




# IMMUNOLocalIZATION OF PECTINS AND ARABINO GALACTAN PROTEINS IN YOUNG OVULES OF SELECTED AMPHIMICTIC AND APOMICTIC SPECIES OF THE ASTERACEAE FAMILY

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Cell wall components, especially arabinogalactan proteins (AGPs) and pectins as the source of signaling molecules active in cell-to-cell communication, are involved in many biological processes, including plant growth and development. Understanding the mechanisms of intercellular communication is particularly important in the context of reprogramming cell fate and transition from somatic to germline identity. The present study focuses on immunodetection of some pectic epitopes and AGPs in young ovules of selected *Hieracium*, *Pilosella* and *Taraxacum* species. The purpose of this research was to answer the questions: (1) whether the distribution of pectins and AGPs is related to the mode of reproduction and (2) whether their spatial and temporal distribution in young ovules may herald a later differentiation of the nutrient tissue present in the integument of mature ovules. We analyzed the localization of low and highly methyl-esterified pectins and AGP epitopes using monoclonal antibodies, i.e., LM19, LM20, JIM13, respectively. Our research found no significant differences in the localization of pectins and AGPs in young ovules of sexual and apomictic species, and the initial distribution pattern of these compounds did not appear to be related to the subsequent differentiation of the pericarpal nutrient zone. The presented findings may confirm the existence of a general developmental trend in the spatial and temporal distribution of pectins and AGPs during the maturation of ovules in angiosperms.

**Keywords:** apomixis, *Hieracium*, megasporogenesis, monoclonal antibodies, *Pilosella*, *Taraxacum*

## INTRODUCTION

Apomixis, as a natural mode of plant reproduction *via* seed-based cloning, is a highly desirable agricultural trait due to its capacity to fix heterozygosity and hybrid vigor. Most crops, however, reproduce sexually and apomixis has been reported in only few economically important species (Spillane et al., 2004; Barcaccia and Albertini, 2013;

Sailer et al., 2016; Yin et al., 2022). For many years, numerous scientific centers around the world have been conducting advanced research to understand the molecular mechanisms of apomictic reproduction and introduce apomixis to crops. Genetic engineering methods, and more specifically gene-editing, are becoming increasingly important (Wang et al., 2017; Kaushal et al., 2019; Vijverberg et al., 2019; Scheben and Hojs-

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gaard, 2020 and references therein). Despite such an advanced level of molecular research, the processes involved in plant reproduction observed at the cellular level are still intriguing and insufficiently explained. In the ovules of sexually reproducing angiosperms, the cell wall composition is significantly modified at the stage of determining the identity of the generative cell (Tucker and Koltunow, 2014; Lora et al., 2017). Therefore, it is an important aspect of basic research, vital also in terms of apomixis. Cell wall components, especially AGPs and pectins, are the source of signaling molecules that are active in intercellular communication (Acosta-García and Vielle-Calzada, 2004; Wolf et al., 2012; Demesa-Arévalo and Vielle-Calzada, 2013; Tucker and Koltunow, 2014; Lora et al., 2017; Saffer, 2018; Shin et al., 2021). AGPs are plant cell surface glycoproteins that belong to the subfamily of hydroxyproline-rich proteins. They are highly complex due to the variety of glycans attached to the protein backbone as well as the heterogeneity of the protein component (Seifert and Roberts, 2007; Ellis et al., 2010; Su and Higashiyama, 2018). The location of AGPs is diverse, i.e., they are present on the surface of the cell membrane, in the apoplastic space, in the cell walls and in exudates (Ellis et al., 2010). AGPs are involved in numerous life-cycle processes in plants, including cell division (Langan and Nothnagel, 1997), programmed cell death (Chaves et al., 2002), root growth and development (van Hengel and Roberts, 2003), plant-microbe interactions (van Buuren et al., 1999), female sporogenesis and gametogenesis (Acosta-García and Vielle-Calzada, 2004; Coimbra et al., 2007; Demesa-Arévalo and Vielle-Calzada, 2013; Pereira et al., 2015), pollen grain development (Coimbra and Pereira, 2012), and male-female interactions (Majewska-Sawka and Nothnagel, 2000; Leszczuk et al., 2019).

Similarly to AGPs, pectins constitute a complex and ubiquitous group of compounds found in plants. This group of acidic heteropolysaccharides is an essential component of the primary cell wall of land plants (Willats et al., 2001). In terms of chemical composition, pectins are a family of polysaccharides rich in galacturonic acid, including homogalacturonan, rhamnogalacturonan I, and the substituted galacturonans, rhamnogalacturonan II and xylogalacturonan (Mohnen, 2008). Homogalacturonans (HGs) differ in the degree of methyl-esterification and, therefore, highly and low methyl-esterified HGs can be distinguished (Knox

et al., 1990; Wolf et al., 2009). The synthesized and secreted pectins are highly methyl-esterified, but their structure within the cell wall is altered by pectin methyl-esterases, which catalyse the specific de-methyl-esterification (Pelloux et al., 2007). The de-esterified galacturonic acid residues bind  $\text{Ca}^{2+}$  ions and form the 'egg-box' model structure that induces the formation of a gel with other pectic molecules and, therefore, strengthen the cell wall (Caffall and Mohnen, 2009; Wolf et al., 2009). Other mechanical properties of cell walls, such as porosity, adhesion, elasticity, are also conditioned by pectins (Verhertbruggen et al., 2009; Pilarska et al., 2013). Moreover, pectic oligogalacturonides, the products of pectins decomposition, are the signaling molecules involved in many biological processes, including plant growth and development (Wolf et al., 2009 and references therein).

The identity of a given cell may be determined more by its adjacent cells than the mother cell, and therefore cell surface molecules, such as receptor-like protein kinases and AGPs, deserve special attention (Majewska-Sawka and Nothnagel, 2000). The role of specific cell wall components in intercellular communication appears to be crucial during the transition from the diploid phase to the haploid phase, i.e., in the interactions between the somatic cells and cells acquiring competence for gametophyte development (Bencivenga et al., 2011). However, it remains impossible to precisely define their role in plant reproductive processes since relatively few species have been studied in this regard (Leszczuk and Szczuka, 2018). Therefore, detailed studies of spatial and temporal distribution of various cell wall components during key stages of reproductive development in sexual and apomictic species are necessary, especially since the data on the distribution of pectins and AGPs in the developing ovules of apomictic species are only fragmentary.

