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Application of extracellular vesicle-carried antimicrobial peptide LL37 enhances rice resistance to *Magnaporthe oryzae*

Yanhui Xia¹, Wenjing Zhu¹, Tiangu Liu¹, Yunfei Hu¹ and Qiang Cai^{1*}

Abstract

Extracellular vesicles (EVs) are critically involved in cross-kingdom communication and plant defense mechanisms. However, their potential as delivery vehicles for exogenous antimicrobial agents remains largely unexplored. In this study, we show that EVs are essential for rice immunity against *Magnaporthe oryzae*, with their secretion being significantly induced upon infection. Genetic evidence from *Oryza sativa* *Tetraspanin 7* (*OsTET7*) and *OsTET13* overexpression lines and mutants further confirms the role of EVs in rice resistance to *M. oryzae*. Additionally, we demonstrate that rice-derived EVs are internalized by *M. oryzae* and subsequently suppress the hyphal growth. We successfully loaded the anti-fungal peptide LL37 onto rice EVs, forming stable EV-LL37 complexes, and demonstrated its efficacy in suppressing *M. oryzae*. These complexes displayed improved environmental stability and enhanced adhesion to leaf surfaces under stress conditions, resulting in sustained anti-fungal activity. Our results highlight the dual function of rice EVs as innate immune components and efficient nanocarriers for antimicrobial agents, providing a novel platform for crop protection strategies.

Keywords Rice, Extracellular vesicles, Antimicrobial peptide LL37, *Magnaporthe oryzae*

Background

Rice (*Oryza sativa*) is one of the most important staple crops worldwide, providing a primary food source for over half of the global population (Wang et al. 2018). However, rice blast, a devastating disease caused by the fungus *Magnaporthe oryzae*, poses a serious threat to rice yield and grain quality (Skamnioti et al. 2009). Although the development of resistant cultivars and the application of chemical fungicides have contributed to controlling disease outbreaks to some extent, these traditional strategies are increasingly challenged by the rapid mutation of the pathogen, rising resistance to fungicides, and concerns about environmental contamination

associated with agrochemical overuse (Skamnioti et al. 2009). Consequently, there is a pressing need to develop novel, effective, environmentally friendly, and sustainable approaches for the control of rice blast. This has emerged as both a research hotspot and a technological bottleneck in the field of plant disease management.

In recent years, antimicrobial peptides have garnered increasing interest as promising agents for sustainable agricultural disease management, owing to their broad-spectrum antimicrobial activity, low toxicity, and reduced likelihood of inducing resistance (Lazzaro et al. 2020; Nayab et al. 2022). Among them, LL37, a human-derived antimicrobial peptide and a prototypical member of the cathelicidin family, has been widely investigated for its strong antimicrobial effects (Sheehan et al. 2018; Keshri et al. 2025). LL37 acts by disrupting bacterial membranes, inhibiting fungal growth, and interfering with viral replication, underscoring its potential as a broad-spectrum

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therapeutic against diverse infectious agents (Keshri et al. 2025). Previous studies have shown that LL37 effectively suppresses the growth of several clinically important human fungal pathogens, including *Candida auris*, *Candida tropicalis*, *Candida krusei*, and *Candida albicans*, all common opportunistic fungi capable of causing candidiasis (Mori et al. 2021; Pinilla et al. 2022; Rather et al. 2022). However, the direct application of LL37 for plant pathogen control remains challenging, mainly due to its susceptibility to proteolytic degradation, poor environmental stability, and low delivery efficiency.

Extracellular vesicles (EVs) are nanoscale, membrane-bound structures actively released by cells and are ubiquitous in both animal and plant systems. They play crucial roles in intercellular communication and immune regulation (Cai et al. 2018, 2021; Wang et al. 2023; Huang et al. 2025; Wang et al. 2025a, b). Recent studies indicate that pathogen infection significantly upregulates EV secretion in plants, facilitating the trans-kingdom delivery of bioactive molecules, including small RNAs, mRNAs, and defense-related proteins, into pathogens, thereby conferring disease resistance (Cai et al. 2018; Wang et al. 2024; Huang et al. 2025). In rice, EVs have been shown to transport defense proteins to *Rhizoctonia solani*, effectively suppressing fungal infection (Huang et al. 2025). However, it remains unclear whether rice EVs play a functional role in resistance against the rice blast fungus, *M. oryzae*.

In biomedical research, EVs have been extensively exploited as efficient carriers for RNAs, proteins, and therapeutic compounds owing to their high biocompatibility, structural stability, and innate targeting capabilities, which collectively improve cargo stability and bioavailability (Kamerkar et al. 2017; Van et al. 2018; Kalluri et al. 2020). While animal-derived EVs are already applied in targeted therapies and diagnostics, the use of plant EVs for delivering exogenous bioactive agents remains largely unexplored. Although artificial liposomes have been successfully used to encapsulate dsRNAs (Qiao et al. 2023), no studies have yet reported the use of plant EVs to deliver antifungal peptides into fungal cells to suppress infection and protect crops. Given the demonstrated capacity of rice EVs to transport defense proteins into fungal cells, they represent a promising delivery platform for antifungal peptides that could help control pathogen infection.

In this study, we show that EVs are essential components of rice defense against *M. oryzae*, and that EVs isolated from *M. oryzae*-infected rice leaves can be internalized by the fungus and subsequently inhibit the fungal hyphal growth. Furthermore, we successfully loaded the anti-fungal peptide LL37, which exhibits strong efficacy against *M. oryzae*, onto rice EVs, forming stable EV-LL37

complexes. These complexes demonstrated enhanced environmental resilience and improved adhesion to leaf surfaces under stress conditions, resulting in sustained anti-fungal activity. Our findings highlight the dual role of plant EVs as efficient nanocarriers for antimicrobial agents, providing an innovative platform for sustainable crop disease management.

Results

Rice leaf secretes EVs in response to *M. oryzae* infection

In this study, we established an optimized and efficient method for isolating EVs from *M. oryzae*-infected rice leaves by improving upon previously reported protocols for rice leaf sheath EVs isolation (Huang et al. 2025) (Additional file 1: Figure S1). Specifically, we used *M. oryzae*-infected leaves and centrifuged at 100,000×g to collect the P100 EV fraction (Additional file 1: Figure S1). To further purify the EVs, the previously isolated P100 EVs were subjected to iodixanol density gradient centrifugation (Huang et al. 2023; Huang et al. 2025). The gradient was fractionated into six fractions (F1–F6) from the top to the bottom. Transmission electron microscopy (TEM) analysis revealed that rice EVs were predominantly distributed between fractions F2 and F4, with fraction F3 exhibiting the highest abundance (Fig. 1a). This distribution pattern is consistent with the density profile of EVs isolated from *Arabidopsis* leaves (Huang et al. 2023). To further validate the EVs distribution, all fractions were analyzed by Western blot to detect the rice EVs marker protein OsTET7 (Huang et al. 2025). The results corresponded well with TEM observations, showing the highest OsTET7 protein levels in fraction F3 (Fig. 1b). Subsequently, nanoparticle flow cytometry was employed to analyze the size distribution of EVs in fraction F3, indicating that the majority of vesicles had a diameter of approximately 80 nm, with a minor population reaching up to 180 nm (Fig. 1c).

To investigate whether *M. oryzae* infection promotes EVs secretion, EVs were isolated from equivalent amounts of infected and non-infected rice leaves and observed by TEM following uranyl acetate negative staining. The results demonstrated a markedly increased EV level isolated from *M. oryzae*-infected leaves compared with mock-inoculated controls (Fig. 1d). Quantitative analysis using nanoparticle flow cytometry further confirmed a significant elevation in EVs concentration in the infected group relative to the control (Fig. 1e). Additionally, EV membranes were stained with the cationic fluorescent dye FM4-64, corroborating the trend observed in TEM imaging (Additional file 2: Figure S2). Western blot analysis of equal amounts of EVs isolated from leaf tissues showed significantly higher OsTET7 protein levels in the infected group compared with the control (Fig. 1f).

