

ARTICULO INVITADO

PLANT SEXUALITY, CELL EXPRESSION AND PREFERENTIAL
FERTILIZATION¹

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Summary: Flowering plant sexuality in its strictest sense is limited to specific gametophytic cells—two male gametes (sperm cells) in the pollen (male gametophyte) and two female gametes (egg and central cells) in the embryo sac (female gametophyte). Somatic cells—including the pollen vegetative cell that provides translocation and nutritional support for male gametes, and embryo sac cells that receive sperm cells and mediate fusion events—are greatly reduced in the gametophytes. These gametes ultimately participate in genetic rearrangement during double fertilization: one sperm and the egg combine to form the typically diploid zygote, whereas the other sperm fuses with the polar nuclei in the central cell to form the nutritive endosperm. Gamete formation is initiated by a coordinated pattern of development that ultimately differentiates sexual lineages in the male and female gametophyte. In some plants, there is further gametic specialization in the male that is coordinated with preferential fertilization. Studies of preferentiality in sperm cells indicate that male gametes may express differences on a genic level. Perhaps some of the unexpected diversity of the *Arabidopsis* genome, which approaches the number of genes proposed for man, resides in the remarkable sexual life cycle that flowering plants possess.

Key words: flowering plant sexuality, male gametophyte, female gametophyte, preferential fertilization.

Resumen: Sexualidad vegetal, expresión celular y fertilización preferencial. La sexualidad de las plantas con flores, en su sentido más estricto, está limitada a células gametofíticas específicas —dos gametas masculinas (células espermáticas) en el polen (gametófito masculino) y dos gametas femeninas (la oosfera y la célula media) en el saco embrionario (gametófito femenino). Las células somáticas —incluyendo la célula vegetativa del polen que provee translocación y apoyo nutricional a las gametas masculinas, y las células del saco embrionario que reciben a las células espermáticas y que intervienen en los eventos de fusión—están muy reducidas en los gametófitos. Estas gametas finalmente participan en rearrreglos genéticos durante la doble fertilización: una célula espermática y la oosfera se combinan para formar el cigoto típicamente diploide, mientras que la otra célula espermática se fusiona con los núcleos polares de la célula media para formar el endosperma nutritivo. La formación de gametas es iniciada por un patrón de desarrollo coordinado que finalmente diferencia los linajes sexuales en los gametófitos masculino y femenino. En algunos vegetales, hay una especialización gamética masculina adicional que está coordinada con una fertilización preferencial. Estudios de la preferencia en las células espermáticas indican que las gametas masculinas pueden expresar diferencias a nivel génico. Tal vez, parte de la inesperada diversidad del genoma de *Arabidopsis*, que se aproxima al número de genes propuestos para el hombre, reside en el notable ciclo de vida sexual que poseen las plantas con flores.

Palabras clave: sexualidad de las plantas con flores, gametófito masculino, gametófito femenino, fertilización preferencial.

Just 310 years ago, Rudolph Camerarius (1694) revealed in his *De Sexu Plantarum Epistola* that sexuality occurred in plants, identifying stamens and pistils as male and female organs, and the pollen as a fertilizing agent. Later discoveries by Amici and

Hofmeister established an early understanding of pollen tubes as the structures through which the pollen delivered the male component. This was later extended with the discovery of fertilization (fusion of one sperm with an egg) and the discovery of double fertilization (an accompanying fusion of the second sperm with the central cell [and sperm nucleus with polar nuclei]). The concept of double fertilization was accepted quite rapidly (Sargent, 1900) with Nawashin's report entering the literature in 1898 and having clear priority over Guinard's report (1899),

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which cites Nawashin (1898). The nature of the second fusion has increasingly become regarded as having descended from a separate fertilization event that has been dramatically modified in form and function from its original significance and outcome (Friedman, 1995).

In later contributions on double fertilization, Nawashin postulated that the two sperm cells seemed visibly different, but Maheshwari's codifying work, *An Introduction to the Embryology of Angiosperms*, dismissed this possibility (Maheshwari, 1950). No credible work confirmed this observation at the time, and the randomly-oriented sperm cells in paraffin sections were hardly compelling. The high expansion coefficient of paraffin, combined with fixation methods that denatured, extracted and often coagulated cell contents, made the reliability of these observations dubious. Confirmation of sperm dimorphism did not come for many years, until nuclear markers (Roman, 1948) and cytoplasmic markers (Russell, 1984) were separately uncovered. Viewing this research from a more modern perspective indicates that sexual reproduction in flowering plants has advanced beyond myriad other biological systems in terms of its subtlety and precision.

