

CELLULAR LOCALIZATION AND LEVELS OF ARABINOGALACTAN PROTEINS IN *LYCIUM BARBARUM*'S FRUIT

HAN BAO¹, GUO QI ZHENG^{1*}, GUO LIANG QI¹, XUE LING SU¹ AND JUN WANG

¹School of Life Science, Ningxia University, Yinchuan, 750021, China

*Corresponding author e-mail: zhengguoqi1977@163.com

Abstract

Lycium barbarum L. contains several polysaccharides which are identified as arabinogalactan proteins (AGPs) and are considered having extensive biological effects and potential health benefits. The crude polysaccharides were isolated from *L. barbarum* fruits and then AGPs were purified from the polysaccharides by precipitation. Ten kinds of antibodies (JIM7, JIM8, JIM12, JIM94, JIM13, MAC204, JIM11, JIM19, JIM20, JIM5) were selected from 30 kinds of antibodies by reacting with purified AGPs in Elisa screening. Western blot analysis confirmed that the anti-arabinogalactan protein antibodies (JIM94, JIM8, JIM13, MAC204) and anti-pectins antibodies (JIM11, JIM20, JIM5) were effective. Immunolocalization of AGPs showed that these proteins accumulated mainly in the partly parenchyma cells of the exocarp, in the vascular bundles of the mesocarp, and even in the endocarp. Subcellular immunolocalization of AGPs showed that the proteins localized in the plasma and vacuole membranes, vesicles, plastid, mitochondria and other organelles in fruit parenchyma cells. These data suggested that AGPs may take a role as lubricants in fruit parenchyma cell to avoid cell rupture at the rapid growth stage.

Key words: *Lycium barbarum*, Wolfberry, Arabinogalactan protein, Fruits, Monoclonal antibody.

Introduction

Lycium barbarum L., also known as wolfberry or goji berry, is a dicotyledon belonging to the Solanaceae family that produces an orange-red ellipsoid berry with length of 1~2cm. *L. Barbarum* have been used in Chinese medicine for two thousand years and their beneficial effects are claimed to include enhancement of the immune system, eye sight, circulation, and anticarcinogenic properties etc. (Gan *et al.*, 2004; Huang *et al.*, 1998; Li *et al.*, 2007), which is attributed to their fruits contain *L. Barbarum* polysaccharides (LBP) in their fruits (Chia *et al.*, 2009). Recent study shows that these polysaccharides are characteristic arabinogalactan proteins (AGPs) (Robert *et al.*, 2011).

Cell wall is composed of cellulose, pectin, hemicellulose and a small amount of structural proteins. These proteoglycans constitute a net embedded in extracellular matrix and involve in many processes of plant development (Showter, 2001). AGPs are belong to these structural proteins, which are characterized by the extensive O-glycosylation of the protein backbone, containing Hyp, Ala, Ser, Thr and Gly as their main amino acid constituents. Their carbohydrate moiety accounts for 90% w/w of the mass of these glycoproteins and composes predominantly of arabinose and galactose residues, as well as other less-abundant sugars (Nothnagel, 1997; Showalter, 2001). Arabinogalactan proteins (AGPs) is composed of β -1,3- and β -1,6-linked galactans containing urohides and neutral sugars, including arabinose. AGPs are found in leaves, stems, roots, floral parts and seeds at the organ level (Nothnagel, 1997; Fincher *et al.*, 1983) and occurred abundantly in many tissues like xylem, stilar transmitting tissue and cell suspension cultures (Clemens *et al.*, 2010, Qin *et al.*, 2007, Smallwood *et al.*, 1996), being thought to function as lubricants, in nutrition or in some aspect of plant cell

recognition (Fincher *et al.*, 1983; Chasan, 1994). Moreover, AGPs play a role of cell expansion in the Moss *Physcomitrella patens* (Kieran *et al.*, 2005), also participate in the procession of egg cell fertilization and zygotic division in tobacco (Yu & Zhao, 2012).

Several tools and techniques are useful to examine and localize the expression of AGPs in plants at several levels, from single cell to the whole plants. The distribution of AGPs can be studied with the β -D-glucosyl Yariv reagent (β -GlcY), a synthetic phenylglycoside that specifically binds to AGPs, or monoclonal antibodies (MABs) that predominantly reacting with the carbohydrate epitopes within AGPs, such as the JIM, LM, MAC and ZUM series (Showalter, 2001). To further investigate the changes in AGPs and pectins in the developing fruit at different stages of *L. barbarum*, in the present study, the crude polysaccharides were isolated from fruits and then AGPs were purified from the polysaccharides by precipitation using the Yariv reagent. ELISA and Western blotting hybridization were performed to select effective antibodies against AGPs. The immunolocalization by fluorescence microscopy and transmission electron microscope (TEM) was employed to study their distribution and possible function during plant development using selected antibodies.

Material and Methods

Plant materials: The fruits of *L. barbarum* were collected at Ningxia province (China) at three different stages during fruit development according to morphological criteria defined by Zheng *et al.* (2012). The three stages were designed to span 1-8 days (first rapid stage), 8-24 days (slow growth stage) and 24-34 days (second rapid growth stage) after blossoming. The fruits in the mature stage (second rapid growth stage) were used for AGPs extraction, while the fruits in different stages were used for immunofluorescence localization and ultrastructural immunolocalization.

Crude polysaccharides extraction: Approximately 45g of dried fruits were crushed and transferred to Soxhlet extractor with 400 ml petroleum ether, incubated at 80°C until the extract was colorless. The residue was filtered and transferred to a flask with 100 ml of 80% EtOH and refluxed for 2 times at 85°C (300 ml each time, $t=2$ h); The residue was filtered and extracted 3 times with boiling water (200 ml each time, $t_1 = 1.5$ h, $t_2 = 1$ h, $t_3 = 1$ h); the combined extracts were concentrated to 100 ml in vacuum at 40°C; After anhydrous ethanol precipitation (4°C overnight), the residues were washed with 200 ml of 95% ethanol, ethanol, acetone, diethyl ether, and finally dried at 50°C to gain crude polysaccharides.

AGPs purification: Crude polysaccharides consisting of proteoglycan and polysaccharides were extracted more than 10 times to separate the free proteoglycan, then were concentrated under vacuum, precipitated with ethanol, filtrated and frozen to gain polysaccharides without free proteoglycan. The solution (polysaccharides without free proteoglycan dissolved in 7.5 ml of 0.05 M Tris-HCl, pH 8.0) was filtered (Whatman GFA 3 glass fibre) to remove traces of particulate matter before adding 5 ml of Yariv reagent (1 mg/mL in 1% NaCl) into 5 ml of the filtrate. After incubating overnight at 4°C and centrifuged (7000 rpm, 20 min), the supernatant was decanted and the sediment (AGP–Yariv complex) was suspended in 100% methanol by a brief ultrasonication. Following centrifugation again, the supernatant was discarded and the sediment was washed by methanol repeatedly. This sediment was dried in a stream of argon and dissolved in 2 ml of 50% DMSO. Approximately 50–75 mg of sodium dithionite were added and the solution was heated to 50°C to break the Yariv–AGP complex and get the colourless solution. After dialysing for 24 h, AGPs was attained by freeze drying as described (Redgwella *et al.*, 2011)

ELISA screening: Monoclonal antibodies (MABs) were purchased from the University of Georgia's Complex Carbohydrate Research Center (USA) and the MAB kit was from University of Lizz's Paul Knox cell wall lab. 100 μ l of purified AGP (50 mg ml⁻¹) samples in Sodium carbonate buffer was pipetted to each well in a 96-well plate and removed solution containing the antigen following. Negative control wells were only coated with Sodium carbonate. The plates were blocked with 200 ml of 3% (w/v) bovine serum albumin in phosphate buffered solution (PBS, pH 7.4) overnight at 4°C. Blocking agent was removed, and the microtiter plate was immersed in a tray containing PBS and washed by PBS at least 15 min. 100 μ l of primary antibodies (diluted to 1:10 in PBS containing bovine serum albumin) were added to each well and incubated for 1.5 h at room temperature. The supernatant was removed and the wells were washed by PBS at least for 15 min. 100 μ l of secondary antibodies, Peroxidase-conjugated goat anti-mouse IgG antibodies (diluted to 1: 2500 in PBS containing bovine serum albumin, Sigma-Aldrich) were added to each well and incubated for 1.5 h. Wells were then washed five times with PBS at least 15 min. Tetramethyl benzidine (TMB) solution was added 150 μ l per well for color reaction. Following 20 min, the reaction was stopped by adding 35

μ l of 2 N sulfuric acid to each well. The OD of each well was read at 450 nm using a model 680 microplate reader (Bio-Rad). The negative control wells were tested on the same plate that contained the same primary and secondary antibodies but no immobilized glycoprotein.

