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Reproduction Multitasking: The Male Gametophyte

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male gametophyte, heterospory, translation regulation, cell–cell communication, pollen tube guidance, double fertilization

Abstract

The gametophyte represents the sexual phase in the alternation of generations in plants; the other, nonsexual phase is the sporophyte. Here, we review the evolutionary origins of the male gametophyte among land plants and, in particular, its ontogenesis in flowering plants. The highly reduced male gametophyte of angiosperm plants is a two- or three-celled pollen grain. Its task is the production of two male gametes and their transport to the female gametophyte, the embryo sac, where double fertilization takes place. We describe two phases of pollen ontogenesis—a developmental phase leading to the differentiation of the male germline and the formation of a mature pollen grain and a functional phase representing the pollen tube growth, beginning with the landing of the pollen grain on the stigma and ending with double fertilization. We highlight recent advances in the complex regulatory mechanisms involved, including posttranscriptional regulation and transcript storage, intracellular metabolic signaling, pollen cell wall structure and synthesis, protein secretion, and phased cell–cell communication within the reproductive tissues.

Contents

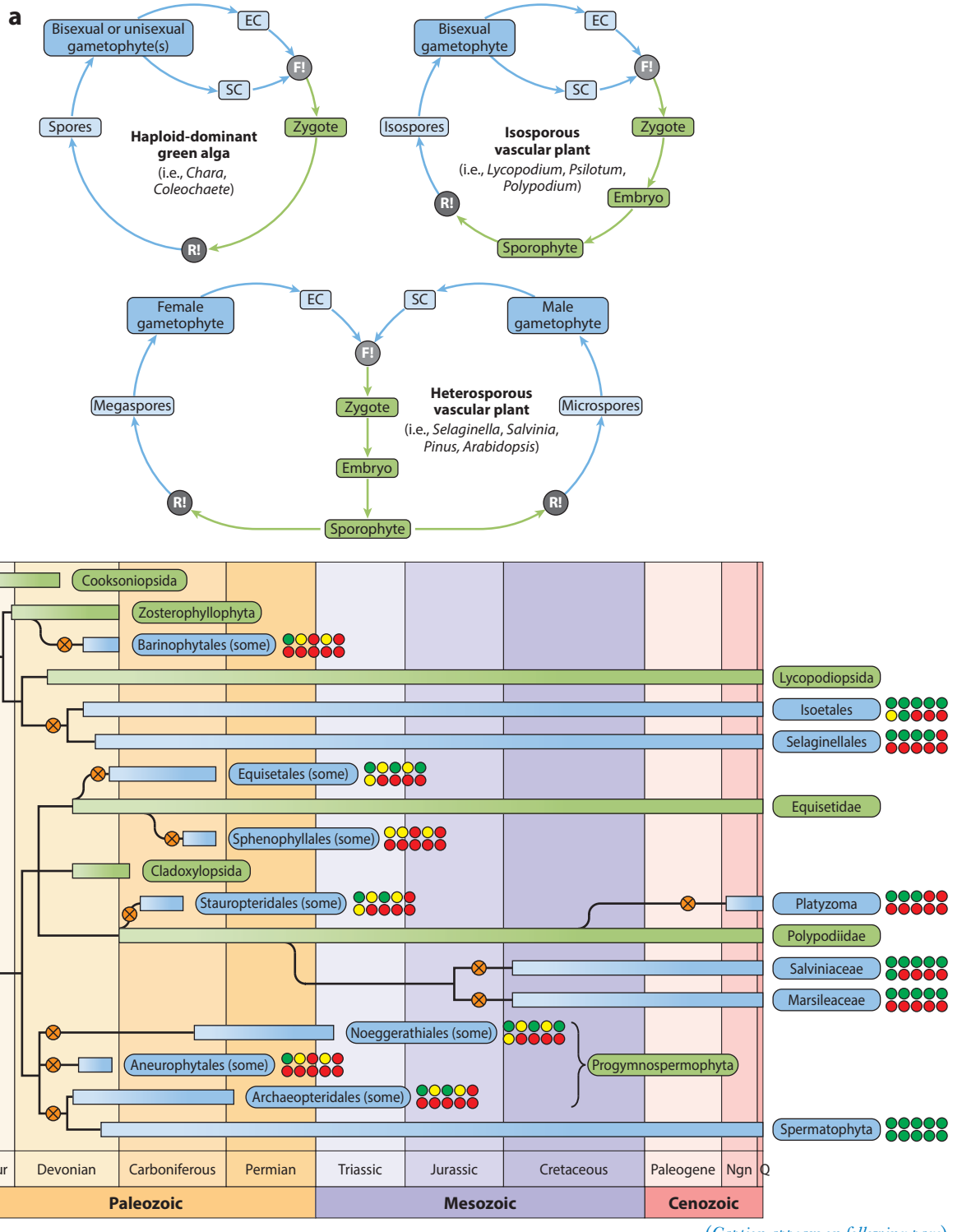
1. INTRODUCTION

The life cycle of land plants oscillates between two alternating generations differing in their ploidy. In the sporophyte, diploid individuals reproduce through haploid spores generated by the meiosis in sporangia. Released spores germinate into the haploid gametophyte, a sexual generation, where specialized reproductive organs differentiate and produce haploid gametes by mitosis. The fusion of male and female gametes leads to the formation of a diploid zygote, the first cell of the next sporophyte generation (reviewed in 65).

Dioecious plants: plants that develop male and female reproductive structures only on different individuals

Alternation of generations: the alternation of sexual and asexual phases in the life cycle of an organism, also known as metagenesis

Plant sexuality has long attracted human attention for its intimate link with crop and honey production. In modern times, the concept of plant sexuality was postulated by Adam Zalužanský ze Zalužan in the pioneering chapter "De Sexu Plantarum" of his book *Methodus Herbaria*, *Libri Tres* in 1592 (55). There, Zalužanský defined male, female, and bisexual plant individuals (termed dioecious and monoecious plants in modern language) and stressed that the individuals of both sexes formed the same species and should not be classified as different taxa or varieties. This postulate was later experimentally confirmed by Rudolf Jacob Camerarius (22) in his famous tract "De Sexu Plantarum Epistola" (55). The next critical milestone was achieved by Wilhelm Hofmeister who explained the plant life cycle that underlies all gametophyte research. He discovered the alternation of sexual and asexual generations in plants (**Figure 1**), including seed plants with their hidden alternation of generations, that made him postulate the unifying theory of plant evolution (75). Interestingly, although Hofmeister made this critical discovery, it was Čelakovský (23) who named the generations as sporophyte and gametophyte for the first time.



(Caption appears on following page)

Figure 1 (*Figure appears on preceding page*)

Evolution of heterospory among land plants. (*a*) Schematic representation of life cycles of vascular land plants and their algae ancestors. Algal ancestors of land plants (haploid-dominant green alga, for example, *Coleochaete*) had a haploid-dominant life cycle, with zygotes being the only diploid cells. Early land plants were isosporous and evolved the fast, apically growing and branching sporophytes, providing them a significant advantage in spore production and dispersal in large numbers. Later, heterospory evolved, resulting in the differentiation of morphologically distinguishable spores that germinated into unisexual gametophytes producing gametes of only one type, male or female. Diploid generation (sporophyte) is depicted in green; haploid generation (gametophyte) is in blue. Panel *a* adapted with permission from Reference 152. (*b*) Simplified phylogenetic tree showing 11 documented heterosporous lineages of vascular plants. Green rectangles represent the main lineages, predominantly isosporous, from which heterosporous lineages evolved. Blue rectangles stand for heterosporous plant lineages. Rounded black branches show the evolution of side lineages when the exact timing is not always precisely resolved. Orange circles with crosses depict the 11 events related to the appearance of heterospory in individual lineages; they phylogenetically represent the evolutionary events, not their timing. Ten circles show whether each of ten selected phenomena associated with heterospory has been identified within individual plant lineages (*green*, yes; *yellow*, probably yes/uncertain; *red*, no). These phenomena are (sequentially) (*upper row*) heterospory, dioecy, heterosporangy, endospory, monomegaspory, (*lower row*) endomegasporangy, integumentation, in situ pollination, in situ fertilization, and PT formation. Panel *b* is based on data from References 6 and 89. Abbreviations: EC, egg cell (female gamete); F!, fertilization; Ngn, Neogene; PT, pollen tube; Q, Quarternary; R!, meiosis; SC, sperm cell (male gamete); Silur, Silurian.

The small size of the gametophytes deeply embedded in sporophytic reproductive tissues made their discovery and detailed examination extremely difficult. Therefore, pollen studies, namely the external appearance of pollen grains and their release from the anthers, marked the beginning of plant reproduction research (114, 158). Further investigation of the pollen and pollen tube (PT) structure and function contributed to the exploration of fundamental phenomena associated with the sexual reproduction of angiosperms, such as the existence of bi- and tricellular pollen (45), differentiation of vegetative and generative or sperm cells (184), and double fertilization (131). Subsequent, considerable progress in pollen research has been driven by the rapid development of cytological and molecular methods. In this review, we discuss the recent advances in complex regulatory mechanisms involved in pollen development and function. These include male germline specification and differentiation, pollen cell wall structure and synthesis, posttranscriptional regulation and transcript storage, intracellular metabolic signaling, protein secretion, and phased cell–cell communication within the reproductive tissues.

Double fertilization: fertilization characteristic of flowering plants in which one sperm cell fuses with the egg nucleus to form an embryo and another fuses with a central cell to form the endosperm

Heterospory: the production of spores of two different sizes by the sporophytes of land plants

Microspore: the smaller of two kinds of spores produced by a heterosporous plant; founder cell of the male gametophyte

2. THE MALE GAMETOPHYTE

2.1. The Evolution of Heterospory in Land Plants

Initially, land plants shed spores of one kind (isosporous = homosporous) that germinated to dioecious gametophytes bearing both male and female gametangia. The conflicting demands for efficient spore dispersal and, at the same time, supplying them with sufficient reserves for nursing the emerging gametophytes provided an evolutionary force for the separation of their roles. It led to the differentiation of two morphological types of spores, known as heterospory. Spores of the first type (male spores, microspores) were optimized for long-distance dispersal and propagation; they were small, light, and low in reserves, and they were produced in large numbers. Parental plants invested much more in the production of spores of the second type (female spores, megaspores). They tended to be larger and rich in reserves, and, therefore, they were produced in smaller numbers. Female spores remained endosporic since their role was to nurse the developing gametophyte and, more importantly, newly emerging sporophyte embryo after fertilization. As such, they enabled the evolution of seeds that was attempted in all terrestrial heterosporous lineages, but the perfection of which was achieved in seed plants. Such functional division of roles contributed significantly to the successful colonization of the land by terrestrial plants, among which seed plants eventually prevailed.

Heterospory has evolved repeatedly in several plant lineages; it has been recorded at least 11 times in the evolutionary history of land plants (6), with four extant groups remaining (**Figure 1**). These comprise spikemosses (*Selaginella* and *Isoetes* clades), water ferns (Marsileaceae and Salviniaceae), a leptosporangiate fern (*Platyzoma microphyllum*), and seed plants (Spermatophyta) (152). Spermatophyta represents by far the largest and most successful group of heterosporous plants that evolved from already heterosporous seedless Progymnospermophyta (6). In contrast, the third group comprises only a monotypic genus *Platyzoma* native to northern Australia (189), although its taxonomical position among Pteridophytes is still unresolved (189; see also **http://www.worldfloraonline.org/taxon/wfo-0001261074**).

