

Paired isolation and comparative characterization of extracellular vesicle populations across the tomato cell wall interface

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Abstract



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Aim: Plant extracellular vesicles (EVs) mediate intercellular communication, defense, and cell wall remodeling, yet how these vesicles traverse the cell wall and distribute between the apoplast and the extracellular environment remains poorly defined. In suspension cultures, EVs that have crossed the cell wall accumulate in conditioned culture medium (CCM-EVs), whereas apoplastic vesicles (AVs) are not readily recoverable without targeted wall loosening.

Methods: Here, we developed an enzymatic method to release AVs from *Solanum lycopersicum* cell suspension cultures by selective pectin degradation, enabling paired isolation of CCM-EVs and AVs from the same biological system. Vesicles were isolated by differential ultracentrifugation and characterized by cryo-TEM, ILM, SDS-PAGE, and LC-MS/MS proteomics.

Results: Both populations exhibited typical bilayer morphology but differed in abundance, size distribution, and protein composition. CCM-EVs were enriched in membrane-associated and vesicle trafficking proteins, whereas AV fractions displayed higher proteome complexity and were enriched in extracellular enzymes, stress-related proteins, and cell wall-associated components. Comparative analyses revealed limited overlap and distinct functional signatures associated with their extracellular context. These findings support the hypothesis that CCM-EVs and AVs represent vesicle populations associated with distinct extracellular locations and suggest that spatial context contributes to EV heterogeneity in plants.

Conclusion: These findings suggest that CCM-EVs and AVs may represent spatially distinct vesicle populations and support a model in which EV composition is influenced by their spatial localization across the cell wall interface.

Keywords: Apoplastic vesicles, extracellular vesicles, plant cell suspension culture, tomato (*Solanum lycopersicum*), conditioned culture medium, functional annotation

INTRODUCTION

Plant cells release membrane-bound vesicles into the extracellular space, collectively termed extracellular vesicles (EVs) (Pinedo *et al.*, 2021). These vesicles play central roles in intercellular communication, defense signaling, and cross-kingdom interactions with

microbes, serving as vehicles for the unconventional secretion of proteins and nucleic acids (Cai *et al.*, 2023; Farley *et al.*, 2022; Y. Huang *et al.*, 2021; Liu *et al.*, 2021; Pompa & Walker, 2016; Qiao & Roth, 2025). Plant EVs are thought to arise from multiple biogenetic pathways, including the well-established MVB-associated route, the less characterised vesicle release via plasma membrane budding, as well as the secretion of EXPO (exocyst-positive organelle)- and PEN1 (PENETRATION1)-associated populations (Ambrosone *et al.*, 2023; Gao *et al.*, 2026; He *et al.*, 2021; Lian *et al.*, 2022; Zhang *et al.*, 2020).

A defining concept in EV biology is that vesicles may occupy a spatial-temporal continuum across cellular and extracellular compartments (Lipinski & Tiemann, 2021); however, whether this framework applies to plant systems, where the cell wall imposes a substantial physical barrier, remains unclear (Shu *et al.*, 2025). Although plant EV secretion is tightly regulated in space and time, with release occurring at specific cellular interfaces and in response to developmental and environmental cues, the presence of the cell wall and the apoplastic boundary introduces additional constraints that may limit or reshape vesicle distribution. As a consequence, it remains uncertain whether EVs detected in different extracellular compartments represent a continuous population or instead correspond to distinct subsets shaped by their localization. Furthermore, EVs residing within the apoplast or associated with the cell wall remain technically challenging to detect, isolate, and track with sufficient spatial and temporal resolution. A central unresolved aspect of this problem is how plant EVs physically traverse the cell wall. Plant EVs typically range from 50 to 200 nm in diameter, whereas the effective pore size of the primary cell wall is estimated to be only 5-20 nm, creating an apparent size-exclusion paradox (Shu *et al.*, 2025). Despite this constraint, vesicles are consistently detected both within the apoplast and in extracellular fluids beyond the wall. Several non-mutually exclusive mechanisms have been proposed to reconcile this discrepancy, including localized cell wall remodeling mediated by EV-associated enzymes, mechanical deformation of vesicles during passage, and transit through transiently loosened regions of the wall during growth or stress (de Marcos Lousa, 2025). However, direct experimental evidence remains limited, and it is unclear whether vesicles

detected outside the cell wall represent the same population as those retained within the apoplast or distinct subsets that differ in composition and function.

After secretion at the plasma membrane, EVs traverse the pectin-rich cell wall-apoplast matrix, where they may transiently accumulate before entering the extracellular milieu. Depending on the methodology and system, EVs can therefore be recovered either from apoplastic washing fluid (AWF) or from the conditioned culture medium (CCM) of cell suspension cultures (Ambrosone *et al.*, 2023). In this context, we use the terms apoplastic vesicles (AVs) and CCM-EVs purely as operational descriptors for EVs sampled at two distinct spatial positions: AVs denote wall-associated EVs embedded within the cell wall-apoplast matrix at the moment of sampling, whereas CCM-EVs are EVs that have already traversed the wall and accumulated in the surrounding medium. These terms do not a priori define distinct biological classes but instead refer to EVs sampled from two different extracellular environments, namely, the cell wall-apoplast matrix and the surrounding medium. While they may reflect different stages of secretion, whether these populations represent the same vesicles in transit or functionally distinct subsets remains an open question.

Plant EVs contain diverse molecular cargo, including proteins, lipids, and RNAs that contribute to stress responses, immunity, and extracellular matrix organisation (Cai *et al.*, 2023; Pinedo *et al.*, 2021; Rutter & Innes, 2017). Consistent with this view, recent analyses of AWF-derived vesicles from *Sorghum bicolor* and *Arabidopsis thaliana* revealed a conserved enrichment in membrane transporters, vesicle trafficking components, defence-related enzymes, and extracellular matrix-modifying proteins, supporting the idea that plant EVs carry functionally specialized but evolutionarily conserved cargo across species (Chaya *et al.*, 2024). EV-mediated communication extends beyond intercellular signalling: several studies have demonstrated that EVs deliver small RNAs and defence-related proteins to invading pathogens, modulating their virulence (He *et al.*, 2023; Y. Huang *et al.*, 2021; Wang *et al.*, 2024). EV-mediated communication is equally relevant in beneficial interactions, such as arbuscular mycorrhizal symbiosis, where EVs contribute to nutrient exchange and extracellular remodelling (Holland & Roth, 2023). Over the past decade, the field has also

recognised the heterogeneity of plant EV subpopulations, including TET8/TET9- positive vesicles, PEN1- associated EVs, and EXPO- derived vesicles (Koch *et al.*, 2026; Langellotto *et al.*, 2025). These vesicle types differ in molecular markers, size distributions (typically 50-300 nm), and functional signatures, underscoring the diversity of EV origin and biogenesis routes.

Several methodological constraints contribute to this gap. AWF-based workflows are restricted to intact tissues and rely on vacuum infiltration and centrifugation to recover wall- and apoplast-associated vesicles (He *et al.*, 2023; C. Y. Huang *et al.*, 2019). Vacuum infiltration itself presents notable limitations, including limited control over the extent and uniformity of infiltration, as well as the risk of cellular damage and contamination from intracellular components. Moreover, such approaches are restricted to intact tissue and cannot be directly applied to suspension cultures, which lack tissue structure and large intercellular spaces. Conversely, CCM-EVs isolated from CCM represent only those vesicles that have completed their passage through the cell wall and accumulated in the extracellular medium. CCM-based methods are valued for their reproducibility and scalability (Ambrosone *et al.*, 2023; Bonaventura *et al.*, 2026; Kırbaş *et al.*, 2025) but can yield low or variable particle numbers, and they do not provide access to vesicles retained within the apoplastic matrix. As a consequence, current methodologies partition EVs according to the system and extraction strategy used, rather than enabling their systematic comparison within a shared biological context. This limitation has hindered efforts to determine whether EVs occupying different extracellular niches, such as the apoplast versus the surrounding medium, represent a continuous population or exhibit differences in abundance and molecular composition, thereby constraining our understanding of how spatial context contributes to EV heterogeneity.

To address this gap, we established *Solanum lycopersicum* CSCs as a controlled *in vitro* platform for plant EVs studies. Tomato represents a biologically and agriculturally relevant model species, widely used in plant research and of importance for human nutrition, while CSCs provide a homogeneous and reproducible sources that minimizes sample variability

associated with tissue complexity and developmental heterogeneity. Using this system, we developed a method enabling paired isolation of vesicles recovered from conditioned medium and from the apoplastic compartment. Vesicles were first enriched from CCM, after which cells were subjected to enzymatic cell wall loosening to release AV. The approach provides operational access to vesicles recovered from two extracellular compartments; however, the precise relationship between these populations remains to be fully established. In this work, we characterize vesicles from both fractions using different analytical techniques, including cryogenic transmission electron microscopy (cryo-TEM), interferometric light microscopy (ILM), SDS-PAGE, and LC-MS/MS-based proteomics, to define their morphology, abundance, and molecular composition. By comparing vesicles recovered from distinct extracellular locations, we aim to determine whether spatial context is associated with differences in abundance and protein composition, and to assess how extracellular localization contributes to vesicle heterogeneity in plants. In doing so, we provide a framework to compare EV populations across distinct extracellular locations.

METHODS

***Solanum lycopersicum* callus and cell suspension cultures**

The TOM5 cell suspension culture used in this study is an in-house line established from leaf explants of *Solanum lycopersicum* cv. M82 and maintained under the conditions described below. M82 is a widely used reference tomato genotype with extensive genetic and genomic resources available. Callus cultures were induced from *Solanum lycopersicum* leaf explants and maintained on solid Murashige and Skoog (MS) basal medium supplemented with Gamborg vitamins (PhytoTech Labs, Lexena, US), 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) (Duchefa Biochemie, Haarlem, The Netherlands), 0.45 mg/L kinetin (Duchefa Biochemie, Haarlem, The Netherlands), 0.1 mg/L naphthaleneacetic acid (NAA) (Duchefa Biochemie, Haarlem, The Netherlands), 30 g/L sucrose (PhytoTech Labs, Lexena, US) and 7g/L plant agar (Duchefa Biochemie, Haarlem, The Netherlands). Cultures were grown at 26 °C in the dark and subcultured every 4 weeks.

Cell suspension cultures (CSCs) were initiated by resuspending 50 mg of 30-day-old callus in 50 mL of liquid medium with the same composition as the callus maintenance medium, excluding the gelling agent. Cultures were maintained at 26 °C at 130 rpm on rotary shaker (Excelsa E24, New Brunswick Scientific, New Jersey, US). Subculturing was performed weekly by transferring 10 mL of suspension into 80 mL of fresh liquid medium. CSCs were stabilized for six months prior to experimental use.

Growth dynamics were characterized in a 12-day time-course experiment by measuring packed cell volume (PCV) and monitoring cell morphology. Three biological replicates were analyzed, with triplicate PCV measurements per flask at each time point. Cell viability was assessed using fluorescein diacetate (FDA) staining as described in section 2.3. CCM-EVs and AVs were isolated (see paragraph 2.4) from cultures three days after subculturing, corresponding to the onset of the logarithmic growth phase when cell viability and metabolic activity were highest.

Enzymatic degradation of cell wall

A commercially available pectinase mixture (Zimopec P110, AGRO M) derived from *Aspergillus niger*, primarily containing pectolytic activity, was used for enzymatic digestion of cell wall. Zimopec P110 is a food-grade enzyme preparation used for depectinization processes and contains predominantly pectinolytic activities. To minimize the contribution of insoluble material, the enzyme preparation was clarified by centrifugation prior to use. Zimopec facilitates the release of intracellular and apoplastic contents by hydrolyzing pectic polysaccharides. Prior to use, Zimopec was clarified by centrifugation at $15,000 \times g$ for 30 min to remove insoluble particles and ensure a uniform enzyme solution. The supernatant was collected and diluted 1:10 isolation buffer (100 mM phosphate, 10mM EDTA, pH 5.6). To determine the optimal digestion time, tomato cells were treated with diluted Zimopec at a 3:1 ratio (v/v, cells:enzyme solution) for various durations (30 min, 60 min, 12 h and overnight). Cell wall degradation was evaluated by observing cell morphology under a Countess™ 3 Automated Cell Counter (Thermo Fisher Scientific, Singapore, USA).

