Two *FAD3* Desaturase Genes Control the Level of Linolenic Acid in Flax Seed

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Industrial oil flax (*Linum usitatissimum*) and edible oil or solin flax differ markedly in seed linolenic acid levels. Despite the economic importance of low-linolenic-acid or solin flax, the molecular mechanism underlying this trait has not been established. Two independently inherited genes control the low-linolenic-acid trait in flax. Here, we identified two genes, *LuFAD3A* and *LuFAD3B* that encode microsomal desaturases capable of desaturating linoleic acid. The deduced proteins encoded by these genes shared 95.4% identity. In the low-linolenic-acid line solin 593-708, both *LuFAD3A* and *LuFAD3B* carry point mutations that produce premature stop codons. Expression of these genes in yeast (*Saccharomyces cerevisiae*) demonstrated that, while the wild-type proteins were capable of desaturating linoleic acid, the truncated proteins were inactive. Furthermore, the low-linolenic-acid phenotype in flax was complemented by transformation with a wild-type gene. Codominant DNA markers were developed to distinguish between null and wild-type alleles of both genes, and linolenic acid levels cosegregated with genotypes, providing further proof that *LuFAD3A* and *LuFAD3B* are the major genes controlling linolenic acid levels in flax. The level of *LuFAD3* transcripts in seeds peaked at about 20 d after flowering, and transcripts were not detectable in leaf, root, or stem tissue. A dramatic reduction in transcript levels of both genes occurred in the low-linolenic-acid solin line, which was likely due to nonsense-mediated decay.

Linolenic acid is produced through the desaturation of linoleic acid by omega-3/delta-15 desaturases. In plants, this reaction occurs both in the plastids and in the endoplasmic reticulum (ER). Arabidopsis (*Arabidopsis thaliana*) carries two plastidial desaturases, *FAD7* and *FAD8* (Browse et al., 1986; Mc Conn et al., 1994), which desaturate both 16:2n-6 and 18:2n-6, and a single ER enzyme, *FAD3* (Browse et al., 1993), which acts only on 18:2n-6. The majority of the linoleic acid desaturation in seeds results from the activity of the microsomal desaturases. For example, in Arabidopsis, the level of 18:3n-3 dropped from approximately 20% of total seed fatty acids in wild-type plants to 1% to 2% in *FAD3* mutant lines (James and Dooner, 1990; Lemieux et al., 1990), whereas leaf 18:3n-3 levels were only slightly reduced (Lemieux et al., 1990; Browse et al., 1993). Although it appears likely that mutations in *FAD3* gene(s) may cause the low-linolenic-acid genotypes in flax, genes for linoleic desaturases have not been isolated from this species. Thus, it has not been determined if both mutations occurred in *FAD3* genes or if one of the mutations arose in a different type of gene, such as a trans-acting factor controlling the desaturase. Here, we present the sequences of two microsomal *FAD3* desaturase cDNAs from flax and demonstrate that these two genes control the low-linolenic-acid trait in flaxseed.

RESULTS

Flax Has Two *FAD3* Genes, *LuFAD3A* and *LuFAD3B*

As a first step in obtaining *FAD3* cDNAs from flax, degenerate primers that targeted the first and third
His-rich motifs of FAD3-like proteins were designed. When these primers were used to amplify first-strand cDNA from developing Normandy (wild-type) seeds, a PCR fragment with high similarity to FAD3/omega-3 desaturase sequences from various plant species was produced. Screening of a Normandy cDNA library with this fragment led to the identification of a full-length cDNA clone, 1,475 bp in length and containing an open reading frame encoding a 392-amino acid protein, LuFAD3A (Fig. 1).

Several primer sets designed to amplify the intron regions of the LuFAD3A gene produced two distinct bands with Normandy genomic DNA. The sequences of these pairs of bands were very similar but not identical, and only one of the two bands had exon sequences matching those of LuFAD3A. This suggested that the additional band represented a second LuFAD3 gene. Primers specific for this second gene were used to amplify overlapping 3’ and 5’ regions from solin line 593-708 adaptor-ligated cDNA, and primers were designed from these sequences to amplify the full-length clones from Normandy and solin line 593-708 cDNA.

