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Research paper

Discovery of a new mechanism for regulation of plant triacylglycerol metabolism: The peanut *diacylglycerol acyltransferase-1* gene family transcriptome is highly enriched in alternative splicing variants

Ling Zheng[a], Jay Shockey[b], Feng Guo[a], Lingmin Shi[a], Xinguo Li[a], Lei Shan[a], Shubo Wan[a,*], Zhenying Peng[a,*]

[a] Bio-Tech Research Center, Shandong Academy of Agricultural Science/Shandong Provincial Key Laboratory of Genetic Improvement, Ecology, and Physiology of Crops, Jinan, 250100, China

[b] United States Department of Agriculture-Agricultural Research Service, Southern Regional Research Center, New Orleans, LA 70124, USA

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ABSTRACT

Triacylglycerols (TAGs) are the most important energy storage form in oilseed crops. Diacylglycerol acyltransferase (DGAT) catalyzes the rate-limiting step of the Kennedy pathway of TAG biosynthesis. To date, little is known about the regulation of DGAT activity in peanut (*Arachis hypogaea*), an agronomically important oilseed crop that is cultivated in many parts of the world. In this study, seven distinct forms of type 1 *DGAT* (*AhDGAT1.1-AhDGAT1.7*) were identified, cloned, and characterized. Comparisons of the nucleotide sequences and gene structures revealed many different splicing variants of *AhDGAT1*, some of which displayed different organ-specific expression patterns. A representative gene (*AhDGAT1.1*) was transformed into wild-type tobacco and was shown to increase seed fatty acid (FA) content by 14.7%–20.9%. All seven *AhDGAT1* s were expressed in TAG-deficient *Saccharomyces cerevisiae* strain H1246; the five longest *AhDGAT1* variants generated high levels of acyltransferase activity and complemented the free fatty acid lethality phenotype in this strain. The alternative splicing that gives rise to *AhDGAT1.2* and *AhDGAT1.4* creates predicted protein *C*-terminal truncations. The proteins encoded by these two variants were not active and did not complement the fatty acid sensitivity in H1246. These results were verified by visualization of intracellular lipid droplets using Nile Red staining. Collectively, the results presented here represent the first comprehensive analysis of the peanut *DGAT1* gene family, which, unlike in other published plant *DGAT1* sequences, shows widespread alternative splicing that may affect the expression patterns and enzyme activities of some members of the gene family.

1. Introduction

TAG is synthesized in the endoplasmic reticulum by at least two pathways. The first is catalyzed by phospholipid:diacylglycerol acyltransferase (PDAT) (Zhang et al., 2009), which transfers the *sn*-2 acyl chain from phosphatidylcholine (PC) to the *sn*-3 position of diacylglycerol (DAG), forming *sn*-1 lyso-PC and TAG. TAG is also produced by DGAT, the terminal enzyme of the Kennedy pathway (Kennedy 1961) via acylation of DAG using acyl-CoA as the donor. While different plants rely on each enzyme activity to different degrees, most published examples to date suggest that DGAT plays an important role in TAG biosynthesis.

Convergent evolution has created three structurally unrelated classes of *DGAT* genes, called type 1 (*DGAT1*), type 2 (*DGAT2*), and type 3 (*DGAT3)*, respectively. DGAT1 and DGAT2 are located in the endoplasmic reticulum (ER) membrane (Shockey et al., 2006) while DGAT3 is a soluble cytosolic enzyme (Saha et al., 2006). DGAT1 was first discovered in mouse, based on homology with mammalian acyl-CoA:cholesterol acyltransferase (Cases et al., 1998). Many plant DGAT1 s were subsequently identified and characterized (Jako et al., 2001; Milcamps et al., 2005; Shockey et al., 2006; Xu et al., 2008; Yu et al., 2008; Misra et al., 2013). DGAT2 was discovered in the fungus *Mortiella ramanniana* (now *Umbelopsis ramanniana*) (Lardizabal et al., 2001) and then identified in other eukaryotes including fungi, humans, and plants (Peng et al., 2013; Xu et al., 2013; Zhang et al., 2013; Chi et al., 2014). DGAT3 was identified from developing cotyledons of peanut (Saha et al., 2006) and later in other plants (Cao et al., 2013; Trenz et al., 2014).