Light microscopy

Cell viability was assessed using fluorescein diacetate (FDA, Sigma Aldrich/Merc, Darmstadt, Germany) assay. FDA solution was prepared at 5 mg/mL concentration in acetone (Romil, Cambridge, UK) and stored at 4 °C. 20 µL of FDA was added to 100 µL of the cell culture (diluted 1 to 10) and incubated at room temperature for 7min at dark conditions. Fluorescence was observed at 470 nm excitation and 510-580 nm emission.

Cell wall integrity and membrane structure were assessed by fluorescent staining. For cell wall staining Direct Red 23 (Sigma-Aldrich, USA), a cellulose binding azo dye 0.01% (w/v) solubilized in PBS and sterilized by filtration through a 0.22 µm PES syringe filter was used. 25 µL of digested or non-digested tomato cells were incubated in 500 µL dye solution at room temperature on a rotary shaker for 30 min in the dark. After staining, cells were centrifuged $1,160 \times g$ for 10 min and washed twice with PBS using the same centrifugation step and resuspended in PBS. For membrane staining, DiI (Thermo Fisher Scientific, USA), a lipophilic tracer, was used at a final concentration of 5 µM. Cells were incubated with DiI for 10 min at room temperature in the dark, followed by two PBS washes under the same centrifugation conditions. Imaging was performed using an inverted Leica DMI8 microscope (Leica Microsystems, Germany) equipped with fluorescence filters appropriate for FDA, Direct Red 23 and DiI.

Isolation of CCM-EVs and AVs

Prior to harvesting and EV isolation, cell cultures were inspected by light microscopy to verify preservation of cell morphology and the absence of extensive cell disruption. CCM, enzymatically digested cells and a control sample containing only the enzyme solution were processed in parallel as three distinct samples. 50 mL of tomato CSC was collected and centrifuged at $400 \times g$ for 30 min at room temperature (RT) to separate cells from CCM, yielding approximately 15 mL of pelleted cells and 25 mL of CCM. After removing the CCM, the cell pellet was washed twice with 20 mL of $10 \times$ diluted isolation buffer (100 mM phosphate buffer pH 5.6, 10 mM EDTA), followed by centrifugation at $400 \times g$ for 10 min

at RT. These washes ensured the removal of any residual EVs originating from the conditioned medium. The washed pellet was then used directly for enzymatic digestion.

In parallel, the control sample containing only 5 mL of the enzyme mixture, and the CCM sample (25 mL) for EVs isolation, were incubated under identical conditions. Zimopex digestion was performed exclusively on the washed cell pellet in the absence of any residual CCM.

All three samples (CCM for EV isolation, digested cells for AV isolation, and enzyme-only control) were processed in parallel using differential centrifugation. Sequential low-speed centrifugation steps were performed at $400 \times g$, $800 \times g$, $2,000 \times g$, and $15,000 \times g$, each for 30 min at 22 °C in an Eppendorf 5804R centrifuge, to remove cells and large debris. Pellets obtained after the $15,000 \times g$ (P15K) were resuspended in 50 μ L isolation buffer for cells and control samples, and 100 μ L for the CCM sample. Supernatants obtained after the $15,000 \times g$ centrifugation (S15K) were transferred into polycarbonate ultracentrifuge tube and centrifuged at $100,000 \times g$ for 2 h at 22 °C using a Beckman Optima XL-A ultracentrifuge (Beckman Coulter, Brea, USA). The obtained pellets of all three samples were resuspended in 20 μ L of isolation buffer.

Characterization of EVs and AVs: Protein quantification and SDS-PAGE analysis

Protein concentration of the three types of samples (CCM-EVs, AVs and control sample) was determined by the Qubit assay. A three-point calibration curve was performed according to the manufacturer's instructions using the Qubit™ Standard reagents (Thermo Fisher Scientific, Singapore, USA). For the estimation 1 μ L of sample was combined with 199 μ L Qubit™ Working Solution and protein concentration was measured on Qubit™ 4Fluorometer (Thermo Fisher Scientific, Singapore, USA).

For SDS-PAGE analysis, samples were lysed prior to loading by adding 4 $\times$ Bolt™ LDS sample buffer and heating at 70 °C for 10 min, ensuring complete disruption of the vesicle membrane and release of luminal and membrane-associated proteins. The lysed samples were

then centrifuged briefly to remove insoluble debris before loading equal amounts of total protein onto the gels. Protein profiles were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using Novex Bolt 4-12% Bis-Tris Plus gel (Invitrogen, Carlsbad, CA, USA) and Bolt MOPS SDS running buffer (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instruction. Proteins were separated using 165V for approximately 45 min. Gels were rinsed with MilliQ water, fixed in 40% (v/v) methanol and 10% (v/v) acetic acid for 1 h and stained with colloidal Coomassie Brilliant blue G-250 (AppliChem GmbH Darmstadt, Germany). Destaining was performed using distilled water.

Interferometric light microscopy (ILM)

Particle size and concentration of CCM-EVs and AVs were determined using interferometric light microscopy (Videodrop, Myriade, Paris, France) (Romolo *et al.*, 2022; Kralj-Iglič and Romolo, 2025). For each measurement, 7 μ L of sample was loaded into the Videodrop sample holder; when necessary, samples were diluted in isolation buffer. A threshold value of 4.2 was applied. Samples were illuminated using a 2 W blue LED, and scattered light from individual particles interfered with the incident light, producing characteristic contrast patterns that were recorded using a high-resolution CMOS camera. The incident light signal was subtracted from the images, and individual interference spots were identified as particles with defined spatial positions. Particle concentration was calculated as the number of detected particles divided by the observation volume (~ 15 pL).

Particle trajectories were tracked over time to analyze Brownian motion. The mean squared displacement of each particle was used to estimate the diffusion coefficient D , according to $\langle d^2(\Delta t) \rangle = 4D\Delta t$ (Kralj-Iglič & Romolo, 2025), where Δt is the time interval between frames. Larger particles exhibit lower diffusion coefficients and consequently smaller displacements.

Assuming spherical particle geometry, the hydrodynamic diameter D_h was calculated using the Stokes-Einstein equation $D_h = \frac{kT}{6\pi\eta D}$ where k is the Boltzmann constant, T is the absolute temperature, and η is the viscosity of the medium.

Data processing and analysis were performed using QVIR software (version 2.6.0, Myriade, Paris, France). ILM measurements were performed in technical replicate for each isolated preparation, and the mean value was reported. Signals from the control zimopec sample were below the detection limit.

Cryogenic transmission electron microscopy (cryo-TEM)

C-flatTM 2/2, copper 200-mesh, holey carbon grids (Protochips, Morrisville, NC, USA) were glow-discharged using a GloQube® Plus system (Quorum Technologies, Laughton, UK) under the following conditions: 20 mA current, 60 s duration, positive polarity, and ambient air atmosphere. A 2 µL aliquot of sample was applied to each grid, blotted immediately, and vitrified in liquid ethane using a VitrobotTM Mark IV (Thermo Fisher Scientific, Waltham, MA, USA). Vitrobot settings were configured to 95% relative humidity at 4 °C, with a blot force of 5 and blot time of 7 s.

Grids were imaged under cryogenic conditions using a 200 kV GlaciosTM transmission electron microscope equipped with a FalconTM 3EC direct electron detector (Thermo Fisher Scientific, Waltham, MA, USA) in TEM mode at different magnifications between 700× and 57,000×. At 57,000× magnification defocus of -4 µm was applied.

Lysis and in-solution trypsin digestion of the samples

Unlike SDS-PAGE, which uses LDS heating for direct denaturation, proteomics samples underwent full solvent-based lysis prior to reduction and tryptic digestion to ensure complete release of vesicle cargo proteins. EV samples corresponding to 10 µg of protein were dried and resuspended in 10 µL 50% of acetonitrile / 50% (v/v) methanol for lysis, followed by sonication and vacuum drying. The resulting pellets were resuspended in 50 mM ammonium bicarbonate (AMBIC). Proteins were reduced by 5 mM dithiothreitol (DTT, Sigma-Aldrich, Saint Louis, MO, USA) in 50 mM AMBIC and alkylated with 15 mM iodoacetamide (Sigma-Aldrich, Saint Louis, MO, United States) in 50 mM AMBIC. Proteolytic digestion was carried on using MS-grade trypsin (Pierce, Thermo Fisher Sci Rockford, IL, USA) at 1:100

protein to enzyme ratio at 37 °C overnight. Tryptic peptides were vacuum-dried prior to LC-MS/MS analysis. Proteomic analyses of CCM-derived EV fractions (EV_P15K, EV_P100K and EV_S100K) were performed on three independent biological preparations. Due to the limited material recovered from AV fractions, LC-MS/MS analyses of AV_P15K and AV_P100K were performed on a single biological preparation. Consequently, comparisons between EV and AV proteomes should be considered descriptive and exploratory.

LC-ESI-MS/MS analysis

Desalted tryptic peptides (Section 2.8) were analyzed using an Ultimate™ 3000 RSLC nano system (Thermo Fisher Scientific, USA) coupled to a Q Exactive™ HF-X hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, USA). The system was equipped with a trap column (Acclaim™ PepMap™ 100 C18, 100 µm × 2 cm, 3 µm; Thermo Fisher Scientific) and an analytical column (EASY-Spray™ C18, 75 µm × 50 cm, 2 µm; Thermo Fisher Scientific). The flow rate was 0.3 µL/min, with mobile phases consisting of solvent A (97% water, 3% acetonitrile, 0.1% formic acid) and solvent B (90% acetonitrile, 0.1% formic acid).

The LC gradient for solvent B was: 2% (0-6 min), 4% (7 min), 31% (7-75 min), 90% (76-80 min), and 2% (81-90 min). Electrospray ionization was performed at 1.8 kV with a capillary temperature of 250 °C. Full MS scans were acquired at 60,000 resolution over m/z 350-1,500, with an AGC target of 3×10^6 and a maximum injection time of 50 ms. Data-dependent MS/MS was performed on the 12 most abundant ions using an isolation window of m/z 1.2. MS/MS scans were acquired at 15,000 resolution with an AGC target of 1×10^6 , maximum injection time of 100 ms, and normalized collision energy of 26%. Precursor charge states of 2-5 were selected, with an intensity threshold of 2.5×10^4 and dynamic exclusion of 30 s.

Proteomics data processing and protein identification

Proteomic analyses of CCM-derived fractions (EV_P15K, EV_P100K and EV_S100K) were performed on three independent biological preparations obtained from separate tomato cell

cultures. Each biological replicate was analyzed independently by LC-MS/MS, and protein identifications were subsequently merged to generate the final EV datasets. Due to the limited quantity of material recovered from the AV fractions, AV_P15K and AV_P100K analyses were performed on a single biological preparation.

Raw LC-MS/MS data were processed using Proteome DiscovererTM software (Thermo Fisher Scientific, USA) with the Sequest HT search engine. Spectra were searched against the *Solanum lycopersicum* (tomato) UniProt protein database (34,663 entries, comprising 508 Swiss-Prot and 34,155 TrEMBL records). Trypsin specificity was specified, allowing up to two missed cleavages. Carbamidomethylation of cysteine was set as a fixed modification, and oxidation of methionine was set as a variable modification. Mass tolerances were set to 30 ppm for precursor ions and 0.02 Da for fragment ions. Peptide-spectrum matches and protein identifications were filtered to a false discovery rate (FDR) of 1% using a target-decoy strategy implemented in Proteome Discoverer. Protein grouping followed the principle of parsimony, and proteins were reported as protein groups. A minimum requirement of ≥ 1 unique or razor peptide was applied for protein identification.

