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Pedro Pérez-Bermúdez, Jesús Blesa, José Miguel Soriano, Antonio Marcilla

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EXTRACELLULAR VESICLES IN FOOD: EXPERIMENTAL EVIDENCE OF THEIR SECRETION IN GRAPE FRUITS

Pedro Pérez-Bermúdez[a], Jesús Blesa[b,c], José Miguel Soriano[b,c] and Antonio Marcilla[c,d]

[a]Departament de Biologia Vegetal, Facultat de Farmàcia, Universitat de València, Burjassot (Valencia), Spain.

[b]Grupo de Ciencias de la Alimentación Basada en la Evidencia y Experimentación (CiAlBEx). Instituto de Ciencias de los Materiales, Parque Científico, Universitat de València, Paterna, Spain.

[c]Unidad Mixta de Investigación en Endocrinología, Nutrición y Dietética Clínica, Instituto de Investigación Sanitaria La Fe-Universitat de València, Valencia, Spain.

[d]Àrea de Parasitologia, Departament de Farmàcia i Tecnologia Farmacèutica i Parasitologia, Universitat de València, Burjassot (Valencia), Spain.

***Corresponding author:** Antonio Marcilla (antonio.marcilla@uv.es)

Abstract

In the last decade, the number of studies related with extracellular vesicles (EVs) has dramatically grown since their role as key part of intercellular communication has been confirmed. EVs, as transporter of distinct bioactive molecules, can take part in different physiological mechanisms and have been gaining attention as potential tools with a wide range of therapeutic effects. Whereas a high number of studies have been published related to mammalian derived EVs, including products as food source, the existence of EVs in plants still is controversial. Recent descriptions of vesicles derived from edible plants show they might contain pharmacological active molecules. In this context, EVs from food are attracting increasing interest due to their relevance in modulating cellular processes (involved in health and disease), as well as therapeutic vehicles. The present work aims to summarize the current knowledge on exosomes in foods, actually limited to only four FAO groups (Milk, Starchy roots and tubers, Nuts and seeds, and Fruits). In addition, we have further characterized EVs isolated from grape berry juice by classical differential centrifugation, and described a preliminary dissection of their secretion *in vivo*.

Keywords: Extracellular vesicles; food exosomes; secretion; grape berry; *Vitis vinifera*

1. Introduction

In the last years, the number of studies related with extracellular vesicles (EVs) has dramatically grown. These vesicles, which can take part in different physiological mechanisms (Yáñez-Mó et al., 2015), have been gaining attention as potential therapeutic guns (Fais et al., 2016; Lener et al., 2015). In this context, the presence of EVs in food is also achieving increasing consideration. A high number of studies have been published related to mammals EVs, including products as food source, whereas still is controversial the existence of EVs in plants.

The first aim of this work was reviewing the description of exosomes (or endosomal-derived vesicles) in foods using FAO/INFOODS databases (FAO, 2014), which could be named "FoodEVs". It is not reviewed the presence of exosomes in parts belonging to edible plants that are not normally consumed (for example leaves of cereals). Therefore, the information about FoodEVs is limited, as shown in Table 1, to four FAO groups, namely Milk, Starchy roots and tubers, Nuts and seeds and Fruits. The second aim of our work was to isolate and characterize grape berry EVs, which has revealed the presence of typical EVs proteins.

1.1. Mammalian products: milk and derivatives

As early as 1973, Plantz et al. showed the presence of vesicles cow skim milk, which were concentrated by differential centrifugation. Recently, many studies have shown the presence of exosomes in cow milk, like in raw milk (Hata et al., 2010; Izumi et al., 2015; Munagala et al., 2016; Reinhardt et al., 2012; Sun et al., 2015), semi-skimmed milk (Arntz et al., 2015; Pieters et al., 2015) or low-fat milk (Kusuma et al., 2016). Different exosome isolation techniques have been employed, including ultracentrifugation (Arntz et al., 2015; Izumi et al., 2015; Kusuma et al., 2016; Munagala et al., 2016; Reinhardt et al., 2012), ultracentrifugation and sucrose gradient (Hata et al., 2010; Sun et al., 2015), or ultracentrifugation and ExoQuick™ kit (Pieters et al., 2015) (Table 1). Different parameters of centrifugation have been used to remove fat globules, casein aggregates and other debris from pasteurized milk: 3000 × g for 15 min; 12,000 × g for 1 h; 35,000 × g for 1 h, 70,000 × g for 1 h (Hata et al., (2010). Izumi et al. (2015) centrifuged twice (1,200 × g, 4°C, 10 min) using the defatted supernatant centrifuged at 21,500 × g, 4°C and passed, clear supernatant (whey), through 0.65-, 0.45-, and 0.22-μm membranes. Kusuma et al. (2016) reported the use of centrifugation at 12,000 x g at 4°C for 30 min, and the fat-free supernatant was mixed with an equal volume of 0.25 M EDTA (pH 7.0), incubated on ice for 15 min to precipitate casein and exosomes coated with casein. The final suspension was ultracentrifuged at 80,000 x g at 4°C for 60 min, to remove precipitated proteins, milk fat globules, and microvesicles larger than exosomes. Munagala et al. (2016) and Reinhardt et al. (2013) centrifuged at 13,000 × g at 4 °C for 30 min, and at 10,000 x g for 15 min at 4ºC, respectively. Furthermore, Hata et al. (2010) applied sucrose-density gradients (5–40%, w/v, solution), and ultracentrifuged at 200,000 x g for 18 h using ExoQuick reagent.

Pieters et al. (2015) used several steps including ExoQuick reagent added to milk supernatant, precipitated overnight at 4°C, centrifuged at 1,500 x g for 30 min at 4°C, and dissolved EVpellets either in PBS or in the appropriate buffer for RNA or protein analysis (Pieters et al., 2015). In these conditions, Arntz et al. (2015) observed around 5×10^9 particles (corresponding to 40 mg protein/200 mL); and Pieters et al. (2015) estimated that the concentration of milk EVs was roughly 5×10^7 particles per μg of protein.

1.1.1. Milk samples processing can affect exosomes

Usually a centrifugation process (from 2,000 to 12,000 x g) removes fat globules, somatic cells and debris to obtain supernatant milk serum. Additional precipitation or filtration processes may be required.

Yamada et al. (2012) carried out a comparison of methods for isolating exosomes from bovine bulk tank milk and human milk. This paper evaluated four techniques including ExoQuick™ kit, with or without ultracentrifugation, ultracentrifugation with density gradient centrifugation, and human milk exosome isolation using the protocol by Admyre et al. (2007), as a control. The highest yield was obtained using ultracentrifugation with the ExoQuick™ kit, with around 100 μg of protein in EVs per mL of milk. On the other hand, ultracentrifugation with density gradient centrifugation rendered high quality exosomes with intact morphological structures.

Dairy technological processes can affect exosomes, as described by Howard (2015), who used Trizol LS® extraction, and compared the concentration of miRNAs in whole, 2% fat, and skim milks, versus store-bought milk. Interestingly, high yield of miRNAs was obtained in the latter. In fermented milk as yogurt, miRNA concentrations decreased due, probably, to the lysis of exosomes during the fermentation and to the large amounts of RNAses produced by microbes used in the fermentation (Howard, 2015).

