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Original article

Simultaneous silencing of *GhFAD2-1* and *GhFATB* enhances the quality of cottonseed oil with high oleic acid

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ABSTRACT

Cottonseed oil has become an important source of edible oil due to its significant cost advantage. However, there is a growing concern over its fatty acid composition and nutritional value. In *Gossypium hirsutum*, *GhFAD2-1* and *GhFATB* encoding the microsomal oleate desaturase and palmitoyl-acyl carrier protein thioesterase, respectively, play critical roles in regulating the proportions of saturated and polyunsaturated fatty acids in cottonseed lipids. In this study, RNAi technology was used to simultaneously inhibit the expression levels of *GhFAD2-1* and *GhFATB* to improve the quality of cottonseed oil by increasing oleic acid content. Transgenic cotton plants with reduced levels of both target genes were successfully generated. In mature seed kernels of transgenic plants, the content of oleic acid was 38.25%, accordingly increasing by 156.96%, while the content of palmitic acid and linoleic acid was 19.15% and 36.68%, decreasing by 21.28% and 33.92%, respectively, compared with that of the control. The total oil content in transgenic and control kernels was 22.48% and 29.83%, respectively. The reduced oil level in transgenic seeds was accompanied by a reduction in seed index, thereby causing disadvantageous effects on seed germination potentiality and seed vigor, particularly under cool stress conditions. Our results demonstrated the feasibility of simultaneous manipulation of multiple genes using RNAi technology and showed the important role of oil content in seed development and vigor. Our findings provide insight into the physiological significance of the fatty acid composition in cottonseeds.

1. Introduction

Oil extracted from cottonseeds (cottonseed oil) has been used in food for hundreds of years (Liu et al., 2015b). Cottonseed oil contains approximately 26% saturated palmitic acid (C16:0), 15% monounsaturated oleic acid (C18:1), and 58% polyunsaturated linoleic acid (C18:2) (Liu et al., 2002). Many reports have shown that cottonseed oil exhibits an unbalanced content of oversaturated, polyunsaturated, and monounsaturated fatty acids (Chapman et al., 2001; Liu et al., 2002; Lu et al., 2011). Consumption of the saturated C16:0 fatty acid increases overall cholesterol levels, specifically low-density lipoprotein (LDL) or "bad" cholesterol, resulting in an increased risk of cardiovascular disease (Baum et al., 2012). Nutritionally, both the C18:1 and C18:2 fatty acid can lower total serum cholesterol; however, the C18:2 fatty acid is relatively more susceptible to oxidation and becomes rancid more quickly. Due to the oxidative instability of polyunsaturated fatty acids, partial hydrogenation is a common procedure in oil production, thus inevitably generating undesirable trans fatty acids. Therefore, increasing the oleic acid content while correspondingly decreasing the proportions of saturated and polyunsaturated fatty acids has become an important objective in the improvement of many oilseed crops. In addition to the applications in food industries, oleic acid-rich oil is also widely used in the production of improved biodiesel, lubricants, and hydraulic oils, as its high oxidative stability is a necessity of these products. Fatty acid biosynthesis in plants is catalyzed by fatty acid synthase, and key enzymes involved in fatty acid metabolism play important roles in regulating the type and content of fatty acid composition during seed development. In plants, the acyl group from acyl-ACP can be hydrolyzed by acyl-ACP thioesterases (FATs) to release free fatty acids and ACP, which is essential for chain termination during de novo fatty acid synthesis and the channeling of carbon flux between the two lipid biosynthesis pathways. Based on amino acid sequence comparisons and substrate specificity, two different classes of acyl-ACP thioesterases have been identified in plants (Voelker et al., 1997). The FATB class encoded by *FATB* has high activity towards saturated acyl-ACP groups (Dörmann et al., 1995; Voelker et al., 1997; Salas and

Abbreviations: FAD2, fatty acid desaturase 2; FATs, acyl-ACP thioesterases; FG, final germination percentage; GP, germination potentiality; GI, germination index; LP, lint percentage; MDA, malondialdehyde; OI, seed oil index; SI, seed index; SVI, simplified vigor index; VI, vigor index

