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ORIGINAL PAPER

# Effects of Monogalactoglycerolipid Deficiency and Diacylglycerol Acyltransferase Overexpression on Oil Accumulation in Transgenic Tobacco

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**Abstract** Engineering accumulation of triacylglycerol (TAG) in vegetative tissues has been recently proposed as a promising strategy for increasing plant oil production. However, little is known about regulatory mechanisms involved in increasing oil production in plant vegetative tissues. In this study, expression of *NtMGD1* encoding a major biosynthetic enzyme for the chloroplast membrane lipid was inhibited by RNAi interference in tobacco. Furthermore, *AtDGAT1*, a rate-regulating gene involved in TAG biosynthesis, was ectopically overexpressed. Results showed that leaf TAG accumulations were significantly increased both by *NtMGD1* RNAi and *AtDGAT1* overexpression. However, combination of *AtDGAT1* overexpression with *NtMGD1* RNAi did not result in additive increase in TAG accumulation in leaves than *AtDGAT1* overexpression or *NtMGD1* RNAi alone. In addition, reduction of monogalactosyldiacylglycerol (MGDG) biosynthesis by *NtMGD1* RNAi was relieved by *AtDGAT1* overexpression. Expression of lipid transfer protein (LTP) was upregulated both by *AtDGAT1* overexpression and *NtMGD1* RNAi and correlated with increased oil accumulation in leaves. Our results indicated that fatty acids deesterified from chloroplast membrane galactolipids could be redirected into TAG. TAG is an energy-dense molecule that might act as a storage pool for carbohydrate. This membrane lipid remodeling may represent an adaptive response that enables plant cells to avoid toxic effects of free fatty acids.

**Keywords** Diacylglycerol acyltransferase · Monogalactosyldiacylglycerol · *Nicotiana tabacum* · Oil accumulation · Triacylglycerol

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**Abbreviations**

| | |
|---|---|
| TAG | Triacylglycerol |
| DGAT | Diacylglycerol acyltransferase |
| MGDG | Monogalactosyldiacylglycerol |
| DGDG | Digalactosyldiacylglycerol |
| DAG | Diacylglycerol |
| TLC | Thin layer chromatography |
| 16:0 | Palmitic acid |
| 18:0 | Stearic acid |
| 18:1 | Oleic acid |
| 18:2 | Linoleic acid |
| 18:3 | Linolenic acid |

## Introduction

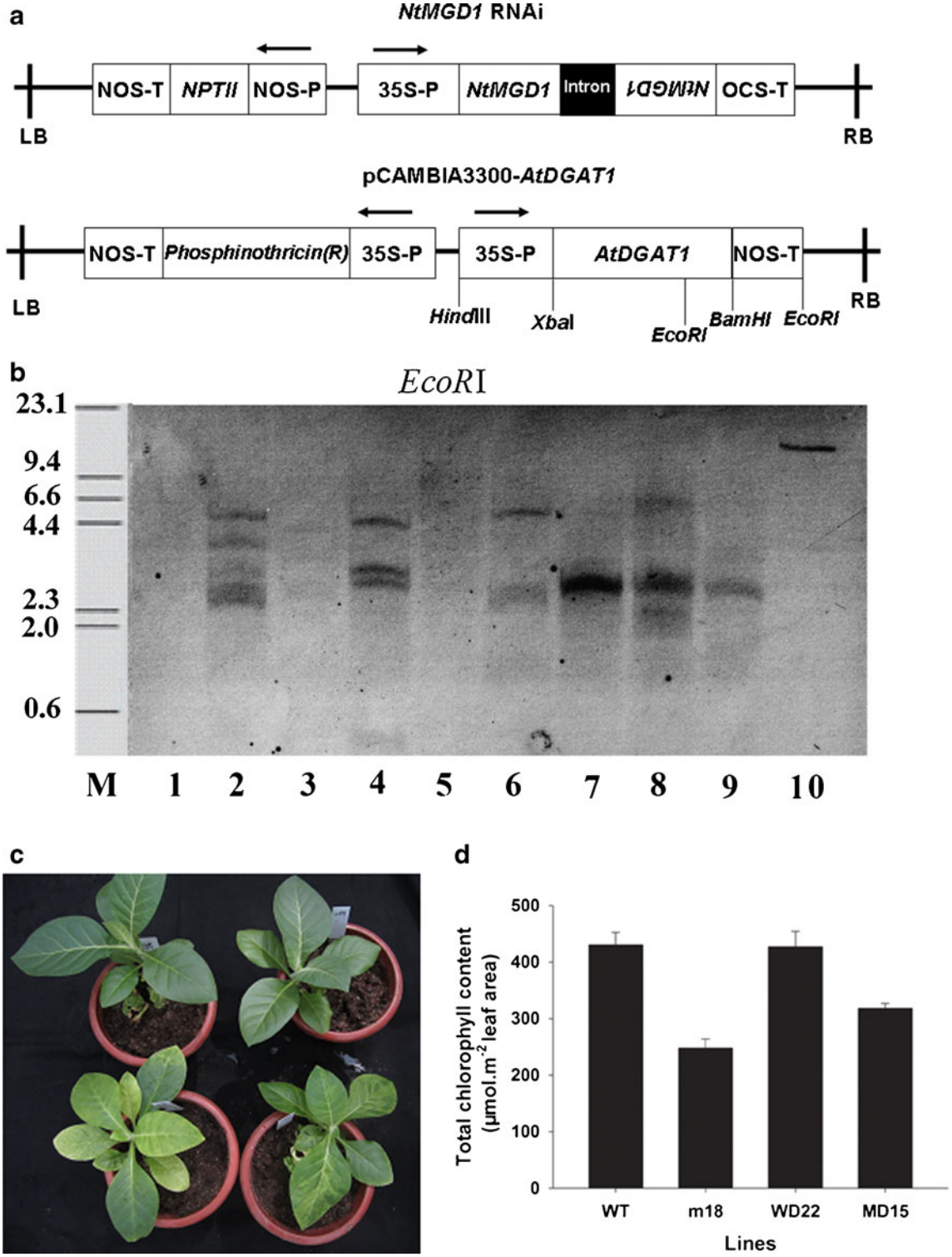
Plant oil triacylglycerol (TAG) plays crucial roles in people's life as main compositions in their diet and is increasingly a source of renewable biomaterials and fuels (Durrett et al. 2008; Andrianov et al. 2010; Chapman and Ohlrogge 2012). To meet growing demand and avoid competition with food, major expansion of oil production using many strategies will be required. Although there has been increasing understanding of plant TAG biosynthesis (Bao and Ohlrogge 1999; Durrett et al. 2008; Nikolau et al. 2003; Ohlrogge and Jaworski 1997; White et al. 2005; Chi et al. 2011; Wang et al. 2007) and its regulation (Baud et al. 2007; Hills 2004; Masaki et al. 2005; Santos Mendoza et al. 2005; Cernac and Benning 2004; Chen et al. 2011), to date, attempts to metabolically engineer oil production in oilseeds by targeting expression of a single gene or combination of multiple genes have met with limited success. One new strategy that has been proposed recently for increasing plant oil production is to elevate oil content in tissues where oil does not normally accumulate, for example, leaves and stems (Andrianov et al. 2010; Ohlrogge and Chapman 2011). Recent research

suggests that overexpressing seed transcription factors, such as LEC1 (Mu et al. 2008), and LEC2 (Slocombe et al. 2009), WRI1 (Slocombe et al. 2009; Mu et al. 2008; Cernac and Benning 2004; Liu et al. 2010; Shen et al. 2010) or upregulating synthesis of fatty acids (Andrianov et al. 2010; Bouvier-Nave et al. 2000; Klaus et al. 2004) or blocking turnover of TAG (James et al. 2010; Slocombe et al. 2009) can all increase the amount of oil accumulating in vegetative tissues. Particularly, overexpression in plants of a key enzyme of TAG biosynthesis, diacylglycerol (DAG) acyltransferase (DGAT), was shown to significantly increase accumulation of TAGs in tobacco leaves (Andrianov et al. 2010; Bouvier-Nave et al. 2000). The specific role for cytosolic leaf TAG has not been proven but participation in carbon storage has been envisaged (Slocombe et al. 2009). For instance, in *Arabidopsis*, blocking starch biosynthesis in combination with upregulation of TAG biosynthesis leads to an enhanced accumulation of TAGs in transgenic seedlings (Sanjaya et al. 2011). Since carbon flux into oil is derived from photosynthesis, occurrence of TAG is dependent on provision of carbohydrates (Cernac and Benning 2004). In the search for new strategies in capturing photosynthetic carbon for the production of oil, it is necessary to understand more regulation of TAG synthesis and accumulation in abundant nonseed tissues.