1.1.2. Livestock milk exosomes

Hata et al. (2010) showed the presence of microvesicles of around 100 nm in diameter in bovine colostrum and mature milk, which contained exosomal markers like the protein MFG-E8. As reported for other mammalian EVs, milk-derived microvesicles were mostly free of ribosomal RNA. Milk specific gene transcripts were clearly amplified only when murine fibroblast cells (NIH-3T3) were incubated with milk-derived microvesicles, but transfer of bovine miRNAs was not concluding, since the sequences in mouse and bovine species were too similar to be distinguished by PCR amplification (Hata et al., 2010).

Recent microarrays analyses have shown that mRNA in milk whey was mostly present in exosomes, whereas 79 miRNAs were detected in supernatant and exosomes (Izumi et al., 2015). The authors investigated whether bovine milk-derived exosomes could exert functional effects in human cells by using human monocytic leukemia cells (THP-1). By mean of flow cytometry and fluorescent microscopy analyses, they revealed that bovine milk exosomes were incorporated into differentiated THP-1 cells, suggesting that bovine RNA present in milk exosomes might affect human cells.

Changes in miRNA content of milk exosomes in response to infection has been described by Sun et al. (2015), providing support for milk exosomes as delivery agents of specific miRNAs involved in immune response. Bta-miR-142, -5p and −223 were identified as potential biomarkers for early detection of bacterial infection of the mammary gland. Additional 22 host-expressed genes (mainly involved in regulation of immune and inflammatory responses) were identified as potential binding targets of the differentially expressed miRNAs (Sun et al., 2015).

The protein composition of milk exosomes reflects the milk protein composition, and the potential significance of exosomes in mammary physiology (Reinhardt et al., 2012). Electron microscopy (EM) analyses evidenced that milk exosomes ranged between 50 and 100 nm in size. Authors also highlighted the role of exosomes as a secondary pathway of cell membrane secretion into milk. The major proteins present in milk fat globule membrane (butyrophilin, xanthine oxidase, adipophilin and lactadherin) were also the most abundant ones in milk exosomes. This diversity could account from exosomes derived from different cells that reside in the milk compartment, such as immune cells and secretory epithelial cells.

Pieters et al. (2015) recently suggested that EVs present in commercial cow milk remain intact in the gastrointestinal tract, and can exert an immune regulatory effect. An examination of the purified EVs using EM revealed that they had the size (80–130 nm) and morphology similar to that of exosomes. Their presence was confirmed by an exosome capture assay using anti-CD63, as well as by nanoparticle tracking analysis (NTA). The authors also examined the stability of milk-derived EVs upon different treatments (low pH, boiling, and multiple freeze-thawing cycles), showing no major differences with untreated EVs. These results support that milk-derived EVs are not degraded in the gastrointestinal

tract. Interestingly, cellular uptake was assessed in cultured cells suggesting that theconsume of commercial milk might expose humans to immune regulatory EVs, which could have implications in health and disease.

Milk exosomes seem to exhibit cross-species tolerance with no adverse immune and inflammatory responses (Munagala et al., 2016). Furthermore, they reported the ability of milk EVs to reach tumour targets.

Baier et al. (2014) reported that a large fraction of miRNAs in milk (including miR-29b and miR-200c) is contained in exosomes, which provide protection against degradation. This study evidenced that the amounts of miRNAs absorbed from milk are sufficient to alter human gene expression. They speculate that milk exosomes enter the human intestine by a shared mechanism for all exosomal miRNAs. Very recently, using an OpenArray assay with samples identical to the ones used in Baier et al., (2014), Auerbach et al. (2016) revealed no significantly altered miRNA (i.e. miR-29b or miR-200c) signals after milk ingestion, making controversial the transfer of dietary xenomiRs into the circulation of adult humans.

Recent report by Kusuma et al (2016) have classified exosomes as bioactive compounds. These authors showed the compatibility of proteins at the surface of milk exosomes with the ones on the surface of human endothelial cells, suggesting the endocytosis of EVs. This opens an interesting field of research as it is the identification and validation of molecular signatures for intake of food vesicles.

Advances in practical applications of milk EVs have been encouraging, in this context, Arntz et al. (2015) showed that treatment with cow milk derived EVs could ameliorate experimental arthritis. EVs were isolated from semi-skimmed milk by ultracentrifugation, rendering particles of 100 nm, which expressed CD63, as well as

immune regulatory microRNAs and mRNAs. This study identified a number of immune-related miRNAs (like miR-30a, -223, -92a), and mRNAs of milk-specific proteins (α-casein, βLG, and elongation factor-1 α). Bovine milk EVs have been recently shown to increase the formation of small osteoclasts in mice, although this effect does not lead to rise bone resorption, probably due to reduced acid secretion (Oliveira et al., 2016). Interestingly, new data suggest that milk exosomes can also serve as a nanocarrier platform to enhance activity of therapeutics by increasing cellular uptake and bioavailability against multiple cancers (Aqil et al., 2016a, 2016b).

Recent studies by Krupova et al. (2016) have shown that the use of commercially available kits for the isolation of milk-based EVs from goats (*Capra aegagrus hircus*) result in the co-precipitation of contaminants with EVs. They have developed a method based on an improved conventional density gradient ultracentrifugation to purify milk EVs free of milk contaminants, at sufficient concentrations to subsequently perform a fully characterization (Krupova et al., 2016).

The presence of EVs in buffalo (*Bubalus bubalis*) raw milk has also been investigated. Kuruppath et al. (2013) isolated exosomes from milk and colostrum through ultracentrifugation and ExoQuick^{TM} kit. The RNA-seq profile of exosomes was very similar to the profile obtained from total skim colostrum.

EVs have been also isolated from dromedary (*Camelus dromedaries*) raw milk by differential ultracentrifugation (Yassin et al., 2015). Transmission EM (TEM) analyses of EVs showed an average size about 30 nm, and evidenced changes with clumping and agglomeration after freezing. Proteomic analyses did not reveal major qualitative or quantitative differences in EVs proteins during mid or late lactation periods. Transcriptomic analyses revealed a stable expression of casein family genes in EVs during

different lactation periods. Additionally, phospholipidomic analyses proved that phosphatidylcholine (PC) is the major phospholipid constituent in dromedary milk exosomes.

1.1.3. Breast milk

The first isolation of human breast milk exosomes, including colostrum and mature milk, was reported in 2007 (Admyre et al., 2007). These authors used differential ultracentrifugation based in 300 x g, followed by 3,000 x g, and filtered by several filters to remove cell debris, being then supernatants centrifuged at 10,000 x g for 30 min at 4°C, and vesicles pelleted at 100,000 x g for 70–90 min. They suggested that milk exosomes might originate from other cells (i.e. macrophages and lymphocytes) present in breast milk, as well as from breast epithelial cells and serum. In fact, colostrum-derived exosomes showed significantly higher levels of HLA-DR than mature milk-derived exosomes, and these authors hypothesized that either tolerizing or immune-stimulating exosomes could be released into breast milk depending on the route of antigen administration and the immune status of the mother. The protein concentration ranged between 41.0-153 μg and 19.8-319 μg of protein per mL of colostrum and mature milk vesicles, respectively. Kosaka et al. (2010) suggested that human breast milk containing exosomes may be important for the development of the infant's immune system, since they were stable even under acidic conditions, indicating that these molecules can tolerate infant gastrointestinal environment and can be absorbed in the intestine. More recently, Zonneveld et al. (2016) have shown that bovine milk EVs reduce immune activation by inhibiting both T-cell responses and endosomal TLR activation, while stimulating epithelial cell proliferation and migration.

