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Arabinogalactan proteins and pectin distribution during female gametogenesis in *Quercus suber* L.

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• **Background and Aims** *Quercus suber* L. (cork oak) is one of the most important monoecious tree species in semi-arid regions of Southern Europe, with a high ecological value and economic potential. However, as a result of its long reproductive cycle, complex reproductive biology and recalcitrant seeds, conventional breeding is demanding. In its complex reproductive biology, little is known about the most important changes that occur during female gametogenesis. Arabinogalactan proteins (AGPs) and pectins are the main components of plant cell walls and have been reported to perform common functions in cell differentiation and organogenesis of reproductive plant structures. AGPs have been shown to serve as important molecules in several steps of the reproductive process in plants, working as signalling molecules, associated with the sporophyte–gametophyte transition, and pectins have been implicated in pollen–pistil interactions before double fertilization. In this study, the distribution of AGP and pectin epitopes was assessed during female gametogenesis.
• **Methods** Immunofluorescence labelling of female flower cells was performed with a set of monoclonal antibodies (mAbs) directed to the carbohydrate moiety of AGPs (JIM8 and JIM13) and pectic homogalacturonans (HGs) (mAbs JIM5 and JIM7).
• **Key Results** The selective labelling obtained with AGP and pectin mAbs JIM8, JIM13, JIM5 and JIM7 during *Q. suber* female gametogenesis shows that AGPs and pectic HG can work as markers for mapping gametophytic cell differentiation in this species. Pectic HG showed different distribution patterns, depending on their levels of methyl esterification. Methyl-esterified HGs showed a uniform distribution in the overall female flower cells before fertilization and a more specific pattern after fertilization. A low methyl-ester pectin distribution pattern during the different developmental stages appears to be related to the pathway that pollen tubes follow to reach the embryo sac. AGPs showed a more sparse distribution in early stages of development, but specific labelling is shown in the synergids and their filiform apparatus.
• **Conclusions** The labelling obtained with anti-AGP and anti-pectin mAbs in *Q. suber* female flower cells showed a dynamic distribution of AGPs and pectic HGs, which may render these molecules useful molecular markers during female gametogenesis. Changes occurring during development will be determined in order to help describe cork oak ovule structural properties before and after fertilization, providing new insight to better understand *Q. suber* female gametogenesis.

Key words: Arabinogalactan proteins, pectins, *Quercus suber*, cork oak, ovule development, immunolocalization, monoclonal antibodies.

INTRODUCTION

Cork oak is one of the most important monoecious tree species in semi-arid regions of Southern Europe, due to its ecological value in environmental protection together with its economic potential due to cork and seed production. The cork oak acorn is crucial for the traditional system of Iberian pig farming and for the quality and certification of its derived products. The Iberian pig-farming system depends on the sustained use of pasture lands of *Quercus suber* and *Q. ilex* in the south-western part of the Iberian Peninsula, where acorns are used to fatten the animals during the mast feeding season, when the animals feed exclusively on fallen acorns and grass (Rodriguez-Estevez *et al*., 2009; Charneca *et al*., 2010).

In cork oak forests of the Iberian Peninsula, the value of this species has facilitated the development of new strategies for reforestation programmes. However, the conventional breeding of cork oak is limited, given their long reproductive cycle, complex reproductive biology and recalcitrant seeds. The inability to predict seed yield, and the existence of fruits with different morphological and physiological features, constitute major problems for research in cork oak plant breeding (Varela and Valdivieso, 1996). The knowledge of *Q. suber* reproductive biology during flowering and fertilization is essential to understand the molecular mechanisms of seed production and identify the natural constraints affecting the reproductive success of this species.

Female inflorescences arise in spikes, with 3–5 flowers, on the axil of new leaves. They are included in a cupule and contain three carpels, with two ovules each (Boavida *et al*., 1999).

Male flowering buds occur in early spring and sometimes also in autumn, whereas female flowers appear in spring and only become fully developed a few months later, if pollinated. Female flowers are partially enclosed by a dome-shaped bud of imbricate scales and, during spike elongation, 3–5 styles emerge from the cupule and the stigma becomes receptive (Ducousso *et al*., 1993). At the time of pollination, the ovary is still undifferentiated and the transmitting tissue extends only to the base of the styles. The wind-driven pollen lays on the receptive stigmatic surface, germinates and the pollen tube grows throughout the transmitting tissue, until it reaches the base of the style. After fertilization, only one of the six ovules develops into a monospermic fruit, which matures during autumn (Ducousso *et al*., 1993; Boavida *et al*., 1999). Such characteristics make *Q. suber* an interesting system for comparative studies of development and sexual reproduction in a non-model forest plant.

The plant cell wall is a highly complex and dynamic structure mostly composed of highly hydrated pectins, structural proteins and diverse soluble proteins such as enzymes, and it is responsible for intercellular communication. In the pistil, cell walls are involved in cell adhesion, cell to cell signaling, defence and processes of nutrition, guidance and protection of the pollen tube along the transmitting tissue (Jauh *et al*., 1997; McCann *et al*., 2000; Chen *et al*., 2006). Pectins are galacturonic acid-rich polysaccharides including HGs, type I and type II rhamnogalacturonan (RG-I and RG-II) and xylogalacturonan. The most common structural model of the cell wall considers a cellulose–hemicellulose network embedded in a pectin matrix (Mohnen, 2008; Dick-Perez *et al*., 2011). AGPs are ubiquitous in the plant kingdom and belong to a large family of glycoproteins highly soluble in water solutions, with high levels of glycosylation (Knox, 2006) and also highly resistant to proteolysis and mainly composed of arabinogalactans (polymers of D-galactose and L-arabinose). AGPs have been implicated in different processes of plant growth and development, are particularly abundant in cell walls, and can be found in the plasma membrane, in the apoplastic space and in the extracellular matrix (Showalter, 2001). Both pectins and AGPs perform common functions in cell differentiation and organogenesis of vegetative and reproductive plant structures (Majewska-Sawka and Nothnagel 2000). Since Keegstra *et al.* (1973) hypothesized that rhamnose residues of arabinogalactan side chains of AGPs might be attachment sites for RG-I, AGPs have been reported to form complexes and interact with pectins (Yamada *et al*., 1987; Saulnier *et al*., 1988; Kwan and Morvan, 1991; Pellerin *et al*., 1995; Yamada, 2000; van Hengel *et al*., 2001; Duan *et al*., 2003, 2004; Tan *et al*., 2012, 2013).

Pectins were shown to be involved in different processes in plants, such as cell expansion, defence, ion regulation, cell orientation and regulation of cell wall porosity (Ridley *et al*., 2001; Harholt *et al*., 2010). On the other hand, AGPs were implicated in cell adhesion and recognition, nutrition and programmed cell death (Seifert and Roberts, 2007), and have been suggested to play a nutritive role and act as chemotropic factors for growing pollen tubes (Hepler *et al*., 2001). Female reproductive organs of angiosperms are especially rich in AGPs, where they have a pattern of distribution that undergoes changes during differentiation. The occurrence of AGPs in ovules of *Brassica napus*, *Amaranthus hypochondriacus*, *Actinidia deliciosa*, *Galanthus nivalis*, *Galtonia candidans*, *Oenothera*, *Sinapis alba*, *Trithuria submersa* and *Arabidopsis thaliana* has been revealed by the use of monoclonal antibodies (mAbs) (Pennell *et al*., 1991; Coimbra and Salema, 1997; Coimbra and Duarte 2003; Chudzik *et al*., 2005*a*, *b*; Pereira *et al*., 2006; Coimbra *et al*, 2007; Costa *et al*., 2013*b*; Pereira *et al*., 2015).

