Plant Cell, Tissue and Organ Culture (PCTOC) (2024) 157:26
https://doi.org/10.1007/s11240-024-02740-6

ORIGINAL ARTICLE

Reconstruction pattern of the cell wall in *Fagopyrum* protoplast-derived hybrid cells

Katarzyna Sala-Cholewa[1] · Anna Milewska-Hendel[1] · Reneé Pérez-Pérez[1] · Ewa Grzebelus[2] · Alexander Betekhtin[1]

Received: 7 December 2023 / Accepted: 21 March 2024

Abstract

The cell wall rebuilding is one of the first stage of protoplast development that enables further mitotic divisions and differentiation. Therefore, this work focuses on the comparison of the cell wall regeneration in the parental protoplasts of *Fagopyrum tataricum*, *F. esculentum* and the *F. tataricum* (+) *F. esculentum* hybrids, which are promising materials in terms of future breeding and research programmes. It is worth emphasizing that the preparation of buckwheat hybrids using electrofusion was described for the first time. The results indicate that cell wall rebuilding exhibited a common mechanism for parent protoplasts and the heterokaryon as all analysed cell wall components recognising arabinogalactan proteins (JIM13, JIM16), extensin (JIM20), xyloglucan (LM25) and pectins (LM20, LM5, LM6) were detected during the process of wall regeneration. However, there were certainly differences in the spatio-temporal appearance or disappearance of individual epitopes during the 72 h of the cell culture, which have been discussed in the paper.

Key message

The hybrid protoplasts similarly restore their wall to *F. tataricum* and *F. esculentum* protoplasts despite some qualitative and quantitative differences in epitope distribution.

Keywords Buckwheat · Cell wall · Heterokaryon · Hybrid · Immunocytochemistry · Protoplasts

Abbreviations

AC	alternating current
AGPs	arabinogalactan proteins
BB	blocking buffer
DC	direct current
dH_2O	distilled water
Fb28	fluorescent brightener 28
Fe	*Fagopyrum esculentum*
Ft	*Fagopyrum tataricum*
Ft+Fe	*F. tataricum* and *F. esculentum* hybrid
HG	homogalacturonan
HRGP	hydroxyproline rich proteins
RG-I	rhamnogalacturonan I
Rhd	rhodamine B
RT	room temperature

Katarzyna Sala-Cholewa and Anna Milewska-Hendel contributed equally to this work.

Communicated by Danny Geelen.

✉ Anna Milewska-Hendel
anna.milewska@us.edu.pl

✉ Alexander Betekhtin
alexander.betekhtin@us.edu.pl

[1] Institute of Biology, Biotechnology and Environmental Protection, Faculty of Natural Sciences, University of Silesia in Katowice, Jagiellonska 28, 40-032 Katowice, Poland

[2] Department of Plant Biology and Biotechnology, Faculty of Biotechnology and Horticulture, University of Agriculture in Krakow, al. Mickiewicza 21, 31-120 Krakow, Poland

Introduction

Buckwheat (*Fagopyrum* spp.) is described as a neglected and underutilised species of cultivated plants; however, it is an attractive crop; it is a good source of, among others, flavonoids, gluten-free proteins, amino acids, dietary fibre, vitamins, minerals (Huda et al. 2021; Li et al. 2019). Of the 23 species of *Fagopyrum*, only two are *F. esculentum* and *F. tataricum* are cultivated (Tomasiak et al. 2022). *F. tataricum* is characterised by over 100-fold higher rutin

Published online: 16 April 2024

content than *F. esculentum* (Fabjan et al. 2003; Zhang et al. 2021). However, low grain content and strong bitter taste limit its use as food. *F. esculentum* is more widely distributed than *F. tataricum;* however the seed yield of this species is often relatively low and unstable (Sytar et al. 2016). One of the most crucial reasons for the low yield is self-incompatibility, as *F. esculentum* plants are dimorphic with two types of flowers: Thrum (pistils shorter than stamens) and Pin (pistils longer than stamens) (Cawoy et al. 2009). *F. tataricum* produces only homostylous flowers capable of self-fertilization.

A somatic hybridization technique has been proposed to overcome breeding barriers in buckwheat (Nešković et al. 1987). Among the different cell fusion methods for developing somatic hybrids in plants, PEG-mediated fusion has been the most popular and used so far. Lachmann et al. (1994) obtained the hybrid calli of *F. esculentum* (+) *F. tataricum* without further plant regeneration. The other method is protoplast electrofusion, which acquired a preferential position due to its rapidity, efficiency and low cytotoxicity (Rems et al. 2013). Since the first description of the electrofusion process by (Senda et al. 1979), it has been successfully applied to different species of agricultural importance (Mackowska et al. 2023; Sedlak et al. 2022).

In *Fagopyrum* spp., plant regeneration from protoplasts has been described for *F. esculentum* from hypocotyl- (Adachi et al. 1989) and callus-derived protoplasts (Zaranek et al. 2023b) while for *F. tataricum* from hypocotyl- (Lachmann and Adachi 1990) and callus-derived protoplasts (Zaranek et al. 2023a). However, to obtain plant regenerants from protoplast cultures, one of the first and crucial stages is cell wall re-synthesis and deposition, which enables the first cell divisions. The cell wall is a dynamic and highly controlled structure mainly composed of cellulose microfibrils, matrix polysaccharides, polyphenolic compounds, and structural proteins in variable amounts (Showalter 1993). Due to its compounds' structural and enzymatic rearrangements, the cell walls play an important role in regulating the morphogenetic processes and maintaining the cellular differentiation status of cells (Knox et al. 1991; Potocka et al. 2018). Cellulose, hemicellulose and pectins, arabinogalactan proteins (AGPs) and extensins (EXTs) all participate (to different degrees) in cell expansion or adhesion and as regulatory molecules they are key determinants of the physical properties of the cell wall (Willats et al. 2001).

Cell wall regeneration from protoplasts has been studied in several species using various techniques (Kuki et al. 2020; Parmentier et al. 1995; Wiśniewska and Majewska-Sawka 2007). However, due to the complexity of the cell wall structure, the most informative method is immunocytochemical detection of specific cell wall antigens (Godel-Jędrychowska et al. 2019; Majewska-Sawka and Münster 2003). To date, there is no data about cell wall reconstruction from protoplasts in any of the *Fagopyrum* species, as well as in their hybrids or the hybrid protoplasts at all, independently of the species. Thus, our goal was to check whether there are differences between those two species and their hybrids (*Ft* + *Fe*) during wall regeneration that could point out the potential recalcitrance of hybrid protoplasts. Since xyloglucan, pectins, extensins, and AGPs are essential components of dicotyledonous walls, the specific antibodies that recognise their epitopes were chosen to follow the process of cell wall rebuilding: LM20, LM5 and LM6 recognising pectic epitopes; JIM13 and JIM16 recognising AGPs epitopes; JIM20 recognised extension epitope and LM25 recognising xyloglucan epitope. It should be emphasized that this is the first study that illustrates and compares the regeneration of the wall of *Fagopyrum* parental protoplasts and their hybrids. Moreover, this is the first record of obtaining hybrids in buckwheat by electrofusion.

Materials and methods

Protoplast isolation

Embryogenic calli of *F. esculentum* and morphogenic calli of *F. tataricum* were used as sources of protoplasts. The callus lines were obtained from immature embryos of both species and maintained in the dark on RX medium (Table S1) at 26 ± 1 °C as Betekhtin et al. (2017) described. The subcultures were carried out every two weeks under the same conditions.

The isolation was performed following the protocol of Zaranek et al. (2023b) with minor modifications. Two grams of 10-day-old callus of *F. tataricum* and 1 g of 12-day-old callus of *F. esculentum* were incubated for 1 h in 10 ml of PSII/F plasmolysis solution (Table S1) in the dark at room temperature (RT). Then, the solution was removed, and 10 ml of E1 and E2 enzyme solutions (Table S1) were added to *F. tataricum* and *F. esculentum*, respectively. The material was incubated for 16 h with gentle shaking (50 rpm, RT). The suspensions were filtered through a 100 µm pore mesh and centrifuged for 5 min (1000 rpm, RT). The pellet was resuspended in 8 ml of Suc/MES solution (Table S1). Next, 2 ml of W5 solution (Table S1) was carefully overlaid to create a gradient and centrifuged for 10 min (1200 rpm, RT). The ring of viable protoplasts between the two phases of the gradient was collected, resuspended in W5 solution up to 10 ml and centrifuged for 5 min (1000 rpm, RT). The pellet was resuspended in 1.5 ml of mannitol solution (Table S1), and the protoplast concentration was adjusted to 8×10^5 cells ml^{-1}. The isolation was performed twice for both species, and one set was kept as control and as nurse culture.

Electrofusion

The electrofusion methodology was adapted from the protocol proposed by Mackowska et al. (2023). One ml of the *F. esculentum* and *F. tataricum* protoplast suspensions were added to the tubes with Rhodamine B (Rhd) and 3,3'-dihexyloxacarbocyanine iodide (DiOC6), respectively and incubated for 10 min in the dark at RT. The preparation of dyes is listed in the Supplementary Information: Table S2. The following washing steps took place on ice. The stained protoplasts were centrifuged for 5 min (1000 rpm, 4 °C), and the pellet was resuspended in 10 ml of ice-cold mannitol solution. These steps were repeated two times more. The pellets were resuspended in 0.9 ml of ice-cold mannitol. The protoplast concentration was adjusted again to 8×10^5 cells ml^{-1}, adding ice-cold mannitol solution if needed. For parental protoplasts analysis, 1 ml of the protoplast solution of each species was stained with 100 µl of calcofluor white (Table S2) and kept in the dark for 10 min.

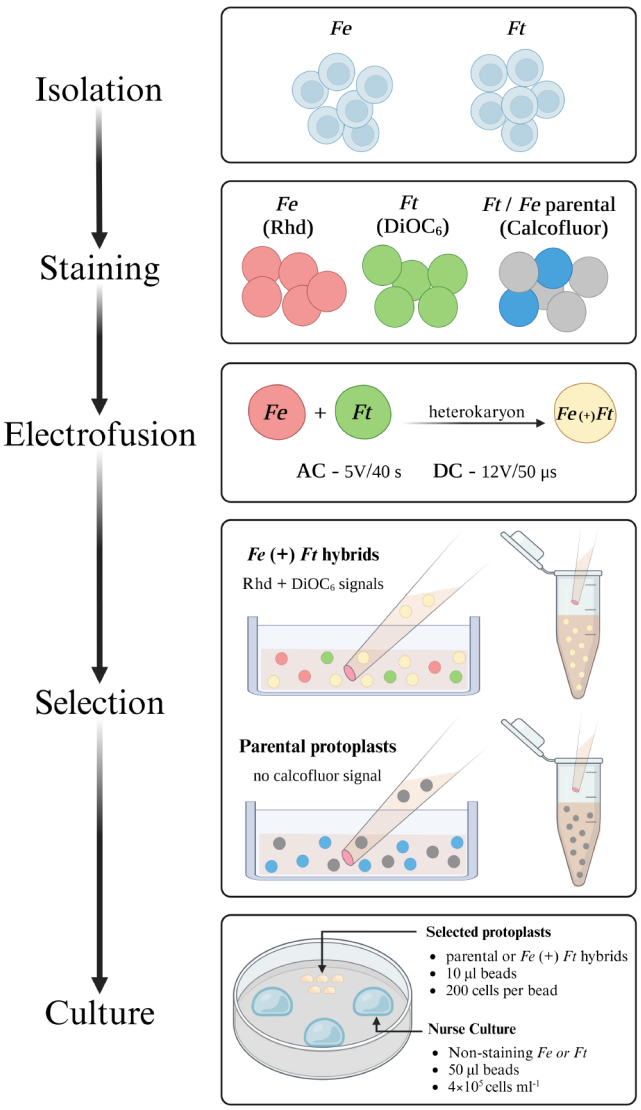

Fig. 1 General scheme of the protoplast electrofusion and selection technique. Once the protoplasts are isolated, they are stained with two contrasting fluorochrome solutions (Rhd– red; DiOC6– green) and fused using periodic electrical pulses. Electrofusion is a random process, so the hybrid protoplasts (yellow) must be selected, one by one, under the microscope. In the case of the parental protoplasts, calcofluor staining allows the subsequent selection of those from which the cell wall was completely removed (no calcofluor signal– grey). The culture of the hybrid and parental protoplasts must be developed after embedding them in agarose beads, and they must be accompanied by a nurse culture that contributes to their development. AC– alternating current; DC– direct current; DiOC6–3,3'-dihexyloxacarbocyanine iodide; Rhd– rhodamine B. This figure was created with BioRender.com. BioRender certificate confirming the publication rights is available upon request from the authors.

