

Outer membrane vesicles from *Agrobacterium rhizogenes* modulate physiological and defense-related responses in *Arabidopsis thaliana*

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ABSTRACT

Outer membrane vesicles (OMVs) are spherical nanostructures secreted by Gram-negative bacteria that encapsulate periplasmic contents along with a diverse array of lipids, proteins, and nucleic acids. Although OMVs are increasingly recognized as important mediators of plant–microbe interactions, their biological effects on plants remain insufficiently understood for many phytopathogenic bacteria. In this study, we provide the first characterization of OMVs produced by *Agrobacterium rhizogenes* and assess their morphology, protein composition, and effects on the model plant *Arabidopsis thaliana*. Proteomic analysis revealed membrane-associated proteins, flagellar components, ribosomal proteins, and proteins potentially related to bacterial fitness and host interaction, indicating that protein cargo may contribute to OMV-mediated communication together with other vesicle constituents. In *A. thaliana*, OMV treatment affected overall plant development and leaf expansion, reduced leaf fresh weight and membrane stability, and altered secondary metabolism, mainly by increasing glucosinolate accumulation. qPCR analysis showed differential expression of genes associated with immune responses and stress-related signaling pathways. In addition, pretreatment of *A. thaliana* leaves with OMVs reduced *Pseudomonas syringae* proliferation and disease symptoms. These findings indicate that *A. rhizogenes* OMVs can modulate host plant physiology and defense responses, highlighting OMVs as previously underappreciated factors in *Agrobacterium*–plant interactions with potential relevance for understanding and modulating plant defense activation.

1. Introduction

Over the past several decades, extensive research has demonstrated that many Gram-negative bacteria secrete outer membrane vesicles (OMVs) from their surface into the environment. OMVs are spherical nanovesicles formed by the bacterial outer membrane and contain a diverse array of periplasmic and cytoplasmic components, including lipopolysaccharides, phospholipids, peptidoglycans, proteins, nucleic acids, and other pathogenicity-associated factors [1–3]. OMVs are produced by both pathogenic and non-pathogenic bacteria, and to date, no Gram-negative bacteria have been identified that do not produce these vesicles [4]. These structures play versatile roles in bacterial survival

and interaction with host organisms. They transport proteins [5] and DNA [6], and participate in toxin delivery, attachment to host cells, stress response, biofilm formation, and bacterial nutrition [7–9].

OMVs also play an important role in pathogen–host interactions by facilitating bacterial attachment, invasion, and the modulation of host defense responses [10]. For instance, OMVs from *Fusobacterium nucleatum* exacerbate periodontitis by triggering inflammation and alveolar bone absorption independent of bacterial colonization [11]. Similarly, OMVs from adherent-invasive *Escherichia coli* influence intestinal inflammation by inducing pro-inflammatory cytokines and altering tight junction proteins in epithelial cells, contributing to inflammatory bowel disease [12]. Moreover, OMVs from *Staphylococcus aureus* cause

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structural changes in host cells, affecting cellular integrity and promoting infection [13]. Their ability to deliver bacterial effectors without direct bacterial contact represents an alternative pathogenic strategy, as shown in *E. coli* O157, where OMVs package and deliver type III secretion system effectors into host cells, bypassing traditional injection pathways [14]. Vesicle formation can be influenced by molecular signals from the host, and OMVs may help bacteria evade immune responses, in some cases by delivering virulence factors directly into host cells [9].

Although much attention has focused on OMV interactions with mammalian systems, recent studies have begun to uncover their importance in plant-bacterial interactions. OMVs produced by phytopathogenic bacteria such as *Pseudomonas syringae* can suppress plant growth, likely due to reallocation of metabolic resources toward defense responses [15]. In addition, OMVs can activate plant immunity and enhance resistance to subsequent pathogen attack. For example, OMVs from *Xanthomonas campestris* induce callose deposition, reactive oxygen species (ROS) production, and transcriptional activation of pathogen-associated molecular pattern (PAMP) receptors [16,17]. These effects have been associated with vesicle-associated MAMPs, extracellular enzymes, and other cargo molecules whose composition depends on bacterial species and growth conditions. The bacterial protein EF-Tu, present in OMVs, acts as an elicitor of plant immunity, with perception mediated by immune coreceptors BAK1 and SOBIR1 [17]. OMVs from *Xylella fastidiosa* may also contribute to disease spread by reducing bacterial attachment to xylem walls, facilitating movement through the vascular system [18]. Despite growing interest in these phenomena, the molecular basis of OMV-mediated effects on plants remains incompletely understood.

Soil pathogenic bacteria of the genus *Agrobacterium* are of particular interest due to their unique ability to transfer a segment of plasmid DNA (T-DNA) into plant genomes [19]. This process induces neoplastic growth, allowing the bacteria to establish a favorable ecological niche in host tissues through a strategy termed genetic colonization [20]. The same mechanism has been widely adapted as a central tool in plant biotechnology and genetic engineering for both fundamental and practical purposes [21,22]. Beyond DNA transfer, members of the *Agrobacterium* complex interact with plants through multiple surface-associated and secreted factors that remain less well characterized. A recent study showed that an *A. tumefaciens* strain releases OMVs containing the surface-exposed Atu8019 protein involved in bacterial adhesion and biofilm-associated interactions [23]. However, compared with other phytopathogenic bacteria, little is known about the biological activity of OMVs produced by *Agrobacterium* species or their influence on plant physiology, and no study has directly addressed the physiological and immune responses of plants to *A. rhizogenes* OMVs. Understanding whether OMVs from these bacteria can modulate plant growth, defense signaling, or resistance responses would broaden current views of *Agrobacterium*–plant interactions beyond the classical T-DNA transfer model.

Therefore, this study aimed to isolate and characterize OMVs produced by *A. rhizogenes* and to assess their effects on the model plant *A. thaliana*. By analyzing vesicle morphology, protein composition, and their influence on plant growth, defense-associated responses, and resistance to *Pseudomonas syringae*, we sought to clarify the role of *Agrobacterium*-derived OMVs in plant–microbe communication.

2. Materials and methods

2.1. Bacterial cultivation, transformation, OMV isolation, and vir operon induction

The study used *Agrobacterium rhizogenes* wild-type strain A4 stored in the Bioresource Collection of the Federal Scientific Centre of East Asia Terrestrial Biodiversity of the Far Eastern Branch of the Russian Academy of Sciences (registration number 2797657). Bacteria were cultured in Luria–Bertani (LB) medium (pH 7.5) at 28°C and 130 rpm in an ES-20

orbital shaker-incubator (BioSan, Latvia).

To assess the response to acetosyringone exposure, the overnight culture of *A. rhizogenes* was diluted 1:100 in fresh LB medium and grown to an optical density at 600 nm (OD₆₀₀) of 1.0, which was reached after approximately 3–4 h. Acetosyringone (AS) was then added to a final concentration of 100 µM, and cultures were incubated for an additional 2 h at 28°C with shaking at 130 rpm.

For OMV isolation, *A. rhizogenes* cells grown either in standard LB medium or in LB supplemented with AS were removed by centrifugation at 20,000 × g for 30 min at 4°C. The resulting supernatant was filtered through a 0.45 µm membrane (Millipore) to eliminate residual cell debris. OMVs were pelleted by ultracentrifugation at 150,000 × g for 1 h at 4°C using an Optima MAX-XP ultracentrifuge equipped with a TLA110 rotor (Beckman Coulter, USA). Typically, 100 mL of clarified culture supernatant was used for a single OMV isolation through sequential ultracentrifugation runs with pooling of the resulting OMV pellets. The pooled OMV pellet obtained after the final ultracentrifugation step was washed twice with sterile phosphate-buffered saline (PBS; pH 7.4) and resuspended in the same buffer. To confirm the absence of bacterial contamination, aliquots of the final OMV suspension were plated on antibiotic-free LB agar and incubated at 28°C for 48 h; no colony growth was observed.