The interactions between pectins control important aspects of plant development, including cell adhesion, wall extensibility, wall porosity and the mediation of defence responses (Krupkova *et al*., 2007; Mohnen, 2008; Caffall and Mohnen, 2009; Wallace and Anderson, 2012). HGs are polymerized and methyl-esterified in the Golgi apparatus, and secreted to the cell wall in a highly methyl-esterified state (Zhang and Staehelin, 1992; Sterling *et al*., 2006), where they can undergo de-esterification by cell wall-associated pectin methyl esterases (PMEs). The removal of methyl groups alters the physical properties of the polymers (Wolf *et al*., 2009; Wolf and Greiner, 2012). After demethylesterification, HGs can form Ca^{2+}–pectate cross-linked complexes (Grant *et al*., 1973), which indicate a denser and more inextensible cell wall (Peaucelle *et al*., 2012). The de-esterification of HGs can lead both to cell wall stiffening and to enzymatic degradation of pectin, influencing its texture and mechanical properties and regulating cellular growth and shape.

Pollination induces changes in both the distribution and metabolism of highly and weakly methyl-esterified HGs localized in the stigma and the style transmitting tract (Lenartowska *et al*., 1997, 2001; Bednarska *et al*., 2005; Dresselhaus and Márton, 2009; Lenartowska *et al*., 2011; Dresselhaus and Franklin-Tong, 2013). In *Petunia hybrida*, appreciable changes induced by pollination, particularly in the distribution of unesterified pectins, were found in the transmitting tissue, with progressive reduction in the amount of strongly Ca^{2+}-binding pectins, showing a correlation with changes in the structure of the extracellular matrix (Lenartowska *et al*., 2001).

The glycosylphosphatidylinositol (GPI) anchor tethers AGPs to the outer layer of the plasma membrane (Borner *et al*., 2002; Schultz *et al*., 2004). As a result, AGPs face the extracellular environment and this is likely to contribute to their proposed signalling function. The model of dynamic flux of AGPs proposed by Lamport *et al.* (2006) states that AGPs upon cleavage of the GPI anchor are liberated into the extracellular space, where they may work as cell wall plasticizers, enlarging the pectin matrix, allowing the extension of the wall and, as a result, promoting cell expansion.

Pectins and AGPs can be localized in tissues and cells through the use of specific mAbs that bind to structurally complex carbohydrate epitopes typical of these proteoglycans (Knox, 1997). AGP-specific mAbs have been essentially useful in revealing the developmental dynamics of the AGP glycan moieties. AGPs have been shown to be involved in sexual plant reproduction of several plant species. For instance, they are present, in a developmentally regulated way, in the extracellular matrix of the transmitting tract of species such as *Gladiolus gandavensis*, *Lilium longiflorum*, *Nicotiana alata* and *Lycopersicon peruvianum* (Hoggart and Clarke, 1984; Sedgley

et al., 1985; Webb and Williams, 1988; Gane *et al*., 1995). AGPs have also been shown to be involved in pollen tube growth in *Amaranthus hypochondriacus*, *Actinidia deliciosa*, *Catharanthus roseus*, *Nicotiana tabacum* and *Arabidopsis thaliana* (Coimbra and Salema, 1997; Cheung *et al*., 1995; Coimbra and Duarte, 2003; Pereira *et al*., 2006; Coimbra *et al*., 2007, 2009, 2010; Costa *et al*., 2013*a*; Pereira *et al*., 2014, 2015). The present work is a contribution to characterize the distribution pattern of AGP and pectin epitopes in *Q. suber* female reproductive tissues, with the available collection of anti-AGP mAbs, and thus to contribute to the understanding of the molecular mechanisms associated with the reproductive development of the female flower structures.

MATERIALS AND METHODS

Plant material, light and scanning electron microscopy

Individual flowers from female inflorescences of *Quercus suber* L., collected from randomly selected trees of two natural populations in the Porto area, were fixed in 2 % (w/v) paraformaldehyde and 2·5 % (w/v) glutaraldehyde in phosphate buffer [0·025 M, pH 7, 0·001 % (v/v) Tween-80], placed under vacuum for 1 h and then at 4 °C overnight. After dehydration in a graded ethanol series, the material was embedded in LR White resin. Thick sections (0.5 µm) were obtained with a Leica EM UC7 Ultramicrotome, placed on glass slides and stained with a solution of 1 % (w/v) toluidine blue (Sigma-Aldrich, St Louis, MO, USA) and 1 % (w/v) safranin for staining of the nuclei. Various slides were left unstained for immunolocalization with mAbs against AGPs and pectin epitopes. Sections for bright-field microscopy were mounted with Eukitt quick-hardener (Fluka). Scanning electron micrographs were taken in freshly collected female flowers using a TM3030Plus scanning electron microscope that allows a low-vacuum observation without advance sample preparation

Immunolocalization and antibodies

A selection of mAbs directed against AGPs and pectins were provided by Professor Paul Knox from the Centre for Plant Sciences, University of Leeds, UK. The mAbs against AGPs used were JIM8 (Pennell *et al*., 1991), JIM13 (Knox *et al*., 1991), JIM16 (Yates *et al*., 1996), LM2 (Smallwood *et al*., 1996), LM6 (which also recognizes a type I rhamnogalacturonan; Willats *et al*., 2000) and M207 (Pennel *et al*., 1989). For pectins, mAbs used were JIM5 (Willats *et al*., 2000), JIM7 (Knox *et al*., 1990), LM5 (Jones *et al*., 1997), LM19 (Verhertbruggen *et al*., 2009) and LM20 (Verhertbruggen *et al*., 2009). Fluorescein isothiocyanate (FITC)-conjugated anti-rat IgG (Sigma-Aldrich F-1763) was used as secondary antibody.

In the slides prepared for immunolocalization, the sections were circled with a PAP pen (Sigma-Aldrich Z672548), and treated as follows: 5 min in phosphate-buffered saline (PBS), pH 7·4, containing 5 % (w/v) non-fat dried milk (blocking solution), followed by incubation with primary antibody (diluted 1:5 in blocking solution), for 2 h at room temperature followed by overnight at 4 °C. After washing with PBS, the sections were incubated with secondary antibody (diluted 1:100 in blocking solution) for 4 h in the dark, and then finally washed with PBS followed by distilled water. Slides were further stained with 0·01 % (w/v) calcofluor white (Fluorescent Brightener 28; Sigma-Aldrich F3543) and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA; ref: H-1000). Control experiments were performed omitting the incubation with the primary antibody (incubation with blocking solution only), and resulted in no staining with secondary antibody.