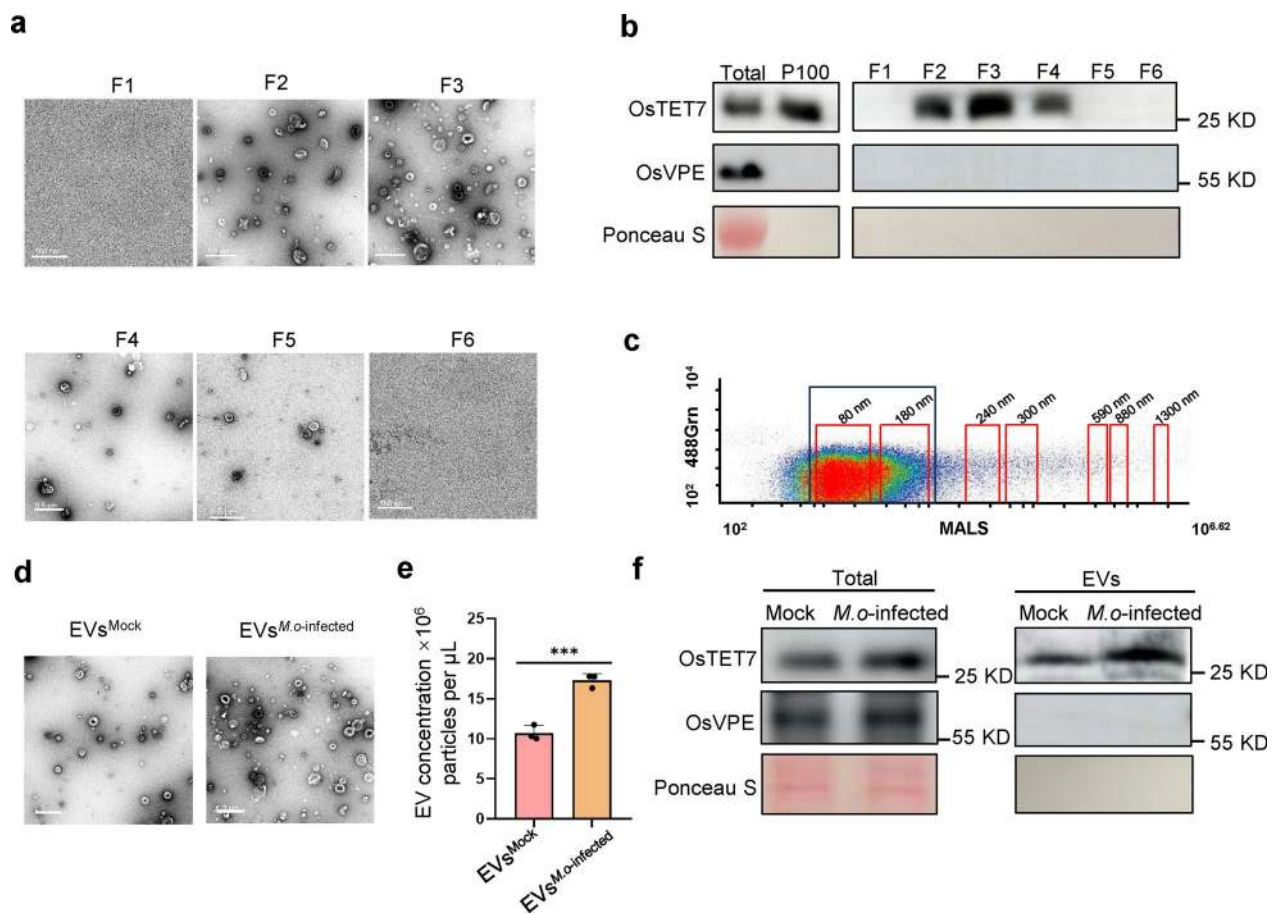


Fig. 1 *M. oryzae* infection promotes the secretion of EVs derived from rice leaves. **a** Transmission electron microscopy (TEM) analysis showing the distribution of rice EVs across different density gradient fractions. EVs were isolated from uninfected rice leaves. Scale bar = 0.5 μm. **b** Western blot detection of the rice EV marker protein OsTET7 predominantly enriched in the iodixanol density gradient fractions F2–F4. **c** Particle size distribution of rice EVs in the iodixanol density gradient fractions F3. Particle size was measured by nanoscale flow cytometry (NFC). Concentration measurement of rice EVs by nanoparticle flow cytometry (nFCM). **d** TEM image of purified EVs. EVs were isolated from equal amounts of uninfected or *M. oryzae*-infected rice leaves. Scale bar = 0.5 μm. **e** The number of EV particles was detected by nano-flow cytometry (FCM), the data are presented as the mean ± standard deviation from three independent measurements. The significant difference was determined using a two-tailed Student's *t*-test: ***, *P* < 0.001. **f** *M. oryzae* infection promotes the secretion of OsTET7-GFP-positive EVs. In **b** and **f**, OsTET7 was a positive EV marker. OsVPE was used as a negative EV marker protein. Ponceau S staining was used for Rubisco protein detection. KD indicates protein molecular weight

Collectively, these findings indicate that the infection induces EV secretion in rice leaves, suggesting a potentially important role for EVs in rice defense responses to *M. oryzae*.

Rice leaf-derived EVs confer resistance against rice blast disease

Tetraspanin (TET) family proteins are well-established key regulators of EV secretion in plants. In *Arabidopsis*, the *tet8/tet9* double mutant exhibits reduced EV secretion, impaired sRNA and mRNA transport to fungal pathogens, and significantly compromised resistance to *Botrytis cinerea* (Cai, et al. 2018; Wang et al. 2024). Similarly, in rice, the *ostet7/ostet13* double mutant

shows decreased EV secretion and reduced accumulation of host defense proteins, leading to enhanced susceptibility to *R. solani* (Huang et al. 2025). To investigate whether OsTET7 and OsTET13 are involved in rice resistance to *M. oryzae*, we evaluated the *ostet7/ostet13* double mutant's response to rice blast infection. The mutant exhibited significantly increased susceptibility to *M. oryzae* (Fig. 2a, b). Consistent with our previous findings for sheath blight disease (Huang et al. 2025), the *ostet13* single mutant also showed increased susceptibility to *M. oryzae* (Additional file 3: Figure S3). In contrast, transgenic lines overexpressing *OsTET7-GFP* and *OsTET13-GFP* showed markedly reduced disease susceptibility compared with the wild type (Fig. 2a, b,