Species from the Asteraceae family were the object of our research. Together with the Poaceae and the Rosaceae, it is one of the three angiosperm families in which apomixis is relatively common (Richards, 2003). In addition, special modifications of the integumentary tissue and the differentiation of periendothelial nutrient tissue have been described in mature ovules of several Asteraceae, including species of *Bellis*, *Chondrilla*, *Galinsoga*, *Helianthus*, *Hieracium*, *Onopordum*, *Pilosella*, *Solidago*, and *Taraxacum* (Cooper and Brink, 1949; Engell and Petersen, 1977; Koltunow et al., 1998; Musiał et al., 2013;

Musiał and Kościńska-Pająk, 2013; Kolczyk et al., 2014, 2016; Płachno et al., 2017). In the case of *Taraxacum*, cells have extremely thickened walls with a spongy structure in the peri-endothelial zone (Musiał et al., 2013), while in *Hieracium* and *Pilosella* the process of gelatinization takes place in this zone; it is associated with the formation of mucus-filled lysogenic cavities (Koltunow et al., 1998; Płachno et al., 2017).

The immunodetection of some pectic epitopes and AGPs in young ovules of selected *Hieracium*, *Pilosella* and *Taraxacum* species and the comparison of their distribution between the analyzed species were the main goal of our research. We further aimed to determine (1) whether the pattern of their distribution may be related to the mode of reproduction and (2) whether this pattern may herald later changes related to the differentiation of nutrient tissue in the integument of older ovules.

## MATERIALS AND METHODS

### PLANT MATERIAL

Young inflorescences of three amphimictic hawkweed species and two taxa of dandelion with different modes of reproduction were used in this study (Table 1). This material was collected from

plants in their natural populations or from plants transferred from natural habitats and grown in an experimental garden. Voucher specimens are stored in the herbarium of the W. Szafer Institute of Botany of the Polish Academy of Sciences (KRAM) and in the herbarium of the Jagiellonian University in Kraków (KRA) (Table 1).

### EMBRYOLOGICAL ANALYSIS

For the embryological analyses that aimed to determine developmental stages in florets, whole inflorescences were fixed in a mixture of glacial acetic acid and absolute ethanol (1:3, v/v) for at least 24 h. Subsequently, plant material was transferred to 70% ethanol and stored at 4°C. Florets isolated from the fixed inflorescences were treated using the tissue clearing technique detailed by Musiał et al. (2015). Briefly, they were dehydrated in a graded ethanol series (70% to 100%) and subsequently incubated in mixtures of absolute ethanol and methyl salicylate (3:1, 1:1 and 1:3, v/v) and in two changes of pure methyl salicylate (over 30 min in each step). Cleared florets were examined using a Nikon Eclipse E600 microscope fitted with Nomarski's interference contrast (DIC optics). At least 10 inflorescences from four different plants of each species were analyzed.

TABLE 1. Locality, somatic chromosome number (2n) and mode of reproduction of analyzed *Hieracium*, *Pilosella* and *Taraxacum* species.

Species (No. of voucher specimens)	Locality	2n	Reproductive mode
<i>H. transylvanicum</i> Heuff. (KRAM00586585-V)	Romania, Southern Carpathians, Mehedinți Mts, Mt. Domugled, <i>Fagus sylvatica</i> forest on limestone	2n=2x=18 (Ilnicki et al., 2010)	amphimictic (Yurukova-Grancharova et al., 2006; Janas et al., 2022)
<i>P. officinarum</i> F.W.Schultz & Sch.Bip. (KRA0570396)	Poland, Cracow, urban lawn	2n=4x=36 (Pogan and Wcisło, 1989)	amphimictic (Pogan and Wcisło, 1995; Janas et al., 2022)
<i>P. pavichii</i> (Heuff.) Holub (KRA0154002)	North Macedonia, Jakupica Mts, siliceous rocks along road from Karadžica chalet to Mt. Krivul	2n=2x=18 (Szelağ and Ilnicki, 2011)	amphimictic (Yurukova-Grancharova et al., 2006; Janas et al., 2022)
<i>T. belorussicum</i> Val.N.Tikhom. (KRA0508446)	Poland, Mścichy, Biebrza National Park	2n=3x=24 (Marciniuk et al., 2010)	meiotic diplospory (Janas et al., 2016)
<i>T. linearisquameum</i> Soest (KRA0460798)	Poland, Siedlce, experimental garden	2n=2x=16 (Mártonfiová, 2013)	amphimictic (Musiał and Kościńska-Pająk, 2013; Musiał et al., 2015)

## IMMUNOCYTOCHEMISTRY

To perform immunocytochemical reactions, capitula of varying sizes were divided into small fragments and fixed in a mixture of 4% paraformaldehyde (Sigma-Aldrich) and 2% glutaraldehyde (Stanlab), both dissolved in a 1 M phosphate-buffered saline (PBS), pH 7.2 (Milewska-Hendel et al., 2017). Plant material was incubated in fixative solution overnight at 4 °C, then rinsed with PBS, dehydrated in an ethanol series (10, 30, 50, 70, 90, and 100%; v/v, 10 min in each solution) and embedded in Steedman wax, according to the protocol described by Vitha et al. (2000). Samples were cut into 7 µm thick longitudinal sections using a Leica microtome (Leica Biosystems, Nussloch, Germany) and aligned on slides covered with poly-L-lysine (Menzel Gläser, Germany). Sections were de-waxed and rehydrated in an ethanol series (100% three times, 90%, 50% in PBS; v/v, 10 min in each solution). As a blocking solution, 2% bovine serum albumin (BSA) in PBS was used; samples were left in this solution for 30 minutes at room temperature (RT). Next, the sections were incubated in primary rat monoclonal antibodies for pectins and AGPs (Paul Knox Laboratory, University of Leeds; formerly the PlantProbes; Table 2) diluted 1 : 20 in BSA solution at RT for 1.5 h. After rinsing the sections three times in BSA (each for 10 min), the subsequent step was performed in darkness. Samples were incubated with Alexa-Fluor 488 goat anti-rat (Jackson ImmunoResearch) as the secondary antibody diluted in 1:100 in BSA for 1.5 h (RT). Further, the sections were rinsed three times in BSA and once in PBS (each for 10 min), and subsequently stained with 0.01% (m/v) toluidine blue for 10 min. After the final rinse (three times in PBS, once in distilled water, 5 min in each), the sections were mounted with the Roti®-Mount FluorCare (ROTH) as the anti-fading medium. Some sections were additionally treated with DAPI (4',6-diamidino-2-phenylindole dihydrochloride; ROTH; 1.25 µg/ml)