Bright-field and fluorescence observations were performed on a Leica DMLB epifluorescence microscope [objectives were Leica N-Plan, and the filters used were 365/445 nm for calcofluor and 4',6-diamidino-2-phenylindole (DAPI); and 470/525 nm for fluorescein stain]. Images were captured with a ProgResVR MF cool (Jenoptik, Jena, Germany) in automatic exposure mode, and processed with ProgResVR CapturePro 2.8.8 software.

RESULTS

At the time of pollination (Fig. 1A), *Q. suber* ovaries are still undifferentiated and the transmitting tissue extends only to the base of the styles, where locules start to appear (Fig. 1C). Three to six stigmata emerging from the dome of imbricate bracts are fully receptive, with green to yellow curved stigmas. After 1–2 weeks, it is expected that the receptive stigmatic surface has collected enough pollen, which germinates and the pollen tubes start to grow throughout the transmitting tissue. Stigmas become brown, start to lose receptivity and close up (Fig. 1B). Inside the locules, the ovules primordia start to develop, but are still undifferentiated at this stage (Fig. 1D). At this early stage of flower development, when performing immunolocalization with two different mAbs, JIM5 and JIM7, that recognize pectic HGs with low and high levels of methyl esterification, respectively, methyl-esterified HGs presented a uniform distribution pattern in the overall female flower cells, but with a more intense labelling in the transmitting tract, as shown by JIM7 (Fig. 1E). Partially methyl-esterified HGs are intensely labelled by JIM5 along the transmitting tract (Fig. 1F). The AGPs are also visible, with epitopes recognized by JIM13/JIM8 widely distributed around locules, especially in the septum (Fig. 1G).

AGP and pectin distribution during macrosporogenesis and macrogametogenesis

At the stage where ovules start to differentiate, it is possible to compare different patterns of development within a single ovary (Fig. 2A, 2B). At the same time, we find locules with similar size, but very different structure, containing in similar spaces two or just one developing ovule. Inside the highly differentiated ovule, inner and outer integuments are distinguishable and the embryo sac, which is not fully differentiated, is visible (Fig. 2C). At this moment, JIM5 labelling shows that partially methyl-esterified HGs are preferentially distributed in the external walls of inner integuments (Fig. 2D, E). In more developed ovules, it is possible to identify JIM5 epitopes in the cells surrounding the micropyle and around the emerging embryo sac (Fig. 2E). Methyl-esterified HG epitopes recognized by JIM7 have a uniform distribution throughout the ovary cells,

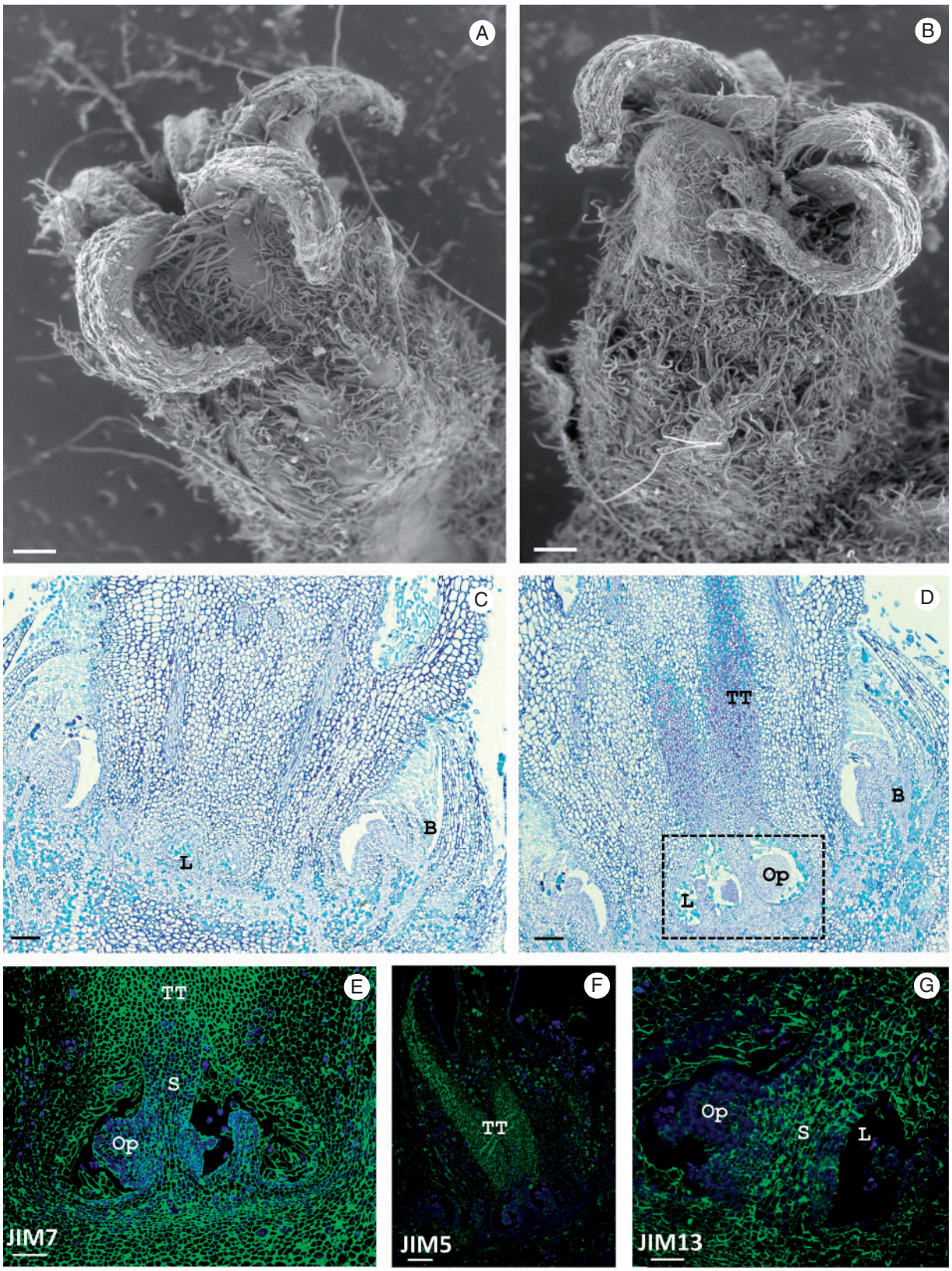

FIG. 1. (A) Scanning electron micrograph (SEM) of the *Q. suber* female flower, when distinct green to yellow curved stigmas appear. Three to six receptive stigmata are emerging from a dome of imbricate scales, providing full receptivity of the flower. (B) SEM of the *Q. suber* female flower when stigmas start to lose receptivity, close up and become brown. (C, D) Bright-field micrographs of cork oak female flowers stained with toluidine blue. (C) Micrograph showing two emerging locules. (D) Micrograph showing development of ovule primordia. (E) Immunolabelling of methyl-esterified pectin showing the uniform distribution of JIM7 epitopes in all cells, with the transmitting tract intensely labelled. (F) Immunolabelling of partially methyl-esterified pectins, with JIM5 strongly located along the transmitting tract. (G) Immunolabelling of AGP epitopes recognized by JIM13 spread over the septum and around locules. (E) and (G) show magnifications of the dotted box section presented in (D). L, locule; B, bracts; S, septum; Op, ovule primordia; TT, transmitting tract. Scale bars (A) = 2 mm, (B) = 1 mm, (C–F) = 100 µm, (G) = 20 µm.

