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Proteomics of Pollen Development and Germination

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In higher plants, pollen grains represent the vestiges of a highly reduced male gametophyte generation. After germination, the pollen tube delivers the sperm cells by tip-growing to the embryo sac for fertilization. Besides the intrinsic importance for sexual reproduction, pollen development and germination serve as an attractive system to address important questions related to cell division, cell differentiation, polar growth, cell–cell interaction, and cell fate. Recently, pollen functional specification has been well-studied using multidisciplinary approaches. Here, we review recent advances in proteomics of pollen development and germination.

Keywords: proteome • pollen • anther • development • germination

Introduction

In flowering plants, pollen is a highly reduced two- or three-cell gametophyte whose development and germination are regulated by a delicate and well-programmed process. Pollen grains are generated in diploid sporophytic plants via meiosis followed by two consecutive mitotic processes. After landing on the stigma of a flower, the metabolically quiescent pollen grains can quickly switch to an active status to interact with the pistil through protein and lipid components in the pollen coat, followed by pollen germination, giving rise to a polarly growing pollen tube whereby two sperms are delivered into the embryo sac for double fertilization.[1] As the male gametophyte in plant sexual reproduction, pollen grains undergo active metabolic changes in the course of pollination processes including signaling, cell recognition, and cell growth. The functional specialization of pollen grains is a key issue in plant sexual reproduction and contributes to the vigor of progeny and genetic diversity of a population. In addition, due to the specific features of pollen development and germination, pollen grains have been considered as an excellent model to investigate the molecular mechanisms underlying cell polar growth, cell differentiation, cell fate determination, and cell-to-cell communication. Furthermore, dissecting the mechanisms of pollen development and fertilization has potential applications in crop breeding and engineering.[2] Thus, as a hot topic in plant science, pollen functional specialization has been studied using multidisciplinary approaches including biochemistry, molecular genetics, and functional genomics in recent years. The completed genome sequences of model plants *Arabidopsis*,[3] *Populus*,[4] and rice;[5] the extensive EST databases and mutant collections; and data sets from microarray experiments present unprecedented resources for dissecting the molecular mechanisms underlying pollen development and germination. To date, more than 150 genes that regulate pollen development have been studied, and some of them have been functionally characterized.[6] The genetic control of pollen development and germination has been well-documented.[1,2,7] Additionally, transcriptomic studies have provided extensive information on *Arabidopsis* pollen development on a genome-wide scale.[8–11] However, gene expression at the transcription level does not always correlate with protein level and activity.[12,13] For example, transcriptomic studies found transcripts related to translation; carbohydrate and energy metabolism are underrepresented in the transcriptome. The finding cannot explain the active carbon and energy metabolism taking place in pollen development and germination. The recent proteomic investigations on pollens from *Arabidopsis*, rice, and gymnosperms[14–17] have provided the first opportunity to overcome the limitations of transcriptomic analysis and offered new insights in the mechanisms underlying pollen functional specification. This review will report the recent advances in pollen proteomics.

Changes of Anther Proteome during Pollen Development

Pollen development takes place in the highly differentiated anther. There are usually four anther locules (pollen sacs), and each consists of a central sporogenous cell and outer anther wall (Figure 1A). The inner layer of anther wall (termed as tapetum) provides nourishment to microspores and enzymes for their release. Sporogenous cells give rise to microsporocytes (or pollen mother cells). Through two meiotic divisions, the microsporocyte is transformed into a tetrad of haploid microspore encased in the callose wall of the tetrad. After breakdown of the callose wall by β-1,3-glucanase secreted from

