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ANALYSIS AND FUNCTIONAL EXPRESSION OF A GENE FOR A COTTON FATTY ACID DESATURASE (FAD2-4)

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Abstract

Over 75% of fatty acids in plants are unsaturated by desaturase enzymes in chloroplast and endoplasmic reticulum membranes. Two fatty acid desaturases designated FAD2 and FAD3 primarily desaturate extrachloroplast lipids and occur as integral membrane proteins in the endoplasmic reticulum. The *FAD2* gene has been shown by several laboratories to be important in the chilling sensitivity of plants, since polyunsaturated phospholipids are essential for maintaining plant viability at lowered temperatures. Cotton genomic libraries harbored in lambda phage were screened to isolate prospective *FAD2* genes, in order to study their regulation of gene expression. The structure and expression of a cotton *FAD2* gene (called the *FAD2-3* gene) was previously analyzed. Recently, the structure and functional expression of a second cotton *FAD2* gene (called the *FAD2-4* gene) was analyzed for comparison with that of the *FAD2-3* gene, to compare the similarities/differences of the genes in the 5'-flanking introns, the promoter elements, and the protein-coding regions. This is to gain insight into how the two cotton *FAD2* genes are regulated for gene expression, and if the 5'-flanking introns really have any bearing on transcriptional regulation of the genes. The *FAD2-4* gene is distinctly different from the *FAD2-3* gene, with minor differences in the coding regions and major differences in the flanking regions. The *FAD2-4* gene is expressed in cotton, since its cDNA was amplified from a cotton cDNA library by PCR. The putative cotton FAD2-4 polypeptide likely is an integral membrane protein in the cellular endoplasmic reticulum, since it has four tentative membrane-spanning domains corresponding to the predicted membrane-spanning domains in desaturase integral membrane protein models. The 5'-flanking region of the *FAD2-4* gene has a number of prospective promoter elements that also occur in the 5'-flanking region of the *FAD2-3* gene. A large intron occurs in the 5'-flanking region of the *FAD2-4* gene, similar in size and location to the large intron in the 5'-flanking region of the *FAD2-3* gene. However, there are substantial differences in the nucleotide sequences of the two introns, indicating that the genes are probably orthologs. These 5'-flanking introns could be important in the transcriptional regulation of expression of the genes. The protein-coding region of the *FAD2-4* gene is 1,155 bp, and is continuous with no introns. The deduced amino acid sequence of 384 amino acids of the putative cotton FAD2 polypeptide has a high identity (about 75%) with the deduced amino acid sequences of other plant FAD2 enzymes. Yeast cells transformed with a plasmid construct containing the cotton *FAD2-4* coding region have an appreciable accumulation of linoleic acid (18:2), not normally present in wild-type yeast cells. Thus, this cotton *FAD2* gene is truly functional, since it encodes an enzyme that catalyses the desaturation of oleic acid (18:1) into linoleic acid (18:2). Two binary plasmid constructs containing the two *FAD2* genes are being generated for transformation of model *Arabidopsis* plants, before beginning transformation of cotton plants. An understanding of the expression patterns of the *FAD2* genes should allow us to genetically engineer these genes in cotton, and possibly modify the membrane fatty acid compositions to improve viability of this crop plant.

Introduction

Fatty acids in plants, as in all other organisms, are the major structural components of the phospholipids in biological membranes and the triacylglycerols of storage oils, and so, understanding the mechanisms underlying the regulation of fatty acid amounts and compositions in membrane phospholipids and oil triacylglycerols in plants is important. The major site of the approximately 30 enzymatic reactions needed to produce C16- and C18-fatty acids in plants is the stroma of plastids, with over 75% of the fatty acids being unsaturated (Ohlrogge and Browse, 1995; Somerville et al., 2000). The individual enzymes of the pathway are dissociable soluble components, perhaps organized into a supramolecular complex. The major membrane phospholipids are first made using acyl groups with palmitate (16:0) and oleate (18:1) in all plant tissues (Ohlrogge and Browse, 1995; Somerville et al., 2000).

