Overexpression of a FAD3 desaturase increases synthesis of a polymethylene-interrupted dienoic fatty acid in seeds of Arabidopsis thaliana L.

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Publisher's version / Version de l'éditeur:
https://doi.org/10.1007/s11745-009-3315-5
Lipids, 44, 8, pp. 753-757, 2009-06-23

COMMUNICATION

Synthesis of a polymethylene interrupted dienoic fatty acid in seeds of *Arabidopsis thaliana* L.

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Abstract

A cDNA encoding the *Arabidopsis* extraplastidic linoleate desaturase (FAD3) was overexpressed in the seeds of wild type *Arabidopsis* and in a mutant line that accumulates high levels of oleic acid. In the transformed wild type plants, linolenic acid (18:3Δ9, Δ12, Δ15) increased from 19% to nearly 40% of total seed fatty acids, with a corresponding decrease in linoleate content (18:2 Δ9, Δ12). In the high oleate mutant, a large increase in the level of a fatty acid identified by gas-chromatography/mass-spectrometry as mangiferic acid (18:2 Δ9, Δ15) was observed. The results demonstrate that the polymethylene interrupted dienoic fatty acid, mangiferic acid, can be produced in seed oil through the over-expression of a fatty acid *n-3* desaturase.

Keywords

Linoleate desaturase, mangiferic acid, polymethylene interrupted dienoic fatty acid, *Arabidopsis*, seed oil.

Abbreviations.

FAMES	Fatty acid methyl esters
PMIFA	Polymethylene interrupted fatty acid.
PUFA	Polyunsaturated fatty acid

VLCFA Very long chain fatty acid

Fatty acid nomenclature:- X:YΔz where X is chain length, Y is number of double bonds and Δz is double bond position relative to the carboxyl end of the molecule. In fatty acids designated using *n-x* or *ωx* nomenclature, the position of the double bond is described relative to the methyl end of the molecule.

Introduction

The predominant dienoic fatty acid of higher plants is linoleic acid (octadeca-9-*cis*,12-*cis*-dienoic acid, 18:2 Δ9,Δ12). The methylene interrupted (MI) *cis*-double bond conformation of this fatty acid is characteristic of most plant polyunsaturated fatty acids. Polymethylene interrupted fatty acids (PMIFA), where double bonds are separated by more than one methyl group, are uncommon, but can be abundant within individual plant families. Seed oils of many conifer species, for example, are rich in Δ5-polyunsaturated fatty acids and contain dienoic PMIFAs such as taxoleic acid (octadeca-5-*cis*,9-*cis*-dienoic acid, 18:2 Δ5, Δ9) and the very long chain fatty acid (VLCFA) eicosa-5-*cis*,11-*cis*-dienoic acid, 20:2Δ5,Δ11. These species also produce a variety of polyunsaturated fatty acids containing double bonds in both MI and PMI configurations (Wolff et al 1996). Fatty acids with Δ5 unsaturation are also the major components of meadowfoam oil, obtained from the seeds of *Limnanthes alba* (Miller et al 1964). This oil contains an unusual VLC-PMIFA, docosa-5-*cis*,13-*cis*-dienoic acid (22:2 Δ5, Δ13) that accounts for up to 16% of total seed fatty acids (Nikolova-Damyanova et al 1990, Knapp and Crane 1995).

Biochemical studies have indicated that in *Limnanthes* species, 22:2 Δ5, Δ13 is synthesized by the action of a Δ5 desaturase on the VLC-monunsaturated fatty acid erucic acid (22:2 Δ13) (Pollard and Stumpf 1980). The PMIFA therefore results from the activity of a desaturase that inserts a double bond at a specific position in the fatty acid on the carboxyl side of an existing double bond. This activity is referred to as "front end desaturation" and is distinct from the "methyl end" desaturation catalysed by the majority of plant desaturases (Sperling et al. 2003). It is likely that Δ5 desaturation of unsaturated fatty acids in conifers also results in the formation of PFIMAs.

In addition to PFIMAs containing Δ5 unsaturation, a novel 18 carbon PMIFA, sometimes referred to as mangiferic acid, has been reported from the pulp of ripe mango fruit (*Mangifera indica* L.) grown in the Philippines (Shibahara et al 1993) and in the seed oil of *Arabidopsis* lines deficient in linoleate desaturase activities (Browse et al 1988, Reed et al 2000). In these reports the fatty acid was suggested to be a result of low level activity of an ω3-desaturase, such as the plastidial or extraplastidial linoleate desaturases, acting on oleic acid (18:1 Δ9). The ωx designation refers to a fatty acid desaturase that catalyses double bond formation at a specific position relative to the methyl end of a fatty acid (Sperling et al 2003).

