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Expression analysis of *VfDGAT2* in various tissues of the tung tree and in transgenic yeast

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ABSTRACT. The tung tree (*Vernicia fordii* Hemsl.; *Vf*) has great potential as an industrial crop owning to its seed oil that has multiple uses. Diacylglycerol acyltransferases (DGATs) catalyze the last and most committed step of triacylglycerol (TAG) biosynthesis. In order to examine the physiological role of the *VfDGAT2* gene in the tung tree, we characterized its expression profiles in different tung tissues/organs and seeds at different developmental stages. Oil content and α-eleostearic acid production during seed development were also examined. Expression studies showed that *VfDGAT2* was expressed in all tissues tested, with the highest expression in developing seeds where the expression was about 19-fold more than that in leaves. *VfDGAT2* showed temporal-specific expression during seed development and maturation. Notably, the expression of *VfDGAT2* in developing seeds was found to be consistent with tung oil accumulation and α-eleostearic acid production. The expression level of *VfDGAT2* was lower in the early

stages of oil accumulation and α-eleostearic acid biosynthesis, rapidly increased during the peak periods of fatty acid synthesis in August, and then decreased during completion of the accumulation period at the end of September. When the *VfDGAT2* gene was transferred to the oleaginous yeast *Rhodotorula glutinis*, its expression was detected along with fatty acid products. The results showed that *VfDGAT2* was highly expressed in transgenic yeast clones, and the total fatty acid content in one of these clones, *VfDGAT2*-3, was 7.8-fold more than that in the control, indicating that *VfDGAT2* contributed to fatty acid accumulation into TAG and might be a target gene for improving tung oil composition through genetic engineering.

Key words: *Vernicia fordii*; Tung oil; Gene expression profile; *VfDGAT2*; Fatty acid accumulation

INTRODUCTION

The tung tree, a member of the Euphorbiaceae family, is an important industrial oil-seed plant. The oil, named tung oil, accumulates in tung seeds mainly in the form of triacylglycerol (TAG) (Chen et al., 2010a). With its 80% α-eleostearic acid content, tung oil is an unusual trienoic fatty acid, making it valuable in numerous industrial applications (Dyer et al., 2002). Tung oil is now widely used in the production of paints, plasticizers, high-quality printing, resins, medicine, chemical regents, and biodiesel (Brown and Keeler, 2005; Li et al., 2010a; Shang et al., 2010). Approximately 80,000 tons of tung oil is produced in China per year. However, the output of tung oil has not kept pace with the demand, and there is increased industrial interest in the yield and characteristics of tung oil (Chen et al., 2010b). Therefore, studying the genes involved in tung oil biosynthesis will provide better understanding of lipid biosynthesis. Furthermore, it will provide the possibility of using genetic engineering to construct improved varieties producing high-value oils.

During the process of tung seed development and maturation, tung oil synthesis starts in July and rapidly increases from mid-August to mid-September (Wang et al., 1985). During the course of TAG synthesis, diacylglycerol acyltransferases (DGATs) catalyze the final and most committed step by transferring an acyl group from acyl-coenzyme A to diacylglycerol (Bouvier-Navé et al., 2000). Three distinct family members, *DGAT1*, *DGAT2*, and *DGAT3*, have been identified in several species such as *Olea europaea* (Banilas et al., 2011), *Jatropha curcas* (Xu et al., 2011), *Ricinus communis* (Cagliari et al., 2010), and *Vernonia galamensis* (Li et al., 2010b; Li et al., 2012), etc. *DGAT* may play an important role in oil accumulation in seeds (Zhang et al., 2005; Chen et al., 2007). Jako et al. (2001) showed that overexpression of *Arabidopsi*s DGAT cDNA enhanced seed oil content and seed weight. Oakes et al. (2011) demonstrated that *NcDGAT2* from *Neurospora crassa* can improve kernel oil production in maize with a 26% (relative) increase. In addition, *DGAT2* was reported to be a potential contributor to the desired accumulation of TAG (Shockey et al., 2006; Cahoon et al., 2007). Li et al. (2010b) showed that both *VgDGAT1* and *VgDGAT2* isolated from *V. galamensis* could increase the accumulation of epoxy fatty acids in soybean oil. Similarly, *RcDGAT2* cloned from castor bean (*R. communis*) could increase hydroxy fatty acid content from 17 to nearly

30% in transgenic *Arabidopsis* (Burgal et al., 2008).