Desaturation of the fatty acid components of the membrane phospholipids is carried out by membrane-bound desaturases of the chloroplast and endoplasmic reticulum membranes. Seven *Arabidopsis* mutants have been found for the fatty acid desaturase enzymes (designated from FAD2 to FAD8) deficient in a specific fatty acid desaturation step (Ohlrogge and Browse, 1995). The FAD4 to FAD8 enzymes affect chloroplast lipid desaturation. The *FAD2* and *FAD3* enzymes primarily desaturate extrachloroplast lipids and occur as integral membrane proteins in the endoplasmic reticulum. The enzymes are phosphatidylcholine desaturases, acting on fatty acids at both the sn-1 and sn-2 positions.

The *FAD2* gene appears to be especially important in the chilling sensitivity of plants (Miquel and Browse, 1994; Okuley et al., 1994; Ohlrogge and Browse, 1995). Thus, polyunsaturated phospholipids in membranes are essential for maintaining cel-

lular function and plant viability at lowered temperatures (Browse and Xin, 2001). Our laboratory has analyzed the structures and functional expression of the first cotton *FAD2* genes, the *FAD2-4* gene and the *FAD2-3* gene (Pirtle et al., 2001). In cotton, a knowledge of the tissue-specific and development-specific regulation of fatty acid compositions of the membranes is crucial in understanding heat and cold tolerance, desiccation and salt tolerance, and disease resistance. A clearer understanding of the expression patterns of the *FAD2* gene(s) should allow us to genetically engineer this gene in cotton, and in turn, ultimately manipulate the fatty acid compositions of cotton membranes predictably to improve the vigor and viability of this important crop plant.

Materials and Methods

Isolation of a Cotton Genomic Clone Encompassing the *FAD2-4* Gene

A cotton (*Gossypium hirsutum*, cv. Acala SJ-2) genomic library harbored in the Lambda FIXII vector (Stratagene), generously provided by Dr. Thea Wilkins of the University of California at Davis, was screened to isolate clones encompassing additional *FAD2* genes, either orthologous or allelic to the *FAD2-3* gene (Pirtle et al., 2001), by the plaque hybridization procedure (Benton and Davis, 1977). A 1.2-kb DNA fragment containing the coding region of the cotton *FAD2-3* gene (Pirtle et al., 2001) was used as template to generate ^{32}P-labeled hybridization probe generated by random priming (Feinberg and Vogelstein, 1983). Prehybridization was done for 4 hours at 60°C in a solution containing 6X SSC (1X SSC is 150 mM sodium chloride and 15 mM sodium citrate, pH 7.0), 5X Denhardt's reagent, 0.5% SDS, and denatured sheared salmon sperm DNA (100 µg/ml). Hybridization was done overnight at 60°C in a solution containing 6X SSC, 5X Denhardt's reagent, 0.5% SDS, 20 mM Tris-HCl (pH 8.0), 2 mM EDTA (pH 7.5), 2.5 mM sodium pyrophosphate (pH 8.0), denatured sheared salmon sperm DNA (100 µg/ml), and ^{32}P-labeled probe. After hybridization, the membranes were rinsed once at room temperature with 2X SSC for 5 min., once with 2X SSC, 0.1%SDS at 60°C for 30 min., and twice with 1X SSC, 0.1% SDS at 60°C for 30 min. The DNA from one genomic clone designated LCFg5b that intensely hybridized to the homologous cotton *FAD2-3* probe was selected for purification for use in physical mapping and subcloning (Sambrook and Russell, 2001). The DNA from the genomic clone was then digested with restriction endonucleases for physical mapping by agarose gel electrophoresis and alkaline blot hybridization. A 8.6-kb *Xba*I fragment of the genomic clone LCFg5b encompassing the hybridizing region was subcloned into the plasmid vector pUC19 for sequence analysis. The resulting recombinant plasmid (designated pCFg5b) DNA was isolated and purified using the Wizard Plus Minipreps DNA Purification System (Promega).

