Plant Physiol. (1995) 108: 399–409

Alteration of Seed Fatty Acid Composition by an Ethyl Methanesulfonate-Induced Mutation in *Arabidopsis thaliana* Affecting Diacylglycerol Acyltransferase Activity[1]

Vesna Katavic, Darwin W. Reed, David C. Taylor*, E. Michael Giblin, Dennis L. Barton, Jitao Zou, Samuel L. MacKenzie, Patrick S. Covello, and Ljerka Kunst[2]

National Research Council of Canada, Plant Biotechnology Institute, 110 Gymnasium Place, Saskatoon, Saskatchewan, Canada S7N 0W9

In characterizing the enzymes involved in the formation of very long-chain fatty acids (VLCFAs) in the Brassicaceae, we have generated a series of mutants of *Arabidopsis thaliana* that have reduced VLCFA content. Here we report the characterization of a seed lipid mutant, AS11, which, in comparison to wild type (WT), has reduced levels of 20:1 and 18:1 and accumulates 18:3 as the major fatty acid in triacylglycerols. Proportions of 18:2 remain similar to WT. Genetic analyses indicate that the fatty acid phenotype is caused by a semidominant mutation in a single nuclear gene, designated *TAG1*, located on chromosome 2. Biochemical analyses have shown that the AS11 phenotype is not due to a deficiency in the capacity to elongate 18:1 or to an increase in the relative Δ15 or Δ12 desaturase activities. Indeed, the ratio of desaturase/elongase activities measured in vitro is virtually identical in developing WT and AS11 seed homogenates. Rather, the fatty acid phenotype of AS11 is the result of reduced diacylglycerol acyltransferase activity throughout development, such that triacylglycerol biosynthesis is reduced. This leads to a reduction in 20:1 biosynthesis during seed development, leaving more 18:1 available for desaturation. Thus, we have demonstrated that changes to triacylglycerol biosynthesis can result in dramatic changes in fatty acid composition and, in particular, in the accumulation of VLCFAs in seed storage lipids.

The fatty acyl composition of seed TAGs determines their physical and chemical properties and, thus, their use in edible oil or industrial applications. TAG composition depends on the interaction of several different groups of enzymes in the lipid biosynthesis pathway. The enzymes of the fatty acid synthase complex in the plastids of developing seeds are responsible for the biosynthesis of fatty acids up to and including oleic acid. Modifying enzymes, such as the extraplastidic Δ12 and Δ15 desaturases, elongases, hydroxylases, and epoxidases, yield polyunsaturated, very long-chain, hydroxy-, and epoxy- fatty acids, respectively. Acyltransferases insert specific acyl moieties onto the glycerol backbone to yield TAGs via the Kennedy pathway (Murphy, 1993; Ohlrogge, 1994).

Most oilseed crops accumulate a limited range of fatty acids in their seed oil. Just six fatty acids contribute more than 95% of world production (Schmid, 1987). Many other fatty acids are of considerable interest as renewable feedstocks for chemical industries. These include the VLCFAs, behenic (20:0), eicosenoic (20:1), and erucic (22:1), characteristic of the seed oils of a number of species within the Brassicaceae. There are currently more than 1000 patented applications for C_{22} oleochemicals and their derivatives (Sonntag, 1991; Taylor et al., 1992c; N.O.V. Sonntag, personal communication). Research to maximize the VLCFA content of industrial rapeseed, for example, is an important effort being pursued in several biotechnology and breeding laboratories (Taylor et al., 1992c; Murphy, 1993).

In the course of characterizing the enzymes involved in the formation of VLCFAs in the Brassicaceae, we have generated a series of mutants of *Arabidopsis thaliana* that are deficient in the accumulation of VLCFAs (Kunst et al., 1989, 1992a, 1992b). Detailed analyses of these mutants contribute to our understanding of the organization of the elongases and their role in the lipid bioassembly pathway. Furthermore, with the development of *Arabidopsis* as a model organism for plant molecular genetics, it is possible to clone genes in the lipid pathway affected by mutations, using the techniques of chromosome walking from RFLP

[1] This is National Research Council of Canada Publication No. 38917.

[2] Present address: Department of Botany, University of British Columbia, 3529–6270 University Blvd., Vancouver, BC, Canada V6T 1Z4

* Corresponding author; e-mail dtaylor@pbi.nrc.ca; fax 1–306–975–4839.

Abbreviations: ACCase, acetyl-CoA carboxylase (EC 6.4.1.2); ACP, acyl carrier protein; cM, centiMorgan; DAG, *sn*-1,2-diacylglycerol; DGAT, *sn*-1,2 diacylglycerol acyltransferase (EC 2.3.1.20); Δ15 desaturase, linoleate (ω-3) desaturase; Δ12 desaturase, oleate (ω-6) desaturase; d.p.a., days postanthesis; EMS, ethyl methanesulfonate; FAME, fatty acid methyl ester; G-3-P, glycerol-3-phosphate; GPAT, glycerol-3-phosphate acyltransferase (EC 2.3.1.15); KAS II, 3-ketoacyl-ACP synthetase II; LPA, lyso-phosphatidic acid; MAG, monoacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; RFLP, restriction fragment length polymorphism; TAG, triacylglycerol; VLCFA, very long-chain (>C_{18}) fatty acid; WT, wild type; 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid, *cis*-Δ9-octadecenoic acid; 18:2, linoleic acid, *cis*-Δ9, Δ12-octadecadienoic acid; 18:3, α-linolenic acid, *cis*-Δ9, Δ12, Δ15-octadecatrienoic acid; 20:0, eicosanoic acid; 20:1, *cis*-Δ11-eicosenoic acid; 20:2, *cis*-Δ11, Δ14 eicosadienoic acid; 22:0, docosanoic acid; 22:1, erucic acid, *cis*-Δ13-docosenoic acid.

sites (Meyerowitz, 1987; Arondel et al., 1992; Dean, 1993) or T-DNA tagging (Dean, 1993; Yadav et al., 1993). One mutant, AC56, has been shown to be deficient in elongase activity (Kunst et al., 1989, 1992b). Here we report the detailed characterization of another mutant, AS11, which has reduced 20:1 and 18:1 and, instead, accumulates 18:3. The results suggest that, rather than a deficiency in the capacity to elongate 18:1, this mutant has a reduced diacylglycerol acyltransferase activity that results in modulation of 20:1 biosynthesis. Thus, we have shown for the first time in *A. thaliana* that changes to lipid bioassembly at the level of the Kennedy pathway can result in dramatic changes in extraplastidic fatty acid modification.

MATERIALS AND METHODS

Plant Material

Several populations of *Arabidopsis thaliana* (L.) Heynh. (ecotype Columbia; WT) were mutagenized separately by soaking the seeds (M_1) for 16 h in 0.3% (v/v) EMS as described by Haughn and Somerville (1986). M_1 seeds were germinated, and plants were grown to maturity and allowed to self-fertilize to produce M_2 seeds. Approximately 9000 randomly chosen M_2 seeds were planted, and plants were again self-fertilized and then harvested individually to give several thousand M_3 families. M_3 seed samples (20–30 seeds) were treated with methanolic HCl (see below) to digest the tissue and convert the fatty acids present in the seed oil to the corresponding FAMEs. The FAME samples were then screened by GC for changes in acyl composition as described by Kunst et al. (1992b). Families with altered VLCFA content were retained. Fifteen seeds from each potentially interesting M_3 family were planted and harvested separately. The FAME composition of a number of M_4 seeds from each of the 15 M_3 plants was analyzed to determine whether the trait was stably inherited and was segregating and to isolate a homozygous line. Selected lines were backcrossed to the WT at least twice before being used for analyses. All plants were grown in growth chambers under continuous fluorescent illumination (150–200 μE m^{-2} s^{-1}) at 22°C on Terra-lite Redi-earth (W.R. Grace and Co., Canada Ltd., Ajax, ON, Canada). Under these growth conditions, one generation took 8 to 10 weeks.

Linkage tester lines in ecotype Landsberg *erecta* were utilized in preliminary investigations to map the mutation. The W-100 line contained two visible markers for each of the five *A. thaliana* chromosomes (Koornneef and Hanhart, 1983; Koornneef et al., 1983, 1987). Line PBI1 contained multiple chromosome 2 markers *sti*, *cp2*, *er*, *as*, and *cer8*. The genetic distances between the various loci were assessed with the computer program LINKAGE-1 (Suiter et al., 1983). The estimates of the recombination percentages (r) for double crossovers were corrected using the Kosambi mapping function, converting them to map distances (D) in cM: $D = 25\ln(100 + 2r/100 - 2r)$ with SD $= 2500s_r/2500 - r^2$ (where s_r is the SD of r) (Koornneef et al., 1983).

