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Review

Genetic and Biochemical Mechanisms of Pollen Wall Development

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The pollen wall is a specialized extracellular cell wall matrix that surrounds male gametophytes and plays an essential role in plant reproduction. Uncovering the mechanisms that control the synthesis and polymerization of the precursors of pollen wall components has been a major research focus in plant biology. We review current knowledge on the genetic and biochemical mechanisms underlying pollen wall development in eudicot model *Arabidopsis thaliana* and monocot model rice (*Oryza sativa*), focusing on the genes involved in the biosynthesis, transport, and assembly of various precursors of pollen wall components. The conserved and divergent aspects of the genes involved as well as their regulation are addressed. Current challenges and future perspectives are also highlighted.

Trends

Pollen wall development exhibits conserved and diversified features.

Genes associated with pollen wall development are coordinately regulated.

The synthesis of exine and anther cutin may share common pathways in rice.

Pollen Wall Development

The pollen wall is the complex multiple-layer outer surface of pollen. It is essential for plant reproduction because of its role in rendering male gametophytes resistant to various biotic and abiotic stresses, as well as its function in male–female interaction, fertilization, and seed production [1]. The underlying genetic, molecular, and biochemical mechanisms of pollen wall development have long defied unraveling, but this is changing fast. Several excellent reviews have summarized the genes and enzymes associated with the biosynthesis and transport of the lipidic and phenolic precursors necessary for the formation of the outer pollen wall named **exine** [1–4] (see Glossary). In the following we address the evolutionary aspects of the pollen wall developmental genes and enzymes from *Arabidopsis* and rice. The syntheses of pollen exine and anther cutin appear to share common pathways in the monocot rice, but not in the eudicot *Arabidopsis*. In addition, we highlight recent advances in understanding the coordinated transcriptional and post-transcriptional regulation of genes involved in pollen wall development.

Based on morphological observations, it has been assumed that the synthesis of pollen wall starts from meiosis when the callose surrounding the microspore is degraded by callases secreted from the **tapetum**, a nutritive somatic tissue enclosing microspores. When programmed cell death (PCD)-induced degeneration of tapetal cells takes place during meiosis, young microspores form **primexine**, a microfibrillar matrix mainly consisting of cellulose that acts as an elaborate template for the deposition and assembly of **sporopollenin** precursors [1,3–5]. At the stage of pollen maturation, although different plant species display a variety of pollen surface morphologies, the mature pollen grains generally contain three layers: the outer exine, the inner **intine**, and the **tryphine** (Figure 1) [3,6]. The exine contains three layers from the outermost side to the inner side: the reticulate layer, the sexine (baculum and tectum), and a flat layer termed the nexine or foot layer. The sexine is usually sculpted in a taxon-specific manner,

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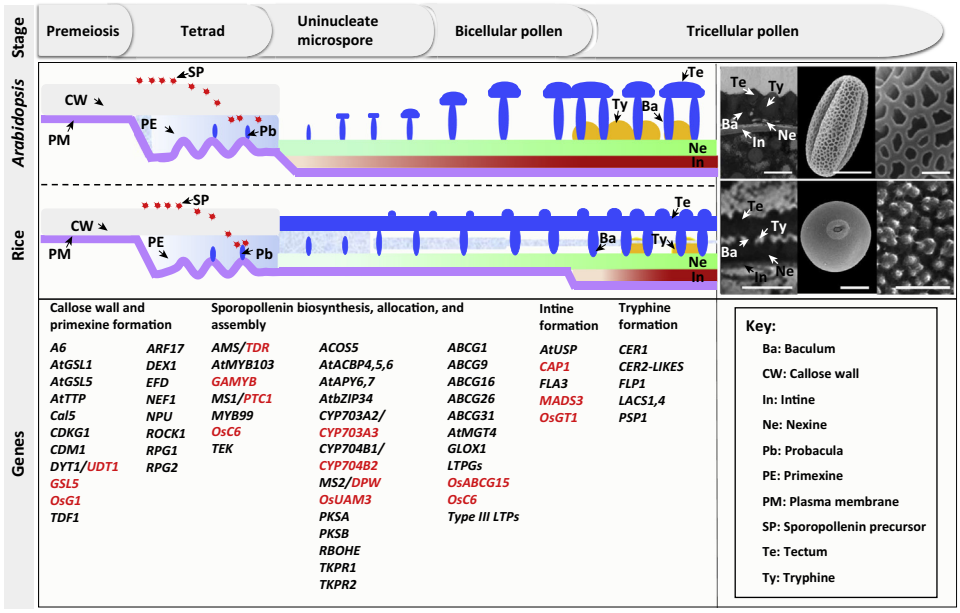

Trends in Plant Science

Figure 1. Comparison of Genes/Enzymes Involved in Various Stages of Pollen Wall Development in *Arabidopsis* and Rice. Stages from pre-meiosis to tricellular pollen are defined in [2]. The scanning electronic microscopy (SEM) and transmission electronic microscopy (TEM) images are both from mature pollen (scale bars from top left to bottom right are 1, 10, 30, 1, 20, and 1 μm, respectively). Pollen wall development-associated genes/enzymes identified in *Arabidopsis* and rice are indicated in black and red, respectively.

and this is one of the important features for species identification. The nexine functions as a skeleton for exine formation. The biochemical nature of exine has remained largely unknown owing to its unusual insolubility and extreme stability. Its major constituent has been found to be sporopollenin, a biopolymer mainly consisting of polyhydroxylated aliphatic compounds and phenolics [7,8]. Although sporopollenin commonly exists in pollens and spores [9], the fine structure of exine and the relative amount of each stratum vary between species. For example, insect- or self-pollinated pollen in *Arabidopsis* has a sculptured surface with reticulate cavities filled with abundant tryphines, whereas wind-pollinated rice or maize pollen has a smooth surface with a continuous tectum and cavities between tectum and nexine that are filled with much less tryphines [3,6,10,11]. This species-specialized pollen wall patterning has also been an intriguing subject of study for researchers.

