EXPRESSION ANALYSIS OF THE FATTY ACID DESATURASE *2-4* AND *2-3* GENES FROM *Gossypium hirsutum* IN TRANSFORMED YEAST CELLS AND TRANSGENIC *Arabidopsis* PLANTS

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Fatty acid desaturase 2 (FAD2) enzymes are phosphatidylcholine desaturases occurring as integral membrane proteins in the endoplasmic reticulum membrane and convert monounsaturated oleic acid into polyunsaturated linoleic acid. The major objective of this research was to study the expression and function of two cotton *FAD2* genes (the *FAD2-3* and *FAD2-4* genes) and their possible role in plant sensitivity to environmental stress, since plants may increase the polyunsaturated phospholipids in membranes under environmental stress events, such as low temperature and osmotic stress. Two *FAD2* cDNA clones corresponding to the two *FAD2* genes have been isolated from a cotton cDNA library, indicating both genes are truly expressed in cotton. Model yeast cells transformed with two cotton *FAD2* genes were used to study the chilling sensitivity, ethanol tolerance, and growth rate of yeast cells. The expression patterns of the two *FAD2* genes were analyzed by reverse transcription polymerase chain reactions (RT-PCR) and Western blot analyses in cotton plants under different treatment conditions. The coding regions of both *FAD2* genes were inserted downstream from the CaMV 35S promoter in the pMDC gateway binary vector system. Five different *FAD2*/pMDC constructs were transformed into the *Arabidopsis fad2* knockout mutant background, and multiple potential transgenic *Arabidopsis* plant lines harboring the cotton *FAD2* genes were generated. The cotton *FAD2* genes were amplified by the polymerase chain reaction (PCR) from the genomic DNAs isolated from

the transgenic *Arabidopsis* T_1 plant lines. Complementation of the putative transgenic *Arabidopsis* plants with the two cotton *FAD2* genes was demonstrated by gas chromatography analyses of the fatty acid profiles of leaf tissues. The cellular localization of cotton FAD2-4 polypeptides with N-terminal green fluorescence protein (GFP) was visualized by confocal fluorescence microscopy. The phenotype of transgenic *Arabidopsis* plants transformed with the cotton *FAD2-4* gene was compared to *Arabidopsis* knockout *fad2* mutant plants and wild type *Arabidopsis* plants regarding their sensitivity to low temperature, and the size and height of the plants.

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CHAPTER 1

INTRODUCTION

Cotton is the most valuable source of natural fiber and is one of the largest crops in terms of economic value in the USA (Zapata et al., 1999; Wu et al., 2005). Cotton is the leading cash crop in Texas, being grown on nearly six million acres. The yield of lint per hectare is a high priority for cotton producers, along with the price per kilogram of lint that the growers receive. Cotton production costs are high, and thus, lint yield per hectare must be maximized for growers to realize a profit. Cotton production costs can also be improved through better host resistance. Low temperature is an environmental stress that adversely affects plant growth and crop production. Improving the host plant resistance of cotton to cold temperatures that annually inflict significant yield-losses will not only reduce production costs, but also contribute positively to sustainable production (Pirtle et al., 2001).

One of the effects of low temperature in plants is the modification of their membrane lipid composition, such that the accumulation of polyunsaturated fatty acids in polar lipids contributes to the preservation of membrane fluidity (Browse et al., 1994). Fatty acids in plants, as in all other organisms, are the major structural components of biological membranes (phospholipid bilayers) and storage oils (neutral lipids or triacylglycerols) (Harwood, 1996). The fatty acid biosynthesis pathway is the primary metabolic pathway because it is essential for growth in every plant cell (Ohlrogge and Browse, 1995). Thus, it is important to understand the mechanisms underlying the regulation of fatty acid compositions in membrane phospholipids in cotton plants. One

of the major control points may occur at the level of gene expression in the regulation and coordination of genes for enzymes of fatty acid biosynthesis in cotton.

The 30 or so enzymatic reactions for the *de novo* synthesis of C16- and C18-fatty acids occur in the stroma of plastids (Ohlrogge and Browse, 1995; Somerville et al, 2000). The first step in fatty acid biosynthesis is transport of acetyl-CoA to the cytosol (Somerville et al., 2000). Acetyl-CoA is formed by β-oxidation of fatty acids or by decarboxylation of pyruvate or degradation of certain amino acids. Acetyl-CoA enters the cytosol by the citrate pathway, and then is carboxylated into malonyl-CoA. The biosynthesis of fatty acids proceeds by the addition of two carbon units to the hydrocarbon chain. The process is catalyzed in many organisms by a large multienzyme complex called the fatty acid synthase complex that includes an acyl carrier protein (ACP). The usual product of fatty acid anabolism is palmitate, the 16-carbon saturated fatty acid. Then, longer fatty acids are formed by elongation reactions catalyzed by enzymes on the cytosolic face of the endoplasmic reticulum (ER) membrane (or alternatively the thiolase reaction in the mitochondria), which add two-carbon units to the carboxyl ends of both saturated and unsaturated fatty acyl-CoA substrates, with malonyl-CoA as the carbon donor.

The major membrane phospholipids in all plant tissues are assembled using palmitate (C16:0) and oleate (C18:1) acyl groups (Harwood, 1980; Ohlrogge and Browse, 1995; Somerville et al., 2000). Membrane glycerolipids have fatty acids attached to both the sn-1 and sn-2 positions of the glycerol backbone and a polar headgroup attached to the sn-3 position. The combination of nonpolar fatty acyl chains and a polar headgroup leads to the amphipathic physical properties of glycerolipids,

which are essential to the formation of membrane bilayers. Desaturation of fatty acids in chloroplast and endoplasmic reticulum (ER) membrane complex lipids is done by membrane-spanning enzymes called fatty acid desaturases (designated from FAD2 to FAD8). The FAD2 and FAD3 enzymes are integral membrane phosphatidylcholine (PC) desaturases in the ER, acting on fatty acids at both the sn-1 and sn-2 positions (Ohlrogge and Browse, 1995; Somerville et al, 2000). The important function of ER 18:1 desaturase, known as FAD2, is to provide 18:2 and (following further desaturation) 18:3 required for the correct assembly of cellular membranes throughout the plant. Another important function of this enzyme is to provide the polyunsaturated fatty acids found in vegetable oils that in turn are the major source of essential fatty acids in most human diets (Okuley et al., 1994).

The introduction of double bonds also takes place in the ER, and the reaction is catalyzed by a complex of three membrane-bound enzymes: NADH-cytochrome b_5 reductase, cytochrome b_5, and a fatty acid desaturase. Fatty acid desaturases are enzymes that catalyze the general reaction:

$$R_1\text{-}CH_2\text{-}CH_2\text{-}R_2 + O_2 + 2e^- + 2H^+ \rightarrow R_1\text{-}CH{=}CH\text{-}R_2 + 2H_20$$

The substrate is generally a fatty acid ester or thioester. Desaturases are found in most animals and plants, with a scattered distribution among eubacteria (Somerville et al., 2000).

Fatty acids desaturases are integral membrane proteins, believed to contain two iron atoms in their active site (Shanklin et al., 1998). While numerous *FAD2* cDNA structures have been analyzed, only very few actual *FAD2* gene sequences have been determined, the first being the single-copy Arabidopsis *FAD2* gene (Okuley et al, 1994).

Our laboratory has characterized the structures and functional expression of the first complete cotton *FAD2* genes, the *FAD2-3* gene (Pirtle et al., 2001) and the *FAD2-4* gene (Zhang et al., 2008). The partial structure of the cotton *FAD2-1* gene has also been analyzed (Liu et al., 1999; Liu et al., 2001). The regulation of *FAD2* gene expression in plants is not well understood, and the post-transcriptional regulation of *FAD2* genes is possible, since the Arabidopsis *FAD2* gene (Okuley, 1994), the cotton *FAD2-1* gene (Liu et al, 2001), the cotton *FAD2-3* gene (Pirtle et al, 2001), and the *FAD2-4* gene (Zhang et al., 2008) have 5'-untranslated region (5'-UTR) introns which appear to be necessary for expression of a number of plant genes, such as the S-adenylosylmethionine decarboxylase genes (Kim et al, 2004; Hu et al, 2005). Furthermore, the potential promoter elements regulating transcription of *FAD2* genes have not been well characterized.

All membrane-bound fatty acid desaturases share a great degree of sequence identity (Zhang et al., 2008). This includes the three histidine-rich sequence motifs, which are thought to be important in forming a di-iron center at the active site. Thus, pending more structural information, membrane-bound fatty acid desaturases can be included in the large group of structurally diverse di-iron proteins, which also includes the soluble stearoyl-ACP desaturase (Shanklin and Cahoon, 1998). In plants, FAD2 is the enzyme that converts oleic acid (18:1) to linoleic acid (18:2) by introducing a double bond at the Δ-12 position. It turns out that FAD2 variants from a variety of plants are capable of catalyzing the formation of hydroxyl-, epoxy-, triple bond- and conjugated double bond-containing fatty acids. Some of these unusual fatty acids are of commercial interest (Somerville et al., 2000).

Based on a current topological model for transmembrane-bound fatty acid desaturases (Shanklin and Cahoon, 1998), fatty acid desaturases are hydrophobic proteins that span the membrane four times as the membrane spanning helices. There is a putative di-iron center, and three histidine-rich structural motifs, which are believed to be involved in coordinating the di-iron catalytic center of the enzyme.

For the last several years, biochemical analysis and immunolocalization studies of FAD2 enzymes indicated them to be located exclusively in the ER and adopt a topological orientation in which their N- and C-termini are exposed to the cytosol (Dyer and Mullen, 2008). A model has been proposed in which FAD2 enzymes are initially targeted to the ER by cotranslational insertion, with retention being mediated by C-terminal peptide signals that act to guide escaped proteins from the Golgi back to the ER (Dyer and Mullen, 2008).

A plant's ability to alter its physiology in response to low temperature to survive lethal temperatures is called cold acclimation (Browse et al., 1994). Surviving chilling stress requires maintenance of the structural and functional integrity of the cellular membranes and it is believed that polyunsaturated membrane phospholipids are essential to maintaining plant viability at lowered temperatures (Browse and Xin, 2001). Saturated fatty acids lack a double bond between carbon atoms. Unsaturated fatty acids, on the other hand, have one or more double bonds, either in the cis or trans configuration. The presence of the double bonds help maintain membrane fluidity by introducing bends or kinks in the fatty acyl chains, thereby inhibiting tight packing of adjacent lipid molecules (Vigh et al., 1998). The fatty acid desaturase 2 (FAD2) is one of the major enzymes for fatty acid biosynthesis and introducing double bonds on 12

position carbon atoms of fatty acid chains. Therefore, the FAD2 polypeptide may be important in the chilling sensitivity of plants. Previous studies have indicated that the *FAD2* gene seems to be important in the chilling sensitivity of plants (Ohlrogge and Browse, 1995; Okuley, 1994; Miquel and Browse, 1994; Browse et al, 1994). Understanding the role of the *FAD2* gene in regulating fatty acid quantities and compositions of membrane phospholipids has important physiological relevance, as well as influences a variety of processes such as the regulation of membrane fatty acid profiles in different tissues, different developmental stages, and in response to abiotic and biotic stresses. For example, a study using seashore Paspalum indicated that that linolenic acid increased significantly during low temperature exposure (Cyril et al., 2002). Thus, the FAD2 enzyme may be involved in cold and heat tolerance, resistance to desiccation, and disease resistance, by being involved in regulation of the fatty acid composition of the cell and organelle membranes of plants. Most of the evidence to date indicates that plant FAD2 regulation occurs primarily at the post-transcriptional level (Miquel et al., 1993). For example, exposure of plants to cold temperature causes an increase in polyunsaturated fatty acid content, and *FAD2* gene expression is not upregulated during the process (Falcone et al., 2004). It is believed that post-tranlational regulation of FAD2 enzymes may rapidly adjust membrane lipid composition in response to sudden environmental changes (Dyer and Mullen, 2008). ER-localized FAD2 enzymes are generally short-lived proteins, and the half-life of FAD2 proteins may be regulated by environmental cues, resulting in changes in protein abundance that correlate with changes in the amount of fatty acid products (Dyer et al. 2001, Horiguchi et al., 2000, Tang et al., 2005).

In cotton, knowledge about the expression patterns of the *FAD2* gene family may permit the genetic manipulation of these genes, and allow for predictable modification of membrane fatty acid profiles to improve the vigor and viability of this important fiber crop. Many plant genes of fatty acid metabolism, including the *FAD2* genes, have already been genetically modified for oilseed improvement (Voelker and Kinney, 2001; Drexler et al., 2002). For example, our research group (Chapman et al., 2001) used a heterologous canola *FAD2* allele to increase the oleic acid content of transgenic cotton plants. Liu et al. (2002b) used hairpin RNA-mediated gene silencing to down regulate the cotton stearoyl-acyl-carrier protein ∆9-desaturase and *FAD2-1* genes in order to produce high-stearic acid and high-oleic acid cottonseed oils, respectively. A clear understanding of the expression patterns of the *FAD2* gene may permit the manipulation of the fatty acid compositions of plant membranes in a predictable manner to improve the vigor and cold-hardiness of the cotton plant. Most of known information of plant desaturases was from the characterization of a series of Arabidopsis mutants with defects in fatty acid desaturation and the genes corresponding to several of the Arabidopsis *fad* loci have been isolated (Ohlrogge and Browse, 1995).

In a previous study from this laboratory, the first cotton *FAD2* gene, designated the *FAD2-3* gene, was isolated from cotton genomic DNA (Pirtle et al., 2001). A cotton genomic library was screened to isolate a second *FAD2* gene (the *FAD2-4* gene), using a hybridization probe generated from the coding region of the *FAD2-3* gene. One genomic clone (designated LCFg5b) that intensely hybridized to the probe was selected for structural analysis by physical mapping and DNA sequence analysis (Zhang et al., 2008). The *FAD2-4* gene is distinctly different from the *FAD2-3* gene, with minor

sequence differences in the coding regions and major differences in the flanking regions. 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 (Pirtle et al., 2000). In addition, 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. 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 deduced amino acid sequences of the two putative FAD2 polypeptides both have 384 amino acids, with only six amino acid differences. The putative FAD2-4 amino acid sequence (Zhang et al., 2008) is shown in Figure 1.

```
        10         20         30         40         50         60
MGAGGRMSVP PSQRKQESGS MKRAPISKPP FTLSELKKAI PPHCFQRSLI RSFSYLVYDF

        70         80         90        100        110        120
ILVSIFYYVA TTYFRNLPQP LSFVAWPIYW ALQGSVLTGV WVIAHECGHH AFSDYQWLDD

       130        140        150        160        170        180
TVGLILHSSL LVPYFSWKYS HRRHHSNTGS LERDEVFVPK KRSSIRWYAK YLNNPPGRTV

       190        200        210        220        230        240
TITIQLTLGW PLYLAFNVAG RPYEGFACHY NPYGPIYNDR ERLQIYISDV GVLAVTYGLY

       250        260        270        280        290        300
RLVLAKGLAW VICVYGVPLL IVNAFLVMIT YLQHTHPALP HYDSSEWDWL RGALATVDRD

       310        320        330        340        350        360
YGILNKVFHN ITDTHLAHHL FSTMPHYHAM EATKAIKPIL GEYYSFDGTP VYKALFREAK

       370        380
ECIYVEPDEG EQSSKGVFWY RNKI*
```

Fig 1. Tentative amino acid sequence of the cotton FAD2-4 polypeptide (Zhang et al., 2008). The locations of three conserved histidine-rich motifs (red) occur in identical locations in both cotton FAD2 proteins.

A hydropathy plot of the FAD2-4 amino acid sequence was done. As shown in Figure 2, there are at least four segments in this sequence that can potentially form a

transmembrane α-helices. For this reason, the putative cotton FAD2-4 polypeptide is probably an integral membrane protein in the endoplasmic reticulum (Shanklin and Cahoon, 1998).

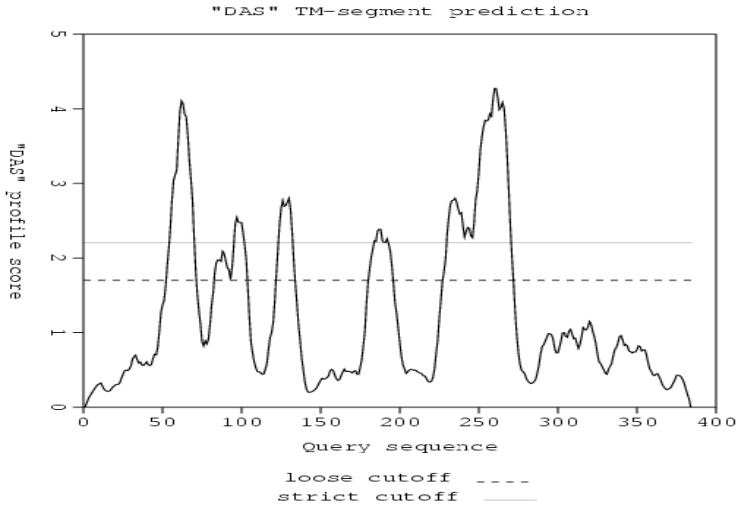

Fig 2. The hydropathy plot of the putative cotton FAD2-4 polypeptide sequence.

Cold tolerance has been correlated with the degree of unsaturation in membrane lipid fatty acids (Miquel et al., 1993). Unsaturated fatty acids are thought to aid in maintaining membranes in a fluid state necessary for biological functioning. It has been suggested that the changes in the membrane fluidity is the initial event of the expression of desaturase genes (Nishida and Murate, 1996). As one of the major enzymes for fatty acid biosynthesis in cotton, fatty acid desaturase 2 (FAD2) synthesizes a polyunsaturated fatty acid called linoleic acid. FAD2 may be involved in regulation of the fatty acid composition of plant cell and organelle membranes, crucial in cold and heat tolerance, resistance to desiccation, and disease resistance. Since polyunsaturated membrane phospholipids seem to be essential to maintaining plant

viability at lowered temperatures, the *FAD2* gene would be important in the chilling sensitivity of plants. A study using seashore *Paspalum* indicated that the triunsaturated linolenic acid (18:3) increased significantly during low temperature, suggesting that accumulation of linolenic acid partly explains the differential response in cold tolerance (Cyril et al., 2002). Another study showed that cold tolerance was enhanced in tobacco plants engineered with the *fad7* gene (Kodama et al., 1994). One of the major goals of this project is to answer the question of whether low temperatures can induce a stronger activity of fatty acid desaturase genes in cotton plants and in yeast cells transformed with the cotton *FAD2* genes.

Characterization of plant desaturases by traditional biochemical approaches has been limited because of the difficulty in solubilization and purification of the membrane proteins. Expression of plant desaturases in the yeast *Saccharomyces cerevisiae* has offered a rapid method to verify enzymatic activity of the desaturases, as well as characterize their substrate/product relationships because of its simple fatty acid composition (Dyer et al., 2001). Research on the tung tree *FAD3* gene (Dyer et al., 2001) showed temperature-dependent synthesis of linolenic acid in yeast cells expressing the FAD3 enzyme and the increase of linolenic acid content at cooler temperatures could be due to cold-inducible, post-transcriptional increase of the plant desaturase enzyme. The yeast cells overexpressing the *Arabidopsis thaliana FAD2* gene (Kajiwara et al., 1996) also showed greater resistance to ethanol than the control cells.

Arabidopsis harbors only a single copy of the *FAD2* gene (**At3g 12120**) and is constitutively and abundantly expressed in the plant (Beisson et al., 2003), while other plants as soybean (*Glycine max*), cotton (*Gossypium hirutum*), corn (*Zea mays*), and

canola (*Brassica napus*) have two or more *FAD2* genes. Because of their unique characteristics, yeast cells and the *fad2-1* mutant knockout Arabidopsis plants were used as model systems to study cotton *FAD2* gene expression. For Arabidopsis plant transformation, the modern binary Gateway vectors pMDC32 (a constitutive overexpression vector) and pMDC43 (with GFP N-terminal fusion) were used to create transgenic Arabidopsis plants with the Gateway cloning technology (Invitrogen). Both plant vectors harbor a dual *Ca*MV*35S* promoter and *Nos* terminator sites, which have been proved to be highly active in most transgenic plant cells (Curtis and Ueli, 2003; Gerald and Labaer, 2004; Hartley and Gary, 2000).

To study the possible roles of the cotton FAD2 enzymes, several experimental approaches were used to study the expression of the two *FAD2* genes. Reverse transcription-polymerase chain reaction assays (RT-PCR) and Western blotting analyses were used to study the *FAD2* gene expression pattern in cotton plants under various environmental and chemical treatments (such as cold, ethanol, abscissic acid, and hydrogen peroxide). Yeast cell and Arabidopsis plant model systems, two widely used model eukaryotic expression systems, were used to characterize the expression of the two cotton *FAD2* genes.

In previous work, the coding regions of both *FAD2* genes (around 1.2 kb) were ligated into the pYES2 yeast transformation vector (Invitrogen) and transformed into yeast cells (Pirtle et al., 2001; Zhang et al., 2008). Yeast cells are eukaryotic, contain an endoplasmic reticulum, and naturally make oleic acid (18:1), a fatty acid with only one double bond. Yeast cells also naturally lack a FAD2 desaturase enzyme to produce the linoleic acid (18:2). The endoplasmic reticulum is necessary for the activity

of plant *FAD2* enzymes, since they are integral membrane proteins in this cellular organelle. Thus, the expression of the cotton *FAD2* gene in yeast cells should be easily detectable, since the *FAD2* protein would generate linoleic acid (18:2) with two double bonds, as assayed by lipid analysis (Covello and Reed, 1996).

One of the goals of this project was to genetically engineer two cotton *FAD2* genes to routinely overproduce the FAD2 protein in transgenic Arabidopsis and cotton plants, as a natural defense against environmental stress. Plant transformation is based on the introduction of foreign DNA into plant cells, followed by the regeneration of these transformed cells into whole plants. Each plant cell has the genetic potential to regenerate an entire plant, and this unique characteristic is the genetic basis for plant tissue culture (Hoekema et al., 1983; Barz and Oksman-Caldentey, 2002). Numerous techniques in molecular biology and gene technology have improved the genetic engineering of plants. With the discovery of *A. tumefaciens* and the development of an efficient T-DNA system for DNA transfer, *Agrobacterium*-mediated transformation has been the most commonly used method for plant transformation (Nain et al., 2005).

Most functional gene analyses in plants rely on the expression of transgenes to manipulate biological processes in transgenic plants, the phenotypic studies by generating gain-of-function or loss-of-function mutants (Curtis and Ueli**,** 2003). To create gain-of-function plants, a gene is placed under the transcriptional control of a constitutive promoter. Another revealing approach to study gene function is to examine the subcellular localization of the corresponding protein by fusing the interested gene with reporter genes (Curtis and Ueli**,** 2003). Each step of characterization requires

subcloning the ORFs (open reading frames) of the genes of interest into one or more specialized vectors.

In this study, recombinational cloning (Curtis and Ueli, 2003; Gerald and Labaer, 2004; Hartley and Gary, 2000) is being used to accomplish this task, in which the DNA segments flanked by recombination sites can be mixed *in vitro* with a new vector also containing recombination sites and incubated with bacteriophage λ integrase recombination proteins to accomplish the transfer of the gene into the destination vectors (Hartley and Gary, 2000). Three vectors from the pMDC group were used as the destination vectors for the plant transformation. The vector pMDC32, a constitutive expression vector harboring a dual 35S CaMV promoter without any terminal protein tag, was used to create transgenic Arabidopsis plants for overexpression and cold treatment studies. Since the GFP protein has become well established as a marker of gene expression and protein targeting in intact cells and organisms (Roger, 1998), the pMDC43 vector was used for GFP (green fluorescent protein from the jellyfish *Aequorea victoria)* fusions, and the pMDC139 vector, the *GUS* (beta-glucuronidase reporter gene from *E.coli*) N-terminal fusion vector, were used for the analysis of subcellular localization of FAD2 proteins (Curtis and Ueli**,** 2003). To express the target genes, all these three vectors contain the promoter and terminator of the cauliflower mosaic virus 35S transcript, because the CaMV 35S promoter is highly active in most plant cells of transgenic plants. Downstream of the promoter, the tobacco mosaic virus leader sequence ensures efficient translation of the inserted coding sequences (Karimi et al., 2002).

Arabidopsis thaliana is a small flowering plant that is widely used as a model organism in plant biology (Meyerowitz and Somerville, 1994). Arabidopsis belongs to the mustard (*Brassicaceae*) family, which includes cultivated species such as cabbage and radish. Although this plant has no major agronomic significance, the rapid life cycle with prolific seed production, as well as the easy cultivation in restricted space, has made Arabidopsis an important plant model for basic research in genetics and molecular biology. An efficient Arabidopsis transformation method utilizing *Agrobacterium tumefaciens* is well developed (Weigel and Glazebrook, 2002), providing an extremely easy method to obtain transgenic plants without the help of specialized equipment. Furthermore, the availability of herbicide resistance genes (Lee et al., 1988) has superceded the need to use antibiotic resistance as a selectable marker and sterile techniques for the selection of transformants. A large number of mutant lines and genomic resources have been made over the years and most of them are available from Stock Centers (Beisson et al., 2003).

A group of Arabidopsis mutants with defects in each of eight desaturase genes (*fad2-fad8*, and *fab2*) was created during the past ten years (Ohlrogge and Browse, 1995; Buchanan, 2000). Plants with different *FAD2* gene mutant lines were generated by T-DNA insertion and were used to study the role of this enzyme in polyunsaturated lipid synthesis and cold acclimation (Okuley et al., 1994). These mutant lines have provided a basis for genetic and molecular studies of membrane structure and function in higher eukaryotes. In this project, the Arabidopsis *fad2* gene mutant was used for plant transformation and study of cotton *FAD2* gene expression and its regulation. We have used the three binary vectors (as mentioned before) pMDC32, pMDC43, and

pMDC139 (Curtis and Ueli, 2003) and the Gateway Cloning system (Curtis and Ueli, 2003; Gerald and Labaer, 2004; Hartley and Gary, 2000) to construct T-DNA insertion vectors harboring both *FAD2-4* and *FAD2-3* cotton genes for Arabidopsis transformation. The transgenic Arabidopsis T_2 and T_3 plants of the pMDC32-*FAD2* line were used to study the gene expression and regulation during cold acclimation. The Arabidopsis pMDC43-*FAD2* lines (with the *GUS* gene as reporter gene) were used to study the subcellular localization of the fusion protein by confocal microscopy.

The goal of this project has been to genetically engineer cotton *FAD2* genes in eukaryotic expression systems such as Arabidopsis and yeast model systems to routinely overexpress the *FAD2* enzymes which catalyze the conversion of oleic acid into linoleic acid (18:1-18:2). Also, the activity of the *FAD2* protein was bioassayed to demonstrate the potential anti-cold and anti-stress efficacy of the cotton *FAD2* genes. Another major objective of this research has been to study the expression and cellular localization of the cotton *FAD2* polypeptides by using a yeast expression system and the Arabidopsis *FAD2* gene knockout mutant plants. This work represents an important step towards a better understanding of the structure, organization, and regulation of the *FAD2* gene family in plants, and provides molecular and genetic information of the gene structure, tissue specific expression, function and cellular location of cotton fatty acid desaturase 2.

CHAPTER 2

MATERIALS AND METHODS

Subcloning and Sequence Analysis of *FAD2-4* and *FAD2-3* cDNAs from a Cotton cDNA Library

In order to prove that *FAD2-3* and *2-4* genes are indeed functional genes in cotton plants, primers were designed from the coding region of both *FAD2* genes and used to amplify the corresponding cDNAs from a cotton cDNA library. Two segments of the putative *FAD2-4* cDNA were amplified by the polymerase chain reaction (PCR) from a cotton cDNA library provided by Dr. Edgar B. Cahoon of Dupont Ag Products, Wilmington, DE. The primers used were designed based on sequence segments unique to the *FAD2-4* genomic sequence and included a segment on the 5'-end to create a *XbaI* site (TCTAGA) or a segment on the 3'-end to create a *SacI* site (GAGCTC). The forward primer #240 (5'-TGTCTAGAGACCAAAGTGAAAGAAAATCGAAG-3') with a *XbaI* site (underlined) compatible with the 5'-flank of the putative *FAD2-4* cDNA, and the reverse primer #1520 (5'-GACGAGCTCCAAAAGCATCTAAAATAGAAGTAACCC) with a *SacI* site (underlined) compatible with the 3'-flank of the putative *FAD2-4* cDNA, were designed to amplify a 1,346 bp PCR product (designated **AY279315**).

The PCR product (designated **AY279315**) was generated from the cotton cDNA library using Platinum *Pfx* DNA polymerase (Invitrogen, Carisbad, CA) and 2 mM $MgSO_4$, after testing several levels of magnesium concentrations to optimize the PCR reaction. The PCR product was purified by precipitation with ethanol (after adding *E.*

coli tRNA carrier) and digested with *Sac I* and *Xba I* restriction enzymes to prepare for ligation into the vector. The pGEM-7Zf(+) vector were isolated using the Wizard *Plus* Minipreps DNA Purification System (Promega, Madison, WI). About 25 µg of the vector was digested with *SacI* and *XbaI* and then precipitated with ethanol (after adding 20 µg of 5S RNA carrier). Both the *FAD2-4* cDNA PCR product and the digested vector were fractionated on a 1.5% agarose gel. The 1,346 bp PCR band and the vector band were excised from the gel and purified using a QIAGEN Gel Extraction Kit for purification.

Both DNA strands of the 1,346-bp *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 for terminator cycle sequencing with BigDye^{TM} using 10% DMSO as denaturant. Analyses and alignments of the DNA and deduced amino acid sequences were done with DNASIS software. The locations of the 5'- and 3'- flanking regions, the 5'- untranslated region (5'-UTR) intron, prospective promoter elements, and the putative CAP binding site of the *FAD2-4* gene were tentatively identified by comparisons with the sequence of the *FAD2-4* PCR product, the structures of the *FAD2-3* gene and cDNA, and consensus motif analyses with DNASIS software. The cDNA sequence was assigned GenBank accession no. AY279315.

The *Xba I/Sac I* digested and purified PCR product and pGEM-7Zf(+) vector were mixed in a 3:1 ratio (insert:vector) and ligated at 10^{o}C using T4 DNA ligase (Invitrogen, Carisbad, CA). The resulting recombinant plasmid DNA was used for transforming

electrocompetent *E. coli* DH5α cells to prepare large quantities of the recombinant plasmid DNA for further use.

Two primers were designed to amplify the coding region of *FAD2-3* cDNA from the cotton cDNA library 2 provided by Dr. Ed Cahoon. The primers used were designed based on sequence segments unique to the *FAD2-3* genomic sequence, but different from the FAD2-4 coding region. The forward primer (5'-GAAAGAAAATCGAAAGTATAGATTTG-3') is compatible with the 5'-flank of the putative *FAD2-3* cDNA, and the reverse primer (5'-GACGACCTCAATTGATGTAACCCAAACGCC-3') is compatible with the 3'-flank of the putative *FAD2-3* cDNA. The PCR reactions were set up as 5 min at 95ºC, followed by 35 cycles of amplification (95ºC for 30 sec, 60ºC for 30 sec, 72ºC for 30 sec), and holding at 72ºC for 7 min. A PCR product with the size of 1216 basepairs was amplified and believed to be *FAD2-3* cDNA. The *FAD2-3* cDNA was purified using gel extraction and the product was sequenced from both the 5' and 3' ends three times. The *FAD2-4* and *FAD2-3* cDNA sequences were aligned with the *FAD2-4* and *FAD2-3* genomic DNA sequences and two other homologous *FAD2* gene sequences from cotton (the *FAD 2-1* gene (Liu et al., 2001) and the *FAD2-2* gene (Yang et al., 2005)).

Chilling-sensitive, Ethanol Tolerance, and Growth Regulation of Yeast Cells Transformed with Cotton *FAD2* Genes.

The FAD2 enzyme converts oleic acid (18:1) into linoleic acid (18:2). Yeast cells do not have this function because they lack a *FAD2* gene, making yeast cells an ideal model system for functional expression of fatty acid desaturases. Previously, the *FAD2-3* and *FAD2-4* 1.2-kb open reading frames were subcloned into the yeast bacterial

shuttle vector pYES2 and then transformed into electrocompetent yeast cells. (Pirtle et al., 2001). The *FAD2* transformed yeast cells were found to have significant accumulation of linoleic acid compared to the control yeast cells transformed with the shuttle vector pYES2 alone (Zhang et al., 2008). The successful expression of *FAD2* genes in yeast cells provided a model to study the functional expression and regulation of cotton *FAD2* genes.

For this project, two cotton *FAD2* gene constructs were made using the pYES2 vector (Invitrogen, Carisbad, CA) nd transformed into yeast cells (Pirtle et al., 2001; Zhang et al, 2008). The yeast transformants and control cells were grown in galactose induction medium to induce lipid synthesis at different temperatures (10ºC, 20ºC). At the same time, the growth curves were recorded by reading the A_{600}. The cells were harvested at mid-log and late-log on the growth curve and the fatty acid methy esters were analyzed by gas chromatography and quantified by flame ionization detection in comparison to an internal heptadecanoic acid (C17:0). To test the expression of these two *FAD2* genes under ethanol tolerance and hydrogen peroxide stress, the viability of *FAD2* transformants and control cells in the presence of ethanol were measured. The yeast cells cultured for four generations were incubated in 67 mM KH_2PO_4 at ethanol concentrations of 0, 5, 10, 15, 20% and 15 mM hydrogen peroxide. The incubation was performed anaerobically at 30ºC for 0, 2, 4, 6 and 8 hours. Plating dilutions on YPD agar plates (1.0% yeast extract, 2.0% peptone, 2.0% agar, 1.5% glucose) and incubating them aerobically at 30ºC for 48 hours determined the viability of the yeast cells. The cell samples were also harvested for the lipid analysis.

Lipid Extraction and Fatty Acid Analysis of Yeast Cells Transformed with Cotton *FAD2* Genes

To extract lipid from yeast cells, the yeast transformants were grown in SC-U (synthetic complete minus uracil) medium (Adam 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 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 (Chilton et al., 1982). The fatty acid methyl esters (FAMES) were analyzed by gas chromatography and quantified by flame ionization detection (FID) essentially as described by Chapman and Trelease (1998), in comparison to an internal heptadecanoic acid (C17:0) standard.

Isolation of RNA from Cotton Plant Extracts

The QIAGEN RNeasy™ extraction procedure was used for the quantitative recovery of intact RNA suitable for the analyses. The total RNA extracted from both control cotton plants and H_2O_2-treated cotton plants was generously provided by Ms. Kimberly Spradling of our laboratory. Organ-specific expression was assessed following isolation of RNA from a variety of organs, including roots, stems, and leaves of two-week old greenhouse grown cotton plants (*Gossypium hirsutum* L. cv. Acala SJ5 or cv. Acala Maxxa). All the treatments were carried out in sealed plastic bags and removed from the bags after an appropriate amount of time. For the H_2O_2 -treated samples, two-week old greenhouse-grown cotton plants were treated with 100 mM H_2O_2 for two hours. Ethanol (37 mM) was used for ethanol treatment. For the cold treatment, two-week old

greenhouse-grown cotton plants were transferred to 25°C and 4°C growth chambers. The plants were pre-conditioned in growth chambers at both temperatures for one week before the initiation of each experiment. The plants were watered once daily but were not fertilized during the treatment period. The treatments lasted seven days, with the plant samples being collected each day. Tissue samples were collected from the control plants and cold-treated plants at one-day intervals during a one-week period. Cotton plants were randomly chosen and removed from the growth chamber. The plants were washed in cold deionized water and excess moisture was removed by blotting on paper towels. Leaf, stem, and root tissues were separated, and were frozen in liquid N_2 and stored at –70°C for later use.

At each collection time point for each tissue and each temperature treatment, around 3-5 grams of tissue were collected. The protocol for RNA preparation was modified from the methods of Chang et al (1993), and McKenzie et al (1997). For each analysis, the total of 1 g of leaf tissue and 1.5 g of each stem and root tissue was ground in liquid nitrogen to fine powder with a mortar and pestle, and then transferred to 50 ml centrifuge tubes for RNA extraction. Then, 15 ml of extraction buffer (2% hexadecyltrimethyl-ammonium bromide, 2% polyvinylpyrrolidone, 100 mM Tris-HCl at pH 8.0, 25 mM Na_2EDTA, 2 M NaCl, 0.5 g/l spermidine (N-[3-aminopropyl]-1,4-butane-diamine)) and 300-µl β-mercaptoethanol were added to each sample and all the tubes were held at 65°C with vigorous shaking to resuspend the tissue. Chloroform (15 ml) was added and mixed well with samples. The samples were centrifuged at 9000 x g for 20 min at 4°C to separate aqueous and organic phases. The top layers were transferred to a fresh tube and the chloroform extraction repeated. A total of 5 ml of 8 M

LiCl was added to each sample. The RNA precipitates were held overnight at 4°C. Then, the precipitated RNAs were pelleted by 30 minutes centrifugation at 8000 x g at 4°C. The supernatants were decanted, and the QIAGEN RNeasy™ extraction procedure was used for the quantitative recovery of intact RNA suitable for the analyses. The pellets were first resuspended in 500 µl of QIAGEN buffer RLT (proprietary composition), containing 5 µl BME, and then mixed with 250 µl of ethanol. The resulting 750 µl mixtures (including any precipitate) were transferred to RNeasy mini-columns (QIAGEN, Valencia, CA), which were placed in 2 ml collection tubes and centrifuged for 1 min at 14,000 x g and 4°C in a microfuge. The columns were washed with 700 µl of QIAGEN buffer RW1 (proprietary composition) and centrifuged for 15 sec at 8,000 x g and 4°C. The second wash was centrifuged for 2 min under the same conditions to dry the columns. The columns were transferred to 1.5 ml centrifuge tubes, and the RNAs were eluted by adding 50 µl of RNase-free water and centrifuging 1 min at 14,000 x g and 4°C. The elution was carried out again with another 50 µl of RNase-free water to ensure all of the RNA was collected from each of the columns. The samples were stored at -70°C for further use. A total of 5 µl of sample was run on an agarose gel to check the quality of RNA. The concentration and purity of each RNA sample was determined by taking spectrophotometric readings at 260 nm and 280 nm using a Varian DMS90 UV-Visible spectrophotometer.

Reverse Transcriptional RT-PCR Analyses of RNA from Cotton Plant Extracts

Reverse transcriptase-polymerase chain reaction (RT-PCR) analyses were performed to determine if cotton *FAD2-4* and 2-3 genes were present in total RNA extracts from leaves, stems, and roots of cotton plants (*Gossypium hirsutum* L., cv.

Acala SJ5) that had been treated with water (as control), hydrogen peroxide, ethanol, and low temperature. Residual DNA was first removed from the RNA samples by incubating 1 µg of each total RNA extract with 1x DNase I Reaction buffer (20 mM Tris-HCl (pH 8.4), 2 mM $MgCl_2$, and 50 mM KCl) and 1 unit of DNase I (Amplification Grade, Invitrogen, Carisbad, CA) for 15 min at room temperature. One microliter of 25 mM Na_2EDTA was added to the reactions to inactivate the DNase, and the reaction mixtures were heated for 10 min at 65º C in a water bath.

Treated RNAs were used for the RT-PCR experiment, and were subjected to PCR amplification using the One-Step Access RT-PCR System from Promega Corp. (Madison, WI) using specific forward and reverse primers designed from both *FAD2* gene sequences. The products of each amplification reaction were examined on agarose gels containing ethidium bromide. The primers that were used for the *FAD2-4* mRNA were FD4FR240: 5'- CTGTCTAGAGACCAAAGTGAAAGAAAATCGAAG-3' and FD4RV1520: 5'-GACGAGCTCCAAAAGCATCTAAAATAGAAGTAACCC-3'. The primers that were used for the *FAD2-3* mRNA were FD3FR220: 5'- GAGA GGGACCAAAGTGAAATCG-3' and FD3RV1540: 5'- CCATGTAACCCAAA CGCCAAAACCC – 3'.

RT-PCR was done using the SuperScript One-Step RT-PCR with Platinum *Taq* Polymerase Kit (Invitrogen, Carisbad, CA), RNaseOUT Recombinant Ribonuclease Inhibitor, the unique oligonucleotide primers, and the DNase I-treated RNA samples. Each RT-PCR reaction of 50 µl contained 1x Reaction Mix (0.2 mM of each dNTP and 1.2 mM $MgSO_4$), 2.5 units of RNaseOUT, 200 ng of DNase-treated RNA template, 0.2 µM of each mRNA-specific primer, and 1 µl of RT/Platinum *Taq* Polymerase Mix. The

reactions were placed in a thermal circler (Perkin Elmer GeneAmp PCR System 2400) and incubated for 30 min at 50ºC for ribonuclease inhibition, followed by a 2 min denaturation step at 94ºC. Once denatured, the reactions were subjected to 35 cycles, including a 15 sec step at 94ºC to denature the double-stranded template, a 60 sec step at 59ºC to allow the gene-specific primers to anneal to the template, and a 1 min step at 72ºC to allow the primers to extend with the available DNA polymerase. After the last cycle, the reactions were held at 72ºC for 10 min and then cooled to 4ºC. The resulting RT-PCR products were mixed with 6x blue/orange loading dye and electrophoresed on a 1.0% agarose gel containing ethidium bromide at a final concentration of 0.5µg/ml, with pGEM DNA Markers (Promega, Madison, WI) to determine the product sizes and detect the presence of the *FAD2* transcripts in each of the tissues examined.

