# Chapter 13
# Role of Lipid Metabolism in Plant Pollen Exine Development

**Dabing Zhang, Jianxin Shi, and Xijia Yang**

**Abstract** Pollen plays important roles in the life cycle of angiosperms plants. It acts as not only a biological protector of male sperms but also a communicator between the male and the female reproductive organs, facilitating pollination and fertilization. Pollen is produced within the anther, and covered by the specialized outer envelope, pollen wall. Although the morphology of pollen varies among different plant species, the pollen wall is mainly comprised of three layers: the pollen coat, the outer exine layer, and the inner intine layer. Except the intine layer, the other two layers are basically of lipidic nature. Particularly, the outer pollen wall layer, the exine, is a highly resistant biopolymer of phenylpropanoid and lipidic monomers covalently coupled by ether and ester linkages. The precise molecular mechanisms underlying pollen coat formation and exine patterning remain largely elusive. Herein, we summarize the current genetic, phenotypic and biochemical studies regarding to the pollen exine development and underlying molecular regulatory mechanisms mainly obtained from monocot rice (*Oryza sativa*) and dicot *Arabidopsis thaliana*, aiming to extend our understandings of plant male reproductive biology. Genes, enzymes/proteins and regulatory factors that appear to play conserved and diversified roles in lipid biosynthesis, transportation and modification during pollen exine formation, were highlighted.

**Keywords** ABC transporter • Exine • Lipid transport protein • Sporopollenin • Tapetum

## Introduction

The alternation of the life cycle of angiosperms relies on the male reproductive development, which forms the haploid male gametes within pollen grains via meiosis and mitosis. During the pollen development, pollination and fertilization, the

D. Zhang (✉) • J. Shi • X. Yang
School of Life Sciences and Biotechnology, Shanghai Jiao Tong University–University of Adelaide Joint Centre for Agriculture and Health,
Shanghai Jiao Tong University, Dongchuan Road 800, Shanghai 200240, China
e-mail: zhangdb@sjtu.edu.cn; jianxin.shi@sjtu.edu.cn; yangxijia1986@163.com



Y. Nakamura, Y. Li-Beisson (eds.), *Lipids in Plant and Algae Development*,
Subcellular Biochemistry 86, DOI 10.1007/978-3-319-25979-6_13

pollen wall, a lipidic structure surrounding pollen grains, provides not only a protective role for male gametes (Scott et al. 2004), but also functions in pollen-stigma communication (Zinkl et al. 1999). Disruption of pollen wall structure frequently causes pollen degradation and/or abortion, leading to partial or complete male sterility (Wilson and Zhang 2009; Li and Zhang 2010). In plant kingdom, pollen grains exhibit remarkable biodiversity in shape and surface patterning. For example, the pollen grains of model dicot plant *Arabidopsis thaliana* display ellipsoidal epidermis covered by reticulate exine, while those of the model monocot plant rice (*Oryza sativa*) exhibit globular epidermis with smooth surface without reticulate cavities (Zhang et al. 2011). The diversity of plant pollen surface results mainly from the structure of the exine (the outer pollen wall), which is also likely associated with pollination, adhesion to vectors and stigmas during pollen-stigma contact, facilitating communication between pollen and stigma and subsequent hydration response during germination (Heslop-Harrison 1979; Murphy 2006). Due to its biological importance, the mechanism underlying the biosynthesis of pollen wall, particularly exine, is an interesting biological question to be addressed for plant biologists.

## Structure and Development of Pollen Wall Structure

Although the morphology of pollen grains varies among different species, the pollen wall is mainly comprised of three layers: the pollen coat, the outer exine, and the inner intine (Blackmore et al. 2007; Zinkl et al. 1999; Edlund et al. 2004). The pollen coat, the outermost stratum of the pollen surface, is extremely hydrophobic, and can be eluted from the exine using organic solvents. It is mainly composed of lipids, and ever-expending list of proteins, pigments, aromatic substances, and other unknown compounds. The pollen coat fills in the depressions or the interspace of the pollen exine, protecting male gametophytes from dehydration and facilitating subsequent pollen-stigma communication and adhesion (Piffanelli et al. 1998; Edlund et al. 2004). Current evidence indicates that mono- and di-cot plants displayed diverse pollen coat proteins (Murphy 2006; Wu et al. 2015), even though limited is known about the lipids in the pollen coat. The report that sterol esters are the major components of pollen coat, indicating that pollen coat is different from membrane lipid, sharing similarity to oil droplet. The intine, an innermost bilayered structure of pollen wall, is mainly composed of exintine and the endintine. The main components of intine are pectin, cellulose, hemicellulose, hydrolytic enzymes and hydrophobic proteins, are required for the maintaining the structural integrity of pollen grains, pollen germination and pollen tube growing into the stigma (Scott et al. 2004; Knox and Heslop-Harrison 1971).

Exine, the most complex layer of the pollen wall, contains two layers, the inner nexine and the outer sexine. The nexine is a bilayer structure, consisting of nexine I (foot layer) and nexine II (endexine) (Ariizumi and Toriyama 2011), and the sexine is composed of tectum and bacula (Zhang et al. 2011; Li and Zhang 2010). Exine has extremely physical and chemical stability resistant to biotic and abiotic stresses such as high temperature, desiccation, ultraviolet (UV) irradiation and mechanical

damage (Scott et al. 2004) due to its major component, sporopollenin, a highly resistant biopolymer of phenylpropanoid and lipidic monomers covalently coupled by ether and ester linkages (Ahlers et al. 2000). Sporopollenin provides the rigid and sculptured framework of the exine, functions to encapsulate and protect the pollen contents, and to aid in stigmatic capture. Despite the evolutionary history of sporopollenin in land plants is not clear, sporopollenin has been found in ferns, moss, fossilized green algae, and even in fungi, suggesting that it has remained evolutionarily conserved since the initial colonization of land (Scott 1994). Since sporopollenin is one of the most durable substances in nature, the biochemical mechanism on the biosynthesis of sporopollenin are still largely uncharacterized (Blackmore et al. 2007). Recent metabolomic analysis with MALDI-TOF mass spectrometer on lipids revealed that plant pollen contains also membrane-associated phospholipids (phosphatidylserine and phosphatidylcholine) and diacylglycerol (Liang et al. 2013).

