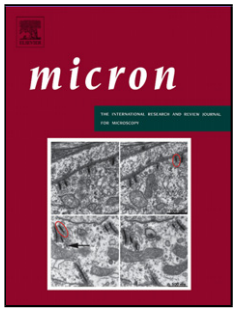

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Authors: Łukasz Baranowski, Wojciech Kurek, Małgorzata Lichocka, Mirosław Sobczak

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A cryotechnique-based method for low abundance protein immunolocalization in tomato (*Solanum lycopersicum*) roots infected with a nematode, *Globodera rostochiensis*

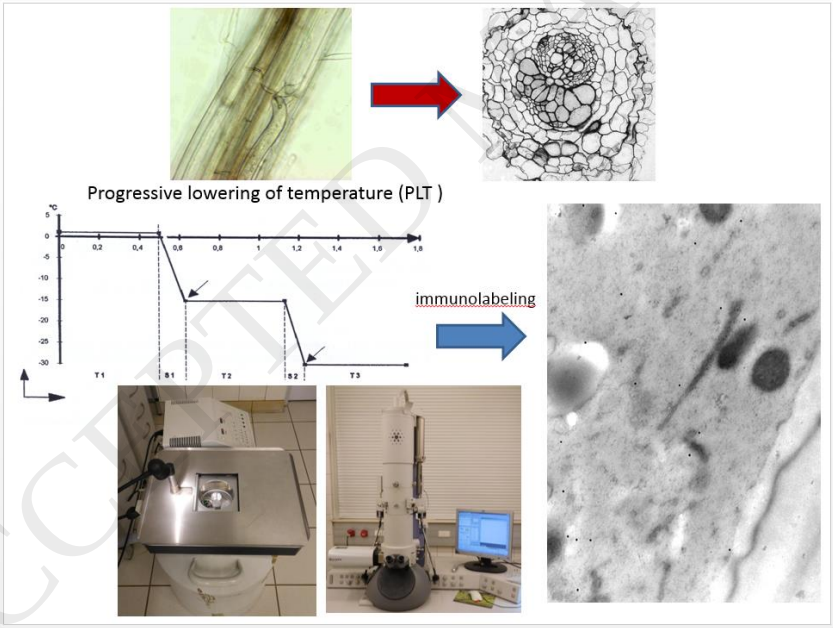
Łukasz Baranowski[a*], Wojciech Kurek[a], Małgorzata Lichocka[b], Mirosław Sobczak[a]

[a] Department of Botany, Warsaw University of Life Sciences-SGGW, owoursynowska 159, 02-766 Warsaw, Poland,

[b] Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawińskiego 5a, 02-106 Warsaw, Poland

[*]Corresponding author: Tel.: ++48 22 593 2654; E-mail address: lbaran85@wp.pl (Ł. Baranowski)

Graphical abstract



Highlights

- Quality of immunolabeling on classical and PLT samples was compared
- Specificity and abundance of immunolabeling were better in PLT-treated sections
- Strong and specific expression of Cellulase 7 in syncytia was shown

- Cellulase 7 is specifically upregulated in syncytium
- Cellulase 7 upregulation is important for syncytium induction and maintenance

ABSTRACT

Plant-parasitic cyst forming nematodes induce in host roots a specific feeding site called a syncytium. Modifications induced by the pathogen in cells incorporated into syncytium include their hypertrophy and changes in apoplast caused by over-expression of plant proteins, e.g. cellulases. As a result cell wall openings between syncytial elements are formed. The major aim of our investigation was to immunolocalize cellulases involved in these cell-wall modifications. Experiments were conducted on tomato (*Solanum lycopersicum* cv. “Money Maker”) infected with *Globodera rostochiensis*. Root segments containing syncytia were processed using two techniques: conventional method of embedding in LR-White resin and cryotechnique of progressive lowering of temperature (PLT). It is believed that the latter is superior to other techniques in keeping in place cell components and preserving antigenicity of macromolecules. It is especially useful when low abundance proteins have to be immunodetected at their place of action. The main principle of the PLT technique is a stepwise lowering of temperature throughout probe dehydration, infiltration and embedding in an appropriate resin. Two-step immunolocalization and visualization using fluorochrome (FITC) at light microscopy level or colloidal gold particles at transmission electron microscopy level was performed in this study. The labeling of cellulase 7 protein at both microscopy levels was more intensive and specific on PLT-treated sections as compared to sections obtained from the classical method. Our results confirm the usefulness of the PLT cryotechnique for plant immunocytochemistry and indicate that in nematode-infected roots cellulase 7 is predominantly present in the syncytia.

