

X. Chi<sup>a,b</sup>, Q. Yang<sup>c</sup>, L. Pan<sup>b</sup>, N. Chen<sup>b</sup>, M. Chen<sup>b</sup>, T. Wang<sup>b</sup>, M. Wang<sup>b</sup>, Z. Yang<sup>b</sup>, X. Guan<sup>d,\vee</sup> and S. Yu<sup>b,\vee</sup>

<sup>a</sup>Key Laboratory of Biology and Genetic Improvement of Oil Crops, Ministry of Agriculture, Oil Crops Research Institute, Chinese Academy of Agricultural Sciences, Wuhan, 430062, P. R. China
<sup>b</sup>Shandong Peanut Research Institute, Qingdao, 266100, P R China

<sup>c</sup>College of food science and engineering of Qingdao agricultural university, Qingdao, 266109, P R China <sup>d</sup>School of Ocean Sciences, China University of Geosciences, Beijing 100083, P R China

<sup>C</sup>Corresponding authors: shanlinyu2012@163.com; guanxy@cugb.edu.cn

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**SUMMARY:** *sn*-Glycerol-3-phosphate acyltransferase (GPAT) catalyzes the committed step in the production of glycerolipids. The functions of GPAT genes have been intensively studied in Arabidopsis, but not in peanuts (*Arachis hypogaea* L.). In this study, six *AhGPAT* genes were isolated from peanuts. Quantitative real-time RT-PCR analysis indicated that the *AhGPAT9* transcript was more abundant in the stems, flowers, and seeds, whereas the transcript abundances of five other genes were higher in the leaves or flowers than in the other tissues examined. During seed development, the transcript levels of *AhGPAT9* gradually increased, whereas the transcript levels of the other five genes decreased. In addition, the levels of *AhGPAT2* transcript were distinctly enhanced after exposure to all four kinds of stress treatments except for ABA-treated leaves. The transcripts of *AhGPAT1*, *AhGPAT6*, *AhGPAT6*, *AhGPAT6*, *AhGPAT8*, *AhGPAT9* and *AhATS1* were slightly higher in leaves under certain stress conditions than under normal conditions. The present study provides significant information for modifying oil deposition and improving the abiotic stress resistance of peanuts through molecular breeding.

**KEYWORDS:** Glycerol-3-phosphate acyltransferase; Peanuts (Arachis hypogaea L.); Phylogenetic analysis; Quantitative real-time RT-PCR

**RESUMEN:** Aislamiento y análisis de la expresión del gen aciltransferasa glicerol-3-fosfato de cacahuete (Arachis hypogaea L.). La aciltransferasa sn-glicerol-3-fosfato (ATGP) cataliza el comprometido paso de la producción de glicerolípidos. Las funciones de los genes AhATGP se han estudiado intensivamente en Arabidopsis, pero no en cacahuete (Arachis hypogaea L.). En este estudio, seis genes AhATGP se aislaron a partir de cacahuetes. El análisis a tiempo real RT-PCR cuantitativa indicó que la transcripción AhATGP9 fue más abundante en tallos, flores y semillas, mientras que la abundancia de la transcripción de los otros cinco genes fueron mayores en hojas o flores que en los otros tejidos examinados. Durante el desarrollo de la semilla, los niveles de transcripción de AhATGP9 aumentaron gradualmente, mientras que los niveles de transcripción de otros cinco genes disminuyeron. Además, los niveles de transcripción AhATGP2 mejoraron claramente después de la exposición a los cuatro tipos de tratamientos de estrés excepto para las hojas tratadas con ABA. Las transcripciones de ATGP1, ATGP6, ATGP8 y AhATS1 aumentaron considerablemente en las raíces expuestas a sal, sequía y estrés de ABA. Las expresiones de AhGPAT6, AhGPAT8, AhGPAT9 y AhATS1 fueron ligeramente más altos en las hojas bajo ciertas condiciones de estrés que en condiciones normales. El presente estudio proporciona información importante para utilizar en la modificación de la acumulación de aceite y mejorar la resistencia al estrés abiótico de maní a través de mejoramiento molecular.

*PALABRAS CLAVE:* Aciltransferasa glicerol-3-fosfato; Análisis filogenético; Cacahuete (Arachis hypogaea L.); PCR cuantitativa a tiempo real (*RT-PCR*)

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#### **1. INTRODUCTION**

Plant lipids are composed of a wide variety of fatty acids and their derivatives, including glycerolipids, lipid polyesters, and sterols. Plant lipids are involved in a diverse range of metabolic reactions and play important physiological roles in plant development, such as major components of cellular membranes, storage reserves, extracellular protective layers, and signaling molecules (Chen et al., 2011a). The biosynthesis of these different types of lipids is controlled by a complex network of genes and proteins. sn-Glycerol-3-phosphate acyltransferases (GPAT) is the first enzyme in the pathway for the *de novo* synthesis of glycerolipids and is involved in different metabolic pathways and physiological processes (Yang et al., 2012). It catalyzes the transfer of an acyl group from acyl-coenzyme A (CoA) or acyl-acyl carrier protein (ACP) to the sn-1 position of sn-glycerol-3-phosphate (G3P). Plants contain three types of GPATs, which are located in plastids, mitochondria, and cytoplasm, respectively (Xu et al., 2006; Li et al., 2011). The enzyme in plastids is soluble and uses acyl-ACP as the acyl donor, whereas the enzymes in the mitochondria and the cytoplasm are bound to membranes and use acyl-CoA as the acyl donor (Murata and Tasaka, 1997).

In Arabidopsis, 10 genes have been identified as encoding GPAT enzymes located in various subcellular compartments, such as plastids (*AtATS1*), mitochondria (AtGPAT1), and the endoplasmic reticulum (ER; AtGPAT8 and AtGPAT9) (Xu et al., 2006; Zheng et al., 2003; Gidda et al., 2009). The soluble, plastid-localized ATS1 (At1g32200) uses acyl-ACP substrates and exhibits sn-1 acyl transfer regio-specifically (Nishida et al., 1993). A second enzyme, GPAT9 (At5g60620), is not related to GPAT1-GPAT8 but is most homologous to the mammalian GPAT3, which is directly involved in the synthesis of triacylglycerols in the adipose tissues (Cao et al., 2006). GPAT9 protein is localized to the ER (Gidda et al., 2009) and may be an acyl-CoA-dependent sn-1 GPAT that enables non-plastid glycerolipid synthesis. The remaining eight GPATs cluster together in a family (Zheng et al., 2003; Gidda et al., 2009; Beisson et al., 2007) which is not required for membrane or storage lipid biosynthesis. Instead, several members of the family clearly

affect the composition and quantity of cutin or suberin. They transfer acyl groups to the sn-2 position with three distinct clades which are associated with key stages in the morphological and functional evolution of land plants and also coincide with a loss in phosphatase activity (Yang et al., 2012). Within the cutin-associated clade, GPAT4, GPAT6, and GPAT8 have been shown to behave as bifunctional *sn*-2 acyltransferase/phosphatase enzymes capable of generating 2-monoacylglycerol (MAG) products. They strongly prefer C16:0 and C18:1 ω-oxidized acyl-CoAs over unmodified or longer acyl chain substrates (Yang et al., 2012). In contrast, suberin-associated GPAT5 and GPAT7 possess sn-2 acyltransferase but not phosphatase activity, and can accommodate a broad chain-length range of  $\omega$ -oxidized and unsubstituted acyl-CoAs. The enzymes GPAT1-GPAT3 represent a distinct clade from the GPAT4/6/8 and the GPAT5/7 clades in the GPAT family of Arabidopsis. Within this clade, phosphatase-minus GPAT1 can use dicarboxylic acyl-CoA substrates, whereas the same activity could not be detected for GPAT2 and GPAT3. Even though GPAT2 and GPAT3 have lost their key amino acids in their phosphatase domain, they retain their HXXXXD and CPEGT conserved acyl transferase domain motifs, and may thus be expected to function as active acyltransferases (Yang et al., 2012).

In Arabidopsis, AtGPAT1 encodes a mitochondrial isozyme that is necessary for pollen development, although AtGPAT1 deficiency does not affect the levels of seed oil (Zheng et al., 2003). Analysis of loss-of-function mutants in Arabidopsis demonstrated an essential role of AtGPAT5 for suberin biosynthesis in the root and seed coat (Beisson et al., 2007). Monomer composition analysis and overexpression of AtGPAT5 in Arabidopsis and tobacco plants caused secretion of MAGs onto the surface of leaves (Li et al., 2007a). Similarly, AtGPAT4 and AtGPAT8 likely encode redundant activities necessary for the assembly of cutin monomers in the stems and leaves (Li et al., 2007b), whereas AtGPAT6 is involved in cutin assembly in sepals and petals (Li-Beisson et al., 2009). In Brassica napus, three homologous GPAT4 genes exhibited different expression patterns and distinct epigenetic features. A phenotypic rescue of a gpat4 gpat8 Arabidopsis double mutant and analysis of

the gpat4 RNAi lines of B. napus suggested physiological roles of GPAT4s in cuticle formation of the rosette leaves, early flower development, pollen development, and the biosynthesis of storage lipids (Chen et al., 2011b). Two homologous GPAT genes isolated from Echium pitardii have high similarity to the AtGPAT4/8 genes of Arabidopsis. Whereas the transcripts of EpGPAT1 were most abundant in seeds, roots, young leaves, and flowers, the transcripts of EpGPAT2 were most abundant in developing leaves and flowers. The ectopic expression of *EpGPAT1* in the leaves of tobacco plants increased the levels of C16 and C18 hydroxyacids and  $a,\omega$ diacids in the cell wall fraction, indicating a role for *EpGPAT1* in the biosynthesis of cutin polyesters (Mañas-Fernández et al., 2010).

In vivo experiments showed that the overexpression of Arabidopsis AtAST1 in tobacco increased both the degree of unsaturation of fatty acids in phosphatidylglycerol (PG) and the resistance of tobacco to chilling stress (Murata et al., 1992). An increase in the level of unsaturation of fatty acids in PG from rice plants transformed with an AtATS1 cDNA improved photosynthetic rates and growth at low temperatures (Ariizumi et al., 2002). The overexpression of *LeATS1* increased the levels of PG cis-unsaturated fatty acids in the thylakoid membranes of tomato, which promoted recovery from chilling-induced photoinhibition of photosystem I (PSI) (Sui et al., 2007). The increase in saturation of thylakoid membrane lipids in transgenic tobacco with expressed ATS1 from sweet pepper enhanced the thermotolerance of the photosynthetic apparatus of transgenic tobacco (Yan et al., 2008).

Given that the members of the GPAT family have several complicated roles during plant development and acclimation to stressful conditions, functional analyses of each member of the gene family should be helpful in elucidating the roles of GPAT isoforms. The peanut (Arachis hypogaea L.) is an allotetraploid species  $(2n=4\times=40, AABB)$ and one of the five most important oilseed crops worldwide. It is grown extensively in tropical, subtropical, and temperate climates. The peanut seed comprises around 50% oil, of which approximately 80% consists of oleic (36–67%) and linoleic (15–43%) acids (Chi et al., 2011). Several molecular studies of lipid biosynthesis in peanuts have been reported in recent years. However, there have been no reports about the function of the GPAT family proteins in peanuts. In the present study, we isolated six novel GPAT genes from peanuts. The expression patterns of these genes were investigated in different tissues and at different stages of seed development. Expressions of these genes were also analyzed under conditions of cold, salt, drought, and ABA stress. Our findings should be of value in efforts

to modify lipid biosynthesis in peanut seeds and to provide a theoretical basis for the study of abiotic stress tolerance in peanut.

