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Developmental evolution of endosperm in basal angiosperms: evidence from *Amborella* (Amborellaceae), *Nuphar* (Nymphaeaceae), and *Illicium* (Illiciaceae)

S. K. Floyd and **W. E. Friedman**

Department of Environmental, Population, and Organismic Biology, University of Colorado, Boulder, Colorado, USA

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Abstract. Because of their basal phylogenetic position, *Amborella*, Nymphaeales, and Illiciales (and allies) are key to reconstructing ancestral character states and to tracing character state transitions that occurred during the earliest radiation of flowering plants. Endosperm is the sexually-derived embryo-nourishing tissue that is unique to the life cycle of angiosperms. We provide detailed descriptions of endosperm development in *Amborella*, *Nuphar* (Nymphaeales), and *Illicium* (Illiciales) and compare patterns within an explicit phylogenetic context for the three basal lineages that they represent. *Amborella* and *Illicium* share a bipolar, cellular pattern of development, characterized by an oblique first division, that was resolved as ancestral for flowering plants. A series of character state transformations occurred within Nymphaeales which led first to a modified cellular pattern with a transverse first division (present in *Nuphar*). The transverse cellular pattern was transformed to a helobial pattern that is present in Cabombaceae. Endosperm ontogeny involves dissociable elements and appears to fit the model of a modular developmental process.

Key words: *Amborella*, angiosperm embryology, development, endosperm, evolution, EvoDevo, helobial, *Illicium*, modularity, *Nuphar*.

Within the last two years remarkable progress has been made toward the resolution of deep angiosperm phylogenetic relationships. The results of several independent, molecular sequence-based analyses have converged on similar results for rooting the angiosperm tree and have identified the three earliest-diverging lineages of flowering plants (Mathews and Donoghue 1999; Parkinson et al. 1999; Qiu et al. 1999, 2000; Soltis et al. 1999, 2000; Borsch et al. 2000; Graham and Olmstead 2000; Savolainen et al. 2000). The consensus of these analyses is that *Amborella trichopoda* is sister to all other extant angiosperms, Nymphaeales (Nymphaeaceae plus Cabombaceae) is the sister group to all taxa except *Amborella*, and a clade including Illiciales, Austrobaileyaceae, and Trimeniaceae (hereafter referred to as the Illiciales clade) is sister to all remaining angiosperms (Fig. 1). With identification of the three, earliest-diverging lineages of flowering plants (the "basal grade"), it is now possible to more reliably reconstruct ancestral character states for the angiosperm clade (Mathews and Donoghue 1999, Soltis et al. 1999, Friedman and Floyd 2001).

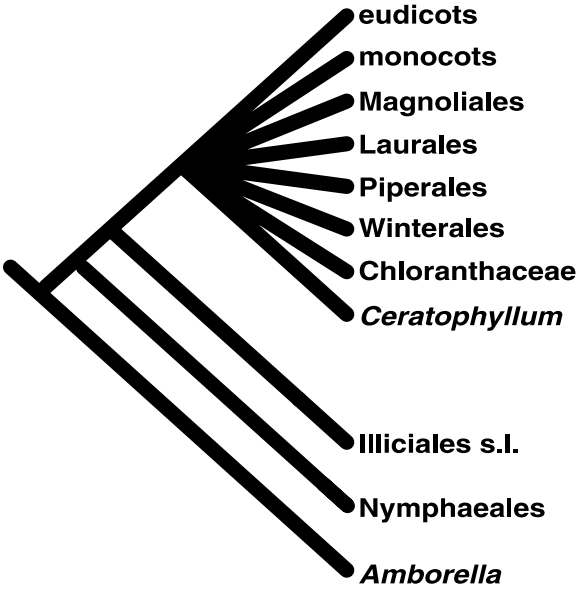


Fig. 1. Consensus of several recent molecular sequence based phylogenies for angiosperms (Mathews and Donoghue 1999; Qiu et al. 1999, 2000; Soltis et al. 1999, 2000; Barkman et al. 2000; Graham and Olmstead 2000)

Reproductive characters constitute the majority of the unique features that separate angiosperms from all other seed plants (Sargant 1908, Crane et al. 1995, Friedman 2001). Thus, knowledge of the reproductive biology of basal lineages of flowering plants is critical to the reconstruction of ancestral character states and key to understanding the origin and early history of the angiosperm clade. One of these unique angiosperm reproductive features is endosperm, the embryo-nourishing tissue that develops following a fertilization event involving a second sperm and the two haploid polar nuclei of the female gametophyte.

Traditionally, endosperm has been classified into three types based on differential patterns of development: "free nuclear," in which early mitotic divisions occur without cytokinesis; "*ab initio* cellular" (cellular), in which cell walls are formed following all mitotic divisions; and "helobial," involving an initial transverse cellular division followed by free nuclear development of the micropylar cell or chamber.

Recent comparative investigations of endosperm in a broad sample of basal flowering plants (Floyd et al. 1999, Floyd and Friedman 2000) revealed new insights into the nature of this unique component of angiosperm reproductive biology that go beyond traditional typology. These analyses demonstrate that endosperms of most ancient angiosperm lineages exhibit a cellular ontogeny that is resolved as ancestral, based on phylogenetic comparative analysis. This pattern is characterized by an unequal division of the first endosperm cell, producing a small chalazal cell and larger micropylar cell. Early development of the micropylar region (transverse cell divisions) results in a few large cells in a uniseriate arrangement. Early development of the chalazal region involves cell divisions in many planes. Characterization of a primitive ontogeny for endosperm provides the basis for tracing the evolution of endosperm within the flowering plant clade.

In addition to proposing an ancestral ontogeny for endosperm, Floyd and Friedman (2000) have shown that three features of early endosperm development define the basic pattern. These are division of the primary endosperm nucleus or cell, development of the chalazal domain, and development of the micropylar domain. Analyzed as characters with variable character states, these three features appear to have evolved independently within angiosperms, resulting in variable endosperm patterns.

Although many basal angiosperms have retained the primitive cellular ontogeny described above, endosperm ontogenetic evolution has occurred in some lineages so that all three endosperm types are represented among early-divergent angiosperms. In particular, helobial development occurs in *Cabomba*, a member of Nymphaeales (Floyd and Friedman 2000), one of the three lineages of the basal grade. The explicit phylogenetic hypothesis for the branching order of *Amborella*, Nymphaeales and Illiciales, and recent resolution of relationships within Nymphaeales (Les et al. 1999), provide the context in which to

reconstruct endosperm character polarity and evolution in general, and more specifically to explore the origin of helobial endosperm in Nymphaeales.

