American Journal of Botany 96(1): 129–143. 2009.

RECONSTRUCTING THE ANCESTRAL FEMALE GAMETOPHYTE OF ANGIOSPERMS: INSIGHTS FROM *AMBORELLA* AND OTHER ANCIENT LINEAGES OF FLOWERING PLANTS[1]

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For more than a century, the common ancestor of flowering plants was thought to have had a seven-celled, eight-nucleate Polygonum-type female gametophyte. It is now evident that not one, but in fact three, patterns of female gametophyte development and mature structure characterize the common ancestors of the four most ancient clades of extant angiosperms: Amborella-type, Nuphar/Schisandra-type and Polygonum-type. The Amborella-type female gametophyte is restricted to a single extant species, *Amborella trichopoda*, and at maturity consists of eight cells and nine nuclei. Development of the Amborella-type gametophyte is essentially identical to the Polygonum-type except that there is an additional and asynchronous cell division at the micropylar pole prior to maturation that produces a third synergid and the egg cell. The Nuphar/Schisandra-type female gametophyte is four-nucleate and four-celled and at maturity contains a typical three-celled egg apparatus and a central cell with a single haploid polar nucleus. This type of gametophyte appears to be universal among extant members of the Nymphaeales (including Hydatellaceae) and Austrobaileyales. Based on explicit reconstruction of character distribution and evolution, the Polygonum-type female gametophyte is certain to be representative of the common ancestors of monocots, eudicots, magnoliids, Ceratophyllaceae, and Chloranthaceae. There are compelling biological reasons to suggest that the four-celled, four-nucleate female gametophyte (as found in Nymphaeales and Austrobaileyales) is ancestral among angiosperms, with transitions to Polygonum-type female gametophytes separately in the Amborellales and in the ancient angiosperm clade that includes all angiosperms except *Amborella*, Nymphaeales, and Austrobaileyales. Subsequent to the evolution of a seven-celled, eight-nucleate Polygonum-type female gametophyte in the Amborellales, we hypothesize that a peramorphic increase in egg apparatus cell number took place and led to the unique situation in which there are three synergids in *Amborella trichopoda*.

Key words: Amborella; Austrobaileyales; double fertilization; endosperm; evo-devo; female gametophyte; Nymphaeales.

For nearly a century, the common ancestor of flowering plants was thought to have had a seven-celled, eight-nucleate Polygonum-type female gametophyte (e.g., Porsch, 1907; Maneval, 1914; Chiarugi, 1927; Schnarf, 1931; Maheshwari, 1950; Johri, 1963; Davis, 1966; Bhandari, 1971; Foster and Gifford, 1974; Stebbins, 1974; Palser, 1975; Takhtajan, 1976; Favre-Duchartre, 1984; Cronquist, 1988; Battaglia, 1989; Haig, 1990; Donoghue and Scheiner, 1992; Johri et al., 1992; Tobe et al., 2000). This conclusion was often reached through the erroneous assumption that "common" features of a clade are likely to be plesiomorphic (more than 80% of angiosperms produce a Polygonum-type female gametophyte; Palser, 1975). Moreover, the prevalence of Polygonum-type female gametophytes among Magnoliales, Laurales, and Winterales (Canellales), along with the phylogenetic assumption that these groups were among the most ancient of angiosperm lineages, was taken as prima facie evidence of the plesiomorphic ("primitive") nature of the monosporic eight-nucleate, seven-celled female gametophyte among angiosperms.

Beginning in the late 1990s, a series of molecular phylogenetic analyses fundamentally altered our thinking about the identities of the most ancient lineages of flowering plants (e.g., Mathews and Donoghue, 1999; Parkinson et al., 1999; Qiu et al., 1999, 2000, 2005, 2006; Soltis et al., 1999, 2000a, b, 2007; Graham and Olmstead, 2000; Graham et al., 2000; Zanis et al., 2002; APG II, 2003; Kim et al., 2004; Soltis and Soltis, 2004; Leebens-Mack et al., 2005; Hansen et al., 2007; Jansen et al., 2007; Moore et al., 2007; Saarela et al., 2007; Doyle, 2008; Qiu and Estabrook, 2008). These diverse phylogenetic analyses continue to provide compelling evidence that *Amborella trichopoda* Baill., Nymphaeales sensu lato (Hydatellaceae, Cabombaceae, Nymphaeaceae), and Austrobaileyales (Austrobaileyaceae, Trimeniaceae, Illiciaceae, Schisandraceae) are three of the four most ancient extant lineages of angiosperms. For the record, the fourth most ancient lineage is the clade that includes all other angiosperms and is sister to Austrobaileyales (*Arabidopsis* (DC.) Hyhnh. and *Zea* L. are members of one of the four most ancient clades of flowering plants). Thus, after a century of concerted effort to study the biological features of Magnoliales, Laurales, and Winterales (Canellales) for the purposes of reconstructing early angiosperm evolutionary history, the focus shifted abruptly to the largely understudied members of the Amborellales, Nymphaeales, and Austrobaileyales. Evolutionary morphologists, anatomists, embryologists, and reproductive biologists interested in deciphering the evolutionary genesis of the currently dominant clade of photosynthetic life on earth began to look carefully at the biology of these newly identified most ancient angiosperm lineages.

The arrival of new (and potentially robust) phylogenetic hypotheses for the most ancient lineages of angiosperms specifically led to an intensive effort to examine the development of the female gametophyte (and other embryological features) in *Amborella* Baill., Nymphaeales and Austrobaileyales. Tobe et al. (2000) were the first to examine *Amborella* and concluded that the female gametophyte was, indeed, Polygonum-type, consistent with the established view that the first angiosperms

[1] Manuscript received 16 September 2008; revision accepted 31 October 2008.

The authors thank P. Diggle for suggestions that improved the manuscript. This research was supported by a grant from the National Science Foundation (IOB 0446191).

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doi:10.3732/ajb.0800311

produced a seven-celled, eight-nucleate female gametophyte. A survey of the embryological literature for Nymphaeales and Austrobaileyales also reported that Polygonum-type female gametophytes were characteristic of these newly identified most ancient angiosperm clades (Tobe et al., 2000). Thus, although a major shift had occurred in angiosperm phylogenetic hypotheses, the century-old hypothesis as to the plesiomorphic condition for the female gametophyte (Polygonum-type) remained unaltered at the turn of the millennium.

In fact, the historical primary embryological literature is far from clear about the developmental and structural characteristics of the female gametophytes of members of the Nymphaeales and Austrobaileyales (see Friedman and Williams, 2003, for a review)—and this realization stimulated a series of new investigations of the embryology of these two clades. In *Nuphar* Sm. (Nymphaeaceae), *Illicium* L. (Illiciaceae), *Kadsura* Juss. (Schisandraceae), and *Austrobailleya* C. T. White (Austrobaileyaceae), mature female gametophytes were definitively shown to contain only four nuclei and four cells at maturity (Nuphar-type or Schisandra-type) (Williams and Friedman, 2002, 2004; Friedman et al., 2003; Tobe et al., 2007). Shortly after the surprising discovery in 2007 that members of the Hydatellaceae (*Hydatella* Diels and *Trithuria* Hook.f.) were not monocots, but rather, water lilies (Saarela et al., 2007), four-celled and four-nucleate female gametophytes were reported in this unusual clade of Nymphaeales (Friedman, 2008; Rudall et al., 2008). Finally, a new examination of the *Amborella* female gametophyte demonstrated that it is not Polygonum-type, but instead a unique nine-nucleate, eight-celled structure, now known as Amborella-type (Friedman, 2006a). Thus, only five years after the outset of a new wave of embryological studies of the ancient angiosperm clades Amborellales, Nymphaeales, and Austrobaileyales, it is clear that none of the representatives of these lineages produces a Polygonum-type female gametophyte—a dramatic reversal of a century of evolutionary thought about this key reproductive feature.

In light of the importance of *Amborella* to the reconstruction of early angiosperm evolutionary history, a thorough investigation of the developmental biology of its female gametophyte has been warranted and is herein presented. Moreover, given the absence of Polygonum-type female gametophytes among three of the most ancient lineages of extant flowering plants, careful analysis of the evolutionary implications of this new set of findings is required. Using recent insights into the developmental biology of the female gametophytes of flowering plants, we will reconstruct explicit hypotheses for the plesiomorphic condition for the angiosperm female gametophyte and the earliest phases of diversification of this egg- and central cell-producing structure that ultimately gave rise to the patterns of sexual reproduction that characterize the vast majority of flowering plants.

MATERIALS AND METHODS

Plant collections—Carpellate buds and open flowers of *Amborella trichopoda* were collected at Plateau de Dogny, New Caledonia (GPS coordinates = 21°37.253′S, 165°52.130′E) between the elevations of 601 m and 949 m a.s.l. (with permission from the Office of the Department of Natural Resources, Southern Province, New Caledonia) and from specimens growing in the University of Colorado greenhouses in Boulder, Colorado. Hand pollinations were performed on flowers in the field and in the greenhouse.

Bright field and fluorescence microscopy—Flowers were fixed for 24 h either in 3 : 1 (95% ethanol : acetic acid) and stored in 70% ethanol or in 4% glutaraldehyde in 50 mM PIPES buffer and stored in PIPES buffer. Specimens were dehydrated through an ethanol series, then infiltrated and embedded in glycol methacrylate (JB-4 embedding kit, Polysciences, Warrington, Pennsylvania, USA). Embedded flowers were serially sectioned into 4-μm thick ribbons. Sectioned flowers were first stained with 0.25 μg/mol of 4′,6-diamidino-2-phenylindole (DAPI) in 0.05 Tris buffer (pH 7.2) and examined. Selected slides were later stained with Schiff's reagent (Fisher Scientific, Fair Lawn, New Jersey, USA) and/or toluidine blue (0.1%) for additional examination under bright field conditions. Digital imaging was performed on a Zeiss Axiocam digital camera using brightfield and fluorescence optics. Fluorescence was viewed with an HBO 100 W burner (Carl Zeiss, Oberkochen, Germany) using a UV filter set (model 48702) with excitation filter (365 nm, band pass 12 nm), dichroic mirror (FT395), and barrier filter (LP397). Images were processed with Adobe (San Jose, California, USA) Photoshop 9.0. Image manipulations were restricted to operations applied to the entire image, except as noted in specific figure legends.

