Developmental Evolution of the Sexual Process in Ancient Flowering Plant Lineages

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INTRODUCTION

Recent investigations of ancient angiosperm lineages are yielding data critical to a fundamental reassessment of the origin and early evolution of flowering plants. To ''reconstruct'' the reproductive features of the earliest flowering plants, biological characters must be examined in an appropriate phylogenetic sampling of extant angiosperms, and their history must then be traced back through 130 million years of time. This task requires, first, a clear formulation of the phylogenetic interrelationships among basal angiosperms. Once robust phylogenetic hypotheses are available, comparative analyses can be used to infer and reconstruct the evolutionary history of a broad range of biological characters. Thus, the study of the origin and early evolution of flowering plants, like that of any group of organisms, relies on the interplay of phylogenetic and comparative biology. Incorrect (or vague) assessments of phylogenetic relationships, as well as misconstrued biological character data, will result in flawed evolutionary historical hypotheses. Both of these pitfalls have plagued the reconstruction of early angiosperm reproductive history for more than a century. After a long period of empirical and intellectual stagnation, critical new reproductive data coupled with more robust phylogenetic hypotheses are radically altering the conceptual landscape. Many of the century-old paradigms about the origin and early evolution of flowering plant reproductive features are in the midst of being substantially overthrown.

For more than a century, the ''defining'' features of flowering plant reproductive biology have been thought to be well circumscribed. Typically, in angiosperms, the pollen grain (male gametophyte) is bicellular when it is shed from the anther and produces two sperm during the growth of the pollen tube within the tissues of the gynoecium. The female gametophyte is almost always monosporic in origin and usually develops into a seven-celled, eight-nucleate organism before fertilization. The fertilization process in flowering plants is marked by two separate gametic fusion events that produce a diploid embryo and a triploid embryo-nourishing endosperm. Finally, in most flowering plants, the embryo initiates a cellular pattern of development and the endosperm proceeds through a free nuclear (syncytial) phase of development (but eventually becomes cellularized)

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(Table 1). Although most angiosperms conform to this suite of reproductive characteristics, the widespread distribution of these features reveals nothing of their evolutionary origin and diversification. As we show here, new data derived from studies of ancient (extant) flowering plant lineages (*Amborella*, Nymphaeales, Austrobaileyales) indicate that the reproductive features of the first flowering plants differed significantly from the suite of gametophyte, embryo, and endosperm characteristics that are common to the overwhelming majority of extant flowering plants.

In this review, we examine the reproductive features of flowering plants associated with the fertilization process from an evolutionary developmental perspective. We address three basic questions. First, did the earliest flowering plants possess a process of double fertilization? The definitive answer (as of now) is maybe. As we show, after a century and a half of study of the plant fertilization process, a key taxon central to reconstructing the ancestral characteristics of extant flowering plants has yet to be examined for this process. Indeed, until two years ago (Williams and Friedman, 2002), there were no definitive studies demonstrating a process of double fertilization in any of the most ancient lineages of angiosperms (for a review of earlier studies, see Friedman and Floyd, 2001). Thus, the long-standing assumption that the first flowering plants expressed a second fertilization event that initiated a genetically biparental endosperm has yet to be proven.

Second, what was the developmental pattern and mature structure of the ancestral angiosperm female gametophyte? We present an analysis of the very recent and surprising discovery that the female gametophyte in the earliest flowering plants probably was not of the Polygonum type (monosporic, seven celled, and eight nucleate) but rather was four celled and four nucleate at maturity (Friedman et al., 2003; Friedman and Williams, 2003; Williams and Friedman, 2004). This finding has led to a fundamental reassessment of female gametophyte developmental evolution. Most importantly, this discovery has provided strong evidence of the fundamentally modular nature of the angiosperm female gametophyte. The female gametophyte developmental module is characterized by the positioning of a single nucleus within a cytoplasmic domain (early in female gametophyte ontogeny), followed by two free nuclear mitoses to yield four nuclei within the domain, and lastly, by the formation of three uninucleate cells accompanied by the sequestration of the fourth nucleus in the central region of the female gametophyte (central cell). Here, we present evidence in support of the hypothesis that the evolutionary diversification of the

Table 1. Developmental Features of the Angiosperm Reproductive Syndrome

Feature	First Angiosperms (20th Century)	First Angiosperms (Current View)	Most Angiosperms	*Zea mays*	*Arabidopsis thaliana*
Pollen grain	Bicellular	Bicellular	Bicellular	Tricellular	Tricellular
Female gametophyte at maturity	Seven-celled, eight-nucleate	Four-celled, four-nucleate	Seven-celled, eight-nucleate	Seven-celled, eight-nucleate[a]	Seven-celled, eight-nucleate
Gamete cell cycle position at fertilization	Unknown	G_1	Unknown	G_1	G_2
Endosperm ploidy	Triploid	Diploid	Triploid	Triploid	Triploid
Endosperm developmental pattern[b]	Free nuclear or cellular	Cellular	Free nuclear	Free nuclear	Free nuclear

[a] With subsequent proliferation of antipodals (Diboll and Larson, 1966).
[b] Floyd and Friedman (2000).

angiosperm female gametophyte has been accomplished largely through the ectopic expression of this basic developmental module.

Third, what were the ploidy and genetic constitution of early angiosperm endosperm? Because endosperm is initiated by the fertilization of the central cell, the ploidy and genetic constitution of endosperm are tied directly to the development and mature structure of the female gametophyte. Thus, any transition from a four-celled, four-nucleate female gametophyte with a haploid central cell to a seven-celled, eight-nucleate female gametophyte with a binucleate (diploid) central cell (Polygonum type) likely marks a transition from the diploid endosperms characteristic of the earliest angiosperms to the triploid endosperms found among most extant flowering plants (Friedman and Williams, 2003). The current data are congruent with the hypothesis that the earliest flowering plants produced diploid biparental endosperms (Friedman and Williams, 2003) and that this condition was followed by a rapid (in geological terms) transition to triploid biparental endosperms in the common ancestor of 99% of extant angiosperms ~10 million years after the origin of flowering plants (Williams and Friedman, 2004). Importantly, the linkage between female gametophyte development and endosperm ploidy and genetics (Friedman and Williams, 2003) indicates that the evolutionary history of endosperm is tied directly to upstream events associated with the development of the female gametophyte.

TWENTIETH CENTURY VIEWS OF THE REPRODUCTIVE BIOLOGY OF THE EARLIEST ANGIOSPERMS

Throughout the 20th century, the broad consensus on the reproductive characteristics of the earliest angiosperms derived from a general phylogenetic hypothesis that ''*Magnolia*-like'' plants and their relatives (magnoliids) constitute a plexus of ancient lineages (in essence, the paraphyletic wellspring) from which all other angiosperms evolved (Bessey, 1897, 1915; Arber and Parkin, 1907; Takhtajan, 1969; Dahlgren, 1980; Cronquist, 1981; Walker and Walker, 1984; Donoghue and Doyle, 1989; Thorne, 1992). As a consequence, members of the Magnoliales and other groups, such as the Laurales and Winterales, frequently were studied to reconstruct the embryological features of the earliest flowering plants (Maneval, 1914; Earle, 1938; Hayashi, 1965; Sastri, 1969; Bhandari, 1971a, 1971b).