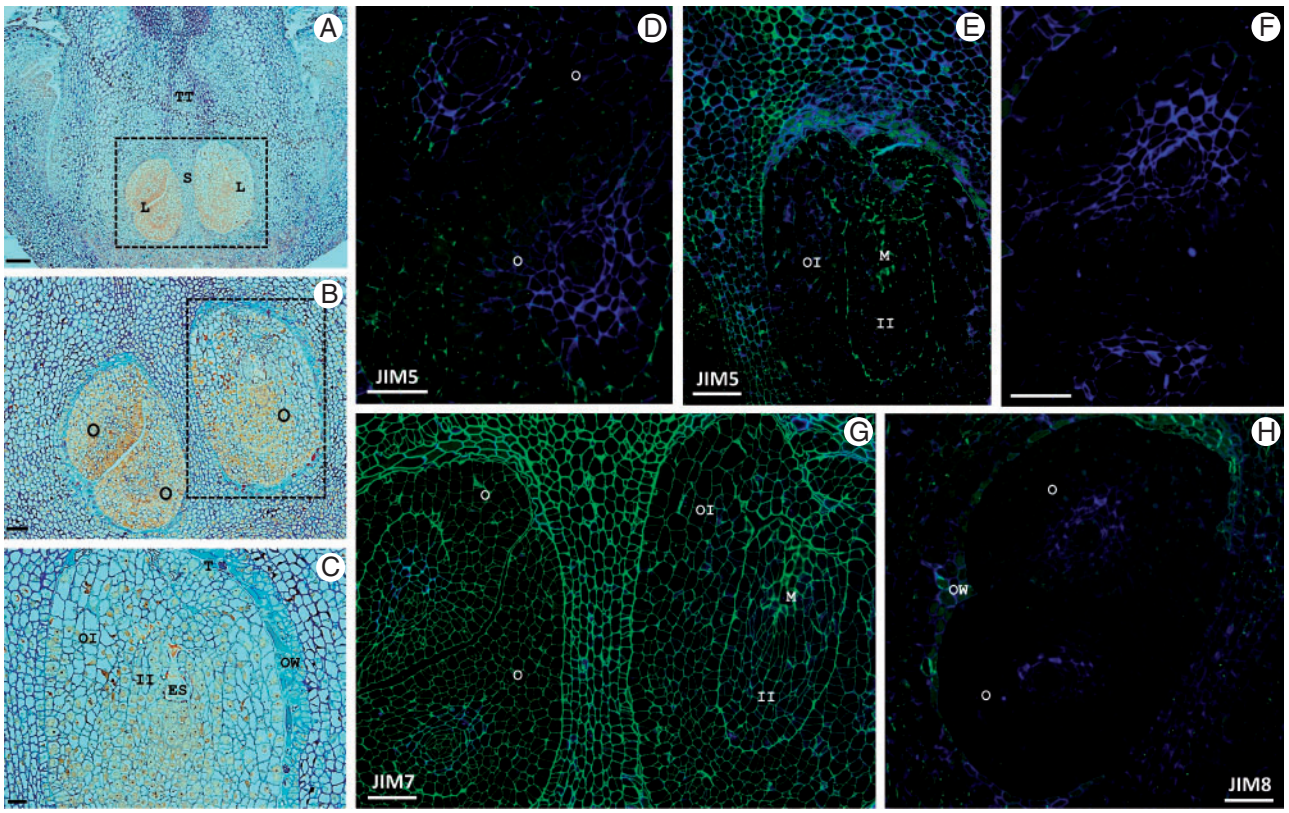

FIG. 2. Cork oak ovules at the megasporocyte stage. (A) Bright-field micrographs of cork oak ovules stained with safranin and toluidine blue, showing loculi with one or two ovules in different stages of development. (B) Detailed magnifications of the dotted box section presented in (A). (C) Detailed magnification of the dotted section presented in (B), showing the more developed ovule, on the right, with inner and outer integuments, micropyle and a developing embryo sac. (D, E) Immunolabelling of pectin epitopes, with JIM5 located around the external wall of inner integuments. (E) An equivalent section to (C). (F) Control image. (G) Detailed magnification of (A), showing uniform distribution of JIM7 epitopes in all cells, labelling the septum and external walls of inner integuments with high intensity. (H) Immunolabelling of AGP epitopes recognized by JIM8 showing a very weak labelling only around the locule wall. (D) and (H) show a detailed magnification of the area outside the dotted box section in (B). L, loculi with one or two ovula; O, ovule; TT, transmitting tract; S, septum; OW, ovary wall; T, trichomes; M, micropyle; OI, outer integument; II, inner integument; ES, embryo sac. Scale bars (A) = 200 μm, (B) = 100 μm, (C) = 50 μm, (D, F) = 20 μm, (E, G, H) = 50 μm.

with strong labelling in the septum and external walls of inner integuments (Fig. 2G).

The AGP epitopes were evaluated using the two antibodies JIM8 and JIM13, with both showing a similar distribution pattern of epitopes. At the beginning of ovule development, JIM8 epitopes are visible at a few points of the locule wall (Fig. 2H).

About 2 months after pollination (Fig. 3A), the *Q. suber* embryo sac has a *Polygonum* type of development that acquires its mature shape. As the ovule development continues with a series of syncytial mitotic divisions and cellularization, immunocytochemistry may help us to identify the molecular and structural developmental changes during embryo sac development (Fig. 3B, C). When synergids are generated (Fig. 3B), AGP sugar epitopes are clearly visible, with a very defined labelling obtained with JIM8 and JIM13 along the synergids and embryo sac walls (Fig. 3D, E). The central cell wall is also labelled by JIM8 and JIM13 mAbs at this stage (Fig. 3F). As for pectic HGs with high levels of methyl esterification, they are present in all cells of the inner and outer integuments (Fig. 3G, H), but no labelling is obtained in the synergids or central cell.

At a more advanced stage of development, when the filiform apparatus is clearly visible at the micropylar end of the synergid cells, the central cell is at the chalazal pole (Fig. 4A) and the immunolabelling results show a similar labelling pattern, with AGP mAbs JIM8 and JIM13 labelling the synergid filiform apparatus (Fig. 4B, C) in a perfectly defined distribution pattern. JIM7 labelling shows a uniform distribution of pectic HG, with high levels of esterification along the inner and outer integument cells, and no labelling is seen in the filiform apparatus or central cell (Fig. 4D). There is no labelling in the central cell with AGP mAbs JIM8 and JIM13 (Fig. 4E, F). Partially methyl-esterified HG epitopes recognized by JIM5, at this stage of ovule development, are clearly present in a well-defined area, along the inner integument and in the cells around the micropyle, thus presenting a similar pattern to the megasporocyte stage (Fig. 4G, H). It is important to highlight that a similar structural pattern was observed in more than one ovule in the same flower at this stage of development.