Isolation of Protein from Cotton Plants for One-Dimensional SDS-PAGE and Western Blot Analyses

An antigenic peptide was designed with 20 amino acids considering antigenicity and hydrophilicity. Since fatty acid desaturases are hydrophobic proteins that span the membrane four times (Shanklin and Cahoon, 1998), a sequence of 20 amino acids at the C-terminal end of cotton *FAD2,* located in the lumen of the endoplasmic reticulum, and containing a histidine-rich region (HVAHHLFS) (Shanklin and Cahoon, 1998) was selected to generate a polyclonal antibody preparation. The polypeptide was prepared commercially by Biosynthesis, Inc., Lewisville, TX. The amino acid sequence of the C-terminal region of the FAD2 polypeptide is: NH_2- (GC) HNITDTHVAHHLFSTMPH-COOH. The polyclonal antibody preparation was used for Western blot analysis to determine the expression profiles of *FAD2* genes in cotton plants, in the yeast cells

transformed with the *FAD2* genes, and in the putative transgenic Arabidopsis plants transformed with *FAD2* genes. This antibody preparation was also used to study the types of environmental signals that induce expression of the *FAD2* genes.

The same plant tissue samples that were used for mRNA extraction were used for total protein extraction. To isolate enough protein from stem and root tissue and lower the background of the protein standard and non-specific bands during Western blotting, the total protein extraction kit from Sigma (Product Code PE0230) was used to generate give qualitative samples of all protein types from any kind of plant tissue, and was effective in preventing protein degradation during the extraction process (Herbert, 1998). Following the instructions of the manufacturer, total protein was extracted from the leaves, stems, and roots of two week-old cotton plants (*Gossypium hirsutum* L., cv Acala Maxxa) that had been treated with water (as control) or other treatment for a period of time. Approximately 400 mg of each tissue were ground to a fine powder with a mortar and pestle under liquid nitrogen. The powdered tissues were then transferred to cold (-20˚C) pre-weighed 2 ml microcentrifuge tubes, and a total of 1.5 ml cold methanol solution (containing a 1:100 dilution of Sigma Protease Inhibitor Cocktail) was added to each sample. The mixtures were vortexed and incubated at -20˚C for 5 min with periodic vortexing. The suspensions were centrifuged for 5 min at 16,000 x g and 4˚C in a microcentrifuge to pellet the proteins and plant debris. The supernatants were discarded, and the methanol wash was repeated three more times. After the final supernatants were discarded, the tubes were inverted over paper towels to allow any remaining methanol solution to drain. After the methanol solution had drained from the

tubes, 1.5 ml of cold acetone was added to each tube and vortexed for 30 sec before being incubated at -20˚C for 5 min.

The mixtures were centrifuged for 5 min at 16,000 x g and 4˚C to pellet the proteins and plant debris. After the supernatants were discarded, the acetone extractions were repeated one more time. The resulting supernatants were discarded, and the pellets were air-dried for 5 min at room temperature. After the pellets were dried, each tube was weighed and its predetermined mass was subtracted to determine the plant tissue mass. The tissue pellets were then suspended in 4 µl of Reagent Type 2 Working Solution (Sigma proprietary composition, a chaotropic reagent to dissolve hydrophobic proteins with a 1:1000 dilution of the Protease Inhibitor Cocktail) per mg of plant tissue by vortexing. The mixtures were incubated for 15 min at room temperature with rocking and intermittent vortexing. The tubes were then centrifuged for 30 min at 16,000 x g and room temperature to pellet the plant debris. The supernatants, which contained the total protein, were finally transferred to clean 1.5 ml microcentrifuge tubes. We have been able to collect qualitative total protein from stem and roots using this kit.

The concentrations of the protein extracts were determined using Bradford assay solution (Sigma-Aldrich, St. Louis, MO). Once the protein concentration was determined, a total of 3-5 µg of protein from each sample was electrophoresed in duplicate on two denaturing SDS polyacrylamide gels at 130 V. The protein samples were mixed with 6x sample buffer (350 mM Tris-HCl (pH 6.8), 30% (v/v) glycerol, 10% (w/v) sodium dodecyl sulfate (SDS), 600 mM dithiothreitol (DTT), and 0.012% (w/v) bromophenol blue) and 2 µl of β-mercaptoethanol (BME). Then they were heat-treated for 5 min in a 95ºC water-bath and cooled down to room temperature. The protein

extracts were resolved on a discontinuous buffer system, consisting of a stacking gel and a separating gel, with 10 µl of Full Range Rainbow recombinant protein molecular weight markers (Amersham Bioscience, Piscataway, NJ) for one dimensional (1-D) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The stacking gel contained 5% acrylamide/bis-acrylamide (29:1), 0.15 M Tris-HCl (pH 6.8), 0.1% SDS, 0.1% ammonium persulfate (APS), and 0.12% TEMED. The separating gel contained 15% acrylamide/bis-acrylamide, 0.375 M Tris-HCl (pH 8.8), 0.1% SDS, 0.1% ammonium persulfate (APS), and 0.04% TEMED. The proteins were electrophoresed at 30 mA per gel using 1x Tris-Glycine Electrophoresis Running Buffer (25 mM Tris base (pH 8.3), 192 mM glycine, and 0.1% (w/v) SDS) in a Mini-PROTEAN 3 cell system (Bio-Rad).

Following electrophoresis, the proteins in one gel was visualized using Coomassie Blue, and the proteins in the duplicated gel were transferred to a nitrocellulose membrane (PROTRAN Pure Nitrocellulose Transfer and Immobilization Membrane, Schleicher & Schuell) using 1x Tris/Glycine Transfer Buffer (48 mM Tris base (pH 9.2), 35 mM glycine, and 20% (v/v) methanol) and a Mini Trans-Blot Electrophoretic Transfer Cell. Each separation gel was equilibrated in the transfer buffer, with the nitrocellulose membrane, two pieces of 3MM filter paper, and two fiber pads, for 45 min before being placed in a gel/membrane sandwish for Western blotting. A Mini Trans-Blot Electrophorestic Transfer Cell was used for electroblotting. The gel/membrane sandwiches, an ice block, and 1x Tris/Glycine Transfer Buffer were then placed in the transfer cell, and the proteins were transferred to the nitrocellulose membranes at 90 mA overnight using a Model 250/2.5 Bio-Rad Power supply.

The following day, each nitrocellulose membrane was placed in a blocking solution of 5% milk: TBS (5% (w/v) dry milk, 20 mM Tris-HCl (pH7.5), and 150 mM NaCl) for 2 hours at room temperature with shaking. This was followed by two washes with TBS-T (TBS with 0.35% (v/v) Tween 20 (polyoxyethylenesorbitan monolaurate)) for 10 min at room temperature with shaking. The membranes were incubated with a polyclonal anti-cotton *FAD2* antibody (prepared commercially by Biosynthesis, Inc., Lewisville, TX). The antibody was used at 1:1500 dilutions in 5% milk: TBS-T for 1 hour at room temperature with gentle shaking. The membranes were then washed with TBS-T as before, and incubated with a 1:3000 dilution of a secondary antibody (Anti-rabbit IgG, peroxidase-linked species-specific whole antibody from donkey; from Amersham Pharmacia Biotech, now part of General Electric Healthcare Life Sciences, Piscataway, NJ.) for 45 min at room temperature with gentle shaking. The membranes were washed one last time with TBS-T as before, and the bound secondary antibodies were visualized after incubating the membranes in a 1:1 mixture of ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ) for 1 min at room temperature and subsequently exposed to Kodak X-OMAT film. To lower the background of protein standard and un-specific bands on the blotting film, we tried different concentrations of primary antibody and secondary antibody to incubate the membrane and different times for film exposure. The 1:2000 dilutions for primary antibody wash and 1:3000 dilution for secondary wash was determined to be the key to gain a specific band.

Vector Design/Construction for Expression of the Cotton *FAD2* Genes in Transgenic Arabidopsis Plants

To further study the *FAD2* genes in transgenic *Arabidopsis* plants, *FAD2* gain-of-function *Arabidopsis* plants were generated, in which the *FAD2* gene was placed under the transcriptional control of a constitutive promoter. The subcellular localization of the corresponding *FAD2* fusion polypeptides was also examined by ligating the *FAD2* genes with N-terminal or C-terminal reporter fusion genes (the *GUS* and *GFP* reporter cassettes).

Initially, the subcloning procedures and the production of the binary constructs was hampered by the large size of the binary plant transformation vector pCAMBIA and the inappropriately positioned restriction sites on the vector. The recently developed Gateway® technology is a powerful system designed to simplify and provide a rapid and highly efficient route for multiple expression and functional analysis options (Gerald and Labaer, 2004). The Directional TOPO® pENTR™ vectors from Invitrogen Corporation take advantage of fast, efficient directional cloning. The PCR products generated from the gene of interest can be cloned into a 5´ to 3´ orientation using a 5 min bench-top ligation reaction. Once the PCR product is cloned into the entry vector, the resulting entry clone can be recombined with any Gateway® destination vector to create an expression clone (Gerald and Labaer, 2004). The *attL* recombination sites flank the PCR product insertion site for efficient recombination with choice of Gateway destination vectors. Once the gene is cloned into the entry vector, the Gateway® LR Clonase™ II enzyme mix is used to catalyze *in vitro* recombination between the entry

clone (containing the *FAD2* genes flanked by *attL* sites) and a destination vector (containing *attR* sites) to generate an expression clone.

To select for the desired recombinant product and exclude the parental plasmids and undesired recombination intermediates, the Gateway system uses an *E. coli* death *ccdB* gene, in combination with drug-resistance markers on the master (Entry) and Destination plasmid vectors. The *ccdB* gene from the *E. coli* plasmid segregation control system allows for negative selection in *E. coli* by its ability to inhibit *E. coli* DNA gyrase. When the products of Gateway recombination reactions are used to transform *E. coli*, the cells transformed by a Gateway Donor or Destination plasmid or by the cointegrate intermediate of the Gateway recombination reaction are unable to grow. Only the desired recombinant product, which lacks the *ccdB* gene and has the appropriate drug selection marker, can give rise to putative transformants (Curtis and Ueli, 2003; Gerald and Labaer, 2004; Harley et al., 2000).

The three pMDC Gateway destination vectors used are available from the ABRC Stock Center (Ohio State University, Columbus, OH). The pMDC32 vector, a constitutive expression vector, harboring a dual 35S promoter without any terminal protein tag, was used to create transgenic Arabidopsis plants and transgenic cotton plants. The pMDC43 vector, used for C-terminal *GFP* fusion constructs, and the pMDC139 vector, used for the N-terminal *GUS* fusion constructs, were used for subcellular localization of FAD2 polypeptides.

In order to directionally clone the PCR product with the *FAD2* coding regions into the vectors, the forward primer has to incorporate the sequence 5'-CACC-3' at the 5'-end with no modification at the 3'-end. The overhang in the cloning vector (GTCC)

attacks the 5'-end of the PCR product, anneals to the added bases, and stabilizes the PCR product in the correct orientation. Also, in order to fuse the PCR product with a C-terminal tag, following recombination of the entry clone with a destination vector, it is necessary to design the reverse PCR primer to remove the native stop codon in the *FAD2* gene open reading frames. The primers used to amplify the coding region of both *FAD2-4* and *FAD2-3* genes are:

5' prime primers: GATEWAY FRI 5'- CACCATGGGTGCAGGTG – 3'

GATEWAY FRII 5'- CACCATGGGTGCAGG – 3'

3' prime primers: GATEWAY RVA1 5'-GATCTTATTTCTAAACCAAAATACACC–3'

GATEWAYRVA2 5'- GATCTTATTTCTAAACCAAAAT ACACCTTTGC-3'

GATEWAY RVB1 5'-TTAGATCTTATTTCTAAACCAAA ATACACC –3'

GATEWAY RVB2 5'-TTAGATCTTATTTCTAAACCAAA ATACACC-3'

One single, discrete PCR product is necessary for the ligation reaction because any PCR cleanup procedure will decrease the efficiency of the ligation reaction, and thus the PCR optimization is mandatory. A proofreading enzyme was used to amplify the *FAD2-4* and *FAD2-3* genes to maintain the sequence identity of the PCR products. The amplifications were set up in 50 µl volumes with 41 µl distilled water, 5 µl 10X *Pfx* mix (Invitrogen, Carisbad, CA), 1.5 µl of each primer, and 100-200 ng of template DNA. The PCR conditions were optimized by screening reactions with different template dilutions and different annealing temperatures. The reaction that resulted in the most intense single, discrete 1.2 kb product was used for the following PCR procedure. The concentration of the PCR product with the *FAD2* coding region was checked by spectrophotometry at A_{260} and diluted to10 ng/µl. All the entry vector ligations were

carried out in 6 µl volumes with 10 ng PCR product, 20 ng Topo cloning vector, and 1 µl 1:4 salt solution. After 30 min incubation at room temperature, 2 µl of the ligation reactions were mixed with 50 µl electrocompetent cells (*E. coli* strain DH5α from Invitrogen, Carisbad, CA). The charging voltage set for the electroporation was 1.3 kV/cm, using the BCM 395 Electroporation System (BTX, Inc. CA). After 250 µl of SOC medium were added, followed by one hour incubation at 37°C with 200 rpm shaking in a shaker/incubator. A total of 200 µl, 100 µl and 50 µl of the transformant mixtures were screened on three LB plates containing 50 µg/ml kanamycin and incubated overnight at 37°C. Colony PCR was used to select the positive pENTR-FAD2 constructs with primers designed to amplify the insert *FAD2* gene. PCR master mixes were made and aliquoted into 50 µl individual reactions. Robust colonies were picked from the transformation plates with pipette tips and dipped into PCR mix. The tips were saved in 5 ml of LB broth containing 50 µg/ml kanamycin and incubated overnight with shaking. Colony PCR products were checked on a large 1% agarose gel. Samples with the 1.2 kb *FAD2* fragments were identified as positive clones.

The destination vectors (Curtis and Ueli, 2003) contain the bacterial *ccdB* gene which encodes an anti-DNA gyrase protein. DNA gyrase (or topoisomerase II) relieves the topological constraints caused by replication and transcription complexes moving along the DNA by introducing a transient double-strand break in the DNA substrate, passing one strand of the DNA through the break and resealing it. The *ccdB* protein disrupts the function of DNA gyrase by interacting with it after it has made the double-strand break in the DNA (Gerald, and Labaer, 2004; Harley et al., 2004) and binds the DNA gyrase in an open configuration such that the DNA gyrase is unable to reseal the

DNA, finally resulting in bacterial death. The *E. coli* strain DB3.1 contains a mutation in the DNA gyrase gene so that the *ccdB* protein is unable to bind the mutant DNA gyrase protein (Gerald, and Labaer, 2004; Harley et al., 2004). In this *E. coli* strain, the DNA is replicated normally and colonies grow. For this reason, all the pMDC vectors have to be transformed into the *E. coli* DB3.1 to obtain viable colonies. The *E. coli* DB3.1 cells were purchased from Invitrogen and grown in LB/streptomycin (100 μg/ml) overnight. The chemical competent cells were prepared by growing *E. coli* DB3.1 cells for 34 hours in a 37°C shaker at 200 rpm. The cells were harvested by 8000 x g centrifugation at 4°C and washed by ice-cold distilled water four times. Nine pMDC vectors were transformed into these competent cells and selected on the LB/streptomycin (100 μg/ml) plates. The plasmids were isolated and the size of each vector (about 2.7 kd) was checked by a single *Spe I* digest.

Before the recombination cloning procedure, one critical problem had to be resolved. Since both the entry clone and the destination vector have kanamycin resistant genes, a technical question of how to lower the background of the entry vector clone when screening for the transformants had to be solved. The ordinary *E. coli* cells transformed with pMDC vector would not survive because of the deadly *ccdB* gene site on the vector, as are the *E. coli* cells transformed with the entry clone that exchanged its *FAD2* insert with the *ccdB* fragment from pMDC vectors. Thus, the negative clone background on the screening plates would be the Topo *FAD2*-entry vector clones that are also resistant to kanamycin. After discussing with Dr Mark Curtis (Institute of Plant Biology and Zurich-Basel Plant Science Centre, University of Zurich, Zurich, Switzerland), who

designed the destination vectors (Curtis and Ueli, 2003), two approaches were used to solve this problem:

1. The entry vectors were linearized with an enzyme that will not cut the insert, but only the vector. Thus, the *FAD2* insert would still be exchanged into the destination vector, but the entry clone will not ligate back to create negative clones. The enzymes chosen were *Not I* (upstream of the *attR2* site) and *EcoRV* (downstream of the *attR1* site). Neither of these sites are present in our two *FAD2* coding regions.
2. The pMDC primers were designed to overlap the *attR1-attR2* region for colony PCR analyses. The transformants were assessed by two PCR reactions to confirm the correct construct, one reaction to amplify the *attR1-attR2* region of the vector, and one reaction to amplify the inserted *FAD2* gene.

Before the recombination reaction, all the entry-*FAD2* plasmids were first digested by *Not I* or *EcoR V* for 2-3 hours. The digests were terminated by 10-15 minutes incubation at 65ºC to denature the enzyme activity. The linearized entry vectors were used for recombination reactions with destination vectors directly without any cleanup procedure. The reactions were performed with 150 ng of cut entry-*FAD* plasmid, 150 ng of destination vector, 2 µl of clonase (Invitrogen, Carisbad, CA), and TE buffer to make a total volume of 10 µl. After two hours to overnight incubation at room temperature, 1 µl of proteinase K was added, followed by 10 minute incubation at 37°C to terminate the reaction. Then 1 µl of reaction mix were added to 50 µl of OminiMax

competent cells (Invitrogen, Carisbad, CA), and heat shocked at 42°C for 30 seconds, followed by two minute incubation on ice. After 250 µl of SOC medium were added to each reaction, one-hour incubation at 37°C with 200 rpm shaking was done. Finally, 100 µl of transformed cells were plated on LB plates containing 50 µg/mL of kanamycin and incubated overnight at 37°C. The next day, 24 robust colonies were picked for colony PCR to amplify both the *attR1-attR2* region on the pMDC vector and the *FAD2* gene insert. Lastly, the potential positive colonies were assayed by *BamH I* digestion. There are three *BamHI* cutting sites in all three vectors. The insertion of the *FAD*2 genes replaces the *attR1-attR2* region that includes two *BamH I* sites. The *FAD2*-pMDC plasmid constructs were linearized while the empty vectors were digested into three bands (12 kb, 0.7 kb and 0.2 kb in size). To confirm the *FAD2*-pMDC constructs, primers were also designed using the sequences from the middle of the inserted genes and sequenced around one thousand base pairs in both the 5' and 3' directions. The sequences also included part of the pMDC vectors. All the constructed pMDC vectors were sequenced from both directions and the sequences were aligned with cotton *FAD2* cDNA sequences and vector sequence.

Transformation, Regeneration, and Screening of Transgenic Arabidopsis Plants

After confirming the sequences of all five *FAD2*-pMDC constructs, we transformed the recombinant plasmid DNA and the pMDC empty vectors were transformed into *Agrobacterium tumefaciens* LBA4404 cells. These competent cells were ordered from Invitrogen and can be transformed by electroporation. LBA4404 cells contain the disarmed Ti plasmid pAL4404, which has only the *vir* and *ori* region of the Ti plasmid. The recombinant DNAs are able to migrate from *A. tumefaciens* cells into plant cells

using components provided by the pAL4404 (Bevan, 1984). The electroporator conditions are set at 2 kV, 200Ω, and 25 μF. The cells were thawed on ice for 20 min, after 20 ul were mixed with 100 ng of each DNA and electroporated in a 0.1 cm cuvette. Immediately, 1.0 ml of room temperature YM medium was added and the solution was transferred to a 15 ml snap-cap tube. The tubes were shaken at 225 rpm at 30˚C for three hours. Then the cells were diluted and spread on YM plates containing 100μg/ml streptomycin and 50μg/ml kanamycin. All the plates were incubated for three days at 30˚C. Colony PCR was used to test the positive transformed cells with primers annealed on the *FAD2* genes only. Empty pMDC vectors were used as the positive controls. All the positive transformants were grown in YM culture overnight and stored at –70˚C with 40% glycerol before the Arabidopsis plants were ready for transformation.

The *FAD2* knockout seeds were ordered from the Arabidopsis Biological Resource Center at Ohio State University (Okuley et al., 1994). The wild type line of *A. thaliana* used in this study is the Columbia ecotype. The *fad2* mutants were derived from the Columbia wild type. By planting these seeds, Arabidopsis knockout plant seeds were obtained for the transformation procedure. For the plant transformation, the simplified Arabidopsis transformation protocol, called the floral dip method (Clough and Bent, 1998), was used. Five *FAD2*/pMDC constructs, along with three empty pMDC vectors as controls, harbored in *Agrobacterium tumefaciens*, were used to transform *Arabidopsis thaliane* (ecotype Columbia) plants using the floral dip method. To prepare for transformation, the inflorescence of Arabidopsis plants was clipped daily to encourage more flowering, which increased the efficiency of transformation. The plants

were then dipped into separate solutions of *Agrobacterium* cells that were prepared as described below.

A total of eight different *Agrobacterium tumefaciens* colonies transformed with the *FAD2*-pMDC and pMDC plasmid DNAs were each inoculated into 2 ml of YEP broth (Yeast Extract Peptone: 10 g/l bactopeptone, 10 g/l yeast extract, and 5 g/l NaCl) containing 50 mg/l kanamycin, and incubated for 24 hr at 28˚C with shaking at 200 rpm in a New Brunswick shaker/incubator. After the 2 ml cultures were added to 50 ml of YEP containing 50 mg/l kanamycin, the cultures were grown at 28˚C with shaking at 200 rpm until an A_{600} of about 1.8-2.0 (turbidity measurement) was reached. The cultures were then centrifuged at 8,000 x g rpm at room temperature using a SA600 rotor for 20 min. All the cell pellets were resuspended in 5% freshly-made sucrose solution to reach an A_{600} of 0.8. Silwet L-77 (VAC-IN-STUFF, LEHLE seeds, Round Rock, TX), a surfactant that enables the *Agrobacterium* cells to penetrate the plant cell walls and membranes, was added to each cell culture to reach a concentration of 0.04%.

The aboveground parts of the Arabidopsis plants were then dipped into the eight separate *Agrobacterium* diluted cell cultures for 2-3 sec with gentle agitation. The dipped plants were then immediately sprayed with distilled water to prevent an overgrowth of *Agrobacterium,* and the plants were covered with Saran wrap and a trash can to maintain high humidity. The plants were kept in the dark with high humidity for 48 hours and then transferred to a 22˚C growth room with a 16 hr photoperiod. Six days after the first dip, the dipping method was repeated using fresh *Agrobacterium* solution. Twenty-four hours after the second dip, the plants were transferred back to

the growth room, where they were grown until the seeds became mature and the dried seeds were harvested and screened for transformants.

The seeds were surface sterilized by vapor-phase sterilization methods (Clough and Bent, 1998). Approximately 50 µl of seeds were transferred to 1.5 ml microcentrifuge tubes. The tubes were then placed in a desiccator jar, which was positioned in a fume hood. Just prior to sealing the desiccator, a 250 ml beaker containing 100 ml bleach was positioned in the desiccator and 3 ml of concentrated HCl was carefully added into the bleach. The desiccator jar with chlorine fumes remained sealed and the sterilization lasted for six hours. To select for transformed plants, sterilized seeds were subsequently dispensed on 100 x 200 mm hygromycin MS selection plates (50 µg/ml) (0.5 x MS (Murashige & Skoog)) salts with micronutrients (iron, manganese, zinc, boron, copper, molybdenum, cobalt; Sigma, Catalog #M0529; Murashige and Skoog, 1962), 0.5 X MS salts with macronutrients (nitrogen, phosphorous, potassium, calcium, magnesium, sulfur; Sigma, CatalogM0654; Murashige and Skoog, 1962), 0.25% Gelrite gellan gum, and 50 µg/ml hygromycin (pH 5.6)). Care was taken to make sure the seeds were evenly separated (the plants grown in colonies have shown higher resistance to selection marker). The plates were than cold-treated in the dark for 3-4 days and transformed to a plant growth chamber under a 16 hr light/8 hr dark cycle regimen. The seedlings were grown in a controlled environment at 24˚C under 23 hr and the petri plates and lids were sealed with Parafilm. Excess moisture during growth was removed by briefly opening the plates and shaking moisture off the lid. Transformants were identified as Hygromycin-resistant seedlings

that produced green secondary leaves and visible well-established roots within the selective medium.

The putative Arabidopisis T1 transformants grow to maturity by transplanting (preferably after the development of 3-5 adult leaves) into heavily moistened potting soil (Clough and Bent, 1998). The transplanting is a very delicate procedure and requires extra care. First, the young Arabidopsis plants were moved to a clear area with gel medium still attached to the root tissue. Distilled water was sprayed on the gel and plant. After carefully separating the roots from medium using a clean scapula, the young plant was transferred to a pot containing autoclaved and moistened Arabidopsis plant soil. The plant, together with the pot, was wrapped with Saran wrap to keep a very moist environment and transferred to the growth room. After two days, a small opening was cut in the Saran wrap to let the air enter. The Saran wrap was taken off after another 2-3 days. By doing this, the young plants could slowly adjust to the environmental change from sealed petri dish to growth chamber with open air. T_2 or T_3 generations of plants were used as seed stock to generate lines of transformed Arabidopsis plants.

PCR Amplification of Cotton *FAD2* Genes using Genomic DNA from Arabidopsis Plant Leaves

To confirm the successful creation of transgenic Arabidopsis plants transformed with cotton fatty acid desaturase genes, the first step was to amplify the desired target genes from the genomic DNA isolated from transgenic Arabidopsis plant T_1 lines. The REDExtract-N-Amp Plant PCR Kit (Sigma-Aldrich, St. Louis, MO) was used to rapidly extract and amplify genomic DNA from plant leaves. The leaf tissue was sampled from

cotton *FAD2* transformed Arabidopsis plant T_1 lines, and Arabidopsis *fad2* knock-out mutant lines (as control). First, the leaf tissues were washed in distilled water three times. Then, a hole punch was used to cut a 0.7 cm disk of leaf tissue. The leaf tissue was transferred into a 2 ml collection tube. A total of 100 µl of Extraction Solution (Sigma proprietary mixture, Sigma-Aldrich, St. Louis, MO) was added to each sample. After a brief vortex, the sample was incubated at 95˚C for 10 minutes. A total of 100 µl of Dilution Solution (Sigma Proprietary mixture, Sigma-Aldrich, St. Louis, MO) was added to each sample and vortexed to mix. The diluted leaf extract was stored at 4˚C in the refrigerator. The diluted leaf extracts were then subjected to PCR amplification using the Sigma REDExtract-N-Amp PCR Reaction Mix (containing a proprietary mixture of buffer, salts, dNTPs, *Taq* DNA polymerase, and TaqStart antibody for specific hot start amplification).

To prove the successful isolation of DNA from Arabidopsis leaf tissues**,** the Arabidopsis *Act8* gene (An et al, 1996) was amplified from all lines. The *Act8* gene is 265 bp, a member of actin subclass (An et al, 1996), and there is strong, constitutive expression of *Act8* in Arabidopsis vegetative tissues, rendering it to be an excellent constitutive control gene to check the quality of genomic DNA isolated from Arabidopsis and to use for PCR reaction controls. The *Act8* PCR reaction was set up with 10 µl of REDExtract-N-Amp PCR reaction mix (containing buffer, salts, dNTPs, *Taq* polymerase and TaqStart antibody), 2 µl of 4 µM *Act8* primers, 4 µl of leaf disk extract and 2 µl PCR grade water. Ms. Kim Spradling of our laboratory generously provided the *Act8* primers: Act8For 5'-GTTAAGGCTGGATTCGCTGG-3', Act8Rev 5'-GTTAAGAGGACCCTCGGTAAG-3'. The reactions were placed in a thermal cycler

(Perkin Elmer GeneAmp PCR system 2400) with the cycling parameters: 3 minutes initial denaturation at 94˚C, 35 cycles of amplification (30 seconds of denaturation at 94˚C, 30 seconds of annealing at 59˚C, and 1 minute of extension at 72˚C), and 10 minutes of final extension at 72˚C. The PCR products were electrophoresed on a 2.0% agarose gel containing ethidium bromide at a final concentration of 0.5 µg/ml, with pGEM DNA Markers (Promega) as standards for size determination. A 265-bp PCR fragment was amplified from both the control DNA (isolated from *fad2* knock out Arabidopsis plants) and the DNA from *FAD2*-pMDC transformed Arabidopsis plants, indicating high quality genomic DNAs.

Since the pMDC139 vector has a *gusA* gene following a *attR2* recombination site, to analyze the *FAD2-3*/pMDC139 Arabidopsis line, genomic DNA isolated from this line was used for PCR reactions to amplify the 366-bp *gusA* gene. The *gusA* primers (generously provided by Ms. Kim Spradling) were For 5'-AATTGATCAGCGTTGGTGGG-3', and Rev 5'-GTCGGTAATCACCATTCCCG-3'. The *gusA* PCR reaction was set up with 10 µl of REDExtract-N-Amp PCR reaction mix (containing buffer, salts, dNTPs, *Taq* polymerase and TaqStart antibody), 2 µl of 4 µM *gusA* primers, 4 µl of leaf disk extract, and 2 µl PCR grade water. The cycling parameters were: 3 minutes initial denaturation at 94˚C, 35 cycles of amplification (30 seconds of denaturation at 94˚C, 30 seconds of annealing at 66˚C, and 1 minute of extension at 72˚C), and 10 minutes of final extension at 72˚C. To prove the successful transformation of the *FAD2-3*/pMDC139 line, two sets of primers were designed. One set of primers annealed to the pMDC139 vectors and overlapped *attR1-FAD2-3-attR2* region to form an amplified fragment of around 1.5 kb.

The other set of primers were used to amplify the *FAD2-3* coding region from basepairs 680 to1050, which is about 400 bp. The primers sequences were: FD2-680FR: 5'-GTTTCCAACGCTCACTTATCCG-3', FD2-1050RV: 5'-GTTGAGGTATTTAGCCCACCATC-3'. The cycling parameters were: 3 minutes initial denaturation at 94˚C, 35 cycles of amplification (15 seconds of denaturation at 94˚C, 30 seconds of annealing at 64˚C, and 1 minute of extension at 72˚C), and 10 minutes of final extension at 72˚C. Purified plasmid *FAD2-3*/pMDC139 DNA was used to set up a two-control reaction. To test the transformed *FAD2-4*/pMDC32 line, primers were designed to anneal to the pMDC32 vectors and overlap the *attR1-FAD2-4-attR2* region; the amplified fragment being around 1.5 kb. A 400-bp fragment from the *FAD2-4* coding region and the entire 1.2-kb *FAD2-4* coding region were also amplified from the Arabidopsis *FAD2-4*/pMDC32 line. The cycling parameters were: 3 minutes initial denaturation at 94˚C, 35 cycles of amplification (15 seconds of denaturation at 94˚C, 30 seconds of annealing at 64˚C, and 1 minute of extension at 72˚C), and 10 minutes of final extension at 72˚C. Purified plasmid DNA *FAD2-4*/pMDC32 was used to set up three control reactions.

Lipid Extraction and Fatty Acid Analysis of Arabidopsis Plant Transformed with Cotton *FAD2* Genes

In order to prove cotton *FAD2* transgenic Arabidopsis plants were generated, and to also demonstrate that the cotton *FAD2* gene is functionally expressed in the transgenic Arabidopsis plants, quantitation by gas liquid chromatography (GLC) of the corresponding fatty acid methyl esters from the membrane phospholipids of transgenic Arabidopsis whole plants and individual leaf tissues was done.

In plants, FAD2 is the enzyme that converts oleic acid (18:1) to linoleic acid (18:2) by introducing a double bond at the Δ-12 positions (Somerville et al., 2000). Arabidopsis *fad2* mutant knockout plants were generated by Okuley et al (1994). The fatty acid compositions were studied using two Arabidopsis *fad2* mutant plants and wild type plants. It was determined that *fad*2 gene knock-out plants had significantly increased 18:1 fatty acid levels and decreased 18:2 fatty acid levels. Since we used Arabidopsis *fad2* mutant knockout plants for the transformation, the most straightforward way to rigorously demonstrate that the cotton *FAD2* gene was functionally expressed in *fad2* mutant Arabidopsis lines was to study their respective fatty acid profiles, especially the content of 18:2 and 18:1. It was hypothesized that complementation with the cotton *FAD2* genes would change the ratio of 18:2 to18:1 fatty acids to the level that similar to wild type Arabidopsis plants if the inserted genes truly functionally expressed.

For the lipid extractions, the method adapted from the paper by Focks and Benning (1998) was used. Before the experiment, the test tubes were rinsed with methanol and allowed to dry. A total of 1 mg of C17:0 powders was measured and dissolved in 1 ml of hexanol. This was used as an internal standard. Four to five young Arabidopsis plants from each supposed transgenic line and wild type plants were rinsed with distilled water, dried, then added to each of the labeled tubes. To each test tube, 5 µl of C17:0 fatty acid standard stock was added. A glass rod that was dipped in acidic methanol (1% HCl in methanol) was used to grind the plant tissues. Then a total of 1 ml acidic methanol was added to each sample. Between the procedures on each sample, the glass rod was cleaned with methanol and dried with a Kimwipe tissue. All the sample

tubes were then placed into a dry heating block at 80˚C for two hours. Then a total of 1 ml of hexane was added, followed by 1 ml of 0.9% NaCl. After the phases separated in the tube, the top organic phase was transferred into a capped vial with a Pasteur pipette. The organic solvent was dried off under a gentle stream of nitrogen gas. The samples were reconstituted in 25 µl of hexane just before loading onto a GC column (SUPELCO, Bellefonte, PA). A total of 1 µl of each sample was injected into the GC column with a syringe. The gas chromatograph (Hewlett-Packard 5890, SUPELCO, Bellefonte, PA) was equipped with a SP-2330 column, and a flame ionization detector. The slow rate of the carrier gas was 4.5 ml per minute. The initial oven temperature was kept for 2 min at 180˚C, then increased to 200˚C, and kept at this temperature for 4 min, and subsequently returned to 180˚C.

Confocal Microscopy using Transgenic Arabidopsis Plants Transformed with Cotton *FAD2* Genes

A *FAD2-4*/pMDC fusion construct was used to investigate the cellular location of the FAD2 polypeptides in transgenic Arabidopsis plants. The functional expression of the *FAD2-4* gene in the individual *FAD2-4/*pMDC43 transgenic plants (T_3 generation) was confirmed by GC analysis. The leaf tissues from these individual plants were sampled, placed on glass slides, and then covered with water and cover slides. The slides were imaged with a Zeiss 200M optical microscope fitted with a CSU-10 Yokogawa confocal scanner (McBain Instruments) and photographed with a digital camera (Hamamatsu, Phoenix, AZ). The location of the FAD2-4/GFP (green fluorescent protein) N-terminal polypeptides was determined. GFP fluorescence was visualized using 488-nm excitation and its emission was detected from 502.5 to 537.5

nm (or 515 to 545 nm if imaged in combination with YFP-yellow fluorescent protein). GFP and YFP were imaged sequentially using a Niptium Spinning disk. The optimal pinhole diameter was set at 2.52 Airy units in all cases. Post-acquisition image processing was done using ImageJ software and the green color was assigned to GFP.

Comparison of Temperature Sensitivity between Wild Type, *fad2* Mutant and Arabidopsis Transformed with Cotton *FAD2* Genes.

The *fad2* knockout mutant Arabidopsis has phenotypes distinct from those of the wild-type plants regarding their pattern of stem growth. At 22˚C, the total stem length of the *fad2* mutant was 80-90% of that for wild-type plants (Miquel, 1993). Another major phenotypic difference between the wild-type and *fad2* mutant Arabidopsis plants is the sensitivity to low temperatures. Miquel et al. (1993) discovered that under 5˚C treatment for 48 days, the *fad2* mutant plants died while wild type survived. The final confirmation for the transformation of cotton *FAD2* genes into the *fad2* mutant Arabidopsis plants, also as the conclusion of this project, would be the comparison of the phenotypes using wild type, *fad2* mutant, and individual plants from the *FAD2*-pMDC-transformed Arabidopsis lines.

The seeds of transgenic Arabidopsis T_3 plants were used to set up the treatment. The seeds were first sterilized by 3 min of 10% bleach, and 5 min of 95% ethanol, and then washed five times with water. Then the seeds of each line were planted in five to ten pots of soil. The plants were kept in the growth room for three weeks until the vegetative tissues were well developed. After three weeks, Arabidopsis plants were transferred into a 5˚C cold room on a growth shelf set up in the room. Photographs of

each line were taken periodically to document the phenotypic differences between each plant line.

CHAPTER 3

RESULTS

Subcloning and Sequence Analysis of Two *FAD2* Genes from a Cotton cDNA Library

In order to prove that the two *FAD2* genes are functional genes in cotton plants, primers were designed from coding regions of both genes and used to amplify a cotton cDNA library provided by Dr. Edgar B Cahoon (then of Dupont Ag Product, Experimental Station, Wilmington, DE). The PCR products (with a size of about 1.2 kb), assumed to be the *FAD2-3* and *FAD2-4* cDNAs, were generated. The PCR products were purified using gel electrophoresis, and then sequenced from both 5'- and 3'-termini for three times (as shown in Fig. 3). The cDNA sequences for the *FAD2-4* and *FAD2-3* cDNAs were aligned with the cognate cotton *FAD2-4* and *FAD2-3* genomic DNA sequences, and cotton *FAD 2-1, FAD2-2* cDNAs (Liu at al., 1999). The alignments showed that both the *FAD2-4* and *FAD2-3* cDNAs have the highest similarities to the genomic DNA sequences, indicating the cDNAs we isolated are indeed derived from the *FAD2-3* and *FAD2-4* genes, and that both genes are transcribed into the corresponding mRNAs in cotton plants.

