OsC6, Encoding a Lipid Transfer Protein, Is Required for Postmeiotic Anther Development In Rice

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Synthesis of lipidic components in anthers, including of the pollen exine, is essential for plant male reproductive development. Plant lipid transfer proteins (LTPs) are small, abundant lipid-binding proteins that have the ability to exchange lipids between membranes in vitro. However, their biological role in male reproductive development remains less understood. Here, we report the crucial role of OsC6 in regulating postmeiotic anther development in rice (Oryza sativa). Found in monocots, OsC6 belongs to a distinct clade from previously identified LTP1 and LTP2 family members found in both dicots and monocots. OsC6 expression is mainly detectable in tapetal cells and weakly in microspores from stage 9 to stage 11 of anther development. Immunological assays indicated that OsC6 is widely distributed in anther tissues such as the tapetal cytoplasm, the extracellular space between the tapetum and middle layer, and the anther locule and anther cuticle. Biochemical assays indicated that recombinant OsC6 has lipid binding activity. Moreover, plants in which OsC6 was silenced had defective development of orbicules (i.e. Ubisch bodies) and pollen exine and had reduced pollen fertility. Furthermore, additional evidence is provided that the expression of OsC6 is positively regulated by a basic helix-loop-helix transcription factor, Tapetum Degeneration Retardation (TDR). Extra granule-like structures were observed on the inner surface of the tdr tapetal layer when the expression of OsC6 was driven by the TDR promoter compared with the tdr mutant. These data suggest that OsC6 plays a crucial role in the development of lipidic orbicules and pollen exine during anther development in rice.

Male reproductive development in higher plants is a complex biological process that includes the formation of anthers with differentiated tissues, in which microspores/pollen form. When anther morphology is established, the anther consists of meiotic cells (also called microsporocytes) at the center of each anther lobe and four surrounding somatic layers: the epidermis, the endothecium, the middle layer, and the tapetum, from the surface to the interior (Goldberg et al., 1995). The successful development of viable pollen grains within the anther requires active cooperative interaction of sporophytic and gametophytic molecules (McCormick, 1993; Ma, 2005).

A lipidic anther cuticle and the pollen exine are thought to be two main protective barriers for microspores/pollen against various environmental and biological stresses (Li et al., 2010). During early anther developmental stages, the anther cuticle plays a crucial role in protecting inner anther tissues, including microsporocytes. During late anther developmental stages, the elaborate exine forms as the outer protective layer of pollen grains, acting as another major barrier. However, the biochemical nature of the anther surface and pollen exine remains less understood, because it is difficult to purify and obtain large quantities of materials from the anther for analysis. Moreover, sporopollenin, the major pollen/spore exine component, is highly resistant to chemical analysis and exceptionally stable (Brooks and Shaw, 1978; Ahlers et al., 2003). Recent investigations indicated that the anther cuticle is mainly composed of a cutin matrix with waxes embedded in (intracuticular) and deposited on the surface (epicuticular) of the matrix, which is similar to the cuticle of other plant organs (Jung et al., 2006; Li et al., 2010). Moreover, limited evidence indicated that sporopollenin is composed of aliphatic polyhydroxy compounds and phenolic hydroxy groups (Ahlers et al., 2000, 2003).

Recent biochemical and genetic investigations revealed several key regulators of aliphatic biopolymers, such as sporopollenin and the anther cuticle, during anther development (Ma, 2005; Wilson and Zhang, 2009). Arabidopsis (Arabidopsis thaliana) mutants including male sterile2 (Aarts et al., 1997), defective exine1
LTP peptides to enter the secretory pathway. Consis-
tently, several LTP1 members from Arabidopsis (Thoma et al., 1993), *Brassica oleracea var italica* (Pyee et al., 1994), *Ricinus communis* (Tsuboi et al., 1992), and *Vigna unguiculata* (Carvalho et al., 2004) are localized at the cell wall, while the extracellular localization of these LTPs was not in line with the assumed function of these proteins in cell cytoplasm (Carvalho and Gomes, 2007).

LTPs were originally defined by their capacity to transfer various lipid compounds between lipid bilayers in vitro (Kader, 1975). Later, these lipid transfer molecules were shown to be able to transport a variety of hydrophobic molecules, such as fatty acids (Han et al., 2001), fatty acyl-CoA (Lercher et al., 1997), lyso-
phosphatidylcholine (Charvolin et al., 1999), and phosphatidylylycerol (Sodano et al., 1997). LTPs in plants are thought to play key roles in many biological processes, such as cutin biosynthesis (Kim et al., 2008; Lee et al., 2009), defense response (Lee et al., 2009; Sarowar et al., 2009), plant signaling (Blein et al., 2002; Maldonado et al., 2002), and seed maturation (Thoma et al., 1994). In the rice genome, 52 LTP genes have been annotated (Boutrot et al., 2008). Rice LTP1 was shown to accommodate a diverse range of lipid molecules (Cheng et al., 2004). LTP2 is likely involved in the transport of more rigid suberin monomers (Samuel et al., 2002).

**Tapetum Degeneration Retardation (TDR)** encodes a putative basic helix-loop-helix (bHLH) transcriptional factor that was shown to be a positive regulator of tapetal degradation during anther development. TDR is mainly expressed in tapetal cells, and *tdr* mutant exhibits delayed degenerated tapetum and abnormal pollen wall formation, causing complete male sterility (Li et al., 2006; Zhang et al., 2008). Moreover, we demonstrated that TDR is directly associated with the promoter region of OsC6 (LOC_Os11g37280; Li et al., 2006). OsC6 mRNA was reported to be abundantly expressed in tapetal cells of the anther (Ishiyama et al., 1992, 1994), and OsC6 was annotated as a protease inhibitor (GenBank), since the typical eight-Cys motif of LTPs is found in some protease inhibitors (Jose-
Estanyol et al., 2004). Recently, OsC6 was grouped as a LTP protein and was found in the locule of rice anther (Boutrot et al., 2008; Huang et al., 2009). In rice, a MYB transcriptional factor, GAMYB, has been shown to be essential for anther development, and *gamyb*-2 mu-
tants displayed abnormal development of exine and orbicules (Aya et al., 2009). Interestingly, the expres-
sion of OsC6 is also down-regulated in *gamyb*-2, and GAMYB was shown to be able to bind the promoter of OsC6 by electrophoretic mobility shift assay (EMSA) analysis (Aya et al., 2009).