Biochemistry of TAG biosynthesis in plants is complex, and the carbon flux into oil may be in competition with other metabolic pathways (Carlsson et al. 2011), such as membrane glycerolipids biosynthesis. It has been investigated that chloroplasts can synthesize their complement of thylakoid membrane lipids de novo in only a limited number of plant species (Maldonado et al. 2002), and nearly all seed plants are able to import glycerolipid precursors assembled at the endoplasmic reticulum (ER) into the plastid for the biosynthesis of thylakoid membrane lipids (Xu et al. 2008). TAG is continuously synthesized and turned over in leaf tissues and shares common enzymatic machinery and pathways with membrane glycerolipids, such that the two processes are highly related (Carlsson et al. 2011; Chapman and Ohlrogge 2012). It has been estimated that most plant leaves contain 5 % fatty acid by weight in the form of polar membrane lipids (Ohlrogge and Chapman 2011), and turnover of membrane lipids in photosynthetic *Arabidopsis* leaves accounts for 4 % of total fatty acids per day (Bao et al. 2000). In drought-stressed cotton leaves, a significant decline in polar lipid and a parallel increase in TAG were observed (El-Hafid et al. 1989). In ozone-treated spinach leaves, TAG synthesis was confirmed to be activated to sequester fatty acids deesterified from galactolipids (Sakaki et al. 1990). In *Arabidopsis*-senescing leaves, 16:3 fatty acids are partitioned into TAG prior to further mobilization (Kaup et al. 2002). TAG production in leaf tissues was presumed to be a way of taking care of carbon already fixed in membrane lipid or perhaps specifically thylakoid galactolipids (Carlsson et al. 2011), and DGAT1 is thought to be instrumental in this process (Kaup et al. 2002; Slocombe et al. 2009).

Unraveling of regulatory mechanisms controlling the flux between TAG and membrane glycerolipids is of special significance. In this study, the relationship between TAG accumulation and thylakoid membrane glycerolipid biosynthesis was analyzed by blocking thylakoid galactolipid biosynthesis through suppressing expression of *NtMGD1*, a gene encoding a major catalytic enzyme monogalactosyldiacylglycerol (MGDG) synthase (MGD; D-galactose: 1, 2-DAG 3-β-D-galactosyltransferase; EC 2.4.1.46) and concurrently overexpressing *AtDGAT1* in the model species *Nicotiana tabacum*. MGDG synthase transfers D-galactose from UDP-galactose to sn-1, 2-DAG, which is the final step in MGDG biosynthesis. In plants, at least two types of MGDG biosynthesis differing in their N-terminal portion were found: type A (such as AtMGD1) and type B (such as AtMGD2 and AtMGD3). MGD1 was suggested to play key roles in maintaining structural organization and normal function of plastidic membranes in plant green tissues (Luo et al. 2006; Jarvis et al. 2000). In our results, we show that TAG accumulates both in leaves of transgenic tobacco lines deficient in chloroplast membrane lipid biosynthesis and overexpressing *AtDGAT1*, and overexpressing *AtDGAT1* contributes to relieving adverse effects of MGDG reduction, implicating that formation of TAG might act as a storage pool for carbohydrate that sequesters fatty acids deesterified from galactolipids and enables plant cells to avoid toxic effects of free fatty acids.

**Fig. 1** The T-DNA of the binary vectors, DNA gel–blot analysis of *AtDGAT1*-transformed tobacco plants and examples for the pale green phenotype in *NtMGD1*-downregulated *NtMGD1* RNAi line. **a** The T-DNA of *NtMGD1* RNAi and 35S-*AtDGAT1* vector. *35S-P* Cauliflower mosaic virus 35S promoter, *OCS-T Ocs* terminator, *NOS-P* nopaline synthase promoter sequence, *NOS-T* nopaline synthase terminator sequence, *NPTII* neomycin phosphotransferase II, *LB* left border, *RB* right border. **b** DNA gel–blot analysis of *AtDGAT1*-transformed tobacco plants. Total genomic DNA samples of plants were digested by *Eco*RI. The blot was hybridized with the DIG-labeled *AtDGAT1* probe, which is specific for *AtDGAT1* allowing for gene-specific detection. *M* DNA marker, *1* wild type tobacco (negative control), *2–4* different 35S-*AtDGAT1* lines: WD5, WD7, and WD22, *5 NtMGD1* RNAi tobacco line (*m18*), *6–9* different 35S-*AtDGAT1*/*NtMGD1* RNAi lines: MD4, MD15, and MD16, *10* positive control (plasmid pCAMBIA3300-*AtDGAT1*). **c** Phenotypes of transgenic tobacco lines compared with wild type control. The wild type tobacco is shown on the *upper left*, the 35S-*AtDGAT1* tobacco is shown on the *upper right*, the *NtMGD1* RNAi line (*m18*) is shown on the *lower left*, and 35S-*AtDGAT1*/*NtMGD1* RNAi tobacco is shown on the *lower right*. **d** Total chlorophyll contents of tobacco plants. Chlorophyll was extracted with 80 % acetone and its concentrations determined by a spectrophotometer as described. Presented values are means from three independent measurements ± SD. (Arnon 1949)




## Materials and Methods

### Plant Materials

Tobacco (*N. tabacum* cv. Wisconsin 38) *NtMGD1* RNAi line (*m18*) was previously described (Luo et al. 2006). It was produced via *Agrobacterium*-mediated transformation using *NtMGD1* RNAi vector as shown in Fig. 1a, in which a hairpin was constructed using a 599-bp fragment of the coding sequence (two to 600) of *NtMGD1* (GenBank accession no. AB047476), placed under the control of *CaMV* 35S promoter, upstream of *Ocs* terminator, with kanamycin as

the selection marker. All tobacco seedlings tested were grown in a growth chamber maintained under a 16 h light/8 h dark photoperiod at 25 °C.

### Vector Construction and Plant Transformation

The 1,563-bp full-length cDNA of *AtDGAT1* from *Arabidopsis thaliana* (accession no. DY641670) was placed downstream of *CaMV* 35S promoter using restriction endonucleases *Xba*I and *Bam*HI, and ligated into the plant transformation binary vector pCAMBIA3300 to get transformation vector pCAMBIA3300-*AtDGAT1*, with phosphinothricin as the selectable marker. The construct was then introduced into *Agrobacterium tumefaciens* strain EHA105. Wild type tobacco as well as *NtMGD1* RNAi line (*m18*) was transformed by pCAMBIA3300-*AtDGAT1* independently via *Agrobacterium*-mediated transformation following standard protocol (Horsch et al. 1985).