Transmission electron microscopy—Flowers were fixed for 24–48 h in a solution of 2% paraformaldehyde, 1% glutaraldehyde, and 2% acrolein in 50 mM PIPES buffer (pH 6.8) and were stored in PIPES buffer. Ovules were dissected from carpels and postfixed in 2% osmium tetroxide at 4°C for 2 h. Specimens were dehydrated through an ethanol series and infiltrated with propylene oxide for 1.5 h. Specimens were then infiltrated with and embedded in Spurr's low viscosity embedding medium (Polysciences). Embedded ovules were sectioned 70 nm thick with a diamond knife and collected onto thin, bar square 200-mesh copper grids. Sections were stained with lead citrate and examined with a Philips CM 10 transmission electron microscope. Images were processed with Adobe Photoshop 9.0. Image manipulations were restricted to operations that were applied to the entire image, except as noted in specific figure legends.

RESULTS

***Female gametophyte development in* Amborella**—A single megasporocyte is produced in the center of each ovule. The megasporocyte nucleus occupies the center of the cell, and a cytoplasmically dense zone forms between it and the chalazal wall (Fig. 1A). This cytoplasmically dense zone persists through the end of megasporogenesis. Meiosis I yields a dyad of two uninucleate cells, typically divided by a transverse cell wall (Fig. 1B). Meiosis II results in a linear tetrad of megaspore cells (Fig. 1C). The chalazal-most cell becomes the functional megaspore, and the other three megaspores degenerate before syncytial development of the female gametophyte is initiated. The functional megaspore has a large centrally placed nucleus that is surrounded by a vacuole. Cytoplasmic strands radiate from the nucleus to the lateral sides, but not the poles, of the cell (Fig. 1D).

The one-nucleate female gametophyte of *Amborella* (Fig. 2A) initiates three rounds of free-nuclear mitotic divisions. Following the first mitosis, the two nuclei migrate to opposite poles of the cell (Fig. 2B) and become situated in the thin band of cytoplasm at the edge of the female gametophyte. At the two-nucleate stage, the female gametophyte is roughly 25% larger than the functional megaspore. After the second set of mitotic divisions, two nuclei reside at each pole of the female gametophyte, which further increases in size (Fig. 2C). The nuclei are smaller at the four-nucleate stage than at the two-nucleate stage. At this point, the nuclei at the micropylar end begin to take on a characteristic torpedo shape. A third round of free-nuclear mitosis produces a quartet of nuclei at each end of the female gametophyte. However, an eight-nucleate syncytial stage was not observed.

Cytokinesis occurs at the eight-nucleate stage. At each pole, three flat cells form abutting the edge of the female gametophyte (Fig. 3). The nucleus of each of these cells is torpedo-shaped and presents a prominent nucleolus. The cells and nuclei

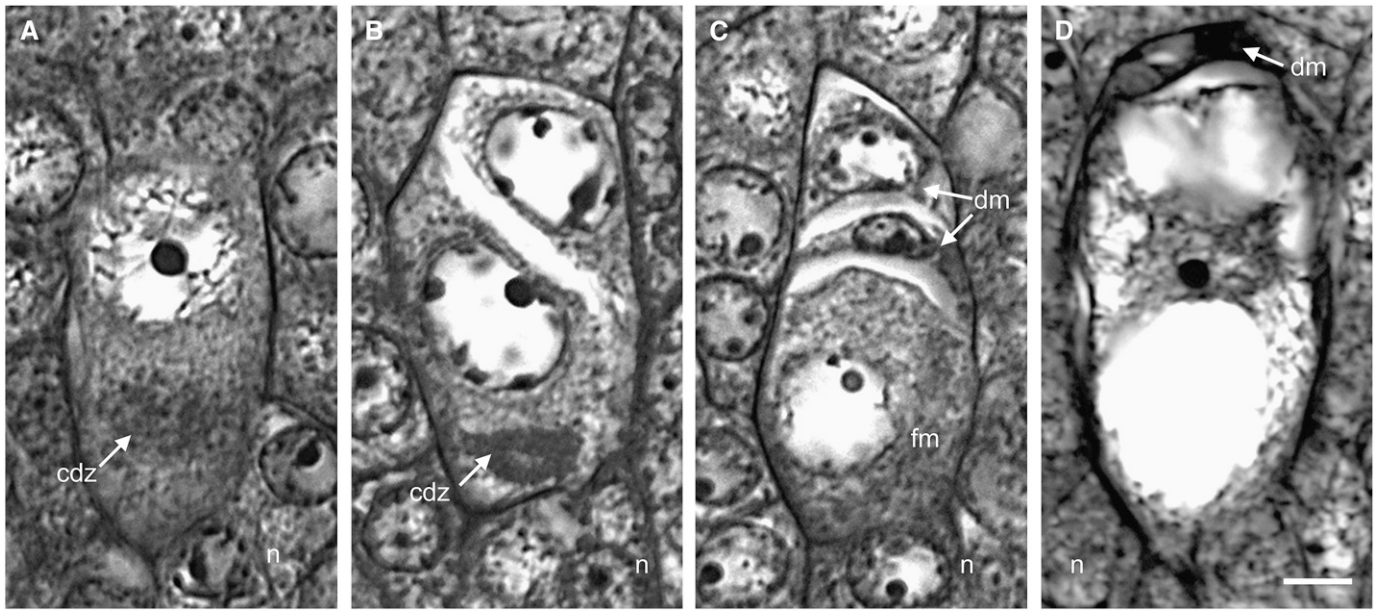


Fig. 1. Megasporogenesis in *Amborella trichopoda*. (A) Megasporocyte with nucleus at micropylar pole (up in figures) and cytoplasmically dense zone in chalazal region. (B) Dyad stage, after meiosis I. (C) Linear tetrad of megaspores after meiosis II. Two degenerate megaspores and the chalazal functional megaspore visible. (D) One-nucleate female gametophyte with crushed degenerate megaspore. cdz, cytoplasmically dense zone; dm, degenerate megaspore; fm, functional megaspore; n, nucellus. Scale bar = 5 μm.

at the micropylar pole are essentially similar in size, shape, and orientation to those at the chalazal pole. In the central cell, two polar nuclei are found midway between the poles of the cell within a parietal band of cytoplasm. At this stage, the female gametophyte contains eight nuclei and seven cells.

The seven-celled, eight-nucleate stage does not constitute the terminus of female gametophyte ontogeny. One of the three cells at the micropylar pole undergoes a final mitotic and cytokinetic division to produce two daughter cells (data shown in Friedman, 2006a) that will contribute to the mature egg appara-

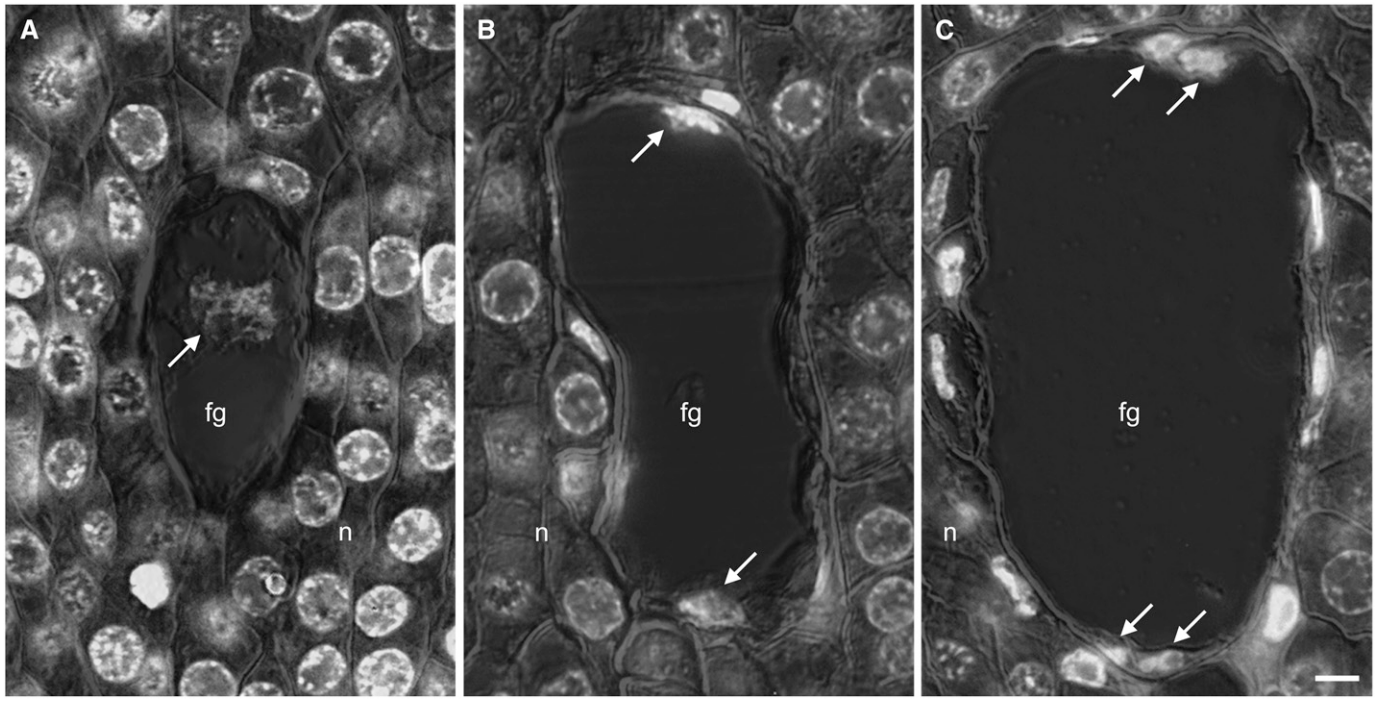


Fig. 2. Syncytial female gametophyte development in *Amborella*. Sections stained with DNA fluorochrome DAPI and viewed with epifluorescence. (A) One-nucleate stage. (B) Two-nucleate stage. Female gametophyte is highly vacuolate. (C) Four-nucleate stage. These nuclei have taken on a characteristic torpedo shape. Arrows identify nuclei of the female gametophyte. Micropylar end up in images. fg, female gametophyte; n, nucellus. Scale bar = 5 μm.