Key words: antigenicity, PLT cryotechnique, cell wall, syncytium, cellulase

1. Introduction

Plant-parasitic nematodes from the cyst forming group (genera *Globodera* and *Heterodera*) induce a specific feeding site in the host plant roots called a syncytium

(Golinowski et al., 1997; Sobczak and Golinowski, 2008). It is composed of highly ultrastructurally modified root cells. Parts of their cell walls creating the outer syncytial wall become thickened whereas in cell walls between syncytial elements cell wall openings are formed (Grundler et al., 1998). These changes in apoplast are caused by over-expression of plant proteins such as cellulases and expansins that have unique ability to reorganize/degrade cell wall (Wieczorek et al., 2006, 2008; Fudali et al., 2008; Karczmarek et al., 2008). Cellulases (endo-β-1,4-glucanases, EGases, EC. 3.2.1.4) are involved in enzymatic degradation of plant cell wall by hydrolysis of the 1,4-β-glycosidic bonds within cellulose molecule whereas expansins catalyze disruption of non-covalent bonds between cellulose and hemicellulose chains on the surface of microfibrils (McQueen-Mason and Cosgrove, 1994; Cosgrove, 2000; Karczmarek et al., 2008). This raises the need for investigations on spatial and temporal expression of genes encoding the cell wall remodeling proteins throughout syncytium development at nucleic acid and protein levels. In respect to proteins a method of choice is immunolocalization leading to observations in light- or electron microscope. In both cases the reliability of final results depends strongly on proper sample preparation. In general, two techniques may be employed at this point: the so called conventional ones (based on chemical fixation and resin embedding) and cryotechniques.

Cryotechniques encompass a range of methods starting either from mild chemical fixation at room temperature or from cryofixation. The latter is a powerful alternative to chemical fixation and performed in different ways results in vitrified material that is further processed employing usually a freeze-substitution method ending with ready for sectioning samples embedded in resin (Hess at al., 2000; Hess, 2003). In the case of mild chemical fixation Tokuyasu cryotechnique may be subsequently implemented (Tokuyasu, 1980, 1986, 1997). It does not demand dehydration of the probe nor their embedding in resin. Instead, probe embedded in cross-linked meshwork of macromolecules (e.g. agarose, albumin) is infiltrated with cryoprotectant, rapidly frozen and cryosectioned at ca. -120ºC and thawed (Liou et al., 1996; Mori et al., 2004). There is another possibility of probe preparation after mild fixation. This technique, called a progressive lowering of temperature (PLT), implements dehydration at stepwisely lowered temperature followed by infiltration with an acrylic resin and polymerization at negative temperature (McCann et al., 1996). Freeze substitution and Tokuyasu method are difficult for plant material and/or

ineffective for structures located deeply inside the specimen. Thus the PLT technique was chosen to immunolocalize cellulase molecules in syncytia induced by a nematode in tomato roots. We report on distribution of the tomato cellulase 7 protein in plant root during development of nematode feeding site and reveal that PLT cryotechnique is a useful tool for such investigations.

2. Material and methods

2.1. Experimental design

Seeds of the potato cyst nematode-susceptible tomato (*Solanum lycopersicum* L.) cv. "Money Maker" were surface-sterilized in 70% (v/v) ethanol for 3 min, then in 5% sodium hypochlorite (w/v) for 15 min and rinsed three times for 15 min in sterile distilled water. Seeds (nine per plate) were germinated on 0.8% (w/v) water agar (Fluka, Buchs, Switzerland) in the dark at 22 °C for 5-7 days. Germinating seeds were transferred (one seed per plate) into Petri dishes containing Gamborg's B5 medium (Sigma-Aldrich, St. Louis, USA) supplemented with 2% (w/v) sucrose and 0.5% (w/v) GelRite (Sigma-Aldrich). The tomato plants were grown in plates at 22 °C under 16/8 h light/dark regime (photon intensity: 220 $E*m^{-2}*s^{-1}$).

Parasitic juveniles of *Globodera rostochiensis* Woll. (J_2s) were hatched from cysts, surface-sterilized in 0.025% (w/v) aqueous mercuric chloride for 1-2 min and rinsed three times for 5 min in sterile distilled water. Approximately 20 J_2s per root tip were inoculated onto 10-day-old tomato seedlings using sterile pipette tips. After inoculation, plants were kept in the dark at 20 °C. Root segments containing syncytia were dissected for further processing at 5 and 9 days post inoculation (5 and 9 dpi).

2.2. Sample processing

Root segments with syncytia were fixed in 4% (w/v) paraformaldehyde (Sigma-Aldrich) and 0.25% (v/v) glutaraldehyde (Fluka) in 50 mM phosphate buffered saline (PBS, pH=7.0) for 2 h at room temperature. Afterwards, they were washed three times in 50 mM PBS for 15 min and embedded in Lowicryl K4M acrylic resin (Sigma-Aldrich) using a modified PLT technique (Table 1, Edelmann, 2001, modified). The procedure was based on a stepwise lowering of temperature throughout the

dehydration process, infiltration, embedding and polymerization of acrylic resin. For comparison a batch of samples was fixed in 2% (v/v) glutaraldehyde and 2% (w/v) paraformaldehyde in 50 mM PBS for 2 h at room temperature, rinsed three times in 50 mM PBS, dehydrated, infiltrated and embedded in LR-White acrylic resin (Sigma-Aldrich). Resin blocks were polymerized at 48 °C for 48 h and serially sectioned on semithin (3 µm thick) cross sections that were collected on slides coated with poly-L-lysine (Polysine® slides, Thermo Scientific, Walthman, MA, USA). Ultrathin (~80 nm thick) cross sections were collected on formvar-coated nickel grids. For comparative anatomical and ultrastructural studies few samples were also embedded in Epon epoxy resin (Sigma-Aldrich) according to procedure described by Grundler et al. (1998).

