

# Distribution of arabinogalactan proteins and pectins in the cells of apple (*Malus × domestica*) fruit during post-harvest storage

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- **Background and Aims** Changes in the arrangement of cell wall components determine cell wall properties (integrity, stiffness), thereby affecting the macro-scale properties of fruits, which are important for consumers and industry. Arabinogalactan proteins (AGPs) are ubiquitous components of the plant cell, in which they have various functions. Currently, AGPs are considered to be one of the less well-known, enigmatic proteoglycans, a consequence of their heterogeneous structure and unclear mechanism of activity.
- **Methods** An immunocytochemical study was conducted to elucidate the distribution of AGPs and pectic polysaccharides contained in apple (*Malus × domestica*) fruit during senescence. De-esterified homogalacturonan (LM19), methyl-esterified homogalacturonan (LM20), processed arabinan (LM16) and three AGP epitopes (JIM13, JIM15, MAC207) were identified in the fruit at three stages: fresh fruit, and fruit at 1 and 3 months of post-harvest storage.
- **Key Results** Microscopy revealed spatio-temporal changes in the localization of all examined epitopes. Changes of fruit cell wall assembly and its degradation were confirmed by determination of the galacturonic acid content in the WSP (water soluble pectins), CSP (chelator soluble pectins) and DASP (dilute alkali soluble pectins) fractions.
- **Conclusions** The results revealed dependencies between AGPs, arabinan and homogalacturonan distribution in apple fruit, which are correlated with changes in microstructure during senescence. We propose that AGPs are involved in establishment of the cell wall – plasma membrane continuum.

**Keywords:** Arabinogalactan proteins, cell wall, fruit senescence, *Malus × domestica*, pectin, plasma membrane.

## INTRODUCTION

The cell wall of fruit plays a significant biological function and determines crop quality, which is important for both the grower and the consumer. The prevailing structural model of the cell wall consists of a cellulose–hemicellulose network, an amorphous matrix composed of pectins, and small amounts of proteins and phenolic compounds (Bidhendi and Geitmann, 2016; Guillon *et al.*, 2017). Among many factors, the distribution of the above constituents, their mutual interactions, cross-linking and multidomain organization determine the mechanical properties of fruit such as firmness (Chen *et al.*, 2009; Geitmann, 2010; Zhang *et al.*, 2010; Jarvis, 2011). Numerous studies describe the presence of de-esterified homogalacturonan (HG) at the corners of intercellular spaces; their presence is related to their function in cell-to-cell contact. Moreover, the correlation with Ca<sup>2+</sup> cross-linking suggests a role of de-esterified HG in the mechanism of intercellular adhesion (Ng *et al.*, 2013). Also, methyl-esterified HG epitopes generally occur at cell junctions and in the middle lamella (Christiaens *et al.*, 2011; Zamil and Geitmann, 2017). By contrast, an entirely different distribution is shown by rhamnogalacturonan type I (RG-I) epitopes, which are restricted to a region close to the plasma membrane, associated with its role in wall rearrangements during cell growth (Guillon *et al.*, 2017).

Proteomic research on dynamic changes occurring during apple fruit ripening and storage indicated that proteins exert an

impact on specific metabolic pathways and in this way have an influence on complex physiological changes, e.g. reduced firmness reduction and cell wall degradation during post-harvest storage. Proteins that are differentially expressed in apple fruit during maturation and storage were characterized by Shi *et al.* (2014). Moreover, it has been established that specific proteins are involved in cell wall synthesis, contributing to the stabilization of the actin cytoskeleton, and are associated with changes in the content of soluble solids (Shi *et al.*, 2014). Furthermore, one of the structural proteins of the plant extracellular matrix, arabinogalactan protein (AGP), is involved in the response to abiotic factors, i.e. mechanical wounding. Studies of AGP gene expression profiles confirmed its involvement in oxygen deficiency adaptation in fruit. Moreover, constitutive expression during developmental processes indicates AGPs functions during fruit ripening (Fragkostefanakis *et al.*, 2012). In addition, as dynamic cell wall components, AGPs are up-regulated by plant growth regulators, and interact with auxin and cytokinin (Seifert and Roberts, 2007).

AGPs are classified as a category of the superfamily of hydroxyproline-rich glycoproteins (HRGPs). Among the HRGPs, there are highly glycosylated members found in cells throughout the plant kingdom, acting as covalent cross-linkers in the polysaccharide–proteoglycan network. Identification of linkages between cell wall polymers provided evidence for the

existence of the protein APAP1, composed of an AGP core, with attached RG-I, HG and arabinoxylan (Tan *et al.*, 2013; Hijazi *et al.*, 2014a). AGPs are distinguishable from other HRGPs (e.g. extensins) based on amino acid composition, the specific arrangement of Ala-Pro, Pro-Ala, Ser-Pro and Thr-Pro, various carbohydrate chains, and anchoring by glycosylphosphatidylinositol sequence to the plasma membrane (Showalter, 2001; Ma *et al.*, 2017). Moreover, Lampion and Varnai (2013) and Lampion *et al.* (2014) described AGPs as a periplasmic  $\text{Ca}^{2+}$  capacitors involved in intramolecular  $\text{Ca}^{2+}$  binding in many biological processes. This feature of AGPs was also associated with their contribution to the generation of an adhesive film by cohesion of the adjacent AGP nanoparticles and  $\text{Ca}^{2+}$  movement in the extracellular space in root cells (Huang *et al.*, 2016). We previously revealed the specific localization of AGPs in apple fruit tissue, and showed that the arrangement of the [ $\beta\text{GlcA}(1\rightarrow3)\text{-}\alpha\text{GalA}(1\rightarrow2)\text{Rha}$ ] epitope depends on the kind of tissue and the stage of post-harvest storage (Leszczuk *et al.*, 2018). Although research on cell wall components is a very active field, little is known about associations between AGPs and other constituents of the cell wall; similarly, the contribution of AGPs to cell wall assembly is still poorly documented.