Recently, it was reported that DGAT1 and DGAT2 from the tung tree are located in distinct dynamic regions of the endoplasmic reticulum, and *DGAT2* is likely to be the main contributor to tung oil production in *Vernicia fordii*, rather than *DGAT1* (Shockey et al., 2006). In the present study, to further elucidate the role of the *VfDGAT2* gene in TAG biosynthesis, we characterized the expression profiles of *VfDGAT2* in different tung tissues/organs and seeds at different developmental stages. Furthermore, we examined total oil content and α-eleostearic acid concentration at each stage of seed development. Transgenic *Rhodotorula glutinis* expressing *VfDGAT2* was constructed to determine the role of *VfDGAT2* in fatty acid accumulation.

MATERIAL AND METHODS

Plant materials

Tung trees were grown in a natural environment at the National Tung Tree Gene Pool (constructed in 1979) in Dongfanghong Forest Farm, Zhejiang Province, China. Tung leaves, petioles, stems, petals, pistils, fruitlets (30 days after flowering), and developing seeds (July 16, July 26, August 11, August 25, September 9, and September 26) were harvested from the cultivar 'Chenjiaxu 9-24'. All samples were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction. All samples were collected in triplicate.

Vectors, strains, and media

The pBI121 plasmid was used to transform yeast. The expression vector pBI121-*VfDGAT2* was constructed and transformed into *Agrobacterium tumefaciens* EHA105 cells and preserved in the laboratory. The oleaginous yeast used in the experiment was *R. glutinis*. The media used were LB, YEPD, induction medium (IM) (Takken et al., 2004), selective medium (SM) (20 g/L glucose, 6.7 g/L yeast extract, pH 6.0), and lipid fermentation medium (LFM) (Liu et al., 2008). All the media were sterilized by autoclaving at 115°C for 15 min.

Transformation of yeast

A. tumefaciens-mediated transformation of pBI121-*VfDGAT2* into *R. glutinis* was by the method of Piers et al. (1996), with some modification. Yeast cells were inoculated into 10 mL YEPD medium and grown in a shaker at 30°C and 180 rpm for 18 h, and then diluted 10-fold with fresh YEPD medium and grown for an additional 6 h. The cells were harvested by centrifugation, washed once with sterile water, and resuspended in IM at a final concentration of 1 x 10^8 cells/mL. *Agrobacterium* clones harboring pBI121-*DGAT2* or pBI121 were used to inoculate 10 mL LB medium (50 μg/mL kanamycin and 50 μg/mL rifampicin) in a 50-mL flask and grown at 28°C and 180 rpm in a shaking incubator for 18 h. The cells were pelleted, resuspended in an equal volume of IM (50 μg/mL kanamycin and 50 μg/mL rifampicin) and incubated for an additional 6 h. The cells were then harvested and resuspended in IM at a final concentration of 1 x 10^{10} cells/mL. Cocultivation of bacteria and yeast was initiated by mixing 50 μL of each in 10 mL IM containing 200 μM acetosyrin-

gone (AS) and grown overnight at 28°C with shaking. The overnight mixture was deposited onto a 25-mm diameter nitrocellulose filter that was then placed on IM plates with AS and incubated for 48 h at 25°C in the dark. The nitrocellulose filter was then transferred to an SM I plate containing 100 µg/mL kanamycin, 200 µg/mL cefotaxime, and 60 µg/mL streptomycin to select transformants. Putative transformants were further selected on SM II plates containing 150 µg/mL kanamycin and 300 µg/mL cefotaxime. Polymerase chain reaction (PCR) with the primers *VfDGAT2*-F/R and *VfDGAT2*-RTF/RTR was used to determine if the genes were successfully integrated into the *R. glutinis* chromosome. A transgenic yeast carrying the empty pBI121 vector was used as a control for further studies. All primers used for gene confirmation are listed in Table 1.