Several theories of the origin and evolution of heterospory have been considered, but it remains unclear why it evolved (152). However, a comparative study of 14 *Selaginella* species, their habitats, and growth characteristics supported the hypothesis that heterospory was initially an adaptation to the increasing height and density of Devonian vegetative canopies that accompanied the diversification of vascular plants with leaves (153). It also seems apparent, at least in extant lineages, that heterospory always involved endospory and the unisexuality of spores (6). It is analogous to evolutionarily older anisogamy (or its reinvention in spores) based on its size differentiation and division of roles between megaspores and microspores. The reduction in microspore size is accompanied by the reduction of male gametophytes (MGs) that culminates in seed plants. In gymnosperms, residual antheridia are represented only by a few prothallial cells, and in angiosperms they are absent. Angiosperms, therefore, require only two cell divisions to produce two nonmotile sperm cells from the microspore, and the germline is directly segregated by a formative asymmetric microspore division (reviewed in 63). The reduction of MGs and the evolution of their features specific to seed plants (see below) were also tightly linked to their coevolution with(in) flowers and their pollinators, predominantly insects (194).

Endospory: the production of spores that germinate into a reduced gametophyte contained within the spore wall

Male gametophyte (MG): haploid plant generation producing male gametes

Antheridium: multicellular organ of the gametophyte producing and containing male gametes

2.2. The Male Gametophyte in Gymnosperms

In the lineage leading from Progymnospermatophyta to the seed plants (**Figure 1**), a sequence of three key MG innovations was associated with the parallel evolution of reproductive structures embedding the gametophytic generation. These were the reversal in microspore polarity, the development of PTs, and the shift from motile to nonmotile gametes (168). All of these innovations achieved perfection in angiosperms, but we can see an exciting variability of solutions and structures, both intermediate and terminal, in gymnosperms. Unlike angiosperms, pollen development within four extant orders of gymnosperms is highly variable.

Gymnosperm pollen grains are usually smooth and spheroidal and exhibit variable thickness of the outer pollen wall, the exine. A smooth surface is usually a feature of wind-pollinated plants, such as the majority of gymnosperms. The first capture surface for pollen is an ovular secretion, the pollination drop, that is exposed at the time of ovule receptivity. Interestingly, studies of pollination drops that later evolved to nectar revealed an increasing number of gymnosperm species being pollinated by insects (197). Insect pollination in gymnosperms is facilitated preferentially by scent rather than by visual attraction (194). The composition of pollination drops differs between wind- and insect-pollinated species, and it also explains the lineage pollination history (133, 156). This pollination mutualism was first described for gnetophytes and cycads (96). Interestingly, *Ginkgo biloba*, the only extant ginkgophyte, is a wind-pollinated species, and it was believed that it was always the case. However, the composition of its pollination drop refers to the likely but previously undisclosed insect pollination of this species or its evolutionary ancestors (133).

Hours to months after pollination, pollen grains hydrate and germinate into relatively short PTs bearing motile or immotile sperm cells. The gymnosperm pollen germination rate and PT

growth are considerably slower than in angiosperms (203). Haustorial and branching PTs of cycads and *Ginkgo* release flagellated sperm cells that swim through pollination drops toward the egg cell. Siphonogamous PTs of conifers and gnetophytes elongate from the nucellus to an archegonium and deliver immotile sperm directly into the egg (50). Siphonogamy likely evolved independently in conifers and gnetophytes (168). Although it also exists in angiosperms, its characteristics differ (168). Mature gymnosperm pollen is shed with varying numbers of cells (ranging from one to five) and does not contain sperm cells or sperm nuclei. The sperm cells originate from the mitotic division of a generative cell in a growing PT (50).

Archegonium: a multicellular organ of the gametophyte that produces and contains the female gamete

Siphonogamy: a mode of pollination in which pollen tubes develop to facilitate the passage of male gametes to egg cells

Microsporocyte: the pollen mother cell; a diploid cell that undergoes meiosis resulting in the formation of four microspores

2.3. The Male Gametophyte in Angiosperms

The MG in angiosperms is rather uniform, and it achieved a maximal structural reduction comprising only two or three cells at its maturity: the vegetative cell, representing the MG somatic cell, and two sperm cells (male gametes), representing the final stage of the male germline (8). The tricellular stage is achieved by two mitotic divisions of the haploid microspore–pollen mitosis I (PMI) and pollen mitosis II (PMII). Such a reduction in complexity while maintaining all necessary functions can be achieved only by tight and exact regulation during pollen development that is composed of two consecutive phases, developmental and functional.

The developmental phase takes place in anther loculi lined up with a layer of tapetal cells. It leads to the release of mature pollen from the anthers (**Figure 2**). It is accompanied by remarkable morphological and physiological differentiation of both cell types, including the synthesis of the specialized pollen cell wall and the storage of protective substances and metabolic reserves (65).

The function starts by pollen activation on the stigma. Pollen grain rehydrates and germinates into a long PT. It grows toward the ovule, where its growth ends with double fertilization. This phase involves the mutual activation of PT and pistil tissues, PT guidance, and the communication between the two gametophytes (reviewed in 42).

3. POLLEN DEVELOPMENT AND MATURATION

3.1. Microspore Development and Pollen Wall Synthesis

The male gametophytic developmental pathway is initiated by the meiotic division of diploid microsporocytes (pollen mother cells) into four haploid microspores forming a tetrad. The second meiotic division is followed by the synthesis of callose walls around and between the individual microspores within the tetrad (37). Callose, chemically β-1,3-glucan synthesized by callose synthase (reviewed in 211), is secreted by the microsporocytes and later by the microspores themselves. Meiosis also represents an important developmental transition from the sporophytic to gametophytic developmental program, and it is associated with global transcriptome reprogramming (132) and cytoplasmic remodeling. It involves the elimination of a significant part of the ribosome complement early in the meiotic prophase and its subsequent restoration during late prophase (39) that may represent a mechanism for the elimination of transcripts persisting from the diploid phase. Messenger RNA (mRNA) elimination is likely to be initiated by mRNA deadenylation, possibly by the activity of the evolutionarily conserved CCR4-NOT deadenylase complex present at the early stages of MG development (150). For an efficient transition, the mRNA elimination must be accompanied by effective protein turnover. Accordingly, stage-specific accumulation of transcripts associated with the ubiquitin proteasome pathway was reported during maize male meiosis (209).

The postmeiotic microspore development period is also when the specialized pollen wall is formed. The pollen wall isolates and protects the MG and mediates its communication with the

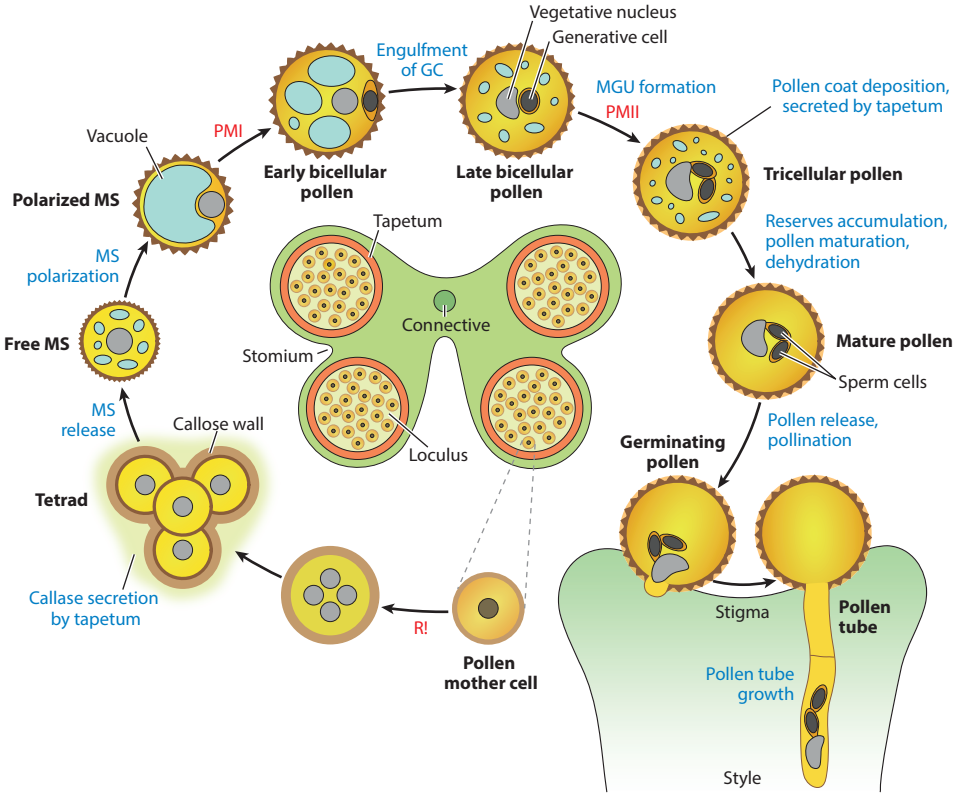


Figure 2

Pollen development and maturation within an anther. During microsporogenesis, the diploid pollen mother cell (microsporocyte) undergoes two meiotic divisions to produce a tetrad of four haploid MSs. The second meiotic division is followed by the synthesis of callose walls around and between the individual MSs within the tetrad. MSs are released from tetrads following callose degradation by callase, an enzyme secreted by the tapetal cells. During microgametogenesis, MSs polarize and undergo highly asymmetric division, PMI, resulting in the formation of two unequal daughter cells with distinct cell fates: a large vegetative cell and a small GC. The GC migrates into the vegetative cell to form a unique cell-within-a-cell structure. The GC undergoes one more cell division, PMII, to produce two male gametes, the sperm cells. The vegetative nucleus remains closely connected to the two sperm cells after PMII, forming the MGU. PMII can occur before or after pollen maturation, and therefore the mature pollen shed is bicellular (as in 70% of species, e.g., tobacco) or tricellular (e.g., *Arabidopsis*). Figure adapted with permission from Reference 78. Abbreviations: GC, generative cell; MGU, male germ unit; MS, microspore; PMI, pollen mitosis I; PMII, pollen mitosis II; R!, meiosis.

stigma surface (175). The pollen wall consists of inner intine, outer exine, and pollen coat/tryphine layers, with the latter two being predominantly of a lipidic nature (212). The pollen wall synthesis is almost finished before PMI (85). The pollen wall is of mixed sporophytic and gametophytic origins since tapetum contributes to the biosynthesis of the exine and pollen coat, and the pollen grain contributes to the intine (2, 59).

After the emergence of the partially formed exine, microspores are released from tetrads following callose degradation by callase (β-1,3-glucanase). Callase is secreted by the tapetal cells (175). Proper timing of callase synthesis and secretion is controlled by MYB-family transcription factor (TF) AtMYB80. It coordinates microspore and tapetum development, resulting in the

programmed cell death of tapetal cells (154). The distortion of this critical coordination mechanism causes male sterility (207). Despite the apparent importance of tapetum, it is possible to achieve the proper maturation of functional pollen in vitro from uninuclear microspores in the presence of essential nutrients (190).

Pollen exine is predominantly composed of sporopollenin, a complex containing fatty acids and phenylpropanoids that is one of the most chemically resistant biopolymers known. Although the pathways involved in sporopollenin formation, as well as its exact structure in angiosperm pollen, remain unknown, its structure was recently solved in *Lycopodium clavatum* spores (122) and *Pinus rigida* pollen (104). The general structure of the *L. clavatum* and *P. rigida* exines is similar, but it is not clear whether the observed differences can be attributed to biological variation or to the methodology used (104, 122).

