The progamic phase of an early-divergent Angiosperm, *Annona cherimola* Mill. (Annonaceae)

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Running title: Progamic phase in *Annona cherimola*

ABSTRACT

• *Background and Aims* Recent studies of reproductive biology in ancient angiosperm lineages are beginning to throw light on the early evolution of flowering plants, but comparative studies are restricted by fragmented and meagre species representation in these angiosperm clades. In this study, the progamic phase, from pollination to fertilization, is characterized in *Annona cherimola*, which is a member of the Annonaceae, the largest extant family among early-divergent angiosperms. Besides its interest due to its phylogenetic position, this species is also an ancient crop with a clear niche for expansion in subtropical climates.

• *Methods* The kinetics of the reproductive process was established following controlled pollinations and sequential fixation. Gynoecium anatomy, pollen tube pathway, embryo sac and early postfertilization events were histochemically characterized.

• *Key Results* A plesiomorphic gynoecium with a semi-open carpel shows a continuous secretory papillar surface along the carpel margins, which run from the stigma down to the obturator in the ovary. The pollen grains germinate in the stigma and compete in the stigma-style interface to reach the narrow secretory area that lines the margins of the semi-open stylar canal and is able to host just one to three pollen tubes. The embryo sac has eight nuclei and is well provisioned with big starch grains that are used during early cellular endosperm development.

• *Conclusions* A plesiomorphic simple gynoecium hosts a simple pollen-pistil interaction, based on a support-control system of pollen tube growth. Support is provided through a basipetal secretory activity in the cells that line the pollen tube pathway. Spatial constraints, favouring pollen tube competition, are mediated by a dramatic reduction in the secretory surface available for pollen tube growth at the

stigma-style interface. This extramural pollen tube competition contrasts with the intrastylar competition predominant in more recently derived lineages of angiosperms.

Key words: *Annona cherimola*, Annonaceae, embryo sac, endosperm, Magnoliid, ovule, pollen-pistil interaction, pollen tube.

INTRODUCTION

Reproductive biology of basal and early-divergent angiosperms is experiencing a renaissance in the last few years and is providing valuable information on evolutionary trends in flowering plants (Friedman and Ryerson, 2009; Rudall *et al.*, 2009). While much remains to be discovered about the basic features of the sexual process in angiosperms, a new understanding of the evolutionary developmental basis is beginning to emerge. Most angiosperms conform to a defined suite of reproductive characteristics but new data derived from studies on ancient extant flowering plant lineages reveal that the reproductive features of the first flowering plants differed significantly from those shown by the majority of extant flowering plants (Friedman and Williams, 2004). Recent work is showing unique features in the female gametophyte of ancient extant angiosperms, such as the egg cell apparatus (Friedman, 2006), the endosperm (Friedman *et al.*, 2008) and the provisioning of ovular resources (Friedman, 2008; Rudall *et al.*, 2008). The study of pollen development and evolution is also emerging as a powerful field to understand the evolution of reproductive characters (Rudall and Bateman, 2007; Lora *et al.*, 2009). While information on the male and the female side is accumulating in ancient lineages of extant angiosperms, there is still much to be learned about the interplay between both: pollen-pistil interaction.

The progamic phase, the period of pollen tube growth through the pistil that elapses from pollination to fertilisation and gamete fusion (Linskens, 1975; Williams, 2009), provides an opportunity for pollen-pistil interaction, which is emerging as a powerful strategy regulating mating in flowering plants (Herrero and Hormaza, 1996; de Graaf *et al.*, 2001; Herrero, 2000, 2003; Rea and Nasrallah, 2008). The molecular mechanisms involved in this signalling are being deciphered (Escobar-Restrepo *et al.*, 2007; Higashiyama and Hamamura, 2008; Hiscock and Allen, 2008), although a

comprehensive view on the events and their implications is still to come. Recent work in *Amborella*, sister to all extant angiosperms, sets the base line for understanding the evolution of pollen-pistil interaction (Williams, 2009) and, in this sense, the study of the progamic phase in ancient angiosperms, that has been performed in a number of species belonging to ancient flowering plant lineages (Vithanage, 1984; Orban and Bouharmont, 1995; Pontieri and Sage, 1999; Thien *et al.*, 2003; Sage and Sampson, 2003; Koehl *et al.*, 2004; Hristova *et al.*, 2005; Lyew *et al.*, 2007), may prove a highly valuable tool to track the evolution of this process (Hiscock and Allen, 2008). Also the evolution and function of the transmitting tissue in extant representatives of early-divergent angiosperm lineages has been recently explored (Sage *et al.*, 2009).