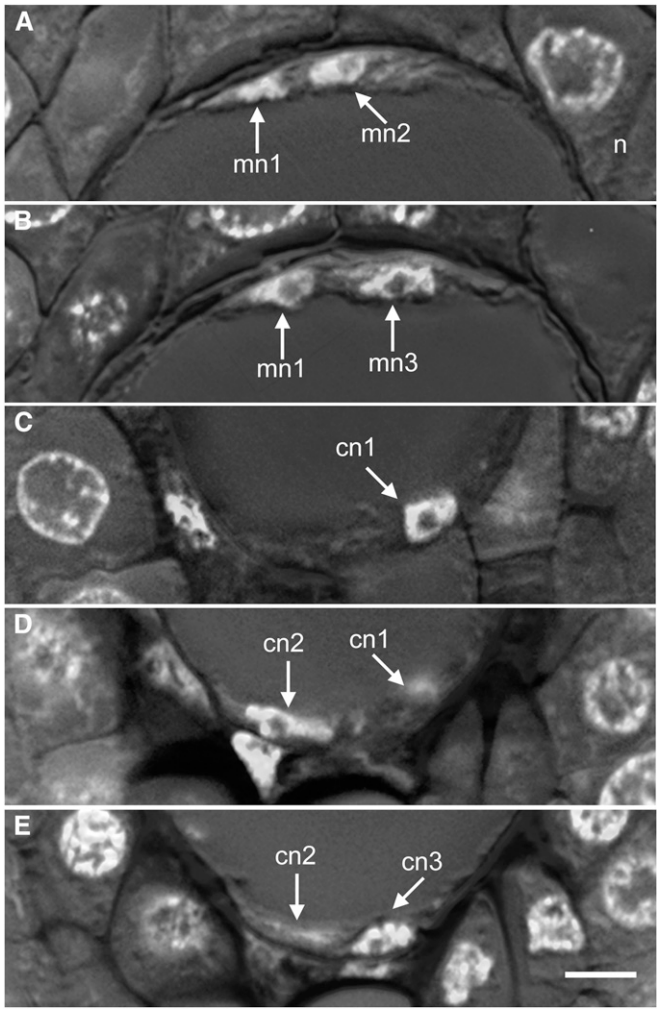


Fig. 3. Serial sections of eight-nucleate stage of female gametophyte development in *Amborella*. (A–E) Following cytokinesis at the eight-nucleate stage of female gametophyte development, three torpedo-shaped nuclei in flat cells are partitioned in (A, B) the micropylar and (C–E) chalazal regions of the gametophyte. The two polar nuclei in the central cell of the female gametophyte not shown. cn, chalazal nucleus; mn, micropylar nucleus; n, nucellus. Scale bar = 5 μm.

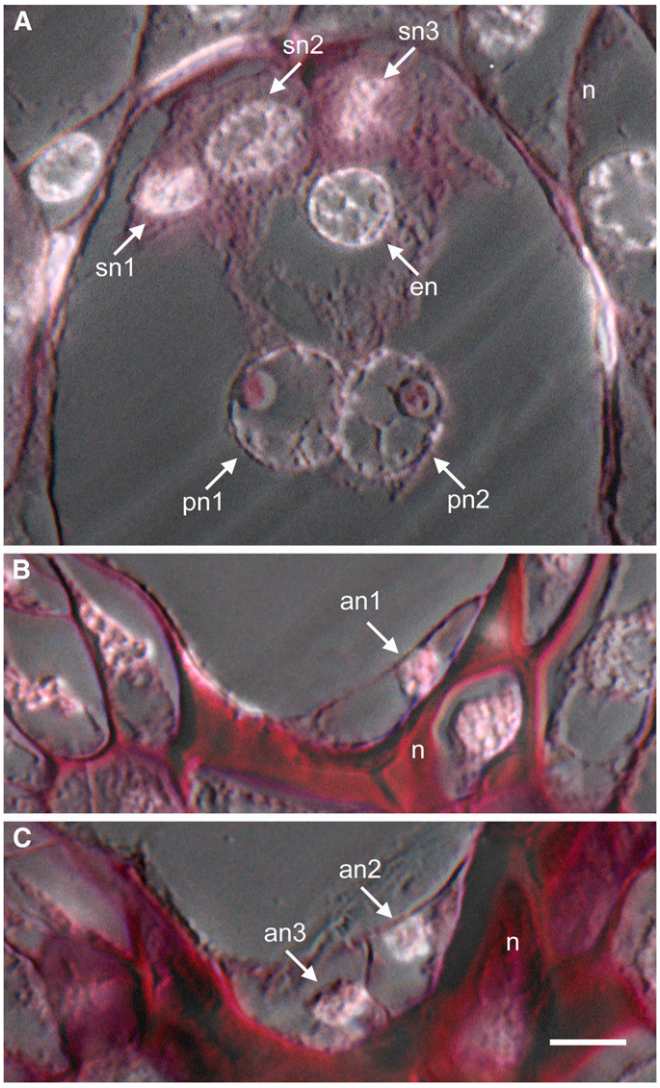


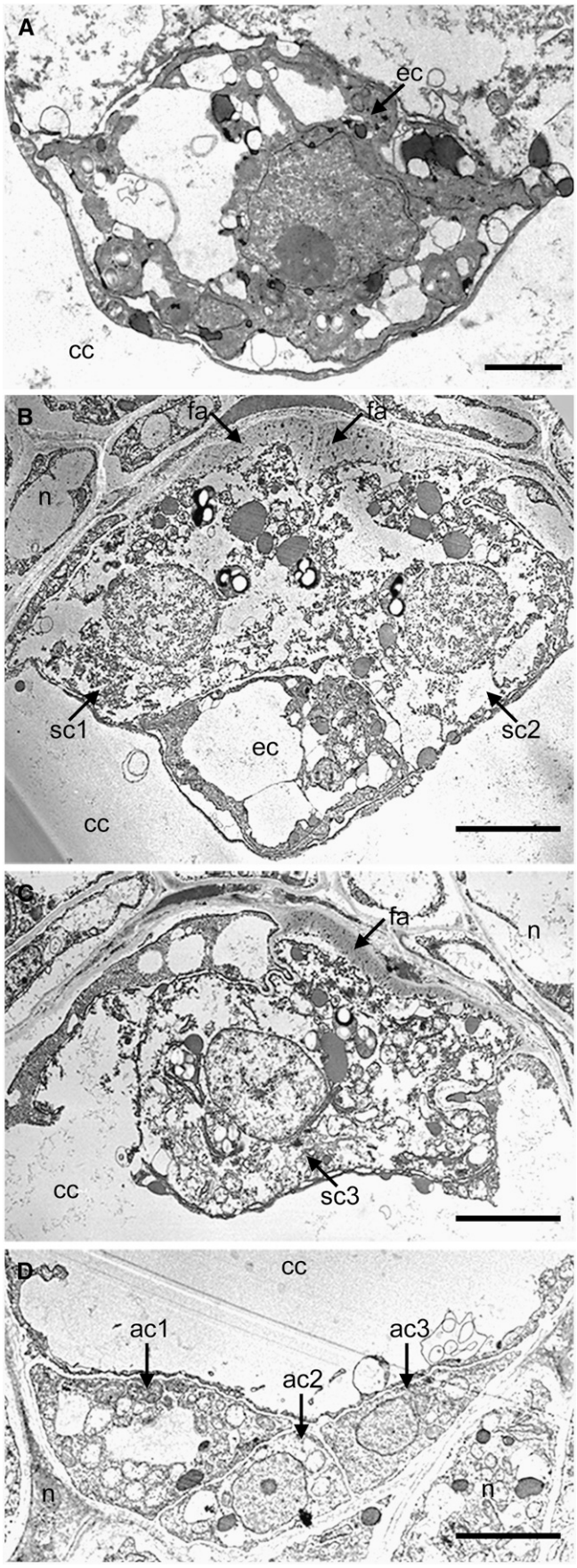
Fig. 4. Serial sections of mature female gametophyte of *Amborella*. (A–C) Eight-celled, nine-nucleate female gametophyte composed of an egg apparatus with three synergids and an egg (A). (A) Central cell with two polar nuclei, and (B, C) three antipodals. an, antipodal nucleus; en, egg nucleus; n, nucellus; pn, polar nucleus; sn, synergid nucleus. Scale bar = 5 μm.

tus (Fig. 4). One of these two daughter cells is positioned chalazal to, and shares interfaces with, the other three cells of the egg apparatus. It also shares an interface with the central cell, but not with the wall of the female gametophyte. This cell will differentiate into the egg cell (described later).

At maturity, the three most micropylar of the four cells at the micropylar pole are similar to one another in cell wall features and cytoplasmic contents. Each contains a thick wedge-shaped, filiform apparatus (Fig. 5B, C). The cytoplasm of these cells has rough endoplasmic reticulum scattered throughout the cell and a few plastids located near the filiform apparatus. Based on these characteristics, the three cells can each be identified as synergids. The fourth micropylar cell, the egg cell (Figs. 4A, 5A), has a thin cell wall and a large central vacuole that takes up approximately half of the cell. Together, the three synergids and the egg cell comprise the egg apparatus in *Amborella*.