Embryological studies throughout the 20th century affirmed that a monosporic seven-celled, eight-nucleate female gametophyte (Polygonum type) is prevalent among magnoliids (Hayashi, 1965; Bhandari, 1971a, 1971b; Tobe et al., 2000) and among angiosperms as a whole (it is estimated to be present in >70% of extant angiosperm species [Maheshwari, 1950; Johri et al., 1992]). The development of the Polygonum type of female gametophyte begins with a single functional megaspore that initiates a series of three free nuclear divisions to yield eight nuclei within a syncytium. The syncytium then cellularizes to produce an egg apparatus (two synergids and an egg cell) at the micropylar pole and three antipodal cells at the chalazal pole. The remaining two nuclei of the female gametophyte are partitioned into the remainder of the syncytium, or central cell. Given the near ubiquity of Polygonum-type female gametophytes among magnoliid lineages, the conclusion was reached early and often in the 20th century that the first flowering plants had a monosporic seven-celled, eight-nucleate female gametophyte (Porsch, 1907; Schnarf, 1931; Johri, 1963; Davis, 1966; 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). Furthermore, all variants of female gametophyte structure and developmental patterns in angiosperms were thought to be derived in some manner from this starting point.

Twentieth century embryological studies also affirmed that a second fertilization event that initiates an endosperm was ubiquitous among flowering plants, including magnoliids (Sargant, 1900; for a review of this topic, see Friedman, 2001a). Thus, soon after its discovery, a double fertilization process was viewed as a defining and ancestral feature of angiosperms. Because the central cell of a Polygonum-type female gametophyte is initially binucleate (the two haploid polar nuclei may fuse before the second fertilization event to yield a diploid ''secondary nucleus''), the product of the fertilization of the central cell is a triploid endosperm with a biparental genetic constitution and a 2:1 ratio of maternal to paternal genomes; this was universally concluded to be the ancestral condition for flowering plants.

The twentieth century views of what characterized the ''primitive'' suite of angiosperm reproductive features were accepted with little dissent. Indeed, based on all of the generally accepted paradigms of flowering plant reproductive biology,

Figure 1. Flowers of Basal Angiosperm Lineages.

(A) Carpellate flower of the dioecious taxon *Amborella trichopoda*.
(B) Hermaphrodite flower of *Nuphar polysepala*.
(C) Staminate flower of the monoecious taxon *Kadsura japonica*.

evolutionary biologists could confidently report that ''[d]ouble fertilization and the development of copious endosperm from a triple fusion nucleus [in a Polygonum-type female gametophyte] are clearly primitive characters within the angiosperms'' (Cronquist, 1988). There was little reason to question these views—that is, until 1999.

THE PHYLOGENETIC SHIFT

The long-standing assumptions about which lineages might contain taxa with ancient characteristics of flowering plants changed dramatically in 1999. At that time, a series of phylogenetic analyses converged on a set of relationships among basal angiosperms that indicated that most magnoliid lineages, although relatively ancient in origin, were not the most ancient lineages of flowering plants. These phylogenetic analyses (Mathews and Donoghue, 1999; Parkinson et al., 1999; Qiu et al., 1999, 2000; Soltis et al., 1999; Graham et al., 2000; Graham and Olmstead, 2000; Zanis et al., 2002, 2003; Borsch et al., 2003) revealed that the specific magnoliid clades *Amborella*, Nymphaeales (Nymphaeaceae and Cabombaceae), and Austrobaileyales (Illiciaceae, Schisandraceae, Trimeniaceae, and Austrobaileyaceae) constitute a basal grade of flowering plants (Figures 1 and 2). There is still ambiguity regarding the precise interrelationships of *Amborella* and Nymphaeales (Barkman et al., 2000; Graham and Olmstead, 2000; Angiosperm Phylogeny Group, 2003); in addition, the phylogenetic positions of other ancient angiosperm lineages, such as Chloranthaceae and Ceratophyllaceae, remain unresolved. However, there is good reason to believe that all angiosperms, to the exclusion of *Amborella*, Nymphaeales, and Austrobaileyales, share a more recent common ancestor (Figure 2). Thus, *Amborella*, Nymphaeales, and Austrobaileyales each originated (and had begun to evolve) before the origin of monocots, eudicots, and eumagnoliids (a clade that includes Magnoliales, Laurales, Piperales, and Winterales). The only recent phylogenetic analysis to suggest that any of these three lineages might not be basal among angiosperms (Goremykin et al., 2003) may suffer from subminimal taxon sampling, which is known to often yield spurious results.

It is now evident that the common ancestor of monocots and eudicots is not the common ancestor of extant angiosperms. Thus, comparative studies of monocots and eudicots (such as *Zea* and *Arabidopsis*) cannot be used to infer directly the ancestral characteristics of flowering plants (Figure 2). With the discovery that *Amborella*, Nymphaeales, and Austrobaileyales are the most ancient extant angiosperm lineages and might (or might not) retain some of the biological features of the earliest angiosperms (Friedman et al., 2003), it was evident that answers
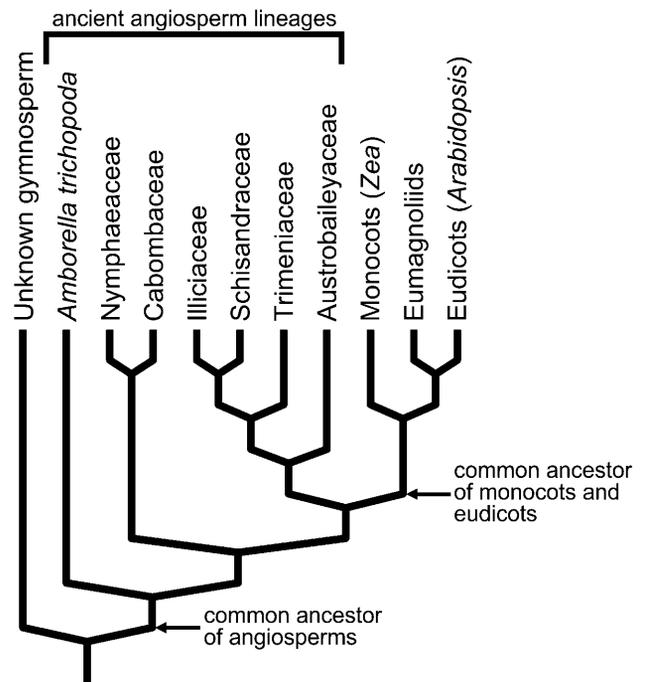

Figure 2. Phylogeny of Angiosperms Based on Recent Molecular Analyses.

Amborella, Nymphaeales, and Austrobaileyales constitute a set of lineages that are more ancient (in terms of time of origin) than monocots, eumagnoliids, or eudicots.

to Darwin's ''abominable mystery'' of the origin of flowering plants (Darwin, 1903) would have to be sought through direct study of these relictual groups (by the few remaining relictual embryologists). Only through careful comparisons of *Amborella*, Nymphaeales, and Austrobaileyales with early divergent lineages of monocots, eumagnoliids, and eudicots can robust hypotheses be formulated regarding the nature of the common ancestor of extant angiosperms.

