Glycerol-3-phosphate acyltransferase 3 (OsGPAT3) is required for anther development and male fertility in rice

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Glycerol-3-Phosphate Acyltransferase 3 (OsGPAT3) is required for anther development and male fertility in rice

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Abstract

Lipid molecules are key structural components of plant male reproductive organs, such as the anther and pollen. Although advances have been made in the understanding of acyl lipids in plant reproduction, the metabolic pathways of other lipid compounds, particularly glycerolipids, are not fully understood. Here we report that an endoplasmic reticulum-localized enzyme, Glycerol-3-Phosphate Acyltransferase 3 (OsGPAT3), plays an indispensable role in anther development and pollen formation in rice. OsGPAT3 is preferentially expressed in the tapetum and microspores of the anther. Compared with wild-type plants, the osgpat3 mutant displays smaller, pale yellow anthers with defective cuticle, degenerated pollen with defective exine, and abnormal tapetum development and degeneration. Anthers of the osgpat3 mutant have dramatic reductions of all aliphatic lipid contents. The defective cuticle and pollen phenotype coincide well with the down-regulation of sets of genes involved in lipid metabolism and regulation of anther development. Taking these findings together, this work reveals the indispensable role of a monocot-specific glycerol-3-phosphate acyltransferase in male reproduction in rice.

Key words: Anther development, glycerol-3-phosphate acyltransferase, lipid metabolism, male sterility, microgametophyte, rice, tapetum.

Introduction

Male reproductive development in higher plants is a complicated biological process that includes the development of the anther and the generation of pollen (Liu and Qu, 2008; Ma, 2005; Sanders et al., 1999; Zhang et al., 2011; Zhang and Wilson, 2009). The developed anther wall has four somatic layers: the epidermis, the endothecium, the middle layer, and the tapetum (Goldberg et al., 1993). The innermost cell layer of the anther wall, the tapetum, which encompasses the meiotic cells (microsporocytes) at the center, plays a crucial role in regulating programmed anther development and microspore/pollen formation (Li et al., 2006; Parish and Li, 2010; Zhang and Yang, 2014). Tapetal cell differentiation and tapetum development are critical for the early events in male reproduction, including meiosis, while tapetal degeneration is vital for formation of viable pollen during late pollen development (Ma, 2005; Wilson and Zhang, 2009; Zhang and Liang, 2016; Zhang et al., 2011). Tapetal cells are characterized by the presence of abundant organelles and vigorous metabolic activities, secreting various monomers or precursors for the synthesis of the anther cuticle, pollen wall, and
pollen intracellular nutrients (Li et al., 2010; Liu and Fan, 2013; Zhang and Li, 2014; Zhang et al., 2011; Zhang and Yang, 2014). The disruption of tapetal function frequently leads to aborted microspore formation and male sterility (Fu et al., 2014; Gómez et al., 2015; Li et al., 2006; Li et al., 2011; Niu et al., 2013; Parish and Li, 2010; Xu et al., 2010; Zhang et al., 2008; Zhang et al., 2010). On the other hand, the mature pollen that is produced within the anther is covered by a specialized pollen wall comprised mainly of three layers: the pollen coat, exine, and intine. Pollen serves as a biological protector of male sperm cells and a communicator between male and female reproductive organs that facilitates pollination and fertilization. The disruption of pollen formation often results in male sterility (Dobritsa et al., 2009; Shi et al., 2015). Recent studies have demonstrated that there is a high level of conservation between anther development and pollen formation in plants, highlighting their joint function in plant reproduction (Gómez et al., 2015; Huang et al., 2015).

Two lipid layers play important protective roles in plant anther development and pollen formation. The first of these is the anther epidermal cuticle and the second is the pollen exine. Both layers share a common lipid metabolic pathway similar to those found in plant cuticular lipids, waxes, and cutin (or suberin) (Jeffree, 1996; Kunst and Samuels, 2003; Li and Zhang, 2010; Li-Beisson et al., 2011; Navrath, 2006; Zhang et al., 2016), which seems to be conserved at least in land plants (Shi et al., 2015; Wallace et al., 2015). Therefore, cuticular lipid metabolism, including biosynthesis, modification, transport, and transcription regulation, is essential for the normal development of anther and microspores (Shi et al., 2015; Zhang and Li, 2014).

During the past decades, significant advances in the understanding of lipid metabolism in plant reproductive development and male fertility have been achieved on the basis of studies of mutants of various lipid metabolic genes, mainly in Arabidopsis and rice; for example, Male Sterile 2 (MS2) (Aarts et al., 1997; Chen et al., 2011a), CYP703A2 (Morant et al., 2007), CYP704B1 (Dobritsa et al., 2009), ACSO5 (de Azevedo Souza et al., 2009), and WBC27 (Dou et al., 2011) in Arabidopsis, and Defective Pollen Wall (DPW) (Shi et al., 2011), CYP703A3 (Aya et al., 2009; Yang et al., 2014), CYP704B2 (Li et al., 2010), Wax-deficient anther1 (WDA1) (Jung et al., 2006), OsC6 (Zhang et al., 2010), PDA1/OsABC1G15 (Qin et al., 2013; Wu et al., 2014; Zhu et al., 2013), DPW2 (Xu et al., 2016), OsDEX1 (Yu et al., 2016), and OsABC2G6 (Zhao et al., 2015) in rice. Except WDA1, most of these genes have relatively high expression levels in the tapetum and/or microspores, and their corresponding mutants exhibit defective anther cuticle and/or pollen exine together with the reduction of anther cuticular lipid components and pollen wall lipid constituents, causing complete or partial male sterility (see review by Shi et al., 2015). Transcription factors that govern tapetum development in plants also play important roles in regulating cuticular lipid-mediated anther and pollen development, such as ABORTED MICROSPORES (AMS) (Xu et al., 2014; Xu et al., 2010) in Arabidopsis and Tapetum Degeneration Retardation (TDR) in rice (Li et al., 2006; Zhang et al., 2008). Nevertheless, none of the abovementioned genes and transcription factors is involved in the metabolism of glycerolipids, an important cutin component in plants (Graça et al., 2002; Li-Beisson et al., 2013), let alone the genetic, biochemical, and molecular mechanisms underlying the involvement of glycerolipid metabolism in anther and pollen development.

