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DISTRIBUTION OF ARABINOGALACTAN PROTEINS DURING MICROSPOROGENESIS IN THE ANTHER OF *BELLIS PERENNIS* L. (ASTERACEAE)

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Using monoclonal antibodies (mAbs) JIM13, JIM15 and MAC207, we investigated the temporal and spatial distribution of some arabinogalactan protein (AGP) epitopes in cells of the *Bellis perennis* L. anther at different developmental stages. AGP epitopes recognized by JIM13 were detected in the protoplasts of tapetal cells, dividing microsporocytes, and microspores; AGP epitopes recognized by JIM15 were present in the cytoplasm of tapetal cells only at the stage with tetrads of microspores in the anther loculus. AGP epitopes recognized by MAC207 were present in the cells of different somatic tissues of the flower bud, but after asymmetric mitosis in the microspore they appeared abundantly in the protoplasts of immature pollen and were still present in mature pollen grains. Callose, revealed by mAb, appeared at the same stage of microsporocyte division as AGPs labelled with JIM13 and JIM15. We discuss the differences in callose and AGP localization and the possible role of the latter during anther development.

Key words: Anther, arabinogalactan proteins, *Bellis perennis*, callose, microsporogenesis, pollen grains.

INTRODUCTION

Glycoproteins sculpt the surface of all eukaryotic cells (Tan et al., 2003). These compounds appear in numerous biological processes, playing diverse functions. Among them, hydroxyproline-rich glycoproteins (HRGPs) are the major surface glycoproteins in plant cells. These naturally and commonly occurring macromolecules have been found mainly in the extracellular matrix (Sommer-Knudsen et al., 1998; Gaspar et al., 2001). Highly glycosylated hydroxyproline-rich glycoproteins, an abundant and heterogeneous class, form a large family of arabinogalactan proteins (AGPs). Their chemical properties and composition are described in comprehensive reviews (Kreuger and van Holst, 1996; Showalter, 2001; Seifert and Roberts, 2007; Ellis et al., 2010).

Besides the biochemical and physical properties of AGPs, research has focused on genetic aspects of these glycoproteins (Coimbra et al., 2009; Costa et al., 2013). AGPs containing a domain responsible for attaching the protein to a glycosylphosphatidylinositol membrane anchor are commonly distributed in different organs of flowering plants and probably participate in all aspects of plant growth and development. This participation starts from a hitherto unclear or poorly known role in determining cell fate during early embryogenesis (Pennel et al., 1992; Kreuger and van Holst, 1993; Toonen et al., 1997; McCabe et al., 1997; Hall and Cannon, 2002), and continues throughout embryogenesis (Qin and Zhao, 2007; El-Tantawy et al., 2013). It is later implicated in plant development, including sexual reproduction.

AGPs have a specific pattern of localization in plant tissues or cells related to plant reproduction (for a review see Showalter, 2001): for example, in the stigma including stigma exudates and in transmitting tissues in styles (Du et al., 1994; Cruz-Garcia et al., 2005; Lee et al., 2008, 2009), pollen grains (Levitin et al., 2008) and pollen tubes (Li et al., 1995; Wu et al., 1995, 2000; Mollet, 2002; Pereira et al., 2006). AGPs have been found not only in many stigma exudates of angiosperms but also in

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the pollination drop of Taxus × media (O'Leary et al., 2004). The functions and evident involvement of AGPs in sexual plant reproduction have been demonstrated in a number of plant species including *Actinidia deliciosa*, *Amaranthus hypochondriacus*, *Catharanthus roseus*, *Lolium perenne* and *Larix decidua* (Coimbra and Salema, 1997; Coimbra and Duarte, 2003; Wiśniewska and Majewska-Sawka, 2006; Rafińska and Bednarska, 2010).

The highly selective labelling obtained with four AGP mAbs (JIM8, JIM13, MAC207, LM2) during *Arabidopsis thaliana* male and female gametophyte development suggests that some AGPs can be regarded as markers for gametophytic cell differentiation (Coimbra et al., 2007a, 2008). Moreover, there are evident differences in the distribution of specific AGP epitopes during both anther and ovule development. Immunolocalization of AGPs (and pectins) was performed in *Actinidia deliciosa* pollen (Abreu and Oliveira, 2004). The *A. thaliana* pollen tube growing in vitro and labelled by MAC207 showed the presence of AGPs all over the pollen tube wall (Coimbra et al., 2008). Those results on the distribution of AGPs during pollen development were confirmed by showing AGP gene expression in *A. thaliana* pollen grains and pollen tubes (Coimbra et al., 2007b). ImmunoTEM also confirmed immunodetection of the AGP epitopes that bind JIM13, JIM8 and LM2 antibodies during pollen exine formation in *Beta vulgaris* (Majewska-Sawka and Rodriguez-Garcia, 2006).

Arabinogalactan proteins have drawn great interest due to their wide use as gums and stabilizers in industry and their presumed action as immune response modulators (Pettolino et al., 2006). The numerous and varied roles of AGPs in a variety of processes in plants, including signaling, make them very frequent study objects as well (for references see Ellis et al., 2010; Tan et al., 2012). BIO OHIO software was recently developed with the aim of organizing the genomic data for HRGPs and to facilitate and guide research (Showalter et al., 2010). Despite all the investigations of AGPs there are still a host of issues that are unclear or insufficiently studied, especially those concerning the participation of particular AGPs in plant reproduction. We studied the temporal and spatial distribution of arabinogalactan protein epitopes during successive stages of anther development in *Bellis perennis* L. (Asteraceae). The results presented here focus mainly on microsporogenesis, the critical transitional period between sporophytic and gametophytic generations and probably the most crucial stage of anther development. We also address the co-localization of AGPs and callose during microsporogenesis in *B. perennis*.