Respectively, 0.4 ml of protoplasts stained with Rhd and 0.4 ml stained with $DiOC_6$ were carefully mixed to combine *F. tataricum* (+) *F. esculentum* (*Fe*+*Ft*, heterokaryon), as shown in Fig. 1. The electrofusion was performed in the Super Electro Cell Fusion Generator 21 (NEPAGEN, Japan) using parameters optimised by our team, standardised for *Fagopyrum* protoplasts. The 0.8 ml CUY497P2 electrode was used with 5 V of alternating current (AC) for 40 s and 12 V of direct current (DC) for 50 µs. The fused protoplasts were transferred into a sterile tube covered with foil and kept on ice.

Selection of protoplasts

These steps were performed entirely in the dark. After electrofusion, 0.25 ml of the protoplast suspension was resuspended in 4.5 ml of ice-cold mannitol and carefully mixed. In a sterile Ibidi µ-Dish $^{35\ mm,\ low}$, 0.3 ml of this suspension was placed and allowed to settle for 5 min in the inverted microscope. The protoplasts that simultaneously showed fluorescence for Rhd and $DiOC_6$ were collected using a stripper tip MXL3-75 (ORIGIO Inc, USA) and micromanipulator TransferMan® 4r (Eppendorf, Germany). About 200 hybrid cells were collected and kept on ice until the next steps were performed. The selection process was applied for the parental protoplasts, *F. tataricum* and *F. esculentum*. Approximately 200 protoplasts that did not show fluorescence for calcofluor white were collected.

Culture of selected protoplasts

The parental and hybrid protoplasts selected into Eppendorf tubes were resuspended in 9 µl of LMPA (Table S1), and about 10 µl beads were made on polystyrene Petri dishes (Ø6 cm × 1.2 cm) at the rate of five beads per dish (Fig. 2). Non-stained *Fe* and *Ft* protoplasts were used as nurse culture during parental and *Fe* (+) *Ft* hybrid cells culture. For this, equal volumes of LMPA and the protoplast solution with a concentration of 8×10^5 cells ml^{-1} were mixed and

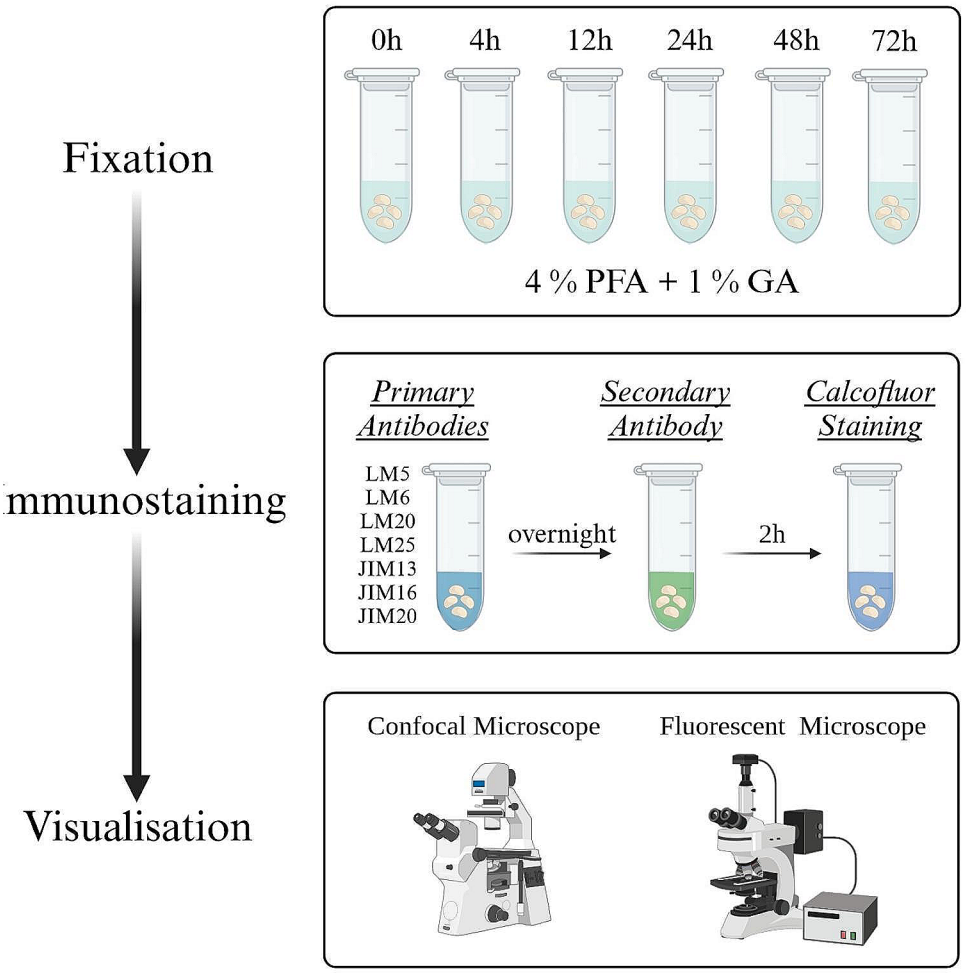

Fig. 2 General scheme of the protoplasts immunostaining process. This figure was created with http://BioRender.com. BioRender certificate confirming the publication rights is available upon request from the authors.

Table 1 Antibodies used for immunostaining of protoplasts and their respective epitopes in the cell wall

Antibody	Epitope	Reference
LM5	linear tetrasaccharide in (1→4)-β-D-galactans (RG I side chain)	(Jones et al. 1997)
LM6	linear pentasaccharide in (1→5)-α-L-arabinans (RG I side chain)	(Willats et al. 1998)
LM20	(1→4)-α-MeGalA (methyl-esterified HG)	(Verhertbruggen et al. 2009)
LM25	XLLG, XXLG and XXXG oligosaccharides of xyloglucan	(Pedersen et al. 2012)
JIM13	Arabinogalactan/Arabinogalactan protein, carbohydrate epitope (β)GlcA1→3(α)GalA1→2Rha	(Yates et al. 1996)
JIM16	Arabinogalactan/ AGP glycan	(Knox et al. 1991)
JIM20	Extensin/Hydroxyproline-rich glycoproteins	(Knox 1995)

RG I: rhamnogalacturonan I; HG: homogalacturonan

placed in three 50 µl beads around the parental and hybrid cells as shown in Fig. 1. Then 4 ml of BM medium supplemented with 4 µl of PSK and 2 µl of Timentin (Table S1) were added to each Petri dish and incubated for 4, 12, 24, 48 and 72 h at 26 ± 1 °C in the dark.

Protoplasts immunostaining

After each time point, the beads with the *F. tataricum* and *F. esculentum* control and hybrid cells were transferred to a 2 ml Eppendorf and fixed with 4% paraformaldehyde (PFA) and 1% glutaraldehyde (GA) in phosphate-buffered saline (PBS, pH = 7.2) overnight at 4 °C (Table S3; Fig. 2). For the 0 h time point; the beads were fixed immediately after they were made. Next, samples were washed with PBS and incubated with blocking buffer (BB) for 30 min (Table S3) and with primary antibodies (Table 1) for 1.5 h.

Negative controls were performed by incubation in BB instead of the primary antibodies. After several rinses with the BB, a secondary antibody conjugated with Alexa Fluor 488 goat anti-rat IgG antibody was applied for 1.5 h in the

dark. Next, the material was washed with PBS and counterstained with 0.01% Fluorescent Brightener 28 (calcofluor white) in PBS solution for 20 min.

Observations

The protoplast beads were individually placed on a glass slide and flattened by the coverslip. The observations and photographic documentation were carried out using an Olympus FV-1000 confocal system (Olympus, Hamburg, Germany) equipped with an Olympus IX81 inverted microscope, a 405-nm diode laser, and a multi-line argon ion laser (excitations 457/488/515 nm; Melles Griot BV, Didam, Netherlands). A series of two-dimensional images of z-stacks (the optical sections through the cells) were taken using two separate photomultipliers. 2D image processing was performed using ImageJ version 1.53s software (Wayne Rasband, National Institutes of Health, USA), and 3D images were performed with Imaris 9.5 software (Bitplane, Zürich, Switzerland). Moreover, 50 cells per antibody were also visualised for statistical analysis with an epifluorescence microscope Nikon Eclipse Ni-U microscope equipped with a Nikon Digital DS-Fi1-U3 camera with the corresponding software (Nikon, Tokyo, Japan). Cells have been scanned in two channels for excitation of Alexa 488 fluorochrome; the 488 nm laser was used, and for FB28, the 405 nm laser. To facilitate the interpretation of the obtained results, the percentage of signal presence from specific epitopes was calculated (number of cells containing signal per total number of cells). Thus, three ranges were specified, where 0–30% means the absence or presence of a signal in a small number of cells; 31–65% occurrence of a signal in the average number of cells; 66–100% occurrence of a signal in most or all cells.

Results

In the presented studies, a spatio-temporal analysis of cell wall regeneration was carried out on protoplasts derived from the callus of two cultivated species of buckwheat (*F. tataricum* and *F. esculentum*) and on their hybrid (heterokaryon: *Ft*+*Fe*). Protoplasts were analysed at various time points: 0 h, 4 h, 12 h, 24 h, 48 h, and 72 h. The wall components that were studied included cellulose (calcofluor staining), pectins (LM5, LM6, LM20), arabinogalactan proteins (JIM13, JIM16), xyloglucan (LM25) and extensin (JIM20). A comparison of protoplasts from two species and hybrids revealed similarities and differences in the cell wall composition at different time points (Table S4).

Arabinogalactan proteins

AGP epitope recognised by the JIM13 antibody occurred in all analysed types of protoplasts (*F. tataricum*, *F. esculentum* and hybrids) and was present at each of the analysed time points, from 0 h to 72 h (Fig. 3A– F, G– L, M– R; Table S4; Supplementary videos S1– S6). This epitope was located within the cell and cell membrane/cell wall at later stages. It was also noticed that the signal from the JIM13 antibody, from 12 h to 72 h, correlated with the arrangement of cellulose microfibrils that were visualised with calcofluor staining (Fig. 3 compare D and D', E and E', F and F', K and K', L and L', P and P, Q and Q'). The presence of JIM13 was also observed outside the cells (Fig. 3, e.g. E, I, J, O). The character of the signal was mainly continuous.