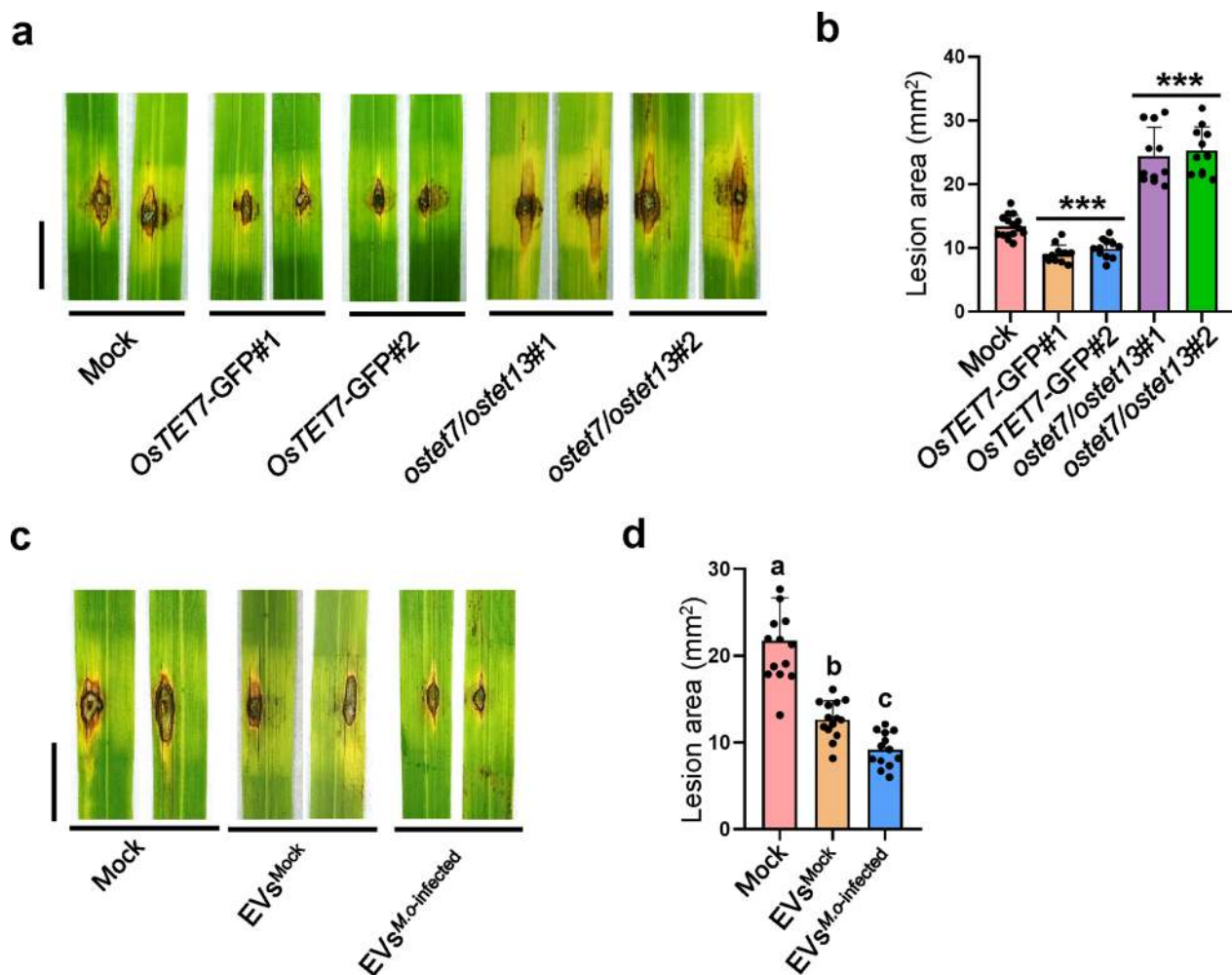


Fig. 2 Rice leaf-derived EVs enhanced rice resistance against *M. oryzae*. **a** Disease susceptibility of the *ostet7/ostet13* double mutant rice and enhanced resistance in *OsTET7-GFP* rice lines against *M. oryzae* infection. Scale bar = 1 cm. **b** Quantification of relative lesion areas corresponding to panel **a**. The significant difference was determined using a two-tailed Student's *t*-test: *** $P < 0.001$. **c** Exogenous application of EVs significantly suppresses the virulence of *M. oryzae*. Rice leaves were sprayed with equal amounts of EVs isolated from mock-treated plants (EVs^{Mock}, 5.0×10^8 particles/mL) or *M. oryzae*-infected plants (EVs^{M.o-infected}, 5.0×10^8 particles/mL). Mock denotes leaves treated with buffer only, without rice-derived EVs. Scale bar = 1 cm. **d** Quantification of relative lesion areas corresponding to panel **c**, different letters indicate significant differences among means as determined by one-way ANOVA followed by Tukey's test ($P < 0.05$). Individual data points are shown as black dots

Additional file 3: Figure S3). These findings indicate that OsTET7 and OsTET13 both contribute positively to rice blast resistance.

To further validate the role of rice leaf-derived EVs in resistance against *M. oryzae*, equal amounts of EVs isolated from healthy plants (EVs^{Mock}) and *M. oryzae*-infected plants (EVs^{M.o-infected}) were sprayed onto rice leaves prior to pathogen inoculation. The results showed that both EVs^{Mock} and EVs^{M.o-infected} treatments significantly reduced lesion area, with EVs^{M.o-infected} providing stronger protection compared to those from healthy plants (Fig. 2c, d). These findings indicate that rice leaf-derived EVs confer protective effects during pathogen

infection and that defense-related cargo molecules are enriched in EVs during plant responses to infection.

Rice leaf-derived EVs are internalized by *M. oryzae* and disrupt its hyphal growth

Previous studies have demonstrated that plant EVs can be rapidly taken up by fungal cells. For instance, *Arabidopsis*-derived TET8-positive EVs are internalized by *B. cinerea*, and rice sheath-derived OsTET7-positive EVs are taken up by *R. solani* cells (Cai et al. 2018; Wang et al. 2024; Huang, et al. 2025). To investigate whether rice leaf-derived EVs can also be internalized by the rice blast fungus, *M. oryzae*, GFP-labeled EVs isolated from

OsTET7-GFP transgenic rice were co-incubated with fungal hyphae for 4 h. Confocal microscopy detected GFP fluorescence inside the fungal cells, which remained even after washing with 1% Triton X-100, indicating that the EVs had been internalized rather than merely attached to the hyphal surface (Fig. 3a). Furthermore, to examine the potential effects of rice EV uptake on fungal cells, equal amounts of EVs^{Mock} and EVs^{*M.o*-infected} were incubated with the fungus. Microscopic observation revealed various hyphal abnormalities, including twisting, swelling, and irregular branching, with the EVs^{*M.o*-infected} treatment causing the most severe morphological distortions (Fig. 3b). These results suggest that rice leaf-derived EVs may disrupt normal hyphal development in *M. oryzae*, potentially contributing to reduced virulence.

LL37 inhibits *M. oryzae* conidia germination and appressorium formation

To employ rice leaf-derived EVs as delivery vehicles for antimicrobial peptides to suppress *M. oryzae* infection, we first synthesized and evaluated the antifungal activity of the antimicrobial peptide LL37 (Additional file 4: Figure S4). The results showed that LL37 effectively inhibited conidial germination and appressorium formation in *M. oryzae* (Fig. 4a). Specifically, the minimum concentration of LL37 that completely blocked conidial germination was 60 μ M, while a lower concentration of 30 μ M significantly reduced appressorium formation (Fig. 4a). To further evaluate the biosafety of LL37 in plants, rice protoplasts were treated with a high concentration of 100 mM LL37. No morphological damage or loss of cellular integrity was observed, indicating that LL37 has minimal phytotoxicity toward rice host cells (Additional file 5: Figure S5).

To elucidate the mode of action of LL37, we prepared its Fluorescein Isothiocyanate (FITC)-labeled derivative (LL37^{FITC}) and incubated it with *M. oryzae* conidia. The results demonstrated that LL37^{FITC} effectively inhibited both conidial germination and appressorium formation, indicating that the fluorescent labeling did not alter its antifungal activity (Additional file 6: Figure S6). Fluorescence microscopy observations revealed that, as early as 10 min post-incubation, strong green fluorescence from LL37^{FITC} was detected on the surfaces of germ tubes and appressoria. This signal co-localized with the membrane-specific dye FM4-64 (red signal) (Fig. 4b), consistent with LL37 targeting the fungal cell membrane.

Given that appressorium formation depends on the effective translocation of energy reserves such as glycogen and lipids (Wang et al. 2005), we further compared glycogen distribution during appressorium development between LL37-treated and control groups. Initial staining showed glycogen presence in conidia for both groups;

however, at 12 h, glycogen was fully translocated into appressoria in controls, whereas in the LL37 treatment group, glycogen remained within the conidia, indicating impaired translocation (Fig. 4c). These results suggest that LL37 disrupts glycogen transport, thereby hindering appressorium maturation and formation, ultimately suppressing the virulence of *M. oryzae*.