Many studies have examined the roles of DGAT in plant TAG biosynthesis. Over-expression, mutation, and RNAi silencing of both

* Corresponding authors.
E-mail addresses: wansb@saas.ac.cn (S. Wan), pengzhenying2005@126.com (Z. Peng).

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Arabidopsis and tobacco DGAT1 reveal strong positive correlations between gene expression level and seed TAG levels (Katavic et al., 1995; Zhang et al., 2005; Bouvier-Navé et al., 2000; Jako et al., 2001), and even in leaf tissue, where *AtDGAT1* was expressed in tobacco behind the strong ribulose-bisphosphate carboxylase small subunit promoter (Andrianov et al., 2010). Yeast expression of tung tree DGAT1 and DGAT2 strongly increased the proportions of TAG species containing two or more eleostearic acid moieties (Shockey et al., 2006).

Peanut oil is commonly used in cooking because of its distinct, attractive flavor and high smoke point relative to many other cooking oils. Three types of peanut *DGAT* genes were identified recently (Peng et al., 2013; Chi et al., 2014). These initial studies showed that *AhDGAT1-1* and *AhDGAT1-2* heterologous expression in a *Saccharomyces cerevisiae* TAG-deficient quadruple mutant could restore lipid body formation and TAG biosynthesis (Chi et al., 2014), and that *E. coli* strains containing AhDGAT2a and AhDGAT2b accumulated markedly higher levels of fatty acids compared to the relevant controls (Peng et al., 2013).

Alternative splicing (AS) of pre-mRNA is an important post-transcriptional regulatory mechanism, whereby pre-mRNA is processed into multiple mature transcripts by regulating the selection and sizes of intron and exon sequences (Kalyna et al., 2012; Kornblihtt et al., 2013; Staiger and Brown, 2013). In plants, AS has been shown to exert significant effects on growth, development, signal transduction, circadian clock function, flowering, and environmental responses (Le Hir et al., 2001; Blencowe, 2006). AS also plays important roles in seed germination and fruit ripening (Tang et al., 2016; Zhang et al., 2016). The common alternative splicing sub-types are exon skipping (ES), alternative exon 5′-ends (5′-AE), alternative exon 3′-ends (3′-AE), intron retention (IR), alternative transcription start site (TSS), and alternative transcription termination site (TTS) (Florea et al., 2013). Some AS isoforms encode distinct functional proteins, while others contain premature termination codons, which are often degraded by nonsense-mediated decay (NMD) processes (Lewis et al., 2003).

In this study, we identified and characterized seven peanut *DGAT1* cDNA variants, here named *AhDGAT1.1-AhDGAT1.7*, which collectively represent three of the four members of the peanut DGAT1 gene family. The rich variety of transcript variants for the genes in this family suggests that AS plays an important role in peanut *DGAT1* gene expression regulation. A summary of the sequence analysis, functional characterization of five active and two inactive variants, as well as a comprehensive search for additional splice variants of *AhDGAT1* expressed in other peanut organs, are presented here.

2. Materials and methods

2.1. Plant materials

The peanut cultivar ‘Fenghua1’ was used for gene cloning and high-throughput sequence analysis. The tobacco (*Nicotiana tabacum*) cultivar ‘SR1’ was used for genetic transformation. The TAG-deficient *S. cerevisiae* strain H1246 (Sandager et al., 2002) was used for yeast transformation.

2.2. AhDGAT1 gene cloning, splice variant detection, and sequence analysis

Total RNA was isolated from peanut seeds 30 days after flowering (DAF) and reverse-transcribed into first-strand cDNAs using a cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). Based on the sequence of existing *AhDGAT1* genes (Genbank accessions KR004113-KR004116), we designed two pairs of primers (AhD1-FS, AhD1-FA, AhD2-FS, AhD2-FA, see Appendix S1 in Supplementary materials) to amplify peanut DGAT1 ORFs.