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FD2-4           ------------------------------GACCAAAGTGAAAGAAAATCGAAG-TATAG 29
FD2-4cDNA       ------------------------------GACCAAAGTGAAAGAAAATCGAAG-TATAC 29
FD2-3cDNA       ------------------------------------------------AAA-TATAG 8
FD2-3           ------------------------------------------------AAA-TATAG 8
FD2-2           TAAAAAAAAAAGGCATTTCTTTCATCTTAAAGAGACAGCGAGGAAGCCACGAAGATAATA 60
FD2-1           ---------------------------------------------TGCTTCGTGTTTCATC 16
                                                              *
FD2-4           ATTTGATTTTCAATCTGCATTTTCAGGGTGTGGAACAATGGG--TGCAGGTGGCAGAATG 87
FD2-4cDNA       ATTTGATTTTCAATCTGCATTTTCAGGGTGTGGAACAATGGG--TGCAGGTGGCAGAATG 87
FD2-3cDNA       ATTTGATTTTCAATCTGCATTTTCAGGGTGTGGAACAATTGGGTTGCAGGTGGCAGAATG 68
FD2-3           ATTTGATTTTCAATCTGCATTTTCAGGGTGTGGAACAATTGGGTTGCAGGTGGCAGAATG 68
FD2-2           GAGTGATTTTCAATCTCCATTTTAAGGGTGTGGAACAATGGG--TGCTGGAGGCAGAATG 118
FD2-1           AACCTGGCGTTAAACTGCTTTCTTTAAAGCCAGCAAAATGGG--TGCCGGTGGTAGGATG 74
                      * ** ** * ** *         * * *** **  *** ** ** ** ***
FD2-4           TCGGTTCCTCCAAGTCAAAGGAAACAAGAATCGGGCTCAATGAAAAGAGCCCCTATATCT 147
FD2-4cDNA       TCGGTTCCTCCAAGTCAAAGGAAACAAGAATCGGGCTCAATGAAAAGAGCCCCTATATCT 147
FD2-3cDNA       TCGGTTCCTCCAAGTCAAAGGAAACAAGAATCGGGCTCAATGAAAAGAGTCCCTATATCT 128
FD2-3           TCGGTTCCTCCAAGTCAAAGGAAACAAGAATCGGGCTCAATGAAAAGAGTCCCTATATCT 128
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(Figure 3 continues)

(Figure 3 continued)

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FD2-2          TCGGTTCCAACGAGTCCAAAAAAACCCGAATTCAACTCACTGAAGCGAGTTCCATACTCA 178
FD2-1          CCAATT--GACG-GTATAAAGGAGGAAAATCGAGGCTCGGTCAATCGAGTTCCGATCGAG 131
                *  **   *  **  **   *     *      ***  * **  ***  **

FD2-4          AAACCACCATTTACTCTCAGTGAAATAAAAAAAGCCATCCCACCACACTGTTTCCAACGC 207
FD2-4cDNA      AAACCACCATTTACTCTCAGTGAAATAAAAAAAGCCATCCCACCACACTGTTTCCAACGC 207
FD2-3cDNA      AAACCACCATTTACTCTCAGTGAAATAAAAAAAGCCATCCCACCACACTGTTTCCAACGC 188
FD2-3          AAACCACCATTTACTCTCAGTGAAATAAAAAAAGCCATCCCACCACACTGTTTCCAACGC 188
FD2-2          AAGCCACCCTTCACTCTGAGTGAAATCAAGAAAGCCATCCCACCACACTGTTTCCAGCGC 238
FD2-1          AAGCCTCCGTTTACGCTCGGTCAGATCAAGCAAGCCATTCCGCCCCACTGTTTTCGCCGC 191
               ** ** ** ** ** **  ** * ** **  ******* ** ** ******** *  ***

FD2-4          TCACTTATCCGTTCATTTTCCTATCTCGTTTACGACTTCATTTTAGTCTCTATCTTTTAC 267
FD2-4cDNA      TCACTTATCCGTTCATTTTCCTATCTCGTTTACGACTTCATTTTAGTCTCTATCTTTTAC 267
FD2-3cDNA      TCACTTATCCGTTCATTTTCCTATCTCGTTTACGACTTCATTTTAGTCTCTATCTTTTAC 248
FD2-3          TCACTTATCCGTTCATTTTCCTATCTCGTTTACGACTTCATTTTAGTCTCTATCTTTTAC 248
FD2-2          TCCGTTTTACGCTCATTCTCATATCTCCTTTACGACTTTATATTGGCCTCTCTTTTTTAC 298
FD2-1          TCCCTCCTTCGATCCTTCTCCTACGTGGTCCATGACCTATGCTTAGCCTCTTTCTTTTAC 251
               **  *  * ** ** ** **  *  *  * *** *    ** * **** * ******

FD2-4          TACGTAGCCACCACTTACTTCCGCAACCTCCCTCAGCCACTATCTTTCGTCGCCTGGCCA 327
FD2-4cDNA      TACGTAGCCACCACTTACTTCCGCAACCTCCCTCAGCCACTATCTTTCGTCGCCTGGCCA 327
FD2-3cDNA      TACGTAGCCACCACTTACTTCCACAACCTCCCTCAGCCACTATCTTTCGTCGCCTGGCCA 308
FD2-3          TACGTAGCCACCACTTACTTCCACAACCTCCCTCAGCCACTATCTTTCGTCGCCTGGCCA 308
FD2-2          CATGTGGCCACCAATTACTTCCCTAACCTTCCTCAGGCTCTCTCCAACGTGGCTTGGCCT 358
FD2-1          TACATTGCAACATCATATTTTCACTTTCTCCCACAACCCTTTTCCTACATTGCTTGGCCT 311
                *  * ** **    ** ** *     ** ** **  *  * **   * * ** *****

FD2-4          ATTTATTGGGCTCTTCAAGGTTCAGTCCTCACTGGCGTTTGGGTTATCGCCCATGAATGC 387
FD2-4cDNA      ATTTATTGGGCTCTTCAAGGTTCAGTCCTCACTGGCGTTTGGGTTATCGCCCATGAATGC 387
FD2-3cDNA      ATTTATTGGACTCTTCAAGGTTCAGTCCTCACTGGCGTTTGGGTTATCGCCCATGAATGC 368
FD2-3          ATTTATTGGACTCTTCAAGGTTCAGTCCTCACTGGCGTTTGGGTTATCGCCCATGAATGC 368
FD2-2          CTTTATTGGGCCATGCAAGGTTGCATTTTGACCGGCGTTTGGGTCATAGCCCATGAATGT 418
FD2-1          GTCTATTGGGTTCTCCAAGGTTGCATCCTCACCGGTGTTTGGGTCATCGCACACGAGTGG 371
                * ******    * *******   *  * ** ** ******** ** ** ** ** **

FD2-4          GGTCACCACGCTTTTAGCGATTACCAATGGATCGATGACACTGTCGGTCTCATCCTCCAT 447
FD2-4cDNA      GGTCACCACGCTTTTAGCGATTACCAATGGATCGATGACACTGTCGGTCTCATCCTCCAT 447
FD2-3cDNA      GGTCACCATGCTTTTAGCGATTACCAATGGATTGATGACACTGTCGGTCTCATCCTCCAT 428
FD2-3          GGTCACCATGCTTTTAGCGATTACCAATGGATTGATGACACTGTCGGTCTCATCCTCCAT 428
FD2-2          GGCCACCATGCTTTCAGTGATTATCAATGGCTTGACGACACCGTGGGCCTTATCCTCCAC 478
FD2-1          GGTCACCACGCTTTCAGAGACTACCAATGGGTTGACGACACCGTCGGGTTGATCCTTCAT 431
               ** ***** ***** ** ** ** ****** * ** ***** ** **  * ***** **

FD2-4          TCATCCCTTCTCGTCCCGTACTTTTCGTGGAAATATAGTCACCGGTCGTCACCATTCCAA 507
FD2-4cDNA      TCATCCCTTCTCGTCCCGTACTTTTCGTGGAAATATAGTCACCGGTCGTCACCATTCCAA 507
FD2-3cDNA      TCATCCCTTCTTGTCCCGTACTTTTCGTGGAAATATAGTCACCGA-CGTCACCATTCCAA 487
FD2-3          TCATCCCTTCTTGTCCCGTACTTTTCGTGGAAATATAGTCACCGA-CGTCACCATTCCAA 487
FD2-2          TCTTCTCTCTTAGTTCCATATTTCTCTTGGAAATATAGCCACCGG-CGTCACCATTCTAA 537
FD2-1          TCCGCCCTTTTAGTCCCGTACTTCTCGTGGAAAATCAGTCACCGC-CGTCACCACTCGAA 490
               **  * **  * ** ** ** ** ** ******   ** *****  ******** ** **

FD2-4          CACTGGTTCCCTTGAACGCGACGAAGTATTTGTTCCGAAGAAACGGAGCAGCATTAGATG 567
FD2-4cDNA      CACTGGTTCCCTTGAACGCGACGAAGTATTTGTTCCGAAGAAACCGAACAACATTAGATG 567
FD2-3cDNA      CACTGGTTCCCTTGAACGCGACGAAGTATTTGTTCCGAAGAAACGGAGCAGCATTAGATG 547
FD2-3          CACTGGTTCCCTTGAACGCGACGAAGTATTTGTTCCGAAGAAACGGAGCAGCATTAGATG 547
FD2-2          CACCGGTTCCCTCGAAAGGGATGAAGTGTTCGTTCCCAAGAAAAAATCTGGTTTAAGATG 597
FD2-1          CACCGGTTCCATGGAGCGTGACGAAGTATTCGTGCCCAAACCCAAGTCTAAATTATCATG 550
               *** ****** * **  * ** ***** ** ** ** **              *   ***

FD2-4          GTGGGCTAAATACCTCAACAATCCACCAGGTCGTTTCGTCACAATCACCATTCAGCTCAC 627
FD2-4cDNA      GTGGGCTTAATACCTCAACAATCCACCAGGTCGTTTCGTCACAATCACCATTCAGCTCAC 627
FD2-3cDNA      GTGGGCTAAATACCTCAACAATCCACCAGGTCGTTTCGTCACAGTCACCATTCAGCTCAC 607
FD2-3          GTGGGCTAAATACCTCAACAATCCACCAGGTCGTTTCGTCACAGTCACCATTCAGCTCAC 607
FD2-2          GTGGGCCAAACACTTCAACAATCCACCGGGTCGGTTTCTGTCAATCACCATTCAACTTAC 657
FD2-1          CTTTGCGAAATACTTAAACAATCCACCCGGTCGAGTTCTATCTCTTGTAGTCACATTGAC 610
                *  **  ** ** * ********** *****  *  *  *  *     *     * **

FD2-4          TCTCGGATGGCCTCTTTACTTAGCATTCAATGTAGCAGGTAGACCTTACGAAGGATTCGC 687
FD2-4cDNA      TCTCGGATGGCCTCTTTACTTAGCATTCAATGTAGCAGGTAGACCTTACGAAGGATTCGC 687
FD2-3cDNA      TCTCGGATGGCCTCTTTACTTAGCATTCAATGTAGCAGGTAGACCTTACGAAGGACTCGC 667
FD2-3          TCTCGGATGGCCTCTTTACTTAGCATTCAATGTAGCAGGTAGACCTTACGAAGGACTCGC 667
FD2-2          CCTTGGTTGGCCGCTTTACTTAGCTTTCAACGTTGCCGGCCGGCCTTACGACAGGTTCGC 717
FD2-1          TCTTGGTTGGCCTATGTACTTAGCCTTCAACGTTTCGGGTCGATACTATGATCGATTAGC 670
               ** ** *****  * ******** ***** **  * **  *    ** **  *  * **
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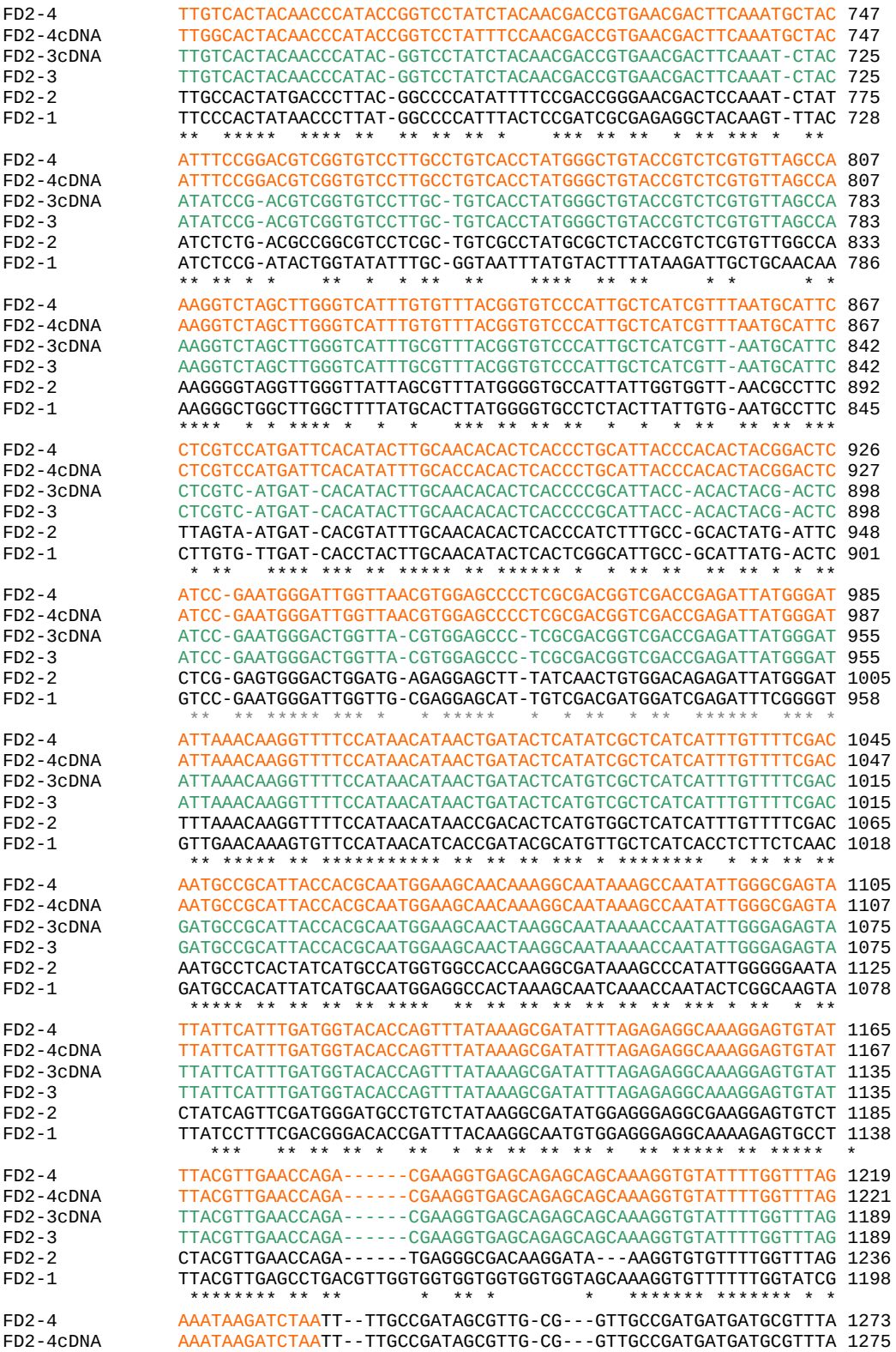
(Figure 3 continues)

(Figure 3 continued)



(Figure 3 continues)

(Figure 3 continued)

```
FD2-3cDNA       AAATAAGATCTAA------------------------------------------------ 1202
FD2-3           AAATAAGATCTAA------------------------------------------------ 1202
FD2-2           AAACAAGCTTTAAATATTTGCATTTTACCTTA-GGCATGTTCTAGTCGTTGATGT-TTTA 1294
FD2-1           TAACAAGTTCTAAAGACCGACCAACTGCCTGATAGCTGGCCGGCGAAATCAACGT-AAAA 1257
                          ** *** * ***
FD2-4           GGAATGTGTTAA-----ATTTGTTACATTATTGTTAAGGA---TTTGGGGTTACTTCTAT 1325
FD2-4cDNA       GGAATGTGTTAA-----ATTTGTTACATTATTGTTAAGGA---TTTGGGGTTACTTCTAT 1327
 FD2-3cDNA       ------------------------------------------------------------
FD2-3           ------------------------------------------------------------
FD2-2           AGGATATTTTAGCCGACATACTTGGTTTTCCTTTTTGGGACTTTTTAGCTTTGTATTTGC 1354
FD2-1           CGTACTTATTAGAC--TAGTGTTAACTAGGGAAGTTAATAATTAATGGTAGGAAAATGTG 1315
```

Fig 3. Nucleotide sequence alignments of the cotton *FAD2-4* cDNA*, FAD2-3* cDNA and the cotton *FAD2-4* and *FAD2-3* genes*,* and the cotton *FAD2-1,* and *FAD2-2* cDNAs. The entire coding region of *FAD2-4* gene (red), *FAD2-4* cDNA (red), *FAD2-3* cDNA (green), and *FAD2-3* gene (green) sequences are colored in alignments.

Chilling-sensitive, Ethanol Tolerance, and Growth Regulation of Yeast Cells Transformed with the Cotton *FAD2* Genes

Previously in our laboratory, the 1.2-kb open reading frames of both the *FAD2-3* and *FAD2-4* cDNAs were subcloned into the yeast bacterial shuttle vector pYES2 and then transformed into yeast cells (Pirtle et al., 2001; Zhang et al., 2008). The transformed yeast cells were found to have a significant accumulation of linoleic acid (18:2) compared to the control yeast cells transformed with the shuttle vector pYES2 alone (Pirtle et al., 2001; Zhang et al., 2008).

At lower temperatures, plants exhibit a significant increase in degree of unsaturation. Research on the tung tree *FAD3* gene (Dyer et al., 2001) showed temperature-dependent synthesis of linolenic acid in yeast cells expressing the *FAD3* gene and an increase in linolenic acid content at cooler temperatures is due to cold-inducible, post-transcriptional increase of the plant desaturase enzyme. Yeast cells

overexpressing the *Arabidopsis thaliana FAD2* gene (Kajiwara et al., 1996) also showed greater resistance to ethanol than did the control cells.

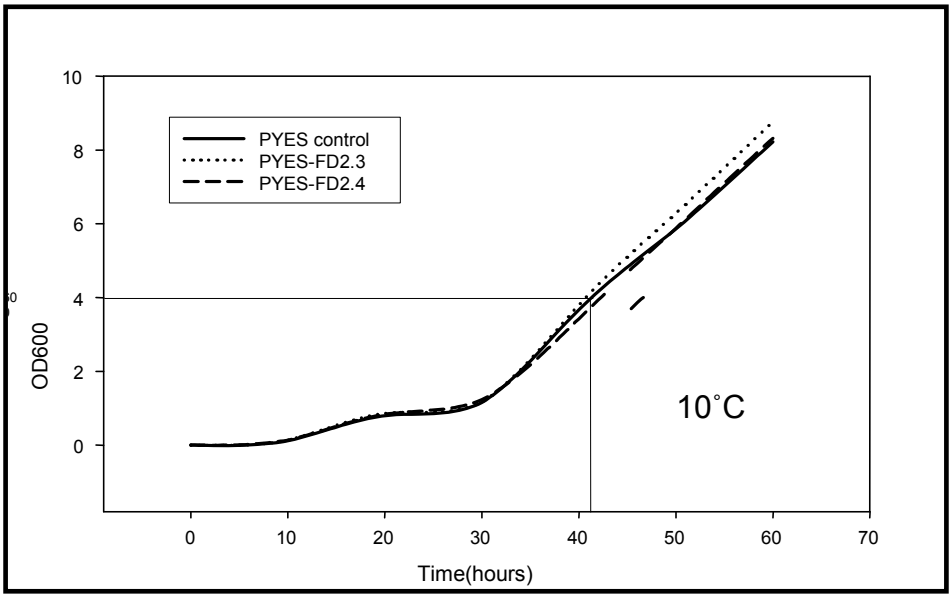
The yeast transformants and control cells were grown in galactose induction medium to induce lipid synthesis at different temperatures (10ºC, 20ºC). At the same time, the growth curves were recorded by turbidity readings at A_{600}. The cells were harvested at mid-log and late-log on the growth curve, and the fatty acid methyl ester profiles were analyzed by gas chromatography and quantified by flame ionization detection in comparison to an internal heptadecanoic acid (C17:0) .

To test the expression of these two *FAD2* genes under ethanol tolerance and hydrogen peroxide stress, the viability of the *FAD2* transformants and control cells in the presence of ethanol were measured. The yeast cells cultured for four generations were incubated in 67 mM KH_2PO_4 with ethanol concentrations of 0, 5, 10, 15, and 20% and 15 mM hydrogen peroxide. The incubations were performed anaerobically at 30ºC for 0, 2, 4, 6 and 8 hours. The viability of the yeast cells was determined by plating dilutions on YPD agar plates and incubating them aerobically at 30ºC for 48 hours. The cell samples were also harvested for lipid analysis (Table 1).

Table 1. The viability of the *FAD2* transformed yeast cells and control cells in the presence of ethanol and hydrogen peroxide

Transformed yeast cells	**Five hours ethanol treatment**		**15 mM H2O2**	
	[15%]	**[20%]**	**4 hours**	**8 hours**
pYES2	**75%**	**19%**	**42%**	**18%**
***FAD2-3*/pYES2**	**96%**	**23%**	**77%**	**43%**
***FAD2-4*/pYES2**	**87%**	**28%**	**65%**	**35%**

The results indicated that at both temperatures, the transformed cell growth rate was close to that of the control cells. These results indicated that the yeast cell growth rate at different temperatures was mainly not affected by the expression of the cotton fatty acid desaturase 2 genes (Fig. 4).



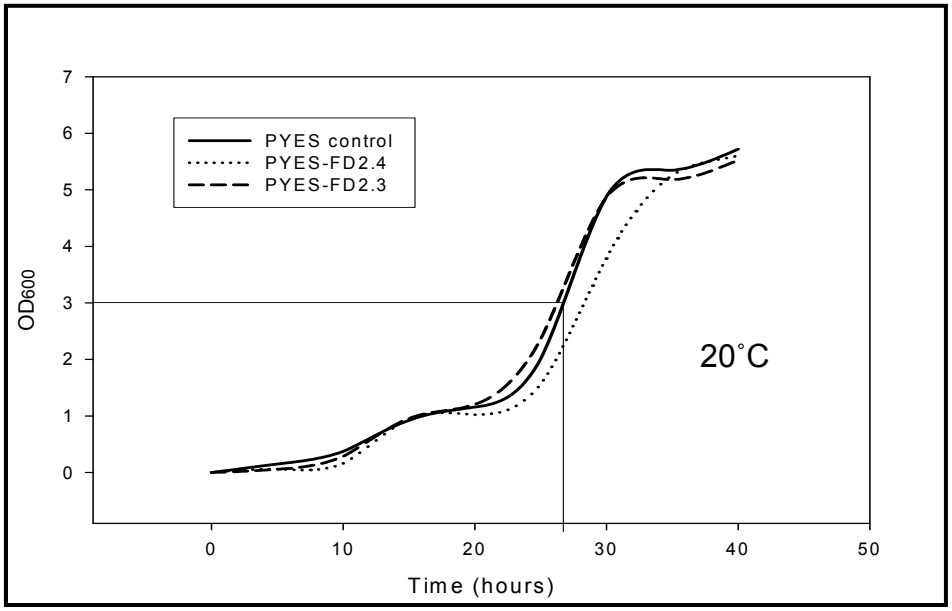
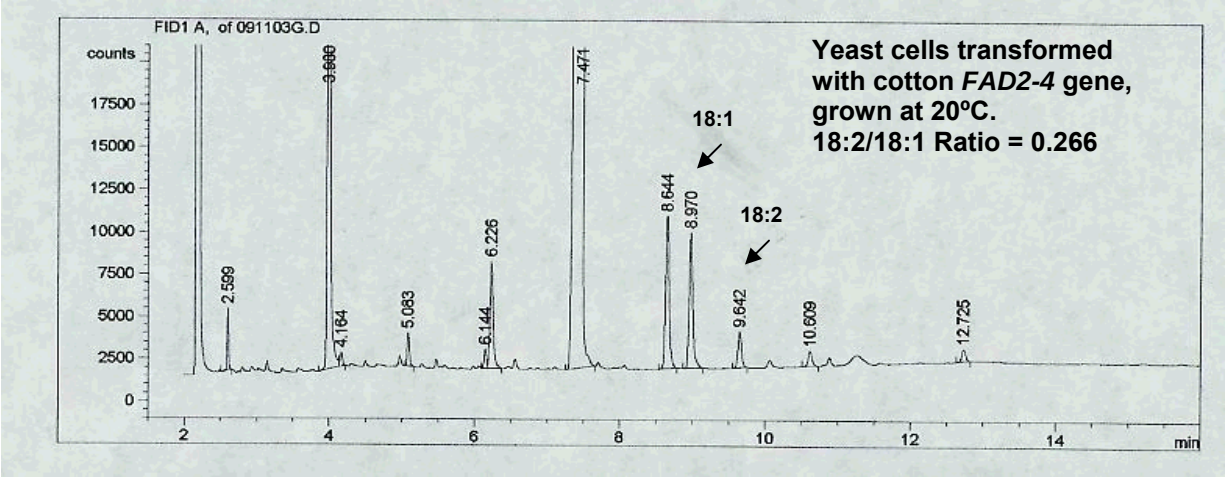


Fig 4. Growth curve of yeast cells transformed with the cotton *FAD2* gene constructs at different temperatures. The cell culture were sampled every two hours for OD_{600} reading.

For the cells transformed with the *FAD2-4* gene, the ratio of linoleic acid to oleic acid was 0.266 at 20°C and 1.940 at 10°C. At 30°C, the amount of linoleic acid was too low to be detected. For the cells transformed with the *FAD2-3* gene, the ratio of linoleic acid to oleic acid was 0.163 at 20°C, and 1.18 at 10°C. These results may indicate that low temperatures induce stronger expression of the fatty acid desaturase gene in transformed yeast cells (shown in Figure 5). However, these cold temperature studies with heterologous desaturase genes in yeast transformants were preliminary, and the experiments need to be repeated.



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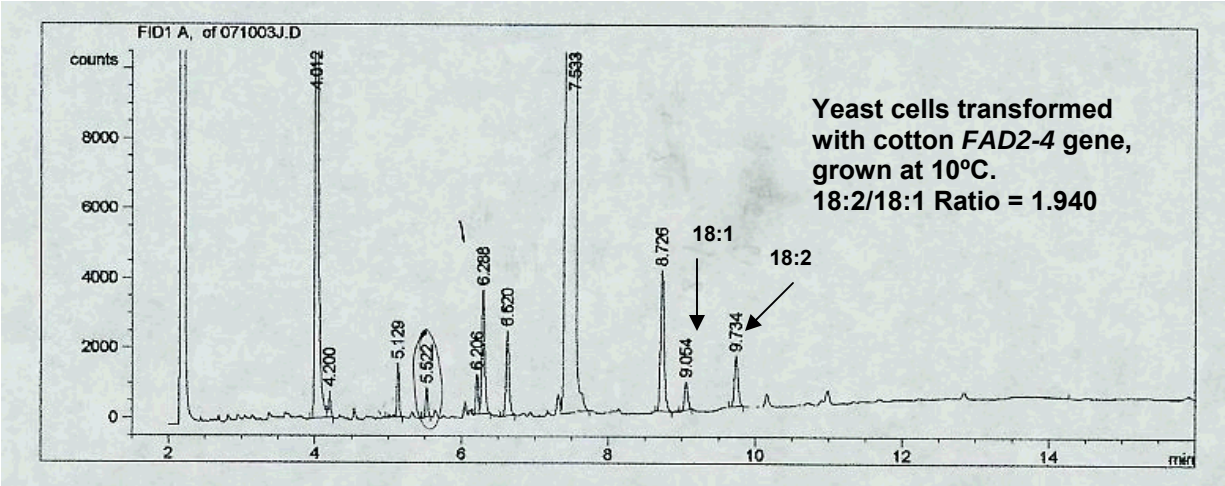
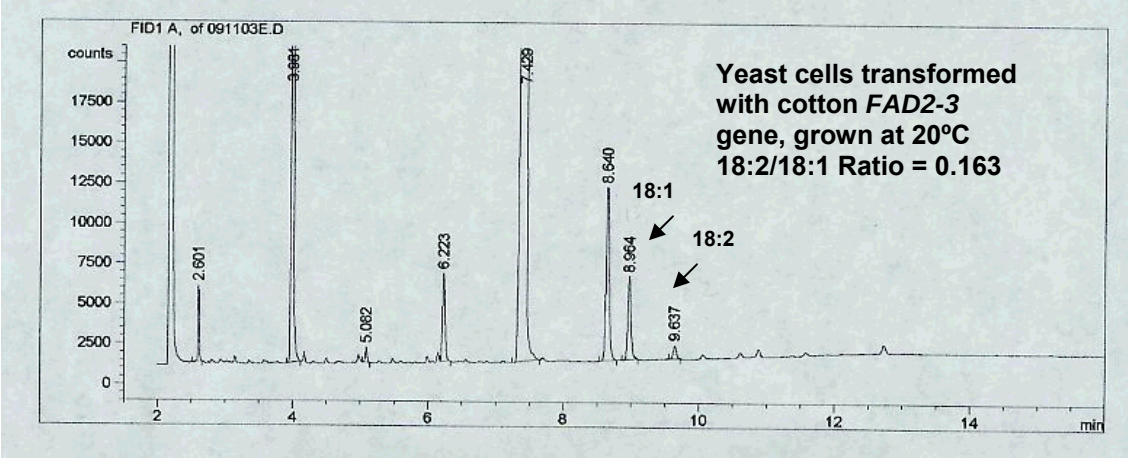
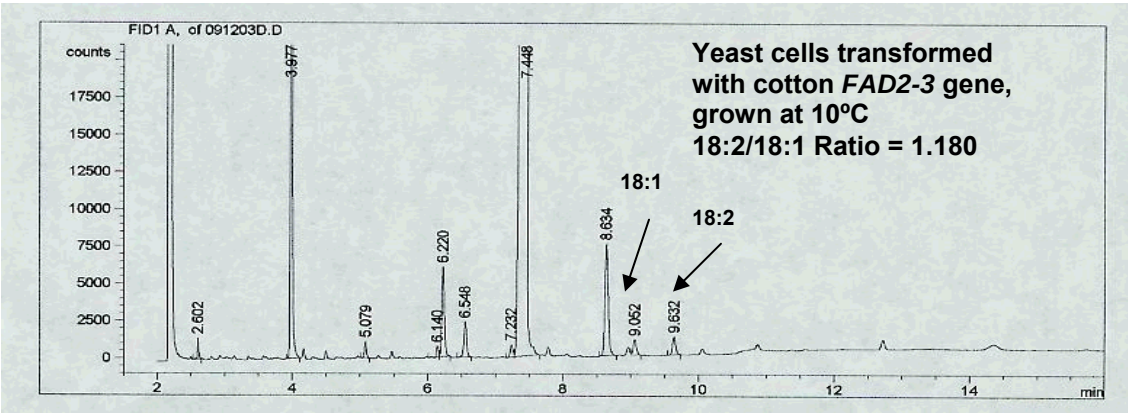


A





B

Fig 5. Fatty acid profiles of yeast cells transformed with pYES2/*FAD2-4* and pYES2/*FAD2-3* plasmids. The yeast cell cultures were incubated at (A) 10ºC, and (B) 20ºC.

For the ethanol tolerance analyses, at the lower concentrations of 5%-10%, both wild type yeast cells and yeast cells transformed with the *FAD2* gene constructs retained their viability. For 15% and 20% ethanol-treated cells, the *FAD2* transformants remained more viable than the control wild type yeast cells. For example, after 5 hours incubation in 20% ethanol, 19% of the control cells survived while 23% *FAD2-3* transformed cells and 28% *FAD2-4* transformed cells survived. When the ability of the *FAD2* transformants and the control yeast cells to survive hydrogen peroxide exposure were compared, the yeast cells expressing the *FAD2* genes survived 8 hours of treatment in 15 mM hydrogen peroxide at a level almost twice as high as that of the control cells under the same conditions (Table 1). These results are consistent with previous studies on the *FAD2* gene expression, which showed the presence of polyunsaturated fatty acids promotes increased tolerance to ethanol and oxidative stresses (Peyou-Ndi et al., 2000; Kajiwara et al., 1996).

Isolation of RNA from Cotton Plant Extracts

Total RNA was extracted from both control cotton plants and treated cotton plants. The QIAGEN RNeasy™ extraction procedure was used for the quantitative recovery of intact RNA suitable for the analyses. Organ-specific expression was assessed following isolation of RNA from a variety of organs, including roots, stems, and leaves of two-week old greenhouse-grown cotton plants.

The *FAD2-4* and *FAD2-3* expression profiles were obtained using roots, stems, and leaves of two-week old greenhouse-grown cotton plants and of cotton plants treated with 0.15 mM H_2O_2. After 30 cycles of RT-PCR of the control plants, only the root tissue could be detected with *FAD2-4* gene expression, and no *FAD2-3* gene expression could be detected from any tissue. After 50 cycles of RT-PCR of the control plants, expression of both genes was observed in all three tissues, with root expression being strongest and leaf expression being the weakest. When testing 30 cycles and 50 cycles of RT-PCR using H_2O_2 -treated plants, both *FAD2-4* and *FAD2-3* expression were detected from all three tissue samples with the same expression level (Fig. 6). These results could indicate that H_2O_2 may play a role in post-transcriptional regulation of cotton *FAD2* genes as a positive factor that can induce *FAD2* expression. This data also helps support the yeast study results of the effect of H_2O_2. Both the yeast cell viability test and the RT-PCR assay of cotton plants were consistent with previous studies on the *FAD2* genes, which indicated that the presence of polyunsaturated fatty acid promotes increased tolerance to oxidative stresses by increasing *FAD2* expression and eventually increasing the cell membrane fluidity (Kajiwara et al., 1996).

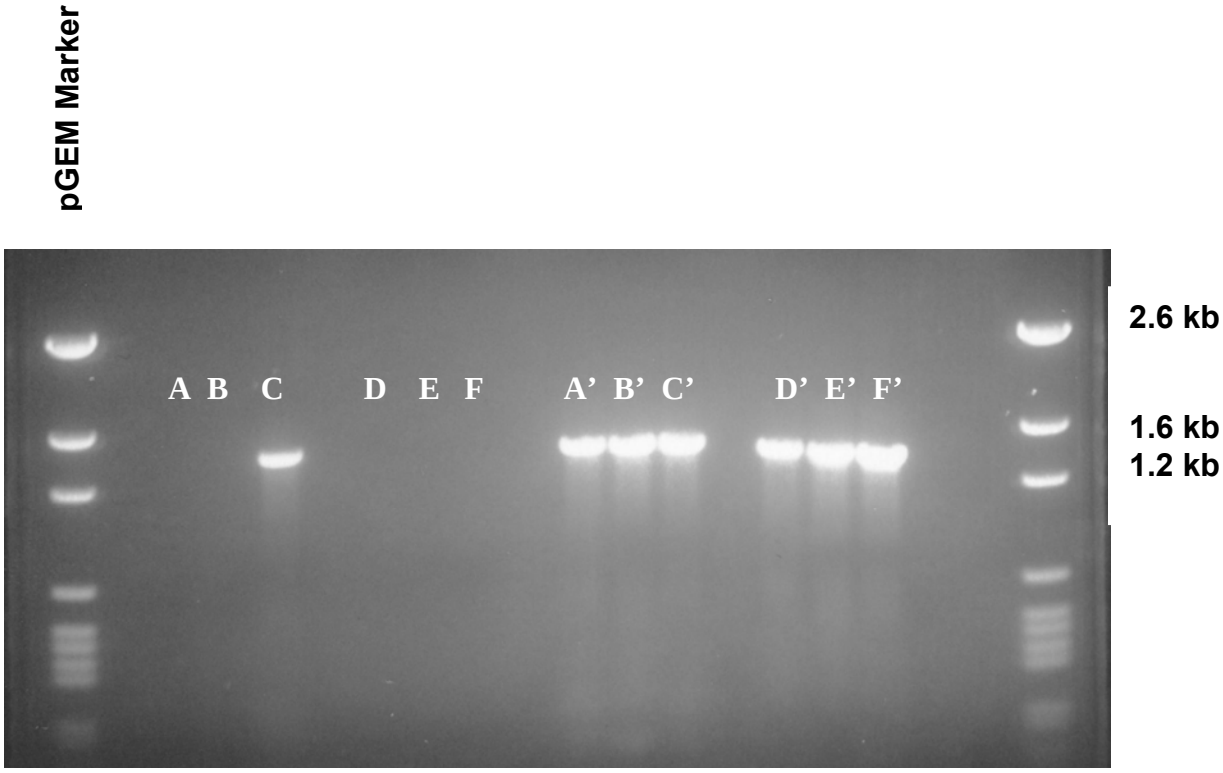


Fig 6. Agarose gel electrophoresis of products derived from 30 cycles of RT-PCR of H_2O_2-treated cotton tissues to assess *FAD2* gene expression.

A – *FAD2-4*/control/leaf, B - *FAD2-4*/control/stem, C - *FAD2-4*/control/root, D – *FAD2-3*/control/leaf, E - *FAD2-3*/control/stem, F - *FAD2-3*/control/root; A' – *FAD2-4*/ H_2O_2 /leaf, B' - *FAD2-4*/ H_2O_2/stem, C' - *FAD2-4*/ H_2O_2/root, D' – *FAD2-3*/ H_2O_2/leaf, E' - *FAD2-3*/ H_2O_2/stem, F' - *FAD2-3*/ H_2O_2/root. The pGEM ladder (Promega) was used as DNA standard.

Both *FAD2-4* and *FAD2-3* gene expression profiles have also been studied using roots, stems and leaves of two-week old greenhouse-grown cotton plants and of cotton plants treated with ethanol. The results indicated that both *FAD2* genes were detected with higher expression levels in all three tissues compared to those of the control plants. These results suggest that, like H_2O_2, ethanol might have some type of effect in the post- transcriptional regulation of cotton *FAD2* genes (Figure 7).

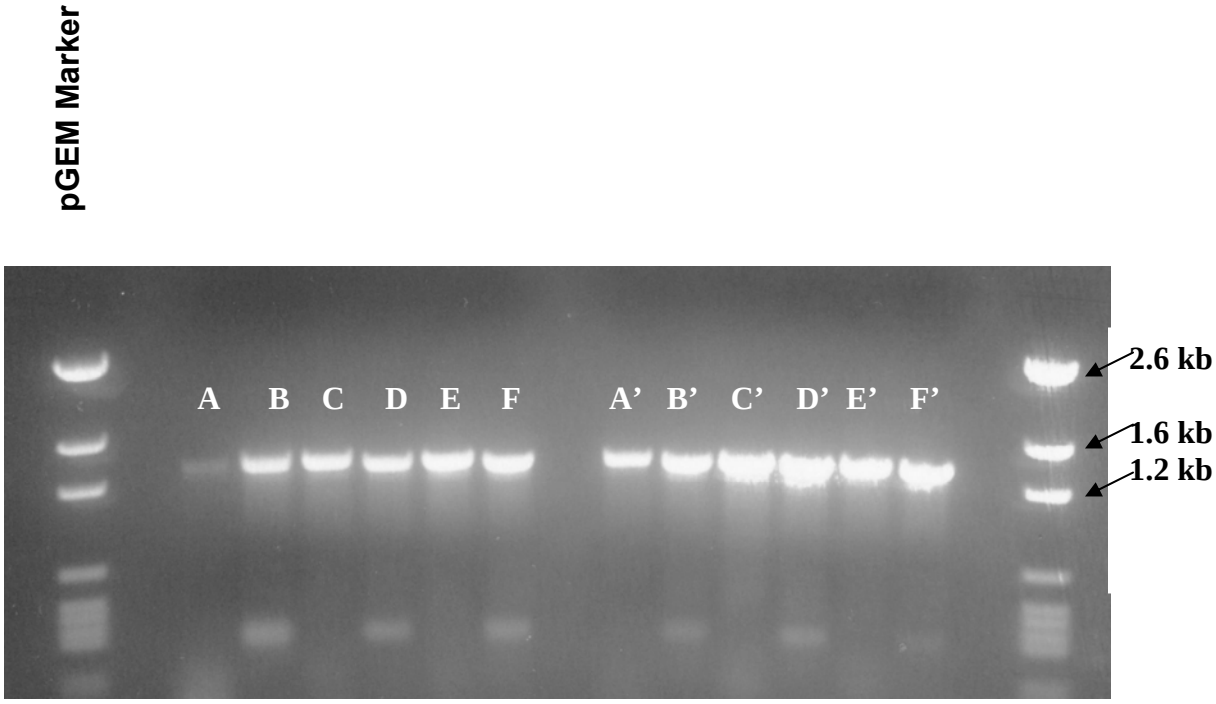


Fig 7. Agarose gel electrophoresis of products derived from 50 cycles of RT-PCR of ethanol treated cotton tissue to assess the *FAD2* expression.

A – *FAD2-4*/control/leaf, B - *FAD2-4*/control/stem, C - *FAD2-4*/control/root, D – *FAD2-3*/control/leaf, E – *FAD2-3*/control/stem, F - *FAD2-3*/control/root; A' – *FAD2-4*/ ethanol /leaf, B' - *FAD2-4*/ ethanol /stem, C' - *FAD2-4*/ ethanol /root, D' – *FAD2-3*/ ethanol /leaf, E' - *FAD2-3*/ ethanol /stem, F' - *FAD2-3*/ ethanol /root. The pGEM ladder (Promega, Madison, WI) was used as DNA standard.

Isolation of Protein from Cotton Plants for One-Dimensional SDS-PAGE and Western Blot Analyses

As described in the Methods section, the cotton FAD2 polyclonal antibody preparation was used for Western blot analysis to determine the expression profiles of FAD2 polypeptides in cotton plants. The total protein extraction kit from Sigma (product

code PE0230) turns out to be the most effective extraction procedure to produce qualitative samples of total proteins from all types of plant tissues, as well as being effective in preventing protein degradation during the extraction process (Herbert, 1998). Plant tissues were ground and methanol and acetone were used to remove polyphenolics, tannins, and other interfering substances. Then the plant tissue was resuspended in the chaotropic reagent. Qualitative total protein extracts from cotton plant stem and roots have been done using the procedure described in the manufacturer's instructions (Sigma-aldrich Chemical Co.) (Figure 8).

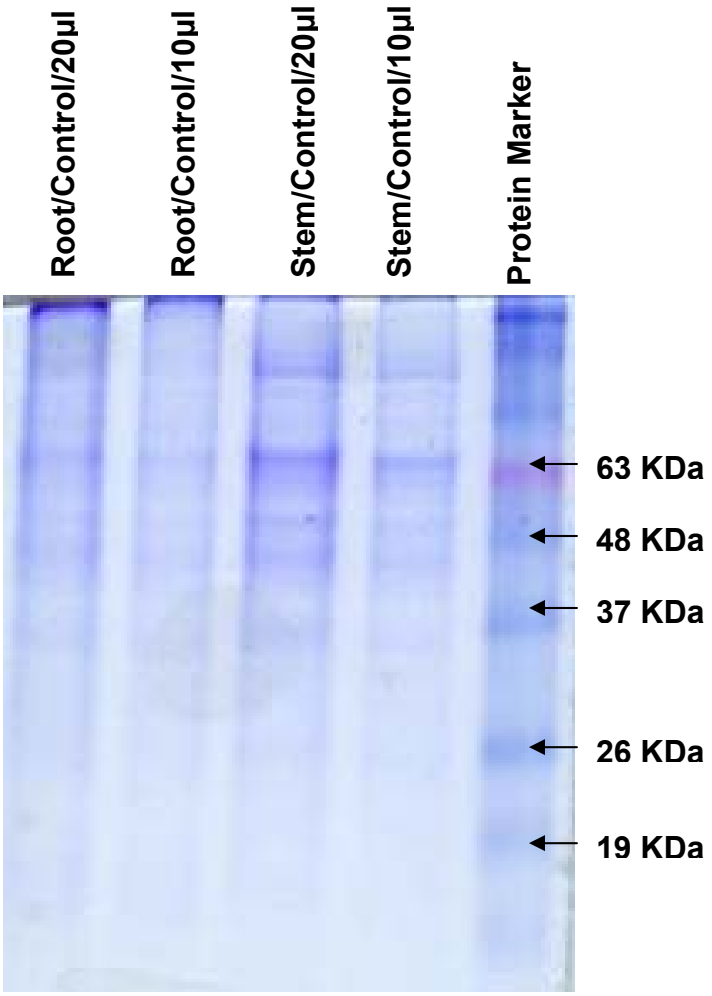


Fig 8. Qualitative total proteins isolated from cotton plants (*Gossypium hirsutum* L., cv Acala SJ5). The plants were treated with water as control and stem and root tissues were sampled for the protein extraction. After determining the concentration of each extract, 1 or 3 µg of total protein were loaded into the well, and the proteins were electrophoresed in a discontinuous buffer system, consisting of a stacking gel and a separating gel, with Full Range Rainbow recombinant protein molecular weight markers.

Plant tissues treated with ethanol and hydrogen peroxide were also used for Western blot analyses to assess the FAD2 protein expression. Insufficient membrane protein samples were obtained from stems and root tissues. However, slightly more of the FAD2 polypeptide band was detected by Western blot analysis of cotton plant leaf tissues treated with ethanol and hydrogen peroxide. The result indicated that hydrogen peroxide may induce the expression of FAD2 polypeptide (Figure 9).

