FEMALE GAMETOPHYTE DEVELOPMENT IN KADSURA: IMPLICATIONS FOR SCHISANDRACAEA, ASTROBAILEYALES, AND THE EARLY EVOLUTION OF FLOWERING PLANTS

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Recent phylogenetic analyses of angiosperms have identified a set of “basal” angiosperm lineages (Amborella, Nymphaeales, and a clade that includes Illiciaceae, Schisandraceae, Trimeniaceae, and Austrobaileyaceae) that are central to the study of the origin and early diversification of flowering plants. Prior to this phylogenetic revelation, much of the work on the embryology of ancient angiosperm lineages focused on core magnoliids (e.g., Magnoliidae, Winterales). It is now apparent that little is known about the basic embryological features of the most ancient extant lineages of flowering plants, particularly with respect to the nature and development of the female gametophyte and the ploidy and genetics of the endosperm. Here, we report that Kadsura japonica (Schisandraceae) develops a four-celled female gametophyte with an egg cell, two synergids, and a uninucleate central cell. The pattern of free-nuclear divisions in the female gametophyte of Kadsura precisely matches what has recently been reported for four-celled gametophytes in the Nymphaeales. Following the first mitosis, migration of one of the two nuclei to the chalazal pole of the female gametophyte, as in Polygonum-type female gametophytes, does not occur. Rather, both nuclei remain close together in the micropylar domain where they undergo one additional mitotic division to yield four free nuclei before cellularization. Microspectrofluorometric analysis of relative DNA content of the central cell nucleus in Kadsura shows that this nucleus is haploid and contains the 1C quantity of DNA prior to fertilization. Thus, the endosperm of Kadsura should be diploid and biparental, as it is in Nuphar and other Nymphaeales. It now appears that four-celled female gametophytes, with consequent production of diploid endosperms, are common among the most ancient lineages of angiosperms, with the sole exception, to date, of Amborella. Finally, based on an analysis of the modular nature of the angiosperm female gametophyte, we provide evidence that four-celled female gametophytes that yield diploid biparental endosperms are likely to be plesiomorphic for flowering plants.

Keywords: angiosperm, evolution, developmental evolution, modularity, gametes, cell cycle, embryology.

Introduction

More than 150 years after “comparative” biology emerged as a central paradigmatic approach to the study of plant diversity and evolution, one could be pardoned for assuming that everything about the basic biology and variation in the reproductive process of plants has been described and cataloged. Yet, despite recent progress in analyzing the phylogenetic relationships of plants, much of the organismic diversity of extant plants has never been studied, even at the most rudimentary level. These “morphological gaps” among plants remain as the largest obstacles to deciphering the key evolutionary historical events that gave rise to the remarkable variation of extant photosynthetic life.

Recent phylogenetic analyses of angiosperms have identified a set of “basal” angiosperm lineages that include Amborella, Nymphaeales, and Austrobaileyales (Illiciaceae, Schisandraceae, Trimeniaceae, and Austrobaileyaceae) (Mathews and Donoghue 1999, 2000; Parkinson et al. 1999; Qiu et al. 1999, 2000; Solis et al. 1999, 2000; Barkman et al. 2000; Doyle and Endress 2000; Graham and Olmstead 2000; Graham et al. 2000; Zanis et al. 2002). It is now clear that these taxa are central to the study of the origin and early diversification of flowering plants. Prior to these recent phylogenetic inferences, much of the work on the embryology of ancient angiosperm lineages had been focused on members of the Magnoliidae, Winterales, and other assorted magnoliid lineages (Maneavel 1914; Earle 1938; Hayashi 1965; Bhandari and Venkataraman 1968; Bhandari 1971a, 1971b; Heo et al. 1998). Given the long-standing impression that Amborella, Nymphaeales (Nymphaeaceae, Cabombaceae), and Austrobaileyales (Illiciaceae, Schisandraceae, Trimeniaceae, and Austrobaileyaceae) were not the most ancient (or potentially plesiomorphic) lineages of flowering plants, relatively little attention was focused on these biologically diverse groups. Thus, little is known about the basic embryological features of members of these flowering plants, particularly with respect to the development and mature structure of the female gametophyte and the ploidy and genetics of the endosperm (Friedman 2001a, 2001b; Friedman and Floyd 2001).

For more than a century, the Polygonum-type (monosporic seven-celled, eight-nucleate) female gametophyte has been as-
sumed to be a defining feature and ancestral condition of the reproductive biology of flowering plants. Recently, however, it was predicted (Friedman 2001b), and then confirmed (Williams and Friedman 2002; Friedman and Williams 2003), that members of some of the most ancient lineages of flowering plants produce mature female gametophytes with only four uninucleate cells (an egg cell, two synergids, and a central cell with a single haploid nucleus) and that the endosperm of these lineages is genetically biparental and diploid.