2.3. Immunocytochemical localization

2.3.1. Immunoblotting

Polyclonal antibody was raised in rabbit using a synthetic peptide PNQNDFFPDERTDYS (which corresponds to the C-terminal 14 amino acids of Sl-CEL7) coupled to KLH (Keyhole limpet hemocyanin) (Sigma-Genosys, Woodlands, TX, USA). The specificity of the antibodies for Sl-CEL7 was evaluated on protein extracts obtained from uninfected tomato roots, *Globodera rostochiensis* infected roots and 2,4-D-stimulated tomato hypocotyls as positive control sample (Català et al., 1997). Plant samples were ground in liquid nitrogen and then thawed in 2 ml of extraction buffer (20 mM Tris-HCl, pH=7.5; 0.5 M NaCl; 3 mM EDTA) per 1 g of tissue powder. Samples were centrifuged at 14,000 rpm at 4 °C for 30 min. Cold (-20 °C) 100% acetone (Sigma-Aldrich) was added to the collected supernatants in 4:1 ratio. After centrifugation precipitated proteins were dissolved in distilled water, fractionated by 10% SDS-PAGE, transferred into PVDF Hybond P membrane (Amersham Pharmacia Biotech) and subjected to immunoblot analysis using polyclonal rabbit anti-cellulase 7 antibodies (anti-Sl-CEL7; Sigma-Genosys) diluted 1:1000 with PBS. Afterwards, membranes were incubated in secondary goat anti-rabbit antibody conjugated to alkaline phosphatase (diluted 1:20,000 with PBS)

(Sigma-Aldrich). Immunoblots were developed using the nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate colorimetric detection solution (Sigma-Aldrich).

2.3.2. Fluorescence microscopy

Semithin cross sections were pre-incubated in 10 mM PBS for 15 min. Afterwards, unspecific epitopes were blocked by incubation in 10 mM PBS supplemented with 3% (w/v) bovine serum albumin (BSA; Sigma-Aldrich) in the dark for 1 h at room temperature. Then, slides were treated with primary rabbit anti-cellulase 7 antibody (anti-Sl-CEL7; Sigma-Genosys) diluted 1:50 with 10 mM PBS supplemented with 1% (w/v) BSA for 1 h at RT. Next, slides were rinsed four times for 10 min in 10 mM PBS with 0.05% (v/v) Tween20 (USB™, Cleveland, Ohio, USA) and once in 10 mM PBS for 10 min. Thereafter, sections were incubated in secondary goat anti-rabbit IgG conjugated with FITC (Sigma-Aldrich) diluted 1:250 with 10 mM PBS supplemented with 1% (w/v) BSA in the dark for 1 h at RT and washed three times in 10 mM PBS (as above). Sections were mounted in polyvinyl alcohol mounting medium (Fluka). In negative controls, primary antibody was omitted. Slides were examined using an Olympus AX70 “Provis” (Olympus, Tokyo, Japan) microscope equipped with a UM61002 filter set. Images were taken with an Olympus DP50 digital camera (Olympus).

2.3.3. Transmission electron microscopy

Ultrathin sections on grids was pre-incubated in 10 mM PBS for 10 min. Afterwards, unspecific binding sites were blocked by incubation in 10 mM PBS supplemented with 2% (w/v) BSA for 45 min. Thereafter, grids were treated with primary rabbit anti-cellulase 7 antibody (anti-Sl-CEL7; Sigma-Genosys) diluted 1:50 with 10 mM PBS supplemented with 2% (w/v) BSA for 1 h at RT. Then, sections were washed in 10 mM PBS with 0.05% (v/v) Tween20 four times for 10 min and once in 10 mM PBS for 10 min. They were incubated for 1 h at RT in secondary goat anti-rabbit IgG conjugated with 15 nm colloidal gold particles (British BioCell International, Cardiff, UK) diluted 1:50 with 10 mM PBS. They were washed three times in 10 mM PBS and rinsed twice in distilled water. Grids were counterstained with 2% (w/v) aqueous solution of uranyl acetate (Sigma-Aldrich) for 10 min and washed in distilled water

five times for 2 min. In negative controls, primary antibody was omitted. Grids were examined in an FEI 268D “Morgagni” (FEI Company, Hillsboro, OR, USA) transmission electron microscope operating at 80 kV. Images were taken with an SIS “Morada” (Olympus-SIS, Münster, Germany) digital camera.

2.3.4. Quantification of immunogold labeling

Immunogold labeling was quantified for 5 dpi syncytia processed either by the conventional technique or by PLT. In both variants, gold particles were counted on 28 micrographs by systematic uniform random sampling. For this purpose, transparent lattices (0.5 x 0.5 µm) were overlaid on each micrograph and gold particles were counted in each second square of the lattice, starting from the upper left corner. Total numbers of grains for given micrographs were taken for further analyses. The two procedures were compared with a generalized linear model, assuming the Poisson distribution of errors, with the pseudo-estimation (so the over-dispersion parameter was not assumed to be 1 but estimated during the modeling) as described by Venables and Ripley (2002).

