Plant Physiol. (1996) 110: 311–319

Developmental and Growth Temperature Regulation of Two Different Microsomal ω-6 Desaturase Genes in Soybeans

Elmer P. Heppard, Anthony J. Kinney, Kevin L. Stecca, and Guo-Hua Miao*

Agricultural Products Department, E. I. duPont de Nemour & Co., Experimental Station, P. O. Box 80402, Wilmington, Delaware 19880–0402

The polyunsaturated fatty acid content is one of the major factors influencing the quality of vegetable oils. Edible oils rich in monounsaturated fatty acid provide improved oil stability, flavor, and nutrition for human and animal consumption. In plants, the microsomal ω-6 desaturase-catalyzed pathway is the primary route of production of polyunsaturated lipids. We report the isolation of two different cDNA sequences, *FAD2–1* and *FAD2–2*, encoding microsomal ω-6 desaturase in soybeans and the characterization of their developmental and temperature regulation. The *FAD2–1* gene is strongly expressed in developing seeds, whereas the *FAD2–2* gene is constitutively expressed in both vegetative tissues and developing seeds. Thus, the *FAD2–2* gene-encoded ω-6 desaturase appears to be responsible for production of polyunsaturated fatty acids within membrane lipids in both vegetative tissues and developing seeds. The seed-specifically expressed *FAD2–1* gene is likely to play a major role in controlling conversion of oleic acid to linoleic acid within storage lipids during seed development. In both soybean seed and leaf tissues, linoleic acid and linolenic acid levels gradually increase as temperature decreases. However, the levels of transcripts for *FAD2–1*, *FAD2–2*, and the plastidial ω-6 desaturase gene (*FAD 6*) do not increase at low temperature. These results suggest that the elevated polyunsaturated fatty acid levels in developing soybean seeds grown at low temperature are not due to the enhanced expression of ω-6 desaturase genes.

Lipids are the major structural components of all cellular membranes of living organisms. Oil-producing crop plants such as soybeans (*Glycine max* L.) and rapeseeds also synthesize and store energy in the form of TAGs, which are composed of a glycerol backbone molecule esterified by three saturated and/or unsaturated fatty acyl groups. The relative composition of saturated and unsaturated fatty acids in seed TAGs is one of the major factors influencing the quality of edible oils. For example, oils high in 18:1 and low in polyunsaturated fatty acids appear to have improved nutritional benefits and increased stability (Liu and White, 1992; Yadav, 1995). Therefore, the food industry has a major commercial interest in understanding how to regulate desaturation of fatty acids within storage lipids in oil seed crops.

Although both plastidial and cytosolic desaturase-mediated pathways leading to synthesis of unsaturated fatty acids are known, the regulation of these pathways is poorly understood. Because of recent successes using genetically based approaches to clone several plant membrane-bound desaturase genes (Arondel et al., 1992; Yadav et al., 1993; Okuley et al., 1994), the question of regulation of these pathways may now be readily addressed. The plastid-soluble stearoyl-CoA desaturase carries out the first desaturation reaction and forms oleoyl-ACP ($18:1^{\Delta 9}$-ACP); the second ($18:2^{\Delta 9,12}$) and third ($18:3^{\Delta 9,12,15}$) double bonds are introduced into 18:1 by both plastidial and microsomal ω-6 and ω-3 desaturases, respectively, after incorporation of the fatty acid into lipids (Somerville and Browse, 1991).

The composition of saturated and unsaturated fatty acids of both membrane and storage lipids also varies depending on environmental temperature. There is a general inverse relationship between polyunsaturation of fatty acids and growth temperature; polyunsaturated fatty acids increase with decreasing temperature in membrane as well as seed storage lipids (Neidleman, 1987; Rennie and Tanner, 1989; Thompson, 1993). Several low-18:3 and/or high-18:1 soybean mutants have been isolated (Ohlrogge et al., 1991; Kinney, 1994), but many of these mutants appear to be temperature sensitive so that the 18:3 content of these plants increased when they were grown at lower temperatures. This might be due to the presence of the second locus of the microsomal ω-3 desaturase gene, expression of which is induced by cold temperature (Kinney, 1994). Indeed, a cold-inducible plastid ω-3 desaturase gene (*FAD7*) has been isolated from Arabidopsis (Gibson et al., 1994). Expression of the cold-inducible desaturase gene may allow plants to make more trienoic fatty acid of the membrane lipids, which is required for plants grown at low temperatures. Similar to the low-18:3 mutants, the high-18:1 soybean mutant A5 is also temperature sensitive (Rennie and Tanner, 1989), indicating potential regulation of ω-6 desaturase genes by growth temperature. In Arabidopsis, microsomal ω-6 desaturase is encoded by a single *FAD2* gene, and expression of this gene is not regulated by low growth temperature (Okuley et al., 1994).

In this paper we report the existence of two different microsomal ω-6 desaturase genes, *FAD2–1* and *FAD2–2*, in soybeans. The cDNA sequences of *FAD2–1* and *FAD2–2* have significant homology with the Arabidopsis *FAD2* gene (Okuley et al., 1994). The two soybean sequences share 73% identity at the deduced amino acid sequence

* Corresponding author; e-mail miaog@esvax.dnet.dupont.com; fax 1–302–695–4296.

Abbreviations: 18:1, oleic acid; 18:2, linoleic acid; 18:3 linolenic acid; ACP, acyl carrier protein; CaMV, cauliflower mosaic virus; TAG, triacylglycerol.

level. The functional identity of the two sequences was confirmed by genetic complementation of Arabidopsis *fad2–1* mutation. The *FAD2–1* gene is specifically induced during seed development when the rate of storage lipid synthesis is at a maximum. In contrast, the *FAD2–2* gene was constitutively expressed in both vegetative tissues and throughout seed development, although the highest expression level was found in leaf tissues. The composition of fatty acids in developing seeds is regulated by developmental stage and growth temperature; however, expression of *FAD2–1* and *FAD2–2* genes controlling the fatty acid desaturation was not induced or enhanced by cold temperature. Thus, we suggest that the decrease in 18:1 content in seed lipids at low temperature may not be due to the transcriptionally induced or enhanced expression of ω-6 desaturase genes in soybean plants.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Soybeans (*Glycine max* L. cv Rye) were grown in growth chambers with a day/night cycle of 32/28, 28/22, 22/18, and 18/12°C. The light/dark cycle was 12/12 h. Developing seeds were harvested 6 to 10 (5–10 mg), 13 (11–30 mg), 17 (31–60 mg), 19 (61–100 mg), 21 (101–150 mg), and 26 (200–300 mg) DAF and stored at −70°C. These seeds represented developing seeds ranging from very young to mature embryos. Leaf, stem, and root tissues were collected from young germinating soybean seedlings.