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Ohlrogge, 2002) and is a major determinant of saturated fatty acid biosynthesis. Furthermore, the $^{\Delta 12}$-desaturase enzyme gene *FAD2-1* encodes a microsomal oleate desaturase, which introduces a double bond at the $^{\Delta 12}$ position of oleic acid on phosphatidylcholine and converts oleic acid into linoleic acid. *FAD2-1* expressed in developing cottonseeds plays an important role in controlling the oleic acid content in seeds (Liu et al., 1999). Genetically modified crops with high oleic acid content, such as soybean, peanut, rapeseed, and cotton, have been generated by silencing the *FAD2* gene using RNAi technology (Buhr et al., 2002; Liu et al., 2002; Peng et al., 2010; Jung et al., 2011; Pham et al., 2012; Qu et al., 2012). Moreover, down-regulation of *FATB* expression in soybean also demonstrates the partial reduction of seed palmitic acid (Wilson et al., 2001; Buhr et al., 2002). Sequences of different genes linked together to form a fusion fragment were also used to produce a single RNA interference construct to simultaneously silence two genes involved in biosynthesis of fatty acids in *Brassica napus* (Peng et al., 2010).

To improve the quality of cottonseed oil in this study, the RNAi technology was used to regulate fatty acid metabolism of cottonseed by simultaneous inhibition of *GhFAD2-1* and *GhFATB* gene expression levels. Our results showed that it is feasible to use a single hairpin structure containing DNA fragments from different genes to down-regulate multiple target genes in *Gossypium hirsutum*. Simultaneous down-regulation of *GhFAD2-1* and *GhFATB* resulted in a significantly elevated level of oleic acid and a reduced level of palmitic acid and linoleic acid in cottonseeds. The results also showed that the regulation of C18:1 content resulted in dynamic changes of the other fatty acid synthesis during the development of cottonseed fatty acids. The effect of changes in fatty acid composition on physiological activity of seed development and mature seed viability was also further analyzed in this study.

2. Materials and methods

2.1. Vector construction and cotton transformation

Genomic DNA was isolated from cotton leaf tissue (*G. hirsutum* cv. Xinluzao 33) using a plant genomic DNA extraction kit (Baitaike, China). To make the RNAi construct targeting the *FAD2-1* and *FATB* genes simultaneously in *G. hirsutum*, a 424-bp *GhFAD2-1* fragment and a 501-bp *GhFATB* fragment were amplified from the 5′ UTR of *GhFAD2-1* and the exons of *GhFATB*, respectively (Fig. A.1 and Fig. A.2 in Supplementary material). The primers used in amplification were 5′-CCC*AAGCTT*TTTAAGGC-TTTTTCTTTTCCTTAGATC-3′ and 5′-CGC*GGATCC*AGTCCTTTTACTG-AAAATTTG-3′ (for *GhFAD2-1*, two restriction sites *Hin*dIII and *Bam*HI were underlined), and 5′-CGC*GGATCC*GCTACTGCTGTGACATCGGCGT-3′ and 5′-C*GAGCTC*CTAGCCAAATAACTGTAACTC-3′ (for *GhFATB*, two restriction sites *Bam*HI and *Sac*I were underlined). The PCR cycles were performed as follows: 94 °C for 5 min, followed by 30 cycles at 94 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min, and a final cycle of 72 °C for 10 min.

The amplified products were individually gel-purified, ligated into vector pMD18-T using the TA Cloning Kit (Takara, China), and subsequently transformed into *E. coli* DH5α competent cells. The amplified fragments were confirmed by sequencing using the ABI PRISM 3730XL DNA Analyzer (Applied Biosystems, USA). The two fragments were finally linked together through the complementary *Bam*HI sticky ends to form a fusion fragment. Based on the expression vector pBI121 and the RNAi vector pANDA35HK, and a combination of the standard DNA recombinant technique and the gateway technology, we successfully constructed the cottonseed-specific dual-target gene-silencing RNAi vector pBISP-*FAD2-FatB* (Fig. 1) with the hairpin driven by a seed-specific promoter. The pBISP-*FAD2-FatB* construct harboring the desired hairpin structure was transferred into the *Agrobacterium tumefaciens* strain LBA4404 by electroporation and was then used to transform hypocotyl explants from *G. hirsutum* cv. YZ1 as previously described (Jin et al., 2006).