### 2. MATERIALS AND METHODS

#### 2.1. Plant materials

Peanut plants (A. hypogaea L. cultivar Huayu 19) were grown in a growth chamber with a 16 h light/8 h dark photoperiod at 26 °C/22 °C day/night temperatures. Leaves, stems, cotyledons, hypocotyls, and roots were sampled from the seedlings at the trefoil leaf stage. Seeds were sampled at 10, 20, 30, 40, 50, and 60 days after pegging (DAP). Flowers were collected when the seedlings were in the flowering phase. For the cold treatment, seedlings in the soil at the trefoil leaf stage were kept at 4 °C, and leaves were sampled separately either before cold treatment (0 h) or after continuous exposure to 4 °C for 1, 3, 6, 12, 24, 48, or 72 h. For stress treatments, the roots of seedlings grown in soil were flushed carefully with tap water to remove all soil, and then submerged in solutions of 200 mM NaCl, 20% PEG-6000, or 100 µM ABA. Leaves and roots were sampled separately after treatment for 0, 1, 3, 6, 12, 24, 48, or 72 h. All samples were immediately frozen in liquid nitrogen and stored at -80 °C until required.

### 2.2. Identification of glycerol-3-phosphate acyltransferase family genes in a peanut cDNA library using bioedit software

The cDNA sequences used in this study came from three cDNA libraries from three institutes (data not shown): Shandong Peanut Research Institute, Oil Crops Research Institute of The Chinese Academy of Agricultural Sciences, and Crops Research Institute of Guangdong Academy of Agricultural Sciences. All expressed sequence tags (ESTs) of the 36,741 cDNA sequences were saved as FASTA format. The amino acid sequences of glycerol-3-phosphate acyltransferase genes of Arabidopsis, AtGPAT2 (NP\_563651), AtGPAT9 (NP\_568925) and AtATS1 (NP\_174499) were used to search for homogeneous genes from the peanut cDNA library. Before searching for members of the GPAT gene family, a local nucleotide database file was created using Bioedi software. A local BLAST procedure was then run to find the homologous genes of the GPAT family. Using this method, we found six genes that may encode GPAT proteins.

#### 2.3. Total RNA isolation and cDNA synthesis

The total RNA was extracted using the RNeasy Plant Mini kit (Qiagen, Valencia, CA, USA). Contamination with genomic DNA was eliminated by treatment with recombinant DNase I (Qiagen), as recommended by the vendor. Only RNA preparations having an A260/A280 ratio of 1.8–2.0 and an A260/A230 ratio >2.0 were used for subsequent analysis. The integrity of RNA was verified by electrophoresis through 2% agarose gels, followed by SYBR Green staining. First-strand cDNA synthesis was carried out with 2 µg RNA using an RT-PCR kit (Promega, WI, USA) according to the manufacturer's procedure.

#### 2.4. Isolation of full-length cDNA sequences

We performed PCR with the LA PCR system (TaKaRa) using 2.5  $\mu$ L of 10×PCR buffer with MgCl<sub>2</sub>, 1  $\mu$ L of each primer (10  $\mu$ M), 4.0  $\mu$ L of 10 mM dNTPs, 1  $\mu$ L of cDNA sample, 0.5  $\mu$ L of LA Taq<sup>TM</sup> DNA polymerase, and 15  $\mu$ L of double-distilled water. The PCR products were separated by electrophoresis through a 1% agarose gel, and purified using a Gel Extraction Kit (Takara) according to the manufacturer's protocol. The purified products were then cloned into the pMD18-T Easy vector (Takara) and sequenced (Shangon, Shanghai).

#### 2.5. Sequence analysis

The open reading frames (ORFs) and encoded amino acid sequences of all the genes were deduced using BioXM 2.6. The physicochemical properties of the deduced protein were predicted using Protparam (http://www.expasy.ch/tools/protparam.html). Active sites of the protein sequence were analyzed by comparison against the PROSITE database. Predicted transmembrane domain (TMDs) in GPAT proteins were identified using the TMHMM Server (version 2.0) (http://www.cbs.dtu.dk/services/TMHMM) and visual inspection. The putative subcellular localizations of the candidate proteins were estimated by TargetP (http://www.cbs.dtu.dk/services/TargetP/) and Predotar (http://urgi.versailles.inra.fr/predotar/ predotar.html).

## 2.6. Gene structure prediction and conserved motif scanning

The gene structure display server (GSDS) program (Guo *et al.*, 2007) was used to illustrate exon/ intron organization for individual desaturase genes by comparison of the cDNAs with their corresponding genomic DNA sequences. To identify the conserved motifs, MEME (Multiple Expectation Maximization for Motif Elicitation) version 4.9.1 (http://meme.nbcr.net/meme/cgi-bin/meme.cgi) was employed with a set of parameters as follows: number of repetitions – any, maximum number of motifs –20, optimum motif width set to ≥6 and ≤200 (Bailey and Elkan 1995). The motifs obtained were recorded using the SMART (http://smart. embl-heidelberg.de/) and NCBI-CDD (National Center for Biotechnology Information Conserved Domain Database) search programs.

#### 2.7. Phylogenetic analysis

Homologs of each member of the Arabidopsis GPAT family were identified by BLASTP searches with datasets from Phytozome v9.1 (www.phytozome.net). Only those sequences with an e-value less than  $e^{-50}$  were considered as members of the GPAT family. In each tree, gene sequences other than Arabidopsis and peanut GPATs were displayed using the nomenclature with the following abbreviations: Ah, Arachis hypogaea; At, Arabidopsis thaliana; Glyma, *Glycine max*; Medtr, *Medicago truncatula*; Pp, Physcomitrella patens; Cre, Chlamydomonas reinhardtii; Vocar, Volvox carteri. Table 1 provides a detailed description of the proteins used and the corresponding accession numbers. Amino acid sequences were aligned using the ClustalX program with the implanted BioEdit (Thompson et al., 1994). The neighbor-joining (NJ) method in MEGA4 (Tamura *et al.*, 2007) was used to construct the phylogenetic tree. Bootstrapping with 1,000 replicates was used to establish the confidence limits of the tree branches. Default program parameters were used. Bootstrap values from the neighbor-joining analyses were listed to the left of each node, and values higher than 50 were shown.

### 2.8. Quantitative real-time RT-PCR

A quantitative Real-time RT-PCR (gRT-PCR) analysis was performed using a LightCycler 2.0 instrument system (Roche, Germany). The alpha tubulin 5 gene (AhTUA5) was taken as a reference gene (Chi et al., 2012). Seven pairs of gene-specific primers (Table 2) were designed after analysis of the sequences of target genes. qRT-PCR reactions were performed using the SYBR Premix Ex Taq polymerase (TaKaRa, Japan) according to the manufacturer's instructions. Each 20-µL reaction comprised 2  $\mu$ L of template, 10  $\mu$ L of 2× SYBR Premix, and 0.4 µL (200 nM) of each primer. The reactions were subjected to an initial denaturation step of 95 °C·10 s<sup>-1</sup>, followed by 40 cycles of 95 °C·5s<sup>-1</sup>, 60 °C·30s<sup>-1</sup> and 72 °C·10s<sup>-1</sup>. A melting curve analysis was performed at the end of the PCR run over the range 60-95 °C, increasing the temperature stepwise by 0.5 °C every 10 s. The baseline and quantification cycle (CP) were automatically determined using the Light Cycler Software. Zero template controls were included for each primer pair, and each PCR reaction was carried out in triplicate. The relative quantification method (delta-delta Cp) was used to evaluate quantitative variation (Livak and Schmittgen, 2001).

Kingdom	Specie	Taxa terminologies	Gene symbol	Database	Access	Length (aa)
Viridiplantae	Arabidopsis thaliana	At	ATS1	JGI	AT1G32200.2	459
			GPAT1	JGI	AT1G06520.1	585
			GPAT2	JGI	AT1G02390.1	530
			GPAT3	JGI	AT4G01950.1	520
			GPAT4	JGI	AT1G01610.1	503
			GPAT5	JGI	AT3G11430.1	502
			GPAT6	JGI	AT2G38110.1	501
			GPAT7	JGI	AT5G06090.1	500
			GPAT8	JGI	AT4G00400.1	500
			GPAT9	JGI	AT5G60620.1	376
	Glycine max	Glyma	ATS1	JGI	Glyma01g01800.1	253
	-	-	ATS1	JGI	Glyma09g34110.1	470
			GPAT1	JGI	Glyma02g45600.1	539
			GPAT1	JGI	Glyma08g42210.1	552
			GPAT1	JGI	Glyma14g03210.1	540
			GPAT1	JGI	Glyma18g12750.1	527
			GPAT2	JGI	Glyma03g37970.1	522
			GPAT2	JGI	Glyma03g37990.1	481
			GPAT2	JGI	Glyma02g01400.1	555
			GPAT2	JGI	Glyma10g01420.1	553
			GPAT2	JGI	Glyma19g40590.1	537
			GPAT3	JGI	Glyma14g33830.1	417
			GPAT3	JGI	Glyma14g33860.1	534
			GPAT3	JGI	Glyma13g02250.1	446
			GPAT4	JGI	Glyma07g07580.1	499
			GPAT4	JGI	Glyma03g01070.1	500
			GPAT5	JGI	Glyma02g41660.1	467
			GPAT5	JGI	Glyma14g07290.1	512
			GPAT6	JGI	Glyma01g27900.1	492
			GPAT6	JGI	Glyma18g42580.1	539
			GPAT6	JGI	Glyma20g16980.1	501
			GPAT6	JGI	Glyma10g23560.1	489
			GPAT6	JGI JGI		362
			GPAT6 GPAT6	JGI JGI	Glyma03g14180.1 Glyma07g17720.1	496
			GPAT0 GPAT9	JGI JGI	Glyma05g26140.1	238
				JGI JGI	Glyma08g09080.1	
			GPAT9 GPAT9		Glyma09g21150.1	373 376
	M. L	Maden		JGI		
	Medicago truncatula	Medtr	ATS1	JGI	Medtr5g029110.1	457
			GPAT1	JGI	Medtr5g098930.1	537
			GPAT1	JGI	Medtr3g062190.1	277
			GPAT2	JGI	Medtr1g106370.1	542
			GPAT4	JGI	Medtr8g031940.1	505
			GPAT5	JGI	Medtr5g087710.1	523
			GPAT6	JGI	Medtr3g024620.1	496
			GPAT6	JGI	Medtr1g059560.1	504
			GPAT9	JGI	Medtr8g129160.1	371
	Arachis hypogaea	Ah	ATS1	NCBI	KC762933	451

TABLE 1. The GPAT enzymes used for the phylogenetic analyses

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Kingdom	Specie	Taxa terminologies	Gene symbol	Database	Access	Length (aa)
			GPAT1	NCBI	JN032676	555
			GPAT2	NCBI	HQ589243	544
			GPAT6	NCBI	HQ589244	499
			GPAT8	NCBI	JX843442	505
			GPAT9	NCBI	JX843441	376
	Physcomitrella patens	Рр	ATS1	JGI	Pp1s136_120V6.1	494
			GPAT6	JGI	Pp1s9_453V6.1	510
			GPAT6	JGI	Pp1s134_51V6.1	504
			GPAT6	JGI	Pp1s72_49V6.1	516
			GPAT6	JGI	Pp1s117_125V6.1	517
			GPAT6	JGI	Pp1s42_150V6.1	513
			GPAT6	JGI	Pp1s281_69V6.1	529
			GPAT6	JGI	Pp1s117_135V6.1	538
			GPAT9	JGI	Pp1s150_100V6.1	276
			GPAT9	JGI	Pp1s138_27V6.1	389
	Chlamydomonas reinhardtii	Cre	ATS1	JGI	Cre02g143000.t1.2	410
			GPAT9	JGI	Creg6130.t1	456
	Volvox carteri	Vocar	ATS1	JGI	Vocar20013783m	406
			GPAT9	JGI	Vocar20002974m	435

 TABLE 1 (continued)

#### 2.9. Seed lipid analysis

Lipid content in seed was determined by a standard Soxhlet extraction method (Harwood, 1984). From each cultivar, 1 g sample was ground and then extracted with petroleum ether in a Soxhlet apparatus for 8 h. Petroleum ether was then volatilized in the draft. The experiment was carried out in triplicate. Lipid content was expressed as % of seed dry weight.