Very few detailed analyses of endosperm development have been published for taxa in the basal grade. Tobe et al. (2000) described some basic aspects of endosperm development for *Amborella* within the context of a broader embryological study. Floyd and Friedman (2000) also reported that *Amborella* and *Illicium* exhibit bipolar, cellular endosperm development. Limited data were previously available for the Illiciales clade (Hayashi 1963a, b). Nymphaeales have been the subject of several embryological studies (Cook 1902, 1906, 1909; Khanna 1965, 1967; Ramji and Padmanabhan 1965; Padmanabhan 1970; Schneider 1978; Galati 1985; Van Miegroet and Dujardin 1992; Orban and Bouharmont 1995), but remarkably, complete descriptions of endosperm development are lacking for all taxa except *Cabomba* (Floyd and Friedman 2000) and *Nymphaea* (Cook 1906). Endosperm development in *Nuphar*, which has been resolved as sister to all other Nymphaeaceae (Les et al. 1999), has never been described.

We report here for the first time detailed descriptions of endosperm development in *Amborella*, *Nuphar*, and *Illicium*. Endosperm ontogenetic patterns are then compared within the framework of recent cladistic analyses for basal angiosperms. Based on comparative analysis, we elaborate on the hypothesis for the ancestral bipolar, cellular endosperm pattern outlined by Floyd and Friedman (2000). A scenario is then presented for endosperm ontogenetic evolution among the three basal angiosperm lineages (basal grade) that provides the first clear hypothesis for the derivation of a helobial pattern from *ab initio* cellular endosperm based on the interpretation of character distribution within an explicit, phylogenetic context. We then briefly discuss the relevance of the concept of modularity in development to our hypothesis of endosperm ontogenetic evolution.

Materials and methods

Amborella trichopoda was collected in New Caledonia and chemically fixed in either FAA or 4% glutaraldehyde in Sorensen's buffer (pH 6.8; Electron Microscopy Sciences). *Illicium floridanum* and *Nuphar lutea* ssp. *polysepala* were collected in Georgia and Colorado, respectively, and brought to the laboratory in Boulder, Colorado, where specimens were chemically fixed with 4% acrolein in 50 mM Pipes buffer (also 5 mM EGTA and 1 mM $MgSO_4$) at pH 6.8. Specimens of *Illicium mexicanum* were shipped overnight from the University of California Botanical Garden, Berkeley, and chemically fixed with acrolein as described above. Collections are summarized in Table 1.

More than 865 female flowers and developing seeds were serially sectioned for this analysis (Table 1). The presence of proteins, lipids, and starch in mature endosperm tissue was determined with histochemical stains and cross polarization microscopy. Histological methods followed Floyd and Friedman (2000). Endosperm characters were parsimoniously optimized onto published cladograms using MacClade (Maddison and Maddison 1992).

Results

Amborella. Endosperm development in *Amborella* begins with migration of the primary endosperm nucleus to the extreme chalazal end of the large first endosperm cell (central cell) where it undergoes mitosis (Fig. 2A, B). Following the initial mitotic division, an oblique cell wall is formed that unequally partitions the first endosperm cell into a small chalazal cell and a much larger micropylar cell (Fig. 2C).

The larger micropylar cell normally undergoes one or two highly unequal transverse/oblique cell divisions at its chalazal end (Fig. 2D) until a single, large cell at the micropylar pole is defined by a roughly transverse wall that crosses the full width of the former first endosperm cell (Fig. 2E–H). The chalazal cell, derived from the first cell division of the endosperm, also divides, most often in a vertical plane (Fig. 2F).

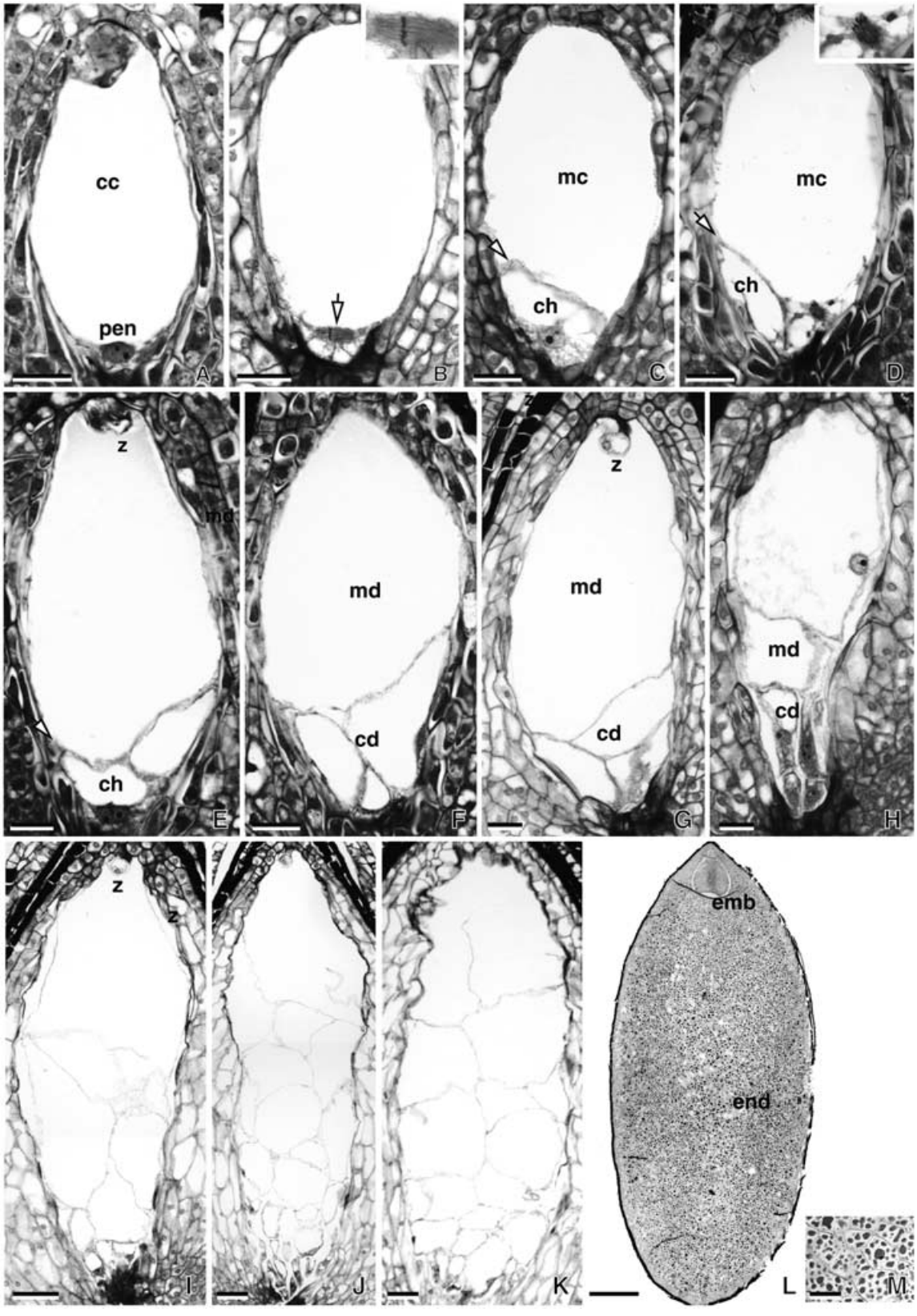
The derivatives of the chalazal cell along with the chalazal derivatives of the micropylar