before mounting. No primary antibodies were used for the negative control, resulting in no fluorescent signal in the controlled sections. The samples were observed under UV light using a Nikon Eclipse E400 microscope with filters for AlexaFluor 488 (a maximum excitation wavelength of 490 nm, barrier filter BA520) and for DAPI (a maximum excitation wavelength of 450 nm, barrier filter BA420). Photographs were taken with a Nikon DS-Fi2 and NIS Elements D ver. 4.0 software. Photographic documentation was graphically processed using CorelDRAW Graphics Suite 2020 and Image-Pro Premier 3D ver. 9.3 software.

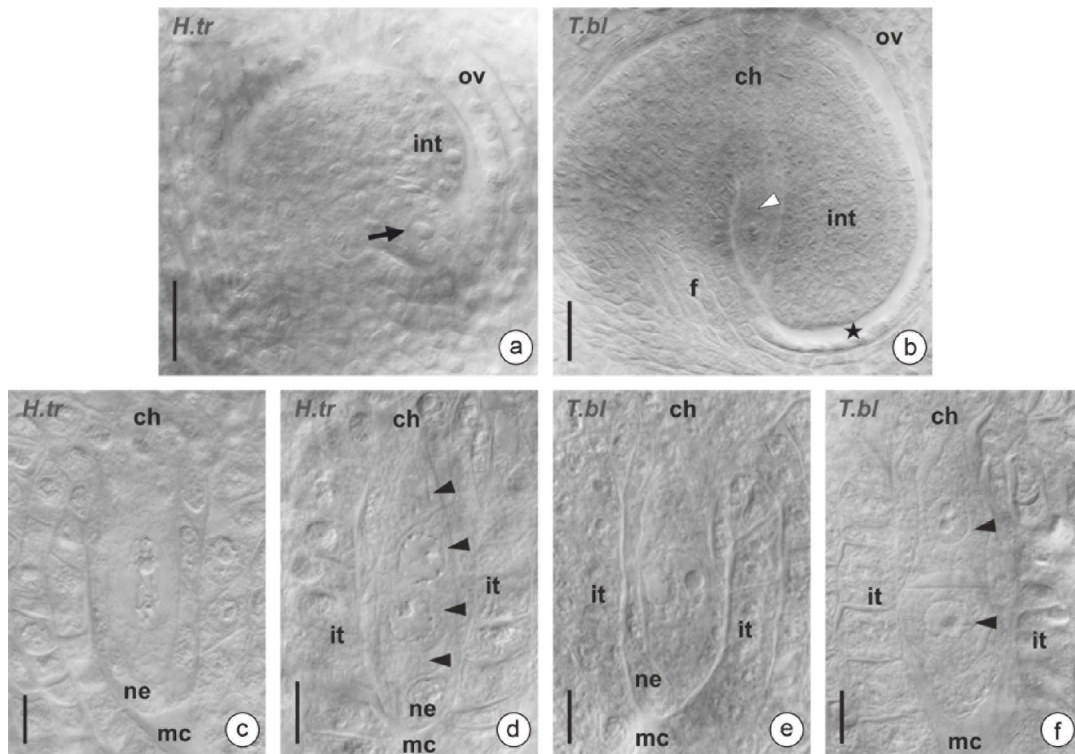
## RESULTS

### ANATOMICAL FEATURES OF YOUNG OVARIES AND OVULES AND EARLY EMBRYOLOGICAL PROCESSES

Florets in the studied species had a bicarpellate gynoecium with an inferior and unilocular ovary in which a single basal ovule developed (Fig. 1a). The ovule was anatropous, tenuinucellate, and had one massive integument with a homogeneous structure showing no zonal differentiation of the tissue (Fig. 1b). In the developing ovule, a single archesporial cell differentiated in the nucellus just below the epidermal layer and directly functioned as the megaspore mother cell (MMC) in amphimictic species or as a diplosporous megaspore mother cell (DMMC) in the apomictic *T. belorussicum* (Fig. 1b,c). In the analyzed sexual species, regular meiosis in the MMC led to the formation of a linear tetrad of haploid megaspores (Fig. 1d). On the other hand, a meiotic diplospory took place in the DMMC of the apomictic *T. belorussicum*, which resulted in a dyad of unreduced megaspores (Fig. 1e,f). The ovules of all studied species were characterized by the presence of an integumentary tapetum (endothelium) that developed from the epidermal cells of the integument directly ad-

TABLE 2. List of primary rat monoclonal antibodies used in this research.

Antibody name	Epitope	Reference
LM19	HG (non-methyl-esterified, partially methyl-esterified)	Verhertbruggen et al., 2009
LM20	HG (methyl-esterified)	Verhertbruggen et al., 2009
JIM13	Arabinogalactan/Arabinogalactan protein	Knox et al., 1991