The exine is not evenly distributed over the pollen surface, and regions with a reduced thickness or even lacking sporopollenin form apertures, sites of a PT emergence. The number and distribution of apertures are strictly controlled, and callose deposition during meiosis plays an essential role in aperture allocation. Since exine synthesis and patterning are under strict sporophytic control, the specification and formation of aperture sites have been enigmatic. Recent research on several specifically localized membrane proteins has started shedding light on this process. In *Arabidopsis*, membrane-associated D6 PROTEIN KINASE-LIKE 3 (D6PKL3) likely defines three distinct aperture domains (99), and INAPERTURATE POLLEN 1 (INP1), a protein of unknown function, aggregates at these domains and prevents exine deposition (40). Strikingly, At-INP1 protein ortholog OsINP1 exists also in rice pollen (214), but there it likely plays a more complex role than in *Arabidopsis*. OsINP1 interacts with lectin receptor-like kinase DEFECTIVE IN APERTURE FORMATION 1 (OsDAF1). Unlike in *Arabidopsis*, the correct localization of OsDAF1 depends on the presence of OsINP1 but not vice versa (215). It suggests that OsINP1 has an additional role in the aperture formation that is absent in *Arabidopsis* (214).

The formation of pollen coatings is completed at later stages of microgametogenesis, during which remnants of degenerating tapetal cells, consisting mostly of lipids and proteins, are deposited onto the pollen surface (162). A broad range of mutants defective in tapetum degradation, therefore, show abnormal pollen wall structure and reduced male fertility (177). Among many others (177, 199), they can be exemplified by recently characterized mutations in rice tapetum-expressed DEFECTIVE POLLEN WALL 3 (DPW3) integrin-like protein (126) and PERSISTENT TAPETAL CELL 2 (PTC2) AT-hook nuclear-localized protein (193). The pollen coat is involved in pollen–stigma signaling, self-incompatibility, pollen hydration, adhesivity, color, and odor. The latter two are essential for its recognition by pollinators. Pollen grains of insect- and other animal-pollinated species are often beautifully decorated with complex structures, whereas the surface of wind-pollinated species tends to be much less elaborate (78).

3.2. Pollen Development and Male Germline Differentiation

The concept of the male germline in plants differs from that described in animals, where the germline initials already differentiate during embryogenesis. The male germline, the cells programmed to produce male gametes, is more difficult to define in flowering plants because of the extreme reduction of the MG (reviewed in 8). However, the male germline must be established within the MG itself, being the haploid, sexual generation. The formative event here is the polarization of microspores and their highly asymmetric division, PMI (reviewed in 63). PMI ensures the fixation of the ongoing MG developmental program, as demonstrated in various plant species by transcriptomic (13, 79, 201) and proteomic studies (26, 52, 83). MGs with abnormal PMI fail to initiate the male germline and do not form gametes (191).

PMI results in the formation of two unequal daughter cells with distinct cell fates: a large vegetative cell and a small generative cell. The generative cell soon migrates into the vegetative cell to form a unique cell-within-a-cell structure (169). The generative cell retains its proliferative activity and is thus defined as a true germline. It divides once more during PMII to produce two male gametes, the sperm cells (8, 169). The vegetative nucleus remains in a close physical connection with the two sperm cells after PMII, forming the male germ unit (97). It plays a vital role not only in the delivery of sperm cells to the female gametophyte but especially in their direct communication (117), perhaps including the modulation of gene expression by noncoding RNAs (115).

Female gametophyte: haploid plant generation that produces female gametes

The establishment of microspore polarity can be impaired by many factors, for example, by centrifugation or cold stress (188), by the application of microtubule-destabilizing agent colchicine (210), or, in particular, by pollen mutants affecting microtubule organization and dynamics. Here, the critical mutation *gem1* disturbs the function of MICROTUBULE ORGANIZATION 1/GEMINI POLLEN 1 (MOR1/GEM1) protein (191). Several other mutations in protein components of the γ-tubulin ring complex also affect microtubule organization in the MG, for example, *gem3/aug6-1* in the AUGMIN complex (138). Disturbance of mitotic spindle orientation in *sidecar* (*scp*) mutants causes delayed mitotic entry and the formation of four-celled pollen grains with an extra vegetative-like cell. SCP encodes a microspore-specific TF, a member of the LBD/ASL family, that is proposed to be involved in determining the timing and orientation of division at PMI (140). Microspore-specific GAMYB TF MYB81 seems to act even earlier since *myb81* mutant microspores fail to undergo PMI and are arrested at the polarized stage with a single central vacuole (137). PMI cytokinesis defects involving phragmoplast organization were reported for *two-in-one* (*tio*) mutants (139) and *kinesin-12a kinesin-12b* double mutants (100), leaving two nuclei in the same cytoplasm with impaired cell fate specification. TIO Fused kinase interacts with KINESIN-12 and, together with kinesins HINKEL and TETRASPORE (136), forms a signaling module involved in phragmoplast expansion (135). Taken together, the disruption of the PMI asymmetry leads to the formation of two similar cells with the vegetative cell fate (44), suggesting that vegetative cell fate is the default developmental program (213).

The generative cell retains its proliferation activity for one more round of cell division, whereas the larger vegetative nucleus exits the cell cycle in the G1 phase (8). The cell cycle exit in the MG is controlled by two closely related regulatory pathways, FBL17-KIP-RELATED PROTEINs (KRPs)-A-type cyclin-dependent kinase (CDKA;1) and RBR1-E2F. In the vegetative cell, KRP CDK inhibitors KRP6 and KRP7 are expressed, and they inhibit the core cell cycle machinery including CDKA;1 (91). In the generative cell, transient expression of F-box-like protein FBL17 triggers proteasome-dependent degradation of KRPs (91) and, as a consequence, maintains the CDKA;1 kinase activity, leading to male germline proliferation (61). Interestingly, FBL17 activity is not restricted to the male germline, but it also controls the cell proliferation in sporophytic tissues via KRP2 (134). The exit of the vegetative cell from the cell cycle is strengthened by the activity of RETINOBLASTOMA-RELATED 1 (RBR1) protein that, together with downregulated E2F/DP family TFs, prevents the G1/S transition (62, 159). The existence of a direct link between cell fate determination and PMI is further demonstrated by the loss of RBR1 protein that causes vegetative cell hyperproliferation and prevents or delays cell determination during plant male gametogenesis (31).

Since its discovery (165), the regulation of the male germline establishment and differentiation has always circulated around the R2R3 Myb-domain protein DUO POLLEN 1 (DUO1) (reviewed in 63). Transcriptomic analyses of DUO1 direct targets (14) revealed genes encoding male germline–specific histone H3.3 variant MGH3 (HTR10) (141) and two sperm cell–expressed membrane-associated proteins essential for gamete attachment and fusion, GAMETE EXPRESSED 2 (GEX2) (127) and GENERATIVE CELL-SPECIFIC 1/HAPLESS

2 (GCS1/HAP2) (128, 198). DUO1 acts as a central integrator of generative cell mitotic division and sperm cell differentiation (14, 18) by forming a regulatory module with two more of its direct targets, DUO1-ACTIVATED ZINC FINGER PROTEIN 1 (DAZ1) and DAZ2 transcription repressors, involving corepressor TOPLESS (16). Interestingly, DUO1 neofunctionalization in stoneworts and its acquired sperm lineage–specific expression in common ancestors of land plants enabled the differentiation of motile sperms. As such, it was the defining event in the evolution of sexual reproduction involving sperm differentiation in the land plant lineage (72). Further rewiring of the DUO1 downstream network enabled the loss of sperm cell motility in seed plants, including angiosperms (72).

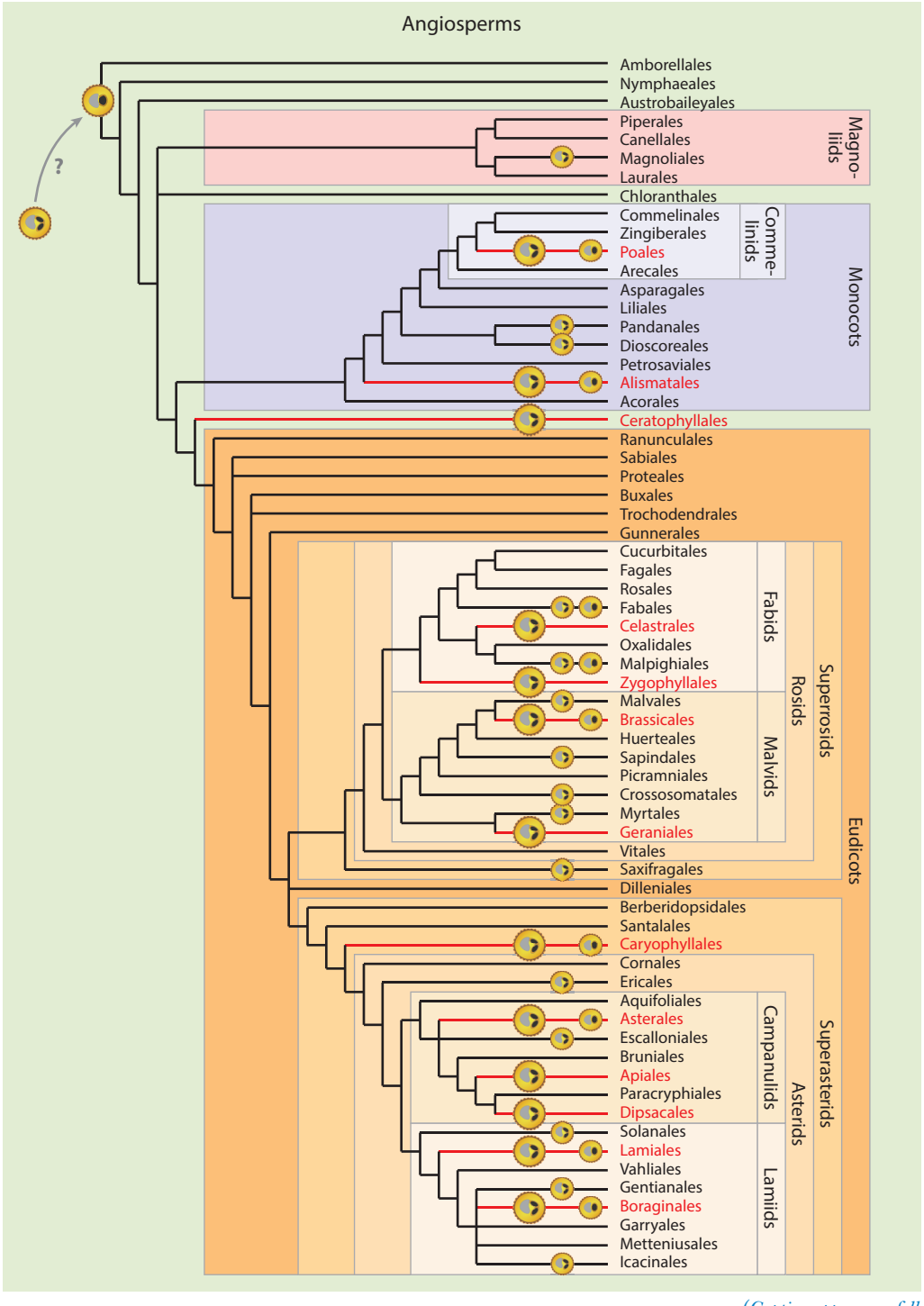
The importance of DUO1 in the definition and differentiation of the male germline highlights the question of its upstream regulation alongside the negative DUO1-DAZ1/DAZ2 feedback loop (16). In *Marchantia polymorpha*, one of the most basal land plants, differentiation of gametangia is regulated by MpBONOBO (MpBNB), a member of the VIIIa subfamily of basic helix–loop–helix (bHLH) TFs (208). Similarly, gamete differentiation is regulated by the RWP-RK TF MpRKD (92, 167). In *Arabidopsis*, homologs of both of these genes exist and are also involved in the germline determination, but individual players acquired specialized roles (73). Several AtRKD genes regulate egg cell differentiation (94), whereas AtBNB1 and AtBNB2 are redundantly involved in generative cell specification and differentiation. They act upstream of DUO1; *bnb1/bnb2* generative cells fail to acquire the male germline cell fate, and they do not express the germ cell lineage marker MGH3; they are not even engulfed within the vegetative cell cytoplasm, suggesting more profound regulatory defects (208). Similar defects were also observed in a pollen-bearing mutation in two *DEFECTIVE REGION OF POLLEN* (*AtDROP1/AtDROP2*) genes, encoding members of the bHLH TF subfamily XI, resulting in the degeneration of generative nuclei and pollen abortion (213).