Therefore, the aim of present study was to describe the specific arrangement of AGPs and pectins with low vs. high levels of methyl esterification in the cell wall – plasma membrane continuum. Details of the localization of AGPs, processed arabinan, HG in the cell wall architecture, and a quantitative analysis of galacturonic acid (GalA) content allow us to make conclusions about changes in the assembly of fruit microstructure.

## MATERIAL AND METHODS

### *Plant material and sample preparation*

The apple (*Malus × domestica*) fruit of the cultivar ‘Gala Must’ were used in this study. Fruit were purchased from a local producer and had been harvested at the optimum maturity time for this cultivar. Samples were taken at three different stages: at the optimal harvest date (1), after storage in a cold room at 2 °C in a normal atmosphere for 1 month (2), and after 3 months of storage (3). The material was sampled from five apple fruit of each examined stage. From each apple, ten cube-shaped samples were taken from the outer part which included the skin and to a depth of approx. 2 cm under the skin. The epidermal and hypodermal layers were examined, as was the parenchymal tissue. For each immunohistochemical staining, a minimum of 20 tissue sections were analysed for each sample of fruit. The experiments were repeated several times for each antibody and typical image sets were selected and shown.

### *Fixation, resin embedding and tissue section*

Prepared fruit segments were fixed in 2 % (w/v) paraformaldehyde and 2.5 % (v/v) glutaraldehyde in phosphate buffered saline (PBS; all from Sigma). PBS was prepared according to the manufacturer’s recommendations. One tablet dissolved in 200 mL of deionized water yields 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride,

pH 7.4, at 25 °C. After placement under a vacuum for 2 h and overnight incubation, the material was rinsed in PBS (three times, 15 min each). Dehydration (in a graded series of ethanol solutions, from 30, 50, 70, 90 to 96 % for 20 min) was carried out at room temperature. The samples were washed with 99.8 % ethanol twice. The ethanol was substituted with 3: 1, 1: 1 and 1: 3 mixtures of EtOH and LR White resin (Sigma) for 2 h and with pure LR White resin overnight. The polymerization was performed at 55 °C for 48 h in gelatin capsules. Semithin (1  $\mu\text{m}$ ) sections were obtained using an ultramicrotome (Leica Reichert Ultracut S) equipped with a glass knife. The sections were mounted on poly-L-lysine-coated glass slides (Sigma). The methods proposed by Wilson and Bacic (2012) were used to prepare samples for microscopy analysis.

### *Toluidine blue staining*

After washing in water, the sections were stained with a 0.5 % (w/v) Toluidine blue aqueous solution at 55 °C for 30 s to visualize the anatomical changes in the fruit tissue during senescence. The observations were carried out and photographs were taken using an N-800M light microscope (Novel) with a Canon PowerShot A640 camera.

### *Yariv reagent staining to detect AGPs*

To confirm the presence of AGPs in the fruit tissue, staining with Yariv reagent was performed (Biosupplies Australia). Yariv reagent ( $\beta\text{-GlcY}$ ) is used as a cytochemical dye to perturb the functions of AGPs and also for the detection, quantification, purification and staining of AGPs (Popper, 2011; Kitazawa *et al.*, 2013).  $\beta\text{-GlcY}$  selectively binds to AGPs, recognizing protein moieties and  $\beta\text{-1,3-galactan}$  chains (Huang *et al.*, 2016).

The sections were incubated in a  $\beta\text{-GlcY}$  solution for 1 h at room temperature and examined by bright field microscopy. The working solution containing 2 mg of  $\beta\text{-GlcY}$  in 1 mL of 0.15 M NaCl was used following the instructions of the supplier.

### *Immunocytochemical analysis of cell wall glycans*

A modified method described by Watanabe *et al.* (2011) and Rydahl *et al.* (2017) was used for immunofluorescence labelling. Slides prepared for immunocytochemistry reactions were circled with a liquid blocker (PAP Pen, Sigma). Sections adhering to poly-L-lysine slides were washed in PBS twice and treated with 1 % bovine serum albumin (BSA) in PBS for 30 min to avoid non-specific binding of antibodies. The sections were then incubated with the primary antibody present at a 1: 50 dilution in 0.1 % BSA at 4 °C for 24 h and washed four times with PBS. The experiment was conducted using monoclonal antibodies: JIM13, JIM15, MAC207 and LM16, LM19, LM20 (Table 1). The applied antibodies directed against AGPs and pectin are available at CarboSource Services (University of Georgia, USA) and PlantProbes (University of Leeds, UK). For visualization of the examined epitopes, incubation with anti-rat antibody conjugated with AlexaFluor 488 (Invitrogen Molecular Probes) was performed. The secondary antibody was diluted 1: 200 in 1

TABLE 1. List of monoclonal antibodies used for the detection of AGP and pectin epitopes in fruit tissue

Antibody name	Antigen	Reference/provider
JIM13	Arabinogalactan protein $\beta$ GlcA(1→3)- $\alpha$ GalA(1→2)Rha	Yates <i>et al.</i> (1996) CCRC, USA
JIM15	Arabinogalactan protein Epitope: unknown	Yates <i>et al.</i> (1996) CCRC, USA
MAC207	Arabinogalactan protein $\beta$ GlcA(1→3)- $\alpha$ GalA(1→2)Rha	Pattathil <i>et al.</i> (2010) CCRC, USA
LM16	Processed arabinan (1→5)- $\alpha$ -L-arabinan	Verherbruggen <i>et al.</i> (2009) PlantProbes, UK
LM19	De-esterified homogalacturonan $\alpha$ -GalA(1→4) $\alpha$ -GalA(1→4) $\alpha$ -GalA(1→4)- $\alpha$ GalA	Verherbruggen <i>et al.</i> (2009) PlantProbes, UK
LM20	Methyl-esterified homogalacturonan $\alpha$ -MeGalA(1→4) $\alpha$ -MeGalA(1→4) $\alpha$ -MeGalA(1→4) $\alpha$ -MeGalA	Verherbruggen <i>et al.</i> (2009) PlantProbes, UK

CCRC, Complex Carbohydrate Research Center.

