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Spatio-temporal distribution and methyl-esterification of pectic epitopes provide evidence of developmental regulation of pectins during somatic embryogenesis in *Arabidopsis thaliana*

K. SALA*, I. POTOCKA, and E. KURCZYNSKA

Laboratory of Cell Biology, Faculty of Biology and Environmental Protection, University of Silesia, Jagiellońska 28, PL-40032 Katowice, Poland

Abstract

The aim of the present study was to describe the occurrence of three pectic epitopes, recognized by JIM7, LM19, and LM5 antibodies, during somatic (SE) and zygotic (ZE) embryogenesis in *Arabidopsis thaliana*. The epitopes recognized by JIM7 and LM19 antibodies showed different distributions during SE stages. Moreover, in the early stages of somatic embryo development, a cytoplasmic occurrence of LM19 epitope was detected. Distribution of a pectic epitope recognized by LM5 antibody corresponded to a vascular system differentiation pattern. Occurrence of LM5 epitope was the same in both zygotic and somatic embryos and often restricted to newly synthesized walls of two adjacent cells. These data suggest that both low and high methyl-esterified pectins (recognized by LM19 and JIM7 antibodies, respectively) are developmentally regulated during SE stages and (1→4)-β-D-galactan epitope (recognized by LM5 antibody) may play a role in cell cytokinesis.

Additional key words: galactan, immunofluorescence microscopy, JIM7, LM19, and LM5 antibodies, pectin methyl-esterification.

Introduction

Somatic embryogenesis resembles the stages of zygotic embryogenesis (ZE) so it is a good model to investigate cell and tissue developmental changes during ZE (Zimmerman 1993, Elviana *et al*. 2011, Pan *et al*. 2011). Changes occurring in the cell wall matrix indicate that the apoplast actively participates in cell differentiation events including embryogenesis (Brownlee 2002, Baluska *et al*. 2003, Bobak *et al*. 2003/4, Kohorn *et al*. 2006, Popielarska-Konieczna *et al*. 2008, Lai *et al*. 2011).

Pectins contribute to a large part of primary cell walls, although in secondary cell walls their amount is severely reduced or they do not occur at all (Mohnen 1999, Willats *et al*. 1999). Pectins comprise a heterogeneous group of polysaccharides composed mainly of galacturonic acid residues (Rose *et al*. 2003). We can distinguish homogalacturonan (HG), substituted galacturonans and rhamnogalacturonans I (RG-I) and II (RG-II).

HG is the most widespread and can constitute up to 65 % of all cell wall pectins (Ridley *et al*. 2001, Wolf *et al*. 2009). It has been proven that HG is mainly responsible for maintaining correct cell adhesion (Bouton *et al*. 2002). Although HG has a simple linear structure, acetylation and methyl-esterification of specific galacturonic acid residues have a strong influence on HG properties (Pauly and Scheller 2000, Willats *et al*. 2001). Changes in the degree of methyl-esterification have been found previously among HGs from different tissues or cells (Liners *et al*. 1992, Femenia *et al*. 1998, Siedlecka *et al*. 2008). On the other hand, the amount and distribution of RG-I are thought to be variable and undergoing dynamic changes during cell or tissue development (Bush *et al*. 2001, Pena and Carpita 2004, Verhertbruggen *et al*. 2009). Previous studies have shown that changes in the composition of side chains composed of neutral sugars like galactan or arabinan are correlated with the status of cell differentiation (Kikuchi *et al*. 1996, Willats *et al*. 1999, Ermel *et al*. 2000, McCartney *et al*. 2000, McCartney and Knox 2002, Leboeuf *et al*. 2004, Ulvskov *et al*. 2005).

The aim of this study was to define temporal and

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Abbreviations: HG - homogalacturonan; IZE - immature zygotic embryo; MS - Murashige and Skoog; PEGs - cuticular pegs; RG - rhamnogalacturonan; SAM - shoot apical meristem; SE - somatic embryogenesis; ZE - zygotic embryogenesis.