LL37 inhibits *M. oryzae* virulence

To further elucidate the role of LL37 in rice disease protection, in vitro assays were performed by exogenously applying the synthetic LL37 peptide to rice leaves. The results demonstrated that treatment with 10 μ M LL37 significantly reduced *M. oryzae* infectivity, while a higher concentration of 60 μ M LL37 resulted in even stronger inhibitory effects (Fig. 5a, b). To substantiate the inhibitory function of LL37 against rice blast, we generated transgenic rice lines (*LL37-flag*) constitutively expressing LL37. In these lines, LL37 was fused with an N-terminal signal peptide (SP) to enable apoplastic secretion and a C-terminal Flag tag for immunodetection (Additional file 7: Figure S7). The resistance of these lines to *M. oryzae* was subsequently evaluated. Consistent with the exogenous application assays, the lesion areas on leaves of *LL37-flag* plants were significantly smaller than those on wild-type controls, confirming that LL37 expression enhances rice resistance against blast disease (Fig. 5c, d). In addition, compared with the wild-type Nipponbare (*Nip*), these *LL37-flag* transgenic plants did not display noticeable differences in plant morphology or growth vigor, indicating that expression of the LL37 fragment does not adversely affect rice growth and development (Additional file 8: Figure S8).

Next, we investigated whether rice leaf-derived EVs could serve as carriers for LL37. Following co-incubation of rice EVs with FITC-labeled LL37 (LL37^{FITC}), confocal microscopy analysis revealed complete overlap between the fluorescence signals of the EV membrane dye FM4-64 and FITC, indicating efficient loading of LL37-FITC onto the EVs (Fig. 5e). Given that the loading was achieved simply by mixing EVs with the peptide, LL37 is likely adsorbed onto the surface of EVs, potentially through physical interactions such as electrostatic binding. To further explore the loading efficiency of LL37 onto the EV surface, a fixed amount of LL37 (0.1 mg) was incubated with increasing volumes of rice EV suspension (at a concentration of 5.0×10^8 particles/mL). Protein gel staining demonstrated that 0.1 mg LL37 was fully loaded when 480 μ L of the EV suspension (5.0×10^8 particles/mL) was used, establishing this ratio as the maximum for stable complex formation. This corresponds to a loading capacity of approximately 0.1 mg LL37 per 2.4×10^8 particles (Fig. 5f).

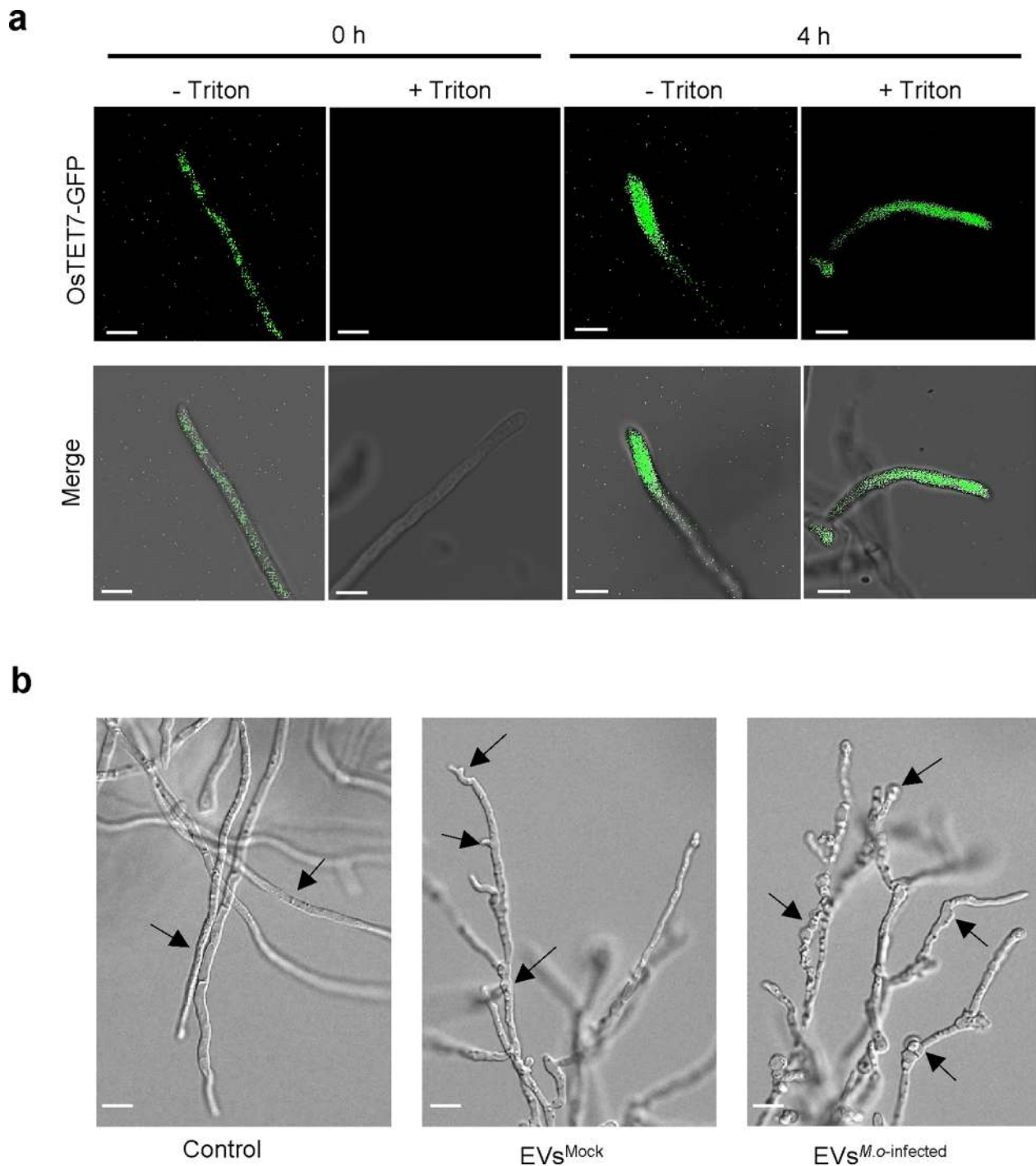


Fig. 3 Rice leaf-derived EVs are internalized by *M. oryzae* and disrupt hyphal growth. **a** *M. oryzae* hyphae internalize EVs isolated from OsTET7-GFP transgenic rice plants. Triton X-100 wash was used to remove EVs outside of the *M. oryzae* hyphae. Scale bar = 10 μ m. **b** Laser confocal scanning microscopy bright-field images showing the morphological characteristics of *M. oryzae* hyphae treated with EVs for 16 h. Black arrows indicate hyphal tips, characterized by twisting, accompanied by a marked increase in hyphal branching. Equal amounts of EVs isolated from untreated plants (EVs^{Mock}, 5.0×10^8 particles/mL) or *M. oryzae*-infected plants (EVs^{M.o.-infected}, 5.0×10^8 particles/mL). Control denotes leaves treated with buffer only, without rice-derived EVs. Scale bar = 10 μ m

EV-mediated loading of LL37 enhances its stability and disease resistance efficacy in rice

Although direct foliar application of anti-fungal peptides can suppress pathogen virulence, the intrinsic instability of peptides under environmental conditions limits their efficacy in open-field agriculture. Enhancing their environmental stability is therefore critical for their practical application. In this study, we first evaluated the retention of EVs-loaded LL37^{FTIC} on rice leaf surfaces under simulated rainfall conditions. Confocal laser scanning microscopy revealed that LL37^{FTIC}-EV complex remained clearly detectable on the leaf surface after rinsing, whereas the fluorescence signal of free LL37^{FTIC} was largely eliminated (Fig. 6a). These results suggest that rice-derived EVs enhance the adhesion of LL37 to leaf surfaces.

To further investigate environmental stability, we compared the disease resistance activity of EVs-loaded versus free LL37 following exposure to UV light or elevated temperature (50 °C) for 5 h. Pathogen inoculation assays demonstrated that free LL37 lost most of its anti-fungal activity after treatment, while EV-LL37 complexes retained substantial capability to suppress *M. oryzae* infection (Fig. 6b, c). We also performed experiments to assess the integrity of OsTET7-positive EVs after UV and heat treatments. Specifically, isolated rice EVs were subjected to UV irradiation or 50 °C heat treatment for 5 h, and then examined by TEM. Our results show that UV-treated and high temperature treated EVs remained largely intact, indicating minimal disruption from both treatment (Additional file 9: Figure S9). Furthermore, under prolonged storage at room temperature, EV-LL37 maintained significantly higher protective activity than free LL37. Notably, EV-LL37 still conferred measurable protection after 7 d, while the activity of free LL37 declined rapidly over the same period (Fig. 6d, e). These findings collectively support the advantage of rice EVs as a delivery system that enhances the stability and durability of LL37. Collectively, these findings indicate that EVs loading not only improves the stability of LL37 on plant leaf surfaces but also maintains its bioactivity under adverse environmental conditions. This EV-based delivery strategy provides a promising avenue for enhancing

the field applicability of anti-fungal peptides in sustainable crop protection.