% BSA at 4 °C and left in darkness. After 24 h, the incubated sections were washed in PBS and deionized water, and finally enclosed in Dako Fluorescent Mounting Medium (Sigma). For better localization of the epitopes, some sections were counterstained with Calcofluor White (Fluka) for cellulose.

Control reactions were carried out by omitting the primary antibody. The sections were also analysed for autofluorescence. All immunofluorescence images were examined by laser scanning confocal microscopy using an Olympus BX51 CLSM equipped with corresponding software FluoView v. 5.0. (Olympus Corporation). The excitation wavelength for the AlexaFluor 488 was 496 nm, and the emission wavelength was 519 nm. All parameters (i.e. laser intensity, gain) were kept constant for all experiments. Photographs and the scheme were edited using the CorelDrawX6 graphics program.

#### Cell wall material extraction

The cell wall material (CWM) for the study was obtained using the method of hot alcohol-insoluble solids with a few modifications as described by Renard (2005) and Szymańska-Chargot *et al.* (2015).

#### Determination of galacturonic acid content

The CWM (0.1 g) was stirred in deionized water (9 mL) overnight. It was then filtered and the supernatant containing water-soluble pectins (WSPs) was collected. The residue dissolved in water was stirred in 0.1 M CDTA (5 mL, pH 6.5) for 6 h, filtered, and again stirred in 0.1 M CDTA (5 mL, pH 6.5) for 2 h. The supernatants (with calcium chelator-soluble pectins, CSPs) from these two steps were collected together. The residue was then stirred in 0.05 M Na<sub>2</sub>CO<sub>3</sub> with the addition of 20 mM NaBH<sub>4</sub> (5 mL) overnight, filtered, and again stirred in 0.05 M Na<sub>2</sub>CO<sub>3</sub> with the addition of 20 mM NaBH<sub>4</sub> (5 mL) for 2 h. The supernatants (with dilute alkali-soluble pectins, DASPs) of these two steps were collected together.

Determination of the GalA content in the WSP, CSP, and DASP fractions was performed in triplicate with an automated wet chemistry analyser, known as a continuous flow analyser (San<sup>++</sup>, Skalar, Analytical). This is an automated procedure for colorimetric determination of GalA based on the total

decomposition of the pectin sample in acidic medium (sulphuric acid). The products obtained are transformed into furfural derivatives, which react with 3-phenyl phenol to form a coloured dye, the colour intensity of which is measured at 530 nm (Cybulska *et al.*, 2015; Chylińska *et al.*, 2016; Szymańska-Chargot *et al.*, 2016). The pectic content is expressed as GalA content per mass of CWM (mg g<sup>-1</sup>).

#### Statistical analysis

The OriginPro 8.5 (Origin Lab v8.5 Pro) program was used for the calculation of average values and standard deviations for chemical compositional changes in the apples. For comparisons of means, an analysis of variance (one-way ANOVA) followed by post hoc Tukey's honestly significant difference (HSD) test was used.

## RESULTS

#### Incurrence of arabinogalactan proteins in cell walls during fruit senescence

During post-harvest storage, microstructural and biochemical changes took place in all fruit compartments. The epidermal layer of fresh fruit at the optimal harvest date for 'Gala Must' is composed of cells that are flattened and stretched to the fruit surface. Intensively stained, thick epidermal cell walls of fruit at harvest maturity indicate an advanced stage of fruit ripening and beginning of fruit softening (Fig. 1A). Deep apertures in the epidermal and subepidermal layers, cuticle penetrating the anticlinal walls of the epidermis, disturbance in the integrity of the epidermal layer and numerous microcracks were visible (Fig. 1B and C). The differences in the morphological features of fresh and stored fruit correlated with the composition of the cell wall. Yariv reagent and monoclonal antibodies against AGPs were used to label fruit cell walls at every examined stage. In fresh fruit, red staining was noticeable in the epidermal layer (e), hypodermis (h) and parenchymatous cells (p) (Fig. 1D). Treatment with Yariv reagent revealed localization of AGPs along the cell wall, mainly in association with the cell membrane. Along with the ageing process in the fruit tissue, the colour reaction was less intense (Fig. 1E and F).