The central cell of the female gametophyte contains a large central vacuole. The two spherical polar nuclei within the central cell each contain one nucleolus. The polar nuclei are typically positioned at the periphery of the central cell, and their placement varies from near the egg apparatus in the micropylar region (Fig. 4A) to near the chalazal pole of the female gametophyte. The position of the polar nuclei within the central cell does not appear to correlate with developmental stage. Female gametophytes with unfused polar nuclei were found in flowers both prior to and following anthesis. Female gametophytes with fused polar nuclei (secondary nucleus) were only seen in post-anthesis flowers, but always prior to fertilization. The secondary nucleus is larger than the individual polar nuclei, contains two nucleoli, and is always found in the chalazal region of the female gametophyte.

The three antipodal cells at the chalazal pole of the mature female gametophyte are densely cytoplasmic and contain rough endoplasmic reticulum and numerous plastids (Figs. 4B, C,



5D). The nuclei are small and retain their torpedo shape. Proliferation of antipodal cells, resulting in seven antipodals in an individual female gametophyte, was observed twice among 166 mature female gametophytes examined. The overall size of the mature eight-celled, nine-nucleate female gametophyte is significantly larger than that of the seven-celled, eight-nucleate stage.

***Fertilization in* Amborella**—In *Amborella*, the pollen tube enters the ovule through the micropyle, proceeds between cells of the nucellus, and enters the egg apparatus via one of the synergids, which appears to be degenerated (Fig. 6). In two instances, we observed three extra nuclei (inferred to be the two sperm nuclei and the tube nucleus), in close proximity to one another, just outside of the tip of a pollen tube that had penetrated the egg apparatus of the female gametophyte (Fig. 7). Sperm nuclei were not observed fusing with the egg nucleus or the secondary nucleus. Nevertheless, circumstantial evidence indicates that *Amborella* appears to undergo double fertilization.

Hand pollinations were performed on open flowers in the field, and these flowers were collected 1, 2, and 3 d after pollination. All the hand-pollinated flowers contained fertilized female gametophytes with clear evidence of a zygote (Fig. 8). Thus, fertilization occurs within the first 24 h after pollination. Compared with the unfertilized egg, the zygote is larger in size and contains a prominent vacuole (Fig. 8). Consistent with a regular second fertilization event, the primary endosperm nucleus has three distinct nucleoli, while the unfertilized secondary nucleus contains two nucleoli and the individual polar nuclei each presents a single nucleolus (Fig. 9).

All three synergids persist after fertilization. Two of the three synergids contain nuclei that remain prominent (Fig. 8), while one synergid typically contains two smaller nuclei. The cell with the two smaller nuclei can be inferred to be the degenerate synergid through which the pollen tube entered; the two nuclei are the tube nucleus and the degenerate synergid nucleus (Fig. 8). The three antipodals persist through fertilization and can be seen after the initial divisions of the endosperm (data not shown). The overall size of the fertilized female gametophyte continues to increase.

The primary endosperm nucleus is always found in the chalazal region of the female gametophyte. The first division of the primary endosperm nucleus produces a pseudotransverse wall at the chalazal end of the female gametophyte, creating two domains of endosperm. Endosperm development in *Amborella* has been described in Floyd and Friedman (2001).

DISCUSSION

The *Amborella* female gametophyte produces eight cells and nine nuclei. This stands in marked contrast with the four-celled, four-nucleate female gametophytes (Nuphar/Schisandra-type)

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Fig. 5. Transmission electron micrographs of serial sections of mature female gametophyte of *Amborella*. (A) Egg cell. (B, C) Three synergid cells; also nonmedian view of egg cell in (B). Each synergid contains a filiform apparatus. (D) Three antipodal cells of the mature female gametophyte at the chalazal pole. ac, antipodal cell; cc, central cell; ec, egg cell; fa, filiform apparatus; n, nucellus; sc, synergid cell. Scale bars A–C = 5 μm; D = 2.25 μm.

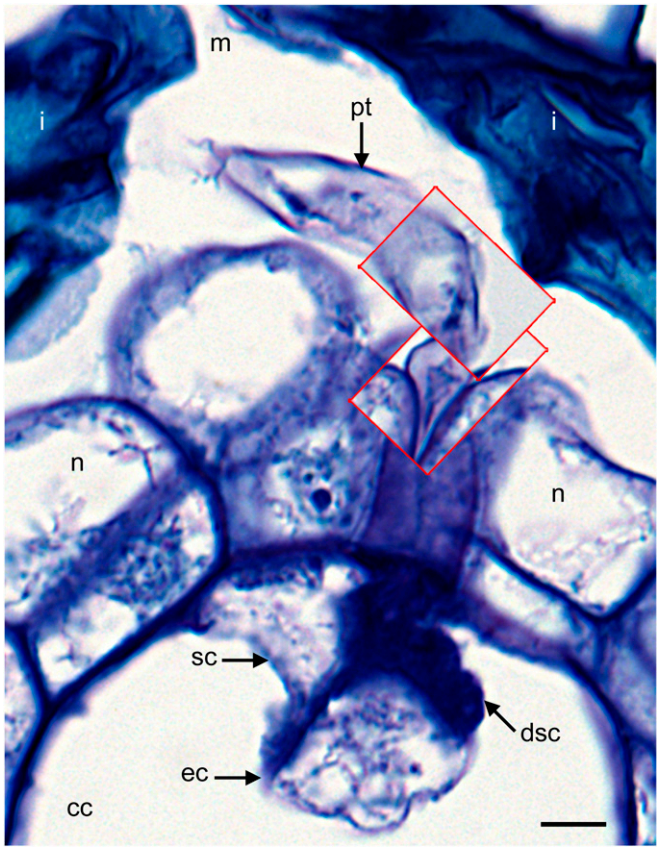

Fig. 6. Light micrograph of pollen tube entry into female gametophyte of *Amborella*. The pollen tube grows between cells of the nucellus and enters the female gametophyte through the degenerated synergid cell. Composite of three serial sections (two sections inlaid in red boxes), stained with toluidine blue. cc, central cell; dsc, degenerated synergid cell; ec, egg cell; i, integument; m, micropyle; n, nucellus; pt, pollen tube; sc, synergid cell. Scale bar = 5 μm.

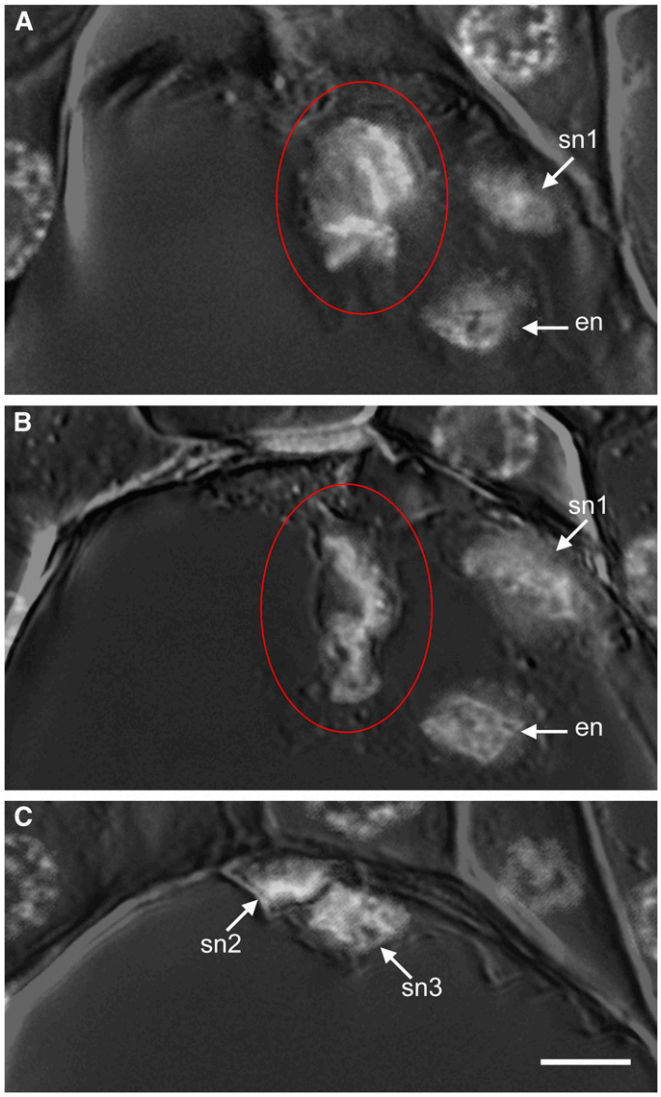

Fig. 7. Serial sections of pollen tube contents in egg apparatus of *Amborella*. (A–C) Deposition of two sperm nuclei and pollen tube nucleus (enclosed in red circle) in the mature egg apparatus results in seven nuclei in the micropylar region of the female gametophyte just prior to double fertilization. cc, central cell; en, egg nucleus; n, nucellus; sn, synergid nucleus. Scale bar = 5 μm.

found in Nymphaeales (Batygina et al., 1982; Galati, 1985; Winter, 1987, 1993; Titova, 1990; Winter and Shamrov, 1991a, b; Van Miegroet and Dujardin, 1992; Shamrov, 1998; Williams and Friedman, 2002; Friedman and Williams, 2003; Friedman, 2008; Rudall et al., 2008) and Austrobaileyales (Yoshida, 1962; Swamy, 1964; Solntseva, 1981; Battaglia, 1986, 1989; Friedman et al., 2003; Williams and Friedman, 2004; Tobe et al., 2007) and differs from the seven-celled, eight-nucleate Polygonum-type common to most monocots, magnoliids, and eudicots (Friedman and Williams, 2003). The absence of Polygonum-type female gametophytes in the most ancient angiosperm lineages is a startling reversal of a century of thought in which it had been universally concluded that this type of female gametophyte characterized the first angiosperms as well as extant members of the most ancient lineages.