However, one of the main limiting factors in studying developmental processes in early angiosperms and placing them in a phylogenetic framework is that most ancestral angiosperm lineages have arrived at the present times in a very fragmented way. Early-divergent angiosperms have a very meagre representation, with some lineages represented by only one or very few species. Annonaceae is the largest living family in the early-divergent angiosperm clade magnoliids (APG II, 2003; Soltis *et al.*, 2005) including about 130 genera and 2300 species with a worldwide distribution (Chatrou *et al.*, 2004). Besides the interest of the family to address questions on early angiosperm evolution, some of the species in the Annonaceae, such as cherimoya (*Annona cherimola*), sugar apple (*A. squamosa*) or soursop (*A. muricata*) were already used as a food source by pre-Columbian cultures in South America (Popenoe, 1989), and now they have a clear niche for expansion in developing countries with subtropical climates. Still, in spite of the importance for both basic and applied studies very little is known on the reproductive biology of this family.

Most works on reproductive biology in the Annonaceae have been focused on descriptive studies of the flowers (Norman *et al.*, 1986, 1992; Carvalho and Weber, 2000; Norman, 2003; Kiill and Costa, 2003), embryology (Svoma, 1998a), and flower development (Decraene and Smets, 1990; Leins and Erbar, 1996). Studies on the pollen tube pathway are limited and restricted just to pollen germination in the stigma (Vithanage, 1984). Recently, we have studied pollen development and release in groups in *A. cherimola* (Lora et al., in press) and we have shown the coexistence of bi and tricellular pollen at anther dehiscence in this species, contributing to the understanding of the heterochronic shift from bicellular to tricellular pollen (Lora *et al.*, 2009).

In this work the progamic phase and early embryo development is characterised in the early-divergent angiosperm *A. cherimola*. The examination of pistil anatomy reveals features showing a simple pollen-pistil interaction that are likely to be plesiomorphic for all angiosperms.

MATERIALS AND METHODS

Plant material

A. cherimola shows protogynous dichogamy (Schroeder, 1971). Flowers are hermaphroditic, but female and male structures do not mature simultaneously hindering self-fertilization in the same flower. Moreover, most flowers of the same genotype are synchronized and, consequently, transfer of pollen between different flowers of the same genotype is also difficult. The cycle of the flower is completed in two days; in the morning of the first day the flower is in preanthesis with the petals tightly closed. Around noon the flower passes to the female stage, petals slightly widen apart and the stigma is receptive. After approximately 30 h, flowers switch to the male stage. Anthers

dehisce at approximately 1700-1800 under our environmental conditions. Concomitantly with anther dehiscence, the petals widen apart and the stigma dries up and loses receptivity.

Adult trees of the *A. cherimola* cultivar Campas located in a field cultivar collection at the EE La Mayora-CSIC, Málaga, Spain, were used in these experiments. Flowers were fixed along the flowering period, during two consecutive years.

Pollination procedures

Anthers and pollen were collected from flowers just before anther dehiscence, stored at 4ºC and used for hand pollination the following day. Fifteen flowers per day were pollinated at 9:00 h. the first day of the flower cycle and the floral tube was then plugged with cotton to prevent further unwanted pollination. To evaluate developmental changes independent of pollination, similarly treated flowers were left unpollinated. The gynoecia of 15 pollinated and 15 unpollinated flowers were weighed and fixed daily from preanthesis (one day before anthesis) to three weeks following hand pollination.

An additional group of flowers were collected in the field, placed in water in the laboratory at room temperature and pollinated at 9:00 h the first day of the flower cycle to study pollen tube growth. Regression analyses were used to describe the relationships between days after anthesis and pistil weight. Several regression models were tested and third-order polynomial regression was selected.