MATERIAL AND METHODS

PLANT MATERIAL

The daisy *Bellis perennis* L. (Asteraceae) growing in a natural habitat near Lublin (eastern Poland) was the source of the study material. Buds and flowers at different developmental stages were collected twice during favorable weather in July and August 2009.

FIXATION AND EMBEDDING

Freshly collected buds and flowers (~50) at different stages of development were immediately fixed in 2.5% paraformaldehyde (P-6148, Sigma Aldrich) and 0.25% glutaraldehyde (G017, Ubichem) in 0.1 M phosphate-buffered saline (PBS, P4417, Sigma Aldrich), pH 7.2, placed in a vacuum for 2 h and then kept at 4°C overnight. Then the material was rinsed twice in 0.1 M phosphate buffer, pH 7.2. Dehydration was performed in an ethanol series (10–70% ethanol) and next in an acetone series (70–100% acetone) for 10 min at each concentration. Dehydrated samples were immersed for 24 h in acetone and LR white acrylic resin (L9774, Sigma Aldrich) mixtures at 3:1, 1:1 and 1:3 proportions successively, and twice in pure LR white acrylic resin. The resin-embedded material was placed in gelatin capsules filled with resin, closed, and left for 24 h at 55°C for polymerization.

For anatomical examination, semi-thin longitudinal sections of anthers cut using glass knives on a Reichert Ultracut S ultramicrotome were stained with 1% toluidine blue and observed under an optical microscope (Nikon Optiphot 2). Microphotographs were taken with a digital camera (Canon Power Shot A640).

IMMUNOLABELLING OF AGPS

Semi-thin sections (2 μm) were mounted on POLYSINE® glass slides (P0425, Meinzel-Gläser, Braunschweig, Germany) to prevent section loss and dried at room temperature. Then the sections were hydrated with distilled water twice for 15 min and washed for 30 min with blocking solution: 1% BSA (bovine serum albumin, O5479, Sigma Aldrich) in 0.2 M PBS, pH 7.4. After that the sections were incubated with primary rat monoclonal antibody diluted 1:50 in 0.1% BSA in PBS (pH 7.4) overnight in a humid chamber at 4°C. After washing in PBS (4 × 20 min) the sections were incubated with secondary rabbit anti-rat FITC (fluorescein isothiocyanate, F5262, Sigma Aldrich)-conjugated antibody (whole molecule) diluted 1:200 with 0.1% BSA in 0.2. M PBS (pH 7.4) overnight in a humid dark

chamber at 4°C. The secondary anti-rat FITC-conjugated antibodies were obtained from Sigma Immuno Chemicals, Saint Louis, USA. The slides were washed in PBS (2 × 15 min) and next in distilled water (5 × 10 min). All manipulations were carried out in darkness. Finally, the sections were mounted in a drop of fluorescent mounting medium (The Binding Site, Ltd.) and immediately examined under a fluorescence microscope (Nikon Labophot 2) with a B2A filter (EX 450–490, DM 505, BA 520).

The monoclonal antibodies used were JIM13 recognizing epitopes on arabinogalactan proteins containing the trisaccharide β-D-GlcA-(1,3)-α-D-GalA(1,2)-α-L-Rha (Knox et al., 1991; Yates et al., 1996), JIM15 (epitope not determined, according to CarboSource and Nguema-Ona et al., 2012), and MAC207 recognizing the epitope β-GlcA-(1,3)-α-GalA(1,2)-Rha (Yates et al., 1996). The monoclonal antibodies were developed by the Complex Carbohydrate Research Center at the University of Georgia, USA.

IMMUNOLABELLING OF CALLOSE

The monoclonal antibody to (1,3)-β-glucan was used to label callose, and the mouse IgG Kappa light (Biosupplies, Australia) recognizes linear (1,3)-β-oligosaccharide segments in (1,3)-β-glucans; it shows no cross-reactivity with (1,4)-β-glucan or (1,3;1,4)-β-D-glucan (Meikle et al., 1991).

Immunolabelling was performed as described for JIM13, JIM15 and MAC207, but the primary antibody was diluted to obtain a final concentration of 10 μg/ml.

CONTROL REACTIONS

Controls were made by omitting incubation with the primary antibody or by preincubating the monoclonal antibody with laminarin (100 μg/ml) to block its binding sites (Meikle et al., 1991). The material was also checked for autofluorescence.

RESULTS

IMMUNOLABELLING OF AGPS WITH JIM13 IN *BELLIS PERENNIS* ANTHERS

An evident symptom of anther tissue differentiation in *B. perennis* is size increase of archesporial cells. The gradually enlarging archesporial cells become markedly bigger, with large, regular-shaped nuclei located in the center, but their features are still meristematic (Fig. 1a). These cells are surrounded by three and later four well-distinguished layers consisting of elongated undifferentiated cells, which will form the tapetum, median layer, endothecium and epidermis.

At the early stages of anther development, epitopes of AGPs recognized by JIM13 were not detectable by immunofluorescence. Epitopes recognized by JIM13 were first detected at early prophase of the first meiotic division in the cell walls of differentiated microsporocytes (Fig. 1b). During the further course of the anther development the cell walls of such microsporocytes were clearly labelled with JIM13 (Fig. 1d). At the same stage of anther development the tapetal cells became differentiated and differed markedly from other cells in the anther wall (Fig. 1c). They were considerably bigger and irregularly shaped, with dense cytoplasm, and vacuoles were not apparent. The noticeably bigger meiotic cells became rounder but still adhered tightly to each other and to the tapetum layer. Their cytoplasm was less dense than the cytoplasm of the tapetal cells. The narrow layer of tapetal cells surrounding and adjacent to the microsporocytes showed weak fluorescence after JIM13 application. Other tissues of developing anthers and the whole flower bud remained unlabelled with JIM13 (Fig. 1d).