Studies of the germination responses of WT and AS11 seeds to exogenously supplied ABA were performed as described by Finkelstein and Somerville (1990), and tests of seed dormancy using developing seeds isolated from WT and AS11 siliques were conducted as described by Karssen et al. (1983).

Substrates and Reagents

[1-^{14}C]oleic acid (58 mCi mmol^{-1}) was purchased from Amersham Canada, Ltd. (Oakville, ON), and [1-^{14}C]erucic acid (52 mCi mmol^{-1}), [1-^{14}C]sodium acetate (59 mCi mmol^{-1}), and L-α-palmitoyl-2-[1-^{14}C]oleoyl-PC (58 mCi mmol^{-1}) were purchased from NEN Research Products (Mississauga, ON). [1-^{14}C]Eicosenoic acid was synthesized as described previously (Kunst et al., 1992b). 1-^{14}C-labeled fatty acids were converted to the corresponding acyl-CoA thioesters using the method described by Taylor et al. (1990). [1-^{14}C]16:0-ACP (56 mCi mmol^{-1}) was synthesized and kindly provided by Dr. John Ohlrogge (Michigan State University). Specific activities were adjusted as required by diluting with authentic unlabeled standards. Unlabeled acyl-CoAs, sodium acetate, ATP, CoA-SH, NADH, NADPH, polyvinylpolypyrrolidone, (±)-ABA, polar lipid standards, and most other biochemicals were purchased from Sigma. Neutral lipid standards were obtained from NuChek Prep, Inc. (Elysian, MN), and FAME standards were supplied by Supelco Canada, Ltd. (Oakville, ON). Mixed TAG and DAG standards for GC, which were not commercially available, were synthesized from the corresponding DAGs or MAGs by condensation with the appropriate acyl chloride and purified as described by Taylor et al. (1991). HPLC-grade solvents (Omni-Solv, BDH Chemicals, Toronto, ON) were used throughout these studies.

Harvests of Developing and Mature Seed, Leaf, and Root Tissue

In all analyses discussed hereafter, the stages of seed development examined (in d.p.a) are described as: "milky" (11 d.p.a. for both WT and AS11), "early green" (13 d.p.a. for WT, 16 d.p.a. for AS11), "green" or "mid-development" (15 d.p.a. for WT, 21 d.p.a. for AS11), "green-brown" (19 d.p.a. for WT, 25 d.p.a. for AS11), and "mature" dry (21 d.p.a. for WT, 28 d.p.a. for AS11). Developing seeds at the desired stages were harvested from siliques and used immediately or frozen in liquid nitrogen for further analysis. Root tissue was obtained from 4-week-old plants grown in liquid culture using a protocol adapted from Valvekens et al. (1988). Fresh leaf tissue was harvested from 4-week-old seedlings and FAME analyses were performed immediately.

Fatty Acid and Lipid Analyses

In some cases, lipids were saponified by treatment of tissues or reaction mixtures directly with 10% methanolic-KOH at 80°C for 2 h. The samples were then acidified with 6 N HCl, and the free fatty acids were extracted with hexane. Following removal of the hexane, the fatty acids were converted to their methyl esters for GC analysis. For FAME analyses, *A. thaliana* M_4 seed, leaf, or root tissues, intact lipid species, or saponified free fatty acid samples were treated with 3 N methanolic-HCl (Supelco Canada,

Ltd.), at 80°C for 2 h after the addition of 1 to 10 μg of 17:0 free fatty acid as an internal standard. FAMEs were extracted and analyzed by GC on a DB-23 column as described previously (Kunst et al., 1992b).

Total lipid extracts were prepared from seeds at the milky and green stages of development and from mature seed, and individual polar and neutral lipid classes were separated and recovered as described by Taylor et al. (1991, 1992a). Following purification by chromatography on silica gel G columns, TAG or DAG samples were spiked with trierucin or 1,2-dierucin as the internal standard, respectively. TAG and DAG separations were performed using a 3-m-wide bore DB-1 column on a Hewlett-Packard 5890 series II GC under the following conditions: injector temperature, 350°C; detector temperature, 375°C; program: 200 to 350°C at 10°C/min, then isothermal for 5 to 10 min; column flow 10 mL/min at a split vent ratio of 10:1. Acyl composition assignments were based on retention times of standards and confirmed by MS. Intact TAGs were analyzed by NH_4^+-CI-MS and product ion MS/MS analyses as described by Taylor et al. (1995). Stereospecific analyses of TAGs were performed using both pancreatic lipase and a Grignard-based method in which MAGs were generated, followed by a chiral separation of di-dinitrophenylurethane-MAG derivatives as reported previously (Taylor et al., 1994, 1995).

Acetate Labeling (in Vivo Studies)

Developing seeds at the green mid-development stage were harvested from 50 siliques and placed immediately in beakers chilled on ice. Intact seeds of a known fresh weight were placed in 10-× 16-mm glass tubes and 40 μM [1-^{14}C]sodium acetate (59 nCi nmol^{-1}), 100 mM Hepes-NaOH, and 320 mM Suc were added to a final volume of 2 mL at pH 7.4. Tubes were shaken at 100 r.p.m. and 30°C under incandescent light (10,000 Lux at the level of the seeds) for 18 h. Following incubation, the medium was aspirated away, seeds were washed several times with 2-mL aliquots of distilled water, and then lipids were saponified or extracted and analyzed as described above.

In Vitro Studies

Cell-free homogenates were prepared from developing green WT and AS11 seeds as described by Kunst et al. (1992b) and adjusted to equivalent protein concentrations with grinding medium. Elongase assays were performed using 40 μM [1-^{14}C]oleoyl-CoA (10 nCi nmol^{-1}) and 1 mM malonyl-CoA with all other reaction conditions as described by Kunst et al. (1992b). Desaturase assays were performed by incubating 150 to 250 μg of homogenate protein with 75 mM Hepes-NaOH, pH 7.4, 1 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM CoA-SH, 1 mM NADH, and 40 μM [1-^{14}C]oleoyl-CoA (10 nCi nmol^{-1}) or 10 μM L-α-palmitoyl-2-[1-^{14}C]oleoyl-PC (58 nCi nmol^{-1}), in a final volume of 0.5 mL in open glass tubes for 30 min at 30°C and 100 r.p.m. For the comparative in vitro elongase and desaturase assays, homogenates prepared from developing green seeds of lines AC56 (elongase mutant) and AJ70 (Δ15 desaturation mutant) were used as controls. KAS II assays were conducted by monitoring the conversion of [1-^{14}C]16:0-ACP (56 nCi nmol^{-1}) to [1-^{14}C]18:0-ACP in the presence of 100 μM malonyl-CoA, 0.5 mM NADH, 0.5 mM NADPH at 30°C and 100 r.p.m., adapted from the method reported by Jaworski et al. (1974). Radiolabeled fatty acyl products of the elongase, desaturase, and β-KAS-II reactions were isolated by saponifying the reaction mixture and converting the fatty acids to FAMEs for analysis by radio-HPLC as described by Kunst et al. (1992b). ACCase assays of developing seeds were carried out using the extraction and assay methods described by Parker et al. (1990). Biosynthesis of glycerolipids via the Kennedy pathway was monitored by supplying homogenates prepared from green developing seeds with G-3-P and radiolabeled acyl-CoAs, followed by separation and quantitation of lipid species as described previously (Taylor et al., 1991, 1992a). Direct assays of DGAT activity were performed by supplying homogenates prepared from developing seeds at various developmental stages with emulsified 1,2-diolein as acyl acceptor, and various [1-^{14}C]acyl-CoAs as acyl donors, followed by radio-HPLC measurement of intact labeled TAGs as described by Weselake et al. (1991). Homogenate protein concentrations were measured by the method of Bradford (1976).

Northern Analyses for Δ12 Desaturase and Δ15 Desaturase Gene Expression

Total RNA was extracted from green developing WT and AS11 seeds using the method of Lindstrom and Vodkin (1991). RNA samples were denatured with formaldehyde and separated on 1.2% formaldehyde agarose gels. The amount of total RNA loaded per lane was calibrated by the ethidium bromide-staining intensity of the rRNA bands. The RNA was transferred onto a Zeta Probe nylon membrane (Bio-Rad) and hybridized with ^{32}P-labeled Δ12 or Δ15 desaturase probes according to the manufacturer's protocol.