SDS-PAGE and Western blotting hybridization: SDS-PAGE was processed as described (Qin *et al.*, 2007). 30 μ g of polysaccharide glycoprotein and AGPs per sample were loaded on 10 % polyacrylamide gels with 3% stacking gels using a Mini-Protean 3 apparatus (Bio-Rad). The separated polypeptides were transferred onto a polyvinylidene difluoride membrane (PVDF) by electroblotting (400 mA, 2 h) in electrotransfer buffer (20 mM Tris-base, 150 mM glycine, 20% methanol). The PVDF membranes were blocked with 5% nonfat dried milk in TTBS buffer (20 mM Tris-base, 500 mM NaCl, 0.05% Tween-20, pH 7.5) for 1 h and then incubated at room temperature with primary antibody selected via ELISA screening (diluted to 1: 100) against AGPs for 2 h. After three washes with TTBS (15min each time), the blots were incubated for 1 h with alkaline phosphatase-conjugated goat anti-rat antibodies (diluted 1 : 10000; Sino-American Biotechnology Co.) and then washed three times (15min each time). The alkaline phosphatase signal was developed using a nitro blue tetrazolium–5-bromo-4-chloro-3-indolyl phosphate kit (Sino-American Biotechnology Co.).

Fluorescence microscopy: Fruit materials (in different developmental stage) were fixed in a mixture of 3.7% formaldehyde (w/v) prepared in 2F4 buffer (PIPES 7.56 g, KOH 2.42 g, 0.1M CaCl₂ 5 ml, pH 6.9-7.0) for 1 h at room temperature, and then were transferred to fresh 2F4 fixative three times, each time 10 min. Following washing in PBS (10 mM, pH 7.0), the material was dehydrated in increasing ethanol concentrations at 4°C and then embedded in Steedman's wax (Vitha *et al.*, 2000). The embedded material was cut into semi-thin sections (16–25 μ m) using a cryo-cut microtome (Leica, RM2235), and were placed on glass slides covered with 0.2% polyethyleneimine (Sigma). Different primary antibodies were used for immunodetection as described above. Sections were dried, dewaxed and rehydrated on glass slides for a week, then were blocked with 50 mM glycine, 2% BSA in PBS for 30 min. Then were washed by 10 mM PBS for 10 min, then the sections were incubated with primary antibody diluted 1: 100 in antibody at 4°C overnight. This was successively washed three time in PBS, and then incubated with secondary antibody conjugated with alkaline phosphatase (1: 100, Sino-American Biotechnology Co.) at room temperature for 1 h at 36°C. After three washes of 10 min each in PBS, the samples were stained with 0.01% toluidine blue for 10 min to remove plant itself autofluorescence, then washed in PBS for 15min. The negative control sections were incubated in PBS instead of the primary antibody. After drying, the sections were covered with a drop of glycerine. The samples were observed and photographed under fluoresce microscope (Leica, DM IRB) equipped with CCD.

TEM immunolocalization: The tissues were fixed separately in a mixture of 4% paraformaldehyde, 0.1% glutaraldehyde, or 4% paraformaldehyde, 0.5% glutaraldehyde, in PBS at 0°C for 4 h, and once transferred to fresh fixatives. The tissue was then washed with PBS four times for 15 min, and dehydrated through a graded ethanol series (50%, 75%, 85% and 95% [v/v] ethanol) for 20 min each time. The dehydrated tissue was moved to 4°C and then gradually infiltrated with cold LR White Resin (Sigma) embedding resin using 50% (v/v) and 66% (v/v) resin in 100% ethanol for 1 h, followed by 100% resin for 1 h. The infiltrated tissue was transferred to gelatin capsules containing 100% resin for embedding to accomplish immunogold location experiments in Sheng Xing Biotechnology Co. Ltd. (Nan Jing, China).

Results

AGPs extraction from *L. barbarum* fruits: A total of 1.5 g crude polysaccharides were obtained from 45g of *L. barbarum* fruits using anhydrous ethanol precipitation, with a yield of 3.3%. 0.31 g of free proteoglycan were separated successively, with a yield of 20.7% from the crude polysaccharide, and a yield of 0.7% from fruits.

Thirty mg of free polysaccharides were used for AGPs extraction using Yariv reagent, and 3.2 mg AGPs were obtained with a yield of 10.7%.

ELISA-based MAB screen for *L. barbarum* immunoassay: The purified proteoglycan and purified AGPs from *L. barbarum* fruits were used as antigen to select suitable antibodies against AGPs in an ELISA assay. MABs that bind epitopes on arabinogalactans (JIM1, JIM4, JIM7, JIM8, JIM12, JIM17, JIM94, MAC266), arabinogalactan proteins (JIM13, JIM14, JIM15, JIM16, LM2, LM14, MAC204, MAC207), pectin containing arabic gum and extensins (JIM3, JIM5, JIM11, JIM19, JIM20, JIM84, JIM93, JIM101, JIM131, JIM132, JIM133, JIM136, JIM137, MAC265 (Pennell *et al.*, 1991; Puhlmann *et al.*, 1994; Dolan & Roberts, 1995; Smallwood *et al.*, 1994, Smallwood *et al.*, 1996) were used as antibodies.

The ELISA result showed that the average OD values at 450 nm were 1.462, 1.357, 1.482 and 1.488 for JIM7, JIM8, JIM12, JIM94, respectively, while the average ODs were 0.411, 0.230, 0.367 and 0.290 for the negative controls (Fig. 1). The average OD value of these four antibodies was higher than that of the control group, indicating that JIM7, JIM8, JIM12 and JIM94 were effective for *L. barbarum* immunoassay.

Meanwhile, the average OD values at 450 nm were 1.926 and 2.589 for JIM13 and MAC 204, respectively, compared to those of controls, 0.576 and 0.159 (Fig. 2).

The average OD values at 450nm were 0.986, 2.078, 2.780 and 2.516 for JIM5, JIM11, JIM19 and JIM20, compared to those of controls, 0.197, 0.305, 0.320 and 0.341 (Fig. 3). These results indicated that JIM13, MAC 204 antibodies and JIM5, JIM11, JIM19, JIM20 antibodies were also effective for *L. barbarum* immunoassay.

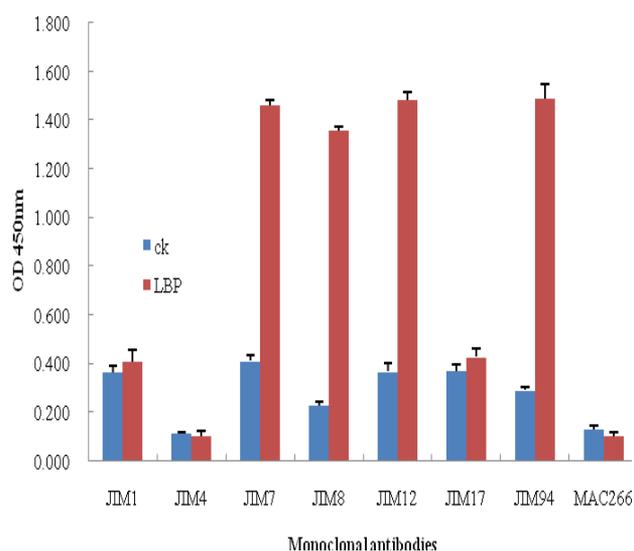


Fig. 1. Screening for antibodies against arabinose and arabinogalactan.

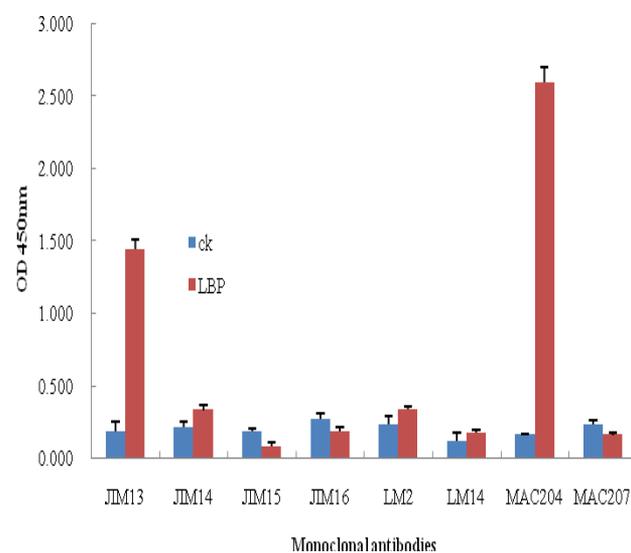


Fig. 2. Screening for antibodies against Arabinogalactan proteins.