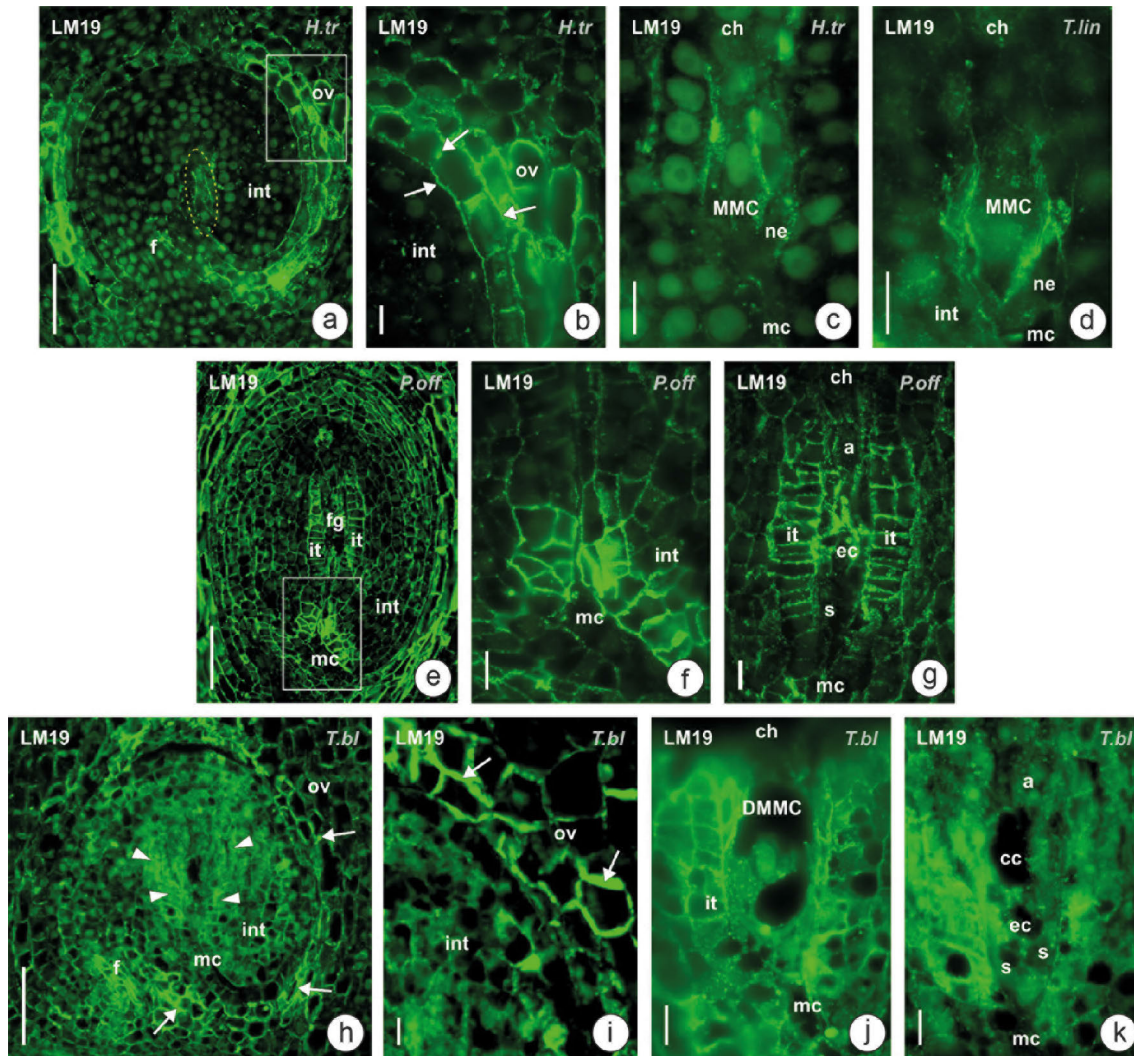
**Fig. 1.** Ovule structure and early female reproductive processes in the amphimictic *Hieracium transylvanicum* (*H.tr*) and the apomictic *Taraxacum belorussicum* (*T.bl*). Images obtained from cleared ovaries using DIC optics. **(a)** Locule of a young ovary with a developing ovule in *H. transylvanicum*; arrow points to a large nucleus in an archesporial cell distinguished just below nucellar epidermis. **(b)** Young anatropous ovule of *T. belorussicum*; arrowhead shows a diplosporous megaspore mother cell (DMMC), asterisk indicates the ovary chamber. **(c-d)** Stages of megasporogenesis in *H. transylvanicum*; prophase I in a megaspore mother cell **(c)** and a tetrad of megaspores **(d)**. **(e-f)** Stages of meiotic diplospory in *T. belorussicum*; prophase I in DMMC **(e)** and young diplodyad of megaspores **(f)**. Abbreviations: **ch** chalazal pole, **f** funicle, **int** integument, **it** integumentary tapetum, **mc** micropylar canal, **ne** nucellar epidermis, **ov** ovary wall, **black arrowheads** megaspores within tetrad and diplodyad. Scale bars: **a** 25 µm; **b-f** 10 µm

adjacent to the nucellus. Depending on the species, however, this layer differentiated at different developmental stages. In the analyzed sexual species, the integumentary tapetum typically began to differentiate towards the end of megasporogenesis (Fig. 1d). In contrast, this layer was distinguished already at the stage of the DMMC in the ovules of the apomictic *T. belorussicum* (Fig. 1e).

#### IMMUNOLocalization of PECTINS

Distribution of low and highly esterified pectic HGs was determined using two monoclonal antibodies, i.e., LM19 and LM20, respectively. In the ovules of amphimictic species, LM19 labeling revealed uneven distribution of low esterified pectins at the stage of the MMC and megasporogenesis. An epitope recognized by LM19 was distributed in the outer walls of integument epi-

dermal cells, while no labeling was observed in the inner layers of the integument tissue (Fig. 2a,b). A particularly intense fluorescent signal, compared to other tissues, indicating the accumulation of this pectin fraction, was also detected in the layers of the ovary wall directly surrounding the ovule-containing locule; the epitope recognized by LM19 was present in both the anticlinal and periclinal walls in the cells of the epidermal and sub-epidermal layer (Fig. 2a,b). LM19 labeling was also observed in the MMC wall as well as in its cytoplasm, in which the fluorescent signal was dispersed (Fig. 2a,c,d). In some ovules, the epitope recognized by LM19 was also discernible in the walls of the nucellar epidermal cells (Fig. 2c,d). This pattern of distribution in the ovules of low esterified pectins persisted during megasporogenesis, but then the spatial distribution of this pectin fraction changed evidently.