Microscopic preparations

Pollen tube growth was documented using squash preparations of pistils from hand pollinated flowers kept in water at room temperature. For this purpose, pistils were fixed in formalin-acetic acid-alcohol (FAA) 3, 6 and 9 hours after pollination. Pistils were water washed and placed in 1N NaOH for 1 hour to soften the tissues. Individual

pistils were dissected and squash preparations were stained with 0.1 % aniline blue in $PO_4\ K_3$ (Currier, 1957; Linskens and Esser, 1957).

Following hand pollination in the field, pistils were also sequentially fixed. Eight flowers per day were collected at 9:00 and fixed in FAA, dehydrated in an ethanol series, and then embedded in paraffin wax. Seven flowers per day were fixed in 2.5% glutaraldehyde in 0.03M phosphate buffer (Sabatini *et al*., 1963). Finally, three flowers per day were fixed for 24 h in 3:1 (V1/V2) ethanol: acetic acid and transferred to 75% ethanol for storage at 4ºC following the method by Williams *et al.* (1999). These flowers and those fixed in 2.5% glutaraldehyde were dehydrated in an ethanol series and embedded in Technovit 7100 (Kulzer) resin.

To observe pollen tubes and callose, squash preparations and paraffin embedded material sectioned at 10 μm were stained with 0.1 % aniline blue in 0.1N PO_4K_3 (Currier, 1957; Linskens and Esser, 1957). Sections were also stained with 0.01% acridine orange in 0.03M phosphate buffer to observe DNA and RNA (Nicholas *et al*., 1986; Dudley *et al*., 1987), with 0.01% auramine 0 in 0.05M phosphate buffer to observe cutine and suberine (Heslop-Harrison, 1977) and with 0.07% calcofluor in water for cellulose (Hughes and McCully, 1975). For general histological examination, paraffin embedded material was stained with a mixed staining in the following order: 0.1% aniline blue in 0.1N PO_4K_3, 0.01% acridine orange in water, 0.01% auramine 0 in water and 0.01% calcofluor in water, for 10 minutes each at 40ºC to accelerate staining and drying. Others sections were also stained with safranin, crystal violet and green light according to Gerlach (1969). Resin embedded material fixed in glutaraldehyde was sectioned at 2 μm and stained with periodic acid-Shiff's reagent (PAS) followed by 0.2% Toluidine Blue in water (Feder and O'Brien, 1968) to observe insoluble carbohydrates and nuclei. Resin embedded material in glutaraldehyde was also stained

with Iodine Potassium Iodide (IKI) for starch (Johansen, 1940). Resin embedded material fixed with 3:1 (V1/V2) ethanol-acetic acid was sectioned at 5 μm and stained with a solution of 0.25 mg/mL of 4′,6-diamidino-2-phenylindole (DAPI) and 0.1 mg/mL *p*-phenylenediamine (added to reduce fading) in 0.05M Tris (pH 7.2) for 1 hr at room temperature in a light-free environment (Williams *et al*., 1999). Preparations were observed under an epifluorescent Leica DM LB2 microscope with 340-380 and LP 425 filters for DAPI. Pollen tube growth rates were calculated as the length of the longest pollen tube divided by the time elapsed from pollination.

RESULTS

Gynoecium anatomy and pollen tube growth

The average number of carpels in the gynoecium of *A. cherimola* is 90.4 ± 5.4 (*N*= 5 flowers) that are fused to form a syncarp which occupies the centre of a conical receptacle. Each of the carpels has a single anatropous ovule that can develop into a single seed. The androecium is located below the gynoecium forming a helicoidal structure with up to 200 stamens. The pistil shows a big stigma and a relatively short style with partial postgenital fusion at the periphery of the innermost side (Fig 1A), forming an open stylar canal. The closing area of this canal is covered in a zip like way by unicellular secretory papillae that result in a continuous secretory carpet starting in the stigma and paving all the way through the style down to the ovary (Fig 1B). Idioblastic solitary oil cells (Fig 1C), thick-walled sclereid cells (Fig 1D) and tanniferous cells were observed along the cortical tissue. Tanniferous cells were also observed in the ovular epidermis.