Table 1. Primers for *VfDGAT2* identification and RTq-PCR.

No.	Primers	Sequences (5'-3')
1	*VfDGAT2*-RTF	TGGCTCTTTCCATTTCATCC
2	*VfDGAT2*-RTR	CGTAAG CACGATCAGAACGA
3	*VfUBQ*-RTF	CCGTGGTGGCTGTTAAGTTT
4	*VfUBQ*-RTR	AAGGCCATTTCAACATCCTG
5	*vfDGAT2*-F	GCCAATGCTTGTTCAGCTAT
6	*vfDGAT2*-R	TAGGCTGTGGATTTTGCTTC
7	*RgACT1*-RTF	ACTTTGAGCAGGAGATGCAG
8	*RgACT1*-RTR	GACATGACAATGTTGCCGTA

RNA extraction and real-time quantitative PCR (RT-qPCR)

Total RNA from six different tung tissues/organs and seeds at six different developmental stages was extracted using the RN38-EASY Spin Plus Plant RNA kit (Aidlab Biotech, Beijing, China). The total RNA of *R. glutinis* with *VfDGAT2* and of the control was extracted using the RN10-EASY Spin Yeast Rapid Extraction Kit (Aidlab Biotech). RNA was concentrated and detected using a NanoDrop 2000 spectrophotometer (Thermo, USA), and verified by loading onto a denaturing 1.0% (p/v) agarose gel. About 3 µg total RNA was used as the template for 1st-strand cDNA synthesis with the SuperScript Ш First-Strand Synthesis System (Invitrogen, USA).

The primers used to detect *VfDGAT2* expression in tung (*VfDGAT2*-RTF and *VfDGAT2*-RTR) were designed using Primer3 (http://frodo.wi.mit.edu/primer3/). Tung *UBQ* gene (JQ680041) served as an endogenous reference gene and was amplified using the primers *VfUBQ*-RTF and *VfUBQ*-RTR (Han et al., 2012). Primers *VfDGAT2*-RTF and *VfDGAT2*-RTR were also used to detect *VfDGAT2* expression levels in transgenic *R. glutinis*, where the *ACT1* gene (AB248916) was used as an endogenous reference gene, which was amplified using the primers *RgACT1*-RTF and *RgACT1*-RTR.

RT-qPCR was carried out using SYBR® Premix Ex Taq™ Kit (TaKaRa, Tokyo, Japan). The 20-µL reaction mix consisted of 2 µL 30-fold diluted 1st-strand cDNA, 10 µL 2X SYBR® Premix Ex Taq™, 0.4 µL each forward and reverse primer (10 µM), 0.4 µL 50X ROX Reference Dye and 6.8 µL DEPC-treated water. Reactions were performed in an ABI7300 Real-Time quantitative instrument (Applied Biosystems, USA). The program used was as follows: initial denaturation step at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, and 60°C for 31 s. A melting curve was performed after the PCR cycles to verify the specificity of

the amplifications. The experiments were performed in quadruplicate, and data were obtained from three independent experiments and analyzed according to Pfaffl (2001).

Lipid extraction and fatty acid composition analysis

Tung oil was extracted from kernels at six different developmental stages by the Soxhlet extraction method (Wei et al., 2004). For analysis of α-eleostearic acid content, tung oil was methylated using the AOCS Official Method Ce 2-66 (1997). The fatty acid methyl esters were analyzed by gas chromatography (GC) on an Agilent 6890N gas chromatograph (Agilent, Santa Clara, CA, USA) equipped with flame ionization detection and an HP-INNOWax column (30 m x 0.25 μm x 0.25 μm (Agilent). The inlet and detector were held at 220°C. The column temperature program was from 140° to 250°C, with a heating rate of 4°C/min, and then held for 5 min at 250°C. The transgenic *R. glutinis* with *VfDGAT2* and the control were fermented in LFM followed by incubation at 30°C for 60 h. Cells were harvested by centrifugation and lipids extracted by the acid-heating method (Li et al., 2001). For fatty acid composition analysis, the lipids were methylated and analyzed by GC, as described above.