Table 1. Source and voucher collection information for floral and fruit materials used in this study

Taxa	Source	Herbarium voucher or garden accession number	Number specimens sectioned
Amborella trichopoda Baillon	Leonard Thien, Tulane University Mt. Aoupinié, New Caledonia	#1000 in liquid specimen COLO Floyd and Lemieux 00-24	> 450
Nuphar lutea Sibthorp & Smith ssp. *polysepala* (Engelmann) Beal	Red Rocks Lake, Colorado	Floyd and Williams 00-36-00-49 in liquid specimen, Boulder, Colorado	> 85
Illicium floridanum Ellis.	Cultivated, Georgia State Botanical Garden, Athens GA	95-0278	> 178
Illicium mexicanum A. C. Smith	Cultivated, University of California Botanic Garden, Berkeley, CA	91.0030	> 157

cell (of the two-celled endosperm) then undergo repeated cell divisions in many planes resulting in a mass of cells at the chalazal end of the endosperm (Fig. 2G, H). During this period of active cellular development at the chalazal end of the endosperm, the large micropylar cell remains undivided or divides once by means of a transverse wall (Fig. 2H). Thus early stages of ontogeny produce two regions that are the result of differential developmental rates and patterns. One of these is a region at the chalazal end of the endosperm that is multicellular and multiseriate and comprises relatively small and cytoplasmically dense cells. We refer to this region as the chalazal domain (Fig. 2G, H). The micropylar domain of the endosperm comprises either a single large vacuolate cell

Fig. 2. Endosperm development in *Amborella*. All sections longitudinal with micropylar end at the top of the page. **A** Primary endosperm nucleus (*pen*) at chalazal end of central cell (*cc*). Scale bar = 25 μm. **B** Primary endosperm nucleus in metaphase (arrow indicates plate); inset shows mitotic figure at higher magnification, Scale bar = 25 μm. **C** Two-celled endosperm, with smaller chalazal cell (*ch*), oblique partitioning cell wall (arrow), and large micropylar cell (*mc*). **D** Nucleus of the micropylar cell in mitosis (half of anaphase figure in view), chalazal cell (*ch*) undivided, original oblique wall indicated by arrow; inset shows mitotic figure at higher magnification. Scale bar = 25 μm. **E** Three-celled endosperm. A cell wall has formed following mitosis of the micropylar cell; chalazal cell (*ch*) remains undivided, original oblique wall indicated by arrow. Zygote (*z*) remains undivided. Scale bar = 25 μm. **F** Four-celled endosperm comprising two derivatives from each of the first two cells. Endosperm is now differentiated into a large, unicellular micropylar domain (*md*) and smaller, multicellular (three cells) chalazal domain (*cd*). Scale bar = 25 μm. **G** Cell division in many planes in the chalazal domain (*cd*) have produced seven cells in a multiseriate arrangement; micropylar domain (*md*) remains unicellular and zygote (*z*) remains undivided. Scale bar = 25 μm. **H** Most advanced differentiated endosperm observed; chalazal domain composed of 12 cells, with those at extreme chalazal end small and densely cytoplasmic. **I–K** Partitioning of micropylar domain into several large, vacuolate cells in a multiseriate arrangement; zygote (*z*) remains undivided. Scale bars = 50 μm. **L** Copious, multicellular endosperm (*end*) and embryo (*emb*) of mature seed (seed coat removed). Scale bar = 500 μm. **M** Higher magnification view of mature endosperm cells showing numerous, angular protein bodies. Scale bar = 50 μm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or two large cells in a uniseriate arrangement (Fig. 2D).

Following this highly differentiated early phase, the micropylar domain becomes developmentally active. The cell or cells of the micropylar domain divide, resulting in a multiseriate arrangement (Fig. 2I–K). Continued growth and cell division occur in both domains to produce a mature endosperm that consists of small, uniform cells that are filled with lipids and protein bodies (Fig. 2L, M). Starch is absent. The mature seed contains copious endosperm and a rudimentary embryo (Fig. 2L).

***Nuphar*.** The primary endosperm nucleus divides in a position slightly nearer to the micropylar end than to the chalazal end of the elongate central cell (Fig. 3A). Following division, a transverse wall is formed that divides the central cell unequally into a large chalazal cell and a smaller micropylar cell (Fig. 3B, C). These two cells precisely define the two regions that will undergo differential development, the chalazal and micropylar domains.