Among basal angiosperms, Nuphar polysepala (and perhaps all members of the Nymphaeaceae and Cabombaceae) produces a four-celled female gametophyte (two synergids, an egg, and a uninucleate central cell), and double fertilization in this taxon yields a diploid zygote and diploid endosperm (Williams and Friedman 2002; Friedman and Williams 2003). It is also evident (based on an extensive analysis of the embryological literature) that most basal angiosperms may yield four-celled female gametophytes and diploid endosperms (Friedman and Williams 2003; Williams and Friedman, in press). The only known currently documented exception to this generalization is found in Amborella, which has recently been shown to produce a Polygonum-type female gametophyte similar to most angiosperms (Tobe et al. 2000).

In this article, we report on the developmental embryology of the female gametophyte of Kadsura japonica, a member of the Schisandraceae and the broader Austrobaileyales clade that also includes Illiciaceae, Trimeniaceae, and Austrobaileyaceae. Little is known about female gametophyte structure in Austrobaileyales. Illicium and Schisandra have been studied embryologically (Yoshida 1962; Hayashi 1963a, 1963b; Kapil and Jalan 1964; Swamy 1964; Solntseva 1981), but there is considerable ambiguity about the specifics of female gametophyte structure in these taxa (Battaglia 1986).

The female gametophyte of Kadsura has been examined once (Hayashi 1963b) and was reported to be of the Polygonum-type. Given the prediction that four-celled female gametophytes are potentially prevalent among basal angiosperms, we decided to reinvigate the development of the female gametophyte in Kadsura. This study provides clear evidence that the female gametophyte of Kadsura is four-celled and should, on double fertilization, yield a genetically biparental diploid endospem. These data, when viewed within the context of the developmental evolution of the female gametophyte, strengthen the conclusion that this pattern of reproductive biology is likely to be plesiomorphic among flowering plants (Friedman and Williams 2003).

Material and Methods

Plant Collection

Kadsura japonica (L.) Dunal is an evergreen woody vine native to Korea, Japan, and Taiwan (Saunders 1998). Kadsura species are generally monoecious; however, K. japonica is occasionally dioecious (Okada 1971). Staminate flowers bear numerous spirally arranged cream-colored tepals and bright red stamens; carpellate flowers also bear numerous spirally arranged cream-colored tepals and green apocarpous carpels. Carpellite buds and flowers at various stages of development were collected in July 2001 and July and September 2002 from specimens in the University of California Botanical Garden in Berkeley, California (accession number 90.0667; Honshu, Japan).

Brightfield and Fluorescence Microscopy

Flowers were fixed for 24 h either in 50 mM PIPES buffer containing 5 mM EGTA and 1 mM MgSO4 with 4% acrolein (pH 6.8) and stored in PIPES buffer or in a solution of 3 : 1 (95% ethanol : acetic acid) and stored in 70% ethanol. Specimens were dehydrated through an ethanol series and infiltrated and embedded with glycol methacrylate (JB-4 embedding kit, Polysciences, Warrington, Pa.). Embedded flowers were serially sectioned into 5-μm-thick ribbons. Sectioned flowers were stained with either 0.1% toluidine blue or with 0.25 μg/mL of DAPI (4′,6-diamidino-2-phenylindole) in 0.05 M TRIS (pH 7.2). Digital imaging was performed on a Zeiss Axioshot microscope equipped with a Zeiss Axiocam digital camera using both brightfield and fluorescence optics. Fluorescence was visualized with an HBO 100 W burner (Carl Zeiss, Oberkochen, Germany), using a UV filter set (model 48702) with excitation filter (365 nm, band pass 12 nm), dichroic mirror (FT395), and barrier filter (LP397). Images were processed with Adobe Photoshop 6.0. Image manipulations were restricted to operations that were applied to the entire image except as noted in specific figure legends.