PCR Amplification of the *FAD2-4* cDNA from a Cotton cDNA Library

To confirm that the *FAD2-4* gene is actually transcribed to generate its mRNA, putative *FAD2-4* cDNA fragments were amplified by the polymerase chain reaction (PCR) from a cotton cDNA library in the plasmid vector pBlueScript II SK(+) (Stratagene), generously provided by Dr. Ed Cahoon of Dupont Ag Products, Wilmington, DE. For this PCR, the primers were derived from gene segments unique to the *FAD2-4* gene. The forward amplimer (5'-TGTCTAGAGACCAAAGTGAAAGAAAATCGAAG-3') was used to create a *Xba*I site (underlined) in the 5'-flanking region of the putative *FAD2-4* cDNA. The reverse amplimer (5'-GACGAGCTCCAAAA-GCATCTAAAATAGAAGTAACCC) was designed to create a *Sac*I site (underlined) in the 3'-flanking region of the putative *FAD2-4* cDNA product. The PCR product, predicted to be 1,328 bp in size, was generated from the cotton cDNA library using Platinum *Pfx* polymerase (Invitrogen), and subsequently digested with *Sac*I and *Xba*I prior to ligation into the *Sac*I/*Xba*I site in the polylinker of pGEM-7Zf(+) vector DNA (Promega).

DNA Sequence Analysis

Both DNA strands of the 8,612-bp *Xba*I fragment encompassing the putative *FAD2-4* gene and the *FAD2-4* PCR product amplified from the cDNA library were sequenced using a primer-based approach by semi-automated procedures on an Applied Biosystems Model 377XL DNA Sequencer by Lone Star Labs (Houston, TX). The sequencing reactions were done according to manufacturer protocols by terminator cycle sequencing with BigDye™ using 10% DMSO as denaturant. Analyses and alignments of the DNA and deduced amino acid sequences were done with DNASIS software (Hitachi). From the sequence of the *FAD2-4* gene, the locations of the 5'-and 3'- flanking regions prospective promoter elements and the CAP binding site were tentatively identified by comparison with the structure of the *FAD2-3* gene and cDNA (Pirtle et al., 2001). The 1,328-bp PCR product derived from the cotton *FAD2-4* cDNA had a sequence identical to 166 bp of the 5'-flanking region of the putative *FAD2-4* mRNA predicted from the *FAD2-4* gene, a coding region of 1,155 bp encoding 384 amino acids corresponding to that predicted from the *FAD2-4* gene, and 107 bp of the 3'-flanking sequence. Thus, the identity of the cotton cDNA corresponding to the *FAD2-4* gene has been confirmed, indicating that the cotton *FAD2-4* gene is indeed transcribed. The genomic sequence has been assigned GenBank Accession Number AY279314 and the cDNA sequence has been assigned GenBank Accession Number AY279315.

Construction of a Plasmid Vector for Functional Expression of the *FAD2-4* Gene in Yeast Cells

For functional expression of the *FAD2-4* gene in a yeast model expression system, the *FAD2*-4 coding region was amplified by PCR using Platinum *Pfx* DNA polymerase (Invitrogen). A forward amplimer was used to create a *Sac*I site adjacent to the *FAD2-4* initiation codon in the open reading frame, and a reverse amplimer was designed to provide an *Eco*RI site in the

3'-flanking region of the coding region. The 1.2-kb PCR product was directionally subcloned into the *Sac*I/*Eco*RI polylinker region of the yeast-bacterial shuttle vector pYES2 (Invitrogen), in the sense direction, downstream from the yeast *GAL1* promoter for inducible expression of genes in the presence of galactose. Both strands of this construct (called pYES2/FAD2-4) were sequenced to confirm that the coding region was indeed identical to that of the gene and that the construct was in-frame relative to the *GAL1* promoter. The 6.8-kb construct designated pYES2/FAD2-4 was electroporated into both yeast INVSc1 cells (Invitrogen) and *E. coli* DH5α cells. In addition, the pYES2 vector was also electroporated into yeast INVSc1 cells for use as a control. Both yeast transformants were grown in SC-U (synthetic complete minus uracil) medium (Adams et al., 1998) at 30°C, washed and suspended in galactose induction medium (SC-U medium containing 2% galactose and 2% raffinose) and grown for three generations. The cells were pelleted and washed four times with water to remove any media or metabolites that could potentially interfere with the lipid analyses. The fatty acids were extracted and transmethylated with 5% HCl in methanol at 85°C for three hours (Christie, 1982). The fatty acid methyl esters were analyzed by gas chromatography and quantified by flame ionization detection (FID) essentially as described by Chapman and Trelease (1991), in comparison to an internal heptanoic acid standard.