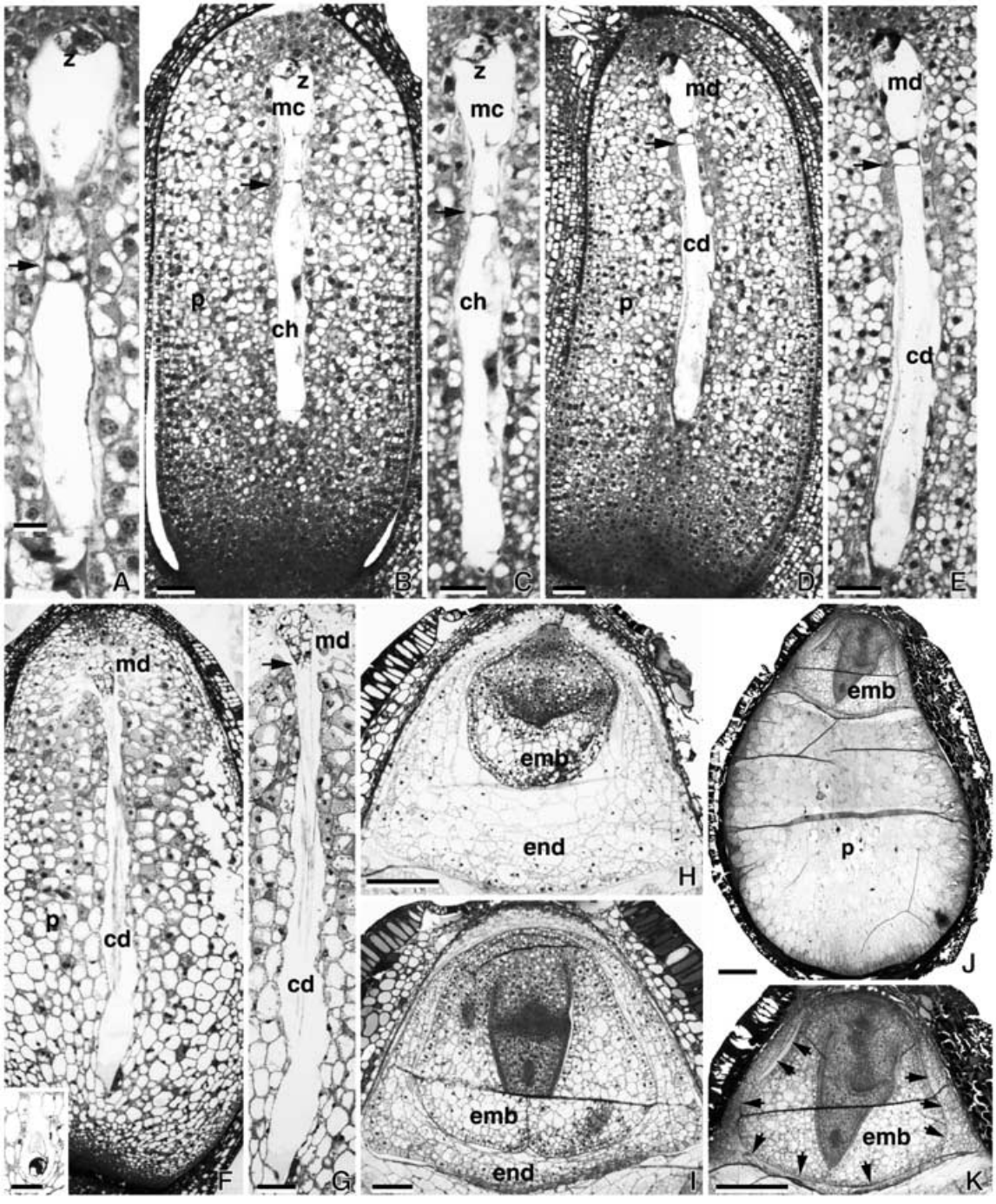
The chalazal domain remains unicellular and uninucleate and continues to grow as an invasive tube (haustorium) to the extreme chalazal end of the massive nucellus (Fig. 3C–G). The nucleus of the chalazal domain cell ultimately migrates to the chalazal end (Fig. 3F). The first cell of the micropylar domain divides once transversely to yield two cells in an uniseriate arrangement (Fig. 3D, E).

Cell divisions in many planes then occur in the micropylar domain leading to a multicellular, multiseriate arrangement that initially retains the narrow, cylindrical shape of the micropylar cell (Fig. 3F, G). Lateral expansion of the micropylar domain then occurs, associated with continued cell divisions. The embryo grows into the micropylar endosperm and consumes all but a thin layer of cellular tissue that remains in the mature seed (Fig. 3H–K). Cells of the mature micropylar endosperm contain lipids and protein bodies. The chalazal domain is still present in the mature seed, but its long tubular shape renders it difficult to see

Fig. 3. Endosperm development in *Nuphar*. All sections longitudinal with micropylar end at the top of the page. **A** Primary endosperm nucleus (arrow) in mitosis (anaphase), zygote (*z*) undivided. Scale bar = 25 μm. **B** Developing seed at the two-celled endosperm stage, with larger chalazal cell (*ch*), transverse partitioning cell wall (arrow), and small micropylar cell (*mc*); zygote (*z*) undivided. The nucleus of the chalazal cell has migrated toward the chalazal end. Narrow, tubular endosperm surrounded by massive nucellus tissue which will develop into perisperm (*p*). Scale bar = 50 μm. **C** Higher magnification of the two-celled endosperm in 3B, scale bar = 50 μm. **D** Developing seed at the three-celled endosperm stage, composed of two derivatives of the micropylar cell and the undivided chalazal cell. Both nuclei of the two micropylar derivatives are in view. Arrow indicates first transverse wall. Endosperm is now differentiated into a uniseriate (two cells) micropylar domain (*md*) and unicellular chalazal domain (*cd*). Narrow, tubular endosperm surrounded by massive nucellus tissue which will develop into perisperm (*p*). Scale bar = 50 μm. **E** Higher magnification of the two-celled endosperm in 3D, scale bar = 50 μm. **F** Developing seed at a later differentiated stage; seed now about twice the size as in 3D. The chalazal domain (*cd*) remains unicellular and has grown closer to the chalazal end of the perisperm (*p*); its nucleus has enlarged and migrated to the chalazal end of the tubular cell. The micropylar domain (*md*) is multicellular and multiseriate. The inset shows different section through the same endosperm to show the nucleus of the chalazal domain at the extreme chalazal end. Scale bar = 100 μm. **G** Higher magnification of the two-celled endosperm in 3F; arrow indicates position of first transverse wall. Scale bar = 100 μm. **H** Micropylar domain has expand laterally, forming a multicellular region of endosperm (*end*) into which the developing embryo (*emb*) has begun to grow. Scale bar = 250 μm. **I** Slightly later stage than 3H, embryo (*emb*) has consumed much of the micropylar endosperm (*end*). Scale bar = 250 μm. J Mature seed with thin layer of multicellular micropylar endosperm surrounding the well-developed embryo (*emb*) occupying the micropylar end of the seed, adjacent to abundant perisperm tissue (*p*). Chalazal endosperm not in view. Scale bar = 500 μm. **K** Higher magnification view of embryo (*emb*) and micropylar endosperm (arrows) in the mature seed shown in 3J. Scale bar = 500 μm

except in serial sections. Perisperm occupies most of the mature seed (Fig. 3J). The perisperm cells are filled with packets of starch grains.

Illicium. Endosperm development begins with migration of the primary endosperm nucleus from its initial position in the first endosperm cell (midway between chalazal and micropylar poles) to the extreme chalazal end (Fig. 4A). Following mitosis of the primary endosperm nucleus, an oblique cell wall is formed that unequally partitions the first



endosperm cell into a small chalazal cell and a much larger micropylar cell (Fig. 4B). Both the chalazal cell and the micropylar cell undergo further divisions (Fig. 4C–F).