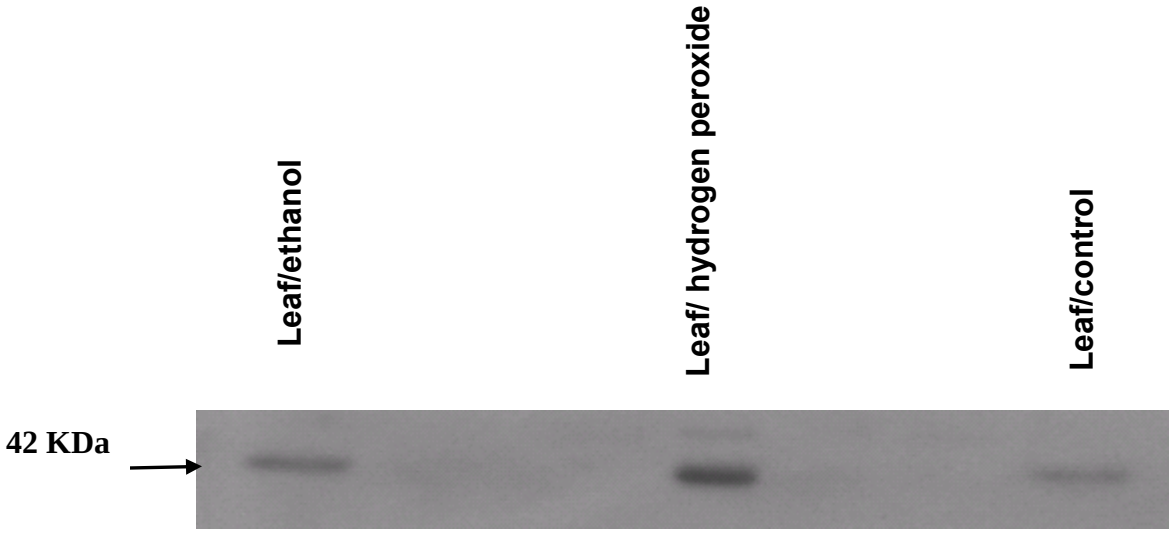


Fig 9. Western blot analysis of membrane protein fractions extracted from two-week old cotton plant (*Gossypium hirsutum* L., cv Acala SJ5) leaves detected with cotton anti-FAD2 antibody preparation. The plants were treated with ethanol, H_2O_2, and water (as control). The plants tissues were sampled for protein extraction. After determining the concentration of each extract, 1 or 3 µg of total protein were loaded into the well, and the proteins were electrophoresed in a discontinuous buffer system. The proteins embedded in the gels were transferred to a nitrocellulose membrane for Western blot analysis with a polyclonal anti-FAD2 antibody preparation. The bound secondary antibodies were visualized with ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ) and exposure to Kodak X-OMAT film for 90 sec. A 42-KDa band was visualized in only leaf samples. The bands correspond to the presumed FAD2 proteins.

It would appear that a comparison of FAD2 polypeptide expression profiles between leaf tissues collected from control cotton plants and cold-treated cotton plants indicated a slight increase of the FAD2 polypeptide at the lower temperature (Figure 10). The fact that both hydrogen peroxide and ethanol may have affected the FAD2 polypeptide expression as part of the response of the cotton plants to abiotic stress (Figure 9). These were the preliminary data of FAD2 polypeptide expression studies. The experiments were repeated and a different protocol was used to extracted total protein from cotton plants.

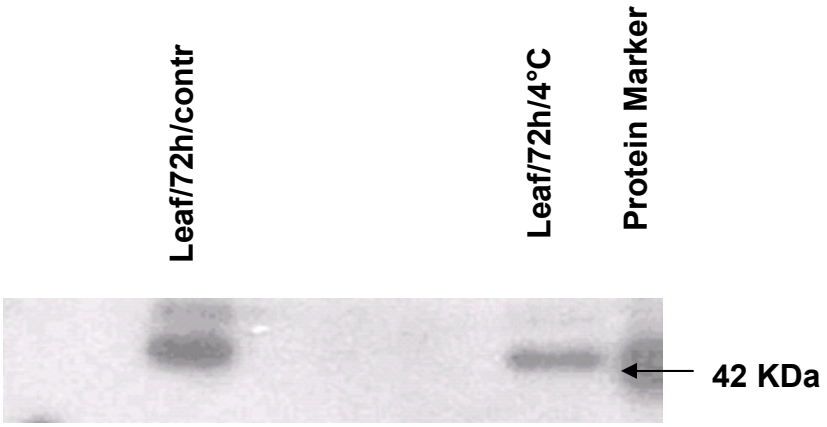


Fig 10. Western blot of membrane protein fractions extracted from two-week old cotton plant (*Gossypium hirsutum* L., cv Acala SJ5) tissues detected with the cotton anti-FAD2 antibody preparation. The plants were grown in a 10°C growth chamber for cold treatment, and a 25°C growth chamber as control. The plants tissues were sampled for protein extraction. After determining the concentration of each extract, 1 or 3 µg of total protein were loaded into the well, and the proteins were electrophoresed in a discontinuous buffer system. The proteins embedded in the gels were transferred to a nitrocellulose membrane for Western blot analysis with a polyclonal anti-FAD2 antibody preparation. The bound secondary antibodies were visualized with ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ) and exposure to Kodak X-OMAT film for 60 sec. A 42-KDa band was visualized in only leaf samples. The bands correspond to the presumed FAD2 proteins.

To study the developmental expression of the cotton FAD2 polypeptides, leaf, stem, and root tissues were collected from one week, two week, and three week old cotton plants. A 1:2000 dilution of primary antibody (anti-*FAD2* antibody) and a 1:3000 dilution of secondary antibody were used to visualize the banding profile. The results seem to

indicate that the three weeks leaf tissues have a steady expression of FAD2 polypeptides, while two-week stem tissues have the strongest FAD2 polypeptides band, and expression declines during the three weeks in root tissues (Figure 11). Although at this point, the factor that regulates the tissue specificity of the expression of FAD2 polypeptides is unknown, the results indicate that the polypeptide expression is in accordance with the growth phase in cotton plant development.

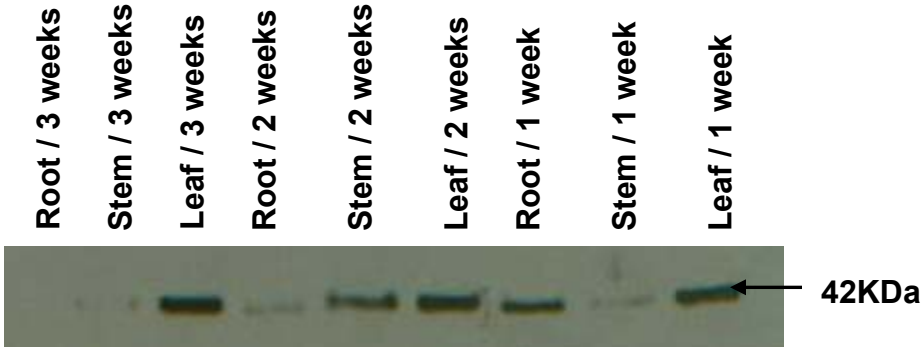
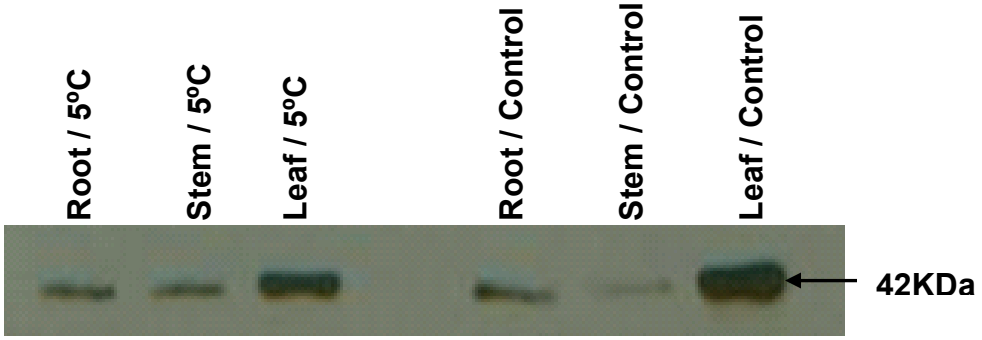


Fig. 11 Western blot of membrane protein fractions extracted from one, two, three-week old cotton plant (*Gossypium hirsutum* L., cv Acala SJ5) tissues detected with the cotton anti-FAD2 antibody preparation. The plants tissues (including leaf, stem and root) were sampled for the protein extraction. After determining the concentration of each extract, 1 or 3 µg of total protein were loaded into the well, and the proteins were electrophoresed in a discontinuous buffer system. The proteins embedded in the gels were transferred to a nitrocellulose membrane for Western blot analysis with a polyclonal anti-FAD2 antibody preparation. The bound secondary antibodies were visualized with ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ) and exposure to Kodak X-OMAT film for 2 min. A 42-KDa band was visualized in only leaf samples. The bands correspond to the presumed FAD2 proteins.

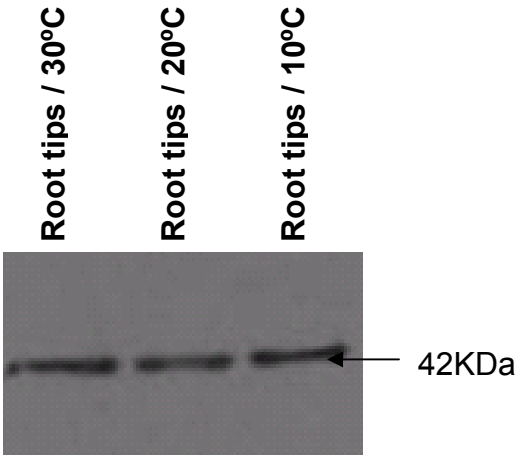
To investigate if the FAD2 expression is increased during cold treatment in all tissues, the three weeks cotton plants that were treated at 5ºC in a growth chamber for 3 days, along with the control plants were used for protein extraction and Western blotting. A new protocol was used to gain significant amount of protein sample from root and stem tissues. The FAD2 expression was analyzed and apparently was not increased by the cold treatment (Figure 12 A). To ensure this data was accurate, the experiment was repeated using only the root tip of 3-day cotton seedlings. Root tips have very abundant proliferating cells that contain continuous membrane biogenesis. Thus, it has been used to study many genes adjustment to the ambient temperature (Horiguchi et al., 2000). The cotton seed were grown on wet filter paper in 10ºC, 20ºC and 30ºC incubators. The root tips of 1 cm were harvested from seeding roots, and then used for protein extraction. The FAD2 expression in root tips under different temperature treatments are of the same apparent level (Fig. 12 B).



A

(Figure 12 continues)

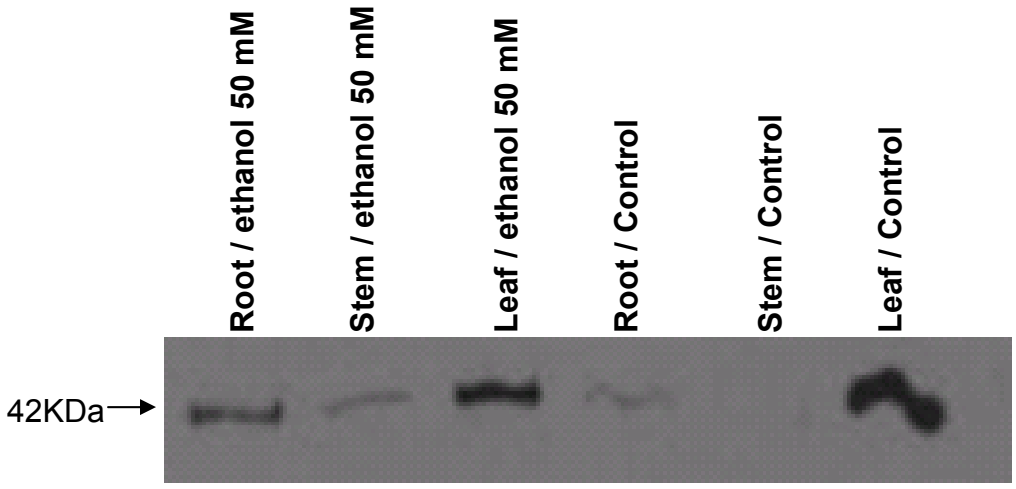
(Figure 12 continued)



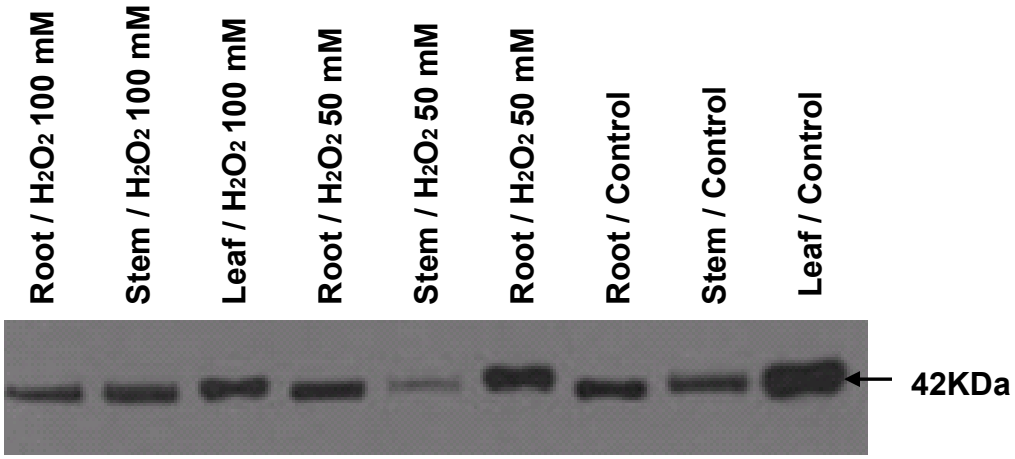
B

Fig. 12 Western blot of membrane protein fractions extracted from (A) low temperature treated (3 days) three week old cotton plant (*Gossypium hirsutum* L., cv Acala SJ5) tissues, and (B) root tips from cotton seedlings grown at 30ºC, 20ºC, and 10ºC, detected with the cotton anti-FAD2 antibody preparation. After determining the concentration of each extract, 1 or 3 µg of total protein was loaded into the well, and the proteins were electrophoresed in a discontinuous buffer system. The proteins embedded in the gels were transferred to a nitrocellulose membrane for Western blot analysis with a polyclonal anti-FAD2 antibody preparation. The bound secondary antibodies were visualized with ECL Western blotting detection reagent (Amersham Pharmacia Biotech, Piscataway, NJ) and exposure to Kodak X-OMAT film for 1 min. A 42-KDa band was visualized in only leaf samples. The bands correspond to the presumed FAD2 proteins.

Three week old cotton plants with treated with 50 mM and 100 mM of H_2O_2, and 50 mM ethanol for three days and the tissues were collected from the treated plants along with the control for Western blot experiments. The results were similar to the experiments using the low temperature treated samples. The FAD2 polypeptide expression was not induced by either of these two treatments (Figure 13).



A. Ethanol-treated sample



B. H_2O_2 treated samples

Fig 13. Western blot of membrane protein fractions extracted from cotton plant (*Gossypium hirsutum* L., cv Acala SJ5) tissues treated with (A) H_2O_2 and (B) ethanol, detected with the cotton anti-FAD2 antibody preparation. Two week old cotton plants were treated with H_2O_2 and ethanol of different concentrations for four hours and leaf, stem, and root tissues were collected for total protein extraction. After determining the concentration of each extract, 1 or 3 μg of total protein was loaded into the well, and the proteins were electrophoresed in a discontinuous buffer system. The proteins embedded in the gels were transferred to a nitrocellulose membrane for Western blot analysis with a polyclonal anti-FAD2 antibody preparation. The bound secondary antibodies were visualized with ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ) and exposure to Kodak X-OMAT film for 1-2 min. A 42-KDa band was visualized in only leaf samples. The bands correspond to the presumed FAD2 proteins.

Changes in the plasma membrane lipid composition as a consequence of cold acclimation represent just one of the many ends of different signaling cascades (Tomashow et al., 2001). In many cases, exogenous application of ABA seemed to substitute for low-temperature exposure, resulting in isothermal improvement of freezing tolerance (Leung et al., 1997). A study has shown that Se FAD2 transcripts can be induced by ABA in developing sesame seeds, and that there are two regions in the *SeFAD2* promoter implicated in ABA-responsive signaling (Kim et al., 2006). Three weeks old cotton plants were treated with 10 mM ABA solution for three days and tissues were collected from the treated plants along with the control for Western blotting

experiments. The data showed that the ABA may be able to qualitatively induce the FAD2 polypeptide expression to 2-3 folds in stem and root tissues (Figure 14).

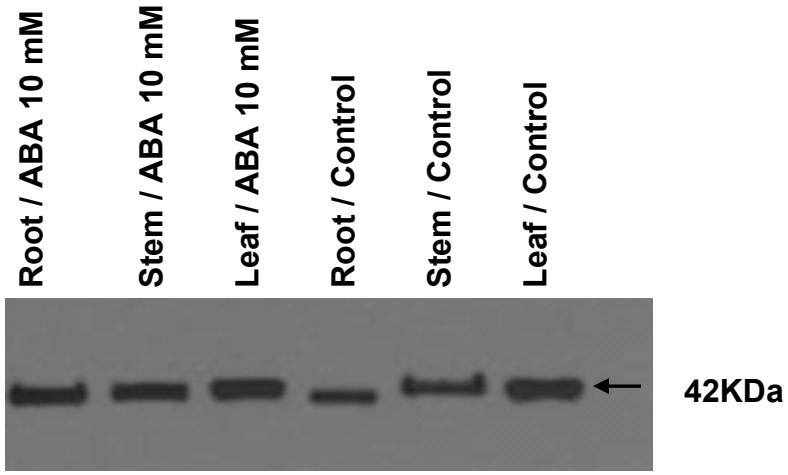


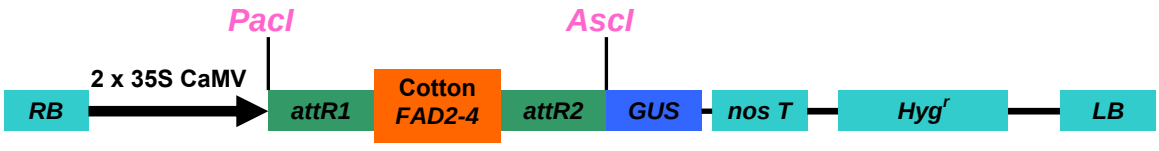
Fig 14. Western blot of membrane protein fractions extracted from cotton plant (*Gossypium hirsutum* L., cv Acala SJ5) tissues treated with 10 mM ABA solution. Two weeks old cotton plants were treated with ABA solution for four hours and leaf, stem, and root tissues were collected for total protein extraction. After determining the concentration of each extract, 1 or 3 µg of total protein were loaded into the well, and the proteins were electrophoresed in a discontinuous buffer system. The proteins embedded in the gels were transferred to a nitrocellulose membrane for Western blot analysis with a polyclonal anti-FAD2 antibody preparation. The bound secondary antibodies were visualized with ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ) and exposure to Kodak X-OMAT film for 45 sec. A 42-KDa band was visualized in only leaf samples. The bands correspond to the presumed FAD2 proteins.

Vector Design/Construction for Expression of the Cotton *FAD2* Genes in Arabidopsis Plants

Three pMDC Gateway destination vectors (Curtis and Ueli, 2003) were used to create transgenic cotton *FAD2*- Arabidopsis plants. The pMDC32 vector, a constitutive expression vector, harboring a dual CaMV35S promoter without any terminal protein tag, was used to create transgenic Arabidopsis plants. The pMDC43 vector, used for N-terminal of FAD2 protein GFP fusion constructs, and the pMDC139 vector, used for the C-terminal of FAD2 protein GUS fusion constructs, was used for subcellular localization of FAD2 polypeptides. The design of the three FAD2-pMDC constructs is shown in Figure 15.

A. *FAD2-4*/pMDC32

B. *FAD2-4*/pMDC43



C. *FAD2-4*/pMDC139

Fig. 15 Structures and organizations of cotton *FAD2* genes in the Gateway destination vectors: A. *FAD2-4*/pMDC32, B. *FAD2-4*/pMDC43, C. *FAD2-4*/pMDC139 (*FAD2-3*/pMDC constructs not shown). All three constructs in the binary vectors were used to transform *fad 2-1 Arabidopsis* knockout mutant plants. The diagrams include the 35S cauliflower mosaic virus (CaMV) cassette in all pMDC vectors from the ABRC Stock Center, Ohio State University, Columbus, OH. The figures were modified and redrawn from the paper by Curtis et al., 2002, and the website http://www.unizh.ch/botinst/Devo_Website/curtisvector/index_2.html. The 1.2-kb *FAD2-3* and *FAD2-4* PCR products were cloned between the two *attR* sites.

A single, discrete PCR product was critical for the following subcloning procedures because any PCR cleaning procedure would affect the ligation reaction. For the PCR optimization, a proofreading enzyme was used to amplify the *FAD2-4* gene and *FAD2-3* gene to maintain the fidelity of the sequence of the PCR product. The reaction that resulted in the best single, discrete 1.2-kb PCR fragment was used. For example, Figure16 shows the PCR amplification of the *FAD2-4* coding region product. Reaction A gave the best result with no primer dimer, smear, or unknown band. Thus, the sample from reaction A was further used for the ligation into the pENTR cloning vector. A total of four PCR products were produced: *FAD2-4*, *FAD2-4-TAA* (with the stop codon), *FAD2-3* and *FAD2-3-TAA*.

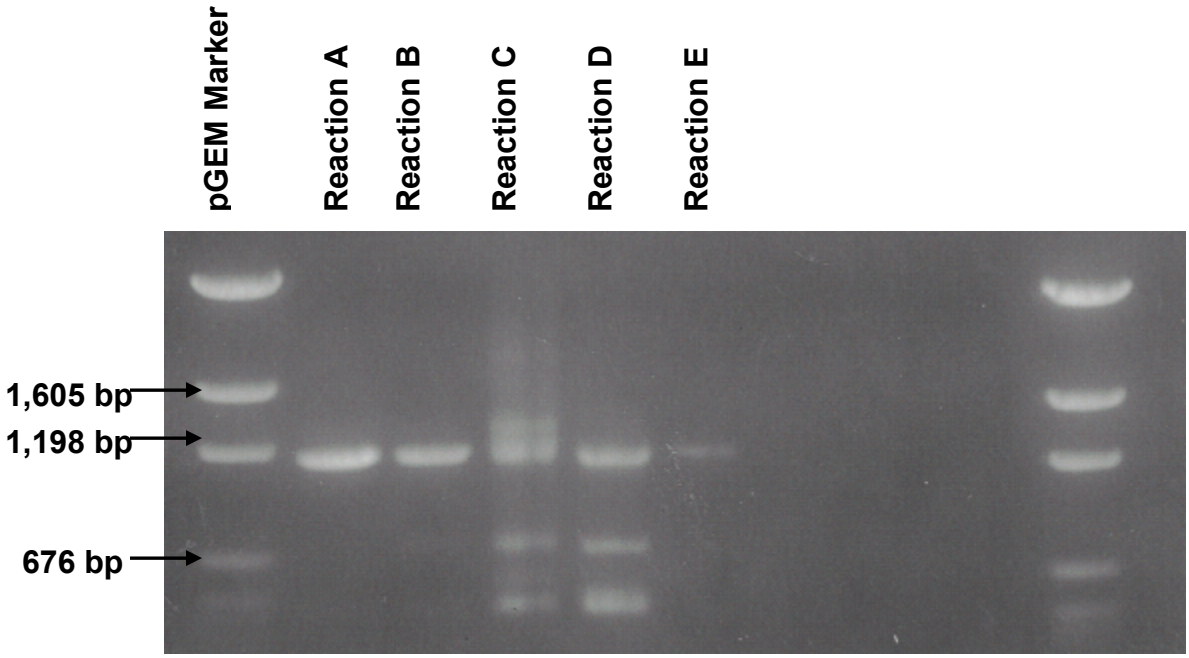


Fig. 16 Agarose gel electrophoresis of the PCR amplification fragment of the *FAD2-4* coding region. The PCR products were electrophoresed on a 1.0 % agarose gel containing ethidium bromide at a final concentration of 0.5 µg/ml, with pGEM DNA Marker standards (Promege, Madison, WI). Reaction (A) results in the product with no primer dimer, smear or unknown band. Thus, the sample from reaction A was used for ligation into the pENTR vector.

Directional TOPO® pENTR™ vectors from Invitrogen take advantage of fast, efficient Directional TOPO® cloning (Invitrogen, , Carisbad, CA, Catalog #K2400-20). PCR products containing the cotton *FAD2-3* and *FAD 2-4* genes were inserted in a 5´ to 3´ orientation using a 5-minute, bench-top ligation reaction. The positive clones were

confirmed by colony PCR and sequencing. The samples with 1.2-kb fragments were identified as the positive clones. As shown in Figure 17, seven out of 24 colonies had the right insert. The plasmid DNAs isolated from each of the positive clones was purified by a Wizard Purification kit (Promega, Madison, WI). Double restriction enzyme digestions were done to further assess the pENTR-*FAD2* construct. The *Not I* site on the pENTR vector is 20 bp upstream of the insert and both the *FAD2-3* and *FAD2-4* genes have a *Bgl II* site near the 3' end that includes the stop codon. Thus, after the double digestion, the clones with the *FAD2* coding regions that include the TAA stop codon would result in two bands: 2.6 kb and 1.2 kb (see sample A3 on Figure 18). The clone with the *FAD2* coding region that excludes the TAA codon would result in only one band, which woud be around 3.8 kb (see samples B1-B5, Figure 18).

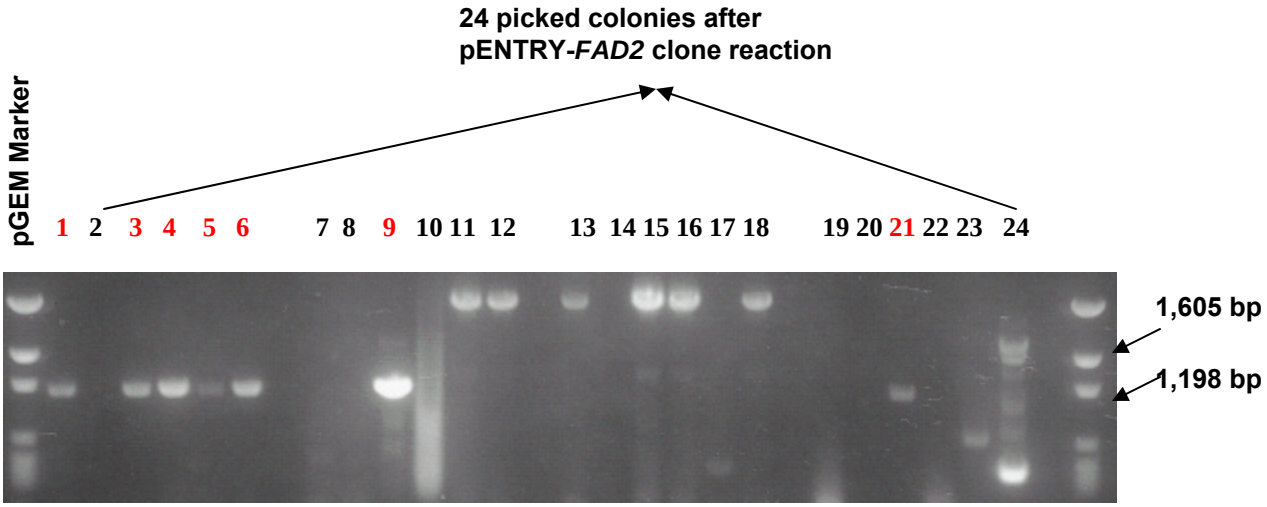


Fig. 17 Colony PCR analysis of positive Entry-*FAD2* clone colonies. A total of 24 colonies were picked for the PCR amplification. The PCR product were electrophoresed on a 1.0 % agarose gel containing ethidium bromide at a final concentration of 0.5 µg/ml, with pGEM DNA Marker standards. The samples with 1.2-kb fragments were identified as the positive clones, and later were used for sequencing. A total of 8 out of 24

colonies have the *FAD2* (1.2-kb) insert (colonies #1, 3, 4, 5, 6, 9, and 21. In red numbers).

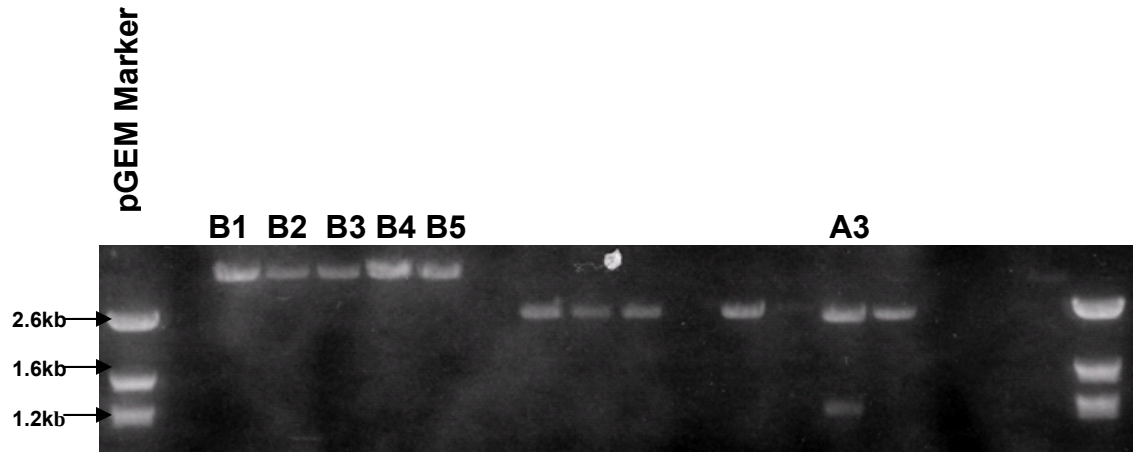


Fig.18 The double restriction enzyme digestion products of the pENTR-*FAD2* construct using *Not I* and *Bgl II*. The digested fragments were electrophoresed on a 1.0 % agarose gel containing ethidium bromide at a final concentration of 0.5 µg/ml, with pGEM DNA Marker standards. The *Not I* site on the pENTR vector is 20 bp upstream of the insert and both *FAD2-3* and *FAD2-4* have a *Bgl II* site near the 3'- end that includes the stop codon. The clone with the *FAD2* coding region that includes the TAA stop codon result in two bands: 2.6 kb and 1.2 kb (A3). The clone with the *FAD2* coding region that exclude the TAA codon was linearized (3.8 kb) (B1-B5). The empty pENTR vector would be linearized to 2.6 kb.

Once the PCR product is cloned into the Directional TOPO entry vector, the resulting entry clone can be recombined with any Gateway® destination vector to create an expression clone (Curtis and Ueli, 2003). The *attL* recombination sites on both

vectors can flank the PCR product insertion site for efficient recombination with our choice of Gateway destination vectors. The *E. coli* strain DB3.1 was used to harbor the pMDC vectors because it contains a mutation in the DNA *gyrase* gene, such that the *ccdB* protein is unable to bind the mutant DNA gyrase protein (Gerald and LaBaer, 2004). In this strain, the DNA is replicated normally and colonies grow. For this reason, all the pMDC vectors have to be transformed into *E. coli* DB3.1 to obtain viable colonies. The DB3.1 chemically competent cells were prepared using standard procedures and dissolved in TSS solution (Transformation and Storage Solution, Invitrogen, Carisbad, CA) for transformation. Nine pMDC vectors were transformed into these competent cells and selected on the LB/streptomycin 100 plates. The plasmids were isolated and the size of each vector was determined by single *SpeI* digestion and agarose gel electrophoreses (data not shown).

The pMDC/*FAD2* positive clones were selected by colony PCR amplification with primers to anneal to the vector and *FAD2* inserts. After the positive colonies were cultured in LB / Kanamycin50 broth, plasmids were isolated, and the constructs were further assessed by restriction enzyme digestions. Figure 19 shows the agarose gel electrophoresis of the pMDC32/*FAD2-3* plasmid constructs by colony PCR and restriction digestions.

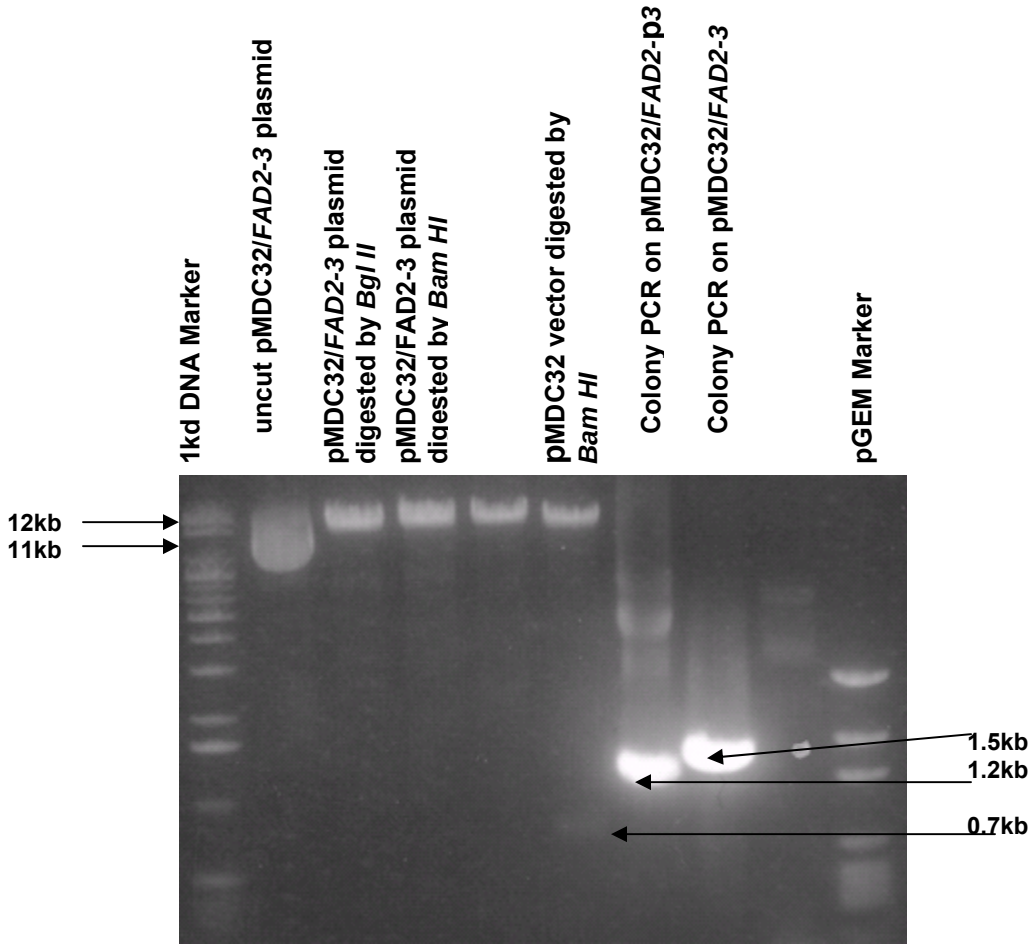


Fig. 19 Agarose gel electrophoreses to assess the *FAD2-3*/pMDC32 plasmid constructs by colony PCR and *Spe I* restriction digestions. The digested fragments and PCR products were electrophoresed on a 1.0 % agarose gel containing ethidium bromide at a final concentration of 0.5 µg/ml, with pGEM DNA Marker standards, and 1 kd DNA Marker standars. The uncut pMDC32/*FAD2-3* plasmid, pMDC32/*FAD2-3* plasmid digested by *Bgl II*, and pMDC32/*FAD2-3* plasmid digested by *Bam HI* all result in a 12 kb band. There are three *Bam HI* restriction sites on pMDC32 vector. The insert would replace two. Thus, the positive clone with the insert will result in only one band (around 12 kb) cut by *Bam HI.* The *pMDC32* empty vector digested by *Bam HI* results in

three bands (11 kb, 0.7 kb, 0.2 kb). The 200 base pair band is too faint to be visualized. Colony PCR on pMDC32/*FAD2-3* plasmid amplified a 1.2 kb band using primers annealing to inserted *FAD2* gene. Colony PCR on pMDC32/*FAD2-3* plasmid amplified a 1.5 kb band using primers annealing to vector. One 1.5 kb band was amplified. The untransformed vector would result in a 1.8 kb fragment in this PCR reaction. For the construction of pMDC43/*FAD2* vectors (Curist and Ueli, 2003), the coding region of the *FAD2* gene with the TAA stop codon at the end were used. The *GFP6* sequence is located at the N-terminal of the *FAD2* insert, and there are several stop codons downstream of the *attR2* region. The gene of interest was placed between *attR1* and a*ttR2*, in frame with the CaMV35S promoter with the GFP fusion. After the LR clonase reactions (for details see page 34), a total of 10 colonies were picked to assay the constructs by the size of the plasmids, then PCR amplification with the primers annealing to *FAD2* genes, and lastly by restriction enzyme digestions. The digested fragments and PCR products were electrophoresed on a 1.0 % agarose gel containing ethidium bromide at a final concentration of 0.5 µg/ml, with pGEM DNA Marker standards, and 1 kd DNA Marker standards. All ten colonies turned out to be positive. For the enzyme digestions, there are three *Bam HI* cutting sites on the pMDC43 vector. The insertion of the *FAD2* gene replaces the *attR1-attR2* region that included two *Bam HI* sites. Thus the plasmid with the right construct will result in one 12,460 band after *Bam HI* digestion (Figure 20).

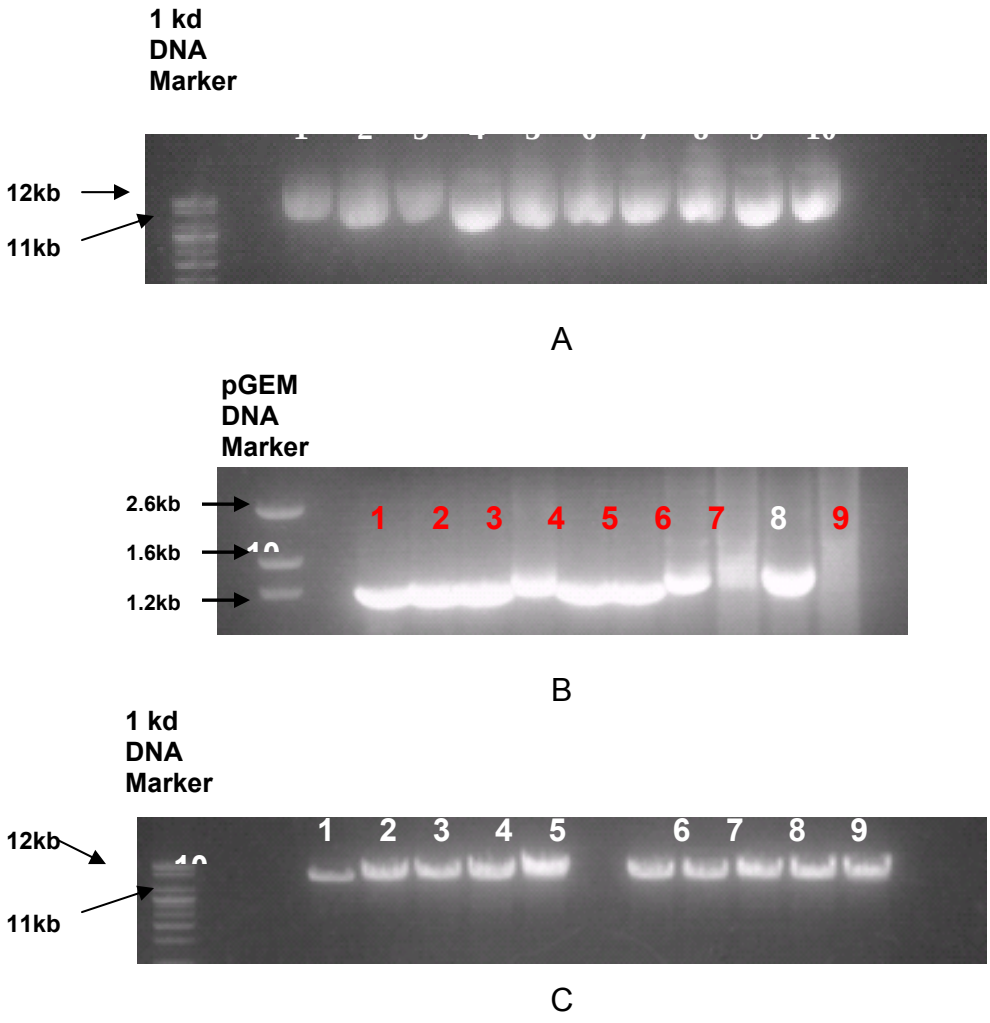


Fig. 20 Agarose gel electrophoresis to confirm the pMDC43/*FAD2-4* plasmid constructs by colony PCR amplification and *Bam HI* restriction digestion. The digested fragments and PCR products were electrophoresed on a 1.0 % agarose gel containing ethidium bromide at a final concentration of 0.5 µg/ml, with pGEM DNA Marker standards, and 1 kd DNA Marker standards.

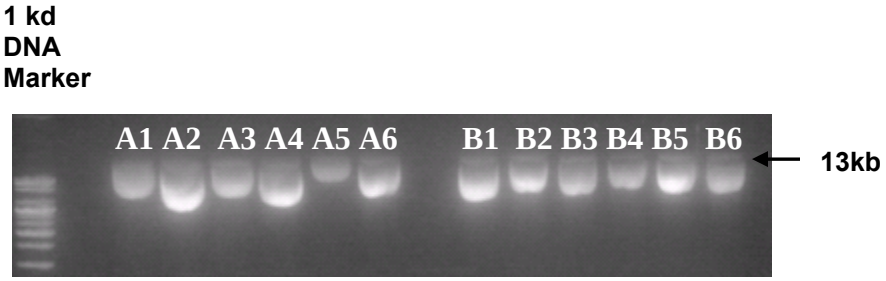
A: The plasmids isolated from ten colonies after LR clonase reaction and transformation. All samples showed the right size of the construct.

B: The same plasmids were used for PCR reactions that amplify only the *FAD2* genes. Eight out of ten samples showed right size of the insert which is around 1.2- kb (labeled with red numbers).