To study the effects of chilling sensitivity on membrane fluidity in the yeast model system, and hence viability at lowered temperatures, yeast cells transformed with the *FAD2-4* gene construct pYES2/FAD2-4, the cotton *FAD2-3* gene construct pYES2/FAD2-3 (Pirtle et al., 2001), and the control yeast cells transformed with only the vector (pYES2), were grown at 10°C, 20°C, and 30°C in SC-U medium (Adams et al., 1998) containing 2% glucose. Cells were washed and suspended in lipid induction medium (SC-U medium containing 2% galactose and 2% raffinose) and grown for three generations. During the third generation, yeast cells were harvested for each of the three transformants at the same A_{600} readings. The cells were pelleted and washed four times with cold distilled H_2O. The yeast samples to be analyzed by gas chromatography were suspended in one ml of distilled H_2O to a final A_{600} of 1.0 and then stored at - 90°C until the lipid extraction procedure described above was done.

Construction of Plasmid Vectors for Transformation of *Arabidopsis* and Cotton Plants

The transformation of *Arabidopsis* plants has recently been started with binary vector constructs containing the *FAD2-4* and *FAD2-3* genes, using co-cultivation with *Agrobacterium tumefacians* for transfer and integration of the constructs into the plant chromosomal DNA, in order to determine if the prospective transgenic model plants express the two cotton *FAD2* genes. The binary vector is called pCAMBIA 2301 (from the Center for Application of Molecular Biology to International Agriculture (CAMBIA), Canberra, Australia). We are generating two pCAMBIA constructs to routinely produce the cotton *FAD2-4* and *FAD2-3* genes in the model *Arabidopsis* plants. For functional expression of the *FAD2* genes, we are inserting the coding regions in-frame into CaMV 35S promoter cassettes of the binary vector. PCR amplification was used to generate DNA fragments with compatible restriction sites for proper alignment of the *FAD2* inserts upstream from the CaMV promoter cassettes of the vector for introduction into *Agrobacterium* LBA4404 cells. We will verify that the two *FAD2* protein-coding regions and promoter cassettes are identical in sequence to the two genes and the pCAMBIA promoter elements.

Results and Discussion

The cotton genomic insert encompassing the *FAD2-4* gene in the clone LCFg5b was deduced to be 17.0 kb by physical mapping and alkaline blot hybridization. The physical maps of the genomic clones encompassing the *FAD2-3* and *FAD2-4* genes are quite distinct, indicating that the genes are either alleles or orthologs in the allotetraploid cotton genome. Both strands of the 8,612-bp *Xba*I fragment of LCFg5b containing the *FAD2-4* gene were sequenced by a primer-based approach. Like the coding region of the *FAD2-3* gene (Pirtle et al., 2001), the coding region of the *FAD2-4* gene has 1,155 basepairs, including the termination codon, and is continuous with no introns. The deduced amino acid sequence of the FAD2-4 polypeptide has 384 amino acids, identical in size to the FAD2-3 polypeptide. The *FAD2-4* gene in LCFg5b is distinctly different from the *FAD2-3* gene in LCFg24, with minor sequence differences in the coding regions and major differences in the flanking regions. There are 18 nucleotide variations between the protein-coding regions of the *FAD2-4* and *FAD2-3* genes in LCFg5b and LCFg24, respectively, leading to six amino acid differences in the deduced sequences of the two putative FAD2 polypeptides (with an identity of 98%). The other 12 nucleotide differences are simply due to variations in the third codon base due to degeneracy of the genetic code, resulting in no amino acid changes at these positions.

