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Vesicular fractions of sunflower apoplastic fluids are associated with potential exosome marker proteins

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ABSTRACT

Based on the presence of phospholipids in the extracellular fluids (EFs) of sunflower seeds, we have hypothesized on the existence of vesicles in the apoplastic compartment of plants. Ultracentrifugation of sunflower EF allowed the isolation of particles of 50-200 nm with apparent membrane organization. A small GTPase Rab was putatively identified in this vesicular fraction. Since Rab proteins are involved in vesicular traffic and their presence in exosomes from animal fluids has been demonstrated, evidence presented here supports the existence of exosome-like vesicles in apoplastic fluids of sunflower. Their putative contribution to intercellular communication in plants is discussed.

1. Introduction

Mammalian cells release membrane vesicles into the extracellular environment. Among them, the best known are exosomes, which are derived from multivesicular bodies (MVB) and can fuse the plasma membrane resulting in the secretion of their luminal vesicles of 40–100 nm [1]. Although the physiological role of exosomes in vivo is still a matter of study, it is apparent that they function in a multitude of processes, including cell–cell communication and tissue developmental processes [2–4]. Particularly, a relevant role of exosomes in intercellular communication is being recognized [5,6]. These secreted membranes have been characterized for their protein composition revealing several transmembrane or peripherals proteins with affinity for ligands on other cell membranes that may direct exosomes to their target cells [7]. In addition, some cytoskeleton related proteins and proteins involved in membrane traffic and fusion have been found [5,8].

With the discovery of exosome-like vesicles in *Drosophila*, it has become clear that they have conserved functions in evolution [9,10]. Moreover, spherical vesicles of unknown function are also released from the outer membrane by certain bacteria [11]. On the other hand, plant exosomes have not been described yet. However, it has recently been shown that MVBs participate in a cell wall-associated defense response in barley leaves attacked by the fungus *Blumeria graminis* f. sp. *hordei* [12]. These MVBs would be able to fuse to the plasma membrane to release their internal content into the paramural space. This evidence has lead to speculate on the existence of exosomes in plants [13], although no demonstration of their presence was reported.

We have recently shown the existence of of phospholipids in extracellular fluids (EF) of imbibed sunflower (*Helianthus annuus*) seeds [14]. Lipid profiling using electrospray ionization tandem mass spectrometry (ESI-MS/MS) revealed a particular phospholipid composition, with phosphatidic acid and phosphatidylinositol as major components. Besides, phosphatidylinositol phosphate was also found in the extracellular milieu of tomato cell suspensions by using a labelling strategy [15]. To our knowledge, these were the first reports on the presence of phospholipids as extracellular components of plants and their origin and significance in an aqueous medium constitutes a relevant issue for plant sciences. The

Abbreviations: EF, extracellular fluid; ESI-MS/MS, electrospray ionization tandem mass spectrometry; FSGTP1, small GTPase from *Fagus sylvatica*; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MALDI-TOF, matrix-assisted laser desorption/ionization – time of flight; MVB, multivesicular bodies; MW, molecular weight; SE, seed extract; TEM, transmission electron microscopy

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occurrence of several phospholipid types in the EF of sunflower seeds may be indicative of the existence of bilayers or micelles, that in an aqueous compartment such as the apoplast, could be organized in vesicular structures, as it is the case in animal fluids. However, to our knowledge this possibility has not been explored in plants. The purpose of this work was to analyze the existence of exosome-like vesicles in sunflower fluids. To that aim, we have applied a biochemical approach already established for exosomes of animal origin, which allowed the isolation of vesicles in EFs of seeds.

2. Materials and methods

2.1. Plant material and collection of extracellular fluid

Intact sunflower seeds (*H. annuus* L., line 10347 Advanta Semillas) were subjected to imbibition during 2 h in water and then carefully peeled to remove the pericarp previous to the extraction of the EF. The EF was collected by a standard technique based in a vacuum infiltration-centrifugation procedure. Briefly, seeds were immersed in 50 mM Tris–HCl pH 7.5, 0.6% NaCl, 0.1% 2-mercaptoethanol (infiltration buffer) and subjected to three vacuum pulses of 10 s, separated by 30 s intervals. The infiltrated seeds were recovered, dried on filter paper, placed in fritted glass filters and centrifuged for 20 min at 400×*g* at 4 °C. The EF was recovered in the filtrate and evaluated for the absence of intracellular contamination as previously described [14].

A seed extract (SE) was obtained by pulverization of decoated seeds previously imbibed in water for 2 h. The resultant powder was immersed in 5 vol. of infiltration buffer and clarified by centrifugation at 10 000×*g* for 15 min.

