Plant Physiology and Biochemistry 139 (2019) 681–690

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Enzymes and vitamin C as factors influencing the presence of arabinogalactan proteins (AGPs) in *Solanum lycopersicum* fruit

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ARTICLE INFO

Keywords:
Arabinogalactan protein
Cell wall
Fruit
Immunocytochemistry
Solanum lycopersicum

ABSTRACT

Arabinogalactan proteins (AGPs) are ubiquitous components of the amorphous plant extracellular matrix. They are characterized by a high proportion of sugar moieties, heterogeneity of their protein backbone and carbohydrate chains. It is known that AGPs form a complex network with other basic constituents in cell wall thus it may also play a role in softening process of fruit. The use of enzymatic degradation and cell wall polysaccharide directed probes are valid analytical tools for the study of developmental modification of the fruit structure. However, it is unknown whether pectolytic enzymes affect AGPs. Thus, the aim of the current work is to detect AGP epitopes *in situ* to understand the impact of selected degradation enzymes on various carbohydrate moieties of AGPs. Secondly, there are no data with clarification of the impact of vitamin C on fruit ripening processes at the cellular level; hence, we also focused on the effect of vitamin C on the arrangement of AGPs as important constituents of the polysaccharide-proteoglycan network in the fruit cell wall. The results indicate that the distribution of the examined AGP carbohydrate moieties differs, which are related to changes in tissue architecture. The absence of glycan chains causes disruption in establishment of correlations between cell wall constituents and rearrangement in the cell wall structure. The induced modifications of cell walls are not comparable to alterations occurring in naturally ripening fruit, which allows a conclusion that the synergistic action of a wide variety of factors influences ripening.

1. Introduction

1.1. Changes of fruit cell during ripening

Textural properties of fruits and vegetables are key features determining quality and becoming selection criteria for consumers. The quality characteristics mainly depend on biochemical machinery for metabolism and biosynthesis of a complex composition of particular fruit cells. All processes leading to fruit ripening and softening are associated with remodeling of the cell wall, mainly with loosening of the polymer networks (Brummell, 2006; Goulao and Oliveira, 2008; Sila et al., 2009). Modifications of the polysaccharide assembly, especially pectins, have a significant role in cell wall changes during fruit growth and ripening. The amount of pectins expressed as a galacturonic acid equivalent decreased during tomato fruit ripening, which was related to enzymatic degradation causing pectin depolymerization. Disruption of the cell wall appearing as a reduction of cell-cell adhesion is also an effect of more homogenous spatial distribution of pectins and their loss from cell corners and cellular junctions (Hyodo et al., 2013; Chylińska et al., 2017). However, not only changes in the pectin structure are responsible for the alteration of the cell wall but also rearrangement of their connections with other components (Wang et al., 2018). One of them are arabinogalactan proteins (AGPs), whose specific distribution is correlated with particular fruit maturity stages (Moore et al., 2014; Leszczuk et al., 2018a, 2019).

1.2. Arabinogalactan proteins

AGPs are highly glycosylated members of the superfamily of hydroxyproline-rich glycoproteins (HRGPs) of plant cell wall proteins, with variable core protein and carbohydrate side chains (Seifert and Roberts, 2007). About 90% of their total molecular mass comes from glycan moieties consisting of (1 → 3)-β-galactan and (1 → 6)-β-linked galactan, decorated with arabinose, rhamnose, D-glucuronic acid, and less frequently with xylose and fucose. Highly branched carbohydrate domains, especially their heterogeneity, are thought to be important for the functional diversity of AGPs (Schultz et al., 2000; Showalter and Basu, 2016; Ma et al., 2017; Su et al., 2018). AGPs are GPI-anchored proteins (GAPs) tightly bound to the plasma membrane by glycophosphatidylinositol at the C-terminus of the protein backbone (Gao et al.,

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https://doi.org/10.1016/j.plaphy.2019.04.035
Received 25 February 2019; Received in revised form 25 April 2019; Accepted 26 April 2019
Available online 27 April 2019

Abbreviations

AG	arabinogalactan
AGPs	arabinogalactan proteins
Ara	arabinose
GalA	galacturonic acid
GAP	GPI-anchored protein
GlcA	glucuronic acid
GPI	glycosylphosphatidylinositol
HRGPs	hydroxyproline-rich glycoproteins
mAb	monoclonal antibody
PG	endo-polygalacturonase
PME	pectin methylesterase
PL	pectate lyase
RG-I	rhamnogalacturonan I
Rha	rhamnose

1999; Liu et al., 2015). Also, they are covalently attached to cell wall hemicellulosic and pectic polysaccharides by their arabinogalactan (AG) moieties, forming the ARABINOXYLAN PECTIN ARABINOGALACTAN PROTEIN1 (APAP1) structure. In APAP1, linkages are present between rhamnogalacturonan I (RG-I) and the Rha residue in the AG domain in AGPs and between arabinoxylan and the Ara residue in the type II AGs domain. AGP interactions with different constituents contribute to their role as an anchor of the cell wall-plasma membrane continuum (Tan et al. 2013, 2018; Hijazi et al., 2014).

1.3. Effect of various factors on the distribution of arabinogalactan proteins in fruit

AGPs are suspected to contribute to the response pathway of plants against biotic and abiotic stresses, from water deficiency to heat stress in both vegetative and reproductive tissues (Mareri et al., 2019). In the case of fruits, that hypoxia and anoxia were reported to regulate mRNA levels of AGP encoding genes, suggesting their roles in adaptation processes and maintenance of cell wall stability in *Solanum lycopersicum* growth (Fragkostefanakis et al., 2012). Moreover, cell expansion events during *Vitis vinifera* ripening were correlated with an increase in the abundance of AGPs. AGP epitopes, β-D-GlcA, recognized by the LM2 antibody are potential biomarkers for ripening in grape berries (Moore et al., 2014). In *Malus* x *domestica* fruit, localization of AGP epitopes depends on the condition of the cell wall-plasma membrane changing during the senescence process as a result of postharvest storage. Also, it has been shown that AGPs are tissue specific, and among others, they are abundant in sclerenchyma tissue in the inner hardened layer of the pericarp surrounding the ovary with seeds (Leszczuk et al., 2018b).