Fig. 1 Megasporogenesis and megagametogenesis in Kadsura. A, The megasporocyte features a micropylar-positioned nucleus and a cytoplasmically dense zone in the chalazal region. B, At the dyad stage, the megasporocyte becomes subdivided by a cell wall after meiosis I. C, Chalazal nucleus of the dyad at metaphase of meiosis II (the micropylar nucleus of the dyad may lag in the cell cycle). The cytoplasmically dense zone can be seen in the chalazal region. D, After meiosis II and cytokinesis, the chalazal-most megaspore cell becomes the functional megaspore, or the one-nucleate female gametophyte. At this stage, the cytoplasmically dense zone no longer appears in the chalazal region. The remaining megaspores degenerate and become crushed (one of three degenerate megaspores [arrow] is visible in this section). E, At the two-nucleate stage of female gametophyte development, the female gametophyte is highly vacuolate, and the two nuclei are positioned within the micropylar domain, typically in perpendicular orientation to the longitudinal axis of the female gametophyte. This image is a composite of two images from different focal planes of the same section (improved resolution). F, G, The four-celled, four-nucleate female gametophyte is composed of two synergids with filament apparatus (arrows) (F), an egg (G), and the large, vacuolated central cell containing a single central cell (polar) nucleus (P). The central cell nucleus is typically positioned near the center or the chalazal third of the mature female gametophyte. All sections were stained with toluidine blue. For all figures, the micropylar pole is at top and the chalazal pole at bottom. c = cytoplasmically dense zone; ccn = central cell nucleus; e = egg cell; fm = functional megaspore (one-nucleate female gametophyte); s = synergid. Scale bar = 10 μm.
The degenerate megaspores are quickly crushed and are often tophyte, the cytoplasmically dense zone disappears (fig. 1). As noted, the one-nucleate stage of female gametophyte development shows considerable vacuolation compared with stages of megasporogenesis. The first mitotic division in the female gametophyte displays a linear tetrad of megaspores of which the chalazal-most cell will develop into the functional megaspore. A cytoplasmically dense zone is prominent in the megasporocyte (in a position chalazal to the nucleus of this cell; fig. 1A) and is ultimately transmitted to the functional megaspore during megasporogenesis (fig. 1C), where it continues to reside at the chalazal pole. The functional megaspore contains small cell vacuoles that begin to coalesce during the growth of the one-nucleate female gametophyte (fig. 1D). Prior to the first mitosis of the female gametophyte, the cytoplasmically dense zone disappears (fig. 1D). The degenerate megaspores are quickly crushed and are often difficult to identify.

Female Gametophyte Development

As noted, the one-nucleate stage of female gametophyte development shows considerable vacuolation compared with stages of megasporogenesis. The first mitotic division in the female gametophyte results in the formation of two free nuclei that are situated either at the extreme micropylar end of the female gametophyte (fig. 1E) or as much as a quarter of the distance in length from the micropylar pole. At no time did we observe any evidence of migration of one of the two free nuclei to the chalazal end of the female gametophyte, as would occur in a typical Polygonum-type female gametophyte during the two-nucleate stage. The second and final round of mitosis within the female gametophyte of Kadsura yields four free nuclei, all of which are located in the micropylar domain. The syncytial four-nucleate stage is apparently quite transitory because we never observed sectioned ovules in this developmental phase, although both young and mature four-celled gametophytes were common (fig. 1F, 1G). However, one female gametophyte yielded the transitional stage from four-nucleate to four-celled female gametophyte, with clear evidence of a phragmoplast depositing a cell plate between the egg cell and the central cell (fig. 2).

Cellularization of the syncytial female gametophyte yields four uninucleate cells: two synergids, an egg cell, and a central cell. The synergids are extremely prominent, with an identifiable filiform apparatus (fig. 1F). The egg cell abuts the two synergids (fig. 3A, 3B). Initially, the central cell nucleus is located very close to the egg apparatus (its original position at the conclusion of the second round of free-nuclear mitosis; fig. 2C), but following cellularization, it migrates near to the mid-point of the length of the female gametophyte (fig. 1F, 1G). None of the more than 300 cellularized female gametophytes that we examined showed any evidence of the presence of antipodal cells or any nuclei at the chalazal pole.

On two occasions (out of a total of 303 mature ovules examined), we observed six nuclei within the female gametophyte. The “extra” nuclei were found in the central cell. In one case, the three nuclei of the central cell were clustered together, and in the other case (fig. 4A–4E), two nuclei were clustered while the third nucleus was found in a different location. Given the rarity (in essence, teratological nature) of this event, we were unable to determine with absolute certainty the developmental preludes to this situation. Neither of these female gametophytes had been fertilized (no pollen tubes in micropyle; no degenerate synergid), indicating that the extra nuclei in the central cell were not derived from sperm. As with all of the other mature female gametophytes observed, neither of these female gametophytes showed any evidence of the presence (or past presence) of antipodals.