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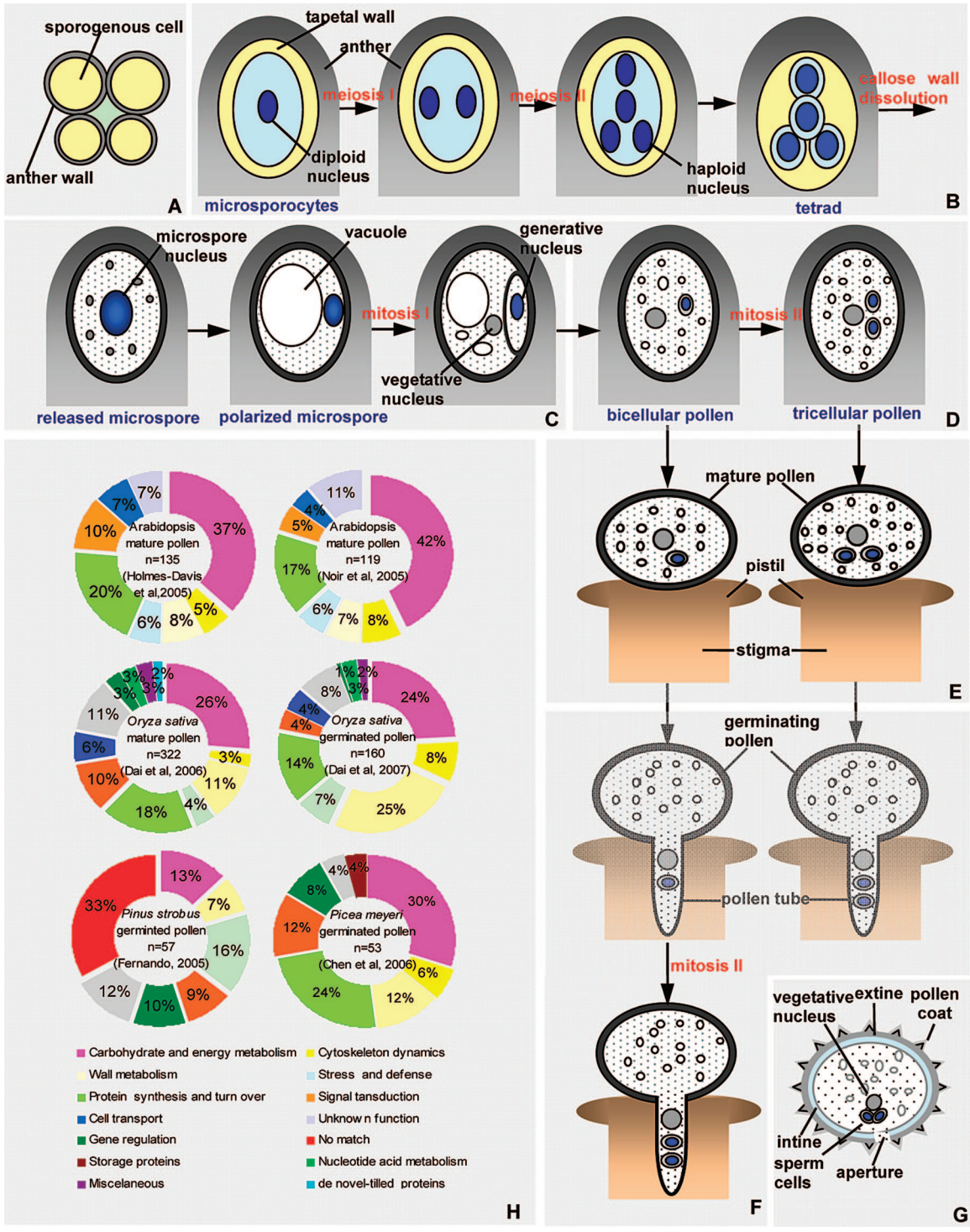

Figure 1. Pollen development process and protein functional categories. (A) Anther with four locules; (B) microspore genesis; (C and D) male gametophyte development (C, microspore development; D, pollen development); (E) mature pollen grains landing on pistil of stigma; (F) pollen germination; (G) mature pollen grains; (H) functional categories of proteins identified in mature and germinated pollens. The data from original literature[8,14–17,26] were reclassified according to published criteria.[16]

the surrounding tapetal cells, the microspores are released into the anther locule. Then, pollen wall and coat deposition starts, utilizing materials secreted from pollen and tapetal cells (Figure 1B). This period is termed microsporogenesis. The released microspores enlarge and undergo cytoplasmic reorganization mediated by the cytoskeleton, resulting in cell polarization with the formation of a single vacuole and a nucleus adjacent to the wall. Subsequently, the polarized cell undergoes an asymmetric division (pollen mitosis I, PMI), forming a big vegetative cell and a small generative cell. This stage is termed the microspore development stage (Figure 1C). For a majority of plant species (approximately 70% of the families), this bicellular structure further develops to produce mature pollen grain, which is called bicellular pollen, whereas for the rest of the species, the generative cell proceeds through one round of symmetrical mitotic division (pollen mitosis II), forming two

sperm cells. This leads to a tricellular pollen (Figure 1D). Bicellular pollen undergoes this division only at a later stage within the elongating pollen tube (Figure 1F). The two sperm cells are closely connected with each other and with the vegetative nucleus to form a male germ unit, which can be delivered into the embryo sac for double fertilization. Thus, mature pollen grains may be considered as a "two sperm cells within a vegetative cell" with specialized functions. The period from two-cell pollen to mature pollen is termed pollen development stage (Figure 1D,E).