1.4. Action of enzymes toward arabinogalactan proteins

Structural modification and degradation of the carbohydrate moieties of AGPs are caused by action of several glycosidases: α-L-arabinofuranosidase, β-galactosidase, and β-xylosidases. The activity of α-L-arabinofuranosidase is correlated with release of L-arabinofuranosyl residues from AGPs and formation of carbohydrate moieties sensitive to β-galactosidase (Kotake et al., 2006). To elucidate the role of AGPs, degradation enzymes of AG sugar chains have been used (Konishi et al., 2008), which led to identification of two genes encoding β-glucuronidase with the capacity to hydrolyze β-GlcA and 4-Me-β-GlcA residues of AGPs. These results allow a conclusion that activation of glycosidase enzymes may lead to damage and release of oligosaccharide chains, which are essential for the physiological functions of these proteoglycans in higher plants. Also, to analyze the structure of AGPs and 'to generate bespoke industrially relevant AGP-derived oligosaccharides for the food industry', a new polysaccharide lyase that cleaves the Rha-α1,4-GlcA linkage, which contributes to removal of 6-deoxy-sugars from the AGP molecule, was provided (Munoz-Munoz et al., 2017).

1.5. Vitamin C as a factor influencing processes occurring in fruit

The non-enzymatic mechanism of fruit softening is also linked with the depolymerization of cell wall polysaccharides. Endogenous ascorbate released to the apoplast by membrane permeabilization promotes non-enzymatic scission of pectins. The reduced firmness of the cell wall observed in red-ripe fruit is correlated with an increase in the breakdown of pectic polymers, which is a mechanism underlying the higher accumulation of ascorbic acid. Ascorbate is involved in solubilization of the arabinogalactan-pectin complex with a high Gal content, leading to wall-loosening during ripening. In turn, solubilization is a result of the action of ascorbate-generated hydroxyl radicals. Metabolic pectin changes associated with ascorbates appear as conversion of D-galacturonic acid into L-galactonic acid (Dumville and Fry, 2003; Di Matteo et al., 2010). It is confirmed that the ascorbic acid content in tomato fruits is associated with the expression of genes involved in pectin degradation. The expression of the *GalUR* gene from strawberry, encoding D-galacturonate reductase, is correlated with changing ascorbic acid content mainly during fruit ripening (Agius et al., 2003). Moreover, the increased expression of *SolyPME*, *SolyPG*, and *UGlcAE* genes influences the enzyme activity, level of pectin de-esterification, and ascorbic acid production at a later stage of tomato fruit ripening (Rigano et al., 2018).

Therefore the aim of the current study is to evaluate the effect of selected factors on various moieties of AGPs such as cell wall degradation enzymes and vitamin C. After enzymatic and vitamin C treatment of prepared fruit material, *in situ* analysis of AGP epitopes at the cellular level was performed. We focused on the modifications of the AGP distribution as important constituents of the polysaccharide-proteoglycan network in the fruit cell wall. The experiment was conducted using an immunocytochemical technique with JIM13, JIM15, LM2, and LM14 monoclonal antibodies against AGP and LM16 recognizing processed arabinans.

2. Material and methods

2.1. Material

Tomato plants (*Solanum lycopersicum* L. cv. Cerise) were grown under greenhouse conditions (Lublin, Poland). Fruit were harvested at the mature green and red stages. Ripening stage was identified by external color and defined according to Batu (2004). Part of tissue of green mature fruits were subjected to hydrolysis by enzymes and ascorbic acid and then prepared to imaging. Rest of the material were prepared to imaging with no previous modifications.

2.2. Sample preparation - enzymatic treatment

The enzymatic treatment of the tissue from mature green tomato fruits was carried out according to Selivanov et al. (2008) with some modifications. In this experiment, pectinase from *Aspergillus niger* L. (Sigma Aldrich, Germany) was used. The mixture of pectinolytic enzymes contained pectate lyase (PL) EC 4.2.2.2, endo-polygalacturonase (PG) EC 3.2.1.15, pectin methylesterase (PME) EC 3.1.1.11, and a small amount of hemicellulases EC3.2.1.8, EC 3.2.1.89, and cellulases EC 3.2.1.4. Hydrolysis was carried out for 3 h at 22 °C in an acetate buffer (pH 5.5) in 3 different concentrations of pectinase. The amount of enzyme was selected as follows: E1 (0.028 U/mL), E2 (0.056 U/mL), E3 (0.084 U/mL).

2.3. Sample preparation - vitamin C treatment

For non-enzymatic hydrolysis ascorbic acid (Sigma Aldrich, Germany) were used according to Dumville and Fry (2003) with some modifications. Hydrolysis was carried out for 18 h at 22 °C in an acetate buffer (pH 3.8) in 3 different concentrations of ascorbic acid: V1 (1.5 mM), V2 (3 mM), V3 (6 mM). In each hydrolysis solution 1.5 μM $CuSO_4$, and 1 mM H_2O_2 were also present.

2.4. Resin embedding and sectioning for microscopic analysis

The material was fixed in 2% (w/v) paraformaldehyde (Sigma Aldrich) and 2.5% (v/v) glutaraldehyde (Chempur) in 0.15 M phosphate buffered saline and placed under vacuum for 3 h at room temperature. Phosphate buffered saline (PBS) was prepared according to the manufacturer's instruction (Sigma Aldrich). One tablet was dissolved in 200 mL of deionized water. Fixed samples were dehydrated in graded ethanol series (from 30%, 50%, 70%, 90%, and 96% for 15 min to 99.8% for 30 min twice). The material was embedded in LR White resin (Sigma Aldrich) and polymerized in gelatin capsules (EMS) for 48 h at 55 °C. 1-μm thick transverse sections were cut under an ultramicrotome (Leica Reichert Ultracut S) equipped with a glass knife. Clues proposed by Wilson and Bacic (2012) were used to prepare samples for microscopy analysis.

2.5. Toluidine blue staining

Semi-thin sections were mounted on poly-L-lysine coated slides (Sigma Aldrich) and circled with a liquid blocker PAP Pen (Daido Sangyo, Japan). To show detailed anatomical changes in the fruit tissue, staining with a 0.5% (w/v) Toluidine blue aqueous solution at 55 °C for 30 s was performed.

2.6. Immunocytochemical analysis

Prior to visualization of AGPs and arabinans, the samples were incubated with sets of rat monoclonal antibodies directed to cell wall matrix proteoglycans/polysaccharides. The monoclonal antibodies used for labeling were purchased from the Paul Knox Cell Wall Lab at the University of Leeds (PlantProbes, UK). The following mAbs were used: JIM series - JIM13 and JIM15 and LM series: LM2, LM14, and LM16 (Table 1).