Discussion

In recent years, EVs have emerged as key mediators of intercellular communication in plant-pathogen interactions. Plant-derived EVs can transport bioactive molecules—including proteins, sRNAs, and mRNAs—across kingdoms to suppress pathogen virulence or growth (Cai et al. 2023; Hamby, et al. 2025; Huang, et al. 2025). Using the rice-*M. oryzae* pathosystem, we established an efficient method for isolating EVs from rice leaves and demonstrated that infection markedly enhances EV secretion. Notably, we show that rice EVs can load the antimicrobial peptide LL37, significantly improving its stability and anti-fungal efficacy.

Under *R. solani* infection, rice EVs selectively enrich defense-related proteins and deliver them into fungal cells, attenuating disease progression (Huang, et al. 2025). Proteomic analyses of rice EVs have also revealed secreted peptides of fewer than 100 amino acids (Huang, et al. 2025), though their functions remain largely unexplored. This finding provides a valuable lead and a potential entry point for future investigations into defense peptides that may be delivered across kingdoms via EVs. From an applied perspective, encapsulating antimicrobial peptides within plant EVs is expected not only to enhance their field stability but also to mitigate environmental inactivation and potential phytotoxicity.

Rice EVs exhibit a stable adherence to plant leaf surfaces, which is likely attributable to their unique surface-adhesive profile. Existing proteomic analyses of plant EVs suggest that rice EVs carry surface glycoproteins with adhesive or cell wall-binding capacities, such as lectin-like proteins, arabinogalactan proteins (AGPs), remorins, and other GPI-anchored proteins (Rutter et al., 2017; De Coninck et al., 2022; Ma et al., 2023; Huang et al. 2025). These molecules can specifically interact with cuticular or cell wall polysaccharides, offering a plausible molecular explanation for the sustained attachment of EVs to rice leaf surfaces. Future studies combining detailed characterization of EV surface proteomes with binding-disruption assays, such as treatment with proteases or

(See figure on next page.)

Fig. 4 Antimicrobial peptide LL37 adsorbs onto the surface of *M. oryzae* conidia and inhibits their germination and appressorium formation. **a** Anti-fungal activity assay of LL37 against the germination and appressorium formation of *M. oryzae* conidia. Scale bar = 10 µm. **b** Enrichment of LL37^{FTIC} at the germ tube and appressorium plasma membrane regions of *M. oryzae* conidia. Conidia were pre-cultured in PDB medium at 28 °C for 6 h, followed by incubation with LL37^{FTIC} and the membrane dye FM4-64 for 10 min. Fluorescence images were acquired using confocal microscopy. Mock indicates conidia treated with buffer only. Scale bar = 10 µm. **c** Observation of glycogen translocation during appressorium formation of *M. oryzae* conidia co-incubated with antimicrobial peptide LL37. Mock refers to conidia cultured with buffer alone. Black arrows indicate *M. oryzae* conidia and appressoria. Scale bar = 10 µm

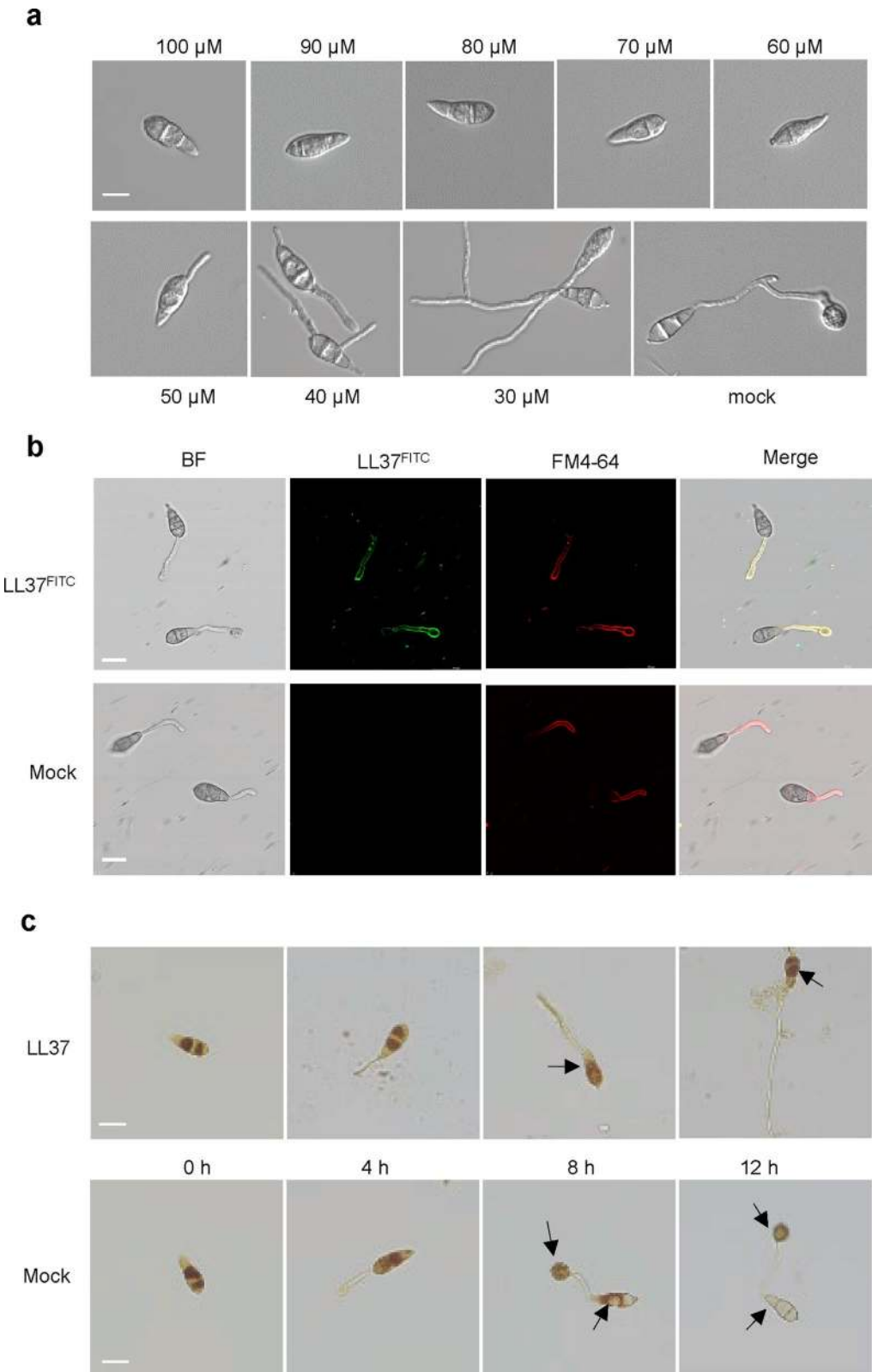


Fig. 4 (See legend on previous page.)