Taken together, their data suggest a role for cow milk EVs in both the development of the intestinal barrier and the immune system.

The function of native populations of exosomes can be recovered from stored breast milk samples. Zonneveld et al. (2014) showed that storing unprocessed breast milk at -80ºC or 4ºC caused death of the cells present in breast milk, leading to contamination of breast milk exosomes with storage-induced exosomes. According to these results, an alternative method based on freezing milk supernatant devoid of cells and cream layer, was proposed to store breast milk samples for exosome analysis at later time points.

Related to composition of breast milk EVs, a recent exhaustive proteomic analysis have identified 1963 proteins, adding 633 new ones that have the capacity to support the infant's developing gastrointestinal tract and its immune system (Van Herwijnen et al., 2016).

1.2. Plant EVs

Although the existence of plant exosomes derived from multivesicular bodies (MVB) is still controversial (Ding et al., 2012; Robinson et al., 2016), near fifty years ago, some works already pointed to the release of small vesicles in non-classical secretory pathways in plants. These works reported the presence of multivesicular structures associated with the plasmalemma of plant cells or the release of vesicles into the apoplast in higher plants (Halperin and Jensen, 1967; Marchant and Robards, 1968).

After the discovery of exosomes in animal cells, increasing evidences support the occurrence of MVB- and exosome‑like vesicles in plants. Such findings are the result of numerous investigations, mainly focused either on cell growth and differentiation, including cell wall-related processes, or on plant responses against a wide range of stresses (An et al., 2007).

The description of new compartments called EXPOs (Wang et al., 2010), spherical double membrane structures resembling autophagosomes, also supports the existence of MVB-derived exosomes. The authors described EXPO as an exosome, although with a different origin to those described in mammalian cells, which represents a form of unconventional secretion unique to plants. In relation with this subject, numerous investigations and reviews have focused on extracellular leaderless proteins and plant secretome in *Arabidopsis*, tobacco, rice, maize, alfalfa, pea, tomato, sunflower, grape, etc., which reflect the importance of the non-classical pathways of protein secretion in plants. Further information on this subject and the applied approaches to study plant secreted proteins can be obtained from various studies (Agrawal, 2010; Alexandersson et al., 2013; Drakakaki et al., 2013; Krause et al., 2013; Regente et al., 2012; Robinson et al., 2016; Tanveer et al., 2014; Tian et al., 2015; Zarsky et al., 2013).

Various recent studies have suggested the existence of plant EVs, so called exosome-like nanoparticles or nanovesicles. Regente et al. (2009) presented evidence of nanosized particles in apoplastic fluids of sunflower seeds. Vacuum infiltration-ultracentrifugation of the seeds allowed the isolation of particles, 50-200 nm in diameter, with apparent membrane organization. A lectin and a small GTPase Rab, also found in exosomes from animal fluids and involved in vesicular traffic, were putatively

identified in these sunflower exosome-like vesicles. The authors proposed a new aspect of intercellular communication, the vesicle-based information transfer in plants.

More recently, Prado et al. (2014) have shown, using a protocol previously described for mammalian exosomes, that olive pollen grains release nanovesicles (pollensomes), during *in vitro* pollen germination and pollen tube growth. Electronic microscopy analysis revealed a heterogeneous population of round-shaped nanovesicles, ranging from 28 to 60 nm in diameter. Proteins were extracted from purified olive nanovesicles and identified using mass spectrometry analysis. Pollensomes contain a large number of proteins, many of them displaying well-known roles in metabolism and signalling, protein synthesis and processing, cell wall expansion, cytoskeleton, and membrane transport. Pollensomes comprise a heterogeneous population of secretory vesicles; their contents suggest that some of the pollensomes could account for Golgi- or endoplasmic reticulum-derived origin. They concluded that alternative routes for pollensome release cannot be discarded, since these vesicles contain several leaderless secretory proteins, which get access to the extracellular space by unconventional secretion.

Putative exosomes have also been isolated from several edible plants. Ju et al. (2013) isolated grape exosome-like nanoparticles (GELNs) from grape juice by ultracentrifugation on a sucrose gradient. They showed that GELNs have unique transport properties and biological functions. Oral administration of GELNs leaded to protection of mice from dextran sulphate sodium-induced colitis, suggesting the use of edible plant-derived nanoparticles as nanosized therapeutic agents or as an alternative drug delivery vehicle.

More recently, Mu et al. (2014) used four different edible plants (carrot, ginger, grapefruit and grape) to isolate and characterize exosome-like nanoparticles (EPDENs), which contain proteins, lipids, and microRNAs. They showed that EPDENs are taken up by intestinal macrophages and stem cells, and mediate interspecies communication by inducing expression of genes crucial for maintaining intestinal homeostasis (anti-inflammation cytokines, antioxidation, etc.).

Zhang and colleagues also analysed grapefruits derived nanovesicles (GDNs) (Wang et al., 2014), which were isolated from the fruit pulp using a sucrose gradient ultracentrifugation method. The isolated vesicles were nanosized with an average diameter of 210 nm. They demonstrated that grapefruit nanovesicles can serve as immune modulators in the intestine, maintain intestinal macrophage homeostasis, and can be used for oral delivery of small molecule drugs to attenuate inflammatory responses in human disease. In fact, there are clinical trials underway analysing their use to prevent cancer progression (www.clinicaltrials.gov/ct2/show/NCT01668849). Supporting these findings, Raimondo et al. (2015) showed that isolated nanovesicles from lemon juice inhibit proliferation in different tumour cell lines, suggesting the possible use of plant-edible nanovesicles as a feasible approach in cancer treatment. These vesicles, between 50 and 70 nm in diameter, exhibited a specific proteomic profile.

A very recent work by Timms et al. (2016) has described the exosome isolation from watermelon by ultracentrifugation and further characterisation by NTA. They observed that these exosomes were internalised into a proliferative subpopulation of Caco-2 cells, concurring with reports of fruit-derived exosome uptake into mouse small

intestinal stem cells. Their findings suggest that cross-kingdom transfer of dietary RNAs into human circulation is likely mediated by exosome uptake by the intestine.

Plants produce pharmacological active molecules and interest has been gained in testing whether these, and other molecules, might be transported in EVs. Grape-derived molecules (i.e. resveratrol) have been shown to inhibit signalling events in cancer, heart disease, degenerative nerve disease, and Alzheimer's disease.

In the present study, we have addressed the characterization of grape berry EVs isolated from juice by classical differential centrifugation. We also describe a preliminary dissection of their secretion by analysing their presence in the outer and inner tissues of the grape berry by TEM. Proteomics analyses of isolated grape EVs confirm the presence of typical EVs proteins including membrane and vesicle-associated ones.

