LETTERS

Embryological evidence for developmental lability during early angiosperm evolution

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Recent advances in angiosperm phylogeny reconstruction[1–3], palaeobotany[4,5] and comparative organismic biology[6–8] have provided the impetus for a major re-evaluation of the earliest phases of the diversification of flowering plants. We now know that within the first fifteen million years of angiosperm history, three major lineages of flowering plants—monocotyledons, eumagnoliids and eudicotyledons—were established[5], and that within this window of time, tremendous variation in vegetative and floral characteristics evolved. Here I report on a novel type of embryo sac (angiosperm female gametophyte or haploid egg-producing structure) in *Amborella trichopoda*, the sole member of the most ancient extant angiosperm lineage. This is the first new pattern of embryo sac structure to be discovered among angiosperms in well over half a century. This discovery also supports the emerging view[9–12] that the earliest phases of angiosperm evolution were characterized by an extensive degree of developmental experimentation and structural lability, and may provide evidence of a critical link to the gymnospermous ancestors of flowering plants.

Nearly 130 years after Darwin proclaimed the origin and early evolution of flowering plants an "abominable mystery" (Charles Darwin's letter to Joseph Dalton Hooker, 23 July 1879), reconstructing the biological features of the first angiosperms and linking them to non-flowering seed plant ancestors continue to challenge evolutionary biologists. Because flowering plants are distinguished from all other plants by diverse aspects of their reproductive biology, the importance of understanding the embryological features of early angiosperm lineages cannot be overstated. For more than a century, the presence of a highly reduced female gametophyte, within which a process of double fertilization initiates an embryo and a sexually formed embryo-nourishing tissue called endosperm, has been viewed as one of a suite of key characters integrally associated with the 'success' of angiosperms[13,14]. Nevertheless, the origin and early evolutionary diversification of the angiosperm female gametophyte has remained enigmatic.

In the overwhelming majority of angiosperms, the female gametophyte contains seven cells and eight genetically identical nuclei at maturity and is referred to as the 'Polygonum-type' (Fig. 1). At the chalazal pole of a Polygonum-type female gametophyte are three sterile cells, the antipodals. At the micropylar pole, a three-celled egg apparatus contains the egg cell and two sterile accessory cells, the synergids. One synergid functions to accept the pollen tube and the initial deposition into the female gametophyte of the two sperm cells that will participate in the double fertilization process. The large central cell, which is the target of the unique angiosperm second fertilization event, is binucleate, and as a consequence, endosperm derived from a Polygonum-type female gametophyte is triploid. Given the near-ubiquity of reports of Polygonum-type female gametophytes among putatively ancient lineages, the conclusion was reached early and often in the twentieth century that the first flowering plants must have had a seven-celled and eight-nucleate female gametophyte[13–19] that initiated a triploid endosperm. In 1999, a series of molecular phylogenetic analyses revealed that many of the century-old assumptions about what constitute the most ancient angiosperm lineages were fundamentally incorrect[20–23]. These and almost all subsequent phylogenetic analyses[1–3] demonstrate that the monotypic New Caledonian taxon *Amborella trichopoda*, Nymphaeales (water lilies) and Austrobaileyales (Illiciaceae, Schisandraceae, Trimeniaceae and Austrobaileyaceae) comprise a basal-most grade of flowering plants whose origins predate the establishment of monocotyledons, eumagnoliids and eudicotyledons. More specifically, these analyses provide compelling evidence that *Amborella* (or *Amborella* plus Nymphaeales) is sister to all other angiosperms, and hence is one of the most ancient lineages of flowering plants. As such, *Amborella* may hold the key to deciphering the earliest phases of angiosperm evolutionary history and character transformation.

In order to examine the early evolution of gametic structures and the fertilization process, I performed a developmental analysis of the female gametophyte in *Amborella trichopoda*. Brightfield, fluorescence, confocal and transmission electron microscopy of *Amborella* ovules (field and greenhouse collections) clearly show that the mature female gametophyte comprises nine nuclei and eight cells. The egg apparatus contains a typical egg cell and three sterile uninucleate cells that share an interface with the egg cell (Figs 2 and 3; see also Supplementary Video). The large central cell is initially binucleate, and there are three antipodal cells that persist through the time of fertilization. Just before the fertilization process, the two polar nuclei of the central cell fuse into a single secondary nucleus (as is characteristic of many angiosperms) that is the target of the second fertilization event; triploid endosperm results (microspectrofluorometric DNA quantification data not shown). This basic structure of

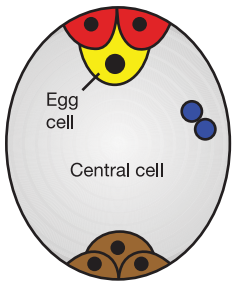

Figure 1 | A seven-celled, eight-nucleate female gametophyte (Polygonum-type) that is common to the vast majority of angiosperms. During the process of angiosperm double fertilization, the egg cell (yellow) and the central cell (grey) with two polar nuclei (blue) are each fertilized by a sperm cell and respectively produce a diploid zygote and a triploid embryo-nourishing tissue (endosperm). Synergid cells, red; antipodal cells, brown.