### **3. RESULTS**

# **3.1.** Isolation of glycerol-3-phosphate acyltransferase genes from peanuts

Six genes that likely encode glycerol-3-phosphate acyltransferase (GPAT) proteins were found using Bioedit software. They were cloned and designated as *AhATS1*, *AhGPAT1*, *AhGPAT2*, *AhGPAT6*, *AhGPAT8*, and *AhGAPT9* according to the homologous genes identified in Arabidopsis. Among the six genes, two genes have the complete open reading frame (ORF) in the peanut cDNA library and cloned by conventional RT-PCR, whereas four genes were cloned using the rapid amplification of cDNA ends (RACE) method. The ORFs of the five genes were 1,356 bp, 1,668 bp, 1,635 bp, 1,500 bp, 1,518 bp, and 1,131bp in length, encoding 451, 555, 544, 499, 505, and 376 amino acids, respectively. The genomic sequences were 5,766 bp,

2,146 bp, 2,209 bp, 3,176 bp, 4,474bp, 4,970 bp in length, respectively (Table 3). The sequence information of six genes was submitted to Genbank, with the Genbank identification numbers KC762933, JN032676, HQ589243, HQ589244, JX843442, and JX843441, respectively.

A search using NCBI BLAST revealed that six GPAT proteins have high sequence similarities with GPATs in Arabidopsis. AhATS1 shares 55.1% sequence similarity with *AtATS1*. *AhGPAT1* shows 55.3% sequence similarity with *AtGPAT1*, *AhGPAT6* shares 78.2% similarity with *AtGPAT6*. The *AhGPAT2* protein shares 52.8% and 52.0% sequence similarity with *AtGPAT7* shows 79% similarity with *AtGPAT9*. The AhGPAT8 protein is most similar to AtGPAT4 (77.9%), and AtGPAT8 (78.4%), both of which have been implicated in the synthesis of cutin polymers (Li *et al.*, 2007b).

As shown in Figures 1 and 2, alignment of the deduced polypeptide sequences of six GPAT proteins demonstrates that they are similar in length and share several features that are characteristic of other plastidial and membrane-bound GPATs from evolutionarily diverse organisms. These features include the presence of four conserved amino acid motifs (AT-I to AT-IV) which are important for acyltransferase activity (Mañas-Fernández *et al.*, 2010). The typical acyltransferase (AT) domain is localized within the C-terminal half of the molecule. Residues implicated in catalysis, such as histidine

 TABLE 2.
 DNA sequences of oligonucleotide

 primers used in this study

Name	Oligonucleotide sequence 5'-3'
Full-length cD	NA sequence cloning
ATS1-F	ATGAACGGGTCTCTCGCTCA
ATS1-R	CTAGTTCCACGGCTGTGACAA
GPAT1-F	ATGGTGTTTCCAATGGTGCT
GPAT1-R	TCACGACAGCAAAGTTTCTC
GPAT2-F	ATGGCTAAAATGTTCAGAGCT
GPAT2-R	CTAAGATTTACCACACGCTC
GPAT6-F	ATGGTCATGGGAGCCTTTTC
GPAT6-R	TTAAGCTTTGTTCTCCTTGTTAG
GPAT8-F	ATGGCAGCGCCGAAACCGA
GPAT8-R	TCACTTCTTGGAACTGTACATGG
GPAT9-F	ATGATGAGGAAGACCAATCC
GPAT9-R	TTACTTTTCTTCCAAGCGCC
Real-time RT-I	PCR
qTUA5-F	CTGATGTCGCTGTGCTCTTGG
qTUA5-R	CTGTTGAGGTTGGTGTAGGTAGG
qATS1-F	TTCCGTGACTGAGCAATATACTGTG
qATS1-R	GGCTGTGACAACGAGACTTTAGG
qGPAT1-F	CCTACTTCACTGGCTTTGTCTCTG
qGPAT1-R	CATTGGGCTTGGATTGTTCACC
qGPAT2-F	GGTGTCAGAAGCAGAGAAGAAGAAG
qGPAT2-R	TGGCGAGGATTAGGGCATAGG
qGPAT6-F	GCTTCCCTCTTAACCTTCCTATGG
qGPAT6-R	TCCGCTTTGCCCTTTCTTTGG
qGPAT8-F	TCACCTACTCCGTCAGCAAGC
qGPAT8-R	GCATTGAACCGCAGCAAGAAG
qGPAT9-F	AACCTAACATTGAAGATTACCT
qGPAT9-R	ATTGACTTGAAGCACCTTAA

and aspartic acid residues in AT-I, glycine residues in AT-III, and a proline residue in AT-IV are all present in peanut GPATs, as are the arginine (AT-II) and glutamic (serine) (AT-III) residues involved in binding to the G3P substrate (Gonzalez-Baró *et al.*, 2007). In addition to the AT region, a haloacid dehalogenase (HAD)-like domain is found in the N-terminal half of AhGPAT6 and AhGPAT8. This conserved domain is present in a super-family of proteins, most of which are phosphohydrolases. Close inspection of this region in AhGPAT6 and AhGPAT8 and their putative orthologues reveal the presence of highly conserved motifs, named HAD-I through HAD-IV (Figure 1), which have been described in HAD-like proteins (Burroughs et al., 2006). They include the typical DXD signature (which contains critical aspartic acid residues that act as a nucleophile during catalysis), the extremely conserved threorine and lysin residues in HAD-II and HAD-III boxes, respectively (both of which contribute to the stability of the reaction intermediates), and a GDXXXD motif in HAT-IV that contains acidic residues required for coordination to the  $Mg^{2+}$  ion in the active site.

The *AhATS1* protein is probably located in chloroplast, as predicted using the TargetP Server and Predotar tools. The N-terminal end of *AhATS1* had a high proportion of hydroxylated and small, hydrophobic amino acids, which is typical of a chloroplast transit peptide. The *AhGPAT1* and *AhGPAT2* proteins also possess an extended N-terminal region that exhibits characteristics of a mitochondrial targeting peptide. All of the other GPAT proteins lack any recognizable N-terminal intracellular targeting signal motifs, but do contain putative C-terminal ER retrieval signals.

# 3.2. Gene structures and distribution of conserved motifs

Genes in the same clade had more similar exon/ intron structures than those genes in the other clades (Figure 3). Both ATS1 and GPAT9 clade members had twelve exons, whereas the GPAT4/8 clade members possessed four exons. All of the remaining six GPAT clade members had two exons, except for AhGPAT1, which possessed three exons.

The MEME motif search tool was employed to identify the conserved motifs present in peanuts and *Arabidopsis* GPAT proteins (Figure 3), and 20 distinct motifs were identified. Most of the motifs belonged to the regions that represented the typical domains of acyltransferase. The motif 4 was found

Protein	Accession	Len (aa)	ORF (bp)	5' upstream region (bp)	3' downstream region (bp)	Genomic sequences (bp)	Molecular mass (kDa)	PI
ATS1	KC762933	451	1356	89	496	5766	49.5908	9.14
GPAT1	JN032676	555	1668	78	137	2146	62.6046	9.05
GPAT2	HQ589243	544	1635	62	61	2209	61.8657	9.34
GPAT6	HQ589244	499	1500	113	10	3176	55.5391	9.19
GPAT8	JX843442	505	1518	96	316	4474	51.642	9.09
GPAT9	JX843441	376	1131	136	158	4970	43.5418	9.09

TABLE 3. Glycerol-3-phosphate acyltransferase genes in peanuts

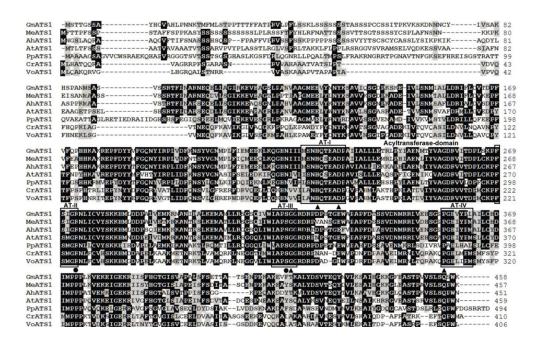


FIGURE 1. Amino acid alignment of peanut plastidial ATS1 proteins and closely related proteins found in the GenBank. Residues shared by a fraction of sequences above 0.5 were shaded, identical residues in black, similar residues in grey. AT-like domains were boxed (ATI to AT-IV). Critical residues previously identified in similar proteins were marked by dots (binding site in AT domain) or triangles (catalytic residues in AT domain). GenBank accession numbers were as follows: Arachis hypogaea (AhATS1, KC762933), Arabidopsis thaliana (AtATS1, NP\_174499), Glycine max (GmATS1, XP\_003612801), Physcomitrella patens (PpATS1, XP\_001771299), Medicago truncatula (MeATS1, XP\_003612801), Chlamydomonas reinhardtii (CrATS1, XP\_001694977), Volvox carteri (VoATS1, XP\_002950506).

in all the members of the GPAT family proteins. Both of the ATS1 proteins had the motifs 4, 11, 13, and 19, whereas the GPAT9 proteins all possessed the motifs 4, 9, and 12. The conserved motifs 1–8 and 14 were present in all of the remaining eight GPAT clade members. Both of the GPAT1 proteins had the motifs 1–8, 10, 14, and 20, whereas another motif 17 was present in AtGPAT1. All proteins belonging to GPAT2/3 clade had the motifs 1–8, 10, and 14, except for AtGPAT2, which had another motif 17. All GPAT4–GPAT8 clade members possessed the motifs 1–8, 14, and 15. The motifs 10 and 16 were present in GPAT4/6/8 proteins, whereas the motifs 17 and 18 existed in GPAT5/7 proteins.

#### 3.3. Phylogenetic analysis

To examine the relationships among different sources of GPAT genes, the neighbor-joining method was used to construct phylogenetic trees and all tree topologies were highly congruent (Figure 4). As shown in the phylogenetic tree, all of the GPATs fell into three distinct clades: the ATS1 clade, GPAT9 clade, and GPAT1–GPAT8 clades.

Searches against prokaryote and nonphotosynthetic eukaryotic sequences, and of the fully sequenced genomes of *Chlamydomonas*, *Volvox* and other algae do not identify any GPATs with significant similarities (BlastX E < $10^{-5}$ ) to the *sn*-2 GPATs found in land plants (Yang et al., 2012). In contrast, plastid-localized ATS1 and GPAT9 were found in the algal genomes. Thus the sn-2 GPAT family clearly belongs to a lineage specific to land plants and evolved to provide pathways for functions not present in other organisms. The AhATS1 protein was grouped with ATS1 enzymes from higher plants and green algae, and lie apart from membrane-bound GPAT clades. AhGPAT9 clustered with GPAT9 from higher plants and green algae, apart from the subgroup comprised of GPAT1-GPAT8 from higher plants. The sn-2 GPAT family also fell into three distinct conserved subfamilies. It was assumed that the GPAT4/6/8 clade is the most ancient and arose early during the evolution of land plants (bryophytes), which is involved in the assembly of cutin or cutin-like polymers in the first land plants (Yang et al., 2012). In contrast, the phosphatase-minus GPAT1-3 and 5/7 clades diverged later with the appearance of tracheophytes (Yang et al., 2012). Whereas AhGPAT8 was grouped with GPAT4 and GPAT8 from higher plants, AhGPAT6 fell into the GPAT6 subfamily. Sequences of the GPAT1-GPAT3 clade were more divergent compared with the GPAT4/6/8 and GPAT5/7 clades. AhGPAT1 and AhGPAT2 were grouped with their respective GPAT1 or GPAT2/3 enzymes from higher plants, and lie apart from the GPAT4-GPAT8 clades.