PMII can occur before or after pollen maturation, and therefore the mature pollen is shed as bicellular (i.e., the vegetative cell engulfs a single generative cell) or as tricellular (i.e., the vegetative cell is associated with two sperm cells). In total, 13 orders of angiosperms shed predominantly tricellular pollen (17). From an evolutionary point of view, the bicellular pollen is considered a plesiomorphic trait because all ancient woody Magnoliales shed bicellular-type pollen (**Figure 3**). Moreover, bicellular pollen is phylogenetically widespread, and tricellular pollen was believed to be restricted to aquatics, grasses, and a few herbaceous perennials (45, 184). However, a reverse transition from tricellular to bicellular pollen has also been suggested (205) as a consequence of various demands on pollen performance concerning the evolution of sporophytic traits (204). Thus, some species shedding bicellular pollen underwent two evolutionary changes (205).

3.3. Epigenetic Control in the Male Germline

Male germline differentiation is also intimately connected with dynamic epigenome modifications (58) and a global reprogramming of epigenetic marks in sperm cells (15).

A dramatic difference exists between the epigenetic status of vegetative and sperm cell DNA. Sperm cell DNA shows condensed chromatin with generally high methylation status, in particular, high symmetrical CG/CHG methylation but lower asymmetrical CHH methylation. Vegetative cell DNA is methylated in the opposite manner (20, 82, 178). There, generally, lower symmetrical but high asymmetrical methylation in heterochromatic transposable elements (TEs) was observed as well as the hypomethylation of retrotransposons (178). Transcription of TEs in the vegetative cell leads to their posttranscriptional silencing by the AGO1/AGO2/DCL4 machinery, resulting in the production of 21- to 22-nt small interfering RNAs (siRNAs) (115). These vegetative cell–derived siRNAs act non-cell-autonomously, where they translocate to sperm cells and inhibit



(*Caption appears on following page*)

Figure 3 (*Figure appears on preceding page*)

Distribution of tricellular and bicellular pollen within angiosperms. Interrelationships are based on APG IV (25). Tricellular pollen is dominant in 13 orders (*red lines* and *large tricellular pollen symbol*, *left pollen column*). Several other orders also produce tricellular pollen (2–44% of species), but the majority are bicellular (*small tricellular pollen symbol*, *middle pollen column*). In nine orders, bicellular pollen evolved secondarily from tricellular ancestors (*small bicellular pollen symbols*, *right pollen column*). The possible tricellular ancestry of angiosperm pollen is reflected by the question mark in the top left corner. The mature pollen grain has a dehydrated cytoplasm, and bicellular pollen is usually shed in a more dehydrated and quiescent state than tricellular pollen (17, 204). The simultaneous presence of both pollen types in one species is very uncommon and more likely in basal angiosperms. For instance, the coexistence of bi- and tricellular pollen grains in the same anther at the same time was observed in early-divergent angiosperm *Annona cherimola*. In *A. cherimola*, the production of the actual pollen type depends on environmental factors such as temperature and humidity during pollen maturation (112, 113). Figure adapted with permission from Reference 52 using data from References 17 and 205. Abbreviation: APG, Angiosperm Phylogeny Group.

TE activities (115). Interestingly, gymnosperm pollen, as in angiosperms, has a unique set of small RNAs derived from TEs, distinctive from sporophytic tissues. Different preferential lengths of pollen-derived small RNAs (24-nt in gymnosperms and 21-nt in angiosperms) point at the diversification of small RNA–induced TE silencing pathways within seed plants (129).

Plants, like mammals, were recently shown to globally reprogram their paternal chromatin. However, rather than demethylation and protamine-mediated histone replacement, the *Arabidopsis* male germline employs a complex mechanism to reset its epigenetic memory by a specific H3K27me3 removal from histone-based sperm chromatin (15). It involves the coordinated silencing of H3K27me3 writers, increased activity of H3K27me3 erasers, and deposition of sperm-specific histone H3.10. Epigenetic resetting of H3K27me3 together with H3K4me3 accumulation not only wipes old generational memories but also facilitates sperm differentiation and likely influences the transcription of zygotic genes, including key regulators of embryogenesis, endosperm development, and flowering (15).

3.4. Accumulation of Reserves

The maturing pollen grain shows considerable metabolic and transport activities, and the vegetative cell accumulates a substantial amount of metabolic reserves, including carbohydrates, proteins, and lipids necessary for the rapid growth of the PT (145). A specific portion of reserves comprise osmoprotectants, e.g., proline protecting cellular membranes and proteins from damage caused by dehydration. However, proline transport from tapetum facilitated by ProT1 (174) is not able to fulfill the high demand, and proline must also be synthesized in developing microspores to ensure pollen development and fertility (116).

In the last decade, research has shown that from PMI to pollen maturity, pollen also significantly accumulates both mRNA and protein reserves (64, 65, 68, 77, 173). Because the whole phenomenon of mRNA storage and activation is tightly connected with PT growth, we cover it in detail in Section 4.3.

4. POLLEN TUBE GROWTH

4.1. Challenges and Constraints of the Initial Checkpoint for Pollen Germination

A PT phase encompasses a postpollination period in which desiccated pollen hydrates on stigmatic papillae and germinates to form a PT transporting sperm cells to an ovule. Female-specific membrane receptors on stigmatic papillae sense pollen-coat-deposited ligand molecules to determine self from nonself pollen and trigger the reception or rejection pathway of a pollen grain

(**Figure 4**). At this phase, pollen performance is fundamental for a successful sperm cell delivery, as a pollen grain faces interpollen competition to germinate, a compatibility test, transmitting tract penetration, and an interpollen tube race in response to ovule attraction signals (204). To fuel all of this, the mobilization of reserves and ability to utilize female-deposited resources determine pollen fitness and influence pollen success in the fertilization of the female gametophyte.

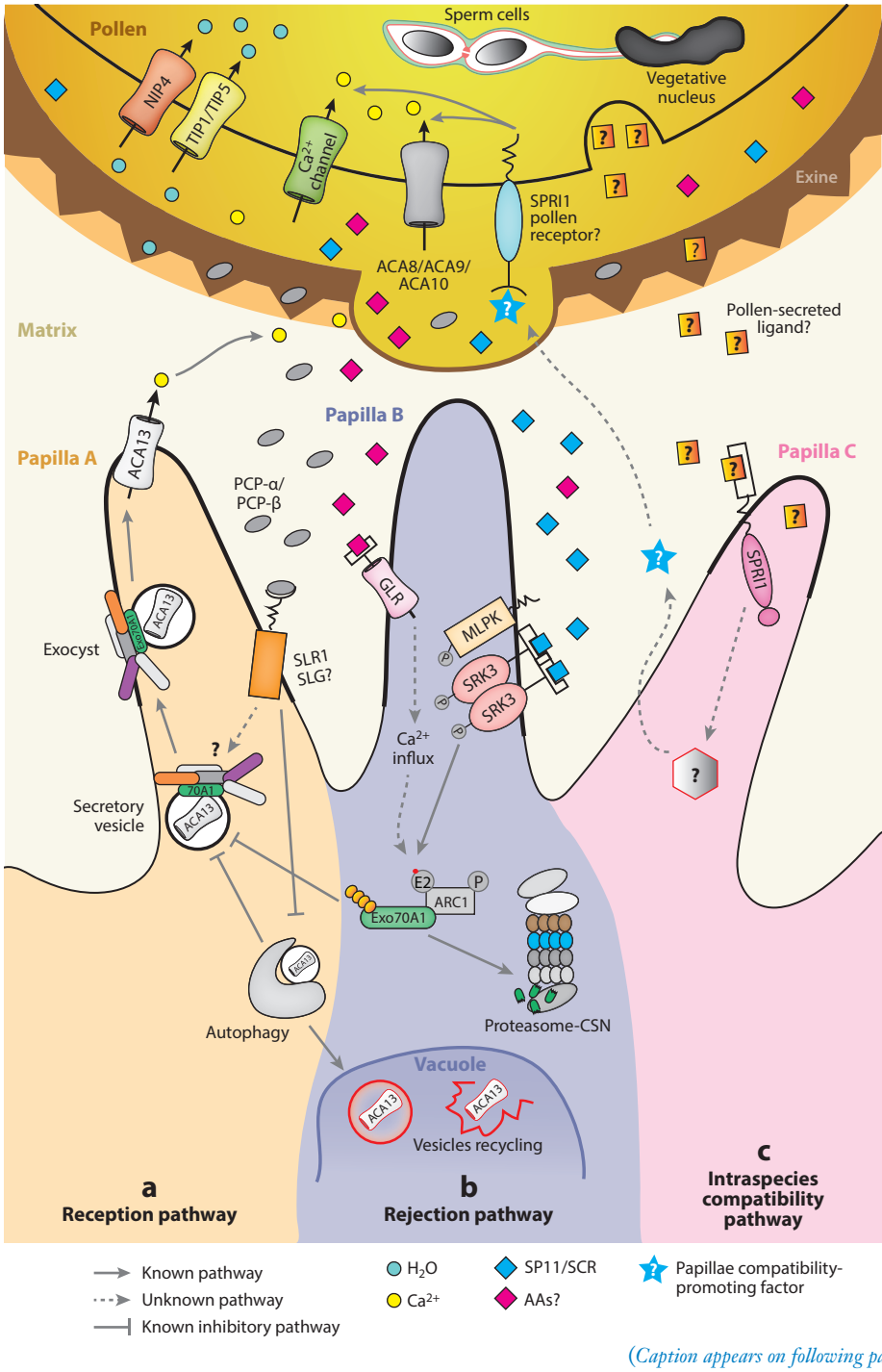
The initial pollen–stigma interaction mostly involves pollen coat and the extracellular matrix of the stigma (1, 86) (**Figure 4**). This initial female–male encounter is also a site of the initial reproductive barrier to prevent inbreeding, whereby the stigma senses and discriminates self from nonself pollen through a self-incompatibility mechanism (11). In flowering plant species, this involves female-secreted S-RNase that generates a cytotoxic pistil that is detoxified by an S-locus F-box (SLF) protein in the male counterpart in the case of self-compatible species (54). To reject self pollen in Brassicaceae and Papaveraceae, a female S-locus receptor kinase (SRK) intercepts male-secreted ligand S-locus protein 11 (SP11), and the pollen grain is prevented from germinating (185, 202). Recently, a completely new mechanism of prezygotic interspecies incompatibility was discovered in *Arabidopsis*. A stigma-specific plasma membrane protein, STIGMATIC PRIVACY 1 (SPRI1), rejects distantly related Brassicaceae PTs to promote intraspecific PT germination and fertilization and is independent of the self-incompatibility mechanism (**Figure 4**). An evolutionary decay of this SPRI1-controlled interspecies incompatibility is likely to have given rise to self-compatible species such as *Arabidopsis* (54).

