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Novel seed lipid phenotypes in combinations of mutants altered in fatty acid biosynthesis in *Arabidopsis*

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Summary. The seed fatty acid (FA) composition of various single mutant combinations of *Arabidopsis thaliana* that affect FA biosynthesis has been examined. Double mutant combinations of *fae,* a mutation affecting C18 elongation, and a series of four other FA biosynthetic mutants were synthesized. The four other single mutants were: *fad2* and *fad3,* which are deficient in 18:1 and 18:2 desaturation, respectively; *fab1,* which is elevated in 16:0 and decreased in 18:1; and *fab2,* which is elevated in 18:0 and decreased in 18:1. The superimposition of two blocks in the FA biosynthetic pathway leads to dramatic changes in the FA content of the double mutants. The ten *Arabidopsis* stocks analyzed to date (wild-type, five single mutants, and four double mutants) make seed oils with a wide range of FA compositions, and illustrate the diversity of oils it is possible to obtain from a single plant species.

Key words: *Arabidopsis thaliana* – Lipid biosynthesis – Fatty acid desaturation – Fatty acid elongation – Oilseed breeding

Introduction

With changes in specific properties brought about by changes in fatty acid (FA) composition, vegetable oils could find manifold extended uses, e.g., different applications as cooking oils, lubricants, or feedstocks in the manufacture of detergents, cosmetics, polymers, and materials for industrial processes (Johnson and Fritz 1989). The goal of our research is to develop a means of customizing FA composition in the seeds of crop plants by altering the expression of genes involved in fatty acid biosynthesis. Towards this end we have initiated a program of gene isolation in *Arabidopsis thaliana* using transposon tagging. Previous studies indicate that the FA composition in seeds can be altered by mutation (Stefansson et al. 1961; Green and Marshall 1984; Bubeck et al. 1989; James and Dooner 1990; Lemieux et al. 1990). Therefore, once the FA biosynthesis genes have been isolated, one should be able to alter expression by transforming the plant of interest with antisense or sense DNA constructs (van der Krol et al. 1988, 1990; Napoli et al. 1990) and thereby affecting the FA composition.

In order to obtain specific FA compositions in seeds, it will be necessary to alter the expression of several genes. It was therefore thought important to determine which FA phenotypes result from combining different single FA mutations in the same nucleus. We have begun by examining the effects of superimposing a block in C18 elongation upon other FA biosynthesis mutants. To that end, we have synthesized double mutants between *fae* (fatty acid elongation) and various other single mutations. In this study we report on the phenotypes of four such double mutant combinations in *Arabidopsis.*

Materials and methods

Plant material

Arabidopsis thaliana (L.) Heynh. cv Columbia ($2n=10$) was used as the wild type in this study. Growth conditions and EMS mutagenesis protocols by which single gene mutants were obtained have been previously described (James and Dooner 1990). Double mutants were obtained by crossing single mutants. Flower buds from which the stigma had not emerged from the sepals were carefully opened, emasculated, and pollinated. Freshly pollinated pistils were covered with plastic wrap to prevent dessication and pollen contamination. After about 4 weeks, mature/ripening siliques were collected and seed were allowed to dry. F_1 seed were germinated and plants were allowed to self-pollinate to obtain F_2 plants. For each cross, approximately 100 F_2 selfed progeny were analyzed for FA composition. The

codominant nature of the mutations enabled assignment of genotypes on the basis of FA phenotypes.