JIM16 antibody, directed against the AGP glycan component, was also observed at every stage of cell wall development for each type of analysed cell (Fig. 4A– F, G– L, M– R; Supplementary videos S7– S12). However, a reduced number of cells (average range) with the presence of JIM16 was observed for *F. tataricum* at 72 h, *F. esculentum* at 24 h and heterokaryons at 48 h (Fig. 4; Table S4). This AGP epitope outside the cell was also observed at each stage of development examined (Fig. 4, e.g., J, K, O, P). Moreover, a difference in the JIM16 distribution was observed. For *F. tataricum*, from 0 h to 24 h, the JIM16 signal was continuous with few areas of higher intensity (Fig. 4A– D). At 48 h and 72 h, the JIM16 epitope did not occur on the entire cell wall but was only present in some regions (Fig. 4E, F). In the case of *F. esculentum*, a continuous signal was observed at 0 h, 4 h, 12 h and 48 h, where areas with increased signal intensity were also observed (Fig. 4G, H, I, K). However, at 24 h and 72 h, the signal was present only in some areas of the cell wall (Fig. 4J, L). For the heterokaryons, the continuous signal was present at 0 h, 12 h, 24 h and 72 h (Fig. 4M, O, P, R); however, for 4 h and 48 h, JIM16 was localised within the walls in a patch-like manner (Fig. 4N, Q).

In summary, the analysed AGP epitopes were present in all cell types, from removing the cell wall (time 0 h) until its reconstitution (time 72 h; Table S4), and were present in the membrane and/or wall. Moreover, the signal outside the cell was also observed. The observed differences concerned the signal's character, especially for JIM16.

Extensin

In *F. tataricum* protoplasts, the JIM20 antibody, which detects extensin epitopes, was present in several cells at the initial stages of wall regeneration (0 h, 4 h; Table S4). In the cells where the signal was detected, it was present in the intracellular compartments (Fig. 5A, B; Supplementary videos S13– S18). An increase in the number of cells

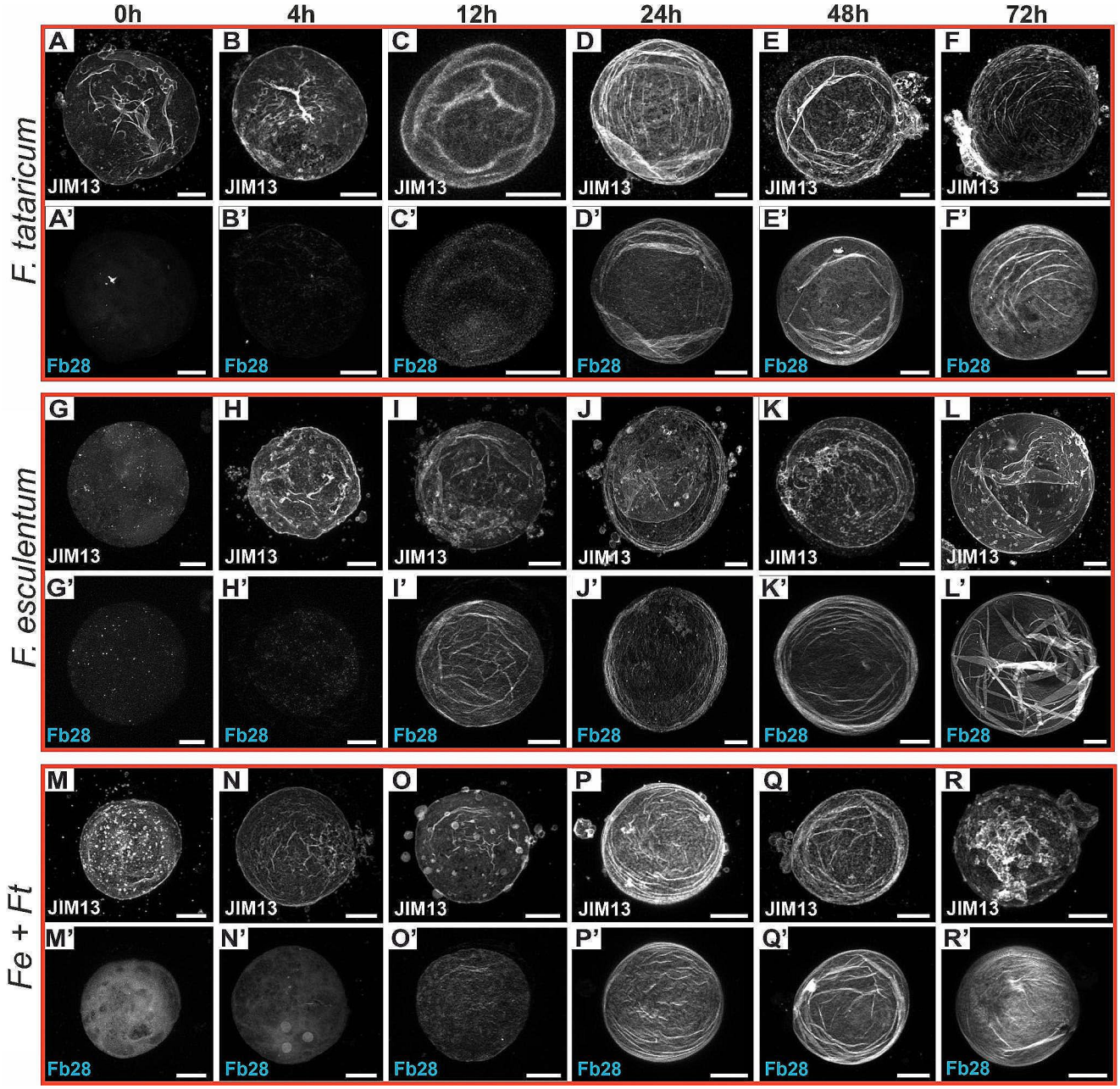

Fig. 3 The presence of JIM13 AGPs epitope during cell wall regeneration in *F. tataricum* **(A-F)**, *F. esculentum* **(G-L)**, and *Ft* + *Fe* hybrid **(M-R)** with the corresponding signal from calcofluor **(A'-R')**

with the occurrence of extensins was observed at 12 h and 24 h, followed by a decrease at 48 and 72 h (Fig. 5 compare C– F). If present, the signal from this antibody was continuous except for 24 h, where it had a patch-like character (Fig. 5D). Moreover, it was noticed that at 12 h, 48 h and 72 h in cells where JIM20 was noticed, the signal from the antibody corresponded to the signal from calcofluor staining (Fig. 5 compare, e.g. E and E').

JIM20 in *F. esculentum* protoplasts at 0 h and 4 h was observed in less than half of the analysed cells. However, when the signal was present, it was localised in the intracellular compartments (Fig. 5G, H). Interestingly, at 12 h there was an increase in cells with the JIM20 signal (Fig. 5), followed by a significant decrease at 24 h, and the signal, if present, was dotted (Fig. 5I, J). At 48 h and 72 h, the extensin epitope was observed in some cells, and its character was dotted and patch-like, respectively (Fig. 5K, L).

During wall reconstruction in hybrid protoplasts, the JIM20 epitope was present in most cells at 12 h and 24 h. However, at 0 h and 72 h, the JIM20 signal was observed

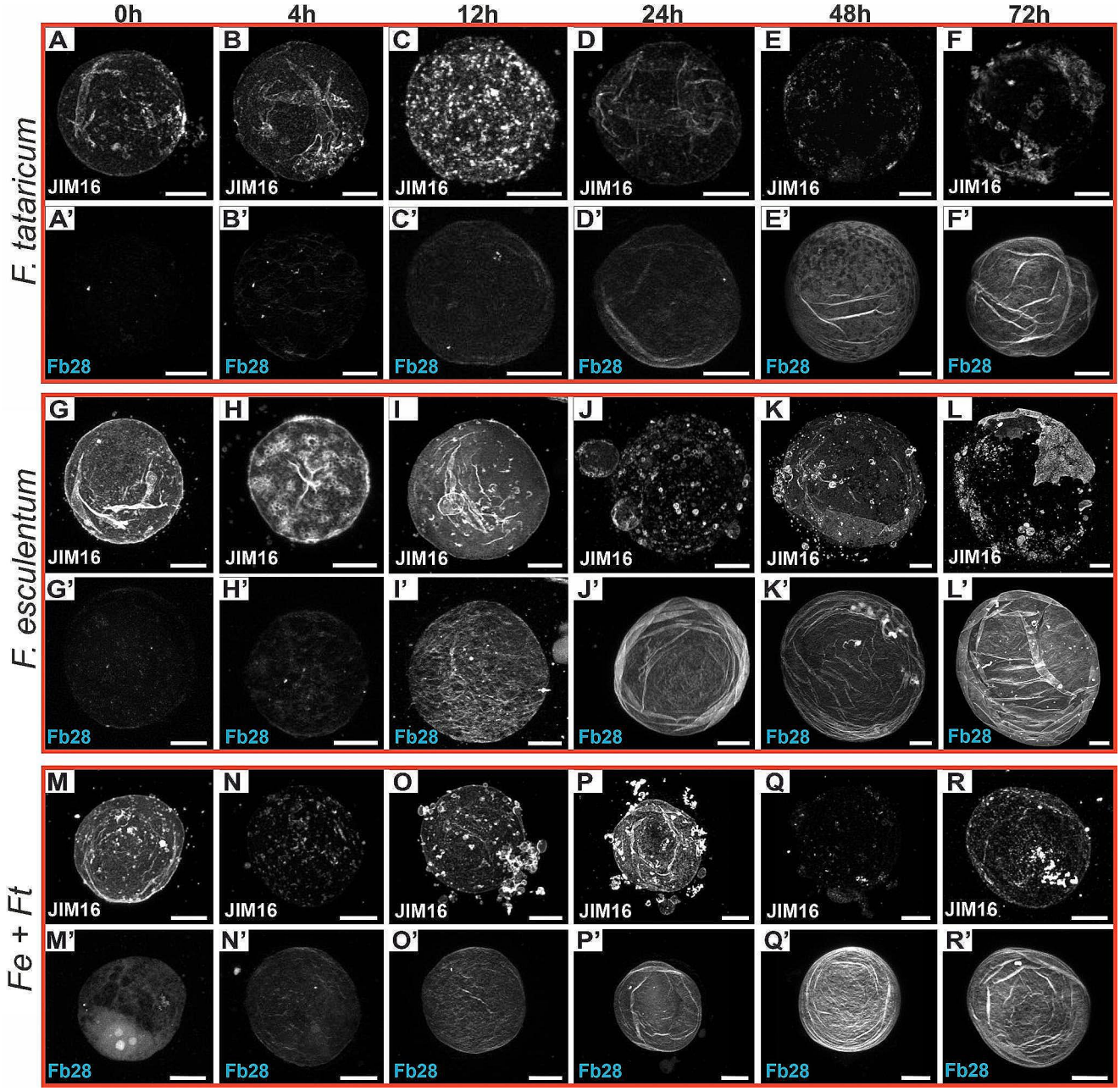

Fig. 4 The distribution of JIM16 AGPs epitope during cell wall reconstruction in *F. tataricum* **(A-F)**, *F. esculentum* **(G-L)**, and *Ft*+*Fe* hybrid **(M-R)** with the corresponding signal from calcofluor **(A'-R')**

only in some cells, while at 4 h it was present in a small number of cells, and at 48 h it was absent. At the initial stages of culture, at 0 h and 4 h, the signal, if present, was detected in intracellular compartments (Fig. 5M, N). At 12 h, 24 h, and 72 h, the epitope was present in areas corresponding to those where the calcofluor signal was observed (Fig. 5 compare O and O', P and P', R and R'). In addition, a punctate signal was observed around the cells at all stages (Fig. 5, e.g. O and P).