A method described in our previous paper was used for immunofluorescence reactions (Leszczuk et al., 2019). Briefly, sections were washed three times in PBS and pre-incubated with 1% BSA in PBS for 30 min at room temperature to block non-specific binding sites. Then, the samples were incubated with the primary rat antibody (mAbs) diluted 1:50 in 0.1% BSA in PBS for 12 h at 4 °C. After washing with several changes of PBS, incubation with the secondary antibody, goat anti-rat IgM (heavy chain) cross – Alexa Fluor 488 (ThermoFisher Scientific, Cat. No. A21212) diluted 1:200 in 0.1% BSA in PBS for 12 h at 4 °C was performed. After labeling, the sections were washed with PBS, deionized water (Mili-Q), and finally enclosed in Dako Fluorescent Mounting Medium (Sigma, USA).

During each labeling reaction, control reactions were carried out by incubation in PBS instead of the primary antibody and keeping the rest of the protocol unchanged. The material was also checked for autofluorescence (data not shown in the paper).

2.7. Calcofluor staining

For counterstaining, and visualization of the presence of cellulose, staining with a 0.01% Calcofluor White aqueous solution (FLUKA) for 10 min in darkness was carried out.

2.8. Imaging

All observations were performed using an Olympus BX51 CLSM microscope equipped with corresponding software FluoView v. 5.0. (Olympus Corporation, Tokyo, Japan). All parameters (i.e. laser intensity, gain) were kept constant for all immunofluorescence experiments. The excitation wavelength for AlexaFluor488 was 490 nm, and the emission was collected at 525 nm. The Calcofluor stained sections were revealed after excitation at 355 nm and emission at 433 nm. For each staining, a minimum of 20 tissue sections were analyzed for each fruit sample. The experiments were repeated several times for each antibody, and representative image sets were selected and edited using the CorelDrawX6 graphics program.

3. Results

3.1. Histological description of fruit structure modification during ripening

The ripening process, in addition to factors such as cell wall-degrading enzymes and vitamin C, induces morphological changes in the structure of the fruit tissue, exocarp, mesocarp, and endocarp, as shown in Fig. 1. A noticeable symptom of ripening is the changes and enlargement of the cuticle surrounding epidermal cells. Similarly, cells forming external layers became larger and rounder. Beside the differences in the epidermal cell histology, examination of the collenchyma layer reveals a difference in its structure. Tightly packed cells in green fruit (Fig. 1 A) disintegrate and loosen during ripening (Fig. 1 B).

After the treatment with the low concentration of enzymes, no changes were observed (Fig. 1 C). The first symptoms appeared after the treatment with the enzyme concentration of 0.056 U/mL (E2). Changes in the cell shape and disorder of the cell walls were visible in all parts of the examined fruit tissue (Fig. 1 D). At the highest enzyme concentration, there was an increase in the disintegration of unevenly thickened cell walls. Additionally, extensive cell wall thickenings of the parenchymal cells were evident (asterisked, Fig. 1 E).

Interestingly, vitamin C-treated sections of the fruit tissue were characterized by subtle textural modifications, which were not distinct in all concentrations used (Fig. 1 F, G). However, at the highest concentration of vitamin C, the cell walls became more undulating (Fig. 1 H).

3.2. In situ analysis of the occurrence of AGPs and arabinans during the ripening process

To examine the occurrence of AGPs and arabinans, we labeled transverse sections of fruit tissue with a range of mAbs recognizing specific carbohydrate epitopes. Also, Calcofluor White with an affinity for β-D-glycans was used for counterstaining and showing the whole surface of the cell wall. All morphological changes described previously are associated with modification of the cell wall composition. During

Table 1
List of primary rat monoclonal antibodies directed against carbohydrate moieties of arabinogalactan proteins and arabinans used in current study.

mAbs	Specificity – recognized epitope	Reference
JIM13	arabinogalactan protein	Yates et al. (1996)
	βGlcA(1 → 3)-αGalA(1 → 2)Rha	Knox et al. (1991)
JIM15	arabinogalactan protein	Yates et al. (1996)
	carbohydrate epitope of AGPs, GlcA	Knox et al. (1991)
LM2	arabinogalactan protein	Yates et al. (1996)
	β-D-GlcA	Smallwood et al. (1996)
LM14	arabinose- and galactose-enriched carbohydrate chains	Moller et al. (2008)
	AG type II	
LM16	arabinans processed further by arabinofuranosidase action (1 → 5)-α-L-Ara	Verhertbruggen et al. (2009)