Beginning at the chalazal end, the large micropylar cell undergoes a few transverse cell divisions that result in one to a few small cells adjacent to the chalazal cell, and a few larger cells in a uniseriate arrangement at the micropylar end of the endosperm (Fig. 4D, E). The results of these first few divisions are the formation of the micropylar and chalazal domains that will undergo differential development.

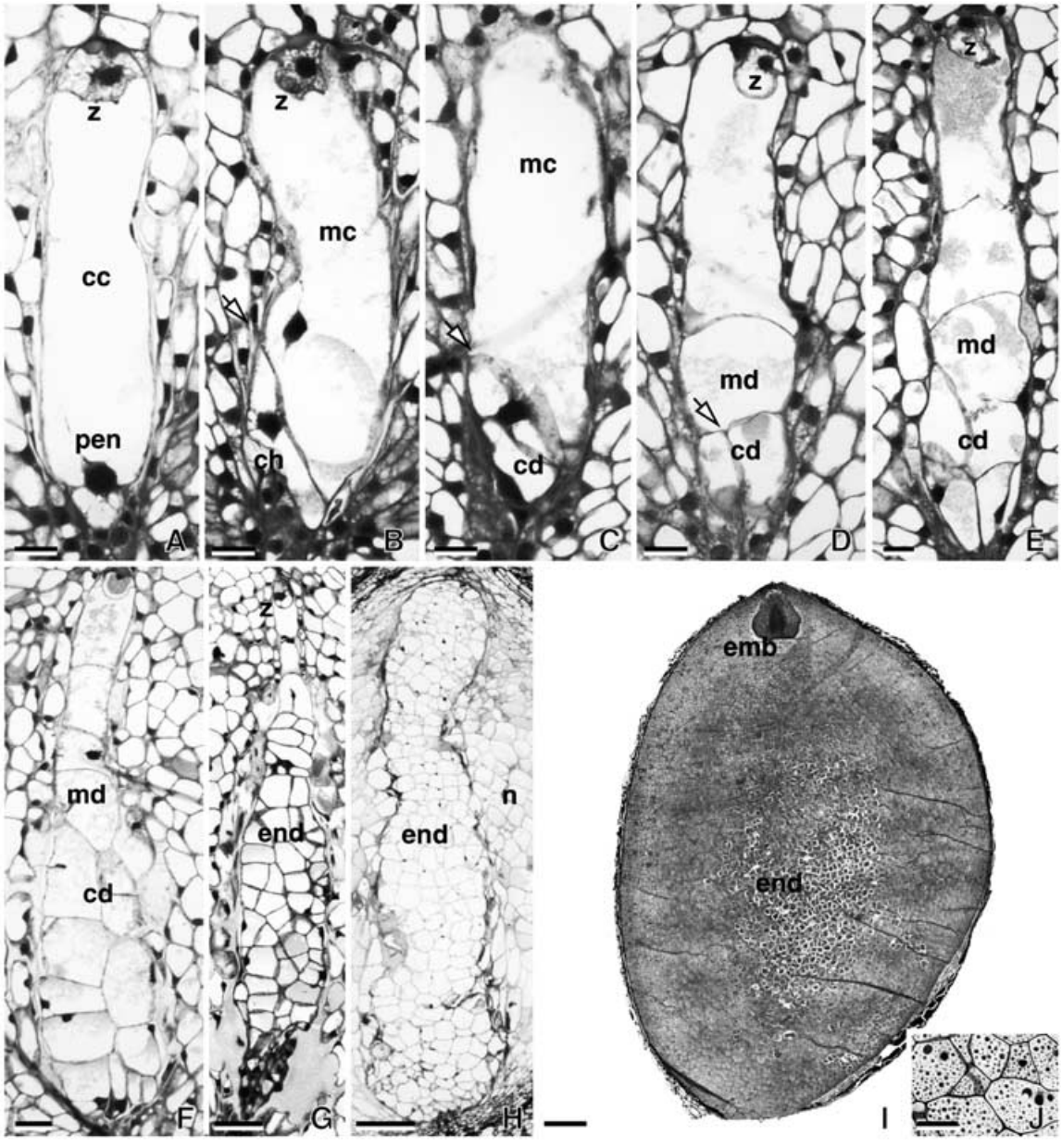
The micropylar domain, comprising the uniseriate derivatives of the micropylar cell, continues limited uniseriate development (Fig. 4D, F). The chalazal domain includes derivatives of both the original chalazal cell and the micropylar cell (of the two-celled endosperm) that both divide in several planes giving rise to a multiseriate mass of cells (Fig. 4C–F). Continued cellular development results in a mass of up to 35 cells in the chalazal domain, while the micropylar domain remains few-celled and uniseriate (Fig. 4F). Following the early phase of differential development, the micropylar domain becomes multiseriate in a chalazal-to-micropylar direction (Fig. 4G). The entire endosperm then undergoes cellular division and growth (Fig. 4H, I). Ultimately the entire endosperm consists of small, uniform cells that are filled with lipids and protein bodies (Fig. 4I, J). Starch is absent. The mature seed contains abundant endosperm and a rudimentary embryo (Fig. 4I).

Discussion

Comparison of endosperm developmental patterns in the basal angiosperm grade. Both *Amborella* and *Illicium* endosperms exhibit all of the features of the bipolar, cellular developmental pattern that was hypothesized as ancestral for angiosperms by Floyd and Friedman (2000). The early pattern-forming phase of this primitive ontogeny involves unequal, cellular partitioning of the first endosperm cell into a large micropylar cell and a small chalazal cell. The initial division takes place at the chalazal end of the first endosperm cell, following the migration of the primary endosperm nucleus to that position. Following the initial division, limited cellular uniseriate development occurs in the micropylar domain (usually just one or no divisions in *Amborella*), and more extensive cellular, multiseriate development takes place in the chalazal domain.