Information is still lacking in genome-wide molecular processes, despite the fact that cytological features of the above process and functions of some of the genes regulating key events of pollen development have been studied. Previous research has shown that the transcripts of "early genes" were detected after meiosis but disappeared in mature pollen grains, while the transcription of "late genes" occurred after PMI and continued to accumulate in mature pollen grains.[18] Recent transcriptomics of *Arabidopsis* pollens revealed the lack of consistency and uniformity in gene expression at several stages of pollen development.[1,10] The number of diverse mRNA species declined, and the proportion of male gametophyte-specific transcripts increased during the transition from bicellular pollen to tricellular pollen.[10] Because of the differences in the purity of the pollen samples and the variations of microarray analysis, the number of transcripts during various stages of pollen development observed by different research groups varies greatly.[1,10] The first proteomics work on early pollen development used rice anthers at the young microspore stage as materials.[19] On the basis of the nondestructive measurement of auricle distance (AD, the distance between the auricle of the flag-leaf (last leaf) and that of the penultimate leaf) and the inspection of anther structure under a light microscope, Imin et al.[19] correlated the AD with the developmental stage of the rice microspore and determined that the AD of −18 to −5 mm for rice cultivar Doongara corresponded to the tetrad and the early stage of microspore development for anthers in the top five spikelets from the top three branches of the panicle. Since it was not possible to separate the two stages, they were combined and termed as the young microspore stage. At this stage, over 4000 anther protein spots were reproducibly resolved on silver-stained 2D gels, including 75 spots representing 62 proteins identified by Edman sequencing and MALDI-TOF MS.[19] Since only a small number of housekeeping proteins were identified, little information could be derived from this proteomics study about the extensive functional categories of proteins in the young microspores. Two years later, this group reported a more detailed study on rice anther proteome.[20] The anther proteins expressed at six different stages of pollen development (pollen mother cell, tetrad, early young microspore, single cell pollen, bicellular pollen, and tricellular pollen) were studied. The comparison of the 2D gels of the developmental stages allowed the selection of 150 protein spots, that displayed consistent changes, for protein identification by MALDI-TOF MS and N-terminal microsequencing. Surprisingly, only 40 spots representing 33 unique proteins were identified. The proteins are mainly involved in carbohydrate metabolism, cell wall, and cytoskeleton. The up-regulated proteins include Jab1/CSN5 homologue, vacuolar acid invertase, fructokinase, β-galactosidase, β-expansin, profilin, and actin. They may contribute to a coordinated process responsible for pollen cytoskeleton remodeling, cell wall expansion, and anther dehiscence at the flowering stage.

It is known that pollen development in rice is susceptible to low-temperature stress. Cold treatment (<20 °C) at the young microspore stage could induce severe pollen sterility and thus large grain yield loss. A recent cDNA microarray analysis of rice anther revealed that approximately 160 transcripts were up- or down-regulated when chilling at the microspore release stage.[21] Proteomics of rice anthers after cold temperature treatment has provided further evidence toward understanding cold-induced male sterility.[22,23] The primary results showed that most of the 3000 protein spots on silver-stained 2D gels of the tricellular stage rice anthers resolved were not responsive to cold treatment (12 °C). Only a small subset of proteins (70 spots, approximately 2%) were differentially displayed, including 12 newly synthesized, 47 up-regulated, and 11 down-regulated spots.[22] The most valuable evidence from the proteins identified was that cold stress at the young microspore stage could enhance or induce protein degradation in rice anthers. Heat shock protein 70 (HSP70), β-expansin, 2,3-bisphosphoglycerate-independent phosphoglycerate mutase, and glycogen phosphorylase were all subjected to degradation or cleavage in response to cold stress. Hence, they are candidate proteins possibly involved in the process leading to infertility of pollen grains. Unexpectedly, none of the abundantly expressed proteins in anthers at a later stage (e.g., vacuolar invertase, profilin, and pollen allergens) were found to be differentially expressed in response to cold stress.[22] A further study revealed that 37 anther protein spots were obviously differentially expressed after 1, 2, and 4 days of cold treatment in a cold-sensitive cultivar Doongara, compared with the relatively cold-tolerant cultivar HSC55.[23] One spot was newly synthesized, 32 up-regulated, and 4 down-regulated. Of these, 13 proteins (3 up-regulated and 10 down-regulated) were identified. Most of the up-regulated proteins were involved in protein turnover, stress responses, lipid biogenesis, cell wall formation, and energy metabolism. These proteins can be considered as candidate molecules that regulate cell metabolism and influence subcellular structures under cold stress. One down-regulated anther-specific protein, T42, with similarity to a lipid transfer protein was proposed to affect the transport of fatty acids and/or other sporopollenin precursors from the tapetum to the microspore at the early stage of exine formation. Another down-regulated protein, calreticulin, was hypothesized to be involved in increasing anther indehiscence. A rice cold-induced anther protein (OsCIA) was identified by proteomics. However, quantitative real-time RT-PCR did not detect any significant changes at the gene transcription level after cold treatment. Thus, OsCIA is a novel protein that is differentially regulated at the post-transcription level.[23]