During further stages of microsporogenesis, dyads were formed after the first meiotic divisions, and finally tetrahedrally arranged tetrads of microspores were formed after second meiotic divisions and simultaneous cytokinesis (Fig. 1e,g). After cytokinesis the dyads and microspores were both still surrounded by a common thick callose wall (Fig. 1e). The tapetal layer underwent further differentiation during microsporogenesis in the anther loculus. The tapetal cells enlarged and flattened out and had very dense cytoplasm (Fig. 1e). The epitopes recognized by JIM13 were located in the thick cell walls surrounding the tetrads of microspores during and sometime after simultaneous cytokinesis. These epitopes were also uniformly distributed in the cytoplasm of dividing cells as well as in tapetal cells (Fig. 1f). The AGP epitopes recognized by JIM13 were not revealed in the cell walls newly formed during cytokinesis and separating the four microspores in the tetrad, nor in the sporoderm forming on the surface of the released microspores. At the same time, the fluorescence of tapetal cells after application of JIM13 gradually diminished and finally disappeared; it was noticeably weaker at the stage with tetrads of microspores in the anther loculus (Fig. 1h).

During the initial process of microgametogenesis, the localization of the AGP epitopes recognized by JIM13 changed considerably. The fluorescence of AGP molecules binding to JIM13 persisted in the cytoplasm of differentiating microspores and the tapetal cells, although the labelling was very weak (Fig. 1i), but gradually disappeared from the binu-

cleate pollen grains (Fig. 1j) and was not observed in the vegetative or generative cells (Fig. 1 k). In mature three-nucleate pollen grains, fluorescence of the cell walls after immunolabelling with JIM13 was no longer observed. At this stage as well as in the control reaction, the sporoderm of pollen grains gave very weak autofluorescence which was not masked by immunolabelling as it was at earlier stages of pollen grain development.

IMMUNOLABELLING OF AGPS WITH JIM15 IN ANTHERS

Similarly to the AGPs revealed by JIM13, the specific AGP epitopes binding JIM15 gave no fluorescence in early premeiosis. Very strong fluorescence was visible at the beginning of the first meiotic division in the cell walls of microsporocytes. In longitudinal section the flower bud usually showed several groups of microsporocytes at the same stages of development (Fig. 2a). All of them were surrounded by strong fluorescence in cell walls containing specific AGP epitopes revealed after JIM15 application. The AGP deposits formed a continuous layer around each microsporocyte. JIM15-binding epitopes were manifested during the entire course of microsporogenesis and were located in the cell walls surrounding dyads (Fig. 2b) and tetrads of microspores before cytokinesis (Fig. 2c) and after cytokinesis (Fig. 2d). JIM15-recognized AGP epitopes were detected until completion of cytokinesis only in the common wall surrounding the tetrads of microspores (Fig. 2e). At this time, small particles of intensely labelled material appeared on the surface of tapetal cells. This material labelled with JIM15 was also located in the loculus, but was accumulated in larger amounts closer to the tapetal cells, whose protoplasts showed subtle fluorescence, indicating a low level of AGPs (Fig. 2e, insert).

After release of microspores from the common wall, JIM15-recognized AGP epitopes were detected as very weak fluorescence which remained in the microspore walls and in tapetal remnants in the anther loculus (Fig. 2f). Fluorescence disappeared gradually. The control reaction omitting the primary antibody showed a lack of AGP-specific fluorescence (Fig. 2g,h). As seen in Figure 2g, only the sporoderm of pollen grains gave autofluorescence.

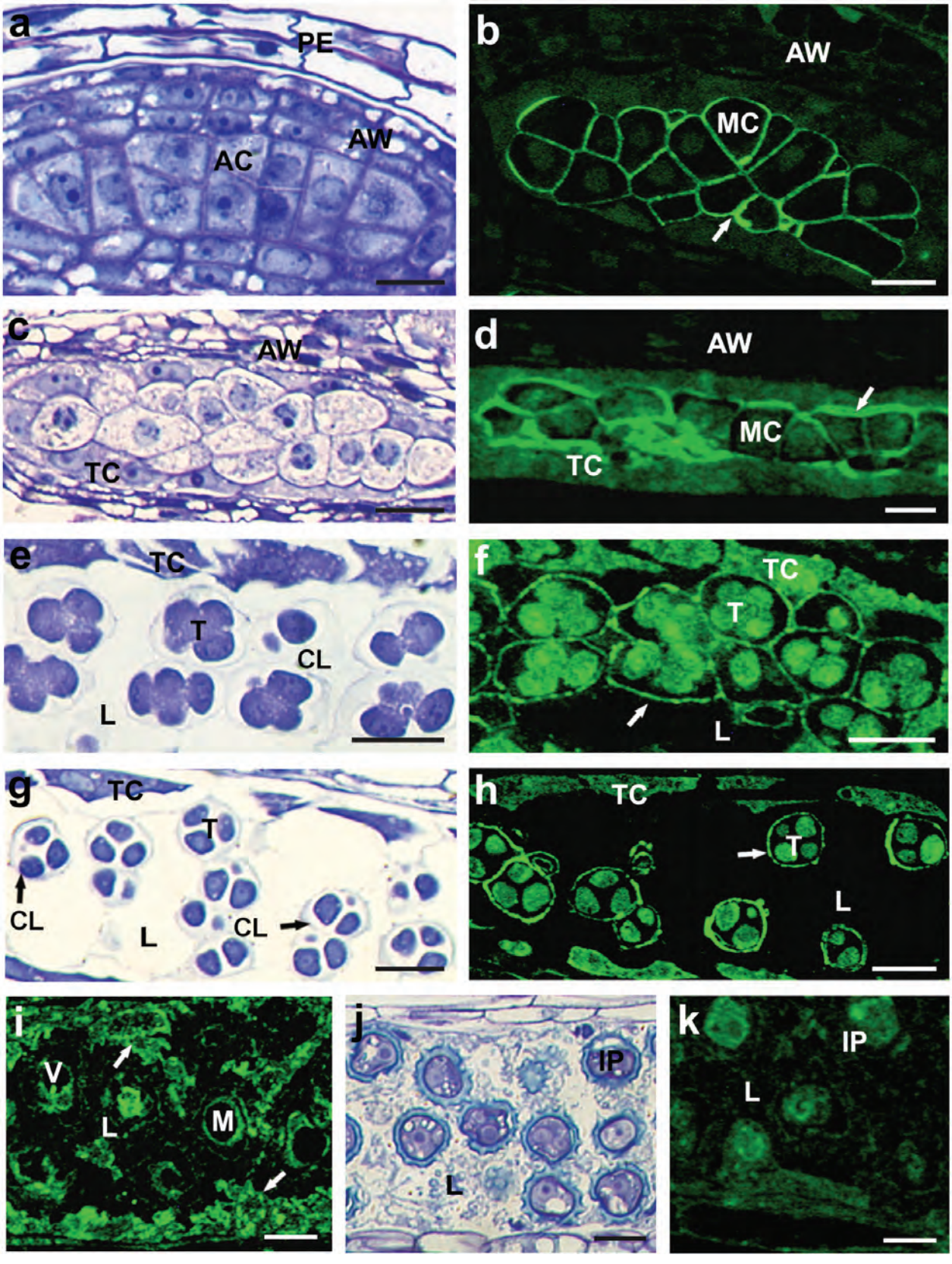
MAC207 IMMUNOLABELLING OF AGPS IN ANTHERS