Unlike members of the Magnoliales, Laurales, Winterales, and many other magnoliids, *Amborella* and taxa within the Austrobaileyales were rarely examined embryologically during the 20th century (Friedman et al., 2003). In the case of the Nymphaeales, many embryological studies were published, although the results often were highly contradictory (Friedman, 2001b; Friedman and Williams, 2003).

IS DOUBLE FERTILIZATION CHARACTERISTIC OF THE EARLIEST ANGIOSPERMS?

After the recent identification of the most ancient lineages of angiosperms, it rapidly became apparent that there was little substantive evidence of a second fertilization event in any of the constituent members of the *Amborella*, Nymphaeales, and Austrobaileyales clades (Friedman and Floyd, 2001). Until 2 years ago, there were just four reports of a fusion of a second sperm nucleus with the two polar nuclei (or their fusion product, the secondary nucleus) of the female gametophyte in the most basal angiosperm lineages: *Brasenia* (Khanna, 1965), *Nymphaea* (Khanna, 1967), *Cabomba* (Galati, 1985), and *Illicium* (Hayashi, 1963a). In contradistinction to these claims, there were reports that some early angiosperm lineages might not have a second fertilization event (e.g., *Nymphaea* [Cook, 1909] and the ancient monocot lineage *Acorus* [Buell, 1938]). Regrettably, none of these studies included photomicrographic evidence in support of their conclusions.

In the last 2 years, the first robust photomicrographic and cell biological evidence of a second fertilization event in basal angiosperms has been published (Williams and Friedman, 2002, 2004). These new data, from studies of the water lily *Nuphar polysepala* (Nymphaeales) and the woody shrub *Illicium mexicanum* (Austrobaileyales), indicate that a regular gametic fusion event forms a genetically biparental endosperm (Figure 3). DNA quantitation of gametes in members of the Nymphaeales and Austrobaileyales demonstrates that the fertilization process conforms to a G_1 karyogamy pattern (sensu Friedman, 1999) in which gametic nuclei (sperm nucleus, egg nucleus, and central cell nucleus) are haploid and contain the 1C quantity of DNA up through the time of nuclear fusion (Williams and Friedman, 2002, 2004; Friedman et al., 2003). Thus, the resulting zygote nucleus and primary endosperm nucleus are both diploid, initially contain the 2C quantity of DNA, and have a 1:1 ratio of maternal to paternal genomic contributions. This stands in marked contrast to the endosperms of flowering plants with Polygonum-type female gametophytes, in which the endosperm is triploid and contains a 2:1 maternal-to-paternal genome ratio.

The G_1 karyogamy pattern in basal angiosperms is fundamentally similar to that of all nonplant eukaryotes that have been studied (reviewed by Friedman, 1999; Corellou et al., 2001) but differs from the cell cycle behavior of *Arabidopsis* (Friedman, 1999) and several other derived angiosperms (Ermakov et al., 1980). These plants, found scattered in Brassicaceae, Asteraceae, and Poaceae, appear to be unique among eukaryotes: the synthesis phase of the cell cycle is expressed in gametes before fertilization (G_2 karyogamy).

Even though a second fertilization event has now been documented conclusively in the Nymphaeales and Austrobaileyales, the determination of whether this process was a feature of the common ancestor of extant flowering plants still depends on an assessment of the condition in *Amborella*. To date, the fertilization event in *Amborella* has not been observed. If *Amborella* is eventually shown to initiate an endosperm from a sexual process, it can be confidently concluded that double fertilization was present in the common ancestor of all extant flowering plants. However, if *Amborella* lacks a second fertilization event, explanations include the possibilities that the first flowering plants lacked a sexual (biparental) endosperm and that double fertilization evolved in the common ancestor of all extant flowering plants other than *Amborella* or that a second fertilization process was present in the first angiosperms but was lost in the lineage that produced *Amborella*.

If double fertilization is eventually shown to be a feature of the common ancestor of flowering plants, this still will not reveal where this process came from and how it evolved. Double fertilization processes, defined as two fertilization events in a single female gametophyte by two sperm from a single pollen tube, also occur in nonflowering seed plants, particularly among members of the Gnetales and possibly some conifers (Friedman, 1990, 1992a, 1992b; Carmichael and Friedman, 1995). However, the second fertilization event in nonflowering seed plants yields a supernumerary diploid zygote, not an endosperm.