Mature Pollen Proteome Exhibits Functional Specialization

Mature pollen grains (MPGs) are highly reduced gametophytes well-prepared for pollination, germination, and fertilization (Figure 1G). Previous work using inhibitors of protein synthesis has demonstrated that protein synthesis is not required for pollen germination, and *de novo* protein synthesis is only needed for later pollen tube growth and fertilization.[24] To prove this notion, researchers have been trying to find how many proteins are present in mature pollens and what proteins are newly synthesized upon pollen germination.[6] Up to date,

more than 150 genes specifically expressed in pollens have been studied in one or more species through reverse genetics.[6] They encode protein kinases, allergen proteins, pollen cell wall proteins, and proteins involved in cytoskeleton, pectin metabolism, transcription, and signal transduction. With the use of biochemical approaches, enzymes involved in pollen metabolism were studied. For example, 12 enzymes (i.e., esterase, acid phosphatase, pectin esterase, malate dehydrogenase, glutamic-oxalacetic transaminase, diaphorase, superoxide dismutase, phosphoglucose isomerase, glutamic-6-phosphate dehydrogenase, alcohol dehydrogenase, shikimic dehydrogenase, and glutamate dehydrogenase) from maize mature pollen grains were separated by 2D gel electrophoresis (2DE).[25]

Recent transcriptomic analysis of mature pollens of *Arabidopsis* identified approximately 14 000 male gametophyte-expressed mRNAs. This finding not only supports the early notion that mature pollens have a storage of presynthesized mRNAs, but also reveals the pollen functional specialization on the whole genome scale; that is, the mature pollen transcriptome displayed reduced complexity, higher proportion of selectively expressed genes than sporophytic tissues, and a functional skew toward genes implicated in cell wall metabolism, signaling, and cytoskeleton dynamics.[8,9,11] The underrepresentation of transcripts related to translation, glycolysis, and energy metabolism and the lack of transcripts encoding ribosomal proteins in *Arabidopsis* mature pollens seem to indicate that protein synthesis and energy metabolism may not be active in mature pollens.[9] However, recent proteomic studies from *Arabidopsis*[15,26] and rice[16] have revealed the active protein and energy metabolism of mature pollens that was misled by transcriptomics results. Proteomics of *Arabidopsis* mature pollens identified more than 135 distinct proteins, about half of which are involved in metabolism, energy, wall metabolism, cell structure, and protein synthesis.[15,26] In addition, there are five pollen-specific proteins (two glycosyl hydrolases, a germin-like protein, a pectin methylesterase inhibitor, and an actin depolymerizing factor), 10 proteins of unknown functions (three of which are flower- or pollen-specific) and nine proteins whose corresponding gene transcripts were not detected in the transcriptomic studies. Seven of the nine proteins are involved in metabolism, energy generation, or cell wall structure. The functional categories of proteins identified in rice MPGs[16] showed similar features as in *Arabidopsis*. In total, 322 proteins were identified from three fractions of mature rice pollens (pollen-coat-associated proteins, pollen-coat/wall-related and -released proteins, and pollen interior proteins). Functional categories of carbohydrate/energy metabolism, wall metabolism, protein synthesis and degradation, and signaling are overrepresented in the proteome.[16] Many of the proteins detected in rice MPGs were discovered for the first time in pollen. They include proteins for signaling (such as protein kinases, receptor kinase-interacting proteins, GDP dissociation inhibitors, C2 domain-containing proteins, cyclophilins), protein metabolism (represented by prohibitin, mitochondrial processing peptidase, ubiquitin fusion degradation protein, AAA1 ATPase), and wall remodeling (such as reversibly glycosylated polypeptides, cellulose synthase-like OsCsLF7).[16] Taken together, the proteomics results have revealed that mature pollens presynthesize a number of proteins (including those implicated in protein synthesis and carbohydrate/energy metabolism) required for pollen function. The proteomics data provide a necessary complement to pollen transcriptomics data and are essential to the understanding of pollen function. In addition, as compared with vegetative organs, the mature pollen proteome obviously is skewed toward functional wall remodeling and signal transduction (Figure 2).[27] This indicates that the stored proteins in MPGs are prepared for pollen–pistil interaction and germination. In addition, the functions of some proteins (14% of 322 rice pollen proteins, 6% of 135 *Arabidopsis* pollen proteins) could be predicted by computational analysis.[15,16] Those pollen proteins with unknown functions or without discernible functional domains (11% of 322 proteins in rice pollen, 27% of 119 proteins in *Arabidopsis* pollen) (Figure 1H) are valuable candidates for future investigation.[16,26]

Proteome Dynamics upon Pollen Germination

The MPGs are released by anther dehiscence at the panicle heading stage and transferred to the stigma by biotic and abiotic pollinators (e.g., insects and wind). The stigma can retain pollen grains rapidly by adhesive interaction. After the initially pollen exine-mediated adhesion depending on the biophysical and/or chemical interactions with the stigma surface, the pollen coat mobilizes its lipids and proteins to contact and recognize the stigma surface. The successful pollen–pistil recognition requires protein–protein interaction to initiate the hydration of metabolically quiescent and dehydrated pollen by importing water, nutrients, and other small molecules from the stigma. With the hydration, the pollen grain is transformed to a highly polarized cell through organization of its cytoplasm and cytoskeleton to support the extension of a single tube. Once the polarity is established, the pollen tube breaks through the wall and carries through the invasion into the stigma by secreting specialized enzymes (Figure 1E,F). Finally, the tip-growing pollen tube reaches the embryo sac and releases two sperms for double fertilization of the egg and the central cell.