Polyunsaturated fatty acids (PUFAs) with double bonds that are not in methylene-interrupted positions are likely to have better oxidative stability and different physical properties compared to their MI-isomers. Very little attention has been paid to these fatty acids as possible raw materials for industrial uses. To attempt to determine whether production of 18:2 Δ9, Δ15 in a seed oil was possible by metabolic engineering, we over-expressed a gene encoding a linoleate desaturase in the seeds of *Arabidopsis*. Transformed plants accumulated this novel dienoic fatty acid in their seed oil to levels of up to 8% of total seed fatty acids.

Materials and Methods

Construction of the plant transformation vector

A full length cDNA encoding the *Arabidopsis* extraplastidic linoleate desaturase (Clone U11701, *FAD3*, At2g29980) was obtained from the Arabidopsis Biological Resource Centre, DNA Stock Centre (Ohio State University; www.arabidopsis.org). An expression cassette comprising the seed specific *Lesquerella* hydroxylase promoter (Broun et al 1998), the *FAD3* cDNA, and an *Arabidopsis* oleosin terminator (kindly provided by Prof. M. Moloney, SemBioSys Inc, Calgary, Canada) were assembled in the binary vector pBar1 (Holt et al 2002) to create the vector pDP5.

Arabidopsis lines and plant transformation.

Wild type (Columbia ecotype) and *fad2/fae1* mutant *Arabidopsis* plants (Smith et al 2003) were transformed with the binary vector pDP5 using the floral dip method (Clough and Bent 1998). For screening, T_1 seeds were sown onto moist potting soil, vernalized at 4°C for 72 hours then transferred to a growth chamber, at 23°C with constant light, and allowed to grow until the first true leaves were clearly visible. Plants were sprayed 3 times at 2 day intervals with a solution containing 0.1ml/L Silwet (Lehle Seeds, Round Rock, Texas, USA) and 80mg/L glufosinate ammonium (WipeOut herbicide, CIL Nu-Gro IP Inc, Brantford, ON). Surviving plants were grown to maturity at 22°C in continuous light.

Gas chromatography of fatty acid methyl esters.

For determination of total seed fatty acid composition, approximately 100 seeds from the individual *Arabidopsis* plants were placed in Pyrex screw cap tubes with 2ml 1M HCl in methanol (Supelco) and 300µl of hexane. The tubes were tightly capped and heated at 80°C for a minimum of 2 hours. After cooling, 2 ml of 0.9% NaCl was added fatty acid methyl esters (FAMES) were recovered by collecting the hexane phase. Gas chromatography of FAMES was conducted using an Agilent 6890N GC fitted with a DB-23 capillary column (0.25 mm x 30 m, 0.25 µM thickness; J & W; Folsom, California, USA) as described previously (Kunst et al 1992).

Identification of fatty acids by mass spectrometry.

For identification of fatty acids by mass spectrometry, diethylamide derivatives were prepared, based on the method of Nilsson and Liljenberg (1991). Free fatty acids were generated from total seed lipids by grinding approximately 200 seeds in methanol and directly saponifying with 8.5% methanolic KOH. Fatty acids were dissolved in 400 µL of chloroform, 50 µL of pyridine was added, followed by the dropwise addition of 150 µL of acetyl chloride with continuous shaking. The mixture was diluted with 600 µL of chloroform, and 500 µL of diethylamine was added dropwise with continuous shaking. Excess reagent was removed by extracting twice with 1 mL of water, and the solvent phase was dried under a stream of nitrogen gas. The sample was dissolved in 200 µL of chloroform and 2 µL was directly injected (40:1 split ratio) onto a 30M DB23 capillary column in an Agilent 7890A GC equipped with a 5975C mass selective detector (ionizing energy of 70 eV). After an initial hold for 1 minute at 160°C, the temperature was ramped at a rate of 4°C until reaching 240°C, and then held for 10 minutes.

Analysis of mango pulp.