In this study, we report the key role OsC6 in the development of orbicules and pollen exine during postmeiotic anther development in rice. Expression of OsC6 was mainly detectable in tapetal cells and weakly in microspores, but OsC6 was observed in the tapetal cell cytoplasm, the extracellular space between the tapetum and the middle layer, as well as in the anther locule and anther cuticle through immunolog-
ical assay. Recombinant OsC6 was shown to have lipid-binding activity. Silencing of OsC6 impairs the formation of orbicules and pollen wall, causing reduced pollen fertility. Strikingly, ectopic granule-like structures on the inner surface of the tapetum were seen in *tdr* when expression of OsC6 was driven by the TDR promoter compared with the *tdr* mutant. These
findings suggest that OsC6 likely acts as a crucial lipid-binding molecule essential for male reproductive development in rice.

RESULTS

OsC6 Encodes a LTP Member

OsC6 (Tsuchiya et al., 1992) consists of two exons and one intron (http://rice.plantbiology.msu.edu/; Fig. 1A). Similar to other LTPs, OsC6 encodes a small protein of 132 amino acids with a 23-amino acid N-terminal signal peptide and eight conserved Cys residues (Fig. 1B). Although the molecular mass of mature OsC6 was estimated to be about 10 kD, which is close to that of type I LTPs, OsC6 is a distinct LTP, because it has less than 30% identity with other rice LTPs and was previously predicted to be a type VII LTP (Boutrot et al., 2008).

To further understand the evolutionary role of OsC6 and get information on its potential function, the full OsC6 sequence was used as a query to search for its closest relatives in published databases. Totally, 82 LTP genes from Arabidopsis, rice, Zea mays, Triticum aestivum, Sorghum bicolor, Vitis vinifera, and Ricinus communis were obtained (Supplemental Table S1). In addition, reported members of the LTP1 subfamily, including OsLTP1 (Cheng et al., 2004), AtLTP1 (Thoma et al., 1994), and AtLTP2 (Clark and Bohnert, 1999), the LTP2 subfamily, including OsLTP2 (Samuel et al., 2002) and TaLTP2 (Douliez et al., 2001), as well as another new subfamily member, AtDIR1 (for defective in induced resistance 1; Maldonado et al., 2002), were used for analysis. The phylogenetic tree of these genes was constructed using the characteristic eight-Cys motif of plant LTPs (C…C…CC…CXC…C…C, where X represents any amino acid) as described by Boutrot et al. (2008; Supplemental Fig. S1). The phylogenetic tree showed that OsC6 was not classified into the LTP1 or LTP2 subfamily, and OsC6 and TAAK330179 (from T. aestivum) were closely grouped with relatively strong support (Fig. 1C, gray clade). A gene from S. bicolor (SBXM002446579) and two genes (AC206507.3FGP004 and GRMZM2G414620) from Z. mays were grouped in the OsC6 clade with relatively lower support (Fig. 1C). Nevertheless, no LTP members from dicots were found to be close to the OsC6 clade, implying that OsC6 likely represents a diversified LTP in monocots.

OsC6 Is Mainly Expressed in Tapetal Cells

To understand the function of OsC6, we analyzed the expression pattern of OsC6. Total RNAs were isolated from different organs of rice (root, shoot, leaf, and floral organs), and quantitative reverse transcription (qRT)-PCR analysis showed that OsC6 expression was detectable in anthers starting from early stage 9 of development, high at stage 10, weak at stage 11, and hardly detectable at stage 13 and was not

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**Figure 1.** Sequence analysis of OsC6 with 87 plant LTPs. A, A schematic representation of the exon and intron organization of OsC6. OsC6 consists of two exons (black boxes) and a 595-bp intron (intergenic region) with a 54-bp 5' untranslated region (UTR; white box) and a 391-bp 3' untranslated region. B, The amino acid sequence and structure of OsC6. The box indicates the signal peptide at the N terminus with 23 amino acids, stars indicate the eight conserved Cys residues, and the underline indicates the AAI domain of LTP protein. C, This phylogenetic tree was constructed using the neighbor-joining method. The number for each interior branch shows the percentage of the bootstrap value (1,000 replicates), and only values greater than 50% are shown. The scale bar indicates the estimated number of amino acid substitutions per site for alignment (see Supplemental Fig. S1).
detectable in root, shoot, leaf, and other floral organs (Fig. 2A).

Previously, we showed that expression of OsC6 was down-regulated in tdr, and chromatin immunoprecipitation-PCR analyses and EMSA indicated that TDR is able to bind to the E-box (5′-CATT TG-3′; −881 to −712 bp) within the promoter region of OsC6 (Li et al., 2006).

Here, we transformed an OsC6pro-GUS fusion construct (for expression of the GUS marker protein driven by the 2,073-bp OsC6 promoter region) into the wild type and tdr, respectively. Histochemical GUS staining of wild-type lines containing OsC6pro-GUS showed that GUS activity was detectable in anthers starting at stage 9, at a maximum level at stage 10, then reduced at stage 11, with no detectable signal in other flower organs (Fig. 2, B and C). Moreover, transverse sections showed that GUS activity was mainly detectable in the tapetum at stage 10 (Fig. 2D). In contrast, no GUS staining was observed in tdr lines containing the OsC6pro-GUS construct, confirming that TDR regulates the expression of OsC6 in vivo (Fig. 2B). Furthermore, RNA in situ hybridization analysis indicated that the expression of OsC6 was strong in tapetal cells and weak in microspores of wild-type anthers at stages 10 and 11 compared with the sense probe control, while no obvious OsC6 expression signal was detected in three other outer anther wall layers compared with the tapetal cells (Fig. 2, E–H). Consistently, recent expression profile data using laser microdissection-mediated microarray analysis indicated the expression signals in both tapetum and microspore at stage 10 (Suwabe et al., 2008).

