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Detection of Early Intermediates of the Glycosylphosphatidylinositol anchor in Liquid-cultured *Arabidopsis*

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Abstract Tissue extracts were prepared from liquid-cultured *Arabidopsis* and reacted with UDP-[^{3}H]-GlcNAc. Phospholipid fractions were then extracted by butanol partitioning. Consecutive thin-layer chromatography identified two glycolipids sensitive to PI-specific phospholipase C, known as early intermediates in glycosylphosphatidylinositol anchor biosynthesis; phosphatidylinositol *N*-acetylglucosamine and phosphatidylinositol glucosamine.

Keywords *Arabidopsis* · glycosylphosphatidylinositol anchor · liquid culture · thin-layer chromatography

Introduction

Many proteins within the eukaryotic cell surface are covalently linked to the plasma membrane through the glycosylphosphatidylinositol (GPI) anchor (Low, 1987; Udenfriend and Kodukula, 1995). Phosphatidylinositol (PI), a component of the membrane lipid bilayer, is sequentially connected to glucosamine (PI-GlcN), glycan (typically three mannoses), ethanolamine, and the carboxy terminus of a protein (Stevens, 1995; Takeda and Kinoshita, 1995). Phosphatidylinositol (PI)-specific bacterial phospholipase C (PI-PLC) can release GPI-anchored proteins (Lisanti and Rodriguez-Boulan, 1990). In addition to a membrane attachment structure, the GPI anchor increases the lateral mobility of membrane proteins (Ishihara et al., 1987; Noda et al., 1987), a signal transduction mechanism of leukocytes (Robinson, 1991), and a sorting signal of apically expressed proteins in polarized epithelial cells (Lisanti and Rodriguez-Boulan, 1990; Brown and Rose, 1992). Deficiencies in GPI biosynthesis can lead to embryonic lethality in mammals (Kinoshita et al., 1997) and conditional lethality in eukaryotic microbes by blocking cell growth, division, and morphogenesis.

The first intermediate of GPI biosynthesis is PI-*N*-acetylglucosamine (PI-GlcNAc), which results after transfer of GlcNAc from UDP-GlcNAc to PI (Kinoshita et al., 1997). The initial step is catalyzed by GPI-*N*-acetylglucosaminyltransferase. This enzyme was isolated from human cells and has been shown to consist of at least six subunits: PIG-A, PIG-C, PIG-H, GPI-1, PIG-P, and DPM2 (Ikezawa, 2002). The PI-GlcNAc form is deacetylated subsequently by PIG-L to yield PI-GlcN.

It has been reported that the *Arabidopsis SETH1* and *SETH2* genes encode homologs of *PIG-C* and *PIG-A*, respectively (Lalanne et al., 2004). Mutations within these two genes specifically block pollen germination and tube growth. The *Arabidopsis PNT1* gene encodes a homolog of mammalian PIG-M, an endoplasmic reticulum-localized mannosyltransferase (Gillmor et al., 2005). In addition, the *OsPIG-F* gene that encodes a homolog of human PIG-F, a subunit of the GPI anchor phosphoethanolamine transferase complexes, was identified in rice (Lee and Kang, 2008). In studies on preassembly of the GPI anchor in mammalian cells, cell-free extracts were incubated with UDP [^{3}H]-GlcNAc (Hirose et al., 1991; Miyata et al., 1993), and the resulting phospholipid products were analyzed by thin-layer chromatography (TLC).

Plant tissues contain high concentrations of various lipids, cutin, and waxes, which hamper the detection of PI-containing molecules on the TLC plate. In the study described herein, tissue extracts prepared from liquid-cultured *Arabidopsis* were used to detect PI-GlcNAc and PI-GlcN. We believe that the procedure

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described in this study will be useful for the purification of GPI-*N*-acetylglucosaminyltransferase, which catalyzes the initial step in GPI-anchor biosynthesis in plants.

Materials and Methods

Culture of *Arabidopsis*. For liquid culture of *Arabidopsis*, the seed (ecotype Columbia) surface was sterilized with 70% ethanol for 2 min, 0.5% NaOCl/0.5% sodium dodecyl sulfate for 15 min, and rinsed five times with sterilized water. Approximately 50 seeds were germinated and grown for 3 weeks in culture medium in a shaking incubator at 22°C, 100 rpm, under a 16-h-light/8-h-dark cycle. The medium contained 1 × MS salt mixture (JRH Biosciences, USA), 1% sucrose, vitamins (1 mg/mL thiamine-HCl, 0.5 mg/mL phyridoxine-HCl, and 0.5 mg/mL nicotinic acid), 100 mg/mL inositol, and 0.5 g/L MES. The pH was adjusted to 5.7 with 1 *N* KOH.

Tissue extracts. Liquid-cultured *Arabidopsis* was ground with glass beads using a mortar according to a procedure described previously (Hirose et al., 1991). The mixture was filtered through two layers of Miracloth, and centrifuged at 200,000 × *g* at 4°C for 20 min. The pellet was washed briefly with extraction buffer (1 mL/1 g tissue) containing 50 mM Na-HEPES, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM phenylmethyl-sulfonyl fluoride dissolved in ethanol, 1 mM ethylene glycol tetraacetic acid, and a protease inhibitor cocktail containing 10 μg/mL pepstatin (in methanol), 5 μg/mL aprotinin, 5 μg/mL leupeptin and 10 μg/mL α_2-macroglobulin, resuspended (0.5 mL/g tissue), and stored at −80°C until use.