RESULTS

Developmental changes in tung oil content and α-eleostearic acid accumulation

To examine the time course of oil synthesis and α-eleostearic acid accumulation in tung seeds, we analyzed the oil content and fatty acid composition at each stage of seed development. As shown in Figure 1, total oil content (%) moderately increased during the early stages (from July 16 to July 26) and then sharply elevated from August 11 to September 9. Subsequently, oil accumulation slowed, reaching a plateau at 37.64% on September 26. The α-eleostearic acid accumulation profile was similar to that for oil: it rapidly accumulated from 7.44% on July 16 to 65.87% on August 11, finally accounting for 76.81% of tung oil on September 26.

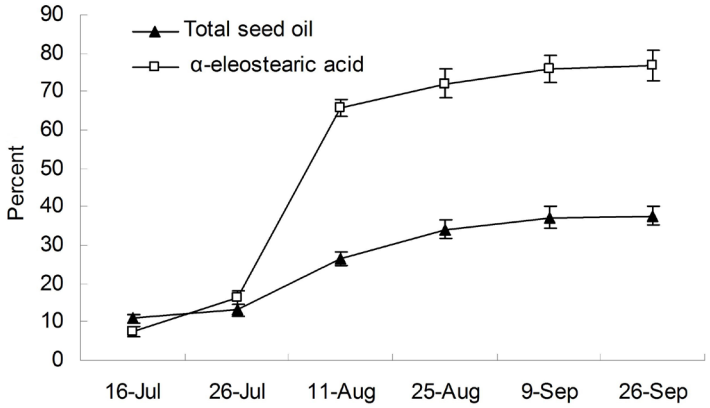

Figure 1. Developmental changes in oil content and α-eleostearic acid production. Tung seeds were harvested at six developmental stages (16 July, 26 July, 11 August, 25 August, 9 September, and 26 September). Changes in total seed oil (% dry weight) and α-eleostearic acid (% of total fatty acid methyl esters) contents are shown. Data are reported as mean values of three independent experiments, and error bars indicate SD.

Expression patterns of *VfDGAT2* in various tissues/organs of the tung tree

To investigate the physiological role of *VfDGAT2*, transcript levels were examined in different tung tissues/organs and seeds at different developmental stages by using RT-qPCR. The results in Figure 2 show that *VfDGAT2* was expressed in all the tissues tested, with the lowest expression level in petioles and the highest transcriptional level in developing seeds (August 25). The expression of *VfDGAT2* in seeds (from August 11 to September 26) was much higher than that in other tissues/organs, where expression varied little except for the slightly higher levels in petals and pistils. Notably, *VfDGAT2* was about 19-fold more highly expressed in seeds (August 25) than in leaves. During seed development, *VfDGAT2* expression changed over time, gradually increasing in the early stages (from July 16 to July 26), reaching its highest level on August 25, and gradually declining from September 9.