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* Author for correspondence: fax (+48)0322009361, e-mail: ksala@us.edu.pl

spatial localization of several pectic epitopes in different stages of somatic *Arabidopsis thaliana* embryo development. We also compared the occurrence of these epitopes in somatic embryos at the cotyledonary stage of development and their zygotic counterparts to find out whether SE is equivalent to ZE in terms of pectic epitopes. The distribution of pectic epitopes in the cell walls of somatic embryos during development provided an insight into the relationship between their occurrence and function.

Materials and methods

Arabidopsis thaliana (L.) Heynh. ecotype Columbia was grown in pots with soil and *Vermiculite* (2:1, v/v) at temperature of 20 - 22 ºC, relative humidity of 40 %, a 16-h photoperiod, and irradiance of 40 μmol m^{-2} s^{-1}. After 8 - 10 weeks, siliques were collected, surface sterilized in 20 % (m/v) NaClO for 20 min and rinsed three times with sterile distilled water. Afterwards, the siliques were dissected under sterile conditions to obtain immature zygotic embryos (IZEs) at the late cotyledonary stage of development (Gaj 2001). IZEs were cultured on Petri dishes (∅ 35 mm) with a B5 (Gamborg *et al*. 1968) liquid medium containing 5 μM 2,4-dichlorophenoxy-acetic acid (2,4-D) (Ikeda-Iwai *et al*. 2002). After 10 d, explants were transferred to phytohormone-free liquid Murashige and Skoog (1962; MS) medium. Cultures were kept on a rotary shaker (90 rpm) at 22 ºC and a 16-h photoperiod. Primary somatic embryos (SEs) formed between 8 - 21 d of culture.

Analysis of cultured explants and developing SEs were carried out under an *Olympus* (Tokyo, Japan) *SZH10* stereomicroscope, then samples were fixed in a mixture of 3 % (m/v) paraformaldehyde (PFA), 1.25 % (v/v) glutaraldehyde (GA), and 0.1 % (v/v) *Triton X-100* in phosphate buffered saline (PBS), pH 7.4 (Satiat-Jeunemaitre and Hawes 2001). Some samples were incubated in fixative at 4 ºC overnight, next rinsed with PBS (three times, each for 20 min), dehydrated in an ethanol series (10, 30, 50, 70, 90, and 100 %; v/v) and embedded in Steedman wax (Vitha *et al*. 2000). Wax-embedded samples were cut using a *Zeiss* (Jena, Germany) *HYRAX M40* microtome into 5 μm thick sections which were collected on microscopic slides covered with Mayer adhesive albumin. For the immunolabelling procedure, sections were de-waxed and rehydrated in a successive ethanol series (three times in 100, 90, and 50 % in PBS, v/v, each for 10 min).

Samples for *LR White* resin embedding were fixed at 4 ºC overnight in a mixture of 4 % PFA, 1 % GA, 0.1 % *Triton X-100*, 2 mM $CaCl_2$, and 1 % (m/v) sucrose in PBS, pH 7.2 (Chen *et al*. 2006). After washing with PBS and dehydration in an ethanol series, samples were infiltrated and embedded in *LR White* resin (*Polysciences*, Warrington, USA) which was polymerized at 50 ºC for 8 h. Serial sections (1 μm thick) were obtained using a *Leica* (Vienna, Austria) *EM UC6* ultramicrotome, collected on poly-L-lysine coated glass slides and dried on a warm plate.

For immunofluorescence labeling, all sections were blocked in a buffer containing 2 % (v/v) fetal calf serum (FCS) and 2 % (m/v) bovine serum albumin (BSA) in PBS, pH 7.2, at room temperature for 30 min, in order to mask non-specific binding sites. These samples were incubated with primary monoclonal antibodies [JIM7 - highly methyl-esterified HG (Knox *et al*. 1990), LM19 - low methyl-esterified HG (Verhertbruggen *et al*. 2009), and LM5 - 4 residues of (1→4)-β-D-galactan (Jones *et al*. 1997)] diluted 1:20 in blocking buffer at room temperature for at least 1.5 h. After rinsing (three times, 10 min each) with blocking buffer, sections were incubated with the secondary antibody (anti-rat IgG-Cy2, *Jackson Immuno-Research Laboratories*, West Grove, USA) and diluted 1:100 in blocking buffer at room temperature for at least 1.5 h. From this moment on, the procedure was carried out in darkness. After washing (three times, 10 min each) with blocking buffer and PBS, sections were stained with 0.01 % (m/v) toluidine blue for 10 min to quench tissue autofluorescence. Finally, sections were rinsed (three times, 5 min for each) with PBS and distilled sterile water, then shaken dry and mounted with *Fluoromount* (*Sigma*, St. Louis, USA) anti-fading medium. Negative controls were performed by omitting the primary antibody. All observations and photography were performed with an *Olympus BX42* microscope equipped with an *Olympus XC50* digital camera using a maximum excitation wavelength of 490 nm and obtaining maximum emission at 590 nm.

