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RESEARCH PAPER

Plant extracellular vesicles are incorporated by a fungal pathogen and inhibit its growth

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Abstract

Extracellular vesicles (EV) are membrane particles released by cells into their environment and are considered to be key players in intercellular communication. EV are produced by all domains of life but limited knowledge about EV in plants is available, although their implication in plant defense has been suggested. We have characterized sunflower EV and tested whether they could interact with fungal cells. EV were isolated from extracellular fluids of seedlings and characterized by transmission electron microscopy and proteomic analysis. These nanovesicles appeared to be enriched in cell wall remodeling enzymes and defense proteins. Membrane-labeled EV were prepared and their uptake by the phytopathogenic fungus *Sclerotinia sclerotiorum* was verified. Functional tests further evaluated the ability of EV to affect fungal growth. Spores treated with plant EV showed growth inhibition, morphological changes, and cell death. Conclusive evidence on the existence of plant EV is presented and we demonstrate their ability to interact with and kill fungal cells. Our results introduce the concept of cell-to-cell communication through EV in plants.

Key words: Antifungal, apoplast, exosomes, extracellular vesicles, fungal growth, intercellular communication, plant defense.

Introduction

Over the past decade, extracellular vesicles (EV) have gained attention due to their multiplicity of functions and evolutionary conservation (Colombo *et al.*, 2014). EV are broadly defined as spherical particles enclosed by a phospholipid bilayer, which are released from cells into their environment. Mainly studied in mammals, they are recognized as novel components of the cell-to-cell communication machinery in both eukaryotes and prokaryotes, since they are vehicles for the transfer of informative biomolecules such as proteins, lipids, and RNAs (reviewed in Yáñes-Mó *et al.*, 2015; Tkach and Théry, 2016; Maas *et al.*, 2017). It has been demonstrated that cells can communicate with both neighboring and distant cells through the secretion of EV. Since they are secreted to the surrounding medium, EV have been detected and purified from conditioned culture media as well as from different body fluids such as plasma, urine, ascites fluid and saliva.

EV include diverse vesicles such as ectosomes, exosomes, and microvesicles, which differ in origin, function, and size (typically 30–1000 nm in diameter) (Raposo and Stoorvogel, 2013; Lötvall *et al.*, 2014). Their biogenesis appears to be diverse and is not yet fully understood. Nevertheless, two main pathways have been described: they can derive directly from the plasma membrane by a budding mechanism, or from an endocytic route (Raposo and Stoorvogel, 2013). As mediators of intercellular communication, EV have been shown to fulfil relevant roles related to cell homeostasis and pathogenesis, which have been extensively described in animal systems (Yáñes-Mó *et al.*, 2015; Maas *et al.*, 2017). They can promote or regulate diverse physiological and pathological processes, such as infections, host immune responses, development, and various diseases, notably neurodegeneration and cancer. For example, tumor EV can alter the cellular physiology of non-tumor cells to allow the dissemination and growth of cancer cells (Peinado *et al.*, 2012). EV cargoes are protected by the surrounding membrane and appear to communicate directives after their uptake by receipt cells. RNA cargo release in particular is an active research field in mammals. EV-mediated transfer of mRNAs into target cells has been demonstrated *in vivo* (Ridder *et al.*, 2014) and miRNAs secreted in EV can be delivered into target cells and modulate their mRNA targets (Wang and Wang, 2016).

Even though most of the current knowledge on EV has been acquired in mammalian systems, vesicles released from bacteria and fungi have also been extensively studied (Brown *et al.*, 2015). So, it is widely accepted that most cells in all domains of life, including eukaryotes, Gram-negative and Gram-positive bacteria, and archaea, actively produce nano-sized membrane vesicles and release them into the extracellular environment. Despite accumulated evidence and the demonstrated conservation of EV through evolution, our knowledge on plant EV remains extremely limited. As in other systems, EV could be expected to be found in the extracellular compartments of plant origin, such as cell culture media or the apoplast, the compartment located outside the plasma membrane and formed by a continuum of cell walls and the extracellular space. The first attempt to isolate exosome-like vesicles in plants was reported in 2009 (Regente *et al.*, 2009). Apoplastic fluids obtained from imbibed sunflower seeds were submitted to a classical procedure used for the isolation of human EV and rendered an ultracentrifugation pellet containing phospholipid vesicles ranging from 50 to 200 nm in diameter (Regente *et al.*, 2009). Interestingly, these vesicles were shown to be enriched in a lectin, later called Helja, which constitutes the only demonstrated case of non-classical secretion of a plant protein to the apoplast (Pinedo *et al.*, 2012, Ding *et al.*, 2014). Strikingly, one of the proposed pathways of non-classical secretion described in eukaryotes involves EV (Robinson *et al.*, 2016). Thus, plant EV seem to mediate the transport of proteins to the extracellular compartment and could partly account for the high number of proteins lacking a signal peptide that are found in extracellular fluids (Regente *et al.*, 2012; Pompa *et al.*, 2017). It must be highlighted that ~50% of the proteins detected extracellularly in diverse plant systems are devoid of signal peptide (Agrawal *et al.*, 2010, Albenne *et al.*, 2013). Further evidence for the presence of plant EV was reported in olive pollen-derived samples. The 100 000 *g* pellet of stigmata exudates and pollen germination medium contained nanovesicles that were partially characterized by transmission electron microscopy (TEM) and Fourier transform infrared analysis (Prado *et al.*, 2014).

Even though plant EV have been ignored for a long time, a recent article by Rutter and Innes (2017) presented new evidence for their existence. EV isolated from apoplastic fluids from *Arabidopsis thaliana* leaves appeared to be enriched in proteins involved in stress responses, and the authors reported that the secretion of these EV was enhanced during infection with a virulent bacterial pathogen, even if the EV proteome showed few changes in response to bacterial infection (Rutter and Innes, 2017). Their participation in plant defense was then proposed. Despite accumulated data, the existence of EV in plants is barely recognized, meaning that we are losing the opportunity to understand novel cellular processes and putative cell-to-cell communication mechanisms. The aim of this work is to present conclusive evidence through the characterization of EV isolated from seedlings of sunflower, a non-model plant, and to develop functional assays to evaluate their ability to interact with cells and control the growth of a phytopathogenic fungus.

Materials and methods

Plant and fungal material

Sunflower seeds (*Helianthus annuus* L., line 10347 Advanta Semillas SAIC, Argentina) were imbibed overnight and then sown in individual pots containing soil:perlite (3:1) and grown at 22 °C with a 16/8 h day/night photoperiod (150 µmol m^{-2} s^{-1} diurnal irradiance) for 17 days. At this stage, plants presented the first leaves of 1–3 cm in length. Plants were watered twice a week.