The glycerolipid triacylglycerol (TAG) and its derivatives are important storage and membrane lipids and indispensable components of biological polymers including cutin and suberin in plants (Pollard et al., 2008). TAG is generated by connecting fatty acids to a glycerol backbone (Coleman and Lee, 2004). Glycerol-3-phosphate acyltransferases (GPATs) catalyze the first step of TAG biosynthesis by acylating glycerol 3-phosphate at the sn-1 or sn-2 hydroxyl with an acyl donor, acyl-CoA or acyl-ACP, and generating lysophosphaticidic acids (LPAs) that can act as signaling molecules in regulating cell growth (Moollenaar et al., 1997; Sheng et al., 2015; Takeuchi and Reue, 2009). Because GPAT displays the lowest specific activity toward a very broad group of substrates, it has been considered to be the rate-limiting enzyme (Wendel et al., 2009; Zheng and Zou, 2001). In animals, GPATs usually acylate glycerol-3-phosphate at the sn-1 position, and are required for membrane lipid synthesis and energy storage. In contrast, in land plants, most GPATs are sn-2 GPATs, which catalyze the reaction in which glycerol is an anchor point for the linear or cross link with fatty acids, playing important roles in the assembly of cutin or suberin in plants (Pollard et al., 2008; Yang et al., 2012).

Arabidopsis has eight sn-2 GPATs with different functions (Chen et al., 2011b). GPAT4 and GPAT8 have high sequence similarity and are suggested to be functionally redundant duplicated genes. Neither the gpat4 nor the gpat8 single mutant showed any obvious cuticle defect, whereas the gpat4gpats8 double mutant exhibited a marked decrease in cutin content in leaves and stems (Li et al., 2007). GPAT6 is highly expressed in flowers (Zheng et al., 2003). Its mutant displayed defective nanoridges on petal surfaces and a significant reduction of cutin monomers in flowers (Li-Beisson et al., 2009). Further biochemical analyses demonstrated that GPAT4, GPAT6, and GPAT8 prefer C16:0 and C18:1 ω-oxidized substrates and have additional phosphatase activity, resulting in the conversion of sn-2 LPA to sn-2 MAG, which is also an important intermediate for polyester assembly (Yang et al., 2012). GPAT5 is required for the synthesis of suberin in seed coat and root, and the gpat5 mutant exhibited strong reduction of very long chain (C22–C24) fatty acid monomers and their derivatives (Beisson et al., 2007). GPAT7, which is phylogenetically most closely related to GPAT5, takes part in suberin synthesis in the wounding response (Yang et al., 2012). GPAT5 and GPAT7 accommodate a broad chain length range of both ω-oxidized and unsubstituted substrates, but they do not possess phosphatase activity. GPAT1, which is mainly expressed in flowers and siliques, also has sn-2 acyltransferase activity utilizing both substituted and unsubstituted substrates, but has no phosphatase activity (Zheng et al., 2003). There is no report yet on the function of GPAT2 or GPAT3 (Yang et al., 2012). Notably, GPAT1 and GPAT6 are essential for male plant fertility. Both gpat1 and gpat6
mutants display altered endoplasmic reticulum (ER) profiles in tapetal cells, as well as severely reduced pollen production and decreased pollen pollination (Li et al., 2012; Zheng et al., 2003). The gpat1gpat6 double mutant exhibited short filaments, defective callose degeneration and microspore release, and complete male sterility (Li et al., 2012). Through database searching, we found 17 GPATs in rice. So far, there is only one report of a rice plastidial GPAT on its substrate selectivity and association with chilling tolerance (Zhu et al., 2009). The involvement of GPATs in rice fertility, particularly anther and pollen development, remains unknown.

In this study, we report the functional analysis of an ER-localized GPAT, Glycerol-3-Phosphate Acyltransferase 3 (OsGPAT3), which plays a crucial role in rice male fertility. The osgpat3 mutant exhibits abnormal tapetum development and defective anther cuticle and pollen exine formation, which is concomitant with a dramatic reduction in aliphatic contents, as well as the down-regulation of genes involved in lipid metabolism and regulation of anther development. We demonstrate that this monocot GPAT plays different roles in male reproduction from its dicot counterpart, providing new insights into the function of glycerolipid biosynthetic enzymes in male fertility.

Materials and methods

Plant materials and growth conditions

Rice (Oryza sativa L.) plants used in this study were in the 9522 background (japonica) and were grown in the paddy field of Shanghai Jiao Tong University. The F2 mapping population was generated from a cross between osgpat3 (japonica) and GuangLuXi 4 (wild type, indica) for gene mapping. Male sterile plants in the F2 population were chosen for gene mapping.