OsC6 Is Localized to the Extracellular Space of Anthers

To understand the in vivo localization of OsC6, we raised polyclonal antibodies against OsC6 using the purified recombinant protein. The specificity of OsC6 antibody was confirmed by protein gel blot (Fig. 3A). In protein extracts of wild-type anthers at stage 10, a specific band with an expected size of 10 kD was detected with the OsC6 antibody, while no signal was detectable in leaves and anthers at stage 13 in the wild type and anthers at stage 10 in tdr and the OsC6-RNA interference (OsC6-RNAi) line (see below; Fig. 3A). Immunogold-labeled antibody localization showed OsC6 signals in tapetal cells and orbicules within the anther at stage 10 (Fig. 3, C and D); more intense staining was observed in the locule, the outer space of epidermal cell wall, and the extracellular spaces of the endothecium, epidermis, and middle layer (Fig. 3, E–H). In contrast, weak labeling was observed on the exine of young microspores, and no detection was observed in young microspores (Figs. 3E). In addition, no obvious signal was observed in the anther when preimmune serum was used (Fig. 3, I–L). Accordingly, these observations suggested that OsC6 is mainly transcribed in the tapetum (Fig. 3M) and then its mature protein is translocated to extracellular spaces (Fig. 3N).

Recombinant OsC6 Has Lipid-Binding Activity

To test whether recombinant OsC6 has lipid-binding activity, we expressed OsC6 without its N-terminal signal peptide and fused it with a His tag at the N terminus in Escherichia coli, using vector pET-32a (Fig.
4A). The recombinant protein was purified using a Ni²⁺-chelating Sepharose column and used for lipid-binding activity using the fluorescence-labeled fatty acid 1-pyrenododecanoic fatty acid (p-96), as described previously (Zachowski et al., 1998; Ge et al., 2003).

Purified OsC6 (10 μM) was mixed with different concentrations of p-96 and placed in a fluorimeter cuvet maintained at 25°C. Rice LTP110 protein (10 μM) was used as a positive control (Ge et al., 2003). When purified OsC6 was incubated with various amounts of p-96, the relative fluorescence intensity increased with increasing concentrations of p-96, relative to negative controls (10 μM), E. coli containing control plasmid pET-32a, and MOPS buffer. The deduced Kₘ for p-96 was about 20 μM by Lineweaver-Burk plot analysis. The fluorescent signal is comparable with that reported for OsLTP110, shown to bind the labeled fatty acid (Ge et al., 2003; Fig. 4B). This result indicated that OsC6 has the ability to bind fatty acid molecules in vitro.

Silencing of OsC6 Reduces Pollen Fertility

To understand the biological role of OsC6 in anther development, we used RNAi. The pHB-OsC6-RNAi construct used a 500-bp OsC6 cDNA fragment including a 135-bp 3’ untranslated region, which has low similarity to other sequences in the rice genome by sequence analysis. Seven independent T0 transformant lines were obtained, and reduction of OsC6 expression was confirmed by qRT-PCR in all lines except line 4 (Fig. 5A). In addition, reduction of endogenous OsC6 in the six RNAi lines was verified by western-blot assays (Fig. 5B), suggesting that OsC6 was reduced in these six transgenic plants. In addition to OsC6, four other LTP genes (Os09g35700, Os01g49650, Os01g12020, and Os08g33290) were reported to be expressed in rice anther (Huang et al., 2009). Our qRT-PCR analysis indicated that the expression level of these four anther-expressed LTP genes was not obviously changed in OsC6-RNAi lines, suggesting that down-regulation of OsC6 by this RNAi approach does not obviously affect the expression of other LTPs (Fig. 5C). Compared with negative control lines transformed with the empty vector, OsC6-RNAi plants were not different during vegetative or floral organ development, and pollen mitosis appeared normal by 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride staining (data not shown). However, 43.2% ± 7.5% of I₂-KI-stained pollen grains was observed in these six independent OsC6-RNAi lines at stage 13 (Fig. 5, D and E). Particularly, only about 3.5% ± 0.47% germination of pollen grains was seen in these OsC6-RNAi lines under in vitro conditions, resulting in reduced seed setting compared with the negative control (Fig. 5, F and G). These observations suggested that knockdown of OsC6 not only alters pollen development but also affects the pollen germination in rice.

To determine the morphological defects of anthers of the OsC6-RNAi lines, transverse sections were examined. At stage 8, pollen mother cells underwent normal meiosis and formed tetrads, and tapetal cells differentiated and were deeply stained (Fig. 6, A and B). At late stage 9, OsC6-RNAi lines had free young microspores released from the tetrad and tapetal cells...
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Grains (43.2% at stage 10 (Fig. 6F) and eventually had aborted pollen condensed tapetal cells and vacuolated pollen grains stage 13 (Fig. 6, E, G, and I), whose tapetal cells became completely degenerated at grains full of starch, lipids, and other nutrients and mitotic divisions forming mature trinucleate pollen. From stage 10 to stage 12, in contrast to the wild type, whose degenerated (Fig. 6C), and the microspores had irregular shapes and became shrunken (Fig. 6D). From stage 10 to stage 12, in contrast to the wild type, whose pollen became vacuolated and underwent normal mitotic divisions forming mature trinucleate pollen grains full of starch, lipids, and other nutrients and whose tapetal cells became completely degenerated at stage 13 (Fig. 6, E, G, and I), OsC6-RNAi lines had less condensed tapetal cells and vacuolated pollen grains at stage 10 (Fig. 6F) and eventually had aborted pollen grains (43.2% ± 7.5% I2-KI-stained pollen grains; Fig. 6, H and J).