In summary, the extensin epitope recognised by the JIM20 antibody showed variable occurrence at different time points in all analysed variants (Fig. 5; Table S4). It can be noted that for all cell types at 12 h, the epitope was detected in most cells. At the remaining time points, differences in the number of cells with a positive JIM20 signal were observed between the hybrid and the two *Fagopyrum* species.

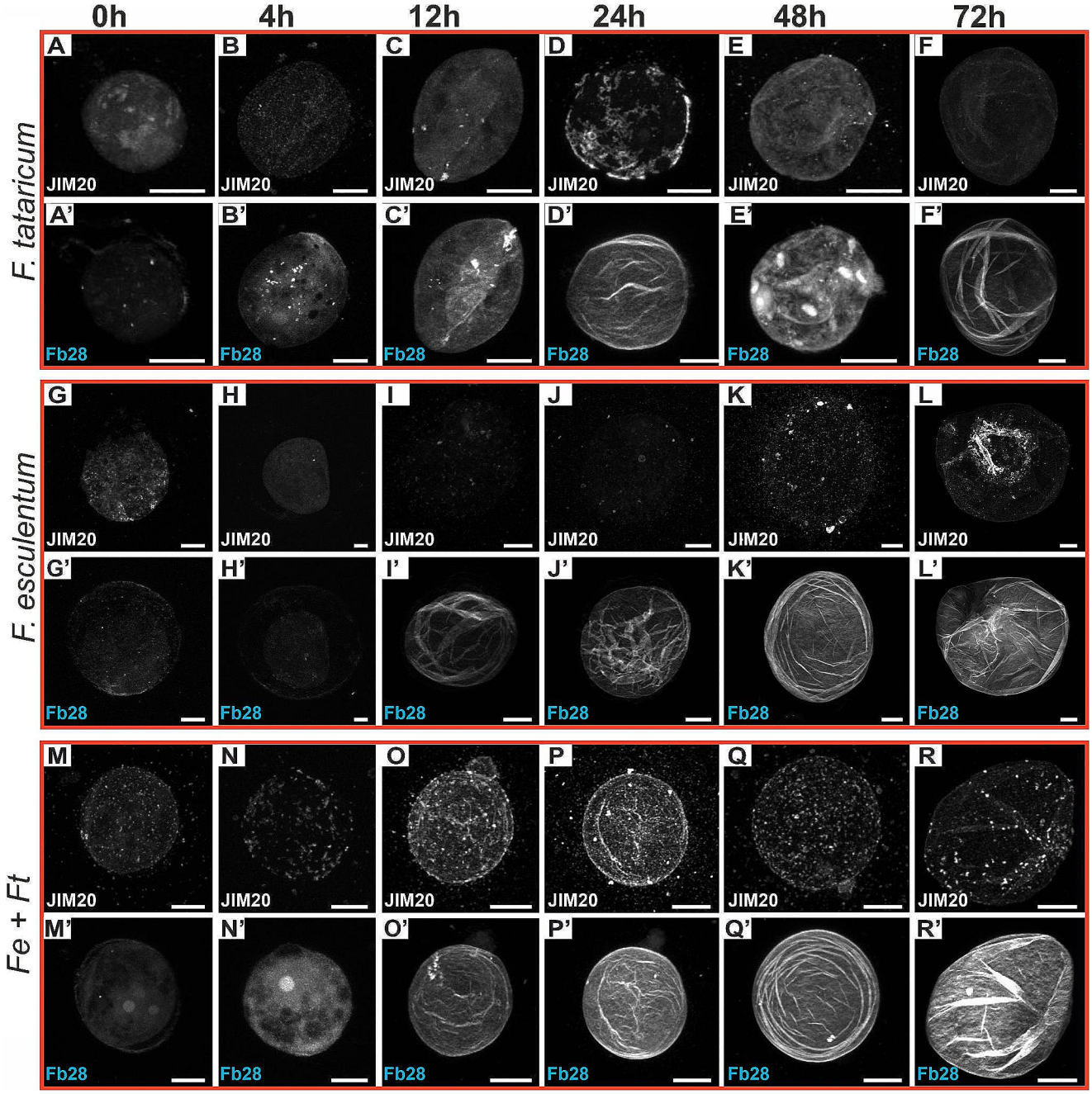

Fig. 5 JIM20 extensin epitope occurrence during wall reconstruction in *F. tataricum* **(A-F)**, *F. esculentum* **(G-L)**, and *Ft* + *Fe* hybrid **(M-R)** with corresponding signal from calcofluor **(A'-R')**

Xyloglucan

At 0 h, the epitope recognised by the LM25 antibody was absent from a minor part of the protoplasts in most of the protoplasts (Fig. 6A– F, G– L, M– R; Supplementary videos S19– S24); however, the fluorescence signal was weak or of moderate intensity (Fig. 6A, G, M), exhibiting a mesh-like pattern. In the next time point, LM25 was detected predominantly in intracellular compartments (Fig. 6B, H, N). From the 12 h time point, the wall was rebuilt by protoplasts (Fig. 6C'– F', I'– L', O'– R') until the 72 h cell wall was restored. The LM25 epitope was present abundantly in all the protoplasts, regardless of genotype, during these time points. The fluorescence signal was observed in the wall matrix as continuous strands that corresponded with the course of cellulose microfibrils (Fig. 6C– F, I– L, O– R).

In summary, at 0 and 4 h, there were differences in the number of protoplasts with detected fluorescence signal

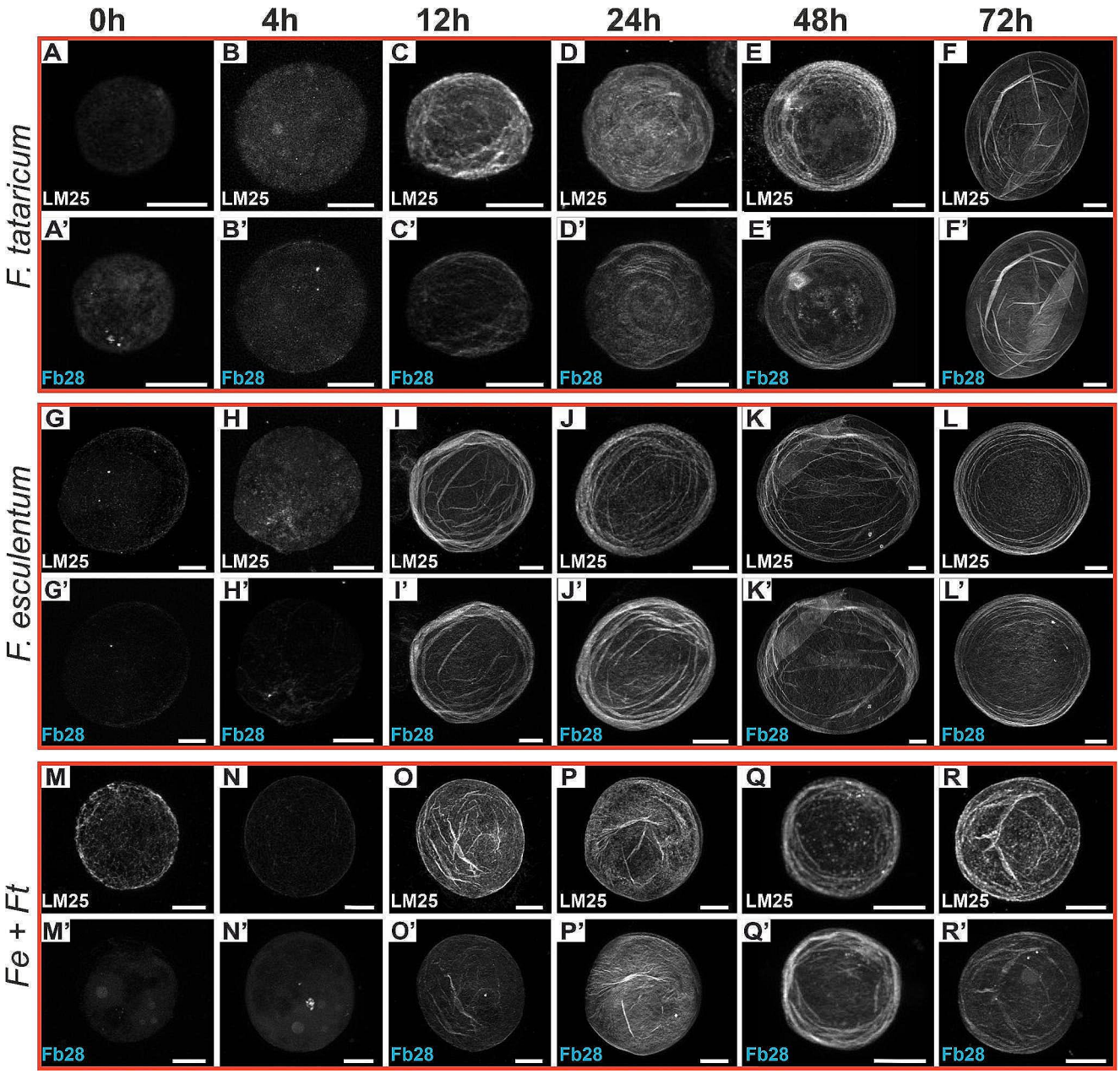

Fig. 6 LM25 xyloglucan epitope distribution during regeneration of cell wall in *F. tataricum* **(A-F)**, *F. esculentum* **(G-L)**, and *Ft* + *Fe* hybrid **(M-R)** with the corresponding signal from calcofluor **(A'-R')**

(Fig. 6; Table S4), with *F. esculentum* in average rate at 0 h and *F. esculentum* and hybrid protoplasts in average rate at 4 h. The LM25 epitope was abundantly observed in most cells during the later stages of wall regeneration regardless of the genotype (Fig. 6; Table S4). As mentioned above, no differences in the distribution pattern within the newly arisen wall matrix were observed.