**Fig. 2.** Localization of low methyl-esterified pectins with LM19 immunolabeling. Longitudinal sections of ovaries and ovules in the amphimictic *Hieracium* (*H.tr*), *Pilosella* (*P.off*) and *Taraxacum* (*T.lin*) species (**a-g**) and in the apomictic *T. belarussicum* (*T.bl*) (**h-k**). (**a-c**) Distribution of the epitope in the ovule and ovary of *H. transylvanicum*. (**a**) An anatropous ovule and a fragment of the ovary wall with a detected pectic epitope; note lack of immunofluorescence signal in somatic ovular cells. (**b**) Magnification of the area marked with a rectangle in Fig. 2a; a fragment of the ovary wall and outer part of ovule integument; note more intense fluorescent signal in the layers of the ovary wall surrounding the ovule-containing locule. (**c**) Magnification of the micropylar pole of the ovule marked with a dashed line in Fig. 2a; note fluorescent signal in the walls of MMC and nucellar epidermis. (**d**) Micropylar part of the *T. linearisquameum* ovule with visible the MMC; clear fluorescent signal visible in the MMC wall and slightly weaker one in the walls of nucellar epidermis cells. (**e-g**) Pectic epitope in *P. officinarum* ovule with a developed layer of integumentary tapetum. (**e**) An anatropous ovule with visible almost even distribution of low esterified pectins throughout the ovule. (**f**) Magnification of the area marked with a rectangle in Fig. 2e; visible accumulation of low esterified pectins in the walls of integumentary cells surrounding the micropylar canal. (**g**) Distribution of the pectic epitope in the cells of a female gametophyte and in the cell walls of the integumentary tapetum layer. (**h-k**) Localization of low methyl-esterified pectin in the ovules of apomictic *T. belarussicum*; LM19 labeling visible in the walls and inside the ovular cells (**arrowheads** **h**), in the cell walls of ovary wall (**i**), in the DMMC and surrounding tissues (**j**), and in female gametophyte cells (**k**). Abbreviations: **a** antipodal cell, **cc** central cell, **ch** chalazal pole, **DMMC** diplosporom megaspore mother cell, **ec** egg cell, **f** funicle, **fg** female gametophyte, **int** integumentary tapetum, **mc** micropylar canal, **MMC** megaspore mother cell, **ne** nucellar epidermis, **ov** ovary wall, **s** synergid, **arrows** intensive fluorescent signal. Scale bars: **a, e, h** 50  $\mu$ m; **b-d, f, g, i-k** 10  $\mu$ m

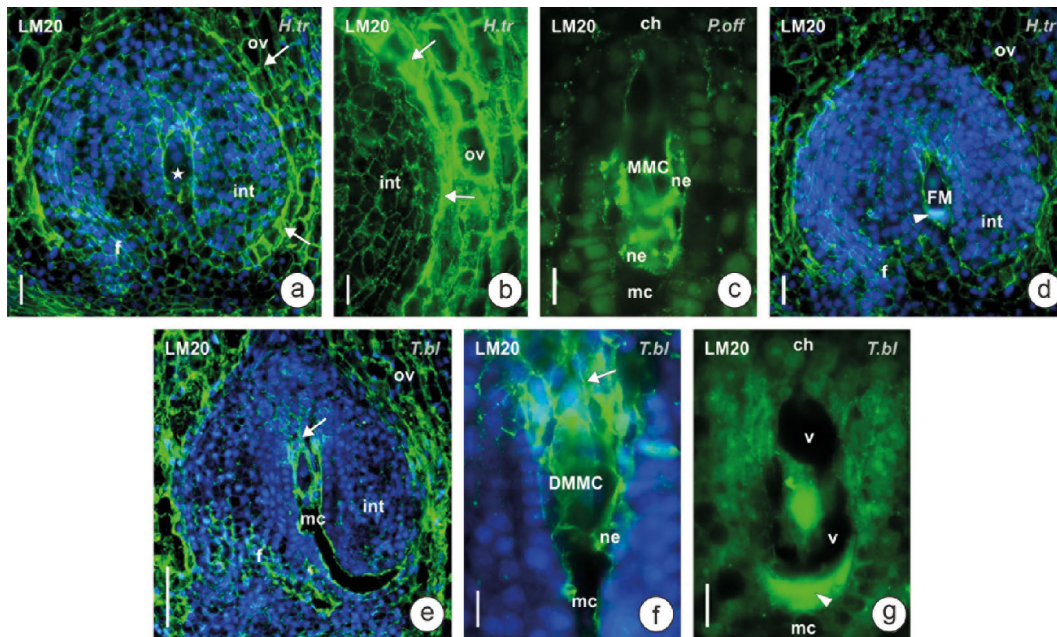
Throughout megagametogenesis in amphimictic species, low esterified pectins also appeared in the walls of somatic cells and were almost evenly distributed across the entire ovule (Fig. 2e). A particular accumulation of this pectin fraction was found in the walls of cells surrounding the ovule micropylar canal (Fig. 2e,f). The pectins labeled with LM19 were also present in the cell walls of the differentiated integumentary tapetum layer and in the walls of the female gametophyte cells (Fig. 2e,g). As at the earlier developmental stages, pectins recognized by LM19 were additionally present in the cell walls of the inner layers of the ovary wall (Fig. 2e). In the case of the apomictic *T. belorussicum*, the distribution of low esterified pectins in the ovary and the ovule was a little different from the pattern described in the analyzed amphimictic species. The epitope labeled with LM19 was distributed in the cell walls of the inner layers of the ovary wall, and a particularly intense fluorescent signal was detected in the area of the placental tissue at the base of the ovary locule (Fig. 2h,i). In the ovules of *T. belorussicum*, the LM19 epitope was visible both in the walls and in the cytoplasm of integument and nucellus cells (Fig. 2h). A similar distribution of low esterified pectins was also observed in the DMMC (Fig. 2j). In the ovules with an unreduced female gametophyte, the LM19 epitope was identified in the cell walls of the integument and the integumentary tapetum. Low esterified pectins were also present in the cytoplasm of the embryo sac cells; however, as indicated by a very subtle fluorescence signal, their walls did not contain significant amounts of this pectin fraction (Fig. 2k).

Pectic HGs with high levels of methyl-esterification, identified by monoclonal antibody LM20, showed a similar distribution pattern in the ovaries of both amphimictic and apomictic species. As in the case of low esterified pectins, the intense fluorescence indicated the presence of the LM20 epitope in the cell walls of the ovary walls' inner layers (Fig. 3a,b,d,e). The distribution of highly esterified pectins in the ovules was in turn slightly different from the pattern of low esterified pectin distribution. In the developing ovules of amphimictic and apomictic species, the epitope recognized by LM20 was uniformly distributed in the cell walls of the integument tissue (Fig. 3a,b,e). In apomictic *T. belorussicum*, however, the fluorescence was much weaker, except for the outer surface of the integumentary epidermis, where the fluorescent signal was relatively strong (Fig. 3e).