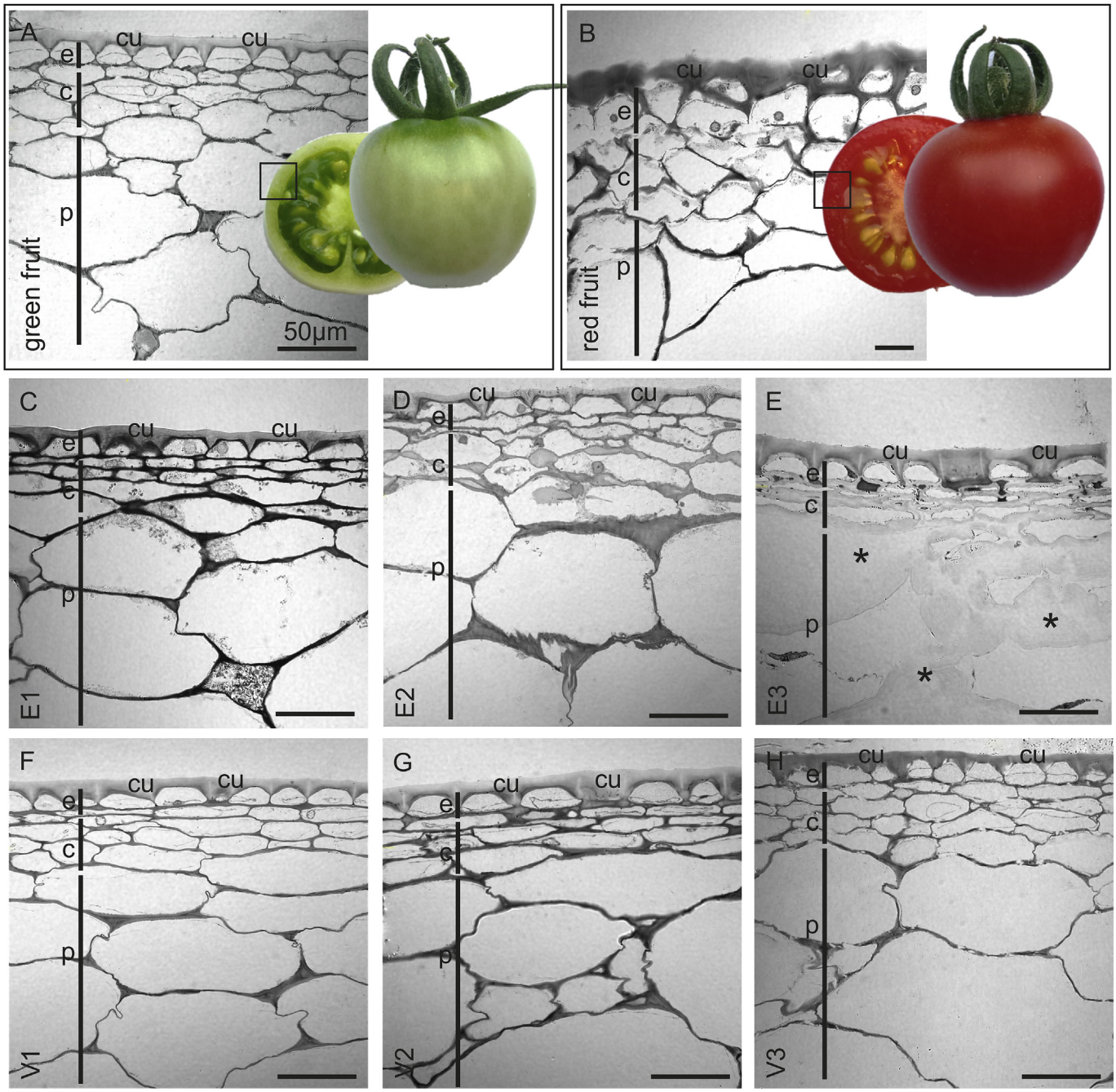

Fig. 1. Histology of fruit tissue at the green stage (A), red stage (B), after enzymatic (C, D, E), and vitamin C (F, G, H) treatments. Toluidine blue stained transverse sections of the fruit pericarp. Scale bars: 50 μm for all figures. Abbreviations: c – collenchyma cells, cu – cuticle, e – epidermal cells, p – parenchymal cells. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

ripening, two specific composition patterns appeared. The first pattern was visible at the green stage, in which the analyzed epitopes labeled the lining of the cell wall (Fig. 2 A–E). In green fruit, the spatial distribution of all examined epitopes was not distinct; however, JIM13 and JIM15 epitopes recognizing βGlcA(1 → 3)-αGalA(1 → 2)Rha residues were more abundant (Fig. 2 A, B). LM2 recognizing the β-D-GlcA residue of AGP chains and LM14 against AG type II bound in a similar pattern but the labeling was less regular (Fig. 2C and D). In the second pattern at the mature red stage, labeling was maintained in the same area, i.e. at the border of the cell wall in a region close to the plasma membrane, but the fluorescence signal was more intense than in the green fruit tissue, indicating that the amount of AGPs and arabinans increased after the ripening process. Moreover, after the process, there were changes in the shape of the exocarp cells, mainly in the epidermal layer, which was composed of cells with a thickened cell wall strongly labeled with Calcofluor White (asterisked, Fig. 2 F–J).

3.3. *In situ analysis of the occurrence of AGPs and arabinans after the enzymatic treatment*

Using enzymatic hydrolysis, we have demonstrated the modifications of the arrangement of AGP epitopes. The disruption of these epitopes as a result of the enzymatic treatment is shown for comparison in Fig. 2 K-Y. There is a relationship, i.e. an increase in the enzyme concentration is accompanied by greater disturbances in the arrangement of all epitopes and lower visibility of the fluorescence signal. The