Characterization of mutant phenotype

Plants or flowers were photographed with a Nikon D90 digital camera and a Leica MZ16FA microscope. Observation of anther development by semi-thin sections and transmission electron microscopy were as described by Li et al. (2006). Anther staging was defined as described previously (Zhang et al., 2011; Zhang and Wilson, 2009). Scanning electron microscopy and analyses of anther waxes, cutin, and internal soluble lipids were performed as described by Shi et al. (2011).

Map-based cloning of the OsGPAT3 gene

For fine mapping of the OsGPAT3 locus, bulked segregation analysis was used to identify markers linked to OsGPAT3 as described by Liu et al. (2005). The primer sequences for InDel markers are shown in Supplementary Table S1 at JXB online. The OsGPAT3 locus was first mapped between two InDel molecular markers, CH1132 and RM6094, on chromosome 11. Then, 3000 F2 offspring from the mapping cross were generated, and five InDel markers (ML2, ML4, ML12, AZ11-2, and YUN1151) were used. OsGPAT3 was finally defined between InDel markers ML4 and ML12, within a 122 kb region. PCR was performed according to Chu et al. (2006). The PCR products were separated on 6% polyacrylamide denaturing gels, and bands were visualized by a silver-staining method (Liu et al., 2005). Sequence data for the genomic DNA and mRNA of OsGPAT3 can be found in the NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and Rice Genome Annotation Project (RGAP; http://rice.plantbiology.msu.edu/) databases under accession numbers Os11g0679700/NM_001074987 and LOC_Os11g45400, respectively.

Complementation of the osgpat3 mutant

For functional complementation, the OsGPAT3 1.632 kb coding DNA sequence (CDS) and the upstream 3 kb promoter were amplified and subcloned into a modified binary vector pCAMBIA1301 with the GUS coding region replaced by an eGFP fragment, which carries a hygromycin resistance marker, using the restriction endonucleases SacI and SpeI. Calluses induced from young panicles of the homozygous osgpat3 plants were used for transformation with Agrobacterium tumefaciens (EHA105) carrying the pCAMBIA1301-OsGPAT3-GFP plasmid or the control plasmid pCAMBIA1301-GFP (Li et al., 2006). For transgenic plants, at least 15 independent lines were obtained for each construct. Transgenic plants were identified by PCR using the primers listed in Supplementary Table S1.

Phylogenetic analysis

Multiple protein sequence alignments were performed using MUSCLE 3.6 (http://www.ebi.ac.uk/Tools/msa/muscle/). A phylogenetic tree was constructed with the alignment of GPAT-like protein sequences of rice, Arabidopsis, and other species. MEGA 4.0 (http://www.megasoftware.net/index.html) and the neighbor-joining method were used, with Poisson correction, pairwise deletion, and bootstrap (1000 replicates; random seed).

Quantitative reverse-transcription real-time PCR assay and in situ hybridization

Total RNA was isolated from various rice tissues, including anthers at different developmental stages, using Trizol reagent (Invitrogen) as described by the supplier. An aliquot of 1 µg RNA per sample was used to synthesize cDNA, using a PrimeScript RT reagent Kit with gDNA eraser (Takara). Quantitative reverse-transcription real-time PCR (qRT-PCR) was performed on a Bio-Rad C1000 machine using Takara SYBR Premix Ex Taq™ GC with a standard two-step protocol, consisting of 95 °C for 30s followed by 40 cycles of 95 °C for 5s and 60 °C for 30s. The expression level of OsACTINI was used as an internal control, and a relative quantitation method (Δ cycle threshold) was used to quantify the relative expression level of target genes. Three biological replicates with three technique replicates each were included for statistical analysis and error range analysis. The GenBank accession numbers of the genes used in the qRT-PCR assay are DPW (Os03g0167600), CYP703A3 (Os08g0131100), CYP704B2 (Os03g0168600), TDR (Os02g0120500), TIP2 (Os01g0293100), and MTR1 (Os02g0491300) (see Supplementary Table S1 for primer sequences). RNA in situ hybridizations were performed as described by Li et al. (2006). A 269 bp cDNA fragment of OsGPAT3 was used for making antisense and sense probes (Supplementary Table S1).

Subcellular localization of OsGPAT3

The full-length OsGPAT3 CDS and OsGPAT3ΔN were cloned into the XhoI and SpeI sites of the pA7-35S::GFP plasmid. The resulting plasmids were coupled with gold particles and bombarded into onion epidermal cells, which were observed as previously described by Liu and Mehdy (2007). A laser scanning confocal microscope (Leica TCS SP5) was used for the analysis. GFP fluorescent signals were imaged at the excitation wavelength of 488 nm and the emission wavelength of 505–530 nm.

Results

Phenotypic analysis of osgpat3

To understand the molecular basis of control of rice male fertility, a completely male-sterile mutant was isolated from our rice mutant library (Chen et al., 2006). This mutant was named osgpat3 because of a deletion of the putative
GPAT gene detected in the mutant by a map-based cloning approach (see below). All the F1 progeny from the backcross between the wild type and osgpat3 displayed the wild-type phenotype, and the F2 progeny had an approximate 3:1 segregation ratio of wild-type (fertile) and mutant (sterile) phenotypes (310:105, \( \chi^2=0.013, P>0.05 \)), suggesting a monofactorial recessive inheritance of the mutation. The mutant exhibited normal vegetative development and inflorescence morphology (Fig. 1A–C), but had pale yellow to white and much smaller anthers compared with those of the wild type (Fig. 1D, E) and lacked mature pollen grains at the late stages of anther development (Fig. 1F, G); these observations indicated that OsGPAT3 is required for anther development and pollen formation in rice.