LM20 labeling was also found in the wall of the MMC and DMMC as well as in the walls of the nucellar epidermis cells (Fig. 3c,f). Furthermore, a greater intensity of the fluorescent signal – indicating the accumulation of highly esterified pectins – was observed in the walls of cells adjacent to the MMC and the DMMC in the ovule's chalazal region (Fig. 3a,e,f). This pattern of spatial distribution in the ovules of highly esterified pectins changed neither during megasporogenesis in amphimictic species nor meiotic diplospory in *T. belorussicum*. At the early stage of female gametophyte development in amphimictic species, the pectins labeled with LM20 occurred in the wall of a developing embryo sac (Fig. 3d), while in *T. belorussicum* the epitope recognized by LM20 was mainly distributed in the cytoplasm of the one-nucleate female gametophyte (Fig. 3g).

#### IMMUNOLocalIZATION OF ARABINOGALACTAN PROTEINS

The distribution of AGPs was identified using the JIM13 antibody. Unlike pectins, the presence of AGPs was neither found in the ovary's wall nor in the somatic cells of the ovule. In the ovules of amphimictic species, AGPs were markers of generative line cells (Fig. 4a–e). AGP epitope labeled with JIM13 was present in both the cell wall and the cytoplasm of the MMC (Fig. 4a). After the first meiotic division, the epitope recognized by JIM13 was localized in dyad cells, and a strong fluorescent signal was also detected in the transverse wall between megaspores (Fig. 4b,c). The same pattern of AGPs distribution was observed following the second meiotic division. A strong fluorescent signal, pointing to the presence of AGPs, was visible in megaspore tetrad cells and in the newly formed transverse walls between megaspores (Fig. 4d,e). In apomictic *T. belorussicum*, the occurrence of AGP epitope labeled with JIM13 was also restricted to the DMMC that initiates the development of an unreduced female gametophyte. JIM13 labeling was detected in the cell wall and the cytoplasm of a functional megaspore within the diplodyad and subsequently in the wall of the female gametophyte (Fig. 4f,g). In addition, a weak fluorescent signal also appeared in the integument cells, which could be the effect of unquenched autofluorescence (Fig. 4g). In the older ovules of *T. belorussicum*, the epitope recognized by JIM13 was distributed in the cells of the female gametophyte (Fig. 4h,i).



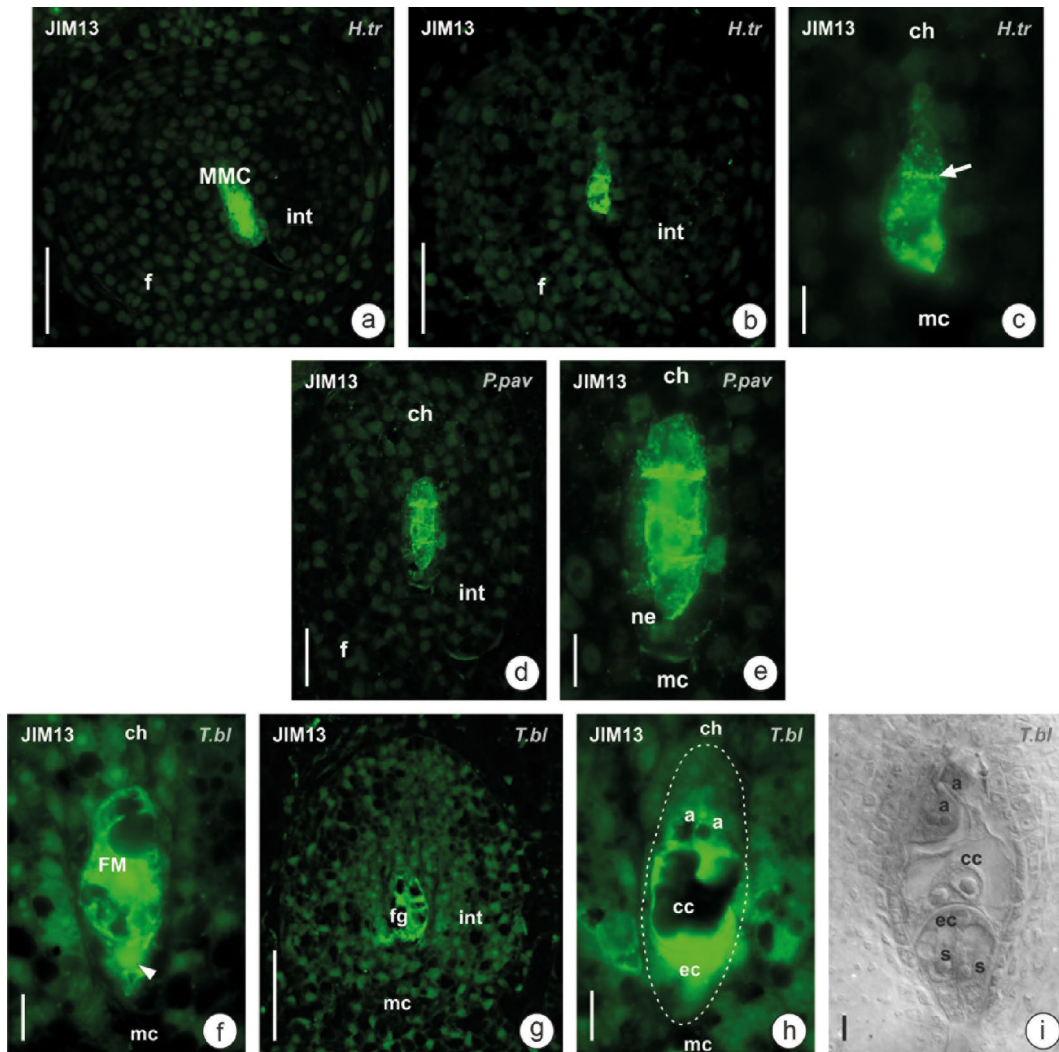
**Fig. 3.** Distribution of highly methyl-esterified pectins with LM20 immunolabeling. Longitudinal sections of ovaries and ovules in the amphimictic *Hieracium* (*H.tr*) and *Pilosella* (*P.off*) species (**a–d**) and in the apomictic *T. belorussicum* (*T.bl*) (**e–g**). Images **a**, **d–f** enhanced with DAPI staining indicating cell nuclei. (**a**) An anatropous ovule at the stage of the MMC (asterisk) and a fragment of the ovary wall in *H. transylvanicum*; note the even distribution of pectic epitope throughout the somatic ovule cells and more intense immunofluorescence in the walls of ovary cells (arrows). (**b**) Magnified part of the ovary wall with visible very strong immunofluorescence from pectins in the cell walls. (**c**) Micropylar pole of the *P. officinarum* ovule with visible MMC; note the fluorescence signal in the MMC wall and in the walls of nucellar epidermis cells. (**d**) An anatropous ovule of *H. transylvanicum* at the functional megaspore stage; visible even distribution of pectin epitope in the walls of somatic cells and in the wall of the functional megaspore, arrowhead shows remnants of degenerate megaspores. (**e–g**) Distribution of highly esterified pectins in the ovules of *T. belorussicum*. (**e**) An anatropous ovule at the DMMC stage and a fragment of the ovary wall; pectic epitope present in the cell wall of DMMC and in the walls of somatic cells, note particularly strong immunofluorescence in the walls of the ovary cells, in the funicle region and in the walls of cells surrounding the chalazal pole of the DMMC (arrow). (**f**) Micropylar pole of the ovule; immunofluorescent signal visible in the DMMC wall, the arrow points to a group of cells adjacent to the chalazal pole of the DMMC, which are distinguished by intense immunolabeling of the walls. (**g**) One-nucleate female gametophyte; pectins labeled with LM20 distributed mainly in its cytoplasm, a visible degenerated micropylar megaspore (arrowhead). Abbreviations: **ch** chalazal pole, **f** funicle, **DMMC** diplosporous megaspore mother cell, **FM** functional megaspore, **int** integument, **mc** micropylar canal, **MMC** megaspore mother cell, **ne** nucellar epidermis, **ov** ovary wall, **v** vacuole. Scale bars: **a**, **d** 20  $\mu\text{m}$ ; **b–c** 10  $\mu\text{m}$ ; **e** 50  $\mu\text{m}$ ; **f–g** 10  $\mu\text{m}$