A plasmid pBNDES3 carrying a cDNA clone of the *Brassica napus* homolog of the *Arabidopsis fad3* (Δ15 desaturase) gene was obtained from the Arabidopsis Biological Resource Center at the Ohio State Biotechnology Center (Columbus, OH). The open reading frame of the *fad2* (Δ12 desaturase) gene (Okuley et al., 1994) was PCR amplified from *A. thaliana* DNA (using the oligonucleotides GCCGAATTCATGGGTGCAGGTGGAAGA and GCCGAATTCCACCATCATGCTCATAACT) and cloned into the plasmid pSE936 (Elledge et al., 1991). The cDNA inserts were excised, and the DNA fragments were purified with the GENECLEAN II kit (Bio 101 Inc., LaJolla, CA), labeled with ^{32}P using the Gibco BRL Random Primers DNA Labeling System as described by the manufacturer (Gibco BRL Life Technologies), and used as probes in northern analyses.

RESULTS AND DISCUSSION

Mutant Isolation and Fatty Acid and Genetic Analyses

Direct GC analysis of 2000 M_3 families resulted in the isolation of 35 mutants with altered seed fatty acid com-

position. Most of the mutants exhibited one of three distinct phenotypes: deficiencies in VLCFA biosynthesis (line AC56), Δ12 desaturation (line AL63), or Δ15 desaturation (line AJ70). The biochemical and genetic characterization of mutant AC56 and its allelles (mutants AK57, AT59, and AX33) was reported previously (Kunst et al., 1992a, 1992b; Taylor et al., 1992b). In AC56, a single semidominant nuclear mutation in the *FAE1* gene resulted in a deficiency in acyl chain elongation from 18:1 to 20:1 and 22:1 and from 18:0 to 20:0. As a result, the proportion of VLCFAs decreased and that of 18:1 increased dramatically.

In the present study, an unusual mutant, AS11, originally reported in 1989 (Kunst et al., 1989), was further characterized. AS11 had much reduced proportions of 20:1 and 18:1, whereas the proportion and absolute amounts of 18:3 dramatically increased (Fig. 1). The proportion of 18:2 remained essentially identical with that of WT. In contrast to the AC56 mutant, the levels in AS11 of the other VLCFAs, 20:0, 22:0, and 22:1, were not reduced, and 20:2 was, in fact, significantly elevated. The ratio of 18:3/18:2 in AS11 seed was 1.39 ± 0.02, whereas in WT seed, it was 0.65 ± 0.01. In having an elevated 18:3/18:2 ratio, AS11 is somewhat similar to a mutant JB11 (*ela1*, enhanced linolenate accumulation; 18:3/18:2 ratio 1.04 ± 0.10) previously described by Lemieux et al. (1990). However, in contrast to JB11, AS11 has a drastically reduced proportion of 20:1; in JB11 this

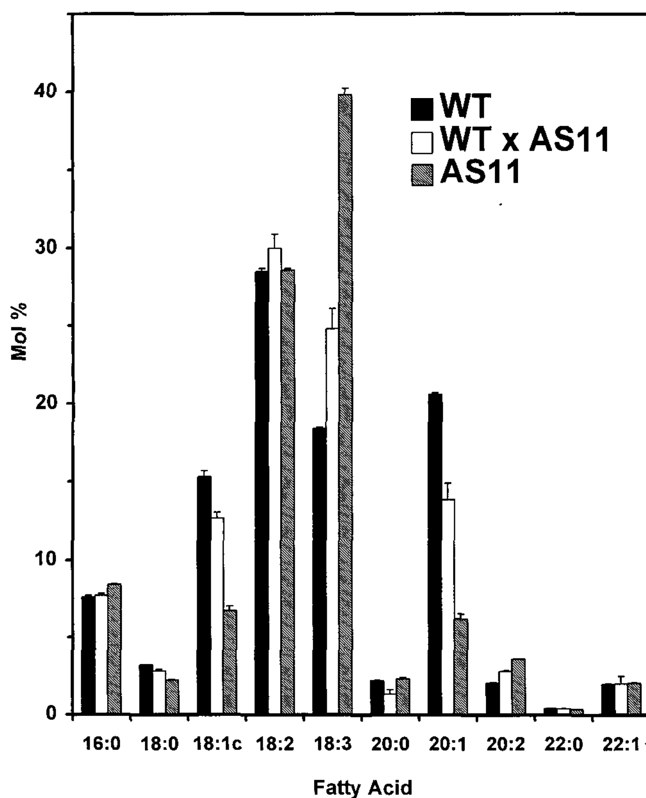

Figure 1. Fatty acid composition of total lipids in mature *A. thaliana* seed of WT, AS11, and WT × AS11 F_1 progeny. Values are shown ± SE (n = 12). 18:1c is primarily the *cis*-Δ9 isomer, oleic acid (greater than 90%) plus a small amount (less than 10%) of the *cis*-Δ11 isomer, vaccenic acid.

fatty acid is unaffected. Furthermore, in the present study, the mutation in AS11 was found to be seed specific, with no significant effect on the fatty acid composition of leaf or root lipids (data not shown). In WT and AS11 leaves, the 18:3/18:2 ratios were 3.30 ± 0.21 and 3.46 ± 0.11, respectively, whereas in WT and AS11 roots, the respective 18:3/18:2 ratios were 0.95 ± 0.05 and 0.99 ± 0.02. In contrast, JB11, in addition to altered 18:3/18:2 seed ratios, showed small but statistically significant increases in the 18:3/18:2 ratio in leaf and root tissues, relative to WT (Lemieux et al., 1990).

To determine the inheritance of the altered fatty acid composition, 10 M_3 seeds of AS11 were sown, plants were grown to maturity and allowed to self-fertilize, and M_4 seeds were harvested and analyzed by GC. The fatty acyl composition of the AS11 M_4 seeds was virtually indistinguishable from that of the M_3 seeds, indicating that the altered fatty acid composition was heritable and that the original AS11 M_3 line was homozygous.

To determine the genetic basis for the alteration in fatty acid composition in AS11, reciprocal crosses were made to WT, and the F_1 seeds were analyzed. The F_1 seeds had intermediate levels of 20:1, 18:1, 18:3, and most other fatty acids, relative to that of the parental lines (Fig. 1, WT × AS11). The intermediacy of these values indicates partial dominance and suggests a gene dosage effect, i.e. that the amount of available gene product is limiting in AS11, resulting in these phenotypic changes in fatty acid proportions. The F_2 progenies derived from self-fertilization of several F_1 plants followed a 1:2:1 segregation pattern (WT × AS11, 56:132:70; χ^2 = 1.42; P > 0.5; degrees of freedom = 2), as expected if the altered fatty acid composition is caused by a single nuclear mutation in a gene we designate *TAG1*.

Complementation tests between AC56 and AS11 clearly indicated that the AS11 mutant represents a different complementation group, suggesting that the elongation of 18:1 in seeds is controlled by several loci. Indeed, two other mutants with reduced capacity to elongate 18:1, AK9 and BB6, were previously shown to be nonallelic, their fatty acid phenotypes being due to mutations in two separate genes that were also distinct from the gene interrupted by the mutation in line AC56 (Kunst et al., 1992b). In preliminary experiments to determine the chromosomal location of the lesion in AS11, the mutant was crossed to a multiply marked line (W-100) containing two visible markers for each of the five *A. thaliana* chromosomes (Koornneef et al., 1983, 1987). An analysis of the F_2 progeny indicated that the lesion was located on chromosome 2. This result was confirmed by analyzing F_2 progeny of a cross between the AS11 mutant and PBI1, a line that carries multiple chromosome 2 phenotypic markers: *sti*, *cp2*, *as*, *er*, and *cer-8*. Examination of 317 F_2 plants indicated that the mutation in AS11 lies in the region between the *sti* and *cp2* morphological markers, approximately 17.5 ± 3 cM from the *sti* locus and 8 ± 2 cM from the *cp2* locus. Thus, the AS11 locus is distinctly different from *FAE1*, which was shown to be about 11 cM from the *cer2* locus on chromosome 4 (Kunst et al., 1992a). However, since there is no indication that loci

belonging to specific phenotypic groups are preferentially located on specific chromosomes (Koornneef et al., 1983), it is not unexpected that loci affecting VLCFA content may be found on both chromosomes 2 and 4.

