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# Methods and Molecular Tools for Studying Endocytosis in Plants—an Overview

Jozef Šamaj[1,2]

[1]Institute of Cellular and Molecular Botany,
University of Bonn, Kirschallee 1, 53115 Bonn, Germany
*jozef.samaj@uni-bonn.de*

[2]Institute of Plant Genetics and Biotechnology,
Slovak Academy of Sciences, Akademicka 2, 949 01 Nitra, Slovakia
*jozef.samaj@uni-bonn.de*

**Abstract** Proteins of the endocytosis machinery in plants, such as clathrin and adaptor proteins, were isolated and characterized using combinations of molecular biological (cloning and tagging) and biochemical methods (gel filtration, pull-down assays, surface plasmon resonance and immunoblotting). Other biochemical methods, such as cell fractionation and sucrose density gradients, were applied in order to isolate and further characterize clathrin-coated vesicles and endosomes in plants. Endocytosis was visualized in plant cells by using both non-fluorescent and fluorescent markers, and by employing antibodies raised against endosomal proteins or green fluorescent protein-tagged endocytic proteins in combination with diverse microscopic techniques, including confocal laser scanning microscopy and electron microscopy. Genetic and cell biological approaches were used together to address the role of a few proteins potentially involved in endocytosis. Additionally, biochemical and/or biophysical/electrophysiological methods were occasionally combined with microscopic methods (including both in situ and in vivo visualization) in plant endocytosis research.

## 1 Introduction

A variety of methods have been used to study endocytosis in isolated protoplasts, suspension cells and intact cells organized within tissues and organs. Among them, microscopic, biophysical/electrophysiological, biochemical, molecular and genetic methods and their combinations have been very helpful in revealing the diversity of the endocytic pathways and molecules involved (reviewed by Holstein, 2002; Geldner, 2004; Šamaj et al., 2004, 2005; Murphy et al., 2005).

# 2
# Biochemical and Molecular Biological Methods

## 2.1
## Isolation of Clathrin-Coated Vesicles

Plant clathrin-coated vesicles (CCVs) were isolated from cucumber and zucchini hypocotyls (Depta et al., 1991; Holstein et al., 1994). CCV components were protected against proteolysis using homogenization media composed of 0.1 M MES (pH 6.4), 1 mM EGTA, 3 mM EDTA, 0.5 mM $MgCl_2$, a mixture of proteinase inhibitors and 2% (w/v) fatty-acid-free BSA (Holstein et al., 1994). The crude CCV fraction (40 000–120 000 g pellet) was further purified by centrifugation in Ficoll/sucrose according to Campbell et al., (1983) and then by isopycnic centrifugation in a sucrose density gradient using a vertical rotor (160 000 g, 2.5 h, Depta et al., 1991). CCV-enriched fractions (collected at 40–45% sucrose) were removed, pooled and pelleted. CCV fractions were stored at – 80 °C for further use. Immunoblotting was performed using monoclonal antibodies against mammalian adaptins and clathrin. Confirmation of the presence of a β-type adaptin in plants was provided by dot and Southern blotting experiments using genomic DNA from zucchini hypocotyls and a β-adaptin cDNA clone from human fibroblasts (Holstein et al., 1994).

## 2.2
## Cloning, Tagging and Interactions between Plant Clathrin and Adaptor Proteins

A full-length cDNA clone for *Arabidopsis* clathrin light chain was isolated and tagged with GST-myc epitopes. It was shown that this construct specifically interacts (binds) with the His-tagged hub region of mammalian clathrin heavy chain using Superose 12 gel filtration and immunoblotting (Scheele and Holstein, 2002). In a similar approach, *Arabidopsis* adaptor proteins AP180 and αC-adaptin were cloned and tagged with His or GST, respectively, and their binding requiring the plant-specific DPF motif was confirmed via pull-down assays and immunoblotting, or alternatively by surface plasmon resonance analysis (Barth and Holstein, 2004). It was also shown in this study using the same approach that AP180 binds to *Arabidopsis* clathrin heavy chain, and αC-adaptin binds several mammalian endocytic proteins such as amphiphysin, epsin and dynamin. AP180 promotes clathrin assembly into cages having almost uniform size and distribution. When the DLL domain was deleted from AP180, its clathrin assembly activity was abolished but its binding to triskelia was not affected, which suggests that this motif is not involved in clathrin binding (Barth and Holstein, 2004). These combined molecular biological and biochemical studies revealed that clathrin and adaptor proteins isolated from plants display the same structural and functional features as their mammalian counterparts.