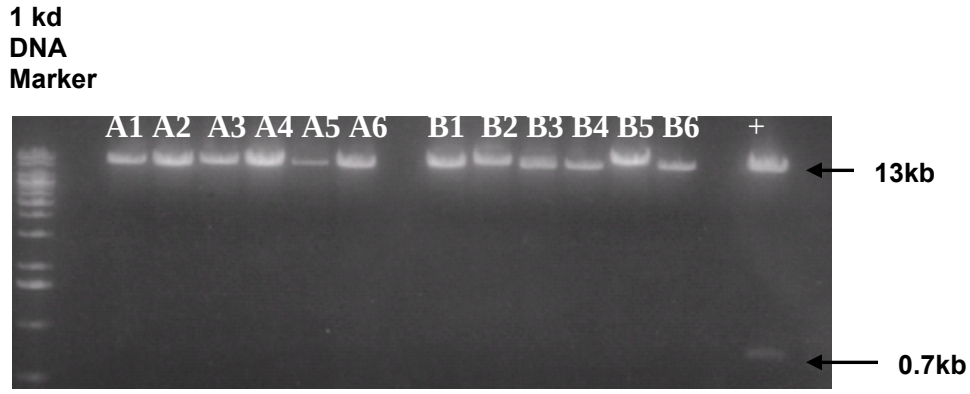
C: The same plasmids were used for the *Bam HI* digestions, all samples had positive results. Two *Bam HI* cutting sites are replaced by insertion of the *FAD2* gene.

The β-glucuronidase (GUS) enzyme from *E. coli* has been well documented to provide desirable characteristics as a marker gene in transformed plants (Karimi et al., 2002). The *GUS* reporter gene system has many advantages including stable expression of *E. coli* GUS enzyme, no interference with normal plant metabolism, and low intrinsic GUS activity in higher plants. The enzyme is also capable of tolerating amino-terminal additions, making it useful for study of plant organelle transport. Various β-glucuronic acid substrates are available for detection of GUS expression, all of which contain the sugar D-glucopyranosiduronic acid attached by glycosidic linkage to a hydroxyl group of a chromogenic, fluorogenic, or other detectable molecule (Karimi et al., 2002). This allows for histochemical, fluorometric, and spectrophotometric measurements of β-glucuronidase gene fusion expression. The vector pMDC139 contains a *GUS* gene downstream of *attR2* site (Curtis and Ueli., 2002). Thus, in order to fuse our genes with the *GUS* gene in frame, driven by the 35S promoter, the coding region of the inserted gene must not have the TAA stop codon. The entry clones of both the *FAD2-4* and *FAD2-3* coding regions (without the stop codon) were used for the LR cloning. After the LR clonase reaction, a total of six colonies from each reaction was used to check the constructs by the size of the plasmids, the PCR amplification of *FAD2* genes, and lastly by restriction enzyme digestion. All colonies turned out to be positive.

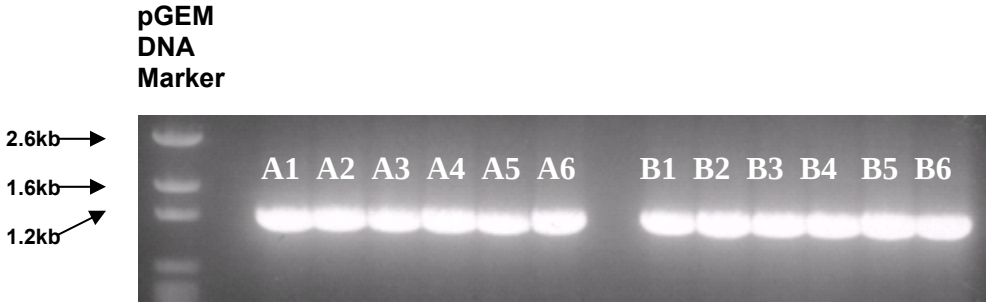
In the pMDC139 vector, there are three *Bam HI* cutting sites at sites 2994, 3697, and 3922. The insertion of the *FAD2* gene replaced the *attR1-attR2* region that includes two *Bam HI* sites. Thus, the plasmids with the right constructs result in single 13,655 fragment after *Bam HI* digestion while the empty vectors yield three fragments with sizes around 12 kb, 0.7kb, and 0.2kb (Figure 21).



A



B



C

Fig. 21 Agarose gel electrophoresis to confirm the pMDC139/*FAD2* plasmid constructs by colony PCR amplification and *Bam HI* restriction digestion. The digested fragments and PCR products were electrophoresed on a 1.0 % agarose gel containing ethidium bromide at a final concentration of 0.5 μg/ml, with pGEM DNA Marker standards, and 1 kd DNA Marker standards.

A: The plasmids isolated from twelve colonies from *FAD2*-pMDC139 LR clonase reactions and transformation. A1-6 are pMDC139/*FAD2-4,* B1-6 are pMDC139/*FAD2-3* constructs. All showed the right size of the construct.

B: The same plasmids were used for the *Bam HI* digestions. All samples have a positive result. Empty vector pMDC139 were used as positive controls which were digested into multiple bands instead of single fragments. The 0.2 kb band is not visualized on the gel.

C: The same plasmids were used for PCR reactions that amplify only the *FAD2* genes. All 12 pMDC139/*FAD2*constructs showed the right size of the insert which is around 1.2 kb.

Both *FAD2-3* and *FAD2-4* were recombined into the pMDC32 vector, the *FAD2-4* coding region with the stop codon TAA was ligated into the GFP fusion vector pMDC43C-GFP and both *FAD2* genes were ligated into the GUS fusion vector pMDC139N-GUS. To doubly confirm the fidelity of the *FAD2*-pMDC constructs, primers were designed using the sequences from the middle of the inserted genes and sequenced around one thousand base pairs in both the 5' and the 3' directions. The sequences also overlapped part of the pMDC vector sequences. Two *FAD2-3*/pMDC constructs were aligned with the *FAD2-3* cDNA to confirm the correct sequence of the

FAD2-3 coding region. The identity between the *FAD2-3* inserts in the pMDC vectors and the *FAD2-3* cDNA are 100% (Figure 22).

```
FD2.3-pMDC32       GGGGCCCCCCCAAGGCTATCAAACAAGTTTGTACAAAAAAGCAGGCTCCGCGGCCGCCTTG 60
FD2.3cDNA          ------------------------------------------------------------
FD2.3-pMDC139      -------TAAATGATTAGCCAAGTGTAAGTTTGTCAAAAGCAGGCTCCGCGGCCGCCTTG 53

FD2.3-pMDC32       TTTAACTTTAAGAAGGAGCCCTTCACCATGGGTGCAGGTGGCAGAATGTCGGTTCCTCCA 120
FD2.3cDNA          ----------------------------------------ATGTCGGTTCCTCCA 15
FD2.3-pMDC139      TTTAACTTTAAGAAGGAGCCCTTCACCATGGGTGCAGGTGGCAGAATGTCGGTTCCTCCA 113
                                                           ***************

FD2.3-pMDC32       AGTCAAAGGAAACAAGAATCGGGCTCAATGAAAAGAGTCCCTATATCTAAACCACCATTT 180
FD2.3cDNA          AGTCAAAGGAAACAAGAATCGGGCTCAATGAAAAGAGTCCCTATATCTAAACCACCATTT 75
FD2.3-pMDC139      AGTCAAAGGAAACAAGAATCGGGCTCAATGAAAAGAGTCCCTATATCTAAACCACCATTT 173
                   ************************************************************

FD2.3-pMDC32       ACTCTCAGTGAAATAAAAAAAGCCATCCCACCACACTGTTTCCAACGCTCACTTATCCGT 240
FD2.3cDNA          ACTCTCAGTGAAATAAAAAAAGCCATCCCACCACACTGTTTCCAACGCTCACTTATCCGT 135
FD2.3-pMDC139      ACTCTCAGTGAAATAAAAAAAGCCATCCCACCACACTGTTTCCAACGCTCACTTATCCGT 233
                   ************************************************************

FD2.3-pMDC32       TCATTTTCCTATCTCGTTTACGACTTCATTTTAGTCTCTATCTTTTACTACGTAGCCACC 300
FD2.3cDNA          TCATTTTCCTATCTCGTTTACGACTTCATTTTAGTCTCTATCTTTTACTACGTAGCCACC 195
FD2.3-pMDC139      TCATTTTCCTATCTCGTTTACGACTTCATTTTAGTCTCTATCTTTTACTACGTAGCCACC 293
                   ************************************************************

FD2.3-pMDC32       ACTTACTTCCACAACCTCCCTCAGCCACTATCTTTCGTCGCCTGGCCAATTTATTGGACT 360
FD2.3cDNA          ACTTACTTCCACAACCTCCCTCAGCCACTATCTTTCGTCGCCTGGCCAATTTATTGGACT 255
FD2.3-pMDC139      ACTTACTTCCACAACCTCCCTCAGCCACTATCTTTCGTCGCCTGGCCAATTTATTGGACT 353
                   ************************************************************

FD2.3-pMDC32       CTTCAAGGTTCAGTCCTCACTGGCGTTTGGGTTATCGCCCATGAATGCGGTCACCATGCT 420
FD2.3cDNA          CTTCAAGGTTCAGTCCTCACTGGCGTTTGGGTTATCGCCCATGAATGCGGTCACCATGCT 315
FD2.3-pMDC139      CTTCAAGGTTCAGTCCTCACTGGCGTTTGGGTTATCGCCCATGAATGCGGTCACCATGCT 413
                   ************************************************************

FD2.3-pMDC32       TTTAGCGATTACCAATGGATTGATGACACTGTCGGTCTCATCCTCCATTCATCCCTTCTT 480
FD2.3cDNA          TTTAGCGATTACCAATGGATTGATGACACTGTCGGTCTCATCCTCCATTCATCCCTTCTT 375
FD2.3-pMDC139      TTTAGCGATTACCAATGGATTGATGACACTGTCGGTCTCATCCTCCATTCATCCCTTCTT 473
                   ************************************************************

FD2.3-pMDC32       GTCCCGTACTTTTCGTGGAAATATAGTCACCGACGTCACCATTCCAACACTGGTTCCCTT 540
FD2.3cDNA          GTCCCGTACTTTTCGTGGAAATATAGTCACCGACGTCACCATTCCAACACTGGTTCCCTT 435
FD2.3-pMDC139      GTCCCGTACTTTTCGTGGAAATATAGTCACCGACGTCACCATTCCAACACTGGTTCCCTT 533
                   ************************************************************

FD2.3-pMDC32       GAACGCGACGAAGTATTTGTTCCGAAGAAACGGAGCAGCATTAGATGGTGGGCTAAATAC 600
FD2.3cDNA          GAACGCGACGAAGTATTTGTTCCGAAGAAACGGAGCAGCATTAGATGGTGGGCTAAATAC 495
FD2.3-pMDC139      GAACGCGACGAAGTATTTGTTCCGAAGAAACGGAGCAGCATTAGATGGTGGGCTAAATAC 593
                   ************************************************************

FD2.3-pMDC32       CTCAACAATCCACCAGGTCGTTTCGTCACAGTCACCATTCAGCTCACTCTCGGATGGCCT 660
FD2.3cDNA          CTCAACAATCCACCAGGTCGTTTCGTCACAGTCACCATTCAGCTCACTCTCGGATGGCCT 555
FD2.3-pMDC139      CTCAACAATCCACCAGGTCGTTTCGTCACAGTCACCATTCAGCTCACTCTCGGATGGCCT 653
                   ************************************************************

FD2.3-pMDC32       CTTTACTTAGCATTCAATGTAGCAGGTAGACCTTACGAAGGACTCGCTTGTCACTACAAC 720
FD2.3cDNA          CTTTACTTAGCATTCAATGTAGCAGGTAGACCTTACGAAGGACTCGCTTGTCACTACAAC 615
FD2.3-pMDC139      CTTTACTTAGCATTCAATGTAGCAGGTAGACCTTACGAAGGACTCGCTTGTCACTACAAC 713
                   ************************************************************

FD2.3-pMDC32       CCATACGGTCCTATCTACAACGACCGTGAACGACTTCAAATCTACATATCCGACGTCGGT 780
FD2.3cDNA          CCATACGGTCCTATCTACAACGACCGTGAACGACTTCAAATCTACATATCCGACGTCGGT 675
FD2.3-pMDC139      CCATACGGTCCTATCTACAACGACCGTGAACGACTTCAAATCTACATATCCGACGTCGGT 773
                   ************************************************************

FD2.3-pMDC32       GTCCTTGCTGTCACCTATGGGCTGTACCGTCTCGTGTTAGCCAAAGGTCTAGCTTGGGTC 840
FD2.3cDNA          GTCCTTGCTGTCACCTATGGGCTGTACCGTCTCGTGTTAGCCAAAGGTCTAGCTTGGGTC 735
FD2.3-pMDC139      GTCCTTGCTGTCACCTATGGGCTGTACCGTCTCGTGTTAGCCAAAGGTCTAGCTTGGGTC 833
                   ************************************************************

FD2.3-pMDC32       ATTTGCGTTTACGGTGTCCCATTGCTCATCGTTAATGCATTCCTCGTCATGATCACATAC 900
FD2.3cDNA          ATTTGCGTTTACGGTGTCCCATTGCTCATCGTTAATGCATTCCTCGTCATGATCACATAC 795
FD2.3-pMDC139      ATTTGCGTTTACGGTGTCCCATTGCTCATCGTTAATGCATTCCTCGTCATGATCACATAC 893
                   ************************************************************
```

(Figure 22 continues)

(Figure 3 continued)

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FD2.3-pMDC32        TTGCAACACACTCACCCCGCATTACCACACTACGACTCATCCGAATGGGACTGGTTACGT 960
FD2.3cDNA           TTGCAACACACTCACCCCGCATTACCACACTACGACTCATCCGAATGGGACTGGTTACGT 855
FD2.3-pMDC139       TTGCAACACACTCACCCCGCATTACCACACTACGACTCATCCGAATGGGACTGGTTACGT 953
                    ************************************************************
FD2.3-pMDC32        GGAGCCCTCGCGACGGTCGACCGAGATTATGGGATATTAAACAAGGTTTTCCATAACATA 1020
FD2.3cDNA           GGAGCCCTCGCGACGGTCGACCGAGATTATGGGATATTAAACAAGGTTTTCCATAACATA 915
FD2.3-pMDC139       GGAGCCCTCGCGACGGTCGACCGAGATTATGGGATATTAAACAAGGTTTTCCATAACATA 1013
                    ************************************************************


FD2.3-pMDC32        ACTGATACTCATGTCGCTCATCATTTGTTTTCGACGATGCCGCATTACCACGCAATGGAA 1080
FD2.3cDNA           ACTGATACTCATGTCGCTCATCATTTGTTTTCGACGATGCCGCATTACCACGCAATGGAA 975
FD2.3-pMDC139       ACTGATACTCATGTCGCTCATCATTTGTTTTCGACGATGCCGCATTACCACGCAATGGAA 1073
                    ************************************************************
FD2.3-pMDC32        GCAACTAAGGCAATAAAACCAATATTGGGAGAGTATTATTCATTTGATGGTACACCAGTT 1140
FD2.3cDNA           GCAACTAAGGCAATAAAACCAATATTGGGAGAGTATTATTCATTTGATGGTACACCAGTT 1035
FD2.3-pMDC139       GCAACTAAGGCAATAAAACCAATATTGGGAGAGTATTATTCATTTGATGGTACACCAGTT 1133
                    ************************************************************
FD2.3-pMDC32        TATAAAGCGATATTTAGAGAGGCAAAGGAGTGTATTTACGTTGAACCAGACGAAGGTGAG 1200
FD2.3cDNA           TATAAAGCGATATTTAGAGAGGCAAAGGAGTGTATTTACGTTGAACCAGACGAAGGTGAG 1095
FD2.3-pMDC139       TATAAAGCGATATTTAGAGAGGCAAAGGAGTGTATTTACGTTGAACCAGACGAAGGTGAG 1193
                    ************************************************************
FD2.3-pMDC32        CAGAGCAGCAAAGGTGTATTTTGGTTTAGAAATAAGATCAAGGGTGGGCGCGCCGACCCA 1260
FD2.3cDNA           CAGAGCAGCAAAGGTGTATTTTGGTTTAGAAATAAGATCTAA------------------ 1137
FD2.3-pMDC139       CAGAGCAGCAAAGGTGTATTTTGGTTTAGAAATAAGATCAAGGGTGGGCGCGCCGACCCA 1253
                    ************************************ *
FD2.3-pMDC32        GCTTCTGTACAAAGTGTCGAACTGAACATT------------------------------ 1290
FD2.3cDNA           ------------------------------------------------------------
FD2.3-pMDC139       GCTTTCTTGTACAAAGTGGTGATAGCTGGCGCGCCTCGACTCAGGTGCCCCCCCTAAAAA 1313

FD2.3-pMDC32        -
FD2.3cDNA           -
FD2.3-pMDC139       A 1314
```

Fig. 22 Two *FAD2-3* sequences from the *FAD2-3*-pMDC constructs were aligned with the *FAD2-3* cDNA sequence to confirm the correct sequences of the *FAD2-3* coding region in the vector constructs. The coding region starts as ATG and ends one nucleotide before the terminator TAA. The identity between the *FAD2-3* inserts and the *FAD2-3* sequences cDNA are 100%.

The sequences of the *FAD2* portion in three *FAD2-4*-pMDC constructs were aligned with the sequence of the *FAD2-4* cDNA to confirm the correct sequence of the *FAD2-4* coding regions. The coding region starts as ATG and ends one nucleotide before the termination codon TAA for pMDC139 constructs and with the TAA termination codon for pMDC43 and pMDC32 constructs (thus the expression of GUS

can occur without the cotton *FAD2* native termination codon). The identities between the *FAD2-4* inserts and the *FAD2-4* cDNA are 100% (Figure 23). The sequences also confirm the construct of *FAD2-3*/pMDC139 by the alignment of part of the *FAD2-3* coding region close to the 5' end (underlined by asterisks) and part of the pMDC139 vector including a partial CAMV 35S promoter and the *AttR1* sequences (underlined bold letters) (Figure 24).

```
FD2.4-pMDC139      ------TTTATACGCCCGGAGTCAGTTTGTACAAAAA-GCAGGCTCCGCGGCCGCCTTGT 53
FD2.4cDNA          ------------------------------------------------------------
FD2.4-pMDC43       -------------------------CCTCAAATTAA-AGAGGCTCCGCGGCCGCCTTGT 33
FD2.4-pMDC32       AGGGCCCAAAGGCGTATCAAACAAGTTTGTACAAAAAAGCAGGCTCCGCGGCCGCCTTGT 60

FD2.4-pMDC139      TTAACTTTAAGAAGGAGCCCTTCACCATGGGTGCAGGTGGCAGAATGTCGGTTCCTCCAA 113
FD2.4cDNA          ----------------------------------------ATGTCGGTTCCTCCAA 16
FD2.4-pMDC43       TTAACTTTAAGAAGGAGCCCTTCACCATGGGTGCAGGTGGCAGAATGTCGGTTCCTCCAA 93
FD2.4-pMDC32       TTAACTTTAAGAAGGAGCCCTTCACCATGGGTGCAGGTGGCAGAATGTCGGTTCCTCCAA 120
                                                          ****************

FD2.4-pMDC139      GTCAAAGGAAACAAGAATCGGGCTCAATGAAAAGAGCCCCTATATCTAAACCACCATTTA 173
FD2.4cDNA          GTCAAAGGAAACAAGAATCGGGCTCAATGAAAAGAGCCCCTATATCTAAACCACCATTTA 76
FD2.4-pMDC43       GTCAAAGGAAACAAGAATCGGGCTCAATGAAAAGAGCCCCTATATCTAAACCACCATTTA 153
FD2.4-pMDC32       GTCAAAGGAAACAAGAATCGGGCTCAATGAAAAGAGCCCCTATATCTAAACCACCATTTA 180
                   ************************************************************

FD2.4-pMDC139      CTCTCAGTGAAATAAAAAAAAGCCATCCCACCACACTGTTTCCAACGCTCACTTATCCGTT 233
FD2.4cDNA          CTCTCAGTGAAATAAAAAAAGCCATCCCACCACACTGTTTCCAACGCTCACTTATCCGTT 136
FD2.4-pMDC43       CTCTCAGTGAAATAAAAAAAGCCATCCCACCACACTGTTTCCAACGCTCACTTATCCGTT 213
FD2.4-pMDC32       CTCTCAGTGAAATAAAAAAAGCCATCCCACCACACTGTTTCCAACGCTCACTTATCCGTT 240
                   ************************************************************

FD2.4-pMDC139      CATTTTCCTATCTCGTTTACGACTTCATTTTAGTCTCTATCTTTTACTACGTAGCCACCA 293
FD2.4cDNA          CATTTTCCTATCTCGTTTACGACTTCATTTTAGTCTCTATCTTTTACTACGTAGCCACCA 196
FD2.4-pMDC43       CATTTTCCTATCTCGTTTACGACTTCATTTTAGTCTCTATCTTTTACTACGTAGCCACCA 273
FD2.4-pMDC32       CATTTTCCTATCTCGTTTACGACTTCATTTTAGTCTCTATCTTTTACTACGTAGCCACCA 300
                   ************************************************************

FD2.4-pMDC139      CTTACTTCCGCAACCTCCCTCAGCCACTATCTTTCGTCGCCTGGCCAATTTATTGGGCTC 353
FD2.4cDNA          CTTACTTCCGCAACCTCCCTCAGCCACTATCTTTCGTCGCCTGGCCAATTTATTGGGCTC 256
FD2.4-pMDC43       CTTACTTCCGCAACCTCCCTCAGCCACTATCTTTCGTCGCCTGGCCAATTTATTGGGCTC 333
FD2.4-pMDC32       CTTACTTCCGCAACCTCCCTCAGCCACTATCTTTCGTCGCCTGGCCAATTTATTGGGCTC 360
                   ************************************************************

FD2.4-pMDC139      TTCAAGGTTCAGTCCTCACTGGCGTTTGGGTTATCGCCCATGAATGCGGTCACCACGCTT 413
FD2.4cDNA          TTCAAGGTTCAGTCCTCACTGGCGTTTGGGTTATCGCCCATGAATGCGGTCACCACGCTT 316
FD2.4-pMDC43       TTCAAGGTTCAGTCCTCACTGGCGTTTGGGTTATCGCCCATGAATGCGGTCACCACGCTT 393
FD2.4-pMDC32       TTCAAGGTTCAGTCCTCACTGGCGTTTGGGTTATCGCCCATGAATGCGGTCACCACGCTT 420
                   ************************************************************

FD2.4-pMDC139      TTAGCGATTACCAATGGATCGATGACACTGTCGGTCTCATCCTCCATTCATCCCTTCTCG 473
FD2.4cDNA          TTAGCGATTACCAATGGATCGATGACACTGTCGGTCTCATCCTCCATTCATCCCTTCTCG 376
FD2.4-pMDC43       TTAGCGATTACCAATGGATCGATGACACTGTCGGTCTCATCCTCCATTCATCCCTTCTCG 453
FD2.4-pMDC32       TTAGCGATTACCAATGGATCGATGACACTGTCGGTCTCATCCTCCATTCATCCCTTCTCG 480
                   ************************************************************

FD2.4-pMDC139      TCCCGTACTTTTCGTGGAAATATAGTCACCGTCGTCACCATTCCAACACTGGTTCCCTTG 533
FD2.4cDNA          TCCCGTACTTTTCGTGGAAATATAGTCACCGTCGTCACCATTCCAACACTGGTTCCCTTG 436
FD2.4-pMDC43       TCCCGTACTTTTCGTGGAAATATAGTCACCGTCGTCACCATTCCAACACTGGTTCCCTTG 513
FD2.4-pMDC32       TCCCGTACTTTTCGTGGAAATATAGTCACCGTCGTCACCATTCCAACACTGGTTCCCTTG 540
                   ************************************************************

FD2.4-pMDC139      AACGCGACGAAGTATTTGTTCCGAAGAAACGGAGCAGCATTAGATGGTGGGCTAAATACC 593
FD2.4cDNA          AACGCGACGAAGTATTTGTTCCGAAGAAACGGAGCAGCATTAGATGGTGGGCTAAATACC 496
FD2.4-pMDC43       AACGCGACGAAGTATTTGTTCCGAAGAAACGGAGCAGCATTAGATGGTGGGCTAAATACC 573
FD2.4-pMDC32       AACGCGACGAAGTATTTGTTCCGAAGAAACGGAGCAGCATTAGATGGTGGGCTAAATACC 600
                   ************************************************************
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(Figure 23 continues)

(Figure 23 continued)

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FD2.4-pMDC139      TCAACAATCCACCAGGTCGTTTCGTCACAATCACCATTCAGCTCACTCTCGGATGGCCTC 653
FD2.4cDNA          TCAACAATCCACCAGGTCGTTTCGTCACAATCACCATTCAGCTCACTCTCGGATGGCCTC 556
FD2.4-pMDC43       TCAACAATCCACCAGGTCGTTTCGTCACAATCACCATTCAGCTCACTCTCGGATGGCCTC 633
FD2.4-pMDC32       TCAACAATCCACCAGGTCGTTTCGTCACAATCACCATTCAGCTCACTCTCGGATGGCCTC 660
                   ************************************************************
FD2.4-pMDC139      TTTACTTAGCATTCAATGTAGCAGGTAGACCTTACGAAGGATTCGCTTGTCACTACAACC 713
FD2.4cDNA          TTTACTTAGCATTCAATGTAGCAGGTAGACCTTACGAAGGATTCGCTTGTCACTACAACC 616
FD2.4-pMDC43       TTTACTTAGCATTCAATGTAGCAGGTAGACCTTACGAAGGATTCGCTTGTCACTACAACC 693
FD2.4-pMDC32       TTTACTTAGCATTCAATGTAGCAGGTAGACCTTACGAAGGATTCGCTTGTCACTACAACC 720
                   ************************************************************
FD2.4-pMDC139      CATACGGTCCTATCTACAACGACCGTGAACGACTTCAAATCTACATTTCCGACGTCGGTG 773
FD2.4cDNA          CATACGGTCCTATCTACAACGACCGTGAACGACTTCAAATCTACATTTCCGACGTCGGTG 676
FD2.4-pMDC43       CATACGGTCCTATCTACAACGACCGTGAACGACTTCAAATCTACATTTCCGACGTCGGTG 753
FD2.4-pMDC32       CATACGGTCCTATCTACAACGACCGTGAACGACTTCAAATCTACATTTCCGACGTCGGTG 780
                   ************************************************************
FD2.4-pMDC139      TCCTTGCTGTCACCTATGGGCTGTACCGTCTCGTGTTAGCCAAAGGTCTAGCTTGGGTCA 833
FD2.4cDNA          TCCTTGCTGTCACCTATGGGCTGTACCGTCTCGTGTTAGCCAAAGGTCTAGCTTGGGTCA 736
FD2.4-pMDC43       TCCTTGCTGTCACCTATGGGCTGTACCGTCTCGTGTTAGCCAAAGGTCTAGCTTGGGTCA 813
FD2.4-pMDC32       TCCTTGCTGTCACCTATGGGCTGTACCGTCTCGTGTTAGCCAAAGGTCTAGCTTGGGTCA 840
                   ************************************************************
FD2.4-pMDC139      TTTGTGTTTACGGTGTCCCATTGCTCATCGTTAATGCATTCCTCGTCATGATCACATACT 893
FD2.4cDNA          TTTGTGTTTACGGTGTCCCATTGCTCATCGTTAATGCATTCCTCGTCATGATCACATACT 796
FD2.4-pMDC43       TTTGTGTTTACGGTGTCCCATTGCTCATCGTTAATGCATTCCTCGTCATGATCACATACT 873
FD2.4-pMDC32       TTTGTGTTTACGGTGTCCCATTGCTCATCGTTAATGCATTCCTCGTCATGATCACATACT 900
                   ************************************************************
FD2.4-pMDC139      TGCAACACACTCACCCTGCATTACCACACTACGACTCATCCGAATGGGATTGGTTACGTG 953
FD2.4cDNA          TGCAACACACTCACCCTGCATTACCACACTACGACTCATCCGAATGGGATTGGTTACGTG 856
FD2.4-pMDC43       TGCAACACACTCACCCTGCATTACCACACTACGACTCATCCGAATGGGATTGGTTACGTG 933
FD2.4-pMDC32       TGCAACACACTCACCCTGCATTACCACACTACGACTCATCCGAATGGGATTGGTTACGTG 960
                   ************************************************************
FD2.4-pMDC139      GAGCCCTCGCGACGGTCGACCGAGATTATGGGATATTAAACAAGGTTTTCCATAACATAA 1013
FD2.4cDNA          GAGCCCTCGCGACGGTCGACCGAGATTATGGGATATTAAACAAGGTTTTCCATAACATAA 916
FD2.4-pMDC43       GAGCCCTCGCGACGGTCGACCGAGATTATGGGATATTAAACAAGGTTTTCCATAACATAA 993
FD2.4-pMDC32       GAGCCCTCGCGACGGTCGACCGAGATTATGGGATATTAAACAAGGTTTTCCATAACATAA 1020
                   ************************************************************
FD2.4-pMDC139      CTGATACTCATATCGCTCATCATTTGTTTTCGACAATGCCGCATTACCACGCAATGGAAG 1073
FD2.4cDNA          CTGATACTCATATCGCTCATCATTTGTTTTCGACAATGCCGCATTACCACGCAATGGAAG 976
FD2.4-pMDC43       CTGATACTCATATCGCTCATCATTTGTTTTCGACAATGCCGCATTACCACGCAATGGAAG 1053
FD2.4-pMDC32       CTGATACTCATATCGCTCATCATTTGTTTTCGACAATGCCGCATTACCACGCAATGGAAG 1080
                   ************************************************************
FD2.4-pMDC139      CAACAAAGGCAATAAAGCCAATATTGGGCGAGTATTATTCATTTGATGGTACACCAGTTT 1133
FD2.4cDNA          CAACAAAGGCAATAAAGCCAATATTGGGCGAGTATTATTCATTTGATGGTACACCAGTTT 1036
FD2.4-pMDC43       CAACAAAGGCAATAAAGCCAATATTGGGCGAGTATTATTCATTTGATGGTACACCAGTTT 1113
FD2.4-pMDC32       CAACAAAGGCAATAAAGCCAATATTGGGCGAGTATTATTCATTTGATGGTACACCAGTTT 1140
                   ************************************************************
FD2.4-pMDC139      ATAAAGCGATATTTAGAGAGGCAAAGGAGTGTATTTACGTTGAACCAGACGAAGGTGAGC 1193
FD2.4cDNA          ATAAAGCGATATTTAGAGAGGCAAAGGAGTGTATTTACGTTGAACCAGACGAAGGTGAGC 1096
FD2.4-pMDC43       ATAAAGCGATATTTAGAGAGGCAAAGGAGTGTATTTACGTTGAACCAGACGAAGGTGAGC 1173
FD2.4-pMDC32       ATAAAGCGATATTTAGAGAGGCAAAGGAGTGTATTTACGTTGAACCAGACGAAGGTGAGC 1200
                   ************************************************************
FD2.4-pMDC139      AGAGCAGCAAAGGTGTATTTTGGTTTAGAAATAAGATC---AAGGGTGGGCGCGCCGACC 1250
FD2.4cDNA          AGAGCAGCAAAGGTGTATTTTGGTTTAGAAATAAGATCTAA---------------- 1137
FD2.4-pMDC43       AGAGCAGCAAAGGTGTATTTTGGTTTAGAAATAAGATCTAAAGGGTGGGCGCGCCGACC 1233
FD2.4-pMDC32       AGAGCAGCAAAGGTGTATTTTGGTTTAGAAATAAGATCTAAAGGGTGGGCGCGCCGACC 1260
                   ************************************    *
FD2.4-pMDC139      CAGCTTTCTTGTACAAAGTGGTGA-TAGCTGGCGCGCCTCGCTCAGGTACCCCCCTAAAG 1309
FD2.4cDNA          ------------------------------------------------------------
FD2.4-pMDC43       CAGCTTC--TGTACAAAGTGTCGAATAGCCCGGA-------------------------- 1265
FD2.4-pMDC32       CAGCTTC--TGTACAAAGTGTCGTATACG------------------------------ 1287

FD2.4-pMDC139      TGCTGA 1315
FD2.4cDNA          ------
FD2.4-pMDC43       ------
FD2.4-pMDC32       ------
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Fig. 23 Three *FAD2-4* sequences from the *FAD2-4*/pMDC constructs were aligned with the *FAD2-4* cDNA sequence to confirm the correct sequences of the *FAD2-4* coding regions in this vector construct. The coding region starts as ATG and ends one nucleotide before termination codon TAA for the pMDC139 constructs and with TAA termination codon for the pMDC43 and the pMDC32 constructs. The identities between the *FAD2-4* inserts and the *FAD2-4* cDNA are 100%.

```
FD2.3-pMDC139       ------------------------------------------------------------
FD2.3cDNA           ------------------------------------------------------------
FD2.3-pMDC139.1     TAAGGGAGGTTCAATTTCATTTGGAGAGGACCTCGGCTCTTAGACTTAGTTTATTAAGAA 60
                       Part of 35S promoter
FD2.3-pMDC139       -------------------------------------------TAAATGATTAGCCAAG 16
FD2.3cDNA           ------------------------------------------------------------
FD2.3-pMDC139.1     TAGCTGCAGCTGCAAGGTCGACTTAGAGATCCCCGGGTACCGAGCTTCGAATTATCACAA 120

FD2.3-pMDC139       TGTAAGTTTGTCAAAAGCAGGCTCCGCGGCCGCCTTGTTTAACTTTAAGAAGGAGCCCTT 76
FD2.3cDNA           ------------------------------------------------------------
FD2.3-pMDC139.1     GTTTGTACAAAAAGCAGGCTCCCGCGGCCGCCTTTGTTTAACTTAAAGAAGGGAGCCCTT 180
                       AttR1
FD2.3-pMDC139       CACCATGGGTGCAGGTGGCAGAATCGGTTCCTCCAAGTCAAAGGAAACAAGAATCGGG 136
FD2.3cDNA           ----------------------ATGTCGGTTCCTCCAAGTCAAAGGAAACAAGAATCGGG 38
FD2.3-pMDC139.1     CACCATGGGTGCAGGTGGCAGAATCGGTTCCTCCAAGTCAAAGGAAACAAGAATCGGG 240
                                           ************************************
FD2.3-pMDC139       CTCAATGAAAAGAGTCCCTATATCTAAACCACCATTTACTCTCAGTGAAATAAAAAAAGC 196
FD2.3cDNA           CTCAATGAAAAGAGTCCCTATATCTAAACCACCATTTACTCTCAGTGAAATAAAAAAAGC 98
FD2.3-pMDC139.1     CTCAATGAAAAGAGTCCCTATATCTAAACCACCATTTACTCTCAGTGAAATAAAAAAAGC 300
                    ************************************************************
FD2.3-pMDC139       CATCCCACCACACTGTTTCCAACGCTCACTTATCCGTTCATTTTCCTATCTCGTTTACGA 256
FD2.3cDNA           CATCCCACCACACTGTTTCCAACGCTCACTTATCCGTTCATTTTCCTATCTCGTTTACGA 158
FD2.3-pMDC139.1     CATCCCACCACACTGTTTCCAACGCTCACTTATCCGTTCATTTTCCTATCTCGTTTACGA 360
                    ************************************************************
FD2.3-pMDC139       CTTCATTTTAGTCTCTATCTTTTACTACGTAGCCACCACTTACTTCCACAACCTCCCTCA 316
FD2.3cDNA           CTTCATTTTAGTCTCTATCTTTTACTACGTAGCCACCACTTACTTCCACAACCTCCCTCA 218
FD2.3-pMDC139.1     CTTCATTTTAGTCTCTATCTTTTACTACGTAGCCACCACTTACTTCCACAACCTCCCTCA 420
                    ************************************************************
FD2.3-pMDC139       GCCACTATCTTTCGTCGCCTGGCCAATTTATTGGACTCTTCAAGGTTCAGTCCTCACTGG 376
FD2.3cDNA           GCCACTATCTTTCGTCGCCTGGCCAATTTATTGGACTCTTCAAGGTTCAGTCCTCACTGG 278
FD2.3-pMDC139.1     GCCACTATCTTTCGTCGCCTGGCCAATTTATTGGACTCTTCAAGGTTCAGTCCTCACTGG 480
                    ************************************************************
FD2.3-pMDC139       CGTTTGGGTTATCGCCCATGAATGCGGTCACCATGCTTTTAGCGATTACCAATGGATTGA 436
FD2.3cDNA           CGTTTGGGTTATCGCCCATGAATGCGGTCACCATGCTTTTAGCGATTACCAATGGATTGA 338
FD2.3-pMDC139.1     CGTTTGGGTTATCGCCCATGAATGCGGTCACCATGCTTTTAGCGATTACCAATGGATTGA 540
                    ************************************************************
FD2.3-pMDC139       TGACACTGTCGGTCTCATCCTCCATTCATCCCTTCTTGTCCCGTACTTTTCGTGGAAATA 496
FD2.3cDNA           TGACACTGTCGGTCTCATCCTCCATTCATCCCTTCTTGTCCCGTACTTTTCGTGGAAATA 398
FD2.3-pMDC139.1     TGACACTGTCGGTCTCATCCTCCATTCATCCCTTCTTGTCCCGTACTTTTCGTGGAAATA 600
                    ************************************************************
FD2.3-pMDC139       TAGTCACCGACGTCACCATTCCAACACTGGTTCCCTTGAACGCGACGAAGTATTTGTTCC 556
FD2.3cDNA           TAGTCACCGACGTCACCATTCCAACACTGGTTCCCTTGAACGCGACGAAGTATTTGTTCC 458
FD2.3-pMDC139.1     TAGTCACCGACGTCACCATTCCAACACTGGTTCCCTTGAACGCGACGAAGTATTTGTTCC 660
                    ************************************************************
FD2.3-pMDC139       GAAGAAACGGAGCAGCATTAGATGGTGGGCTAAATACCTCAACAATCCACCAGGTCGTTT 616
FD2.3cDNA           GAAGAAACGGAGCAGCATTAGATGGTGGGCTAAATACCTCAACAATCCACCAGGTCGTTT 518
FD2.3-pMDC139.1     GAAGAAACGGAGCAGCATTAGATGGTGGGCTAAATACCTCAACAATCCACCAGGTCGTT- 719
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(Figure 24 continues)

(Figure 24 continued)

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FD2.3-pMDC139       CGTCACAGTCACCATTCAGCTCACTCTCGGATGGCCTCTTTACTTAGCATTCAATGTAGC 676
FD2.3cDNA           CGTCACAGTCACCATTCAGCTCACTCTCGGATGGCCTCTTTACTTAGCATTCAATGTAGC 578
FD2.3-pMDC139.1     ------------------------------------------------------------

FD2.3-pMDC139       AGGTAGACCTTACGAAGGACTCGCTTGTCACTACAACCCATACGGTCCTATCTACAACGA 736
FD2.3cDNA           AGGTAGACCTTACGAAGGACTCGCTTGTCACTACAACCCATACGGTCCTATCTACAACGA 638
FD2.3-pMDC139.1     ------------------------------------------------------------

FD2.3-pMDC139       CCGTGAACGACTTCAAATCTACATATCCGACGTCGGTGTCCTTGCTGTCACCTATGGGCT 796
FD2.3cDNA           CCGTGAACGACTTCAAATCTACATATCCGACGTCGGTGTCCTTGCTGTCACCTATGGGCT 698
FD2.3-pMDC139.1     ------------------------------------------------------------


FD2.3-pMDC139       GTACCGTCTCGTGTTAGCCAAAGGTCTAGCTTGGGTCATTTGCGTTTACGGTGTCCCATT 856
FD2.3cDNA           GTACCGTCTCGTGTTAGCCAAAGGTCTAGCTTGGGTCATTTGCGTTTACGGTGTCCCATT 758
FD2.3-pMDC139.1     ------------------------------------------------------------

FD2.3-pMDC139       GCTCATCGTTAATGCATTCCTCGTCATGATCACATACTTGCAACACACTCACCCCGCATT 916
FD2.3cDNA           GCTCATCGTTAATGCATTCCTCGTCATGATCACATACTTGCAACACACTCACCCCGCATT 818
FD2.3-pMDC139.1     ------------------------------------------------------------

FD2.3-pMDC139       ACCACACTACGACTCATCCGAATGGGACTGGTTACGTGGAGCCCTCGCGACGGTCGACCG 976
FD2.3cDNA           ACCACACTACGACTCATCCGAATGGGACTGGTTACGTGGAGCCCTCGCGACGGTCGACCG 878
FD2.3-pMDC139.1     ------------------------------------------------------------

FD2.3-pMDC139       AGATTATGGGATATTAAACAAGGTTTTCCATAACATAACTGATACTCATGTCGCTCATCA 1036
FD2.3cDNA           AGATTATGGGATATTAAACAAGGTTTTCCATAACATAACTGATACTCATGTCGCTCATCA 938
FD2.3-pMDC139.1     ------------------------------------------------------------

FD2.3-pMDC139       TTTGTTTTCGACGATGCCGCATTACCACGCAATGGAAGCAACTAAGGCAATAAAACCAAT 1096
FD2.3cDNA           TTTGTTTTCGACGATGCCGCATTACCACGCAATGGAAGCAACTAAGGCAATAAAACCAAT 998
FD2.3-pMDC139.1     ------------------------------------------------------------

FD2.3-pMDC139       ATTGGGAGAGTATTATTCATTTGATGGTACACCAGTTTATAAAGCGATATTTAGAGAGGC 1156
FD2.3cDNA           ATTGGGAGAGTATTATTCATTTGATGGTACACCAGTTTATAAAGCGATATTTAGAGAGGC 1058
FD2.3-pMDC139.1     ------------------------------------------------------------

FD2.3-pMDC139       AAAGGAGTGTATTTACGTTGAACCAGACGAAGGTGAGCAGAGCAGCAAAGGTGTATTTTG 1216
FD2.3cDNA           AAAGGAGTGTATTTACGTTGAACCAGACGAAGGTGAGCAGAGCAGCAAAGGTGTATTTTG 1118
FD2.3-pMDC139.1     ------------------------------------------------------------

FD2.3-pMDC139       GTTTAGAAATAAGATCAAGGGTGGGCGCGCCGACCCAGCTTTCTTGTACAAAGTGGTGAT 1276
FD2.3cDNA           GTTTAGAAATAAGATCTAA---------------------------------------- 1137
FD2.3-pMDC139.1     ------------------------------------------------------------

FD2.3-pMDC139       AGCTGGCGCGCCTCGACTCAGGTGCCCCCCCTAAAAAA 1314
FD2.3cDNA           ---------------------------------------
FD2.3-pMDC139.1     ---------------------------------------
```

Fig. 24 The DNA sequence alignments that show the 5'-portion of the *FAD2-3* coding region (underlined by asterisks) and part of the pMDC139 vector including the partial CAMV 35S promoter and *AttR1* sequences (underlined bold letters).