2. Materials and methods for grape EVs isolation and characterization

2.1. Plant material

Berries from *Vitis vinifera* L. cv. Bobal were chosen for this study since it has been reported that this grape varietal contains high components with pharmacological activity like resveratrol (Navarro et al., 2008). Bobal is an autochthonous grape variety that occupies more than 70% of the total grapevine area in the D.O. Utiel-Requena (Valencia, Spain). Berries were collected from vineyards grown in a commercial winery located at Camporrobles (Valencia, Spain), within the D.O. Utiel-Requena area.

2.2. Grape EVs isolation

Bobal grape berries (365 g) were surface washed (3 x 15 min) with distilled water, and homogenised in a blender at 15 ºC in the presence of protease inhibitors (Complete Mini EDTA free, Roche®). Solid residuals were removed with a stainless steel sieve (1 mm) and the collected juice was first centrifuged at 16000 x g for 10 min (5 ºC) to exclude debris. Grape juice (150 ml) was concentrated to 10 ml using centrifugal filter devices (Centricon Plus-70, Millipore) at 3500 x g for 40 min (15 ºC); concentrated juice was filtered (0.22 µm), and nanovesicles pelleted at 120000 x g for 60 min (4ºC). Finally, pellets were washed and re-suspended in PBS.

2.3. Grape fixation

Fixation of Bobal grapes was carried out according to Diakou and Carde (2001). Disposable 1 ml syringes and needles (0.30 mm diameter) were used to inject 25% unbuffered glutaraldehyde droplets in the outer and inner tissues of recently collected berries. Eight injections were done in the equatorial plane of the fruit around a 1-2 mm wide circular band. In each injection point, glutaraldehyde was first injected in the endocarp, and successively in the mesocarp and exocarp, until a fixative droplet flowed at the grape surface. After 2 h at room temperature, the equatorial disk was dissected and cut into small pieces that were immersed in fresh fixative for 3 h under vacuum. After fixation, the samples were processed and observed under TEM as described below.

2.4. Sample preparation and TEM observation

For TEM, LR-white resin inclusion was performed by fixing grapes slices and extracellular vesicles with glutaraldehyde 2.5%, as previously described (Bernal et al., 2014). Samples were filtered in resin and polymerized at 60 ºC for 48 h. Ultrathin slides (60 nm) were stained with 2% uranil acetate and viewed by TEM in a Jeol JEM1010 microscope. Images were acquired with a digital camera MegaView III with the Olympus Image Analysis Software.

2.5. Proteomic analysis

Purified EVs were processed in duplicate as previously described (Marcilla et al., 2012) at the SCSIE, Universitat de València Proteomics Unit, a member of ISCIII ProteoRed Proteomics Platform. Five microliters of the suspensions were used to deliver to a trap column, using capillary HPLC. The elution was directly applied to a nanospray source of a QSTAR XL instrument (Applied Biosystems).

2.6. Database searches

Database search on NCBInr databases were performed as previously described (Bernal et al., 2014), using the Protein Pilot® software v4.5 (ABSciex) from data obtained for one sample, analysed independently and combined (total) for database search with the Paragon algorithm (Shilov et al., 2007), restricting taxonomy to plants. Positive identification was considered when at least two peptides were documented.

3. Results and discussion

3.1. Efficient isolation of EVs from grape berry juice

Ju et al. (2013) first identified the isolation of EVs from grape berry juice by mean of differential centrifugation along with sucrose cushion gradient from skin removed fruits (Ju et al., 2013). We now confirmed the ability of the classical ultracentrifugation method combined with previous concentration steps for efficient isolation EVs from grape berries. As shown in Figure 1, EVs with sizes ranging from 30 to 200 nm were characterized by TEM in the final pellets after ultracentrifugation. These sizes are in agreement with both, the described for intraluminal vesicles (ILV) in plants, and for EVs (Mu et al., 2014; Robinson et al., 2016). Different populations of EVs in size, and with double or single membrane were detected (Fig. 1), in agreement with the diversity found in mammalian EVs (Höög & Lotvall, 2015). These results confirmed that fruit juices are a good source of EVs, as had been previously described for grape berries (Ju et al., 2013; Mu et al., 2014), grapefruit (Mu et al., 2014; Wang et al., 2014), or lemon fruit (Raimondo et al., 2015).

We do think it could be also of application to other common fruits. Interestingly, our results further support the notion that exosomes may be released from plants through the production of MVBs, as has been largely controversial (Ding et al., 2012; Robinson et al., 2016).

3.2. Exosomes are released in vivo *by grape berries*

To address the origin of the EVs isolated from grape berry juice we next investigate the presence of EVs in different grape berry structures. We followed an *in situ* fixation protocol, which preserves fine structures of both the thick-walled cells of the outer exocarp, and the thin-walled mesocarp tissue (Diakou & Carde, 2001). As described in the methods section, eight different injections of fixative were used. As shown in Figure 2, the fixative (glutaraldehyde 25% in PBS) was injected under pressure in the outer and inner parts of the fruit, allowing an improved diffusion and high cross-linking of the fixative. We then obtained different cuts of the grape, and structures were analysed by TEM.

Interestingly, in all the tissues analysed we could detect EVs with size and morphology corresponding to exosomes (Fig. 2). Furthermore, several MVBs were detected closed to the plasma membrane (indicated as PMV, or paramural bodies as reviewed by Robinson et al., 2016) (panels C1 and C2), in the cell-cell junctions. In some cases, these vesicles were fused to the plasma membrane, probably to release their content to the neighbour cell (Fig. 2, C1 and C2). We also detected EVs in the outermost space of the grape, extruding from the wall/skin as microvesicles (Fig. 2, C3), suggesting that these EVs could be released by conventional mechanisms (Yañez-Mó et al., 2015).

As far as we know these data represent the first *in vivo* characterization of typical EVs in plants, helping in dissecting this unconventional protein secretion mechanism (UPS). We show evidence of the existence of both, MVBs and microvesicles driven mechanisms for EVs secretion in plants. In this context, previous ultrastructure descriptions of MVB-plasma membrane fusions have been reported (Gruner and Santore, 1991; Robinson et al., 1996), being termed as plasmalemmmasomes, paramural bodies or lomasomes (Robinson et al.,

2016), and their deep characterization as intermediate stages of EVs secretion warrants further investigations.

3.3. Proteomic analysis of grape berries EVs

Previous work by Zhang and colleagues identified what they called GELNs (grape exosome-like nanoparticles), containing different lipids as well as small RNAs (Ju et al., 2013). Along with lipids and RNAs, the identification of a reduced number of proteins was also reported (Ju et al., 2013). To gain further insight on the proteins of grape berries EVs, we next performed a proteomic analysis of the EVs isolated from Bobal grapes.

By mean of LC-MS/MS analysis, a total of 246 peptides corresponding to 121 proteins were identified in EVs from Bobal berries. Of these, 96 corresponded to plant proteins, and 55 different plant proteins were identified positively based on the presence of at least two peptides in the MS/MS analyses (Table 2).