2.2. Nanoparticle tracking analysis (NTA) of OMVs

The size distribution and concentration of isolated OMVs were analyzed using nanoparticle tracking analysis (NTA) on a Nanosight NS500 system (Malvern Instruments, Malvern, UK), as previously described [24]. All procedures were performed according to the manufacturer's instructions. OMV samples were diluted in sterile deionized water to achieve a particle concentration corresponding to 10–20 particles per visual frame. A 60-s video was recorded at 23°C under controlled flow using a syringe pump to ensure consistent sample movement. For each biological replicate, 10 videos were captured and analyzed using NTA software version 2.2 (Malvern). The software automatically tracked Brownian motion of individual vesicles to compute particle size distribution and mean diameter.

2.3. Scanning electron microscopy (SEM)

The morphology of purified OMVs was visualized using field emission scanning electron microscopy (SEM). OMV samples were fixed in 4% (v/v) glutaraldehyde in PBS (pH 7.4) for 20 min at room temperature, followed by two washes with PBS to remove residual fixative. Fixed vesicles were deposited onto carbon-coated SEM grids (EM Resolutions, UK) and allowed to air-dry under a sterile hood. No conductive coating was applied. Imaging was performed using a Sigma FE-SEM instrument (Carl Zeiss, Jena, Germany) operated at an accelerating voltage of 10 kV under high vacuum conditions (~10^{−5} mmHg). High-resolution surface morphology was captured using an InLens secondary electron detector.

2.4. Protein extraction and identification from OMVs

To eliminate extrinsic and surface-bound proteins, OMVs were pre-treated with proteinase K (ThermoFisher Scientific, USA). Briefly, OMVs were incubated with proteinase K at a final concentration of 150 µg/mL for 30 min at 37°C. The enzyme was then inactivated by adding 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich, USA). Following protease inactivation, OMVs were sonicated on ice using a Sonopuls HD 2070 ultrasonic homogenizer (BANDELIN electronic GmbH, Berlin, Germany) at 30% amplitude, in three 5-s bursts (1 s on/1 s off), to disrupt vesicle membranes and release luminal proteins. Proteins were precipitated by adding ice-cold 100% acetone containing 2% 2-mercaptoethanol. Samples were incubated at −20°C for 1 h and then centrifuged at 20,000 × g for 30 min at 4°C. The resulting protein pellet was washed twice with 96% ethanol and

resuspended in Laemmli buffer for electrophoretic analysis. Protein concentration was determined using the Bradford assay, modified with Coomassie Brilliant Blue G-250 and 10% orthophosphoric acid. A standard curve was generated using bovine serum albumin (BSA; Promega, USA) at concentrations ranging from 5 to 100 µg/mL. Absorbance was measured at 465 nm using an xMark™ microplate spectrophotometer (Bio-Rad, USA).

OMV protein samples were resolved by SDS-PAGE using 12.5% and 15% polyacrylamide gels. Protein loading was normalized based on total protein concentration, and 30 µg of protein was applied to each lane. To compare protein profiles, samples derived from proteinase K-treated OMVs and proteins isolated from untreated OMVs were analyzed in parallel. After electrophoresis, gels were stained with Coomassie Brilliant Blue G-250 in a solution containing 50% ethanol and 10% glacial acetic acid, and destained in 10% ethanol with 5% glacial acetic acid until protein bands were clearly visible.

For enhanced sensitivity, silver staining was also performed. The gel was first treated with 10% sodium thiosulfate for 1 min, followed by incubation in 12 mM silver nitrate for 40 min at room temperature. The gel was then transferred to a developing solution containing 3% potassium carbonate, 0.025% formalin, and 0.00125% sodium thiosulfate, and incubated for 10 min with gentle agitation. To terminate the reaction, the gel was placed in a stop solution composed of 4% Tris (hydroxymethyl)aminomethane and 2% acetic acid for 30 min. Finally, the gel was stained with a 15 mM potassium ferricyanide and 50 mM sodium thiosulfate solution at 50°C for 20 min to enhance contrast and protein band visibility.

For proteomic identification, selected bands were excised from stained gels and subjected to in-gel trypsin digestion as described previously [24]. Peptides were analyzed via time-of-flight mass spectrometry (TOF-MS) using an Autoflex II mass spectrometer (Bruker Daltonics, Germany). Protein identification was carried out using Mascot MS Search software (Matrix Science, UK) against the NCBI and Swiss-Prot databases.

2.5. Vacuum infiltration of *Arabidopsis thaliana* with OMVs

For experiments examining the effects of OMVs on plants, sterile seeds of *Arabidopsis thaliana* ecotype Col-0 were germinated on half-strength Murashige and Skoog (MS) medium. OMV preparations used for infiltration were pretreated with proteinase K as described above. After proteolysis and inactivation of proteinase K, OMVs were washed twice with sterile PBS (pH 7.4) by ultracentrifugation and resuspended in the same buffer. Ten-day-old seedlings were subjected to vacuum infiltration in PBS containing OMVs at a concentration of 1.5×10^{11} particles/mL for each treatment. The procedure was performed using a Concentrator Plus vacuum concentrator (Eppendorf, Germany), applying three consecutive vacuum pulsation cycles of 1 min each to ensure uniform uptake. Seedlings infiltrated with sterile PBS served as negative controls. Following treatment, plants were transferred to soil and grown under standard long-day conditions (16 h light/8 h dark, 22°C). Leaf length and width were measured using a ruler at 14, 16, 21, 24, 28, and 42 days post-treatment to assess long-term developmental effects. Leaf size was expressed as a coefficient calculated as length $\times$ width. After 42 days, plants were harvested and immediately used for subsequent analyses.

Possible persistent *A. rhizogenes* contamination was assessed 42 days after OMV treatment. For each treatment group, 100 mg of leaf tissue from PBS-, OMV-, or OMV-AS-treated plants was homogenized in 1 mL of sterile PBS using a sterile mortar and pestle. Homogenate aliquots (100 µL) were plated on LB agar supplemented with rifampicin (90 mg/L) and incubated at 28°C for 5 days. Recovered colonies were screened by PCR using *virD2*-specific primers, with *A. rhizogenes* DNA and sterile water used as positive and negative controls, respectively.

2.6. Membrane stability and electrolyte leakage assays

To assess membrane integrity in plants 42 days after OMV treatment, both membrane stability index (MSI) and relative electrolyte leakage (REL) were measured. For MSI, 0.1 g of *A. thaliana* leaves were immersed in 20 mL of distilled water and subjected to controlled heat stress treatments: 40°C for 30 min (C1) and 100°C for 15 min (C2). Electrolyte conductivity was recorded after each treatment, and MSI was calculated using the formula $MSI = [1 - (C1/C2)] \times 100$, where C1 represents conductivity after moderate heat treatment and C2 represents total conductivity after boiling [25]. For REL analysis, the protocol of [26] was followed. Leaf samples (0.1 g) were incubated in 20 mL of distilled water, and conductivity was measured at the beginning of incubation (C1) and after 3 h at room temperature (C2). REL was calculated as $REL = (C1/C2) \times 100\%$, where C1 represents initial conductivity and C2 represents conductivity after incubation, providing a quantitative estimate of electrolyte leakage from leaf tissues.

2.7. RNA extraction, cDNA synthesis, and gene expression analysis

To examine the expression of the *A. rhizogenes* A4 *virD* gene, bacteria grown in LB or LB supplemented with AS were harvested, and total RNA was extracted using the LIRA RNA Extraction Kit (Biolabmix, Novosibirsk, Russia) according to the manufacturer's instructions. RNA samples were treated with TURBO DNase™ (Thermo Fisher Scientific, CA, USA) to remove residual genomic DNA. First-strand cDNA was synthesized from 1.5 µg of total RNA using random hexamer primers and Reverse Transcriptase (Biolabmix, Novosibirsk, Russia).

Total RNA was extracted from *A. thaliana* plants 42 days after OMV treatment and from corresponding control plants as previously described [27], and first-strand cDNA synthesis was performed according to the same protocol.