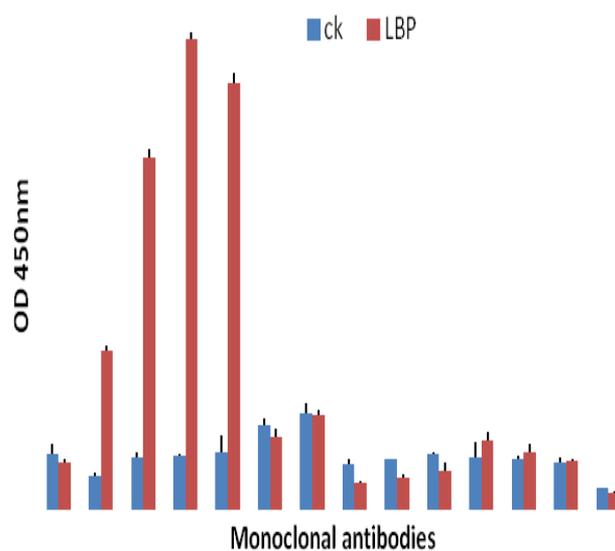


Fig. 3. Screening for antibodies against Pectins.

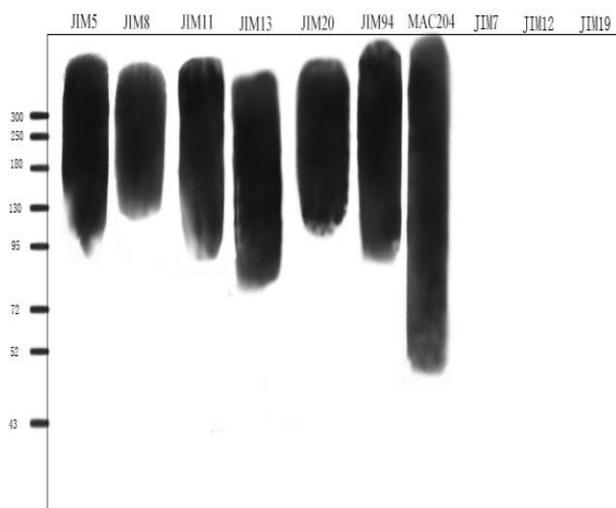


Fig. 4. Western blot analysis of the expression of LBP as detected by JIM5, JIM8, JIM11, JIM13, JIM20, JIM94, MAC204, JIM7, JIM12, JIM19 antibodies in ripe *Lycium b.* fruits. 30 μ g of total protein extract were loaded per lane. Molecular mass is indicated in kD on the left.

Immunodetection of crude polysaccharides and AGPs in *L. barbarum* fruits: The crude polysaccharides and AGPs extracted from *L. barbarum* fruits were separated by SDS-PAGE and Western blotting hybridization was performed using the effective antibodies tested in ELISA-based MAB screen. The results showed that the presence of numerous polysaccharides in a molecular weight ranging from 34 to 300 kDa detected with JIM5, JIM8, JIM11, JIM13, JIM20, JIM94 and MAC204 antibodies (Fig. 4).

Immunofluorescence localization of AGPs in *L. Barbarum* fruits: Two sets of MABs were selected, JIM94, JIM8 and JIM13, MAC204 for arabinogalactan and JIM5, JIM11 and JIM20 for pectin, were used for visual localization of the temporal and spatial distribution of AGPs and pectins at the three different stages (8, 8-24, 24-34 days after blossoming) during the fruit development

1. Immunofluorescence distribution of fruits at the first rapid stage: Using bright field, The complete structure of exocarp, mesocarp and endocarp of fruit at this stage could be observed. The exocarp was composed of a layer of tightly packed, square cells. The mesocarp was composed of parenchyma cells containing large vacuoles and a number of small vascular bundles. At this stage, parenchyma cells did not have significant cell gaps. The endocarp consisted of two layers of cells (Figs. 5-1). Using JIM5, JIM11 and JIM20 for detection of pectins, fluorescence signal in the whole style section of the fruit could be observed that detected by JIM5, with an intense fluorescence labelling in the exocarp, cell wall of parenchyma cells and vascular bundles in the mesocarp, while the signal was weaker in the endocarp (Figs. 5-2). JIM11 and JIM20 gave a strong signal in the whole style section of the fruit, and the signal of JIM11 was stronger

than that of JIM20 (Figs. 5-3, 4). Using JIM94, JIM8, JIM13 and MAC204 for localization of arabinogalactan and AGPs, which was observed that signal mainly in the wall of exocarp, with weaker signal on the mesocarp and endocarp by using JIM94 (Figs. 5-5), in the whole section by using JIM8 (Figs. 5-5). Strong fluorescence signals was also observed in the exocarp and parenchyma cells by using JIM13, comparing with the signal in the endocarp (Figs. 5-7). MAC204 and JIM8 also showed a similar fluorescence labelling pattern (Figs. 5-8).

2. Immunofluorescence distribution of fruit at the slow growth stage: At this stage, an increasing size of parenchyma cells in the mesocarp and a loosening cellular structure were observed (Figs. 6-1). Also using JIM5, JIM11 and JIM20 for detection of pectins, a relatively weak fluorescence signal in the whole section of the fruit was observed by using JIM5 (Figs. 6-2), in the walls of exocarp and some parenchyma cells wall by using JIM11 (Figs. 6-3), and an intense fluorescence labelling in the whole section of the fruit by using JIM20 (Figs. 6-4). Using JIM94, JIM8, JIM13 and MAC204 for localization of arabinogalactan and AGPs, the fluorescence signals using JIM94 were relatively weak in the whole section of the fruit (Figs. 6-5). It gave an intense labelling in the exocarp, wall of parenchyma cells and vascular bundles in the mesocarp by using JIM8 (Figs. 6-6). Strong fluorescence signal was also observed in the inner wall of the exocarp by using JIM13 (Figs. 6-7), and in the exocarp, vascular bundles in the mesocarp and endocarp by using MAC204 (Figs. 6-8), which its signal intensity was stronger than that of JIM13.

3. immunofluorescence distribution of fruit at the second rapid stage: The size of parenchyma cells increased rapidly in the mesocarp and cell structure loosened with evident cell gaps at this stage. The vascular bundles differentiated and arranged in a ring, distributing at the cell layers beside the endocarp (Figs. 7-1). Using JIM5, JIM11 and JIM20. JIM5 for detection of pectins, it was found that the whole section of fruit tissues, had high intensity signals in the exocarp and vascular bundles in the mesocarp (Figs. 7-2). Fluorescence signal was relatively weak in the whole section of the fruit using JIM11 (Figs. 7-3). Similar labelling pattern were showed by using JIM20 and JIM5, but the latter was weaker (Figs. 7-4). Using JIM94, JIM8, JIM13 and MAC204 for localization of arabinogalactan and AGPs, the fluorescence signals using JIM94 were observed in the exocarp and the wall of parenchyma cells nearby (Figs. 7-5). JIM8 showed a similar fluorescence labelling pattern, with an overall signal stronger than JIM94 (Figs. 7-6). JIM13 labelled with a weak intensity signal of the fruit except the exocarp (Figs. 7-7). The overall MAC204 fluorescence signal was stronger than using JIM13 (Figs. 7-8).

According to the result of immunofluorescence localization, it can be confirmed that AGPs were distributed in the exocarp, wall of some parenchyma cells and mesocarp vascular bundles, as well as endocarp.

