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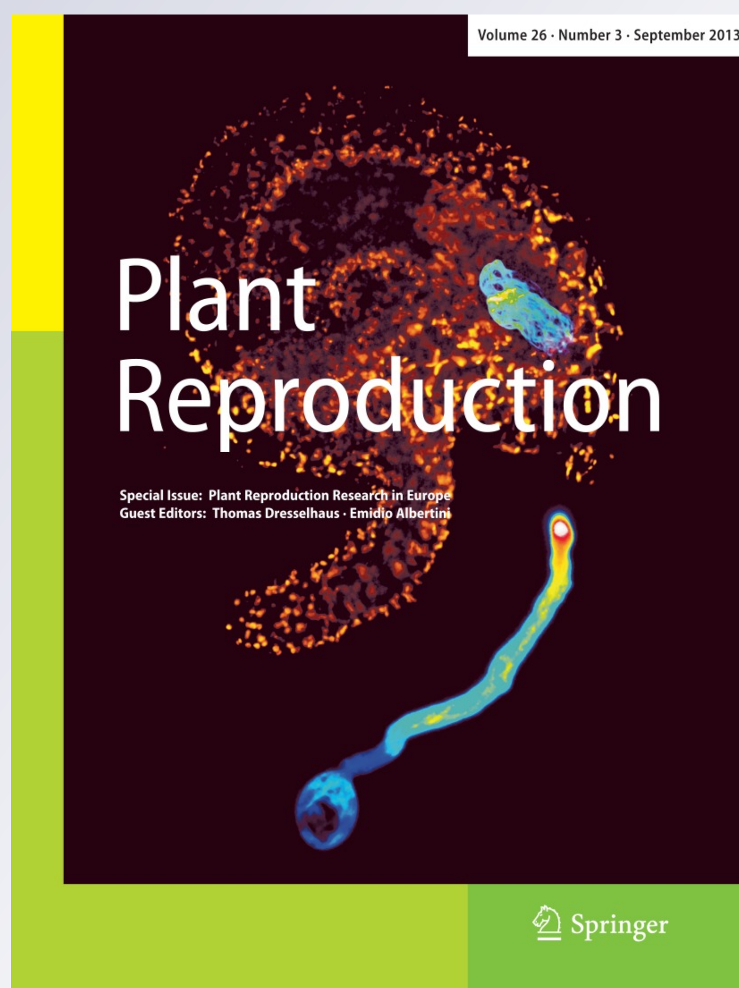

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ORIGINAL ARTICLE

Arabinogalactan protein profiles and distribution patterns during microspore embryogenesis and pollen development in *Brassica napus*

Ahmed-Abdalla El-Tantawy · María-Teresa Solís · Mario L. Da Costa · Silvia Coimbra · María-Carmen Risueño · Pilar S. Testillano

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Abstract Arabinogalactan proteins (AGPs), present in cell walls, plasma membranes and extracellular secretions, are massively glycosylated hydroxyproline-rich proteins that play a key role in several plant developmental processes. After stress treatment, microspores cultured in vitro can reprogramme and change their gametophytic developmental pathways towards embryogenesis, thereby producing embryos which can further give rise to haploid and double haploid plants, important biotechnological tools in plant breeding. Microspore embryogenesis constitutes a convenient system for studying the mechanisms underlying cell reprogramming and embryo formation. In this work, the dynamics of both AGP presence and distribution were studied during pollen development and microspore embryogenesis in *Brassica napus*, by employing a multidisciplinary approach using monoclonal antibodies for AGPs (LM2, LM6, JIM13, JIM14, MAC207) and analysing the expression pattern of the *BnAGP Sta 39–4* gene. Results showed the developmental regulation and defined localization of the studied AGP epitopes during the two microspore developmental pathways, revealing different distribution patterns for AGPs with different antigenic reactivity. AGPs recognized by JIM13, JIM14 and MAC207 antibodies were related to pollen maturation, whereas AGPs labelled by LM2 and LM6 were associated with embryo development. Interestingly, the AGPs labelled by JIM13 and JIM14 were induced with the change of microspore fate. Increases in the expression of the *Sta 39–4* gene, JIM13 and JIM14 epitopes found specifically in 2–4 cell stage embryo cell walls, suggested that AGPs are early molecular markers of microspore embryogenesis. Later, LM2 and LM6 antigens increased progressively with embryo development and localized on cell walls and cytoplasmic spots, suggesting an active production and secretion of AGPs during in vitro embryo formation. These results give new insights into the involvement of AGPs as potential regulating/signalling molecules in microspore reprogramming and embryogenesis.

Keywords Microspore culture · Cell wall · *Sta39-4* gene · *Brassica napus* · AGP epitopes

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Introduction

Arabinogalactan proteins (AGPs) with a key role in several plant developmental processes (reviewed in Seifert and Roberts 2007) are massively glycosylated hydroxyproline-rich glycoproteins that can be found in cell walls, plasma membranes and extracellular secretions. AGPs present a high degree of heterogeneity not only with respect to their protein part but also in their carbohydrate sequences and composition, which have been analysed by a combination of chemical studies and the use of antibodies (Knox 1997). In fact, one of the main tools in the study of AGPs has been

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the use of monoclonal antibodies that bind to AGP-specific sugar epitopes. The setting-up of monoclonal antibodies directed against different AGP epitopes and cell wall polymers has facilitated the analysis of the complex cell wall structure and its dynamics during plant developmental processes. These anti-AGP monoclonal antibodies have been used to unravel the involvement of specific epitopes in controlling cell growth and morphogenesis. Increasing evidence has linked AGPs to many processes involved in plant growth and development, including somatic embryogenesis (Chapman et al. 2000; Thompson and Knox 1998; van Hengel et al. 2001), pollen grain development (Coimbra et al. 2009; Levitin et al. 2008; Pereira et al. 2006) and pollen tube growth (Costa et al. 2013; Cheung et al. 1995; Wu et al. 2001). Despite information gained in recent years with respect to AGPs, their precise functions have not yet been elucidated (for review, see Seifert and Roberts 2007).

Microspore embryogenesis constitutes an intriguing system in which a cell, namely the microspore, has its gametophytic programme redirected towards an embryogenic pathway. After specific stress treatments in vitro, the microspore can reprogramme itself during specific developmental stages and initiate an embryogenesis programme that produces embryos from which haploid and double haploid plants, important biotechnological tools in plant breeding, can finally develop (review in Maluszynski et al. 2003). Isolated microspore cultures, in which microspores are separated from the anther tissues, constitute very convenient systems for studying the mechanisms underlying cell reprogramming and embryo formation. Although, in recent years, increasing amounts of information have been reported on the presence of genes and molecules controlling early embryogenic events, knowledge of the genetic control of the process and the possible involvement of external factors regulating embryo growth and development is still scarce.

It has frequently been hypothesized that AGPs are sources of soluble signal molecules in the form of sugar chain fragments (Johnson et al. 2003; Schultz et al. 1998). Previous findings have revealed changes in cell wall components associated with cell reprogramming, and many of the molecular markers of somatic embryogenesis have also been found in cell walls. Exogenous AGPs are known to affect somatic embryogenesis in different ways (Portillo et al. 2012), namely as stimulating factors for microspore embryogenesis (Yuan et al. 2012). Nevertheless, there is very little information on the presence and possible function of endogenous AGPs in microspore embryogenesis. On the other hand, it has been reported that by adding a Yariv reagent to the culture medium, a synthetic probe that binds to and aggregates AGPs, AGP action is blocked negatively, affecting embryogenesis (Tang et al. 2006) This would suggest that endogenous AGPs are involved in embryo development in vitro.