Scanning electron microscopy was used to further observe the phenotypic changes in osgpat3 at various anther developmental stages as defined previously by Zhang and Wilson (2009). At stage 9, there was no observable anther epidermal difference between wild type and osgpat3 (data not shown). At stage 10, dense array of granular Ubisch bodies were observed on the inner locule surface of wild-type anthers but not on that of osgpat3 (Fig. 2E, F). At stage 12, the outer surface of the wild-type anther was covered by well-formed cutinized nanoridges, while the osgpat3 anther surface was quite smooth and cutinized nanoridges were absent (Fig. 2A–D). In addition, normal pollen grains appeared in wild-type anthers at stage 9, while shrunken and irregularly shaped pollen grains, which became completely aborted at the later stages, were observed in osgpat3 (Fig. 2G, H). These results showed that mutation of OsGPAT3 influences the development of the anther cuticle and Ubisch body, and pollen formation.

Similarly, light microscopic examination of transverse sections of anthers showed that there were no remarkable morphological differences between the wild type and osgpat3 during the early developmental stages (Fig. 3A–D). At stage 8a, ellipsoidal dyads were formed and tapetal cells became vacuolated and shrunken with darkly stained cytoplasm in the wild type (Fig. 3E). In the osgpat3 mutant, although dyads were formed, the tapetal cells were less vacuolated and less darkly stained (Fig. 3F). At stage 8b, even though osgpat3 formed normal tetrads, the cells of the middle layer did not degenerate as did those in the wild type (Fig. 3G, H). At stage 9, the wild-type anther had condensed and deeply stained tapetal cells, and young microspores were freely released from the tetrads. By contrast, at the same stage, osgpat3 had vacuolated tapetal cells, and microspores were still covered with callose and could not be released from the tetrads (Fig. 3I, J).

At stage 10, the wild type displayed degenerating tapetum, as well as vacuolated and round microspores, while the osgpat3 tapetum became swollen and less stained, the microspores started to degrade, and the anther wall started to collapse (Fig. 3K, L). At stage 11, wild-type microspores became falcate in shape and tapetal cells had almost completely degraded into cellular debris, whereas osgpat3 microspores displayed an irregular strip-shaped appearance, and the anther wall collapsed into the locule (Fig. 3M, N). At stage 12, the wild type produced mature pollen grains in the locule, whereas osgpat3 had a flat locule without mature pollen (Fig. 3O, P). These results indicated that OsGPAT3 is essential for the timely differentiation and degradation of the tapetum.

To better understand the developmental abnormalities of anther and pollen in the osgpat3 mutant, transmission electron microscopy was applied. Consistent with the findings of light
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Fig. 2. Appearance of the anther and pollen grain in wild-type (WT) and osgpat3 mutant rice under scanning electron microscopy. (A) WT and (B) osgpat3 anthers at stage 12 of development. (C, D) The outermost surface of the epidermis of (C) WT and (D) osgpat3 anthers at stage 12. (E, F) The inner surface of the anther wall layers of (E) WT and (F) osgpat3 anthers at stage 10. Ub, Ubisch bodies. (G, H) Pollen grains of (G) WT and (H) osgpat3 at stage 9. Bars=200 μm in (A) and (B), 5 μm in (C–F), and 10 μm in (G) and (H).

Fig. 3. Transverse section analysis of anther development in wild-type and osgpat3 mutant rice. Locules from the anther section of wild-type (A, C, E, G, I, K, M, O) and osgpat3 (B, D, F, H, J, L, N, P) from stage 6 to stage 12 of development in sequence (6, 7, 8a, 8b, 9, 10, 11, 12, respectively). E, epidermis; En, endothecium; ML, middle layer; Ms, mature pollen Ms, microsporocyte; Msp, microspores; T, tapetum; Tds, tetrads. Bars=15 μm.
microscopy of transverse sections, no obvious ultrastructural morphological alteration was observed in osgpat3 at the early stages of anther development (Fig. 4A–D). At stage 8a, the osgpat3 mutant tapetal cells showed less stained cytoplasm, and contained swollen ER and more vacuoles and lipidosomes compared with the wild type (Fig. 4E–H). At stage 8b, osgpat3 tapetal cells displayed markedly increased and expanded ER (Fig. 4I, J). More tapetal cells with increased ER profiles are shown in Supplementary Fig. S3. Consistent with the defective release of microspores from the tetrad, a thicker callose wall surrounding the tetrad was observed in osgpat3 (Fig. 4K, L). At stage 9, the wild-type tapetum became condensed, and proliferated ER appeared in the tapetal cells (Fig. 4M). In contrast, at this stage almost no ER but abundant vacuoles and lipidosomes were observed in osgpat3 tapetal cells (Fig. 4N). Additionally, in wild-type anthers the secretory structures Ubisch bodies were seen on the surface of the tapetal cells, fine fibrillar materials released from the tapetum were distributed uniformly throughout the locule, and pollen exine was formed on the surface of the microspores, whereas in the osgpat3 mutant no Ubisch bodies or fibrillar material, and only coarse primexine, was evident (Fig. 4M–P). These observations confirmed that mutation of OsGPAT3 affects the development of tapetum and Ubisch bodies, pollen exine formation, and also callose wall degradation.