Although the fundamental structure of pollen wall shares similarity among species, morphological analysis reveals that the pollen wall of rice is distinct from that of *Arabidopsis* (Li and Zhang 2010). *Arabidopsis* exine has thin nexine layer, semi-open tectum layer and longer baculum, with an abundant pollen coat deposited in the sculptured cavities of exine (Zhang et al. 2011; Edlund et al. 2004). Compared with *Arabidopsis*, rice exine has thicker tectum and nexine, and higher density of bacula, with much less pollen coat filled in the space between tectum and nexine (Li and Zhang 2010; Zhang et al. 2011; Ariizumi and Toriyama 2011). In addition, the outer surface of *Arabidopsis* pollen grains is fully reticulate while that of rice is relatively smooth. This morphological difference may be associated with different pollination methods between rice and *Arabidopsis*. The pollen grains of *Arabidopsis* are mainly distributed by insects, while rice pollen grains are transmitted by wind (Ariizumi and Toriyama 2011).

## *Exine Development*

Current evidence shows that most plant species share similar biological processes in exine development (Fig. 13.1) even though different plants exhibit diversity of pollen grain morphology (Zhang et al. 2011; Ariizumi and Toriyama 2011; Li and Zhang 2010; Blackmore et al. 2007; Wilson and Zhang 2009). Morphological analysis indicates that the development of pollen wall starts from the formation of callose wall before meiosis (Blackmore et al. 2007; Ariizumi and Toriyama 2011; Scott et al. 2004). The callose wall consists of the linear β-1,3-glucan polymer, and it was assumed to act as a temporary cell wall between the anther wall and the plasma membrane from the meiosis stage to the end of the tetrad stage. After the formation of tetrads, the surrounding callose wall is digested by β-1,3-glucanase secreted from the tapetum (Ariizumi and Toriyama 2007; Piffanelli et al. 1998; Verma and Hong 2001; Worrall et al. 1992).

Several genes, such as *AtMYB103* (Zhang et al. 2007), *Callose Synthase* (*CALS5*) (Dong et al. 2005), *AUXIN RESPONSE FACTOR17* (*ARF17*) (Yang et al. 2013) and *CYCLIN-DEPENDENT KINASE G1* (*CDKG1*) (Huang et al. 2013b), have been

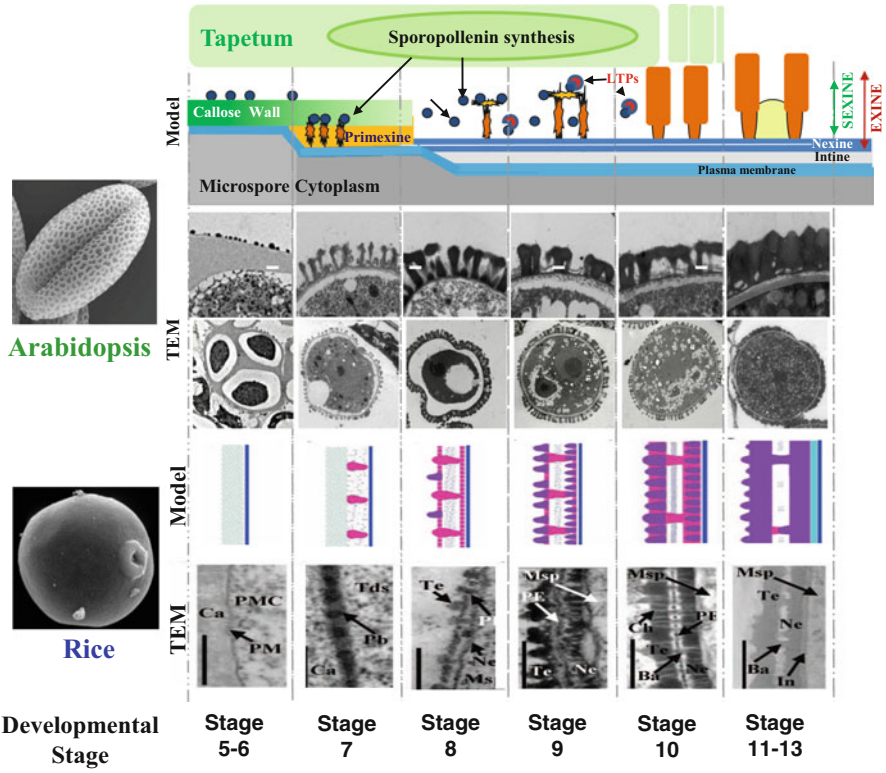


**Fig. 13.1** Current model of pollen wall formation in monocot rice and dicot Arabidopsis (Adopted from Ariizumi and Toriyama 2011; Xu et al. 2014a with modification). Developmental stages for rice and Arabidopsis are defined by Zhang et al. (2011) and Blackmore et al. (2007), respectively

reported to be required for callose formation or dissolution in *Arabidopsis*. Mutations of these genes causes defective pollen exine formation and patterning. It is proposed that the callose wall around the tetrad may recruit the primexine and provide a structural support for the exine formation. During the meiosis, pollen mother cells develop into the tetrad, each containing four newly formed haploid microspores enclosed by callose, and the primexine with the low electron density is synthesized around each microspore, between the callose wall and the plasma membrane (Ariizumi and Toriyama 2011; Blackmore et al. 2007; Paxson-Sowders et al. 1997). Chemical staining experiments show that the primexine is a microfibrillar matrix mainly composed of cellulose components (Paxson-Sowders et al. 1997). Some reports also suggest that the primexine may serve as a template for initial sporopollenin deposition and following polymerization and patterning because of a kind of enzymes called sporopollenin acceptor particles (SAPs), which exist in the primexine and initiate the sporopollenin accumulation and the appearance of the exine pattern (Gabarayeva et al. 2009; Gabarayeva and Grigorjeva 2004) In *Arabidopsis*, there are some genes known to be involved in the primexine formation including *DEX1* (Paxson-Sowders et al. 2001), *RUPTURED POLLEN GRAIN1* (*RPG1*) (Guan et al. 2008; Sun et al. 2013),

*NOEXINEFORMATION1* (*NEF1*) (Ariizumi et al. 2004) and *NO PRIMEXINE AND PLASMA MEMBRANE UNDULATION* (*NPU*) (Ariizumi et al. 2004; Chang et al. 2012; Guan et al. 2008; Paxson-Sowders et al. 2001; Sun et al. 2013). But there is no gene identified to be involved in primexine development in rice.

