Female gamete competition in an ancient angiosperm lineage

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Edited* by Peter H. Raven, Missouri Botanical Garden, St. Louis, MO, and approved May 19, 2011 (received for review March 23, 2011)

Ever since Charles Darwin concluded that the early diversification of angiosperms was an “abominable mystery,” numerous hypotheses have been advanced to explain their extraordinary evolutionary radiation (1, 2). These hypotheses suggest that a number of key flowering plant synapomorphies were intimately associated with their early evolutionary history and subsequent diversification (3). Foremost among the defining features of angiosperms is the carpel, which encloses the ovule/sead and increases the distance of pollen tube growth between the site of pollen reception and point of fertilization. This key innovation fundamentally altered the basic interactions between the male and female components of the reproductive process, and it resulted in higher rates of male gametophyte competition and greater opportunities for maternal selection among potential sperm donors (4).

Recent character state reconstructions show that the common ancestor of angiosperms likely had an extragynoecial compitum, a construction of the carpels that enhances competition among pollen tubes and their selection by the maternal plant before fertilization (5, 6). In addition, the differentiation of a transmitting tissue within the carpel that supports pollen tube growth to the ovule has long been viewed as promoting male competition and increasing maternal choice of sperm donors (7, 8). The formation of callose plugs within pollen tubes recently has been hypothesized to have enabled male gametophytes to grow at higher rates and compete over greater distances than was the case for the immediate ancestors of angiosperms (8, 9). Finally, the evolution of insect pollination in conjunction with the early diversification of floral morphology and anatomy are widely viewed to have led to increased levels of pollen reception and hence, male–male competition (4). Collectively, the evolution of the carpel, extragynoecial compitum, transmitting tissue, callose plugs, and insect pollination seems to have resulted in significantly enhanced levels of prefertilization male competition and maternal choice, and thus may have played a major role in the early diversification of angiosperms (4–9).

Despite all of the attention paid to prefertilization mechanisms of male competition and female choice, there has never been any discussion of the possibility that female gametophytes (haploid egg-producing structures within ovules) might engage in a process of competition. Interestingly, recent embryological studies of several members of the Nymphaeales and Austrobaileyales have revealed that, in contrast with most angiosperms, more than one female gametophyte is occasionally or frequently initiated in each ovule (10–14). For us, this suggests the potential for competition between female gametophyte genomes during the developmental period leading up to the time of fertilization.

To gain insight into whether female gametophytes, in addition to male gametophytes, also engage in intrasexual competition before fertilization among early lineages of flowering plants, we examined ovule and gametophyte development in Trimenia (Tri

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\textsuperscript{2}This Direct Submission article had a prearranged editor.

\textsuperscript{1}Edited* by Peter H. Raven, Missouri Botanical Garden, St. Louis, MO, and approved May 19, 2011 (received for review March 23, 2011)
Female Gametophytes Undergo Tip Growth and Appear to Compete.

Although some megaspores abort, as many as 10 megaspores within an individual ovule initiate female gametophytes that seem to crush and displace the diploid maternal tissue (nucleus) situated above them along the chalazal to micropylar axis (Fig. 1). Shortly thereafter, free nuclear mitosis within several of the one-nucleate female gametophytes yields a set of two-nucleate gametophytes that begin to grow to the distant apex of the nucellus, where fertilization will ultimately occur (Fig. 2 and Movie S1). The nuclei pairs migrate to the growing tips of the female gametophytes. At this stage of development, the base of the nucellus contains several two-nucleate female gametophytes intertwined with each other as well as degenerated megaspores (Fig. 2). More than one tube-like female gametophyte commonly develops beyond this zone and continues to grow between the cells of the central tissue of the nucellus (Fig. 3 and Movie S2). Typically, between two and five female gametophytes extend into the distal half of the nucellus to the micropyle of the ovule (Fig. 3). Computer reconstructed images of multiple female gametophytes within an ovule show that the path of growth may be highly convoluted and that the individual tubes are often entangled with each other. The digital reconstructions also show that, although many female gametophyte tubes are initiated at the base of the ovule, there is attrition, such that only one to three female gametophytes ultimately reach the site of fertilization (Figs. 1–4).