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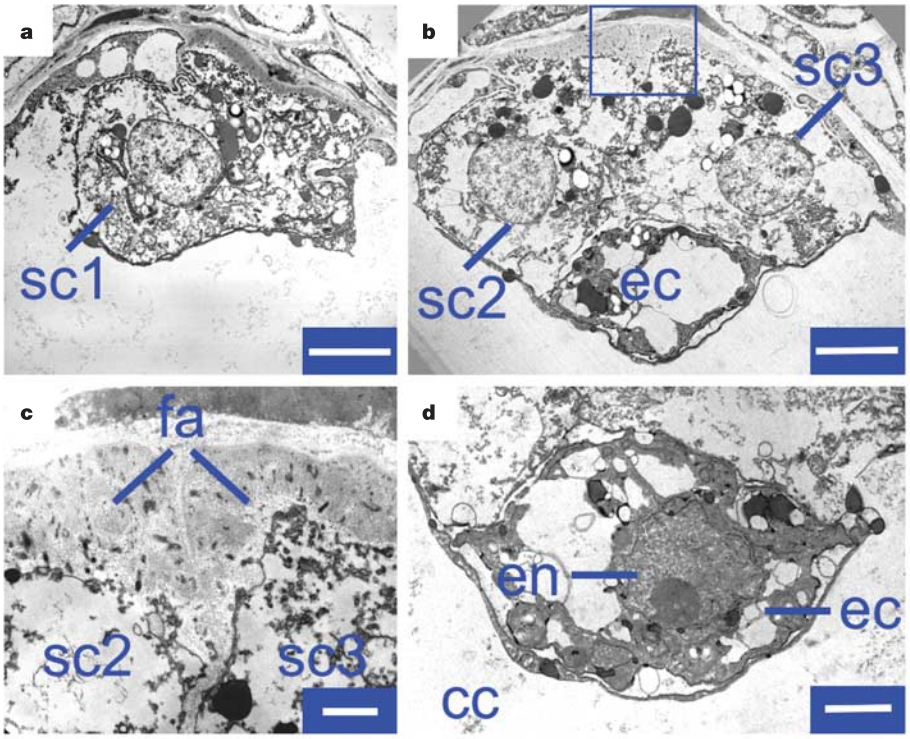

Figure 2 | Transmission electron micrographs of an individual serially sectioned female gametophyte of *Amborella*. **a**–**d**, The egg apparatus contains three synergid cells (sc1, sc2, sc3), each with a distinct nucleus (**a**, **b**), and an egg cell (ec), also with a distinct nucleus (en) (**d**). A filiform apparatus (fa) is present in each of the three synergid cells of the egg apparatus and is shown (boxed area in **b**) at higher magnification in **c**. cc, central cell. Scale bars: **a**, **b**, 5 μm; **c**, 1 μm; **d**, 2.5 μm.

the female gametophyte is characteristic of the more than 220 serially sectioned ovules (and seeds) that were examined.

Ultrastructural analysis of the mature female gametophyte demonstrates that each of the three sterile cells in the egg apparatus has a highly convoluted cell wall (similar to a transfer cell wall) at the micropylar pole that is characteristic of a standard filiform apparatus. Each sterile cell also shares a thin common wall with the pyramidal egg cell (Figs 2, 3). The filiform apparatus, as well as the cytoplasmic contents of the three sterile cells in the egg apparatus of *Amborella*, are essentially similar to each other and resemble those of synergids in other angiosperms that have been examined[24]. Thus, the 'extra' cell in the egg apparatus is a synergid.