At stage 8 during the tetrad formation, according to the classification of rice anther development stage (Zhang et al. 2011), the probaculum is formed and deposited onto the primexine (Paxson-Sowders et al. 1997). At stage 9, when microspores are released from the tetrad, massive sporopollenin precursors putatively secreted by the tapetum are deposited onto the primexine to form a very thin tectum (sexine) (Bedinger 1992; Li and Zhang 2010). Meanwhile, the formation of nexine is seen on the surface of microspores. With the development of microspores, the sporopollenin gradually accumulates to thicken and consolidate the exine structure, a two-layer high density structure with low electron density intervals or channels across the exine is mostly completed by the late stage 10, when the microspores become vacuolated (Li and Zhang 2010). Subsequently, during the first mitosis each microspore generates one generative cell and one vegetative cell, and the microspore starts the synthesis of intine below the exine (Paxson-Sowders et al. 1997; Owen and Makaroff 1995). Finally, at stage 13, after the second mitosis of microspores to form mature pollen grains with two sperm cells and one vegetative nucleus, the pollen exine shape is almost established by the accumulation of sporopollenin compositions and pollen coat deposition (Li and Zhang 2010; Owen and Makaroff 1995). It is notably that different from the exine development, which appears to be actively produced and deposited by intact tapetal cells at the early stages of pollen development, the components of lipidic pollen coat are produced and deposited by degenerating tapetal cells at the late stages of pollen development (Murgia et al. 1991).

Additionally, all abovementioned information associated with the developmental course of pollen wall and anther wall layers are almost obtained from the common light and electron microscopy in which the chemical fixation is used. Increasing evidence demonstrated that the tapetum is a transitory tissue, which is very sensitive to chemical fixation, so do those early emerging ultra-structures of developing pollens. In a recent study, cryo-fixation combined with transmission electron microscopy were employed to examine the sequence of developmental events in anther and pollen development in *Arabidopsis*, and revealed novel information on middle layer, locule fluid and the morphology of tapetal cells (Quilichini et al. 2014). The new features of tapetal cells observed by Quilichini et al. (2014) included: tapetal cell lacks a cell wall as early as the tetrad stage; tapetal cell ultrastructure does not show the sign of active endomembrane secretion system; tapetal cell is distinguishable at the tricellular pollen stage; tapetosomes and elaioplastes, two specific organelles within tapetal cells during the late stage are the major contributors to pollen coat development (Quilichini et al. 2014). In addition, unlike to previous reports from chemical fixation, the middle layer cell persists after the release of pollen and into the late stage of uninucleate microscope development. Furthermore, this analysis revealed a clear and dense presence of locule fluid from the tetrad stage to the early bicellular pollen stage, after which it becomes less dense or absent when locule is filled with tricellular pollen grains, indicating its involvement in the traffic of sporopollenin from tapetal cells to microspores, because at this stage, the locule

fluid is abundant with various lipid transfer proteins (LTPs) (Huang et al. 2013a; Zhang et al. 2010). These morphological features in developing anthers of plants rather than *Arabidopsis* need to be performed to update current knowledge regarding pollen wall formation.

## Tapetum: The Supportive Tissue for Exine Formation

The anther, the male organ of flowering plants, consists of four somatic cell layers, the epidermis, endothecium, middle layer, and tapetum from the surface to interior, surrounding the developing microspores inside. Pollen development relies heavily on the surrounding tissues of the anther wall layers, especially the tapetum, which has the most intimate contact with the microspores and plays an secretory role by supporting developing microspores with enzymes, nutrients, metabolites, sporopollenin precursors and pollen coat components during the exine and pollen coat formation (Blackmore et al. 2007; Pacini and Hesse 1984; Shivanna et al. 1997). The tapetal cytoplasm is usually filled with ribosomes, plastids, mitochondria, Golgi bodies, and both rough and smooth endoplasmic reticulum (ER), and plastids and ER are the places for fatty acid synthesis and modification [i.e., unsaturation, hydroxylation, fatty acid chain elongation, or diversification of aliphatic compounds of very long-chain fatty acid (VLCFAs)] (Yang et al. 2014a, b), respectively, suggesting the essential function of tapetal cells in lipid biogenesis.

The anther primordium in angiosperms is composed initially of three layers, L1, L2, and L3 from the outer to the inner (Poethig 1987), with the L1 developing into the epidermis, and the L3 forming the connective cells and vascular tissues (Ma 2005; Zhang et al. 2011). At the same time, the L2 layer differentiates into the peripheral L2-derived (L2-d) cells dividing and forming the endothecium, the middle layer and the tapetum, and the central L2-d cells developing into the sporogenous cells (Kelliher and Walbot 2011, 2012; Zhang and Yang 2014; Zhang et al. 2011).

When microspore mother cells (MMCs) are formed, the tapetal cell with a single nucleus keep integrity has clear boundaries from MMCs. When entering meiosis stage, the tapetal cells undergo a series of changes to obtain the unique cytological features consisted with the biological function, such as exuberantly synthetized proteins and RNAs, high levels of DNA, and abundant mitochondria, plastids, ERs and other organelles, these characteristics are different from those of other sporophytic cells (Gunning and Steer 1996). At the end of meiosis I, the tapetum cytoplasm becomes condensed and the nuclear DNA becomes fragmented, indicating the tapetum degradation initiated by programmed cell death (PCD) (Li et al. 2006, 2011; Varnier et al. 2005). During this stage, the condensed cytoplasm of tapetal cells contains abundant mitochondria, ribosomes, varisized vacuoles, ERs, and plastids with high electron density substance. After meiosis II, when the tetrads are formed, the tapetum becomes more condensed and vacuolated, but there are still abundant organelles, of which the highly developed ER is the most obvious (Li et al. 2006).

The metabolism in the tapetum is most active during the tetrad stage of anther development, and the β-1,3-glucanase produced by the tapetum breaks down the

callose wall to release the microspores into the locule (Ariizumi and Toriyama 2011; Verma and Hong 2001). With the degradation of tapetum cells after microspores released from tetrads, the tapetum-synthesized nutrition and structural matters, such as sporopollenin precursors, flavonoids and proteins, are provided for pollen development including exine formation. When the trinucleated pollen grains are formed, the tapetum is almost completely degraded, and the proteins and lipids that compose the tryphine as the last materials supplied by the tapetum are deposited on the pollen exine (Edlund et al. 2004; Piffanelli et al. 1998). Throughout the whole process of pollen development, the tapetum, as a source for synthesis, storage and transport of various nutrients and structural compositions, is an important layer of anther wall, and its abnormal structure and function is closely related to pollen abortion (Ariizumi and Toriyama 2011; Wilson and Zhang 2009; Zhang et al. 2011).

Increasing evidence indicates that exine synthesis and patterning rely on the tapetal PCD, which has been shown to be controlled by regulators such as basic helix-loop-helix (bHLH) transcription factors, MYB transcription factors and PHD-finger proteins (Aya et al. 2009; Fu et al. 2014; Ji et al. 2013; Li et al. 2006; Wilson and Zhang 2009; Xu et al. 2010, 2014a; Yang et al. 2007; Zhang et al. 2011). Loss of function of these regulators frequently causes defective tapetal PCD and abnormal exine formation. Emerging evidence suggests that many other genetic factors such as pollen specific mRNA-like non-coding RNA gene (Song et al. 2013) and cysteine protease encoding genes (Lee et al. 2004; Yang et al. 2014b; Zhang et al. 2014) also play important roles in tapetum and microspore development in plants. These findings suggest the possible close link between tapetal PCD and nutritive support for exine biosynthesis. However, it is not clear whether these two processes function in the same pathway or in parallel.

