The transition from somatic to germline identity shows conserved and specialised features during angiosperm evolution.

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Summary: 198

No. of figures: 8

Figs in color: 8

Total word count (excluding summary, references and legends) -

No. of figures: 198

No. of tables: 0

Introduction: No. of Supporting Information files: 6 (Fig. S1-S5; Table S1)

Materials and Methods:

Results:

Discussion:

Acknowledgements: 39

Summary
How and why specific plant cells adopt germline identity during ovule development has proved challenging to address, and the pathways that are active in the ovules of basal/early-divergent angiosperms possessing a multilayered nucellus are still unclear.

Here we compare megasporogenesis between two early-divergent angiosperms (Annona cherimola and Persea americana) and the evolutionary-derived Arabidopsis thaliana studying the three-dimensional spatial position of the Megaspore Mother Cell (MMC), the compositional details of the MMC wall, and the location of PIN1 expression.

Specific wall polymers distinguish the central position of the MMC, and its meiotic products from surrounding tissues in early-divergent angiosperms while in A. thaliana only callose (in mature MMC) and AGPs (in megaspores) distinguish the germline. However, PIN1 expression, which regulates polar auxin transport, is observed around the MMC in the single layer nucellus of A. thaliana and in the multilayer nucellus of A. cherimola, or close to the MMC in P. americana.

Data reveal a similar microenvironment in relation to auxin during megasporogenesis in all three species. However, the different wall polymers that mark MMC fate in early-divergent angiosperms may reflect a specific response to mechanical stress during differentiation, or the specific recruitment of polymers to sustain MMC growth.

**Key words:** Annona cherimola, Arabidopsis thaliana, auxin, megasporogenesis, pectin, Persea americana, ovule evolution.

Introduction
In contrast to animals, individual somatic cells in plants possess the remarkable ability to regenerate into entire new plants, depending on the signals they perceive. This developmental plasticity is critically important for normal plant growth and development, providing sessile plants with the capacity to adapt and respond to environmental and biotic stresses, while retaining the ability to reproduce and give rise to the next generation. This plasticity is particularly important during the establishment of the female germline; while in animals a germline is distinguished early in ontogeny, in plants the germline appears relatively late in development and derives directly from somatic cells. How and why plant cells adopt or change identity has been difficult to determine, particularly in the case of the sub-epidermal female germinal lineage. In flowering plants, the ovule which houses the female germinal cells is typically buried deep within the flower (Maheshwari, 1950; Reiser & Fischer, 1993). Within the ovule, several developmental transitions and complex changes in cell identity must occur over a short developmental window. These transitions are essential for sexual plant reproduction and include the differentiation of a reproductive precursor (Megaspore Mother Cell; MMC) from sporophytic (somatic) ovule cells, formation of the female gamete after meiosis and mitosis, fertilization, embryogenesis, and, finally, the formation of the persistent propagule, the seed.

In diploid plant species, the ovules comprise both diploid and haploid tissues. The diploid generation of the ovule, the sporophyte, usually consists of the proximal funiculus, the central chalaza and the distal nucellus surrounded by the integuments. The germinal lineage initiates in the nucellus with a somatic to germinal line transition (megasporogenesis). As has been reported in shoot and root meristems (Kidner et al., 2000; Brand et al., 2001; Costa, 2016), it is plausible to hypothesize that adjacent cells are initially identical in the nucellus, but positional information allows them to adopt specific fates in response to signals from surrounding cells (Tucker and Koltunow, 2014).

Despite the essential role of megasporogenesis in the reproductive success of flowering plants (Grossniklaus and Schneitz, 1998), little is known about the underlying molecular and genetic mechanisms, particularly in relation to specific microenvironments and positional signals, which determine germinal and somatic cell identity. Moreover, the influence of intercellular communication on this process has been studied mainly in the model plant and eudicot Arabidopsis thaliana, in which only a limited number of germline-establishment mutants have been reported (Bencivenga et al., 2011; Chevalier et al., 2011; Lieber et al., 2011; Tucker & Koltunow, 2014). Known signalling molecules have been localised in the Arabidopsis ovule. For example, both auxin and Pin-formed 1 (PIN1), which regulates polar auxin transport, were observed in the unicellular layer of the nucellar epidermis, adjoining the region where female gametophyte specification occurs (Pagnussat et al., 2009, Benková et al., 2003). Cytokinin was also observed during ovule development in the basal part of the nucellus close to
the MMC (Bartrina et al., 2011; Bencivenga et al., 2012; Zurcher et al., 2013). Thus, in Arabidopsis, megasporogenesis takes place in the presence of auxin and cytokinin, although their exact role during megasporogenesis has not been fully elucidated.

Most of the studies on genetic regulation involved in ovule development have been performed in Arabidopsis and comparative studies in other species are needed to elucidate the evolutionary steps behind ovule development (Mathews & Kramer, 2012). From an evolutionary perspective, the main difference in megasporogenesis among flowering plants is found in the cells surrounding the reproductive precursors. Many evolutionary derived angiosperms of the core eudicots, including the COM clade (Celastrales, Oxalidales, Malpighiales), some Brassicales such as Arabidopsis (Robinson-Beers et al., 1992; Schneitz et al., 1995; Bajon et al., 1999) and the Asterids (Endress, 2011a), show tenuinucellate ovules with a unicellular layer surrounding the MMC. However, basal and early-divergent angiosperms (Lora et al., 2010), monocots (Rudall, 1997), basal grade eudicots and some orders that belong to the rosid clade generally show crassinucellate ovules with several cell layers surrounding the MMC (Sporne, 1969; Endress, 2011b). The archesporial cell of tenuinucellate ovules functions directly as the megaspore. Conversely, in crassinucellate ovules the archesporial cell divides periclinaly at least once and produces an outer parietal cell that contributes to the nucellus and an inner one that becomes the MMC (Bhojwani & Soh, 2001). It remains unclear whether the additional nucellar layers in basal/early angiosperms act collectively as a signal source, similar to the unicellular nucellus in derived angiosperms, or provide independent cues to stimulate and sustain reproductive development.