Given the diversity of plant species and the complexity of pollen structures [3], as well as the limitations of current chemical fixation methods in the preservation of the ultracellular structures of tapetal organelles and pollen wall components [12], it is difficult to draw a single and unified developmental timing-scheme of pollen wall development for all plants. We focus on the recent advances obtained from the dicot *Arabidopsis* and from monocot rice (Figure 1 and Table S1 in the supplemental information online).

Genes Involved in Pollen Wall Development

Callose Biosynthesis and Degradation

Callose is synthesized by callose synthase (CalS). It surrounds the newly formed microspores to act as the mold for primexine, and its degradation by β-1,3-glucanases facilitates the release of microspores from the tetrad. *Arabidopsis CalS5* [13], *GLUCAN SYNTHASE-LIKE 1* and *12*

Glossary

ABC subfamily G (ABCG) transporters: the white–brown complex (WBC) subfamily of ATP-binding cassette (ABC) membrane-transport proteins that facilitate the transport of specific substrates across the membrane in an ATP-dependent manner. They are half-size ABC proteins and form hetero- or homodimers that function as active membrane transporters.

Anther cuticle: the cuticular lipidic layer that covers the anther surface and protects anthers from biotic and abiotic stresses. It is composed predominantly of a polymer matrix cutin consisting mainly of C16 and C18-hydroxy fatty acids that is covered and embedded with distinct very-long-chain (C24–C34) saturated and unsaturated non-polar waxes. Cuticle in the surface of plant tissues other than anther is synthesized exclusively by the epidermal cells. By contrast, anther cuticle seems to be synthesized mainly in the tapetum, the innermost layer of the anther wall.

Callose: also known as β-glucan, is synthesized at the cell wall by callose synthases and degraded by β-1,3-glucanases. This specific cell wall polymer functions in several fundamental biological processes, ranging from plant development to the response to abiotic and biotic stresses. During angiosperm microsporogenesis, callose serves as a temporary wall to separate newly formed microspores in the tetrad from microsporocytes. The development of pollen wall originates from the callose wall, and the callose wall around the tetrad may recruit the primexine and together provide a structural support for exine formation. Abnormal callose deposition and dissolution often leads to male sterility.

Elaioplasts: a type of non-pigmented and specialized plastid (leucoplast) derived from proplastids for the storage of steryl esters, free polar lipids, and plastid lipid-associated proteins in *Brassicaceae* species.

Exine: the most complex and important outer layer of the pollen wall. Generally contains two layers, the inner nexine and the outer sexine. The nexine has a bilayer structure, consisting of nexine I and nexine II, and the sexine is further composed of tectum and bacula. The portions

(*GSL1* and *GSL12*) [14], and rice *GSL5* [15] are required for callose wall formation during microspore formation; mutants of these genes display defective callose synthesis and exine patterning [13]. Knockout or knockdown of the *A6* gene (which encodes a β-1,3-glucanase) in *Arabidopsis*, Brassicas, and rice frequently causes defective callose degradation [16,17].

Primexine Formation and Microspore Plasma Membrane (PM) Undulation

Primexine determines pollen wall patterning, while the undulation of microspore PM guides probacula formation along the primexine. *Arabidopsis* DEFECTIVE IN EXINE FORMATION 1 (DEX1) is a membrane calcium-binding protein and a possible component of the primexine matrix. *dex1* displays severely delayed formation and significantly reduced levels of primexine, reduction in PM undulations, and abnormal exine [18]. Mutants of *Arabidopsis* NO EXINE FORMATION 1 (NEF1), a plastidic integral membrane protein [19], and RUPTURED POLLEN GRAIN 1 (RPG1), a sugar transporter [20], exhibit defective exine phenotypes similar to *dex1*. RPG1 shares a redundant function with RPG2 in regulating the expression of *CalS5* during pollen development. In addition, mutation of NO PRIMEXINE AND PLASMA MEMBRANE UNDULATION (NPU), a PM protein, causes complete absence of primexine deposition and PM undulation [21], whereas mutants of Exine Formation Defect (EFD), a *de novo* DNA methyltransferase, show normal PM undulation but impaired primexine patterning and totally blocked exine formation [22]. However, their metabolic pathways and the regulation of these genes remain unknown.

Biosynthesis of Sporopollenin Precursors

In addition to the extensively reviewed genes and enzymes involved in the biosynthesis of sporopollenin precursors [1,3,4], several sporopollenin biosynthetic genes, including ATP-diphosphohydrolase (Apyrase), cytosolic acyl-CoA-binding proteins (AtACBPs), and UDP-arabinopyranose mutases (UAM) have recently been discovered. The double mutant *apy6 apy7* has much thinner exine that contains few bacula or tectum structures, randomly deposited tryphine, and sticky pollen, possibly due to disturbed polysaccharide production [23]. The triple mutant *acbp4 acbp5 acbp6* shows a slightly smoother pollen surface and irregular bacula and tryphine [24], and RNAi plants of *OsUAM3* have abnormal exine, concomitant with reduced levels of arabinan [25]. The biochemical functions of these gene products are not clear.

