OVER-EXPRESSION OF AN ISOFORM OF THE DELTA-12 FATTY ACID DESATURASE (FAD2-4) IN TRANSGENIC COTTON PLANTS

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Abstract

Embryogenic cell lines of *Gossypium hirsutum* L., variety Coker 312, were transformed with a construct designed to over-express one of its own isoforms of fatty acid desaturase - FAD2-4. In cotton, *FAD2-4* is part of a multi-gene family and this isoform is expressed mostly throughout plant development, albeit at somewhat lower levels than *FAD2-3*. FAD2 introduces the second double bond into fatty acids (converting oleic acid to linoleic acid) and so represents a potential control point for polyunsaturated fatty acid content. We predict that up-regulating FAD2 activity in cotton plants will increase the proportion of polyunsaturated fatty acids in membranes and may impact tolerance of plants to cooler temperatures, which may be especially beneficial for early planting. Here we report the generation of transgenic plants and the initial biochemical characterization of these plants. Several independent transgenic cotton plants were produced via *Agrobacterium*- based transformation. Out of 11 confirmed primary transgenic plants, four plants were fertile and yielded T1 seeds. T1 seeds were analyzed for total protein and oil content using non-invasive, time-domain (TD) - NMR. Transgenic seeds (T1) showed increased protein content compared to wild type (Coker 312). The fatty acid composition of cotton seed oil is about 26% palmitic acid (16:0), 15% oleic acid (18:1) and 55% linoleic acid (18:2), and this seed fatty acid composition was relatively unchanged in transgenics, although a slight increase in 18:2 was noticed in three lines. On the other hand, the fatty acid composition of young leaves from T0 plants showed substantial differences in polyunsaturated fatty acid composition compared to wild type. These transgenic materials will be advanced and used in tests for germination and growth in cool temperatures.

Introduction

Gossypium hirsutum L.*,* commonly called upland cotton, is a dual purpose crop. It is mainly grown in the Southern United States for its fiber and to a lesser extent, its oil and protein meal (www.usda.gov). Cotton crop improvement has mostly concentrated on fiber yield through various breeding strategies and genetic engineering methods (e.g., development of genetically modified *Bt*. cotton, Perlak et al 2001, Ronald 2011). In addition, using genetic engineering approaches, concerted efforts are made to alter the fatty acid composition of cotton seed oil so as to make it more desirable for both food and feed and industrial purposes (Liu et al 2008). The cotton seed oil, used for human consumption, contains about 20% oil by weight. The normal fatty acid composition of cotton embryo oil is about 26% palmitic (16:0), 2% stearic (18:0), 15% oleic (18:1) and 55% linoleic (18:2) acids (Jones and King 1996, Liu et al 2009). The delta – 12 desaturase (FAD2) enzyme that converts oleic acid (18:1) to linoleic acid (18:2) has been extensively investigated in cotton genetic engineering strategies (Chapman et al 2001, Chapman et al 2008, Liu et al 2009, Sunilkumar et. al. 2002, Sunilkumar 2005). Kargiotidou et. al. (2008) have shown that some of the FAD2 alleles (e.g., FAD2-3 and FAD2-4) in cotton are induced upon cold stress. Here we have genetically modified cotton to over-express one of its own isoforms of fatty acid desaturase – a member of the FAD2 multi-gene family. The main objectives of the present work are: 1. to over express a FAD2-4 enzyme in order to enhance the production of polyunsaturated fatty acids in membranes, and 2. study its specific role and impact on seed germination and seedling cold tolerance.

The FAD2-4 cDNA construct cloned in Gateway binary vector system, pMDC32 and pMDC43 (Curtis and Grossniklaus 2003), was transferred into *Agrobacterium tumefaciens* LBA4404. Cotton embryogenic cell lines were generated from cotyledonary tissues of Coker 312 seedlings and were transformed by *Agrobacterium* co-cultivation. T1 seeds of selfed primary transformants were analyzed for changes in morphology, seed fatty acid composition, oil content, and germination/growth at cool temperatures.