We can provide two possible explanations for these rare six-nucleate female gametophytes. Some angiosperms are known to initiate female gametophytes in which the functional megaspore is binucleate (a result of the lack of cytokinesis after meiosis II of the chalazal dyad cell). The more micropylar nucleus of this bisporic cell goes on to produce four nuclei, while the chalazal megaspore nucleus may degenerate, remain intact without dividing, or divide once to yield two nuclei (Haig 1990). In the latter case, female gametophytes with six nuclei will be formed. In our material, we only saw typical uninucleate megasporangial tetrad. However, given the rarity of six-nucleate female gametophytes in Kadsura japonica (<1%), our histological sampling of megasporogenesis could easily have missed rare aberrations.

We believe that a second hypothesis is the more likely explanation for these two anomalous female gametophytes. Twenty-one of the total of 303 (ca. 7%) mature ovules contained a second female gametophyte in addition to the normal four-celled female gametophyte. Of these 21 ovules with two female gametophytes, the second female gametophyte in 15 ovules was binucleate and appears to have arrested development at this stage (fig. 4F–4H). The other six ovules contained two four-celled female gametophytes. It is unclear whether the

Microspectrofluorometry

Sections from flowers fixed in 3 : 1 (95% ethanol: acetic acid) were stained with DAPI (see “Brightfield and Fluorescence Microscopy”) for 1 h in the dark at room temperature. Microspectrofluorometric measurements of relative DNA levels of DAPI-stained nuclei were performed within 2 h. Measurements were made with a Zeiss MSP 20 microspectrophotometer with digital microprocessor coupled to a Zeiss Axioskop microscope equipped with epifluorescence (HBO 100 W burner). A UV filter set (model 48702) with excitation filter (365 nm, band pass 12 nm), dichroic mirror (FT395), and barrier filter (LP397) was used with a Zeiss Plan Neofluar × 40 objective. Before each recording session, the photometer was standardized by taking a reading of fluorescence emitted from a fluorescence standard (GG 17), and this reading was taken to represent 100 relative fluorescence units (RFU). At the completion of each session, an additional reading was made of the fluorescence standard to confirm that little or no deviation in the relative fluorescence value obtained from the fluorescence standard had occurred during the period of data recording. Relative DNA content for each nucleus was determined by summation of individual fluorescence values of each of the serial sections through that nucleus. A net photometric value for each section of a nucleus was obtained by recording an initial reading of the nucleus and subtracting a background value obtained from cytoplasm proximal to the nucleus. Thus, background fluorescence from the glycol methacrylate was removed from the photometric analysis of relative DNA content.

Results

Megasporogenesis

Megasporogenesis typically yields a linear tetrad of megaspores of which the chalazal-most cell will develop into the functional megaspore. A cytoplasmically dense zone is prominent in the megasporocyte (in a position chalazal to the nucleus of this cell; fig. 1A) and is ultimately transmitted to the functional megaspore during megasporogenesis (fig. 1C), where it continues to reside at the chalazal pole. The functional megaspore contains small cell vacuoles that begin to coalesce during the growth of the one-nucleate female gametophyte (fig. 1D). Prior to the first mitosis of the female gametophyte, the cytoplasmically dense zone disappears (fig. 1D). The degenerate megaspores are quickly crushed and are often difficult to identify.
two female gametophytes derive from the initial formation of two megasporocytes or from the development of two functional megaspores from a single megasporocyte.

In one of the two six-nucleate female gametophytes described above, clear evidence of a second (subordinate) female gametophyte was found (fig. 4A–4E); however, there were no nuclei present in this structure, and there appears to be a clear break in the cell wall material separating the two female gametophyte chambers (fig. 4D). We suspect that, occasionally, the degeneration of the subordinate supernumerary two-nucleate female gametophyte may result in the passage of its nuclei into the central cell of the dominant female gametophyte, thus yielding a six-nucleate structure. It is unknown whether these extra nuclei participate in the second fertilization event and thus produce a polyploid endosperm.

During the free-nuclear and cellularization phases of development, the female gametophyte continues to expand in length and in width. At the one-nucleate stage, female gametophytes ranged from 55 to 90 μm long and from 20 to 23 μm wide. By the early four-celled stage, gametophytes ranged from 150 to 195 μm long and from 35 to 50 μm wide. During the final phases of maturation of the female gametophyte, the chalazal domain significantly enlarges in girth to yield a bulbous structure that may be up to 110 μm wide, while the entire gametophyte ranges from 215 to 230 μm long. Often, the central cell nucleus is positioned near the transition zone between the narrower micropylar domain and wider chalazal domain of

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**Fig. 3** Kadsura egg apparatus. In this oblique view of the micropylar end of a mature female gametophyte, the egg cell (A) contains a nucleus near its junction with the central cell. The egg cell abuts the two synergids (B) whose nuclei lie near the micropylar wall of the female gametophyte. All sections were stained with DAPI and visualized with fluorescence and DIC optics. cc = central cell; e = egg cell; s = synergid. Scale bar = 10 μm.