Isolation of cDNA Sequences Encoding Microsomal ω-6 Desaturases

The soybean *FAD2–1* cDNA clone was isolated by screening a developing soybean embryo library (Yadav et al., 1993) using an [α-^{32}P]dCTP-labeled Arabidopsis *FAD2* cDNA fragment (Okuley et al., 1994). Many strongly hybridizing plaques were recovered from a total of 6×10^5 recombinant phages. The clone containing the longest insert, designated pSF2–169K, was fully sequenced from both stands. To isolate other members of the *FAD2* gene family, two oligonucleotide primers, OP1, 5′-TTCCACCTCCTTCCTCAACCC-3′, and OP5, 5′-TGCAACTTTGGATTTTGGTTT-3′ (see also "Results"), were made to amplify the highly conserved region in soybean *FAD2–1* and Arabidopsis *FAD2* cDNA sequences. The PCR-amplified fragment was gel isolated and labeled with [α-^{32}P]dCTP as a probe for screening a soybean epicotyl cDNA library constructed in λZAP II (Statagene). Hybridization was carried out overnight at 50°C as described by Yadav et al. (1993). Several positive plaques were identified and their cDNA inserts were characterized by nucleotide sequence determination in pBluescript following excision from the phagemids.

Genomic DNA Gel Blot Analysis

Genomic DNA was prepared from young soybean leaves according to the method of Dellaporta et al. (1983). Samples of DNA (5 μg) were digested with restriction enzymes and electrophoresed through a 0.8% agarose gel. The gel was soaked in 0.5 M NaOH, 1.5 M NaCl for 30 min and blotted onto a Hybond-N$^+$ nylon transfer membrane (Amersham). The filters were probed with [α-^{32}P]dCTP-labeled *FAD2–1*- and *FAD2–2*-specific DNA fragments corresponding to the 3′ end noncoding sequences, unique to the two genes. The hybridization was performed in 6× SSPE, 10% Denhardt's solution, 0.5% SDS, 100 μg/mL denatured salmon sperm DNA fragments overnight at 65°C, and the filters were washed three times in 2× SSC, 0.5% SDS for 30 min at the same temperature.

RNA Preparations and Analysis

Soybean leaves, stems, roots, and developing seeds were harvested and used for RNA isolation. Total RNA and poly(A)$^+$ RNA preparations were made by using the total RNA and poly(A)$^+$ RNA isolation kits from Promega according to the manufacturer's instructions. The RNAs were electrophoresed in 1% agarose gel containing formaldehyde, transferred to a Hybond-N$^+$ nylon membrane, and probed with [α-^{32}P]dCTP-labeled *FAD2–1*- and *FAD2–2*-specific DNA fragments. Loading of RNA was controlled by probing the blots with a tobacco β subunit of the mitochondrial ATP synthetase (Boutry and Chua, 1985). Hybridization was carried out for 20 h in the same solution as described above for genomic Southern blot hybridization. The filter was washed three times at 55°C.

DNA Sequence Determination and Analysis

The nucleotide sequences of the cDNA clones for *FAD2–1* and *FAD2–2* genes were determined by dideoxynucleotide chain-termination sequencing (Sanger et al., 1977) with Sequenase, version 2.0 (United States Biochemical). The oligonucleotide primers were synthesized and used for DNA sequencing of both strands of the two cDNA clones. The DNA sequence data were compiled and analyzed with MacVector (International Biotechnologies, Inc., New Haven, CT), DNAStar (DNAStar Inc., Madison, WI), and the University of Wisconsin Genetics Computer Group (Madison, WI) programs.

Plant Transformation

The coding sequences of *FAD2–1* and *FAD2–2* genes were first subcloned in a sense orientation into the pSN1 vector at the *Bam*HI site, which is between the CaMV 35S promoter and nopaline synthetase 3′ terminator. The fragments flanked by CaMV 35S promoter and nopaline synthetase 3′ terminator sequences were then cloned into binary vector pBI121 (Clontech, Palo Alto, CA) using the *Hin*dIII and *Eco*RI sites and generated pBI-FAD2–1 and pBI-FAD2–2. These constructs and vector pBI121 were transferred into *Agrobacterium tumefaciens* R1000 and used for plant transformation. An Ri plasmid-mediated, hairy root induction transformation procedure was used as described by Arondel et al. (1992). The hairy roots generated from inoculated mutant explants were assayed for GUS activity, and GUS-positive hairy roots were further propagated on Murashige-Skoog medium (Murashige and Skoog, 1962) containing kanamycin (50 μg/mL). The GUS-positive and

kanamycin-resistant hairy roots were used for lipid composition analysis by GC.

Fatty Acid Analysis

Fatty acid methyl esters were prepared as described by Browse et al. (1986). These methyl esters were separated by GC on a Hewlett-Packard 5890 gas chromatograph.

RESULTS

Isolation and Characterization of Genes Encoding Microsomal ω-6 Desaturases

The Arabidopsis *FAD2* gene was cloned recently by a T-DNA insertional mutagenesis approach (Okuley et al., 1994). Using Arabidopsis *FAD2* cDNA as a probe, we screened a soybean developing embryo cDNA library (Yadav et al., 1993). Several strongly hybridizing plaques were recovered. The restriction enzyme mapping and partial DNA sequence analysis indicated that all of these positive clones have the same sequence identity. The longest clone, designated pSF2–169K, was selected for DNA sequence determination on both strands. The open reading frame of pSF2–169K encodes a predicted peptide of 387 amino acid residues. The pSF2–169K shows significant homology to the Arabidopsis *FAD2* cDNA sequence (68% identity on deduced amino acid sequence level), suggesting that it encodes a soybean microsomal ω-6 desaturase. Thus, we named this cDNA *FAD2–1* (Fig. 1). Sequence comparison of *FAD2–1* with other known desaturase sequences revealed only limited identity (data not shown).