The deduced amino acid sequences of LuFAD3A and LuFAD3B from wild-type (Normandy) flax are 95.4% identical. LuFAD3A carries two additional amino acids in the N-terminal region and lacks one amino acid in the C-terminal region. Besides these differences, only 15 amino acids vary between the LuFAD3A and LuFAD3B proteins (Fig. 1). The LuFAD3A and LuFAD3B cDNA sequences are deposited in GenBank as accession numbers DQ116424 and DQ116425, respectively.

Both FAD3 Genes from Low-Linolenic-Acid Flax Carry Point Mutations

The LuFAD3A cDNA from the solin line was isolated using the same adaptor-ligated cDNA library as that used for the solin LuFAD3B cDNA. Overlapping fragments were isolated using primers specific to the LuFAD3A gene, these fragments were aligned, and a reverse transcription (RT)-PCR employing primers from each end of the alignment was used to obtain the full-length clone.

When compared to the Normandy LuFAD3A sequence, the solin LuFAD3A cDNA sequence contained a single nucleotide substitution 874 nucleotides from the translational start, converting an Arg codon (CGA) to a stop codon (TGA; Fig. 2). Therefore, the protein encoded by solin LuFAD3A was truncated at the C terminus and consisted of only 291 amino acids, as opposed to the wild-type 392-amino acid Normandy LuFAD3A protein. Comparison of the solin-type and wild-type (Normandy) LuFAD3B sequences showed that solin LuFAD3B also carried a nonsense mutation. This substitution was 162 nucleotides from the start site, converting a Trp codon (TGG) to a stop codon (TGA). As a result, the solin LuFAD3B cDNA encoded a very short protein of only 53 amino acids, as opposed to the 391-amino acid protein encoded by the wild-type LuFAD3B gene. The solin-type and wild-type LuFAD3A and LuFAD3B cDNAs were identical except for these point mutations, although the poly-A tail was attached at slightly different positions in the solin- and wild-type LuFAD3B cDNAs. To verify the occurrence of these point mutations, the regions containing the mutations were amplified from solin line 593-708 genomic DNA. The sequences of the genomic PCR products were identical to the corresponding regions of the respective cDNAs (data not shown).

Alignments of the cDNAs with a partial LuFAD3A genomic clone from Normandy indicated that the LuFAD3A mutation occurred in the fifth of six exons, 100 nucleotides upstream of the exon-intron junction, whereas the mutation in the LuFAD3B gene occurred in the first exon.

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**Figure 1.** Comparison of deduced amino acid sequences of LuFAD3A and LuFAD3B. Identical residues are shown on a black background. Arrows indicate the positions of stop codons in the mutated alleles.
The Promoter Regions of LuFAD3A and LuFAD3B Are Similar But Not Identical

A partial wild-type LuFAD3A clone isolated from a Normandy genomic library included the region from the translation start site to approximately 1,000 bp upstream of this site. This region was capable of directing seed-specific expression in flax (Fig. 3). Histochemical staining of transgenic flax plants carrying this promoter region in front of the uidA (β-glucuronidase [GUS]) reporter gene demonstrated strong GUS activity in embryos (Fig. 3). Little or no GUS activity was detected in the seed coat, leaf, root, or stem tissue of these plants (Fig. 3).

When we attempted to amplify this promoter region from Normandy genomic DNA, we obtained two distinct bands. While the sequence of one of the PCR products matched exactly to that of the genomic clone, the other product was similar but not identical, and the alignment of the two products contained a number of gaps. The second product appeared to represent the LuFAD3B promoter. To confirm this, an internal sequence was reamplified using a primer from within the putative LuFAD3B promoter and a primer specific to the LuFAD3B coding region (Fig. 4). LuFAD3A and LuFAD3B upstream regions were also amplified from solin 593-708 genomic DNA, and, while the LuFAD3B upstream regions from each genotype were identical, the LuFAD3A promoter from the solin-type line carried a single substitution (C to T) 559 bp upstream from the translation start site (data not shown).