2.2. Identification of transgenic plants

We used PCR amplification of the fusion fragment to identify positive transgenic plants. Total genomic DNA was isolated from individual transgenic plants and amplified using a pair of primers: 5′-TTTAAGG-CTTTTTCTTTTCCTTAGATC-3′ and 5′-CTAGCCAAATAACTGTAACTC-3′. 5′-GAGTCTGGTAATTGGAATGAG-3′ and 5′-TTCGCAGTTGTTCGTCTT-3′, a pair of primers amplifying the 18S rDNA gene, were used as a control. Southern blot analysis was carried out according to the standard procedures. Briefly, genomic DNA was digested with *Hin*dIII and the resultant fragments were separated by electrophoresis in a 0.8% agarose gel and electroblotted onto Hybond-N$^+$ nylon membrane (Millipore, USA) by Trans-Blot SD semi-dry electrophoretic transfer cell (BioRad, USA). The membrane was hybridized using a 274-bp probe amplified from the *NPTII* gene (Fig. 1). Probe labeling and hybridization were performed according to the instructions of the DIG High Prime DNA Labeling and Detection Starter Kit I (Roche, Germany).

2.3. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cottonseeds at different developmental stages using RNAiso Plus (Takara) and quantified using a GeneQuant™ 1300 spectrophotometer (Biochrom, UK). One microgram of RNA was reverse-transcribed to obtain first-strand cDNA using a PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara, China). qRT-PCR reactions were performed in 10-μl volumes using SYBR Green Master Mix (Takara, China), and amplification reactions were performed using a LightCycler® 480 II (Roche, Germany) under the following PCR conditions: 95 °C for 3 min followed by 45 cycles of 95 °C for 15 s, 56 °C for 15 s, and 72 °C for 15 s. The reactions were performed using the primers 5′-TGGCGTTAAACTGCTTTCT-3′ and 5′-CGGAATGGCTTGCTTGAT-3′ (*GhFAD2-1*), and 5′-TGCTGCTATCACA-ACCAT-3′ and 5′-CCTGAACAATCTTCCCTAT-3′ (*GhFATB*). The cotton poly-ubiquitin gene (*GhUBQ14*, accession number in GenBank: DW505546) was used as an internal control. The primers for *GhUBQ14* were 5′-CAACGCTCCATCTTGTCCTT-3′ and 5′-TGATCGT CTTTCCCGTAAGC-3′. All qRT–PCR reactions were performed in triplicate. Relative gene expression data were normalized against Ct values for *GhUBQ14*, and the fold change ($2^{-\Delta\Delta Ct}$) was determined by comparing with average expression levels according to Livak and Schmittgen (2001).

2.4. Fatty acid analysis

Cottonseeds collected at different developmental stages were de-shelled and ground into powder in liquid nitrogen and used in the analysis of fatty acid composition. The powdered samples were transferred into glass tubes, and then fatty acid methyl esters were prepared by alkaline transmethylation according to Peng et al. (2010). Fatty acid methyl esters were analyzed by gas-liquid chromatography as previously described (Stoutjesdijk et al., 2002). The composition of each fatty acid in the sample was expressed as a percentage of total fatty acids (%).

2.5. Measurements of cottonseed components

Mature cottonseeds after ginning were delinted with H_2SO_4 and then deshelled. The resulting cottonseed kernels were used to determine contents of seed oil, protein, total sugar, and seed moisture using the near infrared grain analyzer DA 7250 SD NIR instrument (Perton, Sweden) with routine analysis and calibration development carried out according to the manual (Perton, Sweden).

2.6. Determination of lipase activity and lipid peroxidation

Cotyledons of three-day-old seedlings were used in lipase assays and determination of lipid peroxidation. For lipase activity assay, frozen samples were ground to fine powder with liquid nitrogen and were