In both microsporogenesis and microgametogenesis the results using MAC207 were the reverse of the results using JIM13 and JIM15. In early stages of development, AGP sugar epitopes recognized by MAC207 were scattered in all floral tissues, with comparatively low intensity and mainly in cytoplasm (Fig. 3a). At the beginning of meiotic division, MAC207-recognized AGP epitopes were uniformly distributed within the cytoplasm of polygonal microsporocytes (Fig. 3b). The distribution was similar in the cytoplasm of tapetal cells and the other cells of the anther wall. AGP epitopes were absent from cells of the anther and floral parts. In the further course of microsporogenesis no signal was observed in cell walls. AGP epitopes recognized by MAC207 were still present in other floral tissues, including the developing style (Fig. 3c,d). Such immunolabelling with MAC207 persisted in the anther loculus to the end of microsporogenesis. The MAC207 signal appeared again conspicuously in the cytoplasm of maturing pollen grains. Fluorescence showed in the cytoplasm of vegetative cells of mature pollen grains, excluding the vegetative nucleus, but not in their generative cells (Fig. 3e,f).

IMMUNOLABELLING OF CALLOSE IN ANTHERS

The use of mAb to (1,3)-β-glucan for the most precise localization of callose in the cells of *B. perennis* anthers showed a lack of fluorescence in the archesporial and anther cell walls at premeiotic stages

Fig. 1. Localization of AGP epitopes recognized by mAb JIM13 in *B. perennis* anthers during microsporogenesis and pollen grains in the loculus. Longitudinal sections. a, c, e, g, j – structure of anther during successive stages of development, toluidine blue staining. b, d, f, h, i, k – immunolabelling. Bars = 20 μm. (**a**) Longitudinal section of anther in premeiotic period. Note the archesporial cells surrounded by 3 (at left) or 4 (at right) layers of elongated undifferentiated cells of the anther parietal layer, (**b**) Early prophase – bright fluorescence of cell walls (arrow) surrounding differentiated microsporocytes, (**c**) Anther at stage of development later than that shown in (a), with differentiated layers of anther wall, (**d**) Fluorescence of microsporocytes: intensive in cell walls (arrow) and less intensive in microsporocyte cytoplasm. Fluorescence in narrow layer of tapetum adjacent to microsporocytes, (**e**) Newly formed tetrads before cytokinesis in anther loculus surrounded by degenerating tapetal cells, (**f**) Intensive fluorescence of cell walls (arrow) around newly formed tetrads of microspores and at microspore protoplast and tapetal cells, (**g**) Microspores after simultaneous cytokinesis (still surrounded by common callose walls) in anther loculus, (**h**) Fluorescence of cell walls (arrow) around each tetrad of microspores and within each microspore, and remnants of tapetal cells. Note the absence of fluorescence in newly formed cell walls separating microspores in tetrads, (**i**) Microspores at vacuolization stage. Fluorescence of all anther tissues and microspore in anther loculus. Arrows indicate disintegrating tapetal cells, (**j, k**) Sections of anther fragments with immature binucleate pollen grains (IP) in loculus (L). All cells in (**k**) show very weak fluorescence. AC – archesporial cells; AW – anther wall; CL – callose wall; PE – petal; MC – microsporocyte; TC – tapetum; L – loculus; T – tetrad of microspores; M – microspore; V – vacuole; IP – binucleate pollen grain.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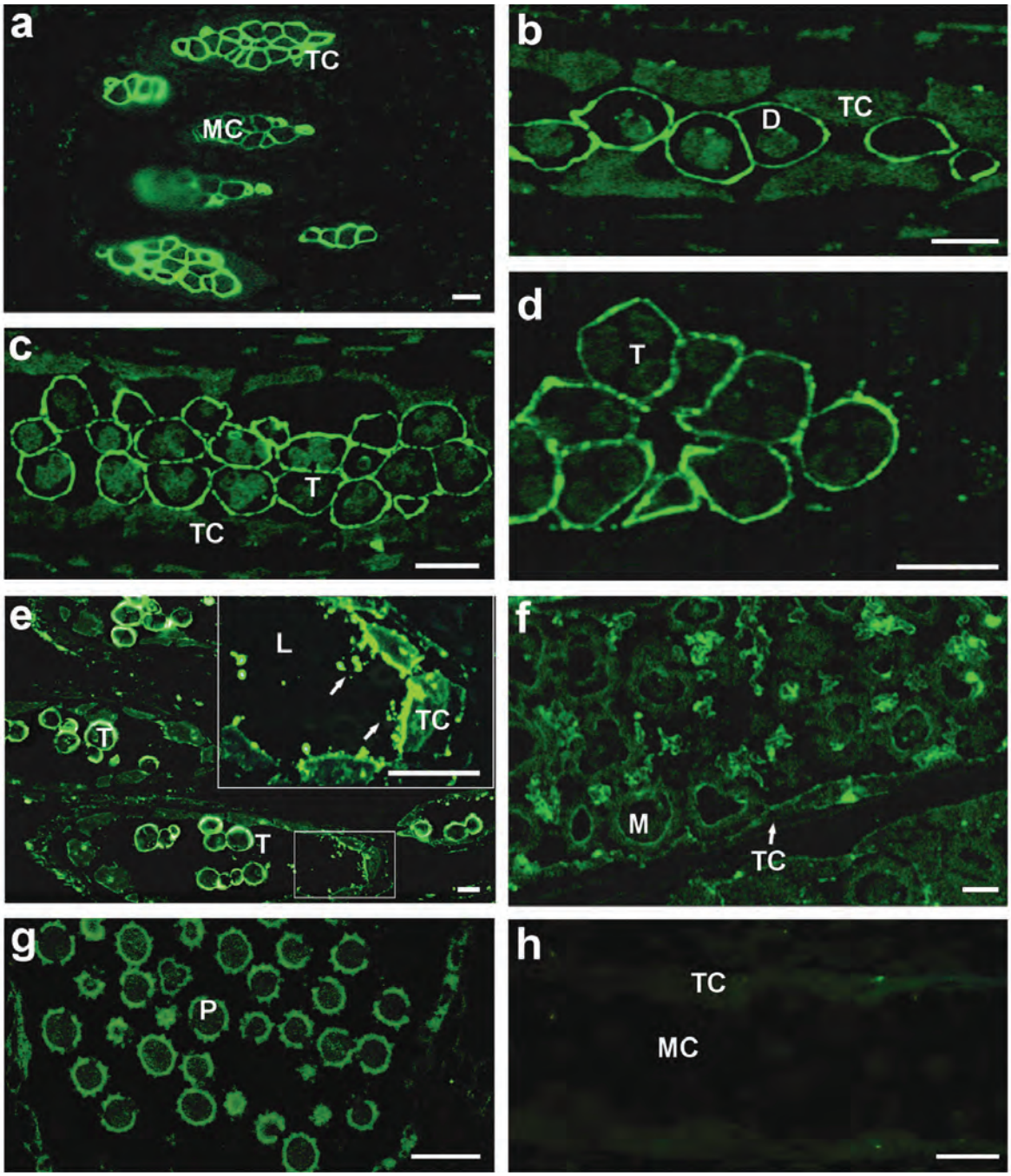