In basal and early-divergent angiosperms, megasporogenesis has been mainly evaluated using two-dimensional (2D) morphological approaches (Igersheim & Endress, 1997; Yamada et al., 2001a,b; Friedman et al., 2003; Tobe et al., 2007; Zhou & Fu, 2008; Endress, 2011a; Povilus et al., 2015), although indeed megasporogenesis occurs in a three-dimensional (3D) space. Information about the spatial arrangement of cells during megasporogenesis, the microenvironment surrounding the reproductive cells and the positional signals involved in megasporogenesis is currently lacking for basal/early-divergent angiosperms, but would contribute significantly to our understanding of the evolution of germline initiation in angiosperms. In order to fill this gap, we chose Annona cherimola (cherimoya) (Lora et al., 2009, 2010, 2011a,b), and Persea americana (avocado) (Chanderbali et al., 2008, 2009), as representative early-divergent angiosperms. A. cherimola belongs to the Annonaceae, a family included within the order Magnoliales, and P. americana belongs to the Lauraceae within the order Laurales. Both orders are included in the early-divergent clade Magnoliid (APGIV, 2016), and show crassinucellate ovules (Schroeder, 1952; Lora et al., 2010), and both species are cultivated in regions with subtropical climates.
In this study, we have utilised a combination of morphometric analysis, immunohistochemistry and molecular analysis to compare the details of megasporogenesis between two early-divergent angiosperms (A. cherimola and P. americana) and an evolutionary-derived angiosperm (A. thaliana), paying special attention to the first steps that commit a cell to initiate a female germline. We reveal both 2D and 3D details of ovule development in A. cherimola and A. thaliana, and show that spatial position is conserved during the transition from somatic to germinal cell identity. The accumulation of PIN1 transcripts in the nucellus adjoining the reproductive precursors is similar in the three species studied. However, subtle differences between the two early-divergent angiosperms analysed suggest that the multi-layered nucellus may act as a collective signal source in some instances, while providing layer-specific information in others. Finally, considerable differences were identified in MMC wall composition. This suggests that different polymers may have been recruited to support differentiation of the MMC in basal and early-divergent angiosperms compared to evolutionarily derived species.

Material and methods

Plant material

Adult trees of A. cherimola and P. americana, located in a cultivar field collection at the IHSM La Mayora-CSIC-UMA, Málaga, Spain and seedlings of the A. thaliana ecotype Colombia-0, were used in these experiments. The relationship between flower bud growth and developmental stages of A. cherimola has been previously established (Lora et al., 2009). A. thaliana plants were grown at a constant temperature of 22°C in a 16 hr light/8 hr dark cycle.

Histology

For the study of 2D ovule development of A. cherimola and A. thaliana, ovules were collected from flower buds covering a range of developmental stages. The ovules were fixed in 4% paraformaldehyde in phosphate buffer saline (PBS) at pH 7.3, dehydrated in an ethanol series, embedded in Technovit 7100 (Kulzer & Co, Wehrheim, Germany), and sectioned at 2 μm. Sections were stained with periodic acid-Shiff’s reagent (PAS) for insoluble carbohydrates, and with PAS and toluidine blue (Sigma-Aldrich, St. Louis, MO, USA) for general histological observations (Feder & O’Brien, 1968). PAS was prepared using pararosaniline base (Sigma-Aldrich), periodic acid (Sigma-Aldrich), charcoal activated pure (Merck, Darmstadt, Germany), CIH 1N and K₂S₂Os. Callose deposition was documented using squashed preparations of pistils of A. cherimola, P. americana and A. thaliana fixed in methanol/acetic acid fixative [50% (v/v)]
methanol, 10% (v/v) acetic acid]. Pistils were washed and squashed preparations were stained with 0.1% aniline blue (Panreac, Barcelona, Spain) in PO₄K₃ (modified from Currier, 1957; Linskens, 1957) and observed with a 340–380 excitation filter and an LP425 barrier filter.

3D reconstruction

For the 3D study of ovule development of A. thaliana and A. cherimola, ovules covering the earliest developmental stages were fixed and stored in methanol/acetic acid fixative [50% (v/v) methanol, 10% (v/v) acetic acid] for up to two months. For staining and clearing, the protocol of Enugutti & Schnitz (2014) was followed with slight changes. The ovules were treated with 1% SDS and 0.2N NaOH solution at room temperature overnight. In the case of A. cherimola, the ovules were additionally treated with 0.01% amylase (Sigma-Aldrich) in PBS and incubated overnight at room temperature (Wuyts et al., 2010). Then, after three washes in H₂O, the ovules were incubated for 2 min in bleach solution (2.5% active Cl⁻ ions), washed in H₂O, and incubated for 60 min in periodic acid (Sigma-Aldrich). After three additional washes in H₂O the ovules were incubated for 60 min with Pseudo-Schiff propidium iodide (PI) containing PI (100 µg/mL, Sigma-Aldrich). The stained ovules were mounted in a drop of chloral hydrate (Sigma-Aldrich) and, after 24 h, the ovules were observed by a Leica TCS SP5 confocal microscope with excitation at 561 nm and detection at 600-700 nm and z-stack images taken. The segmentation and 3D analysis was determined using the open software Lithograph (Barbier de Reuille et al., 2015).

Immunocytochemistry

For immunocytochemistry, ovules from flower buds of A. cherimola and P. americana covering a range of developmental stages were fixed in 4% paraformaldehyde in PBS at pH 7.3, left overnight at 4°C, dehydrated in an acetone series, embedded in Technovit 8100 (Kulzer & Co, Wehrheim, Germany), polymerized at 4°C and sectioned at 2 µm. Sections were placed in a drop of water on a slide covered with 2% 3-aminopropyltrietoxy-silane (Sigma-Aldrich) and dried at room temperature (Satpute et al., 2005; Solís et al., 2008). Different antibodies were used to localize specific cell components; JIM5 and JIM7 rat monoclonal antibodies (Carbosource Service, University of Georgia, USA), which recognize unesterified and methyl-esterified pectins respectively (Knox, 1997), JIM8 (Pennell et al., 1991) and JIM13 (Knox et al., 1991) for AGPs (Carbosource Service, University of Georgia, USA), JIM11 (Carbosource Service, University of Georgia, USA) for extensins (Smallwood et al., 1994) and an anti-callose mouse monoclonal antibody (Biosupplies, Parkville, Australia) for callose. Following the protocol of Lora et al., (2009), sections were incubated in PBS for 5 minutes and later with 5%
bovine serum albumin (BSA) in PBS for 5 minutes. Then, different sections were incubated for one hour with the primary undiluted antibodies JIM5, JIM7, JIM8, JIM11 and JIM13 and diluted anti-callose 1/20 in PBS. After three washes in PBS, the sections were incubated for 45 minutes in the dark with the corresponding secondary antibodies (anti-rat, for JIM5, JIM7, JIM8, JIM11 and JIM13 and anti-mouse, for anti-callose) conjugated with Alexa 488 fluorochrome (Molecular Probes, Eugene, Oregon, USA) and diluted 1/25 in PBS. After three washes in PBS, the sections were stained with DAPI and washed three times in PBS. The sections were mounted in ProLong® Gold Antifade Reagent (Invitrogen, Carlsbad, CA, USA), examined with a Leica DM LB2 epifluorescence microscope connected to a Leica DFC310 FX camera (Wetzlar, Germany). Filters were 470/525 nm for the Alexa488 fluorescein label of the antibodies and with a 340–380 excitation filter and an LP425 barrier filter for DAPI. Overlapping photographs were obtained with the Leica Acquisition Station AF6000 E.