The identities between the deduced amino acid sequence of the FAD2-4 polypeptide and those of the cotton FAD2-3 protein (Pirtle et al., 2001; GenBank AF331163), the cotton FAD2-2 polypeptide (Liu et al., 1997, GenBank Y10112) and the cotton FAD2-1 polypeptide (Liu et al., 1999; GenBank X97016) are 98%, 85% and 74%, respectively. Amino acid identities of 74-78% occur with other FAD2 polypeptides, such as those from soybean, *Arabidopsis*, parsley, *Brassica*, *Borago*, and potato (based on comparisons deduced from the cDNA sequences in the GenBank database). The identities of the DNA sequences of the *FAD2-4* gene with the cotton *FAD2-3* gene (Pirtle et al., 2001), the *FAD2-2* cDNA (Liu et al., 1997), and the *FAD2-1* cDNA (Liu et al., 1999) are 98%, 57%, and 39%, respectively. In addition, the cotton *FAD2-4* gene sequence has significant similarities with numerous plant *FAD2* cDNA sequences, including the *Arabidopsis* FAD2 cDNA (67%) and a *Brassica FAD2* cDNA (63%). There are low sequence similarities between the cotton *FAD2-4* gene and castor bean *FAD2* cDNA (36%) and sunflower *FAD2* cDNA (35%) sequences, with the lowest identity (7%) being that of a soybean *FAD2* cDNA sequence.

A hydropathy plot of the cotton FAD2-4 polypeptide, generated by the method of Kyte and Doolittle (1982) in DNASIS software (Hitachi), indicates that there are four tentative membrane-spanning domains that correspond with the predicted membrane-spanning domains in the desaturase integral membrane protein models of Los and Murata (1998) and Shanklin et al. (1994). Acyl-lipid and acyl-CoA desaturases are hydrophobic proteins that apparently span the membrane four times (Los and Murata, 1998; Shanklin et al., 1994). The enzymes in the endoplasmic reticulum of plant cells accept electrons from an electron transport system composed of cytochrome b_5 and NADH-dependent cytochrome b_5 reductase. In addition, all known desaturases are characterized by the presence of three histidine clusters localized at very conserved locations in the protein sequence. These three histidine clusters are thought to comprise the catalytic center of the desaturase enzymes, since they may form ligands to a diiron cluster in the catalytic site (Shanklin and Cahoon, 1998). The amino acid sequence of the putative FAD2-4 polypeptide has the three histidine-rich motifs in the conserved locations in the protein structure (Los and Murata, 1998), as does the FAD2-3 protein (Pirtle et al., 2001).

A single, large intron occurs in the 5'-flanking region of the *FAD2-4* gene, only 12 basepairs upstream from the ATG initiation codon of the protein-coding region. The locations of the tentative cap site for the start site of the *FAD2-4* mRNA product and the tentative 5'- and 3'-intron splice junctions in the 5'-untranslated region (5'-UTR) were deduced from comparson of the *FAD2-4* and *FAD2-3* gene sequences. The 5'-UTR intron of the *FAD2-4* gene is 2,780 basepairs long, which is 187 basepairs shorter than the 2,967-basepair 5'-UTR intron of the *FAD2-3* gene (Pirtle et al., 2001). The intron has large stretches that are 92-96% identical and of the same length as corresponding regions in the intron of the *FAD2-3* gene. However, there is one region (about 360 basepairs from the 3'-end of the intron) which has a gap of 36 basepairs compared to the *FAD2-3* intron. There are also three other regions in the middle of the intron that have only about 35-59% identity and are shorter than corresponding regions in the FAD2-3 intron. Since the intron would be spliced out to generate the mature *FAD2-4* mRNA, the 5'-untranslated region of the mRNA can be estimated to be about 136 nucleotides in length.

The *Arabiodopsis FAD2* gene (Okuley et al., 1994) has a large 1,134-basepair intron merely 5 basepairs upstream from its ATG initiation codon. Liu et al. (2001) analyzed the *FAD2*-1 gene 5'-UTR intron structures across 31 species of *Gossypium*, including the *Gossypium hirsutum* A and D genome orthologs. The sizes of the 5'-UTR introns of the *Gossypium FAD2-1* genes are about 1,133 bp, and are located 9 bp upstream from the initiation codon. In contrast, the cotton *FAD2-4* and *FAD2-3* genes have much larger 5'-UTR introns in slightly different positions, 2,780- and 2,967-basepair introns located 12 bp upstream from the translation start codons. The variations in the relative positions and the substantial differences in the sizes of the 5'-UTR introns are distinguishing structural differences between the *FAD2-4* and *FAD2-3* genes and the *FAD2-1* gene, which could be important in differential expression of the genes. The presence of large introns in the 5'-flanking regions of plant *FAD2* genes could be important in the regulation of their expression. Introns in 5'-UTRs have been reported to have positive effects on the expression of a number of plant genes. For example, Gidekel et al. (1996) found that the gene for *Arabidopsis* elongation factor 1β has a 5'-UTR intron that is required for high levels of expression, and has an enhancer-like element in this intron. Also, the 5'-UTR intron of a soybean phosphoenolpyruvate carboxylase gene dramatically increases gene expression in plant cells (Kato et al., 1998).