In the past few years, a number of investigations have focused on genes and proteins involved in pollen–stigma interaction and pollen hydration, such as an F-box protein AhSLF-S2 controlling the pollen function of S-RNase-based self-incompatibility in *Antirrhinum*,[28] a cysteine-rich LAT52 protein regulating pollen rehydration,[29] and a Chitinase important for pollen tube penetration into the stigma in *Brassica* and *Nasturtium*.[30] In addition, the mechanisms of pollen tube tip-growth was elucidated using cytology and molecular biology. We have started to understand the importance of cytoskeleton dynamics in polar tube growth after dissecting the roles of actin cables and actin fiber, and the regulation of actin dynamics by profilin and actin depolymerizing factor.[31] Furthermore, calcium gradient and membrane apex-localized GTPases are clearly involved in the polar tube growth.[32–34] Although these investigations helped us understand some of the molecular components involved in the pollen–stigma recognition and tube tip-growth, the intricate molecular network underlying pollination and fertilization is yet to be discovered.

Proteomic analysis of differentially expressed proteins upon pollen germination in rice[17] and two species of gymnosperm (*Pinus strobus* and *Picea meyeri*)[8,14] has shown common features of proteome dynamic changes during the process. This is interesting because pollen germination and tube growth differ in many aspects between flowering plants and gymnosperms including tube growth rate, wall components, and cytoskeletal control.[14] Out of a total of 2300 protein spots detected on 2D gels of mature and germinated pollen grains of rice, only 66 protein spots appeared specific to the two distinct stages.[17] For *P. strobus*, 2D gels resolved 645 and 647