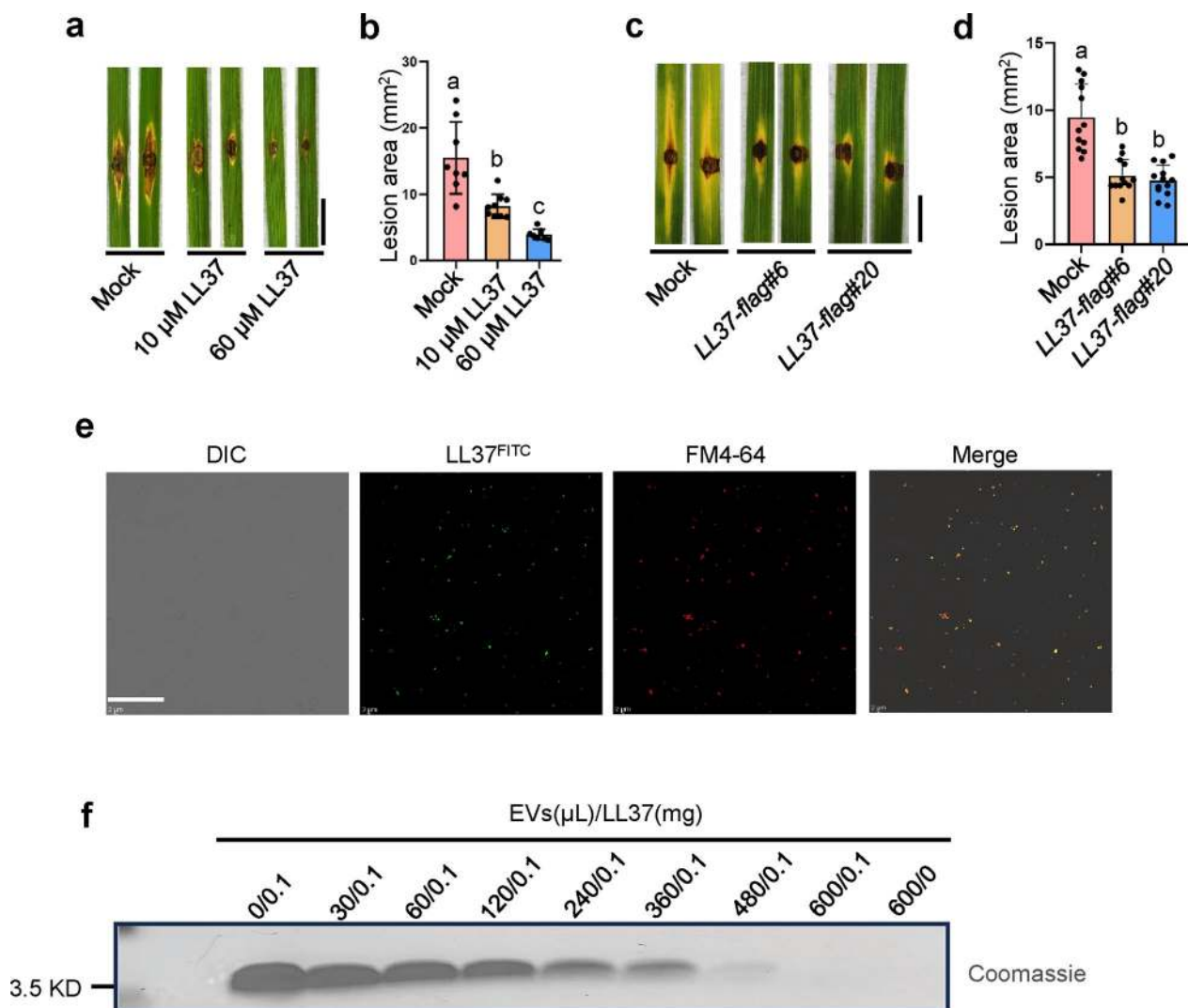


Fig. 5 LL37 significantly inhibits the virulence of *M. oryzae* and rice EVs serve as carriers for the antimicrobial peptide LL37. **a** Exogenous LL37 treatment suppresses the virulence of *M. oryzae* on rice leaves. Scale bar = 1 cm. **b** Quantification of relative lesion area corresponding to panel **a**. Individual data points are represented by small black dots. **c** LL37-flag heterologous expressing rice lines exhibit enhanced resistance against *M. oryzae*, compared with wild-type plants. Scale bar = 1 cm. **d** Quantification of relative lesion area from panel **c**. Individual data points are shown as small black dots. **e** Co-localization fluorescence images of rice EVs (5.0×10^8 particles/mL) stained with FM4-64 (red) and LL37 (10 μ g/mL) antimicrobial peptide labeled with FITC (green). Scale bar = 10 μ m. **f** Protein gel staining showing rice EVs (5.0×10^8 particles/mL) loaded with the antimicrobial peptide LL37 (0.1 mg). In **b**, **d** different letters indicate significant differences among means as determined by one-way ANOVA followed by Tukey's test ($P < 0.05$)

deglycosylation enzymes, are needed to elucidate its biological implications.

Evidence suggests that various fungi can internalize exogenous macromolecules or nanoparticles through canonical endocytic pathways, including clathrin-mediated endocytosis (CME). For instance, *R. solani* EVs can deliver effectors into plant cells via host CME (Wang, et al. 2025b); *B. cinerea* employs EVs to transport Bc-sRNAs, which are subsequently internalized by plant cells through CME (He, et al. 2023). Moreover, *M.*

oryzae has been shown to exploit host CME to translocate its effectors into rice cells (Oliveira, et al. 2023). Therefore, it is reasonable to hypothesize that rice EVs carrying antimicrobial molecules may be recognized by CME and internalized into *M. oryzae* cells. Although our results indicate that EV-mediated LL37 uptake may impair fungal growth and virulence, direct genetic evidence from the pathogen is still lacking. To elucidate the functional significance of this delivery pathway, it will be necessary to construct *M. oryzae* strains

