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Cryopreparation of tobacco Bright Yellow-2 (BY-2) suspension-cultured cells and application to immunogold localization of Green Fluorescent Protein-tagged-membrane-proteins

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Electron microscopy investigations require excellent ultrastructure preservation, coupled with high antigenicity signal in specific applications of Golgi enzyme localization. The optimal detection of membrane-bound proteins needs appropriate specimen preparation techniques, such as cryo-methods known to provide higher cell structure and antigen preservation than conventional techniques. Two cryopreparation methods (high-pressure freezing and Tokuyasu techniques) applied to tobacco cultured cell lines are presented in this study. Two transgenic lines of tobacco cultured cells expressing GFP-fused proteins (Man-I-GFP and MUR3-GFP) were used to assess antigenicity under various conditions of cryomethods. According to cell morphology and immunolabeling detection, pre-freezing treatments are discussed in detail for high pressure freezing and freeze substituted samples embedded in both Spurr's and London Resin White. In a same approach, the effect of two fixative solutions was investigated for cryosectioned suspension-cultured tobacco cells using Tokuyasu technique. Integrity during the thawing step and subsequent immunolabeling were analysed under chemical fixation conditions.

Key words: Cryosectioning, GFP-tagged Golgi proteins, high-pressure freezing, immunogold labeling, tobacco BY-2 cells.

INTRODUCTION

The plant Golgi apparatus is involved in the processing of glycoproteins and also in the synthesis of cell wall matrix polysaccharides, such as hemicelluloses and pectins (Driouich *et al.* 1993; Saint-Jore-Dupas *et al.*, 2004). These biosynthetic functions rely on the activities of glycosidases or glycosyltransferases, the genes of which have been identified (Arabidopsis Genome Initiative, 2000). Information on the distribution and dynamics of these enzymes are currently obtained by using green fluorescent protein (GFP) technologies and confocal laser microscopy (Brandizzi *et al.*, 2004). Transgenic tobacco Bright Yellow-2 (BY-2) suspension-cultured cells (Saint-Jore *et al.*, 2002; Pagny *et al.*, 2003; Yang *et al.*, 2005; Dhanoa *et al.*, 2006; Saint-Jore-Dupas *et al.*, 2006) were often used as a model system for the expression of GFP-tagged proteins. Immuno-electron microscopy is the only approach to study the distribution of GFP-labeling at the organelle level, in contrast to light-based microscopy. Therefore, a central issue for immunogold at transmission electron microscopy (TEM) level is to

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preserve the epitopes of interest while minimizing the loss of ultrastructural details. As previously reported by Follet-Gueye *et al.* (2003) on the distribution of N-glycan-β (1-2) xylosyltransferase, conventional techniques using chemical fixatives have provided valuable information on the subcellular locations of GFP-fused proteins. In order to increase the specific labeling, additional treatments are often necessary after the chemical fixation step, and are known to induce a potential loss of structural details of membranes. To overcome these problems, it is now well established that the cryomethods (mainly cryofixation and cryosectioning) offer the best alternatives to conventional chemical techniques for ultrastructural and immunocytochemical investigations. Under high pressure and extremely low temperature, cryofixation allows immobilization of cellular structures by achieving amorphous state in few milliseconds, thus minimizing disturbance of natural processes (Studer *et al*., 2001). When coupled to specific antibodies, high-pressure freezing (HPF) combined with freeze substitution (FS) have proven their efficiency in the localization of cell wall matrix polysaccharides and glycoproteins (Zhang & Staehelin 1992; Driouich *et al*., 1993; Driouich & Starehelin, 1997), actin cytoskeleton (Lancelle & Hepler, 1992), storage proteins (Jolliffe *et al*., 2004) and vacuolar receptors (Hinz *et al.*, 2007) in many plant species. Compared to HPF/FS, Tokuyasu cryosectioning (Tokuyasu, 1973) remains the most suitable approach for the detection of membrane-bound proteins. This technique optimizes immunolabeling as evidenced by the excellent preservation of the epitopes, while the embedding and dehydration steps are omitted. These promising techniques have, however, scarce applications in plant suspension-cultured cells. Only a few studies were devoted to protein or GFP-tagged protein localization in tobacco BY-2 suspension-cultured cells using the HPF/FS method (Samuels *et al*., 1995; Nebenführ *et al*., 1999; Tse *et al*., 2004; Langhans *et al*., 2007; Toyooka *et al.*, 2009; Chevalier *et al.*, 2010) as well as on other plant cells (Zhang & Staehelin, 1992; Driouich *et al*., 1993; Zhang *et al*., 1993; Driouich & Staehelin, 1997; Lam *et al*., 2007; Ebine *et al.*, 2008). Moreover, the Tokuyasu immuno-cryosectioning method is widely used in animal systems to study the distribution of membrane proteins, such as receptor molecules (Liou *et al*., 1997; Klumperman *et al*., 1998) but few applications are available in plant systems so far. In *Arabidopsis* root cells, immuno-cryosectioning has allowed localization of coat components such as COPI, as well as of many other proteins involved in vacuolar trafficking, including syntaxin, a transmembrane receptor protein called BP-80, μ-adaptin and vacuolar H^+/ ATPase (Conceiçao *et al*., 1997; Sanderfoot *et al*., 1998; Hinz *et al*., 1999; Pimpl *et al*., 2000; Happel *et al*., 2004; Dettmer *et al*., 2006). More recently, Von der Fecht-Bartenbach *et al*. (2007) localized a chloride transporter (AtCLC-d-GFP) to the *trans* side of Golgi stacks in ultrathin cryosections of *Arabidopsis* root epidermal cells. Applied to tobacco BY-2 suspension-cultured cells, the Tokuyasu immuno-cryosectioning method has been published in a limited number of articles (Saint-Jore-Dupas *et al.*, 2006).

In this context, we report an overview of the technical conditions for tobacco BY-2 suspension-culturd cells preparation, using HPF/FS or cryosectioning methods. The impact of different pre-cooling and freeze substitution media prior to HPF and pre-fixative solutions for cryosectioning were assessed on both the quality of endomembrane preservation in tobacco BY-2 cells and immunogold localization of GFP-tagged Golgi proteins. Despite their high level of hydration, we demonstrate that BY-2 cells were successfully prepared by these cryomethods which could be routinely applied for the detection of Golgi-membrane-bound proteins in this plant model.

MATERIALS AND METHODS

Growth conditions

Tobacco BY-2 suspension-cultured cells were grown *in vitro* as described previously (Gomord *et al*., 1998). Cells were harvested by filtration 3 d after subculturing and were immediately used for experiments.

Construction of GFP-tagged proteins and transformation of plant cells

The coding sequence of a xyloglucan-β (1-2) galactosyltransferase (At2g20370) from *Arabidopsis thaliana* (MUR3) (Madson *et al*., 2003) was amplified by PCR without the stop codon, using respectively Forward Kpn1 (5′- GGGGTACCCCATGTTTCCAAGGGTT TCT-3′) and Reverse Nhe1 (5′- CTAGCTAGCCTG TGTCTTATCTCTCTG-3′) primers. The SALK cDNA clones were used as template (S81470). The amplified cDNA was then introduced into the pBLTI221 binary vector containing the sGFP, allowing a N-terminal fusion to the sGFP, and the resulting construct was verified by sequencing. Finally, the cassette containing the chimeric protein was introduced into pBLTI121

binary vector to allow plant transformation. Transformation of *Nicotiana tabacum* (cv. BY-2) cells with *Agrobacterium tumefaciens* was performed as described by Gomord *et al*. (1998). Transgenic lines expressing β (1-2) galactosyltransferase fused to GFP (MUR3-GFP) were obtained as described by Chevalier *et al.* (2010). Transgenic lines of tobacco BY2 suspension-cultured cells expressing the first 99 amino acids of soybean β (1-2) mannosidase I (Nebenführ *et al.*, 1999) membrane protein fused to GFP (ManI-GFP) were obtained as described by Saint-Jore-Dupas *et al.* (2006).

High-pressure freezing, freeze substitution and freeze drying

HPF was performed with the freezer HPF-EM PACT I Leica-microsystems (Studer *et al*., 2001). Prior to freezing, 3 d-old tobacco (BY-2) suspension-cultured cells were treated with different cryoprotectants such as: 20% (v/v) glycerol (Sigma), 0.4 M (w/v) sucrose (Sigma), 20% (w/v) Bovine Serum Albumin (BSA) (Sigma), 20% (w/v) arabic gum (Sigma) or 25% (w/v) dextran 70 KD (Sigma) diluted in culture medium. For glycerol treatment, the cells were concentrated on 50 μm nylon membrane and incubated successively in 5% and 10% glycerol (v/v) for 1 h at +4˚C and under light stirring. Cells were subsequently transferred to 20% (v/v) glycerol and immediately frozen. For the other pre-cooling treatment, cells were incubated for a few minutes in Murashige and Skoog (MS) medium (Sigma) supplemented by cryoprotectants.

Tobacco BY-2 cells pre-treated or not were concentrated on 50 μm nylon membrane and transferred into the cavity of a copper ring used for cryofracture, which is 100 μm in depth and 1.2 mm in diameter. Excess medium was lightly absorbed by filter paper. Using a horizontal loading station, the specimen carriers were tightened securely to the pod of specimen holder. After fixation on the loading device, specimens were frozen according to a maximum cooling rate of 10000˚C sec^{-1}, incoming pressure of 7.5 bars and working pressure of 4.8 bars. Rings containing frozen samples were stored in liquid nitrogen until the freeze substitution procedure was initiated.