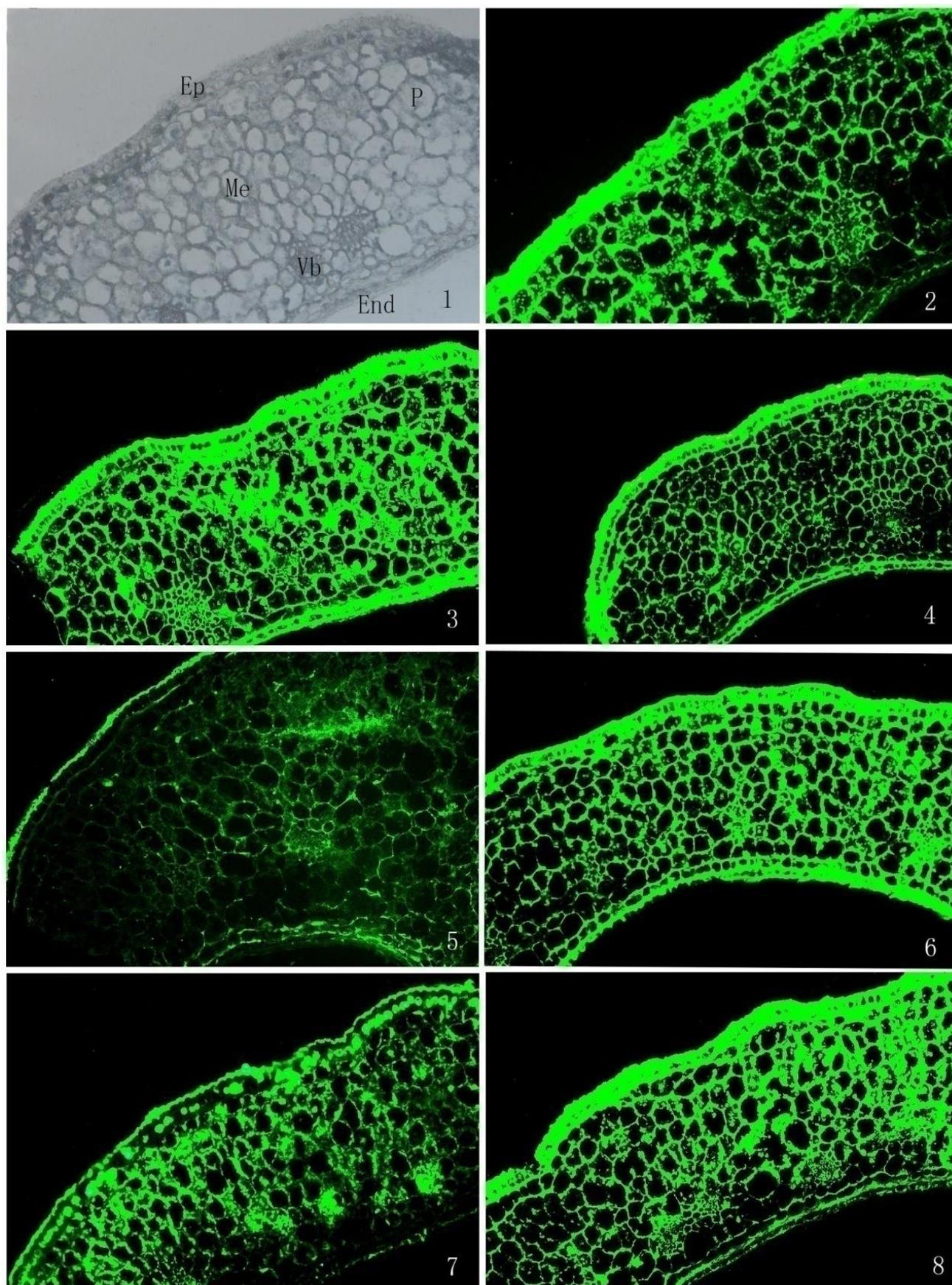


Fig. 5. Immunolocalization of pectins (JIM5, JIM11 and JIM20,2-4) and arabinogalactan and AGPs (JIM94, JIM8, JIM13 and MAC204,5-8) in wolfberry fruit at the first rapid stage ($\times 400$). Brightfield micrographs images show that complete structure of exocarp, mesocarp and endocarp of wolfberry. The Mesocarp was composed of parenchyma cells and a small vascular bundles (1). End, endocarp; Me, mesocarp; Ep, epicarp; P, parenchyma cell; vb, vascular bundle.

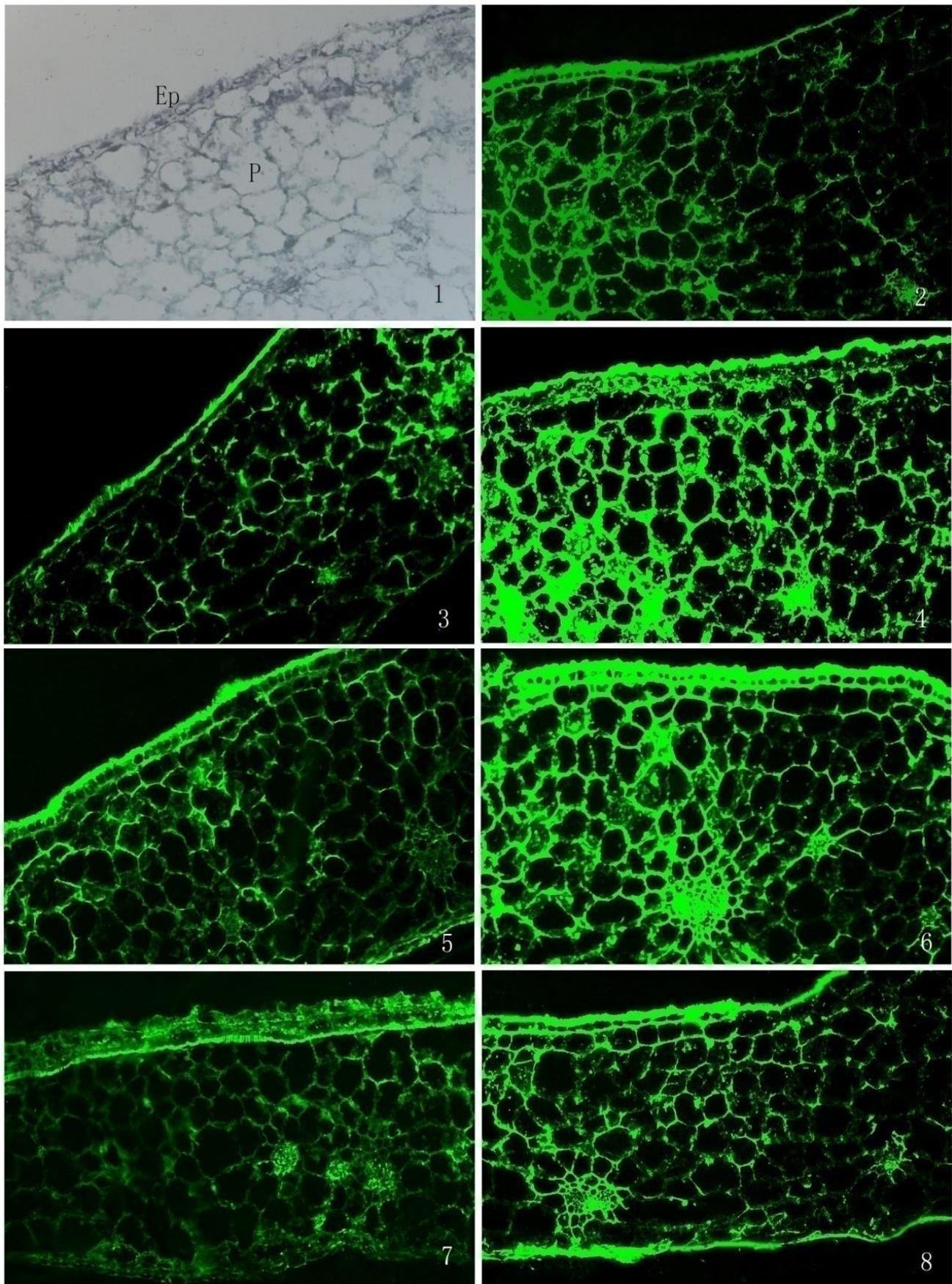


Fig. 6. Pectins (JIM5, JIM11 and JIM20, 2-4) and arabinogalactan and AGPs (JIM94, JIM8, JIM13 and MAC204, 5-8) localization in wolfberry fruit at slow growth stage ($\times 400$) by immunofluorescence microscopy. Brightfield micrographs images showed that the size of parenchyma cells in the mesocarp increased and their structure got loose (1). Ep, epicarp; P, parenchyma cell.