From 1985 through 1998 (Crane, 1985; Doyle and Donoghue, 1986, 1992; Martin and Dowd, 1986, 1991; Zimmer et al., 1989; Loconte and Stevenson, 1990; Hamby and Zimmer, 1992; Chase et al., 1993; Doyle et al., 1994; Rothwell and Serbet, 1994; Doyle, 1996, 1998; Stefanovic et al., 1998), phylogenetic analyses appeared to indicate that Gnetales was the most closely related extant seed plant clade to angiosperms. Thus, it was most parsimonious to interpret double fertilization in Gnetales and angiosperms as homologous, having been derived from a common seed plant ancestor (Friedman, 1990; Carmichael and Friedman, 1996). Recent seed plant phylogenetic analyses either have rejected a close relationship between Gnetales and angiosperms, or have produced highly conflicting topologies, or have been unable to detect any clear (unambiguous) phylogenetic signal of relationships among extant lineages (Hansen et al., 1999; Winter et al., 1999; Bowe et al., 2000; Chaw et al., 2000; Frohlich and Parker, 2000; Qiu et al., 2000; Sanderson et al., 2000; Magallón and Sanderson, 2002; Rydin and Kallersjo, 2002; Rydin et al., 2002; Schmidt and Schneider-Poetsch, 2002; Soltis et al., 2002). For the time being, the phylogenetic placement of Gnetales remains a mystery. Thus, the origin of double fertilization, as represented in angiosperms, and the homology of endosperm (with an antecedent structure) remain shrouded in the depths of evolutionary time.
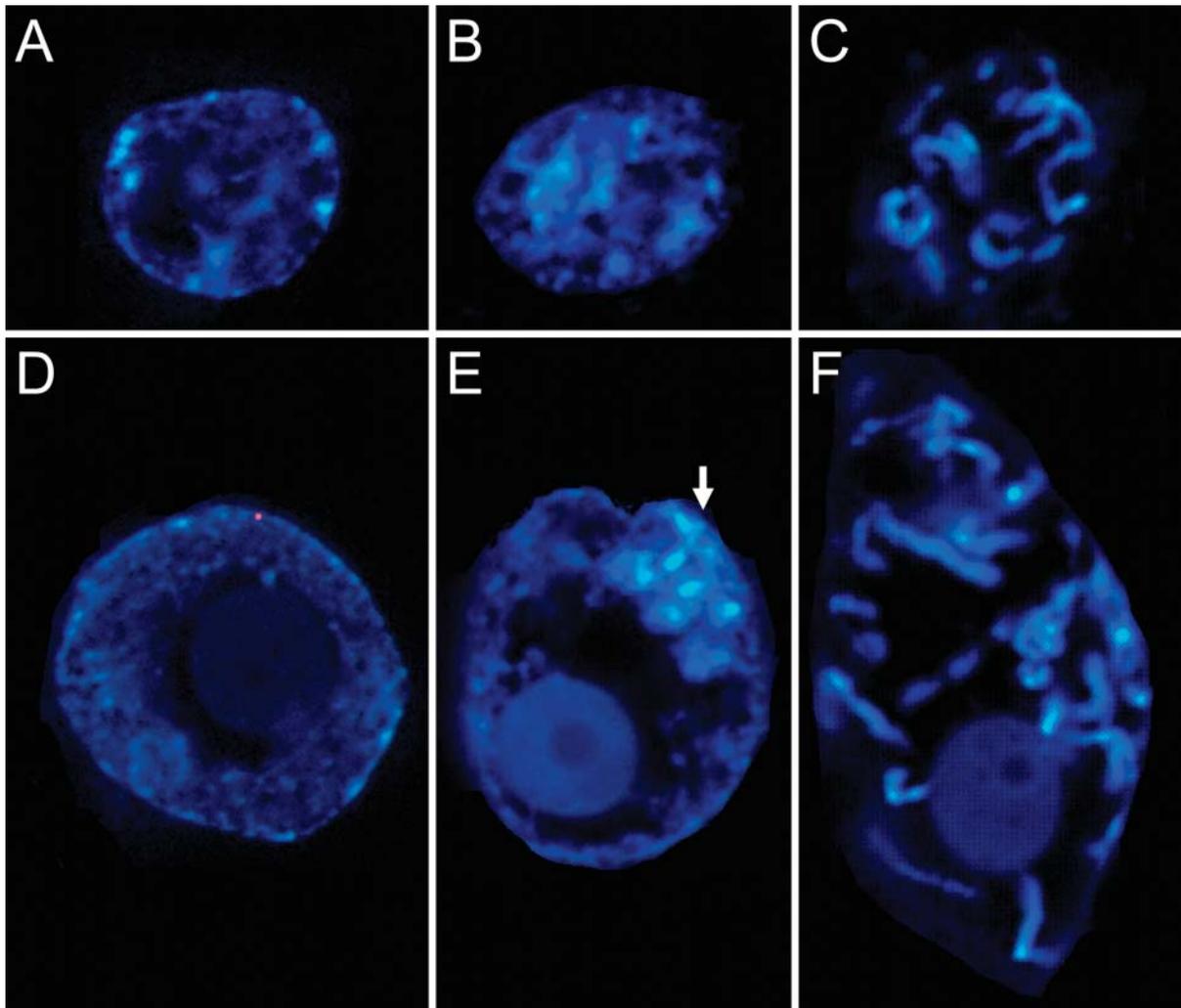
Figure 3. Double Fertilization in the Basal Angiosperm *Nuphar polysepala*.

(A) Egg nucleus (stained with 4′,6-diamidino-2-phenylindole) before the first fertilization event contains the 1C quantity of DNA.

(B) Zygote nucleus before S-phase contains the 2C quantity of DNA.

(C) Zygote nucleus in prophase contains the 4C quantity of DNA.

(D) Central cell nucleus before the second fertilization event contains the 1C quantity of DNA.

(E) Primary endosperm nucleus before S-phase contains the 2C quantity of DNA. The sperm chromatin can be seen (arrow) having entered the central cell nucleus.

(F) Primary endosperm nucleus in prophase contains the 4C quantity of DNA.

STRUCTURE OF FEMALE GAMETOPHYTES AMONG ANCIENT ANGIOSPERM LINEAGES

Subsequent to the 1999 revelation of the basal phylogenetic positions of *Amborella*, Nymphaeales, and Austrobaileyales, Tobe and colleagues (2000) reported that the female gametophyte of *Amborella* produces seven cells and eight nuclei. This finding initially seemed to indicate that although there had been a shift in hypothesis regarding what constituted the most ancient flowering plant lineages, the century-old paradigm that the first angiosperms had a Polygonum-type female gametophyte remained unaltered. Based on a review of the secondary

literature (Padmanabhan, 1970; Johri et al., 1992), Tobe et al. (2000) also noted that members of the Nymphaeales and Austrobaileyales had Polygonum-type female gametophytes, thus perpetuating the long-standing appraisal of the embryological characters of these poorly studied taxa.

In contrast to *Amborella* and what was assumed to be the case in Nymphaeales and Austrobaileyales, there were interesting hints that the female gametophytes in Nymphaeales and Austrobaileyales might not be of the Polygonum type. Beginning with work by Yoshida (1962) on *Schisandra* (Austrobaileyales) and by Batygina et al. (1982) on *Cabomba* (Nymphaeales), several reports (Swamy, 1964; Solntseva, 1981; Galati, 1985;

Battaglia, 1986; Winter and Shamrov, 1991; Van Miegroet and Dujardin, 1992; Orban and Bouharmont, 1998) indicated that the female gametophytes in Austrobaileyales and Nymphaeales contain only four cells at maturity: an egg cell, two synergids, and a uninucleate central cell. However, these findings appeared to be contradicted by other studies that reported seven-celled, eight-nucleate female gametophytes in Nymphaeales and Austrobaileyales (Cook, 1902, 1906; Hayashi, 1963a, 1963b; Kapil and Jalan, 1964; Khanna, 1964, 1965, 1967; Ramji and Padmanabhan, 1965; Schneider, 1978; Prakash, 1998). None of the embryological studies of Nymphaeales and Austrobaileyales included key photomicrographic data that could be evaluated to distinguish between competing hypotheses of female gametophyte structure.

It is now evident that earlier reports of Polygonum-type female gametophytes in Nymphaeales and Austrobaileyales almost certainly are in error (Friedman and Williams, 2003) and that the misinterpretation of data on female gametophyte structure by earlier workers was the result of a subtle "trick" played by nature. In some angiosperms, a transitory seven-celled, eight-nucleate female gametophyte is developmentally transformed into a four-celled, four-nucleate structure before fertilization (Figure 4): the three chalazal antipodal cells undergo cell death and the two polar nuclei within the central cell fuse rapidly to form a single diploid nucleus (the "secondary nucleus"), as is the case in *Amborella* and many magnoliids. As a result, it can be extremely difficult to differentiate a "true" four-celled female gametophyte from a "cryptic" seven-celled female gametophyte (in which antipodals have degenerated and polar nuclei have fused) unless the ploidy of the central cell has been quantitated with DNA microspectrofluorometry (Figure 4). True four-celled female gametophytes have a haploid central cell nucleus, whereas cryptic seven-celled, eight-nucleate female gametophytes in which the antipodals have degenerated and the two haploid nuclei have fused to produce a secondary nucleus contain a diploid central cell.
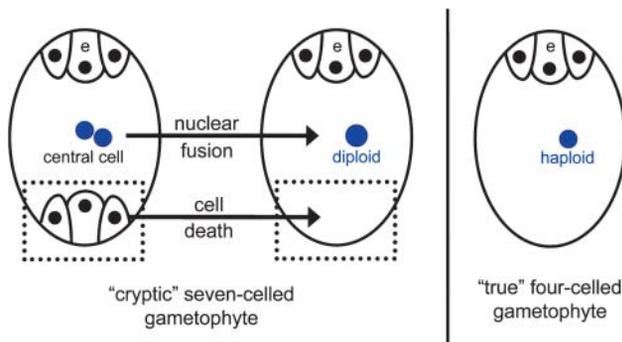

Figure 4. Convergence in the Form of Angiosperm Female Gametophytes at Maturity.