Ripe mangos (*Mangifera indica* L., cultivar unknown) were purchased at a local supermarket. Small pieces of mango pulp (approximately 50 mg) were dropped into Pyrex screw cap tubes and lipids were transmethylated as above, except that 6 ml of 1M HCl in methanol was used. GC of FAMES was conducted as described above.

Results

To demonstrate the activity of the FAD3 desaturase encoded by vector pDP5, we transformed wild type *Arabidopsis* plants with this vector. Transformed plants exhibited increased levels of linolenic acid (18:3 Δ9, Δ12, Δ15) and reduced amounts of 18:2 Δ9, Δ12 in their seed oil (Figure 1B), corresponding to the expected linoleate desaturase activity of the enzyme. Total C18-PUFA (18:2 +18:3) levels, and the levels of other seed fatty acids were largely unchanged (Table 1). The vector was also used to transform a mutant line of *Arabidopsis* deficient in the activities of the extraplastidic oleate desaturase (FAD2) and seed specific β-keto acyl-CoA synthetase; FAE1 (Smith et al 2003). The seeds of these plants contain over 82% oleic acid and less than 3% linolenic acid (Table 1, Figure 1C). They also contain two 18-carbon dienoic fatty acids, linoleic acid (18:2 Δ9, Δ12) and "mangiferic acid" (18:2 Δ9, Δ15) at an average of 0.39% and 1.35% respectively. Preliminary identification of 18:2 Δ9, Δ15 was based on the work of Reed and co-workers (2000) who observed its presence in the seed lipids of a *fad2* mutant line of *Arabidopsis*. *Fad2/fae1* mutant lines expressing the *FAD3* transgene showed increased levels of 18:2 Δ9, Δ15 and a reduction in 18:1Δ9. They also showed lower levels of 18:2 Δ9, Δ12 and an increase in 18:3 Δ9, Δ12, Δ15 (Table 1, Figure 1D). The highest levels of PMIFA (18:2 Δ9, Δ15) observed in the transformed plants was 8.23% of total seed fatty acids. In this line 18:2 Δ9, Δ12 was reduced to 0.27% and 18:3 Δ9, Δ12, Δ15 increased to 2.47%. Total C18-PUFA (total 18:2 +18:3) increased to 10.97% from an average of 2.75%, and a slight increase in total saturated fatty acid content was observed.

To confirm the identity of the putative PMIFA, GC/MS analysis was conducted using diethylamide derivatives of the fatty acid. The mass spectrum (Figure 2) displayed a mass ion at *m/z*= 335 and diagnostic ions differing by 12 amu at m/z = 198 and 210 and m/z = 280 and 292 indicating the presence of double bonds at the Δ9 and Δ15 (ω9 and ω3) positions respectively. An identical mass spectrum was obtained from the diethylamide-derivative of the fatty acid identified as 18:2 Δ9, Δ15 in the seed oil of the untransformed *fad2/fae1* lines. Double bond conformation (*cis/trans*) was not determined.

The fatty acid profile of mango pulp (Table 1) showed palmitic acid as the major fatty acid (30%). The other abundant fatty acids, identified by comparison of retention time to standards, were palmitoleic (16:1 Δ9), oleic (18:1 Δ9), vaccenic (18:1 Δ11), linoleic (18:2 Δ9, Δ15) and linolenic (18:3 Δ9, Δ12, Δ15) acids. The previously reported PMIFA, 18:2 Δ9, Δ15, was not detected in these fruit.

Discussion.

Previous studies have observed the presence of an unusual dioenic acid in the seed oil of *Arabidopsis* linoleate desaturase deficient lines. The fatty acid was identified by GC-MS as 18:2 Δ9, Δ15 and was suggested to result from the ω3 desaturation of oleic acid (Browse et al 1988, Reed et al 2000). We have shown that overexpression of the Arabidopsis FAD3 extraplastidic linoleate desaturase in lines containing high 18:1 content results in a substantial increase in the amount of 18:2 Δ9, Δ15 accumulating in the seed oil. Plant FAD3 desaturases are considered to have ω3 (methyl end) rather than Δ15 (carboxy end) regioselectivity. Preferred substrates have ω6 double bonds, but low level activity is observed on ω9 unsaturated fatty acids (Reed et al 2000). Over-expression of *FAD3* in seeds that have high levels of oleic acid (ω9 unsaturation) and low levels of linoleic acid (ω9 + ω6 unsaturation) leads to the accumulation of the PIMA 18:2, Δ9, Δ15. A corresponding decrease in 18:1Δ9 content was observed suggesting that the PIMA likely results from ω3 desaturation of oleic acid. To demonstrate the activity of the *Arabidopsis* FAD3 desaturase used in this work we used the same construct to overexpress *FAD3* in wild type *Arabidopsis*. Transformed plants synthesized increased amounts of linolenic acid with a corresponding decrease in the substrate fatty acid linoleic acid. Overall levels of PUFAs in these plants were largely unchanged indicating that the limiting activity in C18 PUFA biosynthesis is likely to be the formation of linoleic acid.