Fig. 2. Immunolocalization of AGP epitopes recognized by mAb JIM15 in *B. perennis* anthers during microsporogenesis. Longitudinal sections. Bars = 20 μm. (**a**) Early prophase of first meiotic division. Fluorescence of microsporocyte cell walls. Note the lack of fluorescence in cytoplasm, (**b**) Fluorescence of walls surrounding dyads, (**c**) Fluorescence of walls surrounding tetrads of nuclei, (**d**) An older stage than that in (c); Fluorescence of walls surrounding tetrads of nuclei or microspores after simultaneous cytokinesis. Note the irregular thickness of the fluorescent part of cell walls and lack of fluorescence in newly formed cell walls of microspores shown in (d), (**e**) Fragment of anther with fluorescence in common walls of microspore tetrads in loculus. Note the weak fluorescence in cell walls of anther wall and stronger fluorescence in tapetal cells. Intensely labelled material in loculus (arrows, insert), (**f**) Weak fluorescence of microspore walls and remnants of tapetal cells in loculus (arrow), (**g, h**) Control reaction at stage of mature pollen grain in loculus (**g**) and at prophase of first meiotic division (**h**). Note the autofluorescence of pollen grain sporoderm (g) and the complete lack of fluorescence at the stage shown in (h). MC – microsporocyte; TC – tapetum; D – dyad; L – loculus; T – tetrad of microspores; M – microspore; P – pollen grain.