Nucleic Acid Methods

A PIN1 ortholog from A. cherimola was isolated using primers described in Table S1 and based on the published sequences of A. cherimola (Gupta et al., 2015). Since we could not find an ortholog sequence of PIN1 of Persea americana in public databases, a PIN1 ortholog was isolated using the PIN1 sequence of Persea borbonia (Bennett et al., 2014) (Table S1). Total RNA was extracted from early developmental stages of A. cherimola and P. americana flower buds using the Qiagen RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), and cDNA was synthesized using AccuPower RT/PCR PreMix (Bioneer, Daejeon, Republic of Korea). Polymerase chain reactions were performed by standard methods using BioTaq (Bioline, London, UK) and Phusion (Thermo Scientific, St Leon-Rot, Germany).

Sequence analysis

Sequences of representative PIN proteins were obtained from GenBank (http://www.ncbi.nlm.nih.gov/BLAST, version 2.2.10) and were aligned using Clustal X version 1.82 (Thompson et al., 1997). Alignments were edited to eliminate less conserved regions using GBLOCK version 0.91b (Castesana, 2000; Talavera & Castresana, 2007) following modifications in the parameters for a relaxed selection of blocks with 5 minimum length of blocks, half allowed gap positions, half number of studied species for minimum number of sequence for a flank position and 10 maximum number of contiguous non-conserved positions. Bayesian analysis was performed using MrBayes (Ronquist et al., 2012) that uses a Markov Chain Monte Carlo approach to search for trees. The trees were sampled every 100 generations for 4,000,000 generations, and the first 25% of the trees of each run were discarded as burn-in. Markov chains were converged, as indicated by average standard deviation of split
frequencies < 0.01 after 100,000 generations. Bayesian analysis was performed using the LG + Invariant (I) + Gamma (G) model of amino acid substitutions as recommended by the Prottest 2.5 server (Abascal et al., 2005).

**In situ hybridization**

Tissue preparation and *in situ* hybridization were performed as described previously (Mayer et al., 1998), with the following modifications. For antisense PIN1, the *A. cherimola* probe pGEMEasy-PIN1 *A. cherimola* (JLA3) was linearized with NdeI and transcribed using T7 RNA polymerase (Table S1). For antisense PIN1.1 the *P. americana* probe, pGEMEasy-PIN1.1 *P. americana* (JLP6) was linearized with NcoI and transcribed using SP6 RNA polymerase (Table S1). For antisense PIN1.2 the *P. americana* probe, pJET1.2 PIN1.2 *P. americana* (JLP3) was linearized with NcoI and transcribed using T7 RNA polymerase (Table S1). For antisense PIN11.1 and for antisense PIN11.2 the *P. americana* probes, pGEMEasy-PIN11.1 *P. americana* (JLP8) and pGEMEasy-PIN11.2 *P. americana* (JLP7) respectively, were linearized with NdeI and transcribed using T7 RNA polymerase (Table S1). The slides of *in situ* hybridization were mounted with distilled water and photographed under differential interface contrast using a Leica DM LB2 microscope. For stained tissue sections, paraffin-embedded material was sectioned at 9 μm.

**Results**

**Ovule anatomy**

Ovule development in *A. thaliana* has been studied in detail (Robinson-Beers et al., 1992; Schneitz et al., 1995; Bajon et al., 1999). Female germline development begins with the formation of a single archesporial cell / megaspore mother cell (MMC) in the distal tip of the ovule, which is surrounded by a single epidermal nucellus layer (Fig. S1). After megasporogenesis, the single epidermal layer is consumed by adjoining cells, possibly to support development of the functional megaspore and dividing embryo sac (Fig. S1).

Anatomical details of ovule development have been reported in some species of the Annonaceae, including *Annona muricata* (Igersheim & Endress, 1997), but information on megasporogenesis is still lacking. Thus, we evaluated ovule development in *A. cherimola* using semi-thin resin sections stained with PAS and toluidine blue (Fig. 1). Flower buds of *A. cherimola* develop in the leaf axis after leaf unfolding; the basal nodes are differentiated almost one year prior to flowering and the distal buds differentiate in synchronization with the shoot (Higuchi & Utsunomiya, 1999). Flower bud growth started 39 days before flowering and
carpels developed in a centripetal way and started bending 27 days before flowering. Finally, the mature pistils of A. cherimola showed partially postgenital fusion at the base of the ovary forming a conic shape surrounded at its base by several rows of anthers (Fig. S2). Histological sections revealed dense PAS positive cytoplasm at the base of the pistil 20 days before flowering, where several days later the first placental protrusion was observed (Fig. 1a,b). Early MMC cells were observed under two layers of nucellar cells and coincided with initial inner integument development (Fig. 1c). MMC, and initial outer integument development were observed in the buds 14 days before flowering (Fig. 1d). Carpel differentiation proceeded centripetally and, consequently, developmental differences were observed at this time depending on the position of the carpels in the flower bud. Thus, the most developmentally advanced carpels are those placed in the outermost rows showing the first meiotic division of the MMC (Fig. 1e), and the outer integument starting to partially wrap around the inner integument. The four megaspores generated by meiosis were first observed 10 days before flowering (Fig. 1f). Of these four megaspores, the chalazal megaspore became the functional megaspore and increased in size and transiently accumulated starch (Fig. 1g,h). Successive mitotic divisions subsequently resulted in the development of a Polygonum-type embryo sac with eight nuclei and seven cells organized in three cells forming the egg apparatus close to the micropyle, two polar nuclei and three antipodal cells. Starch grains were again apparent, mainly around the two polar nuclei and the egg cell (Lora et al., 2010).