## Biosynthesis of Exine

As the major component of pollen exine, the chemical compositions of sporopollenin are still elusive due to its highly insoluble and resistant to various degradations (Scott 1994). However, with the application of a series of elaborate physical and chemical techniques, such as nuclear magnetic resonance (NMR) spectroscopy (Bubert et al. 2002) and thiocarbamate herbicides treatment (Wilmesmeier and Wiermann 1995), our knowledge about the chemical compounds in sporopollenin is substantially updated. Increasing evidence indicates that the sporopollenin mainly consists of complex aliphatic monomers including VLCFAs and their polyhydroxylated derivatives, and phenolic compounds, which means the lipid metabolism is critical for sporopollenin biosynthesis (Kim and Douglas 2013; Scott 1994; Piffanelli et al. 1998). Comparison with pollen transcriptomes from lilly (Lang et al. 2015), *Arabidopsis* (Honys and Twell 2003), soybean (Haerizadeh et al. 2009), rice (Wei et al. 2010) and tobacco (Hafidh et al. 2012) clearly shows that similar protein/transcript categories including lipid metabolism and transport are highly expressed as compared with vegetative tissues, indicating the roles of lipid metabolism in pollen development (Fig. 13.2).

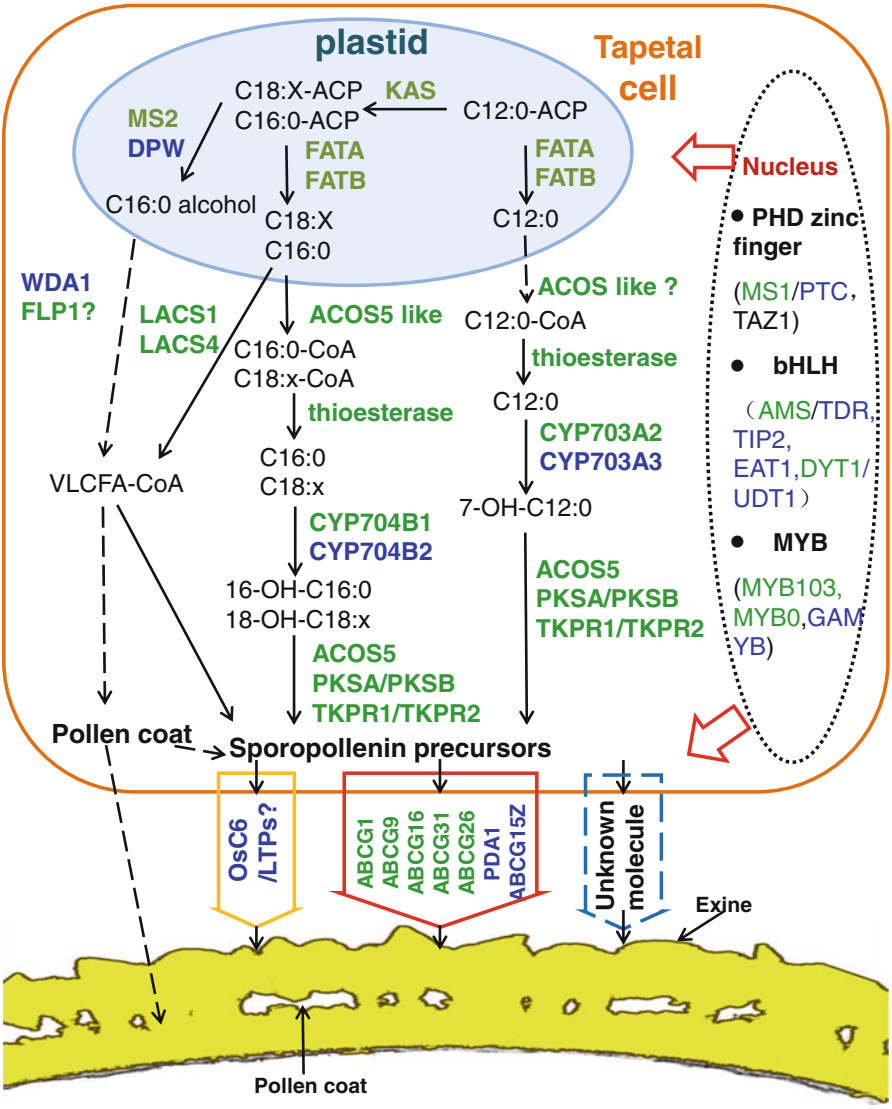


**Fig. 13.2** The metabolic network of pollen wall development in monocot rice and dicot Arabidopsis (Adopted from Yang et al. 2014a with modification). In the endoplasmic reticulum (ER) of tapetal cells, plastid produced fatty acids (C12, C16, and C18) conversed from their corresponding CoAs by thioesterase are hydroxylated by CYP703/704 proteins, and resulting hydroxylated fatty acids are further converted to phenolic compounds via pathway involving ACOS/like, PKS/like and TKPR/like proteins. In ER, VLCFA-CoA pool contributes significantly to aliphatic components of sproropollenin precursors and perhaps pollen coat compositions as well. VLCFA-CoA pool is produced either from fatty acids produced in plastid by the joint activity of LACS proteins or from the likely condensation of fatty alcohols, generated by the plastidic fatty acid reductase activity of MS2/DPW protein, by WDA1 like proteins. The synthesized sproropollenin precursors are transported by several ABCG transporters, LTPs and unknown molecules, and the transportation of pollen coat components remains unknown. There are at least three transcription factor families that are involved in the regulation of exine development and pollen coat formation individually or interactively. *FATA/B* fatty acyl thioesterase A/B, *KAS* ketoacyl-ACP synthase, *VLCFA* very long chain fatty acid. Genes in *blue* and *green* color are ortholog ones from rice and Arabidopsis, respectively

### *Fatty Acid Synthesis*

Biochemical and genetic investigations have recently revealed a number of enzymes catalyzing the metabolism of fatty acids and their derivatives, required for sporopollenin biosynthesis (Aarts et al. 1997; Ariizumi and Toriyama 2011; Chang et al. 2012; Chen et al. 2011b; de Azevedo Souza et al. 2009; Dobritsa et al. 2009, 2010; Grienenberger et al. 2010; Jung et al. 2006; Morant et al. 2007; Shi et al. 2011; Tang et al. 2009). In heterotrophic eukaryotes, the synthesis of fatty acids usually occurs in the cytosol, whereas plants de novo fatty acids are produced in plastids (Li-Beisson et al. 2010, 2013; Ohlrogge et al. 1979), and the elongation (>C18) and modification (i.e., unsaturation, hydroxylation) of fatty acids usually occur in the ER or cytosol (Kunst and Samuels 2003; Li-Beisson et al. 2013).