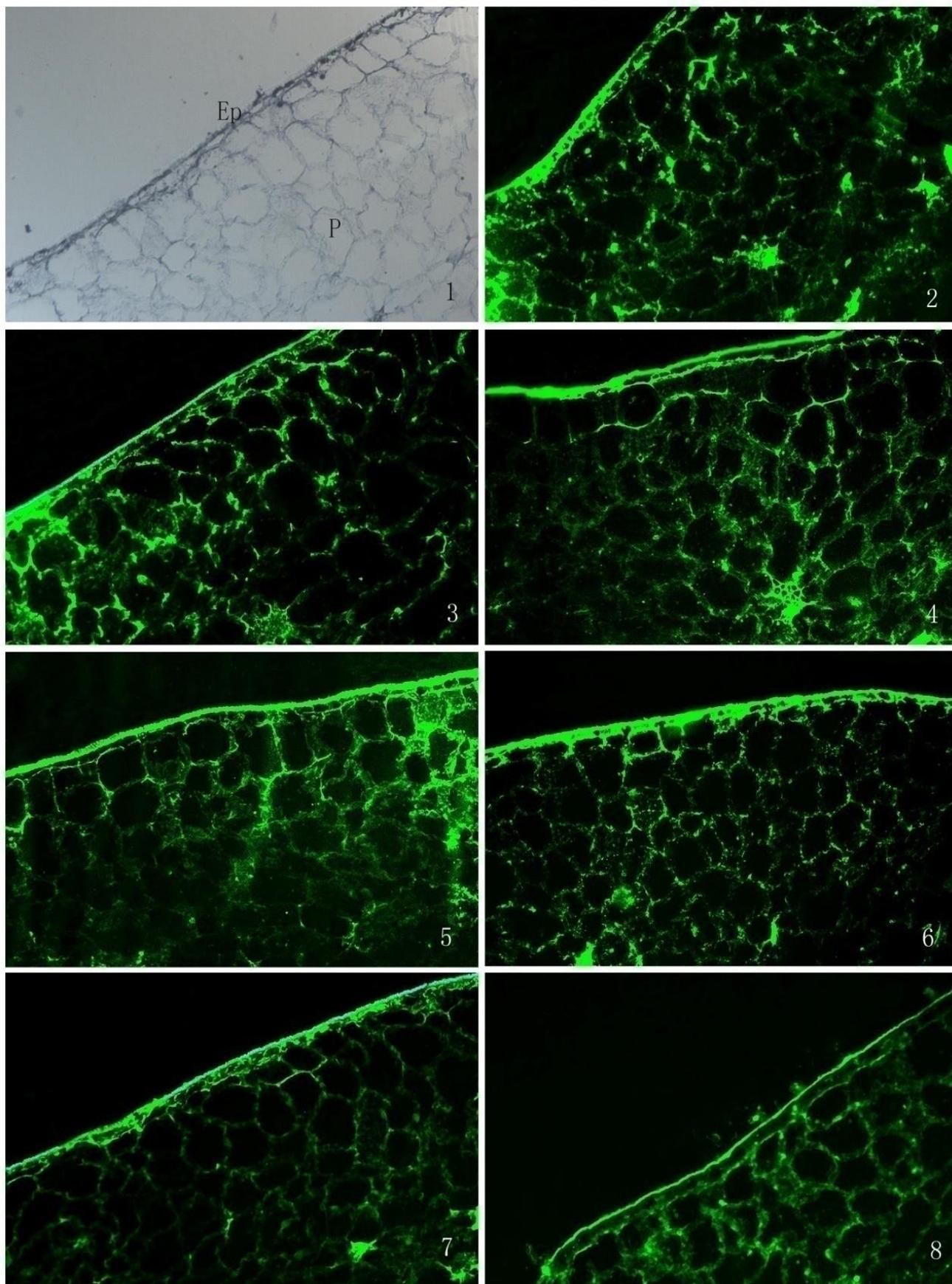


Fig. 7. Localization of pectins (JIM5, JIM11 and JIM20, 2-4) and arabinogalactan and AGPs (JIM94, JIM8, JIM13 and MAC204, 5-8) in wolfberry fruit at the second rapid stage ($\times 400$) by immunofluorescence microscopy. Brightfield micrographs images indicate that the size of parenchyma cells in the mesocarp increased rapidly and the structure loosened, vascular bundles differentiated and arranged in a ring (1). Ep, epicarp; P, parenchyma cell.

Immunogold localization of AGPs and pectin in *L.barbarum* fruits: JIM5, JIM11, and MAC204 were used as primary antibodies in immunogold localization.

1. Immunogold localization in fruits at the first rapid stage: At the first rapid stage, the pectins epitopes recognized by JIM5 were present in the region of cell wall and plasma membrane, plastid membranes, mitochondria and other organelles in parenchyma cell. No immunogold labelling was present in the cell wall. Some osmiophilic particles were present in the cytoplasm of amyloplast, while gold particles did not label in this area (Figs. 8-1, bar=0.2 μm). Immunogold particles distributed in the membrane of vacuolar and plastid, mitochondria of the cytoplasm (Figs. 8-2, bar=0.2 μm), as well as at the edge of vesicles and inside it (Figs. 8-3, bar=200nm).

Extensins epitopes recognized by JIM11 were present in plastid membrane, mitochondria, vacuole and cytoplasm of parenchyma cells (Figs. 8-4, bar=0.2 μm). A strong immunogold labelling was observed in membrane structure in proximity to the chloroplast membrane. Some immunogold particles accumulated inside vesicles (Figs. 8-5, bar=0.2 μm), cell wall and plasma membrane (Figs. 8-6, bar=200 μm).

AGPs epitopes recognized by MAC204 in plasma and plastid membranes, mitochondria and other organelles of parenchyma cells, while no immunogold labelled cell wall or plasma membrane (Figs. 8-7, bar=0.2 μm). A light labelling was observed in the vacuolar membrane, and some particles accumulated beside chloroplast membrane, which its lamellar structure loosened and osmiophilic particles dispersed in it. (Figs. 8-8, bar= 0.2 μm). Immunogold labelling was observed in the vesicles as well (Figs. 8-9, bar=0.2 μm).

2. Immunogold localization in fruits at the slow growth stage: At the slow growth stage, the cell wall of fruit parenchyma cells were twisted and turned, with loosened structure. The pectins epitopes recognized by JIM5 antibody were mainly present in plasma membrane and vacuole membrane. It could be observed that the small vesicles merged at the plasma membrane invagination between the cell wall and plasma membrane, and light immunogold labelling showed in vesicles (Figs. 9-1, bar=0.2 μm). At the meantime, the structure of chloroplast disintegrated and gradually turned to chromoplast, with some immunogold particles located in the plasma membrane and cytoplasm of chromoplast (Figs. 9-2, bar=0.2 μm). Immunogold labelling was visible between plasma membrane and vacuole membrane, with a light labelling in paramural body in close proximity to the cell wall (Figs. 9-3, bar=200nm).

Immunogold labelling using JIM11 showed in vesicles forming by plasma membrane of fruit parenchyma cell. The plasma membrane and vacuole membrane became irregular shape, vesicles forming by vacuole membrane contained some immunogold particles (Figs. 9-4, bar=0.2 μm). Few immunogold

particles distributed in the cytoplasm and at the chromoplast membrane. (Figs. 9-5, bar=0.2 μm). Gold particles scattered in intercellular space between adjacent cell wall (Figs. 9-6, bar=200nm).

AGPs localization using MAC204 labelled in plasma membrane, vacuole membrane and the membrane of plastid and chromoplast inside fruit parenchyma cell (Figs. 9-7, bar=0.2 μm). Some gold particles labelled in chromoplast membranes and its cytoplasm, plastid membrane and mitochondria next to chromoplast (Figs. 9-8, bar= 0.2 μm). Gold particles labelled at plasma membrane invaginations, but did not label in vacuole membranes (Figs. 9-9, bar=200 nm).

3. Immunogold localization in fruits at the second rapid stage: In parenchyma cell, the pectins epitopes recognized by JIM5 were present at the plasma membrane, vacuolar vesicles, plastid membranes, mitochondria and other organelles (Figs. 10-1, bar=0.5 μm). Immunogold particles labelled intensively the edge region of chromoplasts, membrane of plastids, and mitochondria. Gold particle also labelled at vacuole membranes (Figs. 10-2, bar=0.5 μm). Immunogold particles distributed in plastid, around its lumen and vesicles forming by vacuole membrane invagination (Figs. 10-3, bar=0.2 μm).

Extensins epitopes recognized by JIM11 were present in plastid membranes and small vacuole in fruit parenchyma cell. A few gold particles distributed at the membrane of big vacuole (Figs. 10-4, bar=0.2 μm). Weak gold labelling was visible in the in cell wall and plasma membrane (Figs. 10-5, bar=100 nm). Some gold particles were observed along vacuolar membrane and vesicles inside vacuoles (Figs. 10-6, bar=100 nm).

The localization of AGPs with MAC204 antibody labelled the amyloplast membrane in proximity to central vacuole of fruit parenchyma cells (Figs.10-7, bar=0.5 μm). Some immunogold particles were observed in the membrane of vacuole and in fragments of cell wall, while no gold particles were in the intercellular space (Figs. 10-8, bar=100 nm). There were some immunogold particles distributed inside vesicles forming by vacuole membrane fragments (Figs. 10-9, bar=100 nm).