Changes in aliphatic composition in osgpat3

The defective anther cuticle and Ubisch body in osgpat3 suggested that mutation of OsGPAT3 affects anther cuticular lipid profiling. To test this hypothesis, the composition of waxes, cutin, and total soluble lipids in wild-type and osgpat3 anthers was analyzed by gas chromatography-flame ionization detection (GC-FID) combined with gas chromatography-mass spectrometry (GC-MS). We used the approach described by

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**Fig. 4.** Transmission electron microscopy analysis of anthers in wild-type and osgpat3 mutant rice. (A, B) Anthers of (A) the wild type and (B) osgpat3 at stage 6, showing the four layers of the anther wall. (C, D) Tapetal cells of (C) the wild type and (D) osgpat3 at stage 6. (E, F) Anthers of (E) the wild type and (F) osgpat3 at stage 8a. (G, H) Tapetal cells of (G) the wild type and (H) osgpat3 at stage 8a. (I, J) Tapetal cells of (I) the wild type and (J) osgpat3 at stage 8b. Obviously increased and swollen ER can be observed in osgpat3. (K, L) Tetrads in (K) the wild type and (L) osgpat3 at stage 8b. (M, N) Tapetal cells in (M) the wild type and (N) osgpat3 at stage 9. The arrow in (M) indicates an Ubisch body (Ub). (O, P) Young microspores in (O) the wild type and (P) osgpat3 at stage 9. Thick callose surrounds the microspore in osgpat3. No exine, and only a coarse primexine, is formed in osgpat3. E, epidermis; En, endothecium; ML, middle layer; Ms, microsporocyte; Msp, microspores; N, Nucleus; T, tapetum; Tds, tetrads. Bars=5 μm in (A), (B), (E), (F), (K), and (L), 1 μm in (C), (D), (G), (H), (I), and (J), and 2 μm in (M–P).
Li et al. (2010) to calculate the surface area of the anthers, in which the calculated values of surface area were plotted against the weight of each sample (Supplementary Fig. S1). Analytical results showed that the total wax, cutin, and total internal lipid levels in osgpat3 anthers were decreased by 37.5%, 76.8%, and 58.5%, respectively, compared with those of the wild type (Fig. 5D), which was attributed to the significant reduction of almost every lipid molecule in osgpat3 mutant anthers (Fig. 5A–C). These chemical analysis data not only confirmed the defective anther cuticle and pollen exine patterning observed by scanning and transmission electron microscopy, but also indicated that the mutation of OsGPAT3 significantly affects the biosynthesis of these lipid compounds, which are required for anther cuticle and pollen exine formation.

**Map-based cloning and functional complementation of osgpat3**

To identify the mutant gene, a map-based cloning approach was used. Through fine mapping, the mutation was located to a 13 kb deletion on chromosome 11. There are two putative genes in the 13 kb deleted region (Fig. 6A). One is LOC_Os11g45410, encoding a pentatricopeptide repeat-containing protein (PPR) without any functional characterization; the other gene is LOC_Os11g45400, which is presumed to encode a GPAT. To clarify which gene is responsible for the mutant phenotype, genetic complementation was performed in the homozygous osgpat3 mutant. Transgenic lines with the coding sequence of LOC_Os11g45400 fused to GFP driven by its own promoter (3 kb) restored male fertility (Fig. 6D). Moreover, a homozygous mutant PFG_2D-41298.R (Jeon et al., 2000) in which the T-DNA fragment was inserted within the second exon of LOC_Os11g45400, showed a similar sterile phenotype to osgpat3 (Fig. 6D). These results confirmed that the deletion of LOC_Os11g45400 is responsible for the developmental defects observed in osgpat3 plants. According to the annotation from RGAP, LOC_Os11g45400 contains two exons and one intron (Fig. 6B), and is predicted to encode a GPAT protein of 543 amino acids, containing a transmembrane domain and an acyltransferase domain, but no phosphatase domain.

![Fig. 5. Analysis of lipid content in wild-type and osgpat3 mutant anthers. Amount per unit surface area (mg mm⁻²) of (A) cutin monomers, (B) internal lipid constituents, (C) wax constituents, and (D) total anther lipid contents in wild-type and osgpat3 anthers. Error bars indicate SD (n=4). Compound names are abbreviated as follows: C16 FA, palmitic acid; C18 FA, stearic acid; C18:1 FA, oleic acid; C18:2 FA, linoleic acid; C18:3 FA, linolenic acid; C20 FA, eicosanoic acid; C26 FA, cerotic acid; ALK, alkane; C16 ωHFA, 16-hydroxy-hexadecanoic acid; C18:1 ωHFA, 18-hydroxy-oleic acid; C18:2 ωHFA, 18-hydroxy-linoleic acid; cis-9,10-epoxy C18 ωHFA, cis-9,10-epoxy 18-hydroxy-stearic acid; cis-9,10-epoxy C18:1 ωHFA, cis-9,10-epoxy 18-hydroxy-oleic acid; methoxyhydrin of 9,10-epoxy C18 ωHFA, methoxyhydrin of 9,10-epoxy 18-hydroxy-stearic acid; chlorohydrin of 9,10-epoxy C18 ωHFA, chlorohydrin of 9,10-epoxy 18-hydroxy-stearic acid; C16-9/10, 16 DHFA, 9(10), 18 DHFA, 9(10), 18-dihydroxy-stearic acid; C18-9/10, 18 DHFA, 9(10), 18-dihydroxy-stearic acid; C20 2HFA, 1,2-dioic acid; C22 2HFA, 1,2-dihydroxycosanoic acid; C24 2HFA, 1,2-dihydroxydocosanoic acid. (This figure is available in colour at JXB online.)
These results indicated that deletion of the LOC_Os11g45400 gene is responsible for the defective male fertility in osgpat3.