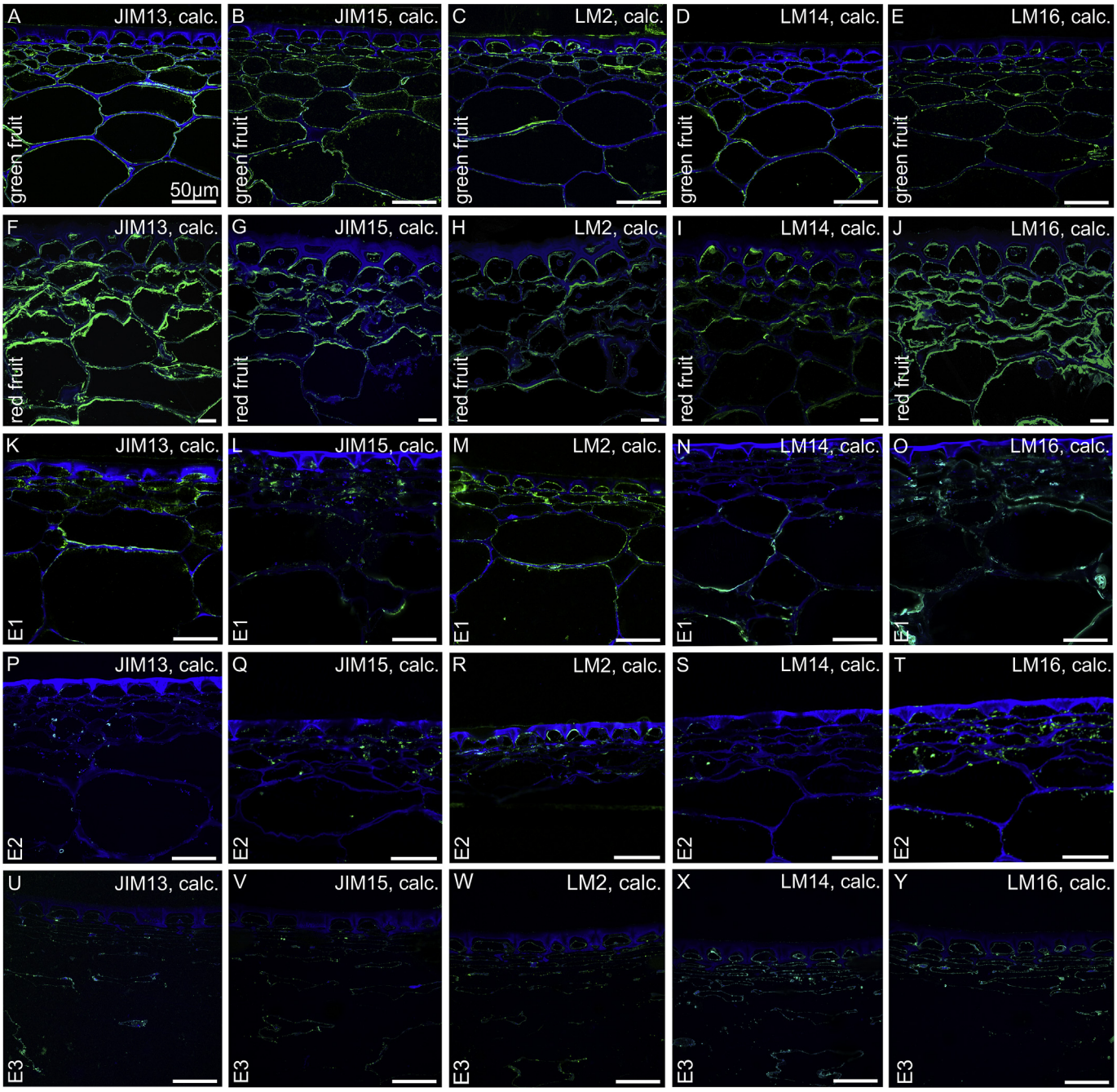

Fig. 2. Changes in the localization of AGP and arabinans in fruit pericarp. Immunolabelling in fruit macrostructure at the green (A–E) and red (F–J) stage. Detection of examined epitopes in fruit tissue after treatment with increasing enzyme concentrations: E1 (K–O), E2 (P–T), E3 (U–Y). Sections after reactions with antibodies and calcofluor counterstaining: JIM13 (A, F, K, P, U), JIM15 (B, G, L, Q, V), LM2 (C, H, M, R, W), LM14 (D, I, N, S, X), and LM16 (E, J, O, T, Y). CLSM. Scale bars: 50 µm for all figures. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

enzymatic treatment (0.028 U/mL; E1) of the fruit tissue reduced but did not completely abolish the epitopes in the cell walls (Fig. 2K–O). Detection of epitopes in the sections treated with 0.056 U/mL (E2; Fig. 2P–T) and 0.084 U/mL (E3; Fig. 2U–Y) amount of enzyme was difficult. In many cases, only small spots of fluorescence were noticeable. Similarly, after the treatment with the highest concentration of the degrading enzymes, Calcofluor White staining revealed regions of cell walls with significantly reduced or weaker fluorescence (Fig. 2U–Y).

Closer look indicated specific localization of the carbohydrate moieties of AGPs recognized by the antibodies used. Regions lining the cell wall periphery connected with the plasma membrane in both the epidermal and parenchymal tissue are the most abundant in the examined AGPs epitopes (arrows, Fig. 3 A, C). Cellulose filled the whole surface of cell wall, which highlights the presence of AGPs at the border of the cell walls in the continuum with the plasma membrane (Fig. 3 B). The ripening process influenced the tissue integrity and cell wall shape (Fig. 3 E), thus cellular dissociation and rearrangement of epitopes were observed. The strongest fluorescence signal appeared in the plasmolyzed plasma membrane adjacent to the inner cell wall (arrows, Fig. 3 D, F).

The enzymatic deconstruction of the cell wall was revealed by the less visible and disordered fluorescence signal. After the treatment with the 0.028 U/mL (E1) concentration of the enzymes, labeling was not restricted to the cell wall/plasma membrane, and the epitopes were