Biochemical and Analytical Characterization of the AS11 Mutant

It was observed that, although plant growth, flowering, and self-fertilization were normal in AS11, seed development was significantly retarded. Whereas WT seed was mature at 21 d.p.a., the AS11 mutant siliques were green and the seeds were still developing, maturing a full 1 week later (28 d.p.a.). In *Arabidopsis* as in other oilseeds, the major accumulation of lipid is known to occur in a well-defined and relatively short period during middle to late embryogenesis (Mansfield and Briarty, 1992). This period of maximal lipid deposition (15–18 d.p.a in WT) was also delayed in AS11 (21–25 d.p.a.). In addition, the total lipid content in mature AS11 seed (195 ± 9 μg/mg dry weight) was significantly reduced, reaching only about 75% of that observed in mature WT seed (262 ± 8 μg/mg dry weight). The fatty acid phenotype of AS11 mature seed lipids showed marked changes in the proportions of several fatty acids: 20:1 and 18:1 decreased by about 60%, whereas the proportions of 18:3 increased by 100% (Fig. 1). Similar trends were observed in the absolute amounts of these fatty acids (μg/mg dry weight) accumulating in mature seed lipids (Fig. 5C). Mature seed weights (2.00 ± 0.05 mg/100 seeds for both WT and AS11) and protein levels (WT, 106 ± 4 μg/mg dry weight; AS11, 112 ± 6 μg/mg dry weight) were not significantly different. Fresh weights and total protein for developing seed at each stage of development were comparable in WT and AS11, varying by ≤10%.

To examine fatty acid biosynthesis in vivo, fatty acid analyses were performed on total lipid fractions isolated from intact green seeds, harvested from siliques of the WT and AS11 lines at mid-development (Fig. 2A). AS11 showed a reduced proportion of 20:1 and 18:1 and an increased proportion of 18:3. Seeds at this stage of development were also incubated with [^{14}C]acetate and the ^{14}C-labeled de novo synthesized fatty acids were assayed (Fig. 2B). Relative to WT, in AS11 the proportion of [^{14}C]20:1 was decreased, whereas the proportion of [^{14}C]18:2 and 18:3 were increased slightly, although not yet at significant expense to the proportion of [^{14}C]18:1. The latter observation is consistent with the known sequential desaturation of 18:1 first to 18:2 and then 18:3. However, it does indicate that a strong decrease in the level of newly synthesized 20:1 was observed prior to a marked increase in the level of 18:3. Of equal importance, the overall level of [^{14}C]acetate incorporated into fatty acids (dpm/mg fresh weight of developing seed) was lower in AS11, averaging only 66% of the rate of incorporation observed in WT embryos. This finding is consistent with the lower lipid content in mature AS11 seed.

In having altered seed development and reduced levels of 20:1, AS11 is somewhat similar to the ABA-insensitive *A. thaliana* mutant *ABI3* (Finkelstein and Somerville, 1990). However, AS11 is also characterized by an accumulation of 18:3 as the major fatty acid, reduced levels of 18:1, and reduced storage lipid in mature seed, whereas in *ABI3*, 18:2 was the major fatty acid, 18:1 was increased, and there was no significant effect on total fatty acid content. In addition, a major characteristic of all ABA-insensitive (*ABI*) mutants is the ability of mature seeds to germinate in the presence of concentrations of exogenous ABA that fully inhibit germination in WT seeds (Finkelstein and Somerville, 1990). However, the inhibition of AS11 seed germination by ABA was similar to that of WT seeds, both being fully inhibited by 3 μM exogenous ABA. Neither did AS11 display any of the phenotypic characteristics of ABA-deficient mutants (nondormant seeds; precocious germination in siliques; Koornneef et al., 1982; Karssen et al., 1983). Dormancy tests in milky, green, and green-brown developing seeds isolated from siliques of AS11 and incubated under conditions of high RH, were negative. The AS11 locus on chromosome 2 is different from the known loci for *A. thaliana* ABA-insensitive mutants (*ABI1*, *ABI2*, and *ABI3*, on chromosomes 4, 5, and 3, respectively) and ABA-deficient mutants (chromosome 5) (Koornneef et al., 1982, 1984). Collectively, these data suggest that the AS11 mutation is not related to ABA-deficient or ABA-insensitive *A. thaliana* mutants.

Based on the fatty acid profile of mature (Fig. 1) and developing (Fig. 2) seed of AS11, a lesion in one of at least

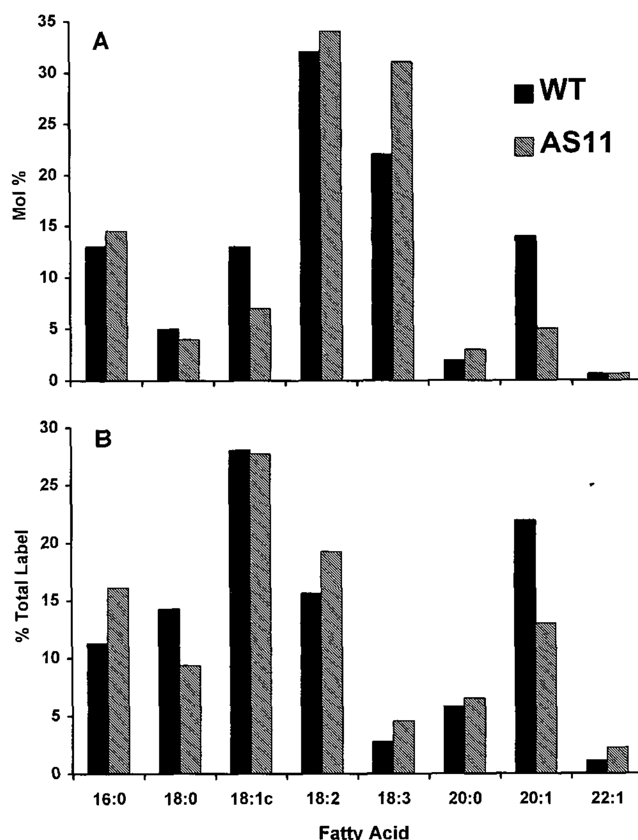

Figure 2. A, Fatty acid composition (mol %) of total lipid extracts from WT and AS11 green seeds at mid-development. B, Proportions of ^{14}C-labeled fatty acids (% total label) produced during an 18-h incubation of WT and AS11 green seeds at mid-development with [1-^{14}C]sodium acetate as described in "Materials and Methods."

five different biochemical steps of fatty acid synthesis/modification was suspected. Since 20:1 was reduced but 18:3 was elevated, the primary candidates were altered 18:1-CoA elongase or Δ15 or Δ12 desaturase activities. Changes in the activity of KAS II, which converts palmitoyl-ACP to stearoyl-ACP in seed plastids, may have caused the minor changes in the ratio of 16:0/18:0. A lesion in extraplastidial (cytosolic) ACCase (Page et al., 1994; Roesler et al., 1994) was a remote possibility but unlikely, since the proportions of other VLCFAs, which also require malonyl-CoA for chain extension, were not adversely affected in AS11.

The results of in vitro biochemical assays performed with homogenates from green developing WT and AS11 seeds at mid-development are summarized in Table I. Similar trends were also observed when data were expressed on a seed fresh weight basis (data not shown). Rather unexpectedly, the elongase activity in AS11 was about 2-fold higher than the corresponding activity in WT seeds. As suspected, the ACCase activity in both lines was not significantly different. Taken together, these results suggest that the phenotype of AS11 is not caused by a mutation directly affecting the integrity of the elongase complex or the supply of malonyl-CoA.

Desaturase activities, measured by following the conversion of ^{14}C- labeled 18:1 to 18:2 and 18:3, were also about 2-fold higher in AS11 than in WT homogenates. However, the proportion of labeled 18:2/18:3 produced was essentially identical, and, more important, the ratio of desaturase to elongase activity was about 3:1 in both the AS11 and WT lines. Northern analyses were performed to determine Δ15 desaturase and Δ12 desaturase transcript levels in green AS11 and WT seeds at mid-development (Fig. 3). The results indicated that there was no increased Δ15 or Δ12 desaturase mRNA in the AS11 mutant. Based on these findings, it is unlikely that the high 18:3/low 20:1 plus 18:1 fatty acid profile of AS11 is caused by a relative up-regulation of extraplastidic desaturase gene expression as has been suggested for mutant JB11 (Lemieux et al., 1990).

