:0; or 18:0/18:3/20:2. However, MS/MS product ions in the $[M - RCOOH]^+$ region showed only M-18:2 (629) and M-20:1 (599) peaks, indicating that the only TAG species present was 18:2/18:2/20:1. Similarly,

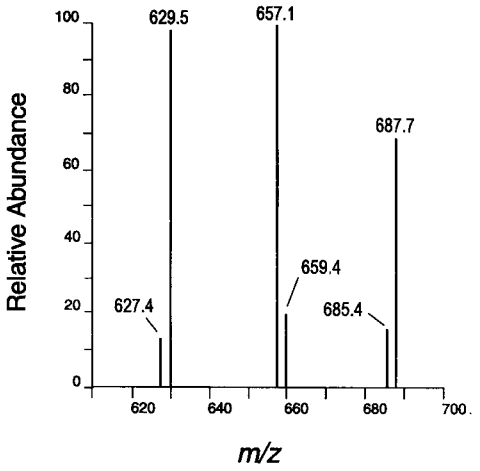

FIG. 1. Partial collision-induced dissociation product ion spectrum of *m/z* 985 $[M + 18]^+$ triacylglycerols adduct ion showing the $[M - RCOOH]^+$ ions for the specific acyl groups present: M-22:0 (627.4), M-22:1 (629.5), M-20:1 (657.1), M-20:2 (659.4), M-18:1 (685.4), M-18:2 (687.7).

NH_4^+–CI–MS/MS analyses of product ions from precursor ions $[M + 18]^+ = 957$ and 955 allowed the structural assignments of 20:1/18:2/20:1 and 20:1/18:3/20:1, respectively, while EI–MS/MS could not distinguish the $[M - RCOO]^+$ product ions. Perhaps the best example of the power of NH_4^+–CI–MS/MS to resolve TAG structural data can be demonstrated in the analysis of CID product ions for $[M + 18]^+ = 985$. Although the parent ion had a relative intensity of only 7% in the magnetic scanned spectrum, the CID product spectrum showed $[M - RCOOH]^+$ fragments for M-22:0, M-22:1, M-20:1, M-20:2, M-18:1, M-18:2 (Fig. 1) and M-24:0 (product ion at 599.4; data not shown). This indicated that there were detectable levels of the following four TAG species: 22:1/18:2/20:1, 20:2/18:2/22:0, 20:2/18:1/22:1, and 18:2/18:2/24:0. Thus, fatty acids present in low (20:2, 22:0, 22:1) or only trace (24:0) amounts (*cf.* Table 1) were detectable in intact TAG species and confirmed by MS/MS.

Arabidopsis thaliana will no doubt be exploited as a model organism for studying the molecular genetics and biochemistry of lipid biosynthesis and for cloning genes encoding enzymes for storage lipid bioassembly by mutant analysis. NH_4^+–CI–MS/MS has been shown to be an improvement over EI–MS in the analysis of TAG species present in low abundance or containing polyunsaturated fatty acids. The detailed stereospecific and mass spectral analyses of *A. thaliana* seed TAGs presented here will provide useful baseline information. By using the two techniques in combination, it should be easier to identify seed lipid mutants with lesions in, for example, the Kennedy pathway acyltransferases.

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