In this work, the presence and distribution of AGPs were studied during pollen development and in in vitro induced microspore embryogenesis in *Brassica napus,* by means of a multidisciplinary approach, combining immunocytochemical, biochemical and molecular techniques. Dot blot assays were carried out, along with immunofluorescence and confocal laser scanning microscopy (CLSM) analyses with several monoclonal antibodies for AGPs: JIM13, JIM14, MAC207, LM2 and LM6, the latter reacting with AGP arabinan epitopes which are also present in pectins. Analysis of the expression pattern of the *BnAGP Sta 39–4* gene (Gerster et al. 1996) by quantitative real-time PCR (qPCR) was also performed.

Results showed the developmental regulation and well-defined localization of the studied AGP epitopes during pollen development and microspore embryogenesis, which revealed different distribution patterns. The dynamics of specific AGP epitopes (JIM13, JIM14, MAC207) were related to pollen maturation, whereas other epitopes were associated with the change of the microspore developmental programme (JIM13, JIM14) and with microspore-derived embryo differentiation (LM2, LM6), suggesting AGPs as potential regulating/signalling molecules involved in these processes.

Materials and methods

Plant material and microspore culture

Brassica napus L. cv. Topas donor plants were grown under controlled conditions at 15 °C day, 16 h photoperiod and 10 °C night. Both isolated microspore culture and embryogenesis induction were performed by a 32 °C treatment, as described by Prem et al. (2012).

Fixation and processing for microscopic analysis

Fresh samples from different culture times and anthers from flower buds at different stages of pollen development were collected and fixed in 4 % paraformaldehyde in phosphate buffered saline (PBS), pH 7.3, overnight at 4 °C and washed in PBS. Isolated microspores and small proembryos were embedded in gelatin and processed as described by Solís et al. (2008). All samples were dehydrated in acetone series and embedded in Technovit 8100 resin (Kulzer, Germany) at 4 °C. semithin resin sections were placed on slides coated with APTES (3-aminopropyltriethoxysilane, Sigma) and stored at 4 °C until used for immunofluorescence. Some sections were stained with

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toluidine blue and examined under bright field microscopy for structural analysis.

Antibodies

The antibodies used in this study were rat monoclonal anti-AGPs: JIM13, JIM14, MAC207, LM2 and the anti-(1 → 5)-α-L-Arabinan LM6 (Plantprobes).

Immunofluorescence

Sections were blocked with 5 % bovine serum albumin (BSA) in PBS and incubated with the primary antibodies JIM13, JIM14, MAC207, LM6 and LM2 for 1 h in 1/5 dilution, except for LM2 which was used in 1/10 dilution in 1 % BSA in PBS. After washing in PBS, the signal was revealed with the Alexa Fluor 488-labelled anti-rat antibody (molecular probes) diluted 1/25 in PBS for 45 min in the dark, as described by Testillano et al. (2013). Finally, sections were counterstained with 1 mg/ml DAPI (4′0.6-diamidino-2-phenylindole) for 10 min and analysed in a laser scanning confocal microscope (TCS-SP5, Leica). Negative controls were obtained by replacing the primary antibody with PBS.

Immunodot blot assay

Proteins were extracted from 60 mg samples of cultures at different time points, and microspores and pollen grains were isolated from anthers according to sizes. Samples were homogenized in liquid nitrogen using a mortar and pestle, in 50 ml of buffer containing 50 mM Tris–HCl pH 7.2, 50 mM trans-1.2-diaminocyclohexane- *N*,*N*,*N*′*N*′-tetraacetic acid (CDTA) and 25 mM dithiothreitol. The resulting supernatant concentrations were determined according to Bradford (1976) (Bio-Rad Protein Assay reagent) using bovine serum albumin (BSA) as calibrator and all samples were adjusted to a concentration of 0.5 mg/ml. For immunodot blot assays, 5 µl aliquots of adjusted supernatants were applied to a nitrocellulose membrane (Millipore; Bedford, MA, United Kingdom) and left to dry for 1 h according to Bárány et al. (2010). Strips were first stained for total protein detection with Ponceau red, and the images of the stained dots were captured.

The membrane was incubated overnight at room temperature, with the primary antibodies (anti-AGPs JIM13, JIM14, MAC207, LM2 and the (1 → 5)-α-L-Arabinan LM6) diluted 1:100, except LM2 which was diluted 1:200, in the blocking buffer (2 % powdered skimmed milk containing 0.05 % Tween-20 in PBS), washed, and incubated for 1 h with alkaline phosphatase-conjugated anti-rat antibody diluted 1:1,000 in the blocking solution. Finally, the epitopes recognized by the antibodies were revealed by treatment with a nitroblue tetrazolium, bromo-chloroindolyl–phosphate (NBT–BCIP) mixture.

Quantification of the relative intensity of the dot blot signals was performed by using appropriate image analysis software. For the quantification, images of three replicates for each antibody and developmental stage were used. Mean values and standard deviations were calculated and the results showed in histograms. *P* values were calculated using Student's *t* test.

Quantitative real-time PCR (qPCR)

RNA was isolated from the different culture and pollen samples at the different stages analysed, according to Solís et al. (2012). One microgram of total RNA was used for the RT reaction using the Superscript TM II reverse transcriptase enzyme (Invitrogen). The oligonucleotides used for Sta39-4 expression analysis were as follows: 5′ GGCA CCCTCAGCTGCTC 3′ and 3′ ATGGTCCATCAACAAC CTCTG 5′ from the sequence of the Sta39-4 gene (L47352.1), one of the first pollen-specific putative AGP genes to be characterized in *B. napus* (Gerster et al. 1996).

cDNA was amplified using SsoAdvanced^{TM}SYBR$^{®}$ Green supermix on an iQ^{TM}5 Real-Time PCR Detection System (Biorad). All qPCRs were run in duplicate. Thermocycle settings were as follows: Initial denaturation of 30 s at 95 °C, followed by forty cycles, each consisting of 5 s at 95 °C, 30 and 30 s at 56 °C. After each run, a dissociation curve was acquired to check for amplification specificity by heating the samples from 65 to 95 °C. Serial dilutions of cDNA were used to determine the efficiency curve of each primer pair according to Costa et al. (2013). β-tubulin (TUB) and glyceraldehyde-3-phosphate dehydrogenase 2 (GAPDH.2) were used as internal reference genes. At the end of the PCR cycles, the data were analysed with the Bio-Rad CFX Manager 3.0 (3.0.1224.1015) (Biorad), using the Livak calculation method (2001).

Results

Temporal AGP distribution patterns during microspore-pollen development and microspore embryogenesis revealed by different antibodies

To analyse the presence and variations in AGPs during the developmental processes studied, a set of five monoclonal antibodies against AGPs: LM2, LM6, JIM13, JIM14 and MAC207, were used for dot blot assays at selected key phases of both pathways, male gametophytic development and microspore embryogenesis. The selected stages of microspore-pollen development for analysis were "vacuolated microspore", "young pollen" and "mature pollen".