After HPF the samples were transferred to a FS automate (AFS, Leica Vienna Austria) pre-cooled to –140˚C. FS conditions followed a modified procedure from D. Studer (personnal communication). For ultrastructure analyses, substitution media consisted of anhydrous acetone, supplemented with 2% (w/v) osmium tetroxide (Polysciences Inc.). The samples were substituted at –90˚C for 72 hrs. The temperature was gradually raised (2˚C/hr) to –60˚C and stabilized during 12 hrs, then gradually raised (2˚C hr^{-1}) to –30˚C (12 hrs) and gradually raised again (2˚C hr^{-1}) to 0˚C for 2 hrs. The samples were washed at room temperature with fresh anhydrous acetone (Fischer). Infiltration was done at +4˚C in acetone-Spurr's resin (Polysciences Inc.) (ratio 2v:1v; 1v:1v; 1v:2v, 8 hrs each step) and with pure resin for at least 2 d. Polymerization was performed at 60˚C for 16 hrs. Samples destined for immunocytochemistry were substituted in anhydrous acetone +0.5% (w/v) uranyl acetate (Polysciences Inc.), using a similar program as that described above, except that the final substitution step was performed at –15˚C. The samples were rinsed twice with anhydrous ethanol (VWR). Infiltration was processed at –15˚C in a solution containing ethanol: London Resin White (LRW) (Polysciences Inc.) (ratio 2v:1v; 1v:1v; 1v:2v, 8 hrs each step) and with pure resin for 2 x 24 hrs. Polymerization was performed into the AFS apparatus at –15˚C under UV light for 48 hrs.

Cryosectioning

For cryosectioning (Tokuyasu, 1973 modified), 3 d-old tobacco BY-2 cells were harvested by filtration on 50 μm nylon membrane and fixed at +4˚C with a mixture of 1.5% (v/v) paraformaldehyde (PAF) (Prolabo) and 0.2% (v/v) glutaraldehyde (GA) (Agar) (fixative solution 1) or 2% (v/v) PAF and 2% (v/v) GA (fixative solution 2) in 0.1 M Na-phosphate, pH 7.2 buffer (PB) for 16 hrs. After successive washing with a mixture containing 0.1 M glycine (Fischer) in PB saline (PBS: 0.01 M Na-phosphate pH 7.2, Sigma), and 0.15 M NaCl (Fischer), the cells were progressively embedded in 12% gelatin (w/v) in 0.1 M PB. Small blocks (1 to 2 mm^3) of gelatin were cut at +4˚C and transferred into 1.2 M sucrose in 0.1 M PB for 1 hr, then into 2.3 M sucrose in 0.1 M PB for 2 hrs, followed by infiltration with fresh 2.3 M sucrose in 0.1 M PB overnight and then into successive solutions of 2.3 M sucrose in 0.1 M PB for 72 hr periods. The sucrose infiltration method mentioned just above was performed at +4˚C with stirring. Gelatin cubes were then mounted onto specimen stubs, frozen in liquid nitrogen by plunging, and cut into 70 nm ultrathin sections at –120˚C with Ultracut UCT microtome (Leica Vienna Austria). Frozen sections were picked up as described by Liou *et al.* (1996) by using a mixture of 2.3 M sucrose in PB and 2% (w/v) methylcellulose and transferred on formvar-coated nickel grids stabilized with carbon.

Immunogold labeling of GFP-tagged membrane protein

Ultrathin sections (50-70 nm; ultracut UCT Leica Vienna Austria) of HPF-prepared tobacco BY-2 cells expressing MUR3-GFP fusion protein were collected on carbon-formvar-coated nickel grids and blocked in Tris-Buffered Saline (TBS: Tris HCl 20 mM pH 7.2, Fischer, 200 mM NaCl) supplemented with 0.2% (w/v) BSA and 0.05% (w/v) Tween 20 (Sigma) for 15 min. Sections were then incubated with the polyclonal anti-GFP antibody (BD living colors A.v peptide antibody – Clontech) at 1:5 dilution in TBS + 0.2% (w/v) BSA and Normal Goat Serum 1:30 (NGS; British Biocell International) for 2 hrs at room temperature. After washing in TBS + BSA, the grids were incubated for 1 hr at room temperature in the anti-rabbit secondary antibody conjugated to 10 nm gold particles (Tebu-British Biocell International). After washing and fixing in TBS +2% (v/v) GA, the sections were first stained with 4% (w/v) vapour osmium followed by classical staining (uranyl acetate and lead citrate).

Cryosections of tobacco BY-2 cells expressing the soybean β (1-2) mannosidase I-GFP fusion protein were immunolabelled with anti-GFP antibodies derived from a rabbit's polyclonal serum (Pagny *et al*., 2003). To this purpose, grids with frozen sections were first incubated in PBS containing 0.1% (w/v) glycine to inactivate any residual fixative. The grids were blocked in PBS with 1% (w/v) BSA for 2 min and then incubated with the anti-GFP antibodies diluted 1:100 in blocking buffer for 30 min. After washing in PBS, the grids were blocked in NGS 1:30 in PBS containing 0.1% (w/v) BSA. After incubation with the secondary antibody (British Biocell International, EM gold conjugates, goat-anti-rabbit IgG-10 nm) diluted 1:25 in blocking buffer for 30 min, the grids were incubated with glutaraldehyde 1% (v/v) in PBS for 5 min and finally washed in water. The specimens were stained for 5 min with cold 2% (w/v) methyl cellulose (Sigma) containing 0.4% (w/v) uranyl acetate, at pH 4. Two controls were systematically done by labeling sections of non-transformed tobacco cells and by omitting the first antibody.

Observations were made with a Tecnai 12 Biotwin TEM (FEI company Eindhoven NL) at 80 KV. Image acquisitions were made with a coupled charge device Megaview II camera controlled by AnalySis software (Eloise Paris France).

RESULTS

HPF/FS and Tokuyasu cryosectioning methods were used to process 3 d-old tobacco BY-2 suspension-cultured cells before TEM investigations. With the HPF/FS approach, five types of components for freezing conditions were tested on subcellular preservation, using a standard osmium tetroxide/acetone condition for FS followed by Spurr's resin embedding. The best conditions were then selected to determine their impact on the detection of xyloglucan-galactosyltransferase fused to GFP (MUR3-GFP), using acetone/uranyl acetate as FS medium followed by LRW embedding. According to the Tokuyasu method, the effect of two fixative solutions were assessed on ultrastructural patterns of the cells as well as immunogold labeling of another glycosyltransferase fused to GFP, the N-Glycan-mannosidase I (ManI – GFP).

Ultrastructural preservation of high pressure frozen and freeze substituted tobacco BY-2 suspension-cultured cells

Freezing medium composition was firstly studied on the subcellular structure of 3 d-old BY-2 cells. Freezing of cells directly in their culture medium MS without any additive induces important ice damage leading to very poor preservation of the cell structure (data not shown). Similar damage was observed in samples in which freeze drying and FS were applied as dehydration processes. Noticeable injuries came from an inefficiency of freezing, including tonoplast disruption, segregation phase and extremely low integrity of organelles within the cytoplasm (data not shown). As for Golgi membrane preservation, those conditions were not sufficient to obtain a well preserved ultrastructure. Thus, to get an optimal freezing of this organelle in BY-2 cells, cryoprotectants are required (Figs 1 and 2).

Pre-freezing treatment with glycerol or sucrose and embedding in Spurr's resin

Figure 1A-E shows the general appearance of a tobacco BY-2 suspension-cultured cells frozen in culture medium containing 20% (v/v) glycerol. The cytoplasm presented no visible ice damage and was deprived of any phase segregation. Its ground appeared densely stained with granular texture and was particularly rich in organelles, demonstrating that no extraction occurred during sample preparation. The nucleus was well preserved, showing a smooth nuclear