Results

At the early globular stage of SE, pectic epitope recognized by JIM7 antibody was abundantly detected in cuticular pegs of protodermal cells (PEGs) and in some inner embryo cell walls where its occurrence was rather dotted (Fig. 1*A*). In globular and heart stage embryos, JIM7 epitope was the most abundantly detected in walls of protoderm and cell layer below protoderm (Fig. 1*B* and *inset 1*). JIM7 epitope also appeared in the walls of some ground tissue cells mainly in the central region of the embryo (Fig. 1*B*, *inset 2*). At the cotyledonary stage, JIM7 epitope was distributed uniformly but its occurrence was the lowest in the outer protodermal cell walls of the embryo with the exception of the shoot apical meristem (SAM) L1 layer (Fig. 1*C*) and hypocotyl protoderm where it occurred abundantly. JIM7 epitope was also present in procambial cell walls (Fig. 1*C*).

In the early globular somatic embryo, pectic epitope recognized by LM19 antibody was detected in the thick outer protodermal walls as well as in the cytoplasm and walls of inner embryo cells (Fig. 1*D*). Distribution in the walls and the cytoplasm had a dotted character. At the globular stage of embryo development, LM19 epitope was still observed in outer periclinal cell walls of the protoderm but cytoplasmic distribution declined largely (Fig. 1*E*). At the heart and torpedo stages of development, LM19 epitope was present in the outer periclinal walls of the protoderm but a strong fluorescence signal was also detected in the area corresponding to