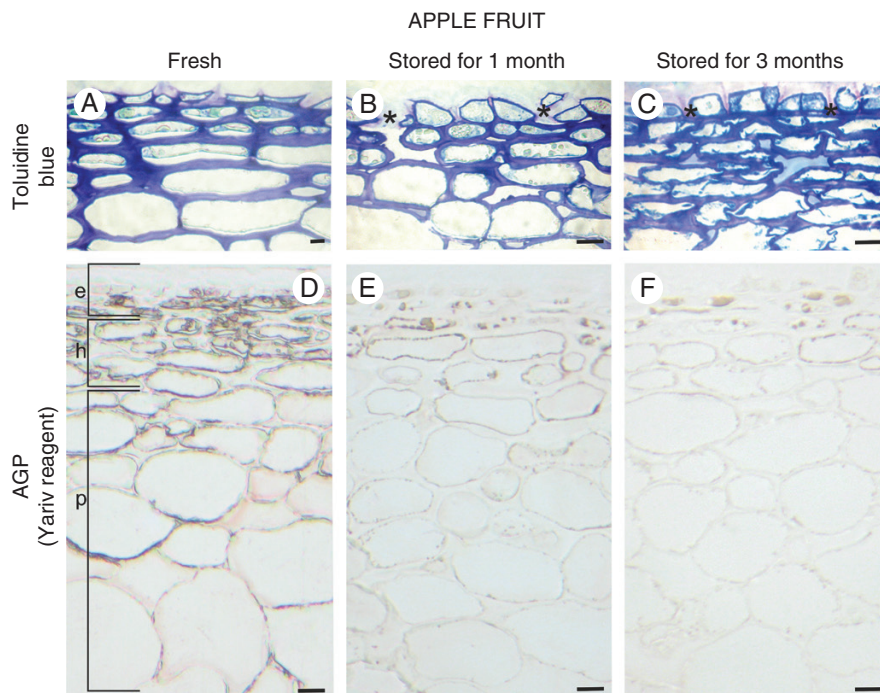


FIG. 1. Histology of fruit tissue during senescence and its correlation with the presence of AGP. Toluidine blue-stained sections of apple fruit: fresh (A), after 1 month of storage (B), and after 3 months of storage (C). The deep apertures in the epidermal layer are asterisked in B and C. Red stain confirming the presence of AGPs in apple fruit: fresh (D), stored for 1 month (E), and stored for 3 months (F), after staining with Yarrow's reagent. Light microscopy. Scale bars = 10 µm (A–C), 20 µm (D–F). Abbreviations: e – epidermis, h – hypodermis, p – parenchymal cells.

#### *Distinctive arrangement of AGPs and pectin epitopes in the external layers of apple fruit*

Most changes associated with fruit senescence and protection of the fruit from environmental conditions take place in the external layers and wax covering of the fruit. Immunofluorescence labelling allowed us to detect a changeable distribution of AGPs and pectins in the cell wall of the epidermal and hypodermal layers. The labelling pattern was similar for all of the antibodies against AGP epitopes in fresh apple tissue. Epitopes recognized by JIM13, JIM15 and MAC207 were found in the inner cell wall layer, in association with the plasma membrane. Moreover, AGP epitopes were absent in the central region of the cell wall (Fig. 2A, D, G). After 1 month of storage, fluorescence indicating the presence of AGPs was also visible in the cytoplasm endomembrane system of epidermal cells (Fig. 2B, E and H). Disorder in the localization of AGP epitopes was found in the fruit after 3 months of storage. Weak fluorescence was visible in the plasma membrane and in the adjacent cytoplasm compartments (Fig. 2C, F, I). The term ‘cytoplasm’ has been used here as a shortcut suggesting a cytoplasmic endomembrane system and/or vesicles of the endoplasmic reticulum and Golgi apparatus.

Immunolocalization of processed arabinan (LM16) and HG (LM19 and LM20) showed different distributions of these epitopes. The peel of fresh apple fruit is rich in domains recognized by LM16 monoclonal antibody (mAb). Detection of the processed arabinan epitope in the adherent walls of the cells of epidermal and subepidermal layers, and its absence from the central region of the cell wall were visible in fresh fruit (Fig. 2J). After reaction with LM16 mAb in sections of fruit after storage, the fluorescence signal was unchanged and originated from the cell walls/plasma membranes (Fig. 2K and L).

The localization of de-esterified and methyl-esterified HG differs significantly from the distribution of previously described cell wall components. The level of esterification of the pectic HG influences its functional properties. LM19 recognizes de-esterified HG with a degree of esterification lower than 50%. For LM20, the binding strength decreased upon de-esterification (Christiaens *et al.*, 2011). LM19 epitopes are abundant in the thickened cell walls, especially at the corners (Fig. 2M). Within 3 months, the fruit exhibited a decrease in fluorescence intensity and the LM19 epitopes are concentrated in the zones of cell adhesion, at the corners and in the anticlinal walls of the epidermal layer (Fig. 2N and 2O). Epitopes of methyl-esterified HG recognized by LM20 were present along whole cell walls of fresh apple fruit, and in the cellular junctions (Fig. 2P). In fruit stored for between 1 and 3 months, the fluorescence signal decreased (Fig. 2Q and 2R). The control reaction performed without the presence of the primary antibody lacked any fluorescence signal (Fig. 2S, T, U).

#### *Distribution of AGP and pectin epitopes in the parenchymal tissue of apple fruit during storage*

More differences between fresh and stored apple fruit cells were observed in the cells of fruit parenchyma (Fig. 3). The labelling signals after reactions with JIM13, JIM15 and MAC207 mAbs were at their most pronounced in fresh fruit, especially in the plasma membrane and in the border of the cell wall (Fig. 3A, D, G). In the pulp of fruit stored for 1 month, a decline in the fluorescence signal was observed but without significant changes in the localization of AGP epitopes (Fig. 3B, E, H). In fruit stored for 3 months, the AGP epitopes were