To gain a more detailed understanding of abnormal anther development and pollen formation in the OsC6-RNAi lines, we used transmission electron microscopy (TEM). Consistent with semisection observation, no differences between the wild type (Fig. 7, A–C) and OsC6-RNAi lines (Fig. 7, D–F) were observed at stage 8. At stage 9, when young microspores develop, wild-type tapetal cells seemed to be condensed (Fig. 7G) and abundant square-edged orbicules were detectable at the inner surface of the tapetal layer (Fig. 7H, arrowhead). Orbicules are thought to supply lipid materials from the tapetum to developing microspores for pollen exine formation (Piffanelli et al., 1998). In agreement with this hypothesis, wild-type microspores developed an electron-dense pollen exine consisting of tectum and nexine (Fig. 7I). In contrast, OsC6-RNAi lines had more endoplasmic reticulum structures and more lipid-like droplets in tapetal cells and fewer normal orbicules formed (Fig. 7K); the pollen exine also appeared disorganized compared with the wild type (Fig. 7J). Consistently, at stages 10 and 13, OsC6-RNAi lines developed fewer normal orbicules and irregular pollen walls (Fig. 7, P, Q, R, and T) compared with the wild type (Fig. 7, M, N, O, and S), causing aborted pollen development at stage 13 (Fig. 7T). Additionally, we confirmed reduced orbicules by scanning electron microscopy (SEM) at stage 10 (Fig. 7, U and V), but there were no significant changes of the outer surface of wild-type and OsC6-RNAi anthers at stage 10 and stage 13, respectively (Supplemental Fig. S2). These results indicated that OsC6 is crucial for the development of orbicules and the lipidic pollen wall during anther development.

Ectopic Expression of OsC6 Controlled by the TDR Promoter Generates Additional Granule Formation in tdr Anthers

TDR is a tapetum-preferentially expressed gene that regulates programmed cell death in rice anther tapetum (Li et al., 2006), and tdr mutants have few orbicules and abnormal pollen walls (Zhang et al., 2008). OsC6 was shown to be the direct target of TDR through chromatin immunoprecipitation-PCR and EMSA analyses (Li et al., 2006). To further identify the biological function of OsC6 and understand its role in the TDR regulatory network during rice anther development, full-length cDNA of OsC6 was cloned under the TDR promoter to produce TDRpro-OsC6. We performed qRT-PCR analysis and observed that the expression level of OsC6 in four tdr lines containing the TDRpro-OsC6 construct was higher than that in tdr (Supplemental Fig. S3, A and B) but lower than that of the wild type (Supplemental Fig. S3B). Furthermore, western-blot analysis indicated the presence of OsC6 protein in these transgenic lines, suggesting that the TDR promoter is able to drive the expression of OsC6 (Supplemental Fig. S3C). However, transgenic plants containing TDRpro-OsC6 could not rescue the male-sterile defect of tdr (Fig. 8, A and B). We assumed that TDR is a master transcription regulator and controls the expression of multiple genes besides OsC6. Consistent with this idea, our previous microarray data showed that a total of 236 genes associated with metabolism, cell death, transcription control, etc., exhibited significant expression alterations in young tdr spikelets (Zhang et al., 2008). We compared the expression levels of TDR and OsC6 in wild-type anthers at stage 10 and found that the transcript level of OsC6

![Figure 4](image-url)

Figure 4. Recombinant OsC6 displays lipid-binding activity. A, The expression and purification of recombinant OsC6 protein. Lane M, Standard protein prestaining marker; lane 1, pET-32a protein from the control bacterium; lane 2, purified OsC6 protein. B, purified OsC6 protein (10 μg) was incubated with increasing amounts of p-96, and relative fluorescence intensities were measured (n = 3). The pET-32a protein from the control bacterium (black triangle) and MOPS buffer without recombinant OsC6 protein (squares) were used as negative controls. Recombinant rice LTP110 protein from Ge et al. (2003) was used as the positive control (black squares).
was slightly lower than that of TDR (Supplemental Fig. S3B).

Strikingly, we observed a number of granule-like droplets on the inner surface of tapetal cells of the transgenic plants by SEM analyses, whereas tdr had smooth inner tapetal surfaces at stage 10 (Fig. 8, C–F). Furthermore, osmium tetroxide fixative TEM analyses showed that these droplets had high electron density of about 1 to 3 μm in diameter (Fig. 8, G and H), which is similar to the lipid body in tapetal cells (Echlin and Godwin, 1968; Suarez-Cervera et al., 1995). This implied that OsC6 likely plays a key role in transporting lipophilic materials from the tapetal cytoplasm to the locule for pollen wall development.

DISCUSSION

OsC6 Is Required for the Formation of Orbicules and Pollen Wall

In hybrid rice breeding, male sterility lines with cytoplasmic and/or nuclear mutations are of agricultural importance for the production of hybrids to improve yield (Ouyang et al., 2009). Here, we showed that a LTP protein, OsC6, plays a critical role in rice postmeiotic anther development. Our biochemical and morphological analyses suggest that OsC6 is able to bind fatty acids and regulate the formation of lipidic structures, orbicules, and pollen exine. Orbicules, discovered by Rosanoff in 1865 (Huysmans et al., 1998),
are spheroid structures thought to transfer sporopollenin precursors from the tapetum to the microspore surface in species with a secretory tapetum. The OsC6-RNAi lines could not undergo normal tapetal development and displayed fewer obvious orbicules along the locular side of the tapetum compared with the wild type, indicating defects in the transport of sporopollenin precursors from the tapetum to the surface of microspores for exine formation. Tapetal endoplasmic reticulum is considered the site of origin for orbicules (El-Ghazaly and Jensen, 1986; Chen et al., 1988). The so-called “gray bodies” in the tapetal cytoplasm are believed to be progenitors of pro-orbicules (Suarez-Cervera et al., 1995). At the young microspore stage, pro-orbicules extrude through the cell membrane of tapetal cells and soon become irregularly coated with sporopollenin (Huysmans et al., 1998). In OsC6-RNAi lines, the endoplasmic reticulum within the tapetal cells became irregularly enlarged and a large number of high-density lipid-like particles were observed in the cytoplasm (Fig. 7K), suggesting a defect in transporting lipid materials from tapetal cells to microspores.