The length of the pollen tube pathway from the stigma to the ovule was 1832.4 μm (± 132.5, *N*= 10). Pollen grains germinated in the stigma and the pollen tubes grew on the stigmatic surface towards the suture line (Fig 1E) to penetrate in the style. The stigmatic suture line formed a furrow all along the stigma continuous with the style secretory area of the semi- open canal (Fig 1F). Germination occurred rapidly and one hour after pollination pollen grains had germinated and grew freely in the stigma. An average of 22.5 pollen grains (± 15.2, *N*= 70 stigmas) were recorded per stigma with and average pollen germination of 43.6 % (± 8.4 %, *N*= 70 stigmas). However, a drastic reduction in the number of pollen tubes occurred at the stigma-style interface (Fig 1F), and only one to three pollen tubes were observed in the style. This reduction is related to the receptive surface available for pollen tube growth. While the stigma is formed by a wide papillar surface that measures 470.1 μm (± 78, *N*= 10) in length and 307 μm (± 69, *N*= 10) in width, the receptive surface in this area is restricted to the narrow stylar semi-closed margins that are lined by secretory papillae continuous with those of the stigma. This stigma-style interface is able to lodge very few pollen tubes which, in their way trough the style, stick to this narrow receptive surface leaving empty the rest of the non-receptive stylar canal. Pollen tubes were first seen growing in the style 2-3 hours after pollination, and travelled over a distance of 836.3 μm (± 77.3, *N*= 10) to reach the ovary locule (Fig 1G) some 4-6 hours after pollination, with an average pollen tube growth rate in the style of 280 μm/h. At the ovary locule, the pollen tubes grew over the obturator and penetrated the hood-shaped ovule after traversing a distance of 526 μm (± 86, *N*= 10). A single pollen tube was observed penetrating each ovule (Fig 1H) and the first fertilized ovules were observed one day after pollination.

Embryo sac and fertilization

A. cherimola shows an anatropous, bitegmic and crassinucellate ovule, with an endostomal micropyle formed by the inner integument that protrudes over the external integument (Fig 2A). The outer integument is vascularised. At anthesis, the embryo sac is mature and shows the Polygonum-type structure with seven cells and eight nuclei, three at the micropylar end [the two synergids (Fig 2A) and the egg cell (Fig 2B)], three at the chalazal end [the three antipodal cells (Fig 2C)], and two polar nuclei in the centre (Fig 2D) which are not fused at anthesis.

The embryo sac contains large starch grains distributed in the central cell (Fig 3A). These starch grains are much bigger than the standard starch grains located in the sporophytic tissues of the ovule. They react both to PAS and IKI stains. Starch accumulates around the egg cell (Fig 3B), and also around the two polar nuclei (Fig 3C) that fuse close to the time of fertilization. Both synergids have a big vacuole at the top of the cell (antipodal side) and the cytoplasm and the nucleus are located at the base (micropyle side) (Fig 3D) where a filiform apparatus is developed (Fig 3E). Starch vanishes following fertilization concomitantly with endosperm development. Endosperm is cellular and starts to develop three days after pollination. It shows a bipolar nature where the first division produces a large cell in the micropylar pole and a smaller cell in the chalazal pole. Starch grains accumulate in the chalazal pole cell (Fig 3F) and decrease with endosperm enlargement (Fig 3G) vanishing three weeks after pollination concomitantly with further endosperm cellular division. After fertilization, a zygote develops and the first cell division could be seen eight days after pollination when the endosperm has already four cells (Fig 3H).

Changes in the pistil

The pollen tube pathway is lined by secretory papillae that form a continuous carpet from the stigma down to the placenta facing the ovule endostome. The papillae in the placenta resemble an obturator since they form a protuberance towards the ovule entrance. The cytohistological features of those papillae are similar to the papillae of the stigma and style (Fig 4A-C) although a basipetal maturation sequence can be seen from the stigma down to the obturator. The papillae in the less mature areas are rich in starch and show no secretion. As papillae mature, starch vanishes concomitantly with the production of a secretion in the surface of the papillae. Thus, while at preanthesis the stigma does not appear to contain starch (Fig 4A), starch is still present in the style (Fig 4B) where a secretion is being produced. At the obturator (Fig 4C) starch is far more conspicuous and the secretion is still not apparent at this time.