AGP and pectic HG distribution after fertilization

After fertilization, the fused polar nuclei form a large primary endosperm nucleus, and continuous division of the primary nuclei generates a free nuclear endosperm. The nuclei with dense cytoplasm connections are peripheral to the central

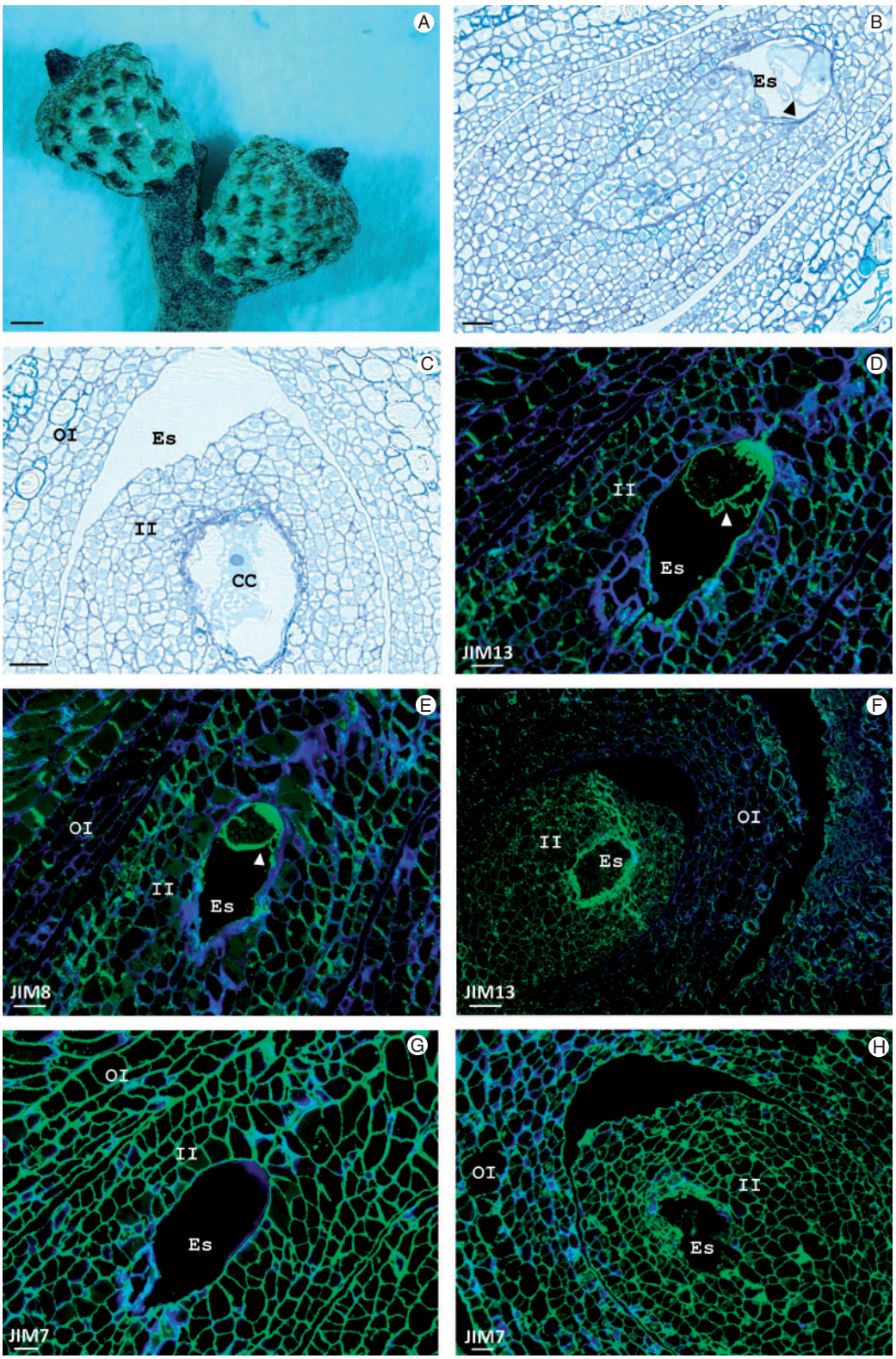

FIG. 3. (A) Pollinized cork oak female flowers. (B) Bright-field micrograph of cork oak ovules stained with toluidine blue, showing the synergid (arrowheads) cells. (C) Bright-field micrographs of cork oak ovules stained with toluidine blue, showing the central cell located centrally in the embryo sac. (D–H) Immunodetection of AGPs and pectic HG epitopes in the cork oak embryo sac. (D, E) JIM8 and JIM13 epitopes labelled the synergid (arrowhead) cell walls more intensively with JIM8 than with JIM13. (F) JIM13 epitopes labelled inner integument and embryo sac wall. (G) Uniform distribution of JIM7 in all sporophytic cells and no labelling in synergids. (H) Uniform distribution of JIM7 in all sporophytic cells and no labelling in the central cell. Es, embryo sac; CC, central cell; II, inner integument; OI, outer integument; (arrowheads), synergids. Scale bars (A) = 1 mm, (B–E, G, H) = 20 µm, (F) = 50 µm.