Bioinformatics and functional annotation

Proteomic datasets generated for EV and AV samples (P15K and P100K fractions), as well as for the EV-depleted soluble fraction (S100K), were subjected to downstream bioinformatic analysis using a multi-step workflow. Protein accession identifiers obtained from Proteome Discoverer were mapped to UniProt entries, and the corresponding FASTA sequences were retrieved using UniProt Retrieve/ID Mapping for functional annotation.

Comparative analyses were performed across the EV P15K, EV P100K, EV S100K, AV P15K, and AV P100K datasets. Functional annotation was carried out using OmicsBox - Bioinformatics made easy (Version 4.0.48), BioBam Bioinformatics. February 3, 2019. <https://www.biobam.com/omicsbox>) Protein sequences were searched against the NCBI non-redundant (nr) protein database, restricted to the Viridiplantae taxonomy (taxid: 33090),

using the Blast2GO workflow with default parameters (e-value $\leq 1 \times 10^{-5}$; maximum hits = 20).

InterProScan was integrated to identify conserved protein domains and domain architectures and to assign domain-based functional descriptors. In parallel, eggNOG-mapper, implemented within OmicsBox, was used to infer orthologous groups and to assign functional categories, including COG/KOG classes, KEGG pathway annotations, and additional Gene Ontology (GO) terms, thereby complementing BLAST- and InterPro-based annotations.

GO terms were subsequently mapped and annotated, followed by GO Slim (Plant Slim) reduction to obtain high-level functional classifications for Biological Process, Molecular Function, and Cellular Component categories. To assess global similarities and differences among EV and AV proteomes, quantified protein abundance data were log₂-transformed and median-normalized prior to unsupervised hierarchical clustering analysis. The resulting normalized protein intensities were used to generate distance matrices and clustering dendrograms for comparative analysis of EV and AV fractions.

RESULTS

Tomato cell suspension culture setup and characterization

CSC of *Solanum lycopersicum* were established from leaf-derived callus and maintained under controlled conditions to ensure stable and reproducible growth [Figure 1A]. A 10-day time-course experiment revealed a reproducible growth pattern across biological replicates [Figure 1B].

Culture exhibited a short lag phase during the first 3 days post sub-culture with PCV increasing modestly from ~93 mg/mL to ~145 mg/mL. From day 3 onwards, cells entered the logarithmic phase characterized by rapid biomass accumulation; PCV increased sharply from ~230 mg/mL to ~500 mg/mL by day 7, reflecting active cell division and metabolic

activity. From day 8 to 10, cultures transitioned to the stationary phase, marked by a growth plateau due to nutrient depletion and early sign of cell death (Komamine *et al.*, 2007).

Microscopic observation confirmed distinct morphological transitions: aggregates dominated the lag phase, while exponential growth was marked by enlarged, elongated cells dispersed into a fine suspension [Figure 1C]. By the stationary phase, irregular-shaped cells and debris were evident, along with the re-aggregation, consistent with stress and nutrient limitation (not shown).

EVs and AVs were harvested at the onset of the logarithmic phase (day 3 post-subculture) when cultures exhibited peak cell division and metabolic activity with minimal cell death. FDA staining confirmed that Day 3 cultures exhibited a high cell viability, with no detectable membrane-compromised cells at this stage [Figure 1C].

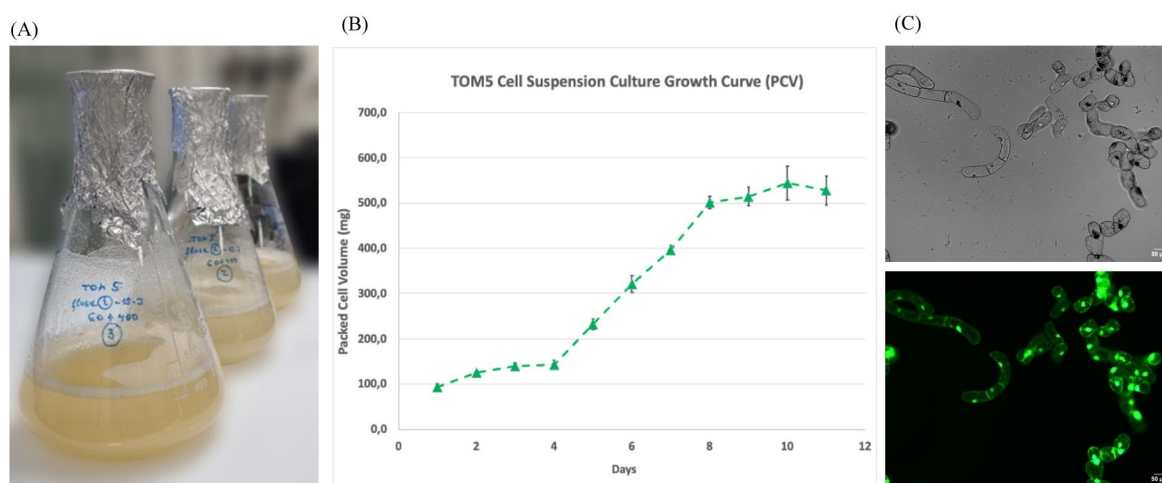


Figure 1. TOM5 *Solanum lycopersicum* cell suspension culture growth curve and viability. (A) Tomato cell suspension cultures on the 4th day after cultivation; (B) Packed cell volume (PCV) growth curves from three biological replicates (individual lines) over 10 days post-subculture, with the average curve (in yellow) showing lag (days 0-3), exponential (days 3-7), and stationary (days 7-10) phases; (C) Bright-field and fluorescein diacetate (FDA) staining of cells at day 3 post-subculture, scale bar-50 µm.

Validation of enzymatic treatment for apoptotic vesicles release

Treatment of *Solanum lycopersicum* suspension culture cells with the pectinase preparation Zimopec resulted in partial cell wall degradation without extensive cell lysis. Preliminary optimization experiments were performed using different Zimopec concentrations and dilution factors. The final treatment condition was selected based on microscopic assessment of cell morphology, wall permeability and preservation of cellular integrity. Time-course experiments were used to establish optimal treatment conditions. At early time points (1 h, 12 h), bright-field microscopy revealed no discernible differences between Zimopec-untreated [Figure 2 A] and treated cells [Figure 2 B-C], with borders remaining sharp and intact like in the control, not treated cells. After 24 h of incubation with Zimopec [Figure 2 D], changes in cell appearance consistent with partial cell wall loosening became apparent in bright-field microscopy. Because light microscopy does not provide sufficient resolution to assess cell wall ultrastructure, these observations were further evaluated by fluorescence staining of cell wall and membrane components.

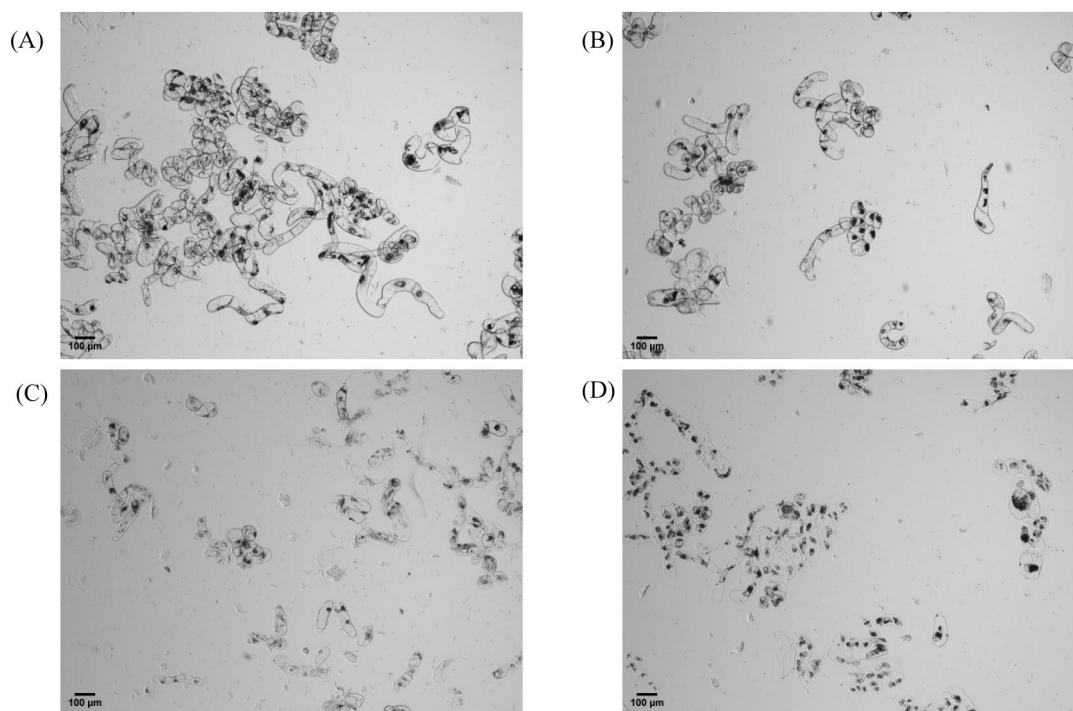


Figure 2. Bright-field images of TOM5 *Solanum lycopersicum* cells after Zimopec treatment acquired on Countess automated counter. (A) Non-digested control cells; (B); (C) and (D) cells after 1h, 12h and 24h of incubation with Zimopec enzyme.

Cell wall integrity was further assessed using dual fluorescent staining. Direct Red 23, a cellulose-specific dye, produced strong red fluorescence in both enzyme treated and non-treated cells, indicating that cellulose remained intact [Figure 3A]. In contrast, DiI, a lipophilic carbocyanine dye that labels plasma membranes, failed to penetrate non-digested cells due to the barrier imposed by the intact cell wall. In digested cells, however, DiI readily permeated and strongly labelled the plasma membrane [Figure 3B], suggesting that enzymatic treatment increased wall permeability without compromising cell membrane integrity.

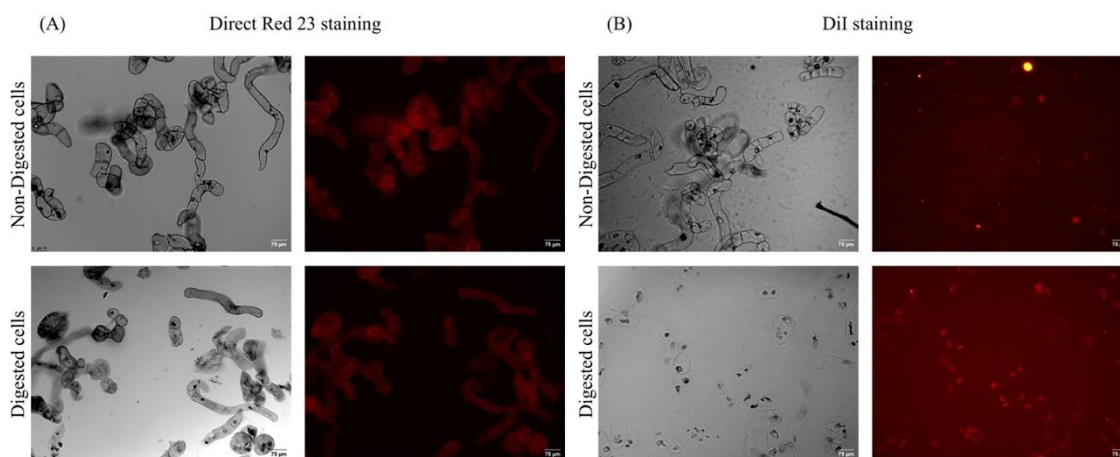


Figure 3. Microscopic images of Zimopec digested and non-digested tomato cells after 24 h of digestion. (A) Bright field and Direct Red 23; (B) Bright fields and DiI staining.