In summary, pectins epitopes recognized by JIM5 were observed that gold labelling in the plasma membrane, vacuole membrane, plastid membranes and vesicles, mitochondria and other organelles in parenchyma cell. The number of immunogold particles decreased slightly at the slow growth stage, and increased slightly at the second rapid stage. Extensin epitopes recognized by JIM11 were observed at plasma membrane, vacuole membrane, vesicles, cell wall and thylakoids in parenchyma cell. It has similar changing trend like JIM5. AGPs epitopes recognized by MAC204 which showed a dense gold labelled at the membranes of plastid, mitochondria and vacuole in parenchyma cells at the first stage. The intensity of the labelling decreased in the slow growth stage and increased slightly at the second rapid stage.

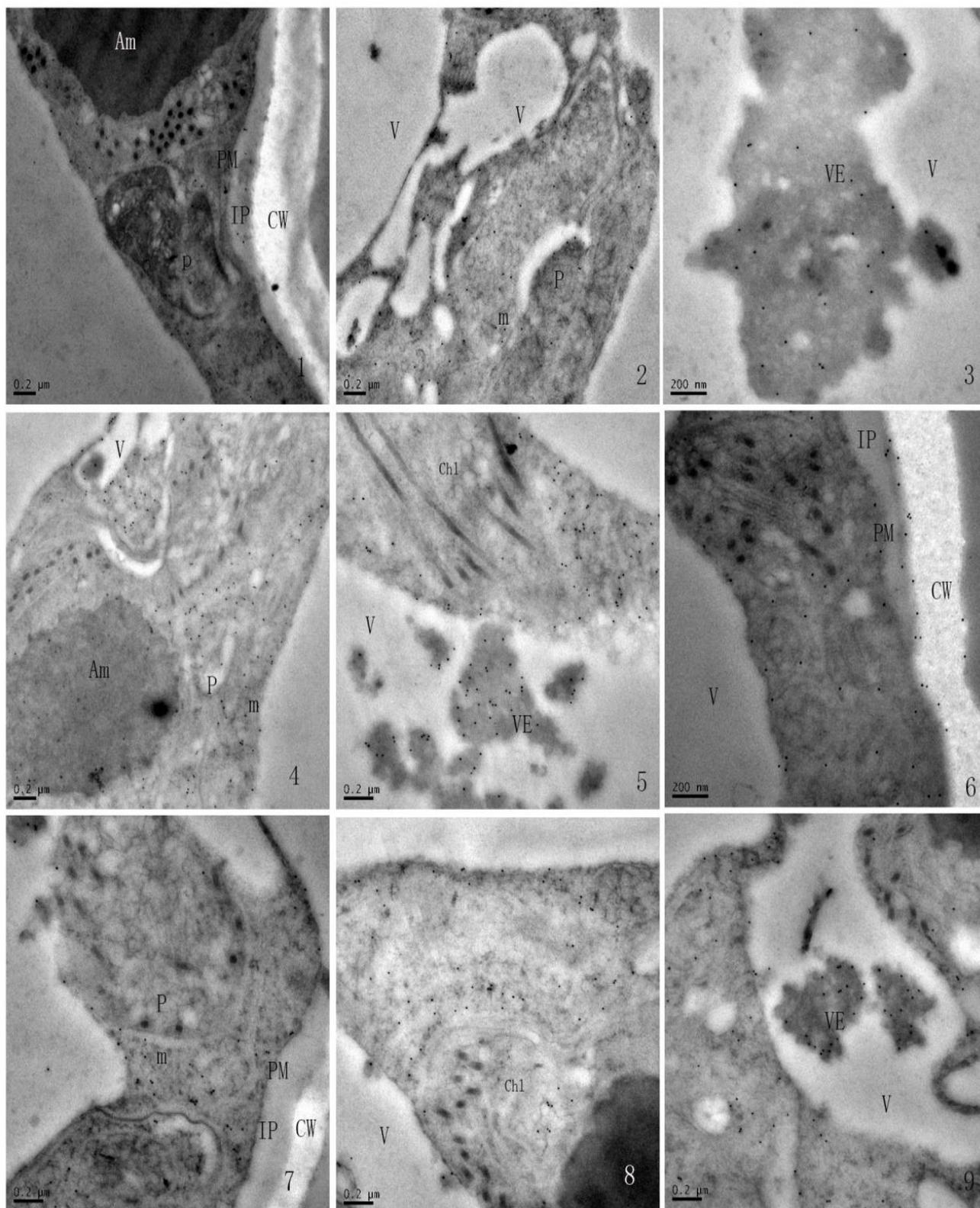


Fig. 8. TEM immunolocalization of pectins recognized by JIM5 in *Lycium b.* fruits at the first rapid stage (1-3). 1: Gold particles were mainly present at the plasma membrane. 2: Immunogold labelling was showed in the organelles. 3: Vesicles contained some immunogold particles in big vacuole. JM11 localization of extensin (4-6). 4: Numerous gold particles were present in the cytoplasm of parenchyma cells. 5: Numerous immunogold particles were located proximity to the membrane of chloroplasts and vesicles. 6: Gold labelling is visible at the cell wall and plasma membrane. Immunolocalization of AGPs using MAC204 antibody at the first rapid stage. (7-9). 7: Gold particles could be observed in plasma membrane and cytoplasm of parenchyma cells. 8: Gold labelling was located in close proximity to the membrane of chloroplast. 9: Immunogold particles was also distributed in vesicles and membrane of vacuole. Am, Amyloplast; Chl, chloroplast; CW, cell wall; m, mitochondria; P, plastid; PI, plasmalemma invagination; PM, plasma membrane; V, vacuole; VE, vesicles.