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**Fig. 2** Cellularization of the four-nucleate female gametophyte in Kadsura. Four sequential sections show the two synergid nuclei directly above one another (A, D) and the egg and central cell nuclei (B, C). The egg and central cell nuclei are in the process of being partitioned into separate cells by the phragmoplast (B–D; arrows). All sections were stained with toluidine blue. For all figures, the micropylar pole is at top and the chalazal pole at bottom. ccn = central cell nucleus; en = egg nucleus; sn = synergid nucleus. Scale bar = 10 μm.
the female gametophyte or may be found in the bulbous chalazal end of the female gametophyte (figs. 1F, 5F).

DNA Content and Ploidy of Central Cell

It is possible that the four-celled mature female gametophyte of *K. japonica* could arise from a Polygonum-type female gametophyte where the three antipodals have degenerated and the two polar nuclei have fused into a secondary nucleus (diploid central cell nucleus); this would yield a four-celled structure. To rule out this possibility, we measured relative DNA contents of egg and central cell nuclei in mature female gametophytes. If the central cell contains only a single haploid nucleus, the DNA content of this nucleus should be identical to that of the haploid egg nucleus, assuming that they occupy similar positions within the cell cycle. If, however, the nucleus of the central cell is the product of an unobserved fusion of two haploid polar nuclei, the ratio of DNA content of the central cell nucleus to the egg nucleus should be 2 : 1.

Although large numbers of female gametophytes were examined with fluorescence microscopy, mitotic figures were never observed; thus, we were unable to directly calibrate the relative fluorescence per C unit of DNA. However, the nuclei of a syncytial female gametophyte (fig. 5A, 5B) are haploid and vary in DNA content by a factor of 2 over the course of the cell cycle (1C during anaphase, telophase, and G1; between 1C and 2C during the S phase; and 2C during G2, prophase, and metaphase). Measurements made on two one-nucleate female gametophytes (fig. 5A) yielded values of 186.83 and 320.39 relative fluorescence units (RFU) per nucleus for each of the gametophytes. Measurements made on three two-nucleate female gametophytes (fig. 5B) yielded average RFU values of 306.09, 305.92, and 160.15 per nucleus for each of the gametophytes. Finally, in the one four-nucleate female gametophyte undergoing cellularization (phragmoplast evident between incipient egg cell and central cell; figs. 2A–2D, 5C–5E), the central cell nucleus emitted 137.60 RFU (the other nuclei were not measurable). Given that the nuclei of this gametophyte have just emerged from mitosis, they should be in G1 of the cell cycle and thus approximate the amount of fluorescence associated with the 1C content of DNA.

The RFU values for the six free-nuclear-stage female gametophytes are distributed in a distinctly bimodal pattern. Three of the values (137.60, 160.15, and 186.83; mean = 161.52) are very close to half of the RFU readings of the other three female gametophytes (306.09, 305.02, and 320.39; mean = 310.50), indicating that the set of lower values closely approximates the 1C DNA content (haploid nuclei in G1 of the cell cycle) and that the higher RFU values represent the 2C DNA content (haploid nuclei in G2 of the cell cycle). Importantly, as shown below, the lower RFU values for syncytial stage female gametophytes are very close to the average RFU values for egg and central cell nuclei, and the higher RFU values for syncytial stage female gametophytes are approximately double the average RFU values for egg and central cell nuclei.

To determine whether the central cell of the female gametophyte of *Kadsura* contains a single haploid nucleus or a diploid fusion product of two polar nuclei (secondary nucleus of a Polygonum-type female gametophyte), we examined 26 unfertilized ovules (no pollen tubes present in micropyle; fig. 5F, 5G). The mean fluorescence of the egg nuclei was 165.0 ± 27.8 (mean ± SD) RFU, strongly indicating that these nuclei are in G1 of the cell cycle and contain the 1C amount of DNA because egg nuclei in sexual plants are haploid. If the central cell nucleus contains a single haploid polar nucleus whose cell cycle is synchronized with the egg nucleus, it should have a mean DNA fluorescence value of ca. 165 RFU, whereas if the central cell nucleus is a cryptic fusion product of two polar nuclei, it will have a mean DNA fluorescence value of ca. 330 RFU. *Kadsura* central cell nuclei expressed a mean RFU of 152.4 ± 25.7 SD, which is clearly more similar to the 1C value of 165 RFU than to the 2C value of ca. 330 RFU. The slight difference in egg nucleus and central cell nucleus means (paired t-test: *P* = 0.014, two-tailed test, *n* = 26 pairs) is probably not an artifact and may reflect subtle, yet real, differences in chromatin conformation between the two types of nuclei (for discussion of these staining issues, see Narayan and Rees 1974; Santisteban et al. 1992; Kapuscinski 1995).