The next constraint for germinating pollen to negotiate is the chemical checkpoint in the form of γ-aminobutyric acid (GABA), a nonproteinogenic amino acid essential for plant reproduction. In a pistil, GABA forms a gradient with an increasing concentration toward the ovary. Lower GABA concentration is stimulating, whereas higher levels inhibits PT growth (146). *Arabidopsis POP2* encodes for the GABA transaminase responsible for GABA degradation. *pop2-1* mutant flowers exhibit massive GABA accumulation, leading to PT growth arrest and infertility (146). Interestingly, POP2 PTs grow normally in the *pop2-1* pistil, suggesting that PT-expressed POP2 is critical and sufficient in turning over increasing physiological GABA levels in the pistil to pave its path toward the ovules (146).

PTs face the physical hurdle of navigating through ovarian tissues in the solid-type style or the hollow-type style of the extracellular matrix. The perception of these compressive forces is crucial to all plant cells during tip growth (28, 36). Therefore, growing PTs within the pistil need to cope with the external mechanical obstacles of the transmitting tract and exert sufficient penetrative force while reconfiguring their growth behavior (19). The advancing PT apex senses physical barriers and adapts its growth rate using mechanoresponse signaling. This behavior has been termed durotropic growth (163). Therefore, PTs need to overcome physical obstacles as a further compatibility check, and the solid matrices function as a filter for nonfit PTs.

4.2. Pollen Tube Growth

A compatible pollen grain hydrates and initiates germination to penetrate the papillae cell. The PT emergence site is selected and marked by the rapid Ca^{2+} influx at the active site (84), likely by actively targeted secretion involving ROP, ROP-INTERACTIVE PARTNER 1 (RIP1), and exocyst subunit SEC3A (106, 107). To prevent the initiated PT from rupture due to hypoosmotic shock, tight regulation of aquaporins and sphingolipids signaling as well as sensors for mechanical stress are essential (29, 84, 151). Pollen coat lipids and pistil-secreted factors such as sulfinylated azadecalins and brassinosteroids accelerate the germination of the compatible pollen (155, 160, 195).



(*Caption appears on following page*)

Figure 4 (*Figure appears on preceding page*)

Pollen–stigmatic papillae interaction marks the beginning of the pollen tube phase. (*a*) In the reception pathway, upon pollen landing, the pollen coat–deposited cysteine-rich PCP-β/PCP-β protein family mediates the recognition of compatible pollen, likely via interaction with an SLR1/SLG receptor that induces pollen adhesion and stimulates Exo70A1-mediated exocytosis of ACA13 Ca^{2+} ATPase and secretion of cell wall–modifying enzymes. Simultaneously, pollen aquaporins NIP4 and TIP1;5 perceive papillae-secreted solutes and initiate the influx of Ca^{2+} and water for pollen hydration and germination. (*b*) In the self-incompatible rejection pathway, SRKs, closely associated with the MLPK, recognize self pollen by interacting with pollen coat–secreted SCR/SP11. This complex, together with an increase in the papillae cellular Ca^{2+} gradient mediated by GLR Ca^{2+}-permeable gated channels, induces phosphorylation of the ARC1 E3 ligase. This results in Exo70A1 ubiquitination and turnover, leading to the block of stigma secretion and pollen hydration (41). AAs could function as agonists for GLR-mediated Ca^{2+} influx (74). SP11 is absent in the secretome of compatible pollen, and, instead, the SRK interacts with stigma-specific THL1 and prevents ARC1 from phosphorylation. (*c*) An independent mechanism from interspecies incompatibility was recently identified involving SPRI1 and an unknown, likely pollen-secreted ligand. SPRI1 also promotes selfing in self-compatible species (54). Once hydrated, the pollen reactivates its translation machinery and the polarization of the secretory complex to mark an exit pore to initiate pollen tube tip growth. Question marks denote unknown factors. Thicker lines indicate membrane lipid bilayers at active fusion sites. Abbreviations: AAs, amino acids; ACA, Putative calcium-transporting ATPase; ARC1, ARM repeat-containing protein 1; CSN, Constitutive photomorphogenesis 9 signalosome; GLR, Glutamate receptor-like channel; MLPK, M-locus protein kinase; NIP, NOD26-LIKE INTRINSIC PROTEIN 4; PCP, pollen coat proteins; SCR, S-locus cysteine-rich protein (synonym SP11); SLG, S-locus glycoprotein; SLR, SLG-like receptor 1; SP11, S-locus protein 11 (synonym SCR); SPRI1, STIGMATIC PRIVACY 1; SRK, S-locus receptor kinases; TIP, TONOPLAST INTRINSIC PROTEIN. Figure adapted with permission from Reference 64.

Parallel mechanisms, including the secretion of digestive enzymes such as *O*-fucosyltransferase, ceramidase, and extensins, facilitate PT penetration (29, 179).

The female gametes' first-come-first-served policy races the PTs toward an ovule to deliver their precious cargo. The speed of PT extension depends on stored nutrition reserves and differs between bicellular and tricellular pollen species (204). The tip growth of a PT is accomplished through cytoskeleton organization, vesicular trafficking, small GTPases signaling, and ion gradient formation, all fueling cell wall extension while maintaining communication with the surrounding female tissues (147). The tip zone (extension zone) of the PT appears clear due to a lack of amyloplasts and is further divided into apical and subapical zones. The apical zone is typical for its inverted cone shape, in which secretory vesicles accumulate. The subapical zone contains the endoplasmic reticulum, Golgi apparatus, and mitochondria (98). Behind the subapical zone, actin filaments are organized in parallel bundles used as highways for myosin-transported vesicles (182). The cortical filaments transport vesicles toward the tip, whereas the central filaments are dedicated to the basipetal vesicular transport, generating a reversed fountain flow (65). Microtubules, on the other hand, are believed to function in mitochondria (164) and vesicular transport (143) and to serve as potential docking sites for RNA storage particles (77).

The PT extension and distribution of PT organelles also rely on ion fluxes. Ca^{2+} ions and protons (H^+) are effectors implementing intracellular PT signaling response (86, 121). The increasing Ca^{2+} gradient influences the dynamics of secretory vesicles by exocytosis and hence affects PT extension (21). A proton gradient is also formed between an acidic pH of 6.5 at the tip and an alkaline pH of 7.5 at the distal band (49, 65). Together with the externally perceived signals, the ion flux dynamics and the Ca^{2+} oscillations define the dynamics of the cytoskeleton and thus could function as a pacemaker for PT growth rate (120) facilitated by massive secretion activities of new cell wall materials and signaling molecules undertaken by the exocyst complex and unconventional protein secretion (65, 161). There, signaling phospholipids have been proposed to

mark the microdomain region of the PT plasma membrane as a new docking site for the exocyst complex and deposition of cell wall precursors (10).

Sequestrome: the population of stored mRNAs repressed from immediate translation in a temporary manner

Monosomes: preassembled large and small ribosome subunits predominantly engaged at the mRNA translation initiation site

4.3. The Mobilization of Reserves: mRNA Storage and Translation Activation

It is well established that MG development is under the control of two subsequent developmental programs, early and late, accompanied by stage-specific gene expression patterns (reviewed in 52, 170). Follow-up translatome (110) and sequestrome (68) studies highlighted that the late developmental program is tightly linked with posttranscriptional regulation of gene expression and mRNA storage (**Figure 5**).

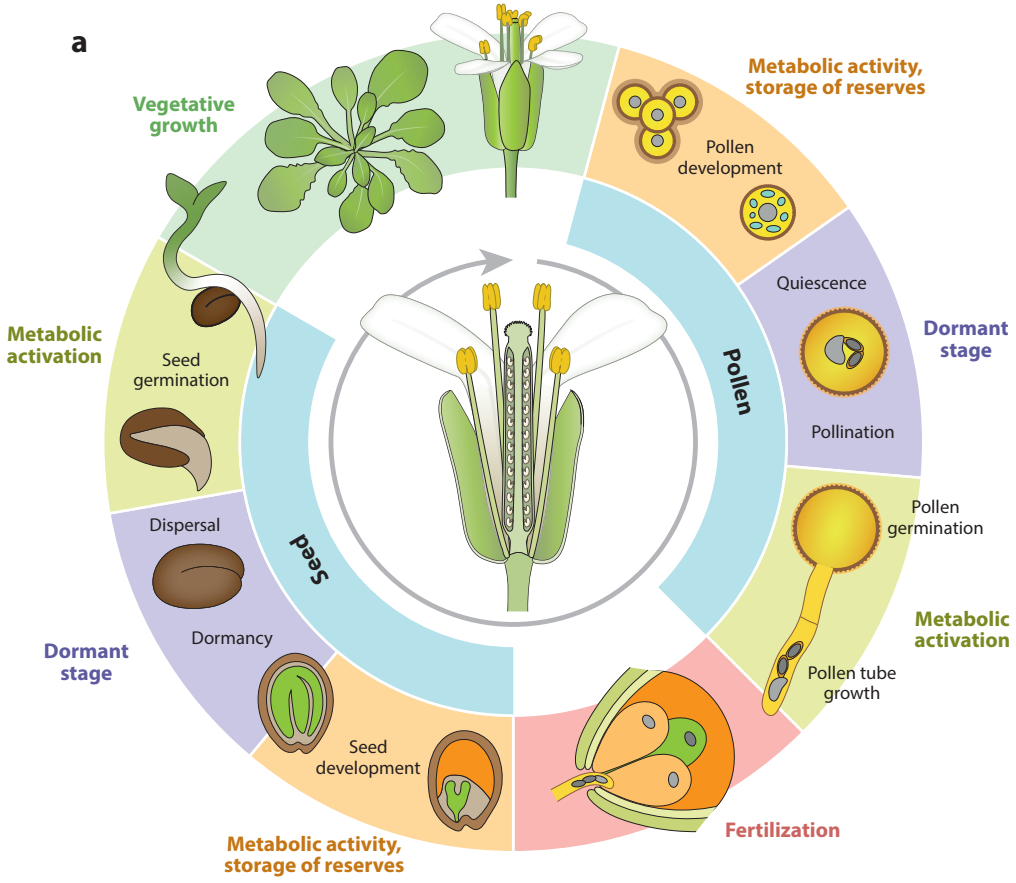
In many model systems, translation was identified as an important regulatory checkpoint under both environmental and developmental control (192). Selective mRNA storage and activation were also observed during tobacco MG development (76), together with the translational repression of stable, pollen-specific transcripts that are stored long-term. The pollen-sequestered transcripts are stored in a novel type of nontranslated monosome (68), previously termed EDTA/puromycin-resistant particles (EPPs) (77) (**Figure 5**). These monosomal particles are associated with the cytoskeleton and are thus likely to be transported toward the tip of the growing PT for tip-localized translation (68, 77). Structural studies should illuminate the nature of sequestered monosomes and how they are formed; however, it is plausible that factors determining the formation of stress granules (118, 119) or processing bodies (173) play this role. Similarly, RNA-binding proteins, classified in several families, were repeatedly identified in association with nontranslating monosomes. These comprised translation initiation factors and elongation factor EF-1A as well as PUM and ALBA proteins (68). Finally, phosphorylation and dephosphorylation likely play a role during translation reactivation as several initiation factors, including eIF4G, eIF4B, and eIF5B, are dephosphorylated upon pollen activation (51).