For high-throughput sequencing, total RNA of each of the four organs (root, leaf, seed1, seed2) was prepared using the NEXTflex™ Rapid RNA Sequencing Kit (Bioo Scientific, Austin, TX, USA) according to the manufacturer’s instructions. Sequencing libraries were generated using NEBNext® UltraTM Directional RNA Library Prep Kit for Illumina® (New England Biolabs, Ipswich, MA, USA) following the manufacturer’s recommendations, and index codes were added to attribute sequences to each sample.

Additional PCR protocols, and other details pertaining to DNA isolation, cloning, and AS variant detection, can be found in the Supplementary materials and methods (Appendix S2).

2.3. Yeast transformation and biochemical activity assay

The ORFs of *AhDGAT1* s were amplified by PCR, digested with *Not*I and *Pac*I, and cloned into multiple cloning site 1 of the galactose-inducible yeast expression vector pESC-URA (Agilent Technologies, Santa Clara, CA, USA). The plasmids were transformed into competent cells of *S. cerevisiae* strain H1246 and selected on SD-URA media. Pooled colonies from each strain were grown in selective liquid media, then back-diluted into liquid media containing galactose and raffinose to induce protein expression. In some cases, the cultures were also diluted and spotted on solid media with 0–3 mM oleic or linoleic acid. The level of growth was photographed with a Nikon camera (model #D7000) at different time points. Empty plasmids lacking a gene or containing *VfDGAT1* (Shockey et al., 2006) served as negative and positive controls, respectively.

2.4. Nile red staining

Aliquots of stationary phase cells (400 µl) were pelleted, washed twice in PBS, and then dispersed in 20 µl of PBS, to which was added 5 µl Nile Red (1 µg μl^{-1}) (Sandager et al., 2002). Stained cells were incubated in the dark for 10 min at 30 °C, then washed twice in PBS, and diluted into 100 µl of PBS. The lipid bodies in stained cells were observed and photographed using a fluorescence microscope (Olympus IX71-A12FL/PH, Japan).

2.5. Tobacco transformation

The ORF of *AhDGAT1.1* was cloned downstream of the *CaMV35S* promoter in plant binary vector pROK II (Transgen, China), which contains a kanamycin resistance gene. The *AhDGAT1.1* plasmid constructs were transformed into *Agrobacterium tumefaciens* LBA4404 by electroporation. Please see Appendix S2 in Supplementary materials for details of plant tissue culture and generation of homozygous transgenic tobacco lines.

The seeds of positive transformants were grown on 1/2 × MS medium with 100 mg l^{-1} kanamycin to select for positive plants (T_1 generation). T_2 and T_3 seeds were transferred to 1/2 × MS medium containing 100 mg l^{-1} kanamycin; homozygous T_3 plants were chosen for molecular identification, western blot analysis, and FA analysis.

2.6. Fatty acid analysis

The FA content of transformed tobacco seeds was analyzed by gas chromatography (GC). Samples were soaked in 2 ml of 2% sulfuric acid in dry methanol for 16 h at room temperature, followed by 80 min of heating at 90 °C to convert the FAs into FA methyl esters (FAMEs). The methyl ester of heptadecanoic acid (Nu-Chek Prep, Elysian, MN, USA) was used as a reference standard. After addition of 2 ml of distilled water and 3 ml of hexane, the FAMEs were extracted and 2-µl samples were analyzed on an Agilent Technologies 6890N gas chromatograph. An initial column temperature of 140 °C was maintained for 5 min, then increased to a final temperature of 240 °C at a rate of 4 °C min^{-1}, followed by a 10-min hold at 240 °C. Injection and detector temperatures were 240 °C and 26 °C, respectively. FAMEs were identified by comparison to standard retention times, and results were analyzed using the Chrom Perfect® LSi system chromatography software (Chrom Perfect®