Sexuality, although associated with pollination, is actually a fairly late phenomenon in the process of reproduction in flowering plants. Pollen is clearly sexual, in its production of the two male gametes, but the process of their transport and the germination and elongation of the pollen tube in the style is hardly sexual. The complex interaction between pollen, stigma and transmitting tissue occurs between vegetative gametophytic tissue (pollen tube) and nutritive sporophytic tissue (gynoecium). Only the male and female gametes are truly sexual. In seed plants, the pollen (microgametophyte) and the embryo sac (megagametophyte) are entirely separate organisms, and it is increasingly evident that they produce signals that trigger complementary behavior and attraction (Lord & Russell, 2002; Weterings & Russell, 2004). Similarly, there is emerging evidence of complexity in the transcriptome of both the vegetative pollen (Becker *et al.*, 2003; Honys & Twell, 2003) and sperm cells (Engel *et al.*, 2003), as well. Comparable surveys of the transcriptome of the embryo sac and egg cells, presumably a more challenging goal because they require microdissection, are yet to emerge.

As a product of meiosis, each pollen grain of each plant is essentially assured being a unique individual because of independent sorting of chromosomes at anaphase I and recombination through crossing over during mid-prophase I. The creation of the pollen and the embryo sac are thus sexless acts of the sporophyte, as are at least initially their meiotic

products, the spores. It is only once the spores have divided that differentiation of the gametes may proceed and the gametophytes (during microgametogenesis and megagametogenesis) enter a truly sexual phase (Figures 1 & 2). Although the gametophytes are highly reduced organisms—the male gametophytes consist of only three cells and the female gametophytes usually consist of typically 7-cells and 8-nuclei—there is a distinction between vegetative and reproductive tissues. In the male gametophyte, the reproductive cells are the sperm cells, which separate from the vegetative lineage with the formation of their predecessor, the generative cell.

In the female gametophyte, the reproductive cells are the central cell, with its polar nuclei, and the egg cell. Interestingly, the sister polar nuclei derive from early divisions of the megaspore (or from metaphase I in the case of megaspores resulting from more than one meiotic product—the traditional bisporic and tetrasporic types). The egg cell and one polar nucleus, however, appear to be immediate sisters (Russell, 1993)—and may be termed properly as gametic and therefore sexual (see Fig. 2). The complementary division of the chalazal nucleus results in a totally vegetative lineage, except for the polar nucleus (or in other embryo sac types, nuclei) that may be subsequently formed. The clearly sexual unit is the one that forms the egg and the central cell, with differentiation of a truly sexual precursor as the mother of the egg and central cell, in other words, the micropylar/central tetranucleus (Fig. 2). This separation of developmental programs would likely coincide with the second mitotic cycle, in a micropylar nucleus located toward the center of the megagametophyte. At this four-nucleate stage, the micropylar tetranucleus—precursor to the synergids—seems to be clearly vegetative in future organization and function. Even as “siren cells” for attracting pollen tubes to the embryo sac (Higashiyama, 2002), the cells perform a sexually related, but clearly non-sexual cellular function.

Thus, the distinction of the sexually functional unit (*i.e.*, female germ unit)—is largely established at the first mitotic division, the truly sexual at the second mitotic division, and the egg cell by the third mitotic division. The development of these cells appears to have strong cytoplasmic influence, as indicated by the behavior of their heritable cytoplasmic organelles during megagametogenesis (Huang & Russell, 1993). Friedman & Williams (2003) propose that these two poles of the megagametophyte function as two modular units characterized by the following common patterns of development: (1) positioning of a single nucleus within a cytoplasmic domain (pole) of the female gametophyte; (2) two free-nuclear mitoses to