To isolate other members of the microsomal ω-6 desaturase gene family in soybean, we screened a cDNA library of soybean epicotyls under low-stringency hybridization conditions using a soybean *FAD2–1* cDNA fragment representing only the highly conserved region between the *FAD2–1* and Arabidopsis *FAD2* cDNA sequences (Fig. 1; see also "Materials and Methods"). Several positive plaques hybridizing to the DNA probe were isolated and characterized. All of these positive clones represented the same class of DNA sequence. The complete DNA sequence analysis of the clone containing the longest cDNA insert (pGH-8) revealed that it encoded a second soybean ω-6 desaturase of 383 amino acid residues. The deduced amino acid sequence of insert in pGH-8 shared 73 and 76% identity with the deduced amino acid sequence of *FAD2–1* and Arabidopsis *FAD2* genes, respectively. Thus, this sequence appeared to be a new member of the microsomal ω-6 desaturase gene family in soybeans and, accordingly, is named *FAD2–2* (Fig. 1). Sequence homology between *FAD2–2* and Arabidopsis *FAD2* is even higher than between the two soybean microsomal ω-6 desaturases (Fig. 1), indicating that *FAD2–1* and *FAD2–2* may have evolved independently. The alignment of the predicted amino acid sequence of

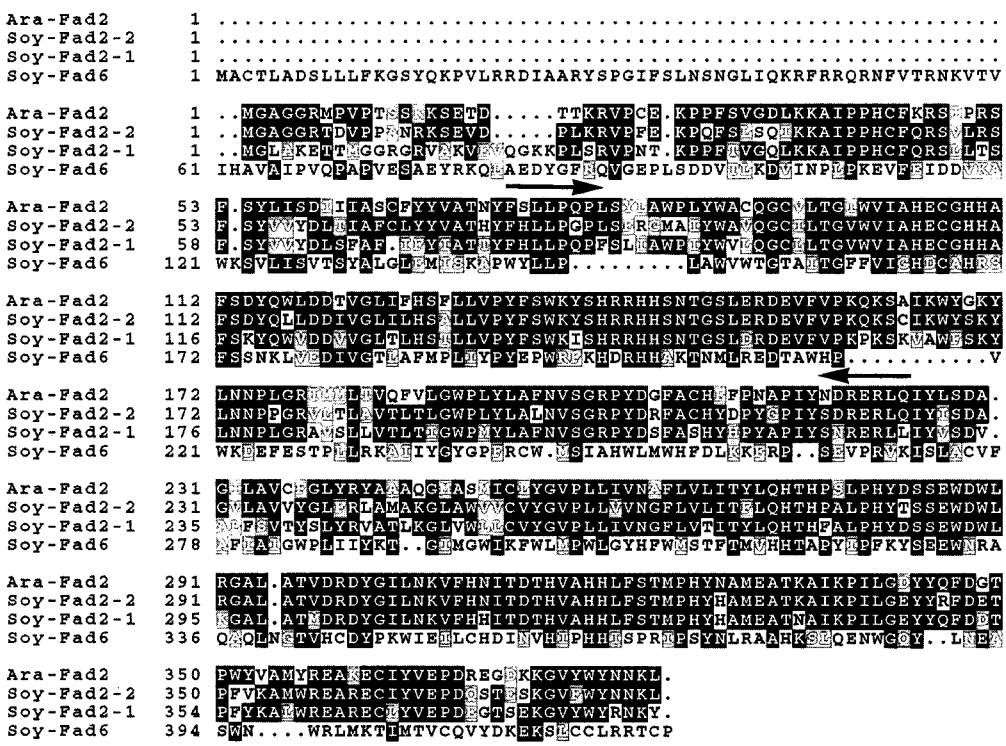

Figure 1. Comparison of the deduced amino acid sequences of soybean (Soy) *FAD2–1, FAD2–2, FAD6* (Hitz et al., 1994), and Arabidopsis (Ara) *FAD2* (Okuley et al., 1994) genes. Identical and similar residues are shown on backgrounds of black and gray, respectively. Solid arrows indicate the regions of the sequence used for making oligonucleotide (see "Materials and Methods") for PCR to amplify the highly homologous region between soybean *FAD2–1* and Arabidopsis *FAD2* genes, respectively.

soybean *FAD2–1*, *FAD2–2*, and plastidial ω-6 desaturase gene *FAD6* (Hitz et al., 1994) with Arabidopsis *FAD2* (Okuley et al., 1994) showed that *FAD2–1* and *FAD2–2* are highly homologous to Arabidopsis *FAD2* (Fig. 1) and canola *FAD2* genes (W.D. Hitz, personal communication). On the other hand, microsomal ω-6 desaturases exhibit limited sequence identity with soybean plastidial ω-6 desaturase (Fig. 1; Hitz et al., 1994) and with other fatty acid desaturases (data not shown).

Complementation of the *fad2–1* Mutation with Soybean Microsomal ω-6 Desaturase Genes

To determine the functional identity of the *FAD2–1* and *FAD2–2* genes, a genetic complementation test was conducted. The coding sequences of the *FAD2–1* and *FAD2–2* cDNA sequences were inserted in the sense orientation behind the CaMV 35S promoter in pSN1 vector. The fusion fragments were then subcloned into the *Hin*dIII and *Eco*RI sites of the binary vector pBI121, which carries the kanamycin selection marker and the GUS visible marker. The resulting constructs, pBI-FAD2–1 and pBI-FAD2–2, were transformed into *A. tumefaciens* R1000. The transformed *A. tumefaciens* R1000 were used to induce hairy root formation on stem explants from the Arabidopsis *fad2–1* mutant plants (Arondel et al., 1992). The vector pBI121 was used to transform *fad2–1* as a control.

Total lipids were extracted from the GUS-positive and kanamycin-resistant hairy roots, and fatty acid compositions of the extracts were measured by GC. As expected the hairy roots transformed by vector pBI121 contain an average of 10% polyunsaturated fatty acid (18:2 plus 18:3) content (Fig. 2), which is similar to the untransformed *fad2–1* mutant plants. In contrast, the 18:2 plus 18:3 content of hairy roots transformed by pBI-FAD2–1 and pBI-FAD2–2 are in the range of 35 to 65% and 61 to 65%, respectively (Fig. 2). The 18:2 plus 18:3 content in roots of wild-type Arabidopsis is about 62%. The increase in 18:2 plus 18:3 in these transformed hairy roots is largely compensated by decreasing 18:1 content (data not shown). Thus, the *FAD2–1* and *FAD2–2* clones are capable of complementing the Arabidopsis *fad2–1* mutation, confirming that both genes encode functional microsomal ω-6 desaturases.

