

Mass spectrometry characterisation of fatty acids from metabolically engineered soybean seeds

André M. Murad · Giovanni R. Vianna · Alex M. Machado ·
Nicolau B. da Cunha · Cíntia M. Coelho · Valquiria A. M. Lacerda ·
Marly C. Coelho · Elibio L. Rech

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Abstract Improving the quality and performance of soybean oil as biodiesel depends on the chemical composition of its fatty acids and requires an increase in monounsaturated acids and a reduction in polyunsaturated acids. Despite its current use as a source of biofuel, soybean oil contains an average of 25 % oleic acid and 13 % palmitic acid, which negatively impacts its oxidative stability and freezing point, causing a high rate of nitrogen oxide emission. Gas chromatography and ion mobility mass spectrometry were conducted on soybean fatty acids from metabolically engineered seed extracts to determine the nature of the structural oleic and palmitic acids. The soybean genes *FAD2-1* and *FatB* were placed under the control of the 35SCaMV constitutive promoter, introduced to soybean embryonic axes by particle bombardment and down-regulated using RNA interference technology. Results indicate that the metabolically engineered plants exhibited a significant increase in oleic acid (up to 94.58 %) and a reduction in palmitic acid (to <3 %) in their seed oil content. No structural differences were observed between the fatty acids of the transgenic and non-transgenic oil extracts.

Keywords Soybean oil · Biodiesel · Metabolic engineering

André M. Murad and Giovanni R. Vianna contributed equally to this work.

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A. M. Murad · G. R. Vianna · A. M. Machado · N. B. da Cunha ·
C. M. Coelho · V. A. M. Lacerda · M. C. Coelho · E. L. Rech (✉)
Embrapa Genetic Resources and Biotechnology,
Laboratory of Synthetic Biology, Parque Estação Biológica, PqEB,
Av. W5 Norte, 70770-917 Brasília, DF, Brazil
e-mail: elibio.rech@embrapa.br

Introduction

Energy needs and environmental concerns have induced worldwide interest in developing fuels based on renewable non-fossil fuel sources. Biodiesels from plants [1], algae [2], bacteria [3] and yeast [4] are rapidly emerging as potential renewable fuel sources. Most plant oils are primarily composed of five fatty acids: palmitate (16:0), stearate (18:0), oleate (18:1), linoleate (18:2) and linolenate (18:3) [1, 5, 6]. Palmitate and stearate are saturated fatty acids, oleate is a monounsaturated fatty acid, and linoleate and linolenate are polyunsaturated fatty acids. However, most plant oils used commercially as biodiesel feedstocks have high levels of polyunsaturated fatty acids, which decreases the stability of the oil, affects the cetane number [7] and directly limits their use as biofuels. Biodiesel with a high monounsaturated fatty acid (oleate) content has desirable characteristics for fuel performance such as ignition quality, nitrogen oxide (NO_x) emissions and oxidative stability [8]. Therefore, from a compositional standpoint, plant oils high in oleic acid and low in palmitic acid are good candidates for developing a high-performance biodiesel [9].

Soybean oil is essentially composed of triacylglycerol, a product of fatty acid esterification, and its performance as a fuel depends directly on its fatty acid composition. The metabolic manipulation of fatty acids in soybeans as it relates to improving oil quality and performance has been studied [1, 10]. Plants have been developed with genetically modified oils via mutation [11] and recombinant DNA technology [10], followed by selective breeding programs. Both approaches require screening to ensure optimal expression, stability across diverse growing environments and acceptable agronomic performance of the derived lines. Mutant soybean lines have been developed and introduced into a breeding program aiming at manipulating their fatty acid composition [11]. However, the results thus far have indicated that in addition to the length of time required to develop advanced varieties via sexual

crossings, the main challenge in developing commercial lines is achieving the required agronomic characteristics, such as high seed yield. Early studies have indicated that these constraints could be due to pleiotropy or unfavourable linkages during sexual crossings [12]. Over the last decade, the fatty acid composition of oil seeds such as soybean and *Jatropha curcas* has been successfully altered via metabolic engineering [13–15, 10, 16]. Down-regulating *FAD2-1* and *FatB* using interference RNA has enabled the production of soybeans with significantly higher oleic acid content (up to 85 %) and reduced levels of palmitic acid (<5 %), which improve its agronomic and biodiesel fuel performances [10]. These results demonstrate that engineering the fatty acid metabolism of soybean seeds allows the production of biodiesel with improved fuel performance, stability and NO_x emissions [10].

Monitoring the composition and structural quality of the fatty acids in the metabolically engineered seed lines of T₀ transgenic plants/T₁ seeds using heterozygous individual segregating seed, while maintaining germination without waiting for further progeny generation, is essential. These possibilities provide the basis by which one can decide whether to propagate advanced progenies for generating homozygous lines. A routine approach to evaluating fatty acid composition was developed using optimised capillary GC-MS [17]. This new ability to determine the structures of the fatty acids was the result of significant technological advances in mass spectrometry [18, 19]. Despite the availability of a range of mass spectrometry procedures [20–22], the structural analysis of fatty acids has been performed on few metabolically engineered oil seed plants [14, 10].

In our study, we have generated metabolically engineered transgenic soybean lines with high levels of oleic acid and decreased levels of palmitic acid by down-regulating the expression of $\Delta 12$ desaturase (*FAD2-1*) and palmitoyl thioesterase (*FatB1a*) under the control of a constitutive promoter and RNA interference technology. We have also determined the composition and structural characterisation of the fatty acids in these lines via mass spectrometry.

Materials and methods

Synthetic fatty acid sequences and the selective marker

To generate the fatty acid down-regulated RNAi soybean construct, we used two gene-specific 300-bp fragments corresponding to the cDNA coding regions of *SoyFAD2-1* (GenBank accession L43920.1, nt 272 to 571) and *SoyFatB1a* (GenBank accession DQ861997.1, nt 65 to 364). These cDNA fragments were assembled in tandem to form a 600-bp fragment. An *in silico* restriction analyses of the 600-bp sequence indicated an *Xba*I site (TCTAGA) at position 533. The first base, T (underlined), was deleted to remove the restriction site.

A synthetic 599-bp fragment corresponding to the *FAD* and *FAT* coding regions flanked by 5' *Xba*I/*Xho*I 3' and 5' *Kpn*I/*Bam*HI 3' restriction sites was produced (DNA2.0 Inc., Menlo Park, CA 94025, USA). The synthetic fragment was inserted in the sense direction into the *Xho*I/*Kpn*I sites and in the antisense direction into the *Bam*HI/*Xba*I sites of pKannibal [23] to generate the pSoybiofuel1 RNAi vector. In the pSoybiofuel1 vector, the total hairpin sequences were under the control of the constitutive 35SCaMV promoter and OCS terminator. The pAC321 vector carrying the mutated *ahas* gene from *Arabidopsis thaliana*, which confers tolerance to the herbicide Imazapyr[®], was used as a selective marker [24].

Soybean gene transfer

The plasmids pSoybiofuel1 and pAC321 [25, 24] were co-bombarded in a 1:1 ratio into the apical meristematic region of somatic embryonic axes from mature soybean seeds (cultivar conquista, Embrapa, Brazil) via particle bombardment as previously described [24]. The putative plants grown in Imazapyr[®]-selective medium were analysed by PCR for both transgenes. The PCR-positive T₀ plants were acclimatised and cultivated in soil under greenhouse conditions until they produced seeds. All seeds were harvested and used individually for molecular and biochemical analyses.