OsGPAT3 belongs to a monocot-specific clade of the plant sn-2 GPAT family

To clarify the evolutionary role and potential function of OsGPAT3, we performed phylogenetic analysis by searching the public databases NCBI, RGAP, and TAIR, using BLASTP with the full length of the OsGPAT3 predicted amino acid sequence as a query. Since the family of GPATs is quite large, we only collected a total of 45 protein sequences of all the GPAT members from Arabidopsis and rice, and the top 18 closely related OsGPAT3 homologs from other 18 plant species. Subsequently, we constructed a neighbor-joining phylogenetic tree of the 45 sequences (Fig. 7), which were grouped into two main clades. Rice plastidial GPAT (LOC_Os10g42720),
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To further understand the function of OsGPAT3 in male sterility, we investigated the expression pattern of OsGPAT3 in anthers at various developmental stages and other tissues in wild-type plants using qRT-PCR. In anther tissues, the expression of OsGPAT3 was detectable as early as stage 7, peaked at stage 8a, declined gradually until stage 10, then increased and peaked again at a lower level at stage 12, and thereafter declined (Fig. 8A). This dynamic expression pattern of OsGPAT3 in anther tissues was also confirmed by GUS staining (Supplementary Fig. S2A), and was consistent with reported data from the Rice Oligonucleotide Array Database (http://www.ricearray.org/; see Supplementary Fig. S2B). Although relatively higher levels of expression of OsGPAT3 were also detected in stem, root, and leaf tissues, and moderate expression was detected in pistil, no obvious morphological abnormalities were observed in these organs or NCBI accession numbers. Bootstrap values are percentage of

OsGPAT3 is mainly expressed in tapetum and microspores

To determine the spatial and temporal expression patterns of OsGPAT3 more precisely, we performed in situ hybridization with wild-type anther sections. The strong signal of OsGPAT3 transcripts was detected mainly in microspores and tapetum, and weak signals were also detected in the middle layer and the endothecium layer of the anther wall (Fig. 8B–K). The tendency to variation in signal intensity at different stages was also in agreement with the results of qRT-PCR and GUS staining analyses. These results supported that OsGPAT3 was also in agreement with the results of qRT-PCR and GUS staining analyses. These results supported that OsGPAT3 classified into three subclades: the AtGPAT4/6/8-related subclade required for the biosynthesis of cutin, the AtGPAT5/7-related subclade associated with the biosynthesis of suberin, and the AtGPAT1/2/3-related subclade, which was less functionally characterized than the other two subclades. LOC_Os11g45400 was relatively close to AtGPAT3, and therefore was named OsGPAT3. The sn-2 clade is a land-plant-specific lineage associated with the landmark transition from aquatic to terrestrial habitats (Yang et al., 2012). Compared with the first two subclades, the third sn-2 GPAT subclade is more divergent and has more members, and is considered to be the evolutionarily more recent clade. So far, only AtGPAT1 in the third subclade has been found to have in vitro enzyme activity, and is essential for tapetum differentiation and male fertility (Zheng et al., 2003). No enzyme activity was detected for AtGPAT2 or AtGPAT3, and their mutants showed no obvious phenotype in organs such as leaves, flowers, or seeds (Yang et al., 2012). There are nine rice GPATs in the third subclade. Among them, OsGPAT3 is closer to members from Brachypodium distachyon, Hordeum vulgare, Triticum aestivum, Zea mays, Sorghum bicolor, Setaria italica, and three other species from rice. Based on the fact that osgpat3 displayed a remarkably defective phenotype in the anther and pollen, we propose that OsGPAT3 and its homologs in rice may have evolved divergently, leading to diversified functions different from those in dicots. Thus, OsGPAT3 may represent one unique sn-2 GPAT member specific to monocots.

Fig. 7. Phylogenetic tree of the GPAT family and OsGPAT3-related orthologs. A neighbor-joining phylogenetic tree was constructed with all of the Arabidopsis and rice GPATs and OsGPAT3 orthologs from other 18 species (E-value less than 7e-132) found in the NCBI, RGAP, and TAIR databases. The proteins are named according to their gene names or NCBI accession numbers. Bootstrap values are percentage of 1000 replicates. The length of the branches is proportional to the amino acid variation rates. Different clades are highlighted in different colors, representing the sn-1 GPAT (blue), the GPAT4/6/8 clade (red), the GPAT5/7 clade (yellow), and the GPAT1/2/3 clade (green). At, Arabidopsis thaliana; Os, Oryza sativa; Cit, Citrus sinensis; Nel, Nelumbo nucifera; Bet, Beta vulgaris; Jatc, Jatropha curcas; Pyr, Pyrus x bretschneideri; Vltv, Vitis vinifera; Glym, Glycine max; Glys, Glycine soja; Seti, Setaria italica; Tria, Triticum aestivum. (This figure is available in colour at JXB online.)
is directly involved in tapetal development, anther development, and pollen formation.