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The vacuolated microspore is the responsive developmental stage for induction of embryogenesis, characterized by a large cytoplasmic vacuole which pushes the nucleus towards a peripheral location (Fig. 1a). The vacuolated microspore exhibited the inner thin wall or intine, surrounded by the outer sporopollenin pollen wall, the exine. During gametophytic development, the vacuolated microspore underwent an asymmetric division leading to the formation of the young pollen grain (Fig. 1b) which is formed by the small generative cell inside the cytoplasm of the large vegetative cell. During later developmental stages, the generative cell divided forming the two sperm cells responsible for the double fertilization. The vegetative cell contains numerous starch granules and other storage products (Fig. 1c) which will be used as energetic and structural substrates during pollen tube growth and fertilization; this is the characteristic morphology of the mature pollen.

After the application of heat treatment for embryogenesis induction in vitro, the responsive vacuolated microspores divided symmetrically forming two-celled embryos (Fig. 1d) which subsequently gave rise, after several divisions, to early embryos (Fig. 1e), still surrounded by the exine. As embryogenesis progressed, the exine broke and embryo growth increased to form typical globular (Fig. 1f), heart-shaped and torpedo-shaped (Fig. 1g, h) embryos, structures which were found developing together in the same plates. Finally, cotyledonary mature embryos formed after approximately 30 days in vitro. The selected stages of microspore embryogenesis for analysis were “early embryos”, “globular and torpedo embryos” and “cotyledonary embryos”.

The results of the immunodot blot for the different antibodies on equal amounts of protein extracted from the selected developmental stages are shown in Figs. 2a, 3a and 4a. A relative quantification of the dot colour intensities for each stage and antibody was performed; results are illustrated in the histograms of Figs. 2b, 3b and 4b. The results identified three main labelling features which suggest three temporal AGP distribution patterns during microspore-pollen development and microspore embryogenesis. The AGP epitopes revealed by LM2 and LM6 antibodies increased progressively with microspore embryogenesis progression and reached their maximum level in cotyledonary embryos, while remaining scarce in microspore-pollen development (Fig. 2). Although the AGPs recognized by JIM13 and JIM14 antibodies showed low levels in microspores, they increased after microspore reprogramming in early embryos, but diminished during later stages of embryogenesis (Fig. 3). Conversely, JIM13 and JIM14 epitope levels were higher in mature pollen (Fig. 3). The MAC207 antibody showed a different temporal distribution pattern with low levels in microspores and all stages of microspore embryogenesis, but with a very high signal in mature pollen (Fig. 4).

The above results indicated that the microspore programme changes from gametophytic development to the embryogenic pathway involved variations in AGP expression. The different AGP epitopes showed different temporal patterns as follows: LM2 and LM6 epitopes were progressively induced during microspore embryogenesis progression (Fig. 2), JIM13 and JIM14 epitopes were induced only during early stages of embryogenesis and with pollen maturation (Fig. 3), and the MAC207 epitope was scarce during microspore embryogenesis but very abundant in the mature pollen stage (Fig. 4).

Subcellular localization of AGP epitopes during microspore-pollen development and microspore embryogenesis

Immunofluorescence and confocal analyses were performed on semithin resin sections of anthers and on in vitro microspore cultures at the selected “vacuolated microspore” and “mature pollen” developmental stages of microspore-pollen development (Fig. 1a, c). The same analyses were carried out on microspore embryogenesis during the “early embryo”, “globular and torpedo embryo” and “cotyledonary embryo” stages (Fig. 1d–h). Merged images of a fluorescent green signal for the epitope and a blue signal for DAPI-stained nuclei were captured, as well as DIC images of the same microscopic field in order to reveal the structure.

LM2 and LM6 antibody labelling on microspores and pollen grains was low. In vacuolated microspores, LM2 labelling was also very low or absent on the intine (Fig. 5a, a′), and in young and mature pollen, the labelling was not only very low but specifically localized on small regions of the generative cell wall (Fig. 5c, c′). A similar pattern of localization was observed for the LM6 antibody in vacuolated microspores (Fig. 5b, b′) and pollen grains (Fig. 5d, d′), but in this case, the labelling on the generative cell wall was higher (Fig. 5d′) though not specific to the wall. No significant labelling was found in other subcellular compartments.

Contrary to the above, during microspore embryogenesis, LM2 and LM6 labelling was higher. In early embryos with few cells, surrounded by the exine, labelling with both antibodies was intense on the walls of every embryo cell (Fig. 5e, e′, f, f′). During the later stages of embryogenesis, the LM6 antibody showed increasing immunofluorescence on the embryo cell walls of small globular, late globular and torpedo stage embryos (Fig. 5h, j, l). In the case of the LM2 antibody, though labelling was lower in cell walls, it was also found on small cytoplasmic spots, resembling secretory vesicles, which were abundant in small globular

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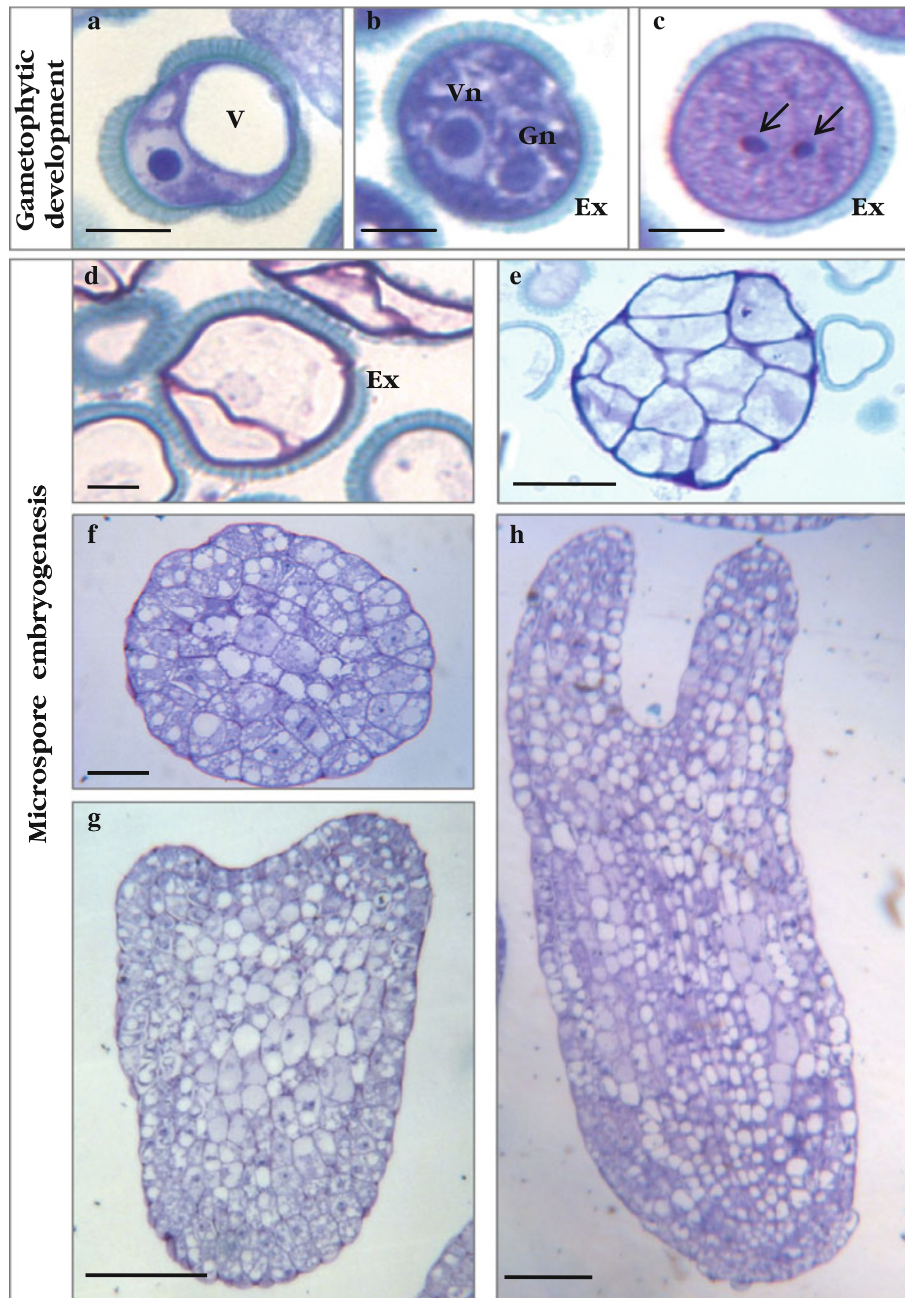