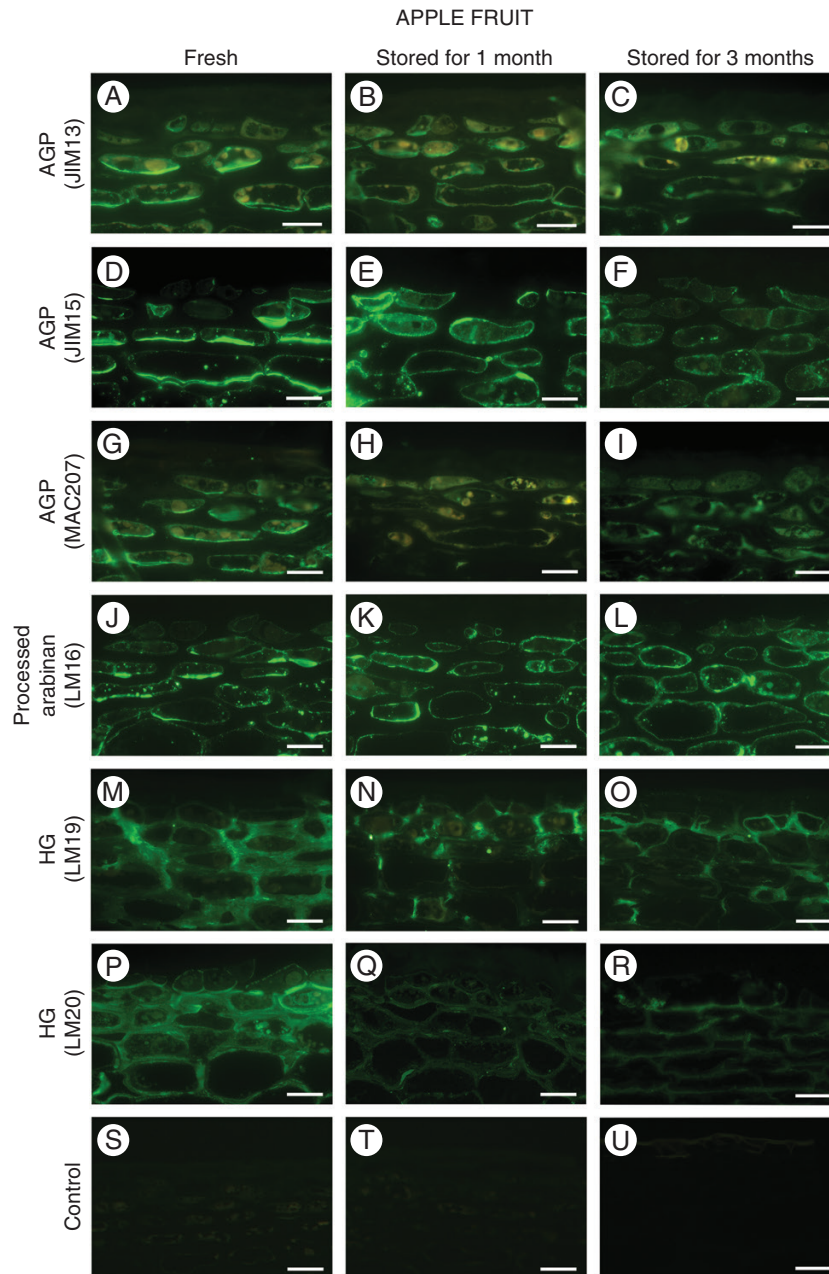


FIG. 2. Changes in the localization of AGP and pectin epitopes in the fruit exocarp during senescence. AGP epitopes recognized by JIM13 antibody in apple fruit: fresh (A), stored for 1 month (B), and stored for 3 months (C). Similar immunofluorescence reaction with JIM15 antibody (D, E, F) and MAC207 (G, H, I). Reactions with LM16-recognized processed arabinan epitopes in apple fruit (J, K, L). Immunofluorescence labelling with LM19 antibody against de-esterified homogalacturonan epitopes (M, N, O) and LM20 antibody against methyl-esterified homogalacturonan (P, Q, R) in apple fruit. Control reactions omitting primary antibody (S, T, U). Confocal laser scanning microscopy. Scale bars = 20  $\mu\text{m}$  (A–R), 40  $\mu\text{m}$  (S–U).

restricted to damaged cell walls, plasma membrane fragments and cytoplasm compartments (Fig. 3C, F, I).

An epitope recognized by the LM16 antibody was distributed in a similar zone of parenchymous cell as the AGP epitopes. In fresh fruit, the LM16-processed arabinan epitope occurred in the cell wall – plasma membrane continuum (Fig. 3J). However, after storage, this epitope was distributed non-homogeneously in regions of the degraded cell walls and plasma membranes (Fig. 3K and L).

Labelling with the LM19 antibody indicated that de-esterified HG epitopes were located in primary cell walls and at the corners of the cell–cell adhesion zones (Fig. 3M). Moreover,

after storage, de-esterified HG epitopes were present within the junctions (Fig. 3N) and after 3 months, they were visible only at the corners of cellular junctions (Fig. 3O).

A different distribution characterized the epitopes of HG with a higher degree of esterification, recognized by LM20. The whole surface of the cell wall of fresh parenchyma was abundant in these kinds of pectin. A strong signal was observed in both the central region of cell wall and the cellular junctions (Fig. 3P and Q). In apple fruit stored for 3 months, the LM20 epitope was dispersed throughout the cell wall and the fluorescence signal monitored was also weaker (Fig. 3R). The control