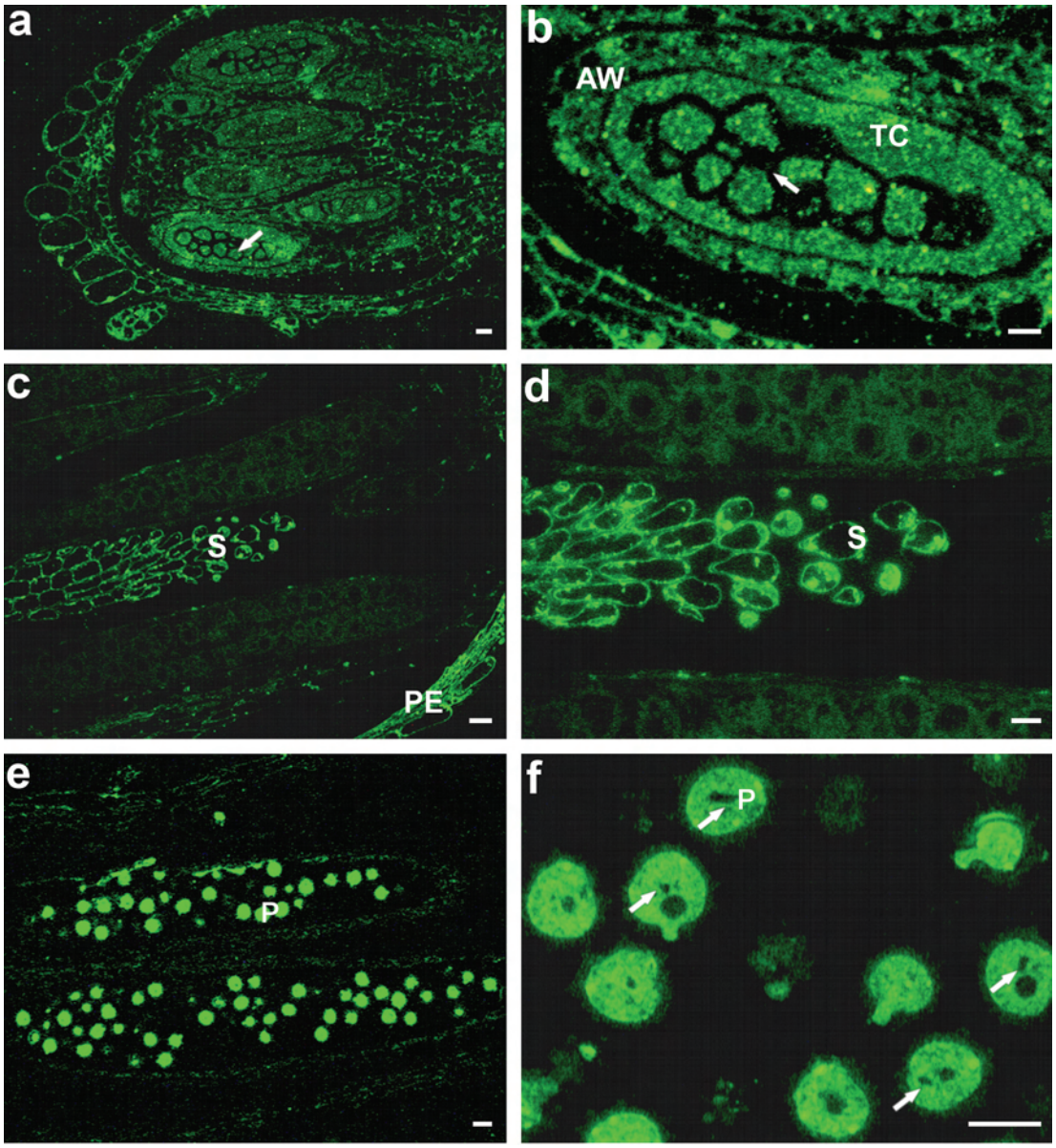

Fig. 3. Immunolabelling of *B. perennis* flower bud with mAb MAC207. Longitudinal sections. Bars = 20 μm. (**a, b**) Flower bud cells – signal is present in cell walls of somatic tissue and in all cells building the anther. Fluorescence of microsporocytes inside its loculus (arrow in b). Note the lack of fluorescence in cell walls of microsporocytes, (**c, d**) Fluorescence of cell walls and cytoplasm of whole cells of progenic tissue and fragments of petal (PE) and stigma (S). Higher magnification shows fluorescence of whole cells except vacuoles (d), (**e, f**) Fluorescence of mature pollen grains (P) within closed anthers. Higher magnification of pollen grains shows the lack of fluorescence in generative cell (arrows), nuclei of vegetative cell, and pollen grain wall. AW – anther wall; TC – tapetum; P – mature pollen grain; PE – petal; S – stigma.

(Fig. 4a,b). First, bright dotted fluorescence appeared in the cell walls surrounding differentiated prophase microsporocytes (Fig. 4d), visible in Figure 4c after toluidine blue staining. Callose deposition fluoresced only in small areas of such microsporocyte cell walls (Fig. 4d,e). As indicated by fluorescence, callose deposition varied in thickness and evidently appeared asynchronously. Some of the microsporocytes showed no fluorescence in the cell wall, while others fluoresced almost throughout the cell wall after mAb application, as in the cell arrowed in Figure 4e

Fluorescence in microsporocyte cell walls after treatment with mAb persisted through the first and