Methods and Observations

*Gh*FAD2-4 gene construct was generated by Zhang et. al. (2009), in the Gateway binary vector system (Curtis and Grossniklaus 2003), and used in cotton plant transformation. Diagrammatic representation of the genetic elements cloned into pMDC32 (no GFP tagged) and pMDC43 (N-terminus GFP tagged, Curtis and Grossniklaus 2003) vectors are presented in Figure 1.

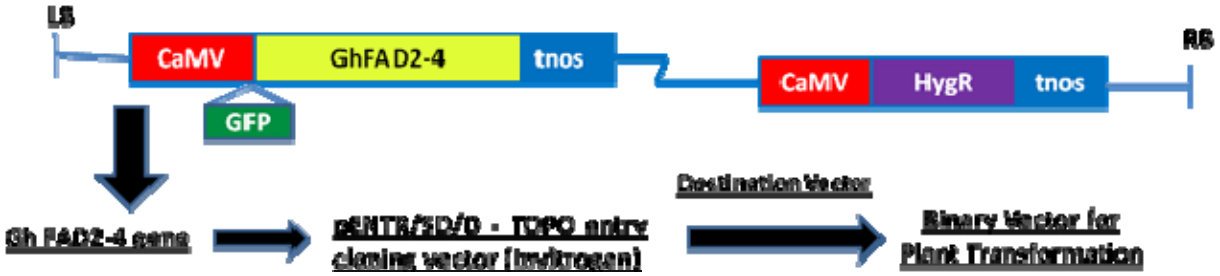

Figure 1. Gh-FAD2-4 cDNA Construct for Plant Transformation. Redrawn from Zhang et al (2009).

Cotton Tissue Culture and Genetic Transformation

Cotton tissue culture and genetic transformation was carried out in two main steps 1. Generation of Embryogenic Cell Lines (ECLs) and 2. *Agrobacterium*-mediation transformation and selection of transgenics with hygromycin (Figure 2). Modified from Leelavathi et. al. (2004) and Rathore et. al. (2006), details of protocols are given in Salimath et al (2010) and Chapman et al (2011).

Figure 2. Transgenic cotton embryos were selected on MSM medium containing hygromycin (15µg/mL) and carbenicillin (400µg/mL). Differentiated plantlets were transferred to magenta boxes on MSM media and allowed to grow until they reached 2-4 leaf stage (Fig. 2A). After about 2-3 weeks the plantlets from magenta boxes were transferred to potting soil and allowed to acclimatize at 28ºC in growth chamber. Once the plantlets acclimatized to soil then they were moved to the greenhouse (Fig. 2B).

DNA Analysis

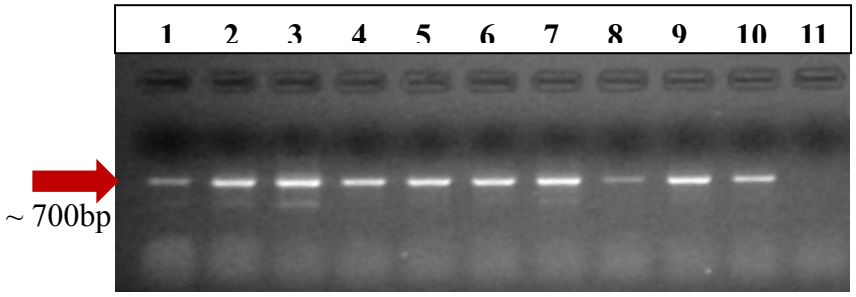

Figure 3. Genomic DNA was isolated from primary transgenic and wild type Coker 312 plants using DNAZol™ method (Invitrogen Inc.) and were subjected to PCR amplification. *CaMV35S* promoter specific primers amplified an expected ~ 700bp DNA fragment from the genomic DNA of ten FAD2-4 primary transgenic plants (Figure 3, Lane 1-10) whereas no DNA fragment was amplified from WT - Coker 312 DNA template (Figure 3, Lane 11.) Lane 1 to 10 respectively represent #1 to #10 FAD2-4 primary transgenics (T0).