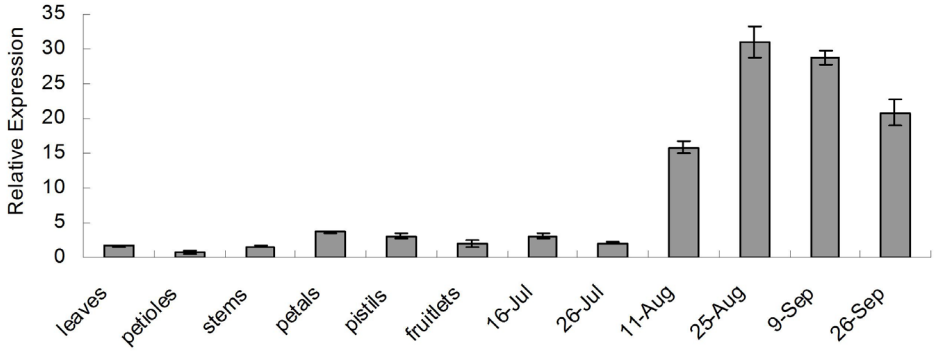

Figure 2. Expression patterns of *VfDGAT2* in tung. *VfDGAT2* gene expression in different tung tissues/organs and developing tung seeds at six developmental stages. Data are reported as mean values of three independent experiments, and error bars indicate SD.

Notably, the expression profiles of *VfDGAT2* in developing seeds were consistent with the accumulation rate of total seed oil and α-eleostearic acid. The gene expression level was lower when oil and α-eleostearic acid slowly accumulated in the early stages (from July 16 to July 26), rapidly increased during the peak periods of fatty acid synthesis in August, and decreased during the accumulation completion period at the end of September (Figures 1 and 2). These data indicate that *VfDGAT2* may be closely involved in oil accumulation and α-eleostearic acid production.

Expression of the *VfDGAT2* gene in transgenic *R. glutinis*

To study the potential function of *VfDGAT2* in fatty acid accumulation, plasmid pBI121-*VfDGAT2* was transformed into the yeast *R. glutinis*. After two screening steps on SM I and SM II media and PCR identification (Figure 3), five independent positive transgenic lines (*VfDGAT2*-1-5) were chosen to measure the expression level of *VfDGAT2*. *VfDGAT2* was highly expressed in all the selected transgenic lines, with *VfDGAT2*-3 showing a 139-fold higher level than the control where little expression was detected (Figure 4). These results show that the *VfDGAT2* gene was successfully inserted and expressed in *R. glutinis*.

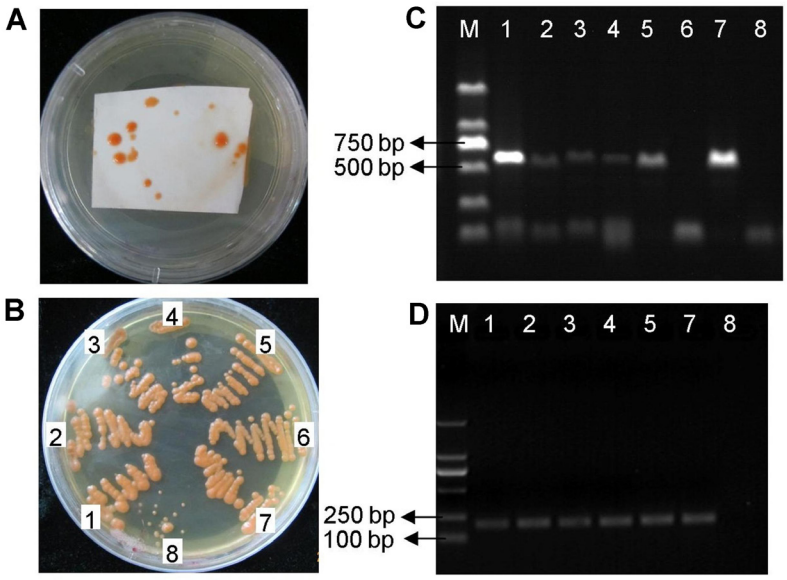

Figure 3. Medium selection and PCR identification of *VfDGAT2* transformants. **A.** *VfDGAT2* transformants grew on the SMI medium containing 100 µg/mL kanamycin, 200 µg/mL cefotaxime and 60 µg/mL streptomycin. **B.** *VfDGAT2* transformants (*lanes 1-7*) and wild-type *Rhodotorula glutinis* (*lane 8*) grew on the SM II medium containing 150 µg/mL kanamycin and 300 µg/mL cefotaxime. **C.** PCR identification of *VfDGAT2* transformants using *vfDGAT2*-F/R. **D.** PCR identification of *VfDGAT2* transformants using *vfDGAT2*-RTF/RTR. *Lane M* = DNA marker 2000.