PCR analyses

A small segment (approximately 1 mm²) of the cotyledon from each T₁ and T₂ seed was excised for DNA isolation. The segment from the seed was removed from the opposite side to the embryonic axis to allow later seed sowing and plant development. The plant DNA was isolated according to the technique of Doyle and Doyle [26]. The PCRs contained 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM MgCl₂, 160 mM each of dNTP, 2 U Taq polymerase (Invitrogen), 20 ng of DNA and 200 nM of one of the following pairs of primers: KANNIf (CCACTATCCTTCGCAAGACC) and PDKr (TAAGTATTTTATACTAAAAGGA); PDKf (TTTC TAAATAATACTTGTAGTT) and KANNIr (CGATCATA GGCGTCTCGCAT) or *ahas*124 (ACTAGAGATTCCAG CGTCAC) and *ahas*500c (GTGGCTATACAGATACCTGG). The DNA was pre-denatured at 95 °C for 5 min and amplified for 35 cycles of 95 °C for 1 min, 55 °C for 1 min and 73 °C for 1 min, with a final cycle at 72 °C for 7 min. The expected amplification products using the given primers contained 728 and 835 bp for the forward and reverse bands, respectively, and 685 bp for the *ahas* gene.

Preparing the oil samples for mass spectrometry

Oil from the soybean seeds was extracted and esterified as previously described [27], with modifications. Soybean pods

Table 1 GC-MS results yielding the oil composition of the transgenic soybean seed line 65 and its sub-lines. Sample number 22 is a non-transgenic event and was used as a negative reference. The standard deviation varies from 0.1 to 0.6 % from injection-to-injection

Sample	Generation	Line/seed	% Methyl palmitate	% Methyl stearate	% Methyl oleate	% Methyl linoleate	% Methyl linolenate
1	T1	65-25	2.12	1.58	94.58	0.71	1.01
2	T1	65-14	3.50	1.47	85.42	7.57	2.04
3	T1	65-01	5.55	2.11	79.29	8.46	4.59
4	T1	65-11	4.33	1.85	85.43	4.98	3.41
5	T1	65-16	3.92	1.56	86.35	5.52	2.65
6	T1	65-17	4.88	1.98	80.47	7.85	4.82
7	T1	65-19	2.80	1.48	93.27	1.28	1.17
8	T1	65-24	1.64	1.62	67.33	23.66	5.75
9	T1	65-26	3.61	1.56	91.79	1.69	1.35
10	T1	65-105	0.40	0.11	85.09	11.57	2.83
11	T1	65-113	3.06	1.30	84.52	6.63	4.49
12	T1	65-114	1.42	1.70	91.74	2.11	3.03
13	T1	65-115	5.05	1.25	86.05	4.36	3.29
14	T1	65-120	2.11	1.65	80.75	10.73	4.76
15	T1	65-121	2.23	1.38	84.63	7.14	4.62
16	T1	65-123	4.33	1.52	82.68	6.75	4.72
17	T1	65-127	2.94	1.19	87.50	4.58	3.79
18	T1	65-135	1.87	1.39	81.78	10.89	4.07
19	T1	65-140	2.78	1.11	92.75	1.21	2.15
20	T1	65-141	2.16	1.28	89.73	3.66	3.17
21	T1	65-144	3.29	1.33	82.56	7.74	5.08
22	T1	122-22	3.37	3.41	24.12	63.22	5.88
23	T2	65-25-13	2.54	0.58	94.74	0.72	1.34
24	T2	65-25-14	1.89	0.20	97.81	0.17	0.13
25	T2	65-25-15	3.72	0.30	92.44	2.07	1.47
26	T2	65-25-18	3.30	0.30	94.37	1.06	0.97
27	T2	65-25-19	2.28	0.38	95.51	0.80	1.03
28	T2	65-25-20	8.02	0.48	81.99	8.13	1.38
29	T2	65-25-33	5.58	0.38	77.41	15.43	1.20
30	T2	65-25-37	7.43	0.38	82.17	9.51	0.51
31	T2	65-25-38	3.50	0.69	90.18	4.00	1.63
32	T2	65-25-40	2.00	0.30	97.04	0.66	NA
33	T2	65-25-55	4.25	0.91	91.35	2.10	1.39
34	T2	65-25-56	2.16	0.66	96.83	0.22	0.13
35	T2	65-25-60	4.30	0.77	88.48	4.63	1.82
36	T2	65-25-66	2.90	0.23	96.15	0.22	0.50
37	T2	65-25-68	2.47	0.31	97.22	NA	NA
38	T2	65-25-69	3.93	0.81	89.82	3.97	1.47
39	T2	65-25-70	5.60	0.77	83.71	8.11	1.81
40	T2	65-25-81	3.36	0.72	89.62	4.87	1.43
41	T2	65-25-82	2.87	0.92	93.82	1.06	1.33
42	T2	65-25-83	5.31	0.73	77.12	13.95	1.89
43	T2	65-25-86	1.72	0.38	96.94	0.30	0.66
44	T2	65-25-94	4.48	0.75	88.04	4.29	2.44

from T₀ transgenic and non-transgenic plants were harvested. The T₁ and T₂ seeds were collected and stored individually below 4 °C. Using a scalpel blade, a 1–2-mm-thick section

weighing approximately 10 mg was excised from the seed cotyledon, as previously described. The section was ground using a mortar and pestle. The sample powder was transferred

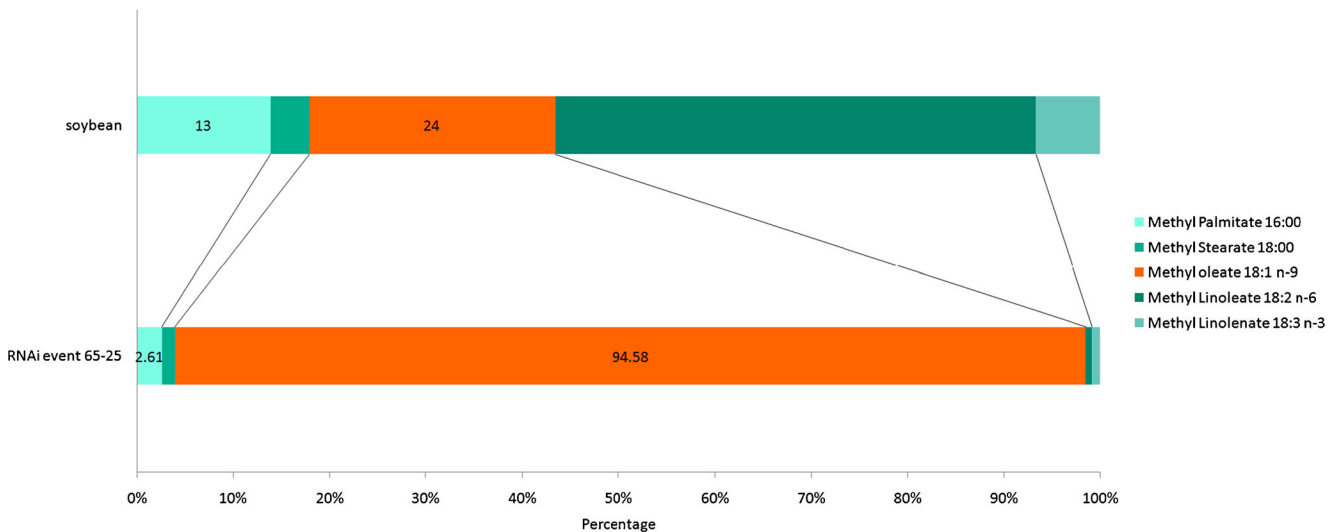


Fig. 1 Soybean fatty acid GC-MS profile. Percentage of soybean fatty acids in the oil extracts from transgenic seed line 65