This 5'-flanking region of the *FAD2-4* gene has a number of possible promoter elements that could be positive regulatory elements. A TATA basal promoter element occurs 34 basepairs upstream from the putative cap site. A basic region helix-loop-helix (bHLH) or E box motif with the consensus sequence CANNTG occurs 103 basepairs upstream from the potential cap site. The E box motif has been shown to be a seed-specific regulatory element in the French bean β-phaseolin gene (Kawagoe et al., 1994). There are also prospective light-responsive elements in the 5'-flanking region of this gene. For example, there are several consensus GT-1 motifs (GRWAAW) and G-boxes (CACGTG), involved in the light-induced expression of many plant genes (Terzaghi and Cashmore, 1995; Guilfoyle, 1997). A G box occurs 130 basepairs upstream from the tentative cap site. Two consensus GT-1 motifs, general features of light-responsive promoters occur 82 basepairs and 115 basepairs prior to the supposed cap site. There are two possible *Dof* core recognition sequences at 8 and 57 basepairs upstream from the tentative cap site. *Dof* transcription factors are associated with genes involved in carbon metabolism in maize (Yanagisawa, 2000). Thus, the promoter elements in the 5'-flanking regions of the cotton *FAD2-4* and *FAD2-3* genes are virtually identical in number and in location.

The 1.2-kb coding region of the cotton *FAD2-4* gene is successfully expressed in transformed yeast cells, indicating that the gene does indeed encode a functional FAD2 enzyme. Yeast cells are eukaryotic and contain an endoplasmic reticulum that is necessary for the activity of plant FAD2 enzymes that are integral membrane proteins in that cellular organelle. Since yeast cells lack a *FAD2*-type gene, they normally do not make linoleic acid (C18:2), a fatty acid with two double bonds. Expression of the cotton *FAD2-4* gene in yeast cells would then be easily detected, since the FAD2-4 enzyme would convert oleic acid (18:1) into linoleic acid (18:2) when assayed by fatty acid analysis. The yeast cells were grown in appropriate media, the fatty acids were extracted from the washed yeast cells, and the fatty acid methyl esters were then analyzed by gas chromatography. The yeast cells transformed with the plasmid construct pYES2/FAD2-4 were found to have a significant accumulation of linoleic acid (C18:2). The oleic acid (C18:1) peak in the transformed cells was noticeably smaller relative to the corresponding oleic acid peak in the control cells, clearly indicating the conversion of oleate into linoleate in the yeast cells containing the plasmid construct with the *FAD2-4* coding region. No linoleic acid was detected in the control yeast cells trans-

formed with the shuttle vector pYES2 alone. Thus, the *FAD2-4* gene has been functionally identified, since it encodes an enzyme that catalyzes the desaturation of oleate to linoleate. We previously used the yeast system to express the cotton *FAD2-3* gene (Pirtle et al., 2001). We plan to use expression of the *FAD2-4* and *FAD2-3* genes in the yeast model system for studying the effect of chilling sensitivity on membrane fluidity and hence plant viability at lowered temperatures.