Pectins

Methyl-esterified homogalacturonan (pectins)

The epitope recognised by the LM20 antibody (Fig. 7A–F, G– L, M– R; Supplementary videos S25– S30) at 0 h occurred predominantly in hybrid protoplasts compared to *F. tataricum* and *F. esculentum* protoplasts (Fig. 7A, G, M). At 0 h, the punctate fluorescence signal was detected within cytoplasmic compartments, and in the further time points

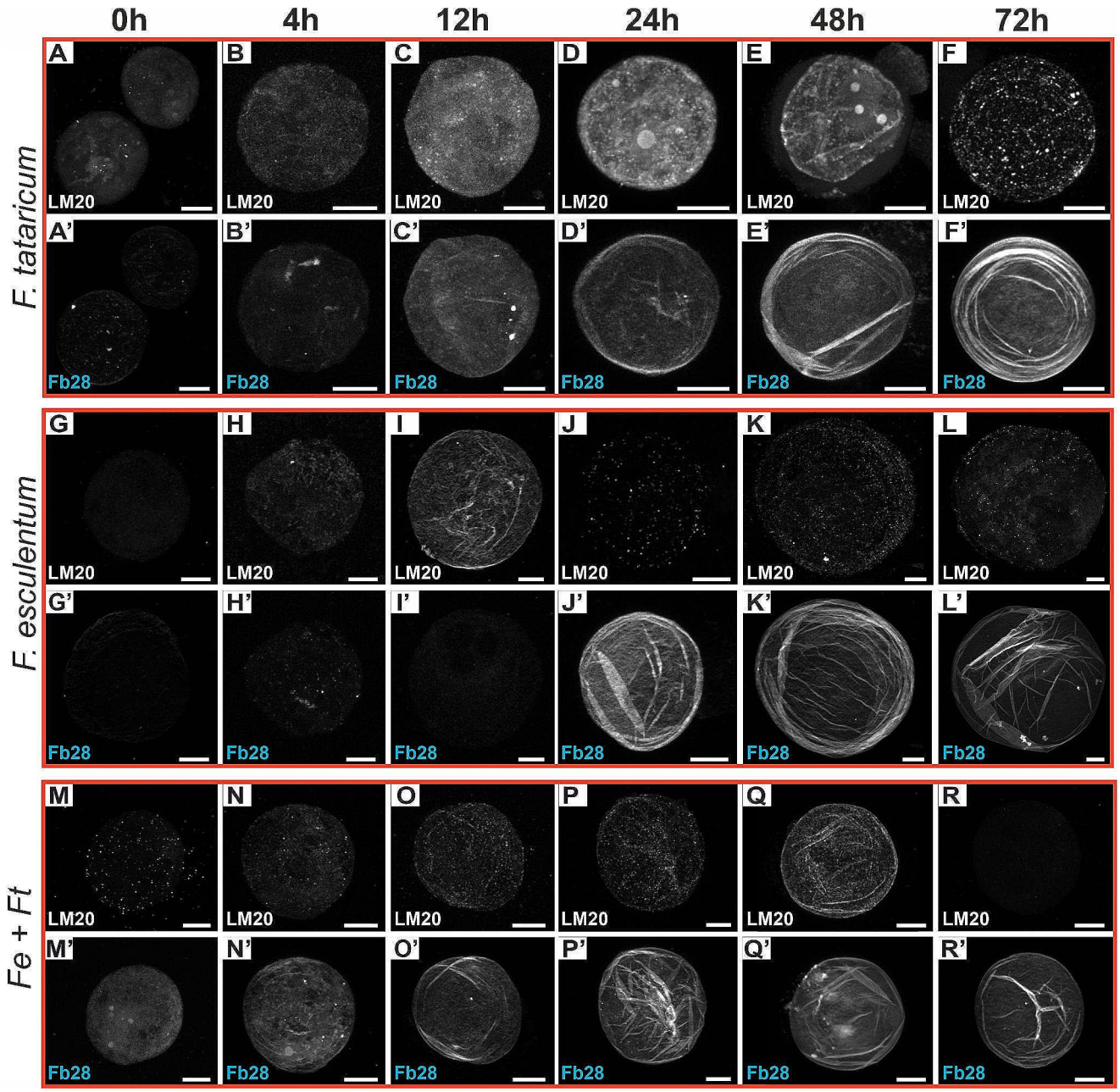

Fig. 7 The presence of LM20 pectic epitope during cell wall rebuilding in *F. tataricum* **(A-F)**, *F. esculentum* **(G-L)**, and *Ft* + *Fe* hybrid **(M-R)** with the corresponding signal from calcofluor **(A'-R')**

(4 h, 12 h), this occurrence became more progressed, if present (Fig. 7 compare A– C, G– I, M– O).

In *F. tataricum* protoplasts, the LM20 epitope was observed abundantly in intracellular compartments in the following 12, 24 and 48 h time points (Fig. 7C, D and E). In 72 h, epitope occurred in the wall matrix and was detected in a punctate manner (Fig. 7F; Supplementary video S26). In *F. esculentum* protoplasts, from 24 h and further, a decrease in LM20 epitope occurrence could be seen; namely, epitope was detected but only as a punctate signal within the matrix (Fig. 7J– L).

In hybrid protoplasts at 24 and 48 h, LM20 epitope was detected in the wall matrix; however, at 72 h, only a low number of protoplasts displayed fluorescence signal, with most of the cells that were devoid of LM20 epitope (Fig. 7R; Supplementary video S30). In some of the analysed protoplasts, the fluorescence signal correlated with cellulose microfibril arrangement (Fig. 7 compare F and F', K and K', O and O', P and P', Q and Q'). The LM20 epitope

mainly occurred in a low or average number of cells (Table S4). At 0 and 4 h, hybrid protoplasts exhibited fluorescence signals more frequently than *F. tataricum* or *F. esculentum* protoplasts. From 12 h, the LM20 epitope was detected in *F. tataricum* more often than in *F. esculentum* or hybrid protoplasts. However, unlike other analysed epitopes, the LM20 epitope was not detected abundantly within the wall matrix.

Galactan (RGI, pectins)

The epitope recognised by the LM5 antibody (Fig. 8A– F, G– L, M– R; Supplementary videos S31– S36) was detected in *F. tataricum* protoplasts in intracellular compartments at 0–12 h (Fig. 8A– C), intracellular compartments/wall matrix at 24 h (Fig. 8D) and in wall matrix in the following 48 and 72 h (Fig. 8E, F; Supplementary video S32). A similar pattern of occurrence was observed in *F. esculentum* and hybrid protoplasts; however, the wall-bound epitope

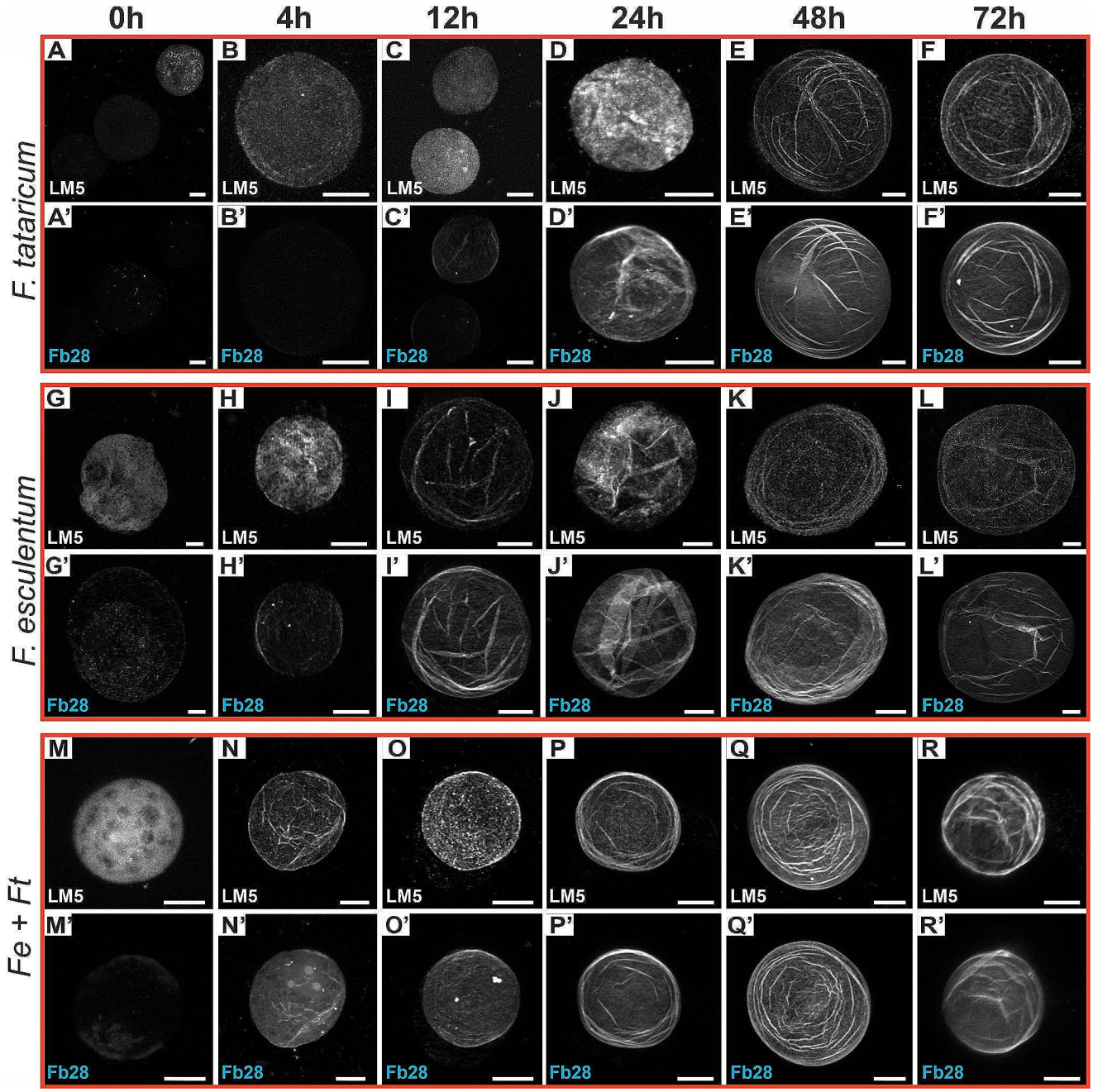

Fig. 8 LM5 pectic epitope distribution during wall reconstruction in *F. tataricum* **(A-F)**, *F. esculentum* **(G-L)**, and *Ft* + *Fe* hybrid **(M-R)** with corresponding signal from calcofluor **(A'-R')**

appeared earlier, from 12 h time point for *F. esculentum* (Fig. 8I– L) and even 4 h for heterokaryons (Fig. 8N– R). The alignment of cellulose microfibrils corresponded with the fluorescence signal, which was depicted in LM5 epitope distribution in different time points regardless of genotype (Fig. 8 compare E and E', F and F', I and I', K and K', L and L', P and P', Q and Q', R and R').

In summary, the fluorescence signal was detected in significant part of the protoplasts (Table S4) with some differences at 0 h (*F. tataricum* and *F. esculentum* belonging to the average range, in hybrid protoplasts– expressed in more prominent number of cells), 24 h (average range for *F. tataricum* in comparison to *F. esculentum* and hybrid) and 48 h (average range for *F. esculentum* in comparison to *F. tataricum* and hybrid).

Arabinan (RGI, pectins)

The epitope recognised by the LM6 antibody (Fig. 9A– F, G– L, M– R; Supplementary videos S37– S42) was detected abundantly in intracellular compartments of *F. tataricum* protoplasts up to 24 h time point (Fig. 9A– D). At 48 and 72 h, the fluorescence signal was localised within the wall matrix (Fig. 9E, E', F, F') and corresponded with the course of some cellulose microfibrils (Fig. 9F, F').

In *F. esculentum* and hybrid protoplasts, the LM6 epitope in the wall matrix was exhibited earlier. Beginning from the 4 h time point, the LM6 epitope appeared at the protoplast surface even before apparent cellulose incorporation (Fig. 9H and H'), and during further stages, the fluorescence signal corresponded with cellulose microfibril deposition (Fig. 9I– L).