3. Results

3.1. Anatomy and ultrastructure of syncytia

Syncytia were induced by infective second-stage juveniles (J_2s) predominantly among cortical parenchyma cells, developed toward the vascular cylinder and finally expanded in the vascular cylinder (Fig. 1A). In such cases a so called ‘cortex bridge’ was formed as a connection between initial syncytial cell (in the cortex) and the main part of the nematode feeding site was located in the stele and expanded longitudinally along vascular elements (Fig. 1A). The syncytium was multinuclear due to protoplasts fusion resulting from partial cell wall dissolutions (Fig. 1A and 1B) caused by the cell wall modifying enzymes. Characteristic features of syncytial ultrastructure were proliferation of dense cytoplasm, enlargement of nuclei, and increase in the numbers of endoplasmic reticulum structures, dictyosomes, mitochondria and plastids (Fig. 1B and 1C). Central vacuoles were replaced by many small ones (Fig. 1B and 1C). Quality of protoplast ultrastructure was better after

classical chemical fixation and embedding in Epon resin (Fig. 2) in comparison to the cryofixation and PLT embedding (Fig. 4), but all organelles were easily recognizable in both procedures.

3.2. Application of PLT method for immunolocalization of cellulases

3.2.1. Fluorescence microscopy

The specificity of the antibody was tested with immunoblot on crude protein extracts from rapidly expanding tomato hypocotyls (2,4-D-stimulated) and from tomato roots: uninfected and infected with *G. rostochiensis* (Fig. 2). Specific band with the appropriate molecular mass for Sl-CEL7 (~50 kDa) was recognized in the positive control sample (2,4-D stimulated hypocotyls) and in the crude protein extract from infected roots (Fig. 2). This finding corresponds to the previous report showing induced expression of cellulase 7 in auxin-stimulated hypocotyls (Català et al., 1997) and proved that the antibody can be used for immunolocalization of Sl-CEL7 in tomato roots infected with *G. rostochiensis*. Immunodetection of cellulase 7 was conducted on semithin sections of roots containing 5 or 9 dpi syncytia induced by *G. rostochiensis*. In negative control experiments primary antibodies were omitted, what resulted in no labeling on sections from classically processed (data not shown) as well as PLT subjected samples (Fig. 3A). It proves specificity of antibodies used and specificity of immunolocalization in treatments where full protocol was applied. Fluorescence microscopy localization of Sl-CEL7 on sections of 5 dpi syncytia indicated that this protein was present predominantly in syncytial elements, especially close to their cell walls and cell wall openings (Fig. 3A and B). This feature is better recognizable on sections of Lowicryl K4M resin (Fig. 3B) while on sections of syncytia embedded in LR-White resin this phenomenon was less evident (Fig 3A). Cells of non-infected roots exhibited less labeling as compared to the infected ones (data not shown). These results were confirmed on sections of 9 dpi syncytia. In this case PLT method enabled specific localization of cellulase 7 in syncytial elements next to the syncytial walls and in the vicinity of cell wall openings (Fig. 3C). In addition to specific localization of the signal generated by the fluorochrome, the PLT technique enabled also more precise localization of lignin autofluorescence in the xylem vessels (Fig. 3B and 3C, versus 3A).

3.2.2. Transmission electron microscopy

Immunogold labeling at transmission electron microscopy level corroborated the results obtained by immunofluorescence microscopy. In the case of both resins (LR-White and Lowicryl K4M), only incidental labeling could be seen on sections of negative controls with omitted primary antibody (Fig. 4A and B). When primary antibody was used in the immunolocalization procedure, gold particles appeared predominantly in the cytoplasm of syncytial elements (Fig. 4C-4H). There was no labeling over the syncytial cell walls, but it appeared more abundant in the syncytial cytoplasm in the vicinity of cell wall stubs delineating cell wall openings (Fig. 4E and 4H). This indicates that cellulase 7 was up-regulated in syncytia and their molecules accumulated near the cell wall openings in syncytia. The labeling in the PLT method was more abundant and apparently more specific that the labeling of the “standard” (LR-White) method (Fig. 4C versus 4D and 4E versus 4F). Comparison of corresponding images obtained from both methods usually shows that the Lowicryl K4M sections contained more gold particles than the LR-White sections. Statistical evaluation of the numbers of gold grains indicated that the micrographs taken from samples processed by the cryotechnique contained in average 2.9 times more gold grains than those taken from the samples processed by the classical method (p<0.001).

The images of Lowicryl K4M sections were less sharp, but it was compensated with a vengeance by the higher sensitivity of the immunolocalization. However, the information loss was small because mitochondria, plastids and ER structures were readily recognizable on the micrographs taken from the Lowicryl K4M sections (Fig. 4D and 4F). Also in 9 dpi syncytia the immunogold labeling was more abundant on the sections obtained from the PLT cryotechnique-treated samples. Gold grains labeling was confined to only syncytial cytoplasm and it was absent in syncytial cell walls (Fig. 4G and H). Additionally, the accumulation of gold particles indicating cellulase 7 molecules close to cell wall openings was evident (Fig. 4H).