### De Novo *Fatty Acid Synthesis in Plastids*

Simply, the fatty acid synthesis is a complex cyclic process, and during each cycle, there are four reactions: condensation, reduction, dehydration, and reduction, catalyzed by fatty acid synthase (FAS), a multi-subunit complex composed of monofunctional enzymes (Brown et al. 2006; Li-Beisson et al. 2013). The plastidial acetyl-coenzyme A (CoA), a two-carbon molecule rapidly generated by the plastidial pyruvate dehydrogenase complex (PDHC) (Johnston et al. 1997), is used as a building block for the process. During the fatty acid synthesis, acetyl-CoA is the starting unit, and malonyl-ACP, catalyzed from acetyl-CoA (Konishi et al. 1996), is used as a carbon donor in each cycle of elongation (Li-Beisson et al. 2013). The condensation reaction is catalyzed by 3-ketoacyl-ACP synthases (KAS) to mediate the combination of two-carbon units provided by malonyl-ACP and the acetyl-CoA (subsequently acyl-ACP acceptors) and release one $CO_2$ (Li-Beisson et al. 2013). There are three KAS isoforms identifies: the KAS I catalyzes the subsequent condensations (up to 16:0-ACP), the KAS II catalyzes the final condensation of 16:0-ACP to 18:0-ACP, and the KAS III catalyzes the condensation of malonyl-ACP and acetyl-CoA at the first step of the fatty acid synthesis (Pidkowich et al. 2007). To complete a whole cycle of fatty acid elongation, additional two reductases and a dehydrase are necessary. At last, fatty acids or alcohols with the carbon chain length up to C18 are exported from the plastids to the ER for further modification or elongation and compose the sporopollenin precursors.

*MALE STERILITY2* (*MS2*) is the first gene identified to participate in the exine development, as evidenced by the aborted pollen grains without exine in the *ms2* mutant (Aarts et al. 1997). Rice *DEFECTIVE POLLEN WALL* (*DPW*), the ortholog gene of *MS2*, is capable of complementing the *ms2* mutant. *dpw* mutant displays completely male sterility and defective pollen exine (Shi et al. 2011), suggesting that DPW/MS2 represents a conserved pathway in pollen wall formation. Subsequent studies revealed that DPW and MS2, expressed in both tapetal cells and microspores, encode a class of plastid-localized novel fatty acid reductases that catalyze

fatty acyl carrier proteins (ACPs) into fatty alcohols, with a strong preference for C16:0-ACP, uncovering a conserved step in fatty alcohols biosynthesis in the plastid for anther cuticle and pollen sporopollenin biosynthesis in monocots and dicots (Chen et al. 2011b; Shi et al. 2011). Rice Wax-Deficient Anther1 (WDA1) (Jung et al. 2006) and its ortholog *FACELESS POLLEN-1* (*FLP1*) (Ariizumi et al. 2003) in *Arabidopsis* are both the orthologs of *ECERIFERUM1* (*CER1*) (Bourdenx et al. 2011) in *Arabidopsis*, which was predicted to encode an enzyme involved in the decarbonylation pathway of alkane biosynthesis. The mutation of both *WDA1* and *FLP1* caused the abnormal sporopollenin deposition, indicating their possible roles in the sporopollenin biosynthesis. Further gene expression and biochemical analysis in *wda1* showed that WDA1 probably participate in the biosynthesis of VLCFAs and affect the exine formation directly or indirectly (Bourdenx et al. 2011).

Among the cytochrome P450 family, members of CYP703A and CYP704B subfamilies play essential roles in hydroxylating fatty acids in ER for sporopollenin biosynthesis (Dobritsa et al. 2009, 2010; Li et al. 2010; Li and Zhang 2010; Morant et al. 2007; Yang et al. 2014a; Yi et al. 2010). CYP703As catalyze the in-chain hydroxylation of fatty acids. The *cyp703a2* mutants can produce partial fertile pollen grains with abnormally developed exine, on which sporopollenin deposited. The heterologous CYP703A2 protein catalyzes in-chain hydroxylase of fatty acids with the chain length from C10 to C16, and the productions are thought to be sporopollenin precursors (Morant et al. 2007). By contrast, the *cyp703a3* mutant is completely male sterile with defective exine formation (Aya et al. 2009; Yang et al. 2014a). *In vitro* enzyme activity assay showed that CYP703A3 catalyzes the in-chain hydroxylation of lauric acid specifically, to produce 7OH-C12 fatty acid (Yang et al. 2014a). While CYP703As encode in-chain hydroxylases with substrates preference for lauric acid, CYP704Bs catalyze the ω-hydroxylation of long-chain fatty acids (Yang et al. 2014a; Dobritsa et al. 2009, 2010; Li et al. 2010; Morant et al. 2007). *Arabidopsis* CYP704B1 (Dobritsa et al. 2009) and rice CYP704B2 (Li et al. 2010) have similar biochemical functions, which can catalyze the ω-hydroxylation of C16 and C18 fatty acids to produce the important precursors involved in sporopollenin synthesis. The *cyp704b1* and *cyp704b2* mutants produce aborted pollens with abnormal exine (Dobritsa et al. 2009, 2010; Li et al. 2010). The mutants of *BnMS1* and *BnMS2*, the orthologs of *CYP704B1* in *Brassica napus*, display delayed tapetum PCD and defective exine without sporopollenin accumulation (Yi et al. 2010). The hydroxylated fatty acids by CYP703A and CYP704B can be further catalyzed by cytoplasm-localized fatty acyl-CoA synthetase (ACOS) to generate CoA esters for sporopollenin synthesis (de Azevedo Souza et al. 2009).

## *Synthesis of Phenolic Compounds*

The other important sporopollenin precursors are the phenolic compounds derived from the phenylpropanoid pathway, involving several key enzymes encoded by the tapetum-expressed Acyl-CoA Synthetase5 (ACOS5) (de Azevedo Souza et al.