Maturation Produces Four-Celled, Four-Nucleate Female Gametophytes. When a dominant tubular female gametophyte reaches the apex of the nucellus (Fig. 3), it enlarges circumferentially at its distal end and crushes the surrounding tissue of the nucellus, thereby exposing it directly to a pollen tube with its sperm (Fig. 4 and Movie S3). The two free nuclei of a dominant female gametophyte undergo a second mitotic division and form two pairs of nuclei arranged transversely to each other. Cellularization of a four-nucleate female gametophyte is followed by differentiation of the four cells into two synergids, an egg cell, and a uninucleate central cell (Fig. 4). Thus, in *T. moorei*, the female gametophyte is four-celled and four-nucleate at sexual maturity (Fig. 4), which is the case with all other extant members of the ancient angiosperm clades Austrobaileyales and Nymphaeales (14, 17).

In 15 of 200 ovules of *Trimenia* that were examined, two or three female gametophytes ultimately reached the site of fertilization at the apex of the nucellus. However, not all of the female gametophytes that emerged from the nucellar apex underwent sexual maturation; some remained at the two-nucleate stage of development. In several cases, we observed two female gametophytes within an individual ovule that had cellularized and differentiated into four-nucleate and four-celled structures (Fig. 4).

Transmitting Tissue for Growth of Female Gametophytes Differentiates Within the Nucellus. The concurrent growth of the multiple tube-like female gametophytes in *Trimenia* occurs in the central tissue of the nucellus. Strikingly, this region of maternal tissue contains conspicuous starch grains and other carbohydrates (Figs. 1B and 2). Both starch grains and carbohydrates accumulate early in the development of the ovule at the time when megaspores start to differentiate. The nutrient reserves disappear progressively as the tube-like two-nucleate female game-
tophytes grow through the central nucellus. After two-nucleate female gametophytes have grown through most of the length of the central tissue of the nucellus, small starch grains and carbohydrates can only be observed in the very distal part of the nucellus (Fig. 2). Callose appears in the cell walls of the central tissue of the nucellus. Even more striking, callose is present in the walls of growing female gametophytes (Fig. 5). However, callose plugs, as found in the pollen tubes of most angiosperms (including those of Trimenia) (Fig. 6), were not observed in the tube-like female gametophytes.

Pollen Tube Development and Male Gametophyte Competition. Pollen grains were rarely observed on the stigma, and often, they seemed to have aborted shortly after germinating and producing a short tube. This behavior is consistent with earlier reports of self-incompatibility in Trimenia (18). When male gametophytes did continue to develop, their pollen tubes extended beyond the receptive stigmatic surface and through the carpel ground tissue. Pollen tubes entered the ovarian cavity (locule) through the open mouth of the ascidiate carpel, which is filled with a secretion. More than one pollen tube rarely entered the locule and extended farther along the base of the funicle. As many as three pollen tubes were observed in our samples to reach the tip of an ovule and enter the micropyle (Fig. 6). However, even if more than one pollen tube entered the micropyle, only one male gametophyte ultimately engaged in a process of double fertilization. In several instances, the dominant pollen tube reached the site of fertilization before the embryo sac had become sexually mature or had completed its growth through the nucellus.

Discussion

Four-Nucleate Female Gametophyte in Trimenia Is Consistent with Most Other Ancient Angiosperm Lineages. Although earlier studies indicated that the female gametophyte in Trimenia was Polygonum-type (11, 15), our results clearly show that it is monosporic in origin and four-celled and four-nucleate at maturity. This finding is consistent with the many recent reversals of embryological reports that originally claimed that ancient lineages of extant flowering plants have a seven-celled, eight-nucleate (Polygonum-type) embryo sac (12–14, 19–22). Indeed, none of the members of the three most deeply divergent clades of extant angiosperms (Amborella, Nymphaeales, and Austrobaileyales) display the common (among angiosperms) Polygonum-type female gametophyte (17). It now seems likely that the first flowering plants were characterized by a four-celled, four-nucleate female gametophyte (14, 17), as found in Trimenia and other members of the Austrobaileyales and Nymphaeales that have been re-investigated during the past decade (12–14, 19, 21, 22).