The secretion of the papillae is present along the whole pollen tube pathway from anthesis to 6 days later. This secretion stains heavily with PAS and with Toluidine blue (Fig 4B). The papillae and secretion are present just in the outermost side of the semi-open stylar canal (Fig 4D). Before anthesis, the unicellular secretory papillae are rich in starch reserves in the style (Fig 4B and E). One day after anthesis, starch vanishes and the secretion increases (Fig 4F) concomitantly with pollen tube passage. The same situation can be seen in the obturator where starch is conspicuous before anthesis (Fig 4G) while it vanishes as secretion increases one day later (Fig 4H). This process does not seem to be triggered by the pollen tube passage, but appears to be developmentally regulated, for it occurs in the same way and at the same time in unpollinated flowers.

While at preanthesis callose is not apparent in the papillae secretory cells, callose layering starts in the papillae one day after pollination (Fig 5A) and is also

present in the obturator (Fig 5B). This callose layering occurs in a similar way in pollinated (Fig 5B) and unpollinated flowers (Fig 5C). However, callose layering in the nucellus at the base of the embryo sac appears three days after pollination only in pollinated flowers (Fig 5D). On the other hand, callose layering in the vascular bundles is only observed in ovules of unpollinated flowers one day after anthesis (Fig 5E). During ovary development, callose also appears in the cell plates forming the walls of the cellular endosperm (Fig 5F and G).

Gynoecium weight increases slowly and is similar in pollinated and unpollinated flowers during the first 4 days after anthesis. Six days after anthesis differences can be observed among pollinated and unpollinated flowers; thus, while unpollinated flowers start to drop, pollinated flowers experience rapid growth (Fig 6). A similar pattern was observed for pollinated and unpollinated flowers in the two years of observations.

DISCUSSION

A. cherimola shows a simple and plesiomorphic pistil, with a short style and a semi-open continuous secretory carpel, which supports a simple pollen-pistil interaction. Interestingly, this interaction exhibits a support-constrain strategy that is prevalent in phylogenetically derived angiosperm species (Herrero and Hormaza, 1996) although in the former case it takes place in the stigma instead of in the style.

Pistil support to pollen tube growth

A. cherimola shows a wet stigma similarly to other closely related species in the genus *Annona* (Vithanage, 1984; Heslop-Harrison and Shivanna, 1977). In *A. cherimola* the common secretory papillar carpet that covers the stigma, style and ovary along the

semi-suture line provides a substrate for pollen tube growth. Secretion along the pollen tube pathway has also been reported in other members of ancient angiosperm clades such as *Trimenia moorei* (Bernhardt *et al*., 2003), *Illicium floridanum* (Koehl 2002 cited by Bernhardt *et al*., 2003), *Amborella trichopoda* (Thien *et al*., 2003), *Saururus cernuus* (Pontieri and Sage, 1999), *Psedowintera axillaries* (Sage and Sampson, 2003) and *Kadsura longipedunculata* (Lyew *et al*., 2007) and appears to be composed of arabinogalactan and arabinogalactan-proteins (Sage *et al.*, 2009). Although dry stigmas are considered as plesiomorphic in flowering plants (Thien et al., 2009) both dry and wet stigmas can be found in taxa of the ANITA and magnoliid clades (Thien et al., 2009). Molecular studies on wet and dry stigmas and their implications in pollen-pistil interaction have been performed only in a limited number of evolutionary–derived angiosperm taxa and, consequently, there is a need for more studies on this topic among early-divergent angiosperm taxa (Hiscok and Allen, 2008).

The production of this secretion is already present before flower opening and reaches a maximum level one day after anthesis, concomitantly with pollen tube passage. Still, the production of secretion is independent of pollination since it occurs in the same way and at the same time in pollinated and unpollinated flowers. This point is different from higher angiosperms, in which pollen tube growth in the style triggers starch degradation (Herrero and Dickinson 1979; Gonzalez *et al*., 1996). But, interestingly, this production of secretion is very similar to the behaviour of the obturator in which secretion occurs at a particular time of development independently of pollination (Herrero and Arbeloa, 1989; Arbeloa and Herrero, 1991). In *A. cherimola* a primitive obturator, formed by the protuberance of the placenta, continuous and with the same cytohistological features of the secretory papillae, appears to be present in the ovary. Similar structures have been described in other ancient angiosperm lineages,

such as in species of the Magnoliaceae that show a funicular outgrowth with papillose cells (Matsui *et al*., 1993; Umeda *et al*., 1994), in Schisandraceae (Lyew *et al*., 2007), Lauraceae (Sedgley and Annells, 1981) and in the monocot *Ornithogalum caudatum* (Tilton and Horner, 1980). In *A. cherimola* the fact that secretion is present right from anthesis at pollination time provides an adequate substrate for a rapid pollen tube growth. This contrasts with longer times for pollen tube growth reported in other species, which are related to waiting times in order to reach the phase where secretion is produced in the pistilar structures (Herrero and Arbeloa, 1989; Herrero, 2000, 2003).