***Female gametophyte development in* Amborella**—As with the overwhelming majority of angiosperms (Maheshwari, 1950) including Nymphaeales and Austrobaileyales, the female gametophyte of *Amborella* is derived from the chalazal-most megaspore. After the first mitotic division of the functional megaspore, the two resulting nuclei migrate to opposite poles of the cell, as is also characteristic in Polygonum-type female gametophytes, but not in the four-nucleate, four-celled female gametophytes of Nymphaeales and Austrobaileyales (Fig. 10). At each pole in the binucleate female gametophyte of *Amborella*, the nucleus undergoes two rounds of mitosis to produce four nuclei. One nucleus from each pole migrates toward the center of the syncytium, after which cellularization of the remaining three nuclei at each pole occurs. After cellularization, a final cell division occurs within the micropylar region of the female gametophyte (Fig. 10). It is this final division that produces the egg cell and a third synergid and represents the final step necessary to prepare the female gametophyte of *Amborella* for fertilization.

As a consequence of the final asynchronous cell division in *Amborella*, the mature female gametophyte contains a four-celled egg apparatus with three synergids and one egg cell, a binucleate central cell (the two polar nuclei fuse to form a secondary nucleus prior to fertilization), and three antipodals that

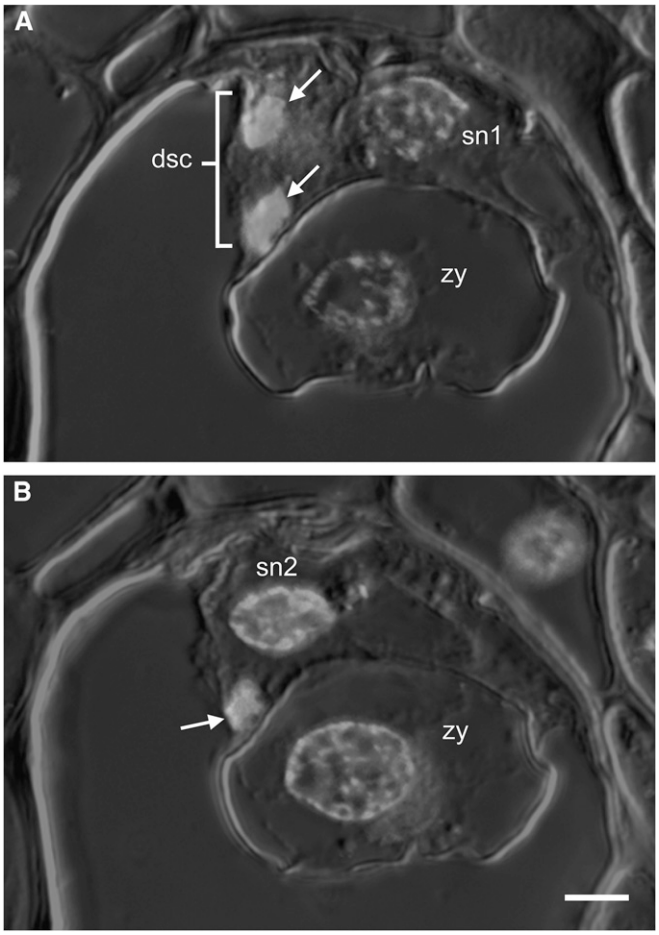

Fig. 8. Serial sections of *Amborella* zygote and synergids after fertilization. (A, B) Two undegenerated synergid cells and the degenerated synergid cell containing the pollen tube nucleus and a synergid nucleus (arrows) in (A) and (B). Unlabeled nucleus (arrow) in (B) is lower nucleus in degenerated synergid seen in (A). Zygote with robust cell wall and highly vacuolated cytoplasm (compared with egg cell). dsc, degenerated synergid cell; sn, synergid nucleus; zy, zygote. Scale bar = 5 μm.

persist through fertilization at the chalazal pole. These results conflict with a previous study of the *Amborella* female gametophyte (Tobe et al., 2000), that reported a seven-celled, eight-nucleate Polygonum-type female gametophyte with antipodals that degenerate before fertilization. However, it is likely that FAA (formalin, acetic acid, ethanol) fixation, coupled with paraffin embedding is unable to provide sufficient resolution to observe the extra cell in the egg apparatus or detect the small antipodals. We conclude, as did Rudall (2006), that the differing results of these two embryological studies, with respect to the number of synergids and persistence of antipodals, are not due to teratology or polymorphic conditions in *Amborella*. Beyond our extensive sampling with light and fluorescence microscopy (over 160 female gametophytes from many genetically distinct plants in the field and greenhouse were observed) to document the developmental basis for the mature structure of the female gametophyte of *Amborella*, serially sectioned female gametophytes prepared for transmission electron microscopy are definitive; the egg apparatus contains four cells (three synergids and an egg cell), and the antipodals are persistent (Fig. 5).

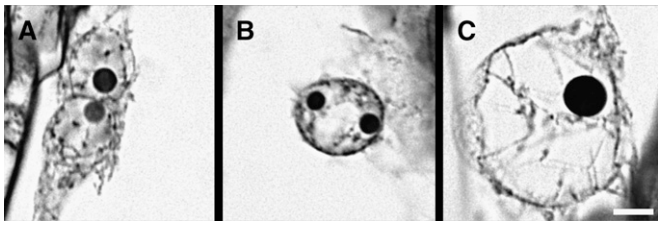

Fig. 9. Polar nuclei, secondary nucleus, and primary endosperm nucleus in central cell of female gametophyte of *Amborella*. (A) Polar nuclei, each of which contains a single nucleolus. (B) Secondary nucleus, resulting from fusion of polar nuclei, with two nucleoli. (C) Primary endosperm nucleus with three nucleoli (only one is visible in image). Scale bar = 5 μm.

It is worth noting that the potential for teratological or polymorphic structures associated with the female gametophytes of *Amborella* does exist. We found two instances, among over 160 mature female gametophytes examined, of antipodal proliferation. Antipodal proliferation has been reported in numerous basal eudicots, magnoliids, and monocots (Johri et al., 1992; Williams and Friedman, 2004; Holloway and Friedman, 2008).

Comparative aspects of female gametophyte development in* Amborella *and other ancient angiosperm lineages—It is now evident that not one, but in fact three, patterns of female gametophyte development and mature structure (Fig. 10) characterize the common ancestors of the four most ancient clades of extant angiosperms (Amborellales, Nymphaeales, Austrobaileyales, and the clade that includes all other angiosperms): Amborella-type, Nuphar/Schisandra-type, and Polygonum-type (Friedman, 2006a, b). The Amborella-type female gametophyte is restricted to a single extant species, *Amborella trichopoda*. The Nuphar/Schisandra-type appears to be universal in the Nymphaeales and Austrobaileyales (Williams and Friedman, 2002, 2004; Friedman et al., 2003; Friedman, 2006b, 2008; Tobe et al., 2007; Rudall et al., 2008), and this specifically includes members of the Hydatellaceae (Friedman, 2008; Rudall et al., 2008), which have recently been discovered to be part of the Nymphaeales (and not monocots, as long believed). Based on explicit reconstruction of character distribution and evolution, the Polygonum-type is certain to be representative of the common ancestor of monocots, eudicots, magnoliids, Ceratophyllaceae, and Chloranthaceae (Friedman and Williams, 2003).

Comparative evolutionary developmental analyses of the four-nucleate, four-celled female gametophytes in the ancient angiosperm lineages Nymphaeales and Austrobaileyales led to the key insight that the female gametophytes of all flowering plants are fundamentally modular entities (Friedman and Williams, 2003, 2004; Friedman et al., 2008) composed of iterative sets of quartets of nuclei (sensu Porsch, 1907; Schnarf, 1936; Cocucci, 1973; Favre-DuChartre, 1976; Battaglia, 1989; Haig, 1990). Development of a standard angiosperm female gametophyte module involves three basic ontogenetic stages: (1) positioning of a single nucleus within a developmentally autonomous cytoplasmic domain of the female gametophyte; (2) two free-nuclear mitoses to yield four nuclei within that domain; and (3) partitioning of three uninucleate cells adjacent to the pole such that the fourth nucleus is confined to the central cell of the female gametophyte (Fig. 11; Friedman and Williams, 2003; Friedman et al., 2008). Angiosperm female gametophytes are