PI-GlcNAc labeling reaction. For the PI-GlcNAc labeling reaction, 100 μL (10 μCi) of UDP [^{3}H]-GlcNAc (Dupont) were placed in an Eppendorf tube and dried using a speed vacuum for 1 h. Tissue extracts (195 μL) were thawed and added to each tube in addition to 0.2 μg/mL tunicamycin (2 μL of 0.02 mg/mL stock), 5 mM $MnCl_2$ (1 μL of 1 M stock) and 1 mM adenosine triphosphate (ATP; 2 μL of 100 mM stock). Next, reaction mixtures were incubated at 37°C for 15 min and terminated by the addition of 1.3 mL chloroform:methanol (1:1, v/v). Reaction mixtures were then centrifuged using a microfuge table-top centrifuge, and supernatants were dried under vacuum. Residues were partitioned in water-saturated 1-butanol:water (200 μL each). Upper solutions were removed and dried, and dissolved in 10 μL chloroform: methanol (2:1, v/v).

PI-PLC digestion. Sample materials were stirred vigorously after adding 100 μL Tris-acetate buffer, pH 7.4, and 0.1% sodium cholate. Next, 10 μL of 0.5 U/μL PI-PLC (Sigma-Al drich, USA), or sterilized water were added to the tubes. Tubes added with water or PI-PLC (at 30°C) were incubated for 4 h.

TLC. Reaction products were analyzed by TLC using a silica gel 60 plate (Merck, USA), after developing with chloroform: methanol: 1 M NH_4OH (10:10:3, v/v) for 1 h. Radioactive spots were detected using EN^{3}HANCE spray (New England BioLabs) and exposed to X-ray film for 20 h.

Results and Discussion

Flurography and autoradiography analyses revealed two spots representing PI-GlcNAc (Rf = 0.76) and PI-GlcN (Rf = 0.70) on the TLC plate (Fig. 1), as identified in an experiment with human JY cells (Miyata et al., 1993), from liquid-cultured *Arabidopsis* tissue extract.

We repeated this experiment with tissue extract from maize coleoptiles, which exhibited three or four bands on the plate (data

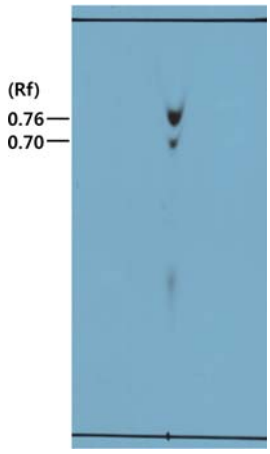

Fig. 1 Detection of early intermediates of the glycosylphosphatidylinositol anchor in liquid-cultured *Arabidopsis*. For PI-GlcNAc labeling, tissue extracts were first reacted with UDP [^{3}H]-GlcNAc. Residues were then partitioned in water-saturated *n*-butanol: water. Upper solutions were removed and dried, dissolved in chloroform: methanol (2:1, v/v), and analyzed TLC using silica gel plates. Chloroform: methanol: 1 M NH_4OH (10:10:3, v/v) was used to develop TLC plates. Radioactive spots were detected using EN^{3}HANCE spray and then exposed to X-ray film. Two spots representing PI-GlcNAc (Rf = 0.76) and PI-GlcN (Rf = 0.70) were appeared on the TLC plate.

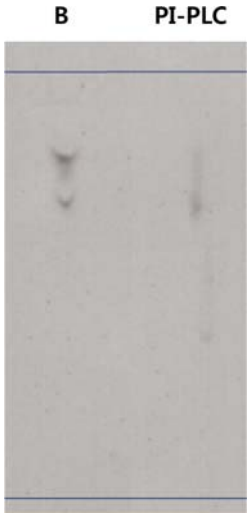

Fig. 2 Phospholipase sensitivity of glycosylphosphatidylinositol spots on TLC plates. Silica powder in the area between Rf 0.6–0.9 on the silica plate (Fig. 1) was excised with a blade and extracted with *n*-butanol. The extract was then reacted with buffer (B) only or PI-specific phospholipase C (PI-PLC), respectively, and developed on TLC plates. Two spots represent PI-GlcNAc (Rf = 0.76) and PI-GlcN (Rf = 0.70), respectively.

not shown). Migration of the products was dependent on the amount of sample loaded on the plate, implying an unidentified interaction between the compounds and the developing solvent.

We also examined the sensitivity of the two *Arabidopsis* glycolipids from the TLC plate to PI-PLC. The two glycolipids in the area between Rf 0.6–0.9 on the silica plate were excised with a blade. TLC and fluorography revealed that the two compounds were sensitive to PI-PLC, thereby revealing their identities as PI-GlcNAc and PI-GlcN, respectively (Fig. 2).

Arabidopsis is a model plant for molecular biology and genomics studies. However, in general, this plant has been regarded as a poor source for biochemical analysis. This is likely because the tissues contain high concentrations of various lipids, cutin, and waxes. In contrast, the procedure described in this study will facilitate the purification of enzyme(s) that catalyze the first step in GPI-anchor biosynthesis in plants, thus representing a direct connection to the genomic information accumulated in the extensive studies conducted using this plant model.

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