Homoplastic Evolution of Tube-Like Female Gametophytes Among Angiosperms. In T. moorei, more than one female gametophyte is regularly initiated within each individual ovule. In addition, the
multiple female gametophytes begin their development at the base of the nucellus in a position remote from the future site of fertilization. These two features of ovule development in *Trimenia* stand in marked contrast to the condition in most angiosperms, where a single functional megaspore produces a single female gametophyte near the nucellar apex (16). As female gametophytes develop in *Trimenia*, they grow concurrently to the apex of the nucellus and form intertwined tube-like structures with callosic walls. Strikingly, the central tissue of nucellus, through which the female gametophytes grow, seems to function as a nutritive transmitting tissue.

Female gametophyte development in *Trimenia*, thus, strongly resembles that of pollen tubes (male gametophytes) in many ways. Female gametophytes seem to undergo tip growth to produce a tubular (rarely branched) morphology, contain callose in their cell walls (as do all flowering plant pollen tubes), and develop within a maternal diploid tissue that is highly reminiscent of the pollen tube transmitting tissue of the carpel. Moreover, as is typical of most angiosperm pollen tubes, a form of competition seems to be the rule: many female gametophytes are initiated within each ovule, but only one can successfully engage in the fertilization process.

The reproductive pattern typified by *Trimenia* in which multiple female gametophytes are initiated far from the site of fertilization, engage in a pattern of tip growth, and seem to compete over a substantial distance within the nucellus is rare among angiosperms but by no means unique. This suite of characters that engage in a form of competition evolved independently several times over the course of flowering plant history.

**Intrasexual Female Gametophyte Competition Is Common Among Ancient Lineages of Angiosperms.** Among angiosperms, the initiation of multiple female gametophytes is not uncommon (24, 29–31). Even in nonflowering seed plants (gymnosperms), a number of taxa are reported to initiate more than one female gametophyte per ovule (24, 30). For years, these observations largely have been treated as mere teratological features of reproductive development. To our knowledge, the concept of competition (before fertilization) between meiotically related female gametophytes within a seed plant ovule has only been noted a few times in the last century (24, 26, 31, 32). However, Haig (31), on the basis of inclusive fitness analysis, predicted that competition between meiotic products in an angiosperm ovule ought to occur and might be a thoroughly underappreciated phenomenon.

Among ancient angiosperm lineages, *Trimenia* clearly represents an extreme case in that its female gametophytes develop in a fashion highly similar to pollen tubes and seem to compete over substantial distances within the nucellus of the ovule. The female gametophytes of most extant members of early divergent angiosperm lineages do not produce extensive tube-like structures. However, this fact does not necessarily imply that female gametophyte (egg) competition is absent in ovules of these clades. In all but one of the extant families of Austrobaileyales and Nymphaeales, multiple female gametophytes per ovule are commonly or occasionally formed (10–14, 21). This same phenomenon also has been reported in the magnoliid lineage Laur...
ales (33) and in Ceratophyllum (34), the sister clade to eudicots. As in Trimenia, more than one female gametophyte may reach maturity, but only one will participate in a double fertilization event to produce an embryo and its associated endosperm. We posit that the potential for direct competition among multiple female gametophytes within an ovule may actually be common in early divergent flowering plant lineages.