Female gametophyte development in *Amborella* parallels that of Polygonum-type female gametophytes until the terminal stage of differentiation of the egg apparatus (Figs 4, 5). Mitotic division of the one-nucleate female gametophyte yields two nuclei, one of which migrates to the chalazal pole. A second wave of mitosis results in two pairs of nuclei at opposite poles of the female gametophyte. A syncytial eight-nucleate stage was never observed, suggesting that this phase is extremely transitory (as is typically the case in other angiosperms[24]); however, a seven-celled, eight-nucleate stage with three cells at each pole of the female gametophyte and a large binucleate central cell was seen in developing (pre-anthesis) floral buds (Fig. 4). At this stage, the female gametophyte of *Amborella* resembles the basic structure of a mature Polygonum-type female gametophyte: there are three antipodal cells at the chalazal pole, a binucleate central cell and three cells at the micropylar pole. In *Amborella*, however, the eight-nucleate, seven-celled female gametophyte represents the penultimate stage of the ontogeny. A single mitotic and cytokinetic event at the micropylar end of the *Amborella* female gametophyte produces the ninth nucleus and eighth cell (Fig. 4). The four cells at the micropylar pole, as well as the three antipodal cells at the chalazal pole, are initially flat, but increase in overall size as the female gametophyte matures (Figs 4, 5).

A four-celled egg apparatus is a definitive and characteristic feature of the female gametophyte of *Amborella* and cannot be viewed as a teratology. Among 147 serially sectioned mature *Amborella* female gametophytes (field and greenhouse collections) that were carefully examined, none could be shown to contain a three-celled egg apparatus.

The terminal cell division in the female gametophyte of *Amborella* produces the egg cell and the third synergid (Fig. 4). This can be inferred from examination of immature female gametophytes with three cells at the micropylar pole: each of the three cells has a face that abuts the boundary wall of the embryo sac (Fig. 4). In light of the fact that the egg cell only shares cell interfaces with the central cell and the three synergid cells (and not the wall of the embryo sac), the egg must be produced from a cell division that cleaves a daughter cell to the inside of the female gametophyte. Thus, the egg cell is the lineal sister cell of a synergid cell, and this developmental relationship is unique

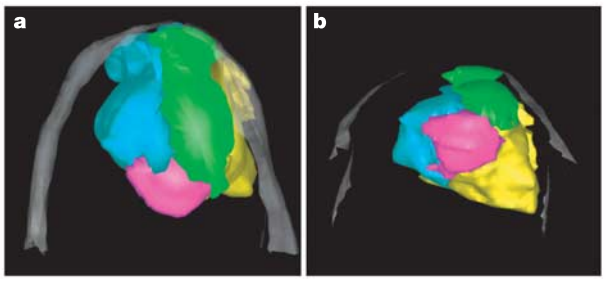

Figure 3 | Three-dimensional reconstructions (from semi-thin serial sections) of the four-celled egg apparatus of *Amborella*. **a**, All three sterile cells (synergids coloured green, blue and yellow) can be seen to abut the micropylar wall (at top) of the female gametophyte (grey) and envelope a portion of the egg cell (pink). **b**, The egg apparatus has been rotated 90° from **a** to show the pyramidal egg cell seated on all three synergids.

among all angiosperms. In all other angiosperm female gametophytes, the egg nucleus is the mitotic sister nucleus of a polar nucleus of the central cell[24].

The eight-celled, nine-nucleate female gametophyte of *Amborella* herein described is the first new type of embryo sac to be discovered among flowering plants in more than half a century of extensive surveys, and is the only one known to produce normally more than two synergids in the egg apparatus. *Indeterminate gametophyte1* mutants in maize frequently produce extra synergids within the egg apparatus, but this is always accompanied by supernumerary egg cells[25].

Until quite recently, *Amborella* and members of the Nymphaeales and Austrobaileyales were generally thought to exhibit a standard seven-celled, eight-nucleate Polygonum-type female gametophyte[26]. However, earlier reports of Polygonum-type female gametophytes in Nymphaeales and Austrobaileyales have now been shown to be erroneous[8,27–29], as is also clearly the case for *Amborella*. Ironically, it is now evident that none of the most ancient lineages of flowering plants produces a seven-celled, eight-nucleate female gametophyte, a stark reminder that much remains to be discovered or correctly circumscribed for the earliest angiosperms. The mature female gametophytes of members of the Nymphaeales and Austrobaileyales contain four cells and four nuclei at maturity: a haploid uninucleate central cell, an egg cell and two synergids[8,27]. The mature female gametophyte of *Amborella* contains eight cells and nine nuclei at maturity.

The issue of whether the eight-celled, nine-nucleate female gametophyte of *Amborella* represents an autapomorphy of the *Amborella* clade or is a plesiomorphic characteristic for all angiosperms cannot be resolved at this time. However, two (among several) evolutionary developmental explanations are particularly intriguing. One possibility is that the Amborella-type female gametophyte is peramorphic and derived from a late developmental modification of a standard Polygonum-type female gametophyte. As such, this would suggest that the Polygonum-type might be plesiomorphic for angiosperms, even though a seven-celled, eight-nucleate female gametophyte is not present in any of the most ancient extant lineages of flowering plants.