Figure 24 shows the alignments of the 3' portion of the *FAD2-3* coding region (underlined by asterisks) and part of the pMDC139 vector including partial *GusA* gene and *AttR2* sequences (underlined bold letters). Lastly, the *FAD2-3* gene with its native stop codon was subcloned into the pMDC32 vector. Three positive *FAD2-3TAA*-pMDC32 clones were sent for sequencing for confirmation (Figure 26).

```
FD2.3-pMDC139       TAAATGATTAGCCAAGTGTAAGTTTGTCAAAAGCAGGCTCCGCGGCCGCCTTGTTTAACT 60
FD2.3cDNA           ------------------------------------------------------------
FD2.3-pMDC139.2     ------------------------------------------------------------

FD2.3-pMDC139       TTAAGAAGGAGCCCTTCACCATGGGTGCAGGTGGCAGAATGTCGGTTCCTCCAAGTCAAA 120
FD2.3cDNA           ------------------------------------ATGTCGGTTCCTCCAAGTCAAA 22
FD2.3-pMDC139.2     ------------------------------------------------------------

FD2.3-pMDC139       GGAAACAAGAATCGGGCTCAATGAAAAGAGTCCCTATATCTAAACCACCATTTACTCTCA 180
FD2.3cDNA           GGAAACAAGAATCGGGCTCAATGAAAAGAGTCCCTATATCTAAACCACCATTTACTCTCA 82
FD2.3-pMDC139.2     ------------------------------------------------------------

FD2.3-pMDC139       GTGAAATAAAAAAAGCCATCCCACCACACTGTTTCCAACGCTCACTTATCCGTTCATTTT 240
FD2.3cDNA           GTGAAATAAAAAAAGCCATCCCACCACACTGTTTCCAACGCTCACTTATCCGTTCATTTT 142
FD2.3-pMDC139.2     ------------------------------------------------------------

FD2.3-pMDC139       CCTATCTCGTTTACGACTTCATTTTAGTCTCTATCTTTTACTACGTAGCCACCACTTACT 300
FD2.3cDNA           CCTATCTCGTTTACGACTTCATTTTAGTCTCTATCTTTTACTACGTAGCCACCACTTACT 202
FD2.3-pMDC139.2     ------------------------------------------------------------

FD2.3-pMDC139       TCCACAACCTCCCTCAGCCACTATCTTTCGTCGCCTGGCCAATTTATTGGACTCTTCAAG 360
FD2.3cDNA           TCCACAACCTCCCTCAGCCACTATCTTTCGTCGCCTGGCCAATTTATTGGACTCTTCAAG 262
FD2.3-pMDC139.2     ------------------------------------------------------------

FD2.3-pMDC139       GTTCAGTCCTCACTGGCGTTTGGGTTATCGCCCATGAATGCGGTCACCATGCTTTTAGCG 420
FD2.3cDNA           GTTCAGTCCTCACTGGCGTTTGGGTTATCGCCCATGAATGCGGTCACCATGCTTTTAGCG 322
FD2.3-pMDC139.2     ------------------------------------------------------------

FD2.3-pMDC139       ATTACCAATGGATTGATGACACTGTCGGTCTCATCCTCCATTCATCCCTTCTTGTCCCGT 480
FD2.3cDNA           ATTACCAATGGATTGATGACACTGTCGGTCTCATCCTCCATTCATCCCTTCTTGTCCCGT 382
FD2.3-pMDC139.2     ------------------------------------------------------------

FD2.3-pMDC139       ACTTTTCGTGGAAATATAGTCACCGACGTCACCATTCCAACACTGGTTCCCTTGAACGCG 540
FD2.3cDNA           ACTTTTCGTGGAAATATAGTCACCGACGTCACCATTCCAACACTGGTTCCCTTGAACGCG 442
FD2.3-pMDC139.2     ------------------------------------------------------------

FD2.3-pMDC139       ACGAAGTATTTGTTCCGAAGAAACGGAGCAGCATTAGATGGTGGGCTAAATACCTCAACA 600
FD2.3cDNA           ACGAAGTATTTGTTCCGAAGAAACGGAGCAGCATTAGATGGTGGGCTAAATACCTCAACA 502
FD2.3-pMDC139.2     ------------------------------------------------------------

FD2.3-pMDC139       ATCCACCAGGTCGTTTCGTCACAGTCACCATTCAGCTCACTCTCGGATGGCCTCTTTACT 660
FD2.3cDNA           ATCCACCAGGTCGTTTCGTCACAGTCACCATTCAGCTCACTCTCGGATGGCCTCTTTACT 562
FD2.3-pMDC139.2     ------------------------------------------------------------

FD2.3-pMDC139       TAGCATTCAATGTAGCAGGTAGACCTTACGAAGGACTCGCTTGTCACTACAACCCATACG 720
FD2.3cDNA           TAGCATTCAATGTAGCAGGTAGACCTTACGAAGGACTCGCTTGTCACTACAACCCATACG 622
FD2.3-pMDC139.2     --------------------------------GGACTCGCTTGTCACTACAACCCATACG 28
                                                    ***************************
FD2.3-pMDC139       GTCCTATCTACAACGACCGTGAACGACTTCAAATCTACATATCCGACGTCGGTGTCCTTG 780
FD2.3cDNA           GTCCTATCTACAACGACCGTGAACGACTTCAAATCTACATATCCGACGTCGGTGTCCTTG 682
FD2.3-pMDC139.2     GTCCTATCTACAACGACCGTGAACGACTTCAAATCTACATATCCGACGTCGGTGTCCTTG 88
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(Figure 25 continued)

(Figure 25 continued)

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FD2.3-pMDC139       CTGTCACCTATGGGCTGTACCGTCTCGTGTTAGCCAAAGGTCTAGCTTGGGTCATTTGCG 840
FD2.3cDNA           CTGTCACCTATGGGCTGTACCGTCTCGTGTTAGCCAAAGGTCTAGCTTGGGTCATTTGCG 742
FD2.3-pMDC139.2     CTGTCACCTATGGGCTGTACCGTCTCGTGTTAGCCAAAGGTCTAGCTTGGGTCATTTGCG 148
                    ************************************************************

FD2.3-pMDC139       TTTACGGTGTCCCATTGCTCATCGTTAATGCATTCCTCGTCATGATCACATACTTGCAAC 900
FD2.3cDNA           TTTACGGTGTCCCATTGCTCATCGTTAATGCATTCCTCGTCATGATCACATACTTGCAAC 802
FD2.3-pMDC139.2     TTTACGGTGTCCCATTGCTCATCGTTAATGCATTCCTCGTCATGATCACATACTTGCAAC 208
                    ************************************************************

FD2.3-pMDC139       ACACTCACCCCGCATTACCACACTACGACTCATCCGAATGGGACTGGTTACGTGGAGCCC 960
FD2.3cDNA           ACACTCACCCCGCATTACCACACTACGACTCATCCGAATGGGACTGGTTACGTGGAGCCC 862
FD2.3-pMDC139.2     ACACTCACCCCGCATTACCACACTACGACTCATCCGAATGGGACTGGTTACGTGGAGCCC 268
                    ************************************************************

FD2.3-pMDC139       TCGCGACGGTCGACCGAGATTATGGGATATTAAACAAGGTTTTCCATAACATAACTGATA 1020
FD2.3cDNA           TCGCGACGGTCGACCGAGATTATGGGATATTAAACAAGGTTTTCCATAACATAACTGATA 922
FD2.3-pMDC139.2     TCGCGACGGTCGACCGAGATTATGGGATATTAAACAAGGTTTTCCATAACATAACTGATA 328
                    ************************************************************

FD2.3-pMDC139       CTCATGTCGCTCATCATTTGTTTTCGACGATGCCGCATTACCACGCAATGGAAGCAACTA 1080
FD2.3cDNA           CTCATGTCGCTCATCATTTGTTTTCGACGATGCCGCATTACCACGCAATGGAAGCAACTA 982
FD2.3-pMDC139.2     CTCATGTCGCTCATCATTTGTTTTCGACGATGCCGCATTACCACGCAATGGAAGCAACTA 388
                    ************************************************************
FD2.3-pMDC139       AGGCAATAAAACCAATATTGGGAGAGTATTATTCATTTGATGGTACACCAGTTTATAAAG 1140
FD2.3cDNA           AGGCAATAAAACCAATATTGGGAGAGTATTATTCATTTGATGGTACACCAGTTTATAAAG 1042
FD2.3-pMDC139.2     AGGCAATAAAACCAATATTGGGAGAGTATTATTCATTTGATGGTACACCAGTTTATAAAG 448

                    ************************************************************
FD2.3-pMDC139       CGATATTTAGAGAGGCAAAGGAGTGTATTTACGTTGAACCAGACGAAGGTGAGCAGAGCA 1200
FD2.3cDNA           CGATATTTAGAGAGGCAAAGGAGTGTATTTACGTTGAACCAGACGAAGGTGAGCAGAGCA 1102
FD2.3-pMDC139.2     CGATATTTAGAGAGGCAAAGGAGTGTATTTACGTTGAACCAGACGAAGGTGAGCAGAGCA 508
                    ************************************************************
FD2.3-pMDC139       GCAAAGGTGTATTTTGGTTTAGAAATAAGATCAAGGGTGGGCGCGCCGACCCAGCTTTCT 1260
FD2.3cDNA           GCAAAGGTGTATTTTGGTTTAGAAATAAGATCTAA------------------------ 1137
FD2.3-pMDC139.2     GCAAAGGTGTATTTTGGTTTAGAAATAAGATCAAGGGTGGGCGCGCCGACCCAGCTTTCT 568
                    ******************************** *
FD2.3-pMDC139       TGTACAAAGTGGTGATAGCT-GGCGCGCCTCGACTCAGGTGCCCCCCCTAAAAAA----- 1314
FD2.3cDNA           ------------------------------------------------------------
FD2.3-pMDC139.2     TGTACAAAGTGGTGATAGCTTGGCGCGCCTCGACTCTAGAGGATCGATCCCCGGGTACGG 628
                     AttR2
FD2.3-pMDC139       ------------------------------------------------------------
FD2.3cDNA           ------------------------------------------------------------
FD2.3-pMDC139.2     TCAGTCCCTTATGTTACGTCCTGTAGAAACCCCAACCCGTGAAATCAAAAAACTCGACGG 688
                              Start for Gus gene
FD2.3-pMDC139       ------------------------------------------------------------
FD2.3cDNA           ------------------------------------------------------------
FD2.3-pMDC139.2     CCTGTGGGCATTCAGTCTGGATCGCGAAAACTGTGGAATTGATCAGCGTTGGTGGGAAAG 748

FD2.3-pMDC139       ------------------------------------------------------------
FD2.3cDNA           ------------------------------------------------------------
FD2.3-pMDC139.2     CGCGTTACAAGAAAGCCCGGGCAATTGCTGTGCCAGGCAGTTTTAACGATCAGTCGCCGA 808
                                        GusA
FD2.3-pMDC139       ------------------------------------------------------------
FD2.3cDNA           ------------------------------------------------------------
FD2.3-pMDC139.2     TGCAGATATTCGTATTTATGCGGGCAACGTCTGGTATCACGGCGAGTCTTTATACCGAAG 868

FD2.3-pMDC139       ------------------------------------------------
FD2.3cDNA           ------------------------------------------------
FD2.3-pMDC139.2     GTGGGCAGGCCAGCGTATCGGGCGCGTTTCGATGCGGCACTCATTACGGCA 919
```
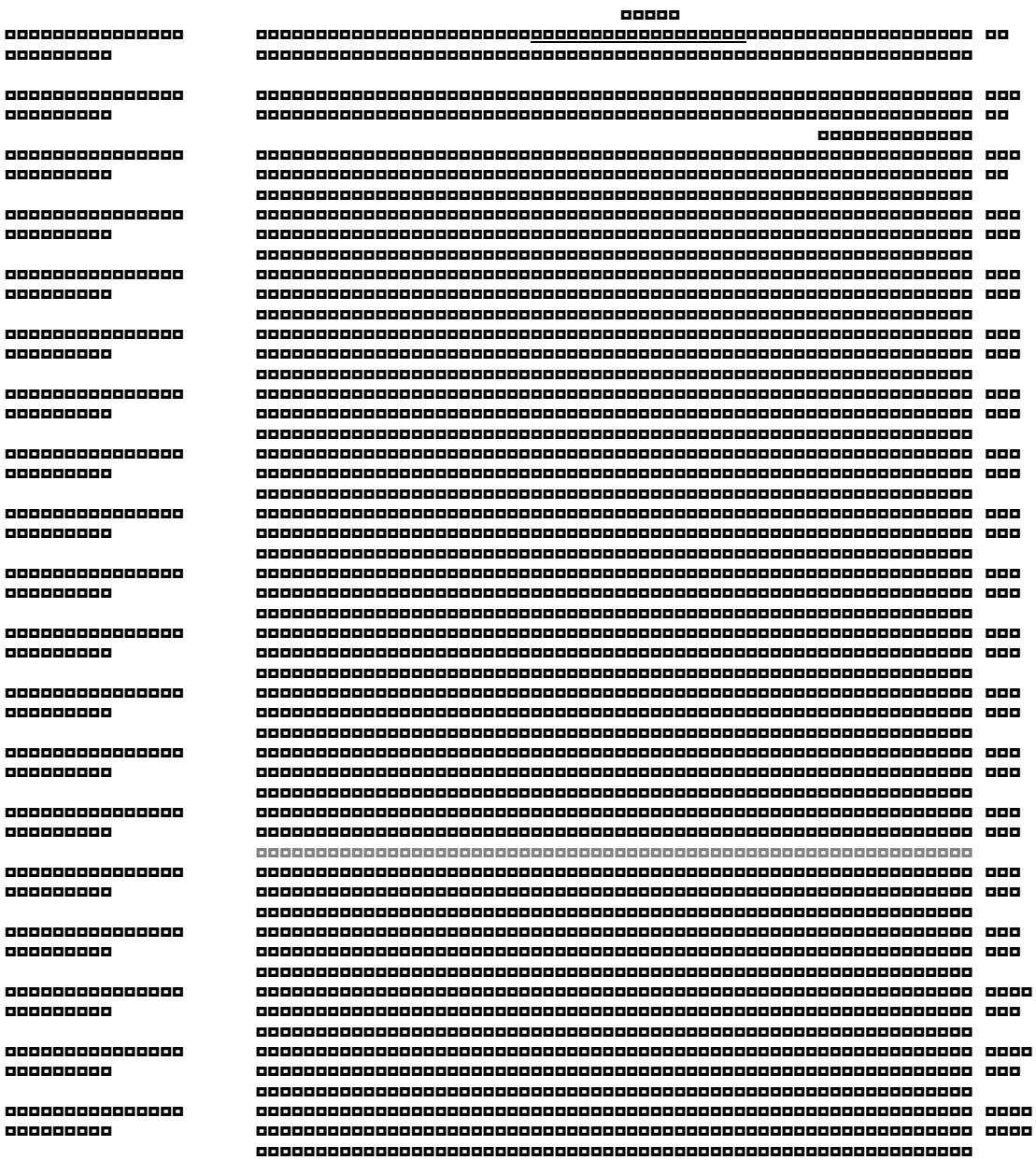
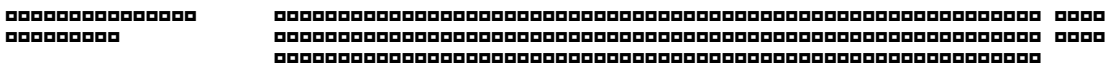

Fig. 25 The DNA sequence alignments that show the 3'-portion of the *FAD2-3* coding region (underlined by asterisks) and part of the pMDC139 vector including the partial sequence of the *GusA* gene and *AttR2* sequences (underlined bold letters).



(Figure 26 continues)

(Figure 26 continued)



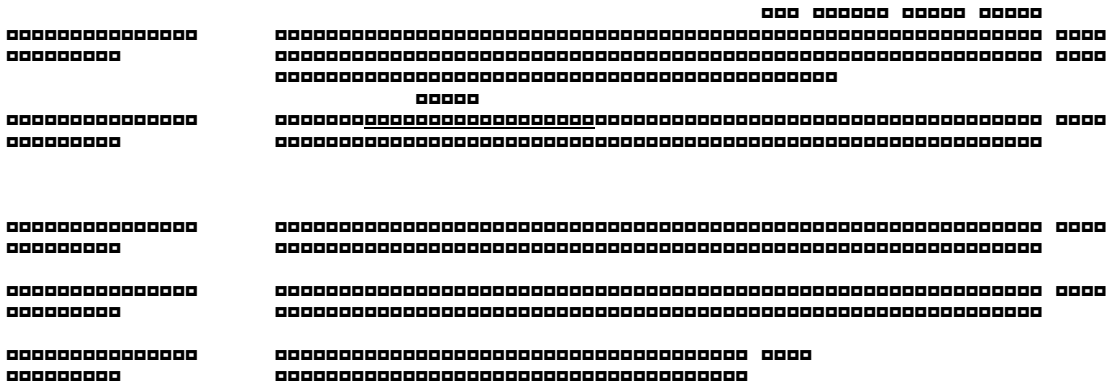


Fig. 26 The sequences of the *FAD2-3*TAA/pMDC32 constructs were aligned with the *FAD2-3* cDNA sequence to confirm the accuracy of the *FAD2-3* coding region. In this vector construct, the coding region starts at ATG and ends after the termination codon TAA. The *AttR* recombination site on the vector is also shown in bold letters. The *FAD2-3* coding region is placed in frame in the correct direction and the identity between the *FAD2-3* inserts and *FAD2-3* cDNA is 100%.

Transformation, Regeneration, and Screening of Transgenic Arabidopsis Plants

After confirming the sequences of all five *FAD2*-pMDC constructs, the cotton *FAD2*-pMDC constructs, along with the pMDC empty vectors as control, were transformed into *Agrobacterium tumefaciens* LBA4404 cells (Bevan et al., 1984). These competent cells were ordered from Invitrogen and were transformed by electroporation. LBA4404 cells contain the disarmed Ti plasmid pAL4404 which has only the *vir* and *ori* region of the Ti plasmid. The recombinant DNAs are able to conjugate from *A. tumefaciens* cells into plant cells using enzymatic components encoded in the plasmid pAL 4404 DNA (Bevan, et al., 1984).

Colony PCR amplification was used to assay the positive transformed cells with unique primers specific for the *FAD2* genes only. Empty pMDC vectors were used as the positive controls (Figure 27).

Fig. 27 Agarose gel electrophoresis of the PCR products generated from colony DNAs of *Agrobacterium tumefaciens* LBA4404 cells transformed with *FAD2*/pMDC recombinant vectors. The PCR products were electrophoresed on a 1.0 % agarose gel containing ethidium bromide at a final concentration of 0.5 µg/ml, with 1 kd DNA Marker standards. All five constructs (*FAD2-3*/pMDC32, *FAD2-4*/pMDC32, *FAD2-4*/pMDC43, *FAD2-3*/pMDC139, *FAD2-4*/pMDC139) were confirmed to be transformed into *Agrobacterium tumefaciens* LBA4404 cells. These colonies were incubated in the LB broth and later used to transform *Arabidopsis* plants.

To transform Arabidopsis plants with *Agrobacterium* cells harboring the *FAD2*-pMDC vectors, the Arabidopsis *fad2* mutant knockout seeds (Browse et al., 1993) were

ordered from the Arabidopsis Biological Resource Center at Ohio State University. For the transformation, the simplified Arabidopsis transformation protocol (called the floral dip method) was used (Clough and Bent, 1998). A total of five *FAD2*-pMDC constructs, along with three pMDC vectors as controls, were used for Arabidopsis transformation. After five days, the dipping procedure was repeated, and the plants were grown in the growth room until the seeds became mature. The dried seeds were harvested and screened for positive transformants.

For Arabidopsis T_1 transformant selection the dried seeds were harvested and surface sterilized by vapor-phase methods. The sterilized seeds were then dispensed onto hygromycin MS plates (50µg/ml). Transformants were identified as hygromycin-resistant seedings that produced green leaves and well-established roots within the selection medium (Figure 28). The transformants grew to maturity by transplantation (preferably after the development of 3-5 adult leaves) into heavily moistened potting soil and the grown plants were used for testing (Clough and Bent, 1998). After T_1 selection, a total of four lines were generated for the *FAD2-4*/pMDC139 construct, as well as three for the *FAD2-3*/pMDC139 construct, five for *FAD2-4*/pMDC32 construct, six for the *FAD2-3*/pMDC32 construct, and five for the *FAD2-4*/pMDC43 construct. These plants were grown into maturity to harvest T_2 seeds.

Fig. 28 Putative transgenic Arabidopsis plants (ecotype Columbia) with *FAD2*/pMDC constructs identified as hygromycin-resistant seedlings with green leaves and well-established roots within the hygromycin MS selection medium. The hygromycin-selected Arabidopsis seedings contains the following *FAD2*/pMDC constructs: *FAD2-3*/pMDC139/T_1 line, *FAD2-4*/pMDC139/T_1 line, *FAD2-3*/pMDC32/T_1 line, *FAD2-4*/ pMDC32/T_1 line. The seeds harvested from each generation of prospective transgenic *Arabidopsis* plants were screened on hygromycin plates. Approximately one month after plating, the seedlings that appeared to contain the hygromycin resistance gene from the pMDC vectors produced green secondary leaves and established good root systems.

To screen the putative Arabidopsis T_2 seedings for the cotton *FAD2*/pMDC transformed lines, the seeds were surface sterilized by vapor-phase methods and sterilized seeds were dispensed onto hygromycin MS plates (20µg/ml). The plates were then cold-treated in the dark for 3-4 days and transferred to a growth chamber in a controlled environment at 22ºC with the petri plates and their lids sealed with Parafilm. After 3 to 4 weeks, about 70% of the seedings showed hygromycin resistance, indicated by green leaves and well-established roots within the selection medium, whereas the other 30% plantlets without the vector constructs were yellow and dying, indicating segregation between the generations (Figure 29). After they became well-developed in the selection medium, the T_2 transformants were transplanted to soil and grown in a 22ºC growth room.

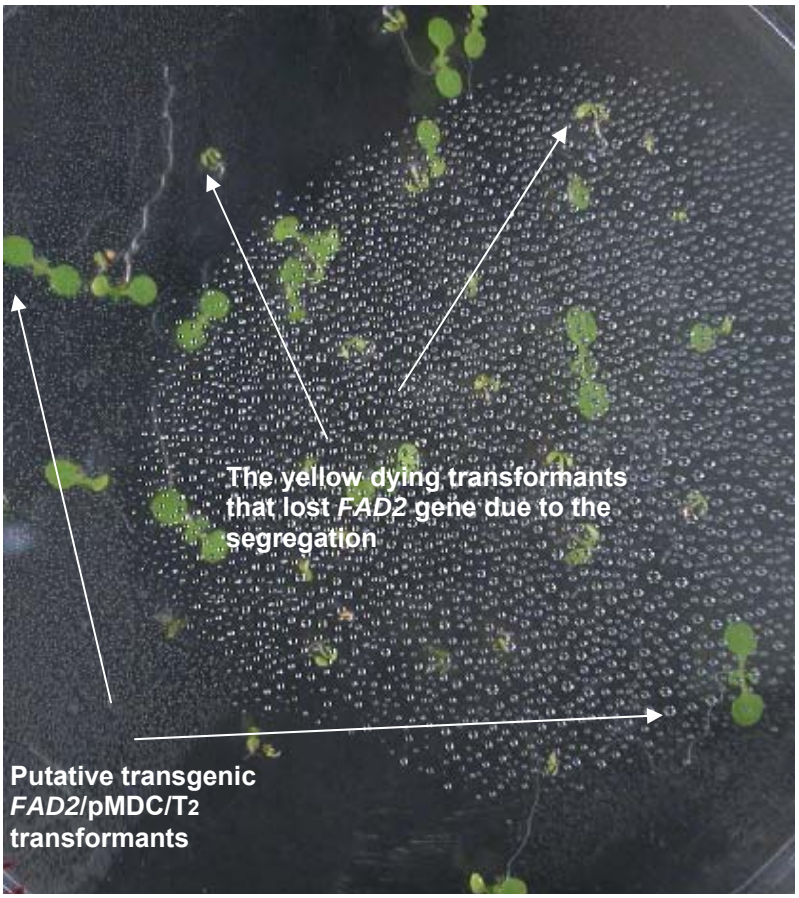


Fig. 29 Hygromycin-selected Arabidopsis T_2 seedings containing the *FAD2-4*/pMDC139 construct. The seeds harvested from the T_1 generation of prospective transgenic Arabidopsis plants were screened on hygromycin plates. Approximately one month after plating, around 75% of the seedlings survived the hygromycin selection, whereas the other 25% plantlets without the vector constructs were yellow and dying, indicating the segregation between the generations.

PCR Amplification of Cotton *FAD2* Genes using Genomic DNAs from Transgenic Arabidopsis Plant Leaves

To confirm that the putative transgenic Arabidopsis plants were indeed transformed with cotton *FAD2* genes, the genomic DNAs isolated from the transgenic Arabidopsis T_1 lines were used as templates to amplify the target gene by PCR.

To isolate genomic DNAs from plant tissues, the REDExtract-N-Amp Plant PCR Kit from Sigma was used to rapidly extract genomic DNAs from plant leaves. The leaf tissues were sampled from all five transformed Arabidopsis *FAD2*/pMDC T_1 or T_2 lines, the *fad2* knockout mutant plants, and the wild type Arabdopsis plants (as control).

To assess the quality of the DNAs isolated from Arabidopsis leaf tissue**,** the Arabidopsis *Act8* gene was first amplified from the plants. The *Act8* gene is 265 bp in length, belonging to the actin subclass (An et al, 1996). There is strong, constitutive expression of the *Act8* gene in Arabidopsis vegetative tissues, which renders it to be an excellent control gene to assess the quality of genomic DNAs isolated from Arabidopsis and used for PCR reactions. As Figure 30 shows, a 265-bp fragment was successfully amplified from both the control DNAs (isolated from *fad2* knockout Arabidopsis plants)

and the DNAs from *FAD2*/pMDC transgenic Arabidopsis plants transformed with the constructs, indicating quality genomic DNAs were isolated for the following analysis.

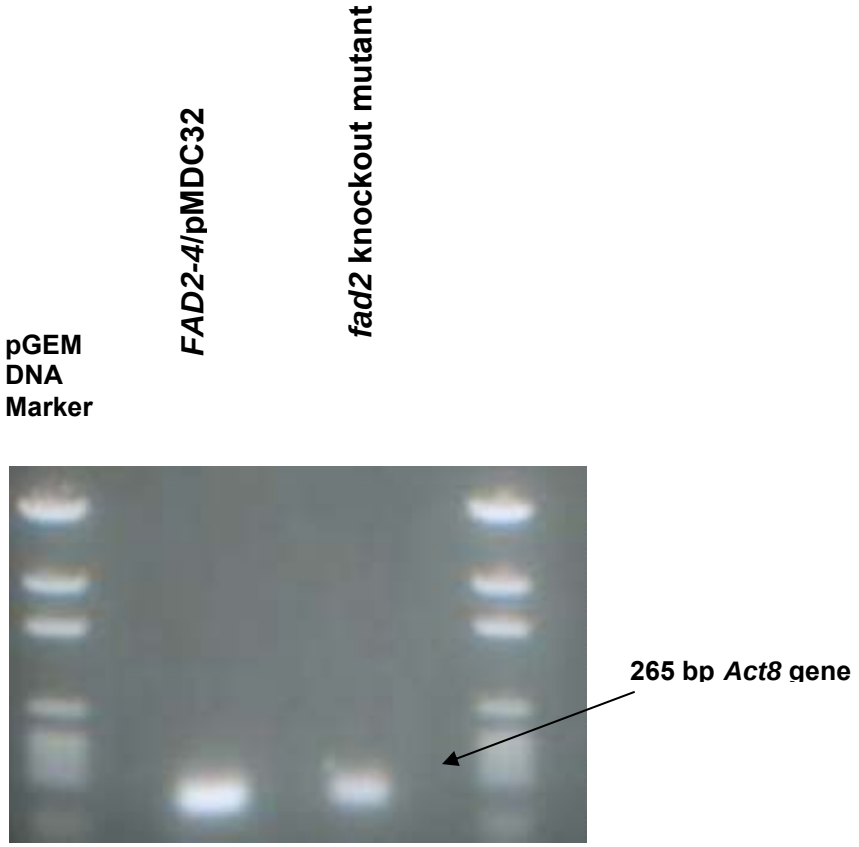


Figure 30. An *Act8* gene was amplified from Arabidopsis genomic DNAs. The *FAD2-4*/pMDC32 line and the *fad2* knockout mutant line were used to extract genomic DNAs. The PCR products were electrophoresed on a 1.0 % agarose gel containing ethidium bromide at a final concentration of 0.5 µg/ml, with pGEM DNA Marker standards. A 265-bp fragment was successfully amplified from both the control DNA (isolated from *fad2* knockout Arabidopsis plants) and the DNA from transgenic Arabidopsis transformed with the *FAD2-4*/pMDC32 construct.

The pMDC139 vectors contain the *gusA* gene following the *attR2* recombination site. Thus, to assess genomic DNAs isolated from this line for PCR amplification, a 366-bp *gusA* gene PCR product was amplified (Figure 31). To further demonstrate that the *FAD2-3*/pMDC139 vector was transformed into this line, two sets of primers were designed.

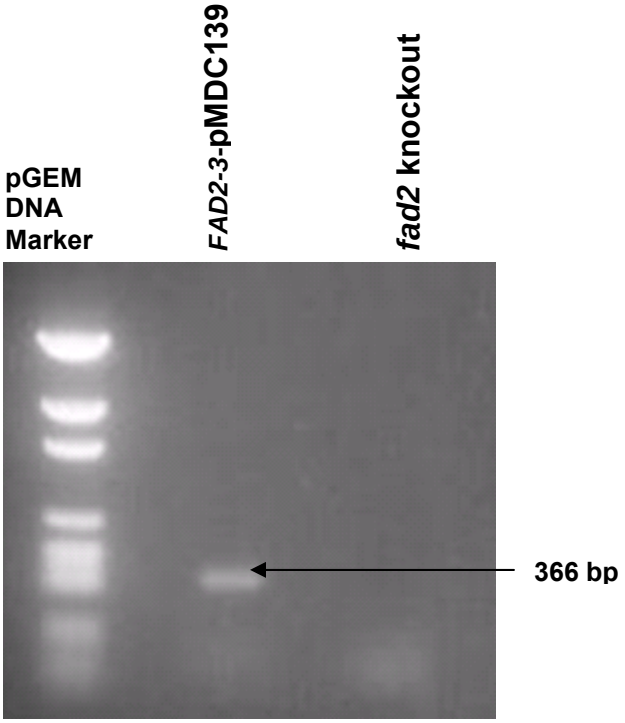


Fig. 31 A *gusA* gene was amplified from Arabidopsis genomic DNAs isolated from *FAD2-3*/pMDC139 lines. The *FAD2-3*/pMDC139 line and the *fad2* knockout mutant line were used to extract genomic DNAs. The PCR products were electrophoresed on a 1.0 % agarose gel containing ethidium bromide at a final concentration of 0.5 µg/ml, with pGEM DNA Marker standards. A 366-bp fragment was successfully amplified using the DNA from transgenic Arabidopsis transformed with the *FAD2-3*/pMDC139 construct, not from the control DNA (isolated from *fad2* knockout Arabidopsis plants), since the pMDC139 vectors contain the *gusA* gene following a *attR2* recombination site.

One set of primers annealed to the pMDC139 vectors and overlapped the *attR1/FAD2-3/attR2* region (Figure 31), yielding an amplified fragment of around 1.5 kb. The other set of primers were used to amplify the *FAD2-3* coding region from residues 680 to 1050, to yield a product of about 400 bp. As Figure 31 shows, the 400-bp fragment generated from the *FAD2-3* gene was amplified from both genomic DNA (isolated from the transgenic Arabidopsis plants transformed with the *FAD2-3*/pMDC139 construct) and the *FAD2-3*/pMDC139 plasmid DNA. A 1.5-kb fragment was also successfully amplified using the primers that cover the *attR1/FAD2-3/attR2* region.

To confirm the transformation of the *FAD2-4*/pMDC32 vector into Arabidopsis plants, primers were designed to anneal to the pMDC32 vectors and overlap the *attR1/FAD2-4/attR2 region* (Figure 32). The amplified fragment would be around 1.5 kb. Also, the primers that anneal to a 400-bp region in the *FAD2-4* gene and the primers designed to amplify the entire 1.2-kb coding region of the *FAD2-4* gene were used for PCR amplifications of Arabidopsis genomic DNA. As Figure 33 shows, a 400-bp PCR fragment from the *FAD2-4* gene and the entire 1.2-kb coding region were successfully amplified from both the genomic DNA (isolated from transgenic Arabidopsis plants transformed with *FAD2-4*/pMDC32 constructs) and the *FAD2-4*/pMDC32 plasmid DNA. A 1.5-kb fragment was also successfully amplified using the primers that anneal to the pMDC32 vector overlapping the *attR1-FAD2-4-attR2* region.

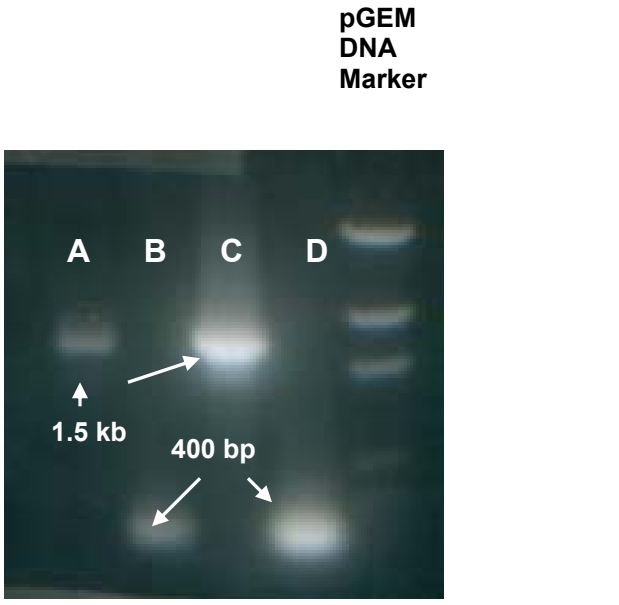


Fig 32. Agarose gel electrophoresis of the PCR products from PCR amplification of the cotton *FAD2-3* gene using transgenic Arabidopsis plants. The PCR products were electrophoresed on a 1.0 % agarose gel containing ethidium bromide at a final concentration of 0.5 µg/ml, with pGEM DNA Marker standards. (A): The 1.5-kb product amplified from Arabidopsis genomic DNA (*FAD2-3*/pMDC139 line) using primers that amplify the *attR1/FAD2-3/attR2* region. (B): The 400-basepair PCR product amplified from Arabidopsis genomic DNA (*FAD2-3*/pMDC139 line). (C), (D): The control reactions using purified *FAD2-3*/pMDC139 plasmid DNA.

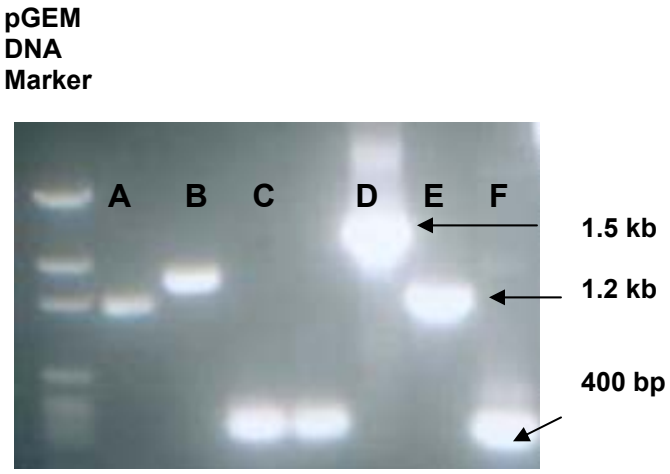


Fig. 33 Agarose gel electrophoresis of the PCR products from PCR amplification of the cotton *FAD2-4* gene using transgenic Arabidopsis plants. The PCR products were electrophoresed on a 1.0 % agarose gel containing ethidium bromide at a final concentration of 0.5 µg/ml, with pGEM DNA Marker standards.

(A): The complete 1.2-kb coding region of the *FAD2-4* gene amplified from Arabidopsis genomic DNA *(FAD2-4*/pMDC32 line).

(B): The 1.5-kb fragment amplified from Arabidopsis genomic DNA (*FAD2-4*/pMDC32 line) using primers that amplify the *attR1/FAD2-4/attR2* region.

(C): The 400-basepair PCR product amplified from Arabidopsis genomic DNA (*FAD2-4*/pMDC32 line).

(D), (E), (F): The control reactions using purified *FAD2-4*/pMDC32 plasmid DNA.

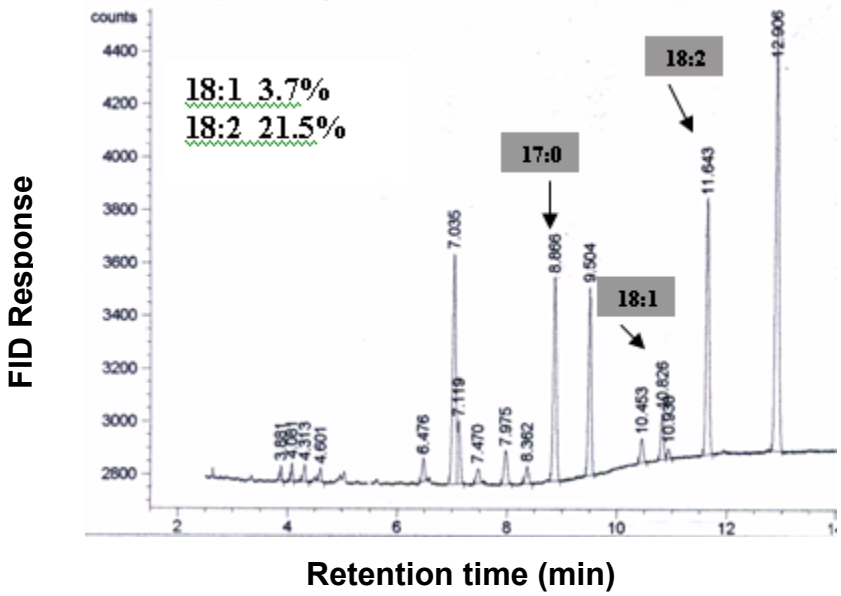
The *FAD2-4* gene is distinctly different from the *FAD2-3* gene, with minor sequence differences in the coding regions and major differences in the flanking regions (Pirtle et al., 2001). Six *FAD2-3*/pMDC32 lines of transgenic Arabidopsis were also generated to observe the function of the cotton *FAD2* gene. The pMDC43 vector contains the GFP (green fluorescent protein from the jellyfish *Aequorea victoria)* reporter genes at the C-terminal fusion of the inserted genes, and can be used for the analysis of subcellular localization of target proteins (Curtis and Ueli**,** 2003). The *FAD2-4* gene coding region was inserted downstream of the CamV 35S promoter and GFP reporter gene. After Arabdsopsis plant transformation and the screening procedure (as described previously), a total of five seedlings had hygromycin resistance. These Arabidopsis T_1 *FAD2-4*/pMDC43 plants were transferred into pots, grown, and the Arabidopsis T_2 seeds were harvested.

Lipid Extraction and Fatty Acid Analysis of Arabidopsis Plants Transformed with Cotton *FAD2* Genes

After successfully screening lines of Arabidopsis plants transformed with the *FAD2-3*/pMDC139 vector, the *FAD2-4*/pMDC139 vector, the *FAD2-4*/pMDC32 vector, the *FAD2-3*/pMDC32 vector, and the *FAD2-4*/pMDC43 vector, genomic DNAs were isolated and the cotton *FAD2* genes were amplified by PCR. All the Arabidopsis lines and their T_2 and T_3 generation plants were grown to maturity and the seeds were harvested for further experiments. In order to prove that transgenic Arabidopsis plants transformed with cotton *FAD2* genes were truly generated and to also demonstrate that the cotton *FAD2* gene was functionally expressed in the transgenic Arabidopsis plants, quantitation of fatty acid methyl esters (FAMESs) derived from the phospholipids in Arabidopsis whole plants and individual leaf tissues was done by gas chromatography (GC). Since *fad2* knockout mutant Arabidopsis plants were used for the transformation, the most straightforward way to rigorously demonstrate that the cotton *FAD2* genes in five different mutant Arabidopsis lines was functionally expressed was to study their respective fatty acid profiles, especially the ratios of 18:2 to 18:1. A method adapted from the paper by Focks and Benning (1998) was used for the lipid extraction procedure.