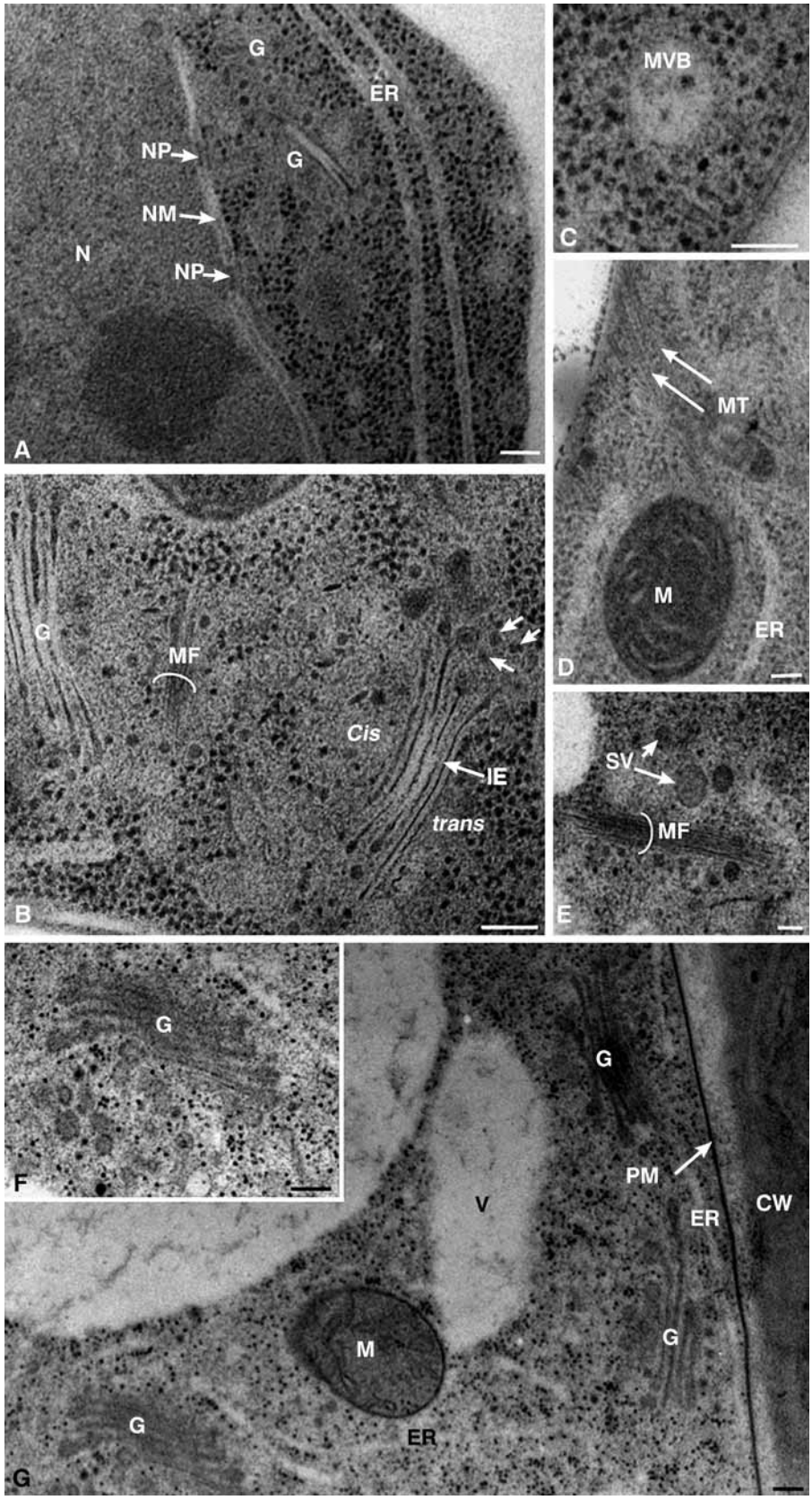

FIG. 1. Electron micrographs of tobacco BY2 suspension-cultured cells cryofixed by HPF. Cells were frozen in 20% (v/v) glycerol (A-E) or in 0.4 M sucrose (F, G). Cryofixed cells were substituted in acetone + 2% (w/v) osmium tetroxide and embedded in Spurr's resin. CW: cell wall; ER: endoplasmic reticulum; G: Golgi stacks; IE: intercisternal elements; M: mitochondria; MF: microfilaments; MT: microtubules; MVB: multi-vesicular body; N: nucleus; NM: nuclear membrane; NP: nuclear pore; PM: plasma membrane; SV: secretory vesicles; V: vacuole. Scale bars = 100 nm.

membrane which exhibited regular disconnections corresponding to nuclear pores. In our conditions, the condensed chromatin showed no evidence of ice crystal damage and appeared as flocculent substances in localized areas. Figure 1C-E depicts the fine ultrastructural details of organelles reported to be particularly susceptible to ice damage. As illustrated in Fig. 1B, Golgi stacks were well preserved. Figure 1B shows *trans* cisternae characterized by a lumen filled with densely stained material and the presence of intercisternal elements. It is worth noting that in plant cells, intercisternal elements are maintained only in cryofixed material showing optimal preservation (see Driouich & Staehelin, 1997). Endoplasmic reticulum membranes were frequently observed with ribosomes lining up their surface. Multi-vesicular bodies and mitochondria both exhibited a typical rounded shape (Fig. 1C-D). Aggregated microfilaments (Fig. 1E) supposed to be actin filaments could often be observed in the vicinity of Golgi vesicles as shown in Fig. 1B. Microtubules were also frequently observed (Fig. 1D) proving that the freezing protocol used is appropriate.

We also assessed the impact of 0.4 M sucrose on the freezing of tobacco BY-2 suspension-cultured cells. This pre-freezing condition provided similar results as glycerol regarding cytoplasm and endomembrane preservation (Fig. 1F, G). The abundance of ribosomes and other organelles throughout the cytoplasm indicated that little or no extraction had occur-

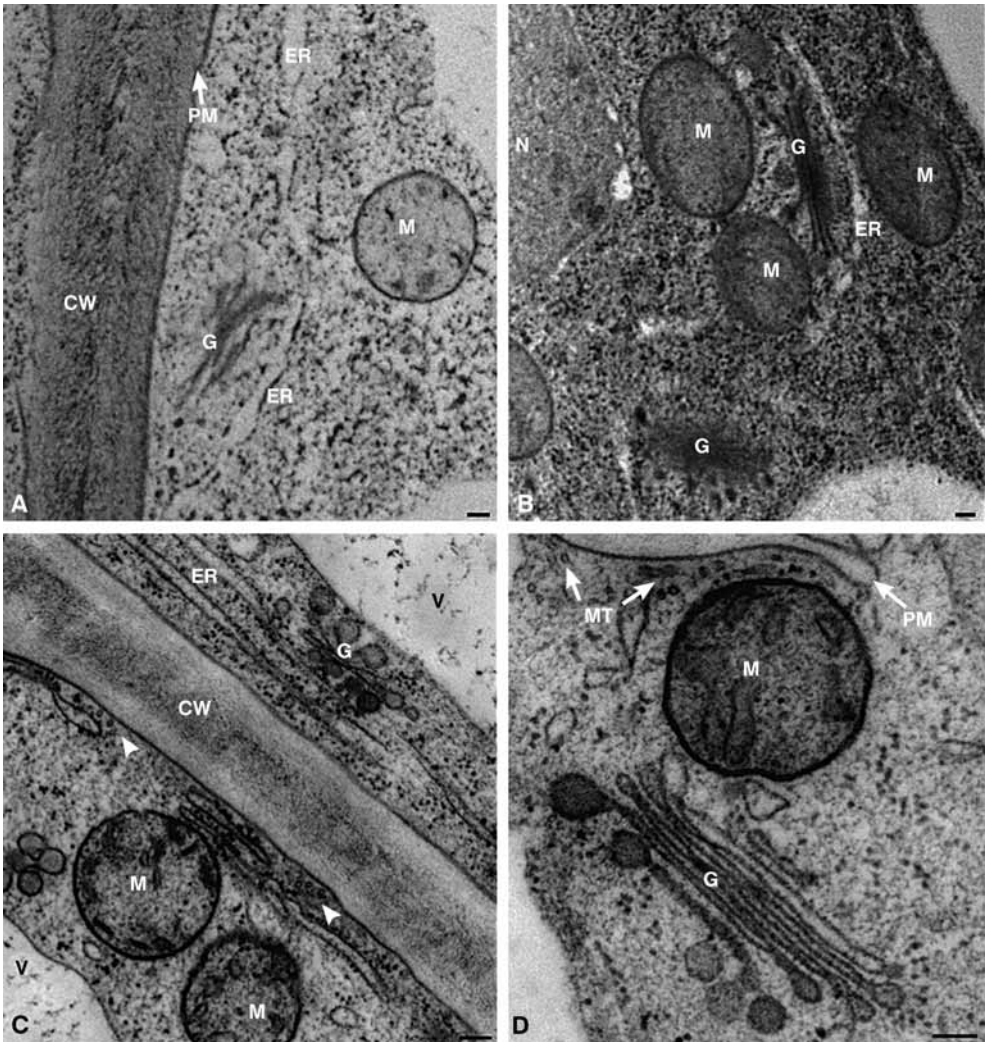

FIG. 2. Electron micrographs of tobacco BY-2 suspension-cultured cells cryofixed by HPF. Cells were frozen in 20% (w/v) BSA (A), 20% (w/v) arabic gum (B) and 25% (w/v) dextran (C, D). Cryofixed cells were substituted in acetone + 2% (w/v) osmium tetroxide and embedded in Spurr's resin. CW: cell wall; ER: endoplasmic reticulum; G: Golgi stacks; M: mitochondria; MT: microtubules; N: nucleus; PM: plasma membrane; V: vacuole. Scale bars = 100 nm.

red (Fig. 1G). Plasma membrane appeared regular as a straight line with no cracks (Fig. 1G). Golgi stacks and associated vesicles were also clearly discernable (Fig. 1F, G). The vacuolar sap, filled with dense material, was also well preserved (Fig. 1G). Comparing images obtained by glycerol (Fig. 1A-E) and sucrose (Fig. 1F, G), constrasting differences appeared on the membranes. It seems that membranes are "negatively" stained by glycerol pre-treatment and positively stained by sucrose.