Importantly, the DNA fluorescence values obtained from central cell nuclei are completely inconsistent with the hypothesis of a diploid central cell nucleus (or a haploid nucleus in G1). The mean fluorescence of *Kadsura* central cell nuclei was significantly different from the predicted diploid 2C DNA fluorescence level (paired t-test: *P* = 7.8 × 10⁻¹⁷, two-tailed test, *n* = 26 pairs). These microspectrofluorometric data in-

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**Fig. 4** Ovules with two female gametophytes (a dominant mature female gametophyte and a subordinate partially developed female gametophyte) that may yield anomalous six-nucleate female gametophytes. A–E, An ovule in which the walls between a subordinate partially developed female gametophyte and a dominant female gametophyte with a mature egg apparatus have been breached. The contents of the subordinate female gametophyte (two free nuclei) appear within the central cell of the mature dominant female gametophyte. In this oblique view of a mature female gametophyte (fg1), the egg (A, B) and synergid (B) occupy the micropylar pole, and the central cell nucleus is in its typical central position surrounded by a denser zone of cytoplasm (D). A cell wall (arrows) indicates the presence of the subordinate female gametophyte (fg2) (C), which has a large rupture in it (D). This subordinate female gametophyte contains no nuclei (other sections not shown). Two small nuclei are, however, found in the chalazal portion of the mature female gametophyte (E), and these nuclei are not surrounded with cytoplasm, as is typical of a central cell nucleus or fusing polar nuclei (in taxa with two or more polar nuclei). For comparison, most mature ovules that contained two female gametophytes possessed a four-celled female gametophyte alongside a subordinate two-nucleate female gametophyte (fg-H). The mature four-celled female gametophyte contains four nuclei: an egg (P), two synergids (G), and a single central cell nucleus (G). Directly adjacent to the mature female gametophyte is the subordinate female gametophyte (fg-H). Two small nuclei (H), similar to those in E, remain within the subordinate female gametophyte. H, Composite of two sections (the upper of the two nuclei, designated with an arrow; is from a separate section, shown with arrow in inset). All sections were stained with toluidine blue. For all figures, the micropylar pole is at top and the chalazal pole at bottom. ccn = central cell nucleus; e = egg cell; fg1 = dominant female gametophyte; fg2 = subordinate female gametophyte; s = synergid. Scale bar = 10 μm.
dependently confirm our developmental observations derived from light microscopy: the Kadsura female gametophyte is four-celled with a uninucleate haploid central cell (fig. 1F, 1G; fig. 5F, 5G). Moreover, the data strongly indicate that the egg and central cell remain in G, before fertilization (see Friedman 1999 for discussion of different patterns of cell cycle expression in plants during gametogenesis and fertilization).

We also measured relative fluorescence values of synergid nuclei in four-celled female gametophytes. The mean RFU of these nuclei was 160.85 ± 23.57 SD. This indicates that similar to the egg and central cell nuclei the synergids are haploid and remain stationed in G, at least until the time of fertilization.

**Discussion**

For the entire twentieth century, it was a central paradigm of flowering plant reproductive biology that the seven-celled, eight-nucleate monosporic female gametophyte (Polygonum-type) was characteristic of the most ancient angiosperm lineages and indeed a synapomorphy of the entire angiosperm clade (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). However, much has changed in light of the recent phylogenetic discovery that Amborella, Nymphaeae, and a clade that includes the Illiciaceae, Schisandraceae, Trimeniaceae, and Austrobaileyaceae (Austrobaileyae) constitute a basal grade of the most ancient flowering plant lineages. Unlike other magnoloids that were carefully and frequently studied embryologically (reviews by Hayashi 1965; Bhandari 1971a, 1971b), members of these recently identified most ancient angiosperm lineages were only rarely examined during the last century.

Recent work on female gametophyte development in basal angiosperms has involved Amborella (Tobe et al. 2000) and members of the Nymphaeae and Cabombaceae (Galati 1985; Winter and Shamrov 1991a, 1991b; Van Miegroet and Dujardin 1992; Orban and Bouharmont 1998; Williams and Friedman 2002; Friedman and Williams 2003). These findings stand in contrast with the conclusions of earlier studies that reported seven-celled, eight-nucleate female gametophytes in Nymphaeales (Cook 1902, 1906; Khanna 1965, 1967; Ramji and Padmanabhan 1965; Schneider 1978). It is now evident that these earlier reports are likely to be in error (Friedman and Williams 2003).