### DNA Gel–Blot Analysis of Transgenic Plants

Aliquots of genomic DNA (15 μg) extracted from tobacco were digested overnight at 37 °C with *Eco*RI, which was predicted to cut within the *AtDGAT1* cDNA and downstream of the *tNOS* terminator, respectively, in the T-DNA region of transformation vector pCAMBIA3300-*AtDGAT1* as shown in Fig. 1a. Genomic DNA was fractionated on 1.0 % agarose gel and then transferred to Hybond $N^+$ filters by capillary transfer. The 899-bp *AtDGAT1* cDNA fragment was labeled as the probe by polymerase chain reaction (PCR) in a reaction mixture containing 2 μL 10× PCR buffer, 0.4 μL dNTP (10 mM), 0.2 μL DIG-11-dUTP (1 mM), 1 μL primer AtDGAT1-1 F (5′-ATGGCGATTTTGGATTCTGCTG-3′, 10 mM), 1 μL primer AtDGAT1-899R (5′-CGTGGATAACTTGGCTGA-3′, 10 mM), 1 μL DNA template (5 ng/μL), 0.2 μL Taq DNA polymerase (5 μm/μL), and 14.2 μL distilled water. Primers for *AtDGAT1* were designed according to the sequence alignment of *AtDGAT1* with its ortholog from other plants allowing for gene-specific detection. Hybridization and detection were performed with DIG system under standard conditions described in the manufacturer's instructions (Roche, Germany).

### RNA Extraction and Semiquantitative RT-PCR Analysis

Semiquantitative reverse transcription PCR (RT-PCR) was performed to determine expression of *AtDGAT1*, *NtMGD1*, and lipid-related genes including biotin carboxylase (BC), pyruvate dehydrogenase E1 alpha subunit (PDH), plastidic pyruvate kinase isozyme A (PKA), plastidic pyruvate kinase isozyme G (PKB), cytosolic pyruvate kinase (PKC), and lipid transfer protein (LTP). Total RNA was extracted from tobacco leaves at 6-week-old stage using the TRIzol reagent (Invitrogen, USA) and treated with RNase-free DNase I (Promega, USA) to eliminate genomic DNA contamination. Quality and concentration of RNA preparations were accurately determined with a UV–visible spectrophotometer and its integrity was visually assessed on ethidium bromide-stained agarose gels. Reverse transcription was carried out using 1 μg of total RNA with MMLV reverse transcriptase (Promega) and degenerate primer OligodT: 5′-GCGGTACCCTTTTTTTTTTTTTTTT-3′ in a 20-μL reaction volume. The newly synthesized cDNA was used as template for PCR amplification. The *AtDGAT1* gene was amplified using primers: AtDGAT1-1 F and AtDGAT1-899R (annealing temperature: 55 °C, 30 cycles). The *NtMGD1* gene was amplified using primers MGD25F (5′-ACTCAAGAACCCACTAACCC-3′) and MGD1374R: (5′-CTGTCCAGCAATGTAATCAT-3′) (annealing temperature: 52 °C, 30 cycles). The primers used for detection of genes encoding BC, PDH, PKA, PKB, PKC, and LTP were listed in Table 1. For normalization of mRNA expression levels, internal control primers (NtTAC9F: 5′-CCCTCCCACATGCT ATTCT-3′; NtTAC9R: 5′-AGAGCCTCCAATCCAGACA-3′) were designed according to the reported cDNA sequence of *NtTAC9* (GenBank accession no. X69885). The PCR procedure was: 5 min at 94 °C, 27 cycles of 30 s at 94 °C, 30 s at 53 °C, 30 s at 72 °C, plus a final extension of 7 min at 72 °C. The described conditions were chosen to ensure that the PCR reaction was terminated within the linear range of amplification.

### Quantitative Real-Time RT-PCR

To quantitatively investigate transcription levels of *LTP* in transgenic tobacco lines with diverse oil accumulation in leaves, quantitative real-time RT-PCR (qRT-PCR) was performed by using the Stratagene (La Jolla, CA, USA) Mx3000P QPCR system, employing SYBR green to monitor double-stranded DNA (dsDNA) synthesis. The gene-specific primers were designed with Primer Express: LTP-F (5′-GAAATCGGCTGCTAATGC-3′) and LTP-R (5′-AGTCTGT GGAGGGGCTGA-3′) for tobacco LTP (GenBank accession no. D13952), NtACT-F (5′-CTGGCATTGCA GATCGTATGA-3′), and NtACT-R (5′-GCGCCACCAC CTTGATCTT-3′) for tobacco actin (*NtACT*, GenBank accession no. AB158612). The qRT-PCR results were analyzed by using MxPro software v4.10 (Stratagene). All data were normalized to the expression of *NtACT*.

### Determination of Total Chlorophyll Content

Leaf discs (16 mm in diameter avoiding veins) were punched from the fourth or fifth leaf numbered starting with the apical bud (position 1) of 6-week-old wild type and transgenic tobacco plants. Chlorophyll was extracted using cold 80 % acetone after ground in a mortar in liquid nitrogen. Pigments were analyzed in a spectrophotometer as described previously (Arnon 1949).

**Table 1** Primers used for semiquantitative RT-PCR analysis of lipid-related genes

| Gene name | GenBank accession number | Primer name | Nucleotide sequences |
|---|---|---|---|
| Biotin carboxlyase subunit (BC) | L38260 | BC-399 F | 5′-AAATGTCTTATCTGCTGCTATC-3′ |
| | | BC-860R | 5′-TACTTGTCCGCCAAAACC-3′ |
| Pyruvate dehydrogenase E1 alpha subunit (PDH) | AB090281 | PDH-9 F | 5′-TGGGATTGTTGGTGCTCA-3′ |
| | | PDH-296R | 5′-AGGGCATCCATACCATCTAC-3′ |
| Pyruvate kinase isozyme A (PKA) | Z28373 | PKA-80 F | 5′-CTTCTTCGTTTCCTCGTCT-3′ |
| | | PKA-562R | 5′-TCATTCAACCTCCTCAGTCT-3′ |
| Pyruvate kinase isozyme G (PKB) | Z28374 | PKB-335 F | 5′-TGGCAGAGGCTGGAATGA-3′ |
| | | PKB-765R | 5′-AGGCAGGGTGGCACTTTT-3′ |
| Cytosolic pyruvate kinase (PKC) | Z29492 | PKC-634 F | 5′-TTTGTTCGTAAGGGTTCAG-3′ |
| | | PKC-1114R | 5′-CTGGGGCATTTGACATAA-3′ |
| Lipid transfer protein (LTP) | D13952 | LTP-24 F | 5′-ATGCTTTGTGGTTTTGTGC-3′ |
| | | LTP-328R | 5′-AGTCTGTGGAGGGGCTGA-3′ |

## Lipid Extraction and Separation

Leaf samples were collected from tobacco plants at 6-week-old stage. Twenty leaf discs (16 mm in diameter avoiding veins) were punched out using a cork borer from the fourth or fifth leaf numbered starting with the apical bud (position 1). Among them, ten were ground to a fine powder under liquid nitrogen for lipid extraction, and another ten were used for determining dry matter content. Lipids were extracted from the samples using the described method (Bligh and Dyer 1959).

Lipids were separated by thin layer chromatography (TLC). TAG was separated by one-dimensional TLC using hexane:ethyl ether:acetate acid (70:30:1, v/v) as the solvent. Polar lipids were separated by two-dimensional TLC using the following solvent systems: chloroform/methanol/water (65:25:4, v/v/v) for the first dimension and chloroform/methanol/ammonia (65:35:5, v/v/v) for the second dimension. Spots were made visible by spaying the plates with 0.01 % primuline in acetone/water (60:40; v/v) and examining the plates under ultraviolet (360 nm) light. Heptacanoic acid was used as the standard.