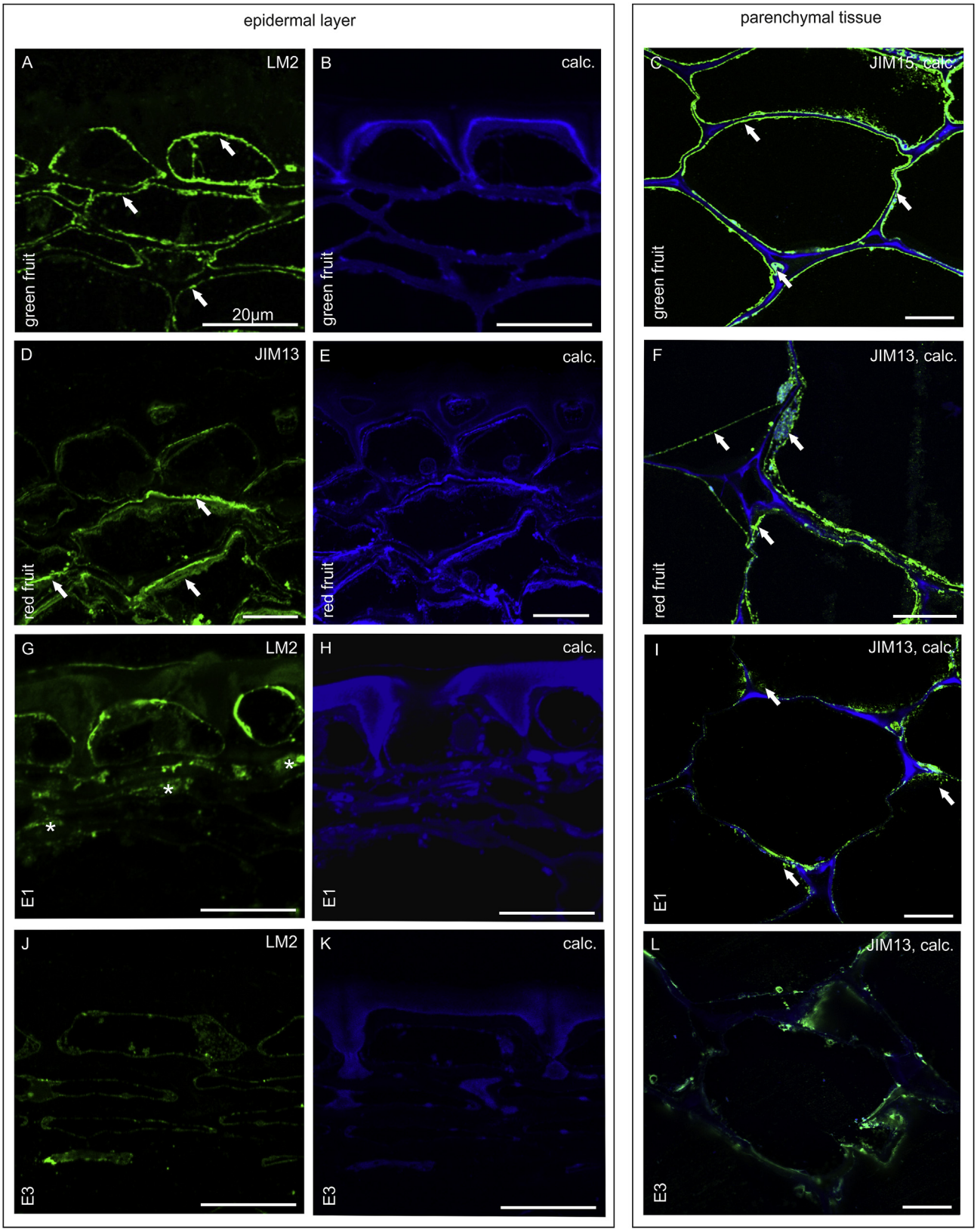

Fig. 3. Changes in the distribution of AGP carbohydrate epitopes at green and red stage, and after enzymatic treatment in the cells of the epidermal layer (A, D, G, J) and parenchymal tissue (C, F, I, L). Staining of cellulose in the exocarp (B, E, H, K) and parenchyma (C, F, I, L). CLSM. Scale bars: 20 μm for all figures. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

distributed on whole surface of the cell wall as well in cellular compartments (asterisks, Fig. 3 G, H). In the parenchymal cells, JIM13 epitopes did not occur along the border of the cell walls, but were present mainly in the destroyed plasma membrane (arrows, Fig. 3 I). In the case of the highest concentration of the enzymes, the binding of all antibodies was rather weak, and even increased exposure times did not improve the visibility of labeling. The disturbance in the distribution pattern and the absence of epitopes are connected with changes in cellulose assembly in all the analyzed types of tissue (Fig. 3J–L).

3.4. *In situ analysis of the occurrence of AGPs and arabinans after the vitamin C treatment*

Treatment with the increasing gradient of the vitamin C concentration resulted in diversity of AGP epitopes detection. Firstly, no differences in the spatial pattern of occurrence of JIM13 and JIM15 epitopes were revealed. The βGlcA(1 → 3)-αGalA(1 → 2)Rha residues were labeled in the lining of the inner cell walls. Additionally, in some regions, we observed binding to internal cellular compartments (Fig. 4 A, F, K, and B, G, L). Unexpectedly, even the lowest vitamin C concentration (1.5 mM) used caused weak binding to a GlcA residue recognized by LM2 (Fig. 4 C, H, M), an AG residue recognized by LM14 (Fig. 4 D, I, N), and a (1 → 5)-α-L-Ara residue recognized by LM16 (Fig. 4 E, J, O).

More detailed comparative analysis of labeling with the JIM and LM antibody series is shown in Fig. 5. Higher magnifications reveal that modifications of the cell wall by the treatment with vitamin C are different from changes induced by the enzymatic treatment, as shown in Fig. 3. An epitope consisting of βGlcA(1 → 3)-αGalA(1 → 2)Rha was detected in the inner cell wall-plasma membrane continuum in both epidermal and parenchymal cells (arrows, Fig. 5 A, C). At the higher vitamin C dose (6 mM), this epitope was visible also inside the cells, in the cellular compartments, and in the disintegrated plasma membrane (asterisks, Fig. 5 G, I). The antibodies from the LM series did not bind or only weakly bound in single regions of the epidermal tissue (Fig. 5 D, J) as well the parenchyma (Fig. 5 F, L). Vitamin C did not induce changes in cellulose arrangement in any case (Fig. 5 B, E, H, K).

Omitting the primary antibody from the immunolabelling procedure on an equivalent transverse section resulted in absence of fluorescence (Fig. 5 M).

4. Discussion

4.1. *Histological changes in fruit tissue*

Ripening-associated changes affecting the fruit texture are correlated with many factors, but one of the most significant determinants is the activity of enzymes (Goulao and Oliveira, 2008; Wang et al., 2018). In this work, the enzymatic and non-enzymatic hydrolysis of the cell wall was performed using various levels of cell wall hydrolases and vitamin C concentrations. Used amount of enzymes relates to their levels present in fruit tissue at it was measured in a previous experiment performed by Chylińska et al. (2017). We showed that these levels were enough to cause microstructural changes of the tissues, however, induced modifications of cell walls were not comparable to alterations occurring in naturally ripening fruit. This observation suggests synergistic action of a wide variety of factors influencing the ripening process.

4.2. *Structural changes in fruit tissue – disruption of AGP arrangement as a result of enzyme action*

A number of studies using biochemical techniques and overexpression technologies investigated the function of cell wall enzymes