yield four nuclei within that domain; and (3) partitioning of three uninucleated cells adjacent to the pole such that the fourth nucleus is confined to the central region of the female gametophyte (central cell). This concept appears accurate to this extent. The chalazal module however appears to be cytoplasmically conditioned by the central cell, as the chalazal module does not seem, in any instance to date, to acquire independent sexual function. Thus, the central cell is sexual by virtue of its participation in the division that formed the egg; however, the nucleus of the chalazal counterpart may be conditioned by its immersion in the central cell, rather than participating in a truly comparable module. Evidence for this is supported in the absence of any compelling evidence for antipodals or synergids attaining sexual function. Although some instances of synergid embryos have been proposed, careful study has frequently resulted in discovery of alternative explanations, such as the subsequent division of the egg cell to produce supernumerary eggs (Secor & Russell, 1988) or various apomictic origins. Thus, it is clear that the two modular units differ in this critical distinction, and thus may not be exchangeable in their sexuality and ability to produce a true gamete. The central cell, in turn, may condition nuclei arising from the non-sexual module to participate in the formation of the endosperm. An examination of expressional and epigenetic factors contributing to these different cell lineages may provide insight into the control of gamete formation—which initiates the truly sexual phase of the gametophytic developmental program.

The male gametophyte truly has an independent life and has had a long evolutionary history. Overall, the male gametophyte generation has two goals: (1) to form two sperm cells and (2) to deliver them very precisely in the embryo sac to the site (egg and central cells) where fertilization occurs. Control by the non-sexual sporophyte generation—which includes the stigma, style and ovary of the gynoecium—provide significant support for the latter process of gamete delivery, but also meddle in how the choice is made. The truly sexual cells have at best a partial role in selection, as the sporophytic parent in many plants determines social interactions of the male and female gametophytes. Incompatibility is rarely determined by gametophytic interactions, but by those between the pollen tube and the ovule-bearing sporophyte. Unlike animal counterparts, the maternal sporophyte is largely successful at the determining paternal gametophyte. The stigma and style in many plants recognize and inhibits self pollen, thus promoting outbreeding. Compatible pollen becomes hydrated

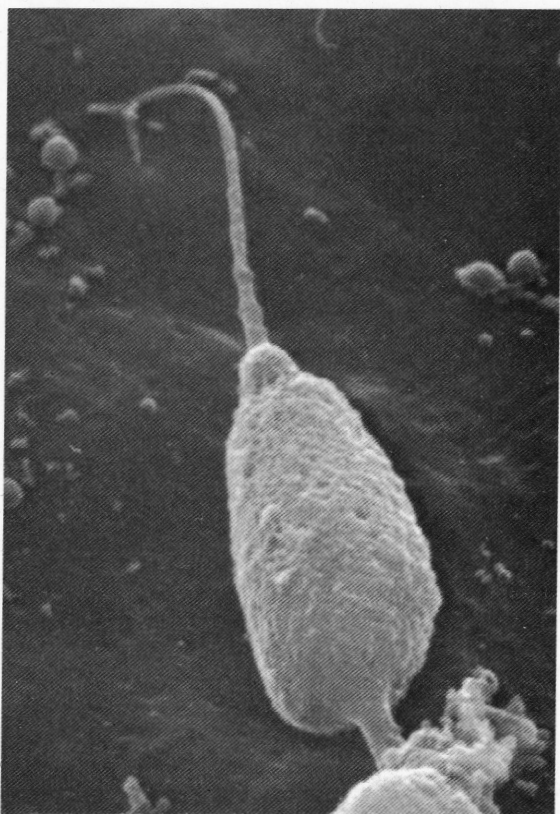


Fig. 3. Scanning electron micrograph of an isolated sperm cell of *Nicotiana tabacum*. Note the bifid cellular extension (above) and, at the opposite end of the cell, the connection with the other sperm cell (below). Reproduced with permission from Zhang *et al.* (1999).

and germinates; incompatible pollen will not in sporophytic incompatibility systems. In gametophytic incompatibility systems, the cessation of the male gametophyte occurs after pollen germination and is far more protracted.

The interactions between the elongating pollen tubes and the gynoecium do not involve entry into any cells, except by mistake or abortion. Instead, the pollen tube pushes between the cells of the stigma and style. Premature discharge terminates the transportation of the male gametes and thus assures that incompatible pollen has no genetic opportunity to mate. Unlike animals, in many cases, the plant successfully terminates nutrient flow to growing pollen tubes. Specific locations in the stylar tissue form sites where abortion is particularly common. Usually many more pollen grains germinate on the stigma than there are ovules in the ovary, but as the too numerous pollen tubes descend within the gynoecium, tubes abort. Often the number of tubes declines until it is very near to the number of ovules.