A seven-celled, eight-nucleate female gametophyte (left) undergoes degeneration of the antipodal cells and fusion of the two nuclei of the central cell to yield a four-celled female gametophyte with a diploid central cell ("cryptic" seven-celled gametophyte). A "true" four-celled female gametophyte contains a single haploid nucleus in the central cell (right). e, egg cell.

Many embryologists, assuming that the particular magnoliid taxon under study had a Polygonum-type female gametophyte (in essence because ancient lineages of flowering plants must have this type of female gametophyte), concluded that they had missed a transitory seven-celled, eight-nucleate stage in their developmental series. Thus, female gametophytes in Cabombaceae, Nymphaeaceae, Austrobaileyaceae, Trimeniaceae, Schisandraceae, and Illiciaceae usually were characterized as possessing "ephemeral antipodals" and undergoing rapid fusion of the two polar nuclei within the central cell (Davis, 1966; Johri et al., 1992; Tobe et al., 2000).

The mature female gametophytes of members of the Nymphaeales and Austrobaileyales are monosporic and now have been shown conclusively to contain four cells and four nuclei at maturity: a haploid uninucleate central cell, an egg cell, and two synergids (Figure 5). When the DNA content of the central cell nucleus is assayed, it is shown to be haploid and to occupy the G_1 phase of the cell cycle (Williams and Friedman, 2002, 2004; Friedman et al., 2003; Friedman and Williams, 2003). These data confirm the handful of earlier reports that were not biased by preconceived notions that Polygonum-type female gametophytes should predominate among basal angiosperms.

MODULARITY AND THE EVOLUTION OF FEMALE GAMETOPHYTE ONTOGENY IN ANCIENT ANGIOSPERM LINEAGES

The female gametophyte of angiosperms is best viewed as a fundamentally modular structure in which individual developmental modules consist of "quartets" of nuclei (Porsch, 1907; Schnarf, 1936; Maheshwari, 1950; Swamy and Krishnamurthy, 1975; Favre-DuChartre, 1976; Battaglia, 1989; Friedman and Williams, 2003). Each module can be characterized by a common developmental pattern: (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 uninucleate cells adjacent to the pole such that the fourth nucleus is confined to the central cell of the female gametophyte (Figure 6) (Friedman and Williams, 2003).

There are only two known female gametophyte ontogenetic sequences present in the most ancient clades of extant angiosperms: the monosporic four-celled, four-nucleate sequence characteristic of Nymphaeales and Austrobaileyales (Friedman et al., 2003; Friedman and Williams, 2003; Williams and Friedman, 2004); and the monosporic seven-celled, eight-nucleate sequence of *Amborella* (Tobe et al., 2000). Although the polarity of evolutionary transition is, on strict grounds of parsimony, unresolved, we have argued that four-celled, four-nucleate female gametophytes may be plesiomorphic among angiosperms (Friedman and Williams, 2003). If this hypothesis is correct, the first angiosperm female gametophytes were composed of a single four-celled, four-nucleate module (Friedman and Williams, 2003). Early in angiosperm history, ectopic expression of this basic developmental module resulted in the initiation of a second developmental module within the female gametophyte.

In basal angiosperm taxa with four-celled female gametophytes, all nuclei are confined to the micropylar domain during
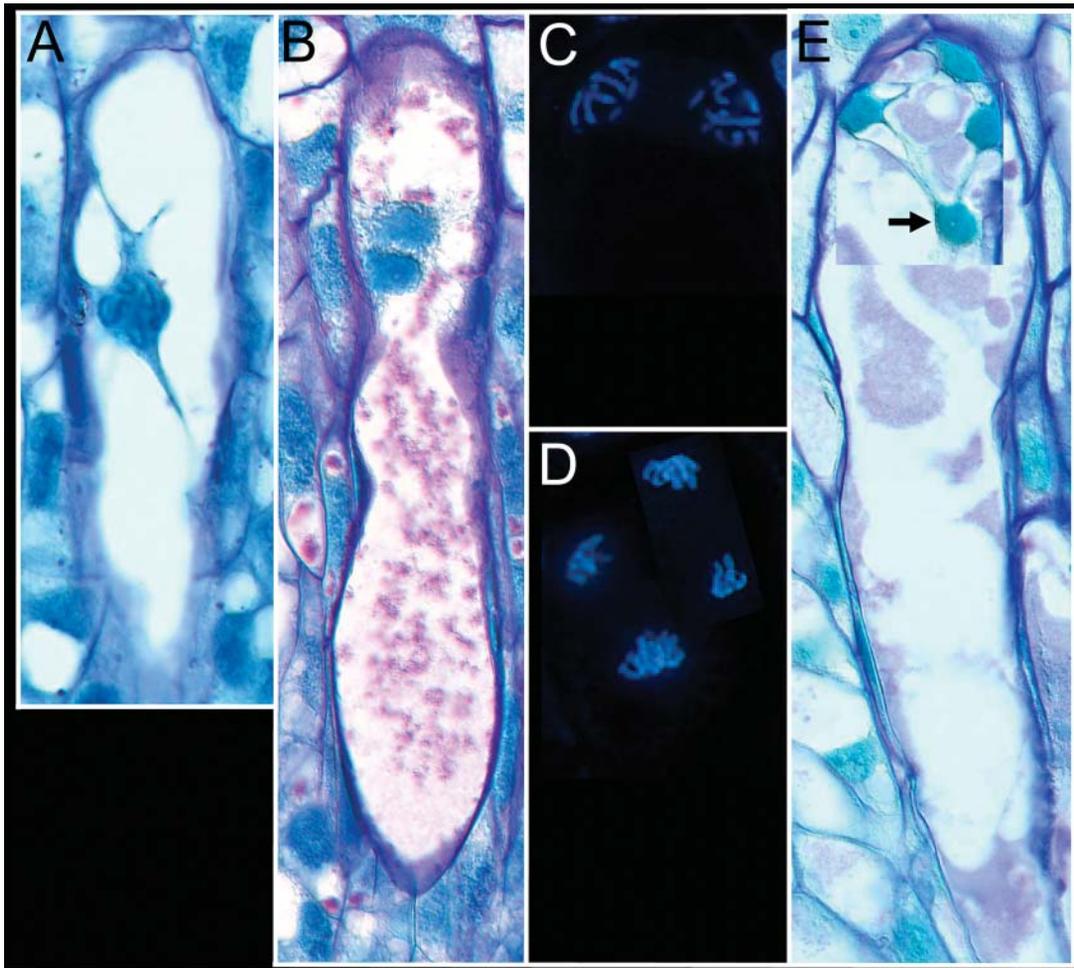
Figure 5. Female Gametophyte Development in a Basal Angiosperm (*Illicium mexicanum*).