4. Discussion

4.1. Role of cellulases in syncytium development

A suggestion that plant endo-β-1,4-glucanases are involved in processes of cell wall rearrangements during nematode feeding site development has been raised by Grundler et al. (1998). It was corroborated by analysis of expression patterns of genes encoding cellulases in syncytia and giant cells which revealed their differential expression during nematode feeding site development. *In situ* localization of a plant EGase gene *Nt-CEL7* transcripts in tobacco roots infected with a tobacco cyst nematode, *G. tabacum* ssp*. solanacearum*, or with a root-knot nematode *Meloidogyne incognita* showed that their expression was specifically and developmentally up-regulated in roots containing syncytia or giant cells and also - in root tips and lateral root primordia (Goellner et al., 2001). Similarly, *Sl-CEL7* gene is up-regulated in tomato roots containing syncytia induced by *G. rostochiensis* (Karczmarek et al., 2008; Święcicka et al., 2009). In the former paper analyses at protein level were also performed for samples embedded in butyl-methyl metacrylate resin (BMM). Consecutive immunolabeling at light microscopy level revealed that cellulase 7 is localized specifically in the syncytium (Karczmarek et al., 2008). Our experiments with application of PLT technique and the same antibody corroborated those results using immunofluorescence and immunogold methods. Moreover, the latter correlated position of CEL7 molecules with the cell wall openings that suggests that the enzyme may be involved in their formation.

Immunolocalization studies reported in this paper indicating over-expression of endogenous cellulases in cells incorporated into syncytium are in accordance with reports on other cell wall modifying proteins and their genes in plant-nematode interactions. In particular, specific up-regulation of several plant expansin genes at both mRNA and protein levels in response to cyst nematode infection was observed (Wieczorek et al., 2006; Tucker et al., 2007; Fudali et al., 2008). Up-regulated endogenous pectinases were also shown to take part in successful development of syncytia (Hewezi et al., 2008). Similarly, transcript profiling of cyst nematode feeding cells revealed up-regulation of several xyloglucan endotransglycosylases (XETs) genes (Ithal et al., 2007). These findings taken together are of crucial importance for understanding mechanisms underlying successful development of the pathogen within the host root.

4.2. Comparison of conventional and PLT method

Methodological studies in last decades revealed that conventional methods of specimen preparation (based on chemical fixation and dehydration at room temperature) can lead to cell artifacts including membrane networks fracture and degradation (Rensing et al., 2002) and deformation of organelles (Zechmann et al., 2005). Moreover, these protocols result often in extraction and/or displacement of labile or diffusible substances within cells/tissues. These disadvantages have been overcome by cryomethods that yield fixed material much closer to what is present in living, undisturbed cells (Allakhverdov and Kuzminykh, 1981). Additionally, it is believed that cryomethods are superior to other techniques not only in maintaining reliable cell ultrastructure but also in preserving antigenicity of macromolecules (e.g. proteins) that is of crucial importance in immunolabeling (Tokuyasu, 1980, 1986, 1997; Liou et al., 1996; McCann et al., 1996; Raposo et al., 1997; Morin and Soll, 1997; Takizawa et al., 2003; Mori et al., 2004; Dettmer et al., 2005; He et al., 2007). There is a vast amount of publications reporting successful application of different cryotechniques for antigen detection. For instance, freeze substitution followed by embedding in resins revealed the structural appearance of chicken skeletal muscles and the antigenicity of the M-line protein (Humbel and Schwarz, 1989). It also enabled immunolocalization of actin in *Nicotiana* pollen (Lancelle and Hepler, 1989), cytoskeleton elements in *Ledebouria* pollen and anther tissue (Hess, 1995, 2003), callose in vascular cambial cells of pine seedlings (Rensing et al., 2002). Similarly, electron microscopy combined with Tokuyasu cryotechnique turned out to be suitable for immunolocalization. With this method, proteins were localized in the chloroplast thylakoids and stroma (Herman, 1988) as well as in the chloroplast outer membrane (Schnell et al., 1990; Morin and Soll, 1997). Tokuyasu cryotechnique enabled also localization of callose in compatible and incompatible interactions between carnation callus and *Fusarium oxysporum* (Trillas et al., 2000). Cryosectioning may also deliver useful material for immunocytochemistry at light microscopy level (Tokuyasu, 1997). The main goal of our experiment was to check if cryomethods are better than conventional techniques for preparation of material for immunolocalization by preserving protein antigenicity. Fluorescence microscopy immunolocalization of cellulase 7 indicated that more abundant labeling was found on Lowicryl K4M-embedded material than on LR White resin sections. Cellulase molecules were localized predominantly in cells incorporated into syncytia, especially next to their cell

walls. These results confirmed advantages of cryomethods in preserving antigenicity of proteins. Additionally, xylem vessels are easier to recognize in fluorescence microscopy on sections from material prepared by PLT cryotechnique. It enables more precise interpretation of results. Many papers report successful application of cryomethods in immunoelectron microscopy studies (e.g. Morin and Soll, 1997; Trillas et al., 2000; Mori et al., 2004). Similarly, in our experiment the PLT technique proved its value in protein localization at electron microscopy level. Although in both methods (conventional one and PLT) gold particles were localized in syncytial cytoplasm mostly in the vicinity of cell walls, their numbers were higher on PLT sections. This way, our results confirmed the usefulness of the cryotechnique for plant immunogold cytochemistry as well as for studies on plant-nematode interactions.

5. Conclusions

Comparison of two methods for sample preparation for immmunolabeling purposes indicated that a PLT cryomethod is superior in preservation of appropriate antigens and provides more reliable localization of macromolecules in the tissue. Additionally, the present study revealed strong, syncytium-specific expression of tomato cellulase 7 protein in syncytia induced upon *Globodera* infection. It corroborates a hypothesis that nematodes employ host gene upregulation to induce and maintain pathogen's feeding structures.

