COTTON PLANTS TRANSFORMED WITH A CASTOR DIVERGED FATTY ACID DESATURASE (FAD2) HAVE REDUCED OIL CONTENT AND EMBRYO SIZE

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Abstract

Gossypium hirsutum L. variety Coker 312 was transformed by a modified *Agrobacterium*-mediated procedure using embryogenic cell lines. A binary plant transformation vector was engineered to contain the cotton alpha globulin promoter (AGP) (1.1 kb) upstream from the codon optimized castor hydroxylase coding region (~1 kb) for seed specific expression. Over two dozen primary transgenic plants (*T*0) were generated (confirmed by PCR). Non-invasive seed oil analysis by 1H-NMR of *T*1 transgenic (progeny) seeds of nine independent transgenic events showed significantly reduced seed weight and lowered oil content compared to that of control non-transgenic Coker 312 cotton seeds. Furthermore, in some cases the *T*1 seeds had shrunken or aborted embryos, suggesting a deleterious effect from the seed-specific, heterologous expression of the castor diverged FAD2. The normal fatty acid composition of cotton seed oil is about 26% palmitic (16:0), 2% stearic (18:0), 15% oleic (18:1) and 55% linoleic (18:2) acids. Although the fatty acid profiles of transgenics revealed little or no accumulation of ricinoleic acid in T1 seeds, there was a substantially elevated percentage in oleic acid content relative to non-transgenic Coker 312 (up to 38%) at the expense of linoleic content (reduced to 38%), suggesting that expression of the castor diverged FAD2 had interfered with endogenous FAD2 activity reducing flux from oleic acid to linoleic acid. Future studies will be aimed at examining the heritability of this altered fatty acid flux and to determine if low levels of ricinoleic acid are being produced in the phospholipid pools of developing embryos and limiting cotyledon development.

Introduction

Among several cultivated species of the genus *Gossypium, G. hirsutum* L. *,* commonly called upland cotton, is the most prominent species grown in the United States, occupying the number one position in acreage and production across the cotton belt (www.usda.gov). The cotton seeds, used to extract edible oil for human consumption and also used as cattle feed, are known to contain about 16-20% oil by weight. The normal fatty acid composition of cotton is about 26% palmitic (16:0), 2% stearic (18:0), 15% oleic (18:1) and 55% linoleic (18:2) acids. (Jones and King 1996) Using genetic engineering approaches, concerted efforts have been 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 Delta – 12 desaturase (FAD2) enzyme that converts oleic acid to linoleic acid has been extensively investigated in cotton genetic engineering strategies in this context (Chapman et. al. 2001, Chapman *et al* 2008, Liu et. al. 2009). Here we have initiated experiments to introduce into cottonseeds a diverged FAD2 enzyme – the delta 12 hydroxylase from castor that converts oleic acid to ricinoleic acid (or 18:1OH). The main objectives of the present work are: 1. To establish a versatile embryogenic cell- based cotton transformation system and 2. To express a codon optimized hydroxylase gene from castor in cotton embryos to test if cotton can produce the hydroxy fatty acid, ricinoleic acid, in a seed specific manner.

Seed specific transgene expression was directed with the cotton α-globulin promoter (AGP) that was cloned from genomic DNA (cv. Coker 312) based on the sequence information from Sunilkumar et.al. (2002). A binary vector, pBincottonGlobRcOHOpt, harboring the AGP promoter (1.1 kb) upstream from the codon optimized castor hydroxylase (1.1kb), was constructed, and 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. T1seeds of selfed primary transformants were analyzed for changes in morphology, seed fatty acid composition and oil content.

Methods and Observations

Cotton tissue culture and genetic transformation methods are modified from Leelavathi et. al. (2004) and Rathore et. al. (2006).