Three-dimensional (3D) analysis of ovules during MMC development

Three-dimensional details of ovule development are often difficult to assess via semi-thin sections, but are required to obtain reliable information on the spatial arrangement of the reproductive cells in the nucellus. To evaluate the role of cell position during the transition of somatic to germinal fate, we performed high-resolution confocal imaging of megasporogenesis in A. thaliana and A. cherimola. The images were processed to obtain information relating to cellular arrangement in 3D and to evaluate cell volume.

The transition from somatic to germinal fate in A. thaliana occurred at stage 1 to stage 2I according to Schneitz et al. (1995). During the earliest stages, the cell volume of somatic nucellar cells was around 100 µm³. Initial archespori-like cells with a volume of 180-220 µm³ were apparent under the most distal nucellar epidermal cells (Fig. 2a). During these early stages, several cells reached this volume, including somatic cells in a lateral position relative to the central archesporial cell. The central archesporial cell increased in volume and became the MMC that was surrounded by a single layer of nucellar cells, concomitant with the increase in the number of somatic nucellar cells (Fig. 2a-e). Interestingly, while the germinal cells showed a volume of 200-400 µm³, the volume of the somatic cells that surrounded the germinal cells was
more variable ranging from 11 to 310 μm³. The volume of the MMC reached 1500-2000 μm³ before the first meiotic division (Fig. 2a, f-i).

In *A. cherimola*, the cell volume of individual somatic cells was similar during the initiation of ovule protrusion from the placental tissue, reaching a value of 130-400 μm³ (Fig 3). When an initial inner integument was observed, the MMC reached a large cell volume of around 900 μm³ and was always positioned underlying 2-3 cell layers of the most distal nucellar cells. The MMC achieved a volume of 1500 μm³ before it was encased by the integuments, which did not allow further high-resolution confocal imaging. The position of the MMC in the nucellus was generally central (n=10) (Fig. 3d).

The 3D confocal imaging showed a stable position of the MMC in a central sub-epidermal region of the nucellus in both *A. thaliana* and *A. cherimola*. While the MMC dramatically increases in volume, it remains surrounded by relatively small nucellar cells. This emphasises the importance of cellular position and, consequently, the remarkable distinction between neighbouring cells in the nucellus during megasporogenesis.

Specific labelling of the MMC

To investigate whether the increase in cell volume of the MMC is accompanied by reinforcement of the cell wall, as occurs in other cells during development (Palin & Geitmann, 2012), pectins and AGPs were studied by immunocytochemical assays in *A. cherimola*, *P. americana* and *A. thaliana*.

Immunocytochemical assays revealed the location of unesterified (JIM5) and methyl-esterified (JIM7) pectins, AGPs (JIM8 and JIM13) and extensins (JIM11). We first evaluated the cell wall components of *A. cherimola* ovule at the earliest stage, before the appearance of a MMC (Fig. 4). Unesterified pectins (JIM5) were barely detectable in the somatic cells of the nucellus prior to the appearance of the germinal cells (Fig. 4a). Conversely, methyl-esterified pectins clearly reacted to JIM7 in the nucellus at the same stage (Fig. 4b) but both types of pectins were observed in the placenta. AGPs revealed by JIM8 were also observed in the placenta and faintly in the nucellus (Fig. 4c), but AGPs did not react to JIM13 (Fig. 4d). Extensins revealed by JIM11 were evenly distributed in all cells of the nucellus at early developmental stages (Fig. 4e). Thus, in the early stages of ovule development of *A. cherimola*, cell walls within the nucellus are rich in esterified pectins and extensins, but lack unesterified pectins and AGPs.

When the MMC was apparent (Fig. 5), JIM5 staining revealed an abundance of unesterified pectins in the MMC wall and reduced levels in the surrounding somatic cells of the nucellus of *A. cherimola* (Fig. 5a). Methyl-esterified pectins (JIM7) were abundant in both the somatic cells of the nucellus and the MMC (Fig. 5b). The pattern observed for JIM8 was similar
to that observed for JIM5, indicating that arabinogalactans were mainly observed in the
germin al cells of *A. cherimola* (Fig. 5c). Arabinogalactans reacting to JIM13 were faintly
observed in the MMC of *A. cherimola* (Fig. 5d), while extensins were more pronounced in the
cell wall of the MMC compared to surrounding nucellar cells (Fig. 5e). Similar patterns were
also observed for *P. americana* (Fig. 5f-j). In contrast to *A. cherimola* and *P. americana*; the
young MMC of *A. thaliana* was not specifically marked by pectins, AGPs, or extensins.
Unesterified and methyl-esterified pectins and extensins were equally observed in the nucellus
and MMC, but the MMC and nucellus did not react to AGPs revealed by JIM8 or JIM13 (Fig.
5k-n).

Pectins, AGPs and extensins were also evaluated during meiosis in *A. cherimola*, *P.
americana* and *A. thaliana* (Fig. 6). In *A. cherimola* (Fig. 6a), most cell walls in the nucellus
appeared to lack unesterified pectins, while signal was abundant in the meiotic cells. Methyl-
esterified pectins were detected weakly in the somatic cells of the nucellus although a more
intense signal was observed in meiotic cells (Fig. 6b). Arabinogalactans that reacted to JIM8
and JIM13 were predominantly observed in cells of the meiotic tetrad and, interestingly, a
stronger signal was observed in the functional megaspore of *A. cherimola* (Fig. 6c,d). Extensins
were detected at relatively low levels in the somatic cells, but a stronger signal was again
observed in meiotic cells of *A. cherimola* (Fig. 6e). The meiosis of *P. americana* was also
similarly marked by pectins, AGPs and extensins (Fig. 6f-j). In *A. thaliana*, the nucellus and the
MMC were equally marked by unesterified and methyl-esterified pectins and extensins but, as
previously shown by Coimbra et al., (2007) the cell wall of the functional megaspore reacted for
AGPs (Fig. 6k-n).