The development of pollen exine starts from the outer surface of the microspore, and the tapetum secretes and deposits lipidic precursors of sporopollenin onto the structure of primexine on the microspore plasma membrane for exine formation (Blackmore, 2007). This process requires cooperative action between the tapetum and microspores (Bedinger, 1992); several genes controlling pollen exine development have been shown to be expressed in both tapetal cells and microspores (Morant et al., 2007; Zhang et al., 2008; Aya et al., 2009; Li et al., 2010). OsC6 is expressed in the tapetum from stage 9 to stage 11 of anther development, and the pollen defect of OsC6-RNAi lines is likely associated with aberrant tapetal development. The failure of pollen wall formation and abortion of microspores in OsC6-RNAi lines led to partial male sterility. Partial pollen grains of RNAi lines with normal I2-KI staining suggest the normal starch accumulation during pollen maturation, while it is not surprising to observe the low germination rate of the pollen grains from RNAi lines. This difference may be from the abnormal pollen development (particularly for pollen wall). Supportively, proteins associated with the pollen wall facilitate pollen tube growth (Suen and Huang, 2007). In addition, the partial male sterility of OsC6-RNAi lines may be explained by the fact that other LTP members may function redundantly with OsC6.

Similar to OsC6, other LTPs have been shown to be expressed in tapetal cells during anther development (Lauga et al., 2000; Xu et al., 2006; Matsuhira et al., 2007). An Arabidopsis mutant, pdd34, with a Dissociation element insertion upstream of a LTP-encoding gene (At5g54740) displayed defective pollen mitosis (Boavida et al., 2009). An anther-specific LTP from sugar beet (Beta vulgaris), BvLTP-1, was expressed in tapetal cells at the young microspore stage, when pollen exine is actively synthesized (Matsuhira et al., 2007). More importantly, the expression level of BvLTP-1 was shown to be correlated with male sterility of plants with Owen cytoplasm: BvLTP-1 expression was expressed in restored plants with viable pollen grains but not in the male-sterile plants (Matsuhira et al., 2007).

OsC6 Is a Lipid Transfer Protein

BLAST searches of Chlamydomonas reinhardtii, Physcomitrella patens, Selaginella moellendorffii, and Volvox carteri genomes showed no LTP family sequences similar to OsC6. Phylogenetic analyses indicate that OsC6 was not grouped in two previously known LTP1 and LTP2 subfamilies (Boutrot et al., 2008), and OsC6
only shares 22% and 17% identity with the anther-specific LTPs YY1 and OsC4, respectively (Supplemental Fig. S4). In rice, YY1 and OsC4 transcripts were observed in tapetal cells and peripheral cells of the vascular bundle at stage 9 (Tsuchiya et al., 1992; Hihara et al., 1996), suggesting that OsC6 may have distinct functions in anther development from other LTPs. 

In dicots, a number of studies showed that anther-specific oleosin-like proteins are likely involved in lipidic transport from the tapetum to the microspore surface (Murphy, 2001). The most significant difference of tapetum between monocots and dicots is that monocots do not form tapetosomes for lipid transport in tapetal cells, while dicots have lipidic tapetosomes, suggesting a difference in the development of lipidic structures of pollen exine between monocots and dicots. Generally, oleosin-like proteins consist of three domains with a size between 14 and 60 kD (Huang, 1996): the N-terminal hydrophilic domain, the central hydrophobic region, and the C-terminal hydrophilic domain. The middle of the domain was assumed to play a key role in oleosin structure and targeting to lipid bodies in tapetosomes or elaioplasts (Murphy, 2001). OsC6 has a similar structure to other LTPs from rice, maize, and wheat, putatively with a hydrophobic cavity for binding a variety of fatty acids, phospholipids, glycolipids, steroids, and acyl-CoA (Sodano et al., 1997; Lee et al., 1998; Hamilton, 2004). Furthermore, the C-terminal domain of oleosin-like protein

**Figure 7.** Transmission electron micrographs of anthers from wild-type and OsC6-RNAi lines. A, Tetrads encased by callose wall in the wild type (WT) at stage 8. B, Magnified image of tapetal cells showing rough wall in the wild type at stage 8. C, Magnified image of tetrad wall showing the primexine at stage 8 in the wild type. D, Normally developing tetrads encased by callose wall in the OsC6-RNAi line at stage 8. E, Magnified image of tapetal cells showing smooth wall in the OsC6-RNAi line at stage 8. F, Magnified image of tetrad wall showing the primexine at stage 8 in the OsC6-RNAi line. G, At stage 9, wild-type tapetal cells became collapsed in conjunction with the dissolution of callose walls for release of the developing microsproes. H, The tapetal cytoplasm continued to agglomerate, and orbicules formed at stage 9. I, The exine of microspore was established in wild-type pollen at stage 9. J, At stage 9, tapetal cells became collapsed in the OsC6-RNAi mutant. K, The tapetal cytoplasm continued to agglomerate, but orbicules formed abnormally at stage 9 in the OsC6-RNAi line. L, The microspore exine in the OsC6-RNAi line at stage 9. M, The wild type developed microsproes with normal pollen wall at stage 10. N, Magnified image of tapetal cell structure with orbicules at stage 10 in the wild type. O, Magnified image of pollen wall structure at stage 10 in the wild type. P, Irregular microsproes were seen in OsC6-RNAi lines at stage 10. Q, Magnified image of tapetal cells with fewer orbicules at stage 10 in the OsC6-RNAi line. R, Magnified image of pollen wall structure at stage 10 in the OsC6-RNAi line. S, The mature pollen grain with disappeared tapetal cells at stage 13 in the wild type. T, The pollen grain at stage 13 in the OsC6-RNAi line. U, Orbicules at the inner side of wild-type tapetal cells at stage 10. V, Orbicules at the inner side of OsC6-RNAi tapetal cells at stage 10. ER, Endoplasmic reticulum; L, lipid body; MP, mature pollen; Msp, microspore; Ne, nexine; O, orbicules; T, tapetum; Tds, tetrads; Te, tectum; UMP, unmature pollen. Bars = 5 μm in A, D, G, J, M, P, S, and T and 1 μm in B, C, E, F, H, I, K, L, N, O, Q, R, U, and V.
can be cleaved, forming a mature protein that constitutes the major protein component of the pollen coat or tryphine in dicots (Roberts et al., 1991, 1993, 1995). OsC6 putatively transfers lipid precursors from the tapetum to the anther locule, playing a key role in sporopollenin formation.