Together, these observations indicate that the diluted Zimopec preparation altered cell wall permeability while preserving detectable cellulose staining and plasma membrane integrity, as assessed by Direct Red23 and DiI staining. The results are consistent with partial loosening of the cell wall rather than complete wall dissolution and support the hypothesis that vesicles associated with the cell wall-apoplast matrix can be released into suspension without extensive cellular disruption CCM-EVs and AVs were isolated using differential ultracentrifugation. EVs were obtained from the CCM, while AVs were recovered from

enzymatically digested tomato cells. A control sample containing only the enzymatic mixture (Zimopec) was processed in parallel under identical conditions [Figure 4].

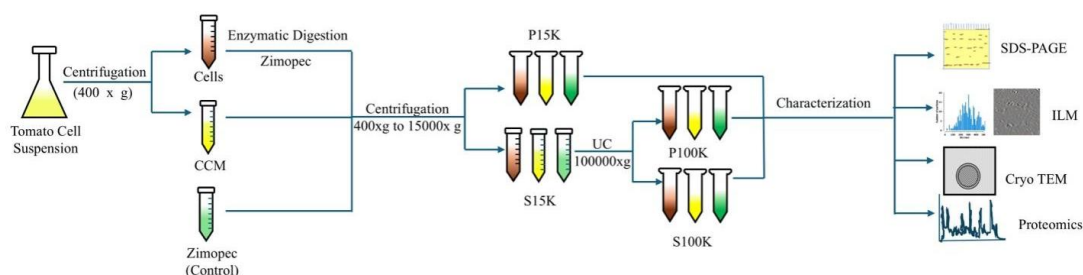


Figure 4. Workflow for the isolation of conditioned-medium extracellular vesicles (CCM-EVs), apoplastic vesicles (AVs) from tomato cell suspension cultures. Tomato cell suspensions were first centrifuged at $400 \times g$ to pellet cells and to collect the conditioned culture medium (CCM) for CCM-EV isolation. The cell pellet was then extensively washed with isolation buffer to remove all residual CCM, ensuring that no pre-secreted EVs remained. Washed cells were subsequently subjected to enzymatic digestion with Zimopec to release apoplastic vesicles (AVs) embedded within the cell wall-apoplast matrix. In parallel, a Zimopec-only control (no cells) was processed identically. Following digestion of the cells, all samples underwent differential low-velocity centrifugation ($400 \times g$ to $15,000 \times g$). S15K refers to the supernatant after the $15,000 \times g$ step; S100K refers to the supernatant after the $100,000 \times g$ step. P15K and P100K were collect as vesicle-enriched fractions. All fractions were analysed by SDS-PAGE, interferometric light microscopy (ILM), cryo transmission electron microscopy (Cryo-TEM), and LC-MS/MS-based proteomics.

Isolation and characterization of CCM-EVs and AVs from tomato cell suspension cultures

Sequential centrifugation steps removed cells and large debris, yielding pellets at $15,000 \times g$ (P15K) and $100,000 \times g$ (P100K). The P15K fraction expected to contain larger particles and residual debris, whereas the P100K fraction represented the vesicle-enriched fraction. All three sample types (CCM, digested cells, and enzyme-only control) produced visible pellets

after ultracentrifugation, which were resuspended in isolation buffer for downstream characterization.

Isolation yield was assessed based on protein concentration in the resuspended pellets. From 45 mL of culture, we obtained 27.5 μg of protein from EVs and 130 μg from AVs. The higher protein-to-particle ratio observed in AV preparations suggests the presence of protein-rich vesicles and/or co-isolated proteinaceous material associated with the enzymatic treatment or the apoplastic matrix.

Characterization and comparison of EVs and AVs

Morphological characterization

Morphological features of CCM-EVs and AVs were investigated by cryo-TEM. Samples were vitrified and imaged under cryogenic conditions using a 200 kV GlaciosTM TEM. Equal protein amounts (2 μL per sample, normalized to 0.2 $\mu\text{g}/\mu\text{L}$) were applied to grids for both preparations to ensure comparability. The resulting micrographs revealed round, membrane-bound structures consistent with plant-derived extracellular vesicles in both CCM-EV and AV samples [Figure 5].

EV samples [Figure 5A] displayed a very high number of vesicles with a markedly heterogeneous size distribution, ranging from approximately 50 nm up to ~ 1000 nm. Many vesicles appeared multilamellar. Most EVs exhibited clear lipid bilayers. Some EVs do not show a clear lipid bilayer; maybe the composition of the membrane is different, or the EVs are coated with some other molecules. We also observed some individual membrane patches. Occasional filamentous structures were also observed in the background of both samples. Their origin cannot be determined from morphology alone, and therefore we avoid assigning them to any specific biological source.

AV samples [Figure 5B] contained a lower concentration of vesicles overall, with a more uniform morphology and size distribution, typically between 50 nm and 250 nm. Lipid bilayer is visible in most vesicles. Occasional clusters of vesicles adhered to the carbon

support. Some vesicles exhibited electron-dense regions. Filamentous structures were also present in the background of AV grids, which may originate from components of the enzymatic digestion mixture (Zimopec). In both samples we observed some undefined or perhaps aggregated unknown material.

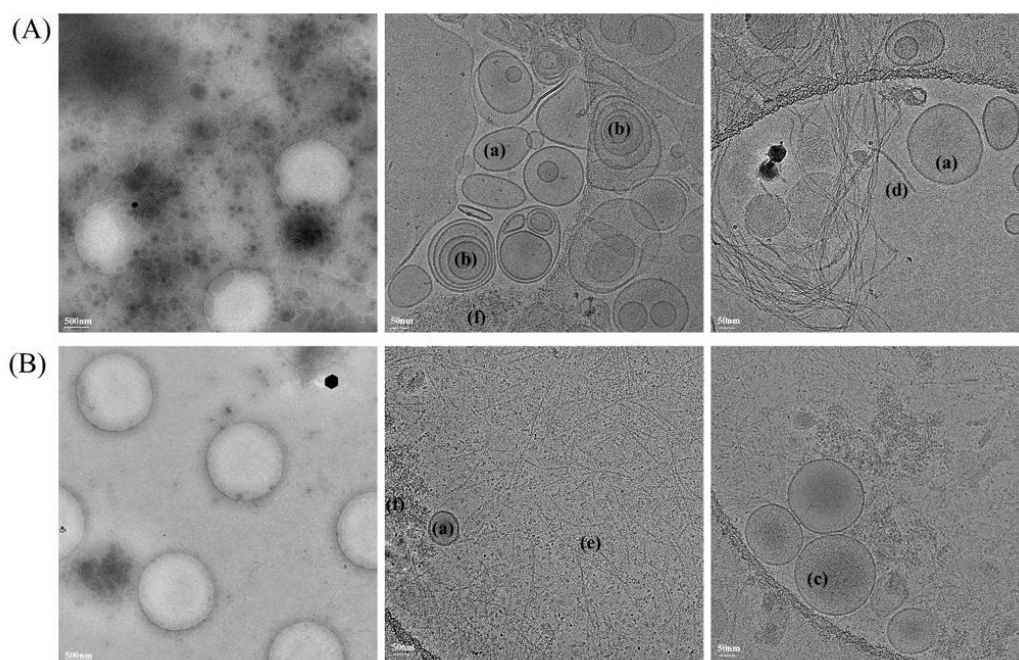


Figure 5. Cryo-TEM micrographs of conditioned cell media- derived extracellular vesicles (CCM-EVs) and apoplastic vesicles (AVs) isolated from tomato CSCs. (A) CCM-EVs (P100K) and (B) AVs released by enzymatic digestion (P100K). Vesicles show typical spherical morphology with visible bilayers. Representative structural features are indicated with letters: (a) unilamellar vesicles; (b) multilamellar vesicles; (c) vesicles with electron-dense lumen; (d) membrane fragments; (e) filamentous structures of unknown origin; (f) aggregated or undefined material. Cryo-TEM micrographs of vesicles isolated from tomato cell suspension cultures, shown at two magnifications (first column: 6,700 $\times$; second and third columns: 57,000 $\times$). (A) CCM-EVs (P100K pellet) display a heterogeneous population, including unilamellar and occasional multilamellar vesicles with clearly visible bilayer membranes; (B) AVs released by enzymatic digestion of the cell wall (P100K pellet) exhibit a more uniform spherical morphology and narrower size distribution compared to EVs. Both panels present representative fields at increasing magnifications to highlight

vesicle integrity and ultrastructural features. In the sample with higher vesicle abundance, unilamellar (a) and multilamellar (b) vesicles with well-defined lipid bilayers are visible, whereas some vesicles lack a clearly resolved bilayer. In panel B, certain vesicles contain electron-dense interiors suggestive of cargo (c); Unidentified filamentous structures are present in both samples (e); along with regions of aggregated or undefined material (f).

Particle size and concentration analysis by ILM

Particle size distributions of CCM-EVs and AVs were analyzed using interferometric light microscopy (ILM). Both vesicle types exhibited broad, right-skewed hydrodynamic diameter (D_h) distributions, consistent with those typically reported for plant-derived EVs [Figure 6A]. In accordance with current EV reporting standards, full distributions are provided alongside median and mean values.

Particle concentrations fell within the optimal measurement range of the instrument ($1-2 \times 10^9$ particles/mL), minimizing the risk of swarm effects (coalescence of particles at high concentrations).

For CCM-EVs, ILM tracked a total of 422 particles across technical replicates, yielding a median D_h of 212 nm and a mean D_h of 205 nm, with a distribution width of 137 nm. For AVs, 499 particles were tracked, with a median D_h of 220 nm and a mean D_h of 217 nm, with a distribution width of 171 nm. We could not detect a difference in the average size within the precision of the measurement.

The broader size distribution observed for the AV sample is indicative of a greater heterogeneity. CCM-EVs derived from conditioned medium exhibited higher particle concentrations (6.3×10^8 particles/mL) compared with AVs (1.8×10^8 particles/mL). When normalized to protein content—a more informative metric for comparing vesicles isolated from matrices of differing biochemical complexity—the values were 2.9×10^9 particles/ μ g protein for CCM-EVs and 6.8×10^8 particles/ μ g protein for AVs (Figure 6A, table).

The enzyme-only control (Zimopec) yielded no detectable particles by ILM, confirming that the diluted pectinase preparation did not contribute detectable particulate material.

Together, ILM and cryo-TEM provide orthogonal evidence of bona fide vesicles in both fractions, with similar size ranges but markedly different abundances, reflecting their spatial origin (CCM-derived vs. apoplast-associated).

