METABOLIC ENGINEERING FOR INCREASED COTTONSEED OLEIC ACID CONTENT

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Abstract

Transgenic cotton plants were developed with altered seed fatty acid profiles aimed at higher monounsaturate levels, specifically oleic acid. Plants were generated by Agrobacterium-mediated transformation, followed by induction of embryogenic calli on kanamycin selection media. A binary vector was designed to suppress expression of the endogenous cottonseed delta-12 desaturase (FAD2) by subcloning a mutant allele of a rapeseed fad2 gene downstream from a heterologous, seed-specific promoter (phaseolin).Fatty acid profiles of total seed lipids from 48 independent transgenic lines were analyzed by gas chromatography. Twenty-five plants showed elevated levels of seed oleic acid content compared with vector-only transformed controls or non-transformed plants of the Coker 312 background. Increased oleic acid content ranged from 21% to 37% (by weight) of total fatty acid content in pooled seed samples from primary transformants. The increase in oleic acid content was solely at the expense of linoleic acid, consistent with reduced activity of FAD2. Collectively, our results extend the metabolic engineering of vegetable oils to cottonseed, and should provide the basis for the development of a family of novel cottonseed oils.

Introduction

Cottonseed is about 16% oil by weight and ranks third behind soy and canola in world oilseed crushings (Update 2000. Inform 11:820). The United States is the second largest producer of cottonseed (www.cottonseed.com), producing 5.8 million metric tons between October 1999 and September 2000. Currently, only about half of the seed is crushed in the United States, leaving a substantial amount of raw material from which to recover added-value processed products. Refined oil is the most valuable cottonseed product (after fiber); it has a distinctive fatty acid composition that is about 26% palmitic (16:0), 2% stearic (18:0), 15% oleic (18:1), and 55% linoleic (18:2) acids (Jones and King, 1996). Although not often thought of as a food crop, cottonseed has certain flavor-enhancing attributes which make it an excellent cooking oil. However, recent trends in the food service industry are tending toward oils high in monounsaturates, primarily due to oxidative stability of these oils and some perceived health benefits (Kinney and Knowlton, 1998). Consequently an increased oleic acid content (with a compensatory reduction linoleic acid) in cottonseed would represent a healthier oil and might promote the additional processing and utilization of raw cottonseed.

Metabolic engineering of oilseeds has resulted in commercial production of new oils in traditional crops (DelVecchio, 1996; Kinney, 1998; Mazur et al., 1999). Alteration of a single, targeted enzymatic step in storage lipid metabolism can produce dramatic changes in fatty acid profiles of the seed oils, and in many cases, these changes have little effect on total oil yield or overall crop performance (Ohlrogge, 1994; Voelker, 1996; Kinney, 1997). With the tremendous diversity of plant fatty acid structures present in nature (Somerville et al., 2000), and increasingly novel strategies for genetic manipulation of crop plants, the possibilities for creating new vegetable oils for edible and industrial purposes are remarkable (Harwood, 1996). Indeed, commercialization of high laurate canola (Del Vecchio, 1996) and high oleic soybeans (Kinney and Knowlton, 1998) highlight the potential of this approach to impact agriculture.

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Materials and Methods

Transformation Vectors

A binary vector, designated pZPHMCFd2, was constructed for use in cotton transformations. This vector contains the Ti-plasmid left and right border (LB, RB) inverted repeat sequences for integration into cotton genomic DNA. The T-DNA segment harbors the selectable marker nptII regulated by the CaMV35S promoter, conferring kanamycin resistance to transgenic plant cells. In addition, a FAD2 suppression cassette is inserted into the T-DNA segment between unique BamHI and EcoRI sites. This cassette (2860 bp fragment) contains a canola mutant (non-functional) fad2 allele subcloned between the 5' and 3' flanking regions of the phaseolin gene. The pZPhMCFd2 binary vector was introduced into Agrobacterium tumefaciens (strain LBA4404) by electroporation and maintained with kanamycin selection conferred by nptI expression. For transformation/ plant regeneration controls, transgenic cotton plants were produced using the binary vector pBI121 (Clonetech) with the same Agrobacterium strain.