Fig. 4. Endosperm development in *Illicium*. All sections longitudinal with micropylar end at the top of the page. **A** Primary endosperm nucleus (*pen*) at chalazal end of central cell (*cc*). Zygote (*z*) remains undivided. Scale bar = 25 μm. **B** Two-celled endosperm, with smaller chalazal cell (*ch*), oblique partitioning cell wall (arrow), and large micropylar cell (*mc*). Zygote (*z*) remains undivided. Scale bar = 25 μm. **C** Three-celled endosperm. Micropylar cell (*mc*) undivided; chalazal cell has divided into two cells which now constitute the chalazal domain (*cd*). Scale bar = 25 μm. **D** Four-celled endosperm comprised of three derivatives of the micropylar cell and the chalazal cell (still undivided). Endosperm is now differentiated into a large, uniseriate (two cells) micropylar domain (*md*) and smaller, multicellular (two cells) chalazal domain (*cd*). The original oblique wall is indicated by the arrow. The zygote (*z*) remains undivided. Scale bar = 25 μm. **E** Later differentiated stage. The chalazal domain (*cd*) comprises several cells in a multiseriate arrangement; the micropylar domain (*md*) comprises 3 cells in a uniseriate arrangement. The zygote (*z*) remains undivided. Scale bar = 25 μm. **F** Most advanced differentiated stage. The chalazal domain (*cd*) comprises about 35 cells; the micropylar domain (*md*) comprises 4 cells. Scale bar = 50 μm. **G** Endosperm (*end*) after elongation and continued cell division. Micropylar domain partially multiseriate. The zygote (*z*) remains undivided. Scale bar = 50 μm. **H** Endosperm (*end*) completely multiseriate and beginning to expand in girth at expense of massive nucellus (*n*). Scale bar = 300 μm. **I** Copious, multicellular endosperm (*end*) and embryo (*emb*) of mature seed (seed coat mostly removed). Scale bar = 500 μm. **J** Higher magnification of mature endosperm cells showing numerous protein bodies, stained blue, surrounded by unstained lipids. Scale bar = 50 μm

Amborella and *Illicium* also share some additional aspects of early endosperm patterning. In both taxa, division of the first endosperm cell occurs by formation of an obliquely-positioned wall, the chalazal domain is formed from derivatives of both of the first two endosperm cells, and the micropylar domain initially comprises a single derivative of the micropylar cell of the two-celled endosperm (Figs. 2, 4). In other words, the first endosperm cell division corresponds imprecisely to the two domains that are subsequently defined. An essentially identical pattern of development was observed in *Drimys winteri* (Floyd and Friedman 2000), and may also occur in another member of Winteraceae, *Pseudowintera colorata* (Bhandari 1963). Thus the endosperm developmental pattern present in *Amborella* and *Illicium* also occurs in at least one later-diverging angiosperm group (Fig. 1). The presence of a pattern of endosperm development characterized by an oblique first



division (that imprecisely defines micropylar and chalazal domains) in two of the three lineages of the basal grade (*Amborella* and the Illiciales clade) suggests that this may represent a shared ancestral condition.

In contrast, early endosperm development in *Nuphar* involves a transverse first division into two cells that directly correspond to the micropylar and chalazal domains (Fig. 3). As in *Amborella* and *Illicium*, the first endosperm wall forms at the position of the primary endosperm nucleus. But in *Nuphar*, the primary endosperm nucleus does not migrate to the chalazal end, hence the first wall is closer to the micropylar end than in *Amborella* and *Illicium*. The chalazal domain develops as a unicellular, uninucleate tubular structure in *Nuphar*, in contrast to cellular multiseriate chalazal development in both *Amborella* and *Illicium*. Micropylar development is cellular, with an initial uniseriate phase in all three taxa described above.

The same basic pattern of development observed in *Nuphar* seems to occur in most other members of Nymphaeaceae, including *Nymphaea*, *Castalia* (Cook 1906), and *Victoria* (Khanna 1967). Free nuclear development has been reported to occur in *Euryale* (Khanna 1964), but additional studies are needed to confirm this finding. Both genera of Cabombaceae, sister group to Nymphaeaceae (Les et al. 1999), exhibit patterns of endosperm development that are very similar to the cellular pattern in Nymphaeaceae with one notable difference. In both *Cabomba* and *Brasenia*, micropylar development begins with a free nuclear stage, followed by centripetal cellularization (Cook 1906, Floyd and Friedman 2000), which constitutes a helobial pattern of development.

Three endosperm developmental patterns may therefore be recognized among taxa in the basal angiosperm grade (Fig. 5): oblique/cellular (*Amborella* and *Illicium*) (Fig. 5A), transverse/cellular (*Nuphar*) (Fig. 5B), and transverse/helobial (*Cabomba*) (Fig. 5C). The transverse/cellular pattern of *Nuphar* bears developmental similarities to both of the other patterns. Transverse/cellular and oblique/cellular ontogenies are initiated by an unequal cellular division, followed by cellular, uniseriate development of the micropylar domain. The transverse/cellular pattern of *Nuphar* is also comparable to the transverse/helobial pattern of *Cabomba* in that both are characterized by an initial unequal, transverse cell division and unicellular development of the chalazal domain. If we accept the hypothesis that the oblique/cellular pattern is ancestral, comparison of patterns suggests a transformational series from the oblique/cellular pattern, through a transverse/cellular intermediate, to a

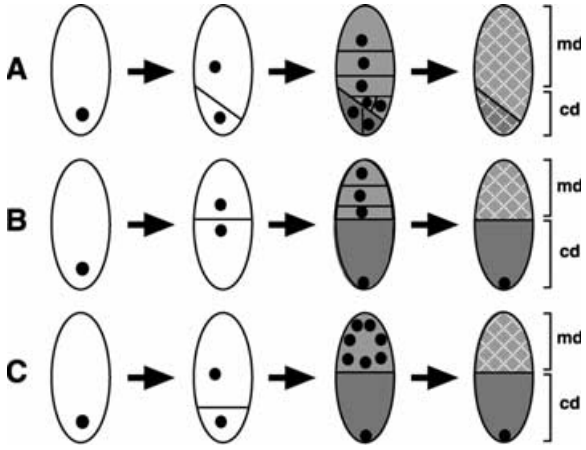


Fig. 5. Diagrammatic comparison of three patterns of endosperm development that occur in lineages of the basal angiosperm grade. Darker gray shading indicates the part of the endosperm derived from the chalazal cell, lighter gray shading highlights micropylar cell development. **A** The cellular endosperm developmental pattern that occurs in *Amborella* and *Illicium*. The first division is oblique, the micropylar domain (*md*) is derived exclusively from a derivative of the micropylar cell and the chalazal domain (*cd*) is derived from both the chalazal and micropylar cells. **B** The cellular endosperm developmental pattern that occurs in *Nuphar* (Nymphaeaceae). The first division is transverse, the micropylar domain (*md*) is derived from the micropylar cell and the chalazal domain (*cd*) is derived the chalazal cell. **C** The helobial endosperm developmental pattern that occurs in *Cabomba* (Cabombaceae). The first division is transverse, the micropylar domain (*md*) is derived from the micropylar cell and the chalazal domain (*cd*) is derived the chalazal cell. Early development of the micropylar domain is free nuclear

derived transverse/helobial pattern (Fig. 5A–C) in Nymphaeales. However, to make a strong statement about ontogenetic evolution, it is necessary to examine developmental character state distribution within an explicit phylogenetic context (Diggle 1992, Raff 1992).