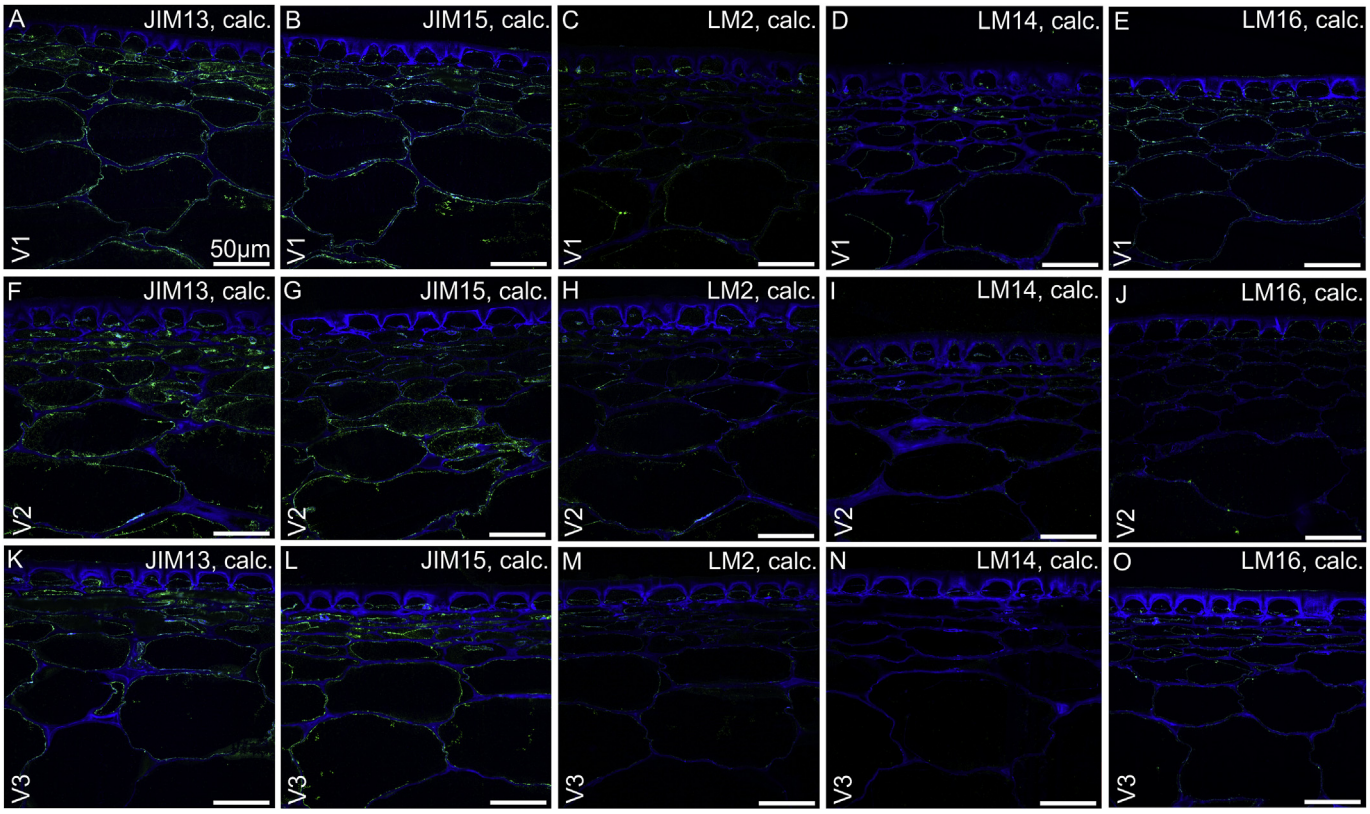

Fig. 4. Detection of AGP and arabinan epitopes in fruit tissue after treatment with increasing vitamin C concentrations: V1 (A–E), V2 (F–J), V3 (K–O). Sections after reactions with particular antibodies and calcofluor counterstaining: JIM13 (A, F, K), JIM15 (B, G, L), LM2 (C, H, M), LM14 (D, I, N), and LM16 (E, J, O). CLSM. Scale bars: 50 µm for all figures.

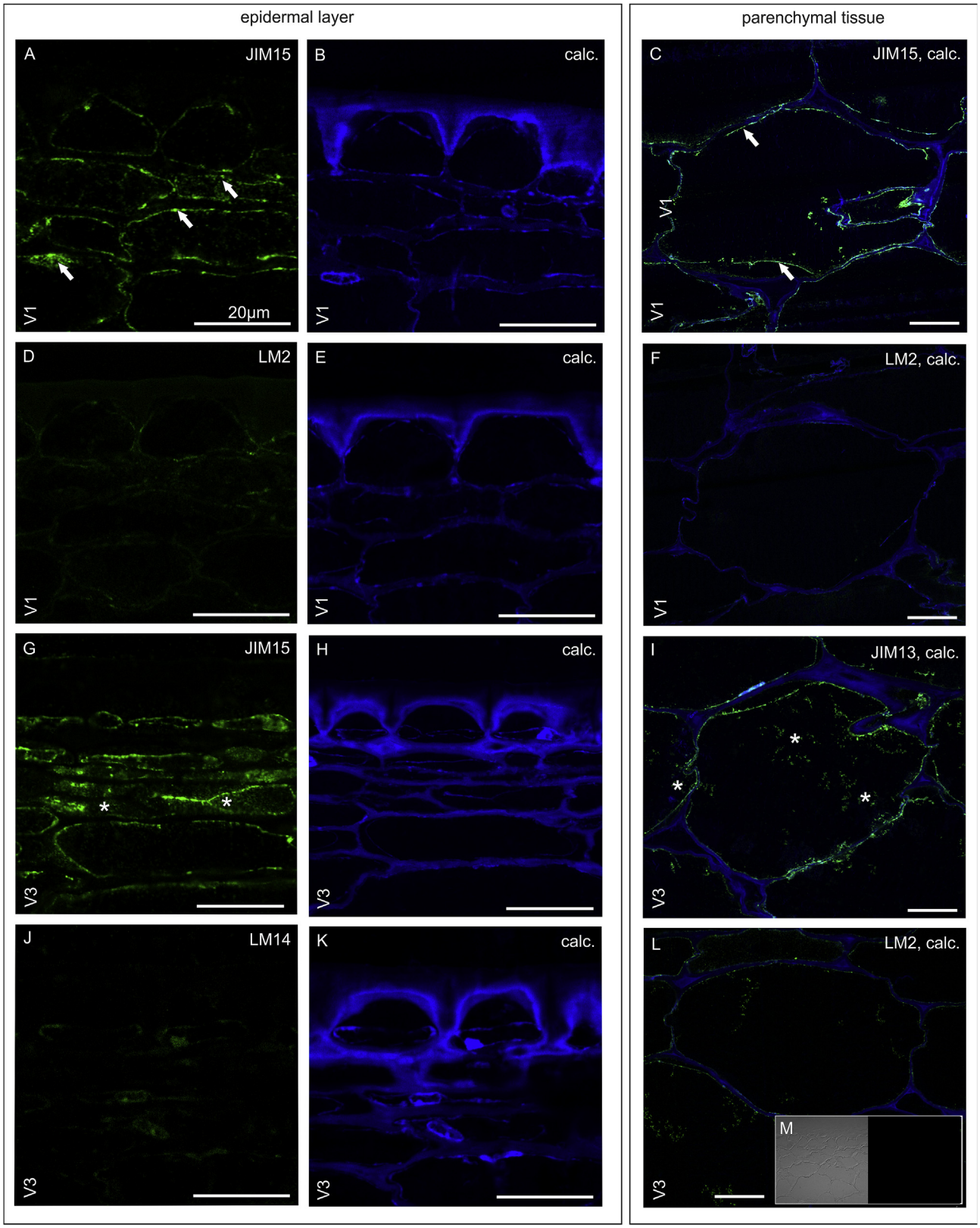

Fig. 5. Changes in the distribution of AGP carbohydrate epitopes in the cells of the epidermal layer (A, D, G, J) and parenchymal tissue (C, F, I, L) after vitamin C treatment. Staining of cellulose in the exocarp (B, E, H, K) and parenchyma (C, F, I, L). Exemplary photography of the control reaction (M). CLSM. Scale bars: 20 µm for all figures.

in fruit ripening. In apple fruits, hydrolysis of three key polysaccharides, such as methylesterified homogalacturonan, crystalline cellulose, and fucosylated xyloglucan side-chains have a major impact on the mechanical properties of the parenchymal tissue (Videcoq et al., 2017). The aim of the present work was to study the induction of cell wall hydrolases and vitamin C in relation to the distribution of carbohydrate moieties of AGPs in tomato fruit as an element of a co-extensive network of cellulose microfibrils cross-linked with hemicelluloses, pectins.