## Fatty Acid Analysis

Fatty acid analysis was carried out as described (Xu et al. 2003). Briefly, spots corresponding to each lipid class separated by TLC were transesterified with 5 % $H_2SO_4$ in methanol at 90 °C for 1 h, and the fatty acid methyl esters (FAME's) were extracted with hexane and separated on a Hewlett-Packard 6890 gas chromatography apparatus supplied with a hydrogen flame ionization detector and a capillary column HP INNOWax (30 m; 0.25 mm i.d.) with $N_2$ carrier at 20 mL/min. The oven temperature was maintained at 170 °C for 3 min and then increased in steps to 210 °C raising the temperature by 5 °C every minute. FAMEs were identified by comparing their retention times with known standards (Supelco 37 Component FAME Mix, 47885-U) and quantified using heptacanoic acid (17:0, from Sigma-Aldrich) as the internal standard.

## Lipid-specific Staining of Cells

The fourth or fifth leaf numbered starting with the apical bud (position 1) from 6-week-old wild type and transgenic tobacco plants was used for lipid droplet (oil body) visualization. Freshly harvested leaf samples stained in a 2.5 μg⁄mL methanol solution of Nile red (Sigma-Aldrich, St Louis, MO, USA) were examined using an Olympus FV1000MPE two-photon laser scanning confocal microscope. Oil droplets were observed at 570–630 nm emission following 559-nm excitation by a solid state laser. Chloroplasts were observed at 655–755 nm emission using the same laser excitation. Images were captured with the Olympus FluoView 1000 ASW software (Olympus, Center Valley, PA, USA).

## Transmission Electron Microscopy

The fourth or fifth tobacco leaf numbered starting with the apical bud (position 1) from 6-week-old plants was fixed in 2.5 % (v/v) glutaraldehyde, 0.1 M sodium phosphate, pH 7.2, on ice followed by a secondary fixation in 1 % (wlv) $OsO_4$ in the same buffer. The specimens were dehydrated in a graded series of acetone and embedded in Spurr's epoxy resin. Ultrathin sections (80 nm) were stained with uranyl acetate and viewed in a Philips 400 transmission electron microscope.

## Results

### Molecular Evaluation of Transgenic Tobacco Plants by DNA Gel–Blot and RT-PCR Analysis

Transgenic tobacco lines deficient in *NtMGD1* mRNA expression by RNAi under the control of the cauliflower mosaic virus (CaMV) 35S promoter, summarized in Fig. 1a, were obtained as described (Luo et al. 2006) and resulted in *NtMGD1* RNAi lines. *NtMGD1* encodes MGDG synthase, a major catalytic enzyme for MGDG biosynthesis. One representative *NtMGD1* RNAi line (*m18*) was selected for further analysis. Furthermore, an overexpression vector pCAMBIA3300-*AtDGAT1*, with full-length cDNA of *AtDGAT1* driven by the *CaMV* 35S promoter as shown in Fig. 1a, was constructed and used to transform wild type tobacco as well as *NtMGD1* RNAi line *m18*. The resulting transgenic tobacco lines were designated as 35S-*AtDGAT1* lines and 35S-*AtDGAT1*/*NtMGD1* RNAi lines, respectively. Three representative lines were selected from each group for further analysis. Tobacco genomic DNA digested with *Eco*RI was subjected to DNA gel–blot hybridization analysis using the probe specific to *AtDGAT1* gene in the vector. The results showed that each positive line contained one to two integrated loci of *AtDGAT1* (Fig. 1b) and no hybridization signal was detected in the untransformed plants as the negative controls (wild type tobacco and *NtMGD1* RNAi line *m18*, respectively). As shown in Fig. 1a, two *Eco*RI restriction sites were found in the T-DNA region of pCAMBIA3300-*AtDGAT1* vector, so that the DNA regions where hybridization signals appeared were of similar molecular weights. Pale yellow leaves have been observed in the *NtMGD1* RNAi line (*m18*) in comparison with the wild type (Fig. 1c), and consistent with the pale yellow phenotype, the total chlorophyll content of the *NtMGD1* RNAi line (*m18*) was almost 40 % lower than that of wild type plants (Fig. 1d). The 35S-*AtDGAT1*/*NtMGD1* RNAi double gene transgenic lines also displayed a pale yellow phenotype, with the total chlorophyll content 24 % lower than the wild type. In contrast, the 35S-*AtDGAT1* lines established well and underwent normal growth and development compared to wild type plants when grown under normal growth conditions.

Abundance of *AtDGAT1* and *NtMGD1* mRNA in transgenic plants was determined with semiquantitative RT-PCR. As shown in Fig. 2, *AtDGAT1* was expressed with high efficiency in 35S-*AtDGAT1* and 35S-*AtDGAT1*/*NtMGD1* RNAi lines in contrast to the

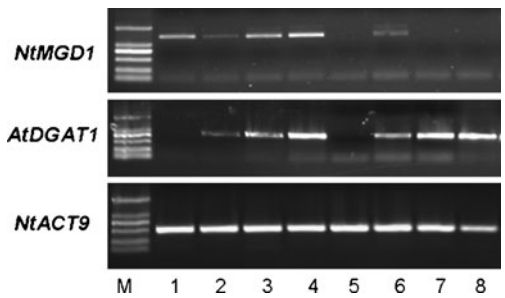



**Fig. 2** Semiquantitative RT-PCR analysis of *NtMGD1* and *AtDGAT1* expressions in tobacco plants. The names of genes analyzed were listed on the *left side*. The *NtACT9* gene was used as the control to show the normalization of the amount of templates in the PCR reactions. *M* DL2000, *1* wild type tobacco, *2–4* different 35S-*AtDGAT1* lines: WD5, WD7, and WD22, *5 NtMGD1* RNAi line (*m18*), *6–8* different 35S-*AtDGAT1*/*NtMGD1* RNAi lines: MD4, MD15, and MD16