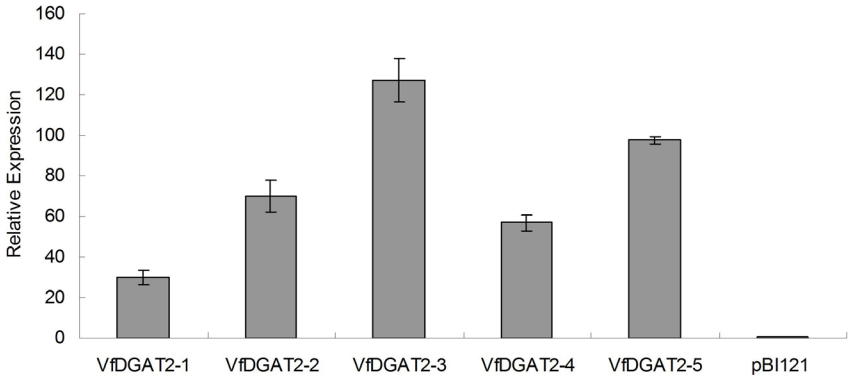

Figure 4. Expression patterns of *VfDGAT2* in transgenic *Rhodotorula glutinis*. Five transgenic *R. glutinis* with *VfDGAT2* were detected. The transgenic *R. glutinis* with empty vector pBI121 was used as control. Data are reported as mean values of three independent experiments, and error bars indicate SD.

Fatty acid profile in transgenic *R. glutinis* with *VfDGAT2*

To determine whether the increased expression of the *VfDGAT2* gene affects fatty acid accumulation, the fatty acid profiles of three different transgenic lines were investigated using GC. Five major fatty acids were identified: palmic acid (C16:0), stearic acid (C18:0),

oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3). As shown in Figure 5, more fatty acids accumulated in the three *VfDGAT2* transgenic lines than in the control. It is notable that the fatty acid content is consistent with the expression level of *VfDGAT2* in the three transgenic lines. The fatty acid content increased the most in *VfDGAT2*-3, which also exhibited the highest expression level of *VfDGAT2*. The total fatty acid content of *VfDGAT2*-3 was 7.8-fold more than that of the control. The palmic acid, stearic acid, oleic acid, linoleic acid, and linolenic acid content improved 14.9-, 2.3-, 6.2-, 13.0-, and 22.9-fold, respectively, as compared with that in the control. *VfDGAT2* may therefore be involved in fatty acid accumulation.

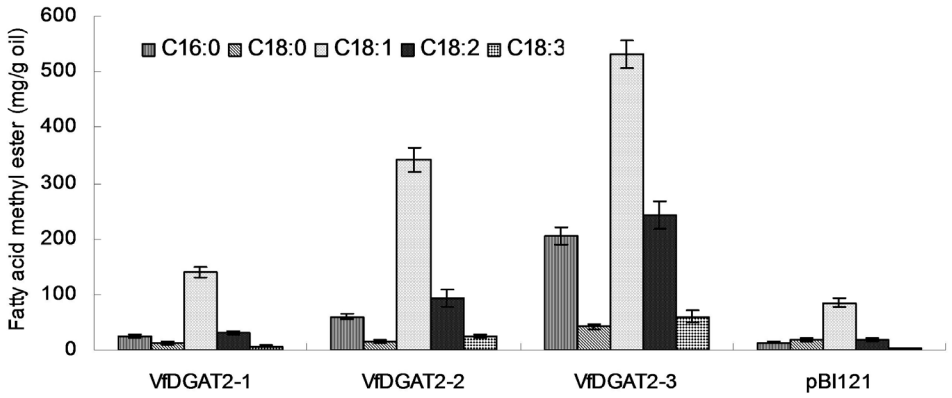

Figure 5. Fatty acid profiles in transgenic *Rhodotorula glutinis* with *VfDGAT2*. Three transgenic lines with *VfDGAT2* were used to examine the fatty acid methyl esters (mg/g oil) including palmic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3). Data are reported as mean values of three independent experiments, and error bars indicate SD.