(A) One-nucleate stage in prophase.
(B) Two-nucleate stage with both nuclei confined to the micropylar pole (top) of the female gametophyte.
(C) and **(D)** 4′,6-Diamidino-2-phenylindole–stained fluorescence views of the mitotic transition from two nuclei (prophase in **[C]**) to four nuclei (telophase in **[D]**).
(E) Four-nucleate stage of the female gametophyte just before cellularization to form an egg apparatus. The future central cell nucleus is indicated with an arrow.

free nuclear development, and this yields a single modular quartet (Figure 7). In *Amborella* and other angiosperms with Polygonum-type female gametophytes, the migration of one of the nuclei to the chalazal pole at the two-nucleate stage results in the establishment of a chalazal developmental module (in addition to the micropylar module) that ultimately forms three antipodal cells and a polar nucleus (Figure 7).

We have hypothesized that the establishment of a duplicate module in the angiosperm female gametophyte was accomplished initially at the two-nucleate stage through the developmental insertion of a cytoskeletal apparatus that separates the nuclei into distinct cytoplasmic domains at opposite poles of the female gametophyte (Figure 7). The net effect of this novel nuclear migration process was the creation of two cytoplasmic/ developmental domains in the female gametophyte and the

parallel initiation of two modular quartets. The result was the formation of a seven-celled, eight-nucleate Polygonum-type female gametophyte (Friedman and Williams, 2003).

The transition to a two-module seven-celled, eight-nucleate female gametophyte from a one-module four-nucleate, four-celled female gametophyte appears to have occurred twice: once in the *Amborella* lineage and once in the common ancestor of the monocots, eudicots, and eumagnoliids (Figure 8). Given that the oldest known angiosperm pollen has been dated to 132 million years before the present (Friis et al., 1999) and that tricolpate pollen (evidence of the presence of eudicots) extends back 121 million years (Friis et al., 1999; for review, see Doyle, 2000), the common ancestor of monocots, eudicots, and eumagnoliids, in which this key developmental transition took place, can be traced to the first
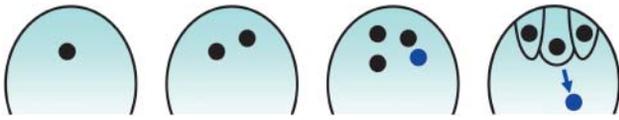
Figure 6. Development of the Basic Modular Quartet of the Angiosperm Female Gametophyte.

A single nucleus is established in a cytoplasmic/developmental domain of the female gametophyte. This nucleus then initiates two free nuclear mitoses to yield four nuclei. Three of these nuclei are partitioned into parietally positioned uninucleate cells, whereas the fourth nucleus is contributed to the common cytoplasm of the central cell of the female gametophyte.

11 million years of angiosperm evolutionary history. It is unknown when the transition to a Polygonum-type female gametophyte occurred in the *Amborella* lineage. Of course, should it turn out that the current phylogenetic placement of *Amborella* is incorrect (and that it is phylogenetically nested among angiosperms with Polygonum-type female gameto- phytes, as suggested by Goremykin et al. [2003]), only a single origin of a chalazal modular quartet (in the common ancestor of monocots, eumagnoliids, and eudicots) would need to be invoked over the course of angiosperm history.

If the modular duplication hypothesis is correct, the chalazal module (or quartet) of monocots, eudicots, and eumagnoliids represents a developmental novelty that arose during the earliest phases of the diversification of angiosperms. Thus, the second polar nucleus and the three antipodal cells of the seven-celled, eight-nucleate female gametophyte are angiosperm innovations and are not strictly homologous with any part of the female gametophyte of nonflowering seed plants. This conclusion stands in marked contrast to the long-standing hypothesis that the antipodal cells of the seven-celled, eight-nucleate female gametophyte are homologous with (and a highly reduced vestige of) the large somatic body (prothallus) of nonflowering seed plant female gametophytes (Sargant, 1900; Battaglia, 1951; Favre-DuChartre, 1984).

The developmental and functional behavior of antipodals and the chalazal polar nucleus in basal monocots and basal eudi- cots suggests that the chalazal module of the Polygonum-type female gametophyte originally consisted of two components: (1) three persistent antipodal cells that lacked conspicuous postfertilization development (Williams and Friedman, 2004), and (2) a polar nucleus that was partitioned into the central cell, where it participated in a second fertilization event. In more recently derived angiosperm lineages, antipodal cells have become modified in diverse ways: these include the proliferation of nuclei to form multinucleate cells, various forms of endopolyploidy, extreme cell growth, and cell mor- phology associated with haustorial function (Huss, 1908; Schnarf, 1931; Kapil and Bhatnagar, 1981; D'Amato, 1984; Willemse and van Went, 1984). The nested phylogenetic placement of taxa with more highly specialized antipodals is in accord with the hypothesis of origin by modular duplication, loss of gametic function (transformation of the egg apparatus

into antipodals), and subsequent co-option for novel functions (Williams and Friedman, 2004).

Evidence for the generality of the modular hypothesis for the angiosperm female gametophyte derives from its ability to account for much of the diversity of mature structures among flowering plant female gametophytes. All angiosperms initiate a micropylar module (which contains an egg). Ninety-nine percent of angiosperms initiate a chalazal developmental module in addition to the micropylar module. In *Penaea*, two additional "equatorial" modules supplement the micropylar and chalazal modules: each module produces three peripheral cells and contributes a nucleus to the central cell. In some angiosperms (e.g., *Plumbago* with two modules and *Plumbagella* with four modules [Gifford and Foster, 1989]), modules have been developmentally reduced to "duets" and undergo only a single mitotic division to produce two nuclei: one nucleus is contributed to the central cell, and the other is partitioned into a separate cell (Figure 9).

The key to transitions between angiosperm female game- tophytes with different numbers of modules lies in the modifica- tion of early development to position nuclei within one, two, or four cytoplasmic and developmentally autonomous domains (Friedman and Williams, 2003). The gain of nuclear migration to opposite poles at the early two-nucleate stage can account for the transition from a four-nucleate to an eight-nucleate female gametophyte in the common ancestor of monocots, eumagno-
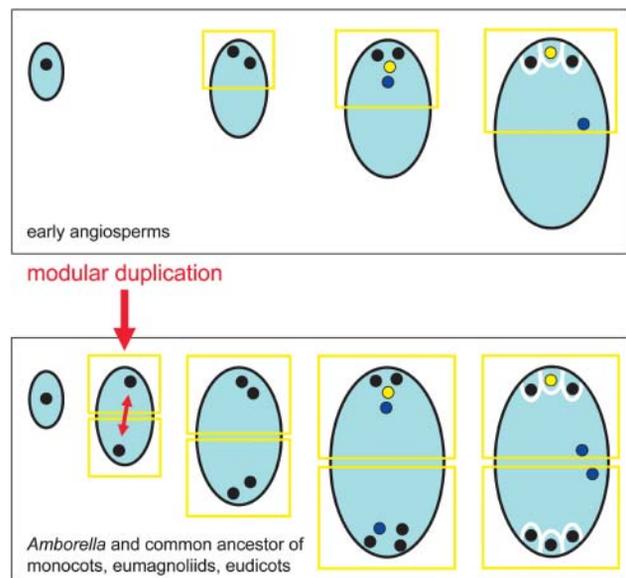

Figure 7. Comparison of the Development of the Four-Celled, Four- Nucleate Female Gametophyte Characteristic of Basal Angiosperm Lineages (One Module) with That of the Seven-Celled, Eight-Nucleate Female Gametophyte Characteristic of *Amborella* and Monocots, Eumagnoliids, and Eudicots (Two Modules).