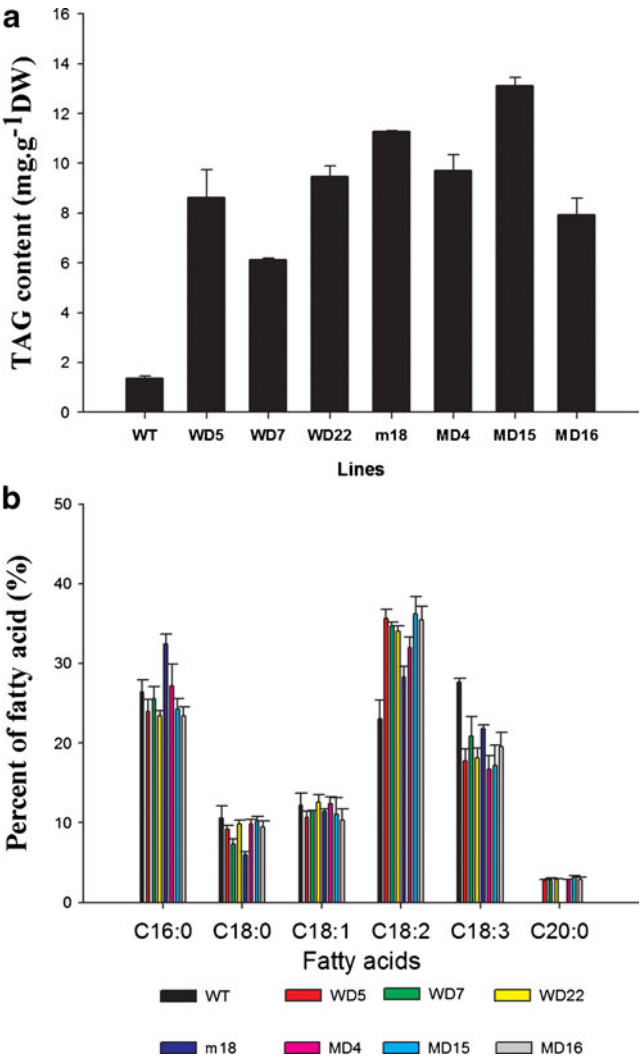



**Fig. 3** Triacylglycerol (TAG) contents and fatty acid profiles of TAG in tobacco plants. **a** Quantitative analysis of TAG in selected tobacco lines. *WT* Wild type tobacco, *WD5*, *WD7*, and *WD22* different 35S-*AtDGAT1* lines, *m18 NtMGD1* RNAi line, *MD4*, *MD15*, and *MD16* different 35S-*AtDGAT1*/*NtMGD1* RNAi lines. TAG amounts are given in equivalents (mean ± SD) of heptacanoic acid (17:0, from Sigma-Aldrich) used as a quantitative standard. **b** Fatty acid profiles of TAG in selected transgenic lines. Fatty acid profiles are given as relative amounts of fatty acids in TAG (mean ± SD)

negative controls, wild type, and *NtMGD1* RNAi line *m18*, in which no *AtDGAT1* signals were observed. Transcripts of *NtMGD1* were shown to be abundant in 35S-*AtDGAT1* lines as well as in the wild type and were negatively observed in *NtMGD1* RNAi line and 35S-*AtDGAT1*/*NtMGD1* RNAi lines (Fig. 2). Thus, irrespective of the *AtDGAT1* overexpression, reduction of *NtMGD1* mRNA in 35S-*AtDGAT1*/*NtMGD1* RNAi lines was similar to that in *NtMGD1* RNAi line *m18*, indicating that no observed negative effects was exerted on the *NtMGD1* RNAi interference by transgenic *AtDGAT1* overexpression.

## *AtDGAT1* Overexpression and *NtMGD1* RNAi Led to TAG Accumulation in Tobacco Leaves

To investigate the effects of *AtDGAT1* overexpression and *NtMGD1* RNAi on lipid metabolism, leaf TAG contents were compared in transgenic plants with negative controls at 6-week stage. The results displayed that the 35S-*AtDGAT1* lines accumulated considerably higher levels of TAG with a 4.5- to 7.0-fold increase compared to wild type plants (Fig. 3a). Even higher TAG accumulation was observed in *NtMGD1* RNAi line *m18* with an 8.3-fold increase relative to wild type. In 35S-*AtDGAT1*/*NtMGD1* RNAi lines 4 and 16, levels of TAG were considerably higher (5.8- and 7.2-fold) than the wild type; line 15 accumulated even more TAG than all other transgenic lines, with a 9.7-fold increase compared to the wild type. These data indicate that either *AtDGAT1* overexpression or *NtMGD1* RNAi alone is sufficient to enhance accumulation of TAG in leaves. However, combination of *AtDGAT1* overexpression along with *NtMGD1* RNAi did not lead to significant additive increase than *AtDGAT1* overexpression or *NtMGD1* RNAi alone.

Increased TAG accumulation in leaves was accompanied by a profound change in fatty acid composition in TAG fraction (Fig. 3b). The proportion of 18:2 was increased by 50–59 % in 35S-*AtDGAT1* lines and 41–58 % in 35S-*AtDGAT1*/*NtMGD1* RNAi lines compared to the wild type. In addition, the amount of 18:3 was reduced by 24–34 % in 35S-*AtDGAT1* lines and 29–33 % in 35S-*AtDGAT1*/*NtMGD1* RNAi lines when compared with the wild type. Relative slighter shifts were observed in *NtMGD1* RNAi line *m18*, with a 22.6 % increase in 18:2 and a 21 % decrease in 18:3 compared to the wild type. In addition, *AtDGAT1* overexpression resulted in the presence of 20:0, which was not found in leaf TAG from wild type plants and *NtMGD1* RNAi line *m18*, with a proportion of 2.7–2.9 % in 35S-*AtDGAT1* lines and 2.8–3.2 % in 35S-*AtDGAT1*/*NtMGD1* RNAi lines.

To examine the intracellular location of the TAG, fresh samples from the fourth or fifth leaf numbered starting with the apical bud (position 1) of 6-week-old wild type, transgenic tobacco plants were stained in methanol solution of Nile red, a lipid-specific fluorescent dye. Confocal microscopy observation showed presence of oil droplets distributed close to the chloroplast in the mesophyll cells, with the numbers of oil droplets much higher in leaf samples of the transgenic lines than the wild type (Fig. 4). The results of light microscopic observation were consistent with quantitative measurement of TAG content in leaves.

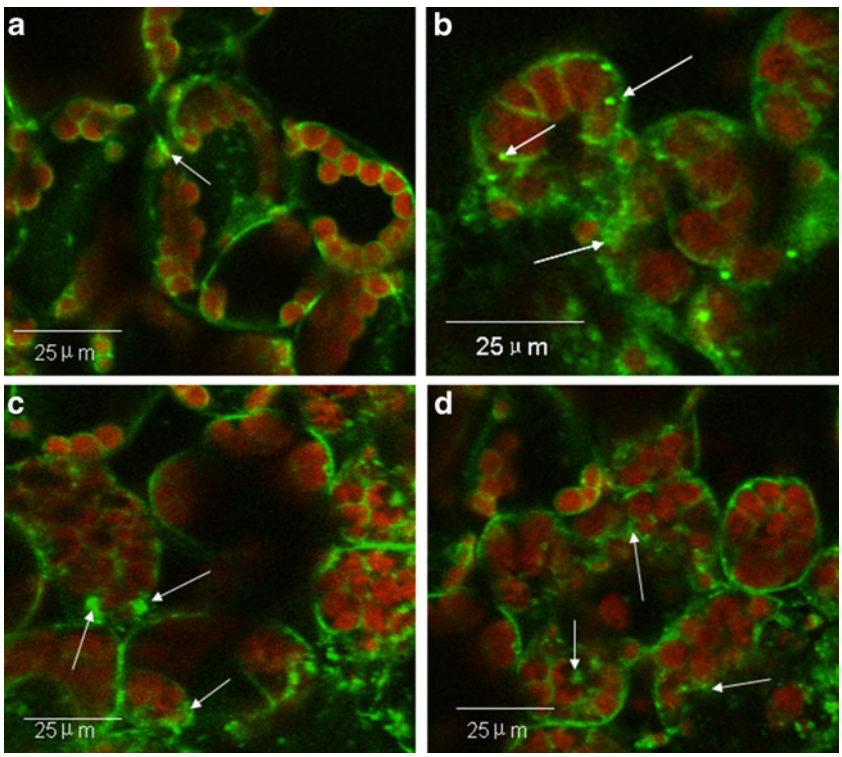



**Fig. 4** Confocal fluorescence image of leaf mesophyll tissues of wild type and transgenic lines stained with Nile red solution. Chloroplasts are shown in *red* and *arrows* indicate oil droplets. **a** Wild type tobacco; **b** 35S-*AtDGAT1* line 22; **c** *NtMGD1* RNAi line *m18*; **d** 35S-AtDGAT1/*NtMGD1* RNAi line 15. *Bars* correspond to 25 µm

*AtDGAT1* Overexpression and *NtMGD1* RNAi Led to Increased Total Fatty Acid Accumulation in Tobacco Leaves

All transgenic plants accumulated higher levels of total fatty acid in leaves, with a 18–77 % increase in 35S-*AtDGAT1* lines and in 35S-*AtDGAT1*/*NtMGD1* RNAi lines in comparison to the wild type, in addition to a 2.07-fold increase in the *NtMGD1* RNAi line *m18* (Fig. 5a). The increase in total fatty acid contents was accompanied by shifts in fatty acid compositions. The main change was the significant increase in the percentage of 18:2, from 13.6 to 20.5–23.2 %, and a decrease in the proportion of 18:3, from 60.8 to 43.4–50.5 %, in transgenic lines compared to the wild type (Fig. 5b).