There have been no recent studies of the female gametophyte in any member of the basal angiosperm clade Austrobaileyales, which includes Illiciaceae, Schisandraceae, Trimeniaceae, and Austrobaileyaceae. Recent molecular systematic analyses indicate that Schisandraceae is apparently monophyletic and comprises members of Kadsura and Schisandra. However, neither of these genera has been shown to be monophyletic, and it may be that both taxa are paraphyletic with respect to each other (Liu et al. 2000; Hao et al. 2001). Thus, for the purposes of our discussion, we will treat the entire Schisandraceae, with an explicit acknowledgement that members of Kadsura and Schisandra are potentially phylogenetically intermingled.

Embryological studies of female gametophyte structure in the Schisandraceae were first undertaken in the early 1960s. Yoshida (1962) published a short article in which he noted that mature female gametophytes in Schisandra chinensis were four-celled and four-nucleate. Yoshida reported that in *S. chinensis*, the single megaspore nucleus divides only twice to yield a total of four free nuclei within the female gametophyte before cellularization and sexual maturation. He also clearly showed (in drawings) that at the two-nucleate stage, the two nuclei are not separated by a large vacuole to the chalazal and micropylar ends, as is the case in the Polygonum-type female gametophyte two-nucleate stage. Rather, in *S. chinensis*, the two free nuclei remain close together in the micropylar end of the female gametophyte, as do the four free nuclei following the next and final round of mitotic division. The result is a four-celled female gametophyte with an egg cell, two synergids, and a central cell with a single polar nucleus (Yoshida 1962).

Swamy (1964) also found that the female gametophyte of *S. chinensis* develops into a four-celled structure at maturity. However, in addition to finding monosporic development, he suggested that 50% of the female gametophytes in this taxon were bisporic. As Battaglia (1986) first pointed out, this report of bisporic development in *S. chinensis* is likely to be based...
on a misinterpretation of some of the early stages of mega-sporeogenesis and female gametophyte development. Our examination of Swamy’s plates also finds no evidence of bisporic development in his drawings of megasporogenesis.

Contemporaneous with the above two reports (Yoshida 1962; Swamy 1964), Hayashi (1963b) reported Polygonum-type female gametophytes in *Schisandra repanda* and *Kadsura japonica*. Importantly, however, Hayashi noted that he typically saw only the egg apparatus (three cells) and what he described as the secondary nucleus derived from the fusion of the two polar nuclei in mature female gametophytes. Nevertheless, he presented drawings of syncytial, eight-nucleate female gametophytes and antipodal cells in Polygonum-type gametophytes for both taxa.

Kapil and Jalan (1964) analyzed female gametophyte development in *Schisandra grandiflora*. Like Hayashi, these workers reported that the female gametophyte is eight-nucleate and of the Polygonum-type. Ironically, all of their figures (drawings) show four-celled female gametophytes with a threcelled egg apparatus and a central cell with a single nucleus. In addition, they stated (incorrectly) that Yoshida (1962) had also found Polygonum-type female gametophytes in *Schisandra*; they assumed that Yoshida, like themselves, had missed witnessing a developmental stage prior to the supposed degeneration of the antipodals and the fusion of the two polar nuclei into a secondary nucleus.

In light of the contradictory reports of four-celled and seven-celled female gametophytes in Schisandraceae, we decided to resolve the characteristics of female gametophyte development in *K. japonica* through a combination of developmental analysis and microspectrofluorometric analysis of the ploidy of the central cell. Our results clearly and unambiguously show that the female gametophytes of *K. japonica* are monosporic and produce four-nucleate, four-celled female gametophytes with an egg cell, two synergids, and a haploid uninucleate central cell. Moreover, the pattern of free-nuclear divisions precisely matches the pattern reported for four-celled gametophytes in the Nymphaeales (Friedman and Williams 2003). Following the first mitosis, there is no migration of one of the two nuclei to the chalazal pole of the female gametophyte (as first reported correctly for Schisandraceae by Yoshida 1962), as would occur in a Polygonum-type female gametophyte. Rather, both nuclei remain close together in the micropylar domain, where they undergo one additional mitotic division to yield four free nuclei (fig. 2). These four nuclei are confined to the micropylar end of the female gametophyte. Only after cellularization does the central cell nucleus migrate to a more chalazal position within the female gametophyte (figs. 1f, 5f).