Genomic Characterization of Microsomal ω-6 Desaturase Genes

The coding sequences of *FAD2–1* and *FAD2–2* cDNA sequences were homologous (Fig. 1), but the 5′ and 3′ nontranslational regions of the two sequences were unique (data not shown), suggesting that *FAD2–1* and *FAD2–2* are two distinct members of the soybean microsomal ω-6 desaturase gene family. Genomic Southern blot analysis by *FAD2–1* and *FAD2–2* gene-specific probes confirmed that *FAD2–1* and *FAD2–2* genes were nonallelic (Fig. 3). Indeed, the two genes have been mapped to different linkage groups of the soybean genome (J.A. Rafaski and S.V. Tingey, personal communication). Two distinct bands were observed from the genomic DNA digested with *Bam*HI and

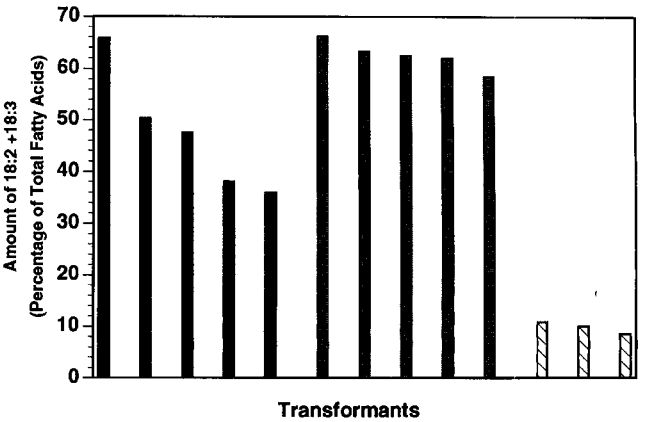

Figure 2. Amount of polyunsaturated fatty acids in transgenic hairy roots of Arabidopsis *fad2–1* mutant plant transformed by soybean *FAD2–1*, *FAD2–2*, and pBI121. Black and gray bars represent polyunsaturated fatty acid (18:2 plus 18:3) content of five independently derived transgenic hairy root cultures expressing *FAD2–1* and *FAD2–2*, respectively. The amount of polyunsaturated fatty acids of three hairy root cultures transformed by vector pBI121 is shown by the stripped bars.

*Hin*dIII enzymes when hybridizing with the *FAD2–1*-specific probe. The *Eco*RI-digested DNA appeared to generate two bands, which migrated to a similar position on the gel (Fig. 3). The *Hin*dIII-digested genomic DNA generated two hybridizing bands when probed with *FAD2–2*-specific DNA. *Bam*HI- and *Eco*RI-digested DNA showed more than two bands (Fig. 3). Based on the intensity of these bands, some of the weakly hybridizing bands may be due to incomplete DNA digestion. These results suggested that there were at least two copies of each of these genes in the soybean genome, which is consistent with the tetraploid nature of the soybean genome. The allelic genes for *FAD2–1* and *FAD2–2* were also isolated. Partial sequence analysis indicated 95 to 97% sequence identity in the 3′ noncoding regions of the allelic genes (E. Hepperd and G.-H. Miao, unpublished data).

Tissue-Specific and Developmentally Regulated Expression of Soybean Microsomal ω-6 Desaturase Genes

To determine the relevance of two microsomal ω-6 desaturase genes in controlling the levels of polyunsaturated fatty acids in developing seeds and other tissues, we measured the steady-state transcript levels of the two genes by northern blot analysis. The RNAs were isolated from leaves, stems, roots, and embryos at different stages of seed development ranging from 6 to 10, 13, 17, 19, 21, and 26 DAF. The RNAs were resolved on the gel and probed with *FAD2–1* and *FAD2–2* gene-specific probes. Both the *FAD2–1* and *FAD2–2* genes were expressed in developing seeds. The expression level of the *FAD2–2* gene was higher than that of the *FAD2–1* gene at the earlier stage (6–10 DAF) of seed development (Fig. 4) when large amounts of membrane lipids are synthesized. However, the transcript of the *FAD2–1* gene rapidly increased during embryo development, peaked in the mid-maturation stages (19–21

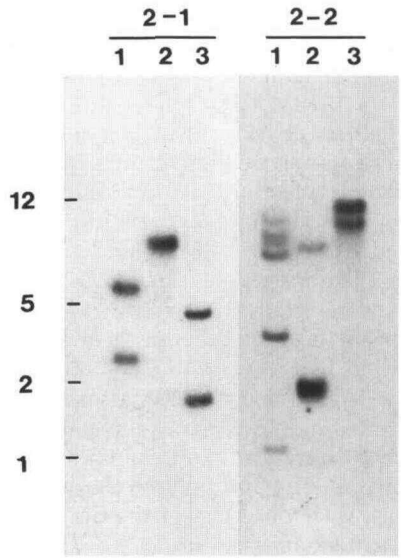

Figure 3. Southern blots of soybean genomic DNA probed with *FAD2–1*- and *FAD2–2*-specific sequence fragments. Because the 3′ noncoding regions of *FAD2–1* and *FAD2–2* are distinct, two pairs of oligonucleotides for *FAD2–1* (S1–1, 5′-GTATTGATGGAGCAACCAAT-3′; S1–2, 5′-GGCAGAAAGCTATAAGCAGA-3′) and for *FAD2–2* (S2–1, 5′-GATTAATGTAGCCGAG GCTT-3′; S2–2, 5′-GACCGGCGCTCAGCTGGAAT-3′) were synthesized to amplify by PCR the 3′ noncoding sequence unique to the two clones. The PCR-amplified fragments were isolated, labeled with [α-^{32}P]dCTP, and used as probes for genomic Southern hybridization. 2–1, *FAD2–1*; 2–2, *FAD2–2*; 1, *Bam*HI; 2, *Eco*RI; 3, *Hin*dIII. Molecular size (kb) of the 1-kb DNA ladder (Life Technologies, Inc.) is indicated on the left.

DAF), and then gradually declined as seeds matured further (Fig. 4). Thus, the timing of *FAD2–1* gene expression coincided with that of fatty acid biosynthesis and oil deposition in developing seeds. Whereas the transcript for the *FAD2–1* gene increased significantly during the embryo development, the expression level of the *FAD2–2* gene appeared to increase only slightly during embryo development. The *FAD2–2* gene was also expressed in vegetative tissues, including leaves, stems, and roots, with the highest level in leaves. In contrast, the transcript of the *FAD2–1* gene was not detected in these tissues (Fig. 4), suggesting that the *FAD2–1* gene encodes a seed-specific microsomal ω-6 desaturase. Hence, *FAD2–1* and *FAD2–2* genes not only differ in structure (Figs. 1 and 3) but also have distinct expression patterns (Fig. 4). The difference in developmental and tissue-specific expression of the two genes may suggest a nonoverlapping role for the genes in desaturation of membrane and storage lipids in developing seeds and vegetative tissues.