Fatty acid analysis

FA analysis was by gas chromatography as previously described (James and Dooner 1990). Briefly, 2.0 mg samples of seed (about 100 seeds) was treated with 1.0 ml of 1 *N* HCl in 100% methanol for 1 h at 80 °C. After incubation, 1.5 ml of 0.9% (w/v) NaCl and 1.0 ml hexane (spiked with 0.1 mg/ml heptadacanoic acid methyl ester, an external standard) was added to the cooled tubes, which were shaken 1 min and centrifuged (1,000 × *g*, 5 min). The hexane phase was transfered to gas chromatography vials for analysis. Samples (1 µl) were injected via autosampler into a 2.0 mm × 3.07 m glass column packed with 3% SP-2310/2% SP-2300 on 100/120 chromosorb WAW (Supelco, Bellefonte/PA). The Perkin-Elmer sigma 300 gas chromatograph was programmed as follows: 160 °C for 2.0 min, ramp to 220 °C at 30 °C/min, held at 220 °C for 12 min. Injector and FID detector were held at 220 ° and 300 °C, respectively.

Results and discussion

FA analysis of double mutants

Table 1 contrasts FA phenotypes of *Arabidopsis thaliana* 'Columbia' wild type, single mutants, and double mutants isolated from selfed F_2 progeny derived from crosses of the single mutants. We have previously described single FA mutants, some of which have now been tested for allelism with the type mutants (James and Dooner 1990). Mutant *4A5* is an allele of the *fad2* (fatty acid desaturation) mutation and *G30* is an allele of *fad3* (J. Browse, personal communication). With respect to wild type, *fad2* shows an increase in oleic acid (18:1) from 15 to 66%, concomitant with a decrease to 1 to 2% in linoleic acid (18:2) and linolenic acid (18:3). This mutant appears to have a deficiency in 18:1 desaturation. The *fad3* mutation shows an increase in 18:2 to about 47%, with a decrease in 18:3 to about 1%, and therefore it appears to be deficient in 18:2 desaturation. The *fae* phenotype shows a decrease to essentially zero in all FAs greater than 18 carbons long, while the concentration of 18:1 is doubled and 18:2 and 18:3 are slightly elevated.

Table 1. FA composition of *Arabidopsis* single and double mutants

Sample	16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:1
Wild type	7.9	2.9	15.0	29.2	18.8	2.1	17.3	1.3
fae	8.8	3.3	28.2	34.6	22.5	0.3	0.2	0
fad2	6.4	2.1	66.4	1.1	1.7	1.2	16.5	0.8
fad2 fae	6.0	1.8	86.9	0.2	1.8	1.0	0.9	0
fad3	6.5	1.9	17.2	47.0	1.3	1.4	17.8	1.8
fad3 fae	9.6	3.4	28.6	52.6	1.9	1.3	0.3	0
fab1	18.9	3.4	7.9	21.7	18.8	3.1	17.2	1.4
fab1 fae	24.4	3.2	19.3	28.0	17.7	1.2	0.4	0
fab2	7.1	7.6	8.6	26.3	21.4	6.2	16.6	1.7
fab2 fae	8.9	13.0	18.9	28.2	26.8	0	0	0

The *fae*:9A1 mutation is deficient in the elongation of 18:1 and stearic acid (18:0). *fab1*:1A9 (fatty acid biosynthesis) is elevated two-fold in palmitic acid (16:0) and decreased by one-half in 18:1; an obvious loss of function is not apparent. *fab2*:2A11 is elevated two- to three-fold in 18:0 and decreased by one-half in 18:1; it may be deficient in desaturation of 18:0.

Crosses of *fae*:9A1 with each of the other single FA mutants mentioned above were performed. Double FA mutants were obtained by analyzing FA composition in segregating populations of selfed F_2 plants derived from the selfed F_1s. Table 1 indicates the FA phenotypes of the double mutants. The double mutant *fad2 fae* has one of the highest percentages of oleic acid (87%) ever reported in plant seeds. This mutant resembles a *fad2* deficient mutant that is unable to elongate FAs beyond C18, with the chief result of approximately 87% 18:1 (the approximate sum of 66.4 and 16.5%) and the almost complete elimination of 18:2, 18:3, arachidic acid (20:0), eicosenoic acid (20:1), and erucic acid (22:1), The double mutant *fad3 fae* follows a similar pattern and looks like a *fad3* mutant deficient in C18 elongation. It has 18:1 elevated to about 29% with a slight increase in 18:2, while 18:3 and FAs > C18 are essentially zero.