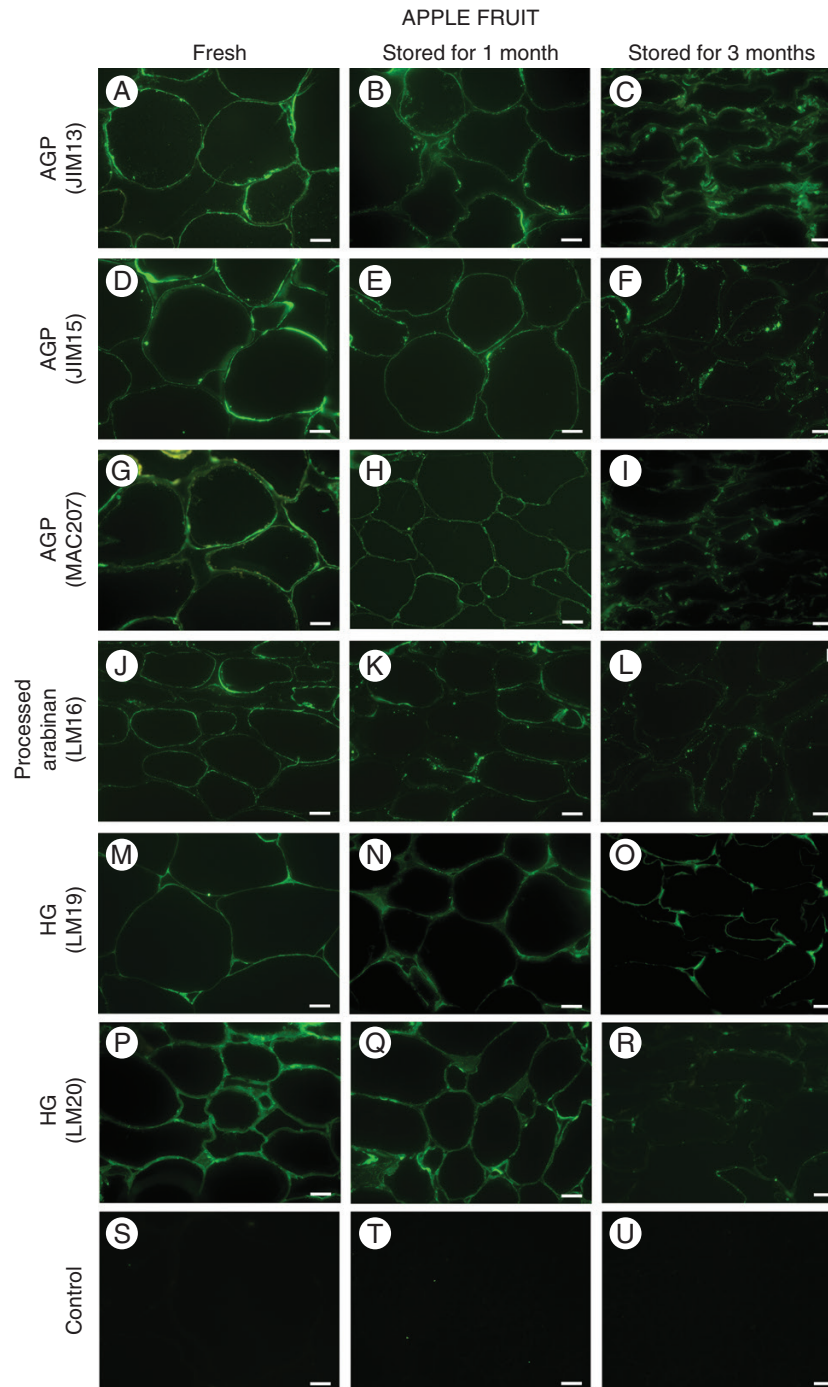


FIG. 3. Changes in the localization of AGP and pectin epitopes in parenchymal cell walls of fruit during senescence. Immunofluorescence labelling for AGP epitopes recognized by JIM13 antibody (A, B, C), JIM15 antibody (D, E, F) and MAC207 antibody (G, H, I) in parenchymal tissue from fresh apple fruit and fruit after storage for 1 and 3 months. Immunocytochemistry reactions with LM16 antibody (J, K, L), LM19 antibody (M, N, O) and LM20 antibody (P, Q, R). Control reactions omitting primary antibody (S, T, U). Confocal laser scanning microscopy. Scale bars = 20  $\mu\text{m}$  (A–U).

reactions performed without the primary antibody lacked any fluorescence signal (Fig. 3S, T, U).

Differences in the location of AGPs, processed arabinan and HG in the cell wall were more noticeable after calcofluor counterstaining, especially in the regions of cell–cell contact (Fig. 4). AGPs and processed arabinan were located mainly in the plasma membrane and at the border of the cell wall (Fig. 4B and F). In

stored fruit, both AGPs and arabinan epitopes occurred at lower amounts in the plasma membrane close to the disintegrated structure of the cell wall (Fig. 4D and H). Fluorescence imaging indicated the presence of HGs in intercellular spaces, which distinguishes them from AGPs and arabinan. Furthermore, de-esterified HGs were concentrated mainly at the corners (Fig. 4J and L), whereas methyl-esterified HGs filled the entire intercellular

spaces. In apple fruit stored for 3 months, the fluorescence signal was weaker (Fig. 4N and P).

To visualize and summarize all of the results described, we created a schematic representation of the localization of the examined epitopes in fresh apple fruit and the changes which occur during fruit storage (Fig. 5).

#### Biochemical analysis of cell wall polysaccharides in fruit during storage

To explain the weaker fluorescence signal in the sections of the stored material, standard chemical analysis of the GalA content of the fruit was carried out for the three stages examined. During fruit storage, the amount of GalA in the WSP and DASP fractions was variable. The WSP fraction contained concentrations varying from 26.48 ( $\pm 0.46$ ) to 62.38 ( $\pm 0.24$ ) mg g<sup>-1</sup> of GalA in CWM during storage. The level of GalA in the CSP fraction was stable, i.e. approx. 28.64 ( $\pm 0.38$ ) mg g<sup>-1</sup> CWM in fresh fruit and 30.88 ( $\pm 0.17$ ) mg g<sup>-1</sup> CWM in the stored material. These results reveal that the content of GalA in the DASP fraction was higher than in the other fractions examined and significantly decreased after 3 months of storage [from 110.87

( $\pm 3.07$ ) to 66.28 ( $\pm 0.74$ ) mg g<sup>-1</sup> CWM]. The observed changes in GalA content are shown in Fig. 6.

#### DISCUSSION

The cell wall is a network composed of polysaccharides and proteins. It is well known that the functions of the components depend on interlinks between them, but their specific distribution in the particular parts of the cell is also crucial for cell wall – plasma membrane properties (Bidhendi and Geitmann, 2016).