to a glass flask and dried overnight in an oven at 90 °C. To each flask, 500 μ L of a 0.5 N KOH-methanol solution was added, and the mixture was vortexed for 15 s. Then, the flasks were heated in an oven at 80 °C for 60 min. To each flask, 2 mL of an acid-methanol solution (6 mL H_2SO_4 in methanol) was added, and the samples were vortexed for 3 min and heated in an oven at 80 °C for an additional 60 min. The samples were cooled at room temperature for 10 min, and 2 mL of hexane was added, followed by vortexing for 3 min. The samples were allowed to separate into phases for 45 min. Then, 1 mL of the supernatant was collected and placed in a capped vial for a GC autosampler. Standards of palmitic acid (Sigma-76119), stearic acid (Sigma-85679), linolenic acid (Sigma-62160), linoleic acid (Sigma-62230) and oleic acid (Sigma-75040) were also esterified using the aforementioned method and used for GC-MS calibration. For the LC-MS analysis, 400 μ L of the GC-MS

prepared sample corresponding to line 65-25 was placed in a new glass vial and dried in a N_2 flux. Methanol (1 mL, LC-MS grade) was added, and the sample was vortexed for 30 s. This solution was directly infused into the MS.

GC-MS analysis

Gas chromatography mass spectrometry was performed using a Shimadzu GC-2010 and a Shimadzu GCMS-QP2010 equipped with an autosampler. Two microlitres was applied to an OmegaWax 250 capillary column (30 m \times 0.25 mm \times 0.25 μ m) from Supelco (Sigma-24136). The following chromatographic conditions were set: initial temperature, 50 °C; final temperature, 240 °C; injection temperature, 250 °C; and rate, 5 °C/min up to 240 °C then hold at 240 °C for 10 min (total run time=58 min). A linear flow of 2 mL/min with a helium pressure of 117.6 kPa was used in a total flow of

Fig. 2 Soybean fatty acid GC-MS profiles for the oil extracts from transgenic line 65 and its sub-lines. Retention times from the different soybean lines. Each colour represents one line

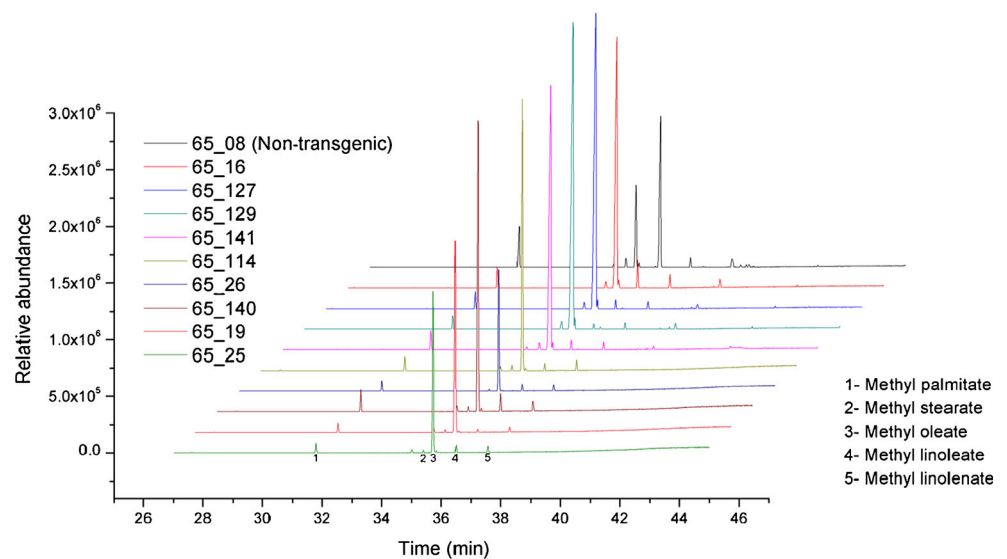
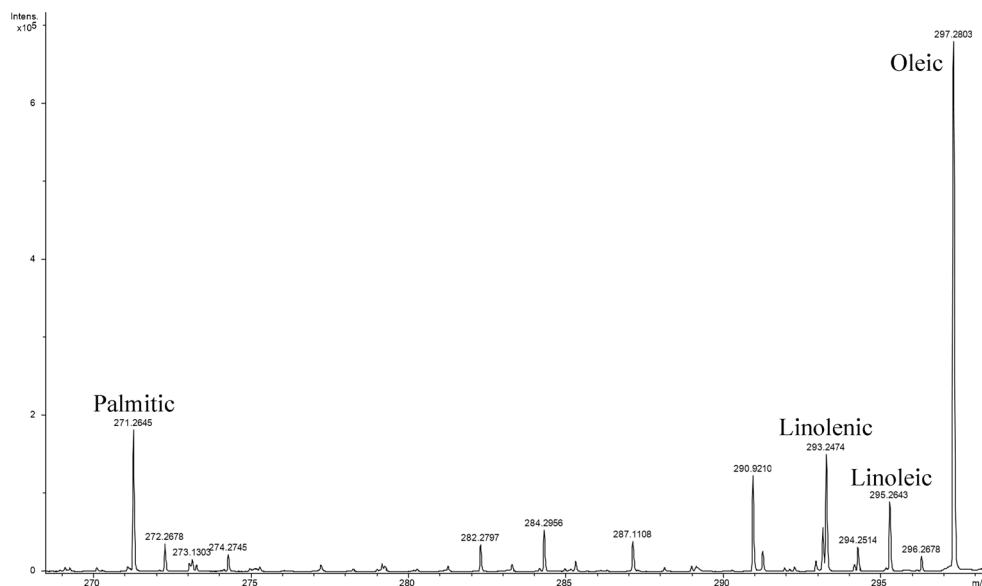


Fig. 3 MicrO-TOF Q exact mass spectra (m/z) from the oil extracted and esterified from line 65-25