Tryphine Formation

The tryphine fills the cavities of the pollen exine and is composed of complex lipids, wax esters, flavonoids, hydroxycinnamoyl spermidine metabolites, and proteins [12,26]. Relatively little is known about the formation of tryphine. Genes related to long-chain fatty acids (LCFA) biosynthesis or transport are essential for tryphine formation. Mutations in *ECERIFERUM (CER)* genes including *CER1* [27], *CER3/FLP1* [28], and three *CER2-LIKE* genes [29], as well as in *Long-Chain Acyl-CoA Synthetases* (*LACS*) 1 and 4 [26], cause abnormal tryphine and exine. Mutants of phosphoserine phosphatase, which catalyzes the last step of the phosphorylated pathway of serine biosynthesis (PPSB), show normal exine without tryphine [30].

Intine Development

It has long been assumed that intine formation is mainly contributed by microspores, but the exact mechanism is not clear. Recently, GLYCOSYLTRANSFERASE 1 (OsGT1), a Golgi-localized glycosyltransferase [31], and COLLAPSED ABNORMAL POLLEN 1 (CAP1), an ARABINOKINASE-like protein [32], were shown to be required for intine development in rice. Mutants of *OsGT1* or *CAP1* display disrupted intine [31,32]. Consistent with the hypothesis that pectin is an important component of intine, overexpression of a rhamnogalacturonan lyase or an endo-α-1,5-L-arabinanase, both of which remove the side chains of pectic arabinan, causes the failure of intine formation in transgenic potato plants [33]. In *Brassica campestris*, silencing of *PECTATE LYASE-LIKE (PLL)*, *BcPLL9* or *BcPLL10*, causes abnormal exintine, endintine, and

between sexine and nexine sculpt the species-specific structure of the pollen grains, while the chemically- and physically-resistant properties of the exine allow plants to adapt to and colonize the land environment.

Intine: an innermost layer of the pollen wall underlying the exine that consists of the exintine and the endintine. The major components of intine are assumed to consist of pectin, cellulose, hemicellulose, hydrolytic enzymes, and hydrophobic proteins. It is required for the maintenance of the structural integrity of pollen grains, pollen germination, and pollen tube growth into the stigma.

Lipid transfer proteins (LTPs): small proteins found in abundance in higher plants that play various roles in plant biology, such as cutin formation, embryogenesis, defense reactions against phytopathogens, symbiosis, and the adaptation of plants to various environmental conditions. LTPs facilitate the movement of lipids between membranes via their binding and solubilizing capacities.

Primexine: a microfibrillar matrix composed largely of neutral and acidic polysaccharide materials, some proteins, and cellulose components. It serves as a template for initial sporopollenin deposition following polymerization and patterning initiated by a enzymes termed sporopollenin acceptor particles (SAPs).

Sporopollenin: a highly-resistant biopolymer of phenylpropanoid and lipidic monomers covalently coupled by ether and ester linkages. Sporopollenin provides the rigid and sculptured framework of the exine, functions to encapsulate and protect the pollen contents, and aids stigmatic capture.

Tapetosomes: a type of lipid-accumulating ER-derived spherical organelles in tapetal cells of *Brassicaceae* species. Tapetosomes accumulate ER-derived flavonoids, alkanes, and oleosins for the formation of the pollen wall, as well as regulating tapetal programmed cell death.

Tapetum: the innermost layer of the anther wall, which functions as a source for synthesis, storage, and transport of various nutrients and structural components for exine and tryphine formation.

Tryphine: also known as the pollen coat, the outermost stratum of the

tryphine [34,35], while silencing of *BcMF8*, a putative arabinogalactan protein-encoding gene, results in misshaped pollen with abnormal intine [36].

Genome-Wide Gene Expression Associated with Pollen Wall Development

It is notable that most of the abovementioned genes known to be involved in pollen wall development have been identified from individual mutants with defective pollen wall development, and this does not provide a genome-wide view of pollen wall development. Transcriptomic profiling will definitely expand our knowledge about the genes involved in this specific process. Unfortunately, currently-available microarray and RNA-seq data are mainly for flowers, anthers, or pollen grains [37–40]. However, these studies indicated that functions of pollen-enriched genes and differentially expressed genes in the male-sterile mutants are mainly relative to two pathways: cell wall and cytoskeletal remodeling [37,40], highlighting possible roles for these genes in pollen wall development. Furthermore, our recent genome-wide coexpression analysis revealed that 98 candidate genes with specific expression in the anther are likely involved in pollen wall formation. These genes encode enzymes/proteins involved in lipidic and phenolic metabolism and transport, as well as in pollen wall patterning [41]. With advances in omics technology, more genome-wide knowledge on pollen wall formation will be generated.