In hybrid protoplasts, the cytoplasmic occurrence was noticed only at 0 h, with a significant decrease in the 4 h time point (Fig. 9; Table S4). From the subsequent time points, the occurrence of LM6 was observed in the wall matrix, where the fluorescence signal corresponded with the deposited cellulose component (Fig. 9O– R).

In summary, the occurrence of LM6 epitope was widespread in protoplasts that regenerated cell walls, except 4 h time point where significant differences were noticed. Namely, only an average number of *F. tataricum* contained this compound, most of *F. esculentum* and a few hybrid protoplasts (Table S4). Also, at 24 h, in *F. tataricum*, a fluorescence signal was detected in an average number of protoplasts compared to the predominant distribution in *F. esculentum* and hybrid protoplasts.

Discussion

Isolated protoplasts can regenerate new walls developed enough in structure and composition to permit cell division and differentiation into specific cellular types, tissues, and even whole plants. The protocols for successful protoplast isolation and plant regeneration from protoplast-derived cells have been established for *F. tataricum* and *F. esculentum* (Zaranek et al. 2023a, b).

Structural proteins

The involvement of different AGP epitopes in wall formation during protoplast culture is well documented. In sugar beet, JIM8, JIM13 and LM2 epitopes were detected abundantly in incipient of cell walls and labelling increased as the rebuilding of the walls progressed (Butowt et al. 1999; Majewska-Sawka and Münster 2003). Similar observations with JIM8 and JIM13 epitopes were reported for *Daucus* protoplast cultures (Godel-Jędrychowska et al. 2019; Mock et al. 1990). Another study that involved guard cell- and mesophyll-derived protoplasts from sugar beet and tobacco implied some species-specific differences (Wiśniewska and Majewska-Sawka 2008). Namely, LM2, MAC207, and JIM13 were detected abundantly in sugar beet protoplasts in contrast to JIM4, JIM8, and JIM15 epitopes, while in tobacco, only JIM13 epitope from all epitopes mentioned was present in abundance (Wiśniewska and Majewska-Sawka 2008). Our results are consistent with these findings– both JIM13 and JIM16 epitopes are present during all stages of wall rebuilding in *Fagopyrum* protoplasts, regardless of protoplast source. AGPs have the properties of binding to β-glycans; thus, these plasma-membrane-associated glycoproteins may act as cell surface attachment sites for cell wall matrix polysaccharides (Pennell et al. 1989). Our findings are also in accordance with the reported extracellular localization of AGPs around the regenerating protoplast (Mock et al. 1990), as we also observed fluorescence signals at the surface and around protoplasts. *Fagopyrum* protoplasts are another system where the significant involvement of AGPs in wall formation is postulated (Cassab 1998; Sadava and Chrispeels 1973; Ye and Varner 1991). Unlike AGPs, which are easy to extract from the wall and are considered "mobile", extensins are glycoproteins incredibly resistant to extraction. After being secreted into the wall, they are immediately immobilised by covalent binding with other extensin molecules (Cooper and Varner 1984; Lamport 1986) or wall polymers, presumably pectins (RG-I, Qi et al. 1995). Recent studies, however, indicate that extensins may also be correlated with the increase in cell size (Moore et al. 2014) or the initiation of growth (Cannon et al. 2008).

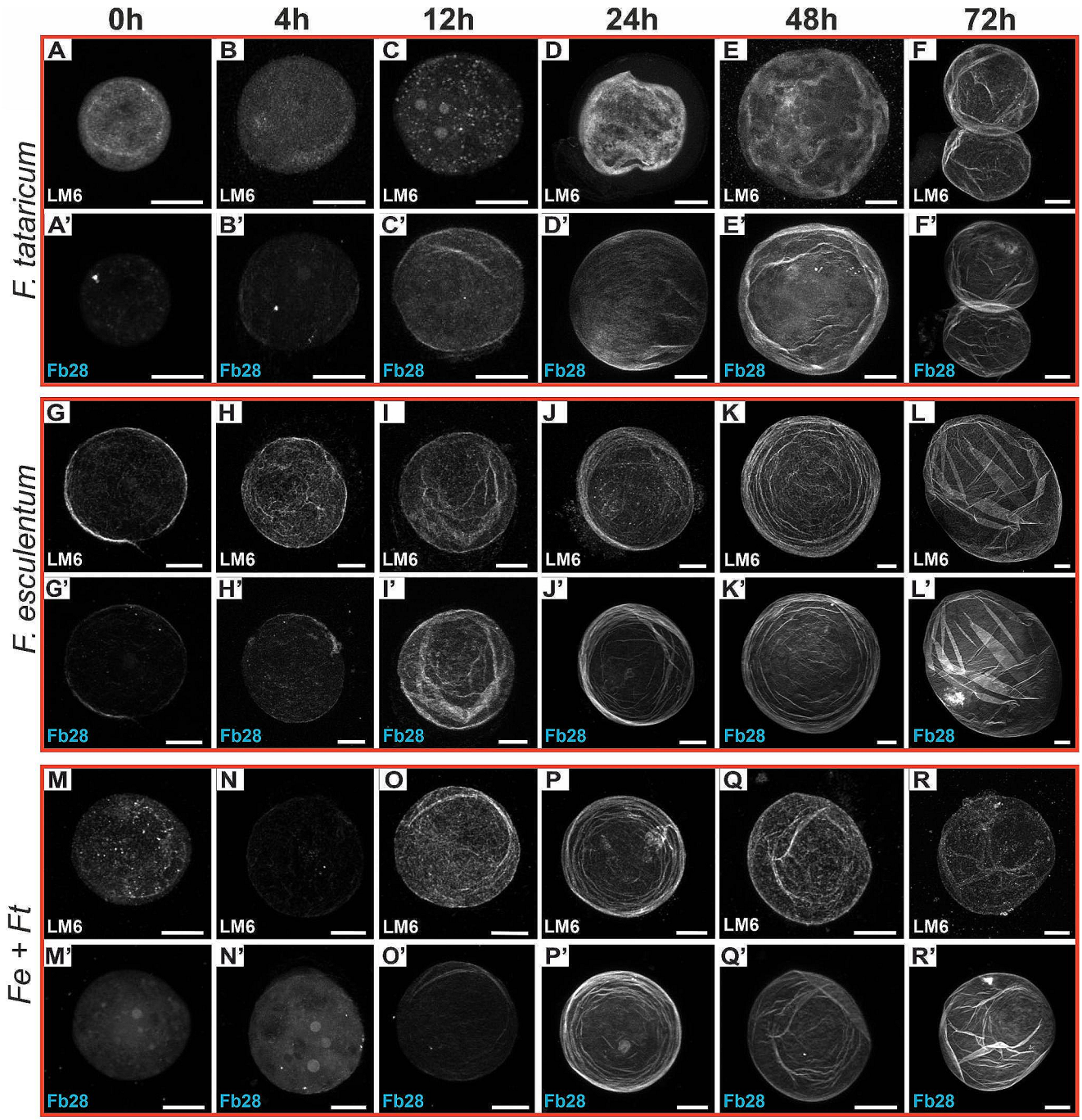

Fig. 9 The occurrence of LM6 pectic epitope during cell wall reconstruction in *F. tataricum* **(A-F)**, *F. esculentum* **(G-L)**, and *Ft* + *Fe* hybrid **(M-R)** with the corresponding signal from calcofluor **(A'-R')**

It was shown that in *Nicotiana sylvestris* protoplasts, the genes encoding the extensins are successively transcribed in a few hours after the isolation procedure (Parmentier et al. 1995). In carrot's protoplast-derived cells, a JIM12 extensin epitope was detected only between 10 and 20 days of culture in the already differentiated cells (Godel-Jędrychowska et al. 2019). Our study shows the occurrence of another extensin epitope, JIM20, in intracellular compartments at the early stages (0 h, 4 h) of wall regeneration and the wall-bound localization during the further time points (12–72 h). Despite differences in the number of protoplasts exhibiting the fluorescence signal, a 12 h time frame is common for all genotypes and points to the most abundant JIM20 occurrence. Thus, it can be stated that JIM20 is involved in the wall regeneration process in *Fagopyrum* species.

Wall polysaccharides

Xyloglucan is the dominant hemicellulose of the primary cell walls of dicotyledonous plants (Brett and Waldron 1996). The network created between cellulose microfibrils and xyloglucan stabilises the spatial structure of the entire cell wall (Park and Cosgrove 2012; Peña et al. 2004; Ryden et al. 2003). However, it was suggested that the formation of a cellulose network during wall rebuilding is independent of xyloglucan as this compound was not essential during the early stages of cellulose assembly and, during the early stages of pea protoplast cultures, it was shown that the binding between cellulose and xyloglucan was not as strong as in the intact pea cell walls (Hayashi et al. 1986). Nevertheless, our results indicate the co-localization of xyloglucan LM25 epitope and cellulose microfibrils. In *Fagopyrum* protoplasts, xyloglucan synthesis and incorporation into the wall matrix accompany the cellulose network formation. Methyl-esterified homogalacturonan (HG) recognized by LM20 was present in intracellular compartments, which indicates the ongoing synthesis. In *F. tataricum* this cytoplasmic distribution was detected even up to 48 h, in contrast to *F. esculentum* and hybrid protoplasts, in which the wall-bound occurrence was observed earlier. However, our results showed a relatively moderate distribution within the wall matrix in the examined time frames. Other results indicate that the amount of esterified HG increases with time during the wall regeneration. For example, in carrot protoplast culture, methyl-esterified pectins were abundantly present after 4 days of culture, and the amount increased along with the culture age (Godel-Jędrychowska et al. 2019). Similarly, in flax hypocotyl-derived protoplasts at 6th day of culture, the amount of methyl-esterified pectins increased compared to analysis from 3rd day (David et al. 1995). Fresh protoplasts of sugar beet showed trace content of methyl-esterified pectin, in contrast to mesophyll protoplast-derived cells (Majewska-Sawka and Münster 2003; Wiśniewska and Majewska-Sawka 2008).

The side chains of RG-I, arabinan and galactan influence the mechanical properties and porosity of walls (Verhertbruggen et al. 2009; Willats et al. 2001) as well as cell adhesion (Iwai et al. 2001; Leboeuf et al. 2004; Orfila et al. 2001; Peña and Carpita 2004). Arabinan and galactan often show different distributions within cells or tissues (Bush et al. 2001; Orfila and Knox 2000) and may have different functions depending on the plant species or cell type (McCartney et al. 2000; Orfila and Knox 2000; Willats et al. 1999). (McCartney et al. 2003; Willats et al. 1999) The occurrence of the LM5 epitope in the walls is correlated with their strengthening (McCartney and Knox 2002; McCartney et al. 2000), in contrast to arabinan (LM6 epitope), the presence of which ensures the elasticity of cell walls (Jones et al. 2003, 2005; Moore et al. 2008, 2013).

Surprisingly, both epitopes were present during wall regeneration in *Fagopyrum* protoplasts. However, it is noteworthy that in hybrid protoplasts, the LM6 occurrence was significantly reduced at a 4 h time point compared to *F. tataricum* and *F. esculentum.* Similarly to the HG, LM5 and LM6 were detected in intracellular compartments and subsequently within the wall, which suggests increased synthesis followed by their incorporation into the wall matrix. So far, LM5 and LM6 have been detected in guard and mesophyll cell protoplasts of tobacco, in contrast to sugar beet protoplasts, in which LM5 was detected in trace amounts, and LM6 occurred only in protoplast-derived callus (Majewska-Sawka and Münster 2003; Wiśniewska and Majewska-Sawka 2008). Thus, the occurrence of galactan and arabinan during wall regeneration seems to be species-specific. Since regenerating walls have to be competent to contain the expansion of the protoplast, withstand the osmotic pressure and, consequently, allow for cell division (Burgess and Linstead 1976), they have to be elastic and strengthened. It could explain the concurrent presence of both LM5 and LM6 epitopes during wall re-establishment in *Fagopyrum* protoplasts.