Fig. 1 Main stages of male gametophytic development and microspore embryogenesis. Semithin sections, Toluidine Blue staining. **a–c** Male gametophytic development in vivo. **d–h** Microspore embryogenesis in vitro. **a** Vacuolated microspore. **b** Young pollen. **c** Mature pollen. **d** Two-celled embryo surrounded by the exine. **e** Small globular embryo. **f** Large globular embryo. **g** Embryo at the heart-torpedo transition. **h** Late torpedo embryo. *Ex* exine, *V* vacuole, *Vn* vegetative nucleus, *Gn* generative nucleus, *arrows* in **c** point to sperm nuclei. *Bars* **a–d** 10 µm, **e**, **f** 20 µm, **g**, **h** 50 µm

(Fig. 5g), late globular, and heart and torpedo-shaped embryos (Fig. 5i). Cotyledonary embryos exhibited intense fluorescence signalling with both LM2 and LM6 antibodies (Fig. 5k, l).

The immunofluorescence assays with JIM13 and JIM14 antibodies showed a progressive increase in labelling as microspore-pollen developed, with low signalling on the intine of the vacuolated microspores (Fig. 6a, a′, b, b′) but higher signalling on mature pollen (Fig. 6c, c′, d, d′). However, JIM13 and JIM14 epitopes exhibited different localization patterns in mature pollen. The JIM13 antibody highly labelled the cytoplasm and wall of the generative and sperm cells (Fig. 6c, c′), while the vegetative cell remained unlabelled. In contrast, JIM14 labelling was

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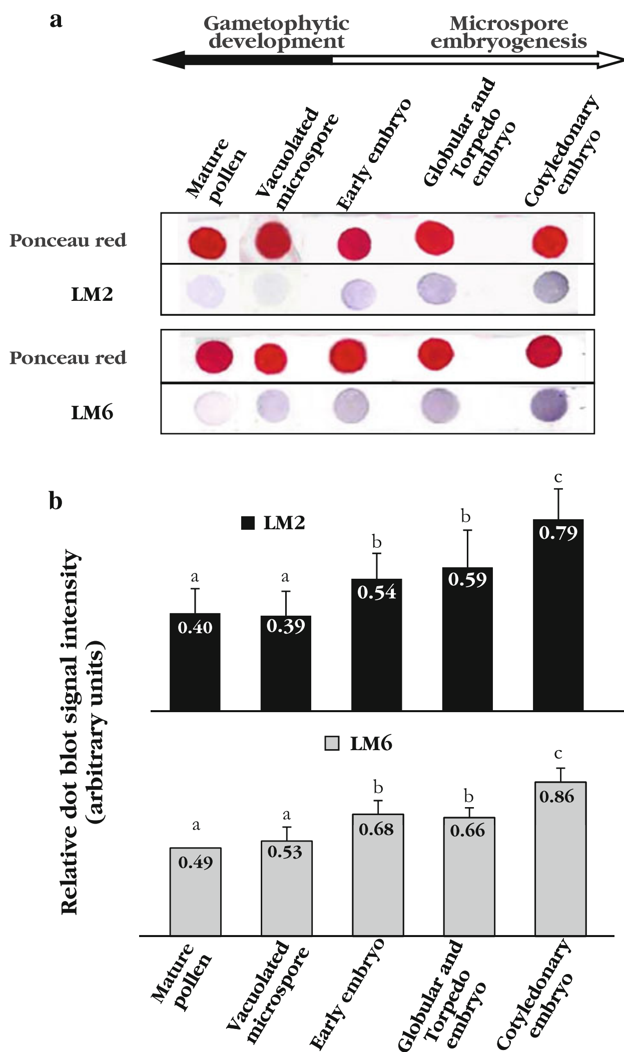

Fig. 2 Temporal distribution patterns of LM2 and LM6 epitopes during male gametophytic development and microspore embryogenesis. **a** Immunodot blot assays at different developmental stages of microspore-pollen development (vacuolated microspore and mature pollen) and microspore embryogenesis (early embryo, globular and torpedo embryo, cotyledonary embryo). Ponceau Red staining for total proteins and immunodot blot of the same strip is shown for each antibody. **b** Histograms representing the mean values of relative *dot colour* intensities in arbitrary units, *numbers in columns* indicate mean values. *Different letters* indicate significant differences at $P < 0.001$

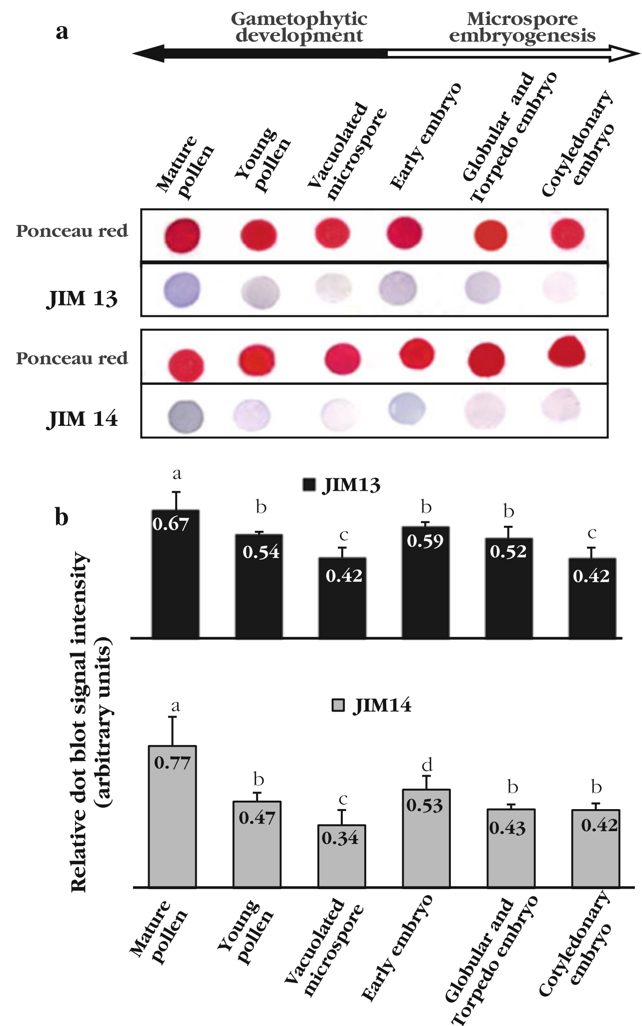

Fig. 3 Temporal distribution patterns of JIM13 and JIM14 epitopes during male gametophytic development and microspore embryogenesis. **a** Immunodot blot assays at different developmental stages of microspore-pollen development (vacuolated microspore, young pollen and mature pollen) and microspore embryogenesis (early embryo, globular and torpedo embryo, cotyledonary embryo). Ponceau Red staining for total proteins and immunodot blot of the same strip is shown for each antibody. **b** Histograms representing the mean values of relative *dot colour* intensities in arbitrary units, *numbers in columns* indicate mean values. *Different letters* indicate significant differences at $P < 0.001$