AtGPAT2	MSCNKISTLCAIVFFLYRFFILRFWCHRSPKQKYQKCPSHGLHQYQCPSHGLHQYQ	46 40
AtGPAT3 AhGPAT2	MSAKISIFÇALVFLFYRFILRRYRNSKPKYCN	40
	PARMFFAFFFALFFFALFFFALFFFALFFLFRQLRALIGURPHILS	60
GmGPAT3	PALFFAFFFASLFFALTAFLFFALTAFLTSASSON ATTYLFAA	57
GmGPAT2 AhGPAT1	KAFEVEMSIFFFAFIFIFWIRFFFALASLERSVSNIII	68
GmGPAT1		62
AtGPAT1		96
AtGPAT5		7
	NVMECAG.	5
AtGPAT7	MESST	18
AtGPAT4	MSFAKKSRSFPFISECKS	
AtGPAT8	.MSPEKKSCNFPPITECRD.	18
AhGPAT8	MAAPKPKRTFPFVTECNGNSP.	21
GmGPAT8		16
AhGPAT6		18
GmGPAT6		18
AtGPAT6		19
GmGPAT9		0
AtGPAT9		0
AhGPAT9		0
	HAD-domain	
	HAD-1	
AtGPAT2	DLSNHTLIFNVEGALLKSNSLFPYFFVVAFEAGGVIRSLFLIVLYPFISIMSYEMGLKTHVMLSFFGVKKESFR.VGKSVLPKYFLE	132
AtGPAT3	DLSRHTLIFNVEGALLKSDSLFPYFMIVAFEAGGVIRSFLIFILYPLISIMSHEMGVRVFVFVSFFGIKKEGFR.AGFAVLPKYFLE	126
AhGPAT2	Elgentivfevenaliksss lfpyfmivafeaggifaivivilyfvacaa.divgqelgikknvmicffgikvosfr.Vgrsvipkflie	145
GmGPAT3	JINDEMLIFEVENALLKSSS LFPYFMIVAFEAGGIVFAIVIVLYPFVC VVGKEMGLKINVNACFFGIHASSFR.VGRSVLPKFFLE	146
GmGPAT2	IINDHTLIFTVEGALLKSSSVFPFFMIVAFEAGGLLBAIVILIVYPFVCIVGDEMGLKMMVMICFFGIREESFR.VGRTVLPKFLLE	143
AhGPAT1	ASOPTIAFCEVHKVLINTHS FFFFFMIVAFEGGSILBALLILLSCPILL IINHCKNLBINTFITECGLRIKCMECUSBAVLPKFYLE	155
GmGPAT1	GSQTIVACDIHFVLLRSHSFFPYFMIVAFEGGSILBALLILLSCEVIWILDHELSLEVMTFITECGLRTSAMENMSFAVLPKFYLE	148
AtGPAT1	VSSDHYRDTFFCDIDGVLLRQHSSKHFHTFFPYFMLVAFEGGSIIFAILLLSCSFLWTLQQETKLFVLSFITFSGLFVKCMCNVSRSVLPKFFLE	192
AtGPAT5	$\dots \texttt{TTSYSVVSEFEGTILKNAD} \dots SFSYFMLVAFEAAGLIRFAILLFIWEVITLLCVFSYKNAALKLKIFVATVGLREPEIESVAFAVLPKFYMD$	97
AtGPAT7	TTSYSVVSELEGTLLKNPKPFAYFMLVAFEASGLIRFATLLFLWPIIALLCVLGYPNGSLKIMIFVATAGLHESEIESVARAVLPKFFMD	95
AtGPAT4	REYDSIAADLDGTLLLSRSSFPYFMLVAIEAGSLFRGLILLSLPIVIIAYLFVSESLGIQILIFISFAGIKIKNIELVSFAVLTRFYAA	108
AtGPAT8		108
AhGPAT8		112
GmGPAT8	TTPCDSVAADLDGTLLISRSSFPVFMLVAVEAGSLLGLLLLSVPFIIFSUFJSESLGIQIIIFISFSGLKIADIEIVSFAVLERFYAA	107
AhGPAT6		109
GmGPAT6	NRFNGVASALDGILLYSRS AFFINI VALGASFLEGULGSVFFVFFTYIFFSTAALSIIFAAGURVIDIEVASSULFRFTA	109
AtGPAT6	DRSNHTVAADLOGTILISRSAFFITHLVALEAGSLEAGULEGVEVIFTIFFSELAAIBSLIFIAFAGLEVKEVLFFTE	110
GmGPAT9	**	0
AtGPAT9	**	0
ALGPAT9 AhGPAT9		0
Angeary		0
	HAD-II HAD-IV-	
AtGPAT2	DVCLEMFQVLKRG.GKEVAVS.DLFQVMICVFLRDYDELEVVVGRDMAVGGYYLGIVEDKKNLEIAFCKVVQEERLGSGRRLIGITSFNSPS	223
AtgPAT3	DVCLEMFEVLKRG.GKKICVSDDLPCVMIEGFLRDY EIEVVVGREMVVGGYYLGIMEDKTKHDLVFCELVRKERINTG.RVIGITSFNTSL	217
AhGPAT2	DVCKEMFDVLKRSSGKVCVT.NMPRINVESFLREY	234
GmGPAT3	DVCAEMFEALKKG.GKTVCVT.NLPHYKVESFLREY	233
GmGPAT2	DVGSEMYEVVKRG.GKKVGIS.KLEFVKVESFLKEYEINFVVGRELKVFNGFYVGIMEERKTVHANLEIVRGKCNSSLMIGISGFBKDL	232
AhGPAT1	NINLHAYEVKASSS.SKVVFT.SVFFVKVEGFLKEY SVCNVIGTELQTFGTYFTGFVSDSG.LIVKHFALKEYFGDRKPDIGIGSSS.FD	242
GmGPAT1	NINLBAYEVLASAG.SKVVFT.SVFVKVEGFLREY <mark>R</mark> SVGAVVGTELBSVGCYFSGIVSGSG.LIVKHFALKDYFGDRKPDIGVGSSS.VH	235
AtGPAT1	NINICVYDIKARTEYSKVYFT.SLECVIVERFLREENADDVIGTKLQEIKVMGRKFYTGIASGSG.EVLKHKSAEDYFFDSKKKFALGIGSSSSPQ	287
AtGPAT5	DV&MDIWRVFSSCK.KRVVVT.PMPFVKVERFAKEH PADEVIGTELIVNRFGFVTGLIRETDVDQSAINFVANLFVGRRPQLGLGKFALIA	187
AtGPAT7	DISMDAWRAFGSCD.KRVVVT.FMFFVEVERFAKDH SADEVIGTEIVVNRFGYATGIIQEINVDQSVFNSVANLFVDRRPQLGLGRHIISD	185
AtGPAT4	DVFKDSFEVFDKCKKRKVVVT.ANFIVKVEPFVKDY GCDKVLGTEIEVNPKTMKATGFVKKPGVIVGDLKRLAILKEFGDDSPDLGLGDRIS	200
AtGPAT8	DVFKDSFEVFDKCK.RKVVVT.ANFIVKVEAFVKDY GCDKVLGTEIEVNPKINPATGFVKKPGVIVGDLKRLAILKEFGNESPDLGLGDRIS	199
AhGPAT8	DVFKESFEVFDSCK.RKVVVT.ANPTVKVEPFVKDFTGCDKVLGTEIEVNPKTKKATGFVKKPGVIVGKLKRLAILKEFGETTSPDIGLGDRES	204
GmGPAT8	DVFKESFEVFERCK.RKVVVT.ANFTVEVEFFVKDFTGCDKVLGTEIEUNPKTKKATGFVKKPGVIVGKWKRLAVLKEFGDDESPLVGLGDRKT	199
AhGPAT6	DUBFVIWKUFNSFG.KEYIVT.ASPRIEVEPFVKTICADKULGTELEATKSGRLTGFVKEPGVIVGEHKKIVIVKEFQSNLPDLGLGDSET	199
GmGPAT6	DUBPESHPUFNSFG.KRYIVT.ASPRVEVEPFVKTFCADRVLGTELEATKSGRFTGFVKEPGVIVGEHKVAVVKEFGGNLFDLGLGDSKS	199
AtGPAT6	EVEPDIWRIFNTFG.KRYIII.ASPRIKVEDFVKTFCCVDKVLGTELEVSKSGRATGFTRKPGIIVCQYKRCVVLREFCGLASDLPDLGLGDSKT	203
GmGPAT9		74
AtGPAT9	MSSTAGRLVTS.KSELDIDHFNIEDY PSGSSIN.EPRGKLSLRDLLDISPTITEAACAIVDDSFTRCFKSNPPEFKNW	77
AhGPAT9	MRKTNPKSQ.STELELOGFNIEDY PSCHTIHCEPHCKLRLCDLLDISPTLSEAACAIVDDSFLRCFKSIRSEFKN.W	77
	* * * *	
AtGPAT2	HRSLFSQFCQEIIFUPNSDKKSWQTLFQDQYPKFLITHDGRIAVKFTPINTIVLFMAAFFAVIAAABIVFGINLFYSIANPFIAFSGIHLTIVN	319
AtgPAT3	HRYLFSQFQEIYFVKKSDKRSWQTLPRSQYPKPLICHDGRIAIKFTLMNTIVLFMWGCFAAAAAARIFVSLCIPYSLSIPIIAFSGCRLTVIND	313
AhGPAT2	DHCLFS.HCKEVYAVSEAEKRSWORLSKOKYPKPLICHDGRIALRFTLFOSIAILMMLYALIIAIIRISIALSLPYNFSTPLIVFTGIHLTISEI	329
GmGPAT3	DHDIFFSIEKEVIVVSEADKRSWOKIARERYPRGLINHDGRIALRETEAESIAMIMWETAIIISVIRISIALSIEFTISTPLIVFSGIRITITTS	329
GmGPAT2	DESEFS. TOKEVENUTEACKKNWKNIARDKYFK PLINHDORIALKETFINTIAMIWIRCOFIIALIBITTALSIPINISTPLLEFTOIRITISRP	327
AhGPAT1	DHIFMS.LEKEGYVUNNPSFMLGREKYPKPLIEHDGRIAFLFTPSATLIMFMUL	333
GmGPAT1	DHSFFS.TCKIVINUTEAEKKNWKNIAHDKYPKPLIDHDORIALKIFFINTIAMINIECOFILALIRITALSLPINISTPLLEFTOIRLISRP DHIFMS.ICKROIVUNNSPRHCGENYFK.PLIDHDORIALFIFFSATIUMFMHIIGILAIYHIVOILEYNAMILCVBSUNNVHGI DHIFFS.ICKRAIVUNNECHKNPSSVLPHDUYFK.PLIDHDORIAFLEFSATIUMFMHIIGILAIYHILOILEYNAMALCVBSUNNUKCU	332
AtGPAT1	DHIFIS.ICKEAYFWNEEESMS.KNNALPRERYFKPLITHDGRIAFLFTFLATIAMFIWLH	383
AtGPAT5	STNFLS.ICEEHIHAPIPENYNHCDQQLQLRPLEVIEHDCRLVKEFTEATALIILINIE PGIILAVIRIFLCAVLPLKATPYVSQIFGCHIVKCK	282
AtGPAT7	SPTFLS.ICECVHAFVPSNYNCHNORLBVOPLEVITHDGRIVKLFTEATALIILIWIT FGIIIAMIRIFVGFLLPIKAIPYVSRIFNTRFIVKGK	280
AtGPAT4	DHERMS.ICRECTNUHETKSATTUPIESLKNRITHDGRIVORETPINALIIVINIRSVFRVYFNLPIPERFVRYTYEILGIHLTIRGH	293
AtGPAT8	DHDEMS.I. KCYNVHATKSATTIPKERLKNRIVEHDGRIACRETPINAIITYIWIE FGFIISIIFVYFNLPLPERFVRYTYEMLGIHLTIRGH	292
AhGPAT8	DHOFMS.AGKECYMVPPSKSAKIVPRORLKSRITHOGRFVCRFODINALFTFLWHRPGFALSIFFVYFNLPLPERIVRYTYELLGIHIVISCH	297
GmGPAT8	DRCEMS.ICKEGINUPPSKSAKAVPQERLKSPMITHDGRFVQRFDEMNALITFTWLIGFVLSIIFVYFNLPLPERIVRYTYEILGIKIVIRGH	292
AhGPAT6	DHORMS.ISKECYNVPRTKCEALAFNKLISPIL HEGREVORFTPLASLLTFLWIEIGIILSILEVVINIPLPERIAWYNYKLLGIRVTVKGT	291
GmGPAT6	DYDEMS.ICKECTNUPRTKCEFLPENKLLSPITHEGREVORFTPLAALLTFLWLGIILSILEVYLNIPLPERIAWYNYKLLGIPUIVKGT	291
AtGPAT6	DHCFMS.ICKEGYNVPRTKCEFLPENKLLSPITEHEGRIVCRFTFIVALLTFINLEVGFVLSIIEVYTNIPLPERIARYNYKLTGIKIVNGH	295
GmGPAT9	NYYEFPIWCCVVVRYL	151
AtGPAT9	NVYLFPINCCCVVVRYL	154
AhGPAT9	NVYLFPINGPGVVIRYL	154
	•	
	Acyltransferase-domain AT-II AT-III	
AtGPAT2	NEN. DLISADRKRGCIPVCNERTIIERLYISYAIRKKNEKAVTYSISRISELLAPIKVVRITEDEVKEGCABEKIESQGC	406
AtGPAT3	YVSSQKQKPSQKKGCLEVCNERTILE PLYVAFALRKKNIKTVTYSLSFVSEILAPIKTVRLTEDBVSEGCAMEKIETEGEVVOTEG	401
AhGPAT2	YVSSORORPSORKGCLEVCHIRTILI ELYVAFALREKNIKTYTYSLSFVSEILAPIX VRLTIDEVSGCAMEKLITEGDIVKOEEGT PREPENNNKTGTLIVCHIRTILI ELYISFTLOR.NLIAVTYSLSFMSEILAPIXTVRLTINROGANMMEHLIAQGDIVKOEEGT	414
GmGPAT3	APTRPENIKCNNKGIVGNINCKIRTII PLYISFSICK.NLTAVTYSISFMSEILAPIKTVRIT NRDELAKMMKNI GQGD VVGIGG	419
GmGPAT2	KTINKE DKFLLKGRIMACNI RTILL RULSFTISS . DIVAVTYSLS FMSEILAPIK VRITENROBAKTMKSIFKH GRIVVOTTO-	413
AhGPAT1	MRS.QSSEQ.KKGVLWCTRTILEFVFLSTSIAK.PLIAVTYSLS.KVSEFIAPIR VRITEDBKGAQTMORIESE	417
GmGPAT1	VLE.EKSEQNKGVLEVCSERTILEFVFLSTCIAK.PLIAVTYSLSKVSELIAPIRTVRLEBREGETHORIEKEGEWVOTTO-	416
AtGPAT1	NIN.NG RPEKENSEVINVCNI RTILLEVFLTTSLER. PLIAVTYSLSKFSEFIAPIX VSIXEDRXKIGEAMCRIJSK	469
AtGPAT5	PPCPEAAGKSCVLEVCTIRTING VULSYVLGE, SIEAVTYSIS, RISETLSPIPVRETRIRCVAAKTROCUSK, CONVUCTOR	366
AtGPAT7	PEACATTONEGVERVCTERTIMENVLSYVLGR, SIFAVTYSIS, RESETLSPIP FRETERDUARMINKETSN. CONVENTION	364
AtGPAT4	RPE PPSPGKPGNLYVINERIAL PIIIAIALGE.KITCVTYSVS. RLSIMLSPITAVALATEDEVALAARMECIPEK	377
AtGPAT8	REF PPSPGTLONEWININIAIAID HI IVAIALGE. KICCVTYSVS. RISINESPILAVALATORATURATURATURATURATURA	376
AhGPATS	REF	381
GmGPAT8	REF	376
AhGPAT4	PPE	375
GmGPAT6	APTEPENTIKUNNIGI VORLÄVINENTILE LIISTSEN NULTAVITSES. PASSELLAPIIVUNIT, MODE ANAMINEL GOGU VOCEZON MTINKEDKFLIGGALACHARILL ELIISTISSE DI VAVITSES. PASSELLAPIIVUNIT, MODE ANAMINEL GOGU VOCEZON MRS.QSSEQKKGVLIVGT RILL VVISTSEAK.PETAVITSES. PASSELLAPIRVUNIT, MODE ANAMINEL GOGU VOCEZON VLE.EK.SEQKKGVLIVGT RILL VVISTSEAK.PETAVITSES. NVSEFLAPIRVUNIT, MODE ANAMINEL SEGU VOCEZON ULE.EK.SEQKKGVLIVGT RILL VVISTSEAK.PETAVITSES.NVSEFLAPIRVUNIT, MODE ANAMINEL SEGU VOCEZON VLE.EK.SEQKKGVLIVGT RILL VVISTSEAK.PETAVITSES.NVSEFLAPIRVUNIT, MODE ANAMINEL SE	375
AtGPAT6	DE DEMONSTRUCTURE TRUCTURE LE CUEVELE CONTRACTOR DE CONTRACTOR DE LE CONTR	375
GmGPAT9	BOT DEPARTMENT AND	244
	PPFPPKPGOPGHLIVCN RIVID VVTAVALGR.KISCVIYSIS.KFSELISPIX/VALT CREW AANIRKIDEEGUVKOTEXG HGGRPSIRPKCVVANTSKITULLECHTAFAVIMCKHPKWVGLLCSTILSVOCIWING FEANGREVANDROHULGANNAD LIPEXG HGGRPSIRPKCVWANTSKITULLECHTAFAVIMCKHPKWVGLLSTILSVOCIWING FEANGREVANDROHUGADANNID LIPEXG HGGRPSMRPKCVWANTSKITULLECHTAFAVIMCKHPKWVGLLSTILSVGCIWING FEANGREVANDROHUGADANNID LIPEXG	244 247
AtGPAT9 AhGPAT9	nor Restark and the set of	247
AUGPALS	BORRESERVECTION AND TRADE I LECHTER AVENUEL CHTERES OUT HENDEL AND RELEARING REBY COLUMN HEIT I DECHTERES	24/