The GC data showed that the 18:2 to18:1 ratio is significantly decreased in Arabidopsis *fad2* knockout mutant plants as compared to the Arabidopsis wild type plants (Figure 34 A, B). The insertion of the *FAD2-3*/pMDC139 and *FAD2-4*/pMDC139 constructs (all lines of each constructs were tested by GC) did not change the mutant profile as expected (Figure 34, C, D), indicating that although the *FAD2* genes were detected by PCR amplification from the Arabidopsis *FAD2*/pMDC139 lines, the FAD2

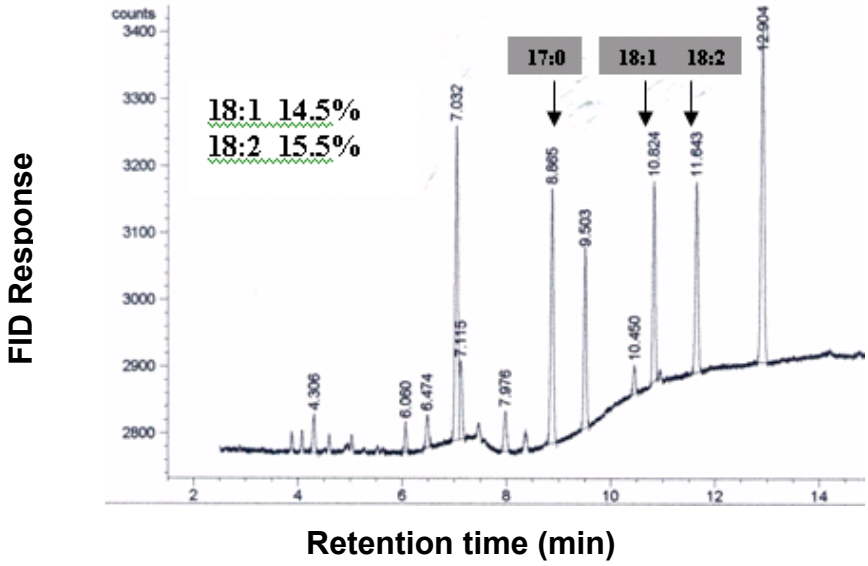
enzymes, the transmembrane protein plus GUS, were not functionally expressed in the these lines. The putative transgenic Arabidopsis transformed with *FAD2-4*/pMDC32 construct was much more promising, since the ratio of 18:2 to 18:1 was significantly increased as compared to the Arabidopsis *fad2* knockout mutant plants (Figure 34E), but not sufficiently to the level of the wild type Arabidopsis plants. These results suggested that the cotton *FAD2* genes were functionally expressed, and that the 18:2/18:1 ratio differences between the wild type plants and the transgenic *FAD*2-4/pMDC32 lines might be caused by segregation during the generation growth, since individual plants were not separated during the GC analyses.



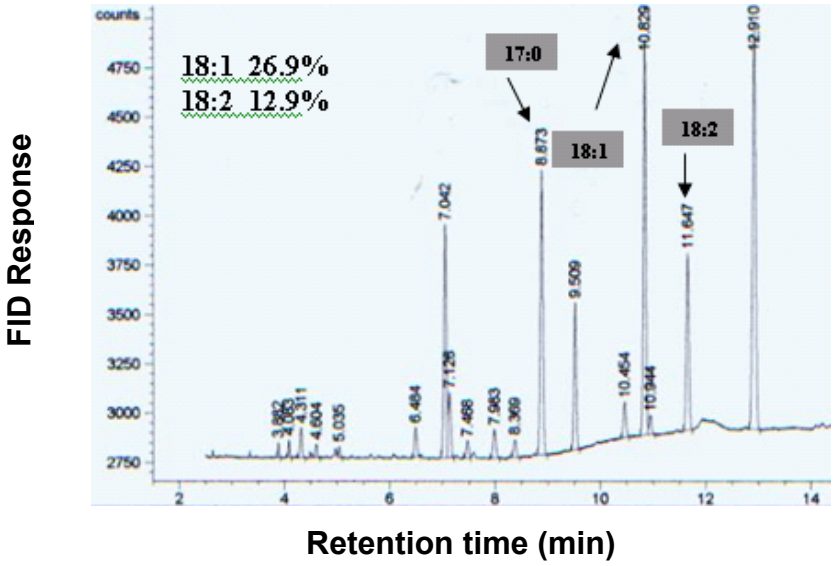
A. Fatty acid methyl ester profile in wild type Arabidopsis whole plants

(Figure 34 continues)

(Figure 34 continued)



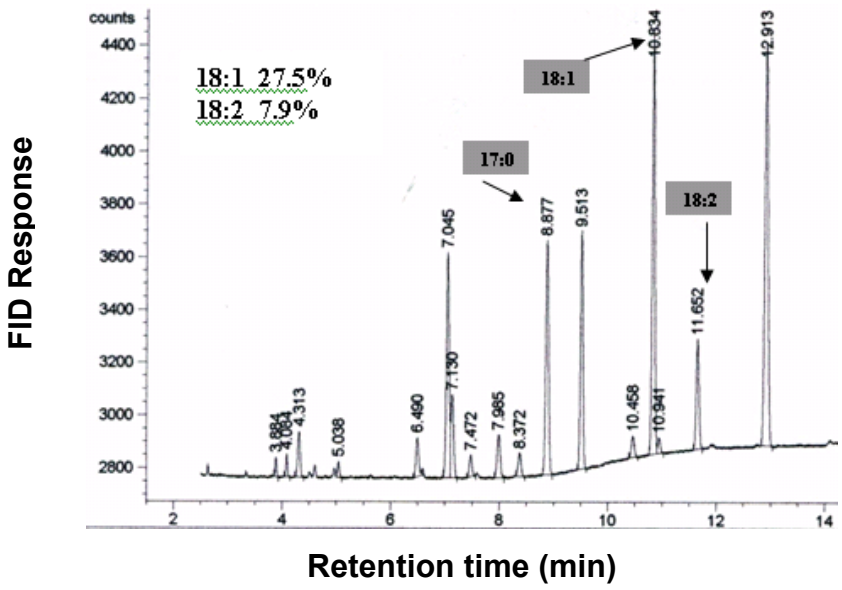
B. Fatty acid methyl ester profile in *fad2* mutant knockout Arabidopsis whole plants



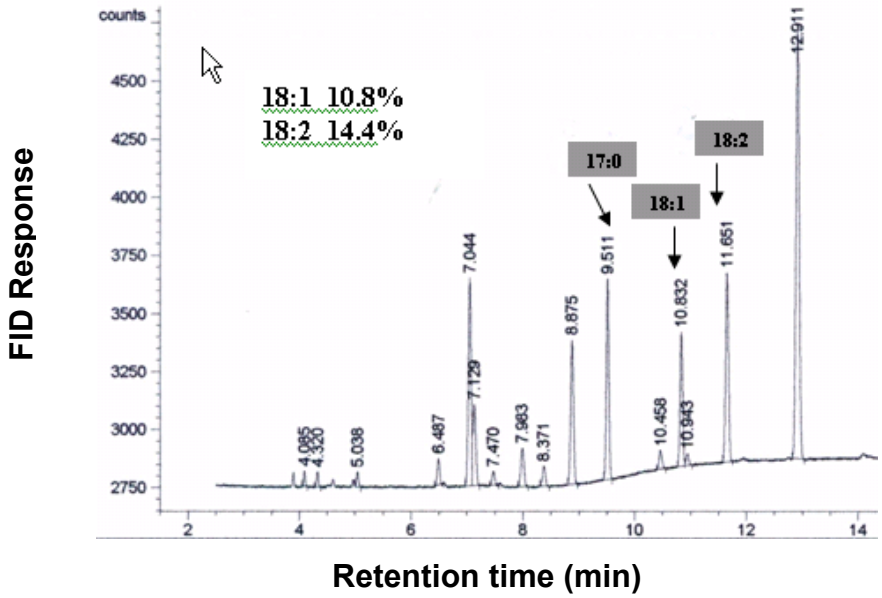
C. Fatty acid methyl ester profile in transgenic Arabidopsis whole plants transformed with the *FAD2-3*/pMDC139 construct

(Figure 34 continues)

(Figure 34 continued)



D. Fatty acid methyl ester profile in transgenic Arabidopsis whole plants transformed with the *FAD2-4*/pMDC139 construct



E. Fatty acid methyl ester profile in transgenic Arabidopsis whole plants transformed with the *FAD2-4*/pMDC32 construct

Fig. 34 The quantitation of fatty acid methyl esters (FAMESs) of the phospholipids in Arabidopsis wild type plants (A), *fad2-1* knock out mutants (B), and cotton *FAD2*/pMDC-transformed *fad2-1* mutant Arabidopsis plants by gas chromatography (GC). The 18:2 /18:1 ratio is significantly decreased in Arabidopsis *fad2* knockout mutant plants as compared to the Arabidopsis wild type plants (A, B). The insertion of the *FAD2-3*/pMDC139 and *FAD2-4*/pMDC139 constructs did not change the mutant profile as expected (C, D). The ratio of 18:2/18:1 in *FAD2-4*/pMDC32 plants was significantly increased as compared to the Arabidopsis *fad2* knockout mutant plants (B, E), but not sufficiently to the level of the wild type Arabidopsis plants (A, E).
plants was used to prepare each sample, and the T_3 seedlings used for this analysis were not screened by hygromycin MS plates.

To test this hypothesis, the experiment was repeated and the leaf tissue was randomly picked from nine transgenic *FAD*2-4/pMDC32 individual plants. Among these individual plants, four showed a fatty acid profiles similar to wild type Arabidopsis plants, while the other five showed fatty acid profile similar to the *fad2* knockout mutant Arabidopsis (Figure 35, and Table 2).

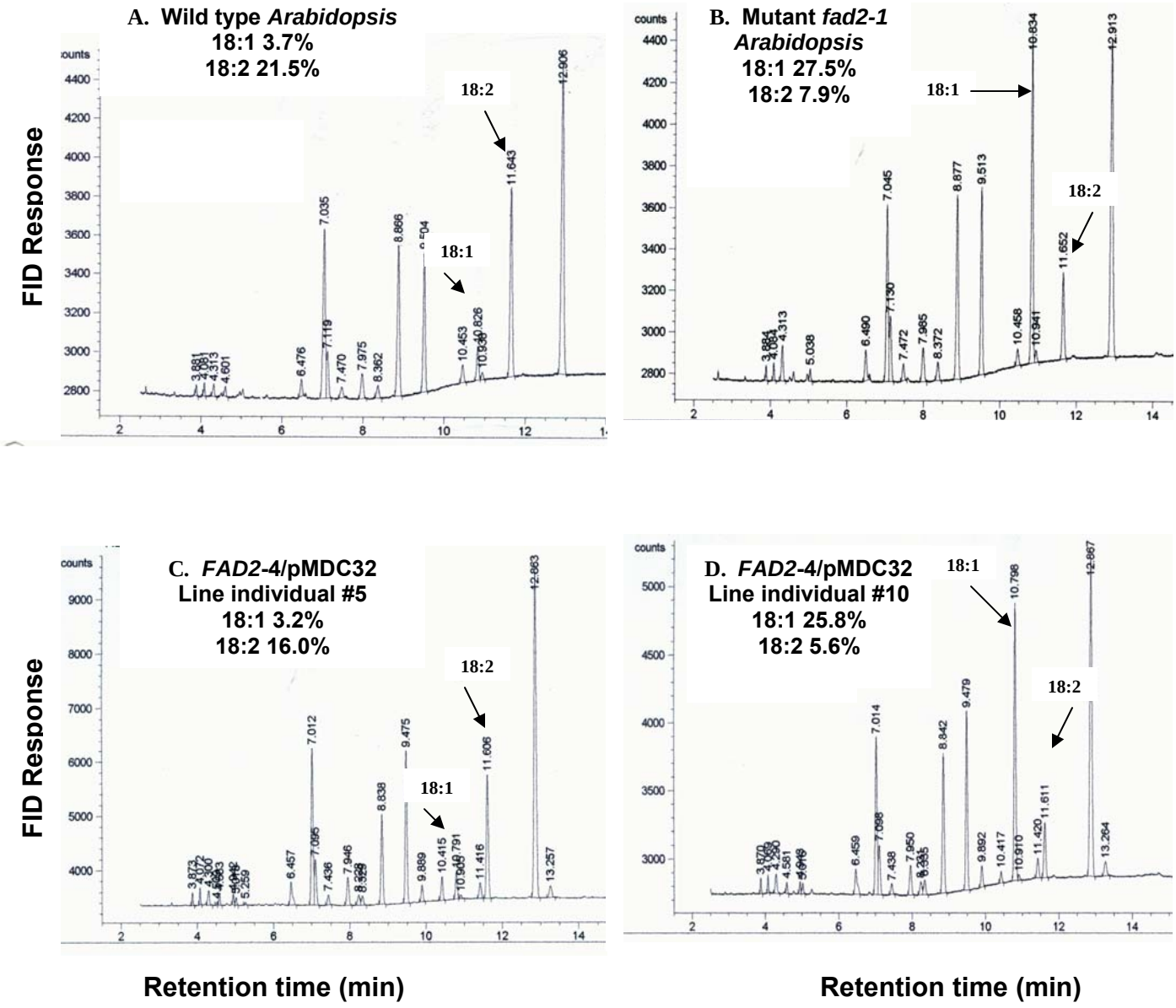


Fig. 35 Fatty acid methyl ester profiles of (A) Arabidopsis wild type, (B) *fad2-1* mutant, and (C, D) *FAD2-4*/pMDC32 transformed Arabidopsis plants. (A), (C): The normal wild type ratio of 18:1 to 18:2 fatty acids was restored in four of the individual Arabidopsis T3 plants transformed with the cotton *FAD2-4* /pMDC32 DNA construct; (D): Other individual T3 plants of the *FAD2-4* /pMDC32 line lost the inserted cotton *FAD2-4* gene due to the segregation and showed similar fatty acid profiles to the mutant *fad 2-1*

Arabidopsis (C). A total of nine individual T_3 plants derived from the same *FAD2-4* /pMDC32 line were used. The profiles of individuals #5 and #10 are shown here as examples.

The fatty acid methyl ester profiles of the wild type, *fad2-1* mutant, and *FAD2-4*/pMDC32-transformed Arabidopsis plants showed that the normal wild type ratio of 18:1/18:2 fatty acids was restored in four of the individual Arabidopsis T3 plants transformed with the *FAD2-4* /pMDC32 DNA construct (Figure 35; A, C). Other individual Arabidopsis T3 plants of the *FAD2-4* /pMDC 32 line lost the insert containing the cotton *FAD2-4* gene due to the segregation, and showed similar fatty acid profiles as did the mutant *fad 2-1* Arabidopsis plants (Figure 35; B, D).

The REDExtract-N-Amp Plant PCR Kit from Sigma was used to rapidly extract and amplify genomic DNAs from leaves of individual plants of the transgenic Arabidopsis *FAD2-4*/pMDC32 line for further confirmation. The *Act8* gene was also amplified as a control. Shown in Figure 36 are six-week old Arabidopsis *fad2-1* plants (yellow arrow), wild type plants (Col-0, turquoise arrow), or transgenic Arabidopsis T_3 progeny of *fad2-1* transformed with the cotton *FAD2-4* gene. Five plants on the left (red bar) exhibit the mutant growth phenotype, whereas the four plants on the right (blue bar) exhibit the wild type growth phenotype. The PCR analyses of genomic DNAs isolated from the plants are also shown in Figure 36 to confirm the presence or absence of the cotton *FAD2* gene.

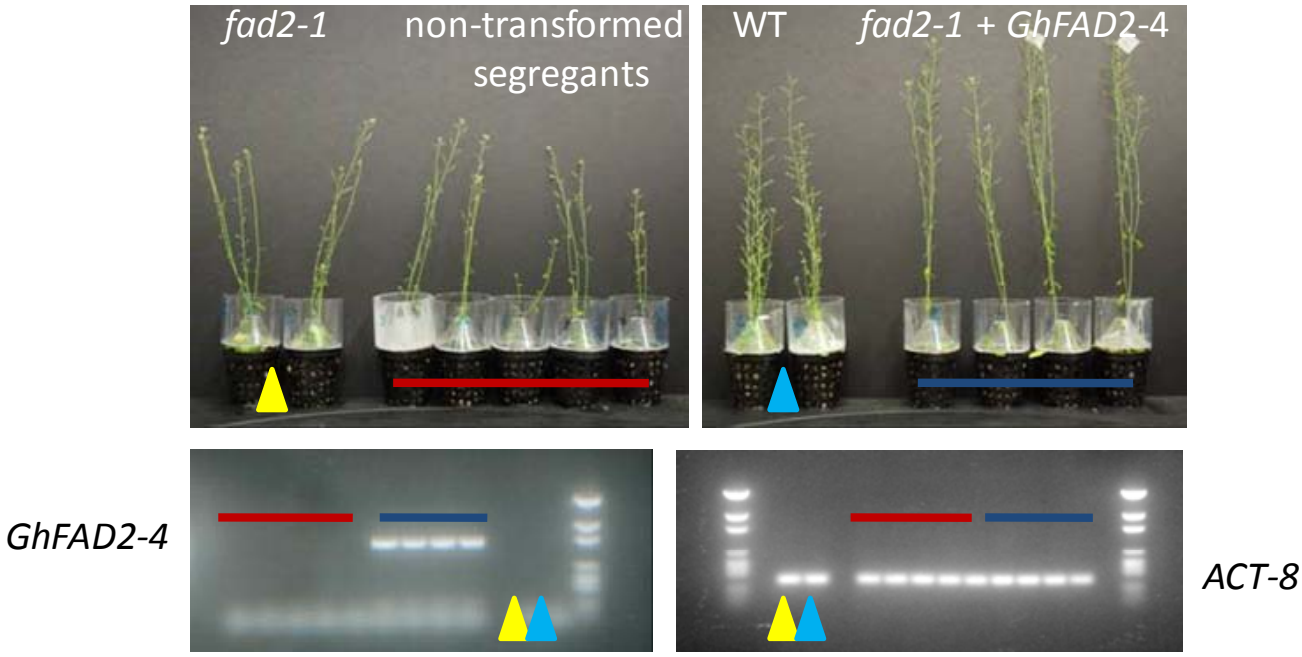


Fig. 36 Six-week old Arabidopsis *fad2-1* knockout (yellow arrow) plants, wild type (Col-0, turquoise arrow) plants or T_3 progeny of *fad2-1* transformed with cotton the *FAD2-4* gene. Five plants on the left (red bar) exhibit a mutant growth phenotype, whereas the four plants on the right (blue bar) exhibit a wild type growth phenotype. Lower panels, PCR analysis of genomic DNAs isolated from plants in upper panels. The red bar represents plants on the left and the blue bar represents plants on the right. A control *fad2-1* mutant and col-0 (WT) are included for comparison. Lower left: cotton *FAD2-4* specific primers; lower right: amplification of actin (*ACT-8*) gene product as a control.

Table 2. Summary of the fatty acid compositions of leaves of Arabidopsis wild type (col-0), *fad2* knockout mutant, and the *fad2* mutant lines transformed with cotton *FAD2-4*/pMDC constructs. Four of the nine T_3 plants sampled have wild type 18:1/18:2 ratios (red font), confirming the functional activity of the cotton FAD2-4 polypeptide in Arabidopsis plants. Fatty acid methyl esters were separated and quantified by GC-FID using 17:0 as a standard. All values are given as mol % of fatty acids. The value for each individual plant is the average of three samples ± SD.

Fatty Acid	Wild type	*fad2-1* mutant	*FAD2-4*/pMDC32 transformed *Arabidopsis fad2-1* plants								
			#1	#5	#7	#9	#3	#4	#6	#8	#10
16:0	16.7 ± 0.12%	13.9 ± 0.10 %	20.5 ± 0.17%	17.2 ± 0.11%	24.0 ± 0.14%	21.0 ± 0.09%	16.2 ± 0.15%	14.9 ± 0.09%	12.5 ± 0.11%	12.1 ± 0.16%	14.7 ± 0.16%
16:1	6.7 ± 0.02 %	5.3 ± 0.05%	7.1 ± 0.08%	4.5 ± 0.04%	5.4 ± 0.05%	5.7 ± 0.07%	6.3 ± 0.05%	6.3 ± 0.15 %	6.0 ± 0.09%	5.7± 0.07 %	4.5 ± 0.03%
16:3	12.4 ± 0.20%	14.8 ± 0.34%	14.5 ± 0.12%	16.0 ± 0.10%	15.0 ± 0.09%	15.2 ± 0.08%	14.5 ± 0.13%	14.1 ± 0.32%	12.6 ± 0.28%	13.8 ± 0.15%	15.2 ± 0.39%
18:0	0.9 ± 0.03 %	1.1 ± 0.03%	2.1 ± 0.02%	2.5± 0.01%	2.9 ± 0.04%	3.0 ± 0.01%	1.1 ± 0.02%	0.9 ± 0.01%	1.1 ± 0.04 %	1.2 ± 0.02%	1.0 ± 0.02%
18:1	2.1 ± 0.11%	20.1 ± 0.72%	2.4 ± 0.09%	3.0 ± 0.03%	2.2 ± 0.01%	2.6 ± 0.19%	22.7± 0.65 %	24.9 ± 0.47%	20.6 ± 0.33%	21.2 ± 0.28%	24.2 ± 0.55%
18:2	14.3 ± 0.15%	7.1 ± 0.25%	15.6 ± 0.13%	14.2 ± 0.12%	13.6 ± 0.10%	14.3 ± 0.12%	7. 2 ± 0.35%	9.4 ± 0.19%	6.8 ± 0.07%	6.6 ± 0.10%	5.2 ± 0.09%
18:3	46.8 ± 0.21%	37.5 ± 0.50%	37.0 ± 0.17%	42.5 ± 0.32%	36.2 ± 0.25%	38.0 ± 0.42%	31.0 ± 0.23%	30.0± 0.38 %	40.4 ± 0.47%	39.3 ± 0.46%	35.0 ± 0.43%

The *fad2* mutant Arabidopsis has phenotypes distinct from those of the wild-type plants regarding their pattern of inflorescence stem growth. At 22˚C, the total stem length of *fad2* mutants was 80-90% of that for wild-type plants (Miquel et al., 1993). When the stem lengths of all the Arabidopsis plants were compared, the T3 generation of Arabidopsis individual plants transformed with cotton *FAD2-4*/pMDC32 constructs (that lost the inserted genes by segregation) showed similar stem lengths to *fad2* mutant knockout plants (Figure 36). The individuals that contained cotton *FAD2-4* (proved by PCR) and functionally expressed the gene (confirmed by GC analysis of the fatty acid profile) showed similar stem lengths to wild type Arabidopsis plants (Figure 36). This observation suggested that the complementation of the cotton *FAD2* gene in *fad2* mutant knock-out Arabidopsis plants was indeed functional. Although the similarity between the cotton *FAD2-4* gene sequence and that of the Arabidopsis *fad2* cDNA is 67% (Pirtle et al., 2001), the function, or maybe the regulation, of the *fad2* gene is highly conserved between the cotton and Arabidopsis plants.

The *FAD2-4* gene is distinctly different from another cotton *FAD2* gene (the *FAD2-3* gene) with minor sequence differences in the coding regions and major differences in the flanking regions (Pirtle et al., 2001). The deduced amino acid sequences of the two putative FAD2 polypeptides both have 384 amino acids, with six amino acid differences. Six *FAD2-3/pMDC32* transgenic Arabidopsis lines were also generated. The leaf tissues from all six lines were used for GC fatty acid analyses. Among all six cotton (ct) *FAD2* gene transformed lines, one line, named Ct*FAD2-3/pMDC32#6*, showed a reversed complementary effect of the 18:2/18:1 ratio (Figure 37). This result indicated that *FAD2-4* and *FAD2-3* may be two cotton isoenzymes with similar functions.

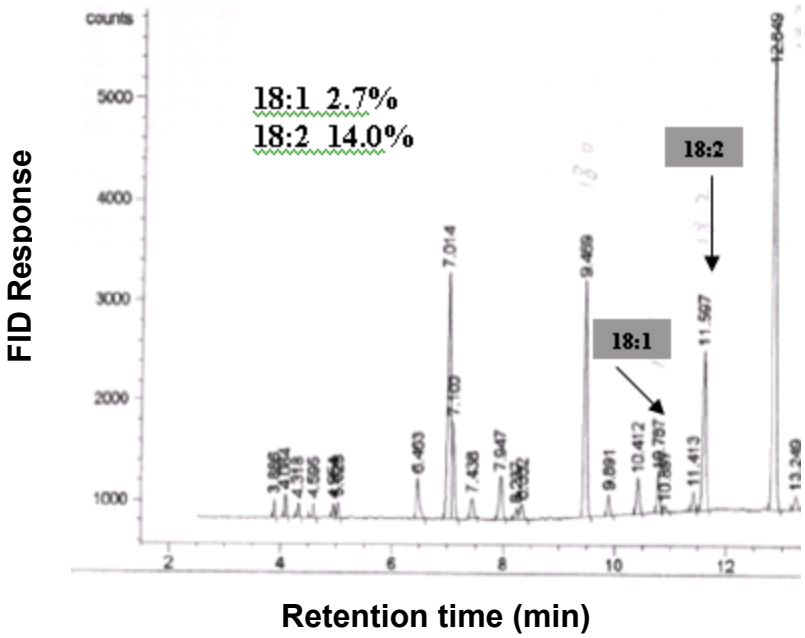
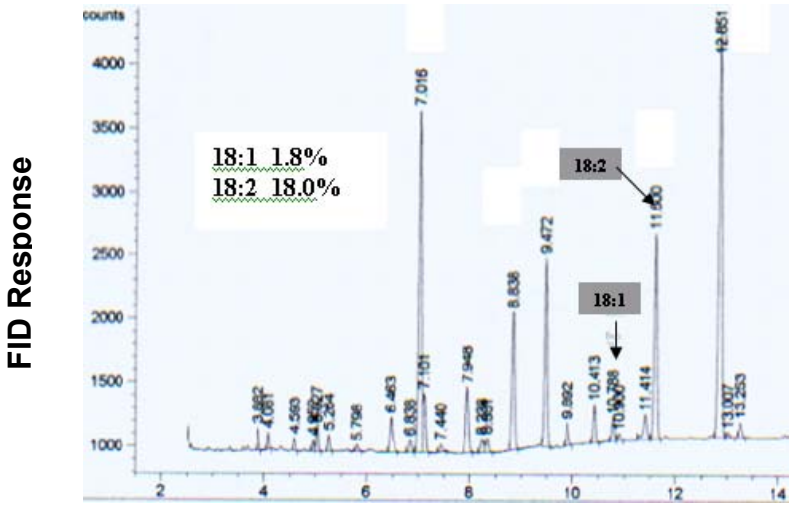
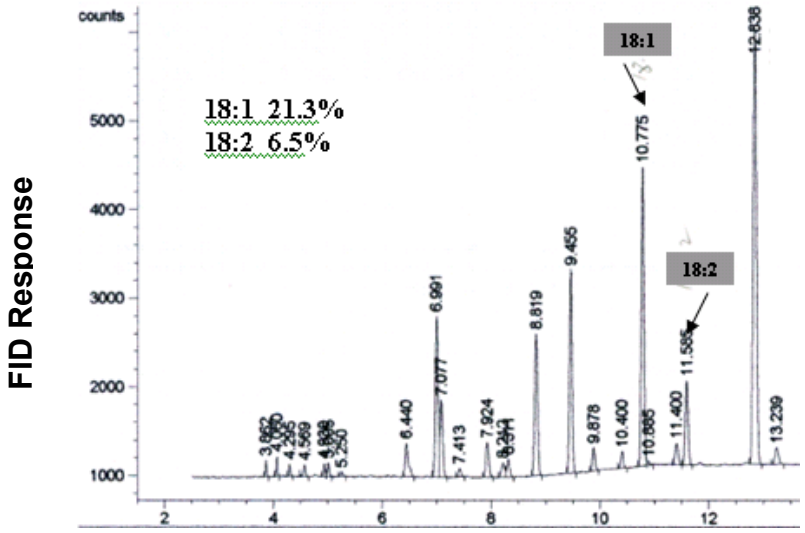


Fig. 37 The quantitation of fatty acid methyl esters (FAMESs) of the phospholipids in Arabidopsis plants transformed with the *FAD*2-3/pMDC32 construct. Among all six lines, individual plant Ct*FAD2-3/pMDC32#6* showed a reversed complementary effect of the 18:2/18:1 ratio. The insertion of the *FAD2-3*/pMDC32 significantly increased the ratio of 18:2/18:1 as compared to the Arabidopsis *fad2* knockout mutant plants and almost to the level of the wild type Arabidopsis plants.

After plant transformation and hygromysin screening, five *FAD2-4*/pMDC43 transgenic Arabidopsis lines were generated. Then GC fatty acid analyses were done using their leaf tissues. Three lines (designated Ct*FAD2-4/pMDC43#1,* Ct*FAD2-4/pMDC43#2,* and Ct*FAD2-4/pMDC43#5*) showed the reversed complementary effect of the 18:2/18:1 ratio, which is similar to the wild type (Figure 38A). These individuals were later used to visualize the GFP localization by confocal imaging. The other two lines (Ct*FAD2-4/pMDC43#3* and Ct*FAD2-4/pMDC43#4)* with fatty acid profiles similar to the Arabidopsis *fad2* knockout mutant (Figure 38B) were used as control plants.



A. Ct*FAD2-4/pMDC43#1*



B. Ct*FAD2-4/pMDC43#3*

Fig. 38 A fatty acid methyl ester profile of leaf tissue of transgenic Arabidopsis plants transformed with *FAD*2-3/pMDC43 construct. Among all five lines, three lines, named Ct*FAD2-3/pMDC43#1, #2, and #5* showed reversed complementary effect of the 18:2/18:1 ratio (A). The other two lines named Ct*FAD2-4/pMDC43#3* and *#4* lost the

inserted cotton *FAD2-4* gene due to the segregation and showed similar fatty acid profiles to the mutant *fad2-1* Arabidopsis (B).

Confocal Microscopy using Arabidopsis Plants Transformed with Cotton *FAD2* Genes

The leaf tissues were sampled from the transgenic Arabidopsis *FAD2-4*/pMDC43 lines, positioned on glass slides with water, and then covered with cover slides. All imaging was conducted on a Zeiss Zoom using a C Aprochro mat lens (8X/0.15, 40X/1.20, 64X/1.25), and attached to a CSU-10 Yokogama Confocal Scanner. GFP was imaged using a 488-nm excitation and its emission was collected from 502.5-537.5 nm, or 515-545 nm if imaged in combination with YFP. GFP and YFP were imaged sequentially using a Niptium Spinning disk. The optimal pinhole diameter was set at 2.52 Airy units in all cases. Post-acquistion image processing was done using ImageJ software and green color was assigned to GFP expression while red and blue represent background.

After GC fatty acid analysis using leaf tissues, the transgenic Arabidopsis *FAD2-4*/pMDC43 lines, like the *FAD2-4*/pMDC32 plants, also showed a reversed complementary effect of 18:2/18:1 ratio, which is similar to the wild type (Figure 38). These individuals were later used to visualize the GFP localization by confocal imaging. The lines with fatty acid profiles similar to the Arabidopsis *fad2* knockout mutant plant were used as control plants. In plants in which the fatty acid and growth phenotypes were restored to wild type levels, the cotton FAD2-4 fusion protein was expressed in all cell types in a pattern resembling an endomembrane (ER), network-like distribution (Figure 39, lower right, green fluorescence) around chloroplasts (reddish in color) and throughout the cytoplasm.

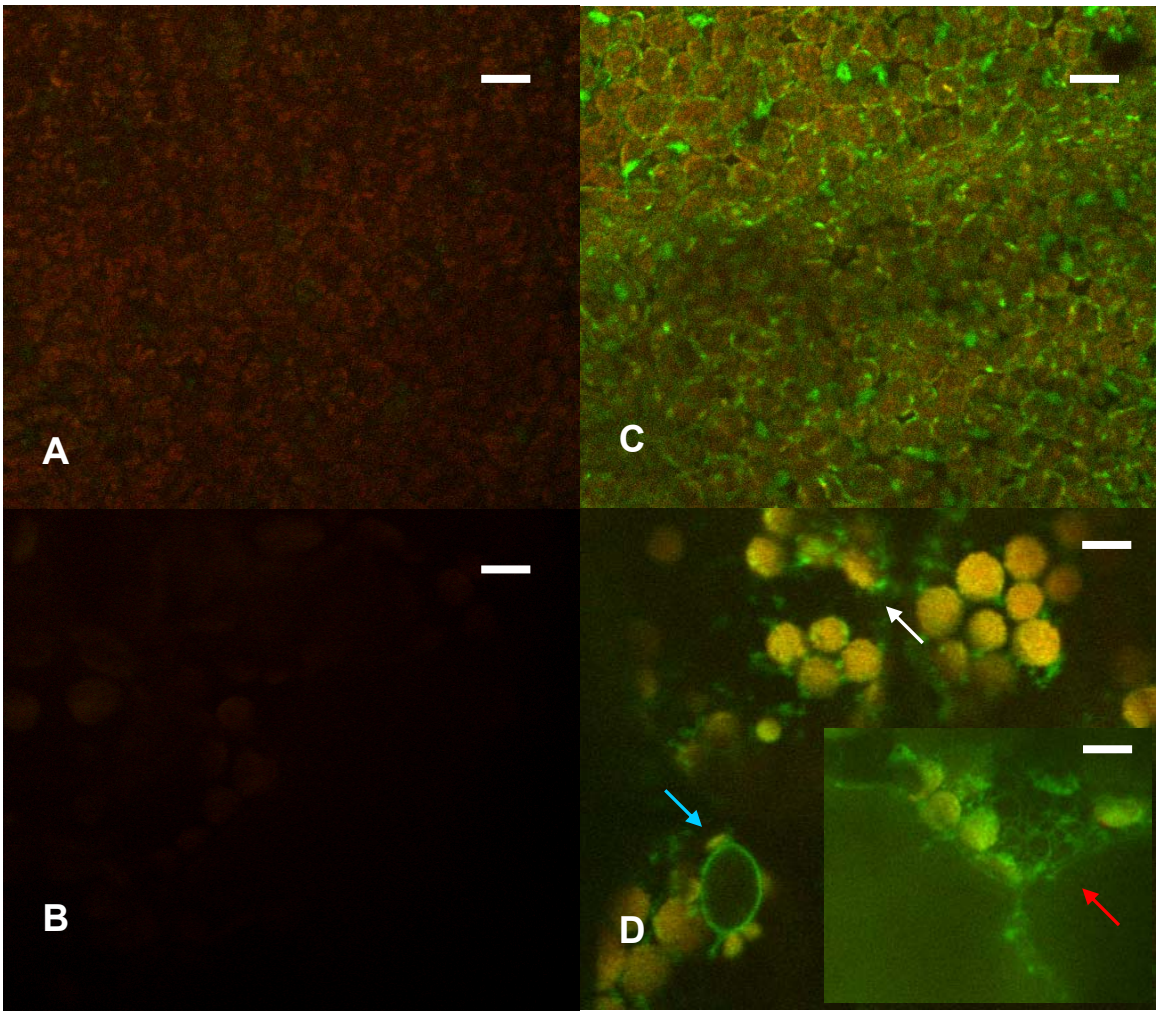


Fig 39. Confocal images of leaf epidermal cells of *FAD2-4*/pMDC43-transformed Arabidopisis T_2 plants. Images at 8X (A), 8X (C), 40X (B), 40X (D), of non-transformed control leaves (left panels) and transgenic leaves (right panels). Transgenic Arabiodopsis (Columbia ecotype) was in the *fad2-1* mutant background, and transgenic plants exhibited a mutant-to-wild type reversal of growth and fatty acid composition (Table 1, Figure 38). GFP expression was detected in cotton *FAD2-4* transformed plants (C), (D) compared to the control (A), (B). The cotton FAD2-4 fusion protein was expressed in a pattern resembling an endomembrane (ER), network-like distribution

outside chloroplasts (reddish in color) and throughout the cytoplasm (D). Bar = 16 μm in Images B, D, and inset on D, Bar = 80 μm in Images A and C.

The distinctive comparison between the images panels in A and C in Figure 39 suggests GFP expression in the transgenic line *CtFAD2-4/pMDC43#1.* The same areas of images A and C were enlarged 40 times. No GFP expression was detected in the control plants (Figure 39. Panel B). In contrast, more detailed cellular localization was detected using a Ct*FAD2-4/pMDC43#1* slide. A network structure of GFP expression was detected (Figure 39. Panel D, indicated by white arrow). It appears to be the ER membrane bound with cotton the GFP-FAD2-4 tagged protein. Image inside Panel D of Figure 39 provides more detail about this network structure in the relative position of chloroplasts (indicated by red arrow). In the same picture, no GFP expression is noticeable in the nucleus (indicated by blue arrow). From all the images, GFP expression can be distinctive in the areas close to the cell membrane.

The FAD2 enzyme is believed to be responsible for desaturation of fatty acids present in extraplastidial membranes (Matos et al., 2007). These data provide information for the subcellular localization of cotton FAD2 protein in the endoplasmic reticulum, and proved again that the FAD2 enzyme genes leads to desaturation of membrane lipids at sites outside the chloroplast.

Comparison of Temperature Sensitivities between Arabidopsis Wild Type, Arabidopsis *fad2* Knockout Mutant, and Cotton *FAD2*-Transformed Arabidopsis Plants

To prove the cotton *FAD2* gene important for low temperature survival, the Arabidopsis *fad2-1* knockout mutant, wild type Arabidopsis, and cotton *FAD2-4* transformed Arabidopsis *fad2-1* knockout mutant plants were used to study whether the biochemical effects of the *fad2* gene knockout could be reversed by the transformation of Arabidopsis *fad2-1* knockout mutant with cotton *FAD2* genes. Seeds of all Arabidopsis lines were sown at half-inch spacing into pots. Seeds were germinated and plants were grown in a 22ºC growth room for 10 days and then transferred to a 5ºC growth room with the same illumination conditions. After three weeks, photographs of individual plants were taken and the phenotypic distinctions between each line were observed. As Figure 40 shows, the leaves of the *fad2-1* mutant, as well as the individuals from the T_3 generation of the *FAD2-4*/pMDC43 line and the *FAD2-4*/pMDC32 line (which lost the inserted gene due to segregation) began to deteriorate, displaying small patches of necrosis

***FAD2-4*/pMDC43-#3** ***FAD2-4*/pMDC32-#5** **Wild type**

(Figure 40 continues)

(Figure 40 continued)

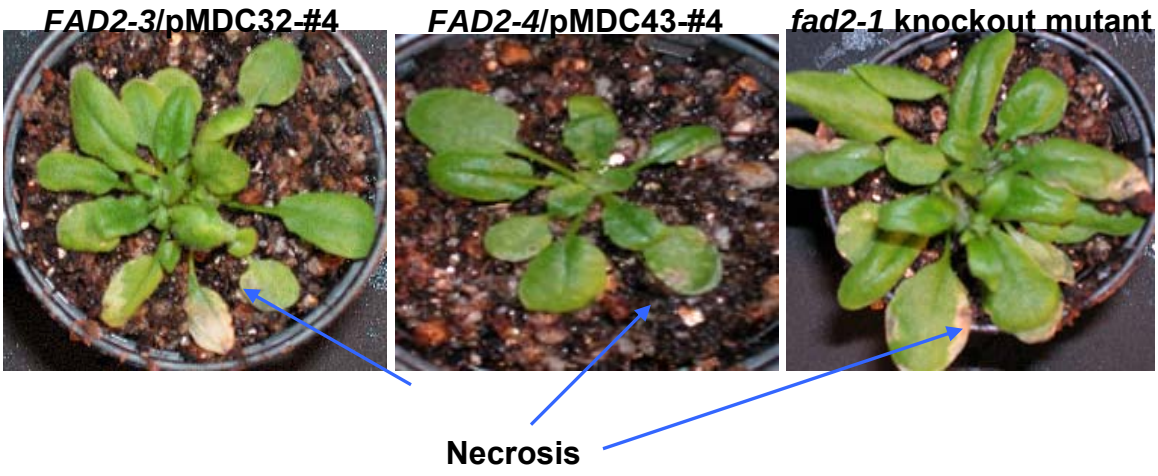


Fig. 40 Images of Arabidopsis plants for a comparison of temperature sensitivities between Arabidopsis wild type, Arabidopsis *fad2* knockout mutant and cotton *FAD2*-transformed Arabidopsis Plants. Seeds were germinated and plants were grown in a 22ºC growth room for 10 days and then transferred to a 10ºC growth room with the same illumination conditions. After three weeks, the leaves of the *fad2-1* mutant, as well as the individuals from the T_3 generation of the *FAD2-4*/pMDC43 line and the *FAD2-4*/pMDC32 line (which lost the inserted gene due to segregation) began to deteriorate, displaying small patches of necrosis (See the three images on the top). These phenomena were not observed in the wild type, in the *FAD2-4*/pMDC43 line, and the *FAD2-4*/pMDC32 line (See the three images at the bottom) of which the functional expression of cotton FAD2 polypeptides by GC analysis and GFP confocal microscopy had been confirmed.

These phenomena were not observed in the wild type, in the *FAD2-4*/pMDC43 line, and the *FAD2-4*/pMDC32 line of which the functional expression of the cotton FAD2 polypeptides by GC analysis and GFP confocal microscopy had been confirmed.

This data suggested the essential role of the cotton *FAD2-4* gene in maintaining cellular function and plant viability at temperatures toward the low end of the physiological range, and the Arabidopsis *fad2* knockout mutant has provided a model system to probe the mechanism of membrane lipid unsaturation in cotton plants.

CHAPTER 4

DISCUSSION

Cotton is a valuable source of natural fiber, feed, and edible oil (Zapata et al., 1999; Zhang et al., 2001; Wu et al., 2005). Environmental stress, such as low temperatur e and drought, destroys much of the cotton crop and subsequently results in large economic losses every year. Traditional breeding methods have produced varieties of cotton with improved agronomic traits, but the lack of useful economic traits in commercial cotton cultivars has been a major challenge (Wu et al., 2005). Therefore, new strategies are being used, based on plant defense mechanisms to improve plant tolerance to low temperature, to improve agricultural production and decrease the cotton yield loses due to cold weather.

In many higher plants, 18:2 and 18:3 fatty acids account for more than 70% of the fatty acids in leaf cells and 55 to 70% of the fatty acid in nonphotosynthetic tissues such as roots (Harwood, 1980). The important function of the ER 18:1 desaturase is to provide unsaturated fatty acids required for the correct assembly of cellular membranes throughout the plant, as well as providing the polyunsaturated fatty acids found in vegetable oils that, in turn, are the major source of essential fatty acids in most human diets (Okuley et al., 1994). Fatty acid desaturases are thought to specifically play important roles in the plant defense system. Due to the wide-ranging temperature effect of the *FAD* genes, it may be possible to use the *FAD* genes as a novel defense that is effective against low temperature damage. Therefore, the ultimate goal of this dissertation research was to try to genetically engineer cotton *FAD* genes to routinely overproduce the *FAD* protein in transgenic cotton plants as a natural defense against low temperature.