## DISCUSSION

It is intriguing to find out whether the distribution of pectins and AGPs in the ovules at the stage of early reproductive processes follows an established pattern common to angiosperms. The second question is whether the mode of reproduction affects the temporal and spatial distribution of pectins and AGPs in the tissues of a developing ovule. Unfortunately, the data available in this regard is limited and no general conclusions can still be drawn. Immunocytochemical studies on

the detection of pectic and AGP epitopes in the female reproductive tissues have mostly concerned later developmental stages and they focused on the distribution of these epitopes in stylar transmitting tissues and in the ovules throughout the progamic phase and embryogenesis (e.g., Jauh and Lord, 1996; Lenartowska et al., 2001; Coimbra and Duarte, 2003; Chudzik et al., 2005b; Kościńska-Pająk et al., 2005; Suárez et al., 2013; Niedojadło et al., 2015; Gawecki et al., 2017; Lopes et al., 2016; Losada and Herrero, 2019; Lora et al., 2019; Płachno et al., 2020,





**Fig. 4.** Immunolocalization of arabinogalactan proteins with JIM13 labeling. Longitudinal sections of ovaries and ovules in the amphimictic *Hieracium* (*H.tr*) and *Pilosella* (*P.pav*) species (**a–e**) and in the apomictic *T. belorussicum* (*T.bl*) (**f–i**). (**a, b**) An anatropous ovule of *H. transylvanicum* at the MMC stage (**a**) and the megaspore dyad stage (**b**); no immunolabeling in somatic cells, fluorescence signal limited to generative cells. (**c**) Magnification of the megaspore dyad from Fig. 4b showing strong immunolabeling in the wall between megaspores (arrow) and in their cytoplasm. (**d**) Immunodetection of AGPs epitope in the ovule of *P. pavichii* at the megaspore tetrad stage; visible intense labeling in megaspores and very weak one in cells of the nucellar epidermis. (**e**) Magnification of the megaspore tetrad from Fig. 4d. (**f–h**) Distribution of AGPs epitope in the ovule of *T. belorussicum*. (**f**) Diplodyad with an increased chalazal megaspore and degenerated micropylar cell (arrowhead); JIM13 epitopes labeled the cell walls and cytoplasm of megaspores. (**g**) An anatropous ovule with a mature female gametophyte; AGPs epitope recognized by JIM13 are detected only in gametophyte cells. (**h**) Mature female gametophyte with visible intense immunolabeling of its cells. (**i**) Structure of a mature female gametophyte. Image obtained from a cleared ovule using DIC optics. Abbreviations: **a** antipodal cell, **cc** central cell, **ch** chalazal pole, **ec** egg cell, **f** funicle, **fg** female gametophyte, **FM** functional megaspore, **int** integument, **mc** micropylar canal, **MMC** megaspore mother cell, **ne** nucellar epidermis, **s** synergid. Scale bars: **a, b, g** 50 µm; **d** 25 µm; **c, e, f, h, i** 10 µm

2022; Leszczuk et al., 2021). The location of these wall components in young ovules at the time of MMC differentiation and megasporogenesis has only been studied in a handful of sexual species,

e.g., in *Pisum sativum* (Pennel and Roberts, 1990), *Arabidopsis thaliana* (Coimbra et al., 2007; Demesa-Arévalo and Vielle-Calzada, 2013; Lora et al., 2017), gymnosperm *Larix decidua* (Rafińska and