OsGPAT3 localizes to endoplasmic reticulum

OsGPAT3 has a 21 amino acid putative signal peptide at the N-terminus that was predicted to be localized to the mitochondrion or chloroplast (SignalP and TargetP; http://www.cbs.dtu.dk/services/). To validate this prediction and to further understand the function of OsGPAT3, we made three constructs: OsGPAT3-GFP containing the full-length cDNA of OsGPAT3, OsGPAT3ΔN-GFP without the sequence encoding the 21 amino acid peptide, and a GFP control, all driven by the CaMV35S promoter, to determine the subcellular localization of OsGPAT3. When these individual constructs were transformed into onion epidermal cells by particle bombardment, OsGPAT3-GFP signal was observed mainly at the ER (Fig. 9D), mimicking the signal of an ER-localized marker protein (CD3-959, a combination of the AtWAK2 signal peptide and the ER retention signal His-Asp-Glu-Leu; Fig. 9E, F) (Nelson et al., 2007). Although OsGPAT3 lacks the classic ER retention signal KDEL, it has one KKXX motif at the C-terminus and three internal RXR motifs, all of which are also well-defined ER retrieval motifs (Gao et al., 2014; Shikano and Li, 2003). Furthermore, the co-transformation of OsGPAT3-GFP with a mitochondrial marker gene did not show overlapping of the fluorescent signals (data not shown), which is consistent with previous reports that there is no mitochondrial localization of Arabidopsis AtGPAT2 or AtGPAT3 (Shen et al., 2003; Zheng et al., 2003). In addition, the OsGPAT3ΔN-GFP construct showed the same fluorescent profile as that of OsGPAT3-GFP (data not shown), suggesting that the 21 amino acid sequence at the N-terminus of OsGPAT3 was not a signal peptide. These data showed that OsGPAT3 is an ER-localized GPAT.

Expression of genes associated with anther development is down-regulated in osgpat3

Because osgpat3 showed a dramatic decrease in most kinds of detectable lipid molecules and defects in anther cuticle and pollen exine formation, we hypothesized that osgpat3 may have impacts on the expression of genes involved in the lipid metabolism required for anther cuticle or exine formation. To test this hypothesis, we compared the expression of a set of genes known to be associated with lipid metabolism in wild-type and osgpat3 anthers using qRT-PCR. The results showed that the expression of not only the three lipid biosynthetic
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genes (DPW, CYB704B2, and CYP703A3) (Fig. 10A–C), but also two transcription factors (TDR and its interaction protein TIP2), which are associated with tapetum degeneration and lipid metabolism (Fig. 10D, E), were significantly down-regulated in the osgpat3 mutant. In addition, the expression level of MTR1 (Fig. 10F), a secretory fasciclin glycoprotein-encoding gene that affects the development of both tapetum and microspores (Tan et al., 2012), was down-regulated in the osgpat3 mutant. These results suggested that OsGPAT3 may play important roles in the early stages of anther development.

**Discussion**

Lipids, their derivatives, and their pathway-related genes are critical for reproductive development and fertility in higher plants. So far as we know, the tapetal lipid metabolism contributes significantly to anther wall cuticle and pollen exine/trypsin formation during anther development. In tapetal cells, de novo synthesized fatty acids were either reduced to fatty alcohols by MS2/DPW in plastid or translocated to ER for activation via ACOS, elongation through long-chain acyl-CoA.

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**Fig. 9.** Confocal microscopic images showing the subcellular localization of OsGPAT3-GFP. (A–C) Localization of GFP control (35S:GFP) in onion epidermal cells. (D) Localization of OsGPAT3-GFP (35S:OsGPAT3-GFP) in onion epidermal cells. (E) Red fluorescence shows the ER localization of an ER marker protein (CD3-959) in the same cell as shown in (D). (F) Merged image of (D) and (E). Bars=100 μm. (This figure is available in colour at JXB online.)

**Fig. 10.** Analysis of the expression of anther development regulatory genes using qRT-PCR at different developmental stages. (A–C) qRT-PCR expression analysis of the cuticle and pollen wall development-related lipid biosynthetic pathway genes (A) DPW, (B) CYP703A3, and (C) CYP704B2 in stage 7 to 13 anthers from wild-type and osgpat3 rice. (D–F) qRT-PCR expression analysis of anther development-related transcription factor genes (D) TDR and (E) TIP2, and (F) a fasciclin glycoprotein protein-encoding gene, MTR1, in stage 7 to 10 anthers from wild-type and the osgpat3 mutant rice. OsActin1 was used as a normalizer control. Error bars indicate the SE of three biological replicates. *P<0.05, **P<0.01, ***P<0.001. (This figure is available in colour at JXB online.)
Glycerol is an essential backbone of plant polyesters as suberin (Pollard et al., 2008); however, its presence in plant cutin was discovered only comparatively recently (Graça et al., 2002). The linking of fatty acids to glycerol that is catalyzed by GPAT was suggested to occur after oxidation (Beisson et al., 2012; Yang et al., 2012); therefore, various substituted fatty acids, such as ω-hydroxylated fatty acids and α, ω-dicarboxylic fatty acid, are thought to be the substrates of those GPATs. For example, the Arabidopsis AtGPAT6 prefers C16 and C18 ω-oxidized acyl-CoA substrates, while AtGPAT1 can use both unsubstituted and substituted acyl-CoAs.