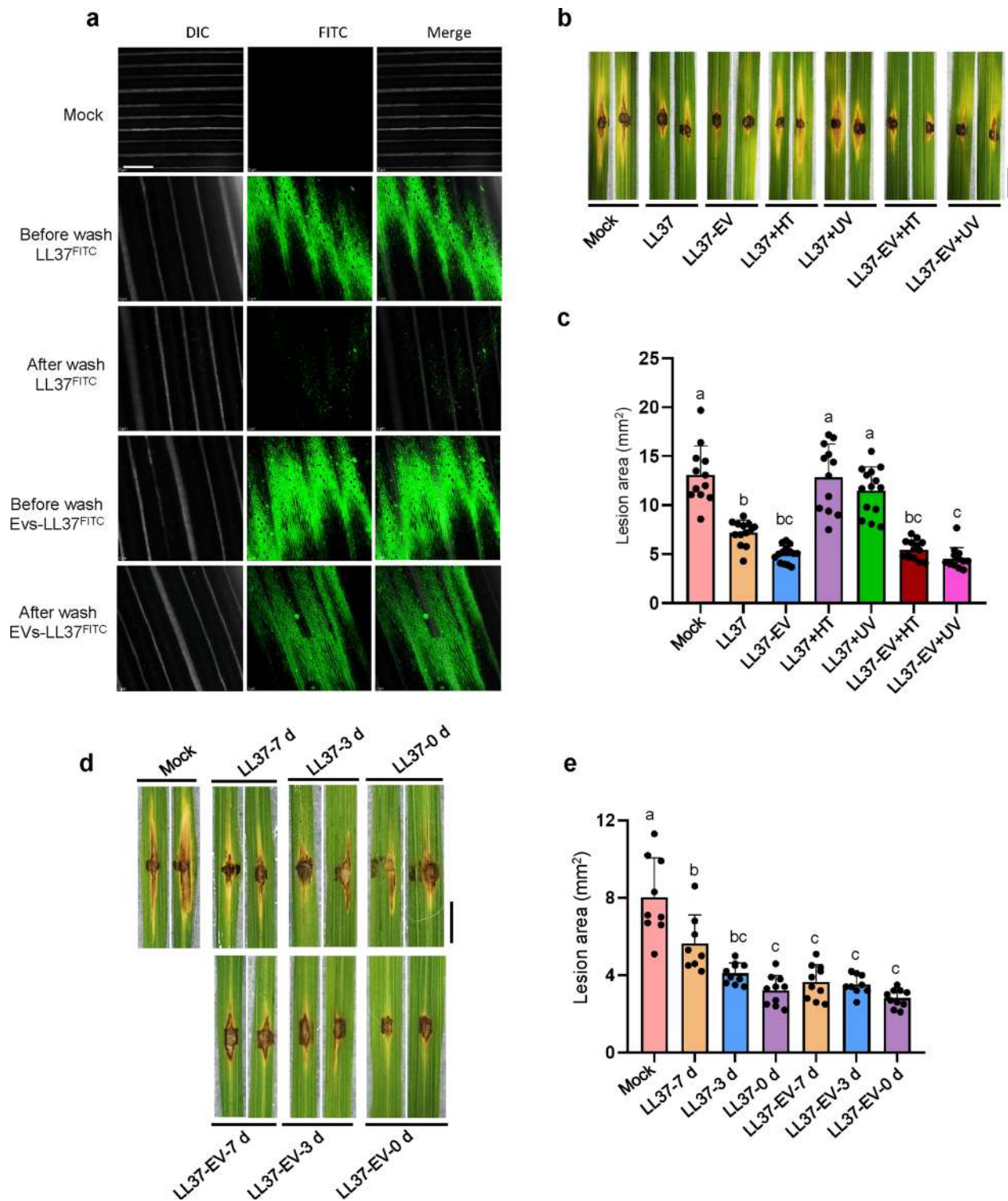


Fig. 6 Rice EVs loading significantly enhances LL37's adhesion and stability. **a** Fluorescence images of EVs-LL37^{FITC} complexes on rice leaf surfaces before and after simulated water wash; Scale bar = 20 μ m. Fluorescence images of LL37^{FITC} complexes on rice leaf surfaces before and after simulated rain wash; Scale bar = 20 μ m. **b** Assessment of anti-fungal efficacy against rice blast fungus of EVs-LL37 complexes and free LL37 following ultraviolet and 50 °C heat treatments; Scale bar = 0.5 cm. **c** Quantitative analysis of relative lesion areas corresponding to **b**. Individual data points are shown as black dots. **d** Assessment of anti-fungal efficacy against rice blast fungus of the long-term stability of LL37 and EV-encapsulated LL37; Scale bar = 0.5 cm. **e** Quantitative analysis of relative lesion areas corresponding to **d**. Individual data points are shown as black dots. In **c** and **e**, different letters indicate significant differences among means as determined by one-way ANOVA followed by Tukey's test ($P < 0.05$)

heterologous expressing LL37 and CME-deficient mutants in the later stage.

Previous studies have demonstrated that EVs released by plant-pathogenic microbes can serve as effective immunogenic stimuli. For example, *Pseudomonas syringae* pv. tomato DC3000-derived EVs carrying the catalase KatB are recognized by host cells and subsequently activate hallmark pattern-triggered immunity (PTI) responses (Deng et al. 2025). These findings indicate that plants are capable of perceiving EV-associated molecular components and initiating immune signaling. Although our study focuses on rice-derived EVs, such evidence raises the possibility that exogenously applied plant EVs, particularly those enriched with bioactive peptides or proteins, could similarly be recognized by the rice innate immune system. Thus, in addition to functioning as nanocarriers for antimicrobial peptides, rice EVs may also contribute to plant defense by modulating immunity.

In animal studies, EVs derived from native or engineered sources have been effectively employed as carriers for small-molecule antimicrobial compounds. For instance, macrophage-derived EVs loaded with linezolid have been shown to enhance therapeutic efficacy against drug-resistant bacterial infections (Yang et al. 2018). Similarly, EVs can be loaded with fluoroquinolones or surface-modified to carry cationic antimicrobial peptides. These strategies not only improve drug accumulation and bactericidal efficiency within target cells but also reduce host toxicity and enhance biocompatibility, underscoring the potential of EVs as advanced drug delivery platforms (Heinrich et al. 2023; Ibrahim et al. 2024). In contrast to their therapeutic use in animals, plant-derived EVs are increasingly recognized as key mediators of innate immunity (Cai et al. 2023; Hamby et al. 2025). Though practical applications are still emerging, plant EVs offer promising potential to enhance cargo stability. Free antimicrobial peptides, despite their broad-spectrum activity, are prone to degradation by proteases, UV light, and rain washout, limiting their stability under natural conditions (Mahlpuu et al. 2016). This study shows EV encapsulation can protect these peptides from degradation and facilitate efficient cross-kingdom delivery into pathogens, thereby improving their persistence and efficacy. Future optimization of EV loading and release mechanisms through nanotechnology may enable sustainable and efficient strategies for crop protection.

Conclusion

In conclusion, this study provides the evidence that rice EVs can be harnessed as natural nanocarriers for the delivery of exogenous antimicrobial peptides. We demonstrate that EV secretion is significantly induced by *M. oryzae* infection, and through genetic manipulation of EV

biogenesis, illustrated by overexpression lines of *OsTET7* and *OsTET13* and corresponding double mutants, we have established a crucial role for EVs in rice resistance to blast disease. Importantly, these rice-derived EVs are actively internalized by *M. oryzae*, supporting their involvement in cross-kingdom communication. Furthermore, EVs loaded with the antifungal peptide LL37 form stable EV/LL37 complexes that exhibit enhanced environmental stability, improved adhesion to leaf surfaces, and sustained antifungal activity under adverse conditions. Collectively, our findings uncover a dual function of rice EVs as intrinsic immune regulators and efficient delivery vehicles, which establishing an eco-friendly, promising strategy for sustainable crop disease management.

Methods

Plant materials and growth conditions

Rice varieties used in this study included Zhonghua 11 (ZH11, *japonica*) and Nipponbare (Nip, *japonica*). The *ostet13* and *ostet7/ostet13* mutants (ZH11, *japonica*) (Huang et al. 2025), *OsTET7-GFP* and *OsTET13-GFP* transgenic lines (ZH11, *japonica*) were described previously (Huang et al. 2025). The *pCAMBIA1301* plant expression vector was constructed by inserting the *SP-LL37* synthetic gene in the *NcoI* and *SpeI* enzyme sites (Lee et al. 2017). The whole sequence of *LL37* is shown in Additional file 7: Figure S7a. *SP-LL37* was controlled by CaMV 35S promoter, followed by 3×Flag tags. The construct was transformed into rice by *Agrobacterium* mediated transformation. All rice plants were cultivated in a growth chamber maintained at 28°C with 80% relative humidity under a 12-h light/12-h dark photoperiod. The fungal strain used was *M. oryzae* P131, cultured on potato dextrose agar (PDA) plates at 28°C.

Peptide synthesis and treatment

The antimicrobial peptides LL37 and N-terminally FITC (fluorescein isothiocyanate)-labeled LL37 were chemically synthesized by GL Biochem (Shanghai) Ltd. The peptides were dissolved in distilled water to prepare working solutions prior to use.

Isolation and purification of EVs from rice leaves

EVs were isolated from apoplastic washing fluids of rice leaves using a differential ultracentrifugation protocol with minor modifications (Huang et al. 2025; Wang et al. 2025a, b). Prior to EV isolation, rice leaves were inoculated with a conidial suspension of *M. oryzae* (1×10^5 spores/mL) and incubated for 48 h. Approximately 300 three-week-old rice plants were required per experiment to obtain sufficient apoplastic washing fluids. After rinsing the leaves three times with sterile water, they were

vacuum-infiltrated with buffer (0.1 M Tris-HCl, pH 7.5, 0.2 M KCl) using a vacuum pump for 2 h. The infiltrated leaves were placed inside 1 mL syringes and inserted into 50-mL centrifuge tubes. Apoplastic washing fluids were collected by centrifugation (Eppendorf 5910 Ri) at 2000 *g* for 10 min at 4 °C and subsequently filtered through a 0.45- μ m membrane. The sample was centrifuged (Eppendorf 5910 Ri) at 10,000 *g* for 60 min at 4 °C to remove larger vesicles. The supernatant was then subjected to a second centrifugation (Hitachi CP80NX) 100,000 *g* for 2 h at 4 °C. The resulting pellet, corresponding to the P100 EV fraction, was resuspended in PBS and either used immediately or stored at – 80 °C for short-term use.