A nuclear migration event at the two-nucleate stage in the Polygonum- type female gametophyte leads to the initiation of a second develop- mental module.
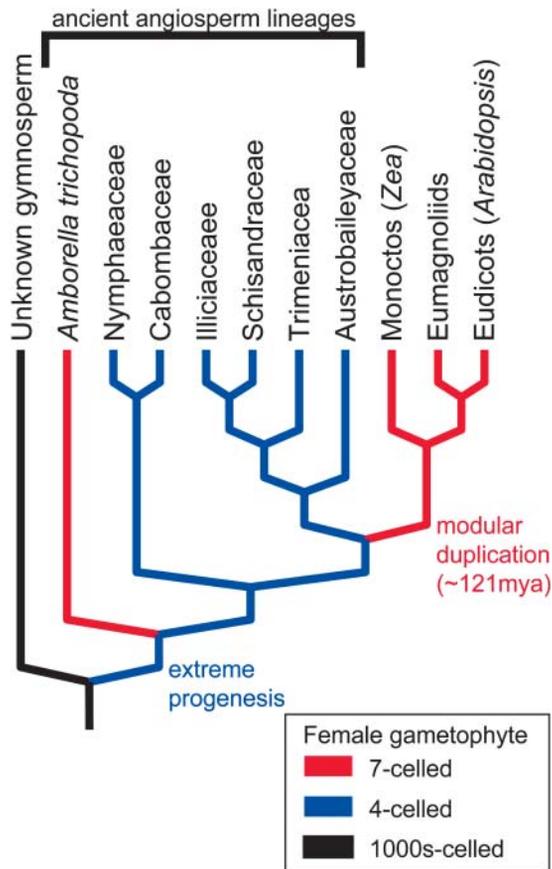
Figure 8. Evolutionary History of the Angiosperm Female Gameto-phyte.

The reduction of the large multi-egg gymnosperm female gametophyte (with thousands of cells) to a four-celled angiosperm female gameto-phyte was accomplished through a process of progenesis (a hetero-chronic effect in which the timing of sexual maturation is advanced within the somatic ontogeny and leads to the truncation of ontogeny at a "juvenilized" stage of the ancestor). Modular duplication in the female gametophyte of the common ancestor of monocots, eumagnoliids, and eudicots (and also in the *Amborella* lineage) resulted in the origin of triploid endosperm. Originally, endosperms in flowering plants were diploid. mya, million years ago.

liids, and eudicots. Conversely, the loss of nuclear migration at the two-nucleate stage can account for the origin of the four-celled, four-nucleate female gametophyte from an eight-nucle-ate ancestor, as is the case in Onagraceae (parsimony analysis indicates that the ancestors of Onagraceae had a Polygonum-type female gametophyte [Tobe and Raven, 1983; Conti et al., 1996, 1997]). Onagraceae is not the only group in which the chalazal module has been lost. In grasses (tribes Paniceae and Andropogoneae), the chalazal module appears to have been lost repeatedly, and in each case this correlates with a shift from sexual reproduction (seven-celled, eight-nucleate female game-tophyte) to apomixis (four-celled, four-nucleate female gameto-phyte) (Warmke, 1954; Brown and Emery, 1958; Battaglia, 1991).

ENDOSPERM GENETICS AND PLOIDY EVOLUTION

The monosporic seven-celled, eight-nucleate Polygonum-type female gametophyte has long served as the baseline for analysis of the origin and subsequent evolution of the angiosperm female gametophyte (Schnarf, 1931; Maheshwari, 1950; Johri, 1963; Davis, 1966; 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). It is now evident that this type of female gametophyte is rare among basal angiosperms (occur-ring in only a single extant species, *Amborella trichopoda*), whereas the four-celled, four-nucleate type of female gameto-phyte typifies the ancient angiosperm lineages Nymphaeales and Austrobaileyales and may be plesiomorphic for flowering plants as a whole. Given that the genetic constitution of endosperm is related directly to the genetic constitution of the central cell of the female gametophyte, changes in female gametophyte development have the potential to directly alter the ploidy and maternal-to-paternal genomic ratios of endosperm.

We believe that major transitions in the modular construction of female gametophytes may have little, if anything, to do with the functional biology of the gametophyte per se. Rather, selection has acted primarily to preserve female gametophyte develop-mental mutants that have created advantageous alterations in the genetics and ploidy of endosperm. If true, the critical classes of mutants associated with evolutionary transitions in endo-sperm genetics and ploidy will be those that alter the distribution of free nuclei during the early stages of female gametophyte development and hence the number of developmental modules that are established.

From this perspective, the key "functional" component of the original chalazal module was the chalazal polar nucleus that participated in a second fertilization event and altered the original genetic constitution and ploidy of endosperm. Thus, the set of three antipodals, in their original manifestation in the earliest Polygonum-type female gametophytes, can be viewed as an evolutionary "spandrel" (sensu Gould and Lewontin, 1979) or, in essence, as a developmental tag-along associated with the evolution of an additional maternal nuclear contribution to the central cell, accomplished through modular duplication (Friedman and Williams, 2003). The iterative (or ectopic) expres-sion of the modular quartet of the angiosperm female gameto-phyte has provided a means for the generation of higher levels of endosperm ploidy and higher ratios of maternal to paternal genomic contributions to endosperm.

A trend toward higher endosperm ploidy and increases in the ratio of maternal to paternal genomic contributions is consistent with and predicted by theoretical analyses of the potential roles of intersexual conflict and kin conflict during the early evolution of endosperm (Westoby and Rice, 1982; Queller, 1983, 1989; Willson and Burley, 1983; Haig and Westoby, 1988; Friedman, 1995). According to these analyses, both intersexual conflict (conflict between pollen- and ovule-bearing parents over the optimal investment of nutrients in the embryo-nourishing tissues of seeds of a maternal sporophyte) and kin conflict (genetically based conflict between sibling embryos for resources from the maternal sporophyte) should favor increases in the maternal
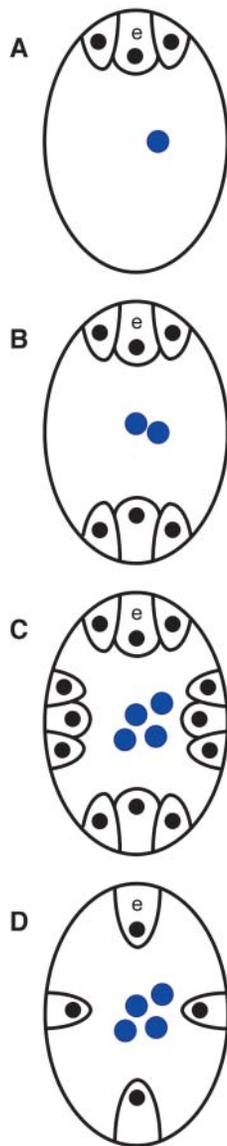
the embryo-nourishing behavior of endosperm must have evolved while the second fertilization product was a diploid entity (Friedman, 1995). Finally, increased ploidy of endosperm has been viewed as an adaptation to promote higher rates of metabolic (embryo-nourishing) activity through enhanced levels of transcription (Stebbins, 1976). Thus, the hypothesis that the first angiosperms had diploid endosperms (as a result of a four-celled female gametophyte) and that this condition rapidly (in geological terms) transitioned to a triploid endosperm with enhanced maternal-to-paternal genomic ratios (as a result of a seven-celled, eight-nucleate female gametophyte) is highly concordant with a wide range of earlier theoretically based analyses of the evolution of endosperm, which, in essence, had predicted this trend.