A seed oil rich in dienoic PMIFAs would have novel chemical properties compared to existing oils where dienoic acids have a MI conformation. Such fatty acids may serve as valuable chemical feedstocks. Recent advances in the development of catalysts for olefin metathesis chemistry, for example, could allow the production of novel hydrocarbon monomers such as a-olefins, α-ω-dienes and short to medium chain ω-unsaturated methy esters (Mol 2002). Metathetical cleavage using ethene (ethenolysis) of fatty acids from meadowfoam oil has been considered as a process for the production of 5-hexenoic acid for polymer biosynthesis (Warwel et al 2004). A second product from this oil was 1,9-decadiene resulting from cleavage of 22:2 Δ5, Δ13. Ethanolysis of mangiferic methyl ester would yield methyl-9-decenoate, 1,7-octadiene, and 1-butene. All of these compounds have potential industrial uses. The C18-PMIFA reported in this work results from the activity of an *ω3* desaturase on a sub-optimal substrate. Levels of PMIFA over 8% of seed fatty acids were however achieved in *Arabidopsis*. Molecular engineering of an *ω3* desaturase to improve its ability to utilize oleic acid offers intriguing possibilities for the generation of new plant oils suitable for use as industrial feedstocks.

The fatty acid profile of the pulp from ripe mangoes purchased locally was very different to that reported previously for ripe fruit obtained from the Philippines (Shibahara et al 1993). It is likely that the samples are not from the same variety of mango, however, the very low levels of ω6 unsaturation observed in the previous study may indicate that those fruit may differ in oleate desaturase activity. Endogenous ω3 desaturase activity with oleate as substrate may account for the mangiferic acid observed in the fruit pulp. The fruit analysed in this study contain abundant unsaturated fatty acids with a double bond in the ω6 position (18:2 Δ9, Δ12 and 18:3 Δ9, Δ12, Δ15) accounting for over 43% of all fatty acids.

References

Broun P, Boddupalli S, Somerville C (1998) A bifunctional oleate 12-hydroxylase: desaturase from *Lesquerella fendleri*. Plant J. 13:201-210

Browse J, Kunst L, Anderson S, Hugly S, Somerville, C (1988) A mutant of *Arabidopsis* deficient in the chloroplast 16:1/18:1 desaturase. Plant Physiol. 90:522-529

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. 16:735-743

Holt BF, Boyes DC, Ellerstrom M, Seifers N, Wiig A, Kauffman S, Grant MR, Dangl JL (2002) An evolutionarily conserved mediator of plant disease resistance gene function is required for normal *Arabidopsis* development. Dev. Cell 2:807-817

Knapp SJ, Crane JM (1995) Fatty acid diversity of section Inflexae *Limnanthes* (meadowfoam). Ind. Crop Prod. 4:219-227

Kunst L,Taylor DC, Underhill EW (1992) Fatty acid elongation in developing seeds of *Arabidopsis thaliana*. Plant Physiol. Biochem. 30:425-434

Mol JC (2002) Application of olefin metathesis in oleochemistry: an example of green chemistry. Green Chem. 4:5-13

Miller RW, Daxenbichler ME, Earle FR, Gentry HS (1964) Search for new industrial oils. VIII. The genus *Limnanthes*. J. Am. Oil Chem. Soc. 41:167-169

Nikolova-Damyanova B, Christie WW, Herslof B (1990) The structure of the triacylglycerols of meadowfoam oil. J. Am. Oil Chem. Soc. 67:503-507

Nilsson R, Liljenberg C (1991) The determination of double bond positions in polyunsaturated fatty acids – Gas chromatography / Mass spectrometry of the diethylamide derivative. Phytochem. Analysis 2:253-259

Pollard MR, Stumpf PK (1980). Biosynthesis of C20 and C22 fatty acids by developing seeds of *Limnanthes alba*; chain elongation and Δ5 desaturation. Plant Physiol. 66:649-655