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Figure legends

Figure 1.

Sections of 9 dpi syncytium induced in tomato roots by potato cyst nematode taken from samples processed according to conventional method and embedded in Epon resin. (A) Light microscopy micrograph of cross section of root with syncytium taken above the nematode head. (B and C) Transmission electron microscopy micrographs of vascular cylinder-derived part of syncytium. Abbreviations: C - cortex; CB - ”cortex bridge”; CW - cell wall; En - endodermis; Ep - epidermis; ER - endoplasmic reticulum; GA - Golgi apparatus; M - mitochondrion; No - nucleolus; Nu - nucleus; Pl - plastid; S - syncytial element; SV - small vacuole; X - xylem. Arrows point to cell wall stubs. Scale bars: 20 µm (A) and 2 µm (B and C).

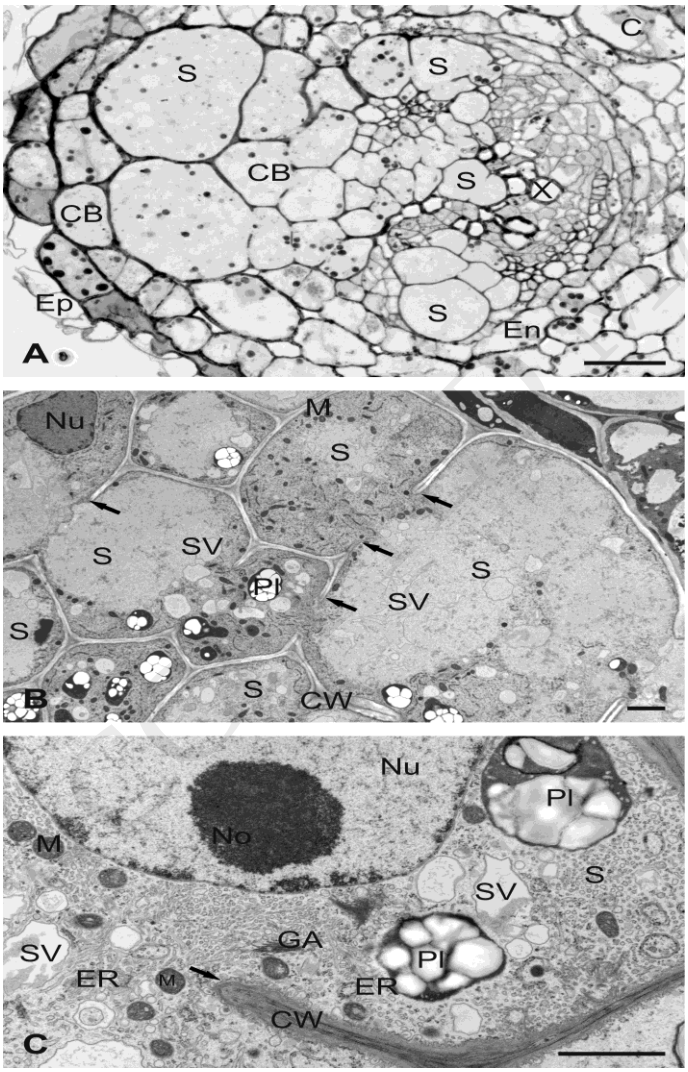


Figure 2.

Immunoblot analysis of the specificity of the polyclonal antibody anti-Sl-CEL7. Immunoblots of crude protein extracts from tomato hypocotyls incubated for 24 h in buffer containing 5 µM of 2,4-D (positive control, Hypocotyl (+2,4-D)), non-treated hypocotyls (negative control, Hypocotyl (-2,4-D)), tomato roots containing syncytia induced by *G. rostochinesis* (Infected roots) and control uninfected roots (Uninfected roots). Arrows indicate position of cellulase 7 protein.