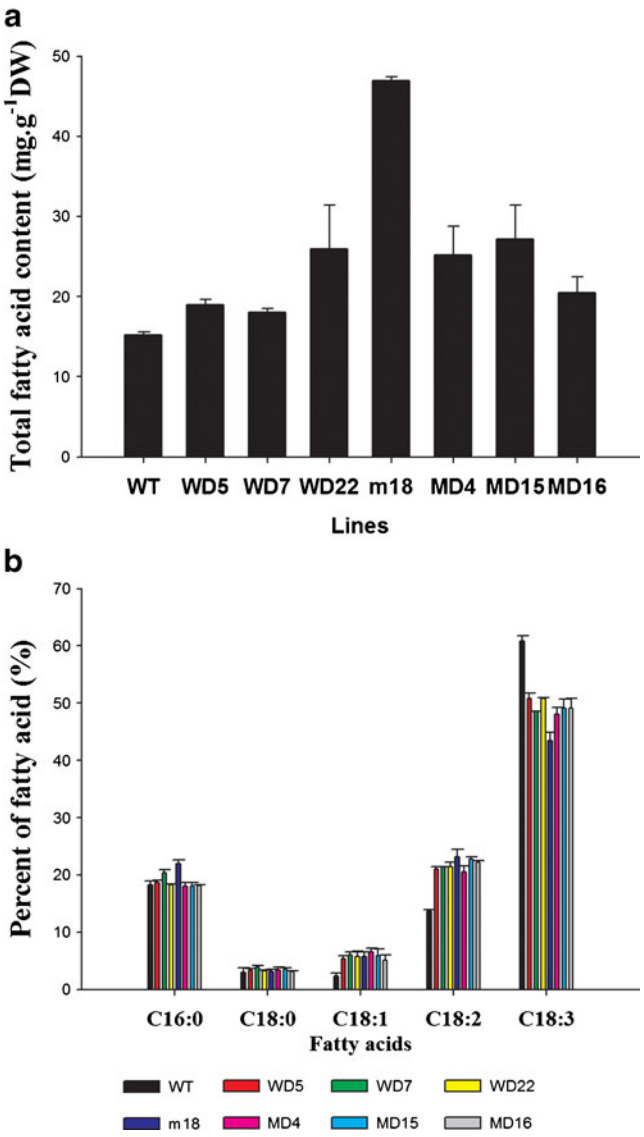



**Fig. 5** Total fatty acid contents and fatty acid profiles in selected tobacco lines. **a** Total fatty acid contents in selected transgenic lines. *WT* Wild type tobacco, *WD5*, *WD7*, and *WD22* different 35S-*AtDGAT1* lines, *m18 NtMGD1* RNAi line, *MD4*, *MD15*, and *MD16* different 35S-*AtDGAT1*/*NtMGD1* RNAi lines. Total fatty acid amounts are given in equivalents (mean ± SD) of heptacanoic acid (17:0, from Sigma-Aldrich) used as a quantitative standard. **b** Fatty acid profiles of total fatty acid in selected transgenic lines. Fatty acid profiles are given as relative amounts of fatty acids in total fatty acids (mean ± SD)

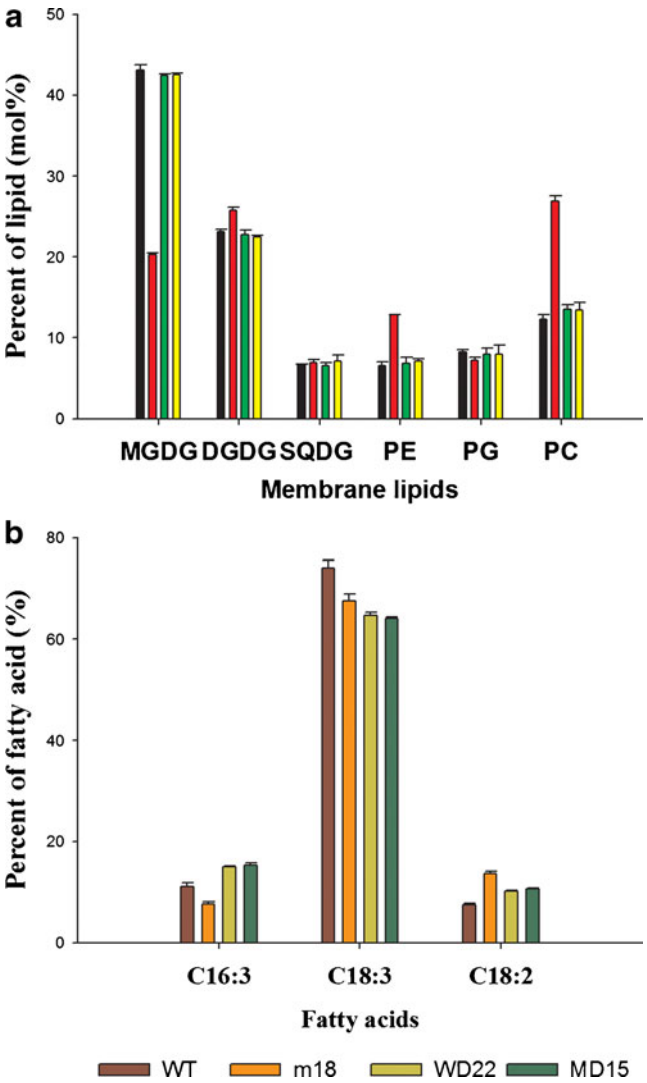



**Fig. 6** Lipid composition and fatty acid profiles of MGDG in selected tobacco lines. **a** Lipid composition of tobacco leaves from selected transgenic lines. *WT* Wild type tobacco, *m18 NtMGD1* RNAi tobacco line *m18*, *WD22* 35S-*AtDGAT1* line 22, *MD15* 35S-*AtDGAT1*/*NtMGD1* RNAi line 15. Total leaf lipids were extracted and the different lipid classes were separated by TLC and quantified. Indicated lipids are monogalactosyl diacylglycerol (*MGDG*), digalactosyl diacylglycerol (*DGDG*), phosphatidylglycerol (*PG*), sulfoquinovosyldiacylglycerol (*SQDG*), phosphatidylethanolamine (*PE*), and phosphatidylcholine (*PC*). **b** Fatty acid profiles of MGDG in tobacco leaves of selected transgenic lines. MGDG isolated in **a** was subjected to fatty acid methyl ester quantification. Presented values are means from three independent measurements ± SD