Protein and Oil content of T1 seeds of FAD2-4 Transgenics (Non-Invasive TD-NMR)

T1 seeds from FAD2-4 primary transgenics and wild type Coker 312 were subjected to non-destructive/non-invasive method of protein and oil content analysis using TD-NMR. Three grams of seeds were randomly selected and two replicates for each sample were analyzed. The mean value of oil (%) and protein (%) are shown in Table 1. In comparison to WT the T1 seeds harvested from 4 independent lines showed increased protein (~5-9%) and reduced oil (~3-8%).

Table 1. Protein and Oil content of T1 seeds of FAD2-4 Transgenics (Non-Invasive TD-NMR)

Number	T1 Seed - Sample ID	Seed Wt (g)	Protein (g) ± SD	% Protein ± SD	Oil (g) ± SD	% Oil ± SD
1	Coker 312	3.0	0.43 ± 0.01	14.30 ± 0.49	0.7 3 ± 0.01	23.90 ± 0.22
2	#1. FAD2-4 T1 seeds	3.1	0.67 ± 0.00	21.50 ± 0.02	0.59 ± 0.01	19.06 ± 0.47
3	#2. FAD2-4 T1 seeds	3.1	0.58 ± 0.01	18.76 ± 0.24	0.63 ± 0.03	20.37 ± 0.95
4	#7. FAD2-4 T1 seeds	3.2	0.71 ± 0.00	22.25 ± 0.15	0.48 ± 0.01	15.08 ± 0.21
5	#11 . FAD2-4 T1 seeds	2	0.48 ± 0.01	23.92 ± 0.47	0.36 ± 0.00	17.79 ± 0.02

FA Content Analysis of Embryo Oil of FAD2-4 T1 transgenics (Seeds Derived From Fertile Primary Transgenics)

Total lipids from cotton embryos were extracted by a modified version of the Bligh/Dyer method using isopropanol and chloroform (Wanjie, 2005). The final lipid extracts, in 2 mL chloroform, were stored under nitrogen (-20°C) for FA analysis. Fatty acid composition analysis of #1, #7 and #11 FAD2-4 transgenic T1 seeds, in comparison to wild type Coker 312, revealed slightly higher-linoleic fatty acid content (Table 2).

Table 2. Fatty acid content of embryo oil of Coker 312 and Transgenic lines (T1)

	Mol % FattyAcid							
Plant /Seed ID	**16:0**	**16:1**	**18:0**	**18:1**	**18:2**	**18:3**	**16 Carbons**	**18 Carbons**
Coker 312	29.7	1.2	2.4	16.2	50.2	0.3	30.9	69.1
#1. FAD2-4 T1 seeds	29.8	1.3	2	13.5	53.2	0.3	31	69
#2. FAD2-4 T1 seeds	30.3	1.2	2.2	17	49	0.2	31.5	68.5
#7. FAD2-4 T1 seeds	24.9	0.8	2.2	19.2	52.8	0.1	25.7	74.3
#11. FAD2-4 T1 seeds	26.2	0.9	2.1	14.3	56.2	0.2	27.2	72.8

Conclusions

Over one dozen independent cotton transgenic plants carrying an overexpression construct of the endogenous GhFAD2-4 were produced *via Agrobacterium* - mediated transformation. Primary transgenic lines were grown to maturity in the greenhouse and they were characterized by molecular analysis, confocal microscopy (GFP expression), and biochemical analysis of leaf and, where fertile, embryo lipids.

T1 seeds from some of the primary transgenic lines showed increased protein content relative to the untransformed Coker 312 background.

Fatty acid composition analysis of Gh-FAD2-4 transgenic T0 leaves revealed increased polyunsaturated fatty acid profiles. Fatty acid methyl esters (FAME) of total seed lipids or analysis of intact triacylglycerols (TAG) from total lipid extracts of T1 seeds showed minor increases of linoleic acid (18:2) in three lines analyzed by conventional gas chromatography.

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