Yeast cells transformed with the *FAD2-4* and *FAD2-3* gene constructs (the plasmid vectors being designated pYES2/FAD2-4 and pYES2/FAD2-3, respectively) and a transformant with only the plasmid vector (pYES2) were grown at three different temperatures (10°C, 20°C, and 30°C) in a glucose-based synthetic medium. The yeast cells were washed, suspended in an induction medium, grown for three generations, and harvested at the same cell density (measured by absorbance at 600 nanometers). The yeast cell samples were subjected to lipid extraction, and the fatty acids were converted to fatty acid methyl esters for analysis by gas chromatography and quantification by flame ionization detection. At all three temperatures, the growth curves of the yeast cells transformed with the *FAD2-4* and *FAD2-3* genes were found to parallel the growth curve of the control yeast cells. From the gas chromatographic lipid analyses, it would seem that the transformed yeast cells produce relatively more linoleic acid at the low temperature of 10°C than at the other two temperatures. For example, yeast cells transformed with the *FAD2-4* gene had a ratio of linoleic acid to oleic acid of 1.9 at 10°C, in contrast to a ratio of 0.32 at 20°C. At 30°C, the amount of linoleic acid was too low for detection. For the yeast cells transformed with the *FAD2-3* gene, the ratio of linoleic acid to oleic acid was 1.2 at 10°C, 0.2 at 20°C, and 0.8 at 30°C, thus showing different ratios from those of the *FAD2-4* gene. From these preliminary results, it would seem that the *FAD2-4* gene is expressed to a greater extent at cold temperature than the *FAD2-3* gene in the yeast model system. An increase in the level of the polyunsaturated linoleic acid would be expected to help maintain membrane fluidity at lowered temperatures. Since this work is still in progress, it is too soon to draw any conclusions.

To confirm that the *FAD2-4* gene is really expressed in cotton plants and to determine the size of the 5'-untranslated region and presumptive cap site of the *FAD2-4* mRNA, we amplified the corresponding *FAD2-4* cDNA by the polymerase chain reaction (PCR) from a cotton cDNA library. Several sets of forward and reverse amplimers were designed from unique sequences of the 5'- and 3'-flanking regions of the *FAD2-4* gene. Using various combinations of the forward and reverse amplimers, PCR products were generated and analyzed by gel electrophoresis. From this analysis, it would appear that the *FAD2-4* gene is actually expressed in cotton, since the predicted PCR fragments were generated, as determined from their mobilities on agarose gels. We subcloned several of these PCR products into a plasmid vector for sequence analysis and confirmed that these products are truly segments of the *FAD2-4* cDNA with the same sequence as the corresponding *FAD2-4* gene.

To express the cotton FAD2 proteins in model transgenic *Arabidopsis* plants, we are inserting the *FAD2-4* and *FAD2-3* coding regions in-frame into CaMV 35S promoter cassettes of the binary vector pCAMBIA 2301, using co-cultivation with *Agrobacterium tumefacians* for transfer and integration of the DNA constructs into plant chromosomal DNA. *Arabidopsis* has only one *FAD2* gene in its genome, and seeds of a *FAD2* gene knockout mutant plant (generated by John Browse and colleagues of Washington State University; Browse et al., 1994) are available from the Arabidopsis Biological Resource Center at Ohio State University. We will use the mutant *FAD2* gene *Arabidopsis* plants for transformation with the plasmid constructs with the cotton *FAD2*-4 and *FAD2*-3 genes. We used PCR amplification to generate DNA fragments with compatible restriction sites for proper in-frame alignment of the *FAD2-4* and *FAD2-3* coding regions upstream from the CaMV 35 promoter cassettes for insertion into the vector pCAMBIA 2301 for transformation of plants.. At that time, the chilling sensitivity of the prospective transgenic *Arabidopsis* plants will be evaluated. We will begin transformation of cotton plants with these constructs when the results from the model *Arabidopsis* plants are deemed satisfactory.

The structural comparison of the two cotton *FAD2* genes has revealed great similarities in the 5'-flanking introns, the promoter motifs, and the protein-coding regions of the two genes. We should be able to gain insight into how the two presumptive *FAD2* genes are regulated for gene expression, and if the 5'-flanking introns really have any bearing on transcriptional regulation of the genes. It is imperative to the long-range understanding of lipid synthesis in cotton to elucidate the mechanisms of regulation of genes for enzymes of fatty acid biosynthesis. This basic information will provide a knowledge base to enable us to understand the pathways of membrane biosynthesis, and potentially modify the membrane fatty acid compositions in cotton plants for the improvement of the vigor and vitality of this important crop plant.

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