intense in the vegetative cytoplasm of mature pollen (Fig. 6d, d′), but the generative and sperm cells appeared negative.

During microspore embryogenesis, the most intense JIM13 and JIM14 antibody labelling was found on early embryos with only a few cells, whereas at later stages of embryogenesis, there were considerably fewer signals. During the first stages, JIM13 labelling was intense in cell walls of early embryos surrounded by the exine (Fig. 6e, e′). JIM14 also labelled the cell walls of these early embryos (Fig. 6f, f′) but displayed lower immunofluorescence intensity than JIM13. In small globular, late globular and torpedo stage embryos, the labelling was lower and localized on small cytoplasmic spots which decreased progressively as the embryos developed (Fig. 6g–j). In some globular embryos, JIM13 labelling was also found in a few discontinuous regions along the embryo cell walls (Fig. 6i).

The labelling pattern of the MAC207 antibody throughout the two microspore pathways was different from the other antibodies. It provided high

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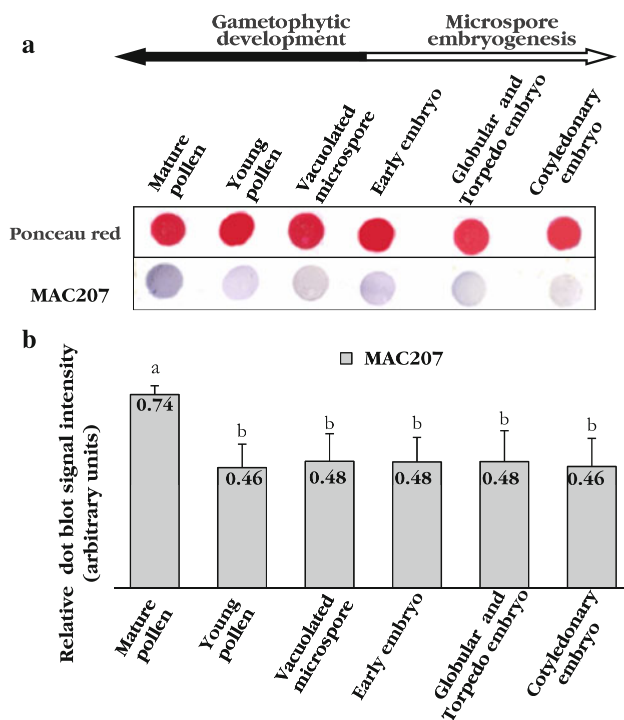

Fig. 4 Temporal distribution pattern of MAC207 epitope during male gametophytic development and microspore embryogenesis. **a** Immunodot blot assays at different developmental stages of microspore-pollen development (vacuolated microspore and mature pollen) and microspore embryogenesis (early embryo, globular and torpedo embryo, cotyledonary embryo). Ponceau Red staining for total proteins and immunodot blot of the same strip is shown. **b** Histogram representing the mean values of relative *dot colour* intensities in arbitrary units, *numbers in columns* indicate mean values. *Different letters* indicate significant differences at $P < 0.001$

immunofluorescence signals at pollen development stages, but very low labelling was found during microspore embryogenesis (Fig. 7). Vacuolated microspores had intense fluorescence signalling on the intine (Fig. 7a, a′). MAC207 labelling increased with pollen development and mature pollen grains exhibited high fluorescence in the vegetative cytoplasm as well as on the intine (Fig. 7b, b′). In the thick intine of the apertural regions (arrows in Fig. 7b′), where the pollen tube will emerge and grow during germination, the labelling was more intense.

However, MAC207 labelling was very scarce during microspore embryogenesis. Only the intine, which remained under the exine at the periphery of 2–4 cell stage embryos, displayed MAC207 antibody labelling (Fig. 7c, c′), whereas no labelling was found on the inner walls separating cells of 2–4 cell stage embryos (Fig. 7d). During later stages of embryogenesis, very low signalling appeared as small cytoplasmic spots on globular (Fig. 7e) and torpedo-shaped embryo cells. Very low or no labelling was observed on cotyledonary embryos.

For all antibodies and developmental stages analysed, the negative controls avoiding the primary antibody did not show any labelling on any subcellular compartment (data not shown). Only the exine, which was clearly distinguished from the intine, along with the more external, thicker and decorated wall layer exhibited low unspecific autofluorescence in negative controls and in some assays with antibodies.

Temporal expression pattern of Bn*AGP Sta 39-4* gene during microspore-pollen development and microspore embryogenesis

To obtain more information on the presence of AGPs and variations in their presence during microspore-pollen development and microspore embryogenesis, the expression of one of the scarce AGP genes identified in *Brassica napu* as Bn*AGP Sta 39-4* (Gerster et al. 1996) was analysed by qPCR at the selected “vacuolated microspore” and “mature pollen” developmental stages of microspore-pollen development, and the “early embryo”, “globular and torpedo embryo” and “cotyledonary embryo” stages of microspore embryogenesis.

The results showed an expression profile with significant changes during microspore-pollen development and microspore embryogenesis (Fig. 8). The expression value of the vacuolated microspore was considered as the unit of comparison for the other stages. *Sta 39-4* expression was highly induced during pollen development reaching its highest expression levels in mature pollen. During microspore embryogenesis, this AGP gene was up-regulated in the early stages of embryogenesis, exhibiting significantly higher levels in early embryos. Nevertheless, as embryogenesis progressed, its expression decreased, being down-regulated at later stages of embryogenesis, in globular, torpedo and cotyledonary embryos (Fig. 8).