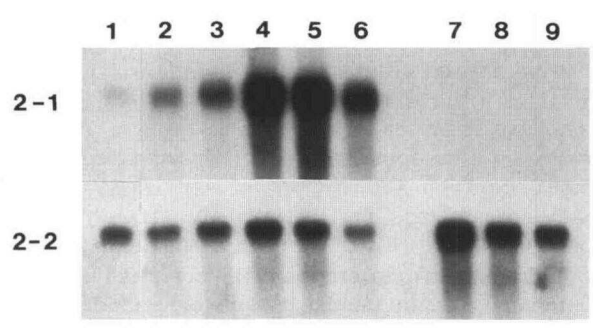

Figure 4. RNA gel blot analysis of the developmental and tissue-specific expression of soybean ω-6 desaturase genes. 2–1, *FAD2–1*; 2–2, *FAD2–2*. Lanes 1 to 6 contain RNAs from developing seeds at 6 to 10, 13, 17, 19, 21, and 26 DAF, respectively. Lane 7, Leaf; lane 8, stem; lane 9, root. The same blot was used for hybridization with *FAD2–1* and *FAD2–2* gene-specific probes.

Fatty Acid Composition in Developing Soybean Seeds

The changes in fatty acid composition during soybean seed development were followed by GC analysis. Both 18:1 and 18:2 levels increased during the seed development, whereas 18:3 content decreased significantly (Fig. 5). The higher level of 18:3 of lipids in young developing seeds may suggest the potential role of 18:3 in maintaining membrane fluidity (Neidleman, 1987) in young embryos in which active membrane biosynthesis takes place. The significant increase in the 18:2 content during seed development is consistent with the observed expression of the *FAD2–1* gene, which was significantly enhanced during seed development (Fig. 4). However, the level of 18:1 of seed lipids also increases during seed maturation (Fig. 5), indicating that microsomal ω-6 desaturase activity may still be limiting during soybean seed development.

Growth Temperature Regulation of Seed Fatty Acid Composition and Expression of Microsomal ω-6 Desaturase Genes

The unsaturated fatty acid level in soybean seeds has been shown to be affected by growth temperature (Rennie and Tanner, 1989; Kinney, 1994). To test whether this temperature effect is due to the enhanced expression of microsomal ω-6 desaturase genes, we determined the changes of both fatty acid composition and the level of transcripts of *FAD2–1* and *FAD2–2* genes. Soybean plants were grown in growth chambers at different day/night temperatures: 32/28, 28/22, 22/18, and 18/12°C. Leaf tissues, developing

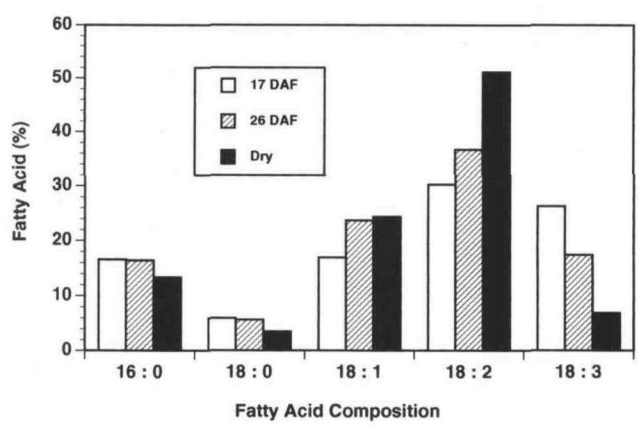

Figure 5. Developmental profile of fatty acid composition in soybean seeds. The values are averages from more than 10 independent seeds at each developmental stage. 16:0, Palmitic acid; 18:0, stearic acid.

seeds, and dry seeds were collected, the fatty acid composition was determined by GC, and the desaturase gene expression was determined by RNA gel blot analysis. In mature (21 DAF) and dry seeds, both 18:2 and 18:3 levels gradually increased as the growth temperature decreased. The increase in polyunsaturated fatty acids of seed lipids at low temperature was largely compensated for by the decrease in 18:1 content. The low temperature appeared to cause an increase in the 18:3 content but not the 18:2 level of seed lipids in young developing seeds (17 DAF; Fig. 6), indicating a different sensitivity to temperature during different stages of seed development. The relatively constant level of 18:2 in the young developing seeds at different temperatures may suggest the existence of a mechanism to retain a certain level of 18:2 by balancing the flux through 18:1 and 18:2 fatty acids. A similar reverse correlation was observed between polyunsaturated fatty acid level and growth temperature in leaf tissues (data not shown).

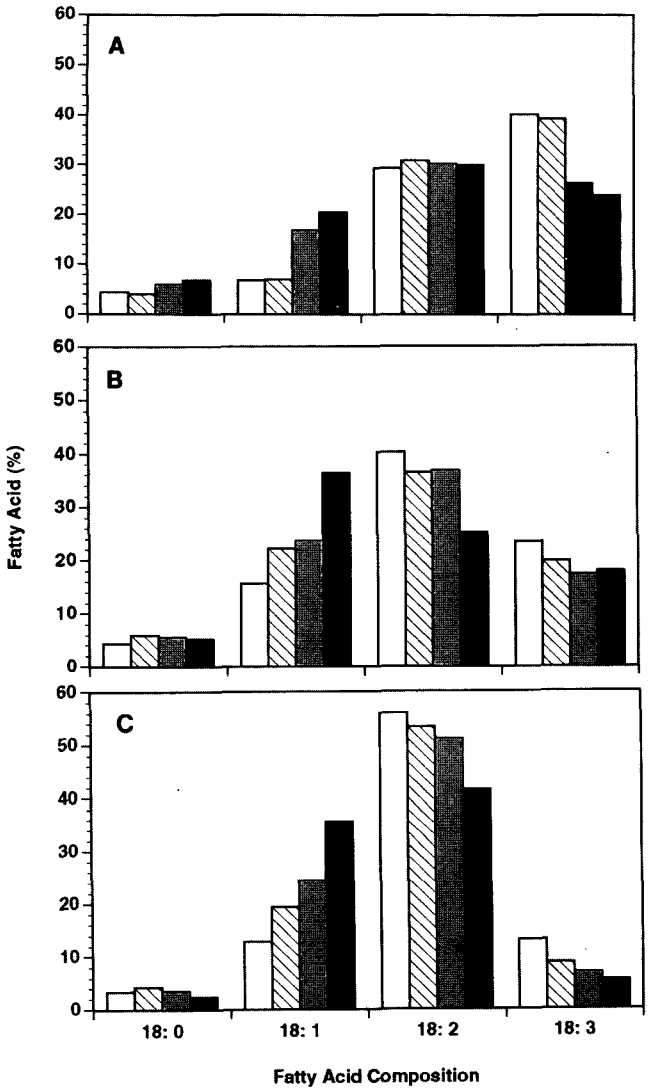

Figure 6. Growth temperature effects on fatty acid compositions of developing soybean seeds. A, 17 DAF; B, 26 DAF; C, dry seed. The results are based on two independent assays, and the values are averages from 6 to 12 independent seeds for each treatment. The variation of independent GC analysis is insignificant. □, 18°C; ▨, 22°C; ▩, 28°C; ■, 32°C.