FIGURE 2. (continued)

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	AT-IV	
AtGPAT2	TCREPTILING PLEASUCE VINNT IS SUTFFY GTTASGLAAFDE IFFLINEFEST VILLOUVSGSSSSTCRGVPLNGRVNFENNNVOHEIGN	502
Atgpat2 Atgpat3	TCREPTING BOLL SOVE UTING IS NOT FOULAS BAR AND IFILM FOST VALLEV SSSSSSSTCK VPLOW MARKAN AND SSS SSS SSS SSS SSS SSS SSS SSS SSS S	492
AbgPAT2	TCREPTILINS FLF SINCELARWYNNY TW TFFIGTIAGUNCLEFYFFINNESFYTYDLLCHYGORARCUR, ANDER ANDRAF MANNASLAF	511
GmGPAT3	TCREPTILIES FLFSSHCDIVG VSRVSHFBGTAGGLKCLDFFFINNEE WIVOLINGVFSCLLSSHTSNTSVLDBCTSFRVNNVGLGG	519
GmGPAT2	TOREPULLES PLESSICUS LIEW. ICCNVINFHOTTAGGLECLDPFFFINNTFRYSVOLLEEWSPSILASVINGVAGFAVENHVQTCIGE	507
AhGPAT1	TCREPULTESSIFATIADEIVEVIA ANVSNFYOTTAAGLECLDPIFFFNNFFCVHIHTLCKVFFELTCACGRSSCEVINVICSCIAL	507
GmGPAT1	TCREPYLEN SSLFADIADE IVITY VNSHVSMFYGTTASGLKCLDPIFFIMNPRPSYVIEVLGKVPXELTCAG	506
AtGPAT1	TCREPYLIN'S PLEASE TEDIVIN'S VIARVSMFY OTTASGLECIDE IFFINNER VYCLETEKKLEVEMTCAG	559
AtGPAT5	TCREDFILES ALFAELTDE IVEVEN YRVGFFHATTARGWKGLDP IFFFENDRE VYELTFINGLEMEATCS	456
AtGPAT7	TCREPFLEMSALFASTEN IVIUM NYRVCFFHATTARCWRCLDPIFFFMNDREVYEVTFINOLEVEATCS	454
AtGPAT4	TCREPYLINSALFARISTER VINCOCOMENCTIVECVEEND FYFFEM PRESTEAT FLORIDEEMTVNC	468
AtGPAT8	TCREEVILINGSALFAELSDAIVEVILMCKOCMENGTIVRCVRENDPYFFFMNPRESVEATFLDRLPEEMIVNG	467
AbGPAT8	TCREPFLLRINALFASLSDSIVEVEVCKOSMFFGTIVRGVKFWDPYFFFMNPREVYEIRFLDRLPEEMSCKA	472
GmGPAT8	TCREPFLIRISALF SZLSDSIVIVEVNCKCNMFFGTIVRGVKFWDPYFFFMNPREVYEVTFLDPLPEEMSCKA	467
AhGPAT6	TCREPFLIR SALFARITES INVENTIVE OF TVERKLLDPY VENTER TYPITFINGLE CS	465
GmGPAT6	TCREPFLIRESALFABLTDSIVEW_INTKOSVFYGTIVCGHKLLDPYFVFKNFMPTYEITFINQLPKELTCS	465
AtGPAT6	TCREPFLIRESALFAELTDSIVEVEINTKOSMENGTITRGYKLLDPYFAFKNPRFTYEITFLKOIFAELTCK	469
GmGPAT9	CVNNHYSVMAKKGAFELGCTICHVAIKYNKIFVDAFWNSRKQSFTTHLLCIMTSWAVVCDVWYLEFCNL.KPGETPIEFAERVRDIISH	332
Atgpat9	CVNNNTTWNKKGAFELDCTVCET IVNKIFVLAFWNSRKQSFIMHLLCIMISWAVVCEVWYLEPQII.RP	335
AhGPAT9	CVNNHYSVMAKKGAFELGCTVCTVTIKYNKIFVDAFWNSRKQSFTKHLLCIMTSWAVVCDVWYLEPCNQ.KPGETSIEFEPVREIISQ	335
AtGPAT2	ALGFECT NLTRRENN LILAGNNG VYKKK.	530
Atgpat3	ALFECTSLTREETVLILAGNNGVVKFN	520
AtGPAT3 AhGPAT2	ALDFECTSLTRKLILLIAGNGVVKFN. ALGFECTKLTRKLILLIAGNEGVVIN.FACGKS	520 544
AtGPAT3 AhGPAT2 GmGPAT3	ALCFECTSLTRKCTLLIAGNRGVNKPN. ALCFECTNLTRKCTLLIAGNEGVNTN. FACGRS ALCFECTNLTRKCTLUNG KOMEDINSTRKCKSKSKS	520 544 556
AtGPAT3 AhGPAT2 GmGPAT3 GmGPAT2	ALCFECTSLTRKETLIIAGNEGVNKN. ALCFECTKLTRKETLIIAGNEGVNIN.RACGKS. ALCFECTKLTRKETLIIAGNEGVNENTKCKSKSKS. ALFFECTGINKKETVIIAGNEGINS.TKSGK.	520 544 556 539
AtGPAT3 AhGPAT2 GmGPAT3 GmGPAT2 AhGPAT1	ALFECTNETNET LILACHNEVWYFN ALGFECTNETNETNETNETNETNETNENNECHSISS ALFFECTNETNETNETNETNETNENNECHSISS ALFFECTURNETTILACHEINYST.TNSGN ALFFECTURNETTILACHEINYST.TNSGN	520 544 556 539 555
AtGPAT3 AhGPAT2 GmGPAT3 GmGPAT2 AhGPAT1 GmGPAT1	ALCFECTSLTRKENLLILAGNEGVYKFN. ALCFECTKLTRKENLLIAGNEGVYKFN. ALCFECTKLTRKENLTKARBEITNNYKTCKSKSKS. ALCFECTQUNRKENLLILAGNEGIYNST.TKSGK. ALCFECTTUTRRENLIMAGNEGIYNSQPNTKINNCKNNKGETLLS ALCFECTTUTRRENLIMAGNEGIYNSQPNTKINGYNNKGETLLS	520 544 556 539 555 540
AtGPAT3 AhGPAT2 GmGPAT3 GmGPAT2 AhGPAT1 GmGPAT1 AtGPAT1	ALFECT KLTRKCT LILACHNEVWYRH ALGFECT KLTRKCT LILACHNEVWYRH ALGFECT KLTRKCT LILACHNEVWYRH ALFECT (LIRKCT) LILACHNEVWYR (NY ST. TRSCK ALFECT (LIRKCT) LILACHNEVWYR (NY ST. TRSCK ALGFECT TLTRKCT) (MACHNEV (NY ST. TRSCK ALGFECT TLTRKCT) (MACHNEV (NY ST. TRSCK) ALGFECT TLTRKCT) (MACHNEV (NY ST. TRSCK)	520 544 556 539 555 540 585
AtGPAT3 AhGPAT2 GmGPAT3 GmGPAT2 AhGPAT1 GmGPAT1 AtGPAT1 AtGPAT5	ALEFECTSLTRKET LILACNEGVYRPN. ALEFECTSLTRKET LILACNEGVYRPN. ALEFECTSLTRKET (MARKETINNERKCSKSSKS. ALEFECTSLTRKET LILACHEIVST.TRSGK. ALEFECTSLTRKET LILACHEIVGENTKINNERCTNNNKGETLLS ALEFECTTLTRKET VLACHEIVMERKURKCY. VLGFECTNLTRKET VLACHEIVR.	520 544 556 539 555 540 585 585 502
AtGPAT3 AhGPAT2 CmGPAT3 CmGPAT2 AhGPAT1 AtGPAT1 AtGPAT5 AtGPAT7	ALEFECTSLTRKET LILACNEGVKFN. ALEFECTKLTRKET LILACNEGVVTN. FACORS. ALEFECTKLTRKET LILACNEGIVST. TKSGK. ALEFECTQINRKET LILACNEGIVST. TKSGK. ALEFECTQINRKET LILACNEGIVST. TKSGK. ALEFECTTLRRET MALACNEGIVST. TKSGK. VLOPECTNETRRET MALACNEGIVR. TLOPECTNETRRET VLACNEGIVR. TLOPECTNETRKET VLACNEGIVS SYLSLLQLKKVVSTFEPCLH. TLOPECTNETRKET VLACNEGIVSILSLLQLKKVVSTFEPCLH.	520 544 556 539 555 540 585 502 500
AtGPAT3 AhGPAT2 GmGPAT3 GmGPAT2 AhGPAT1 AtGPAT1 AtGPAT5 AtGPAT7 AtGPAT4	ALGPECT NUTRICITY LILACING VYCPN. ALGPECT NUTRICITY LILACING VYCPN. ALGPECT NUTRICITY LILACING UND IN NACCHS ALGPECT TURKICITY LILACHE UND ST. TKSGK. ALGPECT TURKICITY LILACHE UND ST. TKSGK. ALGPECT TURKICITY MULACHE UND ST. TKSGK. ALGPECT NUTRICING UND ST.	520 544 556 539 555 540 585 502 500 503
AtGPAT3 AhGPAT2 GmGPAT2 AhGPAT2 AhGPAT1 AtGPAT1 AtGPAT1 AtGPAT5 AtGPAT7 AtGPAT4 AtGPAT8	ALEFECTSLTRKET LILACNEGVYKFN. ALEFECTSLTRKET LILACNEGVYKFN. ALEFECTSLTRKET LILACNEGUYNT. FACORS ALEFECTSLTRKET LILACNEGUYNT. TRSGK. ALEFECTTTRRET LILACNEGUYNT. TRSGK. ALEFECTTTTRRET LILACNEGUYNT. UEFECTNETRET VLACNEGUYNT. TLEFECTNETRET VLACNEGUYNT. TLEFECTNETREKTSVLACNEGUYNT. UEFECTNETREKTSVLACNEGUYNT. VLEFECTETREKTSVLACNEGUYNT.	520 544 556 539 555 540 585 502 500 503 500
