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Heterologous expression of two GPATs from *Jatropha curcas* alters seed oil levels in transgenic *Arabidopsis thaliana*

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ABSTRACT

Oils and fats are stored in endosperm during seed development in the form of triacylglycerols. Three acyltransferases: glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidyl acyltransferase (LPAT) and diacylglycerol acyltransferase (DGAT) are involved in the storage lipid biosynthesis and catalyze the stepwise acylation of glycerol backbone. In this study two members of GPAT gene family (*JcGPAT1* and *JcGPAT2*) from *Jatropha* seeds were identified and characterized. Sequence analysis suggested that *JcGPAT1* and *JcGPAT2* are homologous to *Arabidopsis* acyltransferase-1 (*ATS1*) and *AtGPAT9* respectively. The sub-cellular localization studies of these two GPATs showed that JcGPAT1 localizes into plastid whereas JcGPAT2 localizes in to endoplasmic reticulum. *JcGPAT1* and *JcGPAT2* expressed throughout the seed development with higher expression in fully matured seed compared to immature seed. The transcript levels of *JcGPAT2* were higher in comparison to *JcGPAT1* in different developmental stages of seed. Over-expression of *JcGPAT1* and *JcGPAT2* under constitutive and seed specific promoters in *Arabidopsis thaliana* increased total oil content. Transgenic seeds of JcGPAT2-OE lines accumulated 43–60% more oil than control seeds whereas seeds of *Arabidopsis* lines over-expressing plastidial GPAT lead to only 13–20% increase in oil content. Functional characterization of GPAT homologues of *Jatropha* in *Arabidopsis* suggested that these are involved in oil biosynthesis but might have specific roles in *Jatropha*.

1. Introduction

The *sn*-Glycerol-3-phosphate 1-O-acyltransferase (GPAT: EC 2.3.1.15) is involved in acyl-lipid biosynthesis and plays an important role in plant development. The GPAT enzyme catalyzes the transfer of a fatty acid moiety from the acyl-CoA pool (or acyl-ACP pool in plastids) to the *sn*-1or 2 position of glycerol-3- phosphate (G3P) forming lysophosphatidic acid (LPA) for *de novo* synthesis of numerous glycerolipids, including extracellular lipid polyesters, membrane lipids and storage triacylglycerol (TAG) [1]. Certain GPATs also possess phosphatase activity in addition to acyl transferase activity and can also form monoacyl glycerols (MAGs) from LPA [2].

GPATs belong to small gene family and are studied in different plants such as *Arabidopsis*, Brassica, Pea, Squash etc [3–11]. Ten GPAT members including GPAT1-9 and *ATS1* have been identified in *Arabidopsis* [1,9,12,13]. The sub-cellular localization of these GPATs has been determined by the presence of specific signal peptides and enzymatic activities present in the plastid stroma, mitochondria and endoplsmic reticulum (ER) [14]. Based on sub-cellular localization, three different types of GPATs are present in plants viz., soluble GPATs of plastid (*ATS1*, At1g32200), the endoplsmic reticulum (ER) membrane-bound GPATs (GPAT4-9) and the mitochondrial membrane- bound GPATs (GPAT1-3) [2,9,13]. Mitochondrial GPATs have not been studied much in detail though the studies of Zheng et al. [1] suggested that mitochondrial *AtGPAT1* plays an important role in processes crucial for the development of pollen grains such as tapetal differentiation and nutrient secretion. *ATS1* has been shown to function in production of major phospholipids, required for plastidial membranes by utilizing acyl-ACP as preferred substrates [15–17]. Although the plastidial GPATs of different plant species have been biochemically characterized *in vitro*, the physiological functions of these enzymes were not fully understood until recently. In *Arabidopsis*, few studies suggest that suppression of the plastidial GPAT (*ATS1*) in deficient mutant lines (chemically mutagenized) and RNAi lines resulted in delayed plant growth and aborted seed development which was accompanied by slight reduction of phosphatidylglycerol (PG) content in plastidial lipids [10,15].