The KAS II activities of the AS11 and WT lines were identical (Table I). Thus, the small but significant change in 16:0/18:0 ratios found in AS11 was not explained by a lesion at this biochemical step.

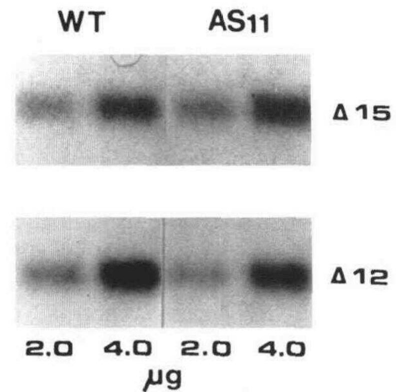

Figure 3. Northern analyses of transcripts for Δ15 and Δ12 desaturases in WT and mutant (AS11) seeds of *A. thaliana* at mid-development. Total RNA was isolated from *A. thaliana* WT and AS11 green seeds and blots were prepared as described in "Materials and Methods." The amount of RNA per lane was verified by staining the gel with ethidium bromide. The probe for Δ15 desaturase was the 1.4-kb *fad3* sequence from *B. napus.* The probe for Δ12 desaturase was the 1.4-kb *fad2* sequence from *A. thaliana.* Both probes were random-primer labeled.

Collectively, the data suggested that the primary biochemical lesion was not at the level of fatty acid biosynthesis. However, the observations of a delayed development in AS11 and lower overall lipid content in mature AS11 seed prompted us to perform more detailed studies of TAG biosynthesis in this mutant. Previous studies in *A. thaliana* and other members of the Brassicaceae had demonstrated that VLCFAs are biosynthesized by successive condensations of malonyl-CoA with 18:1-CoA or 18:0-CoA in the presence of reductant, whereas 18:1-CoA is desaturated to 18:2 and 18:3 while esterified to PC (Agrawal and Stumpf, 1985; Stymne and Stobart, 1987; Fehling et al., 1990; Taylor et al., 1991, 1992a; Kunst et al., 1992b). Then, as in other oilseeds, fatty acyl-CoAs are assembled into glycerolipids via the G-3-P (Kennedy) pathway (Kennedy, 1961; Barron and Stumpf, 1962; Stymne and Stobart, 1987; Kunst et al., 1992b; Taylor et al., 1992c; Taylor and Weber, 1994).

GC analyses of the neutral lipids (primarily TAGs and DAGs) in mature AS11 and WT seeds revealed a reduced

Table I. *Comparison of key fatty acid synthesis and modification enzyme activities in WT versus AS11 A. thaliana seed at mid-development*

Assays were conducted as described in "Materials and Methods." Activities reported ± SD (n = 2–4).

Enzyme(s) Assayed	Enzyme Activity in Vitro		
	WT	AS11	AS11 activity as percent of WT
	pmol min⁻¹ mg⁻¹ protein		
Elongase	135 ± 14	255 ± 12	190
Desaturase			
Total	410 ± 20	730 ± 40	180
% distribution			
[^{14}C]18:2	83.0	84.7	
[^{14}C]18:3	17.0	15.2	
Desaturase/elongase	3.0 ± 0.4	2.9 ± 0.3	n.s.d.[a]
ACCase	1520 ± 100	1380 ± 90	n.s.d.[a]
β KAS II	1.54 ± 0.10	1.58 ± 0.04	n.s.d.[a]

[a] n.s.d., Not significantly different.

TAG level in AS11 and, more important, a qualitative change in the acyl composition of the TAGs that accumulated. The TAG species present in mature seed were confirmed by NH_4^+-CI MS/MS using the method described by Taylor et al. (1995) (data not shown). The shift in the $[M + 18]^+$ peaks for all major TAG species generally reflected an increase in the 18:3 content in AS11, relative to WT, in which 18:2 predominated. Of particular note, a set of C_{56} ($[M + 18]^+ = 927$) and C_{58} ($[M + 18]^+ = 957$) TAGs containing one or two eicosenoyl moieties, respectively, which were prevalent in WT, were less intense in AS11, whereas the relative intensity of C_{52} ($[M + 18]^+ = 871$) and C_{54} ($[M + 18]^+ = 895$) TAGs, containing two or three C_{18} fatty acids, respectively, increased in AS11.

The DAG pool in mature AS11 was highly elevated, representing 8 to 12% of the total lipid fraction, whereas in WT, it was characteristically about 1% or less (Fig. 4). This suggested the possibility that the rate of conversion of DAG to TAG is limiting in AS11. Although all DAG species were increased in AS11, the proportion of DAGs containing C_{20} moieties was most affected.

An examination of the fatty acid composition of AS11 and WT seed lipids throughout development indicated that, even at the early milky stage, the accumulation of all fatty acids was decreased in AS11 (Fig. 5A). However, it is evident that the synthesis of VLCFAs was more dramatically affected than synthesis of 18:2 and 18:3 at this early stage. This supports the acetate-feeding data, discussed previously (compare Fig. 2B), which indicated that by mid-development the elongation of 18:1 to 20:1 diminished

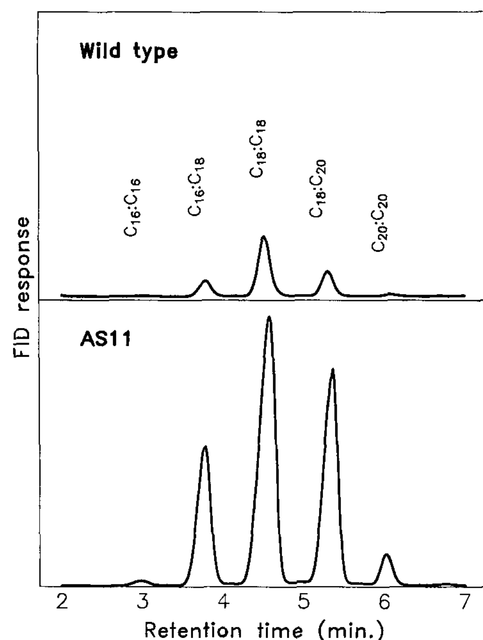

Figure 4. Comparison of WT and AS11 mature seed DAGs by GC. DAG fractions were isolated from equivalent dry weights of mature seed and purified and analyzed by GC as described in "Materials and Methods." Assignments of major DAG species were based on retention times relative to authentic standards. The internal standard (dierucin) is not shown.

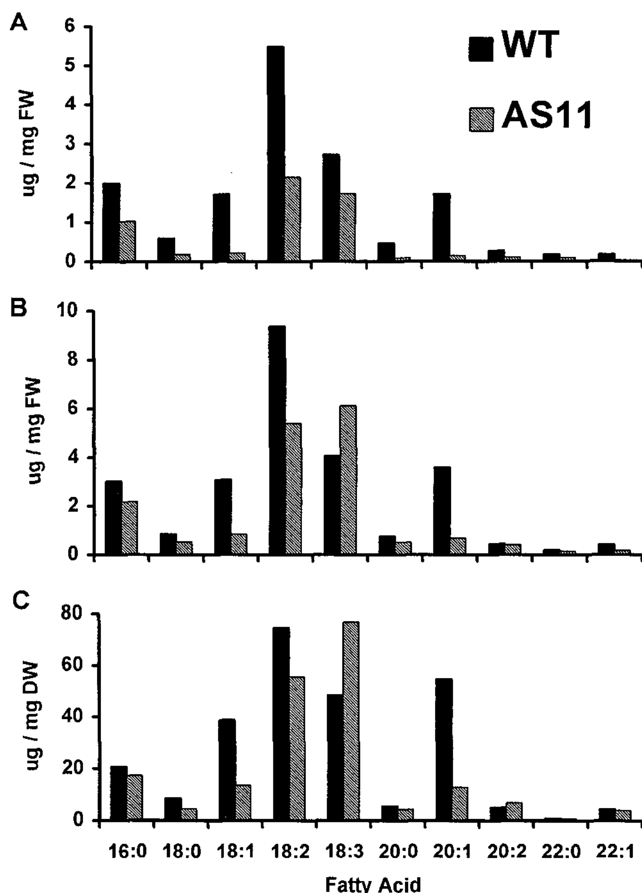

Figure 5. Fatty acyl content of WT and AS11 seed lipids at the milky (A), green (B), and mature (C) stages of development.

before desaturation of 18:1 to 18:3 had increased substantially. By the time maximum TAG deposition began in AS11 at 21 d.p.a., the acyl pool was dramatically different from that of WT seed and reflected a larger concentration of 18:3, relative to 20:1 and 18:1 (Fig. 5B). The result was that at maturity, in AS11, the levels of 20:1 and 18:1 in TAGs were decreased, whereas the level of 18:3 was increased relative to WT (Fig. 5C).