DISCUSSION

Tung oil is accumulated mainly in the form of TAGs, the major storage lipids that serve as an important energy reserve in seeds (Lung and Weselake, 2006). In order to understand the mechanisms of TAG biosynthesis in tung seeds, we measured oil accumulation during tung seed development and maturation. Tung oil synthesis begins in July and rapidly increases from mid-August until its accumulation is completed in mid-September. In seeds, lipids are stored in subcellular lipid particles (oil bodies) in storage tissues such as cotyledons and endosperm (Frandsen et al., 2001). In our research, we found that the number and size of oil bodies in tung endosperm increased markedly during the middle-late stage (Zhan ZY and Chen YC, unpublished results), indicating that there is considerable lipid accumulation in seeds. The trend in oil accumulation in this study was consistent with previous studies (Wang et al., 1985; Hills, 2004) that profiled the rapid accumulation of TAG at the middle-late stage of seed development.

In this study, we measured the expression levels of *VfDGAT2* in various tung tissues/organs, including leaves, petioles, stems, petals, pistils, fruitlets, and developing seeds. We observed that *VfDGAT2* was expressed in all tissues/organs tested, showing that it is widely

distributed in the plant. *VfDGAT2* was highly expressed only in seeds, with uniformly low levels elsewhere, except for slightly higher expression in petals and pistils, suggesting that *VfDGAT2* is expressed mainly in reproductive rather than vegetative organs. These results are similar to those for the expression of *DGAT2* in *Arabidopsis* (Hobbs et al., 1999), *R. communis* (Kroon et al., 2006), and *V. galamensis* (Li et al., 2010b).

As expected, *VfDGAT2* showed temporal expression during seed development and maturation. It is worth mentioning that the expression of *VfDGAT2* in developing seeds is consistent with oil accumulation and α-eleostearic acid production, indicating that *VfDGAT2* may function in fatty acid accumulation and TAG synthesis. He et al. (2004) examined the temporal expression profiles of *RcDGAT* in developing castor bean seeds, and demonstrated that *RcDGAT* was highly expressed during the active phase of oil deposition, and then declined markedly as seed oil content reached a plateau. Similarly in Jatropha, *DGAT1* showed upregulated expression patterns during lipid accumulation in developing seeds (Xu et al., 2011). These data suggest that the expression of *DGAT* may control fatty acid flux into TAG in seeds (Jako et al., 2001). Additionally, previous reports revealed that the amounts of DAG, the substrate from which DGAT forms TAG, increased significantly during the active phase of oil accumulation in *Brassica napus* (Perry and Harwood, 1993; Perry et al., 1999). Probably the high transcription level of *VfDGAT2* is the mechanism for meeting the increasing demand for TAG synthesis in developing seeds. Thus, high activity of VfDGAT2 can be present to convert diacylglycerol and fatty acyl-coenzyme A into TAG during the rapid accumulation period.

In the present study, the fatty acid content accumulated into yeast TAG clearly improved. Our results show that *VfDGAT2* overexpression in yeast can improve the fatty acid content about 7.8 times over that of the control, indicating that *VfDGAT2* may enhance the efficiency of fatty acid accumulation into TAG in yeast. Several studies showed that some desired fatty acids were integrated in phosphatidylcholine and could not successfully accumulate into TAG, and this became a bottleneck in the genetic engineering of plants (Singh et al., 2005; Cahoon et al., 2006). Fortunately, *RcDGAT2* was reported to be a key factor in removing the bottleneck and successfully diverting the phosphatidylcholine-fatty acids into TAG in oilseed plants (Burgal et al., 2008). Our results further support the idea that *VfDGAT2* is a contributor to fatty acid accumulation in TAG. All these data suggest that *VfDGAT2* plays a significant role in fatty acids accumulation and may be a key target for future tung genetic engineering programs seeking to promote α-eleostearic acid accumulation into tung oil.

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