Wild-Type Flax FAD3 Genes Demonstrate Desaturase Activity when Expressed in Yeast

To determine whether the wild-type (Normandy) cDNAs encoded proteins capable of delta-15 desaturase activity and whether the mutations carried by the solin-type cDNAs resulted in the production of inactive proteins, the LuFAD3A and LuFAD3B cDNAs from both genotypes were expressed in yeast (Saccharomyces cerevisiae; Fig. 5). Supplement of yeast cultures carrying either Normandy LuFAD3A or LuFAD3B with linoleic acid led to the production of linolenic acid, while desaturation of linoleic acid did not occur in yeast cultures carrying either of the solin 593-708 FAD3 cDNAs (Fig. 5) or in cultures carrying a vector control (data not shown). The average conversion rate (weight percent product/(weight percent substrate plus product) × 100) in three cultures expressing the wild-type LuFAD3B was 7.79% ± 0.32% SD, which was slightly higher than that seen in cultures expressing LuFAD3A (5.75% ± 0.32% SD).

Figure 2. Mutations in LuFAD3A and LuFAD3B in the solin 593-708 line. The region of exon 5 of LuFAD3A from solin 593-708 (mutated) that carries a point mutation is aligned with the corresponding region from Normandy (wild type). The region of exon 1 of LuFAD3B that carries a point mutation in solin 593-708 (mutated) is likewise aligned with the corresponding region from Normandy (wild type). The deduced amino acid sequences are shown above or below the nucleotide sequences.

Figure 3. Histochemical localization of GUS activity in transgenic flax (cv Normandy) carrying the GUS gene under the control of the LuFAD3A promoter. A, Embryo (20 DAF); B, seed coat (20 DAF); C, leaf; D, stem; E, root.
In control samples, endogenous 18:1n-9 was not desaturated. However, in samples carrying the Normandy LuFAD3 cDNAs without exogenous substrates, a small peak corresponding to the expected position of 18:2 (9,15) was detected (data not shown), which likely resulted from the desaturation of endogenous 18:1n-9; this desaturation activity was also detected with a Brassica napus FAD3 gene (Reed et al., 2000). Although 18:2(9,15) has a slightly longer retention time than does 18:2n-6, it is not detectable in samples from cultures fed 18:2n-6 because its peak overlaps with the very broad linoleic acid peak.

When cultures were fed $\gamma$-linolenic acid (18:3n-6) or arachidonic acid (20:4n-6), no detectable desaturation occurred with either of the wild-type cDNAs (data not shown). This contrasts with the result reported by Reed et al. (2000) where desaturation of both of these fatty acids occurred in cultures of yeast transformed with the B. napus FAD3 gene. However, desaturation levels observed with the Brassica gene were extremely low, and it is probable that conditions in the experiments reported here did not allow us to detect such low levels of activity.

**Heterologous Expression of Flax FAD3A in Low-Linolenic-Acid Plants Rescues the Mutant Phenotype**

In untransformed solin-type flax, the level of linolenic acid is less than 2% of total seed fatty acids.
However, in solin 593-708 flax transformed with the wild-type (Normandy) LuFAD3A gene under the control of the flax 2S storage protein conlinin promoter (Truksa et al., 2003), the mean weight percent of linolenic acid of six seeds randomly selected from a T1 plant carrying the transgene was 41.0% ± 2.91%. Normandy flax seed had linolenic acid levels of about 52%; thus, transformation of solin 593-708 flax with the Normandy gene demonstrated partial complementation of the solin genotype.