Reed DW, Schafer UA, Covello PS (2000) Characterization of the *Brassica napus* extraplastidial linoleate desaturase by expression in *Saccharomyces cerevisiae*. Plant Physiol. 122:715-720

Shibahara A, Yamamoto K, Shinkai K, Nakayama T, Kajimoto G (1993) *Cis*-9, *cis*-15-Octacecadienoic acid: a novel fatty acid found in higher plants. Biochim. Biophys. Acta. 1170:245-252

Smith MA, Moon H, Chowrira G, Kunst L (2003) Heterologous expression of a fatty acid hydroxylase gene in developing seeds of *Arabidopsis thaliana*. Planta 217:507-516

Sperling P, Ternes P, Zank TK, Heinz, E. (2003) The Evolution of desaturases. Prostaglandins Leukotr. Essent. Fatty Acids. 68:73-79

Warwel S, Bruse F, Demes C, Kunz M (2004) Polymers and polymer building blocks from meadowfoam oil. Ind. Crops Products 20:301-309

Wolff RL, Deluc LG,Marpeau, AM (1996) Conifer seeds: oil content and fatty acid composition. J. Am. Oil Chem. Soc. 73:765-771

Acknowledgments

The authors would like to thank Dr Alison Ferrie and Dr Patrick Covello for critical review of the manuscript.

NRC-PBI publication number QQQ

Figure legends.

Figure 1.

Gas chromatographs of FAMES prepared from total seed lipids of *Arabidopsis*. Trace A, wild type; Trace B, wild type expressing the *Arabidopsis FAD3* desaturase; Trace C, *Arabidopsis fad2/fae1* mutant; Trace D *Arabidopsis fad2/fae1* mutant expressing the *Arabidopsis* FAD3 desaturase.

Fatty acids; 1 Palmitic acid (16:0); 2 Palmitoleic acid (16:1 Δ9); 3 Stearic acid (18:0); 4 Oleic acid (18:1 Δ9); 5 Vaccenic acid (18:1 Δ11); 6 Linoleic acid (18:2 Δ9, Δ12); 7 Mangiferic acid (18:2 Δ9, Δ15); 8 Linolenic acid (18:3 Δ9, Δ12, Δ15); 9 Eicosanoic acid (20:0); 10 11-eicosenoic acid (20:1 Δ11); 11 13-eicosenoic acid (20:1 Δ13); 12 11,14-eicosadienoic acid (20:1 Δ11, Δ14), 13 Erucic acid (22:1 Δ13).

Figure 2.