Discussion

Arabinogalactan proteins are regulated during the two microspore developmental pathways, and their production accompanies pollen maturation and embryo development

The results presented in this paper have identified three main patterns of labelling which have indicated three temporal AGP distribution patterns during microspore-pollen development and microspore embryogenesis. The heterogeneous nature of the AGP family suggests that AGPs should have more than one specific role. Carbohydrate-directed monoclonal antibodies are estimated to bind 50–100 different AGP proteins (Ellis et al. 2010). RNA

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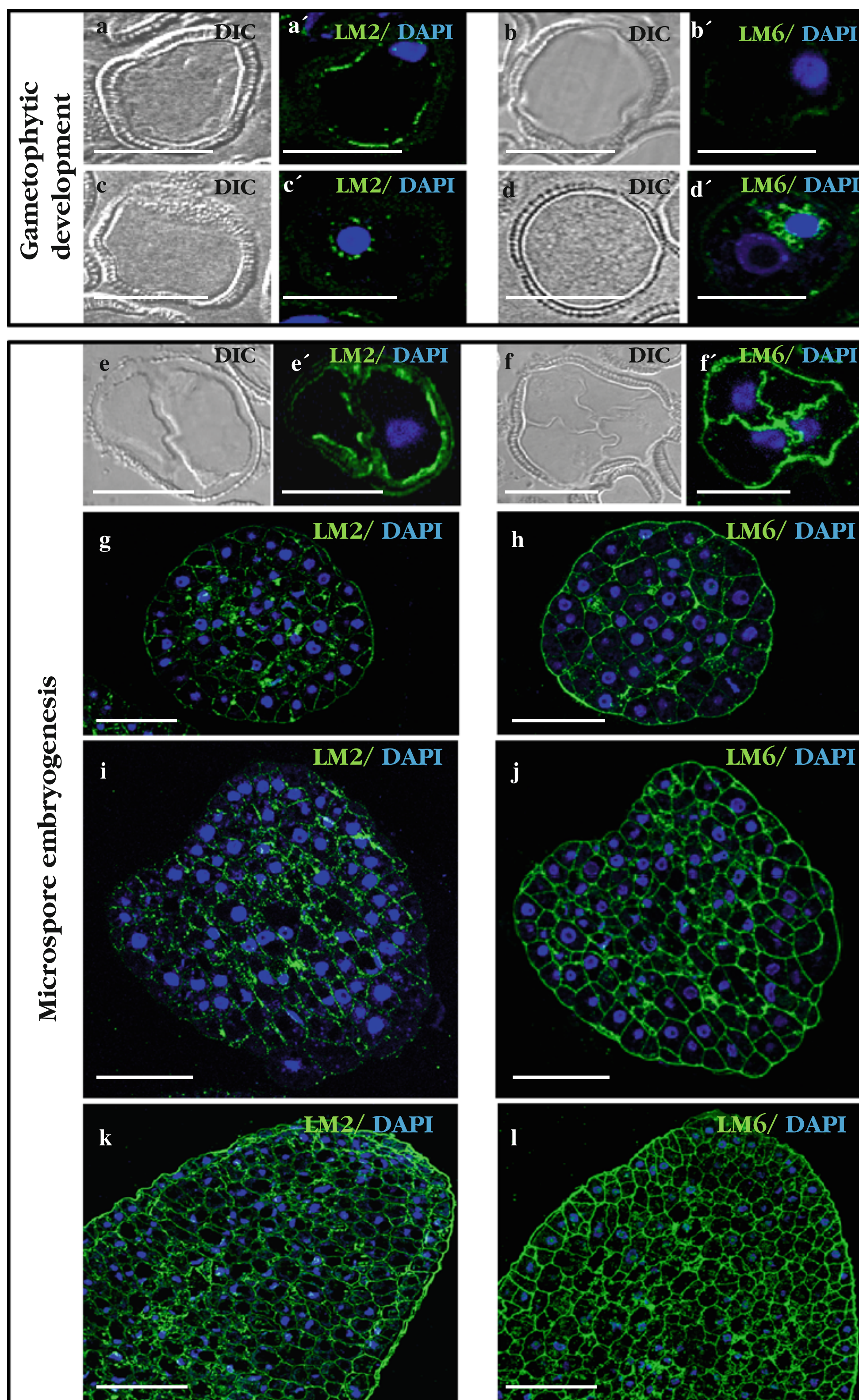

Fig. 5 Immunolocalization of LM2 and LM6 epitopes during male gametophytic development and microspore embryogenesis. Confocal merged images of immunofluorescence signal (*green*) and DAPI staining of nuclei (*blue*). For some stages, a DIC image of the same section is shown to reveal the structure (*left side* for each pair of images). **a**, **a′**, **b**, **b′** Vacuolated microspore, **c**, **c′**, **d**, **d′** mature pollen, **e**, **e′**, **f**, **f′** 2–4-celled embryos confined by the exine, **g**, **h** small globular embryos, **i**, **j** heart-shaped embryos, **k**, **l** cotyledonary embryos. **a′**, **c′**, **e′**, **g**, **i**, **k**) Immunofluorescence of LM2 antigen, **b′**, **d′**, **f′**, **h**, **j**, **l** immunofluorescence of LM6 antigen. *Bars* **a–f** 20 μm, **g–j** 50 μm, **k–l** 75 μm

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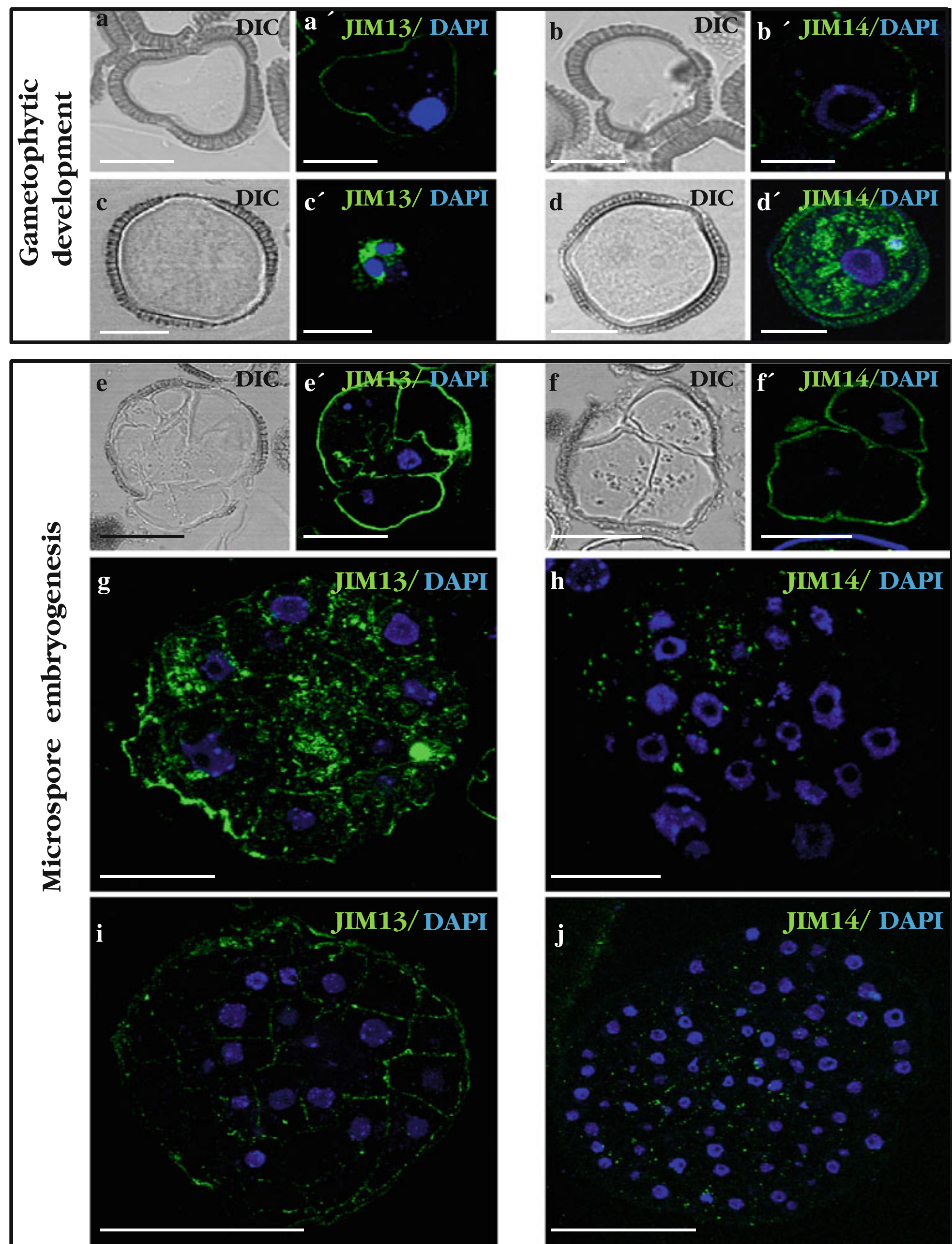