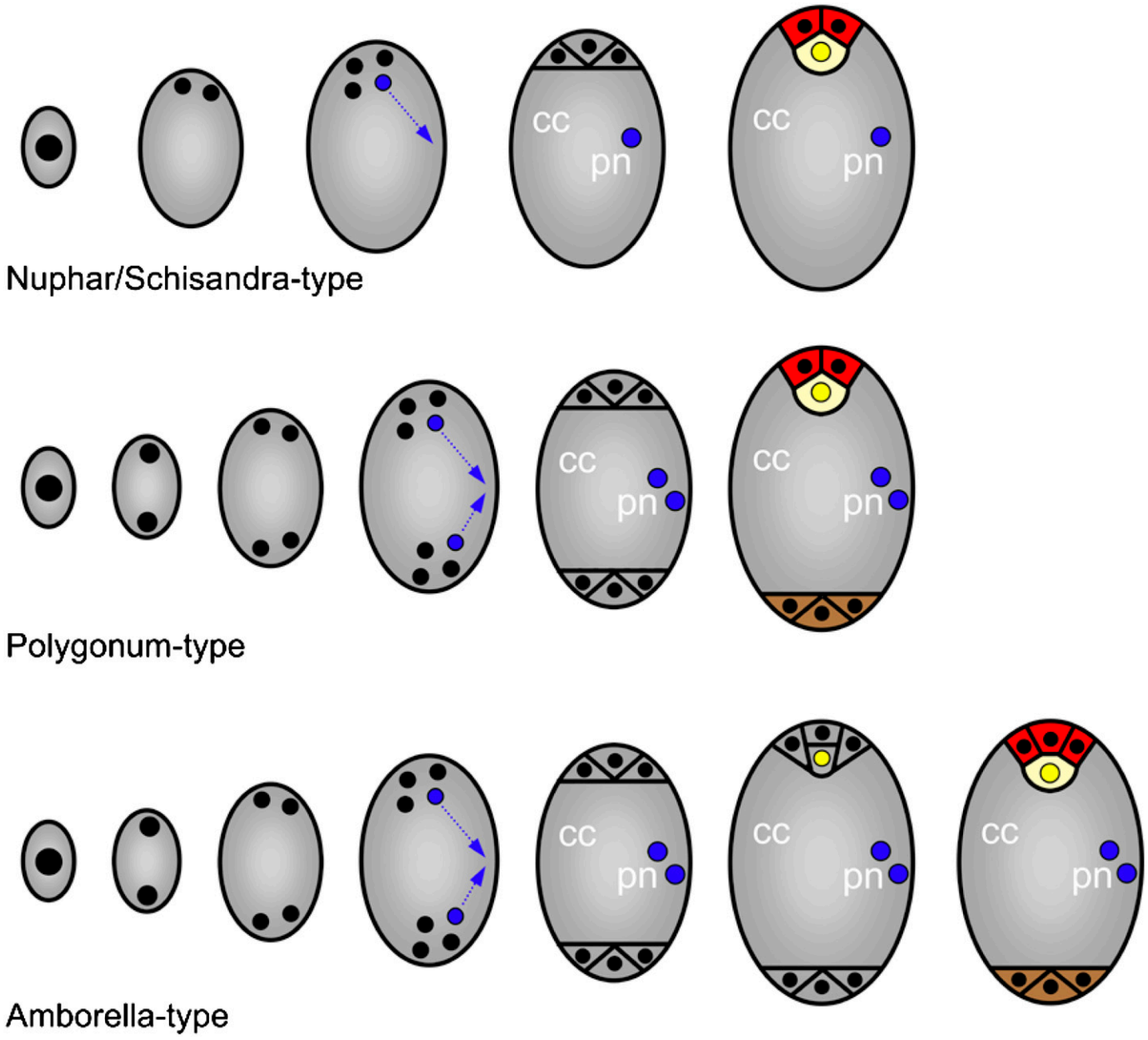

Fig. 10. Comparative development of Nuphar/Schisandra-type, Polygonum-type, and Amborella-type female gametophytes. Micropylar pole is toward top of figure. At the two-nucleate syncytial stage, water lily female gametophytes have both nuclei at the micropylar pole; in Polygonum-type and Amborella-type female gametophytes, a nuclear migration event leads to placement of a single nucleus at each pole. Thus, four-nucleate, four-celled female gametophytes of Nymphaeales and Austrobaileyales have a single developmental module that produces a micropylar egg apparatus and a single polar nucleus. In Polygonum-type and Amborella-type female gametophytes, two developmental modules are initiated at the two-nucleate syncytial stage and cellularization occurs at the eight-nucleate stage to yield three cells at each pole and a contribution of a free nucleus from each module to the central cell. In Polygonum-type female gametophytes, this is the terminal stage of development. In *Amborella*, an asynchronous cell division in the micropylar module yields a third synergid and egg cell. Synergids red, egg cell yellow, antipodal cells brown, polar nuclei blue. cc, central cell; pn, polar nucleus/nuclei.

formed from one (e.g., Nymphaeales and Austrobaileyales), two (e.g., Polygonum-type) or four (e.g., Penaea-type) developmental modules.

In ancient angiosperm lineages with four-celled female gametophytes (Nuphar/Schisandra-type), nuclei are confined to a micropylar domain during free-nuclear development, and this yields a single modular quartet that is partitioned into a three-celled egg apparatus and a uninucleate central cell (Fig. 10). In *Amborella* and angiosperms with Polygonum-type female gametophytes, migration of one of the two nuclei to the chalazal pole at the two-nucleate stage results in the establishment of a chalazal developmental module, in addition to the egg-containing micropylar module, that ultimately forms three antipodal cells and a polar nucleus (Fig. 10). In Amborella-type female gametophytes, the egg apparatus contains four cells; in the Polygonum-type, there are three.

From a comparative perspective, the ontogeny of the Amborella-type female gametophyte is most similar to that of the

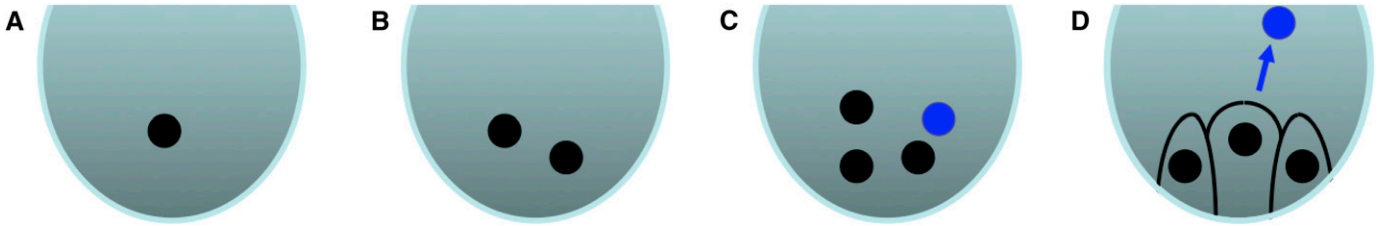

Fig. 11. Development of the basic modular quartet of the angiosperm female gametophyte. (A) A single nucleus is established in a cytoplasmic/developmental domain of the female gametophyte. (B, C) This nucleus initiates two free-nuclear mitoses to yield four nuclei. (D) Three of these nuclei are partitioned into parietally positioned uninucleate cells and the fourth nucleus (blue) is contributed to the common cytoplasm of the central cell of the female gametophyte.

common Polygonum-type. In *Amborella* and in angiosperms with a Polygonum-type female gametophyte, the uninucleate functional megaspore divides mitotically to produce two daughter nuclei that migrate to opposite poles (developmental domains). Each nucleus then initiates an independent developmental module that produces four free nuclei (for a total of eight free nuclei). At the eight-nucleate stage, cytokinesis partitions three nuclei into three cells at each pole, while the remaining free nucleus from each of the two modular quartets is contributed to the common cytoplasm of the central cell. Thus, following cellularization of the syncytium, both Amborella-type and Polygonum-type female gametophytes are seven-celled and eight-nucleate (Fig. 10).

In contrast to angiosperms with Polygonum-type female gametophytes, cellularization of the eight-nucleate syncytium into seven cells in *Amborella* does not constitute the terminus of somatic and sexual ontogeny. After cellularization in Polygonum-type female gametophytes, an egg apparatus with two synergids and one egg cell differentiates (Maheshwari, 1950). However, in *Amborella*, formation of an egg cell and final differentiation of an egg apparatus does not occur until a subsequent cell division yields a nine-nucleate, eight-celled female gametophyte with a four-celled egg apparatus (three synergids and an egg cell). Importantly, the egg cell is formed from this final asynchronous division (Friedman, 2006a). Thus the key (and only) developmental difference between Polygonum-type and Amborella-type female gametophytes involves either the developmental addition of a terminal ontogenetic stage (peramorphosis), assuming that the Amborella-type egg apparatus is apomorphic, or the developmental advancement of sexual maturation (i.e., the formation of an egg) accompanied by truncation of ontogeny (paedomorphosis), assuming that the Polygonum- and Nuphar/Schisandra-type three-celled egg apparatus is apomorphic (Fig. 10).

Development of the Amborella-type female gametophyte differs critically from the Nuphar/Schisandra-type at two key points of ontogeny. In Nymphaeales (Hydatellaceae, Cabombaceae, and Nymphaeaceae) and Austrobaileyales (Austrobaileyaceae, Trimeniaceae, Illiciaceae, and Schisandraceae) after the first mitotic division of the functional megaspore, the two resulting nuclei do not migrate to opposite poles of the female gametophyte to establish two developmental modules. Instead, both nuclei at the two-nucleate stage in Nymphaeales and Austrobaileyales remain in the micropylar region (Fig. 10). As such, only a single developmental module is initiated in the Nuphar/Schisandra-type of female gametophyte. This produces a quartet of nuclei at the micropylar end of the female gametophyte (Friedman and Williams, 2003; Friedman et al., 2008).

The second point of ontogenetic divergence between the Amborella-type and Nuphar/Schisandra-type female gametophyte involves the number of cells and process of differentiation of the egg apparatus (as is the case with the Polygonum-type described earlier). In *Amborella*, the egg apparatus contains four cells and the egg cell is formed by a terminal asynchronous cell division. In members of the Nymphaeales and Austrobaileyales, the egg apparatus is three-celled, and the egg differentiates directly from one of the three cells at the micropylar end of the female gametophyte formed during cellularization (Fig. 10).

Hypothesized plesiomorphic states and character transitions: Analysis of evolutionary history—Female gametophytes of angiosperms, as with any organisms, are an amalgam of biological traits. For the purposes of our analysis, there are two key features of female gametophyte development that vary among ancient lineages of angiosperms: the number of developmental modules initiated (one or two) and the number of cells present in the egg apparatus (three or four).

It is equally parsimonious to hypothesize either character state for the egg apparatus (three-celled or four-celled) as plesiomorphic (Fig. 12). As such, the unique (among angiosperms) asynchronous cell division to form an egg and a third synergid may be viewed as a derived condition that evolved somewhere along the 130 million year long branch that leads to *Amborella trichopoda*. Alternatively, the four-celled egg apparatus could represent the original condition for angiosperms (Friedman, 2006a).

Analysis of the evolution of female gametophyte module number indicates that it is equally parsimonious (two steps or character transitions) to posit that one or two modules is plesiomorphic for angiosperms (Fig. 13). If the four-nucleate, four-celled female gametophytes of Nymphaeales and Austrobaileyales represent the plesiomorphic condition for flowering plants, then there has been the separate and homoplasious evolution of two-module ontogenies in the Amborellales and the common ancestor of monocots, magnoliids, and eudicots. Alternatively, if either the two-module Amborella-type or Polygonum-type female gametophyte is plesiomorphic for angiosperms, then there must have been two separate homoplasious losses of the chalazal developmental module (a result of the loss of nuclear migration at the two-nucleate syncytial stage) to yield, independently, the four-nucleate and four-celled female gametophytes that characterize the Nymphaeales and Austrobaileyales.