Final remarks

We want to highlight that the regenerated walls of protoplasts should not be considered equivalent to the walls of cells from which protoplasts were isolated. The sugar composition of cell walls from protoplasts and walls of their corresponding parent tissue differs, and the type of regeneration media (solid or liquid) affects polysaccharide content (David et al. 1995; Takeuchi and Komamine 1978, 1982). Protoplast walls could be compared, in terms of composition, to a very young cell wall formed during cytokinesis (Franz and Blaschek 1985). Indeed, xyloglucan, pectins, AGPs, and extensins participate in cell plate formation (Shibaya and Sugawara 2009; Sinclair et al. 2022). Additionally, during the early stages of wall formation, wall compounds disintegrate from the surface of the cells and are lost into the medium (Franz and Blaschek 1985) which explains the extracellular occurrence of some analysed epitopes in the current study.

Conclusion

In the presented study, we focused on the process of wall rebuilding for *Fagopyrum* spp., a new yet subsequent system introduced in this basic research area. Surprisingly, the early stages of wall formation in hybrid protoplasts were

not impaired compared to those observed in parental protoplasts of *F. tataricum* or *F. esculentum*. These results can contribute to further research on somatic hybrids, as understanding the development patterns may impact the successful modulation of yield productivity and yield stability for future buckwheat breeding programmes.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11240-024-02740-6.

Author contributions Conceptualization: A.B., K.S-C., A.M-H., R.P.P.; Data curation: A.B., K.S-C., A.M-H, R.P.P.; Formal analysis: K.S-C., A.M-H, R.P.P.; Funding acquisition: A.B.; Investigation: K.S-C., A.M-H, R.P.P; Methodology: A.B, E.G., K.S-C., A.M-H, R.P.P; Project administration: A.B.; Supervision: A.B., K.S-C., A.M-H.; Visualization: K.S-C., A.M-H, R.P.P; Roles/Writing - original draft: K.S-C., A.M-H, R.P.P; and Writing - review & editing: A.B., E.G., K.S-C., A.M-H, R.P.P.

Funding This research was funded by the National Science Centre, Poland. Research project Sonata Bis (2020/38/E/NZ9/00033 awarded to AB) and the Research Excellence Initiative of the University of Silesia in Katowice.

Data availability Data supporting this study are included within the article and/or supporting materials.

Declarations

Ethics approval and consent to participate The use of all plant materials in this study complies with relevant institutional, national, and international guidelines and legislation. Seeds of *F. tataricum* (sample k-17) are from the N. I. Vavilov Institute of Plant Genetic Resources collections, Saint Petersburg, Russia. The Plant Cytogenetic and Molecular Biology Group Institute of Biology, Biotechnology and Environmental Protection, Faculty of Natural Sciences, University of Silesia in Katowice, Poland multiplied the obtained seeds. *F. tataricum* sample k-17 is a common cultivated landrace of *F. tataricum*, and seeds are available upon request from the publication's authors. *F. esculentum* cultivar Panda seeds are commercially available and purchased from the Malopolska Plant Breeding Company (Poland).

Consent for publication Not applicable.

Conflict of interest All the authors declare that there is no conflict of interest. Ewa Grzebelus is one of the journal's associate editors. As such, she was fully excluded during all the evaluation period of this work, had no access to its handling during peer-refereeing, and her status had no bearing on the editorial consideration of the manuscript.

References

Adachi T, Yamaguchi A, Miike Y, Hoffmann F (1989) Plant regeneration from protoplasts of common buckwheat (*Fagopyrum esculentum*). Plant Cell Rep 8(4):247–250. https://doi.org/10.1007/BF00778544

Brett CT, Waldron KW, Brett CT, Waldron KW (1996) 2nd edn. edn. Chapman & Hall, London

Betekhtin A, Rojek M, Jaskowiak J, Milewska-Hendel A, Kwasniewska J, Kostyukova Y, Kurczynska E, Rumyantseva N, Hasterok R (2017) Nuclear genome stability in long-term cultivated callus lines of *Fagopyrum tataricum* (L.) Gaertn. PLOS ONE 12 (3):e0173537.

Burgess J, Linstead PJ (1976) Scanning electron microscopy of cell wall formation around isolated plant protoplasts. Planta 131(2):173–178. https://doi.org/10.1007/BF00389991

Bush MS, Marry M, Huxham MI, Jarvis MC, McCann MC (2001) Developmental regulation of pectic epitopes during potato tuberisation. Planta 213(6):869–880. https://doi.org/10.1007/s004250100570

Butowt R, Niklas A, Rodriguez-Garcia MI, Majewska-Sawka A (1999) Involvement of JIM13- and JIM8-responsive carbohydrate epitopes in early stages of cell wall formation. J Plant Res 112(1):107–116. https://doi.org/10.1007/PL00013851

Cannon MC, Terneus K, Hall Q, Tan L, Wang Y, Wegenhart BL, Chen L, Lamport DTA, Chen Y, Kieliszewski MJ (2008) Self-assembly of the plant cell wall requires an extensin scaffold. Proc. Natl. Acad. Sci. U.S.A. 105(6):2226–2231 https://doi.org/10.1073/pnas.0711980105

Cassab GI (1998) Plant cell wall proteins. Annu Rev Plant Physiol Plant Mol Biol 49(1):281–309. https://doi.org/10.1146/annurev.arplant.49.1.281

Cawoy V, Ledent J-F, Kinet J-M, Jacquemart A-L (2009) Floral biology of common buckwheat (*Fagopyrum esculentum* Moench). Eur J Plant Sci Biotechnol 3(1):1–9

Cooper JB, Varner JE (1984) Cross-linking of soluble extensin in isolated cell walls. Plant Physiol 76(2):414–417. https://doi.org/10.1104/pp.76.2.414

David H, David PB, Savy A, Demazy C C, Van Cutsem P (1995) Pectins in walls of protoplast-derived cells imbedded in agarose and alginate beads. Protoplasma 186:122–130. https://doi.org/10.1007/BF01281322

Fabjan N, Rode J, Košir IJ, Wang Z, Zhang Z, Kreft I (2003) Tartary buckwheat (*Fagopyrum tataricum* Gaertn.) As a source of dietary rutin and quercitrin. J Agric Food Chem 51(22):6452–6455. https://doi.org/10.1021/jf034543e

Franz G & Blaschek W Wall regeneration in protoplasts of higher plants. In, Berlin, Heidelberg, 1985. The physiological properties of Plant Protoplasts. Springer Berlin Heidelberg, p 171–183

Godel-Jędrychowska K, Maćkowska K, Kurczyńska E, Grzebelus E (2019) Composition of the reconstituted cell wall in protoplast-derived cells of daucus is affected by phytosulfokine (PSK). Int J Mol Sci 20(21). https://doi.org/10.3390/ijms20215490

Hayashi T, Polonenko DR, Camirand A, Maclachlan G (1986) Pea xyloglucan and cellulose: IV. Assembly of Beta-glucans by pea protoplasts. Plant Physiol 82(1):301–306. https://doi.org/10.1104/pp.82.1.301

Huda MN, Lu S, Jahan T, Ding M, Jha R, Zhang K, Zhang W, Georgiev MI, Park SU, Zhou M (2021) Treasure from garden: bioactive compounds of buckwheat. Food Chem 335:127653. https://doi.org/10.1016/j.foodchem.2020.127653

Iwai H, Ishii T, Satoh S (2001) Absence of arabinan in the side chains of the pectic polysaccharides strongly associated with cell walls of *Nicotiana plumbaginifolia* non-organogenic callus with loosely attached constituent cells. Planta 213(6):907–915. https://doi.org/10.1007/s004250100559

Jones L, Seymour GB, Knox JP (1997) Localization of pectic galactan in tomato cell walls using a monoclonal antibody specific to (1->4)-beta-D-galactan. Plant Physiol 113(4):1405–1412. https://doi.org/10.1104/pp.113.4.1405

Jones L, Milne JL, Ashford D, McQueen-Mason SJ (2003) Cell wall arabinan is essential for guard cell function. Proc. Natl. Acad. Sci. U.S.A. 100(20):11783–11788 https://doi.org/10.1073/pnas.1832434100

Jones L, Milne JL, Ashford D, McCann MC, McQueen-Mason SJ (2005) A conserved functional role of pectic polymers in stomatal guard cells from a range of plant species. Planta 221(2):255–264. https://doi.org/10.1007/s00425-004-1432-1

Knox JP (1995) Developmentally regulated proteoglycans and glycoproteins of the plant cell surface. FASEB J 9(11):1004–1012. https://doi.org/10.1096/fasebj.9.11.7544308

Knox JP, Linstead PJ, Cooper JPC, Roberts K (1991) Developmentally regulated epitopes of cell surface arabinogalactan proteins and their relation to root tissue pattern formation. Plant J 1(3):317–326. https://doi.org/10.1046/j.1365-313X.1991.t01-9-00999.x

Kuki H, Yokoyama R, Kuroha T, Nishitani K (2020) Xyloglucan is not essential for the formation and integrity of the cellulose network in the primary cell wall regenerated from *Arabidopsis* protoplasts. Plants (Basel) 9(5):629. https://doi.org/10.3390/plants9050629

Lachmann S, Adachi T (1990) Callus regeneration from hypocotyl protoplasts of tartary buckwheat (*Fagopyrum tataricum* Gaertn). Fagopyrum 10:62–64

Lachmann S, Kishima Y, Adachi T (1994) Protoplast fusion in buckwheat: preliminary results on somatic hybridisation. Fagopyrum 14:7–12

Lamport D (1986) The primary cell wall: a new model. Structure, Modification and Hydrolysis, John Wiley & Sons Inc, New York, Cellulose

Leboeuf E, Thoiron S, Lahaye M (2004) Physico-chemical characteristics of cell walls from *Arabidopsis thaliana* Microcalli showing different adhesion strengths. J Exp Bot 55(405):2087–2097. https://doi.org/10.1093/jxb/erh225

Li J, Zhang K, Meng Y, Li Q, Ding M, Zhou M (2019) FtMYB16 interacts with Ftimportin-α1 to regulate rutin biosynthesis in tartary buckwheat. Plant Biotechnol J 17(8):1479–1481. https://doi.org/10.1111/pbi.13121

Mackowska K, Stelmach-Wityk K, Grzebelus E (2023) Early selection of carrot somatic hybrids: a promising tool for species with high regenerative ability. Plant Methods 19(1):104. https://doi.org/10.1186/s13007-023-01080-4

Majewska-Sawka A, Münster A (2003) Cell-wall antigens in mesophyll cells and mesophyll-derived protoplasts of sugar beet: possible implication in protoplast recalcitrance? Plant Cell Rep 21(10):946–954. https://doi.org/10.1007/s00299-003-0612-y

McCartney L, Knox JP (2002) Regulation of pectic polysaccharide domains in relation to cell development and cell properties in the pea testa. J Exp Bot 53(369):707–713. https://doi.org/10.1093/jexbot/53.369.707