2.2. Preparation of vesicles

The EF from 20 g of seeds was filtrated through a 0.5 μm membrane and subjected to fractionation by successive centrifugation steps at 10 000×*g* for 30 min, 40 000×*g* for 60 min and 100 000×*g* for 60 min according to a procedure described previously for animal exosomes [16]. The first pellet was discarded while the 40 000×*g* and 100 000×*g* pellet fractions were suspended in 30 μl of 20 mM Tris–HCl pH 7.5, being the fractions potentially enriched in vesicular material. As a control, the SE obtained from 1 g of seeds was also filtrated and subjected to fractionation by successive centrifugation as described for EF.

2.3. Transmission electron microscopy (TEM)

The pellets obtained by successive centrifugation steps (40 000×*g* and 100 000×*g*) were subjected to phosphotungstic acid negative staining on Formvar carbon-coated electron microscopy grids [17]. Twenty microliters of each sample were applied to the grid and after 5 min excess solution was wicked off the grid with filter paper. An equal part of 2% phosphotungstic acid was added to the grid and after 2 min the grids were placed in a Petri dish containing filter paper for 15 min. The preparations obtained were examined at 90 kV with a JOEL 1200 EXII electron microscope (INTA Castelar, Argentina).

2.4. Protein and lipid analysis

The 40 000×*g* and 100 000×*g* pellet fractions of EF obtained from 20 g of seeds or SE obtained from 20 mg of seeds were suspended in 20 microliters of standard sample buffer [18]. Electrophoretic separation was performed in 12% SDS–PAGE gels [18]. Gels were then stained with Sypro reagent and bands were automatically excised from the gel (Investigator ProPic robotic workstation, Genomic Solutions). The gel pieces were digested with trypsin and MS analyses of peptides were performed in a 4700 Proteomics Station (Applied Biosystems, USA) as previously described [19]. Protein identification was assigned by comparing the obtained peptide mass fingerprinting with the non-redundant plant database, using Mascot 1.9 search engine (Matrixscience, UK). Immunodetection of GAPDH was assayed by Western blot according to standard procedures [14].

Lipid extraction and phospholipid measurement were performed as previously described [14]. Briefly, the vesicular fraction was subjected to lipid extraction with 4.5 vol. of chloroform:methanol:HCl (200:100:1, v/v/v) and 1 vol. of 0.9% (w/v) NaCl. The organic phase was obtained by centrifugation, washed with 4.5 vol. of chloroform:methanol:1 M HCl, 3:45:47, v/v/v and finally evaporated under nitrogen stream. The lipid pellets obtained were analyzed by ESI-MS/MS at the Facility of the Kansas Lipidomics Research Center (Kansas State University, USA).

3. Results

Taking into account our previous results demonstrating the existence of phospholipids in the apoplast of *H. annuus* seeds, we decided to explore whether vesicular structures were present in EFs. To that aim, seeds were imbibed for 2 h and the EF, containing the apoplastic fraction of the seeds, was isolated by infiltration-centrifugation. This fraction was tested for the absence of significant intracellular components as previously described [14] and showed the absence of detectable levels of actin, oleosin and Glu-6-P dehydrogenase. The EF was then fractionated by sequential centrifugation at 10 000×*g*, 40 000×*g* and 100 000×*g*. The first pellet was discarded and the 40 000×*g* and 100 000×*g* pellets, expected to be enriched in exosome-like vesicles [16], were further analyzed. Electron transmission microscopy (TEM) showed the presence of vesicular bodies of 50–200 nm in diameter in both fractions, although a higher density of vesicles was apparent in

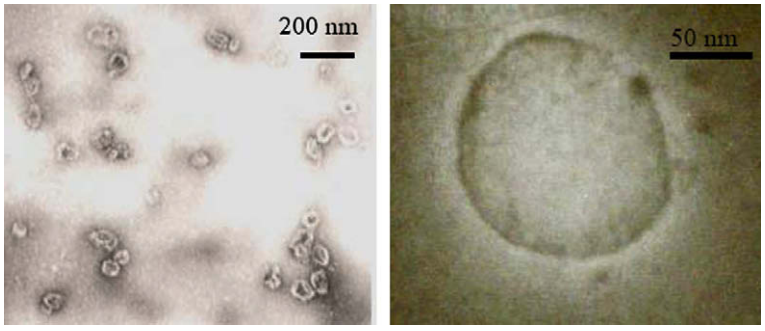

Fig. 1. Presence of vesicles in the extracellular fluids (EFs) of sunflower seeds. TEM images obtained by negative staining with phosphotungstic acid of the EF 40 000×*g* vesicle fraction. Left panel: whole mount. Right panel: detailed visualization of a vesicle. Bars indicate the scale in nm.