Sclerotinia sclerotiorum ascospores from a local isolate were kindly supplied by Advanta Semillas SAIC (Balcarce, Argentina) and were collected in sterile water from Petri dishes containing imprints of apothecia. Ascospore suspensions were quantified in a Neubauer chamber under optical microscopy. Collected spores were immediately used for functional tests.

Purification of EV

Seventeen-day-old sunflower seedlings were cut 1 cm above the level of the soil, pooled, and weighed before immersion in extracellular fluid (EF) extraction buffer (EB: 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1% 2-mercaptoethanol). EF was obtained by vacuum infiltration-centrifugation under minimal-stress conditions of vacuum and salt as previously described by Regente *et al.* (2008). Briefly, seedlings immersed in EB were submitted to a soft vacuum infiltration condition (3 × 10 s, separated by 30 s intervals) at a pressure of 45 kPa, which is half of the standard pressure for apoplastic fluid isolation (Lohaus *et al.*, 2001). Typical preparations were made from 450 g of seedlings, obtaining 95 ml EF.

EF was then subjected to fractionation by successive centrifugation steps at 500 *g* for15 min, 10 000 *g* for 30 min, and 100 000 *g* for 60 min, according to a procedure previously described for animal exosomes. The first pellets were discarded and the 100 000 *g* pellet, which was enriched in extracellular vesicles (EV fraction), was suspended in phosphate-buffered saline (PBS). Approximately 150 µg proteins were obtained in EV prepared from 450 g of seedlings. Samples were immediately used for antifungal activity tests or stored in aliquots at –20 °C. The 100 000 *g* supernatant (S100) was stored at –20 °C for protein content comparison.

Electrolyte leakage

To evaluate the plasma membrane integrity after EF extraction, electrolyte leakage was assessed by measuring electric conductivity (Campos *et al.*, 2003). Freshly cut seedlings and seedlings recovered after EF extraction (10 g) were extensively rinsed with demineralized water and subsequently incubated in 30 ml double-distilled water at room temperature. Electrolyte leakage in the solution was measured after 2 h using a HI8733 conductivity meter (Hanna Instruments, Sigma, USA). Measurements were registered in mS/cm. Total conductivity was obtained in a similar sample incubated at 80 °C for 2 h. Results were expressed as the percentage of total conductivity (Distéfano *et al.*, 2015). Conductivity measurements in isolated EF could not be compared due to the high salt concentration of EB.

Transmission electron microscopy

EV in PBS stored at –20 °C (20 µl) were fixed in 2% paraformaldehyde in PBS and sent refrigerated to a TEM facility for analysis (Servicio de Microscopia Electrónica, CCT Bahía Blanca, Argentina). Procedures used were basically as described in Théry *et al.* (2006). Vesicles were adsorbed on to Formvar-coated copper grids (300 mesh) and submitted to negative staining with 1% (w/v) uranyl acetate for 1 min. Samples were examined in a JEOL JSM 100CX II transmission electron microscope at 100 kV (JEOL USA Inc., Peabody, MA, USA).

Proteomics analysis

EV containing aproximately100 µg proteins were suspended in water and submitted to extraction in methanol/chloroform/water (4/3/1, v/v/v) and then centrifuged for 5 min at 12 000 *g*. Proteins were recovered from the water/organic solvent interface. A quantity of 400 µl methanol was added before centrifugation for 10 min at 12 000 *g*. The pellet, in 50 µl methanol, was sent to a proteomics facility for LC-MS/MS analysis and protein identification (PAPPSO, Gif-sur-Yvette, France). The proteins present in S100 were also isolated. Three (EV samples) or two (S100) biological replicates were analyzed.

Briefly, each sample (40–100 µg proteins) was suspended in ZUT buffer (6 M urea, 2 M thiourea, 10 mM DTT, 30 mM Tris-HCl, pH 8.8, and 0.1% ZALS1) at a final concentration of 4 µg µl^{-1}. Samples of 40 µg of proteins were diluted in loading buffer (50 mM Tris-HCl, pH 8.8, 1% SDS, 10% glycerol, 25 mM DTT) and submitted to short one-dimensional electrophoresis (1 cm). Each lane was cut into three pieces and digested using a standard trypsin-based protocol (Nguyen-Kim *et al.*, 2016). Peptides were analyzed using a NanoLC Ultra 2D system HPLC (Eksigent, Life Sciences Holdings France SAS, Les Ulis, France) coupled to a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Villebon-sur-Yvette, France). Samples were loaded on a C18 trap column (particles of 5 µm, 100 µm in diameter and 2 cm length; NanoSeparations, Nieuwkoop, The Netherlands), and desalted in 0.1% formic acid. Peptides were separated on a C18 analytical column (particles of 3 µm, 100 µm inner diameter and 300 mm length) for 37 min. Eluted peptides were ionized using a nanoelectrospray interface (non-coated capillary probe, 10 µm tip inner diameter; 12 cm length, New Objective Inc., Woburn, MA, USA) before mass spectrometric analysis. The gradient used for elution was as previously described by Nguyen-Kim *et al.* (2016). Peptide ions were analyzed using Xcalibur 2.3 with the following parameters for acquisition steps: (i) full MS scan [mass-to-charge ratio (m/z) 400:1400, profile mode, 70 000 resolution, AGC target set to 3×10^6]; (ii) MS/MS (precursor charge state: 2 to 4, profile mode, 17 500 resolution, AGC target set to 5×10^4, and maximum ion injection time of 120 ms) for 8 major ions detected in the full MS scan. Dynamic exclusion was set to 40 s. Raw data files were converted to mzXML open source format using ProteoWizard software version 3.0 with centroid transformation. Identification of proteins was performed using X!Tandem software (Craig and Beavis, 2004) and the X!Tandem Pipeline (Langella *et al.*, 2017) against the Sunflower database (https://www.heliagene.org/HanXRQ-SUNRISE/, 52 243 entries) (Badouin *et al.*, 2017) and an in-house contaminant database (55 entries including keratins, trypsin, and bovine serum albumin). The following parameters were used: tryptic digestion declared with one possible miscleavage, oxidation of methionine and carbamidomethylation of cysteine set to variable and fixed modification, respectively. Other commonly rare modifications were searched in refine mode (see Supplementary Tables S1–S5 at *JXB* online). Precursor mass tolerance was set to 10 ppm and fragment mass tolerance to 0.02 Da. Identified peptides were filtered using e-value ≤0.01 for peptides and $\log_{10}$ (e-value) < –5 for proteins. False discovery rate was between 0.20 and 0.50% for peptides and 0% for proteins. Proteins were validated if at least two different peptides in the same sample were found in at least two biological repeats.