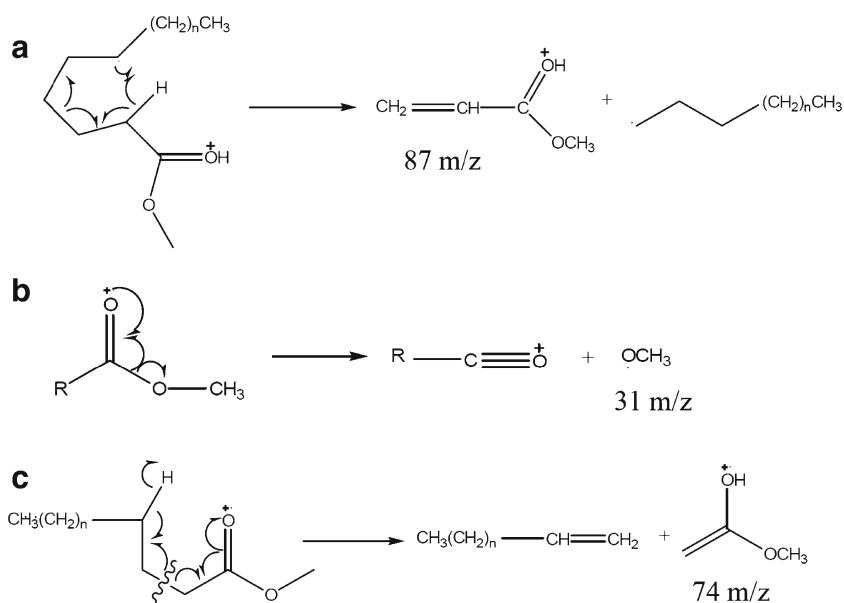
25 mL/min. The linear velocity was set at 51.3 cm/s with a purge at 3 mL/min and a split ratio of 10. The mass spectrometry was operated at a temperature of 250 °C with an 833-Hz scan speed from 100–500 m/z over 58 min. The chromatography peaks and MS fragmentation data were searched in the FAME fragment database.

Exact mass spectrometry and ion mobility mass spectrometry

The samples previously prepared for GC-MS were analysed in a MicrO-TOF Q II electrospray ionisation mass spectrometer (Bruker Daltonics) and a Synapt G2 high definition mass spectrometer (Waters, USA). For the Synapt G2 analysis, a nanospray source (at 600 nL/min) was used in the positive ion

mode with the following conditions: analyser, resolution mode; capillary, 3.0 kV; sampling cone, 30.0; extraction cone, 6.0; source temperature, 70 °C; desolvation temperature, 200 °C; cone gas flow, 0.0 L/h; nanoflow gas pressure, 0.2 Bar; purge gas flow, 800.0 mL/h; desolvation gas flow, 800.0 L/h; LM resolution, 4.9; and HM resolution, 15.0. The mass spectrometry was externally calibrated using ion series from a sodium formate solution. The fragment analyses were performed in the MS/MS mode, and the data were collected from 10 to 300 m/z over 5 min. The collision energy was applied in a trap cell, ramping from 6 to 35 eV, and the LM resolution was set to 23.9. Ion mobility was also evaluated in the resolution mode, adjusting the quadrupole radio frequency to select each FAME with an LM resolution of 23.9. The IMS

Fig. 4 Molecular rearrangements of the fatty acid methyl acids produced in the collision cell. **A** Formation of the base peak of 87 m/z . **B** Loss of OCH₃ (31 m/z). **C** Loss of CH₂COHOCH₃ (74 m/z)



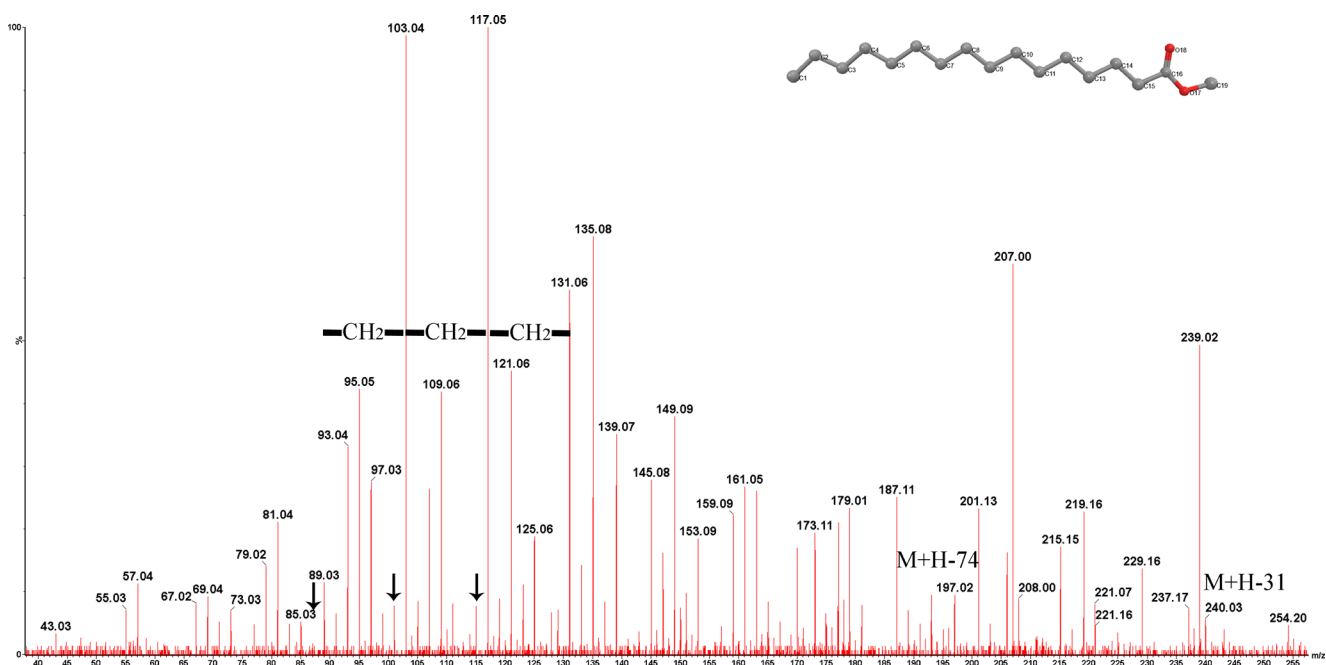


Fig. 5 Palmitic methyl ester fragmentation patterns. The molecular structure of this FAME and the CH_2 loss are shown in detail. The *arrows* indicate the ion series of 87m/z

gas flow was set to 90 mL/min, and the helium gas flow to 180 mL/min. The IMS wave velocity and wave height were set such that the TICs were observed in the centre of the drift tube, and these values were maintained for all samples. The palmitic methyl ester spectra were obtained from 270 to 275 m/z . The linolenic methyl ester spectra were obtained from 290 to 297

m/z . The linoleic methyl ester spectra were obtained from 294 to 300 m/z . The oleic methyl ester spectra were obtained from 295 to 300 m/z . The exact mass and MS/MS experiments were also performed in a MicrO-TOF Q II electrospray ionisation mass spectrometer using a standard electrospray probe and internal calibration for sub-ppm mass errors in the MS mode.