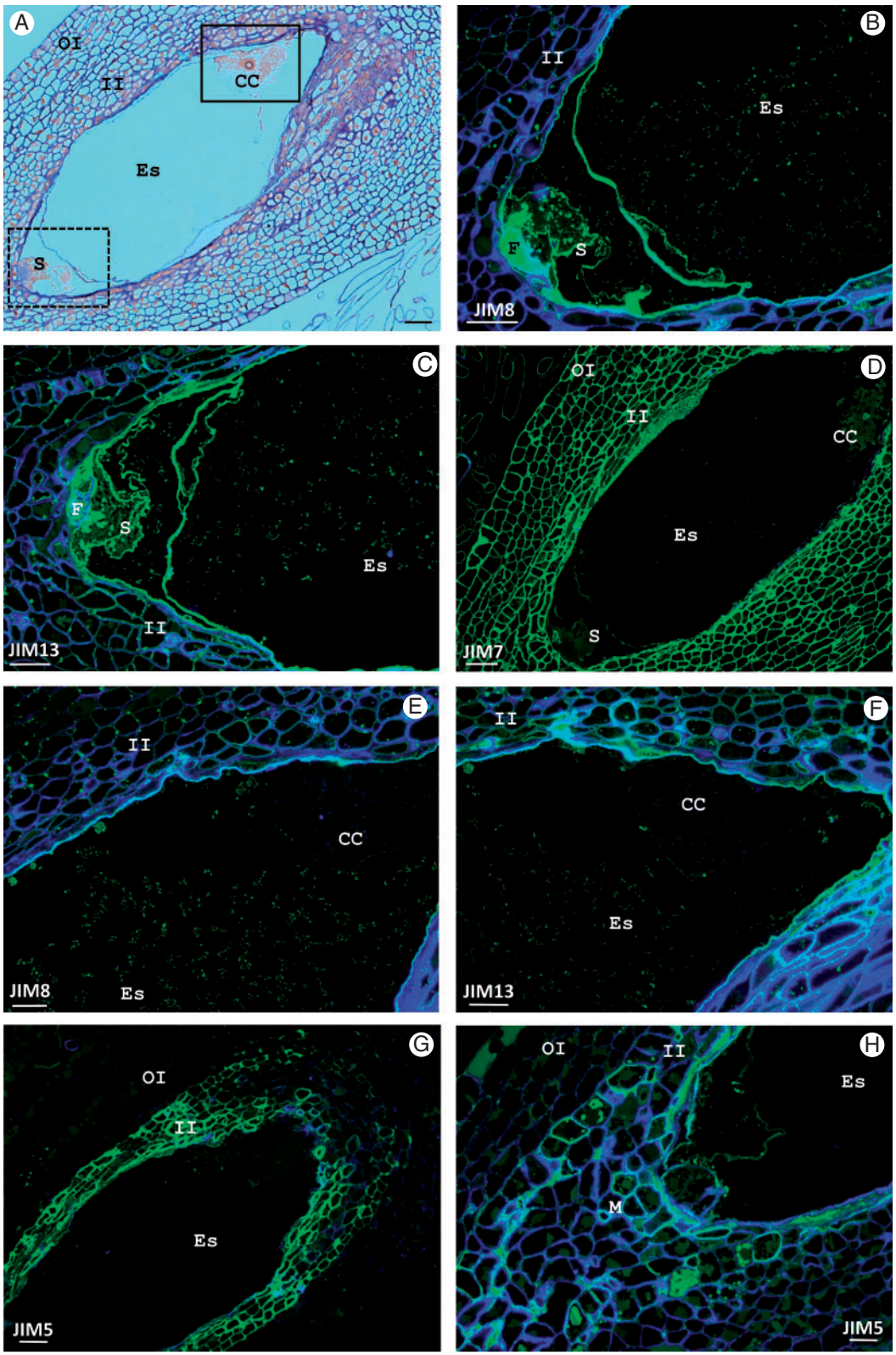

FIG. 4. (A) Bright-field micrographs of cork oak ovule stained with safranin and toluidine blue, showing the embryo sac with the synergids and the central cell located in the chalazal pole close to the inner integument. (B, C) Immunolabelling of AGP epitopes with JIM8 and JIM13 showing an intense and similar signal in the synergids and filiform aparatus. (D) Uniform distribution of JIM7 epitopes in all integument cells. (E, F) Immunolabelling with JIM8 and JIM13, showing no signal in the central cell. (G, H) Detection of JIM5 epitopes clearly visible in a defined area, along the inner integument and in the cells around the micropyle. (B), (C) and (D) are detailed magnifications of the dotted section of (A), and (E) and (F) are detailed magnifications of the straight line section of (A). CC, central cell; S, synergids; Es, embryo sac; II, inner integument; OI, outer integument; F, filiform apparatus; M, micropyle. Scale bars (A, D, G) = 50 µm, (B, C, E, F, H) = 20 µm.

area of the embryo sac. The zygote remains undivided during and after the development of the free nucleate endosperm, whereas the inner integuments degenerate successively (Fig. 5A). At the cellular endosperm stage, the young globular embryo appears at the micropylar end (Fig. 5B).

Considering the proembryo stage, AGP epitopes recognized by JIM8 and JIM13 presented similar labelling patterns, with visible signal in the inner wall of the integument and newly formed endosperm cell walls (Fig. 5D, E). Pectic HGs with high levels of esterification, recognized by JIM7, were detected in the inner wall of the inner integument, in contrast to only a few dots labelled in the endosperm (Fig. 5F).

The zygote enlarges gradually but only starts dividing after the formation of the free nuclear endosperm. This stage is characterized by a uniform and intense distribution of methyl-esterified HG epitopes in integument cell walls and also a defined distribution pattern on the cell walls of globular embryo, with no labelling in endosperm cells (Fig. 5G). JIM13 selectively labelled the basal part of the globular embryo and the micropylar end of the embryo sac (Fig. 5H). A similar pattern of partially methyl-esterified pectins labelled by JIM5 is found at this stage, surrounding the micropylar end of the embryo sac (Fig. 5I).

The other mAbs used in this study to map pectins and AGPs did not show any distinct distribution pattern that could add to the actual gathered information.

A schematic representation of the spatio-temporal patterns of pectins and AGPs throughout the female reproductive structures and tissues in *Q. suber* before and after fertilization is presented (Fig. 6).

DISCUSSION

The patterns and differences found in this study in epitope distribution during *Q. suber* female gametogenesis represent new insights to better understand which functional molecules are present in different cell types, both in sporophytic and in gametophytic tissues. The changes described during cork oak ovule structural development before and after fertilization highlight the importance of these molecules as markers for certain cell or tissue types, in very precise stages of sporogenesis and gametogenesis.

After 1–2 weeks of *Q. suber* female flower pollination, when ovule primordia start to develop, it is possible to define areas where methyl-esterified and non-methyl-esterified HG and AGP are located. Barany *et al.* (2010) suggested in *Capsicum annum* that highly esterified pectins are good markers for proliferating cells, whereas high levels of non-esterified pectins are abundant in walls of differentiating cells. In this work, it was shown that highly methyl-esterified HGs, indicators of cellular proliferation, appear uniformly in all female flower cells, with an intense labelling in the transmitting tract, whereas non methyl-esterified HGs are abundant along the transmitting tract and not on the neighbouring cells. Suárez *et al.* (2013) indicated that pistil development is accompanied by a significant increase in both esterified and de-esterified pectin content, in comparison with previous stages. Bednarska *et al.* (2005) and Lenartowska *et al.* (2001) observed low-esterified HGs in the stigma and in the transmitting tract of the *P. hybrida* style. It has been suggested that HGs in the pistil are the source of nutrition and that oligogalacturonides acting as the signal molecules are responsible for the direction of pollen tube growth (Mollet *et al.*, 2000).

At early stages of pistil development, AGPs are widely distributed around locules, especially in the septum, possibly acting as directional signal molecules for pollen tube growth towards the ovule. In the transmitting tissue of tobacco, a gradient in the level of AGP glycosylation towards the ovules was observed, and their highest level of glycosylation was detected in the base of the ovary (Wu *et al.*, 1995). The cytoplasmic distribution of AGPs may indicate their association with secretory compartments such as the endoplasmic reticulum and Golgi apparatus in cells actively producing and secreting AGPs (Samaj *et al.*, 2000).