Following the production of secretion, callose is layered in the papillar secretory structures in the same way reported in the obturator of peach (Arbeloa and Herrero, 1987), perhaps protecting this area and fulfilling what has been considered as a prophylactic role (Heslop-Harrison, 1999, 2000).

Pistil constraint to pollen tube growth

Pollen tube growth proceeds rapidly and, within one day of pollination, the pollen tubes reach the hood-shaped ovule, which has been considered as a plesiomorphic trait in angiosperms (Soltis *et al*., 2005). Relatively rapid pollen tube growth has also been found in other members of ancient angiosperm clades (Bernhardt *et al*., 2003; Sage and Sampson, 2003; Koehl *et al*., 2004; Hristova *et al*., 2005; Williams, 2008, 2009) and contrasts with the slow growth of pollen tubes in gymnosperms (Gelbart and Von Aderkas, 2002). Both the pollen tube growth rate of *A. cherimola* (480 μm/h) and the length of the pollen tube pathway 1.83 mm are in the range described for basal grade angiosperms (Williams, 2008): ≈ 80-600 μm/h and <0.5 to ≈ 15 mm long. Although in some derived angiosperms delayed fertilization has also been recorded (Sogo and Tobe, 2005, 2006*a*, *b*), an evolutionary trend towards rapid

pollen tube growth in seed plant pollen has been proposed (Pettitt, 1982; Williams, 2008) where the development of callose plugs in pollen tubes could have played a major role (Williams, 2008). Differences in timing also appear to be related to differences in maturation of the pistil (Herrero and Arbeloa, 1989; Sogo and Tobe, 2005, 2006*a*, *b*) and to a requirement for male-female synchrony (Herrero, 2003).

Several pollen grains germinate on the stigma, but only one pollen tube reaches the ovule and achieves fertilization. While pollen grains germinate freely at the stigma and direct their growth towards the semi-open suture line, a clear restriction and reduction in the number of pollen tubes occur at this point of entrance in the short stylar canal. Only 1-3 pollen tubes penetrate the style. This reduction in the number of pollen tubes may be related to the limited space available with only a narrow papillar secretory area that paves, along the semi-open suture line, the carpel margins. Pollen competition and selection appears to be a common fact shared by most angiosperms (Mulcahy, 1979, Hormaza and Herrero, 1992; 1996) and it is usually reflected by a reduction in the number of pollen tubes that continue to grow in the style (Sedgley, 1977; Cruzan, 1990; Hormaza and Herrero, 1996). However, results herein show that in *A. cherimola* the main restriction point appears at the stigma-style interface. This behaviour should be investigated in other early-divergent angiosperms, but interesting recent work in *Amborella trichopoda* (Williams, 2009) shows a very similar behaviour. *A. cherimola* has a semi-open stylar canal similar to that described in *Amborella trichopoda* (Williams, 2009) and in *A. cherimola* only the carpel margins are layered with secretory papillae, paving a narrow way for the few pollen tubes growing in the style. A semi-open stylar canal is a common feature found in other ancient angiosperms (Endress and Igersheim, 2000) and it would be interesting to evaluate if papillar secretory cells restricted to the margins also provide a similar pollen restriction mechanism, in contrast

to the typical pollen tube attrition recorded in the style in evolutionary derived angiosperms. If this is so, during angiosperm evolution, the arena for pollen competition would have changed from the stigma to within the style and, consequently, pollen competition in the style could be considered an innovation in evolutionary derived clades of flowering plants.

Postfertilization events

Three days after pollination callose is layered in the nucellus under the embryo sac micropylar pole only in the ovules of pollinated flowers which appear to have been fertilized. On the other hand, in ovules of unpollinated flowers, deposition of callose in vascular bundles was observed six days after pollination, suggesting impending ovule abortion. This has been shown in other species and explained in terms of blockage of metabolite translocation (Pimienta and Polito, 1982, Herrero and Arbeloa, 1989, Rodrigo and Herrero, 1998).