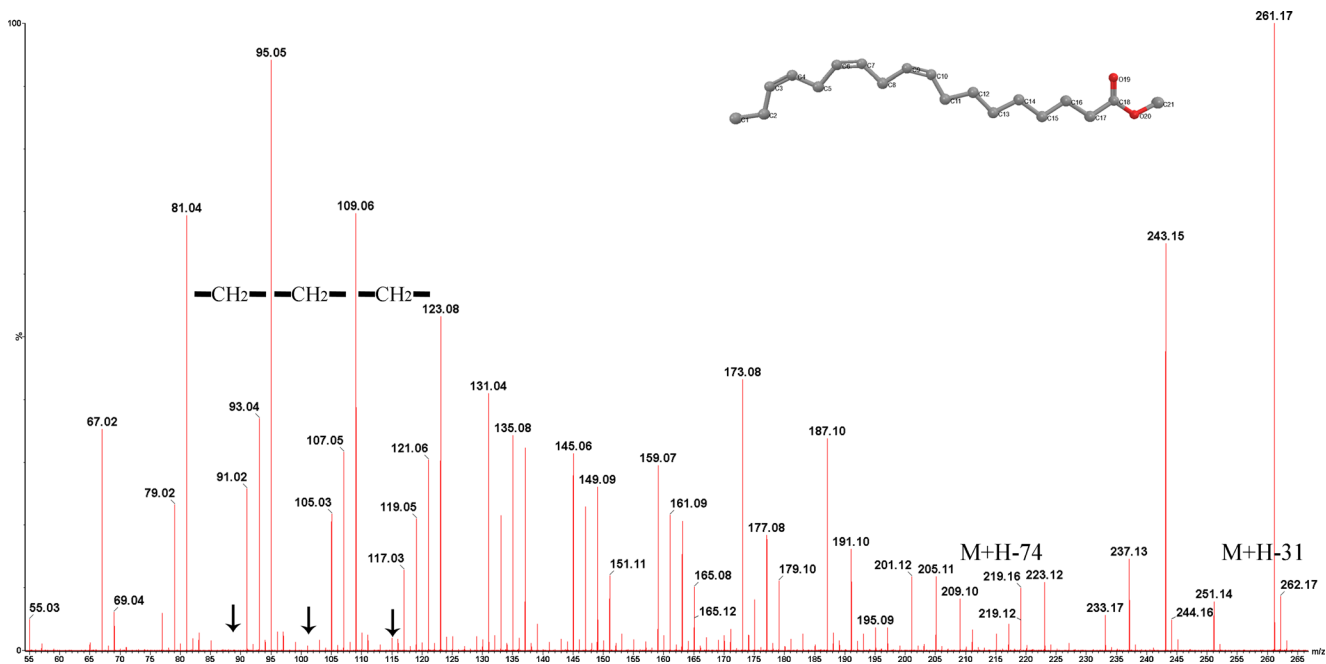


Fig. 6 Linolenic methyl ester fragmentation patterns. The molecular structure of this FAME and the CH_2 loss are shown in detail. The *arrows* indicate the ion series of 87m/z

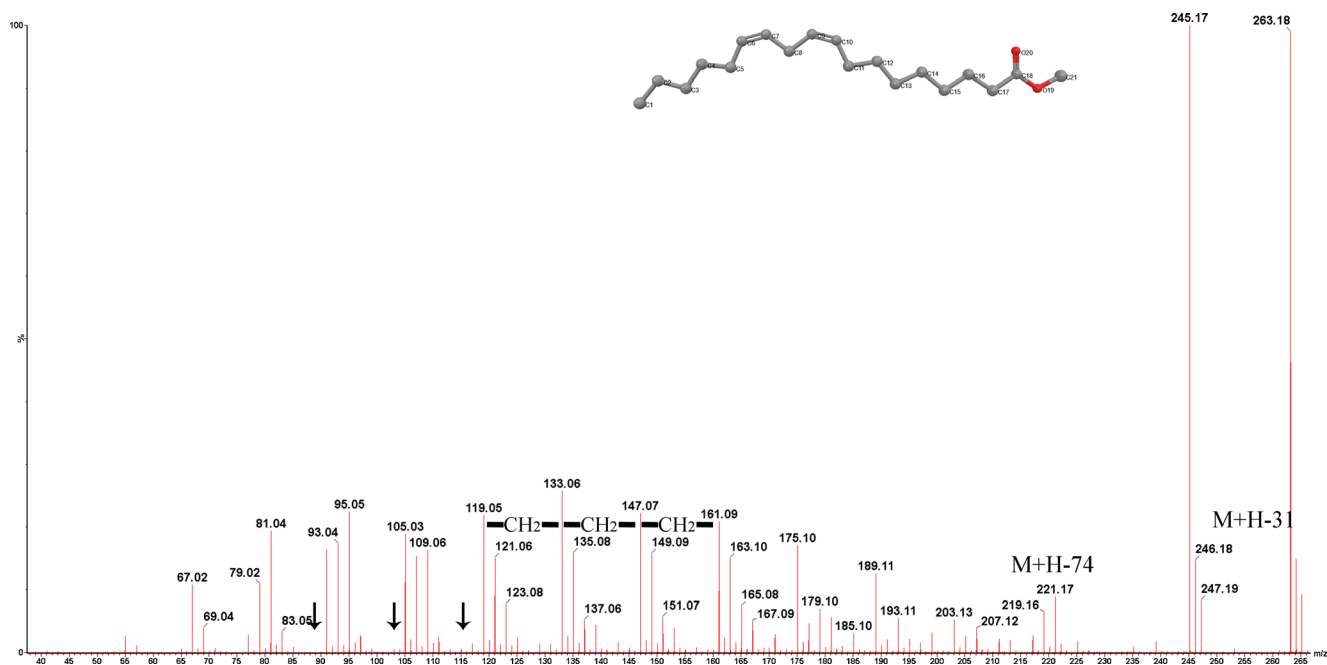


Fig. 7 Linoleic methyl ester fragmentation patterns. The molecular structure of this FAME and the CH₂ loss are shown in detail. The arrows indicate the ion series of 87 *m/z*

Table 2 Mass values (*m/z*) of important ions and series as determined by the mass spectrometry experiments. The ion series (M+H-18) corresponds to a possible water loss. Ion series (M+H-50) corresponds to a possible rearrangement in carbonic chain with a water loss (C₂H₃+3H+H₂O). Ion series 87 was described by McLafferty [28, 18]. Ion series 55 and 53 was observed in the spectra

	<i>m/z</i>	C18	C17	C16	C15	C14	C13	C12	C11	C10	C9	C8	C7	C6	C5	C4	C3	C2	C1	
Palmitic	M+H			271	257	243	229	215	201	187	173	159	145	131	117	103	89	75	61	
	M+H-18			253	239	225	211	197	183	169	155	141	127	113	99	85	71	57	43	
	M+H-50						221	207	193	179	165	151	137	123	109	95	81	67	53	
	Ion series 87			87	101	115	129	143	157	171	185	199	213	227	241	255				
	Ion series 55			55	69	83	97	111	125	139	153	167	181	195	209	223	237			
Linolenic	M+H	293	279	265	251	237	223	209	195	181	167	153	139	125	111	97	83	69	55	
	M+H-18	275	261	247	233	219	205	191	177	163	149	135	121	107	93	79	65			
	M+H-50				243	229	215	201	187	173	159	145	131	117						
	Ion series 87	87	101	115	129	143	157	171	185	199	213	227								
	Ion series 55	55	69	83	97	111	125	139	153	167	181	195	209	223	237	251				
Linoleic	M+H	295	281	267	253	239	225	211	197	183	169	155	141	127	113	99	85	71	57	
	M+H-18	277	263	249	235	221	207	193	179	165	151	137	123	109	95	81	67			
	M+H-50				245	231	217	203	189	175	161	147	133	119	105	91	77			
	Ion series 87	87	101	115	129	143	157	171	185	199	213	227								
	Ion series 55	55	69	83	97	111	125	139	153	167	181	195	209							
Oleic	M+H	297	283	269	255	241	227	213	199	185	171	157	143	129	115	101	87	73	59	
	M+H-18	279	265	251	237	223	209	195	181	167	153	139	125	111	97	83	69			
	M+H-50				247	233	219	205	191	177	163	149	135	121	107	93	79			
	Ion series 87	87	101	115	129	143	157	171	185	199	213	227								
	Ion series 55	55	69	83	97	111	125	139	153	167	181	195	209							
Ion series 53	53	67	81	95	109	123	137	151	165	179										