Cotton Transformation

Cotyledon pieces (approximately $3mm^2$) were excised from 7-14 d-old cotton (Gossypium hirsutum, L, cv. Coker 312) seedlings (germinated asceptically according to Thomas et al., 1995, and Trolinder and Goodin, 1988), except that seedlings were grown at 30°C under a 14 h photoperiod, 60 $\mu mol/s/m^2$). Cotton explants were co-cultivated with $6x10^8$ cells/ml Agrobacterium tumefaciens LBA4404, harboring the binary vector pBI121 for vector-only control experiments, or pZPhMCFd2 for fatty acid modification. Agrobacterium-mediated transformation and plant regeneration were based on a combination of published procedures (Firoozabady et al., 1987; Umbeck et al., 1987; Thomas et al., 1995), with minor modifications. Briefly, cotton cotyledon explants were placed in co-cultivation medium (MS medium, 1.5% w/v sucrose, 40 µM acetosyringone, 2 mM MES-NaOH, pH 5.5) along with an equal volume of Agrobacterium cell suspension. The explant/Agro mixture was placed under vacuum (25" Hg) for 8 min, and equilibrated to 25°C in a water bath for an additional 1 h and 15 min. Explants were then blotted on sterile filter paper and placed on G1 medium (MS salts with 3% w/v glucose) (Thomas et al, 1995) for 3 days at 25°C. Explants were then transferred to G2 medium (MS medium, 100mg/L inositol, 1µM thiamine, 25µM 6-(g-g-dimethylallylaminopurine (2iP), 0.5µM napthaleneacetic acid (NAA), 3% w/v glucose, pH 5.8, 0.2% w/v Phytagel (Sigma)) supplemented with 400 mg/L carbenicillin and 50 mg/L kanamycin (Thomas et al., 1995). The transformed calli were subcultured every 2-4 weeks to fresh G2 medium. Mock transformations (co-cultivation of explants with Agrobacterium that contained no binary vector) were always conducted to verify selection procedures were adequate. After 2-3 months, proliferating transgenic calli were transferred to a modified MSOB medium (modified from Thomas et al., 1995 to contain MS salts, B-5 vitamins, 1.9 mM potassium nitrate, 100mg/L inositol, 3% w/v glucose, pH 5.8, with 0.2% Phytagel) supplemented with 200 mg/L carbenicillin and 50 mg/L kanamycin (Thomas et al., 1995). Developing embryos were recovered after 6-8 weeks and placed on MSOB medium without antibiotics. Elongated embryos were transferred to MS3 medium (MS salts, 0.4µM thiamine-HCl, 0.5µM pyridoxine-HCl, 0.8µM nicotinic acid, 1%w/v glucose, pH 5.8 with 0.8g/L Phytagel and 4g/L agar) for root formation (Thomas et al., 1995). Small plantlets were propagated clonally (Hemphill et al., 1998), or transferred to soil, hardened off, then transferred to glasshouse conditions (14 h photoperiod, supplemented with high intensity Na- and Hg- vapor lamps

when necessary, 35ºC day 25ºC night) for production of flowers and bolls. Plants were fertilized biweekly with a dilute solution of Miracle Gro™, and flower production was stimulated with SuperBloom™ (when necessary). Flowers were selfed, tagged at anthesis, and progress of boll development was monitored daily. Any male sterile primary tranformants (less than 5% of plants) were hand pollinated with Coker 312, wild type pollen to obtain viable progeny.

Lipid Extractions, Preparation of Fatty Acid Methyl Esters, and Gas Chromatography

Whole cottonseeds (pooled 8-seed batches or single seeds) were first frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. Ground seeds were then extracted with hexane. Aliquots of hexane extracts were dried under flowing nitrogen and transesterified with acidic methanol essentially as described by Christie (1982) except that the $KHCO_3$ wash and Na_2SO_4 drying steps were omitted. Fatty acid methyl esters were analyzed by gas chromatography (GC) and quantified by flame ionization detection (FID) essentially as described by Chapman and Trelease (1991), except that a 30 m (0.25 mm i.d.) DB-23 (J&W Scientific) capillary column was employed for separation and the oven temperature was 200ºC.