EV membrane staining

A fresh EV preparation was labeled with FM4–64 stain (Molecular Probes, Thermo Fisher Scientific, Argentina) as described by Marcilla *et al.* (2012) but using the probe in a 5-fold dilution relative to the manufacturer's protocol. Briefly, purified vesicles were suspended in 40 µl PBS and mixed gently with FM4–64 (final concentration 1 µg ml^{-1}) and kept for 60 min on ice. The samples were then diluted to 3 ml and submitted to ultracentrifugation at 100 000 *g* to remove the excess of dye. The pellet was washed again and finally resuspended in 20 µl PBS.

Uptake of EV by S. sclerotiorum

An aliquot of 5 µl of *S. sclerotiorum* ascospores (~10 000 cells) was incubated with 2 µl of FM4–64-labeled EV (obtained from 1 g of fresh tissue) directly on glass slides and observed under confocal microscopy after 3–5 min or 30 min of incubation at room temperature. Microscopic analysis was performed using a Nikon C1 confocal laser scanning microscope (Nikon Instruments Inc., Melville, NY, USA). Control treatments were performed by incubating ascospores with PBS instead of FM4–64-labeled EV. Additionally, *S. sclerotiorum* ascospores were incubated in the presence of FM4–64 (final concentration 5 µg ml^{-1}). All images were acquired with a Super Fluor 40.0x/1.30/0.22 oil-immersion lens. FM4–64 was excited at 488 nm and detected at 650–750 nm. Post-processing of images was performed with the aid of EZ-C1 FreeViewer version 3.2 software.

Effect of EV on fungal spores

A qualitative test for inhibition of germination of fungal spores was performed on microslides using standard protocols. The incubation mixture contained ~1500 spores, 4% sucrose, and the EV sample, in a final volume of 20 µl. After 16 h of incubation at 25 °C and 100% relative humidity, the slides were evaluated for the presence and morphology of hyphae under optical microscopy (Nikon Eclipse 2000) or on a Nikon C1 confocal laser scanning microscope. Controls were performed by replacing EV with the same volume of water or PBS, both controls showing the germination of spores. Additional controls were performed using vesicles prepared by thin-film rehydration according to Zhu *et al.* (2013). Different aliquots of vesicles composed of egg yolk phosphatidylcholine up to 0.8 mg ml^{-1} were tested for the inhibition of spore germination.

Growth inhibition was estimated on enlarged microscopic images, using the scale bar as a tool to measure hyphal length. After evaluation of the antifungal activity, Evans Blue dye was added to a final concentration of 0.05% w/v and incubated for 10 min at room temperature before microscopic observation (Levine *et al.*, 1994). When indicated, assays were observed after a short incubation (3 h) in the presence of EV.

Membrane permeabilization assays were performed on microslides as described above for the test of antifungal activity, but incubated for only 3 h in the presence of EV. After treatment, propidium iodide

was added to a final concentration of 50 µg ml^{-1} and observed under a fluorescence microscope (Nikon Eclipse E200) equipped with an epifluorescence unit and a G-2E/C filter set containing an excitation filter at 540/25 nm, a suppressor filter at 630/60 nm, and a dichroic mirror at 565 nm.

Results

EV in EF of sunflower seedlings

For the isolation of EF from sunflower seedlings, a procedure employing gentle vacuum infiltration-centrifugation was applied. This procedure is based on previously adjusted methods and uses low pressure, low salt, and low centrifugation speed in order to minimize cell disruption (Regente *et al.*, 2008). Before EV isolation, controls were performed to assess contamination of EF with intracellular components that could have originated from cell lysis during apoplastic fluid extraction. Cell lysis should result in electrolyte leakage, since ions rapidly diffuse from the tissue when it is immersed in an aqueous solution. Electrolyte leakage was measured in freshly cut seedlings and compared with measurements made on seedlings recovered after EF isolation. Supplementary Table S6 shows the results of four independent determinations, which revealed that a less than 1% increase in conductivity was observed when comparing intact seedlings with seedlings previously submitted to EF extraction. This evidence confirmed the high quality of the EF obtained, which exhibited minimal contamination with intracellular contents. Once the quality was confirmed, these EF were further used for the preparation of an EV-enriched fraction. A standard procedure employed in animal and plant systems was applied (Regente *et al.*, 2009; Prado *et al.*, 2014). It included sequential centrifugation steps and a final ultracentrifugation at 100 000 *g* for 60 min. The last pellet (EV fraction) was suspended in PBS and used for further characterization. The protein yield of EV obtained in several biological replicates was ~0.3–0.4 µg proteins g^{-1} fresh tissue or 1.4–2 µg proteins ml^{-1} EF.

In a following step, the EV fraction was submitted to fixation with paraformaldehyde for observation under TEM using uranyl acetate for negative staining. TEM images (Fig. 1) revealed a heterogeneous population of typical vesicles, most which had diameters of 30–150 nm. Some of these vesicles showed the cup-shaped morphology frequently observed in EV occurring during the fixation procedure (Théry *et al.*, 2006). In addition, glutaraldehyde fixation also allowed the observation of vesicular structures (data not shown).

EV protein cargo

As a first insight into the features of EV, a proteomics analysis was performed, taking advantage of the recent completion of the sunflower genome (Badouin *et al.*, 2017). Three biological replicates were performed. Proteins were identified by LC-MS/MS and bioinformatics using the genomic sequences of sunflower (Supplementary Tables S1–S3). In total, 237 proteins were unambiguously identified in at least two biological replicates (summarized in Supplementary Table S7). In addition, 41 proteins belonging to different groups were identified. In each of these groups, the proteins shared common peptides because they belonged to the same multigene families, and hence were considered to be ambiguous identifications. Other proteins were detected in only one of the biological replicates and were not retained for this analysis.