AtGPAT3 AhGPAT2 GmGPAT2 GmGPAT3 GmGPAT1 AtGPAT1 AtGPAT1 AtGPAT5 AtGPAT5 AtGPAT4 AtGPAT8 AhGPAT8	ALFFECT SITTERC ILLEACHEVWYFN. ALGFECT KITERC ILLEACHEVWYFN. ALGFECT KITERC ILLACHEVYFT. TRSGK. ALFFECT (INRCI ILLACHEVYFT. TRSGK. ALFFECT (INRCI ILLACHEVYFT. TRSGK. ALFFECT (INRCI ILLACHEVYFT. TRNGK. ALGFECT TITERCI ILLACHEVYFT. TLGFECT NTERKT IVIACHEVYFYT. TLGFECT NTERKT IVIACHEVYFYT. LLGECT NTERKT IVIACHEVYFYT. LLGECT NTERKT ILLCONDGYESINNTKK. VLGFECT ELTERKT ILLCONDGYESINNTKK.	520 544 556 539 555 540 585 502 500 503 500 505
AtGPAT3 AhGPAT2 GmGPAT3 AhGPAT1 GmGPAT1 AtGPAT1 AtGPAT5 AtGPAT5 AtGPAT7 AtGPAT8 AhGPAT8 GmGPAT8	ALEFECT SLTRKET LILACNEG VYRPN. ALEFECT SLTRKET LILACNEG VYNPN. ALEFECT KLTRKET LILACNEG UNNNKCKSKSKS. ALEFECT GUNKKET LILACNEG INNSKKKSKS. ALEFECT DUNKKET LILACNEG INNSKKKSKS. ALEFECT TLTRRET KLACHEGI VNEDKWRKCY. VLCPECT TLTRRET VLACHEGI VNEDKWRKCY. TLCPECT NYTRKET SVLACHEGI VSILSLLQLKKVVSTFEPCLR. TLCPECT NYTRKET SVLACHEGI VSILSLLQLKKVVSTFEPCLR. TLCPECT NYTRKET SVLACHEGI VSILSLLQLKKVVSTFEPCLR. VLCPECT ELTRRET SLLACHEGI VSILSLLQLKKVVSTFEPCLR.	520 544 556 539 555 540 585 500 503 500 505 500
AtGPAT3 AhGPAT2 GmGPAT3 GmGPAT2 AhGPAT1 GmGPAT1 AtGPAT1 AtGPAT5 AtGPAT5 AtGPAT4 AtGPAT8 AhGPAT8 GmGPAT8 AhGPAT6	ALEFECT KLTRKE ILLEACHEVWYFN ALGFECT KLTRKE ILLEACHEVWYFN ALGFECT KLTRKE ILLEACHEVWYFN ALGFECT KLTRKE ILLACHEI WYST. TXSGK ALFFECT QUNKKE ILLGACHEI WACHWYFNHHOTNNNKGETLLS ALGFECT TLTRKE ILLACHEI UNEDWIRKCY VLGFECT NUTRKE IV WIACHEI WHEN KLLLQLKKWVSTFRECLH. TLGFECT NUTRKE IV VLACHEI WST. SLLUQLKKWVSTFRECLH. TLGFECT NUTRKE IV ULACHEI WST. SLLUQLKKWVSTFRECLH. TLGFECT NUTRKE IV ULACHEI WST. SLLUQLKKWVSTFRECLH. VLGFECT LITRKE IV LLLGCHE WSSUS SLLUQLKKWVSTFRECLH. VLGFECT LITRKE IV LLLGCHE WSSINTKK VLGFECT LITRKE IV LLLGCHE WSSINTKK VLGFECT CLTRKE IV LLGCHE MENSINTKK VLGFECT CLTRKE IV LLGCHE MENSINTKK	520 544 556 539 555 540 585 502 500 503 503 500 505 500 499
AtGPAT3 AhGPAT2 GmGPAT3 AhGPAT1 GmGPAT1 AtGPAT1 AtGPAT5 AtGPAT5 AtGPAT7 AtGPAT8 AhGPAT8 GmGPAT6 GmGPAT6	ALGFECT SLTRKET LILACNEGVYEPN. ALGFECT KUTRKET LILACNEGVYEPN. ALGFECT KUTRKET LILACNEG TINNHKCKSKSKS. ALFFECT QUNKET LILACNEGI VYST. TKSGK. ALGFECT TUTRKET LILACNEGI VYST. ALGFECT NUTRKET VMLACNEGI VYST. ALGFECT NUTRKET VMLACNEGI VYST. TLGFECT NUTRKET VMLACNEGI VSST. TLGFECT NUTRKET VMLACGT VSST. TLGFECT NUTRKET VSST. TLGFECT NUTRKET VMLACGT VSST. TLGFECT NUTRKET	520 544 556 539 555 540 585 500 503 500 505 500 505 500 499 496
AtGPAT3 AhGPAT2 GmGPAT3 GmGPAT2 AhGPAT1 AtGPAT1 AtGPAT1 AtGPAT1 AtGPAT4 AtGPAT4 AtGPAT8 GmGPAT8 AhGPAT8 AhGPAT6 GmGPAT6 AtGPAT6	ALEFECT KLTRKE ILLEGENKUWEN ALGFECT KLTRKE ILLEGENKUWEN ALGFECT KLTRKE ILLEGENKUMERK ALFECT QLNRKE IN LIACHEEINGENKUMERT ALGFECT KLTRKE ILLEGENE INGENKEN ALGFECT KLTRKE ILLEGENE INGENKEN VLGFECT KLTRKE IN MERKEN TGFECT NETRKE IN VLGHDET SILS LLDQLKKVVSTFRECLE. TGFECT NETRKE IN VLGHDET SILS LLDQLKKVVSTFRECLE. VLGFECT KLTRKE ILLEGENDERVESINTEKSME. VLGFECT KLTRKE ILLGENDERVESINTEKSME. VLGFECT KLTRKE ILLGENDERVESINTEKSME. VLGFECT KLTRKE ILLGENDERVESINTEKSME. VLGFECT KLTRKE ILLGENDERVESINTEKSME. VLGFECT KLTRKE IN MEGEN SIKK. VLGFECT KLTRKE IN MEGEN SIKKENA. TGFECT KLTRKE IN MEGEN SIKKENA. TGFECT KLTRKE IN MEGEN SIKKENA.	520 544 556 539 555 540 585 500 503 500 505 500 499 496 501
AtGPAT3 AhGPAT2 CmGPAT3 CmGPAT2 AhGPAT1 AtGPAT1 AtGPAT5 AtGPAT5 AtGPAT8 AhGPAT8 AhGPAT8 CmGPAT6 CmGPAT6	ALGPECT NUTRIC ILLEACHNEUWYEN. ALGPECT KUTRIC ILLACHNEUWYEN. ALGPECT KUTRIC ILLACHNEUWYEN. ALGPECT KUTRIC ILLACHNEI WST. TRSGK ALGPECT TUTRIC ILLACHNEI WST. TRSGK ALGPECT TUTRIC ILLACHNEI WHENREKCY UGPECT TUTRIC ILLACHNEI WST.SFLDC VIRVUTFFEPCLH. UGPECT TUTRIC ILLACHNEW SYLSFLDC VIRVUTFFFEPLH. UGPECT TUTRIC ILLACHNEW SYLSFLDC VIRVUTFFFEPLH. UGPECT TUTRIC ILLACHNEW SYLSFLDC VIRVUTFFFEPLH. UGPECT TUTRIC ILLACHNEW SYLSFLDC VIRVUTFFFEPLH. UGPECT TUTRIC ILLACHNEW SYLSFLDC VIRVUTFFFEFLH. UGPECT TUTRIC ILLACHNEW SYLSFLDC VIRVUTFFFFEHL. UGPECT TUTRIC ILLACHNEW SYLSFFFFFEHC VIRVUTFFFFFEHL. UGPECT TUTRIC ILLACHNEW SYLSFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFF	520 544 556 539 555 502 500 503 500 505 500 499 496 501 373
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FIGURE 2. Amino acid alignment of peanut membrane-bound GPAT proteins and closely related proteins found in the GenBank. Residues shared by a fraction of sequences above 0.5 were shaded, identical residues in black, similar residues in grey. Putative trans membrane domains of peanut GPAT proteins were underlined. AT and HAD-like domains were boxed (ATI to AT-IV) or marked by lines (HAD-I to HAD-IV), respectively. Critical residues previously identified in similar proteins were marked by asterisks (HAD domain), dots (binding site in AT domain) or triangles (catalytic residues in AT domain). GenBank accession numbers were as follows: *Arachis hypogaea* (AhGPAT1, JN032676; AhGPAT2, HQ589243; AhGPAT6, HQ589244; AhGPAT8, JX843442; AhGPAT9, JX843441), *Arabidopsis thaliana* (AtGPAT1, NP\_563768; AtGPAT2, NP\_563651; AtGPAT3, NP\_192104; AtGPAT4, NP\_171667; AtGPAT5, NP\_187750; AtGPAT6, NP\_181346; AtGPAT7, NP\_196227; AtGPAT8, NP\_191950; AtGPAT9, NP\_568925), *Glycine max* (GmGPAT1, XP\_003545142; GmGPAT2, XP\_003520759; GmGPAT3, XP\_003536864; GmGPAT6, XP\_003529144; GmGPAT8, XP\_003520970; GmGPAT9, XP\_003533946).