Pre-freezing treatment with high molecular weight compounds and embedding in Spurr's resin

We have also assessed the ultrastructural preservation of BY-2 cells by supplementing the freezing medium with some high molecular weight cryoprotectants (Fig. 2). The addition of 20% (w/v) BSA was not sufficient enough to preserve well the cell structures (Fig. 2A). Similar damage was observed as in samples that were not cryoprotected before freezing. Although scattered ice defaults were present, treatment with arabic gum before freezing enhanced the quality of morphological preservation (Fig. 2B). The cytoplasm was dense with numerous ribosomes and some well preserved and clearly discernable Golgi stacks. Nevertheless, the last freezing medium tested, which consisted of MS medium with 25% (w/v) dextran, yielded the best ultrastructural preservation of BY-2 cells (Fig. 2C, D). In addition to the excellent preservation of different cellular organelles, including Golgi stacks, endoplasmic reticulum, mitochondria and tonoplast, cells appeared turgescent with the plasma membrane tightly appressed to the cell wall (Fig. 2C). Excellent preservation of Golgi stacks was obtained, making the *cis*-to-*trans* polarity very easily recognizable (Fig. 2D). The well preserved cytoplasm was however confined to small areas of the cell.

Pre-freezing treatment with glycerol, sucrose or dextran and embedding in LRW resin

FS for ultrastructural analyses was performed in acetone containing 2% (w/v) osmium tetroxide as a standard substitution medium, according to McCully & Canny (1985). Osmium-free substitution method was secondly investigated for subsequent LRW embedding (Fig. 3). Tobacco BY-2 suspension-cultured cells

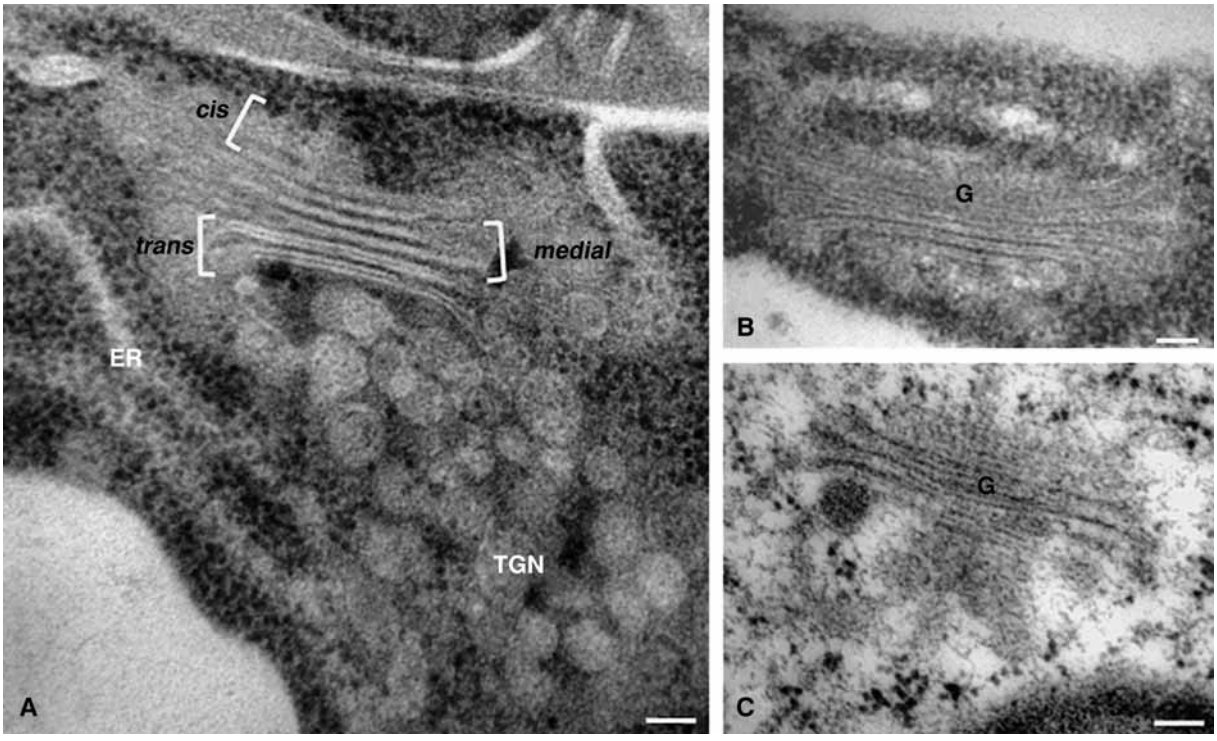

FIG. 3. Electron micrographs of tobacco BY-2 suspension-cultured cells frozen by high pressure in 20% (v/v) glycerol (A), in 0.4 M sucrose (B), and 25% (w/v) dextran (C). Cells were substituted in acetone + 0.5% (w/v) uranyl acetate and embedded in LRW resin. ER: endoplasmic reticulum; G: Golgi stacks; TGN: trans-Golgi network. Scale bars = 100 nm.

frozen in glycerol (Fig. 3A) or sucrose (Fig. 3B) and freeze-substituted in acetone containing 0.5% (w/v) uranyl acetate presented excellent ultrastructural preservation. The well preserved Golgi cisternae were easily distinguishable with *cis* to *trans* polarity and secretory vesicles of various sizes. It is worth noting that, under these conditions of FS, BY-2 cells frozen in glycerol yielded the highest quality of preservation. In contrast, cells frozen in dextran followed by FS in uranyl acetate/acetone presented ice crystal damage throughout the whole cytoplasm. In general, organelle preservation was poor and endomembranes, including Golgi cisternae, were hardly discernable (Fig. 3C).

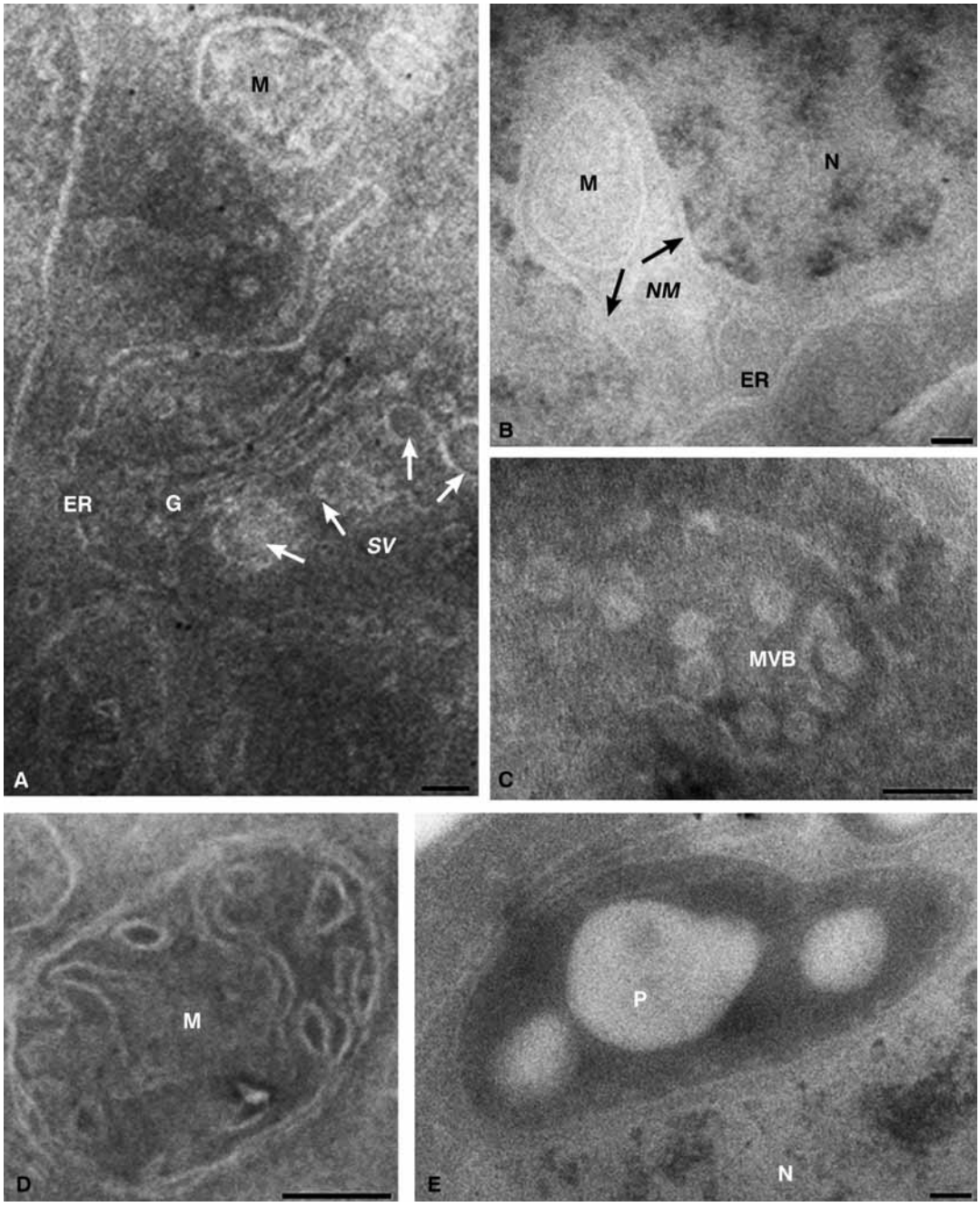

FIG. 4. Electron micrographs of transgenic tobacco suspension-cultured BY-2 cells preserved by cryosectioning method. Cells were chemically pre-fixed with 1.5% (w/v) PAF + 0,2% (v/v) GA (A) or 2% (w/v) PAF+ 2% (v/v) GA (B-E). ER: endoplasmic reticulum; G: Golgi stacks; M: mitochondria; MVB: multi-vesicular body; N: nucleus; NM: nuclear membrane; P: plastid; SV: secretory vesicles. Scale bars = 100 nm.