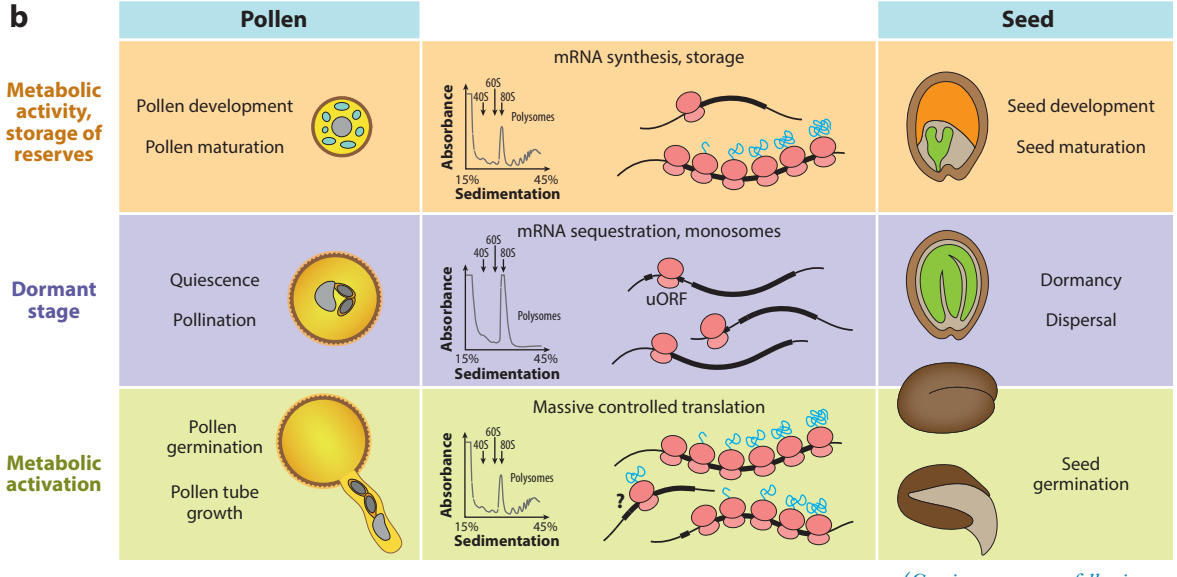
Recently, *Arabidopsis* seed translatomic studies also revealed and characterized nontranslating monosomes as the actual sites of mRNA storage in seeds (3, 5). The evolutionary origin and biological functions of pollen and seeds are different. However, both these entities serve as dispersal units and share analogies in their biology, including an alternate desiccated dormant phase followed by a rapid metabolic activation (144). Both PT growth and seed germination are demanding processes in terms of reserve mobilization, protein synthesis, and formation of new structures. One such mechanism is the regulation of mRNA storage and massive translation activation upon hydration. Recent discoveries showed noteworthy similarities in this regulatory pathway in both pollen (68) and seeds (3, 171, 172) (**Figure 5**).

Strikingly, this role of nontranslating monosomes particularly resembles that of the growing human neuronal axons; therefore, it is not surprising that axons and PTs share more than remarkable mechanistic growth similarities (147). The directional growth in neurons is facilitated by the transport of sequestered mRNAs by neuronal granules to the synaptic surfaces for translation. Neuronal granules are also preloaded with translational machinery and represent the mediators of nerve cell networking, depositing transcripts to the growing tip and catalyzing their efficient translation, thereby promoting directional growth (93). It is very likely that some of the monosome-associated mRNAs might be immediately translated by monosomes to fast-track translation, as was recently shown for the subpopulation of synaptic mRNAs in humans (9).

4.4. Pollen Tube Guidance and Male–Female Communication

Sperm cells of many gymnosperms and all angiosperms lost their motility and solely rely on PTs as carrier cells to reach the female gametophyte. In *Arabidopsis*, this is a few millimeters' journey,





(*Caption appears on following page*)

Figure 5 (*Figure appears on preceding page*)

Translational and posttranslational regulation in the male gametophyte, including the scheme and players involved. (*a*) Simplified angiosperm life cycle highlighting the similar phases of pollen and seed development, including development, quiescence/dormancy, and activation, that are reflected in the accumulation, storage, and activation of mRNA reserves. (*b*) Transcriptome and proteome analyses revealed that pollen and the pollen tube sequestrome comprised diverse mRNA populations that are highly dynamic between the actively translating polysomes and the sequestered monosome subfractions. Indeed, the monitoring of selected mRNAs encoding pollen tube cell wall proteins demonstrated a progressive shift in the majority of these transcripts from the monosomes to polysome fractions upon pollen activation (68). mRNA storage, activation, and translation, in general, are facilitated by numerous RBPs (24); this is also the case in the male gametophyte (68, 77, 110) and in seeds (3, 172). RBPs often bind *cis*-elements of the regulated mRNA sequences, namely their 5′ UTRs, including uORFs. Interestingly, highly similar sequence motifs were identified in 5′ UTRs of monosome-associated stored transcripts in pollen (68) and seeds (3, 4). Therefore, in pollen tubes and perhaps in other tip-growing cell types, the mRNA-bound monosomes likely represent the sequestrome pool of stored mRNAs. The question mark denotes hypothetical translational activation of a specific subtype of monosome-bound mRNA. Abbreviations: mRNA, messenger RNA; RBP, RNA-binding protein; uORF, upstream open reading frame; UTR, untranslated region.

but it can stretch up to 30 cm in maize. Along the route, PTs intensively communicate with the maternal tissues of the stigma, style, and transmitting tract, and they eventually reach the ovule in a phase referred to as preovular guidance. Thereafter, female gametophytic cells, predominantly the synergid cells, take over the communication and guide the PT through the micropyle entry of the ovule in a process referred to as ovular guidance (111).

4.4.1. Pollen tube preovular guidance. The preovular guidance of the PT in the transmitting tract is not unidirectional, and the female gametophyte is unnecessary for PT growth toward the ovule (124). It is suspected at this phase, PTs are kept on track by the physical barriers of the transmitting tissues, adhesin proteins, and chemotropic signals (27). For instance, TRANSMITTING TRACT–SPECIFIC (TTS) glycoproteins TTS1 and TTS2 from *Nicotiana tabacum*, GABA, and D-serine all stimulate PT growth in vitro in a concentration-dependent manner (32, 146). To prevent PTs from wandering, style-originating adhesion molecules such as pectins, STIGMA CYSTEINE-RICH ADHESIN (SCA), and SCA-like lipid transfer protein 5 (LTP5) adhere the PTs tightly to the extracellular matrix (124, 125, 148). Competent PTs then exit the transmitting tract and bend on the funiculus toward the micropylar entry (**Figure 6**). LURE peptides (see Section 4.4.2) accumulate at the base of the funiculus and serve as a possible signal for an abrupt turn and exit from the transmitting tract (186).

PT progression down the style is accompanied by transmitting tract programmed cell death (PCD). Mutants defective in transmitting tract PCD, *ntt* and *hafbee1bee3*, fail to set seeds on the bottom half of the pistil, suggesting difficulties in PT penetration of the transmitting tract (34). It remains unclear whether the transmitting tract is essential for PT guidance; however, ongoing effort to isolate ligands and receptors within the transmitting tract might clarify the role of the transmitting tract in preovular guidance.

4.4.2. Pollen tube ovular guidance. At the proximity of the ovule, ovular guidance takes over the PT reorientation from the transmitting tract toward the micropylar entry. The ovular guidance is subdivided into two phases: funicular and micropylar guidance. No apparent factors have been isolated to exclusively affect the funicular guidance; however, the double mutation of two mitogen-activated protein kinases, *mpk3 mpk6*, results in severe defects in funicular guidance (60). In contrast, the role of micropylar guidance has been extensively dissected, and several ligand-receptor mechanisms have been elaborated.

At first, an R2R3-MYB TF, MYB98, specifically expressed in synergids, switches on the expression and eventual secretion of different groups of cysteine-rich proteins (CRPs) (157). Of them, particularly synergid-specific LURE1 and LURE3 possess PT attraction activities (142)

Micropyle: a small opening in discontinuous ovule integuments serving as a pollen tube entry point

Funiculus: filamentous tissue connecting ovules with the placenta in the ovary

Pollen tube attraction (PT attraction): the growth of a pollen tube toward the embryo sac guided by female-secreted chemoattractants