## 2.3 Cell Fractionation and Isolation of Endosomes

Cell fractionation on sucrose gradients combined with immunoblotting with specific marker antibodies represents the most useful method for isolation of endomembranous compartments (e.g. Boonsirichai et al., 2003; Preuss et al., 2004; Fig. 1a). These methods have been applied to show that peripheral plasma membrane protein ARG1, which localizes to endocytic brefeldin A (BFA) compartments together with auxin efflux facilitator PIN2, cofractionates with the plasma membrane marker H-ATPase and with different en-

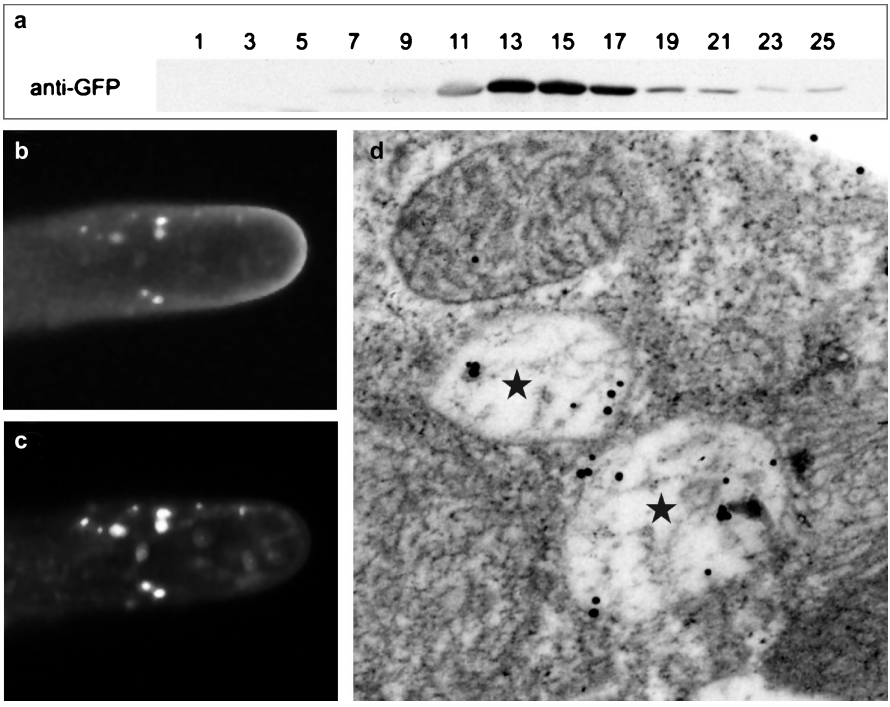


**Fig. 1** Methods for studying endocytosis in plants. **a** Microsomal membranes were isolated from *Arabidopsis* plants stably transformed with endosomal marker RabF2a tagged to GFP and fractionated on sucrose gradients. Subsequently, they were subjected to immunoblotting with GFP antibody. Please note that RabF2a is enriched in endosomal fractions 13–17. **b**, **c** Confocal laser scanning microscopy imaging showing colocalization of endosomal tracer FM4-64 **b** with GFP-tagged endosomal molecular marker RabF2a **c** in actively growing root hairs of *Arabidopsis* roots. **d** Immunogold electron microscopy localization of arabinogalactan proteins (AGPs) within pre-vacuolar compartments (indicated by *stars*) of *Drosera* glandular cells using monoclonal antibody JIM13. AGPs represent cell wall cargo, which is internalized from plasma membrane and delivered via pre-vacuolar compartments to lytic vacuole for degradation and turnover

domembranes including endosomes labelled with PEP12 antibody (Boonsirichai et al., 2003). Membrane fractionation on sucrose density gradients also revealed that the small GTPase RabA4b localizes to a novel endomembrane compartment associated with tip growth of root hairs (Preuss et al., 2004), which turned out to be related to both secretion and endocytosis. Recently, this method was also used for the isolation and identification of prevacuolar compartments (PVCs) representing late endosomes in plants (Tse et al., 2004). Cell extracts from protoplasts of tobacco BY-2 cells were collected and loaded on discontinuous sucrose gradients (consisting of 5 mL each of 25, 40, 55 and 70% (w/v) sucrose solution in basic buffer). The gradient was centrifuged at 110 000 g for 2 h. Immunoblotting with antibodies against vacuolar sorting receptor (VSR) was used to identify PVC/endosomal fractions enriched with VSRs. Further, these fractions were pooled, diluted and separated on a second, continuous 25–50% (w/v) linear sucrose gradient. Each fraction (1 mL) of this gradient was subjected to immunoblotting with specific marker antibodies directed against VSR. In this way, PVCs/late endosomes were isolated and biochemically characterized. Moreover, immunogold electron microscopic (EM) labelling with VSR antibodies revealed that the PVC/late endosome-enriched fractions in fact possess multivesicular bodies (MVBs). Thus, MVBs were identified as PVCs/late endosomes in tobacco suspension BY-2 cells (Tse et al., 2004).