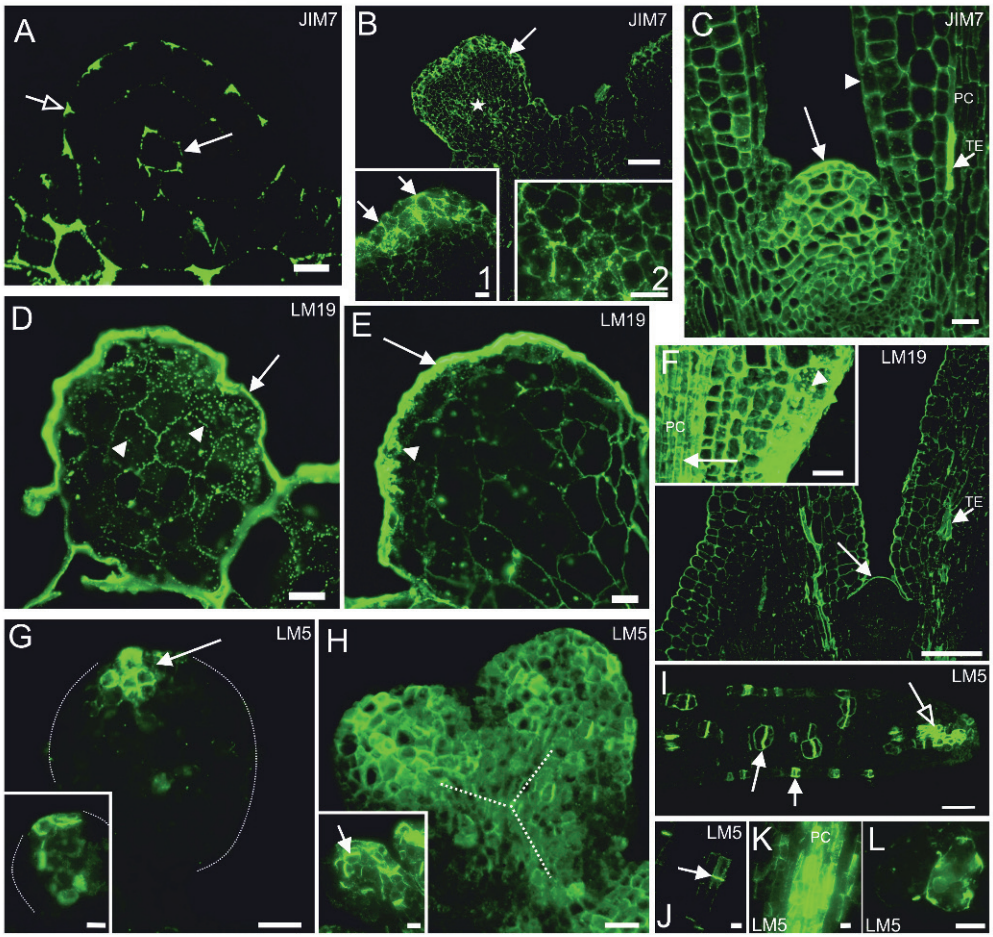

Fig. 1. Immunolabelling of *A. thaliana* somatic embryos. *A* - early globular stage of embryo development with JIM7 epitope occurrence restricted to PEGs (*open arrow*) and some inner cell walls (*arrow*); *B* - heart stage of embryo development with most abundant JIM7 epitope in protodermal and subprotodermal cell walls (*arrow*), *inset 1*: JIM7 epitope present in PEGs and some outer periclinal walls of protoderm (*arrows*), *inset* 2: occurrence of JIM7 epitope in ground tissue cell walls in the central region of the embryo, magnification of the area marked by the *asterisk* in *B*; *C* - SAM of cotyledonary stage embryo with JIM7 detected uniformly in embryo cell walls. Periclinal cell walls of L1 contained much more JIM7 epitope (*arrow*) than the rest of outer protodermal cell walls (*arrowhead*); *D* - early globular stage of development with LM19 epitope distributed continuously on outer periclinal protodermal walls (*arrow*) and in cytoplasm and walls of inner cells in a dotted manner (*arrowheads*); *E* - globular embryo with abundant LM19 epitope in outer periclinal walls of protoderm (*arrow*). Cytoplasmic distribution of LM19 epitope is most abundant in protoderm (*arrowhead*) of all embryo cells; *F* - SAM of cotyledonary stage embryo with LM19 epitope in protoderm outer periclinal cell walls (*arrow*), *inset*: basal part of mature somatic embryo hypocotyl with dotted occurrence of LM19 epitope in cytoplasm of some protodermal cells (*arrowhead*) and in walls of procambial cells (*arrow*); *G* - globular stage of embryo development showing LM5 epitope in some protodermal and subprotodermal cell walls at a pole opposite to the point of embryo attachment to an explant (*arrow*), *inset*: globular embryo with cytoplasmic distribution of LM5 epitope in some cells. *Dotted line* represents outlines of the embryos; *H* - heart stage of embryo development showing LM5 epitope in cotyledon primordia and embryo inner region (*dotted line*), *inset*: developing cotyledons at torpedo stage of embryo development with LM5 epitope in walls of dividing cells (*arrow*); *I* - somatic embryo cotyledon with LM5 epitope in walls of dividing cells (*arrows*) and provascular cells (*open arrow*); *J* - cells of hypocotyl ground tissue with LM5 epitope in anticlinal walls (*arrow*); *K* - procambium in hypocotyl with abundant LM5 epitope in all cell walls; *L* - cotyledon parenchyma cells with both cytoplasmic and wall distribution of LM5 epitope. PC - procambial cells, TE - tracheary elements. *Bars*: *F* = 100 μm, *B* = 50 μm, *H*, *I*, and *F inset* = 25 μm, *A*, *C-E*, *G*, *J-L*, *B inset 1,2*, *G inset*, and *H inset* = 10 μm.