The presence of starch grains has been reported in mature embryo sacs in some ancient angiosperm lineages (Cook, 1902; Kimoto and Tobe, 2001; Friedman, 2008), including species in the Annonaceae (reviewed in Svoma, 1998*b*), and also in higher angiosperms (Evans, 1919; Maheshwari, 1950). But the abundance and big size of the starch grains observed in this work is striking. Recent results in Hydatellaceae (Friedman, 2008; Rudall *et al*., 2008), a family recently recognized among early-divergent extant angiosperms (Saarela *et al*., 2007), shows a maternal seed-provisioning strategy similar to that observed in gymnosperms. The provision of starch grains reported here in the embryo sac before fertilization in *A. cherimola* could respond to a similar plesiomorphic strategy. Through a different accumulation pattern, the accumulation of starch reserves either in the sporophytic of gametophytic tissue would

constitute an accumulation of reserves before fertilization to support early postfertilization processes.

While zygote cell division does not start until 8 days after pollination, endosperm cell division starts 3 days after pollination. Division of the endosperm is bipolar giving rise to a big cell close to the zygote and a small cell full of starch at the chalazal end. This situation persists during the first endosperm divisions. While this behaviour in relation to starch accumulation has not been previously reported, a bipolar endosperm cellular division has been shown in several species of the Winteraceae (a sister group to Annonaceae in the Magnoliales) such as *Drimys winteri* (Bhandari and Venkatar, 1968; Floyd and Friedman, 2000), *Pseudowintera axillaries* (Sampson, 1963 cited in Bhandari and Venkatar, 1968) and *Zygogynum bailloni* (Swamy, 1952). The presence of a similar cellular endosperm with unequal division has been also reported in other ancient angiosperms (Floyd and Friedman, 2000; Tobe *et al*., 2000) and seems to be a plesiomorphic feature in angiosperms. The prominence of endosperm development in *A. cherimola* contrasts with an underdeveloped embryo that in the mature seed is embedded in abundant ruminate endosperm, similar to other species of the Annonaceae (Corner, 1949; Svoma, 1998*b*), where this slight embryo development has been postulated as an ancestral feature (Hayat, 1963; Finch-Savage and Leubner-Metzger, 2006).

CONCLUDING REMARKS

Flowers of *A. cherimola* present a number of ancestral characteristics of angiosperms such as the semi-open simple carpel, the hood-shaped ovule, the cellular endosperm and the seed type with a rudimentary embryo. Still this primitive carpel host a support-constrain strategy for pollen tube growth conserved in phylogenetically derived

angiosperm lineages. Support is provided by the continuous secretory papillar carpet that paves the pollen tube pathway and that provides evidence for a common ontogenetic origin for this tissue as well as for a conserved basipetal maturation that encompasses pollen tube growth. Constraint and restriction in the number of pollen tubes occurs at the stigma-style interface and is mediated by a dramatic reduction in the secretory surface available for pollen tube growth from the stigma to the margins of the semi-open stylar canal. It will be worthwhile to evaluate in other ancient lineages of angiosperms with a similar pistil anatomy if this extramural pollen competition is conserved as compared to the stylar intramural competition in modern angiosperms.

ACKNOWLEDGEMENTS

This work was supported by the Spanish Ministry of Education (Project Grants AGL2004-02290/AGR, AGL2006-13529 and AGL2007-60130/AGR), GIC-Aragón 43, Junta de Andalucía (AGR2742), and the European Union under the INCO-DEV program (Contract 015100). J.L. was supported by a grant from Junta de Andalucía.