For more than a century, the question of what endosperm is, from an evolutionary/homological point of view, has confounded plant embryologists and evolutionary biologists. The prevalence of the diploid condition of early angiosperm endosperms may help to address this difficult question. From an evolutionary-historical perspective, there are only three possible answers to the question of what endosperm is, all of which have been known for more than a century (Sargant, 1900; Strasburger, 1900; Thomas, 1907; Coulter, 1911). Either endosperm is the sterilized homolog of an embryo (both Sargant [1900] and Friedman [1995] favor this hypothesis), the sexualized homolog of a portion of the female gametophyte (favored by Strasburger [1900] and Coulter [1911]), or an entity that arose de novo during the earliest phases of angiosperm evolution and is, in essence, not homologous with any previously existing component of the life cycle.

If double fertilization is a feature of the common ancestor of angiosperms, the fact that diploid endosperms predominate among basal angiosperms and are likely to be plesiomorphic strongly suggests that before the acquisition of embryo-nourishing behavior, the ancestral second fertilization product was diploid. A diploid ancestral condition is congruent with an embryo homology for endosperm, because (obviously) embryos are diploid. Although double fertilization events in Gnetales may not be evolutionarily related to those of flowering plants, it is certainly notable that the second fertilization product in *Ephedra* and *Gnetum* is a diploid embryo (Friedman, 1992a; Carmichael and Friedman, 1995), as appears to be the case in conifers that are reported to have a second fertilization process (Friedman, 1992b). Inclusive fitness theory shows that the loss of individual fitness by an embryo could have been compensated for by overall gains in the inclusive fitness of a sibling embryo that is the beneficiary of the embryo-nourishing behavior of its altruist partner (Friedman, 1995).

Imprinting and parent-of-origin effects in the endosperms of *Arabidopsis* and *Zea* (Kermicle, 1970; Lin, 1982, 1984; Haig and Westoby, 1989, 1991; Grossniklaus et al., 1998; Scott et al., 1998; Adams et al., 2000; Luo et al., 2000; Vielle-Calzada et al., 2000; Leblanc et al., 2002; Danilevskaya et al., 2003) have become topics of intense interest. Although it is unknown whether imprinted genes are present in all triploid endosperms with a 2:1 ratio of maternal-to-paternal genomes, an interesting question arises regarding whether imprinted genes eventually will be discovered among basal angiosperms with diploid endosperms and a 1:1 ratio of maternal-to-paternal genomes.

Figure 9. Modular Basis for the Diversity of Angiosperm Female Gametophytes.

(A) One module (quartet) found in Nymphaeales and Austrobaileyales (plesiomorphic) as well as in Onagraceae and several apomictic grasses (apomorphic, reversal). The central cell nucleus is shown in blue. e, egg cell.

(B) Two modules (quartets) found in Polygonum-type female gametophytes. The central cell nuclei are shown in blue.

(C) Four modules (quartets) found in the *Penaea* female gametophyte. The central cell nuclei are shown in blue.

(D) Four modules (duets) found in the *Plumbago* female gametophyte. Each module undergoes a single mitotic division before the cellularization phase. In this female gametophyte, there are no synergids. The central cell nuclei are shown in blue.

genomic contribution to endosperm and, hence, the increased overall ploidy of endosperm. It also is worth noting that if the antecedent structure that gave rise to endosperm (see below) is an embryo, inclusive fitness analysis has definitively shown that

DEVELOPMENTAL EVOLUTION OF THE SEXUAL PROCESS IN ANCIENT FLOWERING PLANT LINEAGES

A comparative analysis, based on the most recently published phylogenies of angiosperms, supports the following evolutionary developmental hypothesis (Friedman and Williams, 2003; Williams and Friedman, 2004). The earliest angiosperms possessed a four-celled, four-nucleate female gametophyte that lacked antipodals and a second polar nucleus. The developmental reduction of the large gymnosperm female gametophyte (thousands of cells with multiple archegonia) to the four-celled condition of the first angiosperms involved the loss of archegonial structure, the reduction of the number of eggs from many to one (per female gametophyte), and the complete elimination of the somatic body (prothallus). This condition still is to be found among Nymphaeales and Austrobaileyales. Approximately 10 million years after the origin of angiosperms, ectopic duplication of the basic modular quartet that produces the egg apparatus and a uninucleate haploid central cell generated a second developmental module expressed in the chalazal domain of the female gametophyte of the common ancestor of monocots, eumagnoliids, and eudicots (modular duplication also occurred in an ancestor of *Amborella trichopoda*). Thus, the antipodals of the seven-celled, eight-nucleate female gametophyte (Polygonum type) represent a duplicated and sterilized egg apparatus.

Because the ploidy and genetic constitution of endosperm is always tied directly to the development and mature structure of the female gametophyte, the transition from a monosporic four-celled, four-nucleate female gametophyte with a haploid central cell to a monosporic seven-celled, eight-nucleate female gametophyte with a binucleate (diploid) central cell also marked the transition from the diploid genetically biparental endosperms of the earliest angiosperms to the triploid genetically biparental endosperms found among most extant flowering plants, including *Arabidopsis*, *Antirrhinum*, and *Zea*. Finally, although the issue of whether the first angiosperms possessed a process of double fertilization remains unresolved, the answer will be revealed as soon as the condition is investigated in *Amborella*. If double fertilization is found to be a defining feature of the first angiosperms, this should refocus attention on the century-old question of the evolutionary origin of double fertilization and, most importantly, the homology of endosperm.

More than a century after the beginning of intensive study of the reproductive biology of flowering plants, much remains to be discovered about the basic features of the sexual process in angiosperms in general and, more specifically, in the most ancient lineages of this remarkable clade of plants. Nevertheless, a new understanding of the evolutionary developmental basis for the diversification of flowering plant gametophyte structure, fertilization process, and endosperm genetics is beginning to emerge from studies propelled by recent insights into angiosperm phylogeny and a more developmentally dynamic sense of how plant structure evolves.

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Developmental Evolution of the Sexual Process in Ancient Flowering Plant Lineages

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