When ovules start to differentiate, different patterns of ovule development on the same ovary could be observed, which, despite showing locules with similar size, were in different stages of differentiation. Methyl-esterified HGs are uniformly distributed throughout the ovary cells, with high intensity in the septum and walls of inner integuments facing the outside, while partially methyl-esterified HGs are preferentially distributed in the external cellular layer of inner integuments and, if we consider more developed ovules, they appear in the cells surrounding the micropyle and around the emerging embryo sac. For *Larix decidua*, when pollen was present in the micropylar canal, de-esterification of HGs and Ca^{2+} binding occurred in the external cellular matrix of the nucellus, creating an optimal calcium environment for pollen tube growth (Rafinska *et al.*, 2014). Considering that pollination induces an increase in Ca^{2+} levels in the transmitting tract extracellular matrix (Lenartwoska *et al.*, 1997), the major constituents of which are unesterified pectins (Vennigerholz, 1992; Jauh and Lord, 1996; Lenartowska *et al.*, 1997), this category of pectins constitutes not only the store but eventually also the source of Ca^{2+} for growing pollen tubes. The distribution pattern found in this study for unesterified pectins in cork oak appears to have a strong connection to this Ca^{2+} exploitation.

The *Q. suber* embryo sac acquires its mature shape about 2 months after pollination. On the nucellus inside the ovule primordium, the megaspore mother cell develops and will give rise to the female gametophyte, the embryo sac. When synergids are produced, AGP sugar epitopes are clearly visible, with a very defined and intense labelling obtained with JIM8 and JIM13 along the synergids, the embryo sac walls and the central cell wall. Methyl-esterified pectin epitopes are present in all cell walls of inner and outer integuments, but not in the synergids or central cell.

In the mature ovule of *Amaranthus hypochondriacus*, AGPs (JIM8 and JIM13 epitopes) were detected in the egg apparatus and integuments lining the pollen tube pathway (Coimbra and Salema, 1997). Chudzik *et al.* (2005*b*) showed that the timing of the appearance and localization of AGP expression is mainly observed in the pathway of pollen tube growth and during the period of highest ovule receptivity. This suggests that AGPs present along the pathway of the pollen tube could act as lubricants and/or nutrients to create an environment favourable for pollen tube growth within the extracellular matrix along the pistil (Suárez *et al.*, 2013). In a more advanced stage of development, when the filiform apparatus is clearly visible near the

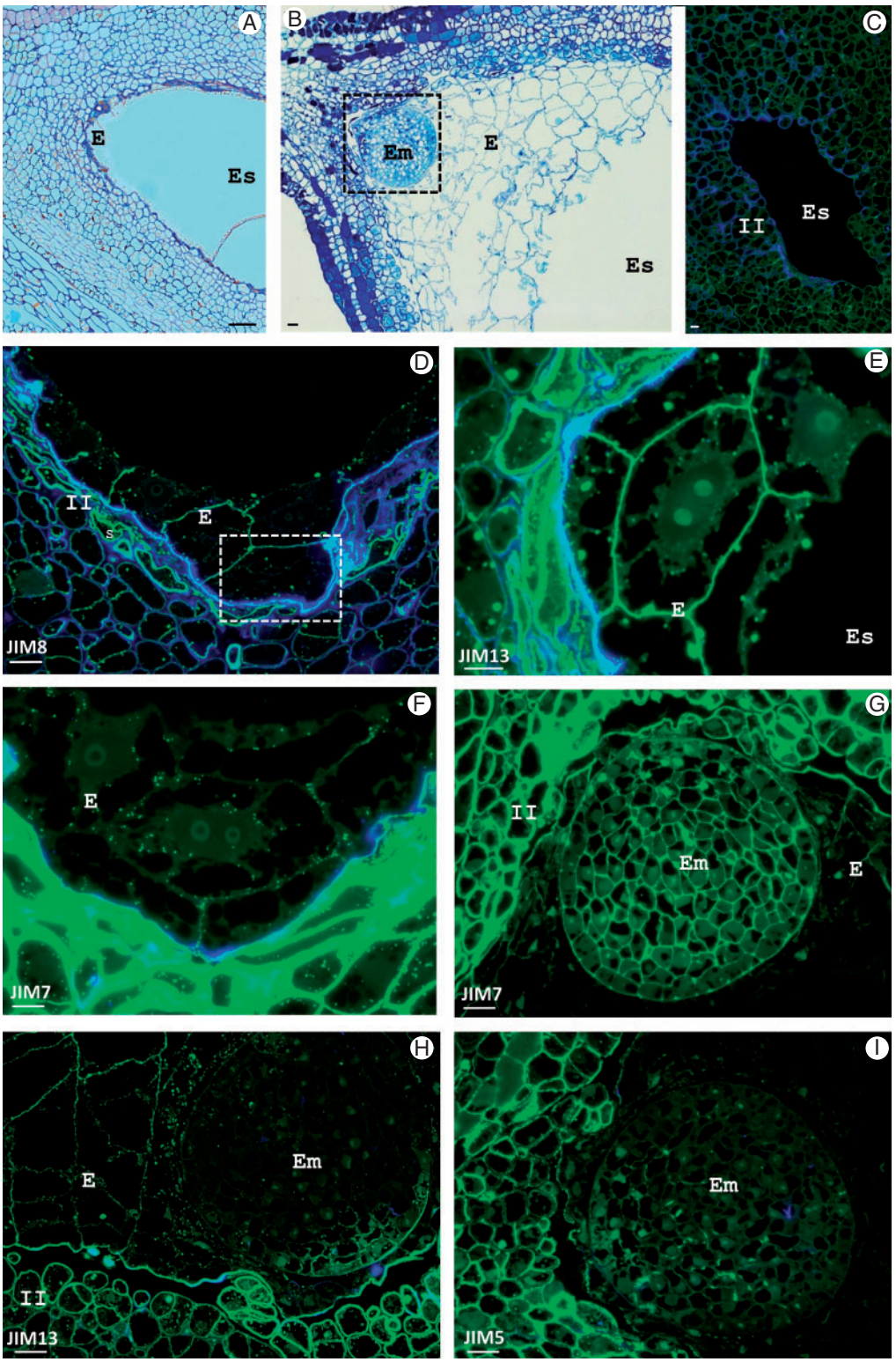

FIG. 5. (A) Bright-field micrograph of a cork oak ovule stained with toluidine blue and safranin at the proembryo stage. (B) Bright-field micrograph of a cork oak ovule stained with toluidine blue at the embryo globular stage. (C) Control image. (D, E) AGP epitopes labelled by JIM8 and JIM13 have a similar distribution, visible at the inner wall of the integuments and newly formed endosperm cell walls. (F) Immunolabelling of methyl-esterified pectin epitopes by JIM7 in a cork oak proembryo, showing an intense signal in the integuments close to the embryo sac and a weak signal in the endosperm (E). (E) and (F) are detailed magnifications of the white dotted section of (D). (G) Uniform and intense JIM7 distribution of methyl-esterified epitopes in integument cells and labelling emerging on the cell walls of the globular embryo, with no labelling at the endosperm cells. (H) AGP epitopes are selectively labelling the basal part of the globular embryo. (I) Partially methyl-esterified homogalacturonans, labelled by JIM5, are visible in the cells surrounding the micropylar end. (G–I) are magnifications of the black dotted section of (B). E, endosperm; Em, embryo; Es, embryo sac; II, inner integument. Scale bars (A) = 50 μm, (B, C, D, G, H, I) = 20 μm, (E, F) = 10 μm.