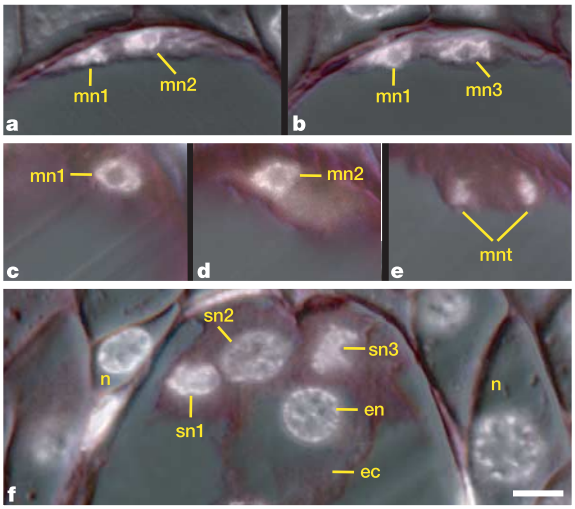

Figure 4 | Terminal developmental stages of the egg apparatus in the female gametophyte of *Amborella*. **a**, **b**, Combined fluorescent and differential interference contrast images of consecutive serial sections showing three DAPI-stained micropylar nuclei (mn1, mn2, mn3). A prominent nucleolus is characteristic of each female gametophyte nucleus at this stage. **c**–**e**, Fluorescent images of consecutive serial sections showing two DAPI-stained micropylar nuclei (mn1, mn2) and a telophase mitotic figure (mnt) that will yield the egg and a synergid. **f**, Mature egg apparatus with three synergids and nuclei (sn1, sn2, sn3) and the egg cell (ec) with egg nucleus (en). n, nucellus. Scale bar, 5 μm.

According to this hypothesis, a terminal addition (a single cell division to create the egg and an additional synergid) to the ontogenetic sequence of the Polygonum-type would have led to the creation of the novel Amborella-type female gametophyte. Within the precepts of this hypothesis, the four-nucleate and four-celled female gametophytes of Nymphaeales and Austrobaileyales would be viewed as derived (and not plesiomorphic) within angiosperms, a conclusion at odds with that recently articulated by Williams and Friedman[27] and Friedman and Williams[8,29].

Alternatively, the unique four-celled egg apparatus in *Amborella* could represent a critical link between angiosperms and gymnosperms. An important difference between female gametogenesis in gymnosperms and angiosperms relates to the fact that gymnosperms never directly form an egg cell; rather they form a mother cell ('central cell' sensu gymnosperms) within an archegonium that later divides to yield a ventral canal cell (or nucleus, if no cytokinesis occurs) and an egg cell[30]. In all flowering plants[24], with the exception of *Amborella*, egg cells are always directly produced at the time of cellularization of the syncytial female gametophyte. One way to account for the four-celled egg apparatus in *Amborella* is that the mother cell that divides to give rise to the egg cell and third synergid is the homologue of the egg mother cell (central cell sensu gymnosperms) within the archegonium of a non-flowering seed plant. In this interpretation, the third synergid cell can be viewed as homologous to the ventral canal cell of a gymnosperm archegonium. Ultimately, if it can be shown that *Amborella* contains vestiges of the basic archegonium of non-flowering plants, this discovery will certainly rise to the standard of a 'missing link'.

It must be noted that should the phylogenetic placement of *Amborella* as a member of the most ancient lineage of angiosperms shift in future systematic analyses, interpretations of the evolutionary developmental significance of the Amborella-type female gametophyte will have to be re-evaluated. Nevertheless, there are now three distinct fully documented ontogenetic sequences present among clades whose origins date to the earliest phases of angiosperm history: the eight-celled, nine-nucleate sequence of *Amborella*, the four-celled, four-nucleate sequence characteristic of Nymphaeales and Austrobaileyales[27–29], and the typical Polygonum-type sequence characteristic of over 70% of extant angiosperms[24], including basal monocotyledons, eumagnoliids and eudicotyledons[29]. Given that only 5 yr ago *Amborella*, Nymphaeales and Austrobaileyales were widely (although not universally) believed to produce a standard Polygonum-type female gametophyte[26,29], these findings are a strong reminder that embryological features are far more diverse among ancient flowering plant lineages than had ever been anticipated. Finally, these data are also strongly congruent with other data sets[4–12]