The relative proportions of the Kennedy pathway intermediates LPA, PA, and DAG, and the product TAG, as well as PC, were examined in seeds of WT and AS11 at the milky, green, and mature stages of development. At all three stages, the ratio of DAG/LPA plus PA and DAG/PC increased, whereas TAG/DAG decreased in AS11 relative to WT. This trend was readily evident in green developing seed (Fig. 6A) and was equally reflected in the ratios of lipid species produced by intact seeds at this developmental stage, during labeling with [^{14}C]acetate (Fig. 6B). There was a concomitant increase in all fatty acids in the DAG pool of AS11 seeds at mid-development, and, to a lesser extent, there was an associated backup of fatty acids into the PC pool (data not shown). TAG accumulation lagged throughout development in AS11, and this was evident even at the earliest milky stage. The TAG/DAG ratio in AS11 was consistently 3- to 5-fold lower than in WT at each

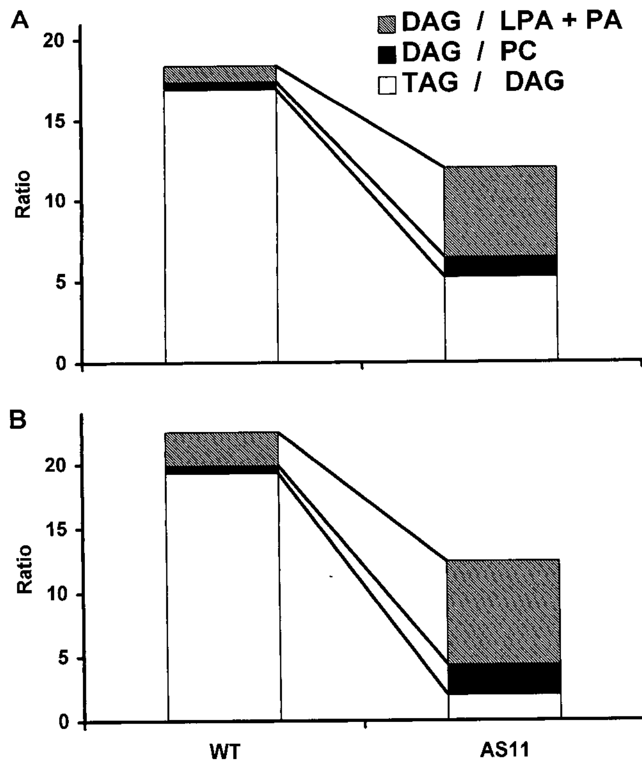

Figure 6. A, Relative proportions of lipid species present in WT and AS11 green developing seeds. Ratios were derived by comparing the levels of all Kennedy pathway intermediates on a μg/mg fresh weight basis. B, Relative proportions of radiolabeled lipid species synthesized during an 18-h incubation of WT and AS11 green seeds at mid-development with [1-^{14}C]sodium acetate as described in "Materials and Methods." Ratios were calculated by comparing the levels of all radiolabeled Kennedy pathway intermediates, TAG and PC, on a dpm/mg fresh weight basis.

developmental stage tested (Table II). This finding, combined with the increase in the levels of DAG relative to other Kennedy pathway intermediates (Fig. 6), strongly suggested that AS11 possessed a lesion that resulted in decreased DGAT activity.

This was confirmed by an in vitro study in which homogenates from WT and AS11 green seeds at mid-development were compared for their ability to incorporate [^{14}C]18:1-CoA into glycerolipids in the presence of G-3-P. 18:1-CoA was chosen as the acyl donor because previous stereospecific analyses of WT *A. thaliana* had shown that 18:1 was incorporated into all three *sn* positions in about equal proportions (*sn*-1, 30%; *sn*-2, 37%; *sn*-3, 33%), suggesting that the Kennedy pathway acyltransferases had similar affinities for 18:1-CoA (Taylor et al., 1995). Labeled DAG/LPA plus PA and DAG/PC ratios remained essentially unchanged between WT and AS11, indicating that the activities of the Kennedy pathway enzymes GPAT, LPA acyltransferase (EC 2.3.1.51), and PA phosphatase (EC 3.1.3.4), as well as CDP-choline:*sn*-1,2-diacylglycerol choline phosphotransferase (EC 2.7.8.2), were similar in the WT and AS11 homogenates. However, the ratio of labeled TAG/DAG was strongly reduced in the AS11 homogenate, suggesting that the DGAT activity was decreased (Fig. 7). Similar differences were observed when [^{14}C]20:1-CoA and G-3-P were supplied to AS11 and WT homogenates (data not shown).

A detailed stereospecific analysis of the TAGs from mature AS11 and WT seeds indicated that the composition at the *sn*-3 position was significantly altered in AS11 (Table III). In particular, relative to WT, there were dramatic decreases in the proportions of 18:1 and 20:1 and increases in the proportions of 18:2 and 18:3 at the *sn*-3 position in AS11 TAGs. These changes generally reflect the major differences in fatty acid content observed in mature seed TAGs (Figs. 1 and 5C) and indicate that the conversion of DAGs to TAGs is affected in AS11.

Direct DGAT assays were conducted using 1,2-diolein as an acyl acceptor and [^{14}C]18:1-CoA as the acyl donor. The results confirmed that, throughout development, the AS11 mutant had much reduced DGAT activity, which was correlated with a decreased TAG and increased DAG content (lower TAG/DAG ratio), compared to WT (Table II). However, in both WT and AS11 homogenates prepared from green seeds at mid-development, the DGAT activity was considerably higher when assayed in the presence of 20:1-

Table II. *Ratio of accumulating TAG/DAG and DGAT activities in WT and AS11 seed at various stages of development*

Developing seeds of WT and AS11 *A. thaliana* were harvested and total lipid extracts or homogenates were prepared. The relative amounts of TAG and DAG were determined as described in "Materials and Methods." DGAT activity in homogenates was assayed in the presence of 200 μM 1,2-diolein and 25 μM [1-^{14}C]18:1-CoA, with measurement of radiolabeled TAG species conducted as described by Weselake et al. (1991). SD ≤ 5% in all cases (n = 2).

Developmental Stage	TAG/DAG Ratio		DGAT Activity	
	WT	AS11	WT	AS11
			pmol min^{-1} mg^{-1} protein	
Milky	1.8	0.6	5.43	2.39
Early Green	n.d.[a]	n.d.	7.05	2.15
Green	16.9	5.3	6.80	2.00
Green-Brown	n.d.	n.d.	0.96	0.55
Mature	91.0	19.0	n.d.	n.d.

[a] n.d., Not determined.

Table III. *Stereospecific analyses of the TAG fraction of mature WT and AS11 seed of A. thaliana*

Seed	Percent Distribution of Each Fatty Acid over All *sn* Positions									
	16:0	18:0	18:1c	18:2	18:3	20:0	20:1	20:2	22:0	22:1
WT										
sn-1	31	21	30	32	38	24.5	35.5	63	19	45.5
sn-2	17	20	37	59.5	50	8.5	5.5	9	20	6.5
sn-3	52	59	33	8.5	12	67	59	28	61	48
AS11										
sn-1	50	34	34	23	21	41	60	74	30	26
sn-2	12.5	19	56	46	41	7	13	16	12.5	5.5
sn-3	37.5	47	10	31	39	52	27	10	57.5	68.5

CoA or 22:1-CoA as compared to 18:1-CoA or 18:2-CoA (data not shown). In competition studies, the DGAT in homogenates from both WT and AS11 exhibited a selectivity for 20:1-CoA over 18:2-CoA, incorporating 20:1 into TAGs at rates greater than 2-fold higher than 18:2. Thus, although DGAT activity was lower in AS11, the preference for incorporating very long-chain acyl-CoAs over polyunsaturated fatty acyl-CoAs in vitro was not altered. This suggests that the shift in *sn*-3 acyl composition observed in the mature seed TAGs of AS11 (Table III) is the cumulative result of reduced DGAT activity throughout development (Table II) and its effect on VLCFA biosynthesis (Fig. 5).