In monocots, another important gene involved in pollen wall formation is OsRAFTIN1. OsRAFTIN1 is required for pollen development, and OsRAFTIN1-silenced lines produced no or only a few seeds due to collapsed pollen grains. TEM observation showed that OsRAFTIN1-silenced lines seemed to have normal orbicular wall and orbicules as the wild type (Wang et al., 2003). Furthermore, OsRAFTIN1-silenced lines displayed delayed tapetal degeneration at stage 10 of anther development compared with the wild type, leading to incompletely degenerated anther wall layers at stage 13 during mature pollen grain formation (Wang et al., 2003). By contrast, OsC6-RNAi lines displayed defective development of orbicules and pollen exine and normal tapetal degeneration. Moreover, OsRAFTIN1 is synthesized in the tapetum, packed in orbicules, transported to microspores, and lastly targeted to the pollen exine (Wang et al., 2003), while OsC6 is widely distributed in the tapetal cytoplasm, the extracellular space between the tapetum and middle layer, as well as the anther locule and cuticle. These results suggest that OsC6 plays a distinct role from that of OsRAFTIN1 in determining tapetum development and pollen exine synthesis in rice.

OsC6 Is Regulated by TDR

As the innermost sporophytic layer within the anther wall, the tapetum undergoes active lipidic metabolism and timely degradation, which is crucial for microspore development and pollen maturation as well as anther cuticle formation in rice (Jung et al., 2006; Li et al., 2006, 2010; Zhang et al., 2008). TDR is a key regulator of tapetum development and pollen formation. tdr forms a normal primexine matrix with about 10- to 20-nm thickness outside the microspore plasma membrane, but no obvious sporopollenin accumulation onto the primexine of tdr microspores was observed (Zhang et al., 2008). There were 18 genes putatively involved in lipid transport and metabolism that were significantly altered in the tdr mutant by microarray analysis. Among these genes, seven were putatively related to lipid transfer: OsC6, OsC4 (Os08g43290), Os03g50960, Os03g46150, Os07g46210, OsRAFTIN1, and OsC6. OsC6 is synthesized in the tapetum, packed in orbicules, transported to microspores, and lastly targeted to the pollen exine (Wang et al., 2003), while OsC6 is widely distributed in the tapetal cytoplasm, the extracellular space between the tapetum and middle layer, as well as the anther locule and cuticle. These results suggest that OsC6 plays a distinct role from that of OsRAFTIN1 in determining tapetum development and pollen exine synthesis in rice.

![Figure 8. Comparison of the inner surface of tapetal cells of tdr and tdr with TDRpro-OsC6. A, The small white anther of tdr at stage 13 after removal of the palea. B, The small white anther of tdr containing TDRpro-OsC6 at stage 13. C, tdr had smooth inner tapetal surface of the tapetal layer at stage 10 by SEM analysis. D, A number of granule-like droplets were observed on the inner surface of tapetal cells of tdr containing TDRpro-OsC6. E, Higher magnification view of C. F, Higher magnification view of D. G, Section of tdr containing TDRpro-OsC6 showing four wall layers (the epidermis, endothecium, middle layer, and tapetum) at stage 10 by TEM analysis. H, Higher magnification view of G. Arrowheads indicate the droplets. E, Epidermis; En, endothecium; M, middle layer; T, tapetum. Bars = 5 μm in C, D, and G and 1 μm in E, F, and H.](image1)

![Figure 9. Proposed biological role of OsC6 in the formation of orbicules and pollen wall. The tapetum-specific expressed OsC6 is directly regulated by a bHLH transcription factor, TDR, and GAMYB during anther development. The OsC6 protein is widely distributed in anther tissues such as the tapetal cytoplasm, the extracellular space between the tapetum and middle layer, as well as the anther locule and anther cuticle. OsC6 is likely to transfer lipidic molecules from metabolically active tapetal cells to other anther cells for orbicule and pollen wall development.](image2)
OsC6 Is Required for Rice Anther Development

OsC6, and Os10g35180 (Zhang et al., 2008). In this study, we observed no expression signal of OsC6-GUS fusion in tdr, confirming that OsC6 is one direct regulatory target of TDR. Moreover, consistent with the expression pattern of TDR, OsC6 mRNA was mainly detectable in the tapetum. Intriguingly, expression of OsC6 using the TDR promoter in tdr caused abundant ectopic granules at the inner surface of tapetal cells, in contrast to the tdr mutant, which is devoid of these lipid-like granules, implying that OsC6 plays a crucial role in assigning lipidic precursors from the tapetum to microspores (Fig. 9).