The localization of pectins in fruit has mainly been investigated using immunolabelling methods (Ng *et al.*, 2013) and confocal Raman microscopy (Szymańska-Chargot *et al.*, 2016; Chylińska *et al.*, 2017). The present study has shown a distinctive pattern of AGPs, arabinan and HG localization in fresh and stored apple fruit. We have shown that AGP and de-esterified and methyl-esterified pectic polymers are located in different parts of the fruit cell. The AGP and arabinan epitopes have been labelled in the plasma membrane and in the border of the cell wall, and their absence in tricellular junctions excludes AGPs having role in intercellular adhesion in fruit tissue.

During the senescence process, the cell wall undergoes numerous modifications causing fruit softening. A change in

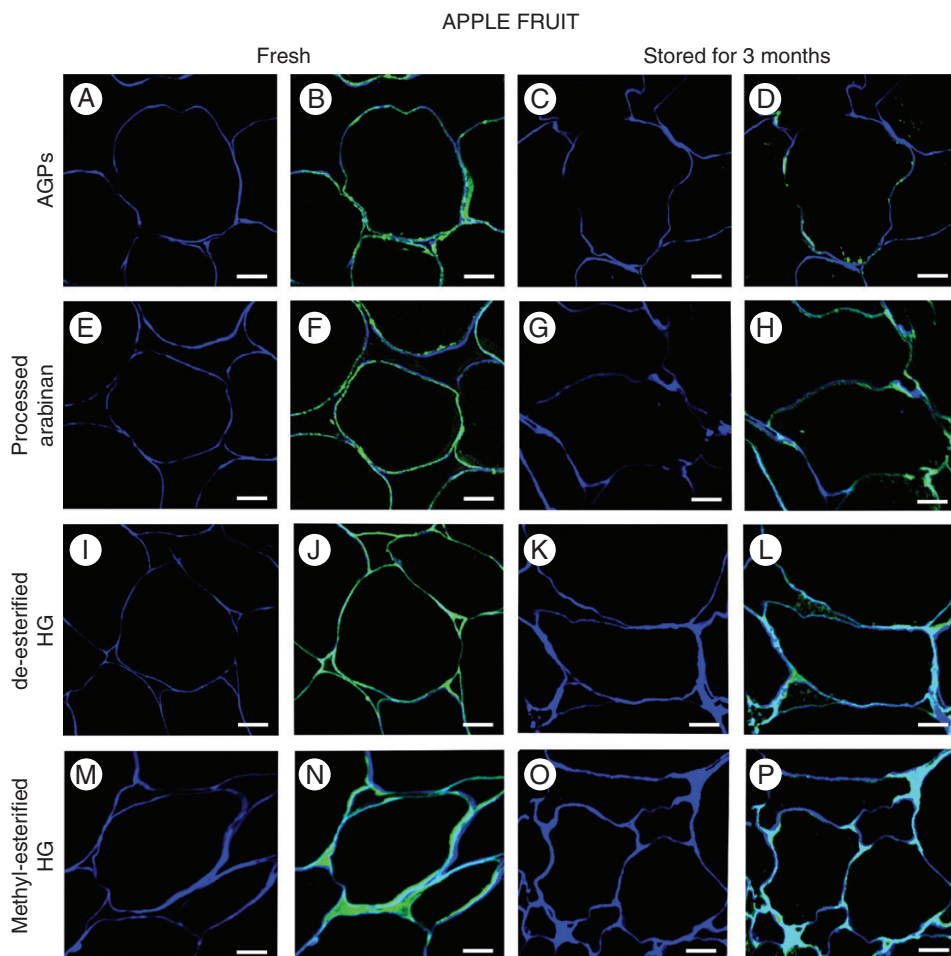


FIG. 4. Localization of AGP and pectin epitopes in cells of fresh fruit and fruit stored for 3 months. Sections after reactions with antibodies and calcofluor counterstaining: JIM13 (A and B, C and D), LM16 (E and F, G and H), LM19 (I and J, K and L), and LM20 (M and N, O and P). Confocal laser scanning microscopy. Scale bars = 20  $\mu$ m (A–P).

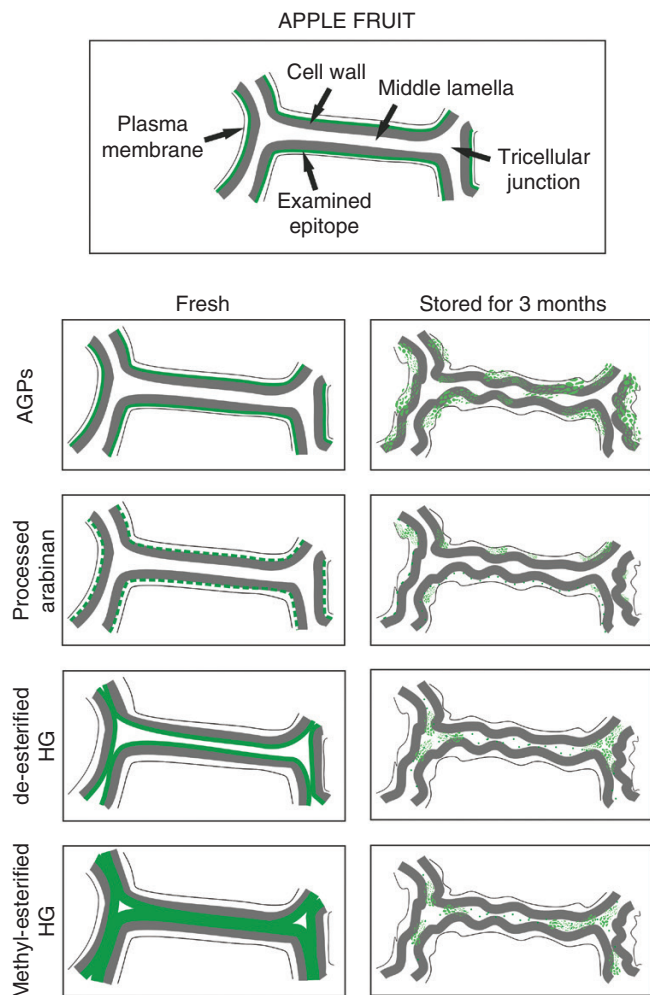


Fig. 5. Schematic representation of the localization of AGP, processed arabinan and HG epitopes in the cell wall of fresh and stored fruit.