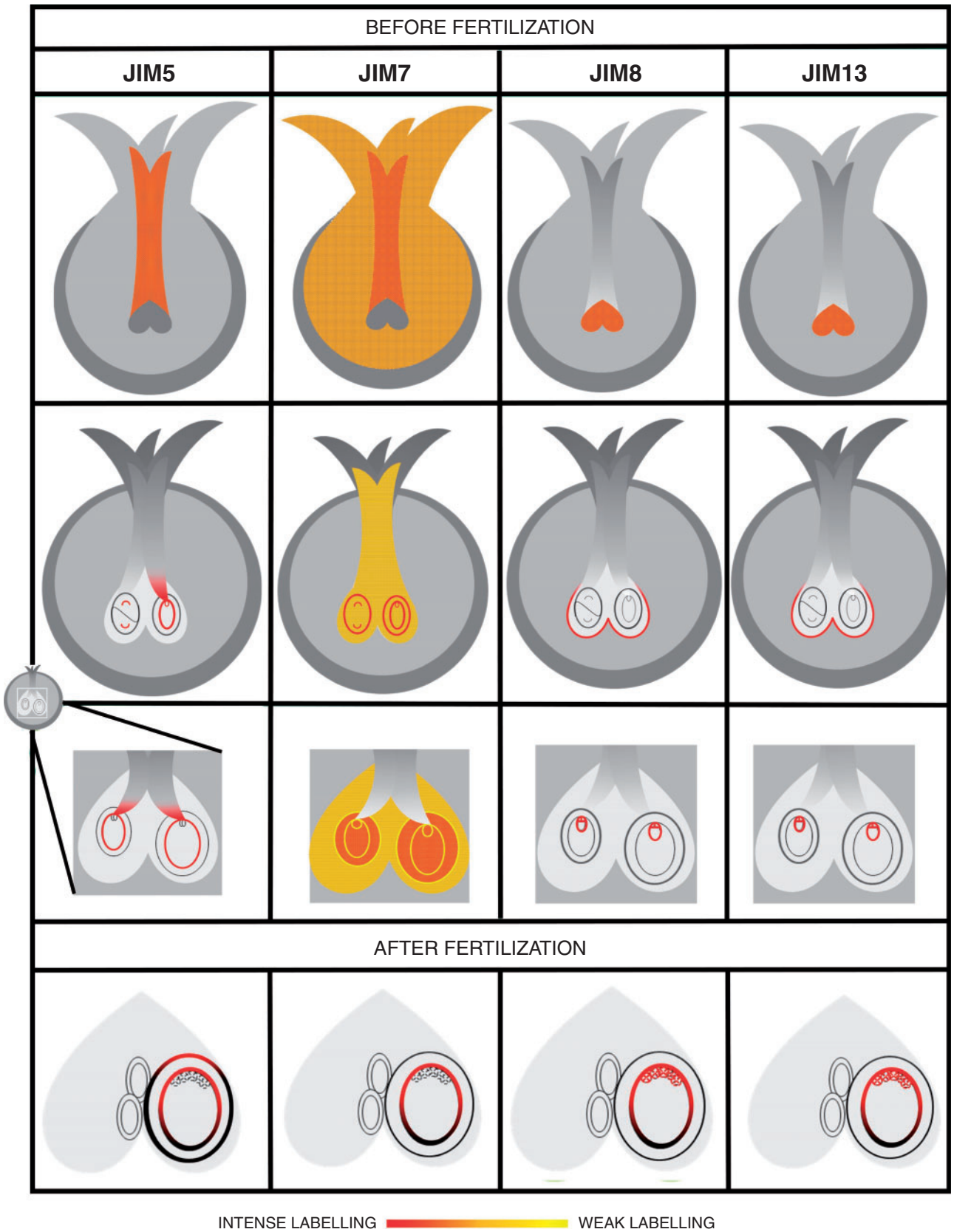

FIG. 6. Spatio-temporal distribution of pectic HGs (JIM5, JIM7 epitopes) and AGPs (JIM8, JIM13 epitopes) throughout the female reproductive structures and tissues in *Quercus suber*, before and after fertilization. Areas in grey have no labelling significance.

micropylar end and the central cell is at the chalazal pole, the immunolabelling pattern is maintained, with AGPs intensely labelled in the synergid filiform apparatus. These results are consistent with those found by Coimbra *et al.* (2007) in *A. thaliana* where JIM8 and JIM13 showed a strong and specific labelling in the embryo sac wall and synergid cells, especially in their filiform apparatus, indicating a possible role related to the secretions by these cells, through the micropyle. The labelling of the integument lining the micropyle suggests that AGP molecules may be related to the attraction phenomena of the pollen tube growth into the embryo sac.

Methyl-esterified pectins appear along the inner and outer integument cells and show weak labelling in the synergid filiform apparatus or central cell. Partially methyl-esterified HGs were only detected along the inner integument and in the cells around the micropyle. The pattern of distribution is maintained and is similar to what was shown at the megasporocyte stage, suggesting a possible contribution in the definition of the pollen tube pathway into the embryo sac.

After fertilization, considering the proembryo stage, a high abundance of pectic HG with high levels of methyl-esterification was found in the integuments close to the embryo sac, in contrast to no signal in the endosperm. AGPs had visible signal in the inner wall of the integument and newly formed endosperm cells.

The young globular embryo appears to be surrounded by the cellular endosperm at the micropylar end. A uniform and intense distribution of methyl-esterified pectins in integument cells and also some presence on the emerging cell walls of the globular embryo, with no presence in endosperm cells, was shown after labelling with JIM7. Pectic HG with low levels of esterification maintains its localization surrounding the micropylar end. AGP epitopes are selectively labelled in the basal part of the globular embryo, suggesting that these types of molecules start to disappear when the embryo structure is established.

Qin and Zhao (2007) showed that JIM13-reactive AGPs are the most fundamental sub-set of AGPs in embryos. The accumulation of AGPs in the basal part of the globular embryo may be an indicator of the embryo transition from the globular to heart-shaped stage, which may reflect the importance of AGPs in the interaction between these structures, including cell communication, signal transduction and transportation of material. Characterization of arabidopsis developmental mutants showed that interaction between the embryo proper and the suspensor is of central importance for embryo development (Vernon *et al.*, 2001; Qin and Zhao, 2006).

The importance and involvement of AGPs in sexual plant reproduction haves been known for a long time, and cork oak is one of the most important forest species in Portugal due to its ecological and socio-economic significance.

The importance of the results presented in this work arises not only from a developmental perspective but also because they provide a framework for more in-depth stepwise research to characterize the expression of individual AGP genes in each of the developmental stages considered.

To continue the present work trying to learn more about the reproductive biology of this species, further immunolocalization assays will be conducted to explore the involvement of other cell wall components in each developmental stage and a comparative transcriptomic analysis will be performed in order to evaluate the expression level of putative AGP-like genes predominantly and preferentially expressed in cork oak female flowers.

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