To answer the question of whether OsGPAT3 works downstream of the previously reported lipid metabolic enzymes, such as DPW, CYP704B2, and CYP703A3, which are involved in the regulation of rice male fertility, we performed an enzyme activity assay of OsGPAT3 with substrates that are the products of DPW, CYP704B2, and CYP703A3 or the dominant components of plant biopolymers. This assay did not provide any positive results (data not shown); this is consistent with findings in Arabidopsis, in which AtGPAT2 or AtGPAT3 also showed no activity to tested fatty acid-CoA substrates (Yang et al., 2012; Zheng et al., 2003). It is possible that an appropriate substrate has not yet been tested or that unidentified factors are missing from the assay system, and these possibilities deserve further investigation in the future. However, the significantly altered cuticular lipid profile in osgpat3 mutant anthers, together with the high amino acid sequence similarity of OsGPAT3 to other known GPAT family members, suggests that OsGPAT3 is an active enzyme that is required for male fertility in rice. In addition, our comparative gene expression analysis through the Rice Oligonucleotide Array Database found that the expression of OsGPAT3 peaks earlier than that of DPW, CYP704B2, and CYP704A3, consistent with previous studies (Li et al., 2010; Shi et al., 2011; Yang et al., 2014) and our qRT-PCR data (Fig. 8A; Supplementary Fig. S2B). Combining the findings that the expression of DPW, CYP704B2, and CYP703A3, and of tapetum programmed cell death regulatory genes such as TDR and TIP2, were significantly down-regulated in the osgpat3 mutant, we propose that OsGPAT3 may not work directly downstream of DPW, CYP704B2, or CYP703A3. Rather, OsGPAT3 may affect male fertility via its function at earlier developmental stages, with a mechanism that differs from that of these previously reported lipid metabolic genes, which are usually expressed at relatively late anther developmental stages. It is possible that a gene like LOC_Os1g63580, a homolog of AtGPAT6, is likely to work downstream of DPW, CYP704B2, and CYP704A3 (Supplementary Fig. S2B).

In Arabidopsis, AtGPAT1 and AtGPAT6 were reported to be required for male fertility. The atgpat1 mutant showed perturbed tapetum degeneration and massive arrest of pollen development, and the atgpat6 mutant exhibited large-scale abortion of pollen grains and defective pollen wall formation. Both atgpat1 and atgpat6 single mutants were semi-sterile and displayed reduced ER proliferation in the tapetal cells, while the atgpat1atgpat6 double mutant showed a defect of microspore release from tetrads and complete male sterility (Li et al., 2012; Zheng et al., 2003). Our results showed that loss of function of OsGPAT3 expression in tapetum and microspores disrupts tapetum development and metabolism, leading to defective anther cuticle and pollen exine formation (including the absence of Ubisch bodies), and eventual complete abortion of pollen grains. Unlike atgpat1 or atgpat6, the osgpat3 mutant exhibited increased ER expansion and proliferation in tapetal cells at anther developmental stage 8, and almost complete absence of ER but abundant vacuoles and lipidosomes (probably generated from the degradation of ER) at stage 9, indicating that the lesion of OsGPAT3 may lead to abnormal ER development and degradation, which may affect tapetum metabolism and function. This functional difference between OsGPAT3 and AtGPAT1/6 may reflect structural differences in tapetum; the tapeta of rice and other cereals exhibit characteristic orbicules/Ubisch bodies, which have not been observed in members of the Brassicaceae family, including Arabidopsis, which have unique secretory tapeta containing specialized organelles including elaioplasts and tapetosomes (Zhang et al., 2011).

It was reported that the land plant GPATs belong to a sn-2 GPAT family that differs from animal GPATs (Yang et al., 2012). Clade classification of this family reflected their biochemical functions, which are evolutionarily associated with the plants' adaptation during the transition from aquatic to terrestrial habitats. While the first and second clades are conserved among the tracheophytes in the biosynthesis of cutin or suberin, the function of the third clade remains poorly known. Our phylogenetic analysis showed that the monocotyledon subclade to which OsGPAT3 belongs includes three other rice members with unknown function (Fig. 7). While atgpat2 or atgpat3 mutants showed no obvious macroscopic or chemical phenotype (Yang et al., 2012), the osgpat3 mutant showed significant changes in anther cuticular lipid profiling and was male sterile, suggesting that OsGPAT3 plays an indispensable role in male reproduction that is distinct from the role of its dicot counterpart. Further functional characterization of the third land plant sn-2 GPAT clade will facilitate our understanding of the evolutionary and molecular aspects of the grass GPAT proteins in plant evolution and adaptation.

In conclusion, we identified a land plant sn-2 GPAT family member, OsGPAT3, in rice, which encodes an ER-localized GPAT. The dysfunction of OsGPAT3 significantly affects the anther cuticle and pollen exine formation, and leads to eventual male sterility. This work has expanded our understanding of lipid metabolism, particularly the poorly known glycerolipid metabolism, in plant male reproductive development. The conservation and divergence of the sn-2 GPAT family in land plants merit further investigations, which would help us to explore the evolutionary and biochemical functions of lipid metabolism in plant fertility.

**Supplementary data**

Supplementary data are available at *JXB* online.

Fig. S1. The ratio of weight/surface area of the anthers in the wild type and osgpat3 mutant.
Fig. S2. Expression patterns of OsGPAT3 in wild-type anthers.

Fig. S3. Transmission electron microscopy analysis of tapetal cell development in the wild type and osogpat3 mutant at stage 8.

Table S1. Primer sequences used in this study.

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Author contributions

DZ and WL conceived the original screening and research plans; DZ and WL supervised the project and experiments; XM performed most of the experiments; QZ and GL participated in primer design, vector construction, and lipid analysis; JS, SQ, LZ, ZL, and MC provided technical assistance to XM; JS and XM conceived and wrote the paper, with contributions from all the authors; DZ supervised and complemented the writing of the paper.

References