Fig. 4. Diagrammatic reconstruction of sperm discharge from the pollen tube. Left: Sperm cells and pollen tube nucleus approach the tip of the pollen tube prior to the arrival of the pollen tube. Center: Upon the bursting of the pollen tube, sperm cells are released from the tube and their surrounding pollen membranes. As the outer pollen plasma membrane disintegrates, the inner membrane of the pollen is also disrupted, releasing the sperm as naked cells. Right: As the sperm cells are expelled from the pollen tube, the male germ unit breaks down and sperm cells separate from the tube nucleus. Basal to the sperm cell formerly associated with the tube nucleus (S_{vn}) are numerous cytoplasmic bodies. Sperm are clearly true cells as they arrive in the embryo sac. Reproduced with permission from Russell (1994).

An interesting point of comparison for reproduction can be made between flowering plants and mammals. Obviously, the life cycle is totally different: meiotic products of mammals directly form gametes, but the scale of competition and likelihood of success are dramatically different. Mammals require, at minimum, tens of millions of sperm cells to be delivered to the female for the male to be considered fertile, and sperm placement is usually no more precise than within centimeters of the egg cell. Of all of these millions, only one sperm cell fertilizes the egg.

In flowering plants, however, the precision of sperm placement through the delivering pollen tube is within micrometers, and both of the two sperm cells that are delivered result in fusion products.

In the course of evolution, seed plant sperm cells have lost the ability and the necessity to produce flagella. Angiosperm sperm cells are joined by a physical association that forms a so-called "male germ unit" consisting of two sperm cells and the nucleus of the pollen tube. These three structures are physically associated during their descent in the tube, with the male germ unit representing the functional unit of male DNA transmission. In some cases, sperm cells may appear irregular in shape or even somewhat rounded. Often they retain tapered regions and in unusual cases, the region that is associated with the vegetative nucleus may even become more complex and display a bifid cellular extension (Fig. 3).

In addition to providing nutrition for the male gametes, the tube mediates their migration toward the ovary, embryo sac and egg cell. The pollen tube reads a chemical signal from the interior walls of the gynoecium and directs growth at the tube tip toward the ovary and ovules (Lord & Russell, 2002). The sperm and the tube nucleus are propelled by a cellular motor consisting of actin and myosin—the same proteins that together form muscle in animals. Directionally-oriented filamentous actin is located in the cortical cytoplasm of the pollen tubes and interacts with myosin, which is present in association with the generative cell (or its descendent sperm cells) and the surface of the tube nucleus. The myosin surrounding the male germ cells is actually present on the surface of an internal pollen plasma membrane

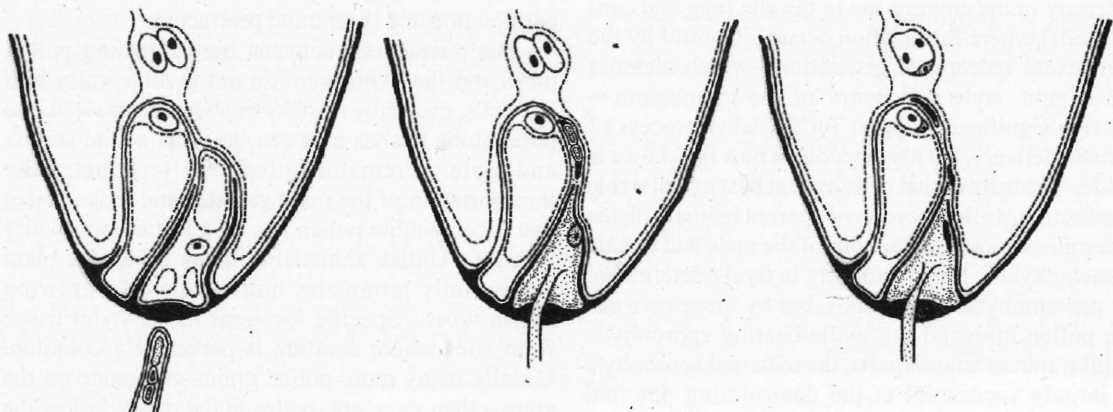


Fig. 5. Diagrammatic reconstruction of fertilization events from the penetration of the embryo sac to the delivery of the sperm cells. Left: Late pre-fertilization stage showing the approach of the pollen tube and degeneration of one synergid. Center: Deposit of the sperm cells in the degenerated synergid and their migration to the chalazal part of that cell. Right: Fusion of the two sperm nuclei with their respective female nuclei. Reproduced with permission from Russell (1994).

that surrounds the male gametic cells (see Fig. 4). Myosin is also present on the outer envelope of the tube nucleus. By virtue of actomyosin translocation and male germ unit associations, the male germ cells are passively moved toward the ovules within the pollen tube. The sperm cells are truly dependent cells in this process; they are entirely surrounded by and reliant on the pollen tube for transportation and nutrition.