Most of the ER bound GPATs from *Arabidopsis* are responsible for

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controlling cutin or suberin levels in different tissues. GPAT 4 and 8 are involved in cutin synthesis in leaves and stems, *GPAT5* in suberin synthesis in root and seed and *GPAT6* in cutin synthesis of the flower petals [1,18–20]. In addition to these ER localized GPATs which are essential for plant extracellular lipid polyester synthesis; an ER-bound GPAT (GPAT9) has been reported which plays a crucial role in TAG biosynthesis in *Arabidopsis*, wherein down-regulation of GPAT9 led to decreased seed oil content [11,21]. Recently, two isoforms of GPAT9 from sunflower (*HaGPAT9-1 and HaGPAT9-2*) have been identified and over-expression of these isoforms in to GPAT deficient *gat1* yeast strain led to enhanced TAG content [22].

Jatropha curcas, a member of the family *Euphorbiaceae,* is an important non edible seed oil crop found in tropical and subtropical countries. It is promoted as a renewable biofuel crop due to high level of oil reserves in seed [23,24]. However, it is not very well studied at molecular level. Limited information is available about functional genomics of *Jatropha* [25]. Therefore, studies of the oil biosynthetic pathway in *Jatropha* could be useful for improvement of seed oil. In one of our previous studies, we have shown that the expression of *JcDGAT1* in a heterologous system resulted in a significant increase in seed oil content without any developmental abnormalities [26].

In the present study we identified two members of GPAT gene family (*JcGPAT1* and *JcGPAT2*) from *Jatropha* seeds. Both the genes were functionally characterized in heterologous system of *Arabidopsis thaliana* with the aim of deciphering their role in oil biosynthesis. Based on our findings, we discuss the role of two *Jatropha* GPATs in TAG and in the fatty acid (FA) biosynthesis in *Arabidopsis* seeds.

2. Materials and methods

2.1. Materials

The plant material used in this study was *Jatropha curcas* (Acc. No. NBRI-UA-Alm-0406). Developing seeds from different developmental stages 1–7 were harvested from *plants* grown in CSIR-National Botanical Research Institute (CSIR-NBRI) field [27]. Other tissues like flowers, leaves, stem and seed coat were also used in the study. Different primers used in the study are mentioned in Table 1.

2.2. RNA isolation and cDNA synthesis

Total RNA from the above mentioned stages was extracted according to Singh et al. [27]. In order to remove the contaminating genomic DNA, the RNA preparation was treated with RNase free DNase I (RFD). 3 µg of RFD treated RNA was used for first strand cDNA synthesis by MMLV reverse transcriptase (MBI Fermentas USA) according to manufacturer's instruction.

2.3. Isolation of GPAT1 and GPAT2

Degenerate primers were used for isolation of GPAT gene fragment from *Jatropha*. The PCR reaction mix comprised of 1X PCR buffer (MBI Fermentas), Ist strand cDNA (pooled cDNA of stage 2 and 4 of *Jatropha* seed development), 5 pmoles each of primer pair (JcGPATF1 and JcGPATR1), 1 mM dNTP mix and Taq polymerase (MBI Fermentas USA) in total volume of 20 µl. Reaction conditions for PCR were: denaturation at 94°C for 3 min, 35 cycles of 10 s denaturation at 94°C, 15 s annealing at 55 °C and extension at 72°C for 50 s and final extension at 72 °C for 5 min. 1 µl aliquot from 200 times diluted reaction product was used for second PCR as a template and different primer combinations were used for amplification. Two different fragments were obtained using primer combination GPATF4-GPATR2 and GPATF1-GPATR2. The PCR-amplified fragments were cloned in pTZ57R vector (PCR MBI, Fermentas USA). Cloned fragments (*JcGPAT1* and *JcGPAT2)* were sequenced on ABI477 system placed at CSIR-NBRI, Lucknow. Based on the sequence obtained, specific primers were designed for 5′ and 3′ RACE (rapid amplification of cDNA ends) and amplification reactions for obtaining 5′ and 3′ end were carried out as described in the SMART (Switching Mechanism at 5′ End of RNA Template) cDNA synthesis kit manual (BD Biosciences Clontech, USA). Amplified fragments were cloned and sequenced and based on composite sequence, gene specific F_0 and R_0 primers were designed to get the complete open reading frames (ORF) by RT-PCR.