2009), LAP6 (LESS ADHESIVE POLLEN)/PKSA (POLYKETIDE SYNTHASE A) and LAP5/PKSB (Dobritsa et al. 2010; Grienenberger et al. 2010), and TETRAKETIDE α-PYRONE REDUCTASE1 (TKPR1) and TKPR2 (Grienenberger et al. 2010) in *Arabidopsis*. ACOS5 functions *in vitro* as a fatty acyl-CoA synthetase taking medium- to long-chain fatty acids including hydroxylated fatty acids as the substrates. The fatty acyl-CoA esters are considered as a central intermediate during the sporopollenin precursor synthesis, and the CoA esters are likely essential for the fatty acids traveling across the membrane (de Azevedo Souza et al. 2009). Loss of function mutant of ACOS5 displays defective pollen exine without sporopollenin (de Azevedo Souza et al. 2009). The products of ACOS5, fatty acyl-CoA esters, are further utilized as the substrates of ER-localized LAP6/PKSA and LAP5/PKSB to yield tri- and tetra-ketide alpha-pyrones required for sporopollenin synthesis (de Azevedo Souza et al. 2009; Dobritsa et al. 2010; Kim et al. 2010). The *pksa/pksb* double mutant lacks pollen exine and sporopollenin deposition (Kim et al. 2010). Next, the reductase encoded by *TKPR1* and *TKPR2* takes tri- and tetra-ketide α-pyrones generated by PKSA/B as the substrates to produce the hydroxylated alpha-pyrone for sporopollenin synthesis (Grienenberger et al. 2010). In accordance with the direct upstream and downstream relations in the common sporopollenin synthesis pathway, *ACOS5*, *PKSA/B* and *TKPR1* (Grienenberger et al. 2010) are tightly co-expressed as predicted, which suggests the possibility that these enzymes function as a metabolon for sporopollenin biosynthesis. Interestingly, it was recently reported that the ACOS5, PKSA/B and TKPR1 are all localized on the ER of the tapetal cells and can interact together *in vivo*, which strongly supports the existence of sporopollenin metabolon (Lallemand et al. 2013). Notably, none of the orthologs of these *Arabidopsis* synthetic genes of phenolic compounds has been reported in rice.

As compared with exine biosynthesis, studies on pollen coat biosynthesis are much less. It is well known that in *Arabidopsis*, the biosynthesis of pollen coat is derived from the tapetum degenerative debris, which shares the same fatty acid CoA pools as those of VLCFAs, in which long-chain acyl-CoA synthetases (LACS), such as LACS1 and LACS4, play important roles. It is reported that the biosynthesis of pollen coat depends on the combined activity of both LACS1 and LACS4 proteins (Jessen et al. 2011). It is not clear whether other genes affecting long chain fatty acid CoA pools in plants affect pollen coat development in plants or not.

## Potential Carriers for Sporopollenin Precursors

How tapetum derived chemicals are exported into the locule for the assembly of sporopollenin is a mysterious question for biologists. Up to date, ABC transporters, LTPs and other molecules (Zhang and Li 2014) have been proved to be responsible for cargo transport of exine constituents (Fig. 13.2).

## *ABC Transporters*

Plant ABC transporters have been shown to be involved in a wide range of cellular activities, such as hormone homeostasis, detoxification, antibiotics resistance and secondary metabolism (Kang et al. 2011). Among these ABC transporters, some G subfamily members such as *Arabidopsis ABCG26* (*WBC27*), ABCG9 and ABCG31, and ABCG1 and ABCG16 are reported to be responsible for transport of sporopollenin precursors from tapetal cells into microspore surface for exine development. Knockdown of either one of those ABCG proteins leads to the aborted exine formation (Choi et al. 2011, 2014; Dou et al. 2011; Kuromori et al. 2010; Quilichini et al. 2010; Xu et al. 2010; Yadav et al. 2014). These findings suggest that the ABC transporter plays a key role in exine formation, likely secreting the sporopollenin precursors from the tapetum to developing microspores.

Recently, rice ABCG15 (also called *Post-meiotic Deficient Anther 1*, *PDA1*), an ortholog of Arabidopsis *ABCG26*, has been shown to be required for exine development (Hu et al. 2010; Niu et al. 2013a; Qin et al. 2013; Wu et al. 2014; Zhu et al. 2013). Mutation of the tapetum-expressed *ABCG15*/*PDA1* leads to aborted microscope without exine formation and vanished orbicules on tapetal cells (Niu et al. 2013a; Qin et al. 2013; Wu et al. 2014; Zhu et al. 2013). Besides, chemical analysis data shows that *ABCG15*/PDA1-mdeidated transport of sporepollenin precursors in turn affects the synthesis of lipidic components. Consistently, the expression of two pollen exine biosynthetic genes *CYP704B2* and *CYP703A3* was decreased in *pda1* anthers (Zhu et al. 2013). These findings suggest that the tapetum-expressed ABC transporters play a conserved role in secreting lipidic sporopollenin precursors from the tapetum to developing microspores. However, the biochemical evidence on how ABC transporters translocate sporopollenin precursors and what are the exact substrates remain to be investigated. Future biochemical function assay of ABCG26/ABCG15 may help us in better understanding of the roles of ABC transporters in plant pollen exine development.

## *Lipid Transfer Proteins*

Beside the possible role of transmembrane ABCG transporters in actively delivering the lipid molecules across the membranes, other lipid transporters such as low molecular weight (LTPs) have also been shown to be involved in lipid trafficking (Huang et al. 2013a, b; Hu et al. 2010; Yeats and Rose 2008; Kader 1996). LTPs were termed as they have the capacity to transfer phospholipids and fatty acids between membranes *in vitro* (Kader 1996). Expression analysis showed that some tapetal PCD regulators affect the expression of LTPs (Aya et al. 2009; Ariizumi and Toriyama 2011; Brown et al. 2006; Jung et al. 2005), implying that LTPs regulate the pollen development. Nevertheless, CaMF2, an anther-specific LTP gene, affects pollen development in *Capsicum annuum* L (Chen et al. 2011a). *OsC6*, a rice LTP,

was shown to function as lipidic transporter from tapetal cells to anther wall layers and microspore surface (Zhang et al. 2010). OsC6 has the typical eight-Cys motif, and its transcription is highly detectable in tepetal cell. However, the localization of OsC6 protein is seen in tapetal cells, anther locule, anther epidermis, as well as the extracellular space between the tapetum and the middle layer, indicating that the tapetum expressed OsC6 can be secreted among anther cells. In addition, the recombinant OsC6 protein has the bind ability of fatty acid molecules (Zhang et al. 2010), suggesting that OsC6 is involved in distribution of lipidic molecules for male reproduction. Furthermore, knock-down of *OsC6* caused aborted exine and orbicule development in rice. This work demonstrates the key role of LTPs in secreting lipidic molecule from the tapetum for exine development. Recently, Huang et al. (2013a) also demonstrated that the tapetum-expressed type III LTP plays a role for the lipid molecular trafficking from tapetum into the pollen exine in *Arabidopsis*, confirming the novel vesicular trafficking mechanism for pollen exine development (Huang et al. 2013a). However, the substrates and molecular mechanisms of LTPs remain to be analyzed in the future.