Quantitative real-time PCR (qPCR) reactions were performed using HS-qPCR 2 $\times$ SYBR Blue Master Mix (Biolabmix, Novosibirsk, Russia) on a CFX96 Real-Time PCR System (Bio-Rad Laboratories, USA). Specific primers targeting *A. rhizogenes* A4 *virD* gene or selected *A. thaliana* genes were used for expression profiling, with *pyrG* and *GAPDH* serving as internal reference genes for bacterial and plant samples, respectively (primer sequences provided in [Supplementary Table 1](#)). Each analysis included three biological replicates derived from independent RNA extractions, and each biological replicate was analyzed in three technical replicates. Amplification efficiency and melt curve analysis were assessed using CFX Manager Software (version 1.5; Bio-Rad Laboratories, USA), and relative gene expression levels were calculated using the 2- $\Delta\Delta C_t$ method [28].

2.8. Secondary metabolite profiling by HPLC-ESI-MS

Secondary metabolites were identified in dried aerial parts of *A. thaliana* plants collected 42 days after OMV treatment and from corresponding control plants using a 1260 Infinity high-performance liquid chromatography (HPLC) system (Agilent Technologies, Santa Clara, CA, USA) coupled with an electrospray ionization (ESI) ion trap mass spectrometer (HCT ultra PTM Discovery System; Bruker Daltonik GmbH, Bremen, Germany). Chromatographic separation was carried out using a Zorbax C18 column (Agilent Technologies) under reversed-phase conditions. Standards of kaempferol, quercetin, sinigrin hydrate, and sulfatase (type H-1 from *Helix pomatia*) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The procedures for sample preparation, glucosinolate desulfation, and the specific chromatographic and mass spectrometric parameters were performed as described previously [29]. Quantification was conducted using a photodiode array detector calibrated with authentic reference compounds. Data acquisition and analysis were performed using Agilent OpenLAB CDS software (v.01.06.111). Representative mass spectra were recorded in negative ionization mode to validate metabolite identification. Mass

spectrometry data acquisition and processing were carried out using Bruker Daltonics Compass 1.3 esquireControl software (v.6.2.581.3 and v.4.0.234.0).

2.9. Plant infection assay with *Pseudomonas syringae* after OMV pretreatment

For this assay, sterile seeds of *A. thaliana* Col-0 were germinated on half-strength MS medium. Ten-day-old seedlings were transferred to soil and grown under standard long-day conditions (16 h light/8 h dark, 22°C). OMVs isolated from untreated *A. rhizogenes* cultures or from acetosyringone-induced cultures (OMV-AS) were used for pretreatment. OMV preparations used for infiltration were pretreated with proteinase K and purified as described above. Leaves of 21-day-old *A. thaliana* plants were detached and subjected to vacuum infiltration in PBS containing OMVs at a concentration of 1.5×10^{11} particles/mL for each treatment. The infiltration procedure consisted of three consecutive vacuum pulsation cycles of 1 min each. Leaves infiltrated with sterile PBS served as negative controls. Following OMV or buffer treatment, leaves were incubated for 24 h under controlled conditions.

Subsequently, the same leaves were infiltrated with a *Pseudomonas syringae* suspension ($OD_{600} = 0.02$) prepared in 10 mM $MgCl_2$ using the same vacuum infiltration method. Infected leaves were maintained for four days at 22°C until visible symptom development.

For bacterial quantification, six leaves per treatment group (PBS + *P. syringae*, OMV + *P. syringae*, and OMV-AS + *P. syringae*) were sampled. In addition, leaves treated with PBS only were analyzed. Identical circular leaf discs were collected using a sterile 0.5 cm diameter metal punch, homogenized in 1 mL of sterile PBS, serially diluted (1:100), and 20 μ L of each dilution was plated on LB agar using a sterile spatula. Plates were incubated at 25°C for 48 h, after which colony-forming units (CFUs) were counted to estimate bacterial proliferation. Colonies were identified based on the characteristic morphology of the inoculated *P. syringae* strain (small, circular, convex, creamy-white, opaque colonies with smooth margins). No colonies with this morphology were observed on plates from leaves not inoculated with *P. syringae*; therefore, these samples were not included in CFU quantification.

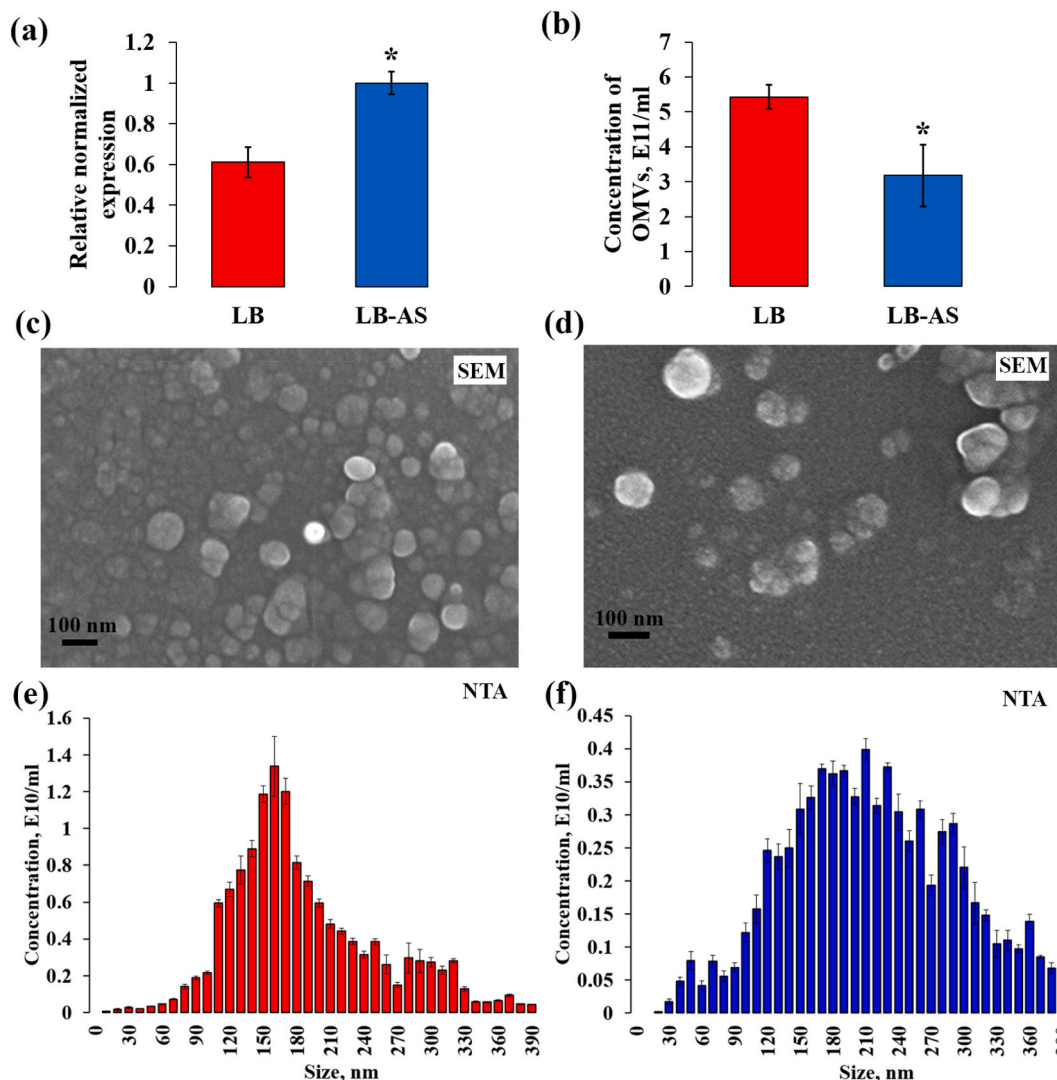


Fig. 1. Characterization of outer membrane vesicles (OMVs) isolated from *Agrobacterium rhizogenes* grown under normal conditions and from cultures exposed to acetosyringone (OMV-AS). (a) Relative expression of the *virD2* gene in *A. rhizogenes* grown in LB and LB + AS. (b) Estimated concentration of OMVs normalized to the original clarified culture medium from LB and LB + AS cultures. (c, d) Scanning electron microscopy (SEM) images of OMVs and OMV-AS, respectively. (e, f) Nanoparticle tracking analysis (NTA) showing size distribution profiles of OMVs and OMV-AS, respectively. Data are shown as mean $\pm$ SE. Asterisks (*) indicate statistically significant differences between groups according to Student's *t*-test ($p < 0.05$).