In summary, the data indicate that the EMS-induced lesion in a single nuclear gene in AS11 affects the conversion of DAG to TAG throughout seed development. Although the FAME phenotype would seem to suggest a lesion in the elongase complex or an up-regulation of desaturation, there is no biochemical evidence of a lesion directly affecting the integrity of the elongase or desaturase enzyme systems, although, ultimately, the fatty acid composition of seed TAGs is dramatically affected. We propose that the mutation in AS11 results in a decreased DGAT activity, such that TAG biosynthesis is impeded (locus affected by the mutation is designated *TAG*). Since 20:1 is normally incorporated preferentially into the *sn*-3 position by DGAT (see Table III), in AS11 there may be an accumulation of unesterified 20:1-CoA, which could feedback inhibit 18:1 elongation. This appears to lead to increased conversion of 18:1 to 18:2 and 18:3. By the time maximum lipid deposition begins at about 21 d.p.a. in AS11, the acyl-CoA pool available to DGAT is already enriched in 18:3, to the detriment of 18:1 and 20:1 (see Fig. 5B). Combined with a lower DGAT activity throughout seed development, the result is that at maturity, AS11 has reduced TAG levels containing proportionally less 18:1 and 20:1 and more 18:3 compared to WT. Although the amounts of most VLCFAs are depressed during development in AS11 as compared to WT (compare Fig. 5, A and B), the effect is most conspicuous by the decrease in 20:1, normally the predominant VLCFA in *A. thaliana*. An exception to this trend is 20:2, the product of 20:1 desaturation, which is present in higher proportions in mature AS11 than in WT (Figs. 1 and 5C). This may primarily reflect the fact that, since DGAT activity is reduced, some 20:1 is available for desaturation. The 20:2 is preferentially inserted into the *sn*-1 position (Table III), presumably by GPAT, displaying a most atypical distribution pattern for a VLCFA in the Brassicaceae (Taylor et al., 1995).

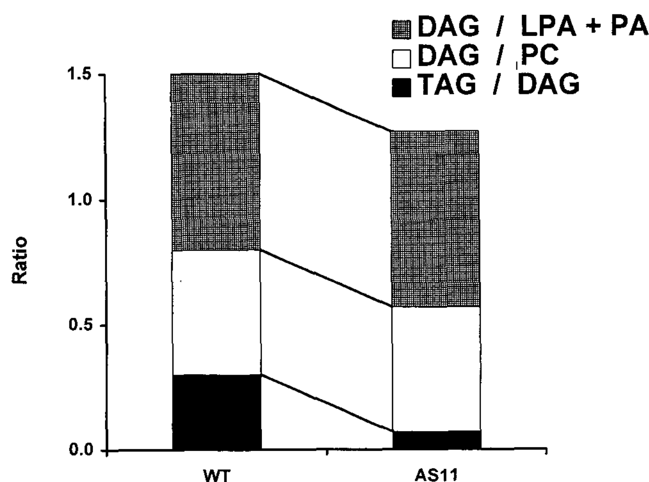

Figure 7. Relative proportions of glycerolipids synthesized in vitro during incubation of [1-^{14}C]18:1-CoA and G-3-P with homogenates prepared from WT and AS11 green developing seed. Ratios were calculated by comparing the levels of [1-^{14}C] 18:1 incorporated into Kennedy pathway intermediates, TAG and PC, expressed on a pmol min^{-1} mg^{-1} protein basis.

CONCLUSIONS

To our knowledge, the present study has shown for the first time in *A. thaliana* that reduced DGAT activity can strongly affect seed development and the pattern of fatty acid biosynthesis. In AS11, a decreased DGAT activity is correlated with delayed seed development, a reduced TAG content, and a repression of VLCFA biosynthesis with an accumulation of 18:3 in TAGs. These results suggest that overexpression of DGAT activity earlier in development may provide a means for channeling more carbon into VLCFAs and, ultimately, into TAGs. Such indirect regulatory mechanisms may have important implications for efforts to maximize the VLCFA content of other members of the Brassicaceae through biotechnology (Taylor et al., 1992b). The effects of the AS11 mutation on DGAT activity throughout development and on the acyl composition and amount of TAG that accumulates are unequivocal. However, it is not yet clear whether the lesion resulting in

decreased DGAT activity is in the DGAT gene itself or in a gene encoding a regulatory factor that directly controls DGAT. Alternatively, it is possible that the lesion in AS11 is in a gene regulating seed development, which results in pleiotropic effects on DGAT activity, fatty acid modification, and TAG bioassembly. Cloning the gene identified by the mutation in AS11, using chromosome walking (Kunst et al., 1992a, 1992b) or subtractive cloning techniques, will help to address these questions. High resolution RFLP mapping will reveal the RFLP marker closest to the gene identified by the AS11 mutation. This RFLP will serve as the starting point for chromosome walking, one of our future research goals.

Many researchers have reported EMS-induced mutations affecting seed and leaf fatty acid biosynthesis in *A. thaliana* (James and Dooner, 1990, 1991; Lemieux et al., 1990; Ohlrogge et al., 1991; Arondel et al., 1992; Browse and Miquel, 1992; Yadav et al., 1993). In an *A. thaliana* mutant with a deficiency in leaf chloroplast GPAT, the cytoplasmic (or ER) pathway was found to largely compensate for the deficiency in plastid GPAT, providing near-normal amounts of all of the lipids required for chloroplast membrane biogenesis (Kunst et al., 1988). That seminal investigation was important in providing a greater understanding of the regulation and interaction of the prokaryotic and eukaryotic pathways during leaf membrane lipid biosynthesis. However, to our knowledge, the current study is the first to document a mutation affecting the extraplastidic Kennedy pathway for storage lipid bioassembly in *A. thaliana* seeds. Our findings demonstrate the dangers inherent in characterizing complex seed lipid mutants based only on fatty acid composition obtained by a simple GC analysis, and we advocate a more rigorous application of analytical, biochemical, and genetic techniques in the analysis of EMS-induced mutants to identify strategic target genes in this model system. Indeed, it may be prudent to re-examine a number of mutant collections, heretofore characterized as fatty acid biosynthesis mutants (e.g. JB11 *ela1*; Lemieux et al., 1990) based solely on FAME analyses of seed lipids. It may be that some of these are, in fact, mutants with alterations to genes encoding other key lipid bioassembly (Kennedy) pathway enzymes or regulatory elements affecting their expression.

ACKNOWLEDGMENTS

The authors thank the Arabidopsis Biological Resource Center at the Ohio State Biotechnology Center for providing the plasmid containing the *B. napus* Δ15 desaturase cDNA. Critical reviews of the manuscript were kindly provided by Drs. W. Keller and A. Cutler. The additional technical assistance of L. Hogge and D. Olson is gratefully acknowledged.

Received October 7, 1994; accepted February 6, 1995.
Copyright Clearance Center: 0032-0889/95/108/0399/11.

LITERATURE CITED

Agrawal VP, Stumpf PK (1985) Elongation systems involved in the biosynthesis of erucic acid from oleic acid in developing *Brassica juncea* seeds. Lipids **20:** 361–366

Arondel V, Lemieux B, Hwang I, Gibson S, Goodman HM, Somerville CR (1992) Map-based cloning of a gene controlling omega-3 fatty acid desaturation in *Arabidopsis*. Science **28:** 1353–1354

Barron EJ, Stumpf PK (1962) The biosynthesis of triglycerides by avocado mesocarp enzymes. Biochim Biophys Acta **60:** 329–337

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Browse J, Miquel M (1992) *Arabidopsis* as a model to develop strategies for the modification of seed oils. *In* DD Bills, S-D Kung, eds, Biotechnology and Nutrition: Proceedings of the Third International Symposium. Butterworth-Heinemann, Boston, pp 435–459

Dean C (1993) Advantages of Arabidopsis for cloning plant genes. Phil Trans R Soc Lond-Biol Sci 189–195

Elledge SJ, Mulligan JT, Ramer SW, Spottswood M, Davis RW (1991) λ YES: a multifunctional cDNA expression vector for the isolation of genes by complementation of yeast and *Escherichia coli* mutations. Proc Natl Acad Sci USA **88:** 1731–1735

Fehling E, Murphy DJ, Mukherjee KD (1990) Biosynthesis of triacylglycerols containing very long chain monounsaturated acyl moieties in developing seeds. Plant Physiol **94:** 492–498

Finkelstein RR, Somerville CR (1990) Three classes of abscisic acid (ABA)-insensitive mutations of *Arabidopsis* define genes that control overlapping subsets of ABA responses. Plant Physiol **94:** 1172–1179

Haughn G, Somerville CR (1986) Sulfonylurea-resistant mutants of *Arabidopsis*. Mol Gen Genet **204:** 430–434