The location of these proteins was predicted using the recently described FunRich software (Pathan et al., 2015). When using the 96 plant proteins, 38% of proteins were predicted to localize in the plasma membrane, and most of them corresponded to protein transport and GTPases as biological function categories (Figure 3). FunRich analysis of the 55 proteins confirmed that most of them corresponded to membrane-associated location (45%), followed by vacuole location (23.5%), and the major biological processes involved were again protein transport and signalling through GTPases (Figure 3).

Interestingly, most of the 55 identified proteins had already been reported in plant EVs proteomes (Table 2). In fact, 32 out of 55 proteins (58,2 %) were previously identified in plant EVs by various authors (Ju et al., 2013; Prado et al., 2014; Raimondo et al., 2015; Wang et al., 2014), from these, twelve proteins were previously detected in grape berry juice EVs by

Ju et al. (2013), which confirms their presence in EVs. We have now added 23 new proteins as present in EVs, including six vesicle-associated ones as well as six Rab-related proteins (Table 2).

The search of these proteins in the EVpedia database (http://www.evpedia.info; Kim et al., 2015), where recently the list of the top 100 identified proteins in EVs from different organisms has been updated, allowed to assign definitely 21 proteins (plus another 8 highly similar to those in EVpedia) as typical EVs ones (Table 2).

Among the identified proteins, the largest group corresponded to enzymes (13 proteins), including proteins involved in sugar metabolism like enolase, GAPDH, malate dehydrogenase, phosphoglycerate kinase, and aldolase, molecules also described in the top 100 proteins found in EVs (as in EVpedia).

A recent proteomic analysis of specific grape berry components has identified 60 proteins from Sauvignon Blanc grapes (Tian et al., 2015). We have found that 14 of these proteins, including the cytosolic proteins malate dehydrogenase, GAPDH, enolase or aldolase, but not others like the Rubisco protein (a typical chloroplast marker), are secreted in grape EVs. In this sense, the first cell wall proteomic study of fruit pericarp performed in tomato also detected GAPDH, enolase, and aldolase, as well as HSP70 (Konozy et al., 2013).

The secretion of these cytosolic enzymes outside the cell have raised the questions of their mechanism of export and their function in the extracellular space. In fact they are called “moonlighting proteins” (a term which, colloquially, means to have more than one job with the second being done at night) (Henderson and Martin, 2011).

Interestingly, out of the 55 identified proteins in grape EVs, only two of them have a signal peptide. Similar results have been recently reported in yeasts, where most of the *C. albicans* proteins identified in the extracellular media (depleted of EVs) carry signal peptide (90%), whereas the proteins identified in EVs do not. Furthermore, some proteins involved in metabolism, with no signal peptide, were exclusively present in EVs, like enolase1 or the GAPDH homolog Tdh3 (Gil-Bona et al., 2015). They showed that it was also the case for all proteins lacking a signal peptide involved in protein folding (i.e. Hsp70), or protein synthesis (i.e elongation factor Eft2) (Gil-Bona et al., 2015). We detected Hsp70 along with other signalling molecules like Calcineurin B and 14-3-3 proteins, all previously identified as secreted in plants (Tanveer et al., 2014).

Our identifications are in agreement with the published proteomic descriptions of plant EVs (Ju et al., 2013; Prado et al., 2014; Raimondo et al., 2015; Wang et al., 2014). In fact, enolase and GAPDH are among the top 10 identified proteins in EVs from several organisms (EVpedia.info).

Marcilla and co-workers have also identified these glycolytic enzymes lacking signal peptides in helminth EVs, supporting the notion that their unconventional secretion is an evolutionary conserved mechanism (Bernal et al., 2014; Marcilla et al., 2012).

Other enzymes related to protein and lipid metabolism were also detected in grape berries EVs, like Peptidyl-prolyl cis-trans isomerase, Chalcone-flavonone isomerase 1, or Delta-(24)-sterol reductase (Table 2). A Thaumatin-like protein was also identified in grape EVs, confirming their presence in grapes (Tian et al., 2015). This molecule is a typical apoplastic marker (Kim et al., 2013), detected among the predominant pathogenesis-related

proteins in grape juice which may cause haze formation in wine, and which origin seems to be the pulp (Tian et al., 2015).

The second largest group of proteins identified in grape berry EVs were GTPases, mostly members of the Rab protein family, with 12 proteins. These proteins participate in vesicle traffic by cycling between an active GTP bound state associated with membranes and an inactive GDP bound state present in the cytosol (Nielsen et al. 2008; Rutherford and Moore 2002; Yalovsky et al. 2008). They regulate the vesicle trafficking in different ways. In association with SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein Receptor) proteins, they participate in the docking step of transport vesicles to the target membrane. In our assays, a v-SNARE molecule has been identified, supporting this notion of coordinated functions in EVs. Small GTPases also participate in post-Golgi secretion of immunity-related proteins to the PM and regulate defense responses (reviewed by Wang et al., 2016). The abundancy of GTPases has been also detected in EVs from *C. albicans*, suggesting their presence in EVs is an evolutionary conserved feature of their secretion, which allows to save the cell wall (Gil-Bona et al., 2015).

Another abundant group of proteins present in grape berry EVs corresponded to proteins involved in membrane/transportation (10 proteins), and vesicle related ones (8 proteins). Among the latest, typical proteins involved in exosome formation on MVB, like the ESCRT-related protein CHMP1 was found, confirming the specific cargo in EVs of these proteins. As already mentioned, we also detected v-SNARE and clathrin related proteins in grape berries EVs (Table 2). All these proteins participate in different secretion pathways in plant cells.

Other proteins involved in secretory pathways in plants are VAMPs (vesicle associated membrane proteins), which three of their members, VAMP711, 713 and 726, have been identified in this study. These molecules either act as transporters or regulate cargo of proteins (Chen et al., 2012; Fujiwara et al., 2014; Heard et al., 2015).

Our data support the notion that fruits release EVs by both MVB and microvesicle formation, and their membranes can protect their content from lysis (Gupta et al., 2015).

4. Future directions: usefulness of food EVs as therapeutics agents

Cow milk exosomes have been recently suggested as good vehicles for delivery therapeutics (Munagala et al., 2016; Aqil et al., 2016a, 2016b). Along with milk, plant EVs provide a more convenient system for delivering therapeutic agents, so FoodEVs can be delivered from our homedoors to our cells. Although there are very few reports regarding this use, current clinical trials by Zhang and co-workers are underway, supporting the use of oral administration of grape EVs as an effective and safe treatment to prevent cancer progression (www.clinicaltrials.gov/ct2/show/NCT01668849). Supporting this notion, Raimondo et al. (2015) recently showed the effect of lemon fruit EVs inhibiting proliferation in different tumor cell lines.

Interestingly, Zhang and colleagues reported that plant EVs resist gastric pepsin solution and intestinal pancreatic and bile extract solutions, indicating their potential for influencing cell biology through ingested foods (Ju et al., 2013; Wang et al., 2014), and suggesting the possible use of food EVs as safe therapeutic agents. As mentioned previously, these authors have demonstrated that edible plant EVs can be taken up by intestinal macrophages and stem cells, which in turn prevent colitis induced by dextran sulphate sodium in a murine model (Ju et al., 2013; Mu et al., 2014). As a proof of

concept, a grapefruit-derived nanovector has been developed which meets all the requirements for safety, targeting and delivery (Wang et al., 2013, 2014). These studies also open new areas of research, related to cell-cell communications including cross-kingdom communications.