The same experimental approach was used to identify proteins present in the apoplastic fraction recovered after the final ultracentrifugation step (i.e. S100). In these samples, 226 proteins were unambiguously identified, as well as nine proteins belonging to multigene families (Supplementary Tables S4 and S5 and summary results in Supplementary Table S8). Comparative analysis of the proteins unambiguously identified in EV and S100 revealed different compositions for each fraction. Altogether, 349 different proteins were identified, among which 114 were common to both fractions, 123 were specific to EV, and 112 were specific for S100 (Fig. 2A). In addition, 47 groups of ambiguously identified proteins were found, among which 38 were found only in EV, 6 only in S100, and 3 in both samples (Fig. 2B). Cell-wall-related proteins were found in both the EV and S100 fractions. All of these proteins were predicted to be secreted via the canonical

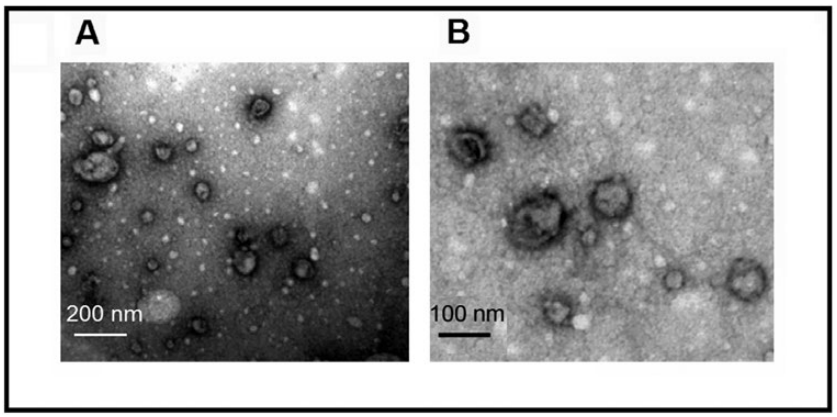

Fig. 1. (A, B) Transmission electron micrographs (TEM) of extracellular vesicles (EV) from apoplastic fluids of sunflower seedlings. EV obtained in the 100 000 *g* pellet of extracellular fluids were fixed in 2% paraformaldehyde and stained with 1% uranyl acetate. The images are representative of several TEM. Bars=200 nm in A and 100 nm in B.

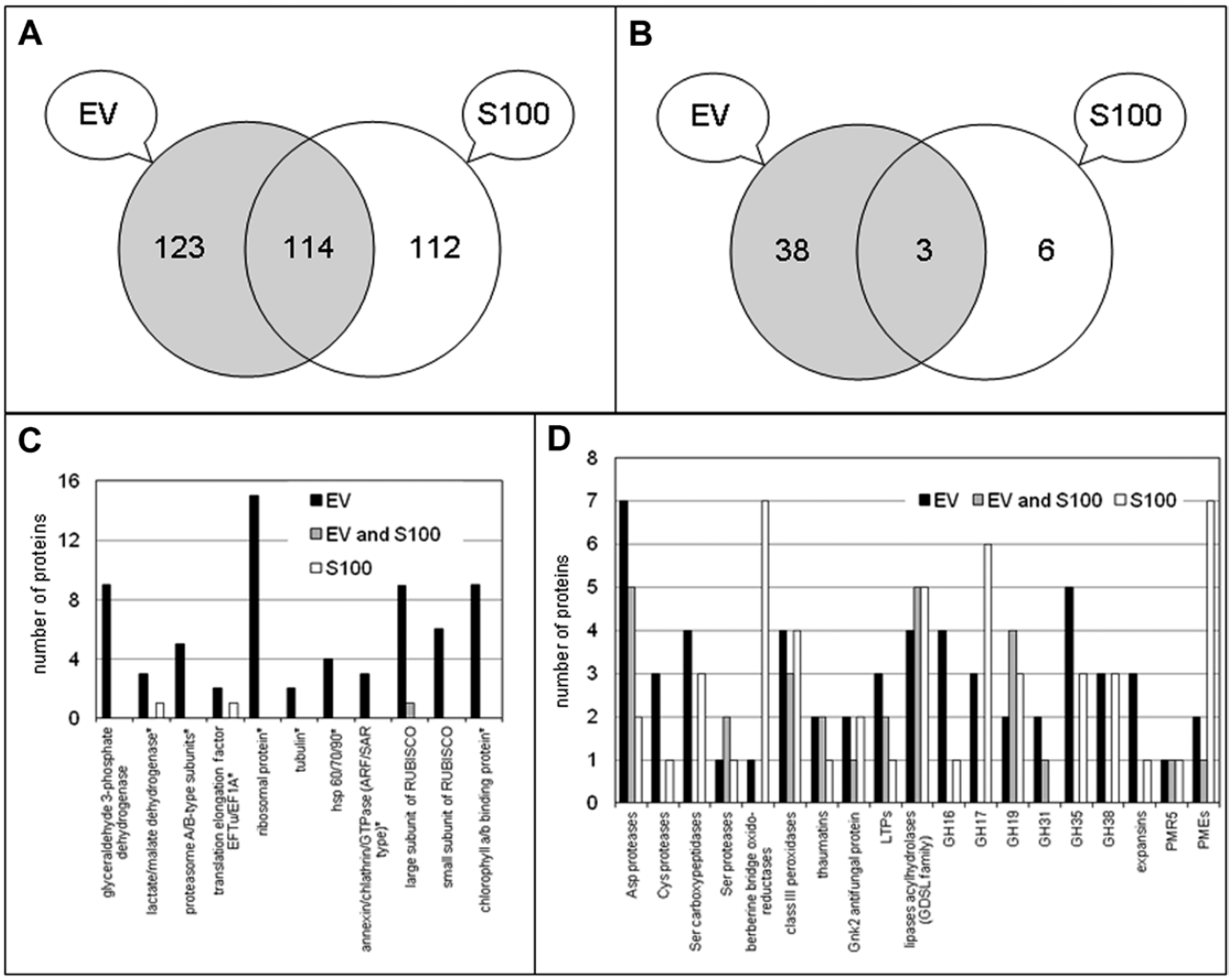

Fig. 2. Proteomics analysis of the extracellular fluids of sunflower seedlings after fractionation into extracellular vesicles (EV) and S100 by a 100 000 *g* ultracentrifugation. (A) Overall distribution of the unambiguously identified proteins. (B) Overall distribution of the groups of ambiguously identified proteins. (C) Distribution of the families of proteins usually found in animal EV and those involved in photosynthesis. Asterisks indicate groups of ambiguously identified proteins. (D) Distribution of the families of cell-wall-related proteins.

secretion pathway through the endoplasmic reticulum and the Golgi apparatus.