### Overexpression of *AtDGAT1* in *NtMGD1* RNAi Line Relieved MGDG Reduction

To investigate the effects of *NtMGD1* RNAi and *AtDGAT1* overexpression on lipid composition, total leaf lipids extracted from 35S-*AtDGAT1* line 22, *NtMGD1* RNAi line (m18), and 35S-*AtDGAT1*/*NtMGD1* RNAi line 15 were separated by TLC and compared with the wild type. Similar to previous reports (Luo et al. 2006), abundance of MGDG lipid in leaves of *NtMGD1* RNAi line was reduced by 52.9 %, from 43.1 % in the wild type to 20.2 % in *NtMGD1* RNAi line *m18* (Fig. 6a). Reduced abundance of MGDG was accompanied by alterations in other major plastid lipids, with digalactosyldiacylglycerol (DGDG) content increased by 11.7 %, from 23.1 % in the wild type to 25.7 % in *NtMGD1* RNAi line *m18*, and phosphatidylcholine (PC) increased by 1.17-fold in addition to phosphatidylethanolamine (PE) increase by 97 % compared to the wild type. Abundance of all major membrane lipids remained unchanged in 35S-*AtDGAT1* lines compared to the wild type. Interestingly, reduction of MGDG proportion in *NtMGD1* RNAi line *m18* was relieved by *AtDGAT1* overexpression. The MGDG proportion was increased from 20.3 % in *NtMGD1* RNAi line *m18* to 42.5 % in 35S-*AtDGAT1*/*NtMGD1* RNAi line 15, associated with the decreased abundance of DGDG, PC, and PE in 35S-*AtDGAT1*/*NtMGD1* RNAi line 15 compared to *NtMGD1* RNAi line *m18*. Lipid compositions in the *AtDGAT1*/*NtMGD1* RNAi line 15 were recovered to levels similar to the wild type. Alterations in the lipid composition were accompanied by shifts in the fatty acid compositions of MGDG lipids (Fig. 6b). MGDG from the *NtMGD1* RNAi line *m18* was found to contain 31 % less 16:3 (7.6 % compared to 11.1 %), 8.6 % less 18:3 (67.6 % compared to 74 %), and 84.9 % more 18:2 (13.7 % compared to 7.4 %) than that from the wild type. In MGDG from 35S-*AtDGAT1* line 22 and 35S-*AtDGAT1*/*NtMGD1* RNAi line 15, the percentage of 16:3 was increased by almost 30 % in addition to 18:2 increase by about 37 % and 18:3 decrease by about 12 % compared to the wild type.

Cross sections of mesophyll cell chloroplasts from the wild type and transgenic lines were analyzed by transmission electron microscopy to compare the ultrastructure of the thylakoid membrane system. As shown in Fig. 7, the ultrastructure of chloroplasts from 35S-*AtDGAT1* lines was not found to be altered compared to the wild type. However, *NtMGD1* RNAi resulted in decreased number of granal stack in chloroplasts when compared to the wild type. Granal stack number in chloroplasts from 35S-*AtDGAT1*/*NtMGD1* RNAi lines was higher than that in the *NtMGD1* RNAi line *m18* and slightly lower than the wild type, indicating that *AtDGAT1* overexpression plays a role in restoring deficiency in chloroplast ultrastucture caused by *NtMGD1* RNAi. Such result was consistent with the data of total chlorophyll contents (Fig. 1d).

## Discussion

Engineering plants to accumulate TAG in vegetative tissues has been recently proposed as a promising strategy for increasing plant oil production. In the present study, we show that TAG production in tobacco leaves could be improved by two different mechanisms, *DGAT1* overexpression and *MGD1* RNAi. Tobacco leaves overexpressing *DGAT1* under the control of a strong ribulose biphosphate carboxylase small

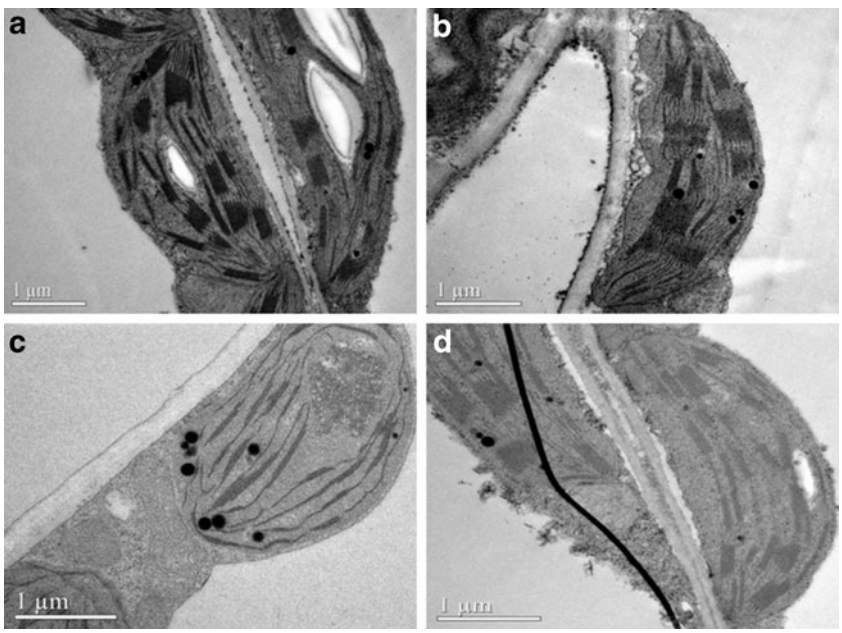



**Fig. 7** TEM indicating changes in plastid morphology in the transgenic tobacco plants compared with wild type control. **a** Wild type tobacco; **b** 35S-*AtDGAT1* tobacco line 22; **c** *NtMGD1* RNAi line *m18*; **d** 35S-*AtDGAT1*/*NtMGD1* RNAi line 15

subunit promoter has been previously reported to accumulate significant levels of oil (Andrianov et al. 2010). DGAT1 was thought to be instrumental in partitioning plastidial 16:3 fatty acids into TAG (Kaup et al. 2002). Consistent with these results, our results revealed that AtDGAT1 overexpression under the control of constitutive promoter *CaMV* 35S led to at least a fourfold increase in oil accumulation in tobacco leaves. We demonstrate the potential of DGAT1 in partitioning fatty acids to TAG in tobacco green biomass. Since TAGs are continuously synthesized and turned over in leaves and share common pathways with membrane lipids (Carlsson et al. 2011), DGAT1 overexpression is efficient for sequestering the fatty acids released from turnover of membrane lipids and protecting plant cells from toxic effects of free fatty acids. TAG production in leaf tissue is presumed to be a way of taking care of carbon that has already been fixed in membrane lipids or perhaps specifically thylakoid galactolipids (Carlsson et al. 2011). In our results, interference of *NtMGD1* mRNA could also result in an 8.4-fold increase in leaf TAG accumulation. We speculate that excess fatty acids caused by thylakoid galactolipids biosynthetic deficiency could be redirected for oil biosynthesis. The data demonstrate the roles that cytosolic leaf TAG acts were to participate in carbon storage and/or membrane lipid remodeling, as proposed previously (Murphy and Parker 1984; Murphy 2001; Kaup et al. 2002; Lin and Oliver 2008).

Increase in TAG accumulation was accompanied by a sharp shift in fatty acid composition. The amount of 18:2 in TAG fraction was detected to be increased from 23 % in wild type tobacco to 28–36 % in all the transgenic lines, accompanied by the amount of 18:3 reduced from 27 % in wild type tobacco to 18–21 % in transgenic lines. Similar increases in the percentage of 18:2 and decreases in 18:3 were also observed in total fatty acids in addition to the percentage of 18:1 increased from 2.2 % in the wild type to 5.3–6.5 % in transgenic lines. Although the mechanism that caused the observed shifts in fatty acid composition is unclear, our results are consistent with previous reports that DGAT1 overexpression resulted in a drastic shift in fatty acid (FA) composition in tobacco leaves (Andrianov et al. 2010). One possible explanation might be limited availability of desaturase, which leads to accumulation of more mono- and diunsaturated FA when the total amount of FA increases, as proposed previously (Kinney et al. 2002). Screening of *Arabidopsis* mutants clearly demonstrated that deficiencies in single genes have profound effects on seed oil FA composition (Millar and Kunst 1999).