The double mutant *fab1 fae* has the highest percentage (25%) of palmitic acid (16:0) yet seen in cruciferous seeds (Kumar and Tsunoda 1980). With respect to the *fab1* parent, the double mutant is elevated in 18:1 and 18:2, in addition to 16:0, and is very much reduced in FAs > C18. A comparison between the wild-type and the double mutant FA composition is interesting. The net effect of the double mutant combination is to shift the content of FAs > C18 into the palmitic acid content. The last double mutant synthesized, *fab2 fae*, has the highest percentage (13%) of stearic acid (18:0) ever reported in cruciferous seeds. Comparing *fab2 fae* to its *fab2* parent, it is approximately doubled in 18:0 and 18:1, whereas FAs > C18 are undetectable.

Assignment of genotypes

All of the single mutants used in the study exhibit incomplete or codominance when crossed to wild type. Because of this feature, genotypes can be assigned on the basis of the FA phenotype. For example, in segregating progeny derived from selfed *fad3*/+ plants, one can distinguish the homozygous mutant and wild-type individuals from the heterozygotes on the basis of the percentage of 18:3. For *m*/*m* individuals the amount of 18:3 is 1–2%, for *m*/+ it is 8–11%, and for +/+ it is 16–18% (James and Dooner 1990). Similarly, *fae* genotypic classes can be assigned on the basis of phenotypes. With *fae* mutants the concentration of eicosenoic acid (20:1) is diagnostic, because mutants deficient in elongation of FAs > C18 show an almost complete loss of 20:1. The percentage of

Table 2. F_2 segregation data for FA composition obtained by selfing a *fad3*/+; *fae*/+ double heterozygote

Phenotype (average % FA concentration ±SE)					Assigned genotype		No. of individuals	Expected ratio
18:1	18:2	18:3	20:0	20:1	*fad3*	*fae*		
13.6±1.2	31.3±1.4	17.6±0.9	2.5±0.5	17.4±0.8	+/+	+/+	6	1
20.7±1.3	32.5±1.4	17.9±1.9	1.9±0.4	10.3±0.7	+/+	+/m	25	2
28.9±1.5	35.4±0.9	17.5±1.4	0.8±0.4	0.1±0.2	+/+	m/m	9	1
15.9±1.9	36.5±2.4	10.7±2.0	2.6±0.5	16.8±0.6	+/m	+/+	18	2
21.3±1.6	39.4±1.3	10.3±1.2	1.8±0.4	10.4±0.9	+/m	+/m	28	4
28.9±1.3	41.9±0.6	11.8±0.9	1.1±0.2	0.3±0.2	+/m	m/m	12	2
16.4±0.9	44.7±0.8	1.8±0.1	2.7±0.2	16.9±0.7	m/m	+/+	5	1
21.0±1.4	47.8±1.0	1.9±0.2	2.0±0.4	10.6±0.9	m/m	+/m	9	2
27.9±2.9	51.7±1.7	1.8±0.2	1.1±0.3	0.1±0.2	m/m	m/m	7	1
							119	16

Table 3. F_2 segregation data for FA composition obtained by selfing a *fab1*/+; *fae*/+ double heterozygote