Clustering of the proteins identified in EV according to Gene Ontology (GO) terms was unsatisfactory since more than 30% of them had no predicted GO term for biological processes (data not shown). Nevertheless, among the proteins identified in EV (Supplementary Table S7), many have also been detected in EV isolated from mammalian sources (see Extracellular Vesicle Database, EVpedia: http://www.evpedia.info). This was the case for those involved in glycolysis/citric acid cycle (glyceraldehyde-3-phosphate dehydrogenase, L-lactate/malate dehydrogenase, isocitrate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase), proteolysis (proteasome subunits, ubiquitin), protein synthesis (elongation factors, ribosomal proteins), cytoskeleton (tubulin), and heat-shock responses (Hsp60, Hsp70, Hsp90). Notably, proteins involved in vesicle trafficking, such as annexin, clathrin, and small GTPases, were detected in EV (Fig. 2C). Interestingly, a comparative analysis revealed that 24 protein families detected in sunflower EV were also identified in EV isolated from Arabidopsis leaves. These results, presented in Supplementary Table S9, highlight the consistency of the results obtained. Other proteins characteristic of plants were also identified; these mainly included some chloroplast proteins (large and small subunits of ribulose bisphosphate carboxylase, photosystem II proteins, chlorophyll A-B binding proteins, carbonic anhydrases) (Fig. 2C) and cell-wall-related proteins (Fig. 2D). Some protein families were enriched in EV, such as proteases (Asp proteases, Cys proteases, Ser carboxypeptidases), lipid transfer proteins (LTPs), xyloglucan endo-transglycosylases (GH16), and expansins. The mannose-binding lectin Helja, previously detected in EV isolated from sunflower seeds (Regente *et al.*, 2009) was also identified in EV (Supplementary Table S7, HanXRGChr02g0047121). Others were mostly identified in S100, such as berberine-bridge oxido-reductases, glucan endo-1,3-β-glucosidase (GH17), and pectin methylesterases (PMEs) (Supplementary Table S8). Finally, some protein families were found in both fractions, e.g. Ser proteases, class III peroxidases, thaumatins, Gnk2-antifungal proteins, lipase acyl hydrolases of the GDSL family, chitinases/lysozymes (GH19), α-mannosidases (GH38), and proteins homologous to *A. thaliana* PMR5

(Powdery Mildew Resistant) assumed to be involved in carbohydrate acylation.

Several proteins identified in EV have been considered to be pathogenesis-related (PR) proteins (van Loon *et al.*, 2006): chitinases II (PR-4), thaumatins (PR-5), proteinase inhibitors (PR-6), peroxidases (PR-9), and lipid transfer proteins (PR-14). Other proteins detected were possibly related to plant defense, such as dirigent protein-disease resistance, PMR5, and Gnk2 antifungal protein, as well as GDSL lipase acylhydrolases, lectins, and germin-like proteins (Vogel *et al.*, 2004; Ralph *et al.*, 2006; Manosalva *et al.*, 2009; Grienenberger *et al.*, 2010; Wang *et al.*, 2013; Miyakawa *et al.*, 2014).

Uptake of sunflower EV by fungal cells

The high content of defense proteins found in EV prompted us to analyze whether plant vesicles could participate in plant defense toward fungal attack by direct interaction with fungal cells. This hypothesis also took into account the current knowledge in animal systems documenting that EV released by donor cells can be taken up by recipient cells (Maas *et al.*, 2017). We focused our study on the interaction between sunflower EV and the phytopathogenic fungus *S. sclerotiorum*, which has a wide host range and can cause severe rot. EV uptake by target cells can take place either by fusion with the plasma membrane or following endocytic pathways (Mulcahy *et al.*, 2014), and can be visualized by EV membrane staining using fluorescent dyes. To test whether sunflower EV could be internalized by fungal cells, the vesicles were labeled with the probe FM 4–64 and further washed and ultracentrifuged to eliminate the residual non-bound probe. The labeled EV obtained were then incubated with *S. sclerotiorum* spores and analyzed to detect whether the label was transferred from the vesicles to the fungal cells. While *S. sclerotiorum* spores did not exhibit red fluorescence when incubated with PBS (Fig. 3A), spores appeared red in the presence of labeled EV, suggesting that the EV were internalized (Fig. 3B). This uptake was extremely rapid, since the label was detected inside the cells as early as 3–5 min after treatment, the minimum time required to initiate the incubation and register the observation. On the other hand, direct treatment of the spores with FM4–64 resulted in a different labeling pattern that required at least 20 min of incubation to be detected (Fig. 3C). In this case, fluorescence was mainly observed at the cell surface. In conclusion, uptake assays suggest that sunflower EV could be incorporated by the fungus, opening up new possibilities concerning the putative role of EV during plant–pathogen interactions.

Plant EV inhibit fungal growth and cause cell death

Functional assays were designed to evaluate the effect of the uptake of EV by *S. sclerotiorum*. A classical qualitative spore germination test was performed in order to assess whether