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FIGURES

FIG. 1. Gynoecium anatomy and pollen tube growth in *A. cherimola*. (A) Pistil showing the stigma (stg), short style (stl) and ovary (ov) with partial postgenital fusion at the periphery of the innermost side (arrow). (B) Longitudinal section of the pistil showing pollen tube growth (arrow) through the short open stylar canal that leads to an anatropous ovule (ov). (C) Oil cells. (D) Thick-walled sclereid cells. (E) Pollen tubes growing on the stigma towards the stigmatic furrow (arrows) that leads to the stylar canal. (F) Stigma-style interface, with the stigmatic furrow leading to the narrow receptive closing margins of the stylar canal (arrow) and pollen tube growing through (pt). (G) Pollen tube (arrow) reaching the locule over the continuous papilar secretory zone. (H) Pollen tube growing through the micropyle formed by the inner integument (ii) that protrudes over a hood-shaped outer integument (oi), and reaching the nucellus (nu) 24 hours after pollination. (A) Wholemount of a dissected pistil stained with aniline blue. (B) (H) Aniline blue staining of a 10 μm paraffin section. (C) (D) Dapi staining of a 5 μm resin section. (E) (F) Aniline blue staining of squash preparation. (G) Mixed staining of a 10 μm paraffin section. Scale bars: (A) 200 μm; (B) 200 μm; (C) 20 μm; (D) 20 μm ; (E) 20 μm; (F) 100 μm; (G) 20 μm; (H) 20 μm.

FIG. 2. Embryo sac in *A. cherimola*. (A) Ovule showing the micropyle (asterisk) formed by the inner integument (ii), the shorter outer integument (oi) and the embryo sac with two synergids (sy). (B) Egg cell (ec). (C) Two of the three antipodal cells (arrows). (D) Two polar nuclei (pn). Dapi staining of 5 μm resin sections. Scale bar = 20 μm.

FIG. 3. Embryo sac and endosperm development in *A. cherimola*. (A) Embryo sac of *A. cherimola* flower in preanthesis with starch grains (sg) around the two polar nuclei and showing the three antipodal cells (arrow). The difference between the standard starch grains (sg) located in the sporophytic tissues of the ovule and the big starch grains in the female gametophyte is apparent. (B) Egg cell with a big vacuole at the base of the cell. (C) Two polar nuclei surrounded by big starch grains. (D) Two synergid cells with nucleus and cytoplasm at the micropylar end and a big vacuole at the top of the cell. (E) Filiform apparatus (fa) of a synergid cell. (F) Starch grains (sg) accumulating in the chalazal pole of the embryo sac in a fertilized ovule four days after pollination. (G) Cellular endosperm eight days after pollination with starch (sg) accumulated at the cell of the chalazal end. (H) Zygote first division eight days after pollination. PAS (A, F, H) and PAS and toluidine blue (B-E, G) staining of a 2 μm resin section. Scale bars: (A) 20 μm; (B) 10 μm; (C) 10 μm; (D) 10 μm; (E) 10 μm ; (F) 20 μm; (G) 20 μm; (H) 10 μm.

FIG. 4. Secretion along the pollen tube pathway in *A. cherimola*. Longitudinal section of secretory papillae of the (A) stigma, (B) style and (C) obturator in preanthesis with the same cytohistological features although with differences in maturation and starch content: while at the stigma (A) starch has already vanished, in the style (B) starch is still present and a rich secretion is apparent; and the obturator (C) is still full of starch. (D) Transverse section of the style showing a semi-open stylar canal (arrow) lined only in the outermost side with papillar cells (pc) with secretion. Stylar longitudinal section in the outermost papillar secretory zone at preanthesis (E), showing starch grains. Same area, one day after pollination (F), shows less starch in the cells and secretion. Transverse section of obturator (ob) cells at preanthesis (G) with starch; (ii: inner

integuments) and one day after pollination (H) with little starch and abundant secretion (ii: inner integument; oi: outer integuments). PAS and toluidine blue staining of 2 μm resin sections. Scale bar = 20 μm.

FIG. 5. Callose layering along the pollen tube pathway and during early endosperm development in *A. cherimola*. Callose one day after pollination in papillar cells (arrow) of style (A) and obturator (arrow) of a pollinated (B) and an unpollinated (C) flower. (D) Callose in the nucellus micropylar pole 3 days after anthesis. (E) Deposition of callose in vascular bundles of the ovule (arrow) of unpollinated flowers one day after pollination. Callose in the cell plates (arrow) forming the walls of the cellular endosperm, four days after pollination (F) and eight days after pollination (G). Aniline blue staining of 10 μm paraffin sections. Scale bar = 20μm.

FIG. 6. Mean pistil weight after anthesis in pollinated and unpollinated flowers of *A. cherimola*. Bars indicate SD.