Table 1
List of primers used in this study.

Primer name	Sequence (5'-3')
JcGPAT1Fo SmaI	CCCGGGATGACACTTTCTGCTTTTCC
JcGPAT1Ro SmaI	CCCGGGTTAATTCCATGGTTGTGACA
JcGPAT1Fo XbaI	GTCTAGAATGACACTTTCTGCTTTTCC
JcGPAT1Ro SacI	GGAGCTCTTAATTCCATGGTTGTGACA
JcGPAT1Fo BamHI	GGATCCATGACACTTTCTGCTTTTCC
JcGPAT1Ro BamHI	GGATCCTTAATTCCATGGTTGTGACA
JcGPAT1F4	GTCAAAYCAYCAAASTGAAGCWGATCC
JcGPAT1F7	TAGGCTTGCTGAACATTCTGGTGCTC
JcGPAT1F6	GATGCGGAGTTTGAAGGAGATGG
JcGPAT2F1	ATCCTCCAGAACCWTGGAAYTGGAA
JcGPAT1R2	TCCCTWCCACCRCTTGGWGCAATCC
JcGPAT2F3	CCTCTTCTCATATTTCCTGAAGGA
JcGPAT1Rq	TCCTTGAGAAGGTGAGCCACGTTGGCG
JcGPAT2F0 SmaI	CCCGGGATGACACTTTCTGCTTTTCC
JcGPAT2R0 SmaI	CCCGGGTTAATTCCATGGTTGTGACA
JcGPAT2Fq	CTTTCATGCTACATTCCTGTGC
JcGPAT2Rq	ATGATGCCACAAAGAAGCTG
JcActinFq	GCCCCTGAGGAACACCCAGTGC
JcActinRq	GCAGGCACATTAAAGGTCTCAAAC
AtATS1Fq	ATGATGCCACAAAGAAGCTG
AtATS1Rq	GCCGCCGGTGAGACGCCGAG
AtGPAT9Fq	CCCTTGAGGTGCTTCACTTTAGCT
AtGPAT9Rq	CGTGATATTTGACAACTCCGGTCC
CaMV35SF	GTAAGGGATGACGCACAATCC
NOST Rev	GGACTCTAATCATAAAAACCC
M13-REV	TGTAAAACGACGGCCAG
M13U	CAGGAAACAGCTATGACC
SSPF2	TAGCTAAGCTTAGCCACTTCATCAAAG
SSPR2	TCCTAGTGTGATCCCAAGCTTCATTTTACC

2.4. Sequence analysis

Basic Local Alignment Search Tool (BLAST, NCBI Bethesda, MD) and SIGNALP version 1.1 (Centre for Biological Sequence Analysis, Technical University of Denmark) respectively were used for homology searches and signal peptide predictions [28]. For Multiple sequence alignments Clustal Omega programme [29] was used. Phylogenetic analyses of putative GPAT proteins were carried out using MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 [30]. GPAT protein sequences were aligned by Clustal Omega and a Neighbor-Joining (NJ) tree was constructed with 1000 bootstrap value.

2.5. Transcript analysis by q-RT PCR

Transcript analyses of *JcGPAT1* and *JcGPAT2* in different tissues were carried out by real time q-PCR using gene specific primers (GPAT1F0 and GPAT1Rq; GPAT2Fq and GPAT2Rq respectively). A reaction was setup with template, forward primer, reverse primer and SYBR Green mix (ABI, NY, USA) in ABI 7500 Fast Real-Time PCR System (ABI, NY, USA). Run method conditions were set as: 50 °C for 20 s, 95 °C for 10 min for holding and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Gene specific primer sets were designed (Table 1) and housekeeping gene *(Actin)* was taken as an endogenous reference gene to normalize the data. The fold change in expression levels was calculated by ΔΔCT value (Fold Change = $2^{-(\Delta\Delta CT)}$) [31].