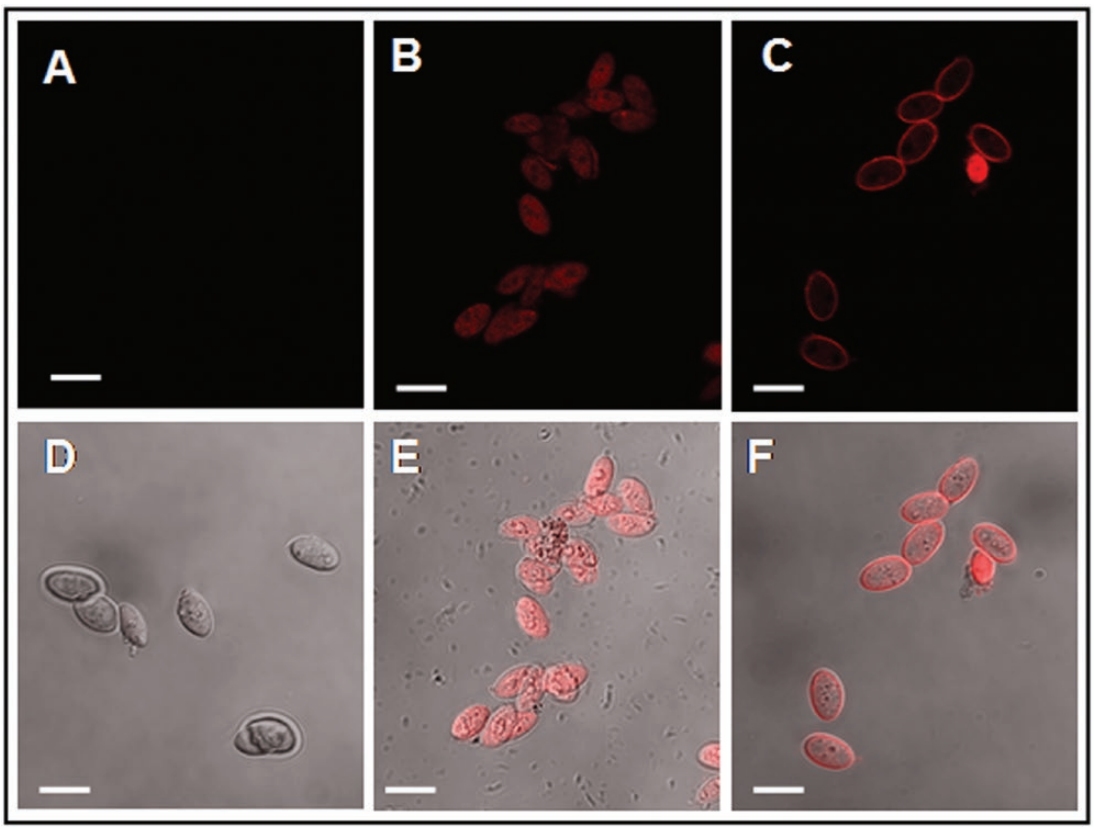

Fig. 3. Uptake of extracellular vesicles (EV) by *S. sclerotiorum* spores. (A) Control spores in PBS showing negative red autofluorescence. (B) Spores incubated for 5 min with FM4–64-labeled EV. (C) Spores treated with FM4–64. (D), (E), and (F) show the merge of panels (A), (B), and (C) with their respective bright-field images. The images were obtained with a confocal laser scanning microscope at 650–750 nm. Bars=10 μm. (This figure is available in colour at *JXB* online.)

EV produced any morphological effect on fungal cells. Spores were incubated in the presence of EV for 16 h and then submitted to microscopic observation. Fig. 4A shows that in a control treatment (incubation with water) spores germinated, producing profuse hyphae with straight elongation. In contrast, spores incubated in the presence of EV showed severe morphological changes; we observed a reduction in hyphal growth together with abnormal shapes, mainly wavy/curly hyphae (Fig. 4B–D). Hyphal length measurements revealed a reduction of 78 ± 8% in EV-treated samples. Additionally, accumulation of unknown materials around hyphae (Fig. 4B, C), as well as non-germinated spores (Fig. 4D), were detected, although they could not be accurately quantified due to the limitations of the test. Complementary assays suggested that the antifungal effect of EV is specific, since spores incubated with different concentrations of artificial vesicles made of phosphatidylcholine, up to 0.8 mg ml^{-1}, showed normal germination and hyphal growth, indistinguishable from the control treatment (data not shown).

Results presented in this section were obtained using freshly prepared EV samples. The antifungal activity appeared to be rather unstable, since it was reduced or lost upon storage of EV at –20 °C or 4 °C, an effect previously described for EV by Lőrincz *et al.* (2014).

Since only a fraction of the spores seemed to germinate in the presence of EV, another approach was used to analyze their viability in these conditions. Staining of the fungus with the vital dye Evans blue revealed that upon incubation for 16 h with EV, most fungal hyphae appeared to be stained, indicating that they had become non-viable (Fig. 5B). We then explored whether this loss of viability was an early response to the treatment. In fact, fungal samples treated for only 3 h began to reveal some non-viable cells and exhibited accumulation of cellular materials around the remaining fungal structures (Fig. 5D), probably as a consequence of cellular lysis. Another approach was used to assess a putative early fungal membrane permeabilization in the presence of EV. Cells with compromised membrane integrity are known to take up the red fluorescent dye propidium iodide. *S. sclerotiorum* spores were incubated in the presence of EV for 3 h and stained with the dye before observation under fluorescence microscopy. Fig. 5G shows that some of the EV-treated fungal cells appeared to be labeled red, while control samples remained unstained (Fig. 5E), indicating the occurrence of alterations in cell membrane permeability and subsequent penetration of the fluorescent probe. Counting of labelled spores in three replicates indicated that 60% of the EV-treated cells took up the probe within 3 h. In conclusion, the uptake of propidium iodide revealed that EV produced fungal membrane permeabilization and loss of viability. Taken together, our results demonstrate that sunflower EV could exert an antifungal effect.

Discussion

Plants do secrete EV

The existence of EV in plants has been neglected until now, probably because of the long-standing preconception that the membrane structures and putative cytosolic proteins found extracellularly are a consequence of cell disruption during apoplastic fluid extraction. Even if a proportion of the proteins found in extracellular compartments may have this origin, other accumulated evidence cannot be ignored. Unconventional protein secretion in plants has begun to be documented and exosome-like vesicles have been recognized as putative vehicles for protein secretion to the extracellular compartment (Ding *et al.*, 2014; Robinson *et al.*, 2016; Pompa *et al.*, 2017). Particularly in sunflower, the lectin Helja has been shown to be extracellular even if it lacks the classical N-terminal signal peptide (Pinedo *et al.*, 2012, 2015), and this protein was found to be enriched in exosome-like vesicles (Regente *et al.*, 2009). Another principle likely contributing to discounting of the presence of EV in plants is related to the existence of the plant cell wall, which might prevent EV passage. However, this concept has also been overcome for other cell-walled organisms, such as Gram-positive bacteria, fungi, and mycobacteria, in which EV are recognized as key components in microbial physiology and pathogenesis, even

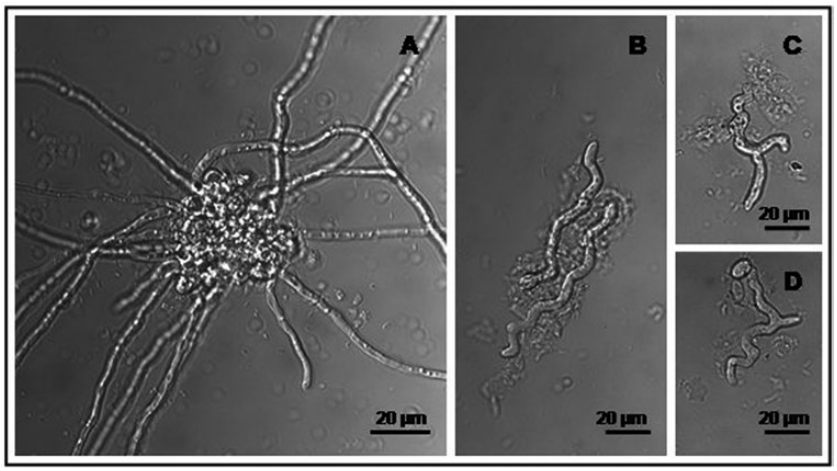

Fig. 4. Extracellular vesicles (EV) inhibit *S. sclerotiorum* mycelial growth. *S. sclerotiorum* spores were incubated for 16 h with water (A) or EV (1.5 µg of proteins) (B–D) and bright-field confocal laser scanning microscopy images were obtained. Bars=20 µm.