To test whether the increase of 18:2 and 18:3 fatty acid levels in these tissues by low temperature was due to enhanced gene expression, we determined the level of transcripts by northern blot analysis for both microsomal and plastidial (Hitz et al., 1994) ω-6 desaturase genes in these plants. The transcript levels of *FAD2–1*, *FAD2–2*, and *FAD6* genes were relatively constant in both young and mature soybean seeds at different growth temperatures. The decrease in temperature did not result in enhanced expression of *FAD2–1* and *FAD2–2* genes (Fig. 7), although low temperature did increase significantly the levels of polyunsaturated fatty acids in seed lipids (Fig. 6). Unexpectedly, increased transcript levels of *FAD2–1* and *FAD2–2* genes were observed in the developing seeds at the elevated growth temperature (32/28°C), particularly in the young, developing soybean seeds. In leaves, the transcript levels of *FAD2–2* and *FAD6* remained relatively constant at different growth temperatures (Fig. 8). The seed-specific *FAD2–1* gene did not seem to be induced in leaf tissues by low temperature. The expression of *FAD6* was significantly lower than that of the *FAD2–2* gene in leaves (Fig. 8), consistent with the current theory that the ER is the major route for polyunsaturated fatty acid biosynthesis (Browse and Somerville, 1991). These results demonstrated that the elevated polyunsaturated fatty acid level in developing soybean seeds grown at low temperatures was not due to enhanced expression of ω-6 desaturase genes. Another regulation mechanism such as translational or post-translational modification of ω-6 desaturase may be responsible for the higher 18:2 level in soybean seeds at low temperatures.

DISCUSSION

Microsomal ω-6 desaturase is encoded by a single gene in Arabidopsis, *FAD2*, and this gene is expressed in both vegetative tissues and developing seeds (Okuley et al., 1994). Thus, one gene is presumably responsible for conversion of 18:1 to 18:2 fatty acids in both membrane and seed storage lipids in these tissues. As with the Arabidopsis *FAD2* gene, the soybean *FAD2–2* gene was also constitutively expressed in both vegetative tissue and developing seeds (Figs. 4 and 7). In contrast, the soybean *FAD2–1* gene was not expressed in vegetative tissues but was specifically activated in developing seeds (Fig. 4). Therefore, it is apparent that there are at least two different classes of microsomal ω-6 desaturase genes in soybeans. *FAD2–2*, encoding a constitutively expressed microsomal ω-6 desaturase, may be responsible for desaturation of 18:1 of membrane lipids in both vegetative tissue and seed development. The seed-specific gene, *FAD2–1*, may play the major role in desaturation of 18:1 of storage lipids synthesized during the mid-maturation stages of seed development, because the expression level of the *FAD2–1* gene is much higher than that of the *FAD2–2* gene during the period of storage lipid biosynthesis (Fig. 4). This suggestion is further supported

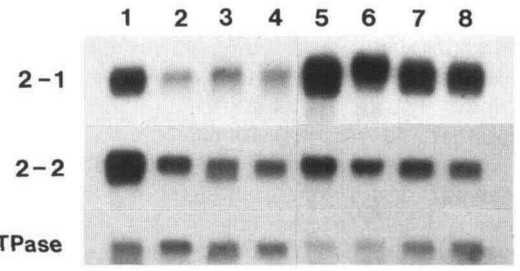

Figure 7. Growth temperature effects on expression of soybean ω-6 desaturase genes in developing seeds. 2–1, *FAD2–1*; 2–2, *FAD2–2*; ATPase, tobacco β subunit of the mitochondrial ATP synthetase (Boutry and Chua, 1985). Lanes 1 to 4 contain RNAs from seeds 17 DAF grown at 32/28, 28/22, 22/18, and 18/12°C, respectively; lanes 5 to 8 contain RNAs from seeds 26 DAF grown at 32/28, 28/22, 22/18, and 18/12°C, respectively.

by our observation that the reduced expression of the *FAD2–1* gene in a seed-specific manner by antisense RNA resulted in a significant increase of 18:1 content of seed lipids of transgenic soybean plants. On the other hand, reduced expression of the *FAD2–2* gene in developing embryos caused only a slight increase in the 18:1 level of storage lipids (A.J. Kinney and G.-H. Miao, unpublished data). Although both *FAD2–1* and *FAD2–2* genes are expressed in developing seeds, the tissue- and cell-type specificity of these genes may not be identical. The precise localization of expression of the two genes in developing seeds may provide additional insight into the role of each gene product in controlling polyunsaturated fatty acid synthesis in membrane and storage lipids in developing soybean seeds.

The extensive oil seed breeding program has led to the isolation of mutants with altered fatty acid composition (Ohlrogge et al., 1991; Yadav, 1995). Some of these mutants have a phenotype expressed in seeds but not in vegetative tissues (Garces and Mancha, 1989). These results may suggest the possibility of the existence of seed-specific isoforms of some of the fatty acid biosynthetic genes or of preferential incorporation of certain fatty acids in TAG in seeds. We have demonstrated that the *FAD2–1* gene is specifically expressed in developing soybean seeds (Fig. 4) and thus provide the molecular evidence that the desaturation of 18:1 in soybean seeds is mainly due to the expression of a seed-specific ω-6 desaturase gene. By extension, it is also likely that a similar seed-specific ω-6 desaturase gene in sunflowers may be responsible for desaturation of 18:1 during oil biosynthesis and deposition, because changes in the fatty acid composition of a sunflower high-18:1 mutant are restricted to the seeds (Garces and Mancha, 1989). The Arabidopsis *FAE1* gene encoding a fatty acid-condensing enzyme, which synthesizes the long-chain fatty acids (C_{20} and C_{22}) in seeds, has recently been isolated (James et al., 1995) and is also specifically expressed in developing seeds. This finding is in agreement with the observed phenotype of the *fae1* mutation, which is only expressed in the seeds. Hence, some genes encoding fatty acid biosynthetic enzymes are regulated by a seed developmental program. These genes appear to play a major role in determining the composition of storage lipids in developing seeds.