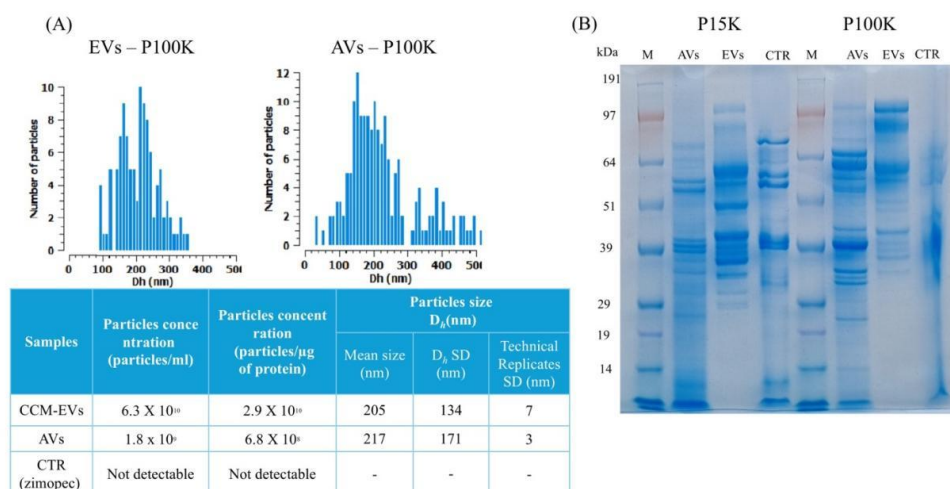


Figure 6. Protein concentrations and SDS-PAGE profiles of EVs, AVs, and enzyme control samples. (A) Summary of Qubit-measured protein and Interferometric Light Microscopy (ILM, Videodrop)-measured particle concentrations and size distribution in the P100K EVs and AVs samples. ILM analyses were performed on technical replicates; (B) SDS-PAGE analysis of 10 μg protein per lane for EV and AV samples (P15K and P100K pellets). The Zimopec control lane (P100K pellet) contains the entire amount of protein recovered from its P100K fraction (~5 μg), resulting in lighter staining compared with the biological samples. The diluted enzyme preparation did not yield a visible pellet and contained no detectable particles by ILM analysis, confirming that it did not contribute vesicle-like material to the preparations. Distinct banding patterns are evident between EV and AV fractions across the P100K pellets.

Effect of zimopec treatment on isolated CCM-EVs

To evaluate whether prolonged exposure to the pectinase preparation could directly affect EVs, isolated CCM-EVs were incubated with diluted Zimopec under the same conditions used for AV release (24 h). Untreated EVs incubated in buffer alone served as controls.

ILM analysis revealed a gradual decrease in particle concentration over time in both conditions. However, the particle concentration profiles of Zimopec-treated and untreated samples remained comparable throughout the experiment, and no substantial differences attributable to enzyme treatment were observed. After 24 h of incubation, particle recovery remained similar in both groups, indicating that the enzymatic treatment used for cell wall loosening does not markedly affect EV particle recovery under the conditions employed in this study. These results suggest that the major differences observed between CCM-EVs and AVs are unlikely to arise solely from direct exposure of vesicles to the enzyme preparation.

Protein profiles by SDS-PAGE

SDS-PAGE protein profiles are shown in Figure 6B. Equal amounts of protein (10 µg protein per lane) were loaded for CCM-EVs and AVs. In contrast, the Zimopec control obtained only about 5 µg total protein in the corresponding P100K fraction; therefore, the entire amount was loaded onto gel, resulting in a lighter control lane. Importantly, ILM analysis detected no particles in the P100K Zimopec control, confirming that the diluted pectinase solution did not contribute vesicle-like material to the samples.

The compact pellets obtained from CCM-EV and AV preparations did not resemble the diffuse or fibrous pectin flocculates reported for pectin-rich ultracentrifugation pellets (Kankaanpää *et al.*, 2024), indicating that pectin carry-over was minimal. Overall, CCM-EV and AV samples displayed clearly distinct protein profiles across P15K and P100K pellets, while the enzyme-only control lane reflected its low protein content rather than technical issues with SDS-PAGE migration.

In the P15K fractions: AVs displayed a more homogeneous distribution of different proteins (typical characteristic of EVs) bands across a wide molecular weight range, whereas EVs

showed fewer bands with higher intensity including strong signals around 39-64 kDa. The control sample exhibited several bands, notably in the 50-64 kDa region, likely corresponding to proteins from the enzymatic mixture. In the P100K fractions: AVs exhibited a richer and more complex protein profile compared to EVs, with intense bands in the 29-64 kDa range and additional lower molecular weight proteins (< 20 kDa). EVs in P100K showed fewer bands overall, with moderate intensity around 39-51 kDa. The control lane for P100K contained very faint bands, indicating minimal protein carryover from the enzyme solution. Moreover, we could see similarities between P15K and P100K protein profiles patterns, both in EVs and AVs. Notably, neither the EV nor AV preparations displayed a dominant protein band at ~55 kDa, which is characteristic of RuBisCO large subunit and indicative of cytoplasmic contamination. This visual profile supports limited cell disruption during the EV/AV isolation workflow. Overall, EV and AV fractions exhibited markedly different protein profiles, suggesting distinct vesicle-associated protein compositions and possible contamination from enzymatic components in AV preparations. Because the P100K pellet from the diluted Zimopex control contained only approximately 5 µg of total protein, the entire amount was loaded onto the SDS-PAGE gel. The lower intensity of the control lane therefore reflects its lower protein content rather than technical issues with gel migration. Consistent with this observation, ILM analysis showed that particle counts in the P100K fraction of the enzyme control were below the detection limit, indicating that the pectinase preparation did not contribute detectable vesicle-like particles to the samples. The diluted Zimopex control did not form a visible pellet after ultracentrifugation at $100,000 \times g$, and the total protein recovered from this fraction (~5 µg) was substantially lower than that of EVs or AVs. ILM analysis also detected no particles in the P100K enzyme control, confirming that the pectinase solution did not contribute to detectable vesicle-like material.

Proteomic analysis of tomato CCM-EV and AV samples

LC-MS/MS analysis was performed on EV and AV samples, including P15K and P100K fractions, as well the EV-depleted soluble fraction (S100K). An overview of protein identifications, annotation efficiency, and dataset overlap is presented in Figure 7. EV datasets were generated from three independent biological preparations.

A total of 1,576 unique non-redundant protein groups were identified across all analyzed datasets [Supplementary Tables 1-5] after merging redundant protein groups detected in multiple fractions and applying the defined identification criteria (≥ 1 unique or razor peptide at 1% false discovery rate). AV fractions contained substantially higher numbers of identified proteins than EV fractions at both the P15K and P100K steps, indicating greater proteome complexity associated with AVs [Figure 7A]. Functional annotation using OmicsBox resolved the majority of initially uncharacterized proteins across all datasets, enabling a comprehensive downstream comparative analyses [Figure 7B]

Comparative overlap analysis further highlighted clear differences between vesicular and non-vesicular extracellular protein pools [Figure 7C]. EV_P100K and AV_P100K shared a limited common vesicular core (73 protein groups), while each fraction contained a large number of unique proteins, with AV_P100K displaying the largest fraction-specific proteome. In contrast, the EV-depleted soluble fraction (S100K) showed minimal overlap with either vesicular dataset, with only a small subset of proteins shared with EV_P100K or AV_P100K and a limited group of proteins detected across all three samples. Together, these data confirm that EVs and AVs represent related but compositionally distinct vesicle populations originating from different extracellular spaces (i.e. apoplast and culture supernatant), whereas S100K corresponds predominantly to a non-vesicular, soluble secretome.

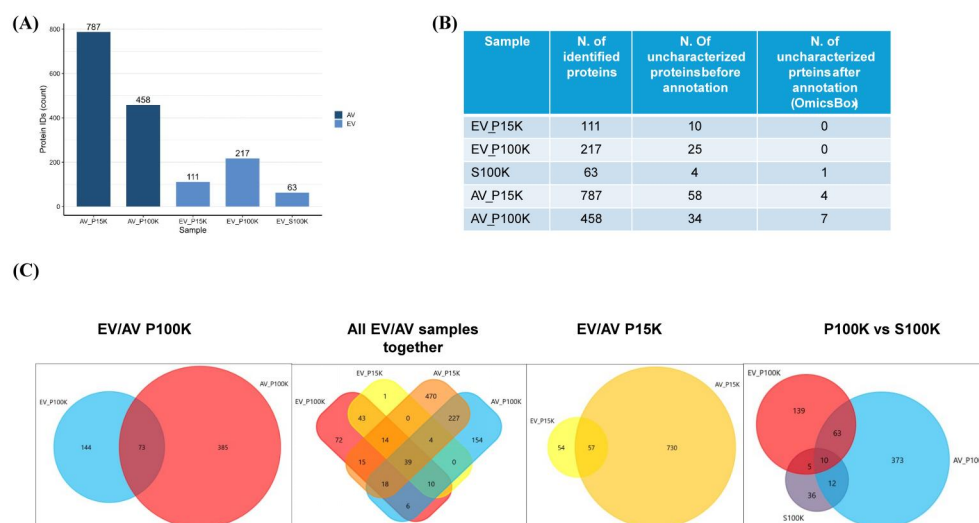


Figure 7. Overview of proteomic datasets from CCM-EV and AV fractions (P15K and P100K) and EV-depleted soluble fraction (S100K) isolated from tomato CSC. (A) Number of identified protein groups in CCM-EV (denoted as EV in the figure) and AV fractions obtained by differential ultracentrifugation (P15K and P100K) and in the EV-depleted soluble fraction (S100K); (B) Summary of functional annotation efficiency, showing the number of uncharacterized proteins before and after OmicsBox annotation for each dataset; (C) Venn diagram analyses illustrating overlap of identified protein groups between CCM-EV and AV datasets at P100K, across all analyzed EV and AV fractions, between EV and AV fractions at P15K, and between vesicular P100K fractions and the EV-depleted soluble fraction (S100K). EV fractions were isolated from the extracellular culture medium, AV fractions from the apoplasmic space, and S100K represents the EV-depleted soluble secretome of tomato cells. Datasets represents three biological preparations.

Proteomic profile of extracellular vesicles (EVs)

Proteomic characterization of EVs was performed primarily on the EV_P100K fraction [Supplementary Table 1], which showed a strong proteomic overlap with EV_P15K [Supplementary Table 2] and represents the most refined EV population isolated in this study. To identify proteins with the highest identification confidence in EVs, proteins detected in EV_P100K were ranked based on their Sequest HT score, and the top-scoring proteins are summarized in Table 1.

The highest-ranking EV proteins included several plasma membrane-associated and vesicle-related components, consistent with a membrane-derived vesicular origin. Among the top-scoring proteins were multiple plasma membrane H⁺-ATPases, reflecting proton transport activity and membrane energization, as well as an ABC/PDR transporter, associated with lipid transport and defense-related compound secretion. Proteins involved in cell wall remodeling and extracellular matrix interactions were also prominent, including α -xylosidase/ α -glucosidase, FAS1 domain-containing proteins, and SKU5-like monocopper oxidases. The latter two are GPI-anchored proteins implicated in glycan turnover, cell wall structural organization, and signaling at the plasma membrane-apoplast interface.

Several proteins associated with membrane microdomains and stress or defense signaling were also detected with high confidence, including neutral ceramidase, involved in sphingolipid metabolism, and a hypersensitive-induced reaction 1 (HIR1)-like protein containing an SPFH/Band-7 domain, a recognized marker of sterol-rich membrane rafts. Together, the highly ranked EV_P100K proteins delineate an EV proteome enriched in plasma membrane-derived, raft-associated, and cell wall-interacting proteins, supporting a role for tomato CCM-EVs in extracellular communication, membrane trafficking, and cell wall remodeling processes.

Table 1 Top-10 proteins identified in EV_P100K sample. Proteins are ordered according to decreasing Sequest HT score as a measure of identification confidence. For each protein, UniProt accession, annotation, sequence coverage, Sequest HT score, number of unique peptides, and inferred functional class are reported.