Given our very large sample size of four-celled female gametophytes (>300) and that no one, including Hayashi (1963b) and Kapil and Jalan (1964), has ever presented photomicrographic evidence, as opposed to drawings, of an actual Polygonum-type seven-celled, eight-nucleate stage in any species of Schisandraceae, it seems very likely that Hayashi (1963b) and Kapil and Jalan (1964) misinterpreted their embryological data. Thus, we conclude that, like the Nymphaeales (Williams and Friedman 2002; Friedman and Williams 2003), all evidence points to the presence, and prevalence, of monosporic four-celled, four-nucleate female gametophytes in the Schisandraceae.

**Evolution of Female Gametophyte Ontogeny in Ancient Angiosperm Lineages**

Only two known female gametophyte ontogenetic sequences are present in the most ancient clades of extant angiosperms: the monosporic four-celled, four-nucleate sequence characteristic of Schisandraceae, Nymphaeales, and possibly the Illiciaceae (further study of *Illicium* is needed to resolve contradictory reports in the earlier literature) 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, Friedman and Williams (2003) argued that four-celled, four-nucleate female gametophytes are likely to be plesiomorphic among angiosperms, with seven-celled, eight-nucleate female gametophytes derived.

As recently (and long ago) argued, the female gametophyte of angiosperms is best viewed as a fundamentally modular structure in which individual developmental modules consist of “quartets” of nuclei. Each module can be characterized by a common developmental pattern: (1) positioning of a single nucleus within a cytoplasmic domain (pole) of the female ga-
Sperm female gametophyte (Schnarf 1931; Maheshwari 1950; analysis of the origin and subsequent evolution of the angiosperm female gametophyte has long served as the baseline for Friedman and Williams 2003). Angiosperm female gametophytes may initiate one (most basal angiosperms), two (most angiosperms), or even four modules (e.g., Penaea-type female gametophyte) (Porsch 1907; Schnarf 1936; Maheshwari 1950; Swamy and Krishnamurthy 1975; Favre-DuChartre 1976; Battaglia 1989; Haig 1990; Friedman and Williams 2003). In K. japonica and other basal angiosperm taxa with four-celled female gametophytes, all nuclei are confined to the micropylar domain during free-nuclear development, and this results in the establishment of a single modular quartet (fig. 6). Conversely, in Amborella and other angiosperms with Polygonum-type female gametophytes, 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 (second) polar nucleus (fig. 6).

The evolutionary developmental analysis of Friedman and Williams (2003) posited that the first angiosperm female gametophytes were composed of a single four-nucleate and four-celled module that, on double fertilization, yielded a diploid endosperm. Early in angiosperm history, ectopic expression of this basic developmental module resulted in the initiation of two developmental modules within the female gametophyte and the formation of a seven-celled, eight-nucleate structure that yields a triploid endosperm with the 2:1 maternal to paternal genome ratio characteristic of most flowering plants. The means of establishing a duplicate module was the developmental insertion of a cytoskeletal apparatus after the first mitotic division that resulted in the positioning of the nuclei (at the two-nucleate stage) in distinct cytoplasmic domains at opposite poles of the female gametophyte. The net effect of this novel nuclear migration process was the parallel initiation and development of two modular quartets and the creation of a seven-celled and eight-nucleate Polygonum-type female gametophyte (Friedman and Williams 2003).

Conclusions

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 may be rare among basal angiosperms (namely, Nymphaeales and Austrobaileyales), while the four-celled, four-nucleate type of female gametophyte characteristic of Schisandraceae, Nymphaeaceae, and Cabombaceae may be typical of the most ancient angiosperm lineages as well as plesiomorphic for flowering plants as a whole. Although we did not observe fertilization in K. japonica, the clear documentation of a uninucleate haploid central cell indicates that if double fertilization occurs in this taxon (and in other members of the Schisandraceae), the endosperm will be diploid with a 1:1 maternal to paternal genome ratio. This would stand in marked contrast with 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.

A careful investigation, and reinvestigation, of the most basic embryological features of early divergent angiosperm lineages is clearly needed. The female gametophytes of Austrobaileyales have never been studied. Embryological studies of the female gametophytes of Illicium are contradictory (one report of a four-celled female gametophyte [Solntseva 1981] and one report of a seven-celled female gametophyte [Hayashi 1963a]). Moreover, even a cursory examination of the literature on female gametophyte structure and fertilization biology of basal monocots (e.g., Acorus, diverse alismatids) indicates that virtually nothing can be inferred with any certainty. Until the reproductive biology of these phylogenetically key basal angiosperm taxa is analyzed anew, the “morphological gaps” that are the result of our own ignorance will continue to prevent us from fully reconstructing the major evolutionary historical events that have produced the remarkable diversification of flowering plants during the last 130 million years.

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