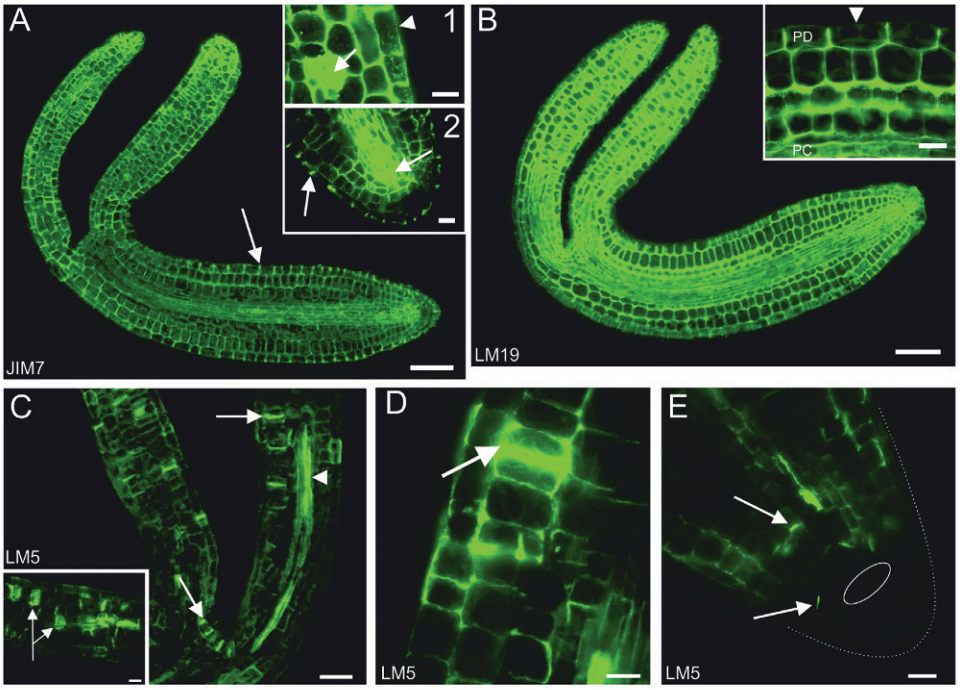

Fig. 2. Immunolabelling of *A. thaliana* zygotic embryos. *A* - uniform distribution of JIM7 epitope except protoderm, where PEGs and anticlinal walls contained noticeable amount of this epitope (*arrow*) whereas in outer periclinal walls only trace amounts of this epitope were detected, *inset 1*: cotyledon, abundant occurrence of JIM7 epitope detected in provascular cell walls (*arrow*) in contrast to outer periclinal walls of protoderm (*arrowhead*), *inset 2*: root, distribution of JIM7 epitope in PEGs and provascular tissue (*arrows*); *B* - widespread occurrence of LM19 epitope with exception of outer periclinal cell walls of protoderm, *inset*: hypocotyl, no LM19 epitope detected in the outer periclinal walls of protoderm (*arrowhead*); *C* - distribution of LM5 epitope in adjacent walls of some cells belonging to different tissues (*arrows*) and in procambial cells of cotyledon provascular system (*arrowhead*), *inset*: cotyledonal parenchyma cells with LM5 epitope in cytoplasm (*arrows*); *D* - hypocotyl, abundant LM5 epitope occurrence in newly formed wall of adjacent cells (*arrow*); *E* - root, occurrence of LM5 epitope restricted to walls of some cells (*arrows*). *Dotted line* represents an outline of the root, circle - QC. PC - procambial cells, PD - protoderm. *Bars*: *A* and *B* = 50 μm, *C* = 25 μm, *D*, *E*, *A inset 1 and 2*, *B inset*, and *C inset* = 10 μm.

provascular tissue and this pattern of LM19 epitope distribution was continued in the cotyledonary stage of embryo development, especially visible in the SAM and provascular tissue of the embryo root (Fig. 1*F* and *inset*). However, a dotted cytoplasmic occurrence of LM19 epitope was also observed in some protodermal cells of the basal part of hypocotyl (Fig. 1*F*, *inset*).