In addition to ABCG transporters and LTPs, multidrug and toxic efflux (MATE) transporters are also involved in trafficking of sporopollenin precursors across tapetal cells for exine development in plants (reviewed by Zhang and Li 2014).

## Regulation Network

Pollen wall development is one of the most important events of plant organ development, involving hundreds of genes and proteins (Honys and Twell 2003, 2004; Xu et al. 2010, 2014a). The transcription factor is the regulatory point of many genes. In higher plants, a number of conserved transcription factors have been shown to be associated with tapetal function and pollen development. So far the known transcription factors involved in pollen development were fallen into following families, forming specific and crosstalk regulatory networks governing the microspore development in plants, which will continue to be the hotpot of this research area (Fig. 13.2).

*MALE STERILITY 1* (*MS1*), is a tapetum-expressed gene encoding a plant PHD-zinc finger transcription factor (Ito et al. 2007; Ito and Shinozaki 2002; Wilson et al. 2001). Loss of function of *MS1* results in severe vacuolation of tapetal cells, exine structural defects and microspore degradation. By analyzing the microarray data of *MS1*, over 260 genes, the majority of which are required for tapetum development and exine formation like the genes encoding cysteine proteases and *LTP*s, are directly or indirectly regulated by *MS1* (Ito et al. 2007; Ito and Shinozaki 2002; Wilson et al. 2001). *PERSISTENT TAPETAL CELL1* (*PTC1*), an ortholog of *MS1* in rice, encodes a PHD-zinc finger transcription factor that is specifically expressed in tapetum and microspores (Li et al. 2011). The *ptc1* mutant exhibited many similar phenotypes to those of *ms1*, such as lack of DNA fragmentation in tapetal cells, delayed tapetum PCD and pollen exine defect. In addition, *ptc1* mutants also

showed uncontrolled proliferation of tapetal cells and abnormal orbicules (Li et al. 2011). It is reported that mutation of *PTC1* significantly alters the expression of 2,417 genes involved mainly in tapetum development and exine formation (Li et al. 2011). In addition, mutation in *TAPETUM DEVELOPMENT ZINC FINGER PROTEIN1* (*TAZ1*), an anther-specific TFIIIA-type zinc finger transcription factor in petunia, leads to defective in exine formation due to precocious degeneration of tapetum (Kapoor et al. 2002), which is similar to *cp51* (Yang et al. 2014b).

*ABORTED MICROSPORE* (*AMS*) in Arabidopsis, encoding a bHLH transcription factor, affects the tapetum development and PCD (Sorensen et al. 2003). The *ams* mutants display enlarged tapetal cells and aborted microspore development. In-depth analysis on *AMS* revealed that *AMS* plays a central role in tapetal PCD and pollen exine formation by directly regulating target genes involved in different developmental events of pollen formation, those affected developmental processes include the separation of the microspore mother cell, callose dissociation, and sporopollenin precursor biosynthesis and secretion at the transcription level, such as *ABCG26* (Xu et al. 2010, 2014a). *Tapetum Degeneration Retardation* (*TDR*/*bHLH5*) (Li et al. 2006), the ortholog of *AMS* in rice, is preferentially expressed in the tapetum from the meiosis stage to the young microspore stage (Li et al. 2006), which functions as a key regulator in tapetum PCD and pollen exine development (Li et al. 2006; Zhang et al. 2008). The pollen wall was severely defective in the *tdr* mutant. Similarly, the expression of a group of genes encoding putative enzymes involved in lipid metabolism is altered in *tdr*, including *OsC6*, *CYP703A3*, both are confirmed to be directly targeted by *TDR* (Li et al. 2006; Yang et al. 2014a; Zhang et al. 2010). In addition, TDR can affect the pollen development by regulating other transcription factors. Rice *ETERNAL TAPETUM 1* (*EAT1*), another bHLH transcription factor, is also a direct target gene of *TDR* (Niu et al. 2013b). *eat1* mutant showed delayed tapetum PCD and abnormal pollen development. The third rice bHLH transcription factor, *TDR INTERACTING PROTEIN2* (*TIP2*), functions as an upstream regulator of *TDR* and *EAT1*, and determines the differentiation of the inner three layers of anther wall and tapetum PCD (Fu et al. 2014). Recently, it is reported that TDR controls tapetal cell development and pollen formation via triggering the possible ADF-mediated proteolysis pathway via its target gene *OsADF*, an anther development F-Box protein (Li et al. 2015). Those data demonstrate that TDR play important roles in the regulation of tapetal and pollen development in rice.

Moreover, *AMS* directly regulates the expression of *ABCG26* via binding to *ABCG26* promoter and coordinates the synthesis of aliphatic molecules in the tapetum (Xu et al. 2010). *Delayed Tapetum Degeneration* (DTD), the rice bHLH 141 transcription factor (EAT1), functions coordinately with *TDR* to regulate lipidic orbicules and exine synthesis, through *OsC6* and *OsC4*, two LTPs, to affect pollen development (Ji et al. 2013; Niu et al. 2013b). In addition, the *bHLH142* coordinates with *TDR* to modulate the expression of *EAT1* and regulate pollen development in rice (Ko et al. 2014). Recently, it is reported that *MS10*$^{35}$ encodes a tomato ortholog of *AtDYT1* and *OsUDT1* (*bHLH164*) (Jung et al. 2005), positively regulates genes for lipid metabolism, cell wall modification/degradation, pollen wall/coat proteins, and transporters. There was neither orbicules nor normal sporopollenin

structures in *ms10*$^{35}$ and the expression of sporopollenin biosynthesis-related genes, such as Solyc12g010920.1.1 (LCFA reductases) and Solyc04g081780.2.1 (lipase), was also reduced in *ms10*$^{35}$ (Jeong et al. 2014).

*AtMYB103*, a R2R3 MYB family member, is important for tapetum and pollen development. Cytological observation showed that the mutation of AtMYB103 caused the defective tapetum development and blocked callose degradation, further leading to aborted microspores without exine. RT-PCR analysis showed that *AtMYB103* regulates the *A6* gene relative to the callose degradation (Zhang et al. 2007). Rice *GAMYB* plays an important role in pollen development (Aya et al. 2009; Kaneko et al. 2004). Loss-of-function of *GAMYB* results in defective tapetum PCD and exine development, and abnormal orbicules (Kaneko et al. 2004). It is reported that cytochrome P450 hydroxylase *CYP703A3* was directly regulated by *GAMYB*, indicating an essential role of *GAMYB* in sporopollenin biosynthesis (Aya et al. 2009). In addition, MYB80, a known MYB protein required for the regulation of tapetal programmed cell death and pollen development in Arabidopsis (Phan et al. 2011), is conserved in its structure and function in all tested monocot and dicot species (Xu et al. 2014b).