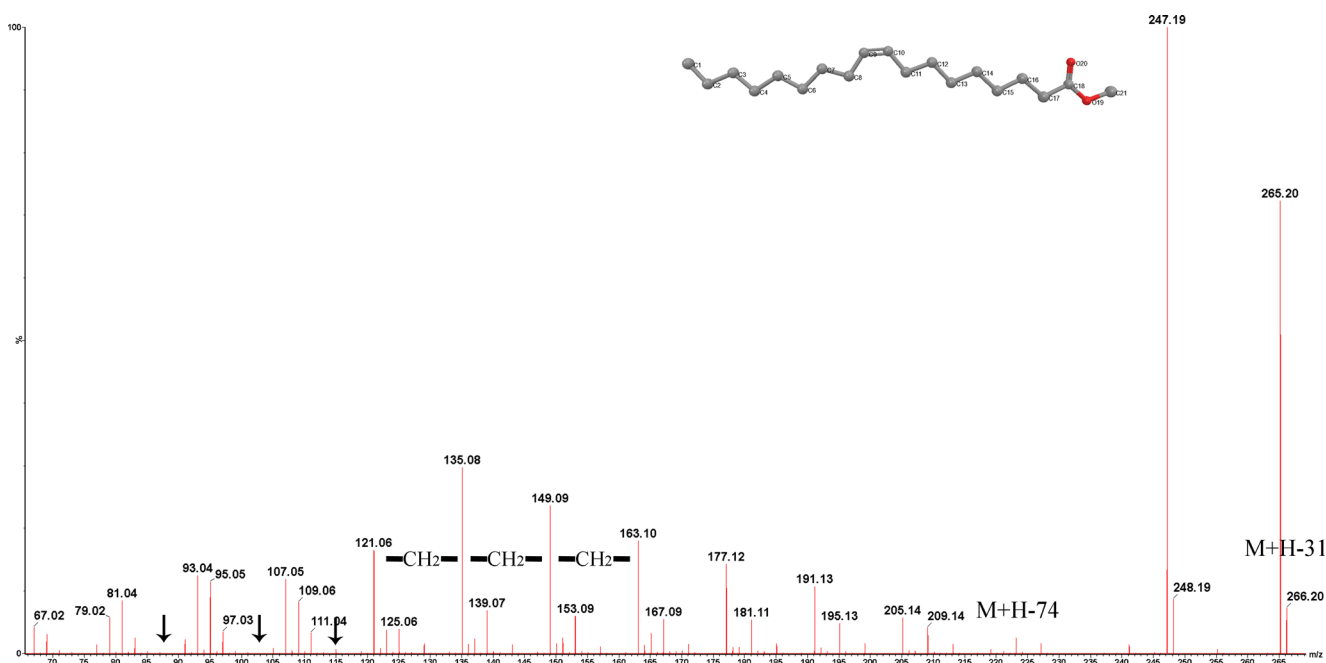


Fig. 8 Oleic methyl ester fragmentation patterns. The molecular structure of this FAME and the CH_2 loss are shown in detail. The *arrows* indicate the ion series of 87 m/z

Results and discussion

Soybean transformation

Transgenic soybean plants were generated by co-bombardment using a developed protocol as previously described [24]. The microparticle bombardment experiments included 3,600 embryonic axes. The embryonic axes were cultured in vitro for 6 weeks, and the putative transgenic plantlets were analysed via PCR. Eight independent soybean T_0 lines were obtained and presented both the 728 and 835 bp fragments corresponding to two internal regions of the pSoybiofuel1 vector expression cassette that were amplified using the KANNIf/PDKr and PDKf/KANNIr primer combinations, respectively. The transgenic plants were also analysed for the presence of the selectable marker gene, *ahas* [25]. All transgenic plantlets presented the 685 bp fragment corresponding to the amplification of the 5' internal segment of the mutated *ahas* gene. The transgenic plants were transferred to soil under greenhouse conditions to set seeds. The T_1 heterozygous individual seed lines were then analysed by mass spectrometry to evaluate the oil composition and structure.

GC-MS analysis

After soybean oil extraction and derivatisation, the methyl esters were analysed by GC-MS (Table 1; Figs. 1 and 2). Different T_1 lines exhibited an improved profile for production of oleic acid and a reduction in palmitic acid (Electronic Supplementary Material, Table S1). Seed line 65-25 presented

the highest performance with 94.58 % oleic acid and 2.12 % palmitic acid (Table 1). To verify the extraction and derivatisation stability over time, lines 65-25, 65-14 and 122-22 were injected into the GC-MS once a week throughout the analytical experiments. The standard deviations (Table 1) demonstrated that the analyses can be performed with a week between the extraction, derivatisation and GC-MS analysis without significant variation in the results. Furthermore, the 65-25 line presented higher concentrations of oleic acid and lower concentrations of palmitic acid than the oil extracted from the non-transgenic seeds (Fig. 1) [10], suggesting that the structural oil characteristics determined in line 65-25 meet the international standards for biodiesel production [10]. Thus, all T_2 seeds (Table 1) were generated from line 65-25.