McCartney L, Ormerod AP, Gidley MJ, Knox JP (2000) Temporal and spatial regulation of pectic (1–4)-β-D-galactan in cell walls of developing pea cotyledons: implications for mechanical properties. Plant J 22(2):105–113. https://doi.org/10.1046/j.1365-313x.2000.00719.x

McCartney L, Steele-King CG, Jordan E, Knox JP (2003) Cell wall pectic (1→4)-β- D-galactan marks the acceleration of cell elongation in the *Arabidopsis* seedling root meristem. Plant J 33(3):447–454. https://doi.org/10.1046/j.1365-313X.2003.01640.x

Mock HP, Emmerling M, Seitz HU (1990) Cell wall synthesis in carrot cells: comparison of suspension-cultured cells and regenerating protoplasts. Physiol Plant 79(2):347–353. https://doi.org/10.1111/j.1399-3054.1990.tb06752.x

Moore JP, Farrant JM, Driouich A (2008) A role for pectin-associated arabinans in maintaining the flexibility of the plant cell wall during water deficit stress. Plant Signal Behav 3(2):102–104. https://doi.org/10.4161/psb.3.2.4959

Moore JP, Nguema-Ona EE, Vicré-Gibouin M, Sørensen I, Willats WGT, Driouich A, Farrant JM (2013) Arabinose-rich polymers as an evolutionary strategy to plasticize resurrection plant cell walls against desiccation. Planta 237(3):739–754. https://doi.org/10.1007/s00425-012-1785-9

Moore JP, Fangel JU, Willats WGT, Vivier MA (2014) Pectic-β(1,4)-galactan, extensin and arabinogalactan–protein epitopes differentiate ripening stages in wine and table grape cell walls. Ann Botany 114(6):1279–1294. https://doi.org/10.1093/aob/mcu053

Nešković M, Vujičić R, Budimir S (1987) Somatic embryogenesis and bud formation from immature embryos of buckwheat (*Fagopyrum esculentum* Moench). Plant Cell Rep 6(6):423–426. https://doi.org/10.1007/BF00272773

Orfila C, Knox JP (2000) Spatial regulation of pectic polysaccharides in relation to pit fields in cell walls of tomato fruit pericarp. Plant Physiol 122(3):775–782. https://doi.org/10.1104/pp.122.3.775

Orfila C, Seymour GB, Willats WG, Huxham IM, Jarvis MC, Dover CJ, Thompson AJ, Knox JP (2001) Altered middle lamella homogalacturonan and disrupted deposition of (1–5)-alpha-L-arabinan in the pericarp of Cnr, a ripening mutant of tomato. Plant Physiol 126(1):210–221. https://doi.org/10.1104/pp.126.1.210

Park YB, Cosgrove DJ (2012) Changes in cell wall biomechanical properties in the xyloglucan-deficient *xxt1*/*xxt2* mutant of *Arabidopsis*. Plant Physiol 158(1):465–475. https://doi.org/10.1104/pp.111.189779

Parmentier Y, Fleck J, Jamet E (1995) A novel wound-inducible extensin gene is expressed early in newly isolated protoplasts of *Nieotiana Sylvestris*. Plant Mol Biol 29(2):279–292. https://doi.org/10.1007/BF00043652

Pedersen HL, Fangel JU, McCleary B, Ruzanski C, Rydahl MG, Ralet MC, Farkas V, von Schantz L, Marcus SE, Andersen MC, Field R, Ohlin M, Knox JP, Clausen MH, Willats WG (2012) Versatile high resolution oligosaccharide microarrays for plant glycobiology and cell wall research. J Biol Chem 287(47):39429–39438. https://doi.org/10.1074/jbc.M112.396598

Peña MJ, Carpita NC (2004) Loss of highly branched arabinans and debranching of Rhamnogalacturonan I accompany loss of firm texture and cell separation during prolonged storage of apple. Plant Physiol 135(3):1305–1313. https://doi.org/10.1104/pp.104.043679

Peña MJ, Ryden P, Madson M, Smith AC, Carpita NC (2004) The galactose residues of xyloglucan are essential to maintain mechanical strength of the primary cell walls in *Arabidopsis* during growth. Plant Physiol 134(1):443–451. https://doi.org/10.1104/pp.103.027508

Pennell RI, Knox JP, Scofield GN, Selvendran RR, Roberts K (1989) A family of abundant plasma membrane-associated glycoproteins related to the arabinogalactan proteins is unique to flowering plants. J Cell Biol 108(5):1967–1977. https://doi.org/10.1083/jcb.108.5.1967

Potocka I, Godel K, Dobrowolska I, Kurczynska EU (2018) Spatio-temporal localization of selected pectic and arabinogalactan protein epitopes and the ultrastructural characteristics of explant cells that accompany the changes in the cell fate during somatic embryogenesis in Arabidopsis thaliana. Plant Physiol Biochem 127:573–589. https://doi.org/10.1016/j.plaphy.2018.04.032

Qi X, Behrens BX, West PR, Mort AJ (1995) Solubilization and partial characterization of extensin fragments from cell walls of cotton

suspension cultures (evidence for a covalent cross-link between extensin and pectin). Plant Physiol 108(4):1691–1701. https://doi.org/10.1104/pp.108.4.1691

Rems L, Usaj M, Kanduser M, Rebersek M, Miklavcic D, Pucihar G (2013) Cell electrofusion using nanosecond electric pulses. Sci Rep 3:3382. https://doi.org/10.1038/srep03382

Ryden P, Sugimoto-Shirasu K, Smith AC, Findlay K, Reiter W-D, McCann MC (2003) Tensile properties of *Arabidopsis* cell walls depend on both a xyloglucan cross-linked microfibrillar network and Rhamnogalacturonan II-borate complexes. Plant Physiol 132(2):1033–1040. https://doi.org/10.1104/pp.103.021873

Sadava D, Chrispeels MJ (1973) Hydroxyproline-rich cell wall protein (extensin): role in the cessation of elongation in excised pea epicotyls. Develop Biol 30(1):49–55. https://doi.org/10.1016/0012-1606(73)90047-X

Sedlak P, Sedlakova V, Vasek J, Zeka D, Cilova D, Melounova M, Orsak M, Domkarova J, Dolezal P, Vejl P (2022) Phenotypic, molecular and biochemical evaluation of somatic hybrids between *Solanum tuberosum* and *S. bulbocastanum*. Sci Rep 12(1):4484 https://doi.org/10.1038/s41598-022-08424-5

Senda M, Takeda J, S A, Nakamura T (1979) Induction of cell fusion of plant protoplasts by electrical stimulation. Plant Cell Physiol 20:1441–1443

Shibaya T, Sugawara Y (2009) Induction of multinucleation by β-glucosyl Yariv reagent in regenerated cells from *Marchantia polymorpha* protoplasts and involvement of arabinogalactan proteins in cell plate formation. Planta 230(3):581–588. https://doi.org/10.1007/s00425-009-0954-y

Showalter AM (1993) Structure and function of plant cell wall proteins. Plant Cell 5(1):9–23. https://doi.org/10.1105/tpc.5.1.9

Sinclair R, Hsu G, Davis D, Chang M, Rosquete M, Iwasa JH, Drakakaki G (2022) Plant cytokinesis and the construction of new cell wall. FEBS Lett 596(17):2243–2255. https://doi.org/10.1002/1873-3468.14426

Sytar O, Zivcak M, Brestic M (2016) Noninvasive methods to support metabolomic studies targeted at plant phenolics for food and medicinal use. In: Hakeem KR, Tombuloğlu H, Tombuloğlu G (eds) Plant Omics: Trends and Applications. Springer International Publishing, Cham, pp 407–443

Takeuchi Y, Komamine A (1978) Composition of the cell wall formed by protoplasts isolated from cell suspension cultures of *Vinca rosea*. Planta 140(3):227–232. https://doi.org/10.1007/bf00390252

Takeuchi Y, Komamine A (1982) Effects of culture conditions on cell division and composition of regenerated cell walls in *Vinca rosea* protoplasts. Plant Cell Physiol 23(2):249–255. https://doi.org/10.1093/oxfordjournals.pcp.a076344

Tomasiak A, Zhou M, Betekhtin A (2022) Buckwheat in tissue culture research: current status and future perspectives. Int J Mol Sci 23(4). https://doi.org/10.3390/ijms23042298

Verhertbruggen Y, Marcus SE, Haeger A, Ordaz-Ortiz JJ, Knox JP (2009) An extended set of monoclonal antibodies to pectic homogalacturonan. Carbohydr Res 344(14):1858–1862. https://doi.org/10.1016/j.carres.2008.11.010

Willats WG, Marcus SE, Knox JP (1998) Generation of monoclonal antibody specific to (1–>5)-alpha-L-arabinan. Carbohydr Res 308(1–2):149–152. https://doi.org/10.1016/s0008-6215(98)00070-6

Willats WGT, Steele-King CG, Marcus SE, Knox JP (1999) Side chains of pectic polysaccharides are regulated in relation to cell proliferation and cell differentiation. Plant J 20(6):619–628. https://doi.org/10.1046/j.1365-313X.1999.00629.x

Willats WGT, McCartney L, Mackie W, Knox JP (2001) Pectin: cell biology and prospects for functional analysis. In: Carpita NC, Campbell M, Tierney M (eds) Plant cell walls. Springer Netherlands, Dordrecht, pp 9–27

Wiśniewska E, Majewska-Sawka A (2007) Arabinogalactan-proteins stimulate the organogenesis of guard cell protoplasts-derived callus in sugar beet. Plant Cell Rep 26(9):1457–1467. https://doi.org/10.1007/s00299-007-0348-1

Wiśniewska E, Majewska-Sawka A (2008) The differences in cell wall composition in leaves and regenerating protoplasts of *Beta vulgaris* and *Nicotiana tabacum*. Biol Plant 52(4):634–641. https://doi.org/10.1007/s10535-008-0124-2

Yates EA, Valdor JF, Haslam SM, Morris HR, Dell A, Mackie W, Knox JP (1996) Characterization of carbohydrate structural features recognized by anti-arabinogalactan-protein monoclonal antibodies. Glycobiol 6(2):131–139. https://doi.org/10.1093/glycob/6.2.131

Ye ZH, Varner JE (1991) Tissue-specific expression of cell wall proteins in developing soybean tissues. Plant Cell 3(1):23–37. https://doi.org/10.1105/tpc.3.1.23

Zaranek M, Pérez-Pérez R, Milewska-Hendel A, Betekhtin A, Grzebelus E (2023a) Promotive effect of phytosulfokine - peptide growth factor - on protoplast cultures development in *Fagopyrum tataricum* (L.) Gaertn. BMC Plant Biol 23(1):385. https://doi.org/10.1186/s12870-023-04402-9

Zaranek M, Pérez-Pérez R, Milewska-Hendel A, Grzebelus E, Betekhtin A (2023b) Efficient and rapid system of plant regeneration via protoplast cultures of *Fagopyrum esculentum* Moench. Plant Cell Tiss Organ Cult 154(3):673–687. https://doi.org/10.1007/s11240-023-02542-2

Zhang L-L, He Y, Sheng F, Hu Y-F, Song Y, Li W, Chen J, Zhang J, Zou L (2021) Towards a better understanding of *Fagopyrum dibotrys*: a systematic review. Chin Med 16(1):89. https://doi.org/10.1186/s13020-021-00498-z