Rank	Accession	Protein (OmicsBox)	UniProt Description	Coverage (%)	Sequest HT Score	Unique Peptide	Functional class / process
1	A0A3Q7GF G8	Alpha- xylosidase 1-	Alpha- glucosidase	11	44.65	8	Carbohydrate metabolism;

		like					glycan degradation
2	A0A3Q7H6 52	Plasma membrane ATPase 4	Plasma membrane ATPase	16	36.55	14	Proton transport; membrane potential and pH regulation
3	A0A3Q7FD 22	Neutral ceramidase 1	Neutral ceramidase	5	30.55	5	Lipid metabolism; sphingolipid signaling
4	A0A3Q7FN 06	Berberine bridge enzyme- like 15	FAD-binding PCMH-type domain- containing protein	8	29.43	5	Oxidoreductase/ redox-related activity
5	A0A3Q7H0 Y2	Plasma membrane ATPase 2	Plasma membrane ATPase	13	23.27	8	Membrane proton transport and cellular pH regulation
6	A0A3Q7HV 28	Monocopper oxidase-like protein SKU5	Uncharacterize d protein	9	16.08	6	Cell wall organization and developmental signaling
7	Q9SPD5	Plasma membrane ATPase 2	Plasma membrane ATPase	7	14.65	5	Ion transport and membrane energization

8	A0A3Q7HF X0	Fasciclin-like arabinogalactan protein 1	FAS1 domain- containing protein	3	13.72	3	Cell adhesion and extracellular matrix/cell wall interactions
9	A0A3Q7H1 A3	Hypersensitive- induced reaction 1 protein-like	Band 7 domain- containing protein	7	11.16	2	Plant defense and stress response signaling
10	A0A3Q7J41 1	Pleiotropic drug resistance protein 1	ABC transporter domain- containing protein	2	10.24	3	ABC transporter involved in transport of lipids and defense-related compounds

Proteomic profile of apoplastic vesicles (AVs)

Proteomic characterization of AVs was performed on both the AV_P100K [Supplementary Table 4] and AV_P15K fractions [Supplementary Table 5]. Compared with CCM-EV fractions [Supplementary Tables 1-2], a higher number of proteins was identified in AV preparations overall. Notably, the AV_P15K fraction yielded a greater number of identified proteins than AV_P100K. This difference likely reflects the higher complexity of particles and/or co-purifying structures recovered at lower centrifugal force in AV_P15K, whereas AV_P100K represents a more refined vesicular subset.

Table 2 lists the top-10 proteins detected in AV_P100K ranked according to their Sequest HT score. This ranking highlights proteins that detected with high peptide support in the AV fraction. In parallel, the expanded protein repertoire detected in AV_P15K [Supplementary Table 5] reflects the presence of additional components co-pelleting at lower centrifugal force.

The highest-ranked proteins in AV_P100K were predominantly associated with cell wall metabolism, apoplastic remodeling, and defense-related processes, consistent with the functional specialization of the apoplast. These included multiple carbohydrate-active enzymes, such as β -fructofuranosidases (invertases), pectinesterases, pectate lyases, and glycosidases, involved in polysaccharide modification and cell wall restructuring. Proteins linked to plant defense and redox regulation were also prominent within the AV proteome, including class III peroxidases, purple acid phosphatases, chitinases, and glucan endo-1,3- β -D-glucosidases, all of which are well-established apoplastic components involved in stress responses and pathogen interactions.

Compared with CCM-EVs, plasma membrane transporters and vesicle-trafficking components were less dominant among the highest-ranked AV proteins, whereas enzymes and structural proteins associated with the extracellular matrix were more prevalent. This distinction was particularly evident when comparing AV_P100K with EV_P100K, supporting compartment-specific differences in vesicle cargo composition.

Overall, the AV proteome delineates a vesicle population enriched in cell wall-active enzymes, defense-related proteins, and extracellular matrix-associated components.

Table 2. Top-10 proteins identified in AV_P100K sample ranked according to decreasing Sequest HT score. For each protein, UniProt accession, annotation, sequence coverage, Sequest HT score, number of unique peptides, and inferred functional class are reported. AV_P100K represents the apoplastic vesicle fraction [Supplementary Table 4] used for comparative analysis with EVs, whereas the proteins detected in AV_P15K is reported separately [Supplementary Table 5].

Rank	Accession	Protein (OmicsBox)	UniProt Description	Coverage (%)	Sequest HT Score	Unique Peptide	Functional class / process
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							Cell wall polysaccharide biosynthesis; arabinose metabolism
1	Q6IV07	UDP-arabinopyranose mutase 1	UDP-arabinopyranose mutase	46	462.85	8	
							Cell wall non-cellulosic polysaccharide biosynthesis
2	A0A3Q7FS Q6	UDP-arabinopyranose mutase 2	UDP-arabinopyranose mutase	62	259.73	13	
							Likely vesicle trafficking / protein-protein interaction scaffold
3	A0A3Q7GM W1	WD repeat-containing protein	Uncharacterized protein	25	258.97	10	
							Phosphate metabolism; stress and defense responses
4	A0A3Q7GF N0	Purple acid phosphatase 1	Purple acid phosphatase	33	197.03	21	
							Catalytic phosphatase activity; phosphate scavenging
5	A0A3Q7IRE 3	Purple acid phosphatase 27	Purple acid phosphatase	23	115.68	12	
							Defense and cell wall degradation enzyme
6	A0A3Q7EE Z8	Glucan endo-1,3-beta-glucosidase B precursor	Glucan endo-1,3-beta-D-glucosidase	19	102.75	6	

							involved in stress response
		UDP-	UDP-				Cell wall polysaccharide biosynthesis and remodeling
7	A0A3Q7FL M7	arabinopyrano se mutase 1	arabinopyranos e mutase	24	95.56	3	
		putative transcription factor					Regulation of stress- and development-related gene expression
8	A0A3Q7IY K0	Hap3/NF-YB family	Histone H4	60	91.45	11	
		Plasma membrane ATPase 4	Plasma membrane ATPase				Proton transport, pH regulation, and membrane energization
9	A0A3Q7H65 2			19	79.9	11	
			phosphopyruvate hydratase				Glycolysis and stress-related energy metabolism
10	A0A3Q7IN8 1	enolase		35	71.62	4	

Notably, comparison of the Top-10 ranked proteins in EV_P100K [Table 1] and AV_P100K [Table 2] revealed no direct overlap at the level of individual protein accessions. While both datasets contained proteins associated with cell wall-related processes, the highest-ranking CCM-EV proteins were dominated by plasma membrane- and membrane microdomain-associated components, whereas the highest-ranking AV proteins were primarily extracellular enzymes and defense-related proteins.

Comparative analysis of EV and AV proteomes

To delineate similarities and differences between EV and AV proteomes, comparative analyses were performed integrating i) Gene Ontology (GO) term distribution, ii) hierarchical clustering, and iii) targeted inspection of candidate protein biomarkers. GO term distribution analyses were carried out across CCM-EV and AV samples recovered at different centrifugation steps (P15K, P100K) as well as the EV-depleted soluble fraction (S100K), enabling assessment of functional trends across vesicle populations and recovery steps. In contrast, hierarchical clustering and biomarker-oriented analyses were conducted primarily on the refined EV_P100K and AV_P100K datasets, which represent the most vesicle-enriched fractions derived from the extracellular medium and apoplastic space, respectively.

1. Gene Ontology term distribution analysis

To compare the functional composition of EV and AV proteomes, GO term distribution analyses were performed for Cellular Component (CC), Molecular Function (MF), and Biological Process (BP) categories. GO distributions comparing the refined EV_P100K and AV_P100K fractions are shown in Figure 8, while the Top-15 selected GO terms and numerical summaries are provided in Supplementary Table 6.

Analysis of CC categories [Figure 8A] revealed clear differences in subcellular association between EVs and AVs. EV_P100K was strongly enriched in plasma membrane and membrane-associated proteins, supporting a vesicular membrane origin. In contrast, AV_P100K exhibited relatively higher proportions of proteins annotated as cytosolic, chloroplast-associated, cell wall-related, and extracellular region categories.

The MF distribution [Figure 8B] identified hydrolase activity and nucleotide binding as the most abundant functional classes in both proteomes. EV_P100K showed a pronounced enrichment of nucleotide-binding, kinase, and transporter activities, consistent with roles in signaling, transport, and regulatory processes. In contrast, AV_P100K displayed comparatively higher representation of protein binding, transferase activity, and

RNA-binding functions, suggesting broader binding and functional diversity within the apoptotic vesicle fraction.

Analysis of BP categories [Figure 8C] showed that protein modification, transport, response to stress, and carbohydrate metabolic processes were among the most represented functions in both datasets. EV_P100K exhibited a relatively higher contribution of proteins associated with protein modification and transport, whereas AV_P100K showed increased representation of carbohydrate metabolic processes, cellular component organization, and stimulus-responsive functions.

Taken together, CC, MF, and BP GO term distributions indicate consistent divergence between EV and AV proteomes at the P100K level. EVs are preferentially associated with membrane-related, transport, and regulatory functions, whereas AVs display broader metabolic, structural, and stress-related functional profiles, consistent with their distinct spatial origins. Extended GO term distributions across additional fractions (EV_P15K, AV_P15K and S100K) are reported in Supplementary Figures 1-3.

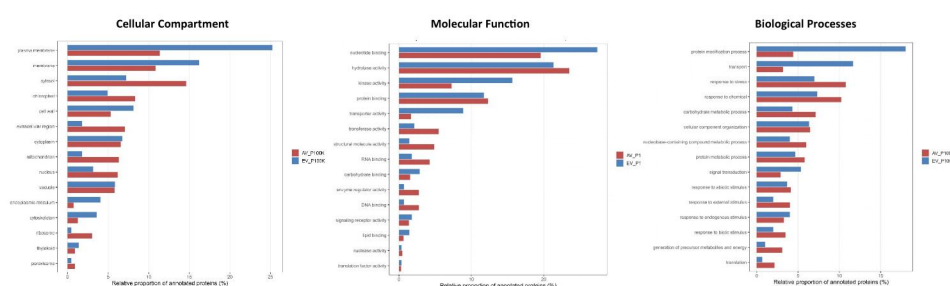


Figure 8. Gene Ontology (GO) term distribution analysis of EV and AV proteomes at the P100K level. Relative distribution of GO terms for Biological Process (BP) (A); Cellular Component (CC) (B); and Molecular Function (MF) (C) categories comparing EV_P100K and AV_P100K protein sets. The analysis highlights distinct GO term profiles between EVs and AVs, with EVs enriched in membrane-associated, transport, and regulatory functions, and AVs showing broader metabolic, stress-related, and binding activities. Detailed GO

distributions across additional fractions are provided in Supplementary Figure 1-3 and Supplementary Table 6.

2. Hierarchical clustering analysis of EV and AV proteomes

Hierarchical clustering analysis revealed clear global differences between EV and AV proteomes [Figure 9]. The normalized protein abundance values used for hierarchical clustering analysis are provided in Supplementary Table 7. EVs and AVs samples segregated into distinct clusters, indicating divergent protein abundance patterns between vesicles isolated from the extracellular medium and those recovered from the apoplastic space.

Proteins enriched in EV and AV fractions formed distinct clusters, whereas shared proteins contributed less to the overall separation, indicating that fraction-specific proteins drive the clustering pattern. Within each vesicle group, additional separation between P15K and P100K fractions was observed, reflecting fraction-specific differences in protein composition. EV_P15K and EV_P100K clustered more closely to each other than AV_P15K and AV_P100K.

Together, hierarchical clustering analysis demonstrates that EV and AV proteomes differ not only in protein identity but also in global abundance patterns, corroborating the compositional differences observed through overlap analysis and high-confidence protein ranking.

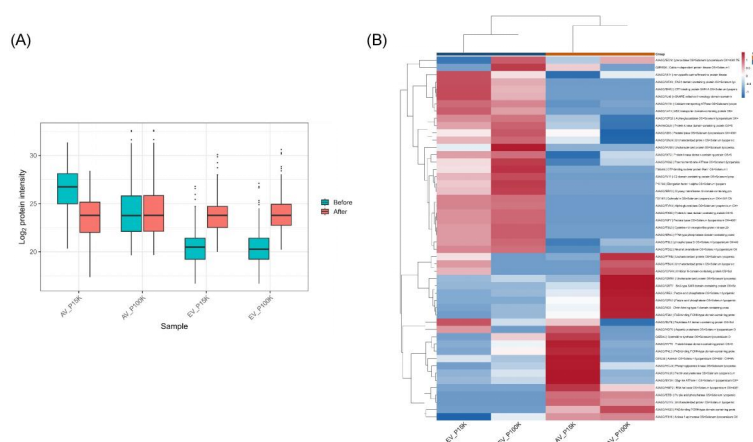


Figure 9. Median normalization and hierarchical clustering analysis of EV and AV proteomes. (A) Boxplots showing log₂-transformed protein intensity distributions for EV and AV fractions before and after median normalization; (B) Heatmap with unsupervised hierarchical clustering of normalized protein abundance values for EV_P15K, EV_P100K, AV_P15K, and AV_P100K samples. Samples segregate according to vesicle type, with EV and AV fractions forming distinct clusters and additional separation between P15K and P100K fractions. Protein identities corresponding to heatmap rows are provided in Supplementary Table 7.

