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# Silencing of *DGAT1* in tobacco causes a reduction in seed oil content

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**Abstract**

The 679-bp fragment corresponding to nt 615–1293 of tobacco *DGAT1* (GenBank, AF129003) was used to create intron-containing construct expressing hpRNA for silencing endogenous *DGAT1* gene of *Nicotiana tabacum*. The oil content in mature seeds of transgenic lines was reduced by 9 to 49%. The transgenic tobacco, designated *Sil7*, is correlated with reduced triacylglycerol (TAG) contents in different tissues, including leaves, stems, roots, petals and mature seeds. We show that the reduction of *DGAT1* transcript level in transgenic tobacco mediated by hpRNA is correlated with the decrease of oil content in different tissues. In company with the decrease of average seed weight, seed oil content reduced, whereas protein and sugar content increased in the seeds of transgenic lines. There is a reversed relation between the deposition of oil and the synthesis of protein and sugar in the seeds of transgenic lines.


*Keywords:* Gene silencing; Tobacco; Diacylglycerol acyltransferase (DGAT); Triacylglycerol (TAG)

## 1. Introduction

Diacylglycerol acyltransferase (DGAT; EC 2.3.1.20) drives the final and only committed acylation step in the synthesis of TAG [1]. TAGs are quantitatively the most important storage form of energy for eukaryotic cells. In plants, TAG mainly accumulates in seeds, pollen and fruits of many species [2,3]. However, *DGAT1* transcripts are also detected in most plant tissues, including roots, leaves, stems, petals, flowers, anthers, developing siliques, young seedlings and germinating seeds [4–7]. The ubiquitously expressed *DGAT1* suggests that TAG can be synthesized in these tissues and it may also play other roles besides its role as the storage reserve in plants. In seeds, TAG biosynthesis occurs in the membranes of the endoplasmic reticulum and is channeled to the oil bodies, which are generated through budding of the outer ER membrane [8]. TAG is also present in lipid bodies localized in the stroma of chloroplasts [9]. Kaup et al. demonstrate that *DGAT1* is up-regulated during senescence of *Arabidopsis* leaves and indicate that the increased levels of TAG during leaf senescence may be an intermediate step in the mobilization of membrane lipid carbon to phloem-mobile sucrose [6]. The chloroform extract of the ATP control from isolated pea root plastids contained about 11% of TAG [10]. This result demonstrates that that root plastids are capable of synthesizing TAG.

*DGAT1* has been quite extensively studied in *Arabidopsis*. Two mutants, AS11 [5,11,12] and ABX45 [13] have been analyzed, and these studies show that dysfunctional DGAT1 protein results in a decrease in stored TAG and modified TAG fatty acid profile. These results provide strong evidence that DGAT1 is a key enzyme in catalyzing TAG formation. Seed-specific over-expression of an *Arabidopsis DGAT1* gene enhances oil deposition and average seed weight, suggesting that DGAT catalyzes the rate-limiting step in TAG biosynthesis [12]. In addition, ABX45, an inactivated *DGAT1* mutant of *Arabidopsis*, still synthesizes triacylglycerol indicating that other enzymes may also be involved in TAG accumulation [13].

Three DGAT-like proteins, DGAT1, DGAT2 [14] and phospholipid:DAG acyltransferase (PDAT) [15] have been identified. They are all capable of catalyzing the final

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acylation step during TAG synthesis. The proteins encoded by these separate gene families may regulate TAG synthesis at different stages of plant development and possibly with different cellular localizations.

It has demonstrated that self-complementary hpRNA-mediated gene silencing, a kind of post-transcriptional gene silencing (PTGS), results in high efficiency and efficacy of gene silencing in many plant, such as tobacco, *Arabidopsis*, cotton and rice [16–18].

In this report, we used a partial coding sequence of tobacco (*Nicotiana tabacum*) *DGAT1* gene to make an hpRNA-producing construct to specifically silence endogenous *DGAT1* gene. The silenced transformants presented different reduction degree of TAG contents in different tissues. To dissect if the pathway(s) of carbohydrate metabolism in the silenced transformants is defective and to identify if *DGAT1* gene is essential for TAG biosynthesis in tobacco seeds, we characterized the amounts of triacylglycerols, proteins, and soluble sugars in mature seeds of transgenic lines.