Ion mobility analysis

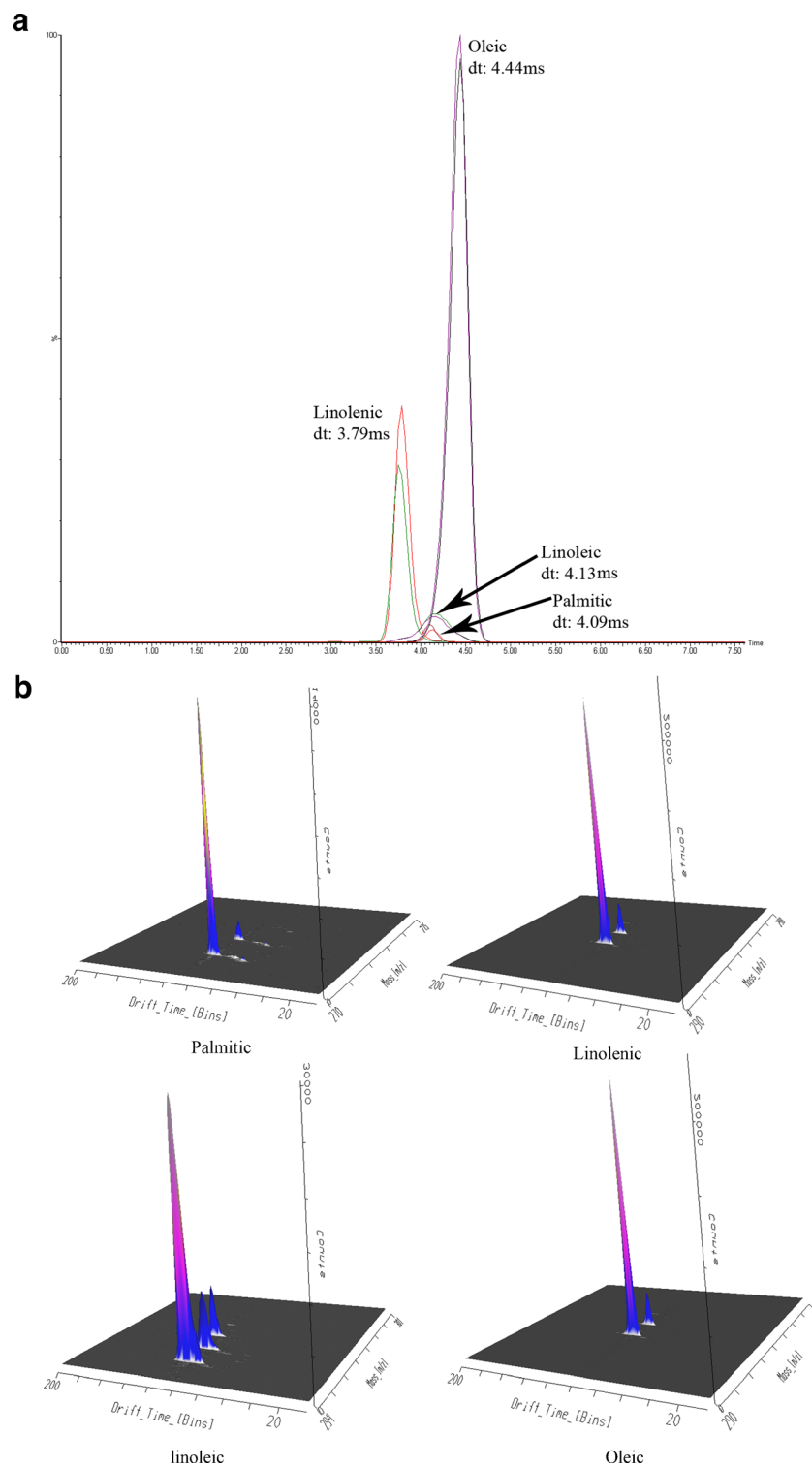
The structural characterisation of the fatty acid profile in the transgenic and non-transgenic soybean seeds was performed using high-resolution/accurate mass spectrometry and ion mobility-MS. The exact mass spectra of line 65-25 (Fig. 3) indicated no change in the masses of palmitic methyl ester (271.2 [M+H] m/z), linolenic methyl ester (293.2 [M+H] m/z), linoleic methyl ester (295.2 [M+H] m/z) and oleic methyl ester (297.2 [M+H] m/z). However, the mass of stearic methyl ester (299.2 [M+H] m/z) could not be determined because it matched that of one isotope of oleic methyl ester, resulting in a contaminated fragmentation spectrum.

Figures 5, 6, 7 and 8 demonstrate the fragmentation of the fatty acid methyl esters (FAMES). The results indicate no

differences between the standard spectra and those of the soybean 65-25 esterified oil. The fragmentation process in the collision cell produces ions that can be assigned as signatures for the FAMES. Figure 4 shows the three most common FAME rearrangements due to the fragmentation process. The McLafferty rearrangement often occurs [28, 18], forming ions

with $M+H-31$ and $M+H-74$ (Fig. 4b, c). The hydrocarbon chain has a signature of $M+H-14$, corresponding to the consecutive break of CH_2 . A series of ions was also detected based on the 87 m/z ion (Fig. 4a), forming ions with masses of 101, 115, 129 and 143 m/z ($\text{CH}_3\text{OCO}[\text{CH}_2]_N$) with low intensity. These signatures can be observed in Figs. 5, 6, 7 and 8,

Fig. 9 Ion mobility analysis of the FAMES from transgenic and non-transgenic seeds. **A** Superimposition of the drift time for each analysed FAME. **B** 3D representation of the positions of the FAMES in the drift tube and their isotopes



which depict ions 197 *m/z* and 240 *m/z* for palmitic acid, 225 *m/z* and 268 *m/z* for stearic acid, 223 *m/z* and 266 *m/z* for oleic acid, 262 *m/z* and 219 *m/z* for linolenic acid and 264 *m/z* and 221 *m/z* for linoleic acid, representing M+H-31 and M+H-74, respectively. In addition, Table 2 contains a list of all relevant masses found in the spectra. We found a series of ions that have M+H-18, M+H-50 as precursors with two other series that start with ions of 55 *m/z* and 53 *m/z*. These ions were also detected in the GC-MS spectra (data not shown). We can theorise that M+H-18 represents the resulting water loss in the compound, $\text{CH}_3(\text{CH}_2)_N\text{CCHOCH}_2 + \text{H}_2\text{O}$. The M+H-50 series could be a multiple rearrangement of the hydrocarbon chain following water loss ($\text{CHCCC}[\text{CH}_2]_N\text{COCH}_2 + \text{C}_2\text{H}_5 + 3\text{H} + \text{H}_2\text{O}$). The ion series starting at 55 *m/z* could begin with a compound modified from Fig. 4b, $\text{CH}_2\text{CH}(\text{CH}_2)_N\text{CO}$. We do not believe that these ions could be formed by contamination either in the ion source or in the sample because the ion mobility of each methyl ester exhibited only one conformation (Fig. 9), indicating that the observed fragments were obtained from ions isolated from the quadrupole.

Furthermore, the ion mobility of each FAME was evaluated for multiple structures and/or contamination between line 65-25 and a non-transgenic line. The results are provided in Fig. 9, with the following drift times for each FAME: palmitic=4.09 ms, linolenic=3.79 ms, linoleic=4.13 ms and oleic=4.44 ms (Fig. 9a). Figure 9b depicts the isotopes of each FAME, showing a uniform distribution. These data indicate that a single structural conformation is formed, and no difference is observed between the FAMES from line 65-25 and those from the non-transgenic line.

These results are in agreement with the analysis and comparison of the soybean oil samples from the transgenic and non-transgenic lines previously performed by other groups using different techniques [23, 9]. For example, Luna et al. [29] analysed the oil content from transgenic and non-transgenic soybean seeds using infrared spectroscopy to distinguish and classify the soybean oil for process monitoring and control. Their results indicated no significant differences among the samples. Similar results were observed by Graef et al. [10], who compared oil performance from transgenic soybean seeds and demonstrated improved quality in transgenic lines for the production of oil for biofuel.

Historically, vegetable oils have been of great industrial importance in the preparation of margarines and other foodstuffs. They have now become important as raw materials for biofuel. The worldwide demand for vegetable oil for both foods and biofuels requires increased production. Biotechnology presents a variety of solutions to improve the fatty acid composition for food and industrial uses. Through metabolic engineering, the oil content can be altered in a variety of crops, such as soybeans, to produce the oils needed by industry. For example, the omega-3 polyunsaturated fatty acids found in fish oil can now be produced in plants [30], improving the quality of food for humans

and animals. Each gene responsible for the production of oil in soybeans has been identified [10, 27, 31–33] and we have developed a soybean line that expresses an oleic acid content of up to 95 % and a palmitic acid content below 4 %. The oils from the metabolically engineered soybean seed lines produced have similar structural characteristics to those from non-transgenic seeds. These lines may form the foundation for the next generation of homozygous lines and the large-scale production of improved performance oils for the bioenergy sector.

