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Strategies to improve the antigenicity, ultrastructure preservation and visibility of trafficking compartments in *Arabidopsis* tissue

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ABSTRACT

Immunolabelling of (ultra)thin thawed cryosections according to Tokuyasu is one of the most reliable and efficient immunolocalisation techniques for cells and tissues. However, chemical fixation at ambient temperature, a prerequisite of this technique, can cause problems for samples, like plant tissue, because cell walls, hydrophobic surfaces and intercellular air slow down diffusion of fixative molecules into the sample. We show that a hybrid technique, based on a combination of cryofixation/freeze-substitution and Tokuyasu cryosection immunolabelling, circumvents the disadvantages associated with chemical fixation and results in an improved ultrastructure and antigenicity preservation of Tokuyasu cryosections used for light and electron microscopic immunolabelling (as shown for Myc- or mRFP-tagged proteins, KNOLLE and carbohydrate epitopes). In combination with the most sensitive particulate marker systems, like 1-nm gold or quantum dot markers, we were able to obtain a differentiated labelling pattern which allows a more detailed evaluation of plant Golgi, trans-Golgi network and multivesicular body/prevacuolar compartment markers (COPI-specific γCOP, the ADP-ribosylation factor GTPase ARF1, ARA7/RabF2b and the vacuolar sorting receptor VSR). We also discuss possibilities to improve membrane contrast, e.g., of transport vesicles like COPI, COPII and clathrin-coated vesicles, and of compartments of endosomal trafficking like the trans-Golgi network.

Introduction

Plants pose considerable problems in many respects if one aims at a life-like preservation of cellular fine structure, e.g., for transmission electron microscopy. In general, plant tissues are difficult to preserve by chemical fixation at ambient temperature for both ultrastructural analysis and immunolocalisation experiments. The cellular turgor pressure, which stabilises the living cells and tissues, immediately collapses during chemical fixation, thereby causing the collapse or rupture of the often thin cytoplasmic strands in vacuolated cells. Intercellular air spaces, cell walls and hydrophobic surfaces, like waxes (e.g., of pollen grains, anthers, ovules, leaves), drastically slow down diffusion of fixatives. This increases the inherent limitations of chemical fixation like the time consuming diffusion of fixatives into the sample, the selectivity of the different fixatives used for cross-linking, and the pH-related and osmotic changes caused by the fixation buffer, the properties of which can never be correct for all cellular compartments.

Immunolabelling of (ultra)thin thawed cryosections according to Tokuyasu is one of the most reliable and efficient immunolocalisation techniques for cells and tissues (Griffiths, 1993; Griffiths et al., 1983; Griffith and Posthuma, 2002; Humbel and Stierhof, 2008; Liou et al., 1996; Slot and Geuze, 2007; Tokuyasu, 1973, 1978, 1997; Webster et al., 2008) for two main reasons. Firstly, antigens are fixed with low concentrations of aldehydes only and remain in an aqueous environment prior to immunolabelling (provided they are not extracted) and secondly, the accessibility of antigens at the thawed cryosection surface is better when compared to that of the resin section surface because antigens are not embedded in a crosslinked resin matrix. However, chemical fixation is a prerequisite for stabilising the ultrastructure before cryoprotectant infiltration (= partial dehydration) and after thawing of the cryosections for immunolabelling. The disadvantages of chemical fixation at ambient temperature are especially obvious when tissues, like anthers containing pollen grains or developing seeds containing embryos, have to be processed for immunolocalisation experiments because structures and antigens can be dislocated or get lost before proper arrest by chemical crosslinking (Ripper et al., 2008).

High-pressure freezing, the most important cryofixation technique for plants, followed by freeze-substitution (Humbel, 2008; Otegui et al., 2001; Studer et al. 1989) circumvents the

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disadvantages of chemical fixation and dehydration at ambient temperature, provided intercellular air (which is not compatible with high-pressure freezing because it can be compressed, in contrast to the liquid cytoplasm) can be removed and the sample size is compatible with the inherent physical restrictions of this cryofixation technique, as visible ice crystal damage can be avoided only up to specimen thickness of 200 μm.

It has been shown for resin section immunolabelling that cryofixation/freeze-substitution can preserve sensitive antigens and structures better than chemical fixation and dehydration at ambient temperature (Hess, 2007; Humbel and Schwarz, 1989). However, there are also antigens that cannot be immunolabelled either in thawed cryosections or after cryofixation, freeze-substitution and resin embedding. Those antigens may be sensitive to chemical fixation at ambient temperature or solvents and resin components or they cannot be detected after cryofixation and resin embedding due to the limited number of epitopes accessible at the resin section surface. Therefore, a hybrid technique was developed for both, difficult-to-fix antigens and difficult-to-fix specimens by combining the Tokuyasu thawed cryosection labelling method with an initial cryofixation step in order to benefit from the advantages of both methods (Ripper et al., 2008; Stierhof et al., 2008). After cryofixation, e.g., by high-pressure freezing, the entire sample is dehydrated and simultaneously fixed during freeze-substitution. In contrast to the conventional procedure, freeze-substitution allows the application of different fixatives and fixative combinations. In addition to formaldehyde (FA), glutaraldehyde (GA) and acrolein, uranyl acetate (UA) and osmium tetroxide (OsO_4) can be used (Stierhof et al., 2008; van Donselaar et al., 2007). Then, after raising the temperature to 0 °C, the sample has to be rehydrated and postfixed with aldehydes, before it is further processed for cryosection labelling, namely infiltrated with a cryoprotectant followed by conventional freezing and cryosectioning.