Phenotype (average % FA concentration ±SE)							Assigned genotype		No. of individuals	Expected ratio
16:0	18:0	18:1	18:2	18:3	20:0	20:1	*fab1*	*fae*		
8.5±0.3	3.1±0.2	14.5±1.0	30.6±1.5	17.6±1.8	2.7±0.3	18.0±0.9	+/+	+/+	12	1
9.3±0.4	3.5±0.3	20.0±1.4	33.0±1.4	17.8±1.5	2.0±0.3	10.8±1.0	+/+	+/m	20	2
10.0±0.4	4.0±0.2	27.8±1.4	35.5±1.1	19.4±1.5	1.2±0.1	0.3±0.1	+/+	m/m	11	1
12.7±0.7	3.1±0.3	12.4±1.2	28.9±1.3	16.3±1.2	3.0±0.3	17.2±1.0	+/m	+/+	26	2
13.5±0.6	3.2±0.4	17.1±1.4	30.4±1.5	17.9±1.5	2.1±0.3	11.0±0.8	+/m	+/m	42	4
14.9±0.7	3.5±0.3	25.3±1.1	32.1±1.5	19.9±1.8	1.1±0.1	0.3±0.1	+/m	m/m	17	2
19.4±1.0	3.2±0.3	8.8±1.0	23.7±1.4	16.8±1.6	3.3±0.2	16.4±0.8	m/m	+/+	10	1
20.7±0.9	3.1±0.3	13.2±1.2	25.8±1.5	18.0±1.7	2.3±0.4	10.2±0.7	m/m	+/m	20	2
23.7±0.7	2.8±0.2	19.0±1.1	28.2±1.2	19.4±1.1	1.1±0.1	0.3±0.1	m/m	m/m	8	1
									166	16

20:1 in segregating progeny from seed of selfed *fae*/+ plants is normally 16–18% for +/+ individuals, 9–11% for *m*/+ individuals, and <1% for *m*/*m* individuals (data not shown).

In the segregation of progeny from selfed individuals from the cross *fad3*×*fae* (Table 2), the 18:3 and 20:1 concentrations assort independently and at levels comparable to the single mutants above, indicating that these genes are not linked ($\chi^2=11.4$; $0.1<P<0.25$). Within each of the three 18:3 phenotypes, three 20:1 phenotypes can be recognized (and vice versa; notice also that the 20:0 concentration is proportional to the 20:1 concentration in the different classes). For example, within the phenotypic class having an 18:3 content of 1–2% (*fad3* *m*/*m* genotype), there are three 20:1 phenotypic classes (16–18%, 9–11% and <1%), which conform to the phenotypic assignments of the single *fae* mutants (above).

A similar situation can be seen in Table 3. Selfed progeny from the cross *fab1*×*fae* were scored for phenotypes based on palmitic acid (16:0) and 20:1 concentration. Within each 16:0 phenotypic class, three 20:1 classes can be recognized. For example, within the 8–10% 16:0 phenotypic class, the three familiar 20:1 phenotypic classes can be readily observed. Tables 2 and 3 therefore demonstrate that with these double FA mutants, genotypes can be assigned on the basis of phenotypes. The best demonstration of this is that all expected classes of individuals were recovered from each of the crosses in Table 1, and in each case the number of the individuals observed in each genotypic class falls within the ratios expected for two genes segregating independently ($\chi^2=3.2$: $P>0.95$). From these data it is clear that none of the genes *fad2*, *fad3*, *fab1*, *fab2* are linked to *fae*. *fab1* and *fab2* were crossed and, although the double mutant was not recovered, these two genes were not linked either.

The physical characteristics of the individual FA mutants in comparison to the wild-type plants varied depending upon the FA mutation. *fad3*:G30 and *fae*:9A1 are very similar in appearance to the wild-type *Arabidopsis thaliana* 'Columbia', except that in *fae*:9A1 the siliques tend to be slightly shorter than the wild type. Figure 1 A shows a typical *fae*:9A1 plant. In comparison

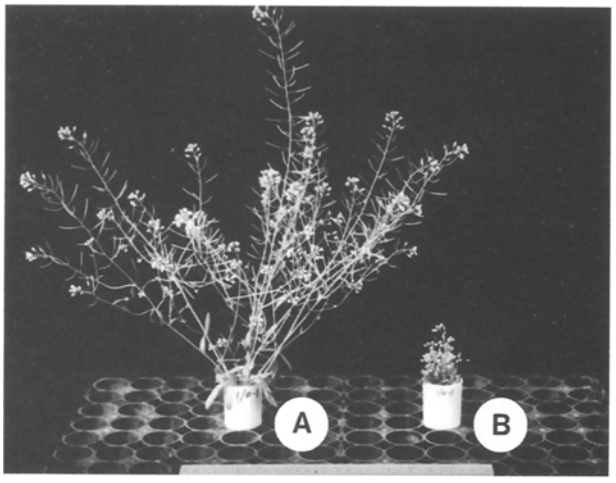

Fig. 1 A and B. Comparison of *fae*:9A1 single mutant (**A**) with *fae*:9A1 *fad2*:4A5 double mutant (**B**)