Conservation and Diversification of Genes/Enzymes Involved in Pollen Wall Development

Lipid Metabolism

Lipids and their derivatives, including fatty acids, waxes [26,42], and phospholipids [30,43], are important components of the pollen wall (Figure 2 and Table S1). Although rice and *Arabidopsis* have different types of secretary tapetal cells, the synthesis of the sporopollenin and tryphine precursors (fatty acids and LCFA derivatives) is controlled by the sporophytic genes expressed in tapetal cells [6,44]. In tapetal plastids, *de novo* synthesized fatty acids are esterified to long chain acyl carrier proteins (ACPs), which are either cleaved and translocated to endoplasmic reticulum (ER) for direct elongation or reduced to hexadecanols by reductases such as *Arabidopsis* MALE STERILITY 2 (MS2) [45] and rice DEFECTIVE POLLEN WALL (DPW) [46] in plastids. Findings of DPW and MS2 represent a new and conserved plastid-mediated pathway for the reduction of fatty acids to fatty alcohols in plants [45,46]. Hexadecanols may diffuse freely or be transported into the hydrophilic phase in the cytoplasm [46], where they are converted into fatty acids, and transported into locule as sporopollenin precursors directly or after further hydroxylation [47].

Notably, mutants of rice orthologs of *Arabidopsis* sporopollenin biosynthetic genes such as DPW [46], CYP704B2 [48], and CYP703A3 [49] show defective sporopollenin biosynthesis, similar to *Arabidopsis* mutants [45,47,50]. These rice mutants also show defective **anther cuticle**, which is not seen in the *Arabidopsis* mutants, suggesting that, during evolution, the functions of the relevant genes and/or enzymes in rice have diversified to contribute to anther cuticle formation [6]. Recently the conserved and diversified function of MS2/DPW in sporopollenin synthesis was also observed in moss [51]. Moreover, rice CYP703As and *Arabidopsis* CYP704Bs both belong to the ancient and conserved P450 gene family in terrestrial plants. These genes form a single gene subfamily in rice, *Arabidopsis*, and other land plants, but have not been identified in green algae, suggesting an essential and conserved role for CYP703As and CYP704Bs in mediating fatty acid oxygenation in plant male reproduction [47–50].

Phenolic Metabolism

Phenolic compounds (Figure 2 and Table S1), especially lignins, coumarins, stilbenes, and flavonoids synthesized from phenylpropanoid pathways, are important components of pollen exine and tryphine [52,53]. In *Arabidopsis* ER, fatty acids undergo consecutive modifications,

pollen surface, tryphine is extremely hydrophobic and can be eluted from the exine using organic solvents. It is mainly composed of lipids, flavonoids, 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.

Ubisch bodies: specialized lipidic structures which are proposed to arise from the pro-obicule and that carry tapetum-derived sporopollenin precursors across the hydrophilic cell wall to the outer surface of the microspores in the anther locule. Ubisch bodies are present in cereals and have an electron-dense outer layer with an undulating surface, and the center is hollow or is composed of lipids; Ubisch bodies are readily observable by scanning electron microscopy of mature anthers.