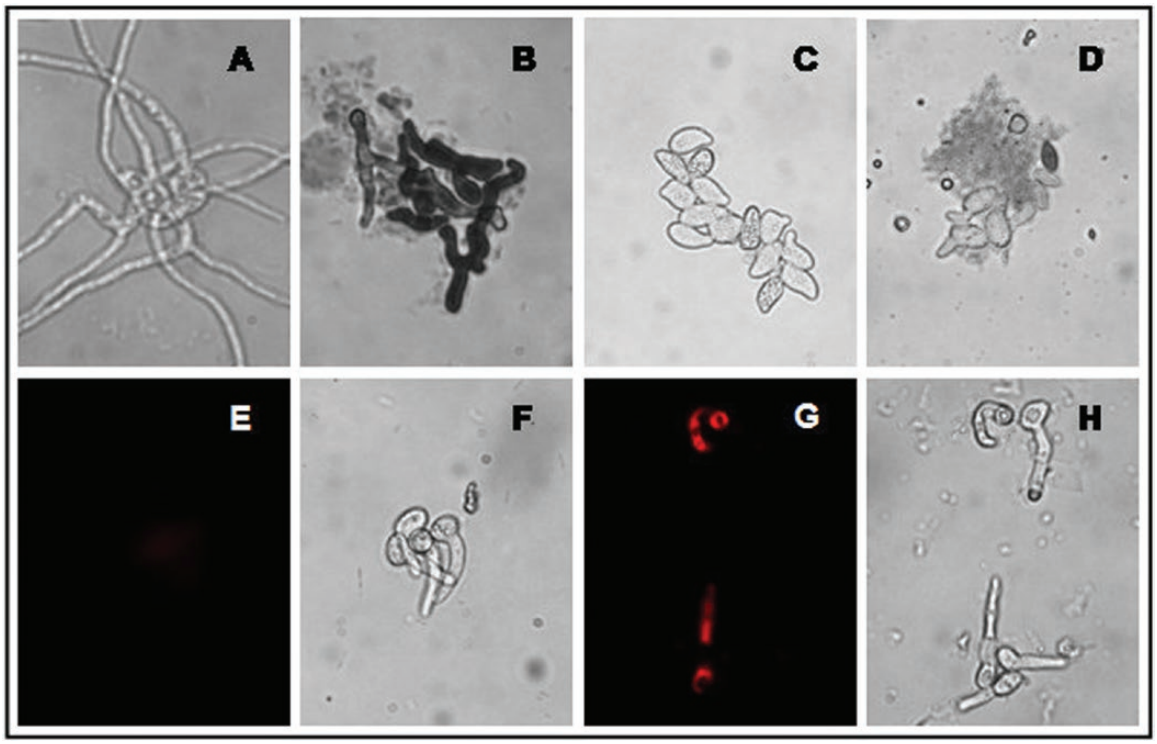

Fig. 5. Extracellular vesicles induce *S. sclerotiorum* cell death. Spores of *S. sclerotiorum* incubated for 16 h (A, B) or 3 h (C–H) with water (A, C, E, F) or EV (1.5 μg of proteins) (B, D, G, H) were stained with Evans blue (A–D) or propidium iodide (E–H). (F) and (H) are the bright-field images of (E) and (G), respectively. Images were obtained using a fluorescence microscope at 40x magnification. (This figure is available in colour at *JXB* online.)

though their mechanisms of release are not fully understood (Brown *et al.*, 2015).

In addition to the reports mentioned above where the isolation of plant EV was described, other experimental approaches have demonstrated the existence of plant EV. For instance, Wang *et al.* (2010) reported EXPO, an intracellular organelle that mediates cytosol to cell wall exocytosis. EXPO was shown to fuse with the plasma membrane, expelling a vesicle into the apoplast. EV have also been observed under electron microscopy in certain plant–pathogen interactions (An *et al.*, 2006; Micali *et al.*, 2011).

In this work, we present evidence that the gentle procedure used for the extraction of EF from sunflower seedlings causes only minimal lysis of cellular structures, but nonetheless EV are detected in apoplastic fluids. In fact, vesicles with the typical size and shape of human EV were observed by TEM, and a proteomic analysis confirmed that their particular protein composition was different from the proteome of the soluble apoplastic compartment. EV have now been isolated at least from sunflower seeds (Regente *et al.*, 2009), germinating pollen grains from olive (Prado *et al.*, 2014), *A. thaliana* leaves (Rutter and Innes, 2017), and here we characterize EV from sunflower seedlings. We expect this list to grow in the coming years, demonstrating the wide phylogenetic distribution of EV and their delivery and/or dissemination in the whole plant and in different growth and developmental stages. Our results, together with the bulk of information already reported by different teams who observed EV in diverse plant systems, confirms their presence in the plant apoplast.

Sunflower EV protein composition reveals common proteins with Arabidopsis and human EV

Here we report the first high-throughput proteomics analysis to identify a large number of plant EV proteins (278), giving clues to EV function. Two previous studies have partially analyzed the EV proteome in olive pollenosomes (Prado *et al.*, 2014) and *A. thaliana* rosettes (Rutter and Innes, 2017). The latter identified 598 EV proteins, although only 170 were validated in two biological replicates.

Many of the proteins identified in the present study are frequently found in mammalian EV samples, including those involved in basic metabolic processes, proteolysis, vesicle traffic, and the cytoskeleton (Choi *et al.*, 2015), which may reflect a conserved function and/or origin. Even though any prediction of function based only on protein identification is rather speculative, some hints emerge for future investigations. Plant EV seem to present a particular subset of proteins related to cell wall remodeling and defense. Cell wall remodeling enzymes are enriched in sunflower EV and have also been detected in the two previous proteomic analyses of plant EV (Prado *et al.*, 2014; Rutter and Innes, 2017). Their presence in EV could be related to the assembly of cell wall components, although the mechanisms involved have yet to be unraveled. Another hypothesis is related to a striking coincidence: proteins acting on polysaccharides are also abundant in EV isolated from fungi and Gram-positive bacteria (Albuquerque *et al.*, 2008; Lee *et al.*, 2009; Oliveira *et al.*, 2010). Since those organisms also have a thick cell wall, it has been hypothesized that crossing the cell wall may require its remodeling using still-unknown mechanisms (Brown *et al.*, 2015).