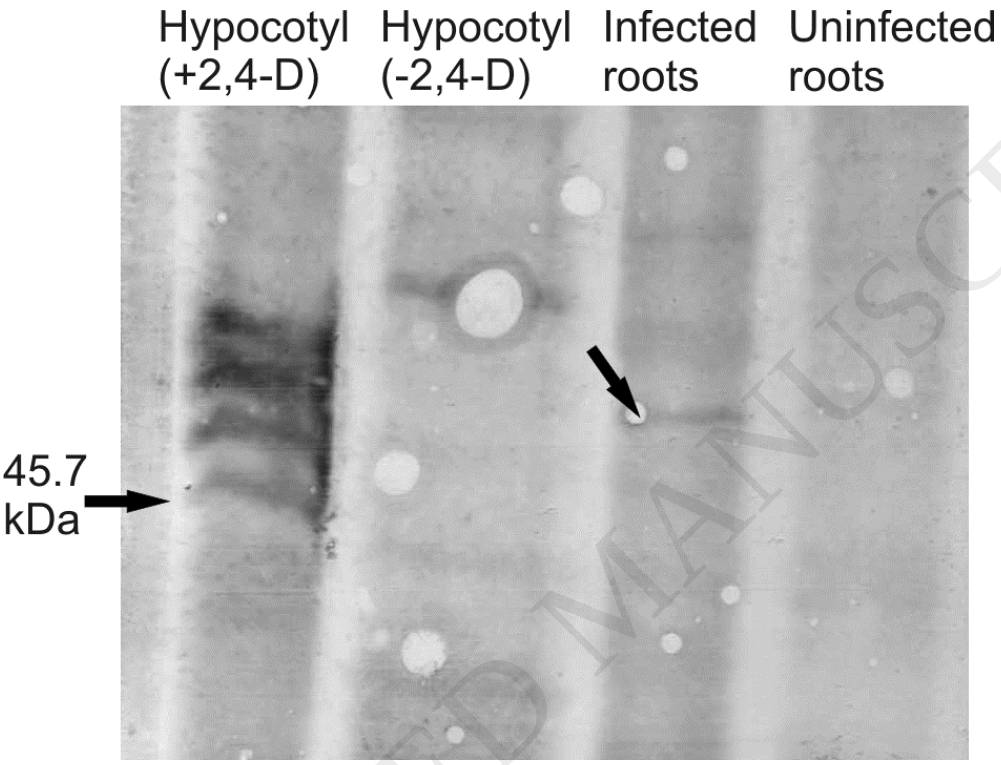


Figure 3.

Immunofluorescence labeling of Cel7 (green signal) on sections prepared by conventional technique (A) and PLT cryotechnique (B and C). Cross sections of tomato roots containing 5-day-old (A and B) or 9-day-old (C) syncytia. Abbreviations: En - endodermis, Ph - phloem elements, S - syncytium, X - xylem vessels (recognized by autofluorescence of lignin). Some selected sites where Cel7 was localized are indicated by arrows. Scale bars: 20 µm.

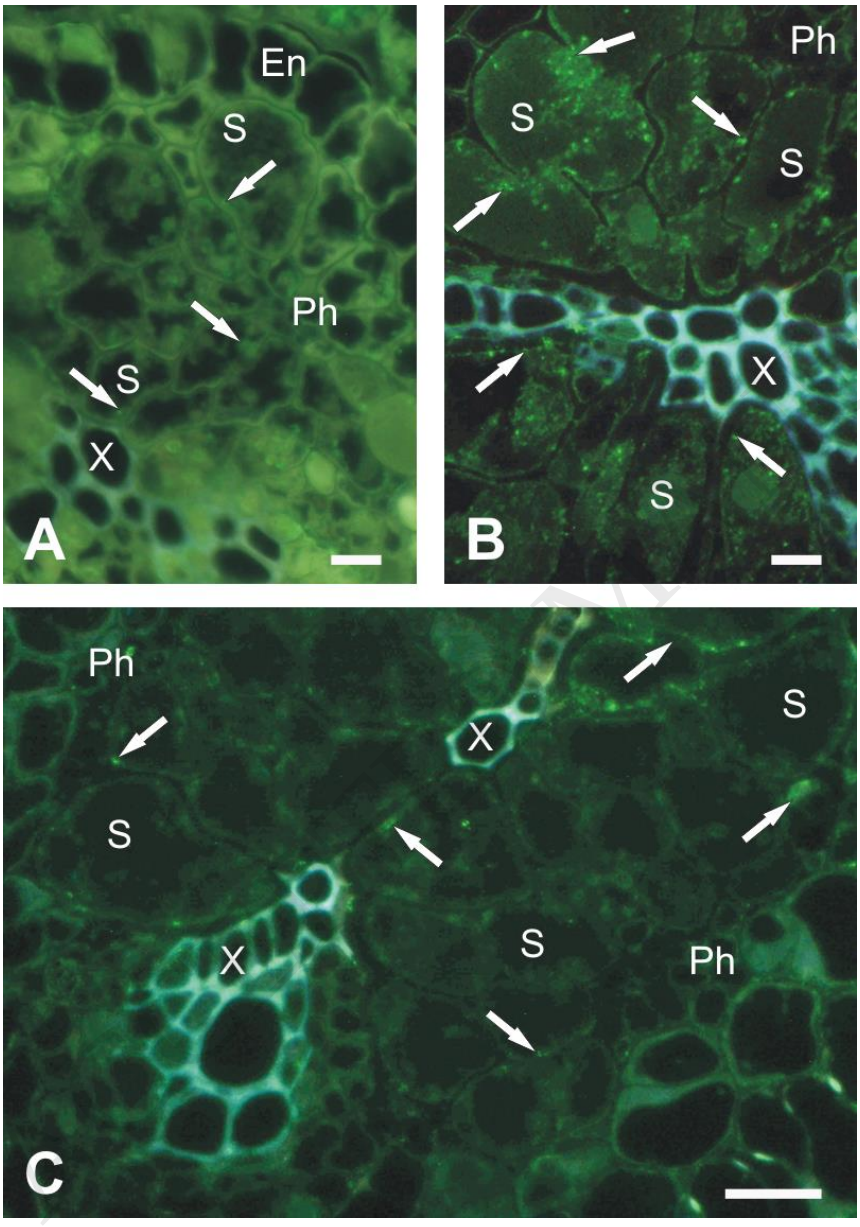
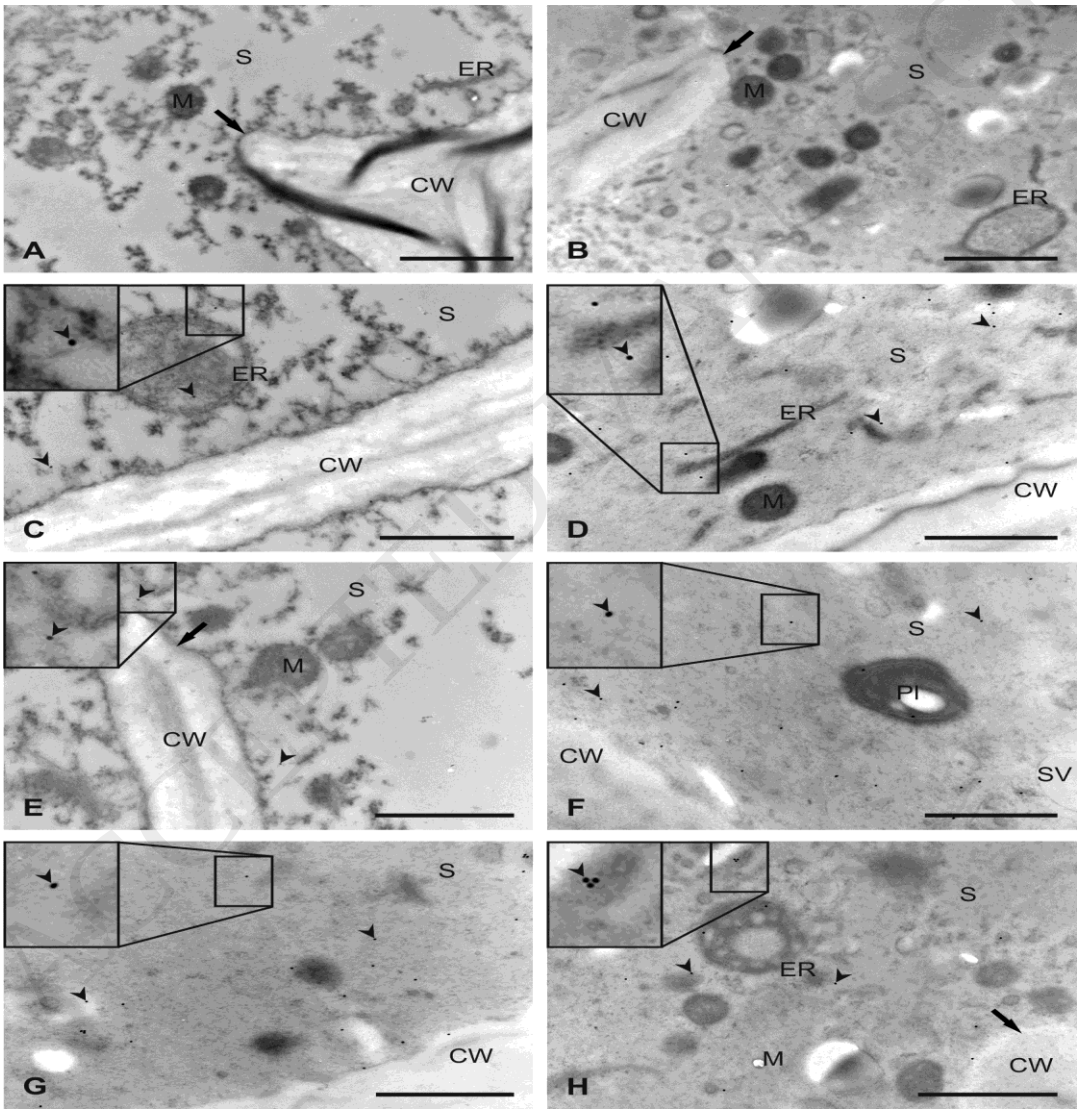