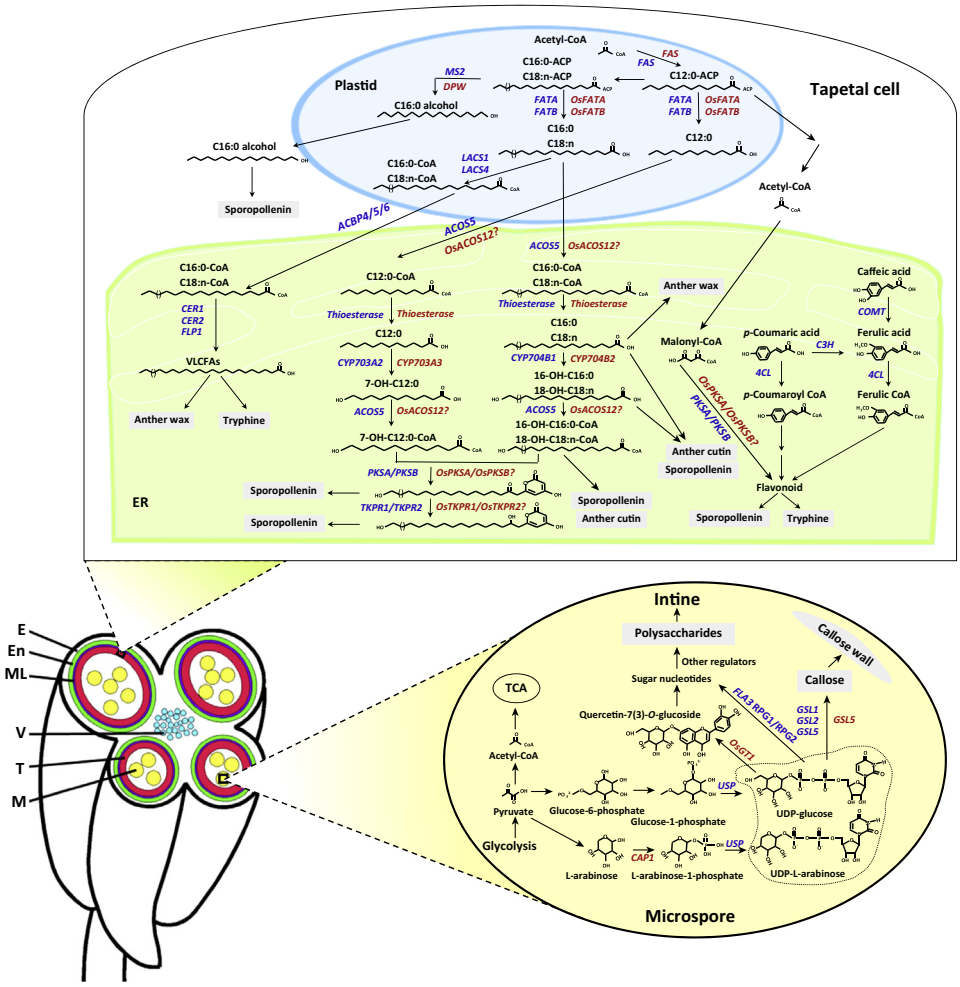

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Figure 2. Pathways in the Biosynthesis of Pollen Wall Components in *Arabidopsis* and Rice. Pollen wall development-associated genes/enzymes identified in *Arabidopsis* and rice are indicated in blue and red, respectively. Note that the tapetum in the anther contributes significantly to pollen wall formation, predominantly via exine and tryphine formation, whereas the microspore contributes to intine formation and callose wall formation. Although the sporophytically regulated lipidic and phenolic pathways are highly conserved between mono- and dicot plants, sharing of conserved lipid metabolic pathways between anther cuticle and pollen wall is observed in rice but not in *Arabidopsis*. In both species, gametophytically regulated intine shares similar polysaccharide building-blocks of the primary cell wall. For full names of other genes/enzymes refer to Table S1 in the supplemental information online and the text. Abbreviations: COMT, caffeic acid *O*-methyltransferase; C3H, coumaric acid 3-hydroxylase; 4CL, 4-coumarate:CoA ligase; E, epidermis; En, endothecium; M, microspore; ML, middle layer; n, number of unsaturated bonds (usually equals 1–3); T, tapetum layer; V, vascular bundle.

including hydroxylation by fatty-acyl-CoA synthetase (ACOS5) [54], condensation by malonyl-CoA by polyketide synthase (PKSA and PKSB) [52,55], and reduction by tetraketide reductase (TKPR1 and TKPR2) [56]. ACOS5, PKSA, PKSB, TKPR1, and CYP704B proteins form a sporopollenin metabolon in the ER to effectively produce lipidic and phenolic precursors for sporopollenin biosynthesis [57]. The function of PKS is conserved in St John's wort (*Hypericum perforatum*, a herb plant) [58]. Whether this pathway is also conserved in rice or other plants remains unknown. In addition, the function of BAHD-like acyltransferases, which are involved in the synthesis of the tryphine components flavonol glycosides and hydroxycinnamic acid amides, is also conserved in angiosperm plants [53].

Polysaccharide Metabolism

Polysaccharide metabolism is believed to be essential for pollen wall development, because mutations in polysaccharide metabolism-associated genes often lead to abnormal pollen wall [1,4]. Pectin polysaccharides are important components of pollen wall, and mutants in genes encoding pectin polysaccharide sysnthetic and degrative enzymes, such as pectate lyases [34,35] and UAMs [25], and other pectin-modifying enzymes [33], usually show defective primexine, or intine, or other pollen wall structures (Figure 2 and Table S1). In addition, disturbing the biosynthesis and degradation of **callose** often results in defective pollen wall development [16,17]. Furthemore, mutation in genes encoding other polysaccharide-associated enzymes also causes abnormal pollen wall development [31,32,36]. Recent transcriptomic analyses also indicated that the expression of polysaccharide metabolism-associated genes in a male-sterile soybean line was significantly downregulated compared to the male-fertile line; these genes encode invertases, hexokinases, and pectin lyases [37]. However, little is known about the biochemical mechanism.

Transport of Precursors Across Anther Tissues

The tapetum is the major tissue for synthesizing sporopollenin precursors, as evidenced by expression analysis. However, how these chemical compounds are allocated for pollen wall development largely remains unclear. Recent investigations indicate that ATP-binding cassette (ABC), **lipid transfer protein** (LTP), and multidrug and toxic efflux (MATE) proteins, may be responsible for the transport of sporopollenin precursors (Figure 3) [5]. Rice OsABCG15/PDA1 is believed to transport lipidic precursors for anther cuticle and exine development because the mutant displays defective cuticle and exine development [59], while its *Arabidopsis* ortholog AtABCG26 transfers both lipid precursors and polyketides for exine formation [60,61]. AtABCG9 and AtABCG31 are involved in the transport of steryl glycosides for tryphine deposition [62], while AtABCG1 and AtABCG16 transfer both lipidic precursors for nexine formation and polysaccharides for intine formation [63]. Whether these **ABC subfamily G (ABCG) transporters** form homo- or heterodimers that mediate the transport of the pollen wall component precursors, and their exact substrates, remain to be elucidated (Figure 3).

Interestingly, *Arabidopsis* type III LTPs function not only as exine precursor distributers but also as exine components [64], whereas glycosylphosphatidylinositol (GPI)-anchored non-specific LTPs (LTPGs), another type of LTPs, only serve as exine precursor transporters [65]. Other pollen wall development-associated transporters include sugar transporter RPG1 for primexine deposition [20], magnesium transporters (AtMGT4, AtMGT5, and AtMGT9) for pollen development [66], and REPRESSOR OF CYTOKININ DEFICIENCY 1 (ROCK1), which transfers UDP-GlcNAc and UDP-GalNAc for sporopollenin and primexine formation [67]. These findings suggest that the functions of proteins responsible for pollen wall precursor allocation are diversified in plants, and need to be characterized further at the biochemical and anatomical levels.