Ultrastructure of cryosectioned suspension-cultured BY-2 cells

Chemical fixation is a prerequisite for cryosections to maintain integrity during thawing and subsequent immunolabeling. Therefore, the effect of two fixative solutions was investigated on subcellular features of BY-2 cells that were fixed for 16 hrs with 1.5% (v/v) PAF – 0.2% (v/v) GA (fixative solution 1) or 2% (v/v) PAF – 2% (v/v) GA (fixative solution 2) prior to cryosectioning.

The use of fixative solution 1 affected drastically the preservation of cellular organelles (data not shown). Membranes were not clearly discernable and the cytoplasm did not appear uniformly preserved, suggesting that extraction occurred. As illustrated in Fig. 5C, similar results were observed for Golgi stacks whose *cis* to *trans* polarity was not preserved.

Increasing concentration of GA to 2% (v/v) (fixative solution 2) enhanced the quality of ultrastructure (Fig. 4A-E) and the visualization of membranes limiting different organelles, including the nuclear membrane (Fig. 4A), endoplasmic reticulum (Fig. 4B), multi-vesicular bodies (Fig. 4C), mitochondria (Fig. 4D) and plastids (Fig. 4E). Compared to the first fixative solution, Golgi stacks have preserved their structural integrity with distinct compartments as well as the a-

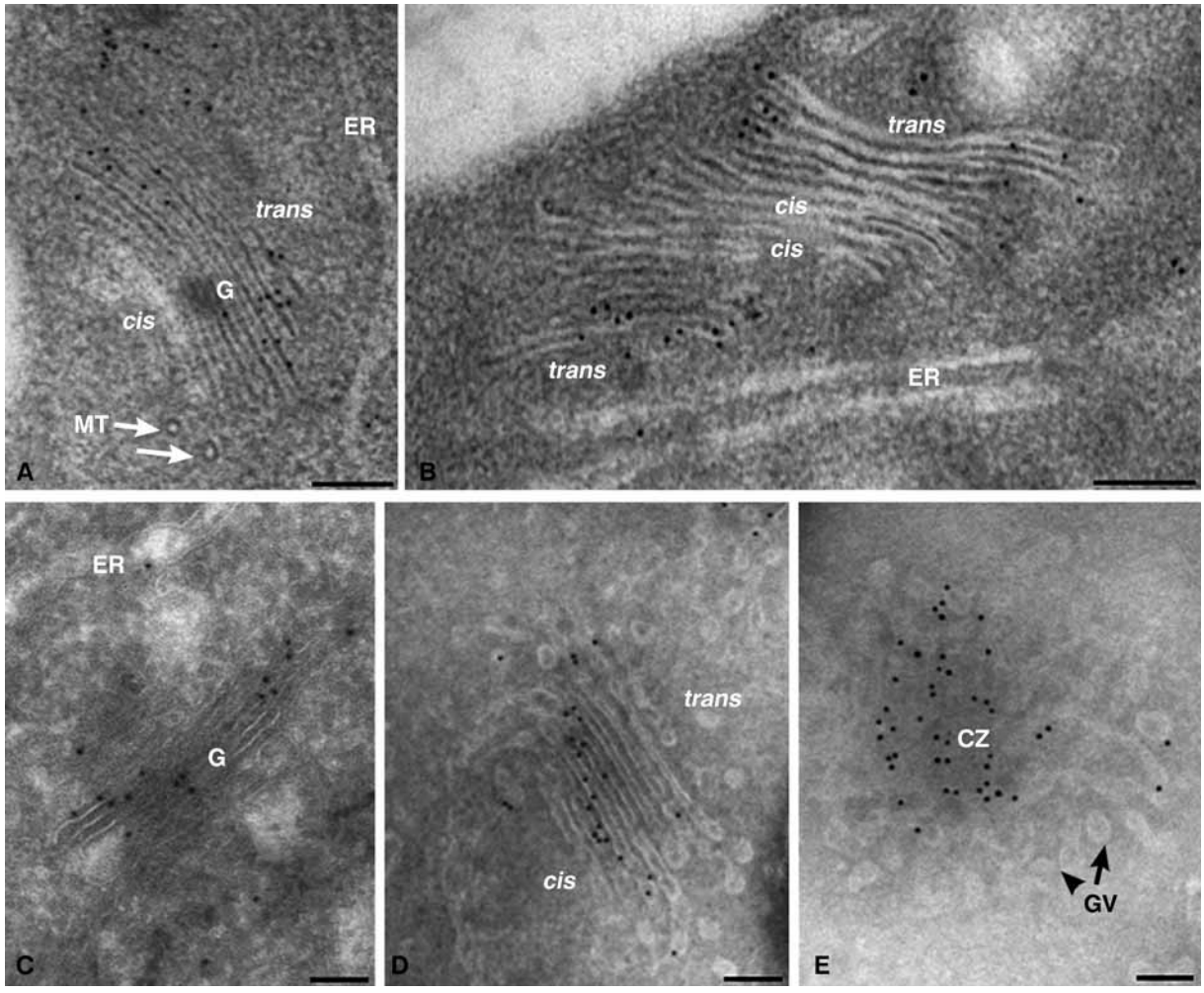

FIG. 5. Electron micrographs showing immunogold localization of GFP-tagged Golgi proteins under cryopreparation methods in transgenic tobacco BY-2 suspension-cultured cells. (A, B) Distribution of MUR3-GFP in cells prepared with HPF/FS, pretreated by 0.4 M sucrose (A) or 20% (v/v) glycerol (B), shows labeling in Golgi stacks (A) and in *medial*/*trans* Golgi margins (B). (C-E) Distribution of ManI-GFP in cryosectioned cells pre-fixed with 1.5% (w/v) PAF + 0.2% (v/v) GA (C) or 2% (w/v) PAF + 2% (v/v) GA (D, E). A cross-section view (D) of Golgi apparatus or a top view (E) of cisternae showing abundant labeling of the central zone as compared to the margins. CZ: central zone; ER: endoplasmic reticulum; G: Golgi stacks; GV: Golgi vesicles; MT: microtubules. Scale bars = 100 nm.

bundance of Golgi-derived vesicles (see Fig. 4A and Fig. 5D, E). It appeared that the original structure of Golgi stacks is better stabilized with high concentration of GA, allowing the identification of different subtypes of cisternae (Fig. 5C, D) according to the *cis*, *medial* and *trans* polarity previously described (Staehelin *et al*., 1990; Zhang & Staehelin, 1992; Driouich *et al*., 1993). Moreover, Figures 4B and 5D, E showed the presence of numerous secretory vesicles in the vicinity of Golgi, illustrating that the quality of endomembrane preservation was improved using those conditions.

Immunogold localization of GFP tagged-Golgi proteins under HPF/FS method

To assess antigenicity preservation of our samples prepared under various conditions, we used transgenic lines of BY-2 cells expressing GFP-fused proteins. A xyloglucan-galactosyltransferase from *Arabidopsis* (MUR3-GFP) and a soybean mannosidase I (ManI-GFP) were localized respectively using HPF/FS and cryosectioning procedures. Since the best conditions of HPF/FS amenable to immunocytochemistry were obtained by using sucrose and glycerol as freezing medium, only these two treatments were applied to the MUR3-GFP detection (Fig. 5A, B). As shown in Figure 5B, labeling with the polyclonal GFP-antibodies was found specifically over Golgi stacks and mostly detected in the margins of *medial*/*trans* cisternae. Similar results in terms of localization were observed for samples frozen in sucrose (Fig. 5A).

Immunogold localization of GFP tagged-Golgi proteins with Tokuyasu method

The impact of the two fixative solutions on the immunogold localization of ManI-GFP within the Golgi stacks was also examined. Detection of GFP-labeling over Golgi stacks was possible in both conditions as shown in Figure 5C-E. With an improved ultrastructure quality, Figure 5D illustrates specifically the distribution of the ManI-GFP with most of the labeling over the *cis* moiety of the Golgi stacks, on cryosectioned transformed BY-2 cells prepared with 2% (v/v) PAF-2% (v/v) GA (fixative solution 2). In addition, the top view of the Golgi cisternae (Fig. 5E) showed that the majority of gold particles were associated with the central zone of the cisternae. The density of labeling was compared and found to be higher in conditions with great concentration of GA (Fig. 5D) than in low GA concentration (Fig. 5C): an average of 21 ± 4.2 gold particles per Golgi stack was numbered with 2% (v/v) GA, compared to 9 ± 1.4 with 0.2% GA (v/v).

DISCUSSION

HPF/FS and cryosectioning are recognized respectively as the best established fixation and immunodetection methods. Nevertheless, in spite of the availability of those tools, relatively few studies on plant suspension-cultured cells have been reported to date. In our work, technical conditions in HPF/FS and cryosectioning for tobacco BY-2 cell preparations were overviewed with a particular focus on the Golgi apparatus and their impact on GFP-tagged protein localization is discussed below.