The timing of *FAD2–1* gene expression appears to be earlier than of seed storage protein genes (Goldberg et al., 1989). Similarly, the *Brassica napus* ACP gene is also expressed earlier than the seed storage genes encoding napin and cruciferin (Kridl et al., 1993). Therefore, regulation of genes encoding fatty acid biosynthetic enzymes and seed storage proteins may be different. Comparison of the *FAD2–1* gene with seed storage protein genes reveals no similarity in their promoter regions (G.-H. Miao, unpublished observation). It will be interesting to determine whether *FAD2–1*, *FAE1*, and other fatty acid biosynthetic genes highly expressed in seeds share any common structure features in their regulatory elements.

In the Arabidopsis *fad2* mutant, 12 to 15% of the normal level of *FAD2* transcript was still able to convert more than half of the 18:1 to 18:2 (Okuley et al., 1994). Therefore, it was suggested that the transcript of microsomal ω-6 desaturase was severalfold in excess of the amount needed to account for 18:1 desaturase activity (Okuley et al., 1994). Overexpression of *FAD2–1* and *FAD2–2* cDNA sequences did not lead to production of 18:1 plus 18:2 content significantly higher than in the root of wild-type Arabidopsis (Fig. 2), confirming that the substrate 18:1 level rather than ω-6 desaturase activity may be limiting in Arabidopsis. In soybean, the expression level of the *FAD2–1* gene significantly increased during the period of maximum storage lipid synthesis and accumulation (Fig. 4), which is consistent with the increased level of 18:2 during the same period (Fig. 6). However, the monounsaturated fatty acid level also increased during the same period (Fig. 5), indicating that the increased *FAD2–1* transcript may not be sufficient to convert the newly synthesized 18:1 to 18:2 of storage lipids. Indeed, the reduced level of the *FAD2–1* transcript by antisense RNA or co-suppression led to a significant increase of 18:1 content in transgenic soybean seeds (A.J. Kinney, unpublished observation). The observed discrep-

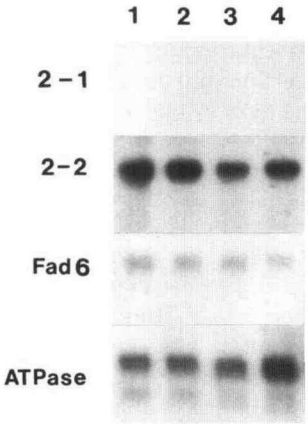

Figure 8. Growth temperature effects on expression of soybean ω-6 desaturase genes in leaves. 2–1, *FAD2–1*; 2–2, *FAD2–2*; *FAD6*, plastidial ω-6 desaturase (Hitz et al., 1994); ATPase, tobacco β subunit of the mitochondrial ATP synthetase (Boutry and Chua, 1985). Lanes 1 to 4 contain RNAs from leaves grown at 32/28, 28/22, 22/18, and 18/12°C, respectively.

ancy may be partly due to the much higher rate of TAG synthesis in seeds of soybean than of Arabidopsis.

The fatty acid composition is greatly influenced by growth temperature; the fatty acid polyunsaturation level increases in response to low growth temperature. This appears to be a general phenomenon for both prokaryotic and eukaryotic organisms (Neidleman, 1987). Unsaturated fatty acids have lower melting points than their saturated counterparts and thus may confer greater membrane fluidity, which may allow bacteria and plants to maintain membrane function under lower growth temperature conditions (Neidleman, 1987; Thompson, 1993). In cyanobacteria, expression of the ω-6 desaturase gene (*desA*) is markedly enhanced by low growth temperature (Los et al., 1993). Introduction of *desA* from a chilling-resistant cyanobacterium, *Synechocystis* PCC6803, into a chilling-sensitive cyanobacterium, *Anacystis*, increased the tolerance of the recipient to low temperature (Wada et al., 1990). This provided direct evidence for the contribution of the unsaturation of fatty acids to low-temperature tolerance in cyanobacteria. The fatty acid composition of soybean seed lipids is also regulated by growth temperature (Fig. 6). High-18:1 mutants are temperature unstable, and the instability of the high-oleate phenotype thus limits the practical applications of these otherwise valuable mutant lines (Rennie and Tanner, 1989). These high-18:1 soybean mutants appeared to have reduced activity of microsomal ω-6 desaturase, presumably because of mutation in one of the soybean microsomal ω-6 desaturase genes. By analogy with cyanobacteria, it was logical to reason that the enhanced expression of a second ω-6 desaturase gene by cold temperature may have been responsible for the instability of the high-18:1 mutants.

Our results confirmed the significant effect of temperature on fatty acid composition in developing soybean seeds (Fig. 6; Rennie and Tanner, 1989). However, the increase in polyunsaturated fatty acids of seed lipids at low temperature is apparently not due to enhanced expression of microsomal ω-6 desaturase genes in soybean (Figs. 7 and 8), as was the case in cyanobacteria (Los et al., 1993). *FAD6* is also not regulated by cold temperature (Fig. 8), although the structure and function of plant plastidial ω-6 desaturase is more similar to *desA* of cyanobacteria (Hitz et al., 1994). At the elevated growth temperature, the transcript levels of *FAD2–1* and *FAD2–2* genes are even higher than at lower temperature (Fig. 7). This effect is more apparent in the young soybean seeds. One possible explanation for this unexpected observation is that more transcript for ω-6 desaturase is made to meet the needs of desaturation of fatty acids at higher temperature, because ω-6 desaturase is highly unstable at high temperature (Cheesbrough, 1989). Expression of Arabidopsis *FAD2* is also not regulated by cold temperature (Okuley et al., 1994). On the other hand, the Arabidopsis gene encoding a chloroplast ω-3 fatty acid desaturase (*FAD7*) appears to be induced by cold temperature (Gibson et al., 1994). Expression of this gene in transgenic tobacco plants resulted in increased levels of trienoic fatty acids and, consequently, enhanced cold tolerance in the transgenic plants (Kodama et al., 1994). Therefore, different mechanisms may be involved in temperature regulation of fatty acid composition in different organisms. The increased level of polyunsaturated fatty acids of soybean seed lipids by cold temperatures is likely the result of translational and posttranslational regulation, such as altered desaturase enzyme activity (Cheesbrough, 1989), rather than transcriptionally induced or enhanced expression of ω-6 desaturase genes in soybean plants.