The initial research presented in this dissertation was done in collaboration with Dr. Irma Pirtle of our laboratory. It involved the DNA sequencing and analysis of the structure of the *FAD2-4* gene, depicted in the physical maps shown in Appendix A. Ms. Stacy Park also contributed a major component of this project, which was the transformation of both cotton *FAD2* genes into the yeast cells. A cotton genomic fragment harboring the *FAD2-4* gene in the clone LCFg5b was deduced to be 17.9 kb by physical mapping and alkaline blot hybridization, as shown in Appendix A. A large, solitary intron occurs in the 5'-untranslated region (5'-UTR) of the gene, followed by the coding region. The physical maps of the genomic clones encompassing the *FAD2-4* gene (Zhang et al., 2008) and the *FAD2-3* gene (Pirtle et al., 2001) are quite different, suggesting they diverged from a common ancestral gene, although due to the allotetraploid nature of the cotton genome, the precise relationship of these two genes to each other (e.g., orthologous, paralogous, or homeologous) remains uncertain.

Once the *FAD2-4* gene was amplified from cotton cDNA, the locations of the tentative cap site, the 5'- and 3'-intron/exon splice junctions in the 5'-UTR, and the coding region of the *FAD2-4* gene were inferred from comparisons with the sequence of the *FAD2-4* cDNA PCR product and the *FAD2-3* gene and cDNA (Pirtle et al, 2001). Thus, the identity of a cotton cDNA corresponding to the *FAD2-4* gene has been confirmed, indicating that the cotton *FAD2-4* gene was indeed transcribed (Figure 3).

As shown in Appendix C, the identities between the deduced amino acid sequence of the FAD2-4 polypeptide and those of the cotton FAD2-3 desaturase (GenBank **AF331163**) (Pirtle et al., 2001), the cotton FAD2-2 protein (GenBank **Y10112**) (Liu et al., 1997), and the cotton FAD2-1 enzyme (GenBank **X97016**) (Liu et al., 1999) 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 of amino acid sequences deduced from the cDNA sequences in the GenBank database)(Tang et al., 2005). The identities of the DNA sequences of the *FAD2-4* gene with the cotton *FAD2-3* gene, the cotton *FAD2-2* cDNA, and the cotton *FAD2-1* cDNA 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.

Regulation of the expression of the *FAD2* gene is still not well understood, and the tissue specific expression pattern of these genes will help to understand the mechanisms behind heat and cold tolerance, salt tolerance, and disease resistance. RT-PCR analyses of leaf, stem, and root tissue of cotton (*G. hirsutum*- 213) with different growth times and under different treatments showed different patterns of expression for *FAD2-3* and *FAD2-4* genes. High-level expression of the *FAD2-3* gene was observed in root, leaf, flower bud, and fiber explants while lower level expression was observed in stem, seeds and hypocotyl explants. The *FAD2-4* is expressed in all the explants, including lower level expression in seeds (Appendix E). Based on the data in this dissertation, the *FAD2* gene expression is more likely to be regulated by plant hormones like ABA then by environmental stress like low temperature or oxidative stress (the H_2O_2 treatment) (Figure 12, 13, 14). The research on *SeFAD2* provides the

similar result that the FAD2 transcript can be induced by ABA treatment. ABA can induce *FAD2* and *FAD3* genes in rapeseed. Under all the treatments, leaf tissue shows the most stable expression of the FAD2 polypeptides and root tissue shows the most drastic change (Figure 11, 12, 13, 14). Linoleic acid (18:2) is part of the lipid exchange between the chloroplast and endoplasic reticulum, and the *FAD3* gene can be induced by low temperature (Houriguchi et al., 2000). It is possible that under environmental stress, to increase the polyunsaturated lipid in the cell membrane, the plant would rather use more 18:2 exchanged from chloroplast and turn on the *FAD3* gene to generate more FAD3 polypeptide and hence18: 3 in the cell membrane, than to induce the *FAD2* gene to produce FAD2 enzyme, which converts 18:1 to 18:2. This hypothesis has also been indicated by Somerville's research (1992), in which wild type Arabidopsis plants were transferred from a 22˚C growth room to 5˚C and their lipid fatty acid profiles were compared, the 18:2 FA decreased while 18:3 increased. Chapman et al (2005) determined that the 18:3 was present in the highest percentage among the total fatty acid extracted from cotton plants. This can also possible explains why root shows the most changed *FAD2* expression pattern. Because in the tissues like root that lack chloroplasts (Miquel et al., 1993), there is no 18:2 backup for the endoplasic reticulum membrane and the *FAD2* gene is more likely to be regulated to produce polyunsaturated fatty acid. Evidence from several Arabidopsis mutants indicates that lipid exchange between the ER and the chloroplast is reversible to some extent (Browse et al., 1993), because extra chloroplastic membranes in mutants deficient in ER desaturases contain polyunsaturated fatty acid derived from the chloroplasts. The study of cotton FAD2 expression in this dissertation suggested no transcriptional regulation,

but possible post-translational regulation, and this data also suggests that the tissue specificity of the expression of *FAD2* gene was modified in accordance with the growth phase in plant development (Figure 11). The *FAD2* genes appear to be regulated in a complex way in response to changes in the environment or other stress-induced factors. Okuley (1994) presented another theory that in Arabidopsis, the *FAD2* transcript may be present several fold in excess of the amount needed to account for the enzyme activity, and that this excess is maintained to ensure that the enzyme activity is never limited by availability to transcript. This concept is consistent with our observations on cotton *FAD2* expression.

The genetic approach has been very useful in studies of metabolic pathways, including the pathways of lipid biosynthesis (Carman and Henry, 1989; Twyman et al., 2002). Many of the enzymes are membrane-bound proteins that have been refractory to purification and characterization by traditional biochemical techniques (Zambryski et al., 1983; Zupan, 2000). Since the same barriers exist to the investigation of membrane lipid synthesis in higher plants, yeast cells and Arabidopsis *fad* mutants have contributed to the knowledge of the biochemistry and regulation of cotton lipid synthesis (Dyer et al., 2001).

The 1.2-kb coding region of the cotton *FAD2-4* gene was successfully expressed in transformed yeast cells, indicating that the gene does indeed encode a functional FAD2 enzyme. Yeast cells are eukaryotic and contain an ER 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). Hence, yeast expression systems are commonly employed for the functional

identification of eukaryotic fatty acid desaturases in the ER, such as the *Arabidopsis* FAD2 (Covello and Reed, 1996; Kajiwara, 1996), the *Caenorhabditis elegans* ∆-12 desaturase (Peyou-Ndi et al., 2000), a white spruce ∆-9 desaturase (Marillia et al., 2002), and a fungal ∆-6 desaturase (Zhang et al., 2004). The yeast system was previously used to functionally analyze the cotton *FAD2-3* gene (Pirtle et al., 2001). Similarly, the yeast cells transformed with the plasmid construct pYES2/*FAD2-4* produced a substantial amount of linoleic acid (C18: 2), clearly indicating the conversion of oleate into linoleate in the yeast cells containing the plasmid construct with the *FAD2-4* coding region (shown in Appendix D). No linoleic acid was detected in the control yeast cells transformed with the pYES2 shuttle vector alone. Thus, the cotton *FAD2-4* gene has been functionally identified, since it encodes an enzyme that catalyzes the desaturation of oleate into linoleate.

Yeast cells transformed with the *FAD2-4* gene construct, the *FAD2-3* gene construct, or with the plasmid vector (pYES2) were grown at three different temperatures (10^{o}C, 20^{o}C). At both temperatures, the growth curves of the yeast cells transformed with the *FAD2-4* and *FAD2-3* genes were found to parallel the growth curves of the control yeast cells (Figure 4). Peyou-Ndi et al. (2000) examined the low-temperature effects of heterologous expression of the *C. elegans* ∆-12 desaturase gene in yeast cells, and found that increased membrane polyunsaturation confers a growth rate advantage to transformed yeast cells grown at 12^{o}C, but not at higher temperatures. They stated that membrane polyunsaturation might be necessary for growth of yeast cells at low temperatures, but that it is only one of the factors involved in survival or growth at low temperatures. In this study, the chilling of yeast cells expressing cotton

FAD2 from 20 to 10˚C resulted in significant increases in the amount of respective desaturase products (Figure 5), which is 18:2 fatty acid, indicating a post-transcriptional mechanisms involved in regulation of cotton desaturase. Thus, cold temperature studies of heterologous desaturase genes in the yeast model system would appear to vary with the particular gene used and even the host yeast strain employed, among other variables. It seems that the yeast model system is excellent for qualitatively assessing the functional expression of heterologous desaturase genes, but cold temperature studies with different heterologous desaturase genes in yeast transformants should be interpreted with caution.

The *FAD2-4* and *FAD2-3* genes may be paralogs, since the gene sequences are strongly conserved, but the 3'-flanking regions and 5'-UTR intron sequences are somewhat different in size and sequence. It is possible that one of the *FAD2* genes may have been duplicated to give rise to the other *FAD2* gene, and hence be homologs, but, if so, the genes have greatly diverged, based on the differences between the 3'-flanking regions and 5'-UTR introns. In contrast, the two *FAD2* open reading frames, and the FAD2 polypeptides have 98% identities, reflecting strong conservation of the FAD2 structure-to-function relationships. Liu et al. (1999) estimated there to be at least five *FAD2* genes in the cotton genome, at least two copies each of the *FAD2-1* and *FAD2-2* genes, corresponding to the A and D genome orthologs (Liu et al., 1999; Liu et al., 2001). Our laboratory detected two possible *FAD2-3* genes in the cotton genome by genomic blotting (Pirtle et al., 2001). Since the physical map and sequence around the *FAD2-4* gene is quite different from that around the *FAD2-3* gene, it is possible that there are two *FAD2-3* genes and two *FAD2-4* genes in the cotton genome. Thus, there

could be as many as eight or more *FAD2* genes in the allotetraploid cotton genome. The allotetraploid soybean genome seems to have four *FAD2* genes, two copies each of the soybean *FAD2-1* and *FAD2-2* genes (Heppard et al., 1996). Scheffler et al. (1997) estimated there to be four to six copies of the *FAD2* gene in the *Brassica napus* genome. Mikkilineni and Rocheford (2003) found four different map loci for *FAD2* cDNAs in maize, indicating four possible *FAD2* genes in that diploid genome. The structural comparison of the two cotton *FAD2* genes has revealed both similarities and differences between the 5'-UTR introns and the 3'-flanking regions (Appendix B), but great similarities in the promoter motifs and the protein-coding regions of the two genes, thus making it possible to gain insight into how the two *FAD2* genes are regulated, and if the 5'-UTR introns really have any bearing on transcriptional regulation of the genes.

In plants, FAD2 is the enzyme that converts oleic acid (18:1) to linoleic acid (18:2) by introducing a double bond at the Δ-12 positions (Somerville et al., 2000). The fatty acid composition was studied using two Arabidopsis *fad2* mutant plants and wild type plants. They determined that Arabidopsis *fad 2-1* mutant plants had significantly increased 18:1 fatty acid levels and decreased 18:2 fatty acid levels (Okuley et al., 1994). To aid in the evaluation of the function of cotton *FAD2* genes, *FAD2-4* and *FAD 2-3* genes were inserted into two pMDC binary vectors (Figure 26), and Arabidopsis *fad 2-1* (*FAD2* knockout) plants without endogenous *FAD2* gene activity are being used for transformation experiments. This way cotton FAD2-4 and 2-3 can be evaluated for their ability to complement the knockout phenotype at the biochemical level (restoration of fatty acid composition) and physiological level (restoration of normal growth and temperature sensitivity).

To demonstrate that the cotton *FAD2* gene is functionally expressed in the Arabidopsis plants, the quantity of fatty acid methyl esters (FAMESs) of the phospholipids in Arabidopsis leaf tissues were measured by gas chromatography (GC). Because T-DNA-containing lines segregated for the insert, it was necessary to sample several individuals from each line. When using the Arabidopsis T_3 generation of cotton *FAD2-4*-transformed Arabidopsis individual plants for GC analyses, the results indicated that the cotton *FAD2-4* gene can functionally substitute for the Arabidopsis *FAD2* gene in the *fad2-1* mutant background, and segregations happened during the growth from the T_2 Arabidopsis to the T_3 generation (Figure 35). This was demonstrated by fatty acid composition, in which the normal wild type ratio of 18:1 to 18:2 fatty acids was restored in four of the individual Arabidopsis T_3 plants transformed with the cotton *FAD2-4*/pMDC32 DNA construct (highlighted in red type, Table 1; Figure 35, A and C). Due to segregation, some of the individual T_3 plants of the *FAD2-4*/pMDC32 line lost the inserted cotton *FAD2-4* gene and showed similar fatty acid profile to the mutant *fad 2-1 Arabidopsis* (in black letters, Table 1; Figure 35, B and D). As reported by Miguel et al. (1992), in phosphatidylcholine of leaves of *fad2* plants, the decrease in polyunsaturated fatty acids was accompanied by a 40-60% reduction in the level of 16:0, as compared with wild type plants. A significant increase in the proportion of 16:0 was observed in all individual Arabidopsis T_3 plants of *FAD2-4* /pMDC32 with Arabidopsis wild type plant ratio of 18:1/18:2 (Table 1, plants numbered as #1, #5, #7, #9). These results indicated that the complementary effect of the cotton *FAD2* gene in Arabidopsis *fad2* mutant is complete, and that both of the changes in fatty acid composition, the change of the 18:1/18:2 ratio and the increase of 16:0, are the result of a single gene insertion.

The Arabidopsis *fad2-1* mutant has phenotypes distinct from those of the wild-type plants regarding their pattern of stem growth. At 22˚C, the total stem length of *fad2-1* mutants was 80-90% of that for wild-type plants (Miguel et al., 1993). The growth phenotypes of the individual Arabidopsis T3 plants transformed with the cotton *FAD2-4*/pMDC32 DNA constructs (Figure 36, upper panel) matched either the *fad2-1* mutant background (Figure 36, upper left) or wild type (Figure 36, upper right), consistent with their fatty acid composition phenotypes (Table 1). PCR analyses of the genomic DNA extracted from all nine plant lines confirmed that those with the ratios of 18:1/18:2 fatty acids most resembling wild type indeed contained the *FAD2-4* DNA sequence integrated into the Arabidopsis genome (Lower left panel, under blue solid line), whereas the five T_3 plants that retained the mutant growth and fatty acid phenotypes did not contain the cotton sequences, indicating that these null phenotypes were non-transgenic segregants from the original transgenic T_1 parent (under red solid line). These data provide compelling evidence that the cotton FAD2-4 can function biochemically to restore the Arabidopsis *fad2* mutant to wild type fatty acid composition and growth. Although the similarity between the cotton *FAD2-4* gene sequence and the Arabidopsis *FAD2* cDNA sequences is only 67% (Pirtle et al., 2001), the function, or maybe the regulation of the *FAD2* gene, is highly reserved between cotton and Arabidopsis. These cotton *FAD2-4*-expressing plants exhibited close to wild type temperature sensitivity by the later test.

The *FAD2-4* gene is distinctly the *FAD2-3* gene, with minor sequence differences in the coding regions and major differences in the flanking regions (Pirtle et al., 2001). Similar results in transgenic Arabidopsis (functional complementation of *fad2-1* fatty

acid and growth phenotypes) have been obtained with the cotton *FAD2-3*/pMDC32 transformed lines (Figure 37), indicating that FAD2-4 and FAD2-3 polypeptides may be two cotton isoenzymes with the same function.

Expression of GFP-tagged *FAD 2-4* gene in Arabidopsis plants was accomplished to visualize the subcellular distribution of the cotton FAD2-4 polypeptide in the Arabidopsis *fad2-1* mutant background (Figure 39). This was accomplished with the pMCD43 binary vector, which features an in-frame fusion to the GFP at the N-terminus of the FAD2 protein. The *FAD2-4* gene coding region was inserted downstream of the CaMV 35S promoter and GFP reporter gene (Figure 15, B). After GC fatty acid analysis using leaf tissues, the *FAD2-4*/pMDC43 lines, like *FAD2-4*/pMDC32 plants, also showed a reversed complementary effect of a 18:2/18:1 ratio to the level of the wild type (Figure 38). These individuals were later used to visualize the GFP localization by confocal imaging. The lines with fatty acid profiles similar to *fad2* mutants were used as control plants. In plants in which fatty acid and growth phenotypes were restored to wild type levels, the cotton FAD2-4 fusion protein was expressed in all cell types in a pattern resembling an endomembrane (ER), network-like distribution (Figure 39, lower right, green fluorescence) around chloroplasts (reddish in color) and through out the cytoplasm. This distribution facilitated functional complementation of fatty acid and growth phenotypes, so likely represents the proper localization of the cotton FAD2 polypeptides in Arabidopsis plants. This is reasonable to expect, since the cotton FAD2-4 C-terminus possesses an ER-retrieval motif similar to other plant FAD2 proteins (Appendix B), but it was important to confirm. It is believed that since both cotton FAD2 enzymes lack a N-terminal hydrophobic signal

sequence, addition of the GFP epitope tag to the N-terminus of the protein would not likely affect targeting to the ER. FAD2 is believed to be responsible for desaturation of fatty acids present in extraplastidial membranes (Matos et al., 2007). The data from this dissertation provides strong support for the subcellular localization of the cotton FAD2-4 protein in the endoplasmic reticulum, and that the *FAD2* gene expression most likely controls desaturation of membrane lipids at sites outside the chloroplast.

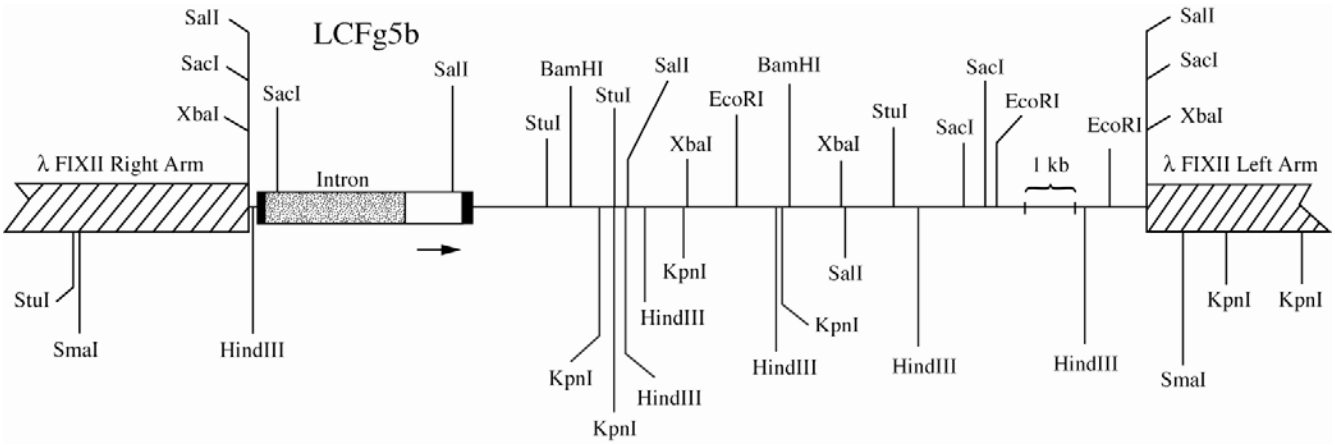
The phenotype studies using wild type, *fad2* mutant, and cotton *FAD2*-transformed Arabidopsis plants indicated that, in cotton plants, the polyunsaturated lipids very probably have an essential role in maintaining cellular function and plant viability at temperatures toward the low end of the physiological range because of the striking phenotypes distinct between the lines (Figure 36, 40). The absence of the FAD2 enzyme can disrupt specific processes in membrane metabolism, especially at low temperatures for cotton plants. This dissertation research on cotton FAD2 has indicated that its gene might not be a cold responsive gene, but the *FAD2* gene has an essential role in the long term due to its function in polyunsaturated lipid synthesis and could be regulated at either the translational or enzyme level. Overall, the regulation of fatty acid desaturation of membrane lipids appears to be intimately related to the wide range of mechanisms that allow plants to adapt to their environment throughout development.

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. Determining what accounts for the differences in freezing tolerance between plant species and the molecular basis of cold acclimation is of basic scientific interest especially and has the potential to provide new approaches to improve the freezing

tolerance of plants. This basic information will provide a knowledge base to help 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. The Arabidopsis plants, together with the Gateway Technology, have provided an excellent model system for studying cotton fatty acid desaturases and to manipulate tissue fatty acid compositions through over-expression and the use of antisense techniques. Such approaches will contribute to our understanding of how membrane lipid composition affects cotton plant function and may lead to the useful manipulation of cottonseed lipids to produce modified vegetable oils. This study has profound effects on the ability to produce engineered cotton plants with increased polyunsaturated fatty acids.

APPENDIX A

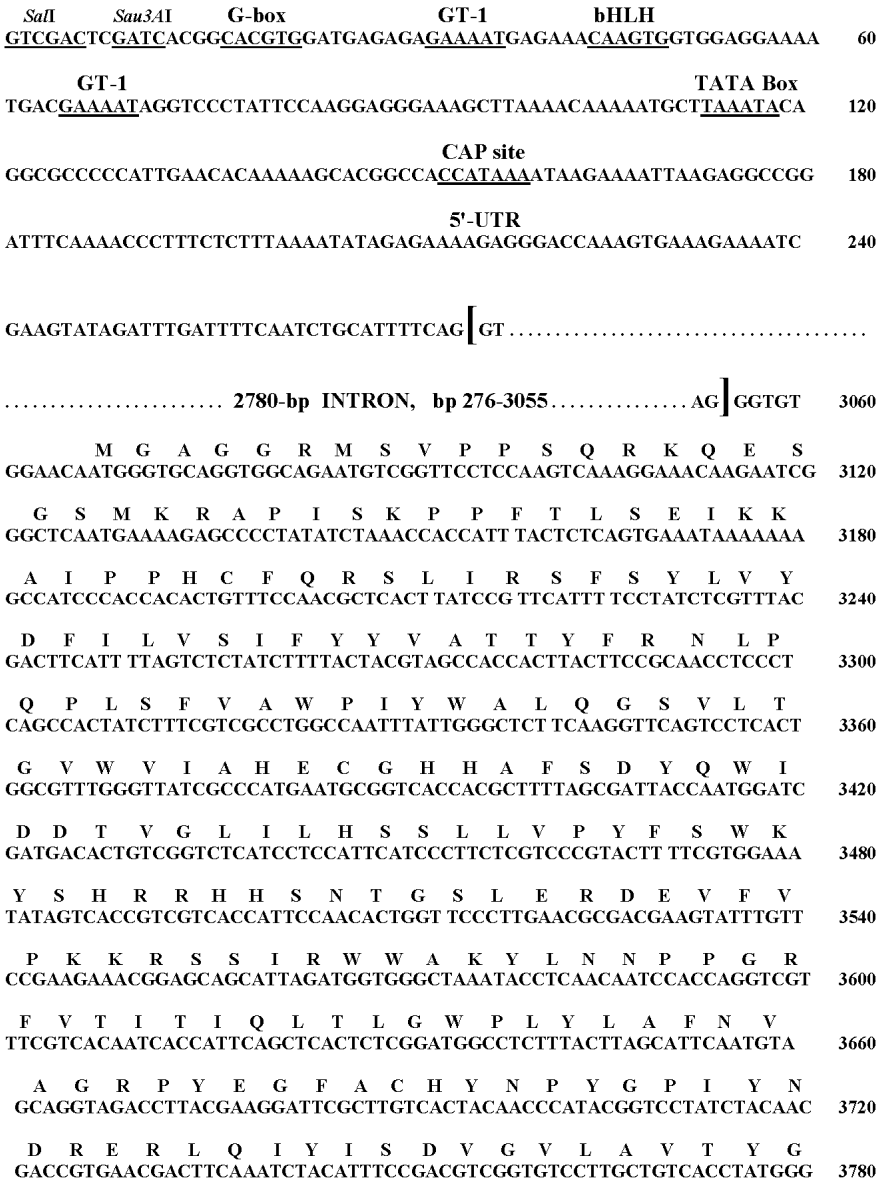
PHYSICAL MAP OF THE COTTON GENOMIC CLONE DESIGNATED LCFg5b ENCOMPASSING THE COTTON FATTY ACID DESATURASE (*FAD2-4*) GENE



The 17.9-kb cotton DNA segment is represented by the horizontal line, and the right and left arms of the Lambda FIXII (Stratagene) vector are represented by the cross-hatched areas. The white rectangle indicates the size and location of the 1.2-kb coding region of the gene, and the arrow denotes its relative polarity from 5' to 3'. The 5'- and 3'-untranslated regions (UTRs) of the mature *FAD2-4* mRNA are indicated by three small black rectangles. The single 2.8-kb intron in the 5'-UTR is depicted by the large stippled rectangle.

APPENDIX B

DNA SEQUENCE OF THE NONCODING STRAND OF AN 8.6-kb *XbaI* FRAGMENT IN A PLASMID SUBCLONE DESIGNATED pCFg5b ENCOMPASSING THE COTTON *FAD2-4* GENE

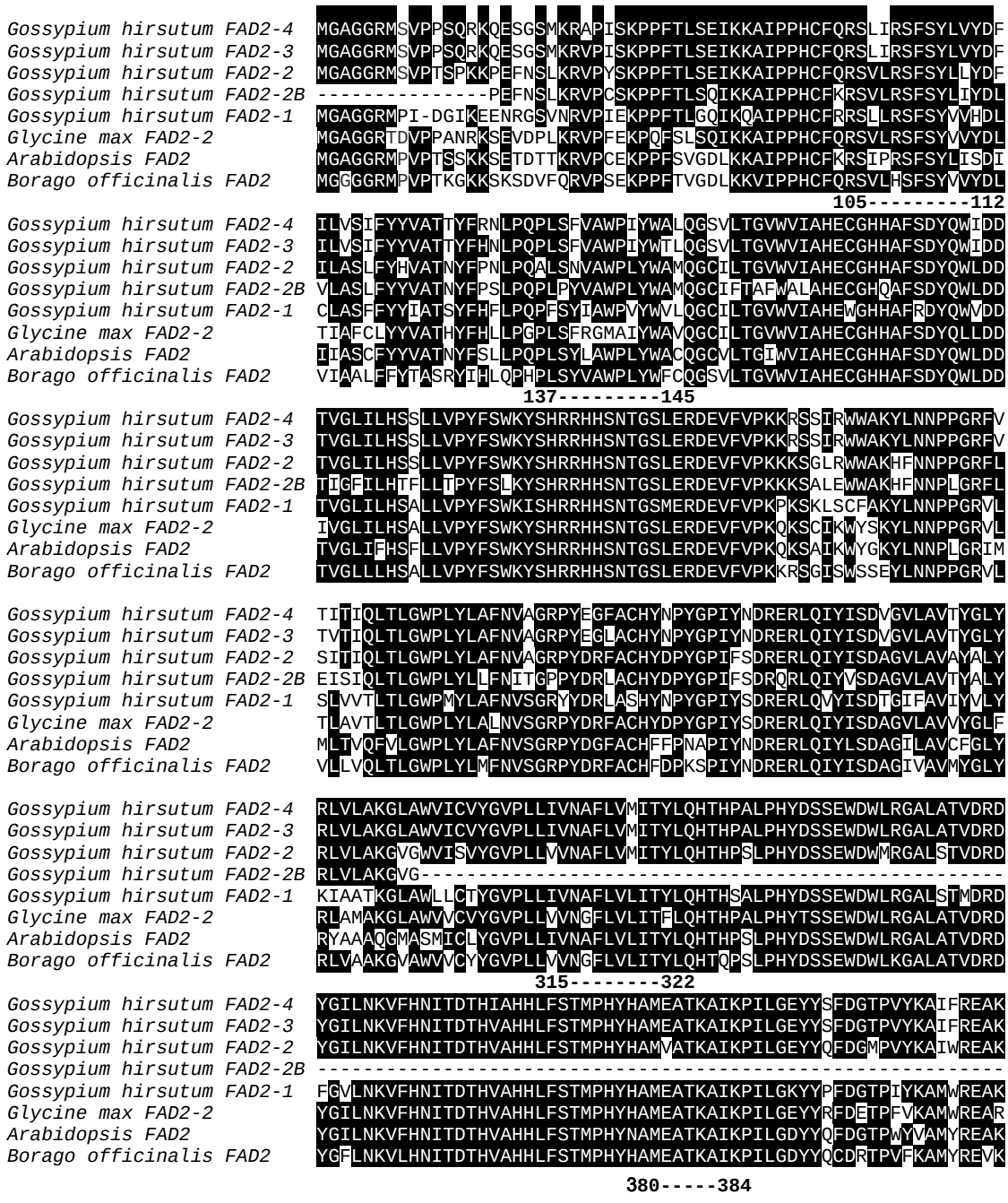

SalI
Sau3AI
G-box
GT-1
bHLH
GTCGACTCGATCACGGCACGTGGATGAGAGAGAAAATGAGAAACAAGTGGTGGAGGAAAA 60
GT-1
TATA Box
TGACGAAAATAGGTCCCTATTCCAAGGAGGGAAAGCTTAAAACAAAAATGCTTAAATACA 120
CAP site
GGCGCCCCCATTGAACACAAAAAGCACGGCCACCATAAAATAAGAAAATTAAGAGGCCGG 180
5'-UTR
ATTTCAAAACCCTTTCTCTTTAAAATATAGAGAAAAGAGGGACCAAAGTGAAAGAAAATC 240
GAAGTATAGATTTGATTTTCAATCTGCATTTTCAG[GT
2780-bp INTRON, bp 276-3055
AG]GGTGT 3060
M G A G G R M S V P P S Q R K Q E S
GGAACAATGGGTGCAGGTGGCAGAATGTCGGTTCCTCCAAGTCAAAGGAAACAAGAATCG 3120
19 G S M K R A P I S K P P F T L S E I K K
GGCTCAATGAAAAGAGCCCCTATATCTAAACCACCATT TACTCTCAGTGAAATAAAAAAA 3180
39 A I P P H C F Q R S L I R S F S Y L V Y
GCCATCCCACCACACTGTTTCCAACGCTCACT TATCCG TTCATTT TCCTATCTCGTTTAC 3240
59 D F I L V S I F Y Y V A T T Y F R N L P
GACTTCATT TTAGTCTCTATCTTTTACTACGTAGCCACCACTTACTTCCGCAACCTCCCT 3300
79 Q P L S F V A W P I Y W A L Q G S V L T
CAGCCACTATCTTTCGTCGCCTGGCCAATTTATTGGGCTCT TCAAGGTTCAGTCCTCACT 3360
99 G V W V I A H E C G H H A F S D Y Q W I
GGCGTTTGGGTTATCGCCCATGAATGCGGTCACCACGCTTTTAGCGATTACCAATGGATC 3420
119 D D T V G L I L H S S L L V P Y F S W K
GATGACACTGTCGGTCTCATCCTCCATTCATCCCTTCTCGTCCCGTACTT TTCGTGGAAA 3480
139 Y S H R R H H S N T G S L E R D E V F V
TATAGTCACCGTCGTCACCATTCCAACACTGGT TCCCTTGAACGCGACGAAGTATTTGTT 3540
159 P K K R S S I R W W A K Y L N N P P G R
CCGAAGAAACGGAGCAGCATTAGATGGTGGGCTAAATACCTCAACAATCCACCAGGTCGT 3600
179 F V T I T I Q L T L G W P L Y L A F N V
TTCGTCACAATCACCATTCAGCTCACTCTCGGATGGCCTCTTTACTTAGCATTCAATGTA 3660
199 A G R P Y E G F A C H Y N P Y G P I Y N
GCAGGTAGACCTTACGAAGGATTCGCTTGTCACTACAACCCATACGGTCCTATCTACAAC 3720
219 D R E R L Q I Y I S D V G V L A V T Y G
GACCGTGAACGACTTCAAATCTACATTTCCGACGTCGGTGTCCTTGCTGTCACCTATGGG 3780

The sequence has been assigned GenBank accession no. AY279314. The numbering on the right refers to the nucleotide (nt) residues, and the numbering on the left alludes to the amino acid residues in the deduced sequence of the conceptual FAD2-4 polypeptide. The *FAD2-4* open reading frame has 1,155 bp encoding 384 amino acids, from nt 3,067 to nt 4,221. The presumptive cap site of the *FAD2-4* mRNA is underlined at nt 153, with a 5'-UTR of about 135 nt. The 2,780-bp intron in the 5'-UTR is demarcated by brackets for the 5'- and 3'-splice sites [GT…AG] at nt 276 and nt 3,055, respectively. The 3'-polyadenylation site occurs at nt 4,449, and thus the 3'-UTR of the mRNA would be 228 residues in length. The near-upstream polyadenylation signal at nt 4,413 is underlined. Presumptive upstream promoter elements (underlined) include a

TATA box at nt 113, a basic region helix-loop-helix (bHLH) or E-box motif at nt 44, and a G-box element at nt 17. Also, two tentative GT-1 motifs are underlined at nt 65 and 32. The locations of the putative cap site, the intron-exon junctions, and the 3'-poly (A) site were deduced from comparison with the DNA sequence of the 1,328-bp PCR-amplified *FAD2-4* cDNA product (GenBank accession no. AY279315) and the *FAD2-3* gene and cDNA sequences. The 5'-flanking UTR intron sequence and a large segment of the 3'-flanking region sequence (both included in GenBank accession no. AY279314) have been omitted for brevity.

APPENDIX C

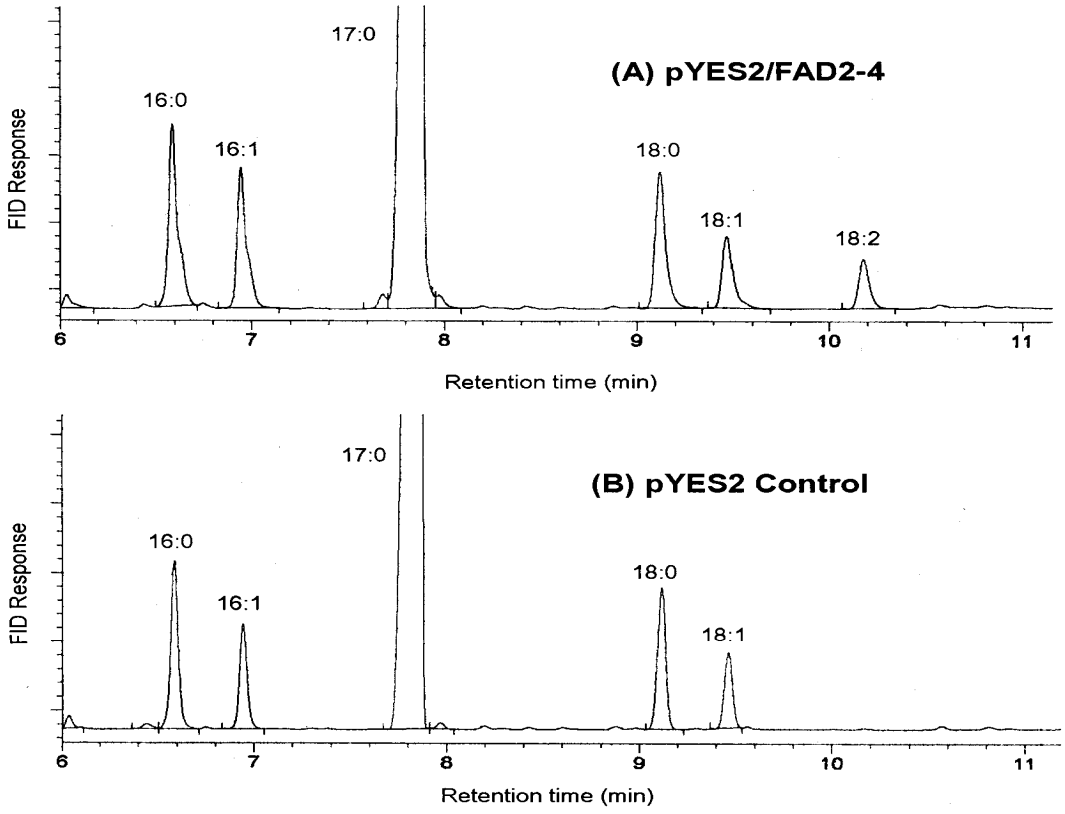
ALIGNMENT OF THE DEDUCED AMINO ACID SEQUENCES OF PLANT FAD2 POLYPEPTIDES


Gossypium hirsutum FAD2-4
Gossypium hirsutum FAD2-3
Gossypium hirsutum FAD2-2
Gossypium hirsutum FAD2-2B
Gossypium hirsutum FAD2-1
Glycine max FAD2-2
Arabidopsis FAD2
Borago officinalis FAD2
105---------112
137---------145
315--------322
380-----384

The alignment was done using DNASIS software (Hitachi), and conserved amino acids are indicated by reverse contrast. The amino acid sequences (with GenBank accession numbers) were derived from: *Gossypium hirsutum FAD2-4* gene (AY279314), *Gossypium hirsutum FAD2-2(B)* gene (EU363790), *Gossypium hirsutum FAD2-3* gene (AF331163), Go*ssypium hirsutum FAD2-2* cDNA (Y10112), *Gossypium hirsutum FAD2-1* cDNA (X97016), *Glycine max FAD2-2* cDNA (L43921), *Arabidopsis FAD2* cDNA/gene (L26296), and *Borago officinalis FAD2* cDNA (AF074324).

APPENDIX D

ANALYSIS OF FATTY ACID METHYL ESTERS (FAMEs) EXTRACTED FROM YEAST TRANSFORMANTS USING GAS CHROMATOGRAPHY WITH FLAME IONIZATION DETECTION (FID)

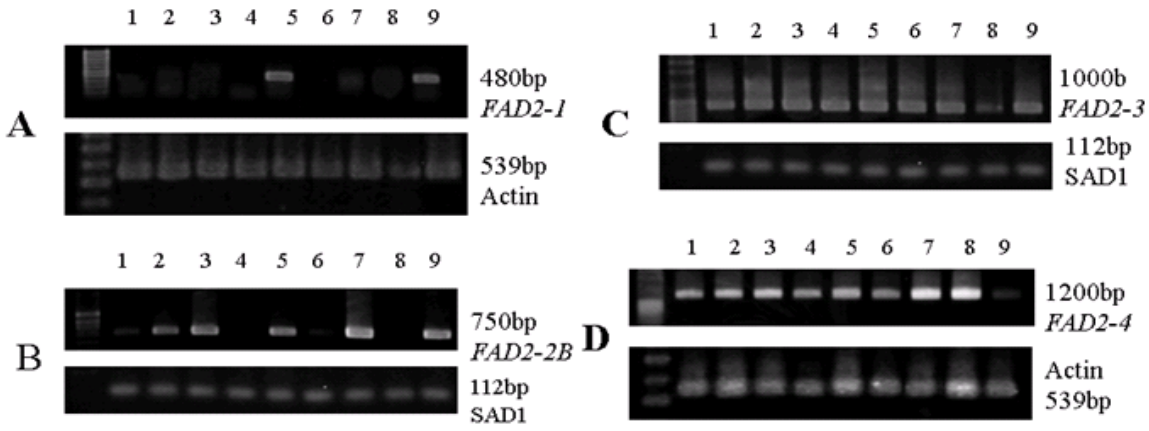


(A) Analysis of *Saccharomyces cerevisiae* strain INVSc1 cells transformed with the recombinant plasmid pYES2/*FAD2-4*, grown in SC-U (synthetic complete minus uracil) medium containing 2% galactose and 2% raffinose at 30°C for three generations. (B) Analysis of the pYES2 control. *Saccharom ces cerevisiae* INVSc1 cells transformed with the shuttle vector pYES2, were grown in SC-U medium containing 2% galactose and 2% raffinose at 30°C for three generations. Heptadecanoic acid (C17:0) was used as an internal standard for quantification and comparison of fatty acid content. The

peak with a retention time of linoleic acid (C18:2) is seen in the yeast cells transformed with (A) the *FAD2-4* gene as expected, and not in the control cells transformed with (B) only vector DNA. In this particular analysis, the yeast cells expressing the cotton FAD2-4 enzyme had a linoleic acid content of 9.0% (normalized weight percent).

APPENDIX E

AMPLIFICATION OF THE *FAD2-1*, *FAD2-2B*, *FAD2-3* AND *FAD2-4* TRANSCRIPTS FORM VARIOUS COTTON ORGANS/TISSUES



FAD2 transcript	Stem	Young leaf	Mature leaf	Root	Fl. bud	Fiber	Cotyledonary-leaf	Hypocotyl	Seed
FAD2-1	–	–	–	–	++	–	–	–	+++
FAD2-2B	+	+++	+++	–	++	+	+++	–	++
FAD2-3	++	+++	+++	+++	+++	+++	+++	+	++
FAD2-4	++	++	++	++	++	++	+++	+++	+

1-Stems; 2-Young leaves, 3-Mature leaves, 4-Roots, 5-Developing flower buds, 6-Developing fibers, 7-Cotyledons, 8-Hypocotyls, and 9-Seeds (*G. hirsutum*, cv. Coker 312). Panel A shows *FAD2-1* transcripts in seeds and developing flower buds. Panel B shows *FAD2-2B* transcripts in all leaf tissues, somewhat less in flower buds and seeds, and barely detectable transcripts in stems, hypocotyls, roots, and fibers. Panel C shows *FAD2-3* transcripts in root, leaves, flower buds, and fibers with somewhat lower transcript levels in stems, seeds, and hypocotyls. Panel D shows robust expression of

FAD2-4 in all tissues except for modest transcription levels in seeds. Amplification of actin transcripts (539 bp) and stearoyl-ACP desaturase (*SAD1*) transcripts (107 bp) by RT-PCR served as a control for the samples. Table inside summarizes the transcript level by RT-PCR analysis performed on various different explants from the cotton plant (*G. hirsutum*, cv. Coker 312). The size of the transcript was determined by the relative motilities in a 1% agrose gel with a MassRuler DNA standard ladder (Fermantas, Maryland). The relative transcription level was estimated based on the intensity of each band in the gel.

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