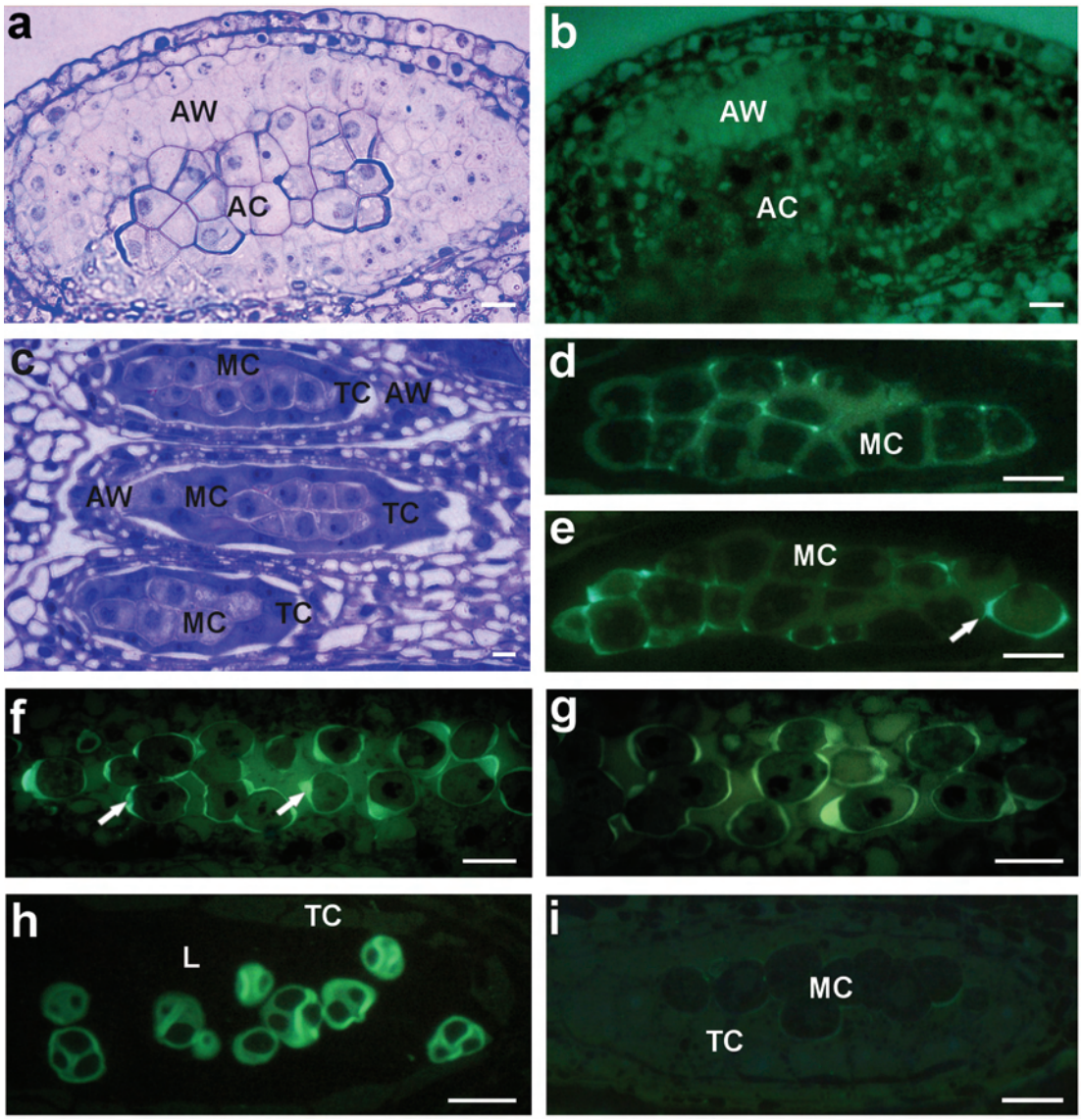

Fig. 4. Localization of callose epitopes recognized by mAb in anthers of *B. perennis* during microsporogenesis in loculus. Longitudinal sections. a, c – structure of anther during early stages of development, toluidine blue staining. b, d–i – immunolabelling; Bars = 20 μm. (**a, b**) Anther at pre-meiotic period of microsporogenesis. Note lack of fluorescence in archesporial and anther wall cells after treatment with mAb (b), (**c**) Anthers at stage with early prophase microsporocytes in loculus surrounded by differentiated layers of anther wall, (**d, e**) Bright, mainly limited to spot fluorescence in cell walls surrounding differentiated microsporocytes. Note the fluorescence of almost the entire microsporocyte cell wall (arrow in e), (**f**) Fluorescence of microsporocytes during first meiotic division; intensive in some parts of cell walls (arrows). Note the lack of fluorescence in cells of tissue adjacent to microsporocytes, (**g**) Fluorescence in wall around dyads, (**h**) Tetrads of haploid microspores. Intensive fluorescence of walls surrounding all newly formed microspores in tetrad and separating them, (**i**) Control reaction. Prophase microsporocytes in anther loculus and other cells of anther show no fluorescence. AC – archesporial cells; AW – anther wall; MC – microsporocytes; TC – tapetum; L – loculus.

second meiotic divisions (Fig. 4f,g). It indicated the presence of callose as a continuous layer around each of the dividing microsporocytes. Such layers are irregular and show considerable variation of the thickness and shape of individual microsporocytes. The tetrads of haploid microspores formed as a result of simultaneous cytokinesis showed intensive fluorescence of the cell walls surrounding and separating the newly formed tetrads of microspores (Fig. 4 h). Control reactions omitting the first antibody showed no fluorescence at any stage of anther development (Fig. 4i).

DISCUSSION

We employed mAbs JIM13, JIM15 and MAC207 to determine the distribution of AGPs during development of the *Bellis perennis* anther. These antibodies are commonly used in plant biology research and have been characterized and used to reveal AGP epitopes in different plant organs (Showalter, 2001). JIM13 and JIM15 were characterized and used decades ago (Knox et al., 1991), as was MAC207 (Pennell et al., 1989).

Immunolocalization of specific AGP epitopes recognized by three different mAbs (JIM13, JIM15, MAC207) in developing *B. perennis* anthers showed clear differences in the temporal and spatial distribution of AGPs, evident in both the generative line investigated and in somatic cells and tissues. The general pattern of AGP distribution revealed by JIM13 and JIM15 in specimens of daisy anther tissue is similar to that obtained in *Arabidopsis thaliana* using JIM8 and JIM13 (Coimbra et al., 2007a). The spatial distribution of AGPs we found at the different developmental stages of *B. perennis* anthers leaves no doubt that the AGPs revealed by JIM13 and by JIM15 can be used as specific molecular markers for cells or tissues strictly connected with reproduction processes. There are certain resemblances between *B. perennis* and *A. thaliana* in the distribution of AGPs in anthers but differences between those species regarding the presence of AGPs in nongenerative parts of the anther. For example, in *B. perennis* the epitopes recognized by JIM13 showed first in the cell walls of microsporocytes, while AGP labelling with the same mAb in *A. thaliana* appeared first in epitopes located preferentially in the median layer. The results using JIM13 and JIM15 in daisy anther did not show any labelling of the endothecium, unlike in *A. thaliana*. The use of MAC207 did not reveal AGP epitopes in any cells undergoing meiosis or generative and sperm cells in pollen grains of *B. perennis*. The chemical composition of the generative cell wall awaits further detailed study, especially the roles of AGPs, which are still not clear in cells that are extremely important in plant reproduction (Qin et al., 2007).