Phylogenetic interpretation of endosperm character state transitions among basal angiosperms. The strategy of Floyd and Friedman (2000) was applied to dissecting the three endosperm developmental patterns of basal grade flowering plants (oblique/cellular, transverse/cellular, and transverse/helobial) into characters and character states (Table 2). Initiation of endosperm development may entail division of the primary endosperm nucleus without wall formation (free nuclear), or nuclear division may be followed by formation of either an oblique cell wall (oblique/cellular) or a wall that is transverse (transverse/cellular). Early development of the chalazal domain may be cellular/multiseriate, cellular/uniseriate, free nuclear, or involve no additional nuclear or cellular proliferation (unicellular). Finally, early development of the micropylar domain may be uniseriate with one or more cells (cellular/uniseriate), cellular/multiseriate, or free nuclear. These character states were parsimoniously optimized onto a simplified angiosperm phylogeny in order to trace endosperm character evolution in the basal angiosperm grade (Fig. 6).

For division of the first endosperm cell, oblique/cellular was resolved as ancestral and this state was retained in *Amborella*, the Illiciales clade, and the most recent common ancestor of all remaining angiosperms. A transformation to transverse/cellular occurred in the most recent common ancestor of Nymphaeales (Fig. 6A). Cellular/multiseriate chalazal development was resolved as ancestral and this state was also retained in *Amborella*, the Illiciales clade and the ancestor of all other angiosperms. A transformation to unicellular chalazal development occurred in the ancestor of Nymphaeales and was retained throughout that clade (Fig. 6B). Cellular/uniseriate micropylar development was resolved as ancestral and this state was retained in all lineages except for Cabombaceae in which there was a transformation to free nuclear (Fig. 6C).

Table 2. Three characters and possible character states associated with early endosperm pattern formation

First division	Chalazal development	Micropylar development
Oblique/cellular	Cellular/ multiseriate	Cellular/ multiseriate
Transverse/cellular	Cellular/ uniseriate	Cellular/ uniseriate
Free nuclear	Unicellular Free nuclear	Free nuclear

Ontogenetic evolution and the origin of helobial endosperm in Nymphaeales. Together, the patterns of character evolution for division of the primary endosperm nucleus/cell, chalazal development, and micropylar development indicate the following scenario for endosperm ontogenetic evolution among the earliest diverging angiosperms. The endosperm pattern characterized by an oblique, unequal, cellular first division (that yields imprecise definition of micropylar and chalazal domains), cellular uniseriate development of the micropylar domain, and cellular multiseriate development of the chalazal domain (Fig. 5A) represents the ancestral angiosperm ontogeny that was retained in *Amborella*, the Illiciales clade, and the most recent common ancestor of all other angiosperms.

A transformation from an oblique/cellular first division to transverse/cellular occurred in the most recent common ancestor of Nymphaeales and resulted in an ontogeny in which the micropylar and chalazal domains are precisely determined by the first division. Additionally, chalazal development was transformed from cellular/multiseriate to unicellular (with invasive growth as a haustorial tube). Those two evolutionary changes produced a modified cellular developmental pattern (Fig. 5B) that was retained in Nymphaeaceae. Finally, micropylar development changed from cellular/uniseriate to free nuclear in the

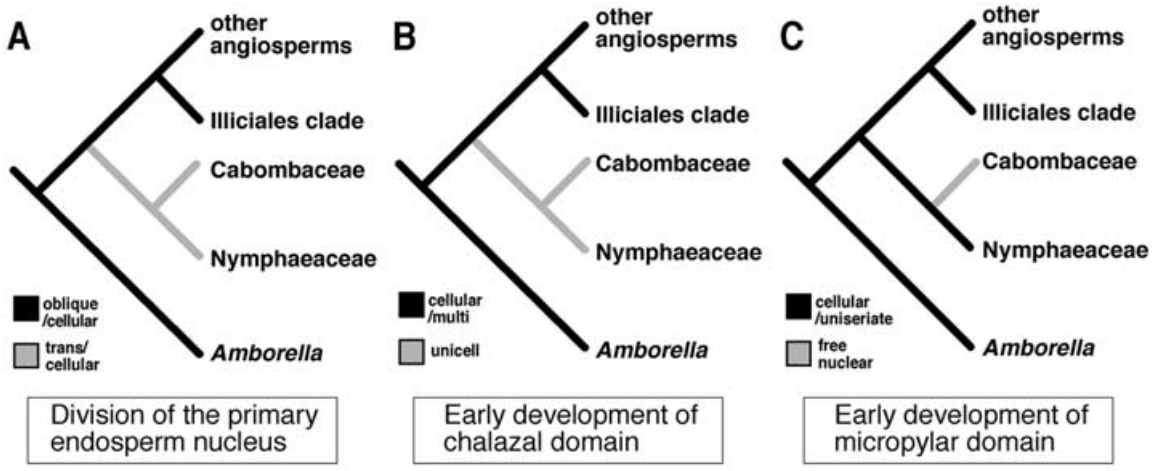


Fig. 6. Three pattern forming endosperm characters mapped onto simplified angiosperm phylogenies. **A** Division of the primary endosperm nucleus/cell is coded either as oblique/cellular, transverse/cellular, or free nuclear. Oblique/cellular division is resolved as ancestral for angiosperms and was retained in *Amborella*, the Illiciales clade, and the ancestor of all other angiosperms diverging above the three basal nodes. Transverse/cellular division evolved in the common ancestor of Nymphaeales. **B** Chalazal development is coded either as cellular/multiseriate, unicellular/haustorial, or free nuclear. Cellular/multiseriate development is resolved as ancestral for angiosperms and was retained in *Amborella*, the Illiciales clade, and the ancestor of all other angiosperms diverging above the three basal nodes. Unicellular/haustorial chalazal development evolved in the common ancestor of Nymphaeales. **C** Micropylar development is coded either as cellular/uniseriate, cellular/multiseriate, or free nuclear. Cellular/uniseriate development is resolved as ancestral for angiosperms and was retained in *Amborella*, the Illiciales clade, Nymphaeaceae, and the ancestor of all other angiosperms diverging above the three basal nodes. Free nuclear micropylar development evolved in the common ancestor of Cabombaceae

most recent common ancestor of Cabombaceae, resulting in the helobial developmental pattern (with a haustorial, unicellular chalazal domain) that typifies that family (Fig. 5C). If free nuclear endosperm does occur in *Euryale* as reported by Khanna (1964), it was likely derived from cellular endosperm since *Euryale* was resolved as highly nested within Nymphaeaceae (Les et al. 1999).