Figure 4.

Immunogold labeling of Cel7 in syncytia. Images were obtained from samples processed by conventional chemical fixation (A, C, E and G) or PLT cryotechnique (B, D, F and H). Negative controls with omitted primary anti-Cel7 antibody labeling (A and B) and labeled with anti-Cel7 antibody (C-H). Section were taken from syncytia collected at 5 dpi (A-F) and 9 dpi (G and H). Abbreviations: CW – cell wall, ER – endoplasmic reticulum, M – mitochondrion, Pl – plastid, S – syncytium, SV – small vacuole. Arrows point to cell wall stubs. Arrowheads point to some selected gold grains. Insets (C-H) show higher magnification images of boxed regions. Scale bars: 1 µm (A-H).



Table

Table 1. Procedure of PLT cryotechnique (Edelmann, 2001, modified)

Step*)	Chemicals/treatment	Time [h]	Temperature [°C]	Temperature slope [°C/h]
T1	Ethanol 30%	0,5	0	
S1	Ethanol 50%	0,5		-20
T2	Ethanol 70%	0,5	-20	
S2		1		-10
T3	Ethanol 100%	1	-30	
T3	Ethanol 100%	1	-30	
T3	Ethanol:Lowicryl 3:1	1	-30	
T3	Ethanol:Lowicryl 1:1	1	-30	
T3	Ethanol:Lowicryl 1:3	1	-30	
T3	Lowicryl 100%	1	-30	
T3	Lowicryl 100%	12	-30	
T3	Polymerization (UV irradiation)	48	-30	

*) T1, T2, T3 - constant temperature, S1, S2 – decreasing temperature