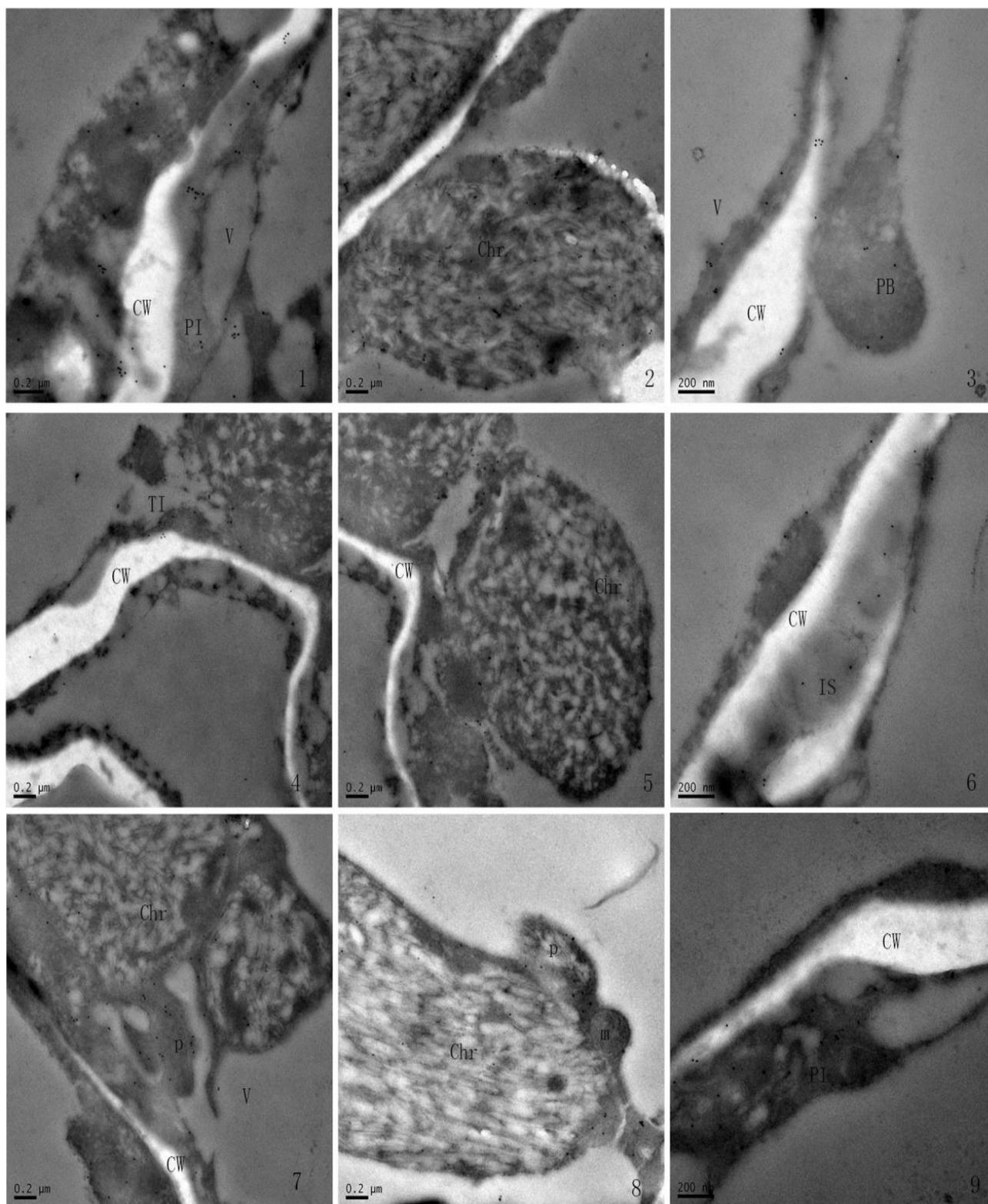


Fig. 9. TEM immunolocalization of pectins by JIM5 in *Lycium b.* fruits at the slow growth stage (1-3) 1: The pectins localized at plasma membrane and vacuole membrane. 2: The cytoplasm and membrane of chromoplast contained immunogold particles. 3: Gold labelling was located in paramural body in close proximity to the cell wall. Immunodetection of extensin at slow growth stage using JIM11 antibody (4-6). 4: Few immunogold particles located in vesicles forming by plasma membrane and tonoplast of fruit parenchyma cell. 5: There are gold labelling on lumen and plasma membrane of chromoplast. 6: Intercellular space between adjacent cell wall contained some gold particles. Immunolocalization of AGPs using MAC204 antibody at slow growth stage (7-9). 7: In fruit parenchyma cell, gold particles labelled at tonoplast and other organelles. 8: Gold particles were labelling at plastid membranes. 9: Gold particles were located at invaginating plasma membranes. CW, cell wall; Chr, Chromoplast; IS, intercellular space; m, mitochondria; P, plastid; PB, paramural body; PI, plasmalemma invagination; PM, plasma membrane; TI, tonoplast invagination; V, vacuole; VE, vesicles.

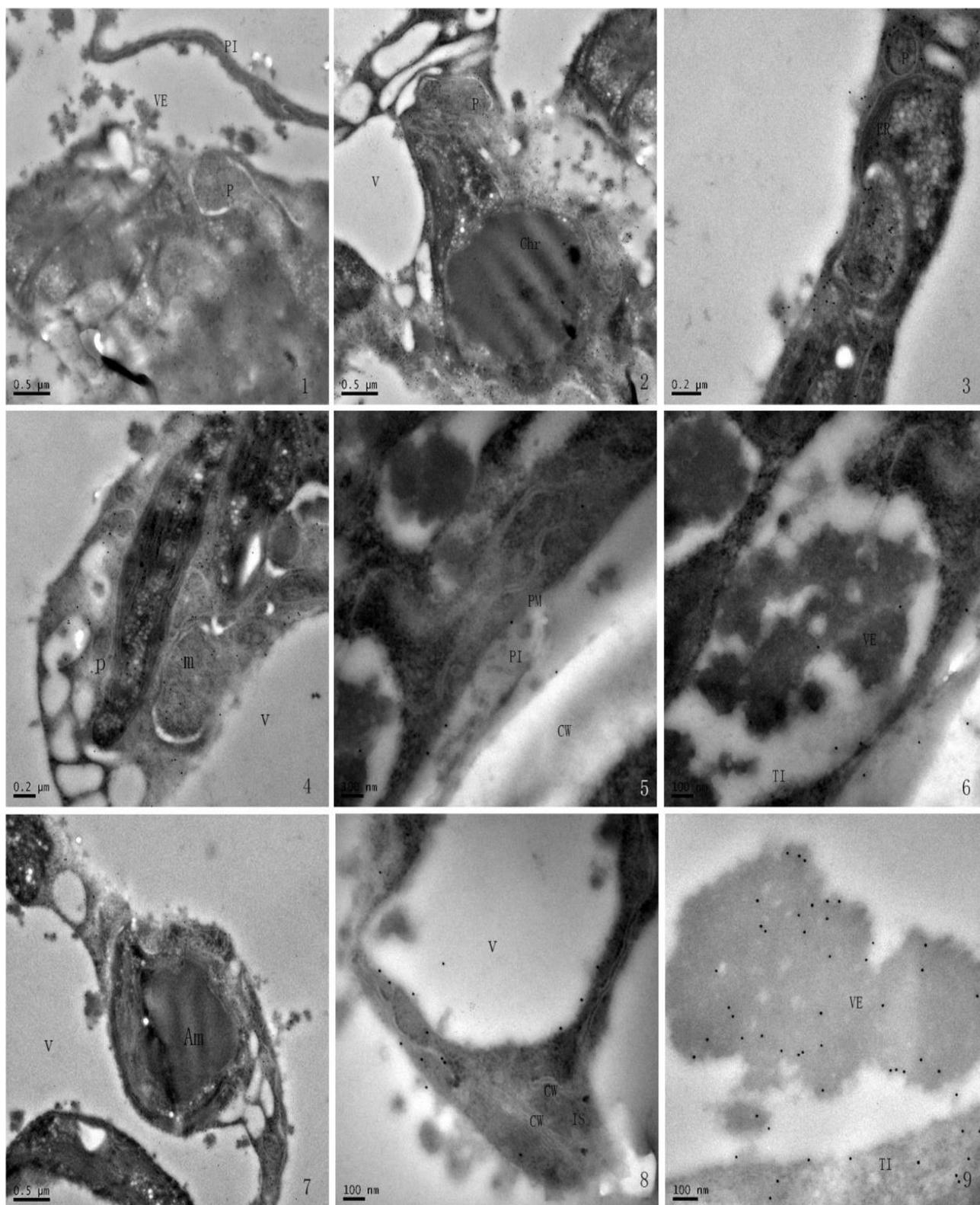


Fig. 10. TEM immunolocalization of pectins recognized by JIM5 in *Lycium b.* fruits at the second rapid stage (1-3) 1: Immunogold particles were present in plasma membrane, vesicles and organelles in parenchyma cell. 2: Gold labelling labelled membrane of chromoplast and vacuole. 3: Immunogold particles were distributed in plastid around cytoplasm and vesicles. Immunodetection of extensins at the second rapid stage using JIM11 antibody (4-6). 4: In fruit parenchyma cell, immunogold particles labelled organelles. 5: Gold labelling was showed at the cell wall and plasma membrane. 6: Gold particles labelled tonoplast and vesicles inside vacuole. Immunolocalization of AGPs using MAC204 antibody at second rapid stage. (7-9). 7: Gold labelling was detected in the membrane of amyoplast. 8: Immunogold labelled tonoplast and cell wall fragment. 9: Immunogold particles distributed inside vesicles. Am, Amyoplast; CW, cell wall; Chr, Chromoplast; ER, endoplasmic reticulum; IS, intercellular space; m, mitochondria; P, plastid; PI, plasmalemma invagination; PM, plasma membrane; TI, tonoplast invagination; V, vacuole; VE, vesicles.

Discussion

We selected 10 antibodies (JIM7, JIM8, JIM12, JIM94, JIM13, MAC204, JIM11, JIM19, JIM20, JIM5) from 30 kinds of antibodies by reacting with purified AGPs and LBP in Elisa screening, and confirmed their effects by Western blotting. Seven effective antibodies were divided into 3 groups: (1) JIM8, JIM13, JIM94, and MAC204, were used for detection of arabinogalactan and arabinogalactan protein; (2) JIM11 and JIM20 were used for detection of extensin and arabia gum; (3) JIM5 were used for detection of homogalacturonan. A previous research on tobacco pollen tube illustrated that extracted proteins from mature anthers could be labelled by JIM13 and LM2, but not by JIM4 (Qin *et al.*, 2007). The difference may result from the various component of the cell wall recognized by the different antibodies.

AGPs were generally present in plasma membranes, cell walls or as secretions to intercellular spaces and culture media, and were also found in intracellular, multivesicular bodies such as vesicles (Showlater, 2001). Immunolocalization of arabinogalactan proteins (AGPs) using JIM13 has been previously reported, showing that AGPs mainly accumulate at the early stages of anther development in tobacco. In pollen tubes, abundant AGPs were present in the transmitting tissue of styles, and immunogold particles were mainly distributed in cell wall and cytoplasm, especially around the peripheral region of the generative cell wall (Qin *et al.*, 2007). AGPs were detected by JIM13 which are regulated developmentally and immunogold labelling displayed a strong signal for AGPs at the juncture of the EP and suspensor cells at the cell wall (Qin *et al.*, 2007). Another report showed an increasing expression of AGPs pollination in olive (*Olea europaea*) in pistil tissues and decreased significantly after pollination. The AGPs localized predominantly in the stigmatic exudate, secretory cell wall of the stigma, as well as in the transmitting tissue of the pistil during pollination (Suárez *et al.*, 2013). In recently studies, some arabinogalactan proteins like Arabidopsis ROOT HAIR SPECIFIC 10 (RHS10), is a Ser/Thr protein kinase with arabinogalactan protein (AGP) motifs in its extracellular domain (ECD), which is a inhibitory in root hair tip growth (Cho HT, 2016). SOS5 and FEI2, encoding a fasciclin-like arabinogalactan protein or a receptor-like kinase, respectively, Jonathan found that FEI2 served to localize SOS5 at the plasma membrane where it established interactions with mucilage polysaccharides, notably pectins, required for mucilage adherence prior to SOS5 being released into the apoplast (Griffiths *et al.*, 2016).