Although *Arabidopsis* and rice both have the secretory type of tapetum, different lipidic organelles are involved in the traffic of lipid molecules to microspores during late pollen development. Orbicules (**Ubisch bodies**), which are located on the inner side of the tapetum facing the developing microspores, are the carriers of sporopollenin precursors in rice and other Poaceae plants [59,68]. By contrast, **elaioplasts** and **tapetosomes** are transporters for the tryphine components in *Arabidopsis* and other Brassicaceae plants [12,41,69]. How these lipid-accumulation organelles are involved in pollen wall development remains to be investigated (Figure 3). We proposed that the formation of pollen exine and anther cuticle in rice share common lipid-synthesis pathways in tapetal cells, because some mutants of tapetal genes show both defective pollen wall development and abnormal anther cuticle formation [46,48,49]. This feature is not observed in *Arabidopsis* [45,47,50]. Whether this difference is caused by the tapetal structure or by specific molecules needs to be elucidated.

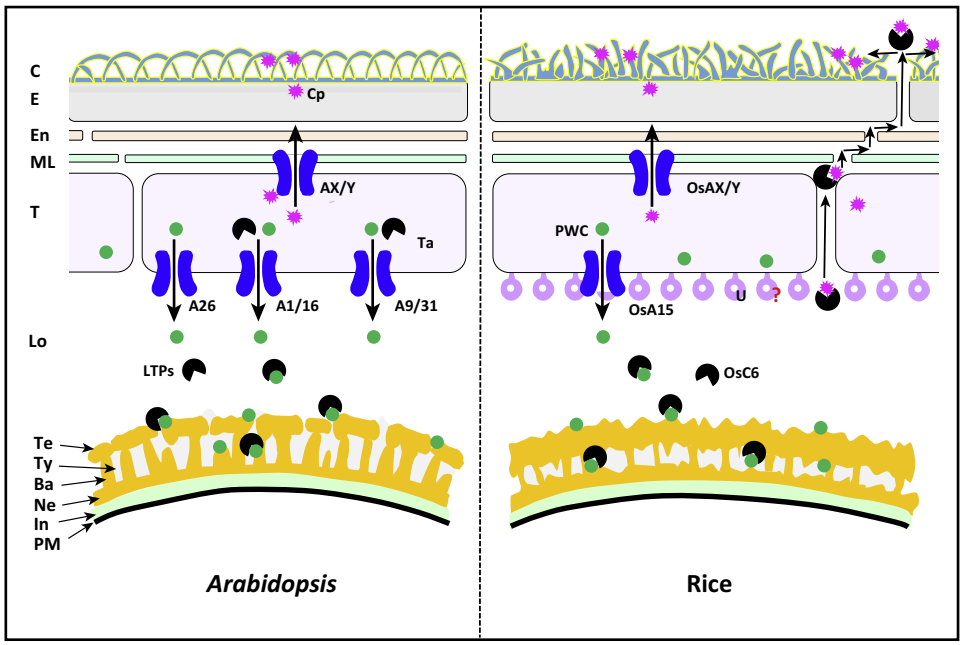

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Figure 3. Proposed Model for the Localization of Pollen Wall Components within Anther Tissues. Facilitated by ATP-binding cassette (ABC) transporter G subfamily (ABCG) proteins and/or lipid transport proteins (LTPs), fatty acids, alcohols, poly- and tetra-ketides, flavonoids, and other tapetum-derived monomers are trafficked across tapetal cells to the locule on the pollen surface for the assembly of exine and tryphine. The same set of transporters, or other unknown transporter-like molecules, may transport lipidic and phenolic precursors for anther cuticle formation. Although it is known that *Arabidopsis* tapetosomes and elaioplasts and rice Ubisch bodies transport different pollen wall components, the underlying mechanisms are unknown ('?' indicates the unknown function of Ubisch bodies in the process). Pink burst dots and green circles indicate anther cuticle precursors and pollen wall component precursors, respectively. Abbreviations: A, ABCG; Ba, baculum; C, cuticular wax and cutin (anther cuticle); Cp, cuticle precursors; E, epidermis; En, endothecium; In, intine; Lo, locules; ML, middle layer; Ne, nexine; PM, plasma membrane; PWC, pollen wall constitutes; T, tapetum layer; Ta, tapetosome; Te, tectum; Ty; tryphine; U, Ubisch bodies; X/Y, other unknown transporters.

Coordinated Regulation of Pollen Wall Development

Transcriptional Regulation

Gene expression data show that hundreds of genes, at least, have putative functions in pollen wall development. How the expression of these genes is coordinated for the sub-sequential development of pollen wall has not been well investigated. Recent evidence suggests that several transcription factors (TFs) form regulatory networks to control pollen wall development (Figure 4, Key Figure) [1,3,4]. *Arabidopsis* MS1 and its rice ortholog PERSISTANT TAPETAL CELL 1 (PTC1) are PHD-finger proteins that regulate pollen exine formation through the exine formation-associated genes [70,71]. PTC1 functions downstream of GAMYB and in parallel with TAPETUM DEGENERATION RETARDATION (TDR) [71,72]. TDR and GAMYB share common target genes for pollen wall development, including *OsC6*, *CYP704B2*, and *CYP703A3* [71].