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FIGURE LEGENDS

Figure 1. Isolated EVs from grape berries characterized by TEM. Grape berries EVs were isolated by ultracentrifugation, and fixed with 2.5% glutaraldehyde, as described in material and methods. Samples were examined under TEM at x80000 magnification (A); and EVs size was measured (B). In panel C, at larger magnification at x100000, typical double membrane EVs are indicated by arrows.

Figure 2. EVs are produced by different cells in grape berries. Whole grape berries were fixed *in situ* to preserve fines structures injecting the fixative (A). After fixation, equatorial slices of grape berries were obtained (B); and three different cuts were processed for TEM (C): from the most internal part (C1), to an intermediate cut (C2), and the most external cut (C3). TEM magnification is showed accordingly to size bars. OS:

outermost space; EV: extracellular vesicle; ICW: intercellular wall; PMW: paramural bodies; MVB: multivesicular bodies; V: vacuole.

Figure 3. FunRich analysis of the proteins identified in grape berries EVs. Comparative analysis by FunRich software (Pathan et al., 2015) of the proteins from grape berries EVs after LC-MS/MS and identified by ProteinPilot, including the 96 plant proteins identified with more than 1 peptide (Grape 96), and the 55 corresponding to more than 2 peptides detected (Grape 55).

Table 1. Evs isolated and described in food. Classification has been made following Food and Agriculture Organisation of the United Nations (FAO), 2014. International Network of Food Data Systems (INFOODS): International food composition table/database directory

FAO GROUP		Species	Sample source	Isolation method	Reference
2	**Starchy roots and tubers**				
	Carrot	*Daucus carota*	root juice	Ultracentrifugation (UC)+sucrose gradient (SG)	Mu et al., 2014
	Ginger	*Zingiber officinale*	root juice	UC+SG	Mu et al., 2014
4	**Nuts and seeds**				
	Sunflower	*Helianthus annuus*	seeds extracellular fluids	UC	Regente et al., 2009
6	**Fruits**				
	Lemon	*Citrus limon*	fruit juice	UC+SG	Raimondo et al., 2015
	Grape	*Vitis vinifera*	fruit juice	UC+SG	Ju et al., 2013
			fruit juice	UC	This study
			fruit pulp	UC+SG	Mu et al., 2014
	Grapefruit	*Citrus paradisi*	fruit pulp	UC+SG	Mu et al., 2014
	Watermelon	*Citrullus lanatus*	fruit	UC	Wang et al., 2014 Timms et al. 2016
10	**Milk**				
	Cow	*Bos taurus*	raw milk	UC	Izumi et al., 2015 Munagala et al., 2016 Plantz et al., 1973 Reinhardt et al., 2013
			raw milk	UC+SG	Hata et al., 2010
				ExoQuick, UC+ExoQuick, UC+SG	Sun et al., 2015 Yamada et al., 2012
			semi-skimmed pasteurized milk	UC	Arntz et al., 2015
				UC+ExoQuick	Pieters et al., 2015
			low-fat (1% fat) commercial milk	UC	Kusuma et al., 2016
			commercial milk	UC	Oliveira et al., 2016
	Goat	*Capra aegagrus hircus*	milk	UC+density gradient	Krupova et al., 2016
	Buffalo	*Bubalus bubalis*	raw milk	UC+ExoQuick	Kuruppath et al., 2013
	Dromedary	*Camelus dromedarius*	raw milk	UC	Yassin et al., 2015
	Human	*Homo sapiens*	mature milk	UC+density gradient	Van Herwijnen et al., 2016
			mature milk	OptiPrep	Zonneveld et al., 2016
			mature milk	UC	Kosaka et al., 2010
			mature milk	UC	Zonneveld et al., 2014
			mature milk and colostrum	UC+SG	Admyre et al. 2007

Table 2. Proteomic analysis of Bobal grape EVs

Accession Membrane	Name	Species	Peptides (95%)	ProteinPilot values	Plant EVs	EVpedia
Q03194	Plasma membrane ATPase	*Nicotiana plumbaginifolia*	24	19,41	R* P*	*
Q9SH76	ATPase 6, plasma membrane-type	*Arabidopsis thaliana*	17	8,68	R* P	*
Q08435	Plasma membrane ATPase	*Nicotiana plumbaginifolia*	12	13,45	R* P*	*
P21616	Pyrophosphate-energized vacuolar membrane proton pump	*Vigna radiata*	9	15,59	nd	no
Q9ZNT0	Suppressor of K(+) Transport growth defect 1	*Arabidopsis thaliana*	8	12,87	nd	no
Q9SZN1	Vacuolar-type H+ ATPase subunit B	*Arabidopsis thaliana*	7	14,90	R*	yes
P09469	Vacuolar-type H+ ATPase subunit A	*Daucus carota*	7	12,03	R	yes
Q8RU33	Vacuolar-type H+ ATPase subunit d	*Oryza sativa*	3	2,09	R	yes
Q658H5	SPX domain-containing membrane protein Os06g0129400	*Oryza sativa*	2	4,00	nd	no
Q9FY75	Potassium transporter 7	*Arabidopsis thaliana*	2	2,15	nd	no
Vesicle associated						
O49377	Vesicle-associated membrane protein 711	*Arabidopsis thaliana*	10	8,09	nd	no
Q9LFP1	Vesicle-associated membrane protein 713	*Arabidopsis thaliana*	5	4,26	nd	no
Q9MAS5	Vesicle-associated membrane protein 726	*Arabidopsis thaliana*	4	3,62	nd	no
Q9LVP9	Vesicle transport v-SNARE 13	*Arabidopsis thaliana*	3	4,00	nd	no
P93798	Alpha-soluble NSF attachment protein	*Vitis vinifera*	2	4,58	R	yes
Q0WNJ6	Clathrin heavy chain 1	*Arabidopsis thaliana*	2	3,96	R	yes
O65421	Vacuolar protein sorting-associated protein 28 homolog 1	*Arabidopsis thaliana*	2	3,02	nd	no
Q84VG1	ESCRT-related protein CHMP1	*Oryza sativa*	2	2,92	nd	no
Signaling						
P29357	Chloroplast envelope 70 kDa heat shock-related protein	*Spinacia oleraceae*	15	21,32	nd	no
P11143	Heat shock 70 kDa protein	*Zea mays*	12	18,24	R W P	yes
Q9SRH6	Hypersensitive-induced response protein 3	*Arabidopsis thaliana*	5	8,00	nd	no
Q9CAR7	Hypersensitive-induced response protein 2	*Arabidopsis thaliana*	4	6,08	nd	no
P93209	14-3-3 family (multifunctional chaperone)	*Solanum lycopersicum*	3	5,70	nd	yes
Q75LU8	Calcineurin B-like protein	*Oryza sativa*	2	4,10	nd	yes
Nuclear						
Q9SHE7	Ubiquitin-NEDD8-like protein RUB1	*Arabidopsis thaliana*	17	11,28	R*	no
Q39256	Polyubiquitin 8	*Arabidopsis thaliana*	13	7,49	nd	no
Q94A97	Ubiquitin-conjugating enzyme E2	*Arabidopsis thaliana*	5	6,57	R	yes