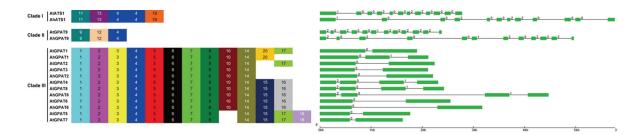


FIGURE 3. The conserved motifs and exon/intron structures of the peanut and Arabidopsis GPAT genes. Schematic representation of motifs identified in peanut GPAT proteins using MEME motif search tool. Each motif was represented by a number in a colored box. Length of box did not correspond to length of motif. Boxes represented the exons and lines represented introns. The sizes of exons and introns could be estimated using the scale at the bottom. The numbers above the boxes and lines indicated the splicing phases of the GPAT sequences, 0 referred to phase 0,1 to phase 1, and 2 to phase 2.

#### 3.4. Tissue-specific expression patterns

Quantitative real-time RT-PCR (qRT-PCR) was employed to confirm the expression patterns of the six novel genes in different peanut tissues and at different stages of seed development. The alpha tubulin 5 (AhTUA5) gene was used as an internal reference control for total RNA input (Chi *et al.*, 2012). As shown in Figure 5, these six genes displayed specific temporal and spatial expression patterns across different tissues and developmental stages. *AhATS1* showed higher transcript abundance in flowers and leaves than in any of the other tissues tested. The highest abundance of *AhGPAT1* transcript was in leaves and the lowest was in stems and flowers. Levels of *AhGPAT2* transcript were highest in leaves, followed by stems and seeds, with the lowest levels in roots and flowers. *AhGPAT6* and *AhGPAT8* had similar expression patterns, showing higher transcript abundance in leaves and roots. *AhGPAT9* exhibited

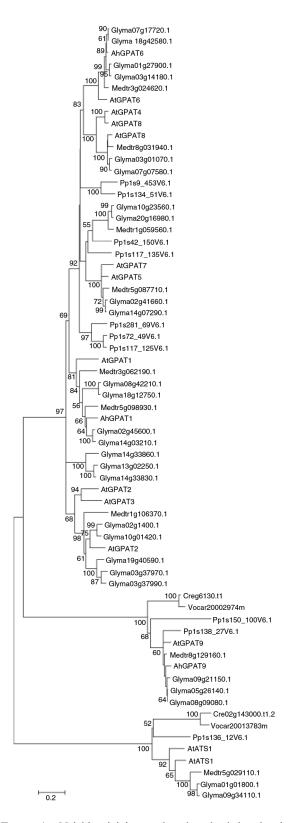


FIGURE 4. Neighbor-joining tree based on the deduced amino acid sequences of GPATs. Gene sequences other than Arabidopsis and peanut GPATs were shown by their nomenclatures found at www.phytozome.org, with the abbreviations. Bootstrap values from neighbor-joining analyses were listed to the left of each node, with values higher than 50 shown. its highest transcript accumulation in stems followed by flowers and seeds.

The expression patterns of six GPAT genes across six developmental stages of seeds are also shown in Figure 5. Levels of AhGPAT1 transcript were maximal at 10 days after pegging (DAP) and decreased gradually thereafter. The expression patterns of AhGPAT2 and AhGPAT6 were similar over the course of seed development, with higher levels of AhGPAT2 and AhGPAT8 transcripts seen at 10 DAP and 40 DAP. The expression levels of *AhATS1* and AhGPAT6 were highest at the initial stage of seed development but dramatically decreased in abundance during later stages. The AhGPAT9 transcript remained relatively low at the initial stage of seed development but increased gradually during later stages of seed development. In peanut cultivar Huayu19, seed lipid content was low in the first period of lipid accumulation, but was characterized by a drastic increase during the initial four stages after pegging (Figure 5). The seed lipid content reached a maximum value of 49.75% at 50 DAP and decreased thereafter at 60 DAP. The expressions of the AhGPAT9 gene coincided with the lipid accumulation rate in peanut seed, whereas the expressions of other AhGPAT genes were not in complete agreement with seed lipid accumulation rate, especially in the earlier stages of seed development like the period from 10 to 30 DAP. These results indicated that AhGPAT9 may be an important component in the lipid biosynthesis process.

# 3.5. Expression patterns of AhGPATs in peanut under abiotic stress

To confirm the expression patterns of six GPAT genes under cold, salt, drought and ABA stress, we monitored the changes in these transcripts in peanut leaves and roots. Figure 6 shows the expression patterns of six GPAT genes in peanut leaves upon cold treatment. Transcript levels of AhGPAT1 in the leaves decreased distinctly and rapidly between 1 h and 6 h after cold treatment, and increased thereafter. The levels of AhGPAT2 transcript gradually accumulated between 1 h and 24 h after cold treatment, and then decreased drastically, with a peak level of about 4-fold increase at 24 h. The expressions of AhGPAT6 and AhGPAT8 were slightly increased under cold stress, with a peak level at 1 h, and then decreased gradually. The expression of AhATS1 and AhGPAT9 gradually decreased under cold stress, while the lowest level was detected at 72 h.

The expression patterns of *AhGPATs* in peanut leaves and roots after treatment with 200 mM NaCl were also monitored (Figure 6). The expression patterns of *AhGPAT1*, *AhGPAT9* and *AhATS1* were different in leaves and roots. Transcript levels of *AhGPAT1* decreased distinctly and rapidly from 1 h to 48 h in the leaves of seedlings subjected to salt

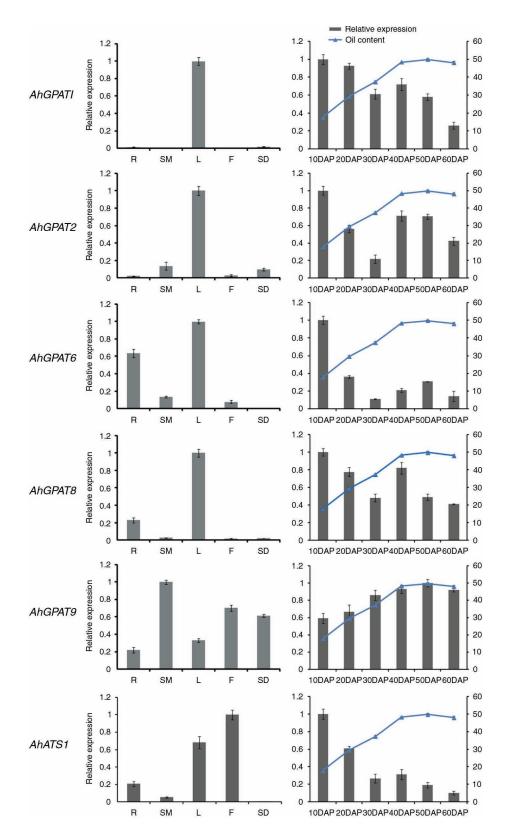


FIGURE 5. Expression analysis of six AhGPAT genes using qRT-PCR in five peanut tissues and at six stages of seed development. R, root; SM, stem; L, leaf; F, flower; SD, seed. The relative mRNA abundance was normalized with respect to the peanut AhTUA5 gene. The bars were standard deviations (SD) of three technical repetitions.

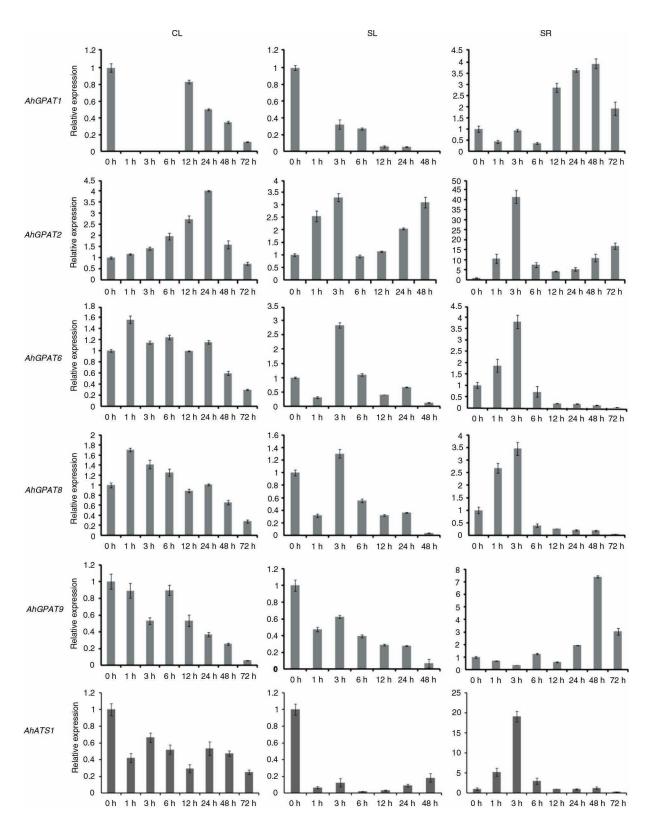
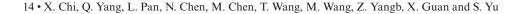


FIGURE 6. Expression analysis of six AhGPAT genes using qRT-PCR under cold and salt stress. The relative mRNA abundance was normalized with respect to the peanut AhTUA5 gene. The bars were standard deviations (SD) of three technical repetitions. CL (0 h to 72 h), leaves exposed to cold (4 °C) treatment; SL (0 h to 48 h), leaves exposed to high salt (200 mM NaCl) treatment; SR (0 h to 72 h), roots exposed to high salt (200 mM NaCl) treatment.



