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

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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.