Q9CAI7	Eukaryotic initiation factor 4A-III	*Arabidopsis thaliana*	2	3,17	nd	yes
GTPase related						
Q39433	Ras-related protein RAB1BV (Rab1)	*Beta vulgaris*	8	12,62	nd	no
P92963	Ras-related protein RABB1c (Rab1)	*Arabidopsis thaliana*	8	11,82	nd	no
O04486	Ras-related protein RABA2a (Rab2)	*Arabidopsis thaliana*	5	9,10	R*	*
Q9LS94	Ras-related protein RABG3f (Rab3)	*Arabidopsis thaliana*	5	8,24	nd	*
P49104	Ras-related protein Rab-2-B (Rab2)	*Zea mays*	5	7,47	R*	no
O24466	Ras-related protein RABE1a (Rab1)	*Arabidopsis thaliana*	4	8,12	nd	*
Q9SEH3	Ras-related protein RABD2c (Rab2)	*Arabidopsis thaliana*	3	7,05	R*	no
Q96283	Ras-related protein RABA2c (Rab2)	*Arabidopsis thaliana*	3	5,48	R*	*
P28188	Ras-related protein RABD2a (Rab2)	*Arabidopsis thaliana*	3	5,00	R*	no
Q40195	Ras-related protein Rab11E (Rab11)	*Lotus japonicus*	2	3,22	R*	*
Q9SMR4	Ras-related protein RABH1c (Rab1)	*Arabidopsis thaliana*	2	4,00	nd	no
P42697	Dynamin-related protein (predicted GTPase)	*Arabidopsis thaliana*	2	2,84	nd	yes
Enzymes						
P42896	Enolase	*Ricinus communis*	9	9,80	R J	yes
P25861	Glyceraldehyde-3-phosphate dehydrogenase	*Antirrhinum majus*	7	10,24	R P	yes
Q9LD57	Phosphoglycerate kinase	*Arabidopsis thaliana*	5	6,89	R J	yes
Q08062	Malate dehydrogenase	*Zea mays*	5	5,51	R J	yes
Q39613	Peptidyl-prolyl cis-trans isomerase	*Catharanthus roseus*	5	5,13	R J W P	yes
P51117	Chalcone-flavonone isomerase 1	*Vitis vinifera*	4	8,61	W*	no
P46257	Fructose-bisphosphate aldolase	*Pisum sativum*	3	5,68	R J	yes
P57751	UTP--glucose-1-phosphate uridylyltransferase	*Arabidopsis thaliana*	3	3,70	R W*	no
P81370	Thaumatin-like protein	*Actinidia deliciosa*	3	3,16	W	no
Q42662	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	*Plectranthus scutellarioides*	2	4,73	R P	yes
O65572	Carotenoid cleavage dioxygenase	*Arabidopsis thaliana*	2	4,00	R	no
Q43497	Monodehydroascorbate reductase	*Solanum lycopersicum*	2	2,99	R J P	no
P93472	Delta(24)-sterol reductase	*Pisum sativum*	2	2,41	R	yes
Cytoskeleton						
P53492	Actin	*Arabidopsis thaliana*	5	10,26	R W P	yes
Q9LEI8	Profilin	*Hevea brasiliensis*	2	2,98	R J W	yes

J: Ju et al., 2013; R: Raimondo et al., 2015; P: Prado et al., 2014; W: Wang et al., 2014. Asterisks indicate similar proteins; nd: not described