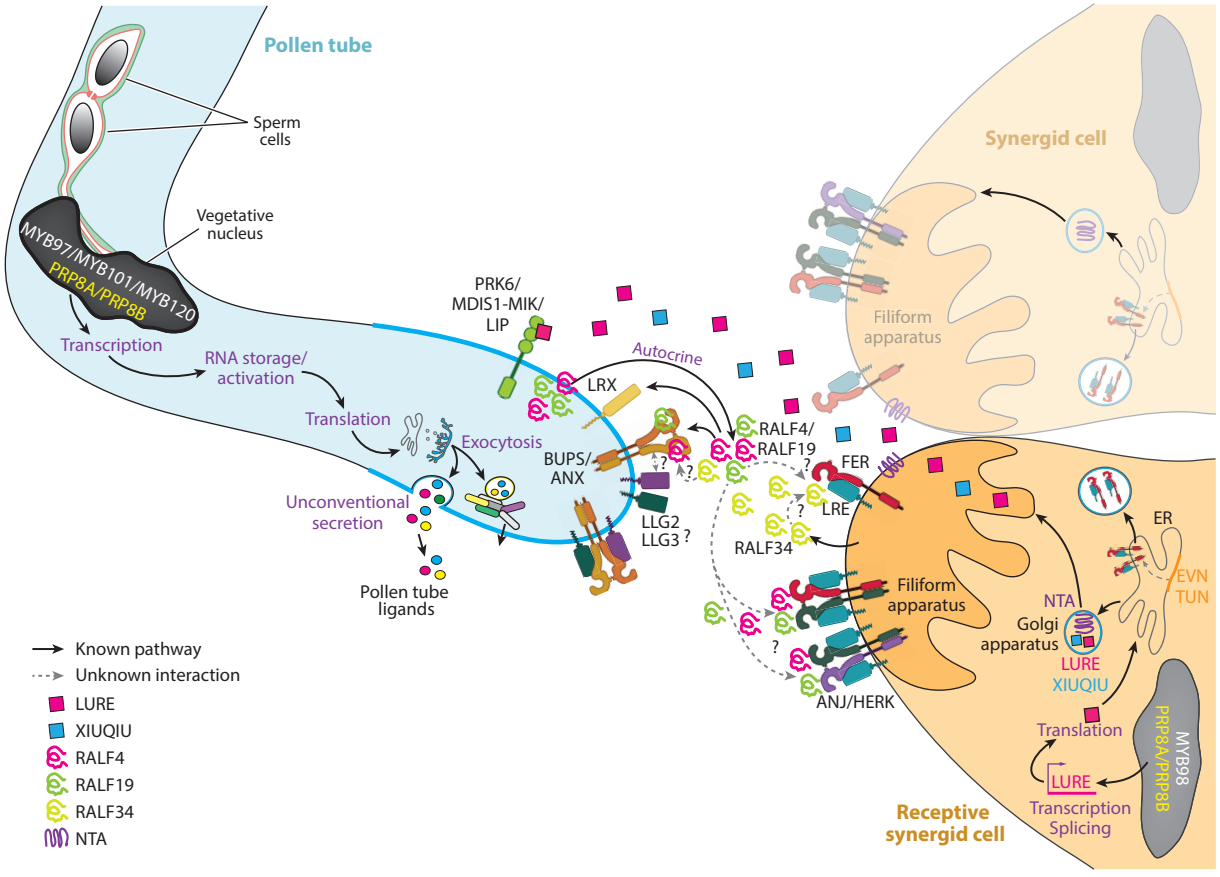


Figure 6

Pollen tube ovular guidance and attraction by the receptive synergid cell (*bottom*, *in darker colors*). MYB98 TF and PRP8 splicing factors promote the expression of PT-attracting CRPs. ER-localized EVN and TUN are likely involved in the *N*-glycosylation of synergid-secreted proteins. To exit the transmitting tract, species-non-specific CRPs, XIUQIUs, attract the PT to emerge from the septum into the funiculus. Once emerged, species-specific CRPs, LUREs, function as a gametophytic barrier and attract only PTs of the same species. LUREs are perceived by PT PRK6/MDIS1-MIK/LIP receptors. As the PT approaches, the FER-LRE-ANJ/HERK1 complex functions as a coreceptor to perceive a yet-unknown ligand. The putative ligands could be the synergid-secreted RALFs, RALF34 or others. LRE-FER signaling induced by a PT-derived ligand promotes the relocalization of NTA from the synergid endomembrane into its plasma membrane. In the PT, MYB97, MYB101, MYB120, and PRP8 prime the PT reception by the synergid cell. To prevent PT premature bursting, the RALF14/RALF19/ANX/BUPS complex, LRXs, and MSL8 impose an autocrine homeostasis control over the PT cell wall. Once correctly received by the receptive synergid cell, synergid-secreted RALF34 outcompetes RALF4/RALF19 to induce PT burst and sperm cell release inside the receptive synergid cell at the proximity of the egg cell and the central cell for gamete fusion. Thicker blue line indicates the membrane lipid bilayer at the active fusion site.
Abbreviations: ANJ, ANJEA; ANX, ANXUR; BUPS, BUDDHA'S PAPER SEAL; CRP, cysteine-rich protein; ER, endoplasmic reticulum; EVN, EVAN; FER, FERONIA; HERK, HERCULES; LIP, LOST IN POLLEN TUBE GUIDANCE; LLG, LORELEI-LIKE GPI-ANCHORED PROTEIN; LRE, LORELEI; LRX, LEUCINE-RICH REPEAT EXTENSIN; MDIS1, MALE DISCOVERER 1; MIK, MDIS1-INTERACTING RECEPTOR-LIKE KINASE; MSL8, MECHANOSENSITIVE-LIKE CHANNEL 8; NTA, NORTIA; PRK6, POLLEN RECEPTOR-LIKE KINASE 6; PT, pollen tube; RALF, RAPID ALKALINIZATION FACTOR; TF, transcription factor; TUN, TURAN. Figure adapted with permission from Reference 86.

Filiform apparatus (FA): the fibrous membrane-enriched cell wall modification at the micropyle end of synergids responsible for the secretion of pollen tube chemoattractants

(**Figure 6**). To perceive LURE1 attraction signals, PT-specific plasma membrane–localized Leucine-rich repeat receptor-like protein kinases (LRR-RLKs), including PRK6, MALE DISCOVERER 1 (MDIS1) and MDIS2, and MIDS-INTERACTING RLK 1 (MIK1) and MIK2, directly interact with LURE1 peptides (187, 200). Additionally, *Arabidopsis* central cell and maize egg apparatus also participate in micropylar control of PT attraction. *Arabidopsis* CENTRAL CELL GUIDANCE (CCG) interacts with CCG BINDING PROTEIN 1 (CBP1) and regulates the expression of synergid as well as several central cell–expressed CRPs to promote PT micropylar guidance (30, 105). This implies the existence of a mobile signal coordinating central cell- and synergid-mediated PT attraction activities (**Figure 6**).

Recently, the precursor-mRNA-processing-SPLICING FACTOR 8, PRP8A/PRP8B, was shown to be a potential upstream regulator of PT attraction and PT navigation (95). In *prp8a prp8b* double mutant ovules, synergid cell fate is lost, as indicated by the loss of expression of MYB98, LURE1.1–1.5, XIUQIU, and several other CRPs from the central cell, resulting in abolished PT attraction (95) (**Figure 6**). On successful PT attraction and micropyle penetration, the PT reception module takes over as a checkpoint of the gametophyte species-specific prehybridization barrier.

4.4.3. Pollen tube reception. Inside the micropyle, the first contact with the receptive synergid arrests further PT growth. The receptive synergid undergoes oxidative degeneration and the PT ruptures to release the two sperm cells. The events leading to the culmination burst of a PT are precisely controlled by continuous communication between the approaching PT and the female reproductive tissues. A family of CRPs, RAPID ALKALINIZATION FACTORs (RALFs), and their receptors from the *Catharanthus roseus* RLK1-LIKE (CrRLK1L) family play an essential role in PT reception (57, 102, 108). FERONIA (FER)/SIRENE is the first female receptor from the CrRLK1L family to perceive PT arrival (47, 81). FER localizes at the filiform apparatus (FA), and *fer* ovules exhibit PT overgrowth, suggesting that FER is critical for PT growth arrest (166). RALFs act as FER ligands, and the RALF-FER complex has been inferred to be involved in several regulatory processes. RALF1 regulates root expansion (70), whereas fungus-secreted RALF23 controls plant immune signaling after fungal infection (183). On the contrary, no PT-secreted RALF has yet been identified to bind FER and to regulate PT reception, although this interaction is the most likely to play a role (**Figure 6**).

Nevertheless, triple mutants of pollen-specific *myb97 myb101 myb120* TFs exhibit a PT overgrowth phenotype reminiscent of a *fer* mutant. The MYB97/MYB101/MYB120 complex, therefore, might act upstream of putative PT-secreted FER ligands (101, 109) (**Figure 6**). In synergids, FER interacts with a glycosylphosphatidylinositol (GPI)-anchored protein, LORELEI (LRE) (103). LRE–FER interaction is critical for the relocalization of FER and a MILDEW RESISTANCE LOCUS O (MLO) protein NORTIA from the endoplasmic reticulum to the FA that is necessary for Ca^{2+} spiking during PT reception (90).

Successful PT reception culminates the epic PT journey and triggers a double suicide of the PT and the receptive synergid cell that further facilitates double fertilization, a unique feature of flowering plants.

5. THE DOUBLE FERTILIZATION MYSTERY

During double fertilization, two dimorphic female reproductive cells (the egg cell and central cell) within the embryo sac are fertilized by the two sperm cells to produce a diploid zygote and a triploid nutritive endosperm (recently reviewed in 71). Exceptionally, in a few orchid species and Podostemaceae (Malpighiales), only single fertilization occurs between a sperm cell and an egg

cell to generate a diploid embryo; the second sperm cell is either not formed or it disintegrates (176).

Plasmogamy: the fusion of the cytoplasm of two gametes bringing together two compatible haploid nuclei

Polytubey: the attraction of more than one pollen tube by a single ovule

Heterofertilization: the fertilization of two female gametes within an ovule by genetically different sperm cells delivered by two pollen tubes

5.1. Sperm Cells Ejection and Repositioning

Upon successful PT reception, the PT bursts next to the receptive synergid and ejects two sperm cells into the proximity of the female gametes. This process is regulated by a PT-specific CrRLK1L receptor complex and its dynamic interaction with regulatory RALF peptides. Two pollen-specific pairs of CrRLK1Ls, ANXUR1 (ANX1) and ANX2 (12, 123), and BUDDHA'S PAPER SEAL1 (BUPS1) and BUPS2 (56) form a heteromeric receptor complex ANX-BUPS that regulates PT integrity (**Figure 6**). *anx1 anx2* double mutant PTs rupture prematurely in vitro and fail to set seeds in vivo (12, 123). During the PT journey, RALF4/RALF19 peptides bind the ANX-BUP complex in an autocrine fashion and keep the PT intact before its reception (56). Double mutant *ralf4 ralf19* PTs rupture prematurely, reminiscent of *bups1 bups2* and *anx1 anx2* double mutant phenotypes. A set of coreceptors, PT-specific LORELEI-like GPI-anchored proteins 2 (LLG2) and LLG3, interact with BUPS and ANX to perceive RALFs and maintain PT integrity (57). The arrest of the PT growth at the receptive synergid then induces a female-specific secretion of RALF34 that antagonizes RALF4/RALF19, binding to the BUPS/ANX receptor complex, and RALF34-BUPS/ANX culminate the PT rupture to eject the sperm cells (56) (**Figure 6**).

Once sperm cells are ejected, two mechanisms are proposed to lead to the repositioning of sperm cells prior to their activation and fusion. Each sperm cell is capable of fertilizing either female gamete (69). Minutes after their discharge, each sperm cell adheres to one female gamete and assembles and relocalizes the cell fusion machinery into close proximity to the plasma membrane (86). If the two sperm cells inadvertently become associated with the same female gamete, their positions are readjusted, suggesting an adhesion-induced communication between sperm cells pairs and the sperm–egg–central cell trio (80, 180). Preferentially, sperm–egg plasmogamy takes place first (69). This short pause suggests possible intercellular signaling to coordinate the two gamete fusion events (180) (**Figure 7**).

Successful gamete fusion triggers the degeneration of the second synergid to prevent polytubey (86). This process involves arabinogalactan protein 4, JAGGER (149). In a situation where one or both gamete fusions fail, the persisting synergid attracts a second PT for the delivery of additional sperm cells, a phenomenon known as fertilization recovery, which also results in heterofertilization (7, 87).

5.2. Sperm Cell Activation and Gamete Fusion

Since two sperm cells are attached and fused independently with the female gametes, sperm activation refers to the membrane separation of the male germ unit to release the pair and to the gaining of fusion competence. The only molecules that have been directly linked with sperm activation are the *Arabidopsis* egg-secreted EC1 CRPs (181). EC1 proteins are retained on secretory vesicles and are only released via exocytosis upon the sperm cells' arrival (181) (**Figure 7**). Mutant *ec1* ovules are defective in sperm cell fusion, resulting in unfertilized ovules (12, 180, 181). A search for an EC1 receptor in sperm cells is currently ongoing.