In rice, the expression of GAMYB is positively regulated by GA in anthers, which is essential for anther development, and gamyb-2 mutants exhibited abnormal development of exine and orbicules. A cytochrome P450 hydroxylase-encoding gene, CYP703A3, was shown to be directly regulated by GAMYB (Kaneko et al., 2004; Aya et al., 2009). Interestingly, the expression of OsC6 is also down-regulated in gamyb-2 mutants, and GAMYB is able to bind the promoter of OsC6 (~388 to ~301 bp) as indicated by EMSA analysis (Aya et al., 2009), while a 170-bp DNA fragment (~881 to ~712 bp) containing an E-box in the OsC6 promoter can be bound by TDR (Li et al., 2006). These observations suggest that OsC6 is a key component in regulating pollen exine formation and is regulated by both TDR and GAMYB. In addition, the expression level of TDR is also decreased in gamyb-2, suggesting that TDR might function downstream of GAMYB (Aya et al., 2009; Liu et al., 2010). It has been shown that in higher plants, MYB transcription factor is able to bind bHLH transcription factors (Ramsay and Glover, 2005; Gonzalez et al., 2008). Therefore, TDR and GAMYB likely coregulate OsC6 by forming a complex or separately controlling the expression of OsC6.

CONCLUSION

Here, we show the role of a tapetum-specific gene, OsC6, in the formation of pollen exine and orbicules. OsC6 belongs to a unique LTP clade and is regulated by two anther developmental regulators, TDR and GAMYB. The OsC6 protein is secreted into extracellular spaces of the anther, including anther cuticle, anther locule, and the space between the tapetum and middle layer (Fig. 9). This work provides new insights into the role of LTPs in anther development and pollen formation.

MATERIALS AND METHODS

Phylogenetic Analysis

Full-length OsC6 protein was used as the query to search for its closest relatives in published databases: National Center for Biotechnology Information, genomic database in Joint Genome Institute, Eukaryotic Genomes (http://genome.jgi-psf.org/), J. Craig Venter Institute, The Arabidopsis Information Resource, and Gramene (http://www.gramene.org/). Each of the databases was searched using TBLASTN (Supplemental Table S1), and the sequences with E-values below 1e-5 were obtained and redundant sequences with identity of at least 95% were removed from our data set. The remaining sequences were combined as our data set. To verify that the sequences we obtained encoded LTP homologs, the predicted amino acid sequences of the encoded proteins were searched for the eight-Cys motif (C…C…CC…CC…C…C…C…C…C, where X represents any amino acid) manually. The protein sequences that lacked the motif were excluded from further analyses. To understand the relationship of OsC6 with LTP1 and LTP2 subfamilies and other LTP members, six additional sequences (OsLTP1, AtLTP1, AtLTP2, OsLTP2, TaLTP2, and AtD1R1; Supplemental Table S1) were added into the data set.

Protein sequence alignment was performed using muscle 3.6 (Edgar, 2004) with the default settings; the results were adjusted manually using GeneDoc (version 2.6.002) software (Pittsburgh Supercomputing Center; http://www.psc.edu/biomed/genedoc/). A neighbor-joining tree was constructed using MEGA software (version 3.1; http://www.megasoftware.net/index.html; Kumar et al., 2004) with the following parameters: Poisson correction, pairwise deletion, and bootstrap (1,000 replicates, random seed).

Conventional and Quantitative RT-PCR

Total RNA was isolated using Trizol reagent (Ganery Biotech) from rice (Oryza sativa) tissues: root, shoot, leaf, glume, lemma, palea, and pistil at stage 13 of anther development as well as anthers at various stages (stages 8–13). Stages of anthers were classified according to Zhang and Wilson (2009). After treatment with DNase (Promega), 0.3 μg of RNA was used to synthesize the oligo(dT)-primed first-strand cDNA using the ReverTra Ace-a-First Strand cDNA synthesis kit (Toyobo). Two microliters of the RT product was then used as template for conventional and quantitative RT-PCR analysis. PCR primers are listed in Supplemental Table S2, with OsACTIN1 as an internal control.

GUS Staining Assay

A 2,073-bp upstream DNA fragment of OsC6 was amplified from bacterial artificial chromosome (BAC) clone OSJNBa006K21 (kindly provided by the National Center for Genome Research) with the primers OsC6-F and OsC6-R (Supplemental Table S2). The PCR product was cloned into pMD18-T vector (TaKaRa), and then the sequencing-confirmed fragment digested with HindIII and Ncol was subcloned into the binary vector pCAMBIA1301 (kindly provided by Richard Jefferson) fused to the GUS gene to generate OsC6-GUS. The OsC6-GUS construct was transformed into the callus induced from wild-type and tdr mutant line with Agrobacterium tumefaciens (Hiei et al., 1994). GUS activity was visualized by staining the root, stem, leaf, and flowers from spikelets of transgenic lines overnight in X-Gluc (Willselen et al., 1998), then tissues were cleared in 75% (v/v) ethanol. The cleared anther was observed and photographed.

In Situ Hybridization

A 399-bp fragment of OsC6 cDNA was amplified from clone AK064672 kindly provided by the Rice Genome Research Center (RGRC) with the primers OsC6-F and OsC6-R (Supplemental Table S2). The PCR product was cloned into pMD18-T vector (TaKaRa), digested with HindIII and Xhol, and subcloned into the vector pBluescript II SK+. Subsequently, the vector was transcribed in vitro under the control of T7 or SP6 promoter with RNA polymerase using the DIG RNA labeling kit (Roche). This mixture was prepared for the digoxigenin-labeled RNA antisense or sense probe. RNA hybridization and immunological detection of the hybridized probes were performed according to the procedure of Li et al. (2006).