Although expression in yeast is a useful test for enzymatic activity, occasionally the activity of an enzyme when expressed in yeast will not exactly mirror its activity in plants. For example, Heilmann et al. (2004) noted a change in regiospecificity of Arabidopsis desaturases (ADS1, ADS2, and ADS3) when these genes were expressed in plants versus yeast; this difference was greatly enhanced with plastidial retargeting. However, the activity of LuFAD3A in flax mimicked its activity in yeast, confirming that LuFAD3A is a delta-15/omega-3 desaturase.

**DNA Markers Can Distinguish among FAD3 Genotypes and Segregate with Linolenic Acid Content**

To confirm that the two genes we identified were the major genes responsible for controlling the level of desaturation of linoleic acid in flax, we designed markers to distinguish plant genotypes, allowing us to correlate genotypes with phenotypes. A comparison of the sequences of the wild-type and mutated genes indicated that the point mutation in the LuFAD3A gene resulted in the loss of a PvuII site, whereas a BsaJI site was lost due to the point mutation in the LuFAD3B gene. Primers were designed for each gene such that the amplification product would contain the mutation, and digestion of the product with the appropriate enzyme would produce fragments with distinguishable sizes. Using these codominant markers, it was possible to classify plants as homozygous mutant, homozygous wild type, or heterozygous at each of the two LuFAD3 loci (Fig. 6).

These markers were used to analyze progeny from a number of F7 plants originating from a cross between a wild-type flax line and a solin type (593-708) flax. Since flax is strictly self-pollinated, it is likely that the F7 plants were homozygous at most loci. Three seeds from each plant were analyzed by cutting seeds in half and performing gas chromatography analysis on one half-seed while germinating the other half and subjecting the seedling to marker analysis. The markers for LuFAD3A and LuFAD3B identified several lines that were homozygous mutant for one gene and homozygous wild type for the second gene (Fig. 7). Gas chromatography analysis of the seed from which these plants originated confirmed that the LuFAD3 markers segregated with plant phenotype. Plants with less than 2% of the total fatty acids in seed as linolenic acid carried markers for both mutated genes, and plants with linolenic acid contents above 50% carried markers for both wild-type genes (Table I). Plants carrying a homozygous mutation at a single LuFAD3 locus had
intermediate levels of linolenic acid, and the lack of active LuFAD3B seemed to have a greater effect on linolenic acid content than did the lack of LuFAD3A. While the ratio of 18:2n-6/18:3n-3 was less than one in null LuFAD3A/wild-type LuFAD3B seeds, it was greater than one in null LuFAD3B/wild-type LuFAD3A seeds (Table I).

Transcript Levels of LuFAD3A and LuFAD3B Are Dramatically Reduced in Low-Linolenic-Acid Lines

Northern-blot analysis of RNA from various tissues of Normandy and solin 593-708 was performed using a probe amplified from the coding region of the LuFAD3A gene (Fig. 8). LuFAD3A and LuFAD3B share 95% identity at the nucleotide level in the coding region; therefore, this probe should hybridize well to both mRNA species. LuFAD3 expression in wild-type seeds peaked at around 20 d after flowering (DAF; Fig. 8A). While flax seed reaches physiological maturity at about 60 DAF, the exponential phase of linolenic acid accumulation begins at about 20 DAF, and by 32 DAF one-half of the total seed lipids have been synthesized (Stymne et al., 1992). Expression of the LuFAD3 genes appears to be largely limited to seed; the probe did not hybridize to leaf, root, or stem tissue in either genotype (Fig. 8B). Although LuFAD3 transcripts were present in seeds of both genotypes, the expression in solin 593-708 was much lower than that in Normandy (Fig. 8B). However, because of the high homology between the two genes, it was not possible to determine if this was due to a reduction in expression of one or both of the LuFAD3 genes. To clarify this, a real-time PCR experiment using primers specific for each gene was conducted. Although the expression of both genes was greatly reduced, the level of LuFAD3A transcripts in solin 593-708 seed was 4.7% (SD 2.3%) of that in Normandy, whereas the level of LuFAD3B in solin 593-708 was even lower, at 0.75% (SD 0.4%) of the Normandy level.