I. Embryogenic cell lines derived from cotton
- Initiated cotton seed germination on MSM (Murashige and Skoog basal salts + D-Maltose) solid media in petri-plate(s) - (3-5 days).
- Transferred germinated cotton seeds to MSM liquid media to aid in cotyledon expansion – (2-5 days).
- Harvested expanded leaves and continued culture of cotyledon pieces on MSMK (MSM + KNO3) plates - (2-3 weeks).
- Sub-cultured to fresh medium until callus production - (every 4-8 weeks) (Fig. 1A).
- Collected Somatic Embryogenic Cells (SECs) from callus cultures and initiated generation of SECs in MSM liquid medium (3 mL per well) in 12 well tissue culture plates on a gyratory shaker (110rpm, 16hr light/8hr dark) – (4-6 weeks) (Fig. 1B).
- Multiplied SECs in MSM liquid medium (3mL) in 12 well tissue culture plate for use in Transformation experiments – (4-8 weeks) (Fig. 1B).

II. *Agrobacterium*-mediated cotton transformation
- Actively growing cotton Somatic Embryogenic Cell (SEC) lines, maintained at 30oC, were used for *Agrobacterium* mediated transformation (Fig. 1B).
- *Agrobacterium tumefactions* strain LBA4404 cells (containing gene of interest in binary vector) were cultured at 28C for 36-40 hours in LB medium with kanamycin (50 µ g/mL) and streptomycin (100 µg/mL).
- SECs and *Agrobacterium* cells were co-cultured in the dark for 48 hours on MSM solid plates layered with sterile filter paper disc containing MSM medium with 100µM acetosyringone.
- SECs, in small (2-5mm) tissue mounds, were transferred to MSMK (MSM + KNO3) solidified media containing carbenicillin (400µg/mL) and kanamycin (50 µg/mL) to select for developing transgenic embryos (Fig. 1C).
- Collected differentiating embryos onto fresh MSMK media with carbenicillin + selection agent ± charcoal (Fig. 1D).
- Transferred plantlets at 2-4 leaf stage to MSM in magenta boxes (Fig. 1E).
- Established and acclimatized plants to soil and then moved them to the greenhouse for seed production (Fig. 1F-G).

  Following these methods we were able to move the T0 transgenics to the greenhouse as soon as 3 months and harvest the T1 seeds (Fig. 1H) within 6-8 months of co-cultivation.

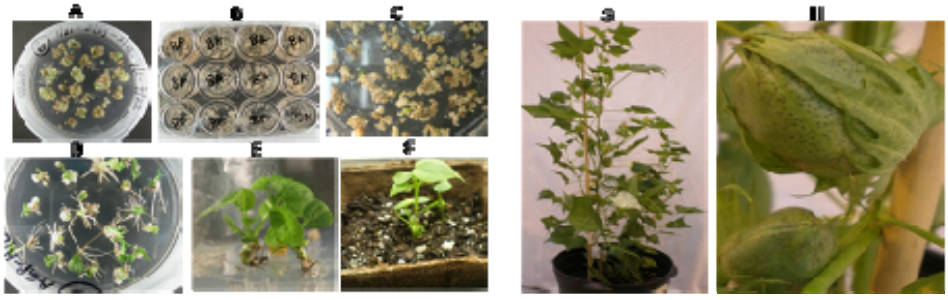

Figure 1A-H: A. Callus B. SECs in 12 well plate C. Transgenic embryos D. transgenic plantlets E. transgenics at 2-4 leaf stage F. transgenics transferred to soil G. transgenic in greenhouse H developing bolls.