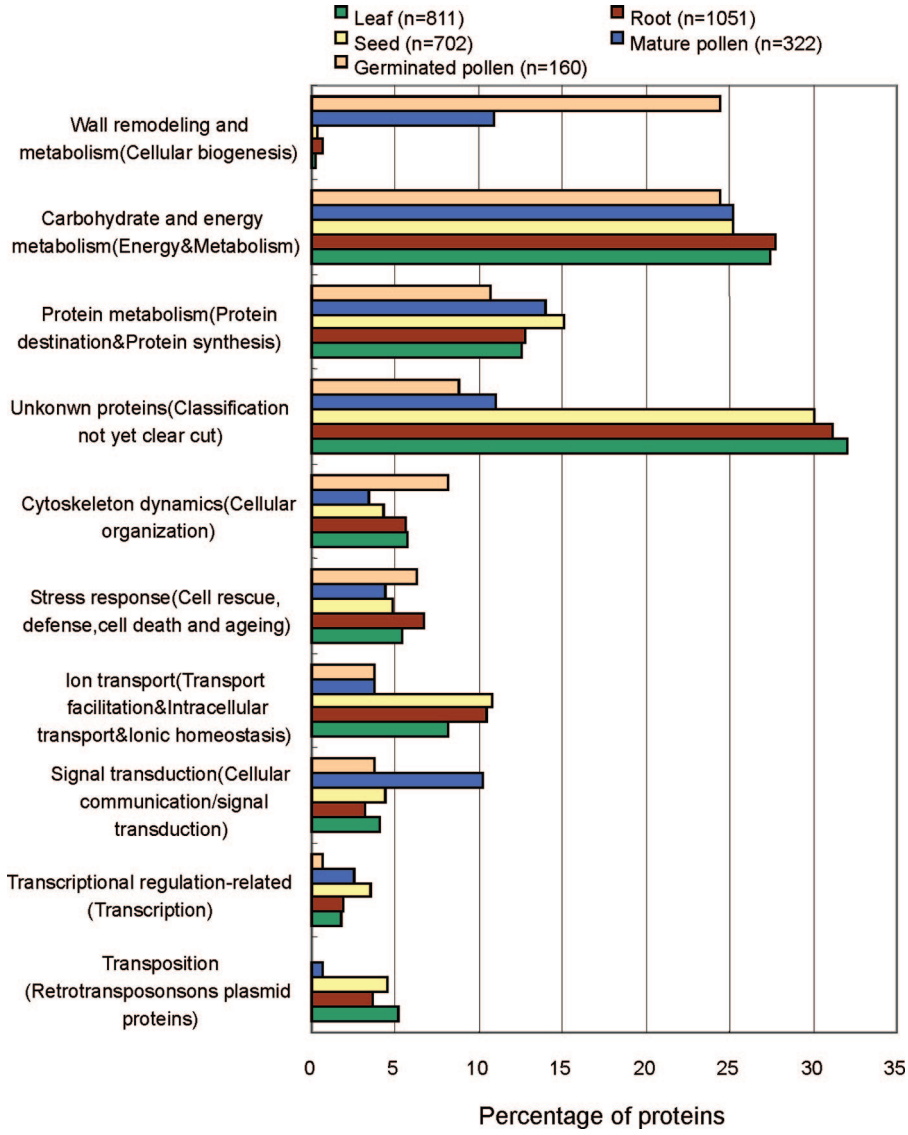

Figure 2. Comparison of function categories of proteins identified from pollen and vegetative organs (leaf, root, and seed) of rice. The data were summarized based on studies of mature pollen proteome,[16] germinated pollen proteome,[17] and proteomes of vegetative organs.[27] Nine of 15 function categories of pollen proteins were matched with 13 of 16 categories of vegetative organ proteins. The names of vegetative organ protein categories were included in parentheses, and the names of combined categories were indicated with "&". The *y*-axis is the reclassified functional categories, and the *x*-axis shows the percentage of identified proteins.

protein spots of mature and germinated pollen, respectively. A large number of proteins (94%) expressed in germinated pollens were also present in mature pollens.[14] These results support the notion that the mature pollens and germinated pollens have similar proteomes in flowering plants as well as in gymnosperms.[35] Interestingly, the differentially expressed proteins displayed functional skew, which can be illustrated by the functional categories reclassified based on the comparative proteomics data obtained from rice, *P. strobus*, and *P. meyeri*.[8,14,17] First, wall-metabolism-related proteins were highly represented. The differentially expressed wall-related proteins account for 25% and 7% of identified proteins in germinated rice and *P. strobus* pollen, respectively,[14,17] and 12% in *P. meyeri* pollen germinated under pollen tube inhibitor latrunculin B (LATB) (Figure 1H).[8] Some wall-metabolism-related proteins, such as UDP-glucose pyrophosphorylase (UGP), reversibly glycosylated polypeptide (RGB), members of class III peroxidase, exopolygalacturonase, and β-expansin in rice pollen and α-expansin, ADP-glucose pyrophosphorylase, and UGP in the pollen of *P. strobes* were up-regulated upon germination,[14,17] whereas type IIIa membrane protein cp-wap13, RGB, Golgi-associated protein se-wap41, UGP, β-expansin OsEXPB13, and proline-rich protein were down-regulated in *P. meyeri* pollen germinated under LATB.[8] Furthermore, 41 proteins were presumed to be released from pollen interior to medium during *in vitro* germination. They include eight unexpected enzymes (e.g., enolase, triose-phosphate isomerase, and phosphoglycerate kinase) involved in carbohydrate and energy metabolism.[17] Some proteins like members of class III peroxidase, polygalacturonase, β-1,4-xylanase, and some pollen allergens may be involved in loosening and hydrolyzing the pistil surface cell and transmitting track of stigma. Some, like calreticulin and C2 domain containing proteins, may function in pollen–pistil interactions.[17] Second, proteomic analysis discovered that active protein synthesis and selective degradation was necessary for pollen tube growth. The proteins involved in protein synthesis and processing have been presynthesized in mature pollen of rice (accounting for 18% of the 322 identified proteins)

(Figure 1H).[16] Upon germination, 14% and 24% of differentially expressed proteins in rice pollen and in *P. meyeri* pollen, respectively, have functions in protein metabolism (Figure 1H).[8,17] Among them, eIF4A and eIF4G involved in protein synthesis were up-regulated, and a translationally controlled tumor protein which suppresses protein synthesis was down-regulated in rice pollen. In addition, members of 26S proteosome, which were stored in mature pollen, and some subunits of the proteosome were significantly up-regulated during pollen germination.[16,17] In *P. meyeri*, pollen germinating under LATB, protein elongation factor Tu, and seven proteins involved in protein processing (luminal binding protein, members of HSP family, molecular chaperone, protein disufide isomerase, and mitochondrial processing peptidase) were up-regulated.[8] Third, proteins involved in active cytoskeleton dynamics and energy metabolism were highly represented in germinating pollens. They were discovered in the comparative proteomic analysis. Among the 120 differentially expressed proteins upon pollen germination, proteins involved in carbohydrate and energy metabolism (24%) and cytoskeleton dynamics (8%) were the majority in rice.[17] This applies to proteins identified in gymnosperms as well, where 13% of the expressed proteins in pollen tubes from *P. strobus* were involved in carbohydrate and energy metabolism (Figure 1H).[14] In addition to *in vitro* germinated pollen of *P. meyeri* growing under LATB, 17 proteins (30% of differentially expressed proteins) were involved in carbohydrate and energy metabolism (Figure 1H). There were three cytoskeleton proteins and several signaling and wall-related proteins that were down-regulated.[8] Compared with vegetative organs, germinating pollen proteome also displayed preferentially expressed wall and cytoskeleton-related proteins (Figure 2).[27] All these features discovered from large-scale proteomic analysis illustrate that germinating pollens undergo active cytoskeleton-mediated wall remodeling based on carbohydrate, energy, and protein metabolism.