For EV purification, a discontinuous iodixanol density gradient was prepared using OptiPrep™. Iodixanol solutions of 10%, 20%, and 30% (v/v) were layered sequentially into ultracentrifuge tubes (4.8 mL of 30%, 2.1 mL of 20%, and 2 mL of 10% from bottom to top). Then, 400 μ L of P100 EVs were loaded on top of the gradient and ultracentrifuged (Hitachi CP80NX) at 100,000 $\times g$ for 12 h at 4 °C. After centrifugation, the top 400 μ L was discarded, and six sequential fractions (1.4 mL each) were collected from top to bottom. Each fraction was diluted to 10 mL in PBS, followed by ultracentrifugation (Hitachi CP80NX) at 100,000 $\times g$ for 1 h at 4 °C. The resulting pellets were resuspended in PBS to obtain purified EVs.

Characterization of rice EVs

EV particle concentrations were determined using nano-flow cytometry (nFCM) (Deng et al. 2025). The instrument was calibrated using a mixture of polystyrene and silica reference beads with diameters of 80, 180, 300, 522, 590, 880, and 1300 nm, respectively. EV samples were diluted 50-fold in PBS (phosphate-buffered saline) and analyzed at a flow rate of 1.5 mL/min for at least 30 s. Morphological features of EVs were observed using transmission electron microscopy (TEM; JEM-1400plus, JEOL, Tokyo, Japan) and confocal laser scanning microscopy (TCS SP5, Leica, Wetzlar, Germany) as described previously (Huang et al. 2023).

Assessment of *M. oryzae* uptake and growth modulation by rice EVs

Purified *OsTET7-GFP*-labeled EVs were co-incubated with freshly harvested *M. oryzae* hyphae at room temperature for 4 h. To remove non-specifically bound EVs, fungal cells were treated with 1% Triton X-100 for 15 min. Equal amounts of EVs derived from *M. oryzae*-infected and non-infected rice plants were then co-incubated with fungal hyphae for 16 h. All samples were visualized using confocal laser scanning microscopy.

Spore germination inhibition assays

Spores of *M. oryzae* strain P131 were harvested from sporulating potato dextrose agar (PDA) plates using sterile water and adjusted to 1×10^5 spores/mL. Spore suspensions were mixed with different concentrations of LL37 solution to form a treatment gradient. A 10- μ L aliquot of each mixture was dropped onto a hydrophobic slide, placed in a humidity-saturated Petri dish, and incubated at 28 °C in the dark for 6 h. Spore germination was then observed and imaged.

Glycogen relocation assay in spores of *M. oryzae*

Spores of *M. oryzae* were harvested using ddH₂O, adjusted to a concentration of 1×10^5 spores/mL, and inoculated onto hydrophobic plastic coverslips. The inoculated coverslips were maintained in a humidified chamber under dark conditions. Samples were collected at designated time points for staining. Glycogen was detected by treatment with KI/I₂ solution (60 mg/mL KI, 10 mg/mL I₂), followed by microscopic imaging (Leica STELLARIS 5).

Localization of LL37FITC in *M. oryzae*

M. oryzae spores were cultured in PDB medium at 28 °C with shaking for 12 h. Fungal cells were pelleted by centrifugation at 6,000 *g* for 2 min and washed three times with sterile water. Ten μ g/mL LL37-FITC (excitation at 488 nm and emission collected at 500–530 nm) and 5 μ M FM4-64 membrane dye (excitation at 552 nm and emission collected at 570–600 nm) were added to the fungal suspension and incubated in the dark at room temperature for 10 min (Wang et al. 2022). Samples were observed using confocal laser scanning microscopy (Leica STELLARIS 5).

Determination of LL37 loading efficiency by rice EVs

To determine LL37 loading efficiency, EV suspensions (5×10^5 particles/ μ L) were aliquoted into tubes (0, 30, 60, 120, 240, 360, 480, and 600 μ L). Each tube received 0.1 mg LL37, and PBS was added to a final volume of 1 mL. A control sample (600 μ L EVs + 400 μ L PBS) was included. Samples were incubated at room temperature for 10 min, followed by ultracentrifugation (Optima MAX-TXP Ultracentrifuge) at 100,000 $\times g$ for 2 h at 4 °C. The supernatants were collected and analyzed using Tricine-SDS-PAGE. Gels were stained with EXBlue™ rapid protein staining solution, and LL37 bands were visualized for quantification.

Stability and leaf adhesion assays of EV-LL37 complex

To compare the stability of EV-LL37, 10 μ M of free LL37 and EV-encapsulated LL37 (EV-LL37) were

subjected to heat (50 °C for 5 h) or UV light (5 h) treatment, followed by *M. oryzae* pathogen assays. Following each treatment, EV morphology and membrane integrity were examined using TEM. Untreated EVs processed in parallel served as controls. To evaluate the long-term stability of LL37 and (EV-LL37), aliquots of each sample were stored at room temperature for 0, 3, and 7 days, respectively. At each time point, the samples were applied to rice leaves, and their protective activity against *M. oryzae* was assessed using a standard rice blast inoculation assay. To assess leaf surface retention, LL37^{FITC} and LL37^{FITC}-loaded EVs (both at 10 µM) were sprayed onto three-leaf-stage rice seedlings. After 10 min of incubation, the leaves were subjected to simulated rain by spraying with water mist for 5 min. Leaves were then collected and examined under confocal microscopy for green fluorescence to assess surface adsorption.

M. oryzae infection assays

Detached leaf assays were performed using a wound-inoculation method (Sha et al. 2023). Fully expanded leaves from four-leaf-stage rice plants were placed adaxial side up in Petri dishes. Leaves were gently wounded with a sterile needle, and 10 µL of *M. oryzae* conidial suspension (2×10^5 conidia/mL) was applied to each wound site. Plates were sealed and incubated at 28 °C in the dark for 24 h, then transferred to a growth chamber under a 16 h light/8 h dark cycle at 95% humidity. Disease symptoms were documented at 7 days post-inoculation by photographing the infected leaves. For each experiment, a minimum of eight plants per treatment showing clear and evaluable symptoms were considered as valid biological replicates and were included in the final dataset for statistical analysis.

Statistical analysis

Data from different biological treatments are presented as the mean $\pm$ SD of biological replicates. Statistical significance was determined using a two-tailed Student's *t*-test or one-way ANOVA followed by Tukey's multiple comparison test.

Abbreviations

CME	Clathrin-mediated endocytosis
EVs	Extracellular vesicles
FITC	Fluorescein isothiocyanate
FM4-64	N- (3-Triethylammoniumpropyl)-4- (6- (4- (Diethylamino) Phenyl) Hexatrienyl) pyridinium dibromide
nFCM	Nano-flow cytometry
<i>M. oryzae</i>	<i>Magnaporthe oryzae</i>
PDA	Potato dextrose agar medium
PBS	Phosphate buffered saline

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s42483-026-00421-0>.

Additional file 1: Figure S1. Schematic workflow for the isolation of EVs from rice leaves.

Additional file 2: Figure S2. FM4-64 staining of rice EVs.

Additional file 3: Figure S3. OsTET13 positively regulates rice resistance to *M. oryzae*.

Additional file 4: Figure S4. Amino acid sequence and schematic structural representation of the antimicrobial peptide LL37.

Additional file 5: Figure S5. Cytotoxicity assessment of 100 mM LL37 and 1% Triton on rice protoplasts after co-incubation.

Additional file 6: Figure S6. Anti-fungal activity assay of LL37^{FITC} against the germination of *M. oryzae* conidia.

Additional file 7: Figure S7. The expression construct of LL-37 and identification of LL37-*flag* heterologous expressing rice lines.

Additional file 8: Figure S8. Phenotypic comparison of plant height between the wild-type plants and LL37-*flag* lines.

Additional file 9: Figure S9. Transmission electron microscopy analysis of EV-LL37 integrity following UV and high-temperature treatments.

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Author contributions

QC conceived the idea and supervised the project. QC and YX designed the experiments. YX performed most of the experiments. YX and WZ contributed to the *M. oryzae* infection and the collection of rice materials. TL and YH helped with the isolation of EVs from rice. QC and YH wrote the manuscript. QC, YX, WZ, TL, and YH reviewed and revised the draft. All co-authors approved the manuscript.

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Availability of data and materials

The authors declare that the data supporting the findings of this study are available within the paper, its supplementary information files.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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