DISCUSSION

Flax is unusual among crop plants in its very high level of seed linolenic acid. This appears to be due at
least in part to the presence in this species of two genes encoding very similar enzymes capable of desaturating linoleic acid. These genes have additive effects, and lines carrying a mutation in a single gene have intermediate levels of linolenic acid (Table I). Lesions were identified in the coding regions of both of these genes in the low-linolenic-acid line solin 593-708. This low-linolenic-acid line was selected following EMS mutation of a wild-type line (Rowland, 1991). The mutagen EMS is efficient at causing point mutations, commonly G/C to A/T transitions (Anderson, 1995). A C to T transition in $\text{LuFAD3A}$ and a G to A transition in $\text{LuFAD3B}$ produced premature stop codons in these genes, resulting in inactive enzymes. These mutations occurred in a single M2 seed (Rowland, 1991), demonstrating the efficiency of EMS as a mutagen and suggesting the possibility of isolating mutations in other fatty acid biosynthesis genes from the original material.

Even when both $\text{LuFAD3A}$ and $\text{LuFAD3B}$ were mutated, there was still a low level of linolenic acid (<2%) present in seeds. This suggests that one of the $\text{FAD3}$ genes may have some residual activity. However, although the mutation in the $\text{LuFAD3A}$ gene is closer to the C-terminal end of the gene, we did not detect desaturase activity when this gene was expressed in yeast (Fig. 5). Therefore, it appears more likely that the activity of another enzyme, probably a plastidial desaturase, results in the accumulation of low levels of linolenic acid in the solin line. A second low-linolenic-acid line obtained independently by EMS, which also carries two mutations, had linolenic acid levels similar to those found in solin lines (Tonnet and Green, 1987). This is in accordance with the residual activity originating from a different enzyme since it is highly unlikely the mutations carried by both lines are identical. Furthermore, Stymne et al. (1992) noted that the very low level of desaturation occurring in their mutant during embryo development was similar in magnitude to that occurring in wild type before the exponential phase of TAG biosynthesis and linoleic desaturation. We also observed higher relative proportions of linolenic acid in the solin line at early stages of seed development (data not shown). This suggests that an enzyme with a low level of delta-15 desaturase activity in seeds, such as a plastidial enzyme, is contributing the residual levels of linolenic acid. Arabidopsis lines carrying mutations in the single microsomal $\text{FAD3}$ gene also retain low levels of linolenic acid in seeds (James and Dooner, 1990; Lemieux et al., 1990), likely due to the activity of the plastidial omega-3 desaturases $\text{FAD7}$ and $\text{FAD8}$.

Both $\text{LuFAD3A}$ and $\text{LuFAD3B}$ transcript levels were dramatically lower in the low-linolenic-acid line solin 593-708 compared to the wild-type Normandy line. The presence of a premature stop codon in a transcript has been associated with a reduction in transcript levels in plants, yeast, and animals. This RNA degradation functions as an mRNA quality control system, known as nonsense-mediated mRNA decay (or NMD; for review, see Schell et al., 2002; Wagner and

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**Table I. Seed fatty acid composition of F8 seeds from a cross between solin- and wild-type flax**
The values are the means of three half seeds ± se.