The *Arabidopsis* tapetum-expressed bHLH transcription factor ABORTED MICROSPORES (AMS), ortholog of rice TDR, is a master regulator of pollen wall development by directly regulating the expression of 23 genes involved in callose dissociation (*A6*), fatty acid hydroxylation (*CYP703A2*, *CYP704B1*, and *CYP86C3*), phenolic synthesis (*PKSB* and *TKPR1*), tryphine formation (extracellular lipase genes, *EXLs*, and glycine-rich protein genes, *GRPs*), or lipid transport (*ABCG26*) [41,73]. AMS also directly promotes the expression of *TRANSPOSABLE*

Key Figure

Transcriptional Regulatory Networks of Pollen Wall Development in *Arabidopsis* and Rice

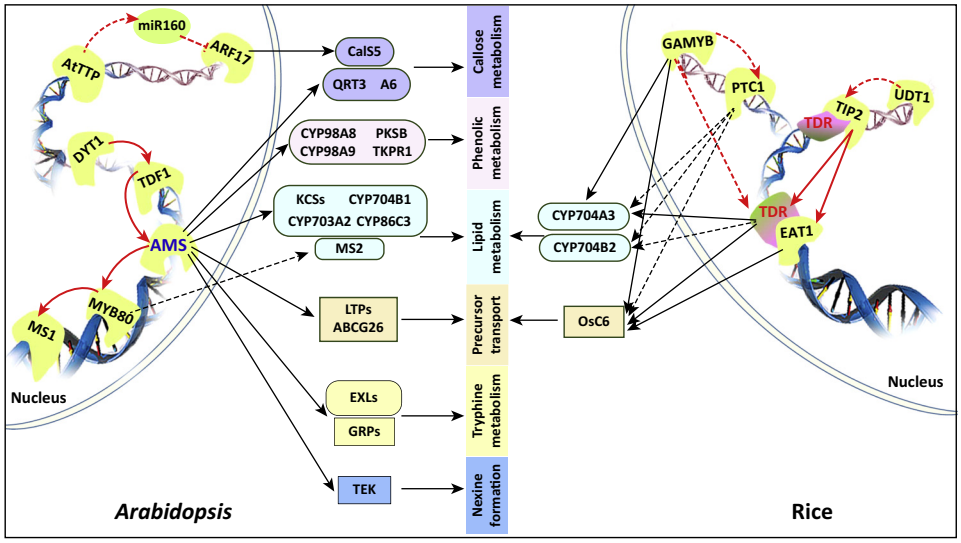

Figure 4. Solid and dotted arrows indicate direct positive regulation supported by promoter binding (solid arrows) and transcriptomic data (dotted arrows). Positive and negative regulatory actions are indicated by arrows and lines with bars, respectively. TDR (TAPETUM DEGENERATION RETARDATION) interacts directly with both TIP2 (TONOPLAST INTRINSIC PROTEIN 1) and EAT1 (ETERNAL TAPETUM 1).

ELEMENT SILENCING VIA AT-HOOK (*TEK*) in nexine formation and *AtMYB103/MYB80* in sexine formation [74]. Consistently, TDR modulates rice pollen exine development through directly regulating the expression of downstream genes such as *OsC6* [72] and *CYP703A3* [49]. Furthermore, we recently revealed that three bHLH proteins, TDR INTERACTING PROTEIN 2 (TIP2)/bHLH142 [75,76], TDR, and ETERNAL TAPETUM 1 (EAT1)/DELAYED TAPETUM DEGENERATION (DTD) [77,78], form a regulatory cascade in determining the differentiation and development of anther wall and pollen exine.

AtMYB103/MYB80, a R2R3 MYB family member, likely regulates pollen wall development by acting upstream of *A6*, *MS2*, *GLOX1* (*glyoxal oxidase 1*), and *VANGUARD 1* (*VGD1*, a pectin methylesterase-encoding gene) [79,80]. GAMYB, an important component in gibberellin (GA) signaling, regulates pollen wall development by activating the expression of *CYP703A3* [81]. Notably, the function of AtMYB103/MYB80 in tapetal and pollen development is conserved among land plants [82,83], as is the regulation of *CYP703A3* by GAMYB [84]. In addition, *Arabidopsis* DYSFUNCTIONAL TAPETUM 1 (DYT1) [85], Defective in Tapetal Development and Function 1 (TDF1) [86], AMS, AtMYB103/MYB80/MS188, and MS1 form a genetic pathway that regulates pollen wall development [87]. DYT1 affects pollen wall development by acting upstream of AMS, MS188/MYB80, TEK, and MS1, and directly regulates the expression of *TDF1* [88]. The identification and characterization of MYB80 and TDF1 orthologs in rice have not yet been reported. $MS10^{35}$ is the tomato ortholog of *Arabidopsis* DYT1, which positively regulates genes for lipid metabolism, cell wall modification/degradation, pollen wall/coat

proteins, and transporters [89]. The *ms10*^{35} mutant shares a total of 65 downregulated target genes with *dyt1* and *udt1*, highlighting the conserved transcriptional control of pollen wall development [89].

In addition, two CCCH zinc-finger proteins affect callose metabolism and exine development. CALLOSE DEFECTIVE MICROSPORE 1 (CDM1), acts on callose biosynthetic and degrading genes [90], while AtTTP (TRISTETRAPROLINE) forms a genetic pathway including AtTTP-miR160–ARF17-CalS5 [91]. Furthermore, AtbZIP34 was shown to modulate pollen wall development by targeting six genes associated with lipid metabolism and/or transport, including *AtABCB9* [92].