The first occurrence of pectic epitope recognized by LM5 antibody was observed at the globular stage in some protodermal and other embryo cell walls of a pole opposite to the point where the embryo was attached to an explant (Fig. 1*G*). At this stage, cytoplasmic distribution of LM5 epitope in a few cells was also detected (Fig. 1*G*, *inset*). At the heart stage, LM5 epitope was detected in the protoderm, the subprotoderm, the central region of the embryo, and the cotyledon primordia. However, it could not be determined whether LM5 epitope was restricted to the cell walls or to both the cell walls and the cytoplasm (Fig. 1*H*). At the torpedo stage of development, LM5 epitope occurred in provascular tissue and cells of developing cotyledons and was localized mainly in cell walls (Fig. 1*H*, *inset*). In a cotyledonary stage embryo, LM5 epitope was present in the walls of cells belonging to different tissues and most abundantly in the walls of dividing cells (Fig. 1*I*,*J*) and provascular tissue (Fig. 1*I*,*K*). LM5 epitope can be observed in the cytoplasm of some cells (Fig. 1L).

Regarding zygotic embryos, pectic epitope recognized by JIM7 antibody was detected in the cell walls of all tissues although it occurred at the lowest rate in the outer periclinal walls of the embryo protoderm (Fig. 2*A* and *inset 1*). An especially abundant occurrence of JIM7 epitope was observed in the walls of provascular cells in a cotyledon (Fig. 2*A*, *inset 1*) and a root (Fig. 2*A*, *inset 2*), in PEGs and in the walls of columella and lateral root cap cells (Fig. 2*A*, *inset 2*).

The pattern of LM19 epitope distribution was very similar to JIM7 epitope with one difference in protodermal outer periclinal walls where its occurrence was not detected (Fig. 2*B*, *inset*). It could also be noticed on the basis of fluorescence intensity that LM19 epitope occurred in higher amount than JIM7 epitope (Fig. 2*A*,*B*).

Pectic epitope recognized by LM5 antibody was detected in all embryo tissues. In the SAM, LM5 epitope occurrence was restricted to anticlinal cell walls (Fig. 2*C*). LM5 epitope occurred abundantly in newly formed walls of adjacent cells (Fig. 2*D*) but sometimes it was also present in the cytoplasm (Fig. 2*C*). Even in the meriste-

matic part of the root, where the occurrence of LM5 epitope was observed at the lowest rate, LM5 epitope appeared in walls of some cells, but not in the quiescent center (QC) (Fig. 2*E*).

Discussion

It is well established that modifications of pectin cell wall components contribute to cell adhesion and mechanical properties of plant tissues (Knox 1992, McCartney *et al*. 2000, Jarvis *et al*. 2003). One of these modifications is methyl-esterification of HG carried out by pectin methylesterases (PMEs, Micheli 2001). The degree of methyl-esterification and distribution of methyl groups along the HG chain, both dependent on PMEs action, affect the properties of HG (Micheli 2001, Willats *et al*. 2006).

Observations reported in this study indicate that pectic epitopes recognized by JIM7 and LM19 antibodies have distinct localization at similar stages of somatic embryo development. In the early globular somatic embryos, high methyl-esterified HG domains, corresponding to JIM7 epitope, were detected in PEGs of outer cell walls, whereas in the inner cell walls only some of them contained JIM7 epitopes and distribution had a dotted character. In contrast, low methyl-esterified HG, recognized by LM19 antibody, occurred most abundantly in the thick outer periclinal cell walls of the early globular embryo protoderm. Presence of low methyl-esterified HG during early SE events was also described in other species (Chapman *et al*. 2000, Ramirez *et al*. 2003, Xu *et al*. 2011). However, our study showed that low methyl-esterified HG was also present in the inner cell walls of the early embryo and, surprisingly, in the cell cytoplasm. How to explain the cytoplasmic localization of this kind of pectic epitope? It is believed that pectins are synthesized in high methyl-esterified form and methyl-esterification is carried out during cell growth and differentiation (Jarvis 1984, Liners *et al*. 1992, Zhang and Staehelin 1992, Femenia *et al*. 1999, Bush *et al*. 2001, Willats *et al*. 2001). If so, there are two possible reasons that could explain the cytoplasmic occurrence of low methyl-esterified HG: either HG was synthesized in low methyl-esterified form, or demethylo-esterification was carried out after synthesis but before HG was secreted to the cell wall. The second possibility correlates with the suggestion of Micheli (2001) that there is co-transport of pectins and PMEs in secretory vesicles to the cell wall. However, cytoplasmic accumulation of low methyl-esterified HG has been observed only in the early stage of embryo development and subsequently disappeared.