Upon reaching the ovary, the pollen tube is attracted to and enters a specific synergid cell. Interestingly, the pollen tube appears to respond to a "siren's call" that emanates from a living synergid (Higashiyama, 2002). Within a receptive synergid cell, which appears to be selected to receive the pollen tube, the tube ruptures and discharges its nucleus and the two sperm cells. During pollen tube discharge, the cell membrane breaks down and the sperm cells become *au naturel* – surrounded by neither the pollen membranes nor by an external cell wall (Fig. 4). At this stage, they are truly naked cells and this is the first stage at which the cells are directly exposed to their environment. Within the synergid, the sperm cells have access to the interior of the female gametophyte – with interfaces to the egg cell and central cell. In the normal case, both of the sperm cells come into contact with the egg and the central cell simultaneously prior to fusion (Fig. 5). From this location, the sperm nucleus is transmitted, and sometimes co-transmitted with either all, part or none of the sperm cytoplasm, which may include heritable organelles (Russell, 1993).

Sperm cells inside the embryo sac, though, still have a very large journey, considering that they are non-motile cells located within the cytoplasm of an apoptotic synergid. But a pathway has been made for them: there are filamentous actin bands known as "actin coronas" – two of them – that are located at the edges of the receptive synergid. One extends from the middle of the synergid, leading to perinuclear regions of the egg cell on one side of the synergid and a second that extends from the middle of the synergid to the perinuclear region of the central cell on the other side (Russell, 1996). Associated with the exterior of the sperm cells is myosin that appears to originate from the pollen tube and potentially, the synergid. Thus, the passive sperm cells appear to be actively transported to their fusion sites on the egg and central cell.

The fusion of one sperm with the egg produces the zygote and initiates the cellular lineages of the embryo and young sporophyte. The fusion of the other sperm with the central cell and its polar nuclei

results in the formation of the nutritive endosperm. These events constitute double fertilization, and both events are required for the production of seed: the zygote forms the embryo and the young plant, whereas the endosperm is required for their nutrition.

The sperm cells might be expected to be identical cells since they arise from the same "father" cell. If during their origin, there are defects in division, or the generative cell is strongly polarized, the sperm cells may differ, resulting in heterospermy. Nuclear heterospermy results when non-disjunct chromosomes fail to separate during the mitotic division that forms the sperm nuclei. This is quite unusual, as the non-disjunction must occur specifically during generative cell division, but this commonly does occur in some B-chromosome-containing strains of maize (Roman, 1948). Cytoplasmic heterospermy results from unequal organelle location during sperm cell formation. The latter case occurs when the generative cell cytoplasm is polarized prior to division.

The most extreme case of cytoplasmic heterospermy in flowering plants is *Plumbago zeylanica*, in which plastids typically occur in one sperm cell, but not the other (Russell, 1984). Since plastids are heritable organelles that may become chloroplasts, this may have important genetic consequences. In this plant, one sperm cell (the one associated with the tube or vegetative nucleus) contains many of the mitochondria (about 150-325 mitochondria and at most, two plastids. Since this sperm cell is the one associated with the tube or vegetative nucleus, it is designated as the S_{vn} . The other sperm cell contains few mitochondria (20 to 60 mitochondria), whereas the number of plastids in the other sperm cell is much higher – from: 8 to 48 plastids. This second sperm cell is unassociated with the vegetative nucleus and therefore known as the S_{ua} (Russell, 1984).

In this plant, double fertilization also occurs preferentially. The sperm cell with plastids (S_{ua}), fuses with the egg cell, forming the zygote and embryo, whereas the other sperm cell (S_{vn}) with more mitochondria, fuses with the central cell and undergoes nuclear fusion with the polar nuclei to form the nutritive endosperm. This pattern occurs in over 95% of the cases observed and is strong evidence that sperm cells are targeted to fuse preferentially with a given sperm cell during fertilization. Thus the sperm display a potential separation of function.