Epigenetic Control

Emerging evidence demonstrates the existence of pollen-specific or pollen stage-specific microRNAs in *Arabidopsis* [93], rice [94], and Chinese cabbage (*Brassica campestris*, syn. *Brassica rapa*) [95], suggesting their possible roles in pollen wall development. Notably, silencing of *BcMF11*, a non-coding RNA gene in Chinese cabbage (*Brassica campestris*), leads to aborted pollen [96]. Furthermore, *Arabidopsis* CYCLIN-DEPEDENT KINASE G1 (CDKG1), a member of the cyclin-dependent protein kinase family, affects pollen wall development by regulating *CalS5* pre-mRNA splicing [97]. Further exploring those epigenetic regulators and their corresponding targets will be important for a deeper understanding of pollen wall development.

Other Regulators

Increasing evidence shows that many other regulators besides TFs are also involved in pollen wall development. Reactive oxygen species (ROS) affect pollen intine properties [98], thus *mads3-4* exhibits disorganized intine owing to the loss of temporal ROS kinetics [99]. RESPIRATORY-BURST OXIDASE HOMOLOG E (RBOHE), a PM-localized NADPH oxidase that is crucial for the temporal ROS pattern in tapetum, also affects exine formation [100]. ROS likely act on redox-sensitive kinases and a subset of TFs, including MYBs [100].

Phytohormones such as auxin, GA, cytokinin (CK), brassinosteroid (BR), ethylene, abscisic acid (ABA) and jasmonic acid (JA) affect pollen wall development [101]. AUXIN RESPONSE FACTOR 17 (ARF17) controls callose biosynthesis and primexine deposition by directly modulating the expression of *CalS5* and *RPG1* [102]. In flowering plants, GA regulates pollen wall development via GAMYB [81,84], and GAMYB activates targets such as *CYP703A3* [81], and the double mutant *rga-28 gai-td1*, which is defective in the key GA signaling repressor DELLA proteins, displays defective pollen wall development [103]. Cytokinin receptors in the sporophyte are indispensable for pollen maturation [104], and loss of function of ROCK1 (REPRESSOR OF CYTOKININ DEFICIENCY 1) enhances cytokinin response and induces defective exine formation [67]. BR receptor mutant *bri1-116* and biosynthetic mutant *cpd* lack an obvious bacula/tectum structure in the pollen exine, and BES1, an important TF in BR signaling, regulates the expression of *SPOROCYTELESS/NOZZLE* (*SPL/NZZ*), *TDF1*, *AMS*, *MS1*, and *MS2*, genes essential for pollen development [105]. Although ethylene [106], ABA [107], and JA [108] are associated with pollen development, their roles in pollen wall development remain poorly understood.

Concluding Remarks and Future Prospects

Pollen wall development is a complex and well-coordinated process governed by genetic and epigenetic machineries and by other factors such as phytohormones. Progress in past decades has significantly advanced our understanding of this unique and important process, particularly the conserved and diversified enzymes/genes and their regulatory network. However, further investigations on the biochemical function of these proteins and new players involved in pollen wall formation in various plant species are still needed (see Outstanding Questions).

Outstanding Questions

How closely does the current morphological knowledge about pollen wall structural components, obtained from chemical fixation, match those obtained from cryo-fixation?

What are the distinct roles of unique organelles (such as tapetosomes present in dicot *Arabidopsis* and Ubisch bodies in monocot cereals) in pollen wall development?

Are there any unknown metabolic pathways and/or chemicals involved in the pollen wall development?

What are the evolutionary relationships of the genes involved in the biosynthesis, modification, transport, and assembly of pollen wall components?

How do gametophytic and sporophytic cells work together to coordinate the process of pollen wall development?

What signals or molecules upstream of currently known networks regulate pollen wall development?

Advances in anatomic, biochemical, genomic, transcriptomic, metabolomic, and proteomic tools will allow us to obtain further insights into the physical and chemical properties of the pollen wall and its mechanisms of development. In addition, high-quality ultrastructural characterization of pollen wall components (particularly tapetosomes and elaioplasts, and Ubisch bodies) will be necessary for future comparative studies on pollen wall development among various plant species and mutants. Recent cryo-fixation studies from rice, maize [69], and *Arabidopsis* [12] have already demonstrated the power of cryo-fixation in obtaining novel ultrastructural features, mainly owing to improved sample preservation. Moreover, previous omics studies indicate the appearance of stage-specific or tissue-specific proteins [11], genes [95], and metabolites [43,56] during pollen wall development, highlighting the importance of systems biology in research on pollen wall development. Together with *in vivo* imaging, scanning, and cell-specific expression systems, omics studies may help us to fully understand the dynamic molecular and ultrastructural nature of pollen wall development.

Plant hormones are known to affect many aspects of reproductive development in plants, but their functions in pollen wall patterning are poorly understood, and this merits in-depth investigation. In addition, both tapetal PCD and pollen wall development appear to be well coordinated, but the underlying mechanism is not clear. Could tapetum-derived lipidic molecules be the signals that harmonize the two processes?

It remains uncertain whether other cell layers of the tapetum, namely middle layer, endothecium, and epidermal layer, also play important roles in pollen wall development. Mutation in rice *Wax-deficient anther 1* (*WDA1*), an epidermis-specific expressed gene, causes defective exine [42], while mutation in *Arabidopsis ECHIDNA* shows partial male sterility owing to reduced and disorganized thickening of anther endothecium [109]. In kiwifruit (*Actinidia deliciosa*), delayed PCD in the middle layer induces male sterility [110]. Given these findings, the contribution of non-tapetal anther layers to pollen wall development is expected to be further unraveled in the future.

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