## 2.4 Isolation of Plasma Membrane Lipid Rafts

Recently, lipid raft plasma membrane domains were identified in plants based on their insolubility with the detergent Triton X-100 (Berczi and Horvath, 2003; Mongrand et al., 2004; Borner et al., 2005). First results obtained using thin-layer chromatography revealed that both quantitative and qualitative differences exist between the lipid composition of plant plasma membranes isolated from etiolated bean hypocotyls and green *Arabidopsis* leaves (Berci and Horvath, 2003). Later, protocols for the preparation of Triton X-100 detergent-resistant membranes (DRMs) from *Arabidopsis* callus were developed by Borner et al., (2005). Further, a proteomics approach using two-dimensional gel electrophoresis and liquid chromatography–tandem mass spectrometry revealed that the DRMs were highly enriched in specific proteins. Among them, eight glycosylphosphatidylinositol (GPI)-anchored proteins, several plasma membrane (PM) ATPases, multidrug resistance proteins and proteins of the stomatin/prohibitin/hypersensitive response family, were identified, suggesting that the DRMs originated from PM domains. Further analysis has shown that PM contains phytosterol and sphingolipid-rich lipid domains with a specialized protein composition. DRMs were prepared by low-temperature detergent extraction. According to Borner et al., (2005), membranes were resuspended in cold TNE (25 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, pH 7.5)

containing 4–6 : 1 (detergent-to-protein, w/w) excess of Triton X-100 (no detergent was used in the control extractions). The final concentration of Triton X-100 was approximately 2%. Extractions were performed on ice with shaking at 100 rpm for 35 min. Extracts were adjusted to 1.8 M sucrose (Suc)/TNE by addition of 3 volumes of cold 2.4 M Suc/TNE. Extracts were overlaid with Suc step gradients 1.6–1.4–1.2–0.15 M and centrifuged at 240 000 g in a Beckman SW50.1 rotor for 18 h at 4 °C. DRMs were visible as off-white to white bands near the 1.2/1.4 and 1.4/1.6 M interfaces. Control fractions had a grey-green tinge. Fractions of 1 mL (0.5 mL above and 0.5 mL below the centre of the bands) were collected to harvest the DRM fractions and control fractions. Membranes were diluted with 4 volumes of cold TNE and pelleted at 100 000 g for 2 h in a Beckman 50Ti rotor.

## 3
## Genetic Approaches

Site-directed mutagenesis of important amino acids and truncated versions resulted in mislocalization of the mutated Rab5 proteins, Ara6 and Ara7, which were preferentially localized either to the plasma membrane or to the tonoplast but not to endosomes (Ueda et al., 2001). This mutational analysis revealed that Ara6 requires N-terminal fatty acid acylation, nucleotide-binding and a C-terminal amino acid sequence for its correct targeting to endosomes (Ueda et al., 2001).

Stably transformed plants carrying constitutively active mitogen-activated protein kinase (MAPK) SIMK mutant (carrying a point mutation preventing dephosphorylation) are able to overcome root-hair growth inhibition caused by the MAPK inhibitor, UO126, which is linked to downregulated endo/exocytosis in inhibitor-treated control root hairs (Šamaj et al., 2002).

Point mutation within the catalytic Sec7 domain of the endosomal protein, GNOM, which is an explicitly BFA-sensitive guanosine exchange factor for ADP-ribosylation factor (ARF-GEF), renders this protein BFA-insensitive. Transgenic plants carrying such a mutated GNOM version show defects in endosomal recycling of the auxin efflux facilitator PIN1. Additionally, the inhibition of polar auxin transport upon BFA treatment is rescued, and endosomes show morphological changes in this mutant (Geldner et al., 2003).

ARF1, the reaction partner of ARF-GEF, is a small GTPase involved in vesicular trafficking, and constitutive active, GTP-locked (Q71L) mutant localized to both Golgi and endosomes (similarly to wild-type protein). The dominant negative, GDP-locked form (T31N) was rather diffusely distributed throughout the cytoplasm and the nucleus (Xu and Scheres, 2005). Recently, it was reported that overexpression of constitutive active mutant of another small GTPase, RAC10, in *Arabidopsis* plants abolished normal endocytic uptake of FM dye into root hairs (Bloch et al., 2005).

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