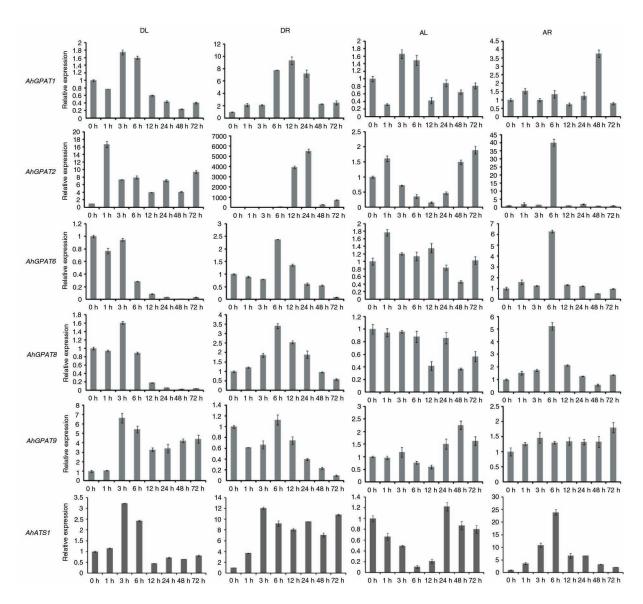


FIGURE 7. Expression analysis of six *AhGPAT* genes using qRT-PCR under drought and ABA stress, The relative mRNA abundance was normalized with respect to the peanut *AhTUA5* gene. The bars were standard deviations (SD) of three technical repetitions. DL (0 h to 72 h), leaves exposed to 20% PEG-6000 treatment; DR (0 h to 72 h), roots exposed to 20% PEG-6000 treatment; AR (0 h to 72 h), roots exposed to 100 uM ABA treatment.

treatment, but increased obviously in roots after 12 h treatment, with a nearly 4-fold increase 48 h after the salt treatment. The levels of *AhGPAT9* transcript decreased gradually in leaves under salt stress, but increased obviously after 24 h treatment and exhibited nearly a 7-fold increase after the roots were treated for 48 h. The transcript levels of *AhATS1* decreased distinctly and rapidly from 1 h to 48 h in salt-treated leaves, but increased obviously in roots after 1 h treatment, with a peak level of about 20-fold observed at 3 h. The expressions of *AhGPAT2* were increased under salt stress, with a peak level at 3 h in both leaves and roots, where the greatest increases were about 3-fold and 41-fold,

respectively. In leaves, the expressions of *AhGPAT6* and *AhGPAT8* decreased rapidly after 1h treatment and then increased to the peak level at 3 h. After 3 h, the levels of *AhGPAT6* and *AhGPAT8* transcripts decreased distinctly. In the roots, the expressions of *AhGPAT6* and *AhGPAT8* gradually increased under salt stress, with a maximum increase of about 4-fold observed at 3 h, and then decreased substantially.

A 20% solution of PEG-6000 was used to mimic drought stress to monitor the expression patterns of AhGPATs in peanut leaves and roots (Figure 7). In the leaves, the expressions of AhGPAT1 slightly increased 3 h after treatment, and then decreased from 6 h to 72 h. In PEG-treated roots, the levels of

AhGPAT1 transcript were distinctly enhanced relative to the peak level (an approximately 9-fold increase) which was observed at 12 h. The transcript levels of AhGPAT2 were obviously increased in both leaves and roots under drought stress, with peak expression levels at 1 h in leaves and 24 h in roots. The greatest increase was about 16-fold in leaves and 5,537fold in roots. Within 6 h after treatment, AhGPAT6 and AhGPAT8 genes were slightly down-regulated in leaves and obviously up-regulated in the roots of peanut seedlings subjected to drought stress. The expressions of AhGPAT9 in leaves increased rapidly with a peak level of about 6-fold increase at 3 h under drought treatment, whereas in roots the expressions increased slightly after 6 h treatment and then decreased from 6 h to 72 h. The expressions of AhATS1 were obviously increased in both leaves and roots under drought stress, with peak levels for both at 3 h. The greatest increase was about 3-fold in leaves and 12-fold in roots.

We also examined the response of *AhGPAT* genes to exogenously applied ABA, which is a plant signaling molecule involved in plant defense signaling pathways (Figure 7). There was no obvious change in the levels of AhGPAT1 transcript in peanut leaves following ABA treatment, although the levels of AhGPAT1 transcript in roots were obviously higher 48 h after initial exposure to exogenous ABA. In leaves, the expressions of AhGPAT2 increased slightly after 1 h treatment with ABA and then decreased from 3 to 12 h. After 24 h, the levels of AhGPAT2 transcript remained slightly higher than in untreated leaves. The Levels of AhGPAT2 transcript were higher in ABA-treated roots than in untreated roots observed 6 h after treatment, with a maximum increase of approximately 40-fold. There were no obvious changes in the abundances of AhGPAT6, AhGPAT8 and AhATS1 transcripts in peanut leaves after ABA treatment. However, the levels of three transcripts increased in roots, where they reached maximum levels 6 h after ABA treatment, with the greatest increases observed being about 6-, 5- and 25-fold, respectively. The expressions of AhGPAT9 were slightly increased in both the leaves and roots of seedlings subjected to ABA stress, with peak levels at 48 and 72 h, respectively.

The above results indicate that *GPAT* transcripts from peanuts are differentially expressed following exposure to abiotic stresses or abscisic acid. The levels of *AhGPAT2* transcript were distinctly enhanced after exposure to all four kinds of stress treatments except for ABA-treated leaves. The transcripts of *AhGPAT1*, *AhGPAT6*, *AhGPAT8* and *AhATS1* increased substantially in roots exposed to salt, drought, and ABA stress. The expressions of *AhGPAT6*, *AhGPAT8*, *AhGPAT9* and *AhATS1* were slightly higher in leaves under certain stress conditions than under normal conditions. These results suggest that these genes may play an important role in enhancing peanut resistance to abiotic stress. Some genes were obviously down-regulated after stress treatments, such as *AhGPAT1* and *AhGPAT9* transcripts in cold- and salt-stressed leaves. This indicates that these genes may have a negative function in peanut abiotic stress regulation.

### 4. DISCUSSION

*sn*-Glycerol-3-phosphate acyltransferase (GPAT) is an important enzyme in glycerolipid synthesis, and is involved in different metabolic pathways and physiological functions. In this study, six genes were identified. These genes likely represent the peanut homologues of Arabidopsis genes involved in the synthesis of cutin, suberin, membrane lipids, or storage lipids. Phylogenetic analysis showed that AhATS1 fell into the plastidial ATS1 subgroup and showed a high sequence similarity with AtATS1. AhGPAT1 and AhGPAT2 belonged to the GPAT1-3 subfamily and shared high sequence similarities with AtGPAT1 and AtGPAT2/3, respectively. Sequence analysis indicates that the NH<sub>2</sub>-terminal domain of the three genes contains four acyltransferase motifs (Pfam 01553) that are conserved among glycerolipid acyltransferase family members, which include GPATs, AGPATs, and a dihydroxyacetonephosphate acyltransferase (Takeuchi and Reue, 2009). It has been suggested that motifs I and IV are important for catalysis, and that motifs II and III are important for substrate binding. The COOHterminal domain is also necessary for enzyme activity and appears to physically interact with the NH<sub>2</sub>-terminal domain to contribute to either catalysis or substrate binding (Pellon-Maison et al., 2006).

The AhGPAT6 and AhGPAT8 proteins belonged to the GPAT4/6/8 clades and shared high sequence similarity with AtGPAT4/8 and AtGPAT6, respectively. Sequence analysis reveals that AhGPAT6 and AhGPAT8 each contain an N-terminal HAD-like domain attached to the acyltransferase moiety. The HAD domain is widespread over the three superkingdoms and is found in a very diverse range of enzymes with hydrolytic activities. Maximum homology of the HAD domain from GPATs out of plants is seen for members of the "PSP/P5N-1 assemblage" (Burroughs et al., 2006), which are characterized by the presence of a C1-type cap module with a fourhelix arrangement. This group includes enzymes with activities as diverse as those of phosphoserine phosphatases (PSP family) and nucleotidases (P5N-1 family). The presence of this typical hydrolytic domain in plant GPATs allows them to behave as bifunctional enzymes that catalyze the dephosphorylation of glycerol in addition to acyl transfer, thus yielding MAGs as the reaction product (Mañas-Fernández et al., 2010).

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The AhGPAT9 protein showed high sequence similarity to AtGPAT9, which was identified in Arabidopsis by a bioinformatics approach, and exhibits a much closer evolutionary relationship with mammalian GPATs. Although the enzymatic activity of AtGPAT9 has not been directly confirmed and its physiological function is unknown, polypeptide sequence alignment, phylogenetic analvsis, conserved domain analysis and gene expression data have all suggested that AtGPAT9 may play an essential role in the synthesis of membrane and storage lipids in plants (Gidda et al., 2009; Chen et al., 2011b). Expression profiling revealed that the levels and tissue-specific accumulations of AhGPAT9 transcript are distinct from those of other GPAT family members, which is consistent with the more diverged nature of the AtGPAT9 gene. Notably, the expression patterns of AhGPAT9 coincided with the lipid accumulation rate in peanut seed. This suggests a potential role for AhGPAT9 in glycerolipid metabolism in developing seeds, although this possibility remains to be tested experimentally.

Cutin and suberin are extracellular lipid barriers deposited by certain types of plant cells (Yang et al., 2012). They are both fatty acid-and glycerol-based extracellular polymers that are insoluble in water and organic solvents (Beisson et al., 2007). These insoluble polymers and other associated waxes function to control water, gas, and ion fluxes and serve as physical barriers to protect plants from pathogen invasion (Schreiber, 2010). The seed coats of Arabidopsis gpat5 mutants were substantially more permeable to tetrazolium salts than those of wildtype seeds. Furthermore, the germination rate of gpat5 seeds under high salt was reduced, and gpat5 seedlings were less tolerant of salt stress than wildtype seedlings (Beisson et al., 2007). The lines of B. napus in which GPAT4 expression was suppressed using RNAi exhibited alterations in cuticle load and stomatal structure, resulting in increased water loss (Chen et al., 2011b). Our results indicated that AhGPAT2 was distinctly enhanced under all four kinds of stress treatments except for ABA-treated leaves. The levels of AhGPAT1 transcript and cutinassociated AhGPAT6 and AhGPAT8 transcripts increased substantially in the roots of seedlings subjected to salt, drought, and ABA stresses. Thus, we infer that these GPAT genes may be involved in regulating some kinds of abiotic stress in peanuts.

GPAT family proteins play crucial roles in the synthesis of cutin, suberin, membrane lipids, and storage lipids (Chen *et al.*, 2011a). Better the understanding of this enzyme family will be valuable to efforts to modify the content and composition of seed oils or to improve abiotic stress resistance in plants. The information generated in our study has improved our understanding of the involvement of these genes in lipid synthesis and opens the way to selecting candidate genes for functional validation studies in peanuts.

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