3. Analysis of candidate plant EV protein markers

To further investigate differences between EV and AV proteomes, the presence, distribution, and relative abundance of selected protein families commonly proposed as plant EV associated markers were examined in the EV_P100K and AV_P100K fractions. The analyzed protein families included fasciclin-like arabinogalactan proteins (FLAs), tetraspanins, germin-like proteins, patellins, heat shock proteins (HSPs), aquaporins, exocyst complex components, clathrin-associated proteins (including calreticulins), phospholipase D, Rab GTPases, V-ATPase subunits, 14-3-3 proteins, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the latter included as a predominantly cytosolic reference protein. Analysis of marker family distribution across samples [Figure 9A] showed that several protein families, including Rab GTPases, 14-3-3 proteins, HSPs, and SNARE proteins, were detected in both EV and AV fractions, indicating partial overlap of these protein groups

between vesicle populations. In contrast, exocyst components, clathrin-associated proteins, and aquaporins were predominantly observed in AV samples, whereas FLAs were more frequently detected in EV fractions.

Comparison of average protein abundance [Figure 9B] revealed distinct quantitative trends. FLAs, Rab GTPases, SNARE proteins, and phospholipase D showed higher abundance in EV fractions, while HSPs, clathrin-associated proteins, and 14-3-3 proteins were relatively more abundant in AV samples. GAPDH displayed low and variable abundance across fractions, consistent with its role as a cytosolic reference. Relative enrichment of marker families, expressed as $\log_2(\text{EV}/\text{AV})$ ratios [Figure 9C], further highlighted these differences. FLAs, SNARE proteins, and phospholipase D showed EV-biased enrichment, whereas Rab GTPases displayed AV-biased enrichment at the P100K level.

Hierarchical clustering of marker family abundance profiles [Figure 9D] showed clear separation between EV and AV fractions. Proteins enriched in EVs (e.g., FLAs, SNARE proteins, phospholipase D) grouped separately from those more abundant in AVs (e.g., HSPs, clathrin-associated proteins, 14-3-3 proteins). Together, these results indicate that EV and AV fractions display distinct marker family profiles, consistent with compositional differences between the two vesicle populations.

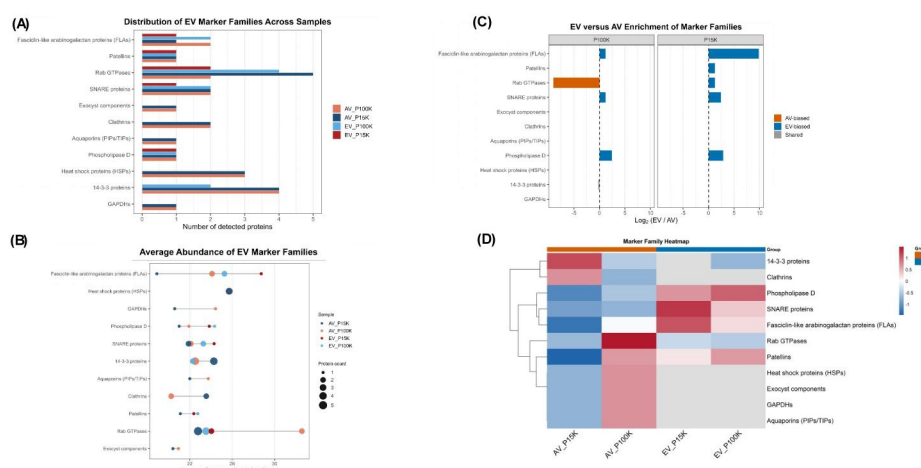


Figure 10. Analysis of candidate EV- and AV-associated protein marker families. (A) Distribution of EV marker families across samples. Presence and detection frequency of selected EV-associated protein families across EV and AV fractions. Rab GTPases, 14-3-3 proteins, and HSPs were among the most frequently detected families in both vesicle populations, whereas exocyst components, clathrin-associated proteins, and aquaporins were more prevalent in AV-derived samples; (B) Average abundance of EV marker families. Average normalized abundance of selected marker protein families across EV and AV fractions. FLAs, Rab GTPases, SNARE proteins, and phospholipase D showed higher average abundance in EV fractions, while HSPs, clathrin-associated proteins, and 14-3-3 proteins were more abundant in AV fractions. GAPDH is shown as a predominantly cytosolic reference protein; (C) EV versus AV enrichment of marker families. Relative enrichment of marker protein families calculated as $\log_2(\text{EV/AV})$ abundance ratios. Positive values indicate EV-biased enrichment, whereas negative values indicate AV-biased enrichment; values close to zero represent similar abundance in both vesicle populations; (D) Marker family abundance heatmap. Hierarchical clustering of marker family abundance profiles across EV and AV fractions. EV-associated clusters were characterized by enrichment of FLAs, SNARE proteins, and phospholipase D, whereas AV-associated clusters showed higher representation of HSPs, clathrin-associated proteins, and 14-3-3 proteins.

Notably, tetraspanins, which have been proposed as canonical extracellular vesicle markers in mammalian systems and have been associated with plant EVs in *Arabidopsis*, were not detected in either EV or AV datasets from *Solanum lycopersicum* under the conditions analyzed.

DISCUSSION

This study introduces an enzymatic strategy to release AVs from *in vitro* plant cells and compares them with EVs recovered from the CCM of the same cultures [Figure 4]. In a controlled system of tomato CSC [Figure 1], we combined cryo-TEM morphology [Figure 5], ILM sizing and counting, SDS-PAGE profiling [Figure 6], and LCMS/MS proteomics of CCM-EV and AV samples [Figure 7-8] to obtain data that extends current understanding of

plant vesicle spatial distribution across the plasma membrane -cell wall-apoplast interface and heterogeneity.

Accessing putative apoplast-associated vesicles following cell wall loosening

A major challenge in plant EV research is the recovery of vesicles retained associated with the apoplast-cell wall matrix, while avoiding contamination from intracellular contents. Classical AWF-based approaches rely on vacuum infiltration and centrifugation (Y. Huang *et al.*, 2021; Rutter & Innes, 2017), which cannot be directly applied to *in vitro* CSCs due to the lack of organized tissue architecture and interconnected apoplastic spaces. Here, we optimised an enzymatic treatment using a pectinase-rich preparation (Zimopec), containing both pectolytic and accessory cell wall-degrading activities, which selectively loosened the pectin-rich wall matrix while preserving the cellulose network (as assessed by Direct Red 23 staining) and plasma membrane integrity (DiI staining). This approach resulted in controlled cell wall permeabilization without extensive cellular rupture [Figures 2-3]. As the present study was conducted using dedifferentiated tomato cell suspension cultures, references to pectin-rich components are made in the context of the cell wall-apoplast environment rather than a defined middle lamella structure typical of differentiated tissues.

Importantly, conditioned medium was removed prior to enzymatic digestion and cells were washed, ensuring that the AV fraction primarily reflects vesicles originally retained in the apoplast/cell-wall continuum. This aligns with the spatial-temporal continuum model of plant EVs, in which vesicles may reside transiently within the wall matrix before completing their passage into the extracellular environment. Mild secretion during the treatment cannot be excluded; however, such vesicles would enter the digestion buffer only after wall loosening begins and thus represent a minor component relative to wall-embedded AVs.

An additional control experiment performed showed that incubation of isolated CCM-EVs with diluted Zimopec under the same experimental conditions used for AV release resulted in particle concentration profiles comparable to those of untreated EV controls. Although particle numbers gradually decreased during prolonged incubation, this effect was observed

in both control and enzyme-treated samples. These results suggest that the pronounced differences observed between CCM-EVs and AVs are unlikely to be explained solely by direct exposure of vesicles to the enzyme preparation [Supplementary Table 8].

Consistent with this controlled release, both CCM- EVs and AVs produced compact P100K pellets after ultracentrifugation, whereas the enzyme- only control yielded only ~5 µg of total protein and no detectable particles by ILM. Cryo-TEM analysis confirmed the presence of membrane-bound vesicles in both CCM-EV and AV preparations. However, filamentous and undefined material was also observed, particularly in AV samples. We note that the prolonged enzymatic wall-loosening treatment used in this study may itself induce physiological responses that contribute to the observed AV proteome. In particular, cell wall degradation may activate signalling pathways associated with oligogalacturonide-derived DAMPs and other stress-related responses. Consequently, some of the proteins enriched in AV fractions may reflect treatment-induced cellular responses in addition to their localization within the cell wall-apoplast environment. A direct comparative analysis of untreated and enzymatically treated cells would be required to determine the extent of such treatment-induced proteomic changes.

Morphology and size: Shared bilayer architecture, distinct abundance

Cryo- TEM imaging revealed membrane- bound vesicles with well-defined bilayers in both CCM-EV and AV preparations [Figure 5]. EV grids displayed abundant vesicles spanning a broad size range (~50-1000 nm), including apparent multilamellar morphologies. Phenomena reported in plant vesicle imaging and other EV systems when particle populations are polydisperse and concentrated. AV grids contained fewer vesicles with a more uniform size distribution (~50-250 nm) and occasional electron- dense cargo inside. ILM measurements [Figure 6A] provided complementary hydrodynamic sizing and particle counts. CCM- EVs showed a median D_h of 212 nm and a mean D_h of 205 nm, while AVs showed median 220 nm and mean 217 nm, consistent with the polydisperse nature of plant vesicles detected by interferometric methods. EVs were significantly more abundant (6.3×10^8 particles/mL) than AVs (1.8×10^8 particles/mL). When normalized to protein, the

difference remained pronounced (EVs: 2.9×10^9 particles/ μg protein; AVs: 6.8×10^8 particles/ μg protein), reflecting their spatial origin: CCM- EVs accumulate freely in medium, whereas AVs must be enzymatically released from the apoplastic matrix.

Together, cryo- TEM and ILM provide orthogonal evidence that both CCM- EVs and AVs are bona fide vesicles with characteristic plant EV morphology, while differing in abundance and matrix association. This difference in abundance likely reflects not only differences in vesicle release but also their spatial dynamics, as CCM- EVs might accumulate over time in the extracellular medium, whereas AVs are retained within the cell wall-apoplast matrix and require enzymatic release.

Protein profiles and EV/AV proteomes: Plasma membrane/raft signatures and extracellular remodeling

SDS- PAGE profiles revealed clear differences between CCM- EV and AV fractions across P15K and P100K pellets [Figure 6B]. In P15K, AVs displayed a broad, uniform distribution of bands, while EVs showed fewer but more intense bands (notably 39-64 kDa). In P100K, AVs exhibited a richer and more complex banding pattern than EVs, including < 20 kDa components, whereas EVs presented fewer bands with moderate intensity around 39-51 kDa. These patterns support distinct protein compositions between CCM- EVs and AVs.