DGAT1 catalyzes the enzymatic process that located downstream in the synthesis of TAG. Although it is possible that increased oil accumulation is solely due to increased activity of this one enzymatic step, it is also conceivable that altering DGAT1 activity results in secondary metabolic or regulatory effects that contribute to increased TAG accumulation, so that DGAT1 levels might also affect steps upstream in the fatty acid and TAG biosynthetic pathways (Sharma et al. 2008). It was speculated that increases in DGAT1 activity may lower the size of the acyl-CoA pools, thereby signaling a need for enhanced fatty acid synthesis (Jako et al. 2001). In an attempt to ascertain possible secondary effects associated with transgenic *AtDGAT1* overexpression, relative transcription levels of genes involved in lipid metabolism including pyruvate dehydrogenase E1 alpha (PDH, EC1.2.4.1), BC, pyruvate kinase (PK,

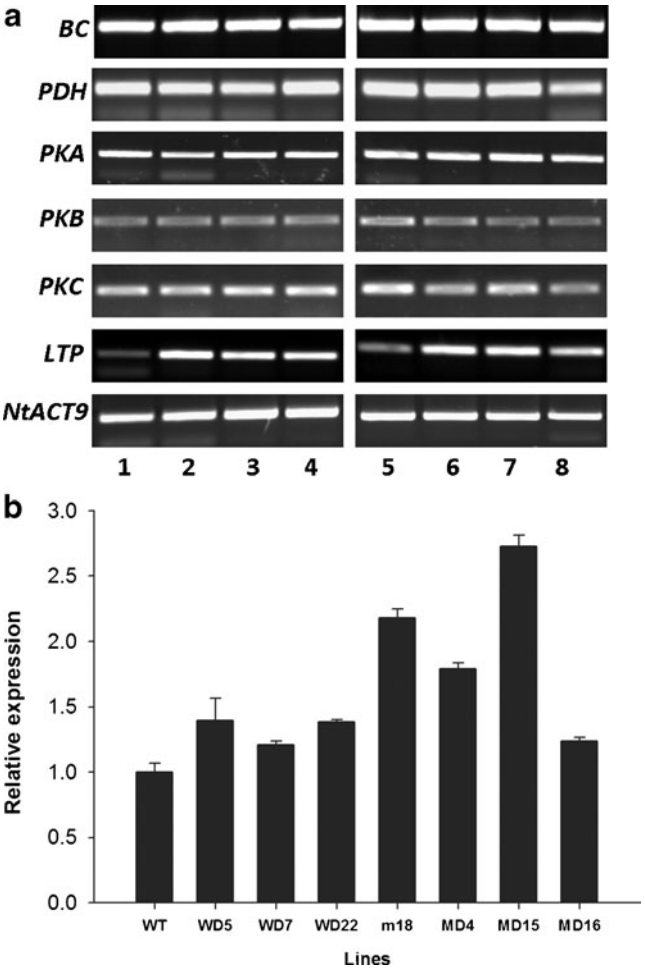



**Fig. 8** Transcription levels of lipid-related genes in leaves of selected tobacco lines. **a** Semiquantitative RT-PCR analysis of lipid-related genes. The names of genes analyzed were listed on the *left side*. The *NtTAC9* gene was used as the control to show the normalization of the amount of templates in the PCR reactions. *1* Wild type tobacco, *2–4* different 35S-*AtDGAT1* lines: WD5, WD7, and WD22, *5 NtMGD1* RNAi line (*m18*), *6–8* different 35S-*AtDGAT1*/*NtMGD1* RNAi lines: MD4, MD15, and MD16. **b** Quantitative analysis of *LTP* expression in leaves of selected tobacco lines. Expression of *LTP* was quantified by using qRT-PCR and normalized to the expression of the *NtACT* gene. Transcript accumulation of the *LTP* gene was shown as the relative expression compared with the *LTP* mRNA expression of wild type tobacco. Presented values are means from three independent measurements ± SD

EC 2.7.1.40), and transfer protein (LTP) were analyzed. Our results showed that transcriptions of genes encoding BC, PDH, and PK encoding genes *PKA, PKB*, and *PKC* were consistent in all the transgenic lines compared to the wild type (Fig. 8a). However, relative expression levels of the gene encoding LTP were found to be upregulated in all transgenic lines (Fig. 8a). LTPs are basic 9-kDa proteins that present in high amounts (as much as 4 % of the total soluble proteins) in higher plants and were reported to enhance in vitro transfer of phospholipids between membranes and participate in regulation of intracellular fatty acid pools. Results of qRT-PCR analysis confirmed the changes of *LTP* expression levels. Consistent with improvement of oil accumulation in leaves, relative *LTP* mRNA levels were increased by 1.2- to 2.1-fold in transgenic lines compared to the wild type (Fig. 8b). We suggest that LTP might participate in the process of lipid remodeling in which excess fatty acids deesterified from galactolipids were sequestered into TAG. Upregulation of *LTP* was also identified in transgenic *Brassica* overexpressing *DGAT1*, with increased oil accumulation in seeds (Sharma et al. 2008). LTPs can enhance in vitro transfer of phospholipids between membranes and were thought to participate in membrane biogenesis and regulation of intracellular fatty acid pools (Ohlrogge and Chapman 2011; Sharma et al. 2008).

The main goal of our study in tobacco was to test the hypothesis that the two biosynthetic mechanisms could lead to additive increase in oil accumulation in vegetative tissues. Intriguingly, the combination of *AtDGAT1* overexpression along with *NtMGD1* RNAi did not lead to additive increase in leaf TAG accumulation than *AtDGAT1* overexpression or *NtMGD1* RNAi alone. Such results demonstrated that the two mechanisms for generating TAG in leaves are nonadditive, possibly because of limited fatty acid supply. For example, evidence suggests that oil synthesis in plant tissues may be limited by production of fatty acids (Bao and Ohlrogge 1999; Ohlrogge and Chapman 2011).

The mechanism underlying restoration of MGDG biosynthesis by *AtDGAT1* overexpression is not clear. One possible explanation is that transgenic overexpression of *AtDGAT1* in 35S-*AtDGAT1*/*NtMGD1* RNAi lines might enable plants with transient increased synthesis of TAG, which acts as an intermediate product, to be subsequently translocated and provides precursors for thylakoid membrane lipids. For example, it has been suggested that the TAG produced during senescence is not accumulated but there is a rapid turnover of fatty acids and other components during this phase (Carlsson et al. 2011), and nearly all seed plants that have been investigated are able to import glycerolipid precursors assembled at the ER into the plastid for biosynthesis of thylakoid membrane lipids, in particular for glycoglycerolipids MGDG, DGDG, and sulfoquinovosyldiacylglycerol (SQDG) (Xu et al. 2008). Since the interference of *NtMGD1* transcription by RNAi was not affected by the transgenic *AtDGAT1* overexpression, restoration of MGDG synthesis in *NtMGD1* RNAi line might be catalyzed by its isoenzymes encoded by other genes (such as MGD2 and MGD3) or by the relieved enzyme activity of NtMGD1 on the basis of residual *NtMGD1* transcription. It was probable that the enzyme activity of NtMGD1 was increased by the temporary shortage of fatty acids because of accelerated TAG synthesis, possibly through feedback effects. Increased transcription of isoenzymes of NtMGD1 might complement with the function of NtMGD1. Given the correlation of relieved synthesis of thylakoid membrane lipids such as MGDG, it is not surprising that overexpression of *AtDGAT1* did not lead to additive TAG accumulation in leaves of *NtMGD1* RNAi line because of limited availability of fatty acids. Further research should be performed on the mechanism controlling the balance between TAG and membrane lipid synthesis, and their remodeling.

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