Our analysis of the phylogenetic distribution of multiple female gametophytes per ovule among ancient lineages of flowering plants indicates that this character may have arisen in the common ancestor of all angiosperms or have been highly homoplastic in its origins during the earliest phases of angiosperm evolution (Table S1). If the production of multiple competing female gametophytes is not an ancestral trait of flowering plants, the high levels of homoplasy certainly provide circumstantial evidence that the production of multiple female gametophytes per ovule was advantageous during the initial diversification of angiosperms—and would, once again, be a strong reminder of the tremendous developmental lability manifest among ancient angiosperms (3, 20). Irrespective of whether this condition is plesiomorphic or highly homoplastic, competition between meiotically related but genetically unique female gametophytes (eggs) within an ovule seems to have been a key reproductive feature of the earliest phases of flowering plant evolution.

Brief History of Intrasexual Competition. In 1794, Erasmus Darwin, Charles Darwin’s grandfather, first proposed that competition among males was an important driver of evolutionary change (35). Sixty-five years later, Charles Darwin devoted a section of On the Origin of Species to the “struggle between males for possession of the females” (36). From its conceptual origins, intrasexual competition has, thus, largely but not exclusively been viewed as a highly asymmetric process in which females mate with and may choose among the winners of battles among potential male metazona partners.

Concepts of male competition and female choice were first applied to the life cycles of plants in the latter half of the 20th century (4, 7, 9, 31, 37–39). Since then, a number of developmental and genetic studies of angiosperm reproduction have shown that pollen tube (male–male) competition and female selection among pollen tubes (sperm donors) can result in increased levels of fitness among progeny (40). The insights derived from our discovery of competing tube-like female gametophytes within an ovule in Trimenia coupled with the prevalence of the production of multiple female gametophytes per ovule among many other ancient angiosperm lineages strongly suggest that male gametophytes were not alone in the competition to parent progeny within seeds. Rather, a combination of intrasexual competition among male gametophytes and among female gametophytes may have played an important role in enhancing the overall fitness of progeny during the earliest phases of flowering plant evolution.

Materials and Methods

Material. Reproductive material of T. moorei (Oliv. ex Benth.) Phillipson was collected at different stages of development in October 2009 and 2010 from plants growing in the subtropical rainforest of New South Wales in Australia (Table S2). Plant material was fixed for 24–48 h in 4% glutaraldehyde, FA (formaldehyde/acetic acid/alcohol), or 3:1 95% ethanol/acetic acid.

Bright-Field and Fluorescence Microscopy. Dissected floral buds, open flowers, and fruits were prepared for embedding in glycol methacrylate and were serially sectioned into 3- to 5-μm-thick ribbons (17). Subsets of serial sections were stained with either 0.25 μg/mL DAPI in 0.05 M Tris buffer (pH 7.2) to detect DNA or aniline blue in 0.15 M K2PO4 buffer (pH ~11) to detect callose before being stained with 0.1% toluidine blue and/or periodic acid Schiff’s reaction (17). Digital imaging and fluorescence were performed as in previous studies (17).

3D Computer Reconstructions. Digital images of selected transverse serial sections of ovules and female gametophytes at different stages of development were imported in a single file and manually aligned with Adobe Photoshop CS3. Aligned images were compiled in Z-stacks and modeled in 3D using the IMOD software package (Boulder Laboratory for 3-D Electron Microscopy of Cells). Pictures of each model were generated using IMOD and compiled into a movie using the Windows Live Movie Maker software from Microsoft. Each movie features the model of an ovule presented in the text. Each model is presented as shown in the corresponding figure and rotated counter-clockwise along the y axis through 720°. During the second full rotation, the nucellus is faded to show the interwoven development of the tubular female gametophytes.

ACKNOWLEDGMENTS. We thank P. K. Diggle, P. K. Endress, D. L. Marshall, R. H. Robichaux, and J. H. Williams for critical reading of the manuscript; J. J. Bruhl and Ian R. H. Telford for key assistance with many aspects of the research; and J. J. Bruhl and Ian R. H. Telford for key assistance with many aspects of the research. We also thank two anonymous reviewers for their comments on our manuscript. This work was supported by the National Science Foundation Grant IOS-0919986 (to W.E.F.).