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

James DW Jr, Dooner HK (1991) Novel seed lipid phenotypes in combinations of mutants altered in fatty acid biosynthesis in *Arabidopsis*. Theor Appl Genet **82:** 409–412

Jaworski JG, Goldschmidt EE, Stumpf PK (1974) Fat metabolism in higher plants. LXIII. Properties of the palmityl acyl carrier protein:stearyl acyl carrier protein elongation system in maturing safflower seed extracts. Arch Biochem Biophys **163:** 769–776

Karssen CM, Brinkhorst-van der Swan DLC, Breekland AE, Koornneef M (1983) Induction of dormancy during seed development by endogenous abscisic acid: studies on abscisic acid deficient genotypes of *Arabidopsis thaliana* (L.) Heynh. Planta **157:** 158–165

Kennedy EP (1961) Biosynthesis of complex lipids. Fed Proc **20:** 934–940

Koornneef M, Hanhart CJ (1983) Linkage marker stocks of *Arabidopsis thaliana*. Arabidopsis Inf Serv **20:** 89–92

Koornneef M, Hanhart CJ, Van Lonen Martinet EP, Van Der Veen JH (1987) A marker line that allows the detection of linkage of all *Arabidopsis* chromosomes. Arabidopsis Inf Serv **23:** 46–50

Koornneef M, Jorna ML, Brinkhorst-van der Swan DLC, Karssen CM (1982) The isolation of abscisic acid (ABA) deficient mutants by selection of induced revertants in non-germinating gibberellin sensitive lines of *Arabidopsis thaliana* (L.) Heynh. Theor Appl Genet **61:** 385–393

Koornneef M, Reuling G, Karssen CM (1984) The isolation and characterization of abscisic acid-insensitive mutants of *Arabidopsis thaliana*. Physiol Plant **61:** 377–383

Koornneef M, Van Eden J, Hanhart CJ, Stam P, Braaksma FJ, Feenstra WJ (1983) Linkage map of *Arabidopsis thaliana*. J Hered **74:** 265–272

Kunst L, Browse J, Somerville C (1988) Altered regulation of lipid biosynthesis in a mutant of *Arabidopsis* deficient in chloroplast glycerol-3-phosphate acyltransferase activity. Proc Natl Acad Sci USA **85:** 4143–4147

Kunst L, Reed DW, MacKenzie SL (1992a) Fatty acid elongation in seeds of *Arabidopsis thaliana*. *In* SL MacKenzie DC Taylor, eds, Seed Oils for the Future. American Oil Chemists' Society, Champaign, IL, pp 70–76

Kunst L, Taylor DC, Giblin M, Underhill E (1989) Analysis of seed lipid elongation using *Arabidopsis thaliana* mutants. *In* Ab-

stracts of Symposium on Genetics and Molecular Biology of *Arabidopsis*, Indiana University, Bloomington, IN, October 12–15, 1989

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

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

Lindstrom JT, Vodkin LO (1991) A soybean cell wall protein is affected by seed color genotype. Plant Cell **3:** 561–571

Mansfield SG, Briarty LG (1992) Cotyledon cell development in *Arabidopsis thaliana* during reserve deposition. Can J Bot **70:** 151–164

Meyerowitz EM (1987) *Arabidopsis*: a useful weed. Cell **56:** 263–269

Murphy DJ (1993) Plant lipids: their metabolism, function and utilization. *In* PJ Lea, RC Leegood, eds, Plant Biochemistry and Molecular Biology. John Wiley & Sons, New York, pp 113–128

Ohlrogge JB (1994) Design of new plant products. Engineering of fatty acid metabolism. Plant Physiol **104:** 821–826

Ohlrogge JB, Browse J, Somerville CR (1991) The genetics of plant lipids. Biochim Biophys Acta **1082:** 1–26

Okuley J, Lightner J, Feldmann K, Yadav N, Lark E, Browse J (1994) *Arabidopsis fad2* gene encodes the enzyme that is essential for polyunsaturated lipid synthesis. Plant Cell **6:** 147–158

Page RA, Okada S, Harwood JL (1994) Acetyl-CoA carboxylase exerts strong flux control over lipid synthesis in plants. Biochim Biophys Acta **1210:** 369–372

Parker WB, Somers DA, Wyse DL, Keith RA, Burton JD, Gronwald JW, Gengenbach BG (1990) Selection and characterization of sethoxydim-tolerant maize tissue cultures. Plant Physiol **92:** 1220–1225

Roesler KR, Shorrosh BS, Ohlrogge JB (1994) Structure and expression of an *Arabidopsis* acetyl-coenzyme A carboxylase gene. Plant Physiol **105:** 611–617

Schmid RD (1987) Biotechnology: application to oleochemistry. J Am Oil Chem Soc **64:** 563–570

Sonntag NOV (1991) Erucic, behenic: feedstocks of the 21st century. Inform **2:** 449–463

Stymne S, Stobart AK (1987) Triacylglycerol biosynthesis. *In* PK Stumpf, EE Conn, eds, The Biochemistry of Plants: Lipids, Vol 9. Academic Press, New York, pp 175–214

Suiter KA, Wendel JF, Case JS (1983) LINKAGE-1: a Pascal computer program for the detection and analysis of genetic linkage. J Hered **74:** 203–204

Taylor DC, Barton DL, Rioux KP, MacKenzie SL, Reed DW, Underhill EW, Pomeroy MK, Weber N (1992a) Biosynthesis of acyl lipids containing very-long chain fatty acids in microspore-derived and zygotic embryos of *Brassica napus* L. cv Reston. Plant Physiol **99:** 1609–1618

Taylor DC, Giblin EM, Reed DW, Hogge LR, Olson DJ, MacKenzie SL (1995). Stereospecific analysis and mass spectrometry of triacylglycerols from *Arabidopsis thaliana* (L.) Heynh. Columbia seed. J Am Oil Chem Soc **72:** 305–308

Taylor DC, Kunst L, MacKenzie SL (1992b) Bioassembly of storage lipids in oilseed crops; Target: trierucin. *In* J Janick, JE Simon, eds, New Crops, John Wiley & Sons, New York, pp 181–191

Taylor DC, MacKenzie SL, McCurdy AR, McVetty PBE, Giblin EM, Pass EW, Stone SJ, Scarth R, Rimmer SR, Pickard MD (1994) Stereospecific analyses of seed triacylglycerols from high erucic acid Brassicaceae: detection of erucic acid at the *sn*-2 position in *Brassica oleracea* L. genotypes. J Am Oil Chem Soc **71:** 163–167

Taylor DC, Magus JR, Bhella R, Zou J-T, MacKenzie SL, Giblin EM, Pass EW, Crosby WL (1992c) Biosynthesis of triacylglycerols in *Brassica napus* L. cv Reston; Target: trierucin. *In* SL MacKenzie, DC Taylor, eds, Seed Oils for the Future. American Oil Chemists' Society, Champaign, IL, pp 77–102

Taylor DC, Weber N (1994) Microspore-derived embryos of the *Brassicaceae*—model systems for studies of storage lipid bioassembly and its regulation. Fat Sci Technol **96:** 228–235

Taylor DC, Weber N, Barton DL, Underhill EW, Hogge LR, Weselake RJ, Pomeroy MK (1991) Triacylglycerol bioassembly in microspore-derived embryos of *Brassica napus* L. cv Reston. Plant Physiol **97:** 65–79

Taylor DC, Weber N, Hogge LR, Underhill EW (1990) A simple enzymatic method for the preparation of radiolabeled erucoyl-CoA and other long-chain fatty acyl-CoAs and their characterization by mass spectrometry. Anal Biochem **184:** 311–316

Valvekens D, Van Montagu M, Van Lijsebettens M (1988) *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. Proc Natl Acad Sci USA **85:** 5536–5540

Weselake RJ, Taylor DC, Pomeroy MK, Lawson SL, Underhill EW (1991) Properties of diacylglycerol acyltransferase from microspore-derived embryos of *Brassica napus*. Phytochemistry **30:** 3533–3538

Yadav NS, Wierzbicki A, Aegerter M, Caster CS, Pérez-Grau L, Kinney AJ, Hitz WD, Booth JR Jr, Schweiger B, Stecca KL, Allen SM, Blackwell M, Reiter RS, Carlson TJ, Russell SH, Feldmann KA, Pierce J, Browse J (1993) Cloning of higher plant ω-3 fatty acid desaturases. Plant Physiol **103:** 467–476