<table>
<thead>
<tr>
<th>Line No.</th>
<th>Genotype (from PCR)</th>
<th>18:2n-6</th>
<th>18:3n-3</th>
</tr>
</thead>
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<tr>
<td>256</td>
<td>aabb</td>
<td>54.4 ± 3.8</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>258</td>
<td>aaBB</td>
<td>30.4 ± 1.3</td>
<td>30.8 ± 1.4</td>
</tr>
<tr>
<td>259</td>
<td>AAbb</td>
<td>36.4 ± 0.2</td>
<td>30.7 ± 0.2</td>
</tr>
<tr>
<td>261</td>
<td>aabb</td>
<td>66.2 ± 4.3</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>262</td>
<td>aaBB</td>
<td>26.1 ± 2.5</td>
<td>35.5 ± 1.8</td>
</tr>
<tr>
<td>278</td>
<td>AAbb</td>
<td>36.6 ± 2.3</td>
<td>25.8 ± 2.0</td>
</tr>
<tr>
<td>280</td>
<td>AAbb</td>
<td>37.0 ± 2.1</td>
<td>33.8 ± 2.3</td>
</tr>
<tr>
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</tr>
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<td>AAbb</td>
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<td>28.0 ± 3.0</td>
</tr>
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<td>AAbb</td>
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<tr>
<td>313</td>
<td>AAbb</td>
<td>32.3 ± 0.8</td>
<td>27.2 ± 2.2</td>
</tr>
</tbody>
</table>

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**Figure 8.** Northern-blot analysis of total RNA from flax. The coding region of the $\text{LuFAD3A}$ cDNA was used as a probe. The ethidium bromide-stained gels are shown beneath the autoradiographs. A, Expression of $\text{LuFAD3}$ genes in developing Normandy flax seeds harvested at 10 to 25 DAF. B, Expression of $\text{LuFAD3}$ genes in Normandy and solin 593-708 seeds and vegetative tissues.
Lykke-Andersen, 2002). Translated truncated proteins can cause dominant-negative effects, and NMD reduces the levels of such proteins. The null LuFAD3A and LuFAD3B genes both carry premature stop codons, and the reduction in transcript level observed here appears to be a result of NMD. The deposition of exon junction complexes near the sites of intron removal appear to be involved in NMD, and in mammals stop codons occurring at least 50 nucleotides upstream of the final exon-exon junction in transcripts often result in NMD, whereas stop codons in the final exon do not elicit NMD (for review, see Baker and Parker, 2004). The NMD process in plants and mammals may not be identical; for example, in Arabidopsis, components of the exon-junction complex are present in the nucleolar proteome (Pendle et al., 2005). Although the conditions governing the occurrence of NMD in plants are not well established, the premature stop codons in both the LuFAD3A and LuFAD3B transcripts are more than 50 nucleotides from the final exon-exon junction and thus meet the prerequisites recognized for mammalian NMD. The solin FAD3A promoter differs from that of Normandy by a single base pair, and it is possible that this substitution also contributes to the reduction in LuFAD3A transcript levels.

While the deduced protein of the null LuFAD3B gene is missing 338 amino acids, that of the null LuFAD3A gene lacks only 101 amino acids from its C terminus. However, the C-terminal regions of ER-localized desaturases contain ER retrieval motifs (McCartney et al., 2004). Deletion of just five amino acids from the C terminus of the Brassica FAD3 resulted in a dramatic reduction in activity, as well as mislocalization of this protein to the Golgi (McCartney et al., 2004).

The two LuFAD3 genes have very similar amino acid sequences, and both were capable of desaturating linoleic acid in yeast (Figs. 1 and 4). Furthermore, expression of the wild-type LuFAD3A gene under the control of the conlinin promoter was able to rescue the mutant phenotype. The association of intermediate levels of linolenic acid with single homozygous mutations and low linolenic acid with double null plants (Fig. 7; Table I) provides further evidence that LuFAD3A and LuFAD3B are the major genes responsible for desaturation of linoleic acid in flax. The codominant markers described here are also useful for plant breeding purposes, particularly for the rapid introgression of the low-linolenic-acid trait into adapted genotypes. Developing similar markers for other genes involved in fatty acid biosynthesis and combining them with the markers described here may accelerate the production of “designer” flax oil.

MATERIALS AND METHODS

Plant Materials

F8 plants from a cross between solin line E1747 and the fiber flax (Linum usitatissimum) variety Viking were used for marker analysis. The linseed flax cultivars CDC Normandy and solin line 593-708 were used for all other experiments. Flax plants were grown in a growth chamber at temperatures of 22°C/17°C (day/night) under a 16-h photoperiod.