the content of pectins in fruit is the major factor influencing cell wall properties and thereby the organization of intercellular spaces and texture of the whole fruit structure (Winisdorffer *et al.*, 2015). The enzymatic degradation of pectins through increased pectin methyltransferase expression is connected to the transformation of GalA (Chylińska *et al.*, 2017). Chemical analysis performed on carrot (Cybulska *et al.*, 2015) and apple (Szymańska-Chargot *et al.*, 2016) showed that the GalA content in the DASP fraction decreased, whereas GalA extracted from the CSP and WSP fractions increased during fruit storage. Our results, obtained by calorimetric determination of GalA content, confirm the changes of pectin content in fruit at different times of post-harvest storage. There were substantial differences between the particular pectic fractions, i.e. covalently bonded in DASPs and HG from the middle lamella present in CSPs. The content of the pectin most closely associated with the cell wall (DASPs) decreases during storage. These results are in agreement with the observations of the pectin distribution in cell walls carried out using the immunolabelling technique. In sections of fruit after storage, the fluorescence signal was weaker. Moreover, less intensive fluorescence after JIM13, JIM15 and MAC207 labelling indicates that the glycan moiety of arabinose and galactose sugars of the AGP molecule also

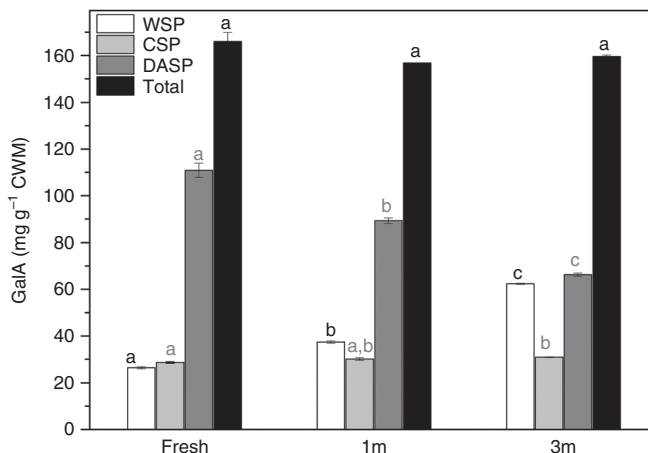


Fig. 6. Content of galacturonic acid in different pectin fractions from apple fruit at three stages: fresh, stored for 1 month (1m) and 3 months (3m). Bars show standard deviation. Different letters in the same colour indicate significant differences between means ( $\alpha = 0.05$ ).

undergoes degradation during fruit senescence. The LM16 antibody is a determinant of arabinan metabolism and reveals the enzymatic removal of arabinan side chains from the RG-I backbone (Verhertbruggen *et al.*, 2009). Thus, detection of the (1→5)- $\alpha$ -L-arabinan epitope recognized by the LM16 antibody in regions of senescing fruit tissue indicates that cell wall degradation is ongoing.

Current knowledge provides evidence that AGPs are interlinked with pectins (Immerzeel *et al.*, 2006; Ma *et al.*, 2017). AGP domains contribute to the cell wall architecture by reinforcing the polysaccharide scaffold (Lamport *et al.*, 2006; Hijazi *et al.*, 2014b). The interactions between AGPs and pectins are crucial for the regulation of cell expansion (Griffiths *et al.*, 2014). As is well known, changes in the spatial arrangement of polysaccharides lead to softening of the fruits (Szymańska-Chargot *et al.*, 2016). In our studies, we observed that the distribution of AGP, processed arabinan and HG changes in fruit tissue during post-harvest storage. The increasing irregularity in the content of pectins and in their localization during the senescence process was combined with alterations in the distribution of AGP and arabinan.

Interestingly, our studies show that the assembly of AGPs changed in various way to the distribution of HG. These results indicate the specific direction of AGP ‘movement’ in the senescing fruit tissue. Migration of AGPs through the cell wall has been the subject of many previous studies, but the mechanism of their release from the plasma membrane is still unclear (Knox, 2006; Lamport *et al.*, 2006; Showalter and Basu, 2016; Olmos *et al.*, 2017). The ‘movement’ of AGPs during plasma membrane – cell wall changes is associated with cell wall – membrane disintegration. We hypothesize that the ability of AGPs to detach the glycosylphosphatidylinositol (GPI) anchor from the plasma membrane and its penetration into the extracellular matrix as a signalling molecule may play a role in the maintenance of contact between the cell wall and the plasma membrane of fruit. Due to the breakdown of fruit microstructure, a non-homogeneous distribution of AGPs confirms disturbance to establishment of the cell wall – plasma membrane continuum. The specific arrangement of AGPs, essential for cell wall – plasma membrane links, is crucial for fruit tissue organization.



## CONCLUSION

We have used an immunomicroscopy approach to examine cell wall changes at the cellular level based on epitope distribution using cell wall antibodies in fresh and stored apple tissue. The immunocytochemistry reactions with mAbs against AGPs, arabinan and HG showed that the examined polysaccharides and proteoglycans occurred in different zones of the plant cell. Our results are in accordance with the well-known function of pectin in cell–cell adhesion and allow us to propose that AGPs are good candidates for cell wall – plasma membrane anchors.

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