Close similarities across organisms were drawn based on the structurally conserved molecules responsible for gamete fusion. GAMETE-EXPRESSED 2 (GEX2) (46, 127) and HAPLESS 2 [HAP2; also known as GENERATIVE CELL-SPECIFIC 1 (GCS1)] (198) are sperm cell–specific transmembrane proteins involved in sperm cell attachment and fusion (48). Recently, two other sperm cell–specific DUF679 membrane fusogen proteins, DMP8 and DMP9, were identified to

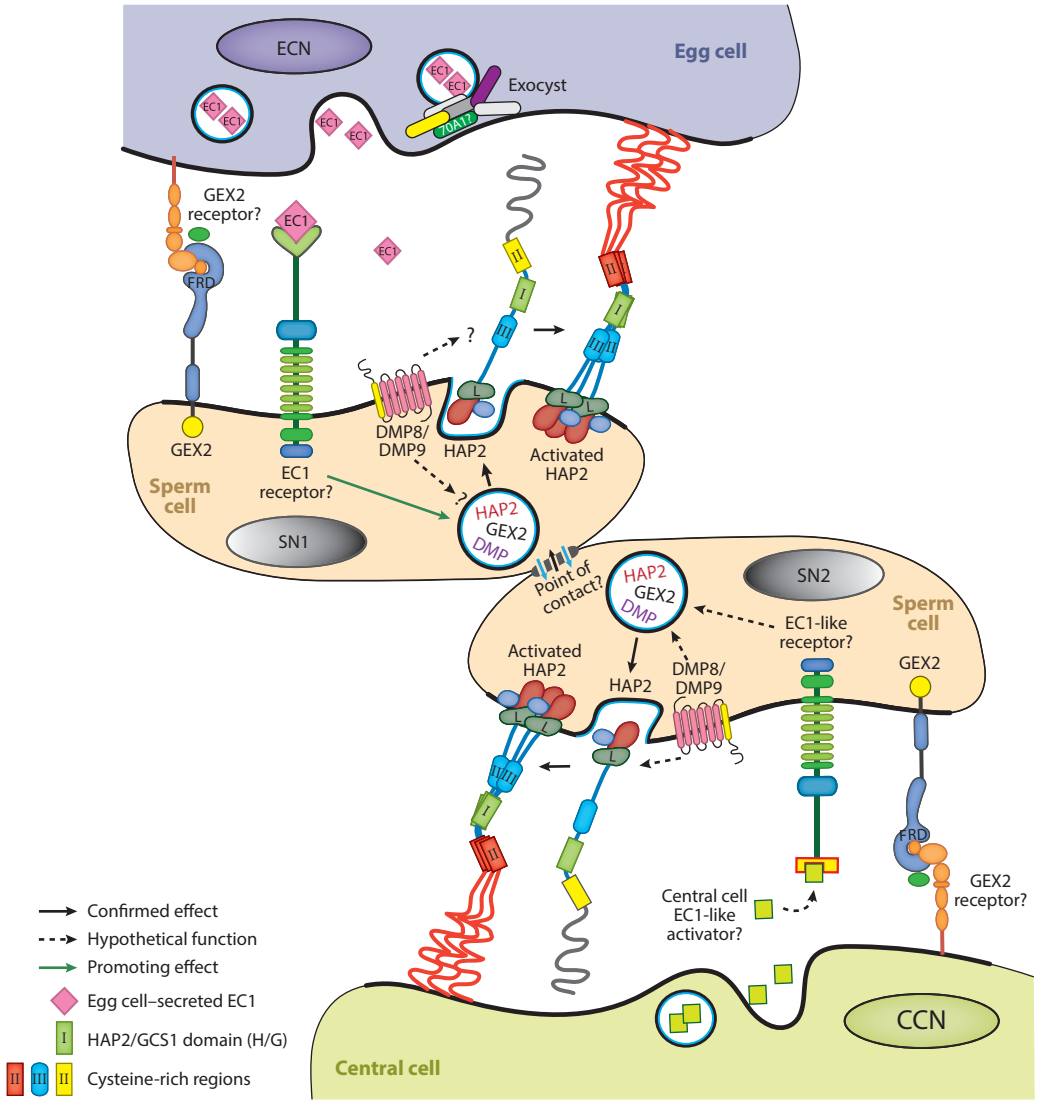


Figure 7

Gamete double fertilization in *Arabidopsis*. Known (and proposed) proteins with a direct role in gamete fusion are outlined. Once ejected by the pollen tube, the sperm cell pair reside at the egg–central cell interphase and make membrane contact. Sperm cell arrival triggers exocytosis of EC1 peptides from the egg cell perceived by an unknown EC1 sperm cell receptor (181). Sperm-specific GEX2 then makes membrane contact and promotes membrane adhesion via an unknown egg cell receptor. These events coincide with the further relocalization of an evolutionarily conserved fusogen HAP2 protein from the endomembrane to the sperm cell plasma membrane. To facilitate gamete fusion, DMP8 and DMP9 likely assist with efficient HAP2 plasma membrane localization, activation (homotrimerization), and possible selection of an HAP2 docking site as a membrane fusion–competent site (35, 48). In the central cell, a similar EC1-like peptide likely triggers a second fusion cycle of sperm–central cell plasma membranes. Readjustment of sperm cell positioning upon association with only one female gamete suggests a contact-induced communication between the two sperm cells. A membrane connection between the sperm cell pair might allow this symplastic communication to coordinate gamete fusions. Thicker lines indicate membrane lipid bilayers at active fusion sites. Abbreviations: CCN, central cell nuclei; EC1, EGG CELL 1; ECN, egg cell nuclei; FRD, two filamin repeat domains; GEX2, GAMETE EXPRESSED 2; HAP2, HAPLESS 2; L, HAP2 linker stretch; SN, sperm cell nucleus. Figure created using data from Reference 180.

facilitate gamete fusion, with a greater effect on sperm–egg fusion than on sperm–central cell fusion (35) (**Figure 7**).

HAP2 is structurally homologous to viral class II fusion proteins and is similar to somatic cell fusogen EFF-1 of *Caenorhabditis elegans* (35, 48). It belongs to a broadly conserved protein family essential for gamete fusion across many organisms, except fungi (48). However, unlike EFF-1, HAP2 proteins and viral class II fusion proteins possess a hydrophobic fusion loop and function unidirectionally in all organisms examined by actively bridging two membranes approximately 10-nm apart (33). HAP2 and DMP8/DMP9 likely function independently, as HAP2-YFP localization is not impacted in *dmp8 dmp9* mutants, but both are essential for sperm cell fusion (35). It has been proposed that DMPs could contribute to influence membrane curvature and promote the action of HAP2 during the merging of the lipid bilayer (35) (**Figure 7**). An equivalent GEX2 structural insight has yet to be reported.

5.3. Polyspermy Block

To prevent an egg cell from fusing with multiple sperm cells (polyspermy), the persistent synergid imposes a polytubey block by cutting off the secretion of PT attractants (7, 130). Nevertheless, up to three PTs have been observed in about 3% of ovules in self-pollinated *Arabidopsis* pistils, but no polyspermy has been reported in angiosperms (7). In humans, after a single sperm cell fusion with the egg cell, a rapid membrane depolarization takes place, and a wall is formed around the zygote to prevent polyspermy (206). In *Arabidopsis*, simultaneous spikes in cytosolic Ca^{2+} in both newly fused sperm and egg cells were observed (38). Whether this Ca^{2+} oscillation is associated with membrane depolarization and polyspermy block remains to be established. Recently, FER was proposed to prevent polytubey by nitrosating LURE1 to prevent its secretion and interaction with its receptor (43). In some fish species, the diameter of the aperture in funnel-shaped micropyles restricts the number of entering sperms across the chorion toward the oocyte plasma membrane, and thus prevents multiple sperm entry (32). It will be interesting to establish whether the micropyle opening of flowering plants could physically prevent multiple PT entry, as observed in other organisms.

5.4. Gamete-Omics to Identify New Players

The fast progress that has been achieved in recent years in understanding essential PT–pistil signaling factors, activators, and fusogen molecules for double fertilization was fast-tracked by multi-omics approaches that facilitated reverse genetic studies. However, efforts have been hampered on the MG due to the inaccessibility of the PTs within the pistil (196). New approaches in capturing PT-secreted proteins following interaction with female reproductive tissues will facilitate the fast identification of equivalent male factors required for PT–synergid communication (66, 67). Genetic tools exist that are invaluable on the quest to isolate new players for cell–cell communication. Similar to the exploration of the *myb98* mutant that paved a path to the identification of synergid cell-specific guidance factors (88), *myb97 myb101 myb120* triple mutants can be utilized to uncover male-specific factors of PT guidance (109). Similarly, a double mutant of a spliceosome subunit, *prp8a;b*, which functions as a potential upstream regulator of PT guidance of both the male and female gametophyte, could facilitate isolation of male–female guidance molecules (95).

On molecular factors for double fertilization, the 4–10 min lag time from sperm discharge to gamete fusion provides an ample opportunity window for all-in-one gamete multi-omics profiling to explore translation-secretion-activation pathways and novel fusogen-mediating factors that play a key role in gamete fusion. Identification of the EC1 family of proteins, for instance, was initially

a result of an EST screen specific to the *Arabidopsis* egg cell, and they were later isolated in rice, maize, tobacco, and lately in *Amborella trichopoda*, the most ancient angiosperm (53). Moreover, achievement by multi-omics will empower structural and crystal structure studies to resolve the respective adherens' and fusogens' exact mechanisms (48).

6. WHERE WE GO FROM HERE: CONCLUSIONS AND SPECULATIONS

The evolution of the multitasking MG to modern angiosperms has economized plant resources, constructed new structures, and adapted to the female guidance avenues to securely deliver sperm cells. The innovation of these new structures, for instance, the PT, also led to the reorganization and specialization of the core cellular machinery to fulfill the demands for the new structures. We may speculate that to fast-track the translation of stored mRNAs in a rapidly extending PT tip, the translation machinery in PTs uses single, prebound monosomes to scan and translate a subpopulation of mRNAs and to bypass the arrangement of polysomes. Future research on these hypotheses will better emphasize how the multitasking role of the MG has been perfected over time, particularly to adapt with the fast-extending PT tip. The dominance of angiosperms has brought about high nutritional, agronomical, and economical values across the world. However, we have only started to dissect the complex molecular mechanisms of pollen development, pollen–pistil interaction, and gamete fusion in flowering plants, particularly in an evolutionary context. Therefore, exploration of the genetic toolbox in combination with fast-developing high resolution imaging, single cell-specific multi-omics, and synthetic biochemistry will unravel the communication network and the adapted mechanisms for successful fertilization in modern angiosperms.

SUMMARY POINTS

1. The ontogenic development of the male gametophyte is accompanied by oscillatory phases of gene expression, with the early phase governing spore development and formation of the male germline to produce sperm cells and the later phase predominantly driving pollen desiccation and supporting nearly the entire progamic phase that involves pollen tube growth and sperm cells delivery for double fertilization.
2. Recent transcriptome coverage of the male gametophyte by RNA-sequencing (RNA-seq) has led to the identification of a number of previously unidentified expressed genes and noncoding RNAs that likely fine-tune the function of the male gametophyte pre- and post-progamic phase. A generation of traceable knocked-in insertion alleles created using CRISPR-Cas9 technology will empower pollen research and build upon the already expanding understanding of the multitasking role of the male gametophyte.
3. The second phase of gene expression is able to support the progamic phase via the translation repression and mRNA storage phenomena that are reactivated upon pollen hydration.
4. Isolation of *cis*-acting factors that play a direct role in mRNA fate and translatability, including splicing regulators, translation initiation subunits, and chaperone mRNA-binding proteins, will facilitate better insight on how the fueling of a fast-growing pollen tube is made possible.
5. The many checkpoints of pollen tube growth through the female gametophyte set control the recognition of self from nonself pollen and trigger reception or rejection

pathways to prevent genome cross-hybridization. A new frontier using exactly the natural checkpoint mechanisms (sporophytic or recently emerging gametophytic barriers) for incompatible pollen/pollen tube rejection to engineer interspecies cross-hybridization offers hope to develop new variants that could be well adapted with the changing environment.

6. How posttranslational modifications [phosphorylation, nitrosylation, glycosylation, glycosylphosphatidylinositol (GPI)-anchoring] and the extracellular matrix environment shape protein function—for instance, disulfide bonds that fold secreted cysteine-rich proteins—is an entirely new chapter to be unraveled in controlling cell–cell communication during pollen–pistil interaction.
7. To date, only EC1 and HAP2 have been recognized as major molecular players in gamete fusion. The recent identification of DMP8/DMP9 proteins as associates of EC1 during sperm–egg cell membrane fusion suggests the existence of other key factors and likely serves as a last defense barrier preventing interspecies hybridization.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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