In globular embryos, high methyl-esterified HG was still restricted to PEGs in protodermal tissue, although in cell walls of inner embryo cells its distribution was uniform. This distribution pattern remained during further SE stages with some increase in occurrence due to tissue expansion. These observations correlate with a recently reported study on SE of bananas (Xu *et al*. 2011). Occurrence of low methyl-esterified HG also proceeds with a specific pattern and most abundant distribution was detected in outer periclinal cell walls during all SE stages. It is possible that occurrence of low methyl-esterified HG in the outer cell walls allows to maintain a definite volume, essential for coordinated cell divisions as reported earlier (Chapman *et al*. 2000). The observed pattern of low methyl-esterified HG distribution also corresponds with results published by Xu *et al*. (2011). We suggest that separate localizations of high and low methyl-esterified HG point to distinct functions of these two epitopes. However, it could not yet be defined exactly which roles they play in cell differentiation during SE. Moreover, similar distributions of mentioned pectic epitopes in monocotyledonous species, such as banana (Xu *et al*. 2011), and dicotyledonous one, such as *Arabidopsis thaliana*, can signify that pectin involvement in SE can be universal.

We also want to emphasize differences between the high methyl-esterified HG occurrence in walls of cells from SAM of somatic embryo at the cotyledonary stage of development and embryo cells which are undergoing differentiation. In all cell walls of SAM, this HG domain occurs abundantly which corresponds with earlier observations of SAM cells of *Sinapis alba* L. (Sobry *et al*. 2005). Moreover, it has frequently been reported that JIM7 epitope occurrence is specific to meristematic cells (Bush and McCann 1999, Iwai *et al*. 1999, Bush *et al*. 2001, Sobry *et al*. 2005). Similarities between meristematic and embryo cells, such as dense cytoplasm and tight adherence to each other, are often misleading when explants are examined by light microscopy and make difficult distinguish one type from another. We postulate that a detected difference in above mentioned HG domain distribution can be used as a marker which allows us to determine the character of analyzed cells.

Additionally, we compared the distribution of high and low methyl-esterified HG in somatic embryos and their zygotic counterparts and detected some differences in the presence of analysed pectic epitopes (JIM7 and LM19). The question arises, what is the cause of these differences? As it was suggested by Mingozzi *et al*. (2011), different growth conditions indeed influence physiological responses and thus morphogenesis. Furthermore, differences in chemical compositions of outer periclinal walls found between *Daucus carota* somatic embryos from solid and suspension cultures support this observation (Dobrowolska *et al*. 2012). Our results also fit to the statement mentioned above and the distinct outer protodermal wall composition may have occurred due to the *in vitro* conditions rather than to developmental differences between somatic and zygotic embryos. However, this issue requires further studies.

The highest abundance of (1→4)-β-D-galactan was

detected in newly formed adjacent cell walls and this specific distribution was observed in different tissues. Galactan side chains of RG-I have previously been shown to play an important role in cell differentiation in many plant species (Willats *et al*. 1999, McCartney *et al*. 2000, 2003). During the developmental switch from proliferation to elongation, the occurrence of galactose-rich pectin side chains is reduced (Willats *et al*. 1999, Leboeuf *et al*. 2004). Moreover, galactan epitopes recognized by LM5 antibody were found in tobacco *in vitro* cell cultures characterized as easily differentiating (Wisniewska and Majewska-Sawka 2008). In addition, side chains of RG-I were proposed to participate in the formation of cell plates during cytokinesis (Freshour *et al*. 1996). Setting all these observations together, our results and those presented by other authors mentioned above suggest that the (1→4)-β-D-galactan occurrence could contribute to *de novo* synthesis of cell walls during cytokinesis and its cytoplasmic localization would indicate cells which are about to divide. It is noteworthy that the same distribution pattern of (1→4)-β-D-galactan was observed in examined zygotic embryos.

Distribution of (1→4)-β-D-galactan during SE also corresponds with the established developmental pattern of procambium differentiation in somatic embryos. However, we could not define whether the presence of LM5 epitope contributes to cell wall differentiation or if its occurrence is characteristic to differentiated procambial cells.

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