Results and Discussion

Fatty acid profiles of seeds of 48 independent lines were analyzed for changes in the relative percentages of oleic acid (18:1) in total lipids extracted from pooled seed samples (Table 1). In general, an increase in seed oleic acid content was noted (compared with wild type; see Table 1) and this appeared to be at the expense of decreased linoleic acid (18:2) content. Palmitic (16:0) and stearic (18:0) acid levels remained relatively unchanged. These results strongly support a targeted suppression of endogenous cottonseed FAD2 enzyme activity in many of the transgenic cotton plants. These results indicate that 1) the molecular manipulation of endogenous FAD2 activity was an efficient process in cotton, 2) changes in oleic acid content were manifested in the appropriate plant part and 3) the modified oleic acid content was heritable in the progeny of the primary transformants.

It will be a priority to verify that other cottonseed quality factors are unaltered in these transgenics, once that stable, back-crossed individual lines can be selected for field trials. Of paramount importance will be to examine fiber yield and quality; however this is not likely to be affected since fiber development and oil accumulation are temporally separated events in cotton boll development (Trelease et al., 1986). Other factors to be examined will be oil and protein quantities. Also important is to ensure that negative attributes such as gossypol content, or cyclopropenoid fatty acid (CPFA) levels remain unchanged in transgenic seeds. This likely will be the case because these metabolites are either produced by other biochemical pathways (polyphenolic metabolism; Croteau et al., 2000), or accumulate in organs other that cotyledons (CPFA occurs exclusively in the axis of mature seeds; Wood, 1986). . It should be pointed out here that the changes we noted in fatty acid composition targeted selectively oleic acid and linoleic acid content in transgenics. Even other storage fatty acids such as palmitic or stearic acid content were unchanged, emphasizing the likelihood that metabolic changes are specific for FAD2 alone. Consequently, it should be possible to identify individual lines with promising characteristics for advancement.

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Table 1. Fatty acid profiles (wt %) of seeds (pooled batches) from nontransformed (Coker 312), vector-only transformed controls (pBI121-1,2), and transformed plants (ZPH lines).

Line	16:0	18:0	18:1	18:2
Coker312	26	2	15	56
pBI121-1	28	3	17	51
pBI121-2	27	3	16	54
ZPH50a	26	3	14	54
ZPH 17	26	3	15	54
ZPH 18b2	26	2	15	55
ZPH 61a	27	2	16	53
ZPH 61c	29	2	16	51
ZPH 18a2	28	3	16	52
ZPH 5b	30	2	16	50
ZPH 50d	30	2	16	50
ZPH 55a	26	3	17	53
ZPH 11a1	26	3	17	53
ZPH 61	26	2	17	53
ZPH 11b	25	3	17	53
ZPH 11j	29	4	18	47
ZPH 11d1	24	3	18	53
ZPH 82	28	3	18	50
ZPH 81e	26	3	18	52
ZPH 5a	29	3	19	46
ZPH 11e	25	2	19	53
ZPH 61d	26	3	19	50
ZPH61b	27	3	19	50
ZPH 64a	26	3	19	50
ZPH 56a	25	3	20	51
ZPH 55c	27	3	20	49
ZPH 63d	24	4	21	49
ZPH 11g	26	3	21	49
ZPH 11f	25	3	22	49
ZPH 83b	25	3	22	48
ZPH 50I	30	3	22	42
ZPH 36a	26	4	23	45
ZPH 7a/b	27	4	23	42
ZPH 72a	25	4	23	46
ZPH 11I	25	3	24	45
ZPH63c1	25	4	24	43
ZPH 11c	25	4	24	44
ZPH82c	23	4	24	47
ZPH 55b	25	4	25	44
ZPH 16	24	4	25	44
ZPH 56d	27	4	25	42
ZPH 36b	28	3	25	43
ZPH 83c	26	4	25	42
ZPH 11a3	25	3	27	42
ZPH 11h	25	4	28	41
ZPH 11a	26	4	28	41
ZPH 84a	25	5	28	39
ZPH 11d2	24	4	29	40
ZPH 11m	29	4	30	35
ZPH 83a	26	4	30	39
ZPH 12b	29	4	37	29

Percentages rounded to the nearest whole number. Only Palmitic (16:0), Stearic (18:0), Oleic (18:1), and Linoleic (18:2) acid content is shown. Others were less than 1% each.