Pollen Coat-Related Proteome and Allergens

Mature pollen grains are enclosed with an outermost coat and an underlying wall (Figure 1G). Pollen coat consists of lipids, proteins, pigment and other small molecule mixtures, which are derived from the release of tapetum cell and the secretion of pollen interior.[36] The underlying wall with unique structures (intine and extine) consists of sporopollenin and other polymers embedded with proteins, which are also derived from the tapetum and pollen interior.[36] Pollen coat/wall-related components, especially proteins, are considered to play vital roles in pollen–pistil recognition during plant sexual reproduction. Some common proteins in pollen coat, such as 35-kDa xylanase, β-glucanase, pectin esterase, and pollenin, are initially present in the cytosol of tapetum cell and then released to the anther locule and deposited on pollen surface following tapetal apoptosis.[36,37] Other pollen coat proteins, such as expansin, extensin, and polygalacturonase, are probably secreted from pollen interior. These proteins are presumed to interact with proteins on the pistil surface for pollen–pistil interaction, initiation of germination, or hydrolysis of the stigma wall for the entry of the pollen tube into the transmitting track.[37] In some species, pollen coat also contains minor proteins interacting with *S* locus related protein of pistil involved in self-incompatibility.[38] This aspect has been well-reviewed.[39] Given the importance of pollen coat proteins, researchers have been working on dissecting the molecular components and functions of the pollen coat. Early quantitative cytochemical analysis have found succinate dehydrogenase and cytochrome *c* oxidase in extine; acid phosphatase, esterase, and ribonuclease in intine; and allergen protein, phosphorylase, amylase, β-fructofuranosidase, β-1,4-glucanase, and polygalacturonase localized in both extine and intine.[40] Recently, additional coat proteins have been characterized in different species.[41–43] However, the exact localization of these proteins, their spatial and temporal expression features, and their association mechanisms are still unknown.

Although 2DE-based proteomic approach has already been applied to compare pollen coat proteins of self-incompatible and self-fertile *Festuca pratensis,* no protein function information is available due to the lack of protein identification data.[43] Proteomics using SDS-PAGE combined with MS identified six lipases and six oleosins containing lipid-binding domain in *Arabidopsis* pollen coat.[41] The most abundant oleosin, GRP17, has been shown to function in pollen rehydration.[34] A recent rice pollen proteomic study has expanded our knowledge of pollen coat- and wall-associated proteins.[16] With the use of SDS-PAGE combined with nanoLC–MS/MS analysis of the diethyl ether-eluted fractions, 37 pollen coat-associated proteins were identified, most of which were involved in wall remodeling and metabolism (e.g., β-1,4-xylanase, β-glucanase, β-galactosidase, β-expansin, β-expansin OsEXPB13, major pollen allergen Ory s1 precursor, group 3 pollen allergen, pollen allergen, UGP, and myoinositol-1-phosphate synthase), signal transduction (e.g., Rab GDP dissociation inhibitor, OsGDI1, calreticulin precursor, C2 domain protein-like, 14-3-3 protein, GF14-d protein, GF14-c protein, and cyclophilin 2), vesicle trafficking (e.g., translocon-associated protein and membrane translocase), stress response (e.g., L-ascorbate peroxidase), and ion transport such as vacuolar ATPase B subunit. There were some unexpected proteins involved in cytoskeleton dynamics (actin-depolymerizing factor 2), protein synthesis, assembly and degradation (e.g., 30S and 40S ribosomal proteins, Eukaryotic initiation factor 4A, translational inhibitor protein, Heat shock protein 82, and prohibitin), carbohydrate and energy metabolism (vacuolar acid invertase, hexokinase I, enolase, ATP synthase alpha chain), and amino acid metabolism (pyrroline-5-carboxylate reductase).[16] Furthermore, the rice pollen wall/coat-related and -released proteins (PRPs) were separated from mature pollen by isotonic elution.[16] With the use of 2D gel and MS, 158 unique proteins were identified. Besides 10 previously reported pollen coat proteins (β-1,4-xylanase, expansin, profilin, pectin methylesterase inhibitor, esterase, amylase, polygalacturonase, pollen allergens, cyclophilin, and calreticulin) and eight excellular matrix proteins (cellulose synthase, pectin acetylesterase, β-glucosidase, β-glactosidase, subtilisin-like serine protease, peroxidase, apospory-associated protein, and thioredoxin), the rest of the 140 proteins were discovered in the fraction of PRPs for the first time. They are mainly involved in signal transduction, wall remodeling, metabolism, carbohydrate and energy metabolism, and stress response.[16] Whether these proteins are permanent or transient components in the PRPs fraction awaits further investigation.