A comparison of our results for the *B. perennis* anther with data for the anther or other organs such as ovules in different plants (Chudzik et al., 2005) indicates that species differ in the temporal and spatial distribution of AGPs. This is supported by work on immunolocalization of AGPs during male and female gametophyte development in Virginia fanpetals *Sida hermaphrodita* (Chudzik et al., 2010). Other results on the presence of AGPs in reproductive organs clearly point to the role of these glycoproteins in reproductive growth and development. For example, AGPs are essential for initiation of female gametogenesis in *Arabidopsis* (Acosta-Garcia and Vielle-Calzada, 2004) and are required for stamen and pollen function (Levitin et al., 2008). In *Lolium perenne*, carbohydrate epitopes of AGPs that react with JIM13 are present within microspores, but anther wall layers are devoid of these glycoproteins (Wiśniewska and Majewska-Sawka, 2006).

Similarly to the AGP epitopes recognized by JIM13, AGP epitopes recognized by JIM15 in the *B. perennis* anther were revealed in the thin cell walls surrounding microsporocytes and were visible until the end of meiosis in the thick cell walls surrounding the four newly formed microspores. These AGPs were not found in the cytoplasm of the tapetal cells or any other cells of the anther during the whole course of microsporogenesis in the loculus. Their appearance in tapetal cell cytoplasm at a comparatively low level coincided in time with the presence of microspore tetrads in the anther loculus, prior to the release of microspores from the tetrads. Except for the latter event, the occurrence of AGPs recognized by JIM15 was strictly limited to the dividing microsporocytes and the tetrad of microspores. Therefore we can suggest the novel idea that AGPs recognized by JIM15 in the *B. perennis* anther are better or more precise markers of male generative line cells than those recognized by JIM13. Due to a lack of data, however, we cannot extend our supposition to species other than *B. perennis*.

Besides the presence of AGPs in the extracellular matrix, we showed the presence of JIM13-recognized AGPs in the cytoplasm of dividing microsporocytes, microspores and tapetal cells. That result is in line with data reported for *A. thaliana* (Coimbra et al., 2007a). In *Nicotiana tabacum*, Qin et al. (2007) found AGPs in the protoplasts of microsporocytes, microspores and tapetal cells. Using immunogold labelling they observed accumulation of AGPs recognized by JIM13 in the whole content of the pollen tube except for the nucleus; besides the pollen tube surface, AGPs were abundantly distributed in the cytoplasm, vacuoles and vesicles. Qin and Zhao (2006) found AGPs in the cytoplasm of the egg cell and proembryo of *N. tabacum*. Rafińska and Bednarska (2010) described the presence of AGP epitopes recognized by JIM8 and JIM13, though at a comparatively low level, in the cytoplasm of larch megasporocytes; they associated the cytoplasmic distribution of these AGPs with the secretory compartments of the cell, such as the endoplasmic reticulum and Golgi apparatus, demonstrated earlier in cells that actively produce and secrete AGPs in *Drosera capensis* (Šamaj et al., 2000). Such an association is supported by the presence of AGPs in the protoplast of anther tapetal cells, which are responsible for secretion of structural and nutritive compounds to the anther loculus.

As described above, the localization of AGPs after JIM13 labelling was usually uniform in the cytoplasm of specific cells. Fluorescence of anther sections labelled with JIM15 was restricted mainly to the walls of specific cells; AGPs gave relatively weak fluorescence within the tapetal walls at only one of the anther developmental stages. At the same stage, bright dot-like material appeared on the surface of tapetal cells and inside the loculus of the *B. perennis* anther, and intensive synthesis of sporoderm components took place (Gabarayeva et al., 2010). Based on this observation we cannot suggest any participation of AGPs in sporoderm formation.

Unlike both of the AGP epitopes recognized by JIM13 and JIM15, the epitopes recognized by MAC207 are characteristic for somatic cells of the daisy anther. They were found in anthers only at the beginning of meiosis in the loculus and were distributed in the cytoplasm in different somatic cells or flower bud tissues. The results obtained using MAC207 did not show any defined or specific pattern of binding throughout the phases of microsporogenesis and pollen grain development.

In *B. perennis* the appearance of AGPs recognized by JIM13 and JIM15 coincides with the beginning of callose (β-1,3-glucan) deposition. This polysaccharide forms a wall around microsporocytes, is considered a prerequisite for meiosis, and further affects the proper course of microgametogenesis in flowering plants (Dong et al., 2005; Li et al., 2010; Rodriguez-Garcia and Majewska-Sawka, 2011). In daisy microsporocytes, initially the callose deposits were limited to small areas in the cell wall but later enveloped the whole dividing cell. It is well known that callose walls appear in other angiosperm microsporocytes at prophase of the first meiotic division (Majewska-Sawka and Rodriquez-Garcia 1999) or even prior to the start of meiosis (Wilson and Zhang, 2009). In the course of meiosis the amount of callose increases but it is degraded in order to release microspores from the tetrads (Chen and Kim, 2009). Our results show that AGPs are maintained in the cell walls surrounding dividing microsporocytes and in the thick, massive, common wall enveloping all four microspores in the tetrads. At that time the location of AGPs coincides with callose deposits, but in the tetrads formed after simultaneous cytokinesis, callose is also deposited in the newly formed cell walls separating microspores, while AGPs are not revealed there. Up to now, co-localization of AGP and callose has been observed only with the use of JIM13. Such co-localization was reported for plasmodesmata (Šamaj et al 1998), pollen tubes (Roy et al., 1998), and tetrads of microspores (Otegui and Staehelin, 2004; Majewska-Sawka and Rodriguez-Garcia, 2006). Although co-localization of some AGPs and callose has been observed in different plant cells and organs as mentioned above, the interrelations between the occurrence of callose and AGPs during microsporogenesis need to be clarified by extensive studies using other plant species.

AUTHORS' CONTRIBUTIONS

SE design, planning and supervision, writing the manuscript; CB performing the immunocytochemical reactions and participation in manuscript preparation; ZB collecting plants, preparing samples and sections; LA preparing figures. All authors participated in data analysis and discussion of results. All authors read and approved the final manuscript.

The authors declare that they have no conflicts of interests.

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