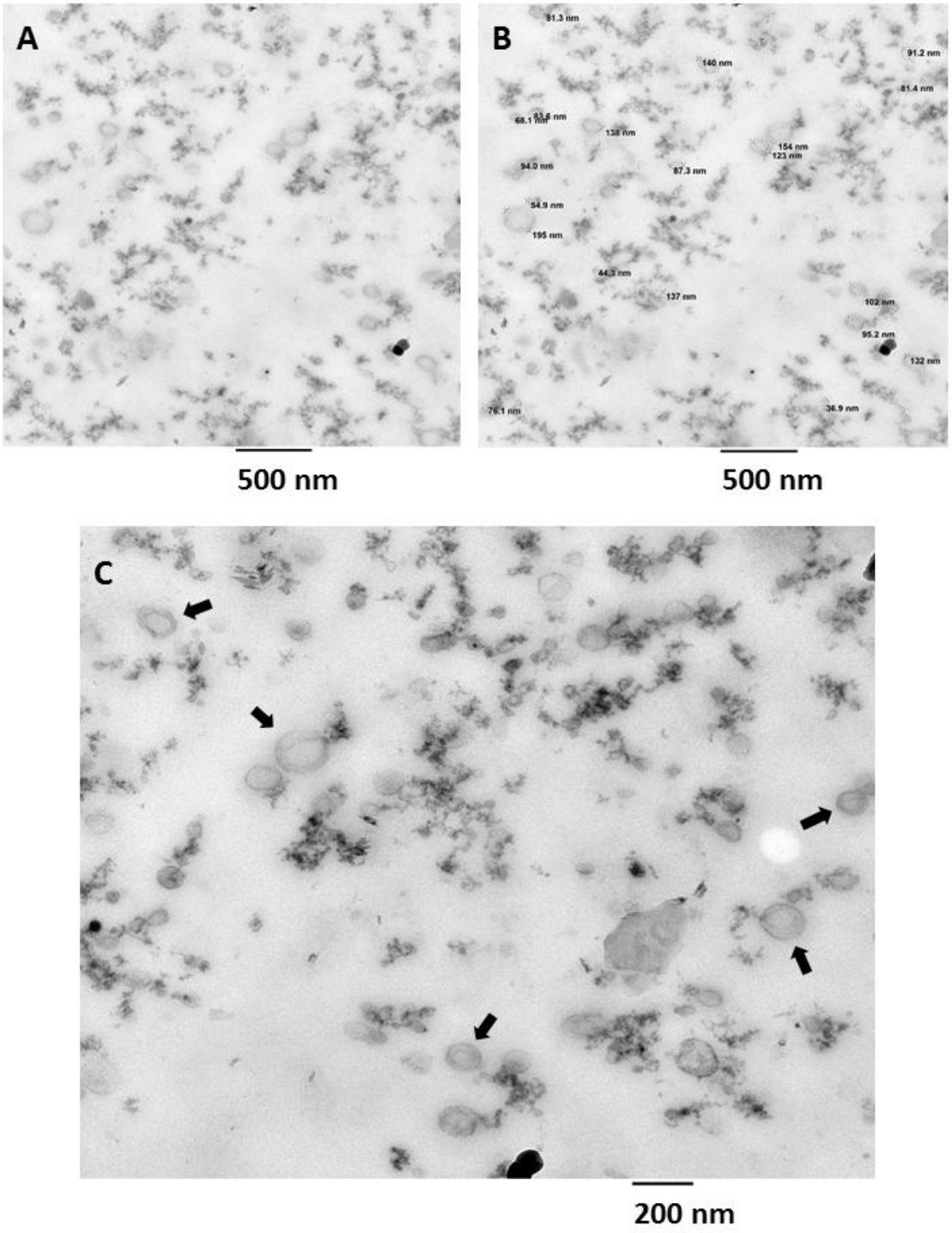

Figure 1

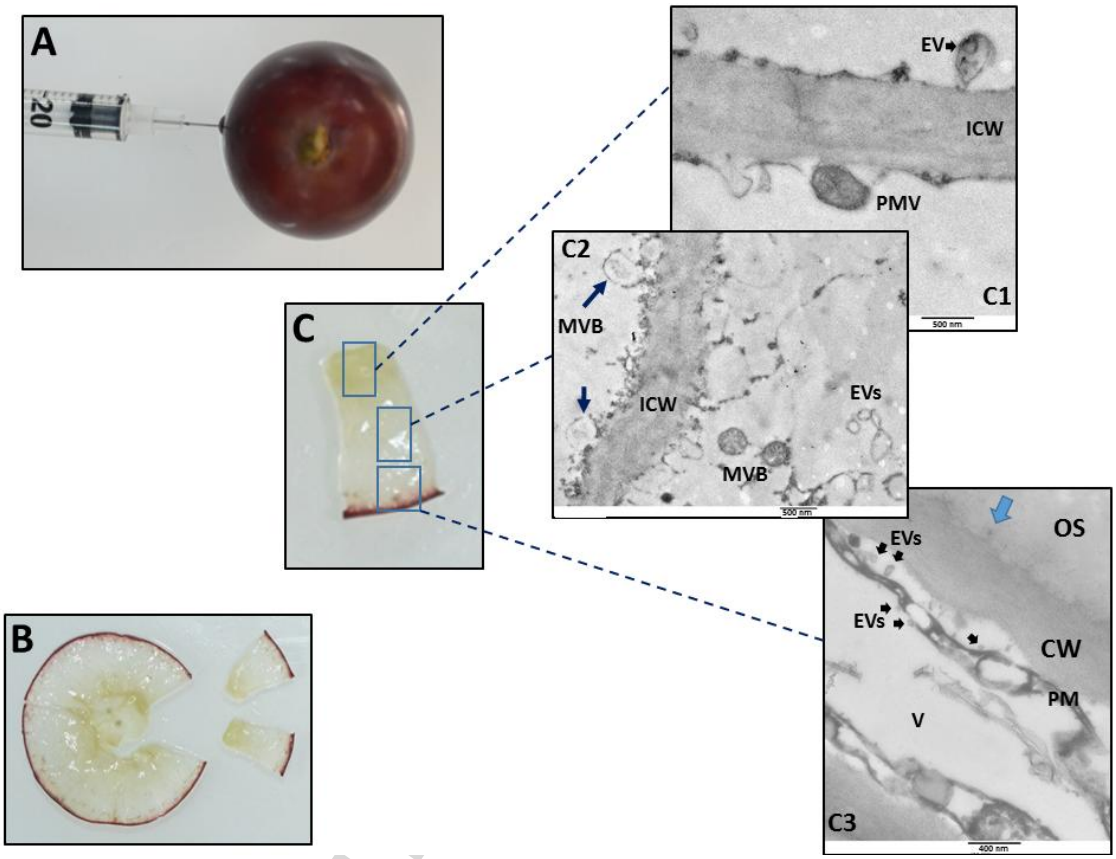

Figure 2

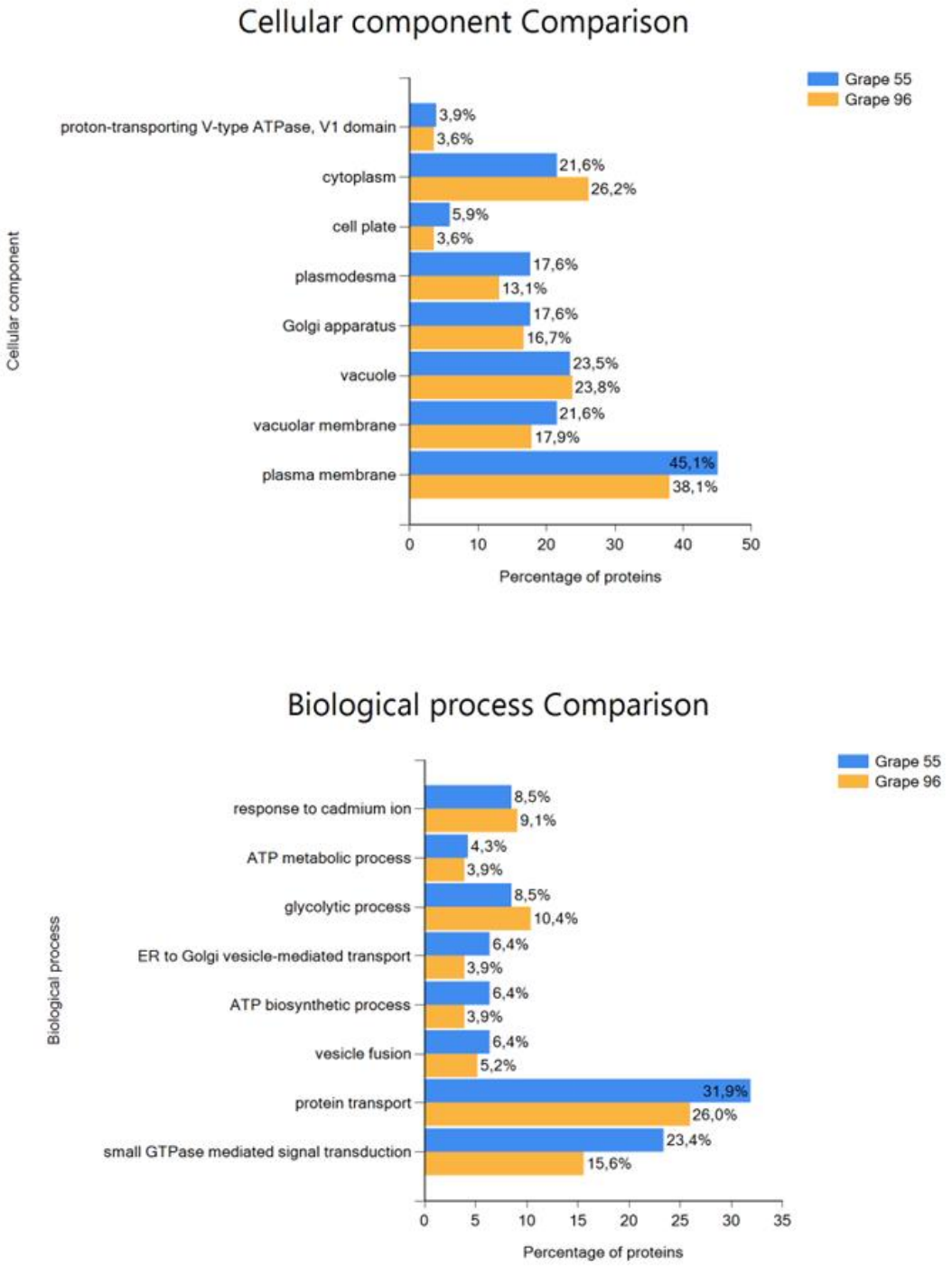

Figure 3

Graphical abstract