In our present study, immunofluorescence localization using JIM13 reveal that AGPs are accumulated mainly at the exocarp and some vascular bundles in the mesocarp, decrease slightly by the development of fruit. During the fruit development, the structure of cell wall loosen, assuming that AGPs may quickly respond to environment changes, through changing the composition of cell wall to adapt with volume increasing on parenchyma cells. Thus, the intensity of immunofluorescence weaken in the process of development.

MAC204 antibody recognized arabinogalactan epitope. With regards of ultrastructural immunolocalization, it can be observed that the intercellular space between fruit parenchyma cells increased and emerged a loosening of the cell arrangement, plasma membrane invaginated and vesicles which were formed by vacuole membrane invagination with development of fruit. Moreover, using MAC204 antibody labeling, numerous immunogold particles were present at the plasma, plastid membranes, mitochondria and other organelles of parenchyma cells at the first rapid stage, located at plasma membrane invaginations at slow growth stage, then emerged in vesicles forming by vacuole membrane at the second rapid stage (Figs. 8-10). With the development of fruit, the walls of parenchyma cells loosed. Therefore, we presume that the polysaccharides and AGPs of *L. barbarum*, may transplant to other part of fruit parenchyma cells and store at vesicles. They may also play a role in protecting cells in the process of expansion that the cell size increasing dramatically. Similar result has been reported in previous studies in cell expansion of *Arabidopsis thaliana* (Willats & Knox, 1996).

Conclusion

Our works indicated that distribution and accumulation in tissue and cellular and subcellular levels of *Lycium b.* polysaccharides and AGPs with immunohistochemical method, AGPs might play a role as lubricants during the cellular growth of fruit parenchyma cell to avoid cells rupture.

Acknowledgments

The authors are thankful to Professor Dr.H.Q.Tian for expert support, Dr X.F.Na for his assistance on experimental techniques on Western Blot and Professor Ch.R for manuscript proofread. We thank the Centre for Plant Sciences (University of Leeds, UK) for the generous gifts of the antibodies. This work was supported by National Natural Science Foundation of China (81360639, 81160517), The National Key Research and Development Plan (SQ2016YFSF030161-07) and Natural Science Funds, Ningxia University (ZR1448).

References

- Chasan, R. 1994. Arabinogalactan-proteins: getting to the core. *Plant Cell*, 6: 1519-1521.
- Chia, C.W., Ch. Shyh and H. Ch. Bing. 2009. Chromatographic determination of polysaccharides in *Lycium barbarum* L. *Food Chem.*, 116: 595-603.
- Cho, H.T. 2016. Arabinogalactan protein motif-containing receptor-like kinases are likely to play the negative feedback factor to maintain proper root hair length. *Plant Signal Behav.* Aug 25:0[Epub ahead of print].
- Clemens, M.A., N.T. Elena, C.J. Michael and J.H. Philip. 2010. Distribution of (1→4)-β-glucans in tracheid cell walls of softwoods. *Tree Physiol.*, 30: 782-793.
- Dolan, L. and K. Roberts. 1995. Secondary thickening in roots of *Arabidopsis thaliana*: anatomy and cell surface changes. *New Phytologist*, 131: 121-128.

- Fincher, G.B., B.A. Stone and A.E. Clarke. 1983. Arabinogalactan-proteins: structure, biosynthesis and function. *Ann. Rev. Plant Physiol.*, 34: 47-70.
- Gan, L., S.H. Zhang, X.L. Yang and H.B. Xu. 2004. Immunomodulation and antitumour activity by a protein complex from *Lycium barbarum*. *Int. Immunopharmacology*, 4: 563-569.
- Griffiths, J.S., M.J. Crepeau, M.C. Ralet, G.J. Seifert and H.M. North. 2016. Dissecting seed mucilage adherence mediated by FEI2 and SOS5. *Front Plant Sci.*, 2016 Jun 29; 7: 1073.
- Huang, L.J., Y. Lin, G.Y. Tian and G.Z. Ji. 1998. Isolation, purification and physicochemical properties of immunoactive constituents from the fruit of *Lycium barbarum* L. *Acta Pharmacologica Sinica*, 33: 512-516.
- Kieran, J.D.L., S. Yoichi, M. Shaio-Lim, P. Filomena, B. Antony S.Q. Ralph, D.K. Celia and J.K. Paul. 2005. Arabinogalactan proteins are required for apical cell extension in the moss *Physcomitrella patens*. *The Plant Cell*, 17: 3051-3065.
- Li, X.L. and A.G. Zhou. 2007. Evaluation of the antioxidant effects of polysaccharides extracted from *Lycium barbarum*. *Med. Chem. Res.*, 15: 471-482.
- Nothnagel, E.A. 1997. Proteoglycans and related components in plant cells. *Int. Rev. Plant Cytol.*, 174: 195-291.
- Pennell, R.I., L. Janniche, P. Kjellbom, G.N. Scofield, J.M. Peart and K. Roberts. 1991. Developmental regulation of a plasma membrane arabinogalactan protein epitope in oilseed rape flowers. *The Plant Cell*, 3: 1317-1326.
- Puhlmann, J., E. Bucheli, M.J. Swain, N. Dunning, P. Albersheim, A.G. Darvill and M.G. Hahn. 1994. Generation of monoclonal antibodies against plant cell wall polysaccharides. I. Characterization of a monoclonal antibody to a terminal α -(1-2)-linked fucosyl-containing epitope. *Plant Physiol.*, 104: 699-710.
- Qin, Y., D. Chen and J. Zhao. 2007. Localization of arabinogalactan proteins in anther, pollen, and pollen tube of *Nicotiana tabacum* L. *Protoplasma*, 231: 45-53.
- Robert, J.R., C. Delphine, W. Juankuan, M.D. Justyna, J.G. Gerrit, P.K. Johannis and P. Bucheli. 2011. Cell wall polysaccharides of Chinese Wolfberry (*Lycium barbarum* L.): Part 2. Characterisation of arabinogalactan-proteins. *Carbohydrate Polymers*, 84(3): 1075-1083.
- Showlater, A.M. 2001. Arabinogalactan-proteins: structure, expression and function. *CMLS Cell Mol. Life Sci.*, 58: 1399-1417.
- Smallwood, M., A. Beven, N. Donovan, S.J. Neill, J. Peart, K. Roberts and J.P. Knox. 1994. Localization of cell wall proteins in relation to the developmental anatomy of the carrot root apex. *The Plant Journal*, 5(2): 237-246.
- Smallwood, M., E.A. Yates, W.G.T. Willats, H. Martin and J.P. Knox. 1996. Immunochemical comparison of membrane-associated and secreted arabinogalactan-proteins in rice and carrot. *Planta*, 198: 452-459.
- Suarez, C., A. Zienkiewicz, A.J. Castro, K. Zienkiewicz, A. Majewska-Sawka and M.I. Rodríguez-García. 2013. Cellular localization and levels of pectins and arabinogalactan proteins in olive (*Olea europaea* L.) pistil tissues during development: implications for pollen-pistil interaction. *Planta*, 237: 305-319.
- Vitha, S., F. Baluška, M. Braun, J. Šamaj, D. Volkmann and P.W. Barlow. 2000. Comparison of cryofixation and aldehyde fixation for plant actin immunocytochemistry: aldehydes do not destroy F-actin. *The Histochem. J.*, 32(8): 457-466.
- Willats, W.G.T. and J.P. Knox. 1996. A role for arabinogalactan proteins in plant cell expansion: Evidence from studies on the interaction of β -glucosyl Yariv reagent with seedlings of *Arabidopsis thaliana*. *The Plant Journal*, 9(6): 919-925.
- Yu, M. and J. Zhao. 2012. The cytological changes of tobacco zygote and proembryo cells induced by beta-glucosyl Yariv reagent suggest the involvement of arabinogalactan proteins in cell division and cell plate formation. *BMC Plant Biol.*, 12: 126.
- Zheng, G.Q., L. Zhang, J. Wang and Z.H. Hu. 2012. The morphology development of fruit and seed in *Lycium barbarum* L. *Guihaia*, 32(6): 810-815.

(Received for publication 30 August 2015)