Defense proteins are the other subpopulation that appeared to be well represented in plant EV. Rutter and Innes (2017) found that EV from *A. thaliana* leaves were enriched in proteins involved in biotic and abiotic stress responses, and these proteins were also found in vesicles isolated from olive pollenosomes (Prado *et al.*, 2014). Likewise, it has been shown that the ultracentrifugation pellet obtained from the extracellular medium of tomato cell suspension was enriched in defense proteins (Gonorazky *et al.*, 2012). The present study provides additional evidence, since several defense proteins were detected. In addition to the well-known pathogenesis-related proteins, we found proteins such as PMR5, an ortholog of the gene conferring resistance to powdery mildew in *A. thaliana* (Vogel *et al.*, 2004), and GDSL lipases, related to the GLIP2 involved in *A. thaliana* susceptibility to the necrotrophic bacterium *Erwinia carotovora* (Lee *et al.*, 2009). Taking into account the fact that our proteomic analyses were performed in plants grown in the absence of biotic stress, the protein composition of EV strongly suggests their involvement in plant innate immunity, as has been proposed for *A. thaliana* rosettes (Rutter and Innes, 2017).

Intriguingly, EV also contain some chloroplastic proteins. A proportion of these could probably be accounted for by cell leakage, but it is also tempting to speculate that EV could participate in waste management. In fact, autophagy has been established as a cellular pathway for chloroplast degradation (Xie *et al.*, 2015), and EV participate in the removal of waste products linked to autophagy in animal systems (Baixauli *et al.*, 2014). Even though our knowledge of plant EV is scarce, the high content of proteases detected in this study may also be related to such a function, and deserves further investigation.

Here we present interesting data arising from the comparison of proteins identified in EV from sunflower (this study) and Arabidopsis (Rutter and Innes, 2017). Not only different species but also different tissues and developmental stages were used in the two studies: apoplastic fluids isolated from rosettes of mature Arabidopsis plants (5–7 weeks) versus sunflower seedlings (17 days after sowing) with long hypocotyls, cotyledons, and growing first leaves. Despite this limitation, we have detected several protein families common to EV from both experimental systems. Moreover, some of these proteins are also present in mammalian EV. So, a core of *bona fide* EV plant proteins has been identified that could serve to improve our understanding of their function and origin.

EV can control fungal growth

EV derived from human cells have been shown to exert an antimicrobial effect (Timár *et al.*, 2013) and the participation of EV in host–pathogen interactions has been widely documented in human systems (for a review, see Schorey *et al.*, 2015). Concerning plant–fungal interactions, no evidence is yet available, even if the plant extracellular matrix is a key component in defense responses (Delaunois *et al.*, 2014). In fact, the early stages of plant–pathogen interactions occur in the intercellular spaces of the plant tissues, but apoplastic defense responses were analyzed for the assumed soluble components, since EV were not yet recognized. However, EV clearly could function as vehicles for information and/or delivery of components directly involved in plant defense.

A recent opinion article has speculated on the possible participation of EV in plant–fungal interactions, based on accumulated evidence in human fungal infections and taking into account some indirect microscopic evidence in plant systems (Samuel *et al.*, 2015). Evidence shows that fungal infection of barley leaves enhances the formation of paramural vesicles (An *et al.*, 2006), which are membranous structures observed between the cell wall and the plasma membrane in plants attacked by fungi. These paramural vesicles proliferate in the periphery of intact cells adjacent to the site of localized hypersensitive response in the incompatible barley–powdery mildew interaction (An *et al.*, 2006). Multivesicular bodies (MVB), which are involved in the biogenesis of certain EV in animal systems, have also been implicated in the interaction between a host plant cell and the invading fungus (An *et al.*, 2006; Micali *et al.*, 2011). The observation that plant MVB and paramural vesicles accumulate during fungal attack has prompted the authors to postulate the release of exosome-like vesicles in barley leaves attacked by the pathogenic powdery mildew fungus (An *et al.*, 2007), although no experimental evidence has been presented yet.

All these observations are consistent with the concept that EV may participate in defense responses, acting as carriers of active proteins through the apoplast. In this respect, we present here functional assays showing for the first time that plant-released EV can be incorporated by fungal cells and can induce severe growth defects in them, finally leading to cell death. Whether this antifungal activity requires EV uptake remains to be determined. Nevertheless, EV produce inhibition of spore germination, stunted mycelial growth, and loss of vitality.

The mode of entry of EV to fungal cells is beyond the scope of this paper, but some data suggest a putative mechanism that remains to be further explored. No concentration of fluorescence was observed on the fungal plasma membrane upon incubation with FM4–64 labeled EV, even at the shortest time that could be analyzed (Fig. 3B). On the other hand, direct labeling of the spores with the dye showed the typical labeling of the cell surface. Although preliminary, these observations suggest that EV may not fuse to the plasmalemma but rather may be incorporated through endocytic pathways.

EV constitute a unique package of information that can provide the simultaneous delivery of multiple messengers and components even to distant sites. Only the protein content of EV has been analyzed here, but according to the accumulated knowledge in other organisms, lipids and RNA might also be delivered to fungal cells, thus contributing to plant protection mechanisms against invading fungi. The question of whether EV are actually involved in plant defense remains to beaddressed. We have attempted functional tests *in planta* that were unsuccessful because we failed to achieve a reliable uptake of EV suspensions through the plant vascular system. Nevertheless, our results contribute to a paradigm shift in how plant and fungus communicate, and should stimulate analysis of the participation of EV in other cell-to-cell

communication events in plants. While we have focused on the function of plant EV in defense, their protein richness may account for other putative functions still waiting to be discovered.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. Identification of proteins in EV (biological replicate 1) by LC-MS/MS and bioinformatics.

Table S2. Identification of proteins in EV (biological replicate 2) by LC-MS/MS and bioinformatics.

Table S3. Identification of proteins in EV (biological replicate 3) by LC-MS/MS and bioinformatics.

Table S4. Identification of proteins in S100 (biological replicate 1) by LC-MS/MS and bioinformatics.

Table S5. Identification of proteins in S100 (biological replicate 2) by LC-MS/MS and bioinformatics.

Table S6. Electrolyte leakage of seedlings.

Table S7. List of proteins identified in at least two biological replicates of EV.

Table S8. List of proteins identified in the two biological replicates of S100.

Table S9. List of EV protein families common to *H. annuus* seedlings and Arabidopsis leaves.

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