to the wild type, *fad2*:4A5 is stunted, bushier, and produces smaller siliques with fewer seeds per silique. *fab1*:1A9 appears to be more branched than wild type and does not obtain the height and vigor of the wild type. *fab2*:2A11 is severely stunted. After delayed germination it produces a small rosette of leaves. It eventually produces one or two racemes, with a limited number of siliques.

Whether the growth and development of the plants is a consequence of the FA mutation alone or of other closely linked mutations needs to be addressed. The FA mutants were originally obtained by EMS mutagenesis followed by selfing and selection of five or six generations to allow other characters to segregate away. This type of mutagenic program usually leads to multiple lesions, and therefore we cannot completely rule out other closely linked mutations. We are reasonably certain, however, that the FA mutations are connected with growth characteristics of the plants, since different alleles of both *fad2* and *fab1* have similar growth habits. With virtually all of the double FA mutant pairings, both the FA phenotypes and the growth habits of the plants appear to be expressed in the same additive manner. An example is shown in Fig. 1 B. The *fae fad2* double mutant resembles the weaker *fad2* single mutant parent in appearance (results not shown).

We have shown that a wide range of FA compositions can be obtained by combining different single FA mutations. All of the resulting plants complete their life cycle under the same conditions that wild-type plants are grown. Some mutants are indistinguishable from the wild type, while several of the plants are compromised with respect to their growth habit (which may be related to the FA mutation). In such instances where loss of function of a FA gene throughout the plant results in a decrease of vigor, one could inhibit its expression only in developing seeds by employing, for example, a tissue-specific promoter to drive the antisense/sense construct. These sorts of considerations will be useful in designing DNA constructs for plant transformation experiments where specific seed FA compositions are desired.

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Note added in proof

fae: 9A1 is allelic with *fae*1 of Lemieux, et al. (1990).

References

Bubeck DM, Fehr WR, Hammond EG (1989) Inheritance of palmitic and stearic acid mutants of soybean. Crop Sci 29:652–656

Green AG, Marshall DR (1984) Isolation of induced mutants in linseed (*Linum usitatissimum*) having reduced linolenic acid content. Euphytica 33:321–328

James DW, Dooner HK (1990) Isolation of EMS-induced mutants in *Arabidopsis* altered in seed fatty acid composition. Theor Appl Genet 80:241–245

Johnson RW, Fritz E (1989) Fatty acids in industry. Marcel Dekker, New York, 659 pp

Krol AR van der, Mol JNM, Stuitje AR (1988) Modification of eukaryotic gene expression by complementary RNA or DNA sequences. BioTechniques 6:958–967

Krol AR van der, Mur LA, Beld M, Mol JMN, Stuitje AR (1990) Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell 2:291–299

Kumar PR, Tsunoda S (1980) Variation in oil content and fatty acid composition among seeds from the Cruciferae. In: Tsunoda S, Hinata K, Gomez-Campo C (eds) *Brassica* crops and wild allies. Japan Scientific Societies Press, Tokyo, pp 235–252

Lemieux B, Miquel M, Somerville C, Browse J (1990) Mutants of *Arabidopsis* with alterations in seed lipid fatty acid composition. Theor Appl Genet 80:234–240

Napoli C, Lemieux C, Jorgensen R (1990) Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes *in trans*. Plant Cell 2:279–289

Stefansson BR, Hougen FW, Downey RK (1961) Note on the isolation of rape plants with seed oil free from erucic acid. Can J Plant Sci 41:218–219