2.10. Statistical analysis

All data are presented as the mean $\pm$ SE. Statistical comparisons between two independent groups were performed using Student's *t*-test. For comparisons involving multiple groups, analysis of variance (ANOVA) was performed, followed by Fisher's least significant difference (LSD) post hoc test. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Isolation and characterization of *A. rhizogenes* OMVs

In this study, OMVs were isolated from *A. rhizogenes* cultures grown either in standard LB medium or in LB supplemented with acetosyringone (AS). LB medium was selected because it provides stable and reproducible growth conditions suitable for comparative OMV production, while allowing assessment of AS exposure under otherwise identical conditions. Although LB is not an optimal medium for maximal *vir* gene induction, AS treatment nevertheless resulted in approximately twofold upregulation of *virD2* expression (Fig. 1a). Therefore, the OMV-AS preparation should be interpreted as derived from AS-exposed cultures rather than from fully virulence-induced bacteria.

The estimated concentration of OMVs normalized to the original clarified culture medium was 5.43×10^{11} particles/mL for untreated cultures, whereas AS-treated cultures yielded 3.18×10^{11} particles/mL (Fig. 1b), indicating a moderate reduction in vesicle production under AS exposure. This decrease in particle concentration is not inconsistent with the slight increase in mean vesicle size, because particle yield and vesicle diameter represent different parameters of OMV biogenesis and may respond differently to culture conditions.

Scanning electron microscopy (SEM) revealed that the OMVs exhibited a predominantly spherical morphology, with an average diameter of 95.55 ± 6.42 nm (Fig. 1c and d), consistent with typical OMV structures reported in other Gram-negative bacteria [1,2]. In contrast, nanoparticle tracking analysis (NTA), which accounts for the hydrodynamic diameter, showed a larger average OMV size of 220.18 ± 23.84 nm (Fig. 1e). OMVs isolated from AS-treated *A. rhizogenes* cultures (OMV-AS) exhibited a slightly increased average size of 244.05 ± 5.46 nm (Fig. 1f); however, this difference was not statistically significant compared to OMVs from untreated bacteria.

The variation in vesicle sizes observed between SEM and NTA measurements can be attributed to the distinct methodologies used in each technique. NTA measures the hydrodynamic diameter, which encompasses not only the vesicle itself but also the surrounding layer of surface-associated molecules. In contrast, SEM sample preparation involves dehydration with acetone and critical point drying, which removes the hydration shell and leads to a reduction in the measured vesicle size. According to previous studies, the hydration shell may include proteins, nucleic acids, lipopolysaccharides (LPS), ion clusters, and polysaccharides, all of which contribute to the increased hydrodynamic diameter observed with NTA measurements [30].

3.2. Protein composition of *A. rhizogenes* OMVs

The protein composition of *A. rhizogenes* OMVs was assessed by polyacrylamide gel electrophoresis (PAGE) under denaturing conditions (Supplementary Fig. 1). The gel profile revealed a broad spectrum of protein bands with molecular weights ranging from approximately 10 to 150 kDa. Treatment of OMV preparations with proteinase K resulted in the disappearance of several prominent bands, indicating degradation of protease-accessible, surface-exposed proteins while preserving proteins protected within vesicles. However, this assay does not by itself identify the specific degraded proteins or prove that all remaining proteins are strictly luminal cargo. Comparative analysis of OMVs from untreated and acetosyringone-treated bacteria (OMV-AS) revealed a generally similar protein profile. MALDI-MS analysis (Supplementary Table 2) of

excised gel bands identified several protein groups, including ribosomal proteins (RplC, RplS), elongation factor Tu (EF-Tu), multiple flagellar components (FlaA, FlaB, FlaC, FlgG, FlgK, FlgE, FlaD, FlhB), outer membrane proteins (OmpV and OmpW families), proteins associated with *Agrobacterium* pathogenesis (VirB4 and AopB), and a peptidoglycan-associated lipoprotein (Table 1). The identified proteins spanned molecular weights from 10 to 90 kDa, consistent with gel-based observations.

3.3. Influence of *A. rhizogenes* OMVs on morphological and physiological responses in *A. thaliana*

The impact of *A. rhizogenes* OMVs on *A. thaliana* morphology and development was evaluated by monitoring both overall plant growth and leaf expansion over time. Plants treated with phosphate-buffered saline (PBS) served as negative controls, while treatment groups received OMVs isolated from either untreated (OMV) or acetosyringone-treated (OMV-AS) *A. rhizogenes* cultures. Plant morphology was examined at selected time points, and leaf length and width were measured to evaluate the influence of OMVs on plant development, and leaf fresh weight was determined at 42 days post-treatment (Fig. 2a, b, c).

Representative images showed that OMV treatment affected the overall growth of *A. thaliana* plants during prolonged cultivation (Fig. 2a). Compared with PBS-treated controls, OMV-treated plants developed more slowly and displayed a more compact rosette phenotype by days 24 and 42 (Fig. 2a). Quantitative analysis of leaf dimensions further confirmed this growth-inhibitory trend (Fig. 2b). Initial assessments at 14 and 16 days post-treatment showed no statistically significant differences in leaf dimensions between PBS controls and OMV-treated plants, except for a slight reduction in leaf width in the OMV-AS group. However, as the plants matured, more pronounced effects emerged. By day 21, leaves from OMV-treated plants exhibited modest reductions in both length and width compared to controls, though the differences remained statistically insignificant. In contrast, plants treated with OMV-AS displayed a significant reduction in leaf size by day 24 compared to both control and OMV-treated plants, indicating a stronger developmental impact from OMVs produced under AS exposure. By day 28, these differences became more pronounced, with the OMV-AS group showing a statistically significant reduction in both leaf length and width. This trend persisted through day 42, where OMV-AS-treated plants had the smallest leaf size among all groups. While the OMV treatment also resulted in reduced growth compared to controls, the effect was consistently milder than that observed with OMV-AS. These findings indicate that *A. rhizogenes*-derived OMVs can influence both overall plant growth and leaf expansion, and that OMVs produced after AS exposure have a more substantial inhibitory effect on plant development.

Consistent with the reduction in leaf expansion, leaf fresh weight measured at 42 days post-treatment was significantly lower in both OMV- and OMV-AS-treated plants than in PBS-treated controls (Fig. 2c). Fresh weight decreased by approximately 28% in the OMV group and by approximately 35% in the OMV-AS group relative to the control. However, no significant difference in fresh weight was detected between the OMV and OMV-AS treatments.

To assess whether OMVs influence cellular integrity, membrane stability and relative electrolyte leakage in *A. thaliana* leaves were evaluated following treatment. Plants exposed to *A. rhizogenes* OMVs exhibited a 17% reduction in membrane stability compared to PBS-treated controls (Fig. 2d), whereas no statistically significant differences were observed in relative electrolyte leakage (Fig. 2e).

Because these morphological and physiological effects were assessed 42 days after a single OMV treatment, we additionally examined whether they could be associated with persistent *A. rhizogenes* contamination. Occasional bacterial colonies were observed in all experimental groups, including PBS-treated controls; however, none of the tested colonies produced a *virD2* PCR signal (Supplementary Fig. 3).

Table 1
Protein composition of *A. rhizogenes* OMVs.