Fig. 6 Immunolocalization of JIM13 and JIM14 epitopes during male gametophytic development and microspore embryogenesis. Confocal merged images of immunofluorescence signal (*green*) and DAPI staining of nuclei (*blue*). For some stages, a DIC image of the same section is shown to reveal the structure (*left side* for each pair of images). **a**, **a′**, **b**, **b′** Vacuolated microspore, **c**, **c′**, **d**, **d′** mature pollen, **e**, **e′**, **f**, **f′** 2–4-celled embryos confined by the exine, **g**, **h** small globular embryos, **i**, **j** late globular embryos. **a′**, **c′**, **e′**, **g**, **i** Immunofluorescence of JIM13 antigen, **b′**, **d′**, **f′**, **h**, **j** immunofluorescence of JIM14 antigen. *Bars* **a–d** 10 μm, **e–h** 20 μm, **i–j** 50 μm

transcript analysis has shown that in many cases there is a clear gene expression pattern both in tissue location and the developmental stage for many individual AGPs, whereas in other cases, specific AGPs are found in several tissue types (Ellis et al. 2010). *Sta 39-4* and *Sta 39-3* genes are among some of the scarce AGP genes isolated from *B. napus* (Gerster et al. 1996), and they were the first characterized pollen-specific putative AGP genes. The temporal pattern of Bn*AGP Sta 39-4* gene expression found during the two microspore pathways, with high expression levels on mature pollen and early embryos, resembled the patterns exhibited by JIM13 and JIM14 epitopes, suggesting that a certain group/family of AGPs could be involved in two different processes of the microspore pathways, namely late gametophytic development, and microspore reprogramming and/or early embryogenesis. The patterns of LM2 and LM6 antibodies revealed that there were other groups of AGPs with increasing expression throughout microspore embryogenesis progression, therefore indicating that AGPs could be involved in other functions related to microspore-derived embryo development.

The involvement of AGPs in pollen ontogeny has been reported in several plant species (Coimbra et al. 2009; Coimbra et al. 2010; Pereira et al. 2006; Qin et al. 2007).

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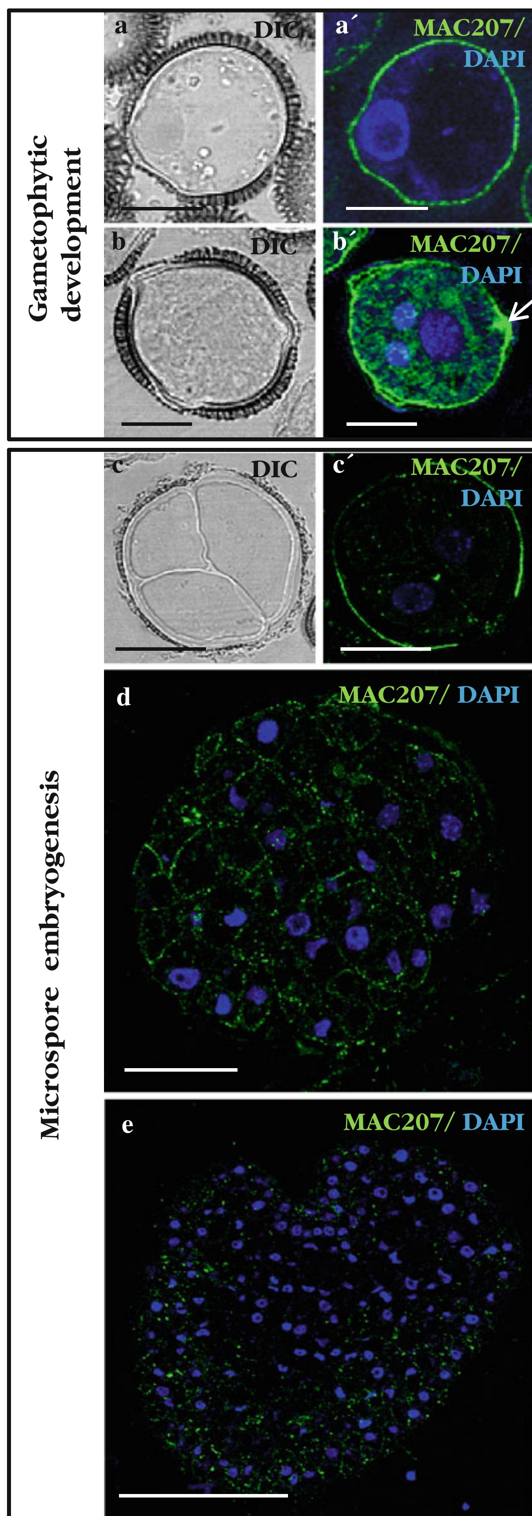

◀ **Fig. 7** Immunolocalization of MAC207 epitope during male gametophytic development and microspore embryogenesis. Confocal merged images of immunofluorescence signal (*green*) and DAPI staining of nuclei (*blue*). For some stages, a DIC image of the same section is shown to reveal the structure (*left side* for each pair of images). **a**, **a′** vacuolated microspore, **b**, **b′** mature pollen, *arrow points* to the pollen aperture **c**, **c′** 2–4-celled embryos confined by the exine, **d** small globular embryo, **e** late globular embryo. *Bars* **a**–**b** 10 µm, **c**–**d**, 20 µm, **e** 50 µm

Microarray data of Arabidopsis sperm cell transcriptome indicated that male gametes have a different gene expression from pollen grains (Borges et al. 2008). Different genetic, immunochemical and biochemical approaches have indicated a role for AGPs in pollen germination, pollen tube growth and male gamete function (Coimbra et al. 2010; Costa et al. 2013; Qin et al. 2007). Our results have also indicated that AGPs are involved in pollen development in *B. napus* and suggest that different AGP families, grouped together by the same polysaccharide antigenic determinants, could act separately in two different processes. In these processes, the AGPs localized in vegetative cytoplasms, intine and apertures (JIM14 and MAC207 epitopes) would be involved in pollen germination and pollen tube growth, whereas the AGPs localized in the generative and sperm cells (JIM13, LM2 and LM6 epitopes) would be related to the gamete function. Even though the AGP molecular mechanism of action is still unknown, AGPs specifically localized in generative and sperm cells have been linked with the signals that are necessary to direct these cells to their targets inside the pollen tube (Coimbra and Pereira 2012). AGP epitopes localized in the intine and pollen tube wall, predominantly associated with the tip region, have been suggested as structural and/or control elements for germination through the modulation of water uptake (Coimbra et al. 2010).