Construction and Screening of a cDNA Library from Normandy Flax

Total RNA was extracted from flax embryos at 15 to 20 DAF using an RNaseasy Plant mini kit (Qiagen), and mRNA was isolated using the Dynabeads Oligo(dT)25 system (Dynal). The library was constructed with the ZAP cDNA synthesis kit and cloned into the UniZap XR vector (Stratagene). As a first step in isolating the LuFAD3A cDNA, the degenerate primer set DI5F [5'-AT(ACGT) TG(CT)(AGCT) GG(AG) AA(ACGT) A(GA)GA TG (AG) TG-3'] and DI5R [5'-AG(T) T(AGCT) GG(AGCT) CA(TC) GAT(C) TG(TC) GG(AGCT) CA-3'] was used to obtain a PCR product that had high homology to FAD3 genes. This fragment was then cloned into pCR 2.1 (Invitrogen), and the plasmid insert was used to screen the Normandy flaxseed cDNA library.

The 3' end of the wild-type (Normandy) LuFAD3B gene was isolated from this cDNA library via PCR using a 17 primer and a LuFAD3B primer based on sequences from the solin-type LuFAD3B sequence (see below), while 5' sequences were obtained by RT-PCR using primers designed based on the solin LuFAD3B sequence.

Construction of a Marathon cDNA Library and Isolation of LuFAD3 Genes from Solin-Type Flax

Seed from solin line 593-708 was collected at 15 to 20 DAF, removed from the pod, frozen in liquid nitrogen, and ground to a fine powder. RNA was extracted according to the method described by Carpenter and Simon (1998). Poly(A⁺) RNA was isolated using a PolyATrac mRNA Isolation System III (Promega) according to the manufacturer’s instructions. Adaptor-ligated cDNA was produced using the Marathon cDNA amplification kit (BD Biosciences CLONTECH) according to the manufacturer’s instructions. The Marathon primer API was used with primers specific to the LuFAD3B or LuFAD3A genes to produce 5' and 3' regions of the LuFAD3 cDNAs from the solin cDNA library.

Flax Genomic Library Construction and Screening

DNA was isolated from 15-d-old flax cv Normandy seedlings using Qiagen Genomic Tips (Qiagen). After partial digestion and size fractionation, fragments between 16 to 21 kb were cloned into the Lambda Fix II vector (Stratagene). The LuFAD3A cDNA from Normandy flax was used as a probe to screen the library.

Expression in Yeast

Precultures were grown at 30°C overnight in minimal media supplemented with 2% Glc and lacking uracil. Precultures were then washed and used to inoculate 20 mL of induction media (minimal media lacking uracil and supplemented with 2% Gal and 1% raffinose) to an OD600 of 0.5. Cultures were induced for 6 h at 20°C, then supplemented with 150 μl linoleic acid (18:2n-6), and grown at 20°C for 3 d.

Fatty Acid Analysis

For analysis of fatty acids from yeast (Saccharomyces cerevisiae), cells were pelleted by centrifugation, washed once with induction media and once with water, suspended in 2 mL of 3 N methanolic HCl, and incubated at 80°C for 30 min. After the addition of 1 mL of 0.9% NaCl, the sample was extracted with 2.5 mL of hexane. The hexane extract was dried and resuspended in 200 μL of hexane and analyzed on a Hewlett-Packard 5890A gas chromatograph equipped with a DB-23 column (30 m × 250 μm × 0.23 μm). The temperature was programmed to hold at 160°C for 1 min, increase to 240°C at 4°C/min, and hold for 10 min.

For analysis of fatty acids from plants, single seeds were cut in half, and one or both pieces were incubated in 1 mL of 3 N methanolic HCl at 80°C for 30 min, after which the extraction essentially followed the procedure described above.
Transformation of Flax

Hypocotyls of 5- to 6-old seedlings of flax were used as explants. Agrobacterium tumefaciens strain GV3101 containing the helper plasmid pMP90 was used as the host for a binary vector containing the LuFAD3A DNA under the control of the flax 2S storage protein conlinin promoter (Truksa et al., 2003) or the uidA (GUS) gene under the control of the LuFAD3A promoter.