LC-MS/MS analysis of CCM- EV fractions revealed a proteome enriched in membrane- associated and vesicle trafficking-related proteins, including ABC/PDR transporters and the t- SNARE syntaxin- 132- like protein, together with small GTPases such as RAB and YPT family members. In addition, membrane-extracellular interface proteins, including FAS1 domain-containing and fasciclin- like arabinogalactan proteins (FLAs), as well as annexin D1, were detected. Cell wall-modifying enzymes, including α - xylosidases and pectate lyases, were also identified, indicating that EVs carry components capable of interacting with or remodeling the cell wall matrix. These features are consistent with GO term enrichment in vesicle- mediated transport, cellular localization, and cell wall organization (Results 3.5.1). An alternative explanation for the enrichment of stress- and

defence-related proteins in AV preparations should also be considered. During enzymatic loosening of the cell wall, pectin degradation may generate oligogalacturonides (OGs), which are recognized as endogenous Damage-Associated Molecular Patterns (DAMPs) in plants (Benedetti *et al.*, 2015; Degli Esposti *et al.*, 2025; Ferrari *et al.*, 2013). OGs are known to activate reactive oxygen species (ROS) production, defence signalling pathways and immune responses resembling those induced during pathogen attack. Consequently, the stress-related protein signature observed in AV fractions may reflect not only the specific extracellular location of these vesicles within the cell wall-apoplast continuum, but also cellular responses induced by pectinase-mediated wall loosening. Therefore, while the marked differences between CCM-EVs and AVs support the influence of spatial context on vesicle composition, some AV-associated proteins may additionally arise from treatment-induced immune responses.

In contrast, AV fractions were dominated by proteins associated with the extracellular region and cell wall environment, including multiple peroxidases, aspartic proteases, serine carboxypeptidases, and carbohydrate-active enzymes, together with proteins involved in stress and defense responses. This composition may reflect both the intrinsic cargo of apoplast-associated vesicles and proteins associated with vesicle surfaces after their residence within the protein-rich apoplastic environment. In the mammalian EV field, externally associated proteins have been described as part of an EV protein corona (Tóth *et al.*, 2021). Although the existence and composition of a protein corona in plant EVs remain largely unexplored, vesicles residing within a protein-rich apoplastic environment may similarly acquire surface-associated proteins. Consequently, some proteins identified in AV preparations may represent proteins associated with the external vesicle surface rather than exclusively membrane-enclosed vesicular cargo.

The prevalence of extracellular enzymes, defence-related proteins and cell wall-associated proteins, together with the higher overall proteome complexity of AV fractions, is consistent with vesicles embedded within and interacting extensively with the environment. Future proteinase K treatment experiments on intact vesicles, combined with membrane

permeabilization controls, will be required to distinguish membrane-protected cargo proteins from externally associated proteins.

CCM-EVs and AVs proteomes show selective overlap with plant EV datasets described in *Arabidopsis*, particularly in the presence of vesicle trafficking components such as syntaxin-like SNARE proteins and Rab family GTPases, membrane-associated proteins including annexins, and cell wall-related enzymes such as α -xylosidases, pectate lyases, and FAS1/FLA proteins, which have been consistently reported in plant EV preparations. However, canonical EV marker EXO70 described in some in agreement *Arabidopsis* datasets, including tetraspanins, were not detected in the present study.

To further contextualize these findings, we compared the tomato CCM-EV and AV proteomes with candidate EV-associated protein families proposed in the literature (Rodríguez de Lope *et al.*, 2025), and summarized in Table 3. Previous studies, largely conducted in *Arabidopsis thaliana*, have identified several protein families as potential plant EV markers, including tetraspanins (TET8 and TET9), PEN1/SYP121 syntaxins, and EXO70E2 associated with EXPO-derived vesicles. However, a consensus set of bona fide EV markers has not yet been established, and the applicability of these markers across plant species remains uncertain.

In our system, tetraspanins were detected only in the AV_P15K fraction and were not present in the vesicle-enriched EV_P100K or AV_P100K fractions, consistent with growing evidence that TET family proteins are not universally conserved EV markers across plant species and may represent *Arabidopsis*-specific EV subpopulations. The detection of a tetraspanin-8-like protein in the AV_P15K fraction but not in P100K fractions suggests that TET-associated structures may be present in heterogeneous assemblies rather than in the refined EV populations analyzed here.

Despite the limited presence of tetraspanins, CCM-EV fractions were enriched in core vesicle trafficking proteins, including syntaxin-like SNAREs and Rab GTPases [Table 3],

supporting a plasma membrane-derived vesicular origin and active exocytic pathways. In contrast, AV fractions displayed a distinct protein signature characterized by extracellular and stress-related proteins, including heat shock proteins and 14-3-3 proteins, together with membrane-associated components such as clathrin and aquaporins, consistent with the complex apoplastic environment.

Plant-specific extracellular interface proteins, notably fasciclin-like arabinogalactan protein 1 (FLA1) family members, were relatively enriched in CCM-EVs [Table 3], highlighting the role of these vesicles at the plasma membrane-cell wall interface. Overall, these results indicate that EV and AV fractions share core vesicle-associated machinery but differ markedly in their associated protein environments, reinforcing the view that plant EV marker composition is strongly dependent on species and extracellular context rather than universally conserved across plant systems.

Table 3. Distribution of candidate EV-associated protein families in CCM-EV_P100K and AV_P100K fractions from *Solanum lycopersicum*. Protein families are grouped according to functional categories consistent with Figure 10, including core EV trafficking machinery, membrane-associated/lipid dynamics proteins, plant-specific extracellular interface proteins, and stress-related/regulatory proteins. Core trafficking components (Rab GTPases, SNAREs, exocyst and clathrin) were detected across fractions, while extracellular interface proteins (e.g., FLAs) were enriched in EVs and stress-related proteins (HSPs, 14-3-3) in AVs. Tetraspanins were not detected in either fraction. Shared proteins are indicated in bold; GAPDH and RuBisCO are included as cytosolic reference and contamination markers, respectively; Tetraspanin-8-like protein was detected in the AV_P15K fraction but not in EV_P100K or AV_P100K fractions.

Protein marker family	Tomato Proteins (SOLLC) EV_P100K	Tomato Proteins (SOLLC) AV_P100K	Enrichment	Functional category
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Rab GTPases	A0A3Q7FXJ2; A0A3Q7I1Z6; A0A3Q7IA56 ; Q41339; A0A3Q7FZ41; A0A3Q7HG43	A0A3Q7FPJ2 A0A3Q7GNM1 A0A3Q7IA56 A0A3Q7HXG9 A0A3Q7HG43	EV ↑	Core EV trafficking machinery
SNARE proteins	A0A3Q7IL48; A0A3Q7EEA9	A0A3Q7JYS6 A0A3Q7EEA9	shared	Core EV trafficking machinery
Exocyst components	n.d.	A0A3Q7GZ09; A0A3Q7IRE8	AV ↑	Core vesicle trafficking machinery (EXPO- associated in plants)
Clathrins	n.d.	A0A3Q7GJG3	AV ↑	Core EV trafficking machinery
Tetraspanins	n.d.	n.d.	n.d.	Canonical EV markers (not detected here)
Patellins	A0A3Q7IXE6	A0A3Q7GYN8	shared	Membrane- associated proteins (plasma membrane origin markers)
Aquaporins (PIPs/TIPs)	K4DFV3; A0A3Q7HL14	A0A3Q7HL14 ; Q08451	AV ↑	Plant-specific PIP/TIP subclasses; plasma

				membrane-associated channels (membrane origin markers)
Phospholipase D	A0A3Q7F6L3	A0A3Q7HNP1 A0A3Q7HVM3 A0A3Q7F6L3	EV ↑	Membrane-associated / lipid dynamics proteins
Fasciclin-like arabinogalactan proteins (FLAs)	A0A3Q7HFX0; A0A3Q7J5H8	A0A3Q7HFX0; A0A3Q7J5H8; A0A3Q7JFW9	EV ↑	Plant specific extracellular interface - potential plant EV marker
Germin like protein	n.d.	A0A3Q7EP74	AV ↑	Plant specific extracellular interface - potential plant EV marker
14-3-3 proteins	A0A3Q7HSM4 A0A3Q7IRT6 A0A3Q7G7H8	A0A3Q7EZ16; P93214; P93212; A0A3Q7IRT6; A0A3Q7G7H8; A0A3Q7HSM4	AV ↑	Stress-related/regulatory proteins
Heat shock proteins (HSPs)	n.d.	P24629; A0A3Q7HZV3 P36181; A0A3Q7HA43	AV ↑	Stress related/regulatory proteins
GAPDHs	n.d.	A0A3Q7HUQ3	AV ↑	Background (cytosolic

		protein)		
ruBisCO	n.d.	A0A3Q7H376	AV ↑	Contamination
		A0A3Q7ECG0		(chloroplastic protein)

CONCLUSIONS

Kocholatá *et al.* (2024) demonstrated that EVs isolated from different tobacco sources, such as leaves, callus, and CSCs, vary substantially in yield and cargo composition. In contrast, the present study focuses on a single, controlled biological system—tomato CSC—enabling the paired analysis of vesicles sampled at two defined extracellular positions: CCM-EVs, which have traversed the cell wall, and cell wall-associated AVs.

To our knowledge, this study presents the first report of an enzymatic cell wall-loosening strategy enabling recovery of AV-enriched fractions from suspension-cultured plant cells. Although both vesicle populations share canonical EV morphology and polydispersity, they differ markedly in abundance and protein composition. CCM-EVs are enriched in membrane-associated and vesicle trafficking proteins, including SNARE components, small GTPases, and membrane-apoplast interface proteins such as fasciclin-like arabinogalactan protein 1 (FLA1) family members. In contrast, AV fractions display greater proteome complexity and are enriched in extracellular enzymes, cell wall-associated proteins and stress-related proteins. This composition may reflect both their association with the cell wall-apoplast environment and physiological responses induced during enzymatic wall loosening. These compositional differences were observed across multiple analytical approaches, including SDS-PAGE, GO term analysis, hierarchical clustering, and marker family comparisons, suggesting meaningful divergence between EV and AV proteomic profiles. The limited overlap among the most abundant proteins further suggests that the observed differences reflect intrinsic variation in vesicle-associated protein repertoires rather than simple quantitative shifts. Together, these findings support the working model that CCM-EVs and AVs may represent spatially distinct vesicle populations derived from a common cellular origin. In this framework, AVs correspond to vesicles retained within or interacting

with the apoplastic matrix, whereas CCM-EVs represent vesicles that have completed their passage into the extracellular medium. This spatial partitioning provides a mechanistic basis for the observed proteomic divergence and highlights the influence of the extracellular microenvironment on vesicle composition.

Overall, the workflow presented here provides a platform for the isolation and comparative analysis of plant EV populations across defined extracellular locations. More broadly, these results highlight the importance of spatial context at the cell wall interface in shaping extracellular vesicle composition in plant systems.

DECLARATIONS

Authors' contributions

Experimental design, tissue culture setup, plant cell growth and characterization, EVs and AVs isolation, writing and editing: Barbulova A;

Tissue culture setup, EVs isolation: Moubarak M.

Plant tissue culturing, sample preparation for proteomics: Fiume I;

Plant cell growth and characterization, sample preparation for proteomics, bioinformatics: Devarakonda P;

Plant tissue culturing and characterization, microscopy: Szász C;

ILM analyses, manuscript editing: Kralj-Iglič V;

ILM analyses: Romolo A;

Cryo-TEM analyses, manuscript editing: Kisovec M, Podobnik M;

Proteomics: Oh J. W, Kim D. H;

Proteomics, bioinformatics: Sajjad A;

Resources, proteomics supervision: Kim K.-P;

Conceptualization, experimental design, resources, supervision, writing the original draft, review and editing: Pocsfalvi G.

Availability of data and materials

The datasets of mass spectrometry proteomics data generated and analyzed for this study have been submitted to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier: PXD078293.

AI and AI-assisted tools statement

Perplexity AI (Perplexity; web-based version, accessed in December, 2025) was used to assist with English-language editing, including improvements in grammar, clarity, readability, and phrasing. Google Gemini (Google; web-based version, accessed on December, 2025) was used to assist with the design and refinement of the graphical abstract.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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