Protein name	Molecular weight, kDa	UniProt ID	Function	Reference
FlaA	31.6	P96307	Flagellum component	[31]
FlaB	32	Q7D188	Flagellum component	
FlaC	32.8	A0A822V2I6	Flagellum component	
FlgG	27.8	A0A4D7WVX8	Component of the basal body of the flagellum	
MipA/OmpV family protein	27.9	A0A546XQX1	Family of outer membrane proteins associated with pathogenesis and antibiotic resistance	[32]
OmpW family protein	24.8	A0AA88EZV0	Transmembrane transport of cations	
AopB	22.8	Q8VRG3	Involved in tumorigenesis <i>A. tumefaciens</i>	[33]
Porin family protein	23.07	A0AAE6BB33	Putative outer membrane channel	[34]
				Bioproject PRJNA534393
FlgE	44.2	A0A1S7PXL0	Component of the basal body of the flagellum	[35]
Tol-Pal system protein TolB	48.09	A0AAE6BHK5	Component of the Tol-Pal system, which plays an important role in cell division	[36]
FlaD	45.1	A0A822V1D2	Flagellum component	[37]
FlgK	51.5	A0AAE6EDU8	Flagellum hook-associated protein	[38]
Chaperonin GroEL	61.9	A0AAP9E144	Protein folding	[39]
ATP synthase beta chain atpD	51.4	Q8UC76	Produces ATP from ADP in the presence of a proton gradient across the membrane. The catalytic sites are hosted primarily by the beta subunits	[40]
FlhB	44.7	A0A4D7YDB3	Flagellum biosynthesis	[41]
50S ribosomal protein uL3	22.6	A0A4D7XWD5	Components of the 50S ribosome subunit	[42]
50S ribosomal protein uL19	9.4	A0A4D7XYP0		
Peptidoglycan-associated lipoprotein	18.8	A0A546XFT8	Part of the Tol-Pal system, which plays a role in outer membrane invagination during cell division and is important for maintaining outer membrane integrity	[43]
Elongation factor Tu	42.5	P75022	Responsible for the binding of aminoacyl-tRNA to the ribosome	[44]
VirB4 family type IV secretion/conjugal transfer ATPase	87.4	A0A2Z2PQN1	ATP hydrolysis; participation in the process of plasmid transfer during conjugation; component of the type IV secretion system	[45]

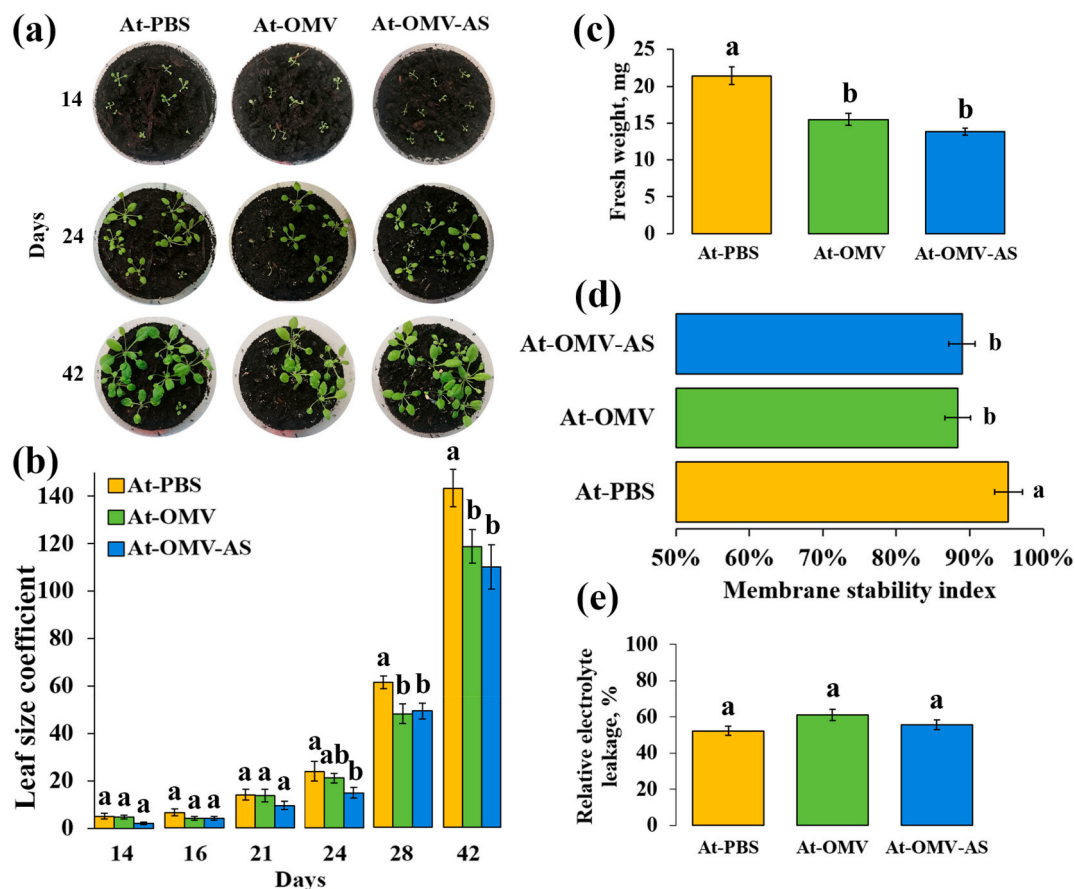


Fig. 2. Effects of OMV treatment on growth parameters and membrane integrity in *Arabidopsis thaliana* plants. (a) Representative images of *A. thaliana* plants treated with phosphate-buffered saline (At-PBS), OMVs isolated from untreated *A. rhizogenes* cultures (At-OMV), and OMVs isolated from acetosyringone-treated cultures (At-OMV-AS), shown at 14, 24, and 42 days post-treatment. (b) Changes in leaf size of *A. thaliana* following OMV treatment. Leaf size coefficient was calculated as the product of leaf length and width. (c) Leaf fresh weight of *A. thaliana* plants measured 42 days post-treatment. (d) Effect of *A. rhizogenes* OMVs on membrane stability in *A. thaliana* leaves measured 42 days post-treatment. (e) Effect of *A. rhizogenes* OMVs on relative electrolyte leakage in *A. thaliana* leaves measured 42 days post-treatment. Data are shown as mean $\pm$ SE. Different letters above the bars indicate significant differences according to Fisher's LSD test ($p < 0.05$).

3.4. Expression of defense-related genes in response to OMVs

To assess the immunomodulatory effects of OMVs from *A. rhizogenes*, we analyzed the expression of several defense-related genes in *A. thaliana* leaves treated with OMVs from untreated cultures (OMV) and from acetosyringone-treated cultures (OMV-AS). As shown in Table 2, treatment with both OMV and OMV-AS altered the expression of a range of genes associated with plant defense and stress responses, with OMV-AS generally inducing stronger responses.

Among the genes analyzed, *WRKY30* and *WRKY40*, transcription factors involved in defense and stress-responsive transcriptional regulation, showed strong upregulation. *WRKY40* expression was approximately 35- and 39-fold higher in OMV- and OMV-AS-treated plants, respectively, than in PBS-treated controls, with no significant difference between the two OMV treatments. *WRKY30* also responded strongly to OMV treatment and showed particularly pronounced induction in the OMV-AS group. Receptor- and signaling-related genes were also upregulated, but the magnitude of induction differed markedly between treatments. In the OMV group, *RPL23*, which encodes a receptor-like protein involved in pattern-triggered immunity, *EFR*, which encodes the EF-Tu receptor, and *RbohF*, which encodes an NADPH oxidase associated with ROS production, showed moderate increases in expression, generally ranging from 2- to 3-fold relative to the control. In contrast, OMV-AS treatment caused substantially stronger induction of these genes, reaching approximately 18- to 21-fold increases. *PAL1*, a key gene of the phenylpropanoid pathway, showed a similar treatment-dependent pattern, with stronger upregulation in response to OMV-AS. Additionally, *SGA1* and *ViP1*, two genes involved in plant responses to microbial signals and stress-related pathways, showed increased expression in response to both OMV types, again with more pronounced changes under OMV-AS, reaching approximately 13- and 12-fold increases relative to the control, respectively.

3.5. Effect of *A. rhizogenes* OMVs on secondary metabolite accumulation in *A. thaliana*

The analysis of secondary metabolite accumulation in *A. thaliana* following OMV treatment showed that glucosinolate accumulation, rather than flavonol content, represented the major secondary-metabolite response (Fig. 3, Supplementary Table 3). Flavonol content showed no statistically significant change after treatment with either OMVs from untreated *A. rhizogenes* cultures or OMVs from acetosyringone-treated cultures (OMV-AS) (Fig. 3a).