Since callose deposits are also found around the MMC in angiosperms and may act as a
physical filter of signalling molecules (Rodkiewicz, 1970; Tucker & Koltunow, 2014), callose
deposition was studied using aniline blue, a fluorochrome that reveals callose deposition, and an
anti-callose antibody. Callose was not observed in the MMC and was only observed in the cell
wall of the megaspores of *A. cherimola* (Fig. 7a) and *P. americana* (Fig. 7b) during meiosis. In
*A. thaliana*, while we could not detect callose in early stages of MMC development, we
confirmed punctate callose deposition around the mature MMC (Fig. 7d,e) and in the cell wall
of the megaspores during meiosis (Fig. 7c, Tucker and Koltunow, 2014).

**PIN-FORMED 1 (PIN1)** expression around the MMC is conserved among
species, but shows differences in the number of cell layers with expression
In *A. thaliana*, PIN1 accumulates in the nucellus and regulates polar auxin transport (Fig. 8a),
leading to an auxin maximum at the tip of the ovule that coincides with MMC formation and
megasporogenesis (Benková et al., 2003; Pagnussat et al., 2009; Ceccato et al., 2013). Studies
suggest that this is critical for the downstream events of female gametophyte development (Ceccato et al., 2013). To determine whether accumulation patterns of PIN1 are similar during ovule development in basal and derived angiosperms that display different nucellar morphologies, PIN1 orthologs were isolated from A. cherimola and P. americana and analysed by in situ hybridisation.

To identify PIN1 sequence from A. cherimola, we examined a recent transcriptome study from the closely related A. squamosa (Gupta et al., 2015). This led to the identification of a single PIN1-like sequence from A. cherimola cDNA. Similarly, two PIN-like sequences (AIF28294 and KJ664280) were identified from P. borbonia (Bennett et al., 2014) with high similarity to PIN1 of A. thaliana. Moreover, we also found two PIN11 sequences [also called SISTER OF PIN1, SoPIN1 (O’Connor et al., 2014)] from P. borbonia (Bennett et al., 2014) that are closely related to PIN1 (Bennett, 2015). Phylogenetic analysis of these genes together with orthologs from other angiosperms showed that the PIN1 orthologs of A. cherimola and P. americana group in a clade exclusively containing PIN1 orthologs. A subclade of this main clade also contains the PIN11 ortholog of P. americana and PIN11 orthologs from other species considered in the phylogenetic study performed by O’Connor et al., (2014) (Fig S3). Thus, we named the AIF28293, AIF28294, AIF28316 and AIF28315 sequences of P. borbonia as PIN1.1, PIN1.2, PIN11.1 and PIN11.2, respectively. To determine PIN1 expression pattern, in situ hybridization was undertaken using probes for cDNA of A. cherimola PIN1 and P. americana PIN1.1, PIN1.2, PIN11.1 and PIN11.2.

PIN1 transcripts in A. cherimola were first observed distally in the protrusion of the ovule before the appearance of the archesporial cell (Figs 8b, S4). In subsequent stages, PIN1 transcripts were detected in all cell layers of the nucellus around the MMC and during meiosis (Figs 8c,d, S4). PIN1 expression was also observed in the vascular tissue concomitant with the appearance of the germline cell (Figs 8c,d, S4). Remarkably, in P. americana, PIN1 transcripts using PIN1.1 cDNA probe were also observed around the MMC but did not extend to all the cell layers of the nucellus (Figs 8e-g, S4). PIN1.1 expression was also observed in the vascular tissue and the chalaza. PIN1 expression using the PIN1.2 cDNA probe was similar to the expression of PIN1.1 but, interestingly, was not observed in the nucellus (Figs S4, S5). The expression of PIN11.1 was observed in all cell layers of the nucellus around the MMC with a stronger signal in the distal layers of the nucellus (Figs 8h,i, S4), but PIN11.2 expression was restricted to the vascular tissue (Figs S4, S5). PIN11.1 expression was also observed in the vascular tissue.

Discussion
In this study, two and three-dimensional analyses of ovule development confirmed the typical cell division patterns of cells within the tenuinucellate ovule of *Arabidopsis*; the archesporial cell functions directly as the MMC and resides in a central, subepidermal position. The MMC was also observed in a central position in the crassinucellate ovules of *A. cherimola* and *P. americana* in which the archesporial cell underwent a periclinal division to form an outer parietal cell and an inner cell that becomes the MMC. The results obtained also show that compositional details of the cell wall of the female germline differ between the eudicot *Arabidopsis* and two early-divergent angiosperms, mostly during the transition of the somatic to germinal cell lineages. However, despite these differences, the female germline becomes isolated and differentiates from the sporophyte similarly in all cases, showing analogous PIN1 expression around the MMC.

**Initiation and commitment of the germinal cell lineage**

The transition from somatic to germline fate is defined by a continuous interaction among a small population of ovule cells. In many ways, the ovule primordia show striking similarities to the shoot meristem, where centrally positioned cells adopt a specific identity based on positional information from adjoining cells. In the shoot meristem, studies have shown that mechanical signals are important for cell identity determination (Hamant *et al.*, 2008; Sampathkumar *et al.*, 2014; Landrein *et al.*, 2015). In this study, we examined ovule development in *A. thaliana* and in an early-divergent angiosperm (*A. cherimola*) to assess the position of the MMC in 3D and determine the volume of adjoining ovule cells during differentiation. Reproductive differentiation in both *A. thaliana* and *A. cherimola* ovules is characterised by rapid expansion of a central nucellar cell, which becomes the MMC. Interestingly, the volume of somatic nucellar cells in *A. thaliana* is more variable at the early stages of megasporogenesis. As the MMC of *A. thaliana* differentiates and expands, lateral cells close to the MMC are usually larger than other nucellar cells. This could reflect an early patterning of sub-epidermal ovule cells that later stabilises concomitant with the increase of volume of the MMC. In this sense, it will be of interest to identify mutants with anomalies in cell division or/and cell volume in the ovule that might disrupt this transition.