Some of the pollen coat/wall and interior components are categorized as allergens because of their allergenic properties.[44] Pollen from many species, especially grass, contains allergens, which are a group of water-soluble proteins or glycoproteins capable of evoking an IgE antibody-mediated allergic reaction.[45] Examples are expansin, profilin, group 3 pollen allergen, and UGP mentioned above. Recently, by a combination of proteomics and immunoblotting techniques, a number of novel

pollen allergens, including enolase, aldolase, elongation factor 2, pathogenesis-related protein, and malate dehydrogenase, were identified and characterized in Bermuda grass (*Cynodon dactylon*),[46] maize (*Zea mays*),[47]and other plants.[48] The information has enhanced our knowledge of IgE-reactive pollen proteome. The question becomes whether these proteins have functions in pollen development and germination.

Multiple Protein Isoforms Expressed in Pollen Proteome

Protein isoforms and homologues have been widely detected and documented in proteomes of different organisms including pollens at different stages.[8,14,17,26,27] The isoforms are derived from splicing variations during transcription, post-translational modification (PTM), or processing. In pollens, isoforms from various proteins were discovered and presumed to have different physiological functions during pollen development and germination.[8,14–17] At the young microspore stage, isoforms of four proteins are likely to represent different phosphorylation states, since they were characterized by similar apparent molecular mass but different isoelectric points on 2D gels.[19] The four proteins are actin 1 with two isoforms, ascorbate peroxidase with four isoforms, GSH-dependent dehydroascorbate reductase I with two isoforms, and protein disulfide isomerase with two isoforms. Another four proteins (endosperm luminal binding protein, enolase, glycine-rich cell wall structural protein, and phosphoglycerate mutase) may contain other PTMs because their isoforms displayed different molecular weights and isoelectric points. Moreover, protein homologues (encoded by different genes) of glycine-rich protein, disulfide isomerase, and pyruvate dehydrogenase E1 beta subunit were identified.[19] In another proteomic analysis of pollen development, multiple isoforms of proteins involved in wall metabolism (vacuolar acid invertase, β-expansin, β-galactosidase), cytoskeleton dynamics (profilin), and carbohydrate metabolism (fructokinase II) were up-regulated. This implies that PTMs of these proteins may be crucial for the protein function in pollen development.[20] In mature pollens of *Arabidopsis* and rice, proteins with multi-isoforms account for 23–30% of the identified proteins.[15,16,26] In addition, 27 proteins in *Arabidopsis* pollen and at least five proteins in rice pollen were homologues.[16,26] The multiple protein isoforms stored in mature pollens indicate that these presynthesized proteins are prepared for functions in germination. Upon pollen germination, 25% of the differentially expressed proteins were present as isoforms.[17] There were 24 proteins that displayed 59 isoforms, 37 of which were up-regulated and 22 down-regulated upon germination. Among them, 14 protein spots were detected as potential glycoproteins and four as potential phosphorylated proteins.[17] These proteins with multi-isoforms in mature and germinated pollens are mainly involved in wall metabolism, carbohydrate and energy metabolism, cytoskeleton dynamics, stress and defense, and ion transport,[15–17,27] implying their important roles in pollen development and germination.

Conclusions

The proteomic analysis of pollen development and germination has provided new insights on the whole genome level into the cellular and molecular mechanisms underlying pollen development, pollen–pistil recognition, and pollen tube tip-growth. Integrated with pollen transcriptomics, the features highlighted by proteomic investigation are significant. In the highly reduced gametophytes, proteins show functional skew toward wall remodeling and metabolism, protein fate, cytoskeleton dynamics, signaling, carbohydrate, and energy metabolism, as compared with vegetative organs (Figure 1H). Especially during germination, the proteins for wall remodeling and cytoskeleton become predominant in the proteome (Figures 1H and 2). Despite recent advancements, current pollen proteomics research is far from complete because of several limitations and difficulties. First, collection of enough quantities of pollen grains, especially developing pollens in anthers, is not easy. Second, 2D-gel-based proteomics generally only identifies abundant soluble pollen proteins unless sample prefractionation is conducted.[49] Third, characterization of different types of PTMs is challenging and usually limited by lack of protein samples. Last and not the least, protein identification success rate of low-abundance proteins and of proteins from species with little genome information is relatively low. It should be noted that up to date gel electrophoresis, mostly 2DE, has been exclusively used for pollen proteomics. Application of "shotgun" proteomics approaches such as the multidimensional protein identification technology (MudPIT)[50] to the analysis of pollen proteome is expected to increase the proteome coverage and enhance the detection of low-abundance proteins.[49] In the future, biochemists and plant biologists should cooperate to overcome the limitations and invest more efforts in the functional analysis of identified candidate proteins in pollen development, germination, and fertilization using multidisciplinary approaches, including cell biology, molecular biology, biochemistry, genetics, and bioinformatics. Pollen subcellular proteomics, for example, proteomics of sperm and vegetative cell nucleus, mitocondria, and membrane, will be attractive areas. Given the pollen sampling limitation, more sensitive and efficient proteomics technologies, for example, MudPIT, should be implemented and/or developed. In addition, protein PTMs, protein–protein interaction, and complex formation in the course of pollen development and germination deserve special attention in future research.

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