In the HPF/FS procedure, the most critical phase of the whole fixation process is undoubtedly the preceding preparation prior to freezing of specimen, as very well described in the recent upgrade published by McDonald *et al.* (2007). In our study, the use of FS media free of strong fixatives amenable for immunoanalysis, provided optimal freezing quality. It is well known that the water content of vacuolated cells (such as tobacco suspension cells) is a crucial factor for vitrification of hydrated specimens. The complex interactions between water and intracellular compounds and the various forms of water present in the different compartments, including vacuoles, cytoplasm and apoplast, greatly influence phase transitions during cooling procedures in spite of high cooling rate generated by HPF. Studer *et al*. (1995) demonstrated the dependence of vitrification on proteoglycan concentration and water content in articular cartilage. Similarly, Lepeault *et al.* (1997) studied vitrification of water and showed that amorphous ice of pure water could be obtained by HPF only by adding at least 20% (w/v) sucrose.

In the case of tobacco suspension-cultured cells, it was already described that freezing without any cryoprotectant gives some sufficient ultrastructural result to immuno-analysis of specific vacuolar or vesicular compartment, and multi-vesicular bodies (Tse *et al*., 2004; Oliviusson *et al*., 2006; Langhans *et al*., 2007). In our work, we showed that freezing tobacco cells without any additive induced important ice damage leading to poor preservation of cell structures, including the Golgi stacks. These ice injuries are not dependent on the dehydration process but result in the lack of efficiency of vitrification. In this context, cryoprotectants become essential to improve the quality of freezing by ensuring a rapid heat removal from the

sample without the formation of ice crystals. Usually, cryoprotectants are divided into two categories, including non-penetrating (e.g. dextran, BSA, arabic gum and sucrose) and penetrating compounds (e.g. glycerol). Extracellular compounds act as an "embedding agent", thereby increasing the viscosity of the outside medium. Thus, by preventing freezing outside the cell, the ice nucleation within the cell is delayed and ice damage is prevented (Echlin, 1992). They are often more suitable than intracellular cryoprotectants, which might change cell physiology. However, another classification of these agents, based on their osmotic activities, should be considered. Dextran, BSA and arabic gum are molecules with low osmotic activity, compared to sucrose or glycerol. Dextran and sucrose have previously been used in large HPF/ FS applications on sycamore or tobacco suspension-cultured cells (Driouich *et al*., 1993; Zhang *et al*., 1993; Samuels *et al*., 1995; Driouich & Staehelin, 1997; Winicur *et al*., 1998; Nebenführ *et al*., 1999) in contrast to glycerol. As shown in our results, dextran could be an effective cryoprotectant when tobacco suspension-cultured cells were freeze-substituted in osmium tetroxide and embedded in Spurr's resin (Fig. 2C, D) but not when FS conditions deprived of strong fixatives followed by LRW resin were applied (Fig. 3C). In this case, segregation compartments caused by crystallization were present. These data are in line with the previous report of Matsko & Müller (2005), who demonstrated that Spurr's components were involved in the stabilization of fine structures after HPF, resulting in higher quality of ultrastructure compared to LRW resin. Nevertheless, the major drawback in the use of dextran is its incompatibility with substitution fluids such as acetone supplemented with uranyl acetate suitable for immunolabeling. The possible interaction of dextran with uranyl salts (Kiss *et al*., 1990; Giddings, 2003) leads to an important reduction of freeze substitution efficiency inducing re-crystallization when temperature increases during the substitution process. In those conditions, ultrastructure is damaged. In addition, high concentration of dextran may exert a non colligative osmotic pressure involved in some membrane shrinkage.

Freezing in glycerol or sucrose provided good preservation with LRW resin (Fig. 3A, B), avoiding the necessity of using osmium tetroxide in the freeze substitution medium as generally proposed in the literature (Nebenführ *et al.*, 1999; Toyooka *et al*., 2009). Glycerol is a natural compound found in the plant cell membranes and known to play a key role in tolerance to various abiotic stresses such as cold or dehydration (Eastmond, 2004). Although glycerol is identified as a penetrating cryoprotectant, its entry into the plant cell may be limited by the cell wall (McGann, 1978; Echlin, 1992; Hayat, 2000). In our experiments, glycerol incubation was performed gradually on non-fixed cells at +4°C, which decreased greatly the cell glycerol penetration (McGann, 1978; Echlin, 1992). Thus, in this context, the glycerol played the role of an osmotic agent, like sucrose. The good ultrastructural preservation of Golgi stacks obtained with sucrose and glycerol treatment is due to an osmotic effect that does not occur with dextran. This finding suggests that the optimal freezing of BY-2 cells requires their light dehydration. To preserve the integrity of endomembranes, osmotical action of sucrose or glycerol is more successful than dextran.

The lack of membrane staining when glycerol is used as cryoprotectant, compared to sucrose, could be explained by the behaviour of membranes during high pressure freezing. Because of the structure and composition (phospholipids closely arranged to each other in a double lamellar layer) of membranes, the ultrarapid freezing process could be modified and became different of free water. Wolfe & Bryant (1999) have shown that membrane vitrification is dependent on the ability of cryoprotectants to diffuse in the interlamellar spaces and each side of phospholipid layers. A good vitrification of membrane can be achieved if the cryoprotectant diffuses efficiently in the interlamellar spaces. Otherwise, the presence of different types of ice in intra- or extra- compartments could induce drastic injuries, such as membrane cracks or swells (Wolfe & Bryant, 1999). We can also speculate that the visibility of membranes could be modified according to vitrification status. Although no link between vitrification quality and their contrast was established, some authors notice the poor staining of membranes after HPF/FS, and added low rate of water in the FS medium to increase membrane contrast (Walther & Ziegler 2002; Giddings, 2003).

When sucrose or glycerol is added to the freezing medium, no difference in immunogold localization of specific Golgi enzymes (Fig. 5A, B) was observed. These findings are illustrated by the localization of the MUR3-GFP fused protein involved in the synthesis of the xyloglucan, a major hemicellulosic polysaccharide of dicotyledonous primary cell wall (Carpita & McCann, 2000). In line with the previous work of Zhang & Staehelin (1992) on the distribution of oligosaccharide linked to xyloglucan, we recently

showed that the enzyme responsible for the galactosylation of this polymer is mostly localized in the *medial*/*trans* margins of Golgi cisternae (Chevalier *et al*., 2010). We demonstrate here that whatever the precooling treatment applied to BY-2 cells, sucrose or glycerol, the localization of these GFP-Golgi fused proteins is the same. Given the fact that ultrastructural preservation is better with glycerol pretreatment, we also used this procedure to describe two other XyG synthesizing enzymes localization in BY-2 cells prepared by HPF/FS (Chevalier *et al*., 2010).

Tokuyasu thawed cryosectioning technique is based on fixation with aldehydes, infiltration with high concentration of cryoprotectant, followed by plunging in liquid nitrogen and finally cryosectioning on a microtome cryo-chamber at –90˚C and –130˚C. During this process, two major difficulties are generally encountered: the cryoprotection step and the retrieval section. The presence of sucrose, widely used in high concentration, ensures the vitrification of the biological material when freezed upon plunging into liquid nitrogen. Thus infiltration of sucrose into the cell seems to be essential to prevent ice damage and to achieve vitrification, in contrast to the HPF/FS approach. Therefore, aldehyde fixation plays an important role by permeabilizing the plasma membrane, in addition to stabilizing cell structure. Despite that fixed cells are permeable to sucrose, the infusion rate into cell is dependent on the fixation intensity (Hayat, 2000). When applied to plant cell material, sucrose infiltration is obviously impaired by the presence of cell wall, which constitutes a great biological barrier. This results generally in severe plasmolysis of the cell, which can be overcome by increasing fixative concentration and length of incubation to 72 hrs, in gradual sucrose solutions.

Section retrieval step in the cryo-chamber is known to be a critical step in the cryosectioning approach. This step can induce damage of the cell endomembranes (Skepper, 2000) and must be performed rapidly to avoid the freezing of the retrieval medium. When 2.3M sucrose solution is used, the time needed to retrieve sections in a cryo-chamber maintained at –100˚C was estimated at 3 sec (Skepper, 2000). An alternative mixture of 2% (w/v) methyl cellulose and 2.3M sucrose helps to avoid tissue over-streching caused by the strong osmotic activity of sucrose (Griffiths, 1993). Consequently, this solution freezes faster than sucrose, shortening the section retrieval time. Thus, we believe that improving pre-fixation will prevent damage occurring at this critical stage. Indeed, fixation with 1.5% (v/v) PAF – 0.2% (v/v) GA for 16 hrs at +4˚C resulted in insufficient ultrastructural preservation (see Fig. 5C) with a quality below that obtained after chemical co-fixation method (Follet-Gueye *et al.*, 2003).