Interestingly, these results suggest that it is equally parsimonious for any of the three female gametophyte patterns (Amborella-type, Nuphar/Schisandra-type, or Polygonum-type) to

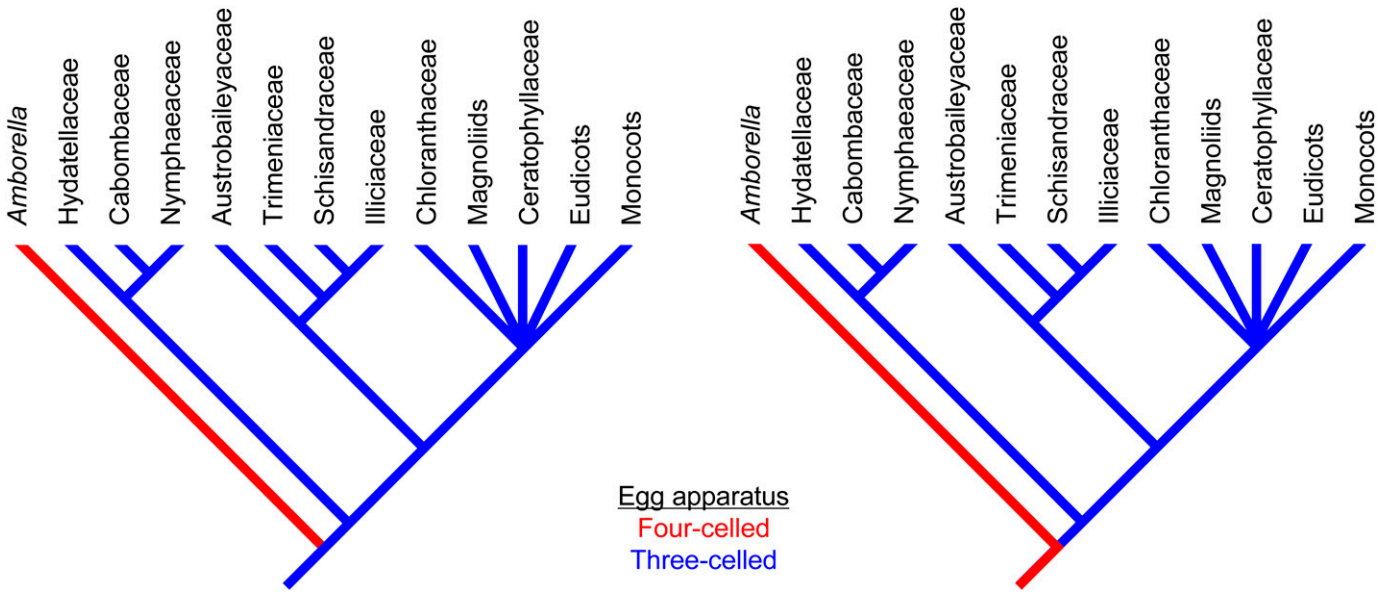

Fig. 12. Parsimony analysis for plesiomorphic number of cells in egg apparatus for angiosperms. The four-celled egg apparatus of *Amborella* may be plesiomorphic (right) or the three-celled egg apparatus common to Nymphaeales, Austrobaileyales, and almost all other angiosperms (left) may be plesiomorphic for flowering plants.

represent the plesiomorphic condition for angiosperms. Thus, from a strict parsimony perspective, the common ancestor of extant flowering plants had an Amborella-type female gametophyte, a Nuphar/Schisandra-type female gametophyte, or a Polygonum-type female gametophyte. For now, and in spite of the fact that none of the members of the Amborellales, Nymphaeales and Austrobaileyales has a Polygonum-type female gametophyte, the possibility that the Polygonum-type female gametophyte is plesiomorphic for flowering plants remains a reasonable conjecture.

Beyond parsimony—Simple parsimony analysis of the distribution and evolution of female gametophyte types among ancient lineages of angiosperms does not appear to provide any resolution of the ancestral condition. Thus, it is worth asking whether insights into the biological consequences (correlates) of the three alternative gametophyte structures can help to resolve this issue. Endosperm in flowering plants is initiated by the fertilization of the central cell of the female gametophyte during the process of double fertilization. For this reason, the genetic constitutions of endosperms among flowering plants are specifi-

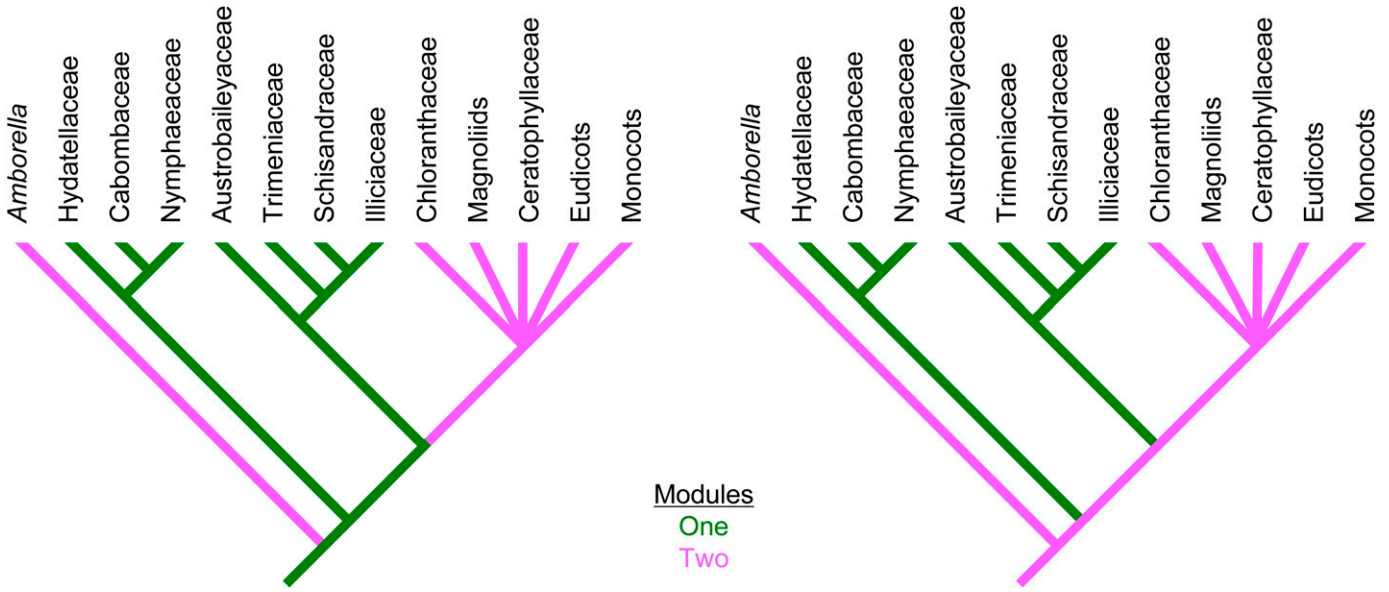

Fig. 13. Parsimony analysis for plesiomorphic number of developmental modules in female gametophytes of angiosperms. One-module female gametophytes of Nymphaeales and Austrobaileyales may be plesiomorphic (left) or two-module female gametophytes associated with Polygonum-type and Amborella-type female gametophytes may be plesiomorphic. Either condition requires two character-state transitions during early diversification of angiosperms.

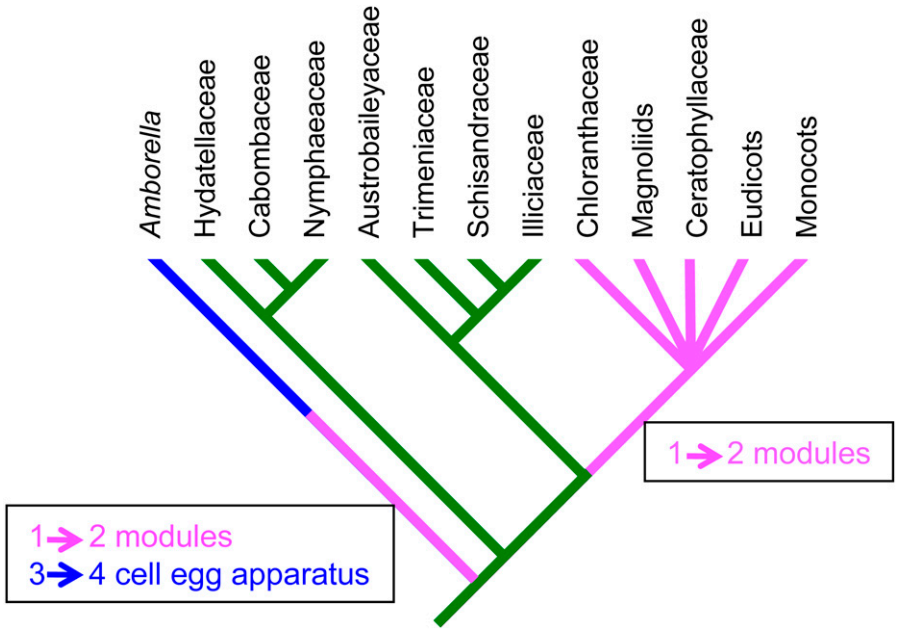

Fig. 14. Best hypothesis for early evolution of angiosperm female gametophytes based on considerations of the functional biology of endosperm (see Discussion). It is likely that the first angiosperms produced four-nucleate, four-celled female gametophytes with a single developmental module (no nuclear migration at the two-nucleate syncytial phase). These female gametophytes (coded in green), as currently found in Nymphaeales and Austrobaileyales yield diploid endosperms from fertilization of a uninucleate haploid central cell. In the common ancestor of all angiosperms except *Amborella*, Nymphaeales, and Austrobaileyales, and in the lineage leading to *Amborella trichopoda*, insertion of a nuclear migration event at the two-nucleate syncytial stage led to initiation of two developmental modules and formation of a seven-celled, eight-nucleate female gametophyte (Polygonum-type, coded in pink). We hypothesize that in Amborellales modular duplication occurred prior to increase in cell number in the egg apparatus (coded in blue) because it would otherwise seem likely that an ectopically expressed chalazal module would have four parietal cells (antipodals) rather than three. Thus, ancestors of *Amborella trichopoda* are likely to have had Polygonum-type female gametophytes at some point in their evolutionary history.

cally determined by (and vary according to) the nuclear complement of the central cell. Variations in female gametophyte development and mature structure have direct consequences on endosperm function and fitness (Friedman et al., 2008).