Pectin is one of the main components of young cell walls (McCann & Roberts, 1994) and has been recently suggested as a primary morphogenic trigger (Bidhendi & Geitmann, 2016). De-methylesterification of pectins is a key process during primordia differentiation of the shoot apical meristem in *Arabidopsis* (Peaucelle *et al.*, 2008) increasing elasticity (Peaucelle *et al.*, 2011); interestingly, cell wall rigidity is also reduced by auxin (Braybrook *et al.*, 2013). Using immunocytochemical assays, cell wall composition was investigated in the ovules of two early-divergent and one evolutionary-derived angiosperm species, revealing changes in
apparently identical somatic cells during the transition from somatic to germinal fate. During ovule initiation, unesterified pectins and AGPs (JIM8) were more evident in the placenta than in the ovule primordia of the young nucellus of *A. cherimola*. Subsequently, during MMC differentiation, the MMC wall of *A. cherimola* and *P. americana* was distinguished by clear labelling of unesterified and methyl-esterified pectins, which persisted in the germinal cells during meiosis. Similar results were also observed during megasporogenesis in the gymnosperm *Larix decidua* (Rafińska & Bednarska, 2011) and during microsporogenesis in different *Annona* species (Lora et al., 2009, 2014) and *Quercus suber* (Costa et al., 2015). In contrast, *Arabidopsis* ovules showed a similar abundance of unesterified and methylesterified pectins in the walls of the nucellus and germinal cells during MMC initiation and subsequent meiosis.

Differences between the two early-divergent angiosperms studied and *Arabidopsis* during megasporogenesis were also found in another cell wall component, arabinogalactan proteins, which are glycosylated proteins that have been identified as important signalling molecules during ovule development (Acosta-García & Vielle-Calzada, 2004; Tucker et al., 2012; Demesa-Arévalo & Vielle-Calzada, 2013; Tucker & Koltunow, 2014). The results herein show that the MMC wall of *A. cherimola* and *P. americana* contains AGPs which bind strongly to JIM8 and weakly to JIM13. Moreover, meiotic cells of both species and, more specifically, the cell wall of the functional megaspores reacted to JIM8 and JIM13. Additionally, extensins, a group of hydroxyproline-rich glycoproteins usually related to cell elongation (Lamport, 1967) that react to JIM11, were also specifically detected in the cell wall of the MMC and the functional megaspore. A previous study performed in the gymnosperm *Larix decidua* also showed AGPs recognized by JIM4 labelling the MMC, but labelling is lost prior to meiosis (Rafińska & Bednarska, 2011). Interestingly, while the functional megaspore in *Arabidopsis* is specifically marked by AGPs (JIM8 and JIM13) (Coimbra et al., 2007), the only polymer identified to date that shows specific labelling in the mature MMC is callose (Tucker & Koltunow, 2014, our results).

Our results from the immunolocalization studies provide compelling evidence to suggest that pectins and AGPs mark germline cell types during megasporogenesis in basal/early-divergent angiosperms, but this feature is lacking in *Arabidopsis*. The mechanical consequences of these differences are currently unknown, but may influence the interactions between the MMC and surrounding cells. Indeed, differences are seen in the size of cells surrounding the MMC in the divergent species, since the nucellar cells adjoining the MMC in *Arabidopsis* are relatively large. Despite this, the female germline cells increase in size in a similar developmental pattern and the MMC resides in a generally similar central position. The conserved central position may be due to signals converging at the tip of the ovule, of which auxin is an excellent candidate (Durbak et al., 2012).
Hormonal changes during megasporogenesis

While members of the PIN family (Viaene et al., 2013) that are involved in cell to cell active transport of auxin (Adamowski & Friml, 2015) are found early and throughout land plant evolution (Chapman & Estelle, 2009), most studies investigating their role have been performed in Arabidopsis. Auxin distribution has been observed in the single cell layer of the Arabidopsis nucellar epidermis during megasporogenesis (Benková et al., 2003; Ceccato et al., 2013), and influences female gamete specification during megagametogenesis (Friedman, 2009; Pagnussat et al., 2009). We show that, although several cell layers surround the A. cherimola and P. americana MMC compared to the single cell layer of nucellus in Arabidopsis, PIN1 expression around the MMC is conserved during megasporogenesis. PIN1 expression has been also reported in the monocot maize, in which a faint signal of ZmPIN1 expression was observed in the nucellus surrounding the MMC (Forestan et al., 2012) and clearly confirmed in all cell layers of the nucellus at the micropylar pole by the auxin synthetic responsive promoter DR5::mRFP:ER and ZmPIN1a::PIN1aYFP (Lituiev et al., 2013). Interestingly, PIN1 expression was observed in all cell layers surrounding the MMC of A. cherimola and restricted to the nearest cell layers around the MMC of P. americana a similar observation to that found in Arabidopsis.

PIN11.1 expression, which is closely related to PIN1 (Bennett, 2015), was similarly observed around the MMC of P. americana although with a strong distal signal and it was also observed in the nucellus cells closest to the chalaza. We also observed a duplication of PIN1 (PIN1.2) and PIN11 (PIN11.2) in P. americana that overlap with the expression domain of PIN1 in the vascular tissue but not in the nucellus. Thus, PIN1 and PIN11 could share a similar function in the vascular tissue and a complementary function in the layers of the nucellus of P. americana. This could represent a case of gene duplication and subsequent sub-functionalization, with the combined expression in the nucellus contributing similar function to PIN1 alone in A. cherimola. Gene duplication and diversifcation has been a source for the evolution of new morphologies (Irish & Litt, 2005) with many examples in flowering plants (Kramer & Hall, 2005). For example, in the ovule, the ETTIN/AUXIN RESPONSE FACTOR3 (ETT/ARF3) gene is involved in integument differentiation (Lora et al., 2015) and can be considered an innovation in angiosperms generated by duplication of the gymnosperm ETT/ARF4 like gene before angiosperm radiation (Finet et al., 2010, 2013). Duplication and loss of PIN genes have also been reported in angiosperms; one example is the loss of the PIN11 clade in Brassicaceae (O’Connor et al., 2014) in which some species such as Arabidopsis show tenuinucellate ovules (Endress, 2011a).
Isolation of the female germline during megasporogenesis

During meiosis, the four megaspores of *A. cherimola* form a linear tetrad and the functional megaspore continues megasporogenesis following a monosporic pattern. We observed that the functional megaspore was marked by starch grains that were also observed in the MMC before meiosis. Accumulation of starch grains that vanish concomitantly with the beginning of cell division, such as meiosis or mitosis, is commonly found during sporogenesis and gametogenesis. It has also been observed during microsporogenesis of *Annona* species (Lora et al., 2009, 2014). The functional megaspore increases in size and organelles are usually polarized in the chalazal cytoplasm in both gymnosperms (Fiordi & Maugini, 1977; Pennell & Bell, 1987; Fiordi et al., 1988) and angiosperms (Medina et al., 1981). At this stage, many studies have reported callose deposition around the MMC and in the cell wall of the four megaspores during meiosis (Rodkiewicz, 1970; Kuran, 1972; Meral Ünal, 2013; Tucker et al., 2001; Musial et al., 2015), although callose has not been observed in angiosperms with tetrasporic embryo sacs that do not show degenerating megaspores (Rodkiewicz, 1970).