## 2. Materials and methods

### 2.1. DGAT1 gene-silencing constructs

A 679-bp fragment corresponding to nt 615–1293 of tobacco *DGAT1* (GenBank, AF129003) was amplified using a forward primer (5′-CCCCTCGAGAAACAGAGTCACG-CAGGC-3′) and a reverse primer (5′-CCGAATTCGGCA-TATAAAAGGTTTCC-3′). The forward primer contains an *Xho*I restriction site and the reverse primer includes an *Eco*RI restriction site, which made the PCR amplified product be digested by *Xho*I/*Eco*RI and cloned into *Xho*I/*Eco*RI-cut pKANNIBAL vector [16] in sense orientation relative to the CaMV35S promoter. Thus, the sense *DGAT1* 679-bp fragment was cloned into pKANNIBAL vector to form pKANNIBAL-679S. The 679-bp *DGAT1* fragment was then ligated into TA Vector I by T/A cloning method to form a plasmid designated TA-679. The TA Vector I was constructed from pBluescript SK digested with *Eco*RV, and followed by adding a dTTP to its 3′ end. The fragment containing the 679-bp *DGAT1* was released from the TA-679 plasmid by digestion with *Hin*dIII and *Xba*I, inserted into the restriction sites *Hin*dIII/*Xba*I of pKANNIBAL-679S plastid in antisense orientation subsequently. Thus, the *DGAT1*-silencing construct with inverted repeat configuration in pKANNIBAL-679S + 679A came into being. Plasmid pART27-S (Fig. 1) was generated by subcloning the *Not*I restricted fragment of pKANNIBAL-679S + 679A into *Not*I restricted binary vector pART27 [19], and then introduced into Agrobacterium tumefaciens strain LBA4404 by tri-parental mating for transformation of tobacco.

### 2.2. Plant transformation and materials

*Nicotiana tabacum* (Wisconsin 38) plants were transformed with pART27-S using Agrobacterium-mediated leaf disc transformation [20] and kanamycin (300 mg/L) selection. Presence of the transgene was conformed by PCR using CaMV35S promoter primers. For consistency in comparison, the non-transformed wild-type (n-t WT) lines and plasmid only control transgenics (transformed with pART27 empty vector without hpRNA insert, pWT) were both undergone the tissue culture process.

Seedlings were grown at 25 °C in the same growth chamber under 16 h light/8 h dark cycle. The roots, stems and leaves of 3-week-old seedlings were used to extract total lipids and RNA. To obtain the materials of flowers and seeds, n-t WT, and plasmid only WT (pWT) and primary transgenic tobacco plantlets were transferred to the greenhouse under natural day length, 26 °C in the day and 20 °C at night. When the plants were in bloom, the petals were harvested for extracting lipids and the fully opened flowers (with calyxes peeled off) for isolating RNA. The developing seeds 10 days after flower (DAF) were harvested for Northern blotting analysis and mature seeds for triacylglycerol analysis.

### 2.3. Lipids analysis

For triacylglycerol quantification, total lipids were extracted from roots, stems, leaves, petals of flowers and mature seeds of tobacco according to the method of Bligh and Dyer [21]. Lipid extracts were fractionated by thin layer chromatography. The plates were developed in hexane/diethyl ether/acetic acid (70:30:1; v/v). The separated TAG bands were visualized with iodine vapor and identified using triacylglycerol standard. Fatty acids of the separated lipid fractions were transmethylated with sulfuric acid/methanol (5:95; v/v) at 85 °C for 1 h after addition of 17:0 fatty acid as an internal standard, and the resultant fatty acid methyl esters were quantified by gas

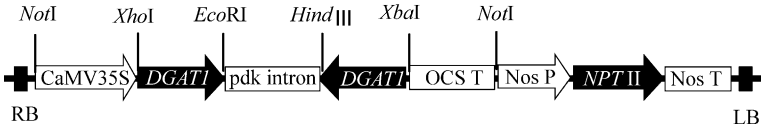


Fig. 1. Schematic diagram (not to scale) of *DGAT1*-silencing construct used to transform tobacco. The construct driven by CaMV 35S promoter consists of an inverted repeat of the 679-bp fragment corresponding to nt 615–1293 of tobacco *DGAT1* (GenBank, AF129003) separated by intron of pdk [16]. The neomycin phosphotransferase selectable marker gene (NPT II) is driven by the Nos promoter (Nos P). The T-DNA left border (LB) and right border (RB) are shown. The positions of enzyme sites are indicated.