Increasing the fixative concentration proved a marked improvement of cellular preservation. In particular, the morphology of the endomembrane system was very well preserved, matching that obtained with HPF technique in BY-2 cells (see Fig. 5). This method also allowed a clear distinction of the *cis* to *trans* Golgi polarity. Despite the good patterns of cell ultrastructural preservation, the use of a high fixative concentration is known to hamper epitope accessibility. In our work, use of 2% (v/v) GA fixative combined to PAF afforded a high density of gold particles (Fig. 5C, D) and the precise GFP localization within Golgi membranes (Fig. 5C, D). We observed that the GFP localization was predominantly associated with the *cis* and less frequently in the *medial* Golgi cisternae compartments, as previously described (Saint-Jore-Dupas *et al*., 2006). Similar distribution of this Golgi membrane protein was obtained using either high-pressure frozen tobacco BY-2 cells (Nebenführ *et al.*, 1999) or chemically co-fixed cells (data not shown), but the density of labeling was much weaker than that obtained in cryosectioned samples. More recently, Staehelin & Kang (2008), in a paper dedicated to EM tomography, showed also that the native β mannosidase was predominantly located to the central zone of the medial cisternae in *Arabidopsis* root tip as presented here in Figure 5D, E.

Impacts of Tokuyasu cryosectioning *versus* HPF/FS preparations on antigenicity are highly dependent either on the sample and the antigen (Lauber *et al*., 1997; Hess, 2007; Stierhof & Kasmi, 2010). Provided that antigens were maintained in their original location and not extracted during chemical fixation and sucrose infiltration, the accessibility of epitopes at the thawed cryosection surface is better when compared to that of the resin section surface. Moreover, in the Tokuyasu cryosectioning approach, because tissues are only fixed with low concentrations of aldehydes and remain in an aqueous environment prior to immunolabeling, epitope detection would sometime turn out to be more favorable. Here, in the Tokuyasu technique, to perform the ultrastructural preservation of tobacco BY-2 cells, we fixed them with an aldehyde mixture containing 2% (v/v) PAF and 2% (v/v) GA and then infiltrated them slowly with 2.3M sucrose. We show here that this process is not deleteri-

ous to GFP-tag detection in transgenic BY-2 cells. On the other hand, we also reveal that the GFP-tag is insensitive to HPF/FS procedure following by LRW resin embedding.

Whereas Tokuyasu cryosectioning is often assumed to be the most suitable approach to label membrane-bound proteins (Liou *et al*., 1996; Griffiths & Posthuma, 2002), the ultrastructural features of BY-2 cells was often inferior to HPF/FS experiments. The major drawback is the great cell plasmolysis which is reduced by a progressive sucrose infiltration and stronger fixation. Alternatively, it would be interesting to combine the quality of the ultra-rapid cooling with the sensitiveness of thawed frozen section technique. In practice, a modified approach was proposed by Van Donselaar *et al*. (2007) and Ripper *et al.* (2008) named the "rehydration method". It combines HPF/FS procedure followed by a rehydration step, infiltration in sucrose and cryo-sectioning. The rehydration method is particularly sufficient to investigate some extractable epitopes like polysaccharides that are often lost during Tokuyasu process and was recently used to immunolocalize epitopes associated with a hemicellulosic compound, the xyloglucan (Viotti *et al.*, 2010). This new approach was also successfully applied to *Arabidopsis thaliana* plant model on pollen grains, cotyledons and root tip (Stierhof & Kasmi, 2010) but never described on tobacco BY-2 cells. Considering the high hydration level of BY-2 cells, successive dehydration and rehydration steps might be delicate. However, it could be of interest to adapt the rehydration method to plant suspension cells like tobacco BY-2 cells.

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REFERENCES

Arabidopsis Genome Initiative, 2000. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature*, 408: 796-815.

Brandizzi F, Irons SL, Johansen J, Kotzer A, Neumann U, 2004. GFP is the way to glow: Bioimaging of the plant endomembrane system. *Journal of Microscopy*, 214: 138-158.

Carpita N, McCann M, 2000. The cell wall. In: Buchanan BB, Gruissem W, Jones RL, eds. *Biochemistry and molecular biology of plants*. American Society of Plant Physiologists, Rockville, Maryland: 52-108.

Chevalier L, Bernard S, Ramdani Y, Lamour R, Bardor M, Lerouge P, Follet-Gueye ML, Driouich A, 2010. Subcompartment localization of the side chain xyloglucan-synthesizing enzymes within Golgi stacks of tobacco suspension-cultured cells. *Plant Journal*, 64: 977-989.

Conceiçao AdS, Marty-Mazars D, Bassham DC, Sanderfoot AA, Marty F, Raikhel NV, 1997. The synaxin homolog AtPAP12p resides on a late post-Golgi compartment in plants. *Plant Cell*, 9: 571-582.

Dettmer J, Hong-Hermesdorf A, Stierhof YD, Schumacher K, 2006. Vacuolar H^+-ATPase activity is required for endocytic and secretory trafficking in *Arabidopsis*. *Plant Cell*, 18: 715-730.

Dhanoa PK, Sinclair AM, Mullen TR, Mathur J, 2006. Illuminating subcellular structures and dynamics in plants: a fluorescent protein tool box. *Canadian Journal of Botany*, 84: 515-522.

Driouich A, Faye L, Staehelin LA, 1993. The plant Golgi apparatus: a factory for complex polysaccharides and glycoproteins. *Trends in Biochemical Sciences*, 18: 210-214.

Driouich A, Staehelin LA, 1997. 7-dehydrobrefeldin A, a natural-occuring BFA derivative, inhibits secretion and causes a *cis*-to-*trans* breakdown of Golgi stacks in plants cells. *Plant Physiology*, 113: 487-492.

Eastmond P, 2004. Glycerol –insensitive *Arabidopsis* mutants: gli1 seedlings lack glycerol kinase accumulate glycerol and are more resistant to biotic stress. *Plant Journal*, 37: 617-625.

Ebine K, Okatani Y, Uemura T, Goh T, Shoda K, Niihama M, Morita MT, Spitzer C, Otegui MS, Nakano A, Ueda T, 2008. A SNARE complex unique to seed plants is required for protein storage vacuole biogenesis and seed development of *Arabidopsis thaliana*. *Plant Cell*, 20: 3006-3021.

Echlin P, 1992. *Low temperature microscopy and analysis*. Plenum Press, New York.

Follet-Gueye ML, Pagny S, Faye L, Gomord V, Driouich A, 2003. An improved chemical fixation method suitable for immunogold localization of green fluorescent protein in the Golgi apparatus of tobacco Bright Yellow (BY-2) cells. *Journal of Histochemistry and Cytochemistry*, 51: 931-940.

Giddings TH, 2003. Freeze substitution protocols for improved visualization of membranes in high pressure frozen samples. *Journal of Microscopy*, 212: 53-61.

Gomord V, Fitchette AC, Denmat LA, Michaud D, Faye L, 1998. Production of foreign proteins in tobacco cell suspension culture. In: Cunningham C, Porter AJ, eds.

Methods in biotechnology. Totowa Humana Press Inc., Vol. 3: 155-164.

Griffiths, G, 1993. *Fine structure immunocytochemistry*. Springer-Verlag, Heidelberg.

Griffiths JM, Posthuma G, 2002. A reliable and convenient method to store ultrathin thawed cryosections prior to immunolabeling. *Journal of Histochemistry and Cytochemistry*, 50: 57-62.

Happel N, Höning S, Neuhaus JM, Paris N, Robinson DG, Holstein SHE, 2004. *Arabidopsis* μA-adaptin interacts with the tyrosine motif of the vacuolar sorting receptor VSR-PS1. *Plant Journal*, 37: 678-693.

Hayat MA, 2000. *Principles and techniques of electron microsocpy: biological applications*. Fourth edition. Cambridge University Press, Cambridge.

Hess MW, 2007. Cryopreparation methodology for plant cell biology. *Methods in Cell Biology*, 79: 57-100.

Hinz G, Hilmer S, Bäumer M, Hohl I, 1999. Vacuolar storage proteins and the putative vacuolar sorting receptor BP-80 exit the Golgi apparatus of developing pea cotyledons in different transport vesicles. *Plant Cell*, 11: 1509-1524.

Hinz G, Colanesi S, Hillmer S, Rogers JC, Robinson DG, 2007. Localization of vacuolar transport receptors and cargo proteins in the Golgi apparatus of developing *Arabidopsis* embryos. *Traffic*, 8: 1452-1464.

Jolliffe NA, Brown JC, Neumann U, Vicré M, Bachi A, Hawes C, Ceriotti A, Roberts LM, Frigerio L, 2004. Transport of ricin and 2S albumin precursors to the storage vacuoles of *Ricinus communis* endosperm involves the Golgi and VSR-like receptors. *Plant Journal*, 39: 821-833.

Kiss JZ, Giddings TH, Staehelin LA, Sack FD, 1990. Comparison of the ultrastructure of conventionnally fixed and high pressure frozen/freeze substituted root tips of *Nicotiana* and *Arabidopsis*. *Protoplasma*, 157: 64-74.

Klumperman J, Kuliawat R, Griffiths JM, Geuze HJ, Arvan P, 1998. Mannose 6-phosphate receptors are stored from immature secretory granules via adaptor protein AP-1, clathrin and synaxin 6-positive vesicles. *Journal of Cell Biology*, 141: 359-371.

Lam SK, Siu CL, Hillmer S, Jang S, An G, Robinson DG, Jiang L, 2007. Rice SCAMP1 defines clathrin-coated, trans-Golgi-located tubular-vesicular structures as an early endosome in tobacco BY-2 cells. *Plant Cell*, 19: 296-319.