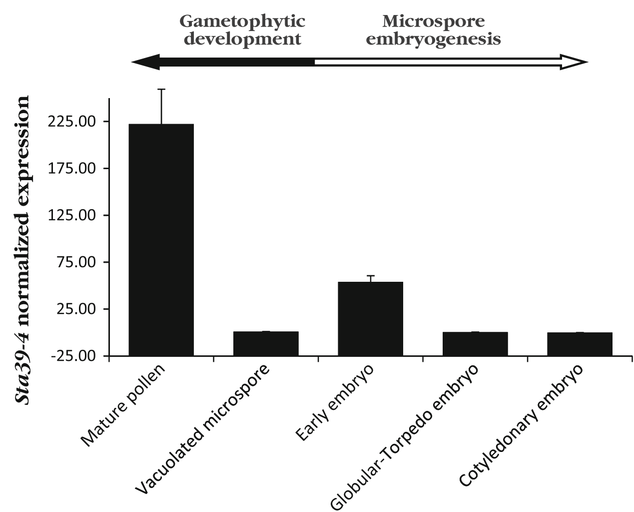

Fig. 8 qPCR analysis of *BnAGP Sta39-4* gene expression during male gametophytic development and microspore embryogenesis. Quantification of real-time RT-PCR amplification products of *Sta39-4* mRNA transcripts at different stages of male gametophytic development (vacuolated microspore and mature pollen) and microspore embryogenesis (early embryos, globular and torpedo embryos and cotyledonary embryos). Each *bar* represents an average of two independent reactions and technical replicates. Transcript levels were normalized to vacuolated microspore levels

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In vitro embryogenic systems are influenced by numerous exogenous and endogenous factors, which can stimulate or inhibit development. Many studies have reported the positive effects on in vitro somatic and microspore embryogenesis by adding exogenous AGPs, normally those contained in gum arabic, to the culture medium (Pandey et al. 2012; Yuan et al. 2012). During *B. napus* microspore embryogenesis, which progressed without the addition of exogenous AGPs (Prem et al. 2012), AGPs progressively increased in embryo cells in line with development and differentiation. Some pectin epitopes, like JIM5 which recognize low-esterified pectins, were found to increase during microspore-derived embryo development, being especially abundant in the cell wall of differentiating embryo cells (Bárány et al. 2010; Solís 2012; Solís et al. 2012). LM6 can recognize a pentasaccharide of (1–5)-a-L-arabinans present in AGPs, but also present in the rhamnogalacturonan I domain of pectins (Willats et al. 1998). In the present work, LM2 and LM6 epitopes increased progressively in embryo cells along with development, but their localization patterns did not differ between proliferating and differentiating embryo cells, being distributed not only in cell walls but also in cytoplasmic spots, especially the LM2 antigen. Previous immunochemical studies have revealed that LM2 recognizes AGPs secreted by suspension-cultured carrot cells (Smallwood et al. 1996), and that this AGP epitope is associated with the subcellular elements of the secretory pathway within plant cells actively producing and secreting AGPs (Samaj et al. 2000). The presence of secreted AGPs in maize microspore and zygote cultures has been reported to be a stimulating factor for embryo development (Massonneau et al. 2005). Several reports have revealed that scavenging cellular AGPs, through the addition of a "Yariv" reagent to the tissue culture media, inhibit somatic embryogenesis in *Daucus carota* (Thompson and Knox 1998) and Cichorium hybrids (Chapman et al. 2000); whereas the exogenous addition of AGPs restores such potential, increasing somatic embryogenesis. These studies, among others, have suggested that secreted AGPs could be the extracellular matrix molecules that control and maintain plant cell fate during somatic embryogenesis (Pandey et al. 2012). Recent work with maize microspore cultures revealed that tunicamycin treatment, that blocks protein glycosylation and therefore secretion, inhibited microspore-derived embryo development, which was subsequently recovered by supplementation with a medium containing all the secreted factors from a well-developed microspore culture (Testillano et al. 2010). A role for AGPs has also been proposed in the initiation and maintenance of microspore embryogenesis (Tang et al. 2006). The present results, with the localization of AGPs on cell walls and cytoplasmic spots which resembled elements of the secretory pathway, suggest an active production and secretion of AGPs during microspore-derived embryo formation and differentiation. This indicates a possible role for endogenous AGPs in sustaining/stimulating in vitro microspore embryogenesis, as reported in somatic embryogenesis.

Microspore reprogramming and early microspore embryogenesis involve AGP expression

Induction of embryogenesis in microspore cultures is a reliable and convenient model for investigating the mechanism of cell fate reprogramming and the onset of embryogenesis. Changes in various cell activities and the structural organization of subcellular compartments have been reported as accompanying the microspore reprogramming process in some herbaceous and woody species (Bárány et al. 2005; Seguí-Simarro et al. 2006; Solís et al. 2008, 2012; Testillano et al. 2005). Information on the biological significance of a protein can be inferred from its differential presence in specific developmental stages, cell types and external conditions. Our results have revealed that a wide group of AGPs (those recognized by JIM13, JIM14, LM2 and LM6 antibodies) were induced and specifically localized in the cell walls of early embryos with just two or four cells which were formed by the first embryogenic divisions of the microspore after reprogramming, whereas they were much less present or absent in the microspore before the programme changed. The qPCR analysis also showed high expression induction of the AGP gene *Sta 39-4,* specifically in the early microspore embryogenesis stages, in early embryos, followed by down-regulation during later embryogenesis stages. The specific expression of AGPs in 2–4 cell stage embryos and their localization in the newly formed embryo cell walls strongly suggests that AGPs are early molecular markers of microspore embryogenesis.

The β-D-Glucosyl Yariv reagent (Yariv et al. 1962) is used in many studies to bind AGPs, thereby interfering with all AGP activity and thus indirectly inferring/elucidating their function. The addition of the Yariv reagent, that specifically reacts with AGPs, to microspore embryogenesis cultures disturbed microspore embryogenesis initiation in a concentration-dependent manner (Tang et al. 2006). Recent studies of in vitro cultured zygotes and proembryos of tobacco have shown that AGP scavenging by the Yariv reagent affected the first zygotic divisions and proembryo pattern formation, indicating the involvement of AGPs in cell division and cell plate formation during the initial embryogenic divisions of the zygote (Yu and Zhao 2012). Furthermore, in tobacco zygote cultures, both immunofluorescence detection with the JIM13 antibody and staining with the Yariv reagent showed that AGPs

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were distributed in the new cell plate during normal in vitro zygotic division (Yu and Zhao 2012). In Arabidopsis zygotic embryogenesis in vivo, JIM13-labelled AGPs have been localized in the embryo proper at very early stages, but they gradually disappeared after the torpedo stage (Zhong et al. 2011). Our study of *Brassica* microspore embryogenesis provides new evidence of the specific association of AGPs with the newly formed walls of two-four cell stage embryos suggesting their involvement in the first embryogenic divisions of the microspore, similar to zygote divisions.

In conclusion, our results have provided new data which indicate that different AGPs are involved in pollen maturation and germination, microspore reprogramming, early embryogenesis and embryo development. Further work will be needed to shed more light on the precise mechanisms of AGP action in these processes.

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