Bednarska, 2011), *Quercus suber* (Lopes et al., 2016), early-divergent angiosperms *Annona cherimola* and *Persea americana* (Lora et al., 2017), *Capsicum chinense* (Pérez-Pastrana et al., 2018), and recently in *Bellis perennis* (Leszczuk et al., 2021). Research findings clearly indicate that AGPs are involved in the early sexual reproductive processes. However, the distribution of AGPs varies between species and changes during ovule development as well as in response to abiotic stresses (Ma and Zhao, 2010; Lora et al., 2019; Leszczuk et al., 2021). For example, JIM13-labeled AGP epitope was not detected in the MMC of *Arabidopsis thaliana*, whereas it was observed in the MMC wall of basal angiosperms, i.e., *Annona cherimola* and *Persea americana* (Lora et al., 2017). Similarly, AGP epitope recognized by JIM13 was detected in the MMC as well as in the megaspores of amphimictic *H. transylvanicum*, *P. officinarum*, *P. pavichii* and *T. linearisquameum* analyzed in this study. With regard to apomicts, immunocytochemical studies of ovular cell walls have been carried out for just a few taxa from the Asteraceae family, i.e., *Chondrilla juncea*, *Pilosella piloselloides*, *P. praealtum*, *Taraxacum officinale* (Chudzik et al., 2005a; Kościńska-Pająk et al., 2005; Kościńska-Pająk, 2006; Gawecki et al., 2017; Juranić et al., 2018; Płachno et al., 2020) and *Fragaria x ananassa* from the Rosaceae family (Leszczuk and Szczuka, 2017, 2018). AGP epitope labeled with JIM13 has been shown to be the marker of the cell that initiates the development of an unreduced female gametophyte in both diplosporous *C. juncea* and aposporous *P. piloselloides* and *P. praealtum* as well as in the facultative apomict *Fragaria x ananassa* (Chudzik et al., 2005a; Juranić et al., 2018; Leszczuk and Szczuka, 2018). Similarly, we found AGPs in the developing unreduced female gametophyte in the young ovules of diplosporous *T. belorussicum*. Previously, the presence of JIM13-labeled AGP epitopes was described in the cells of a mature female gametophyte of *T. officinale* and the facultative apomict *Fragaria x ananassa* (Gawecki et al., 2017; Leszczuk and Szczuka, 2018; Płachno et al., 2020). All of the above examples indicate that AGPs are the early markers of both sexual and apomictic cell lineages. Notwithstanding the mechanism of AGPs' activity, their interactions with other cell wall components and their exact role have not been sufficiently explained (reviewed by Pereira et al., 2015; Leszczuk et al., 2019).

HGs are other cell wall components actively involved in plant reproductive processes. It has been established that the degree of pectin esterification changes dynamically in the ovule at the time of gametogenesis and embryogenesis. The methyl-esterification status affects the cell wall texture and mechanical properties, thus regulating cell growth and its shape (Wolf et al., 2009; Saffer, 2018). Hence, the different degrees of pectin esterification can be regarded as the markers of proliferation and differentiation, as demonstrated by Bárány et al. (2010) during pollen embryogenesis in *Capsicum annuum*. These authors found that highly esterified pectins were characteristic of proliferating cells, while high levels of non-esterified pectins were abundant in the walls of differentiating cells. Many reports indicate that highly esterified pectins prevail in the early stages of ovule development, following which the level of methyl-esterification gradually decreases and low esterified pectins dominate in the mature ovule. This pattern has been documented, for example, in *Olea europea* (Suárez et al., 2013), *Hyacinthus orientalis* (Niedojadło et al., 2015), *Quercus suber* (Lopes et al., 2016), *Capsicum chinense* (Pérez-Pastrana et al., 2018). Our findings were in line with these data and showed that with the development of the ovule (in both the amphimictic *H. transylvanicum*, *P. officinarum*, *P. pavichii*, *T. linearisquameum*, and in the apomictic *T. belorussicum*) the composition of HGs in cell walls changed according to the previously described pattern. Therefore, it seems that the dynamically changing level of pectin methyl-esterification observed in developing ovules may be a common, developmentally regulated feature. Nevertheless, Płachno et al. (2022) suggested that the pattern of spatial and temporal distribution of low and highly esterified pectins in the ovule may be species- or genus-specific. In the integumentary tissue of young ovules of *Hieracium*, *Pilosella* and *Taraxacum*, we did not notice any particular indications of later structural changes related to the differentiation of the periendothelial zone formed by mucilage cells (thickening of the cell walls or accumulation of a specific pectin fraction recognized by LM19 and LM20 antibodies, respectively). We did not detect the presence of AGPs in the somatic tissues of the ovules either, and JIM13-labeled AGP epitope was only the marker of cells initiating the sexual and apomictic development pathways. However, the accumulation of AGPs in mucilage cells was described in

older ovules and seeds of *Pilosella officinarum* and *Taraxacum officinale* (Gawecki et al., 2017; Płachno et al., 2020). According to Płachno et al. (2020), the presence of AGPs in mucilage cells points to the increased activity of these cells and suggests that they may play an important role in communication between maternal tissues and the developing embryo. Moreover, mucilage cells in the ovules of these species differed in terms of pectin content. The results of immunodetection with JIM5 and JIM7 antibodies showed the accumulation of both low and highly methyl-esterified pectins in the periendothelial tissue of *P. officinarum*, while the mucilage cells of *T. officinale* were characterized by a lower pectin content, dominated by low esterified pectins (Płachno et al., 2020). These findings indicate that the different content of low and highly methyl-esterified HGs in mature ovules is species-specific and it may be associated with a different way of forming the periendothelial zone. Nevertheless, pectins and their degree of methyl-esterification are believed to play an important role in seed imbibition and germination by altering the mechanical properties of cell walls (Müller et al., 2013; Francoz et al., 2019).

## CONCLUSIONS

The findings presented in this article complement the still sparse reports on angiosperms and suggest that the dynamically changing level of pectin methyl-esterification in developing ovules and the characteristic location of AGPs are not species-specific. They may, however, be a common, developmentally regulated feature of maturing ovules in angiosperms. Thus, further advanced research is still required to establish the role of these cell wall components in cell-to-cell communication during developmental and reproductive processes in the ovules of amphimictic and apomictic species.

## AUTHORS' CONTRIBUTION

All authors contributed to the study conception and design. ABJ – design of the study, material preparation, microscopic analyses, interpretation of results, data collection, preparation of figures and writing the manuscript; JM – identification and sampling of plant material, proofreading of the manuscript; KM – design of the study, inter-

pretation of results, proofreading of the manuscript. All authors read and approved the final manuscript. The authors declare that they have no conflict of interest.

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