Phylogenetic comparison of endosperm developmental characters supports the hypothesis that a transverse/cellular pattern was intermediate between an ancestral oblique/cellular pattern and a derived helobial pattern. These results indicate that helobial endosperm is neither restricted to the monocot clade as has been asserted (Swamy and Parameswaran 1962, Swamy and Krishnamurthy 1973) nor is this pattern transitional between cellular and free nuclear (Schnarf 1929, Maheshwari 1950, Wunderlich 1959) in Nymphaeales. Furthermore, comparative developmental analysis of endosperm within a phylogenetic context shows a clear and unambiguous relationship between helobial endosperm and cellular endosperm in Nymphaeales, with the derivation of a helobial pattern from a cellular pattern through a series of character state changes.

Implications of alternative topologies for the interpretation of endosperm evolution. Although several different phylogenetic analyses agree that *Amborella*, Nymphaeales, and the Illiciales clade are basal to all other extant angiosperms, there is support for different branching orders of these three lineages (Qiu et al. 2000). Some analyses (Barkman et al. 2000, Qiu et al. 2000) indicate that *Amborella* plus Nymphaeales may form a clade that is sister to all other angiosperms. This hypothesis requires no change in the interpretation of

endosperm character polarity or developmental evolution described above. Graham and Olmstead (2000) found some support for a rooting with Nymphaeales alone. With this topology, we lose the ability to resolve the ancestral state for division of the primary endosperm cell (either oblique or transverse) and for chalazal development (unicellular or cellular/multiseriate). However, rooting of angiosperms with Nymphaeales does not lead to a rejection of the hypothesis that oblique/cellular endosperm development is ancestral for flowering plants, nor does it change the conclusion that helobial endosperm in Cabombaceae is derived from a cellular endosperm pattern.

Modularity in endosperm development. We have shown that endosperm in basal angiosperms exhibits a bipolar ontogeny in which two domains (chalazal and micropylar) undergo differential patterns of development, following an initial cellular or free nuclear division. Furthermore, these three features (first division, micropylar development, and chalazal development) have evolved independently in different angiosperm lineages, leading to different developmental patterns (Floyd and Friedman 2000). For example, in the most recent common ancestor of Nymphaeales, cellular multiseriate chalazal development (ancestral for angiosperms) was transformed to unicellular uninucleate chalazal development (Fig. 6B), without any change in early micropylar development (Fig. 6C). During subsequent evolution of the Nymphaeales clade, micropylar development was transformed from cellular uniseriate to free nuclear in the most recent common ancestor of Cabombaceae (Fig. 6C), but chalazal development did not change (Fig. 6B). Thus at least two of the early, pattern forming stages of endosperm development (micropylar development and chalazal development) can be described as dissociable processes (sensu Raff 1996).

Dissociability of the components of ontogeny has long been recognized (Diggle 1992) and forms the foundation for the concept of developmental modularity (Raff 1996, Wagner 1996, Wagner and Altenberg 1996, Kirschner and Gerhart 1998, von Dassow and Munro 1999, Raff and Raff 2000, Raff and Sly 2000). Modularity, the compartmentalization of development into semiautonomous processes (Wagner 1996), has been described as a fundamental feature of biological organisms that is crucial to the evolution of development and complex adaptations (Bonner 1988, Gilbert et al. 1996, Wagner and Altenberg 1996, Kirschner and Gerhart 1998, Ancel and Fontana 2000, Raff and Raff 2000, Raff and Sly 2000). Although many studies have shown that plant developmental processes are dissociable (reviewed in Diggle 1992), the concept of modularity has been discussed almost entirely within the contexts of animal developmental evolution (see Raff and Raff 2000, Raff and Sly 2000) and evolutionary computation (Wagner 1996, Rotaru-Varga 1999, Calabretta et al. 2000).

Early endosperm ontogeny seems to fit the model of a modular process, as there are individual dissociable components (e.g. chalazal and micropylar development) that have been the "modular unit[s] of evolutionary transformation" (Wagner 1996, p. 38). Without an understanding of the genetic control of endosperm development, endosperm developmental characters do not yet fully meet the genetic criteria of modules defined by Raff (1996) and Wagner (1996). However, there is evidence that micropylar and chalazal endosperm domains exhibit differential gene expression in *Arabidopsis* (Luo et al. 2000) and maize (Hueros et al. 1995, Doan et al. 1996, Opsahl-Ferstad et al. 1997, Olsen 1998). Molecular genetic analysis of endosperm development in these model plant taxa, directed toward revealing the developmental programs of early ontogeny, are needed to fully examine the modular nature of endosperm ontogeny.

Endosperm is a simple organism with relatively few developmental modules compared to mature plant sporophytes and certainly compared to metazoans. Thus endosperm may serve as an ideal organismic system in which to study the role of modularity in evolution by determining the genetic control of modular development and the types of

genetic changes that have led to the transformation of modules. Endosperm can also serve to bring plant development into the discussion of modularity which is important if the concept is to remain a general theme in evolutionary biology. Studies of simple living organisms like endosperm, along with the emerging data from mathematically-based simulations investigating the role of modularity in evolution (e.g. Gruau 1995; Calabretta et al. 1998a, b; Rotaru-Varga 1999; Ancel and Fontana 2000; Calabretta et al. 2000), have the potential to provide significant insights into what is perhaps a fundamental process in organismic evolution.

Conclusions

Analysis of endosperm development in representatives of the three putative basal angiosperm lineages has resulted in a more complete hypothesis for the ancestral ontogeny than previously proposed, in which oblique/cellular and transverse/cellular patterns were not recognized as distinct character states (Floyd and Friedman 2000). Furthermore, the scenario for the origin of helobial endosperm in Nymphaeales described herein clearly illustrates that comparative study of endosperm development within a phylogenetic context can illuminate the evolutionary modifications of ontogeny that link different developmental patterns (helobial and cellular). Endosperm ontogeny involves dissociable elements and thus appears to conform to the definition of a modular developmental and evolutionary process. As our understanding of phylogeny and endosperm development increases for taxa diverging above the three basal nodes of the angiosperm tree, we can continue to piece together the story of endosperm evolution in flowering plants. These results highlight the major impact that the interaction of phylogenetic research and comparative developmental biology can have on our understanding of organismic evolution.

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Addresses of the authors: Sandra K. Floyd* (skfloyd@ucdavis.edu) and William E. Friedman, Department of Environmental, Population, and Organismic Biology, University of Colorado, Boulder, Colorado 80309, USA. *Present address: Section of Plant Biology, One Shields Avenue, University of California, Davis, CA 95616, USA.