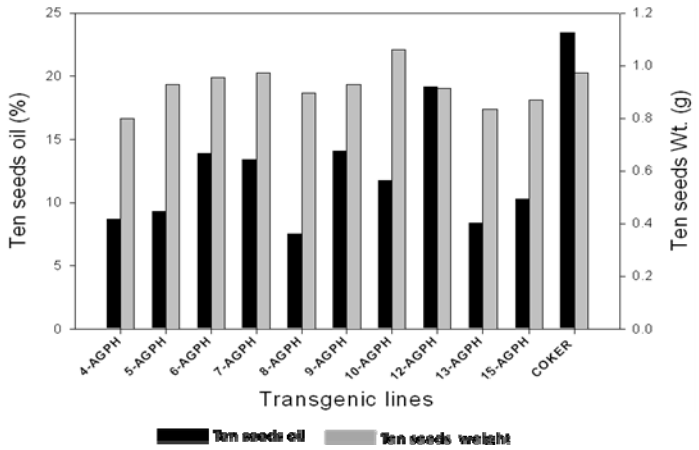

Figure 2. Seed weight and oil content (10-seed pools) in randomly selected progeny (*T*1) of AGP-hydroxylase transgenics. Seed weight and oil per cent given are the mean of two independent readings taken for each sample (10 seed pool). Note decreased oil per cent (>3 fold) in T1 seeds of AGP-hydroxylase transgenics in comparison to wild type Coker seeds.

Table 1. Fatty acid content in AGPH transgenics.

Sample	**14:0 (%)**	**16:0 (%)**	**18:0 (%)**	**18:1 (%)**	**18:2 (%)**
Coker (control)	1.5	25.6	2.7	14.7	55.5
AGPH-4	1	26.4	2.7	28.6	41.3
AGPH-5	0.8	26.7	2.9	26.1	43.4
AGPH-6	1.2	32.3	2.5	17.2	46.8
AGPH-7	1.2	26.5	2.6	22.3	47.3
AGPH-8	0.5	23.2	3	19.2	54.1
AGPH-9	0.5	25.3	2.6	19.9	51.7
AGPH-10	0.7	25.9	3.3	29.8	40.3
AGPH-12	0.9	25.9	2.4	14.6	57.1
AGPH-13	1.1	27.7	2.3	27.4	41.5
AGPH-15	0.5	21.2	3.3	37.7	37.9

Total lipids from cotton embryos were extracted by a modified version of the Bligh/Dyer method using isopropanol and chloroform (Wanjie et al 2005). The final lipid extracts, in 2 mL chloroform, were stored under nitrogen (-20°C) for FA analysis or ESI-MS analysis. Fatty Acid composition analysis of AGP-hydroxylase transgenic T1 seeds revealed *high-oleic fatty acid profiles*. Fatty acid methyl esters (FAME) from total lipid extracts showed relative increases of oleic acid (18:1) at the expense of linoleic acid (18:2)(see Table below), as quantified by GC-FID. Hydroxy fatty acids were not accumulated perhaps due to detrimental effects on embryo development.

Conclusions

More than 15 transgenic lines carrying a diverged FAD2 castor hydroxylase were produced *via Agrobacterium*-mediated transformation, and further grown to maturation in the greenhouse. Transgenic lines were confirmed by molecular analysis of plants and by evaluation of seed morphology and biochemical analysis of embryo lipids. T1 transgenic lines showed significantly reduced seed/embryo size and oil content (Fig. 2). It was evident that T1 seeds contained shriveled and/or aborted embryos compared with non-transformed wild-type seed. Expression of the castor hydroxylase in transgenic cotton may be deleterious to embryo development. There are few lines with reduced oil that do not show highly reduced embryo size or aborted embryos, and perhaps reflect a tolerable level of hydroxylase expression.

Fatty Acid composition analysis of AGP-hydroxylase transgenic T1 seeds revealed *high-oleic fatty acid profiles* (Table 1). Fatty acid methyl esters (FAME) or intact triacylglycerols (TAG) from total lipid extracts showed increases of oleic acid (18:1) at the expense of linoleic acid (18:2, see Table 1) analyzed by conventional gas chromatography. This suggests that the diverged FAD2 from castor is specifically interfering with the metabolic utilization of oleic acid in developing seeds, perhaps by interacting with endogenous cotton FAD2.

Acknowledgements

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