Hiostochimical GUS Assays

Tissues were stained overnight in staining solution (0.5 mg/ml 5-bromo-4-chloro-3-indolyl-beta-d-glucuronide, 50 mM Na phosphate buffer, pH 7, 1 mM K4Fe(CN)6, 1 mM K3Fe(CN)6, 20 mM EDTA), and fixed after staining in 5% v/v formaldehyde, 5% v/v acetic acid, 20% v/v ethanol.

Development of Markers for LuFAD3A and LuFAD3B

Genomic DNA was isolated from flax leaf discs using the method described by Edwards et al. (1991). Each PCR contained 2.5 μL MgCl2 (50 mM), 5 μL Invitrogen PCR buffer (200 mM Tris-HCl, 500 mM KCl), 1 μL Invitrogen Taq DNA polymerase (5 U/μL), 1 μL dNTPs (each at 10 mM), 2.5 μL of the genomic DNA solution, and 50 pmol of each primer in a volume of 50 μL. The PCR program consisted of a 4-min denaturation cycle at 94°C, followed by 25 cycles of 94°C for 45 s, 65°C for 30 s, and 72°C for 2 min, and a final 10 min extension at 72°C. The primer set MutAF2 (5'-CAG-TCAGCTCTGACCACC-GGG-3')/MutAR2 (5'-CCCGCTAGGTTGATCATG-3') was used to distinguish wild-type versus mutant LuFAD3A alleles, while the set Lu15FLF (5'-TTCAAAACTGTGGCTCTGCAG-3')/MutAR2 (5'-CCCGCTAGGTTGATCATG-3') was used to distinguish LuFAD3B alleles. Twenty microliters of PCR product was digested with 20 to 30 units of the appropriate enzyme (PvuII for LuFAD3A, Bgl II for LuFAD3B reactions) in a total volume of 30 μL for 3 h. Digests were run on 1.5% to 2.0% agarose gels.

The markers were tested against 11 F8 lines from a recombinant inbred population from the cross E1747/Viking. Seeds from each line were cut in half and fatty acid analysis was performed on one half-seed, while the second half-seed was germinated and DNA was extracted and analyzed as described above. Fatty acid and PCR analyses were performed on three seeds from each line.

Real-time Quantitative RT-PCR

The gene-specific primer sets RTAF2 (5'-CCACCAACCGCACACCACC-G-3')/ RTAR2 (5'-AATCCAGTAGAACGGCCAGACAG-3') and RTBF2 (5'-CCACCCACCGCCGATGT-3')/RTBR2 (5'-ACCCAGTAAGAGGGCAAGAGG-3') were designed to amplify approximately 250-bp regions from LuFAD3A and LuFAD3B, respectively. To ensure that the measured levels would be specific to a particular gene, a PCR experiment was conducted under the same conditions as those used for the real-time PCR, with plasmids containing the LuFAD3 cDNAs as templates. The LuFAD3 primers were unable to amplify the LuFAD3B DNA and vice versa.

Total RNA was treated with FPLCpure DNAse I (Pharmacia), and cDNA was synthesized using the Superscript III first-strand synthesis system for RT-PCR (Invitrogen) according to the manufacturer’s instructions. Real-time PCR was performed on an iCycler (Bio-Rad). The PCR was performed with Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) according to the manufacturer’s instructions. Primers for a conlinin seed storage protein gene were used for an endogenous control for normalization. Three separate first-strand cDNA reactions were analyzed in duplicate for each sample, and expression levels were calculated as described by Livak and Schmittgen (2001), using the Normandy samples as the calibrators.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers DQ116424 and DQ116425.

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Two FAD3 Genes Control Linolenic Acid Levels in Flax Seed

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