Glucosinolate accumulation was more responsive to OMV treatment. The levels of aliphatic glucosinolates increased significantly in both OMV- and OMV-AS-treated plants (Fig. 3b). A similar pattern was observed for aromatic glucosinolates, which also increased significantly in both treatment groups (Fig. 3c). In contrast, indole glucosinolates remained relatively stable across all treatments, with only minor variations (Fig. 3d). These results suggest that *A. rhizogenes* OMVs primarily affected aliphatic and aromatic glucosinolate accumulation, whereas

flavonol content and indole glucosinolate levels were not significantly altered under the conditions tested.

3.6. OMV-mediated enhancement of *A. thaliana* resistance to *P. syringae*

To investigate the effect of *A. rhizogenes* OMVs on plant resistance to pathogenic bacteria, *A. thaliana* leaves were first infiltrated with OMVs or OMV-AS preparations, followed 24 h later by infiltration with a *P. syringae* suspension. The leaves were incubated in Petri dishes for 4 days under controlled conditions. Subsequently, identical fragments were excised, homogenized, serially diluted, and plated on LB agar without antibiotics. After 48 h, bacterial colony-forming units (CFUs) were quantified (Supplementary Fig. 2). Both OMV and OMV-AS treatments resulted in an approximately 2.6- to 2.8-fold reduction in *P. syringae* CFU numbers compared to the control (PBS-treated) group (Fig. 4a). No substantial growth of unrelated bacterial or fungal contaminants was observed during the assay period (Supplementary Fig. 2). Moreover, disease symptoms were markedly reduced or entirely absent in OMV-treated leaves (Fig. 4b), indicating that OMVs may induce defense responses that limit pathogen proliferation.

4. Discussion

Outer membrane vesicles (OMVs) are increasingly recognized as important mediators of interactions between Gram-negative phyto-pathogenic bacteria and plants, influencing growth, immunity, and stress responses [15,46,47]. However, despite the well-established role of *Agrobacterium* species in inducing neoplastic growth, the contribution of their OMVs to plant-microbe interactions remains poorly understood.

In this study, we isolated OMVs from both untreated and acetosyringone-exposed *A. rhizogenes* cultures. These vesicles displayed a spherical morphology and an average diameter of 95.55 nm, with a slight increase in mean size observed following AS exposure. For plant-associated bacteria, vesicle sizes ranging from 20 to 200 nm have been described in genera such as *Pseudomonas*, *Xanthomonas*, and *Xylella* [15, 17,48]. In addition, smaller vesicles in the range of 20–80 nm were reported for an *A. tumefaciens* strain [23]. Variation in reported OMV size may arise from multiple factors, including strain-specific properties, growth phase, culture medium composition, and environmental conditions. Differences in reported OMV size may also reflect methodological variation among imaging and particle-sizing approaches [49–51].

Proteomic analysis revealed a diverse set of candidate vesicle-associated proteins in *A. rhizogenes* OMVs, including structural components, flagellar proteins, outer membrane proteins, and factors potentially associated with bacterial fitness and host interaction. This overall composition is consistent with previous observations in an *A. tumefaciens* strain, whose OMVs contained proteins ranging from 13 to 60 kDa, including numerous ribosomal proteins and flagellins [23]. OMV-associated ribosomal proteins have been proposed to contribute to protection against antibiotics targeting the translational machinery [52]. Flagellins may play a role in OMV biogenesis [53]. Although flagellins are also well-characterized microbe-associated molecular patterns (MAMPs), in *Agrobacterium* these proteins are highly modified, rendering them largely non-detectable by the FLS2 immune receptor in most plants, with only rare exceptions [54,55]. We also detected EF-Tu, which is a fully active elicitor in Arabidopsis and is recognized through the EFR receptor [56]. Because proteinase K treatment and gel-based analysis cannot conclusively distinguish luminal from tightly associated vesicle-surface proteins, the identified proteins should be interpreted as OMV-associated candidates rather than as definitively internal cargo. These observations suggest that immune responses induced by *A. rhizogenes* OMVs may involve multiple vesicle-associated MAMPs acting in a partially redundant manner rather than flagellin alone, thereby enhancing the sensitivity and robustness of pathogen perception even when individual recognition pathways are weak or evaded [57].

Acetosyringone treatment did not substantially alter the overall OMV

Table 2

Relative expression of selected plant response genes in *A. thaliana* following treatment with *A. rhizogenes* OMV preparations.

Gene	At-PBS	At-OMV	At-OMV-AS
<i>EFR</i>	0.65 ± 0.04 ^C	1.51 ± 0.11 ^B	13.77 ± 0.68 ^A
<i>RPL23</i>	0.68 ± 0.06 ^C	1.88 ± 0.14 ^B	14.31 ± 0.90 ^A
<i>WRKY30</i>	0.14 ± 0.01 ^C	1.12 ± 0.09 ^B	13.06 ± 0.09 ^A
<i>WRKY40</i>	0.26 ± 0.07 ^B	9.12 ± 0.70 ^A	10.14 ± 1.06 ^A
<i>PAL1</i>	0.63 ± 0.08 ^C	1.99 ± 0.59 ^B	14.90 ± 2.92 ^A
<i>RbohF</i>	0.84 ± 0.05 ^C	2.14 ± 0.17 ^B	15.17 ± 0.66 ^A
<i>SGA1</i>	1.01 ± 0.03 ^C	1.70 ± 0.10 ^B	13.11 ± 2.24 ^A
<i>ViP1</i>	1.00 ± 0.19 ^C	2.59 ± 0.20 ^B	11.67 ± 1.89 ^A

Data are shown as mean ± SE. Different superscript letters within each row indicate significant differences according to Fisher's LSD test ($p < 0.05$).

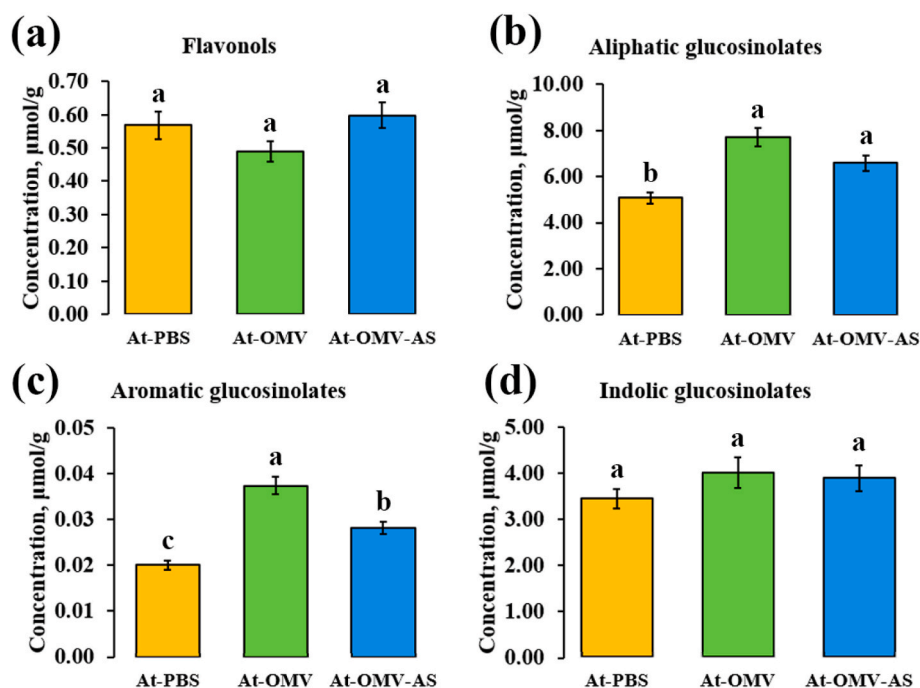


Fig. 3. Effect of *A. rhizogenes* OMVs on the accumulation of secondary metabolites in *A. thaliana*. Concentration of (a) flavonols, (b) aliphatic glucosinolates, (c) aromatic glucosinolates, and (d) indole glucosinolates were measured in plants treated with OMVs from untreated bacteria (At-OMV) and OMVs from acetosyringone-treated cultures (At-OMV-AS). Control plants were treated with phosphate-buffered saline (At-PBS). Data are shown as mean $\pm$ SE. Different letters above the bars indicate significant differences according to Fisher's LSD test ($p < 0.05$).