Most of these studies have been generally performed in eudicots and some monocots, but callose deposition during megasporogenesis has not been evaluated in basal/early-divergent angiosperms. In our study, callose deposition was observed during meiosis of both *A. cherimola* and *P. americana*. Callose has been rarely reported in gymnosperms (Rodkiewicz, 1970; Rafińska & Bednarska, 2011) but thickening of the megaspore cell wall has been commonly observed (Pettitt, 1977; Fiordi & Maugini, 1977; Fiordi et al., 1988, 1991). After meiosis, in monosporic embryo sac species, callose accumulates in the dying megaspores and degrades around the functional megaspore that continues megagametogenesis (Van Hautegem et al., 2015). Callose deposition could potentially act as an apoplastic and symplastic filter of signalling molecules, blocking the exchanges of molecules between cells, mostly to the MMC before and during meiosis. In fact, several studies have reported the influence of callose on cell-cell communication via plasmodesmata (Vatén et al., 2011; Benitez-Alfonso et al., 2013; Han et al., 2014), although callose deposition may not be sufficient to completely block signaling, since the *callose, somatic, and microspore defect 1 (csmd1)* mutant of maize shows excess of callose deposition during meiosis, but meiosis continues (Wang et al., 2011). In some cases, such as the gymnosperm *Taxus baccata*, the megasporocyte plasmodesmata remain unchanged during meiosis and, interestingly, more than one, even up to four megaspores, can survive (Fiordi et al., 1991). Taken together, our results and the reported data from other angiosperm and gymnosperm species suggest that the isolation of the female germline from the sporophyte is morphologically similar between species. However, different aspects of germline specification, such as chemical composition of the cell wall, cell-cell communication with adjacent cells and longer distance hormonal signalling, appear to have evolved independently.
Conclusion

Although there are still only few known genes involved in the transition from somatic to germinal fate, and most have been described in Arabidopsis (Schiefthaler et al., 1999; Yang et al., 1999; Lieber et al., 2011) and the monocots rice (Nonomura, 2003) and maize (Sheridan et al., 1996, 1999), genetic studies suggest an essential role of cross-talk between germline cells and the neighbouring somatic cells in different plant species. Changes in cell wall composition during megasporogenesis are likely to fulfil an underexplored role in cross-talk between different ovule cells, and this study shows differences in cell wall composition between early-divergent angiosperms and Arabidopsis.

Despite the differences in cell wall composition, both the central position of the female germ line and the distribution of a possible positional signal such as auxin are conserved. This first study of auxin distribution in the developing ovule of basal/early-divergent angiosperms shows a similar PIN1 expression profile compared to the eudicot Arabidopsis, although PIN1 is observed in all layers in A. cherimola surrounding the MMC but only in the layers close to the MMC in P. americana. A positional signal together with the mechanical stress of neighbouring cells could determine germinal and somatic identity. It remains to be seen whether the differences in the cell wall composition and similarities in auxin distribution during megasporogenesis observed in this study, between early-divergent angiosperms and the eudicot Arabidopsis, represent a more widespread situation in flowering plants.

Acknowledgements

This work was supported by Ministerio de Economía y Competitividad – European Regional Development Fund, European Union (AGL2012-40239, AGL2013-43732-R, AGL2015-74071-JIN, AGL2016-77267-R and Recupera2020) INIA (RF2012-00010 and RFP2012-00016), and Gob. Aragón A-43. MRT is supported by an Australian Research Council Future Fellowship.

Author contributions

J.L., M.H., M.R.T. and J.I.H. planned and designed the research. J.L. performed the experiments. J.L., M.H., M.R.T. and J.I.H. wrote the manuscript.

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**Figures**

Fig 1. Megasporogenesis of *A. cherimola* in 2D. (a) PAS positive cytoplasm at the base of the pistil (arrow) appears 20 days before flowering. (b) The beginning of the placental protrusion that will become the ovule. (c) Early MMC appears under two nucellar cell layers. (d) Ovule with MMC and the initiation of inner and outer integument development. (e) Ovule showing the first meiotic division. (f) Linear tetrad of megaspores result of meiosis. (g) Three degenerating megaspores and the chalazal megaspore that will become the functional megaspore. (h) Functional megaspore enlarges and stores starch grains. MMC, megaspore mother cell; m1, first meiotic division; m, product of meiosis; FM, functional megaspore. Bars: 20 µm.

Fig 2. Megasporogenesis of *A. thaliana* in 3D. Cell volume is shown with a range of colour from blue for the smaller cells to red for the larger cells. (a) Volume of somatic (red line) and MMC (blue line) cells and number of neighbouring cells during megasporogenesis (green line). (b-c) Protrusion of a young ovule at stage 1III showing different cell volumes. (b) Outermost cells and (c) inner cells. (d-e) Young ovule at stage 2I showing the MMC differentiation (red cell) that increases in size. (d) Outermost cells and (e) inner cells. (f-i) Ovule at stage IV showing the first meiotic division (f-g) and the four megaspores (h-i), where the integuments have been removed from the ovule. (f, h) Outermost cells and (g,i) inner cells. ID* The ovules were arranged in developmental order; MMC, megaspore mother cell; m1, first meiotic division; m, product of meiosis; ii, inner integument; oi, outer integument. The error bars indicate SD. Bars: 10 µm.

Fig 3. Transition of somatic to germinal cell of *A. cherimola* in 3D. Cell volume is shown with a range of colour from blue for the smaller cells to red for the larger cells. (a-b) Early phase of ovule development showing different cell volumes but without MMC differentiation. (a)
Outermost cells and (b) inner cells. (c) Volume of somatic (red line) and MMC (blue line) cells of *A. cherimola*. (d) Young ovule showing the MMC differentiation (red cell) that increases in size and the initiation of the inner integument. ID* The ovules were arranged in developmental order; MMC, megaspore mother cell; ii, inner integument. The error bars indicate SD. Bars: 20 µm.