Selection is hypothesized to favor endosperms with higher ploidy, higher heterozygosity, higher maternal to paternal genome ratios, and reduced opportunity for genetic (interparental and/or parent–offspring) conflict (see Friedman et al., 2008 for a review of this extensive and fascinating literature). Stebbins (1974, 1976) was the first to suggest that higher levels of endosperm ploidy should enable higher rates of transcription in support of the active physiological role of endosperm and, hence, should be adaptive and evolutionarily favored. Thus, transitions from female gametophytes that produce diploid endosperms (Nuphar/Schisandra-type) to those that yield triploid endosperms (e.g., Polygonum-type and Amborella-type) are predicted to be beneficial (Friedman et al., 2008).

A trend toward higher ratios of maternal to paternal genomic contributions to endosperm has also been predicted by theoretical analyses of the potential roles of interparental and/or parent-offspring conflict associated with seed provisioning (Charnov, 1979; Cook, 1981; Westoby and Rice, 1982; Queller, 1983, 1989, 1994; Willson and Burley, 1983; Law and Cannings, 1984; Bulmer, 1986; Haig, 1986; Haig and Westoby, 1989a, b; Friedman, 1995; Friedman et al., 2008). According to these analyses, conflict between pollen- and ovule-bearing parents over optimal investment of nutrients in the embryo-nourishing tissues of seeds of a maternal sporophyte and/or conflict between sibling embryos for resources from the maternal sporophyte should favor increases in the maternal genomic contribution to endosperm.

The triploid endosperms derived from Amborella-type and Polygonum-type female gametophytes are genetically equivalent (a sperm and two genetically identical polar nuclei participate in the second fertilization event). When compared with the diploid endosperms derived from Nuphar/Schisandra-type female gametophytes (a sperm and one polar nucleus participate in the second fertilization event), predicted levels of interparental and/or parent-offspring conflict are significantly reduced in the triploid endosperms derived from both Amborella-type and Polygonum-type female gametophytes (see Friedman et al., 2008 for specific calculations associated with different plant mating systems).

Reduced levels of genetic conflict (as a consequence of increased maternal genomic contributions to endosperm) in a population of seeds on a maternal sporophyte should lead to preferential provisioning (under resource-limited conditions) of a subset of the most fit embryos/seeds (this maximizes maternal fitness through her progeny). Conversely, higher ratios of paternal to maternal genomes in endosperm are predicted to result in more "selfish" behavior in individual endosperms with respect to the acquisition of nutrients for their compatriot embryos; the result being that paternal genetic interests may lead to the abortion of other embryos with greater overall fitness. There is critical experimental evidence to suggest that when paternal genome dosage or expression in endosperm (in *Zea* and *Arabidopsis*) is increased, the endosperms and embryos of these seeds are larger and aggressively acquire nutrients from the maternal sporophyte (Lin, 1982, 1984; Scott et al., 1998; Adams et al., 2000;

see Gehring et al., 2004, and Baroux et al., 2007, for excellent summaries of this literature), potentially at the expense of seeds with more fit embryos.

Assuming the predictions of the endosperm ploidy hypothesis and genetic conflict hypotheses are correct, increases in the number of female gametophyte developmental modules should have been favored over the course of angiosperm evolutionary history. The benefits of higher module number (for example, two vs. one) will be manifest in trends toward increased endosperm ploidy, increased maternal to paternal genomic ratios, increased relatedness of the maternal sporophyte to the endosperms contained within its seeds, and diminished conflict through decreased ratios of relatedness of endosperm to its compatriot embryo vs. other embryos (Friedman et al., 2008).

For these reasons, the most plausible reconstruction of early-angiosperm female gametophyte character evolution is one that begins with a single-module female gametophyte with a haploid central cell that yields a diploid endosperm with a 1 : 1 maternal to paternal genome ratio and high levels of genetic conflict. This stands in marked contrast to the hypothesis that either a Polygonum-type or Amborella-type female gametophyte (with a diploid central cell) is plesiomorphic because this hypothesis requires two independent transitions from triploid endosperm to diploid endosperm (which run counter to the theoretical predictions of Stebbins's ploidy hypothesis and the genetic conflict hypotheses).

We also hypothesize that the common ancestor of angiosperms had a three-celled egg apparatus. If the original (plesiomorphic) female gametophyte module produced four parietal cells and contributed a fifth nucleus to the central cell, it would seem likely that upon modular duplication, an ectopically expressed chalazal module in Amborella-type and Polygonum-type female gametophytes would have had four antipodal cells rather than three. If correct, the Nuphar/Schisandra-type, with its three-celled egg apparatus gave rise to the unique Amborella-type female gametophyte through module duplication (to create a seven-celled, eight-nucleate structure) and a subsequent peramorphic addition of an asynchronous cell division to yield a four-celled egg apparatus (Fig. 14). Thus, the ancestors of *Amborella trichopoda* are likely to have had Polygonum-type female gametophytes at some point in their evolutionary history.

In the common ancestor of the fourth ancient angiosperm clade (all angiosperms except *Amborella*, Nymphaeales, and Austrobaileyales), module duplication (again through nuclear migration at the two-nucleate syncytial stage) gave rise to the Polygonum-type female gametophyte (Fig. 14) that characterizes well over 80% of extant angiosperms (Palser, 1975; and this is likely to be a conservative estimate). Thus, triploid endosperm (derived from a sperm and two genetically identical polar nuclei) appears to have evolved twice: once in the ancestor of *Amborella* and once in the common ancestor of monocots, eudicots, and magnoliids. Of course, should future phylogenetic analyses alter the best hypotheses for relationships among the most ancient lineages of angiosperms (and particularly the position of *Amborella* as sister to all other extant flowering plants), careful reexamination of the evolutionary developmental history of the female gametophyte will be required.

Concluding remarks—For over a century, a specific set of paradigms concerning the origin and early diversification of flowering plant reproductive features was dominant. The first flowering plants were long hypothesized to have had large showy flowers with many parts, conduplicately folded carpels and laminar stamens. At the level of the fertilization process, the female gametophyte was believed to be Polygonum-type (seven cells and eight nuclei) with a second fertilization event that yielded a triploid endosperm. Most workers thought that the endosperm of the earliest flowering plants was free nuclear. Finally, it has long been a matter of "fact" that angiosperms were unique among seed plants in deferring maternal allocation of resources to the seed until after fertilization. In the last five years alone, each of these paradigms appears to have fallen or is poised to do so.

We now know that the flowers of the first angiosperms were extremely small, with ascidiate carpels and nonlaminar stamens (Endress, 2001, 2006; Friis et al., 2006). None of the members of the earliest extant angiosperm clades (specifically Amborellales, Nymphaeales, and Austrobaileyales) produces a Polygonum-type female gametophyte and, as we have argued, the four-nucleate, four-celled Nuphar/Schisandra-type is very likely to be plesiomorphic. With the exception of *Amborella*, members of early-divergent angiosperm lineages, including *Hydatella* (*Trithuria*), form diploid (not triploid endosperm, as had been universally assumed) genetically biparental endosperms from the fertilization of a haploid uninucleate central cell. It is clear that the plesiomorphic pattern of endosperm development is ab initio cellular (not syncytial; Floyd and Friedman, 2000; Holloway and Friedman, 2008). Most recently, with the discovery that maternal plants of *Hydatella (Trithuria)* provision ovules/seeds with embryo-nourishing reserves prior to fertilization (Friedman, 2008), yet another angiosperm-defining biological feature may well be overturned. Only time, and further study of the embryological features of the ancient angiosperm lineages, will tell how many additional longstanding assumptions (dogmas) concerning the earliest flowering plants will fall.

In the roughly ten years since our phylogenetic understanding of the relationships of ancient lineages of angiosperms changed radically, the focus on the biology of *Amborella*, Nymphaeales (including Hydatellaceae), Austrobaileyales, magnoliids, early divergent monocots, and early divergent eudicots has provided an entirely new set of perspectives on the earliest phases of the diversification of flowering plants. The sudden shift in accepted hypotheses governing our understanding of early angiosperm evolution was essentially unexpected; not one of these new hypotheses was even remotely anticipated a decade ago. The paradigm shift has occurred in the blink of an eye.

There is cause for considerable optimism that our understanding of the patterns of early angiosperm diversification will continue to unfold at a remarkable pace. The extraordinary expansion of knowledge of the early flowering plant fossil record over the last thirty years shows no signs of abating. Our current phylogenetic insights into the deeper relationships of angiosperms that began in the late 1990s have been strengthened with further and more comprehensive analyses. Finally, the few extant (perhaps even relictual) morphologists, embryologists, and anatomists have made the study of early-divergent angiosperm lineages a remarkably active field. In so doing, they have uncovered tremendous and heretofore unknown biological diversity among the most ancient clades of flowering plants, have themselves been intellectually energized and may even live to witness a resurgence of these disciplines. Not since the golden age of organismic structural plant biology in the first half of the twentieth century has there been so much to look forward to in

terms of discovery and its potential impact on our understanding of one of the most complicated and interesting evolutionary stories in the history of photosynthetic life, the origin and early diversification of flowering plants.

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