Since sporophytes and gametophytes are independent plants, it would be interesting to know:

(1) how many genes are turned on in the gametophyte, (2) how many of the genes overlap, and (3) how many of the genes are unique. The first available molecular data indicated the presence of some 20,000 to 24,000 pollen-expressed mRNAs, with 2,000 to 7,000 pollen-specific mRNAs (Willing & Mascarenhas, 1984; Willing *et al.*, 1988). When one considers that *Arabidopsis* may possess on the order of 37,363 genes (based on gene models, Garcia-Hernandez *et al.*, 2002), this diversity seems quite remarkable. Recent studies based on the Affymetrix gene chip of *Arabidopsis* have provided the following evidence from the pollen transcriptome: 3,500 to 5,700 mRNAs are pollen-expressed and 600 to 1,400 mRNAs are pollen-specific in *Arabidopsis* pollen (Becker *et al.*, 2003; Honys & Twell, 2003). Of these products, a large number of gene products are expressed in both sporophyte and male gametophyte (~61% overlap according to Honys & Twell, 2003), which is significant in an evolutionary sense. Since there is expressional overlap, competition among gene products in the gametophytes may contribute to improving the fitness of the sporophyte. Without such expressional overlap, competition among gametophytes would be entirely neutral and therefore not capable of improving the sporophyte.

Based on the rather unexpectedly high level of transcript abundance in pollen gametophytes, a comparative examination of the female gametophyte transcriptome would be particularly attractive. Since the female gametophyte consists of four major cell types, whereas the male gametophyte only consists of two, one might assume that the female is more complex. Unfortunately, no information of this type is currently available.

Quite preliminary statements on sperm cell expression, however, may be derived from recent results from Sheila McCormick's lab, published earlier this year (Engel *et al.*, 2003) and from my lab (<http://bomi.ou.edu/russell/>). Sperm cDNA libraries are rich and complex, contain unique expressional elements, and include library members that are distinct from those in pollen. Available EST libraries, as of November 2003, include: ~4400 GenBank entries for *Zea mays* (maize) from the McCormick lab, 59 GenBank entries for *Oryza sativa* (rice) from the Chen lab, 2 entries for *Nicotiana tabacum* (tobacco) from the Mariani lab and 1522 GenBank entries for *Plumbago zeylanica* from the Russell lab.

One question that we can address uniquely with *Plumbago* is whether the two different kinds of sperm cells relate to differences in gene expression.

Therefore, we undertook the step of isolating populations of each type of sperm cell. Each pollen grain contains two sperm cells: the S_{ua} and the S_{vn} . From each pair, we selected a given cell type until we collected 11,000 sperm cells of each type. Then we constructed cDNA libraries to each sperm cell, based on the mRNA present in that cell type. We have placed 893 cDNA sequences representing the sperm type S_{ua} into GenBank, and 629 representing the sperm type S_{vn} . When we examine BLAST results of these data, there are indeed differences between the two sperm cell types. Categorization of these presumptive homologs into functional groups reveals that sperm cells with abundant plastids—the sperm S_{ua} —have significantly more products relating to transcription factors, signal transduction and plasma membrane-associated proteins. In contrast, the sperm type S_{vn} appears to have significantly more products relating to protein turnover and control—for instance polyubiquitin (Singh *et al.*, 2001), more DNA replication and repair products, and more unclassified metabolic gene products (unpublished data; <http://bomi.ou.edu/russell/>). Although *Plumbago* has an extreme form of sperm cell heteromorphism, more subtle forms have been evident in numerous flowering plants, including even tobacco (Tian *et al.*, 2001).

Many sequences exist for which there are no currently known homologies. To place these data in context, we may examine the genomics of best known flowering plant, which is currently *Arabidopsis*. In this plant, we know many of the likely and purported gene products, but we have quite limited information on the tissue, generation or circumstances in which these specific products are transcribed, translated, present or active. Perhaps some of the unexpected diversity of the *Arabidopsis* genome, which approaches the number of genes proposed for man, resides in the remarkable sexual life cycle that flowering plants possess. We may anticipate a better understanding of sexuality in flowering plants, its regulation and expression as we delve into the genes controlling these complex phenomena.

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