Notably, interactions between different group transcription factors play essential roles in the regulation of lipid metabolism during pollen development, In Arabidopsis, a recent study shows that DYT1, a putative bHLH transcription factor, plays a role during the synthesis and transport of pollen wall lipidic materials via TDF1, a putative R2R3 MYB transcription factor, by forming a heterodimer to bind to the E-box motif of the TDF1 promoter (Gu et al. 2014). Further investigation into the correlation network of different regulatory transcription factors governing the lipid metabolism during pollen development would expend greatly our understanding of the molecular interactive network for gametogenesis and reproductive development.

## Evolutionary

The colonization of land by plants was a highly significant event in Earth's history (Beerling 2007), during which key adaptations include rooting structures, conducting tissues, cuticle, stomata, and sex organs such as gametangia and spores/pollen (Menand et al. 2007), play important roles in the successful colonization. Another important innovation was the development of a durable spore wall structure capable of withstanding physical abrasion, desiccation and UV-B radiation environment encountered on land (Wellman 2004). The major component of the spore/pollen wall proposed to be of primary importance in enabling resistance to the conditions described above is the highly resistant biopolymer sporopollenin (Cronk and Cronk 2009; Ito et al. 2007). It seems reasonable to hypothesize that colonization of the land by plants was not possible prior to the evolution of the sporopollenin spore wall, and this adaptation is considered to be a synapomorphy of the embryophytes. Additionally, spore walls are not present in the green algae (Wellman 2004). However, the production of sporopollenin is highly likely to be pre-adaptive as it is

present in a number of different algal groups. Fossil green algae dating back to the Devonian period have been shown to contain sporopollenin (Wall 1962), and there are reports that sporopollenin also occurs in fungi (Shaw 1970), indicating an origin predating the appearance of plants.

Phylogenetic studies and fossil evidence have shown that the most basal living land plants are the paraphyletic 'bryophytes' (Kenrick and Crane 1997). They comprise the liverworts, mosses and hornworts. The moss Physcomitrella patens is the first 'bryophyte' genome to be sequenced. This genome, through comparisons with angiosperm genomes, is proved to be a valuable tool in experimental studies that attempt to reconstruct genome evolution during the colonization of land (Quatrano et al. 2007; Rensing et al. 2008). Bioinformatic studies have suggested that genes implicated in pollen wall development in angiosperms are also present in moss and lycopsids, and may therefore be involved in spore wall development in basal plants (Wallace et al. 2011). This suggests that the molecular genetics of sporopollenin biosynthesis are highly conserved among all land plants, despite the large morphological and functional differences between spores and pollens. Comparative phylogenetic and genomic analyses support this hypothesis, as putative orthologs of ACOS5, PKSA, PKSB, CYP703A2 and CYP704B1 are broadly distributed in flowering plants, and in the moss Physcomitrella patens (Colpitts et al. 2011; de Azevedo Souza et al. 2009; Dobritsa et al. 2010; Grienenberger et al. 2010; Kim et al. 2010). These genes are apparently absent from the green algal lineage, supporting a key role for acquisition of these genes in the progression of plant life onto land (Colpitts et al. 2011; de Azevedo Souza et al. 2009; Dobritsa et al. 2010; Grienenberger et al. 2010; Kim et al. 2010; Morant et al. 2007). Recently, reverse genetic analysis on moss MS2 ortholog gene revealed a core component of the biochemical and developmental pathway required for angiosperm pollen wall development was recruited early in land plant evolution but the continued increase in pollen wall complexity observed in angiosperms has been accompanied by divergence in MS2 gene function. Knock out of moss *MS2* homolog results in defective spore wall and extremely compromised germination. Nevertheless, the moss *MS2* gene could not rescue the *Arabidopsis ms2* phenotype (Wallace et al. 2015). The hypothesis that a highly conserved biochemical pathway leading to sporopollenin biosynthesis exists among land plants has been further supported by genetic and biochemical approaches used to study a variety of plant species (Quilichini et al. 2014). In particular, *Physcomitrella patens* ASCL encodes an enzyme with *in vitro* preference for hydroxy fatty acyl-CoA esters that is capable of hydroxyalkylpyrone synthase activity, suggesting that PpASCL is a functional ortholog of Arabidopsis PKSA and that the pathway to sporopollenin may be conserved among land plants (Colpitts et al. 2011). The similar functions and enzymatic activities of Arabidopsis MS2, CYP704B1, CYP703A2 and there rice orthologs DPW, CYP704B2 and CYP703A3 support the conservation of sporopollenin synthesis among monocotyledonous and dicotyledonous plants (Aya et al. 2009; Chen et al. 2011b; de Azevedo Souza et al. 2009; Dobritsa et al. 2009; Morant et al. 2007; Shi et al. 2011; Yang et al. 2014a).

## Perspective

Lipidic and phenolic molecules secreted from tapetum cell are considered to be the key compounds determining the framework for pollen exine. Even though this process is essential for reproduction success in flowering plants, many questions remain to be addressed. For instance, how about the link between tapetal PCD and the supply of sporopollen precursors? How about the signaling between the microspore and tapetal cells for coordinating the developmental programs, and how about the complex transcriptional regulation of tapetal cell PCD and pollen development? Furthermore, during the microspore development, the microspore always attaches to the inner side of tapetal cells, whether the liquid in the anther locule plays a short-distance transport role for exine formation? Moreover, the substrates of different transport machineries/molecules during exine formation remain to be elucidated. As the advance of the tools for cell biology, biochemistry, in-depth understandings will be revealed on the mechanism underlying the synthesis and transport of sporopollenin precursors during plant exine formation.

In addition, tapetal cell layer is a key player in forming the highly sculptured sporopollenin wall of the exine. However, the dynamic cellular structures of tapetum and developing pollen walls are not fully understood due to the limitation of current microscopy examination. It is reported that those fine structures are sensitive to the chemical fixation (Ariizumi et al. 2003). Combination with the advance in living scanning technology and tapetal cell-specific expression system would facilitate our fully understanding of the dynamic molecular and ultra-structural changes of this specific cell layer occurring along the pollen development in general and of the role of lipids in pollen development in particular.

Finally, although pollen development relies heavily on the tapetum, other cell layers of the anther, namely middle layer, endothecium and epidermal cell layer, may also play important roles in pollen development, particularly the epidermis. Mutation in *WDA1*, an epidermal cell specific expressed gene in rice, causes defective exine development, indicating that *WDA1* might affect pollen development via affecting biosynthesis of lipidic components of sporopollenin.

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