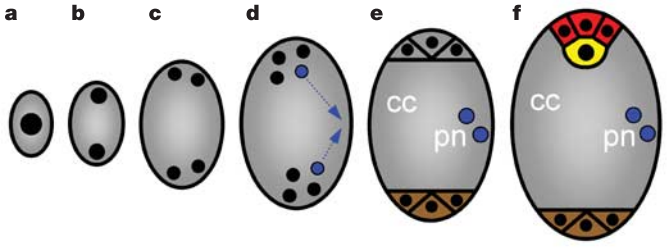

Figure 5 | Development of the female gametophyte of *Amborella*. **a**–**d**, A series of three free-nuclear mitoses yields an eight-nucleate syncytium (**d**). **e**, A brief phase of cellularization creates a structure with three antipodal cells (brown) at the chalazal pole, three cells at the micropylar pole and a binucleate central cell (cc) with two polar nuclei (pn) that resembles a mature *Polygonum*-type female gametophyte. **f**, A single cell division in one of the micropylar cells produces an egg cell (yellow) and a cell that differentiates into one of the three synergids (red) characteristic of the mature eight-celled, nine-nucleate female gametophyte. The micropylar pole is at the top.

that show that the earliest phases of flowering plant evolution were marked by a tremendous degree of structural lability and developmental experimentation.

METHODS

Plant collection. Reproductive material of *Amborella trichopoda* was collected from plants growing in the field in New Caledonia at Plateau de Dogny (GPS coordinates = 21° 37.253′ S, 165° 52.130′ E) and greenhouse populations at the University of Colorado. Supplemental pollinations were performed on a subset of flowers in the field and in greenhouses.

Brightfield and fluorescence microscopy. Carpellate flowers were chemically fixed for 24–48 h in either 3:1 95% ethanol:acetic acid or 4% glutaraldehyde in a solution of 50 mM PIPES buffer (also 5 mM EGTA and 1 mM $MgSO_4$, pH 6.8) or 0.02 M potassium phosphate buffer. Specimens were dehydrated through an ethanol series, infiltrated and embedded with glycol methacrylate, and serially sectioned into 5- or 3-μm-thick ribbons. Flowers fixed in 3:1 95% ethanol:acetic acid were stained with a solution of 0.25 μg ml^{-1} of 4′,6-diamidino-2-phenylindole (DAPI) and 0.1 mg ml^{-1} *p*-phenylenediamine in 0.05 M Tris (pH 7.2). Flowers fixed in glutaraldehyde were stained in 0.1% toluidine blue or periodic acid Schiff's reaction and DAPI. Fluorescence and brightfield digital micrographs were taken on a Zeiss Axiophot microscope equipped with a Zeiss Axiocam digital camera, and images were processed with Adobe Photoshop software. For visualization of DAPI, ultraviolet filter set (model no. 48702) with excitation filter (365 nm, band-pass 12 nm), dichroic mirror (FT395), and barrier filter (LP397) was used with a Zeiss Plan Neofluar 63× objective.

Transmission electron microscopy. Carpellate flowers were chemically fixed for 24–48 h in 4% glutaraldehyde in 0.02 M potassium phosphate buffer or a solution of 2% paraformaldehyde, 1% glutaraldehyde and 2% acrolein in 50 mM PIPES buffer (pH 6.8). Ovules were dissected out of individual carpels and post-fixed with 2% osmium tetroxide at 4 °C for 2 h, rinsed, dehydrated through a standard ethanol series and infiltrated with propylene oxide for 1.5 h. Tissues were then infiltrated with Spurr's resin and embedded. Female gametophytes were serially sectioned with a diamond knife at a thickness of 70 nm, mounted on 200 mesh copper grids, stained with lead citrate, and examined on a Phillips CM 10 transmission electron microscope.

Confocal microscopy. Prepared slides stained with DAPI and periodic acid Schiff's reagent were examined with a Leica TCS SP2 laser scanning confocal microscope and excited with a 405 nm blue diode. DAPI emission was detected within the 440–500 nm range, Schiff's reagent in the 570–630 nm range and autofluorescence in the 650–690 nm range. All images were captured with a Leica oil-immersion 63× objective.

Three-dimensional reconstructions. Tissues embedded in Spurr's resin were serially sectioned with a glass knife at a thickness of 0.5 μm, stained with toluidine blue and digitally photographed (see brightfield microscopy). Outlines of each cell of the egg apparatus, as well as the micropylar portion of the female gametophyte wall, were digitized and serially aligned in IMOD (Copyright, Boulder Laboratory for 3-D Electron Microscopy of Cells). IMOD programs were then used to reconstruct and display three-dimensional images of the egg apparatus.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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