Fig 4. Cell wall components during early ovule development in *A. cherimola* revealed by monoclonal antibodies: JIM5 for unesterified pectin, JIM7 for methyl-esterified pectins, JIM8 and JIM13 for AGPs and JIM11 for extensins. Unesterified pectins (a) and methyl-esterified pectins (b) were observed in the chalaza but, while unesterified pectins (b) were observed in the nucellus, methyl-esterified pectins (a) were faintly detected in the nucellus of the first placental protrusion. Similarly AGPs detected by JIM8 (c) were mainly observed in the chalaza and faintly detected in the nucellus but AGPs were not revealed by JIM13 (d). Extensins (e) were observed indistinctly in the nucellus and chalaza of the first placental protrusion. n, nucellus; p, placenta. Bars: 25 µm.

Fig 5. Cell wall components during megasporogenesis of *A. cherimola* (a-e), *P. americana* (f-j) and *A. thaliana* (k-n) revealed by monoclonal antibodies: JIM5 for unesterified pectins (a, f, k), JIM7 for methyl-esterified pectins (b, g, l), JIM8 (c, h, ll) and JIM13 (d, i, m) for AGPs, JIM11 for extensins (e, j, n). The early MMC of *A. cherimola* showed signal of unesterified (a) and methyl-esterified (b) pectins, AGPs revealed by JIM8 (c) and JIM13 (d), and extensins (e). The nucellus only showed signal of methyl-esterified pectins (b). Similarly, the young MMC of *P. americana* showed signal of unesterified (f) and methyl-esterified (g) pectins, AGPs detected by JIM8 (h) and JIM13 (i), and extensins (j) but the nucellus only showed signal of methyl-esterified pectins (g). Unesterified (k), methyl-esterified (l) pectins and extensins (n) were observed equally in the ovule during megasporogenesis but AGPs were not observed (ll-m) in *A. thaliana*. MMC, megaspore mother cell. Bars: 25 µm (a-j); 10 µm (k-n).

Fig 6. Cell wall components during meiosis of *A. cherimola* (a-e), *P. americana* (f-j) and *A. thaliana* (k-n) revealed by monoclonal antibodies: JIM5 for unesterified pectins (a, f, k), JIM7 for methyl-esterified pectins (b, g, l), JIM8 (c, h, ll) and JIM13 (d, i, m) for AGPs, JIM11 for extensins (e, j, n). Unesterified and methyl-esterified pectins were observed in the cell wall of the meiotic product in *A. cherimola* (a, b) and *P. americana* (f, g) and, interestingly, unesterified pectins were also observed around the meiosis of *A. cherimola* (a). Methyl-esterified pectins were also observed in the nucellus of *P. americana* but signal was weaker compared to that observed in the meiotic product (g). AGPs were also observed in the cell wall of the meiotic product of *A. cherimola* (c, d) and *P. americana* (h, i) and the signal of JIM13 was stronger in...
the functional MMC of *A. cherimola* (d). The cell wall of the meiotic product of *A. cherimola* (e) and *P. americana* (j) also reacted to extensins. Unesterified (k), methyl-esterified (l) pectins and extensins (n) were observed equally in the ovule during meiosis but the meiosis was marked by AGPs (ll, m) in *A. thaliana*. MMC, megaspore mother cell; m, product of meiosis. Bars: 25 \( \mu m \) (a-j); Bars: 10 \( \mu m \) (k-n).

Fig 7. Callose deposition during megasporogenesis in *A. cherimola* (a), *P. americana* (b) and *A. thaliana* (c-e) revealed by anti-calllose monoclonal antibody (a-c) and aniline blue (d-e). Callose was only observed in the cell wall that separates the megaspores in *A. cherimola* (a) and *P. americana* (b). Callose deposition was also observed around the cell wall that separates the megaspores (c) of *A. thaliana*, but, while callose deposition was not observed around the early MMC (white arrow), it appears in the mature MMC (black arrow) (d, e). MMC, megaspore mother cell; m, product of meiosis. Bars: 25 \( \mu m \)

Fig 8. *In situ* hybridization of *PIN FORMED 1* (*PIN1*) gene (b-g) from *A. cherimola* and *P. americana* and *PIN11.1* (h-j) from *P. americana* in the megasporogenesis of ovules of *A. cherimola* (b-d) and *P. americana* (e-j). (a) Schematic representation of *PIN1:PIN1-GFP* expression (green) in the nucellus of *A. thaliana*. (b) *PIN1* expression was observed distally in the nucellus of the first placental protrusion of *A. cherimola*. *PIN1* expression was also observed distally in the nucellus around the young MMC (c) and the four megaspores after meiosis of *A. cherimola* (d). (d) Expression of *PIN1* was also observed in the vascular tissue of *A. cherimola*. Similarly *PIN1.1* expression was observed in the vascular tissue of *P. americana* in the young ovule (e, f) and during meiosis (g) and, interestingly, in the cell layers close to the MMC (e, f) and the four megaspores (g) of *P. americana*. *PIN11.1* expression was observed around the somatic cell that will become the MMC in young ovules (h) and around the MMC in all layers of the nucellus but with a stronger distal signal (i, j). MMC, megaspore mother cell; m, product of meiosis; oi, outer integument; ii, inner integument; v, vascular tissue. Bars: 20 \( \mu m \) (a); 50 \( \mu m \) (b-j).

Supporting Information

Additional supporting information may be found in the online version of this article.

**Fig. S1** Ovule development in *Arabidopsis*.

**Fig. S2** Flower structure of *A. cherimola*.

**Fig. S3** Phylogenetic relationships among PIN proteins inferred by Bayesian analysis.
**Fig. S4** *In situ* hybridization with antisense and sense PIN1 probe in *A. cherimola*, PIN1.1, PIN1.2, PIN11.1 and PIN11.2 in *P. americana*.

**Fig. S5** *In situ* hybridization of *PIN FORMED 1.2 (PIN1.2)* and *PIN11.2* genes from *P. americana* during megasporogenesis.

**Table S1** Primers used in this work.