Preparation of OsC6 Polyclonal Antibody and Immunological Analysis

OsC6-specific fragment with less similarity to other sequences was amplified from cDNA clone AK064672 (kindly provided by the Rice Genome Research Center (RGRC)) with the primers N-OsC6-F and N-OsC6-R and cloned into the Ncol and EcoRI sites of pET-32a prokaryotic expression vector and then transformed into Escherichia coli strain BL21 (DE3) (Novagen). The fusion protein expression and purification were performed according to the manual from Novagen, and antibody preparation was performed as described by Huang et al. (2003). Total proteins of wild-type, tdr, and OsC6-RNAi plants were extracted as described previously (Nelson et al., 1998) and then subjected to SDS-PAGE on 15% gels (50 μg of total protein).
protein per lane). For western-blot analysis, proteins on gels were electro-pherotically transferred to a polyvinylidene difluoride membrane (Pierce) and blocked with a blocking buffer (3% bovine serum albumin in phosphate-buffered saline [PBS] buffer [20 mM Tris-Cl, pH 7.5, and 150 mM NaCl]). After washing with PBS buffer, the membrane was incubated with a polyclonal antibody against OsC6 (1:1,000 dilution) or tubulin (1:5,000 dilution; Sigma) as a control (Zhang et al., 2006) in the blocking buffer for 1 h. After washing with PBS buffer and then incubating for 20 min with goat anti-rabbit IgG-conju-gated alkaline phosphatase (1:2,000 dilution; Santa Cruz), the quantity of PBS buffer and then incubating for 20 min with goat anti-rabbit IgG-conju-gated alkaline phosphatase (1:2,000 dilution; Santa Cruz), the quantity of protein was detected as described previously (Deng and Wang, 2007).

For immunogolabeling, rice anthers were fixed with 1% glutaraldehyde and 4% paraformaldehyde in 0.025 M phosphate buffer (pH 7.2) for 5 h, followed by a rinse with phosphate buffer at 4°C overnight, and microtome sections were analyzed as described previously (Wang et al., 2003). The antibody of OsC6 (1:500 dilution) was used as the primary antibody and probed by the colloidal gold-conjugated (10 nm; Sigma) goat anti-rabbit IgG (1:40 dilution).

**Fatty Acid-Binding Assay**

The activity of lipid binding was detected as described previously (Zachowski et al., 1998; Ge et al., 2003). Purified recombinant OsC6 (10 μmol L⁻¹ in 10 mM MOPS, pH 7.2, and 8 μM β-mercaptoethanol) was mixed with various concentrations of p-β (Invitrogen) and placed in a fluorimeter cuvette maintained at 25°C. The excitation wavelength was set at 343 nm, and the emission spectrum was recorded at 378 nm on a Thermo Scientific Varioskan Flash. The pET-32a protein (10 μmol L⁻¹) and MOPS buffer without OsC6 protein were used as the negative controls. Rice LTP110 protein (10 μmol L⁻¹; kindly provided by Xiaochun Ge) was used as the positive control (Ge et al., 2003).

**TDR<sub>Plm</sub>-OsC6 Construction and Transformation**

The 399-bp OsC6 cDNA fragment was amplified from clone AK064672 (RGRC) with the primers TDR pro-OsC6-F and TDR pro-OsC6-R (Supplemental Table S2) and cloned into pMD18-T vector (TaKaRa), digested by EcoRI and PvuCI, and then subcloned into the binary vector pCAMBIA 1301 (kindly provided by Richard Jefferson) to produce vector p1301-OsC6. A 2,353-bp fragment of the TDR promoter was amplified from BAC clone AP008581 with primers TDR GUS-pF and TDR GUS-pR (Supplemental Table S2), digested with Xhol and BgIII, and subcloned into the vector p1301-OsC6 to generate the vector TDRpro-OsC6. The calli of <i>tbr</i> young panicles were used for transformation as described previously (Li et al., 2006).

**Construction of RNAi Vector and Transformation**

A 500-bp OsC6 cDNA fragment (from +35 to +534 bp) with low similarity to other rice genes after sequence analysis was amplified from the cDNA clone vector AK064672 (RGRC) using primers OsC6-RNAi-F and OsC6-RNAi-R (Supplemental Table S2) and digested with EcoRV/PstI and SphI/SalI, respectively. The two fragments were subsequently inserted in opposite directions into the binary RNAi vector pHB (Ma et al., 2005) to generate the pHB-OsC6-RNAi plasmid with double cauliflower mosaic virus 35S promoter. Then, rice Calli of <i>tbr</i> were transfected with <i>A. tumefaciens</i> and used for transformation as described previously (Hiei et al., 1994).

**Phenotype Analysis of OsC6-RNAi Lines**

Flowers were randomly collected from OsC6-RNAi lines and control plants at stage 13. Anthers were dissected and immersed in L<sub>KI</sub>-solution (1% L<sub>KI</sub>), crushed, and observed. These collected fresh mature pollen grains were transferred into a liquid germination medium (20% Suc, 10% polyethylene glycol, 4000, 5 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 4H<sub>2</sub>O, 40 mg L<sup>⁻¹</sup> H<sub>2</sub>BO<sub>3</sub> and 3 mg L<sup>⁻¹</sup> vitamin B<sub>J</sub>) and cultured for about 10 min at room temperature (30°C) to generate germinated rice pollen grains as reported previously (Dai et al., 2007). TEM and SEM were performed and observed as described previously by Li et al. (2006).

Sequence data from this article for the cDNA and genomic DNA of OsC6 can be found in the GenBank/EMBL data libraries under accession numbers AK064672 and OSJN0At006K21, respectively.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Sequence alignment of OsC6 with 87 related LTP members.

**Supplemental Figure S2.** Comparison of anther surfaces of wild-type and OsC6-RNAi lines.

**Supplemental Figure S3.** Expression level of OsC6 in tbr lines containing the TDR<sub>Plm</sub>-OsC6 construct.

**Supplemental Figure S4.** Sequence alignment of OsC6 with reported tapetum-specific LTPs.

**Supplemental Table S1.** Sequence information of the protein alignment among rice OsC6 (LOC_Os11g37280) and 87 related members in the phylogenetic tree.

**Supplemental Table S2.** Primers used in this article (underlining shows the enzyme restriction sites).

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