protein profile in our experiments. This likely reflects the relatively weak effect of phenolic inducers under rich medium conditions and neutral pH. In agreement with this interpretation, acetosyringone promoted only weak *virD2* induction in our study. Likewise, several previous reports have shown that acetosyringone can exert measurable physiological or regulatory effects under similar rich-medium conditions [58,59]. Full virulence induction, however, generally requires additional environmental signals such as acidic pH, monosaccharides, and phosphate limitation [60–62]. Therefore, the OMV-AS preparation should be viewed as reflecting partial phenolic exposure in rich medium rather than full virulence induction. Interestingly, OMVs from both AS-exposed and untreated cultures contained proteins linked to the *Agrobacterium* type IV secretion system, including the VirB4 ATPase and AopB [34,63]. Previous studies have shown that some Vir proteins, including VirB4, can be detected under non-induced conditions or in rich media, albeit at substantially lower levels than after full induction [64–66]. Taken together, these observations suggest that the cargo of *A. rhizogenes* OMVs reflects both bacterial physiological state and environmental conditions, supporting multifunctional roles in microbial adaptation and host interaction.

OMVs from both animal- and plant-associated bacteria can interact with host cells and deliver proteins, lipids, and regulatory RNAs [47,67]. However, the presence of the plant cell wall makes direct membrane fusion less straightforward than in animal systems. Although preliminary observations indicate that bacterial OMVs can traverse this barrier [68], the precise mechanism remains unclear. One proposed explanation is that cell wall-degrading enzymes associated with some bacterial OMVs [48,69] may facilitate local loosening of the wall matrix, thereby promoting vesicle passage [47]. However, no obvious cell wall-degrading enzymes were detected among the proteins identified in the present *A. rhizogenes* OMV preparations. Alternatively, OMVs may act at the cell wall surface by releasing diffusible bioactive cargo without requiring intact vesicle entry. In the present study, the induction of PRR-related genes and defense responses is most consistent with recognition of OMV-associated MAMPs at, or near, the plant cell surface, whereas direct vesicle uptake remains a possibility that requires further

experimental verification.

The biological activity of *A. rhizogenes* OMVs was further evident in the developmental and physiological responses observed in *A. thaliana*. Although early growth effects were modest, the effects became more pronounced during prolonged cultivation, especially in the OMV-AS group, and included reduced leaf expansion and decreased membrane stability. This growth-inhibitory effect was also reflected in final leaf fresh weight, which was significantly reduced by both OMV treatments. Similar OMV-associated reductions in plant fresh weight were previously reported for *A. thaliana* treated with OMVs from *P. syringae* and *P. fluorescens*, with responses comparable to those induced by flg22 [15]. The mechanisms underlying these physiological responses remain incompletely understood and may involve OMV-mediated interference with plant hormone signaling, disruption of nutrient uptake, or activation of stress-related signaling pathways. The decrease in membrane stability observed in our study may represent another component of this stress-associated response. It may reflect subtle changes in membrane organization or composition rather than severe membrane disruption, consistent with the absence of significant changes in electrolyte leakage. These assays reflect related but distinct aspects of membrane integrity: membrane stability indices estimate resistance to stress-induced membrane injury, whereas electrolyte leakage more directly reflects loss of membrane barrier function and ion efflux [26,70].

The transcriptional changes observed in *A. thaliana* suggest that *A. rhizogenes* OMVs activate multiple plant defense signaling pathways. The receptor-like protein RLP23, which detects necrosis- and ethylene-inducing peptide 1 like proteins, was upregulated more than tenfold in plants treated with OMV-AS, consistent with activation of pattern-triggered immunity [71]. Similarly, *EFR*, a receptor kinase that recognizes bacterial elongation factor Tu, showed elevated expression levels, suggesting enhanced sensitivity to bacterial elicitors [72]. *WRKY30* and *WRKY40*, key regulators of defense signaling [73], were strongly induced. OMV treatment also induced the expression of *SGA1/ASF1*, a histone chaperone linked to chromatin dynamics, and *ViP1*, a bZIP-related signaling factor involved in stress-responsive transcription and defense-related signaling [74–77]. *RbohF*, a gene encoding an

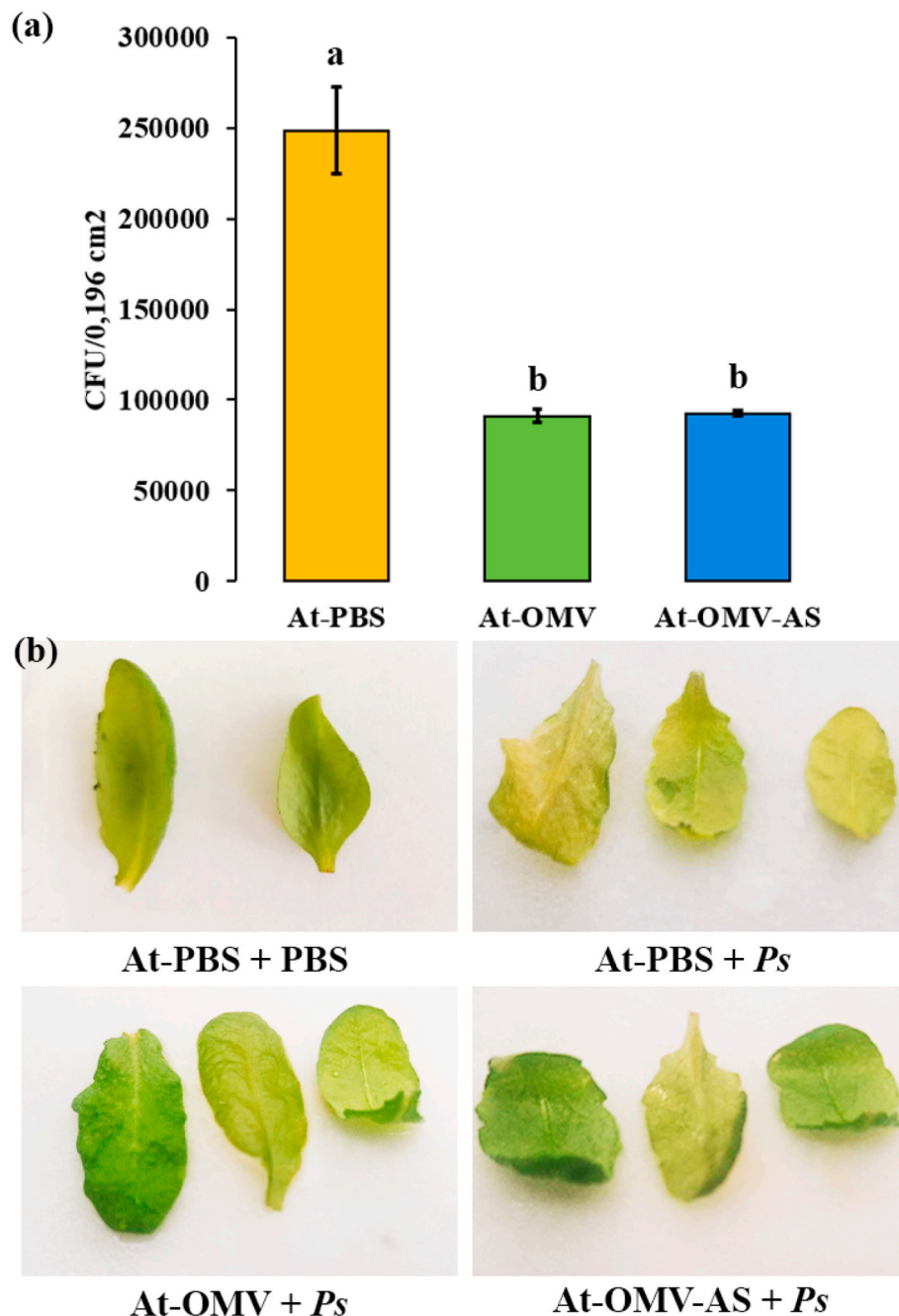


Fig. 4. Effect of *A. rhizogenes* OMV pretreatment on *P. syringae* proliferation and disease symptoms in *A. thaliana* leaves. (a) Quantification of *P. syringae* colony-forming units (CFUs) recovered from *A. thaliana* leaves pre-treated with phosphate-buffered saline (PBS), OMVs, or acetosyringone-treated OMVs (OMV-AS), followed by *P. syringae* challenge. (b) Visual symptoms of infection on leaves treated with PBS, OMVs, or OMV-AS and subsequently challenged with *P. syringae* (+*Ps*). Data are shown as mean $\pm$ SE. Different letters above the bars indicate significant differences according to Fisher's LSD test ($p < 0.05$).