Lancelle SA, Hepler PK, 1992. Ultrastructure of freeze-substituted pollen tubes of *Lilium longiflorum*. *Protoplasma*, 167: 215-230.

Langhans M, Hawes C, Hillmer S, Hummel E, Robinson DG, 2007. Golgi regeneration after Brefeldin A treatment in BY2 cells entails stack enlargement and cisternal growth followed by division. *Plant Physiology*, 145: 527-538.

Lauber MH, Waizenegger I, Steinmann T, Schwarz H, Mayer U, Hwang I, Lukowitz W, Jürgens G, 1997. The *Arabidopsis* KNOLLE protein is a cytokinesis-specific syntaxin. *Journal of Cell Biology*, 139: 1485-1493.

Lepeault J, Bigot D, Studer D, Erk I, 1997. Freezing of aqueous specimen: an X ray diffraction study. *Journal of Microscopy*, 187: 158-166.

Liou W, Geuze HJ, Slot JW, 1996. Improving structural integrity of cryosections for immunogold labeling. *Histochemistry and Cell Biology*, 106: 41-58.

Liou W, Geuze HJ, Geelen MJH, Slot JW, 1997. The autophagic and endocytic pathways converge at the nascent autophagic vacuoles. *Journal of Cell Biology*, 136: 61-70.

Madson M, Dunand C, Li X, Verma R, Vanzin GF, Caplan J, Shoue DA, Carpita NC, Reiter WD, 2003. The *MUR3* gene of *Arabidopsis* encode a xyloglucan galactosyltransferase that is evolutionarily related to animal exostosins. *Plant Cell*, 15: 1662-1670.

Matsko N, Mueller M, 2005. Epoxy resin as fixative during freeze substitution. *Journal of Structural Biology*, 152: 92-103.

McCully ME, Canny MJ, 1985. The stabilization of labile configurations of plant cytoplasm by freeze substitution. *Journal of Microscopy*, 139: 27-33.

McDonald KL, Morphew M, Verkade P, Müller-Reichert T, 2007. Recent advances in high-pressure freezing: equipment and specimen loading methods. In: Kuo J, ed. *Electron microscopy: methods and protocols*, second edition. Humana press Inc, Totowa, NJ: 143-173.

McGann LE, 1978. Differing actions of penetrating and non-penetrating cryoprotective agents. *Cryobiology*, 15: 382-390.

Nebenführ A, Gallagher LA, Dunahay TG, Frohlick JA, Mazurkiewicz AM, Meehl JB, Staehelin LA, 1999. Stop-and-go movements of plant Golgi stacks are mediated by the acto-myosin system. *Plant Physiology*, 121: 1127-1141.

Oliviusson P, Heinzerling O, Hilmer S, Hinz G, Tse YC, Jiang L, Robinson D, 2006. Plant retromer localized to the prevacuolar compartment and microvesicles in *Arabidopsis* may interact with vacuolar sorting receptors. *Plant Cell*, 18: 1239-1252.

Pagny S, Bouissonié F, Sarkar M, Follet-Gueye ML, Driouich A, Schachter H, Faye L, Gomord V, 2003. Structural requirements for *Arabidopsis* alpha1,2xylosystransferase activity and targeting to the Golgi. *Plant Journal*, 33: 189-203.

Pimpl P, Movafeghi A, Coughlan S, Denecke J, Hillmer S, Robinson DG, 2000. *In situ* localization and *in vitro* induction of plant COPI-coated vesicles. *Plant Cell*, 12: 2219-2235.

Ripper D, Scwartz H, Stierhof YD, 2008. Cryo-section immunolabeling of difficult to preserve specimens: advantages of cryofixation, freeze-substitution and rehydration. *Biology of the Cell*, 100: 109-123.

Saint-Jore C, Evins J, Brandizzi F, Moore I, Hawes C,

2002. Redistribution of membrane proteins between the Golgi apparatus and endoplasmic reticulum in plants is reversible and not dependent on cytoskeletal networks. *Plant Journal*, 29: 661-678.

Saint-Jore-Dupas C, Gomord V, Paris N, 2004. Protein localization in the Golgi apparatus and the trans Golgi network. *Cellular and Molecular Life Sciences*, 61: 159-171.

Saint-Jore-Dupas C, Nebenführ A, Follet-Gueye ML, Boulaflous A, Plasson C, Hawes C, Driouich A, Faye L, Gomord V, 2006. The transmembrane domain length plays a key role in intra-Golgi targeting of soybean α-1,2-mannosidase I. *Plant Cell*, 18: 3182-3200.

Samuels AL, Giddings TH, Staehelin LA, 1995. Cytokinesis in tobacco BY2 and root tip cells: A new model of cell plate formation in higher plants. *Journal of Cell Biology*, 130: 1345-1357.

Sanderfoot AA, Ahmed SU, Marty-Mazars D, Rapoport I, Kirchhausen T, Marty F, Raikhel NV, 1998. A putative vacuolar cargo receptor partially colocalizes with AtPEP12p on a prevacuolar compartment in *Arabidopsis* roots. *Proceedings of the National Academy of Sciences USA*, 95: 9920-9925.

Skepper JN, 2000. Immunocytochemical strategies for electron microscopy choice or compromise. *Journal of Microscopy*, 199: 1-36.

Staehelin LA, Giddings Jr TH, Kiss JZ, Sack FD, 1990. Macromolecular differentiation of Golgi stacks in root tip of *Arabidopsis* and *Nicotiana* seedlings as visualized in high pressure frozen and freeze-substitued samples. *Protoplasma*, 157: 75-91.

Staehelin LA, Kang BH, 2008. Nanoscale architecture of endoplasmic reticulum export sites and of Golgi membranes as determined by electron tomography. *Plant Physiology*, 147: 1454-1468.

Stierhof YD, El Kasmi F, 2010. Strategies to improve the antigenicity, ultrastructure preservation and visibility of trafficking compartments in *Arabidopsis* tissue. *European Journal of Cell Biology*, 89: 285-297.

Studer D, Michel M, Wohlwend M, Hunziker EB, Buschman MD, 1995. Vitrification of articular cartilage by high-pressure freezing. *Journal of Microscopy*, 179: 321-333.

Studer D, Graber W, Al-amoudi A, Eggli P, 2001. A new approach for cryofixation by high pressure freezing. *Journal of Microscopy*, 203: 285-294.

Tokuyasu KT, 1973. A technique for ultracryomicrotomy of cells suspensions and tissues. *Journal of Cell Biology*, 57: 551-565.

Toyooka K, Goto Y, Asatsuma S, Koizumi M, Mitsui T, Matsuoka K, 2009. A mobile secretory vesicle cluster involved in mass transport from the Golgi to the plant cell exterior. *Plant Cell*, 21: 1212-1229.

Tse YC, Mo B, Hilmer S, Zhao M, Lo SW, Robinson DG, Jiang L, 2004. Identification of multivesicular bodies as pre-vacuolar compartments in *Nicotiana tabacum* BY-2 cells. *Plant Cell*, 16: 672-693.

Van Donselaar E, Posthuma G, Zeuschner D, Humbel BM, Slot JW, 2007. Immunogold labeling of cryosections from high-pressure frozen cells. *Traffic*, 8: 471-485.

Viotti C, Bubeck J, Stierhof YD, Krebs M, Langhans M, van den Berg W, van Dongen W, Richter S, Geldner N, Takano J, Jürgens G, de Vries SC, Robinson DG, Schumacher K, 2010. Endocytic and secretory traffic in *Arabidopsis* merge in the trans-Golgi network/early endosome, an independent and highly dynamic organelle. *Plant Cell*, 22: 1344-1357.

Von der Fecht-Bartenbach J, Bogner M, Krebs M, Stierhof YD, Schumacher K, Ludewig U, 2007. Function of the anion transporter At*CLC*-d in the *trans*-Golgi network. *Plant Journal*, 50: 466-474.

Walther P, Ziegler A, 2002. Freeze substitution of high pressure frozen samples: the visibility of biological membranes is improved when the substitution medium contains water. *Journal of Microscopy*, 208: 3-10.

Winicur ZM, Zhang GF, Steahelin LA, 1998. Auxin deprivation induces synchronous Golgi differentiation in suspension-cultured tobacco BY-2 cells. *Plant Physiology*, 117: 501-513.

Wolfe J, Bryant G, 1999. Freezing, drying, and/or vitrifcation of membrane-solute-water systems. *Cryobiology*, 39: 103-129.

Yang YD, Elamawi R, Bubeck J, Pepperkok R, Ritzenhaler C, Robinson DG, 2005. Dynamics of COPII vesicles and the Golgi apparatus in cultured *Nicotiana tabacum* BY 2 cells provides evidence for transient association of Golgi stacks with endoplasmic reticulum exit sites. *Plant Cell*, 17: 1513-1531.

Zhang GF, Staehelin LA, 1992. Functional compartmentalization of the Golgi apparatus of plant cells. An immunochemical analysis of high pressure frozen and freeze substituted sycomore maple suspension-cultured cells. *Plant Physiology*, 99: 1070-1083.

Zhang, GF, Driouich A, Staehelin LA, 1993. Effect of monensin on plant Golgi: re-examination of the monensin-induced changes in cisternal architecture and functional activities of the Golgi apparatus of sycomore suspension-cultured cells. *Journal of Cell Science*, 104: 819-831.