Cell wall-modifying enzymes, e.g. pectate lyase (PL) EC 4.2.2.2, polygalacturonase (PG) EC 3.2.1.15, and pectin methylesterase (PME) EC 3.1.1.11, are responsible for remodeling of the pectin network (Marín-Rodríguez et al., 2002; Brummell, 2006; Gwanpua et al., 2014). Pectin methylesterase catalyzes demethylation of the C6 carboxyl group of galacturonosyl residues throughout fruit growth (Tucker et al., 1982; De Assis et al., 2001). Pectate lyase catalyzes the cleavage of demethylated galacturonic acid residues from pectic polymers through β-elimination (Fischer and Bennett, 1991). Polygalacturonase acts in hydrolytic cleavage of α-(1 → 4) galacturonan linkages and its activity is detectable in tomato abscission zones as well as during the ripening-associated softening process (Tucker et al., 1984). Comparative analysis of tomato cell wall mutants performed by Wang et al. (2019) indicated only pectate lyase has a major influence on fruit softening, and other enzymes coordinate cell wall disassembly. Throughout tomato ripening, regions lining the periphery of cell wall with the adjacent plasma membrane are the most abundant in all examined AGP epitopes. The treatment with cell wall-modifying enzymes was associated with the loss or remodeling of AGP epitopes in the fruit cell wall assembly, which is correlated with degradation of AGP carbohydrate domains. The examined epitopes, which are constituents of AGP glycan chains such as βGlcA(1 → 3)-αGalA(1 → 2)Rha, GlcA residue, and AG type II, undergo hydrolysis. Interestingly, deposition of (1 → 5)-α-L-arabinan in pleiotropic tomato ripening mutant - *Cnr* with altered cell-to-cell adhesion properties is disrupted prior to ripening. In case of *rin* and *never–ripe* mutants, changes in the distribution of mentioned epitope was not seen. Taken overall, the variable alternations in (1 → 5)-α-L-arabinan occurrence in tomato mutants are indicated the direct role of arabinans in cell wall swelling. Absence of (1 → 5)-α- arabinosyl residues is correlated with disturbance into cell wall matrix assembly and alternations of cell wall properties (Orfila et al., 2001). It may be significance that (1 → 5)-α-L-Ara epitope recognized by LM16 antibody also undergoes modification in tomato as a result of enzymatic treatment. Thus, specific arabinan deposition in fruit and its attachment to periphery of cell wall is likely to be a key factor leading to maturation.

Fruit softening is associated with cellulose synthesis as well as physical properties of the cellulose microfibrils and its deposition (Goulao and Oliveira, 2008). The cellulose orientation in the extracellular matrix is related to the presence of other components of the cell wall. The GPI-substituted and *N*-glycosylated protein in the cell wall/plasma membrane (COB), acts as a scaffolding protein implicated in deposition of cellulose microfibrils. Partial loss of the *cob* gene expression leads to a degraded orienting mechanism based on reduction of the degree of cellulose polymerization during expansion in most developing organs (Roudier et al., 2005). The cellulose microfibrils are directly tethered by arabinan and galactan side chains, thus the loss of carbohydrate residues is correlated with disassembly of cellulose microfibrils, leading to changes in fruit softening and cell separation (Wang et al., 2019). In our study, the highest concentration of enzymes had an impact on the occurrence of the examined AGP epitopes and also on the presence of cellulose. As is well-known, the cellulose degradation is correlated with the synergistic action of three classes of enzymes: endo-1,4-β-glucanases, exo-1,4-β-glucanases, and β-glucosidases (Horn et al., 2012). Our results showed that also lack of another cell wall component, i.e. proteoglycans leads to cellulose disorganization. These data indicated that the decrease of fluorescence signal as a carbohydrate chains degradation, cell wall swelling and changes in cellulose assembly have been linked.

4.3. Structural changes in fruit tissue – disruption of AGP arrangement as a result of vitamin C impact

Dumville and Fry (2003) showed that ascorbate at physiologically relevant concentrations gradually solubilized a substantial proportion of pectic polysaccharides present in tomato fruits. The occurrence of ascorbate in the apoplast leads to scission of polysaccharides and, consequently, natural fruit softening. Also, the performed immunolocalization experiments in tomato fruit revealed that vitamin C influences the fruit tissue structure. In our work, the modifications of the cell wall caused by the vitamin C treatment are different from changes induced by the removal of pectic polysaccharides by enzymatic hydrolysis. We observed a diversity of fluorescence signals and a distinct labeling pattern of antibodies directed against different AGP carbohydrate epitopes. Epitopes consisting of βGlcA(1 → 3)-αGalA(1 → 2) Rha residues are less susceptible to addition of any vitamin C dose than epitopes composed of AG type II, GlcA, and (1 → 5)-α-L-Ara residues. After the vitamin C treatment, especially LM2 and LM14 antibodies did not bind to the cell wall, indicating that epitopes recognized by mentioned mAbs contain glycans of AGP chains sensitive to modification by non-enzymatic digestion.

5. Conclusions

Overall, the spatio-temporal-related structural heterogeneity of the cell wall, which has implication for the cell wall functions, is also associated with the AGP distribution. The results indicate that the distribution of the examined carbohydrate moieties of AGPs differs due to diverse factors, which are related to changes in the fruit tissue architecture. While the fruit cell wall is composed of cellulose and pectic polysaccharides as a fundamental elements and a key factors in fruit ripening, we assume that the crosstalk between constituents is the most significant in controlling ripening and postharvest physiology. The absence of glycan chains causes disruption and remodeling of the establishment of interactions between all cell wall constituents and induces changes in the whole cell wall structure.

Author contribution statement

AL carried out the immunocytochemical work, analyzed data, prepared data presentation and wrote the manuscript, MC prepared material hydrolysis, AZ revised the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.

Funding

This work was supported by the project no. 2018/02/X/NZ9/01926 funded by National Science Centre, Poland.

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