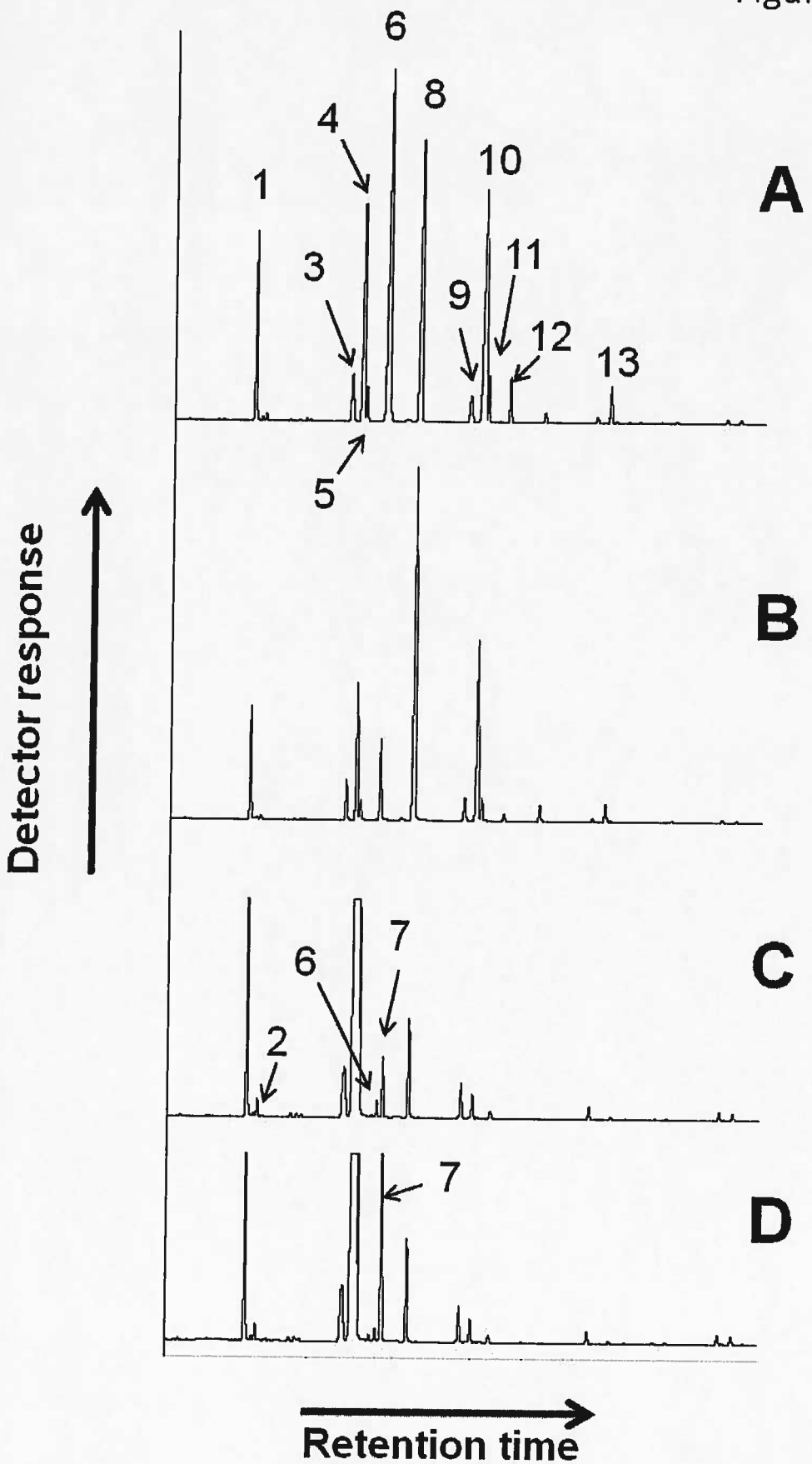
Mass spectrum of diethylamide derivative of 18:2 Δ9, Δ15.

Table 1

Major Fatty Acid	*Arabidopsis* seed fatty acid composition %				Mango Pulp
	WT	WT + *FAD3*	*fad2/fae1*	*fad2/fae1* + *FAD3*	
16:0	7.60 ± 0.03	8.48 ± 0.22	5.61 ± 0.02	6.33 ± 0.06	30.09 ± 0.92
16:1Δ9	0.27 ± 0.01	0.28 ± 0.01	0.34 ± 0.01	0.39 ± 0.01	17.43 ± 0.46
18:0	3.16 ± 0.05	3.40 ± 0.14	2.60 ± 0.10	3.00 ± 0.06	0.12 ± 0.10
18:1Δ9	13.22 ± 0.21	12.36 ±0.20	82.85 ± 0.17	74.60 ±0.28	2.37 ± 0.17
18:1Δ11	1.34 ± 0.05	1.71 ± 0.13	3.03 ± 0.15	2.82 ± 0.22	3.99 ± 0.14
18:2Δ9,Δ12	27.45 ± 0.03	7.58 ± 0.42	0.39 ± 0.02	0.27 ± 0.01	24.69 ± 0.01
18:2Δ9Δ15	ND	ND	1.35 ± 0.04	8.23 ± 0.06	ND
18:3Δ9,Δ12,Δ15	19.38 ±0.14	39.57 ± 0.09	2.36 ± 0.10	2.47 ± 0.01	19.00 ± 0.49
20:0	2.02 ± 0.03	2.08 ± 0.16	0.88 ± 0.03	0.94 ± 0.01	
20:1Δ11	19.19 ± 0.07	18.94 ± 0.51	0.60 ± 0.01	0.60 ± 0.01	
20:1Δ13	1.76 ± 0.05	1.94 ± 0.11			
22:1	1.79 ± 0.04	1.56 ± 0.07			
Total Saturate	12.78 ± 0.10	13.97 ± 0.53	9.08 ± 0.15	10.27 ± 0.12	30.21 ± 0.76
Total C18 PUFA	46.84 ± 0.17	47.14 ± 0.51	2.75 ± 0.12	10.97 ± 0.07	43.69 ± 0.48

ND = not detected

Figure 1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Retention time