Conclusions

Metabolically engineered soybean plants exhibited a significant increase in oleic acid and a reduction in palmitic acid in their seed oil content. Structural characterisation, performed using high-resolution/accurate mass spectrometry and ion mobility-MS, revealed no differences in the fatty acids of the transgenic and non-transgenic oil extracts. Using this approach, it is possible to check potential modifications in the first plant generation, keeping the seeds viable for germination and progeny development

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References

- Durrett TP, Benning C, Ohlrogge J (2008) Plant triacylglycerols as feedstocks for the production of biofuels. *Plant J* 54(4):593–607
- Batten D, Beer T, Freischmidt G, Grant T, Liffman K, Paterson D, Priestley T, Rye L, Threlfall G (2013) Using wastewater and high-rate algal ponds for nutrient removal and the production of bioenergy and biofuels. *Water Sci Technol* 67(4):915–924
- Zhang F, Ouellet M, Batth TS, Adams PD, Petzold CJ, Mukhopadhyay A, Keasling JD (2012) Enhancing fatty acid production by the expression of the regulatory transcription factor FadR. *Metab Eng* 14(6):653–660
- Shi S, Valle-Rodriguez JO, Khoomrung S, Siewers V, Nielsen J (2012) Functional expression and characterization of five wax ester synthases in *Saccharomyces cerevisiae* and their utility for biodiesel production. *Biotechnol Biofuels* 5:7
- Fehr WR (2007) Breeding for modified fatty acid composition in soybean. *Crop Sci* 47:S72–S87
- Koria L, Nithya G (2012) Analysis of *Datura stramonium* Linn. biodiesel by gas chromatography - mass spectrometry (gc-ms) and influence of fatty acid composition on the fuel related characteristics. *J Phytol* 4(1):06–09
- Frankel EN (1980) Lipid oxidation. *Prog Lipid Res* 19(1–2):1–22
- Canakci M, Monyem A, Van Gerpen J (1999) Accelerated oxidation processes in biodiesel. *Trans ASAE* 42(6):1565–1572
- Duffield JA (2007) Biodiesel: production and economic issues. *Inhal Toxicol* 19(12):1029–1031

10. Graef G, LaVallee BJ, Tenopir P, Tat M, Schweiger B, Kinney AJ, Van Gerpen JH, Clemente TE (2009) A high-oleic-acid and low-palmitic-acid soybean: agronomic performance and evaluation as a feedstock for biodiesel. *Plant Biotechnol J* 7(5):411–421
11. Patil A, Taware SP, Oak MD, Tamhankar SA, Rao VS (2007) Improvement of oil quality in soybean [*Glycine max* (L.) Merrill] by mutation breeding. *J Am Oil Chem Soc* 84(12):1117–1124
12. Clemente TE, Cahoon EB (2009) Soybean oil: genetic approaches for modification of functionality and total content. *Plant Physiol* 151(3):1030–1040
13. Qian J, Shi H, Yun Z (2010) Preparation of biodiesel from *Jatropha curcas* L. oil produced by two-phase solvent extraction. *Bioresour Technol* 101(18):7036–7042
14. Berchmans HJ, Hirata S (2008) Biodiesel production from crude *Jatropha curcas* L. seed oil with a high content of free fatty acids. *Bioresour Technol* 99(6):1716–1721
15. Worapun I, Pianthong K, Thaiyasuit P (2012) Two-step biodiesel production from crude *Jatropha curcas* L. oil using ultrasonic irradiation assisted. *J Oleo Sci* 61(4):165–172
16. Qu J, Mao HZ, Chen W, Gao SQ, Bai YN, Sun YW, Geng YF, Ye J (2012) Development of marker-free transgenic *Jatropha* plants with increased levels of seed oleic acid. *Biotechnol Biofuels* 5(1):10
17. Chai C, Ju HK, Kim SC, Park JH, Lim J, Kwon SW, Lee J (2012) Determination of bioactive compounds in fermented soybean products using GC/MS and further investigation of correlation of their bioactivities. *J Chromatogr B Analyt Technol Biomed Life Sci* 880(1):42–49
18. McLafferty FW, Gohlke RS (1959) Mass spectrometric analysis—aromatic acids and esters. *Anal Chem* 31(12):2076–2082
19. Christie WW (1998) Gas chromatography–mass spectrometry methods for structural analysis of fatty acids. *Lipids* 33(4):343–353
20. Cahoon EB, Shanklin J (2000) Substrate-dependent mutant complementation to select fatty acid desaturase variants for metabolic engineering of plant seed oils. *P Natl Acad Sci U S A* 97(22):12350–12355
21. Dormann P, Frentzen M, Ohlrogge JB (1994) Specificities of the acyl-acyl carrier protein (Acp) Thioesterase and glycerol-3-phosphate acyltransferase for octadecenoyl-Acp isomers—identification of a petroselinoyl-Acp thioesterase in Umbelliferae. *Plant Physiol* 104(3):839–844
22. Broun P, Boddupalli S, Somerville C (1998) A bifunctional oleate 12-hydroxylase: desaturase from *Lesquerella fendleri*. *Plant J* 13(2):201–210
23. Wesley SV, Helliwell CA, Smith NA, Wang MB, Rouse DT, Liu Q, Gooding PS, Singh SP, Abbott D, Stoutjesdijk PA, Robinson SP, Gleave AP, Green AG, Waterhouse PM (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. *Plant J* 27(6):581–590
24. Rech EL, Vianna GR, Aragao FJL (2008) High-efficiency transformation by biolistics of soybean, common bean and cotton transgenic plants. *Nat Protoc* 3(3):410–418
25. Aragão FJL, Sarokin L, Vianna GR, Rech EL (2000) Selection of transgenic meristematic cells utilizing a herbicidal molecule results in the recovery of fertile transgenic soybean [*Glycine max* (L.) Merrill] plants at a high frequency. *Theor Appl Genet* 101(1–2):1–6
26. Doyle JJ, Doye JL (1987) Rapid DNA isolation procedure from small quantities of fresh leaf tissues. *Phytochem Bull* 19(1):11–15
27. Primomo VS, Falk DE, Ablett GR, Tanner JW, Rajcan I (2002) Inheritance and interaction of low palmitic and low linolenic soybean. *Crop Sci* 42(1):31–36
28. McLafferty FW (1959) Mass spectrometric analysis—molecular rearrangements. *Anal Chem* 31(1):82–87
29. Luna AS, da Silva AP, Pinho JSA, Ferre J, Boque R (2013) Rapid characterization of transgenic and non-transgenic soybean oils by chemometric methods using NIR spectroscopy. *Spectrochim Acta A* 100:115–119
30. Truksa M, Wu GH, Vrinten P, Qiu X (2006) Metabolic engineering of plants to produce very long-chain polyunsaturated fatty acids. *Transgenic Res* 15(2):131–137
31. Li R, Hatanaka T, Yu K, Wu Y, Fukushige H, Hildebrand D (2013) Soybean oil biosynthesis: role of diacylglycerol acyltransferases. *Funct Integr Genomics* 13:99–113
32. Heppard EP, Kinney AJ, Stecca KL, Miao GH (1996) Developmental and growth temperature regulation of two different microsomal omega-6 desaturase genes in soybeans. *Plant Physiol* 110(1):311–319
33. Tang GQ, Novitzky WP, Griffin HC, Huber SC, Dewey RE (2005) Oleate desaturase enzymes of soybean: evidence of regulation through differential stability and phosphorylation. *Plant J* 44(3):433–446