Polyunsaturated fatty acids in membrane lipids have been shown to be required for low-temperature survival of Arabidopsis (Miquel et al., 1993), and an altered membrane structure of an *fad2–2* mutant plant could not sustain the cellular processes required for seed development at low temperature (Miquel and Browse, 1994). A high level of polyunsaturated fatty acid may be required for maintaining membrane fluidity and membrane-bound enzyme activity for cell division and differentiation during early seed development. The major portion of fatty acids made during the early seed development stages are used for membrane biosynthesis. Indeed, the 18:2 level is maintained relatively constant even at different temperatures in the early stages of seed development (Fig. 6). Significant alteration of fatty acid composition during early seed development may affect the fitness of plants at low temperature. However, reduced expression of microsomal ω-6 desaturase genes only during the period of storage lipid synthesis in transgenic plants may circumvent the poor agronomic performance associated with the high oleate content of membrane lipids and, hence, generate commercially viable, high-oleate germplasms through genetic engineering.

ACKNOWLEDGMENTS

We wish to thank J. Browse for Arabidopsis *fad2–1* seeds; W.D. Hitz, J.A. Rafalski, and S.V. Tingey for unpublished information; and J. Lightner, S.Y. Yamamoto, J.B. Shen, and R.M. Broglie for comments.

Received August 10, 1995; accepted October 16, 1995.

The accession numbers for nucleotide sequences of *FAD2–1* and *FAD2–2* are L43920 and L43921, respectively.

LITERATURE CITED

Arondel V, Lemiewx 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 **258:** 1353–1355

Boutry M, Chua N-H (1985) A nuclear gene encoding the beta subunit of the mitochondria ATP synthetase in *Nicotiana plumbaginifolia*. EMBO J **4:** 2159–2165

Browse J, McCourt PJ, Somerville CR (1986) Fatty acid composition of leaf lipids determined after combined digestion and fatty acid methyl ester formation from fresh tissue. Anal Biochem **152:** 141–145

Browse J, Somerville CR (1991) Glycerolipid synthesis: biochemistry and regulation. Annu Rev Plant Physiol Plant Mol Biol **42:** 467–506

Cheesbrough TM (1989) Changes in the enzymes for fatty acid synthesis and desaturation during acclimation of developing soybean seeds to altered growth temperature. Plant Physiol **90:** 760–764

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep **1:** 19–21

Garces R, Mancha M (1989) Oleate desaturation in seeds of two genotypes of sunflower. Phytochemistry **28:** 2593–2595

Gibson S, Arondel V, Iba K, Somerville CR (1994) Cloning of a temperature-regulated gene encoding a chloroplast ω-3 desaturase from *Arabidopsis thaliana*. Plant Physiol **106:** 1615–1621

Goldberg RB, Barker SJ, Perez-Brau L (1989) Regulation of gene expression during plant embryogenesis. Cell **56:** 149–160

Hitz WD, Carlson TJ, Booth JR, Kinney AJ, Stecca KL, Yadav NS (1994) Cloning of a higher-plant plastid ω-6 fatty acid desaturase cDNA and its expression in a cyanobacterium. Plant Physiol **105:** 635–641

James DW Jr, Lim E, Keller J, Plooy I, Ralston E, Dooner HK (1995) Directed tagging of the Arabidopsis fatty acid elongation (FAE1) gene with the maize transposon activator. Plant Cell **7:** 309–319

Kinney AJ (1994) Genetic modification of the storage lipids of plants. Current Opinion in Biotechnology **5:** 144–151

Kodama K, Hamada T, Horiguchi G, Nishimura M, Iba K (1994) Genetic enhancement of cold tolerance by expression of a gene for chloroplast ω-3 fatty acid desaturase in transgenic tobacco. Plant Physiol **105:** 601–605

Kridl JC, Knauf VC, Thompson GA (1993) Progress in expression of genes controlling fatty acid biosynthesis to alter oil composition and content in transgenic rapeseed. *In* DPS Verma, ed, Control of Plant Gene Expression. CRC Press, Boca Raton, FL, pp 481–498

Liu HR, White PJ (1992) Oxidative stability of soybean oils with altered fatty acid composition. J Am Oil Chem Soc **69:** 528–532

Los D, Horvath I, Vigh L, Murata N (1993) The temperature dependent expression of the desaturation gene desA in *Synechocystis* PCC6803. FEBS Lett **318:** 57–60

Miquel MF, Browse JA (1994) High-oleate oilseeds fail to develop at low temperature. Plant Physiol **106:** 421–427

Miquel MF, James DW Jr, Dooner HK, Browse JA (1993) *Arabidopsis* requires polyunsaturated lipids to low temperature survival. Proc Natl Acad Sci USA **90:** 6208–6212

Murashige J, Skoog F (1962) A revised medium for rapid growth and bio assay with tobacco tissue cultures. Physiol Plant **115:** 473–497

Neidleman SL (1987) Effects of temperature on lipid unsaturation. Biotechnol Genet Eng Rev **5:** 245–268

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, Browse J (1994) The Arabidopsis *FAD2* gene encodes the enzyme that is essential for polyunsaturated lipid synthesis. Plant Cell **6:** 147–158

Rennie BD, Tanner JW (1989) Fatty acid composition of oil from soybean seeds grown at extreme temperatures. J Am Oil Chem Soc **66:** 1622–1624

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA **74:** 5463–5467

Somerville CR, Browse J (1991) Plant lipids: metabolism, mutants, and membranes. Science **252:** 80–87

Thompson GA Jr (1993) Response of lipid metabolism to developmental change and environmental perturbation. *In* TS Moore Jr, ed, Lipid Metabolism in Plants. CRC Press, Boca Raton, FL, pp 591–619

Wada H, Gombos Z, Murata N (1990) Enhancement of chilling tolerance of a cyanobacterium by genetic manipulation of fatty acid desaturation. Nature **347:** 200–203

Yadav NS (1995) Genetic modification of soybean oil quality. *In* DPS Verma, R Shoemaker, eds, Soybean Biotechnology. CAB International, Wallingford, CT (in press)

Yadav NS, Wierzbicki A, Aegerter M, Caster CS, Perez-Grau L, Kinney AJ, Hitz WD, Booth 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