NADPH oxidase associated with reactive oxygen species production and defense signaling [78], was also upregulated. *PAL1* induction is consistent with enhanced phenylpropanoid biosynthesis and stress defense [79]. Transcriptional shifts were accompanied by changes in secondary metabolism. Glucosinolates, characteristic Brassicaceae defense metabolites that accumulate in specialized plant tissues [80,81], were increased in both aromatic and aliphatic fractions. Together, these responses indicate coordinated defense-associated transcriptional and metabolic reprogramming in *A. thaliana*, as summarized in the simplified conceptual model presented in Fig. 5. Although the ecological significance remains unclear, induction of host defenses by OMVs may influence microbial competition, colonization dynamics, or broader host

physiological states.

The persistence of morphological, physiological, metabolic, and transcriptional changes in adult plants after a single OMV exposure of seedlings requires careful interpretation. Because OMVs are non-replicating bacterial structures and no evidence for persistent *A. rhizogenes* contamination was detected at the final time point, the long-term phenotype is unlikely to reflect continuous vesicle production or chronic bacterial exposure. One plausible explanation is that early perception of OMV-associated MAMPs redirected plant development toward a stress- and immunity-associated trajectory. This interpretation is consistent with previous studies showing that bacterial OMVs activate plant immune responses, induce transcriptional reprogramming,

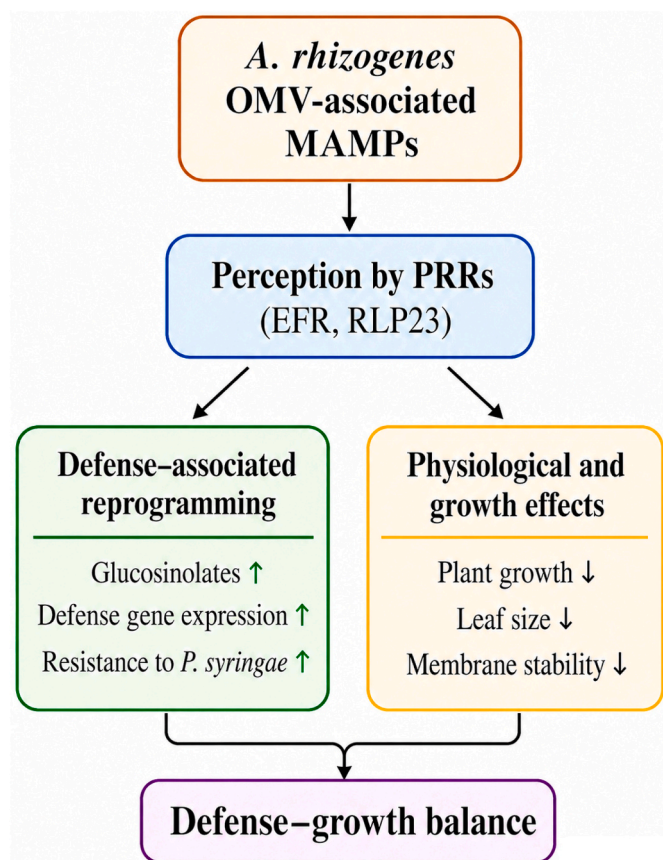


Fig. 5. Simplified model of *Arabidopsis thaliana* responses to *Agrobacterium rhizogenes* OMV-associated MAMPs.

downregulate growth-related processes, and prime *A. thaliana* resistance to subsequent pathogen challenge [17,47,82]. It is also consistent with MAMP-associated growth inhibition and the broader concept of the growth–immunity trade-off [83]. Plant immune priming and stress memory provide an additional framework for such prolonged responses, since transient stimulation can be associated with latent molecular changes, including dormant signaling components, altered primary metabolism, and chromatin modifications [84]. Studies in *A. thaliana* have further shown that transient stress or hormone signaling can produce long-lasting changes in transcriptional responsiveness, epigenomic state, immunity, and specialized metabolism [84–86]. Thus, the long-term phenotype observed here may be best explained by a combination of early OMV-triggered priming-like or stress-memory processes, altered developmental progression, and sustained metabolic reprogramming. It should also be acknowledged that OMV preparations obtained by ultracentrifugation may contain minor co-isolated non-vesicular components; therefore, some contribution from associated material cannot be completely excluded.

Functionally, OMV pretreatment conferred enhanced resistance to *P. syringae* infection, as evidenced by an approximately 3-fold reduction in bacterial proliferation and milder disease symptoms. Similar protection of *A. thaliana* has been reported after treatment with OMVs from *P. syringae*, *P. fluorescens*, and *X. campestris* [15,17]. However, the underlying immune pathways may differ among bacterial species; for example, *P. syringae* OMVs induced isochlorismate synthase, a key enzyme in salicylic acid biosynthesis, whereas *P. fluorescens* OMVs did not [15]. Together, these observations highlight the variability of OMV-mediated immune activation across bacterial species and support the view that OMVs can act as bacterial vesicle-based signals that prime plants for enhanced pathogen resistance.

5. Conclusion

This study provides the characterization of OMVs produced by *A. rhizogenes* and demonstrates their potential relevance in plant–microbe interactions. The isolated OMVs exhibited typical spherical morphology and contained diverse candidate vesicle-associated proteins, including structural components, flagellar proteins, ribosomal proteins, and factors associated with bacterial adaptation and host interaction. Biological assays showed that *A. rhizogenes* OMV preparations influenced the model plant *A. thaliana*. OMV-treated plants showed reduced leaf expansion, decreased leaf fresh weight, and lower membrane stability, together with broad defense- and stress-associated transcriptional responses. These changes were accompanied by metabolic reprogramming, including increased accumulation of glucosinolates. Importantly, pretreatment with OMVs enhanced resistance of *A. thaliana* to *P. syringae*, supporting their role as immune-modulating bacterial structures. Together, these findings expand current understanding of OMV-mediated communication in plant-associated bacteria and identify OMVs as previously underappreciated factors in interactions between *A. rhizogenes* and plants. Future studies should define the specific vesicle components responsible for these effects, clarify OMV uptake and trafficking *in planta*, and determine how environmental conditions shape OMV composition and biological activity. In addition, time-course experiments will be needed to define the duration of OMV-triggered responses and to distinguish between sustained priming, developmental reprogramming, and stress-memory-related effects.

CRedit authorship contribution statement

Maria Sorokina: Writing – original draft, Validation, Investigation, Formal analysis, Data curation, Conceptualization. **Yulia Yugay:** Validation, Project administration, Methodology, Investigation. **Anastasia Mironova:** Validation, Methodology, Investigation. **Dmitry Bulgakov:** Validation, Methodology, Investigation. **Vladimir Silant'ev:** Validation, Methodology, Investigation. **Valeria Grigorchuk:** Validation, Methodology, Investigation. **Olesya Kudinova:** Investigation, Formal analysis. **Yulia Yaroshenko:** Software, Data curation. **Victor Bulgakov:** Supervision, Funding acquisition. **Yury Shkryl:** Writing – review & editing, Validation, Supervision, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.pmpp.2026.103553>.

Data availability

Data will be made available on request.

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