Strategies to improve labelling density in order to localise sparsely distributed antigens are of attracting interest in immunohistochemistry. We will compare the most sensitive particulate EM markers available like 1-nm gold and quantum dot (Qdots) markers and will re-evaluate some controversially discussed plant endosomal markers in the light of improved processing and labelling techniques.

A specific problem of high-pressure frozen and freeze-substituted plant samples is the poor visibility of the bilayer structure of many membranes especially in the case of well frozen samples embedded in resin or processed for cryosection immunolabelling (for resin sections, see (Donohoe et al., 2006; Hess, 2003, 2007; Murata et al., 2002); for thawed cryosections, see (Ripper et al., 2008; van Donselaar et al., 2007)). To improve the visibility of the membrane bilayer, different freeze-substitution protocols were evaluated in respect to the ultrastructural appearance of Golgi stacks, trans-Golgi network (TGN) and endosomes.

Materials and methods

Specimens used in this study

Ecotype Col-0 (wild type) and mutant *Arabidopsis thaliana* seedlings were grown on Murashige and Skoog (MS) medium +1% sucrose for 3-5 days. GFP-KNOLLE- and ARA7/RabF2b-GFP-expressing lines were provided by A. Völker, H. Wolters and G. Jürgens (Developmental Genetics, ZMBP, University of Tübingen) (Reichardt et al., 2007). VHA-a1-GFP- and VHA-a1-mRFP-expressing lines have been described (Dettmer et al., 2006; von der Fecht-Bartenbach et al., 2007). The coding sequence of VAMP727 was cloned into the KNOLLE expression cassette (Müller et al., 2003) in order to tag it with a 1 × Myc tag at the N-terminus and to express it in mitotic cells. Incubation of seedlings with brefeldin A (BFA; 50 μM, 90 min; Invitrogen, Karlsruhe, Germany) was done in cell culture dishes in 1 ml basal medium (Dettmer et al., 2006).

Antibodies and markers used

Primary antibodies

Rabbit anti-mRFP (1:50, anti-DsRed, Clontech, USA), rabbit anti-GFP IgG (1:500; #TP401, Torrey Pines Biolab Inc., East Orange, USA), rabbit anti-KNOLLE serum (1:1500; (Lauber et al., 1997)), mouse anti-Myc monoclonal IgG 9E10 (1:500; Santa Cruz Biotechnology); mouse IgG CCRC-M1 (1:5; (Zhang and Staehelin, 1992); Carbosource Services, University of Georgia, USA), rabbit anti-γCOP serum (1:500; (Movafeghi et al., 1999)), rabbit anti-ARF1 IgG (1:500; (Pimpl et al., 2000)); rabbit anti-clathrin IgG (1:100; antibody against plant clathrin heavy chain peptide; (Kim et al., 2001; Dhonukshe et al., 2007)); rabbit anti-VSR IgG (1:50; (Tse et al., 2004)).

Markers

Goat anti-rabbit F(ab')$_2$ coupled to Nanogold (1:60; Nanoprobes, Stony Brook, USA); goat anti-mouse F(ab')$_2$ coupled to Nanogold (1:60; Nanoprobes), goat anti-mouse IgG coupled to ultrasmall colloidal gold (1:30; Aurion; Wageningen, The Netherlands), goat anti-mouse IgG-12 nm gold (1:30; Dianova, Hamburg, Germany), protein A-6 nm gold (1:200; (Slot and Geuze, 1985)), goat-anti-rabbit F(ab')$_2$-Qdot655 (1:10: Molecular Probes/Invitrogen, Karlsruhe, Germany), goat anti-rabbit F(ab')$_2$-Qdot525 (1:10; Molecular Probes); goat anti-rabbit IgG coupled to Cy3 (1:800; Dianova).

Cryofixation /freeze-substitution for Tokuyasu croysection labelling

Root tips and degassed cotyledons and anthers were high-pressure frozen (HPM 010; Bal-Tec, Balzers, Lichtenstein) in aluminium planchettes filled with 1-hexadecene (Merck Sharp and Dohme) (Studer et al., 1989). Samples were freed from 1-hexadecene (below −100 °C) and transferred into 2-ml cryotubes placed in a Leica FS unit (at −90 °C) filled with acetone containing 2% water, 0.075-0.1% OsO_4 (EMS, Hatfield, PA, USA), 0.5% GA (from a 10% stock solution in acetone; catalogue no. 16530; EMS, Fort Washington, PA, USA), and 0.1–0.2% UA (Agar Scientific, Stansted, Essex, UK), as well as 0.5–2.5% methanol (deriving from UA stock solutions (20% UA in methanol); (Hawes et al., 2007)). After 50–70 h at −90 °C, samples were kept at −60 °C for 8 h, and then further warmed to −35 °C. After 8–10 h at −35 °C, samples were washed five times (30–45 min each) with acetone containing 2% water and 0.5% GA. Thereafter samples were brought to −20 °C (for 10 min), and then further warmed to 0 °C. Between −20 °C and 0 °C, acetone/GA was replaced in two steps (50% acetone, 0.38% GA, −20 °C; 10% acetone, 0.25% GA, 0 °C, 10 min each) by water containing 0.25% GA. Root tips were kept for another 30–45 min in water with 0.25% GA, *Arabidopsis* anthers and cotyledons for 60-90 min at 0 °C. Then samples were washed once with water and twice with water containing 50 mM glycine (to inactivate residual reactive aldehyde groups) and further processed for conventional cryosectioning according to (Tokuyasu, 1997).

In some cases the protocol was changed. Samples were fixed with 0.5% GA and 0.5% UA without OsO_4 in ethanol or acetone and postfixation was omitted.