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Message in a Bubble: Shuttling  
Small RNAs and Proteins  
Between Cells and Interacting  
Organisms Using Extracellular  
Vesicles

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**Keywords**

extracellular vesicles, small RNA, cross-kingdom RNAi, exosome, cell-to-cell communication, plant immunity

**Abstract**

Communication between plant cells and interacting microorganisms requires the secretion and uptake of functional molecules to and from the extracellular environment and is essential for the survival of both plants and their pathogens. Extracellular vesicles (EVs) are lipid bilayer–enclosed spheres that deliver RNA, protein, and metabolite cargos from donor to recipient cells and participate in many cellular processes. Emerging evidence

has shown that both plant and microbial EVs play important roles in cross-kingdom molecular exchange between hosts and interacting microbes to modulate host immunity and pathogen virulence. Recent studies revealed that plant EVs function as a defense system by encasing and delivering small RNAs (sRNAs) into pathogens, thereby mediating cross-species and cross-kingdom RNA interference to silence virulence-related genes. This review focuses on the latest advances in our understanding of plant and microbial EVs and their roles in transporting regulatory molecules, especially sRNAs, between hosts and pathogens. EV biogenesis and secretion are also discussed, as EV function relies on these important processes.

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## INTRODUCTION

Plant pathogens and pests cause serious damage to plant health and productivity, posing a threat to both agriculture and natural ecosystems. Understanding the biological interaction between hosts and pathogens/pests aids in the development of new strategies for disease control in crop production and environmental protection. Communication between plants and pathogens requires the

transport of molecules, such as effector proteins and toxins from pathogens to hosts (72, 156) or antimicrobial peptides and metabolites from hosts to pathogens (60, 88), across cellular boundaries. Recently, it has been found that RNA molecules, particularly regulatory small RNAs (sRNAs), also travel between interacting organisms to induce gene silencing in *trans*, playing an important role in plant–pathogen interactions (30, 63, 180). sRNAs are generated by Dicer or Dicer-like (DCL) proteins and then loaded into Argonaute (AGO) proteins to induce silencing of genes with complementary sequences (18). One of the major pathways of cell-to-cell communication and molecule exchange is through extracellular vesicles (EVs). EVs are lipid bilayer-bound spheres that contain different cargos from the secreting cells, traverse the extracellular environment, and enter interacting organisms to play significant roles in regulating host–pathogen interactions (28, 30, 36, 141, 162).

EVs have been well characterized in animal cells and can be isolated from diverse bodily fluids or cell culture media (77). Cells release a number of heterogeneous EV subpopulations that are differentiated primarily by their intracellular origin, specific marker proteins, biophysical properties, and biological functions. They can be broadly classified into exosomes, microvesicles or shedding vesicles, and apoptotic cell–derived vesicles (1, 36, 162). Exosomes are 30–150-nm vesicles that originate from multivesicular bodies (MVBs). Their secretion into the extracellular space involves the fusion of MVBs with the plasma membrane, resulting in the release of intraluminal vesicles into the extracellular space. Microvesicles are 30 to 1,000 nm in diameter, are more heterogeneous, and originate by outward budding directly from the plasma membrane (36, 162). Another class of EVs is produced during apoptosis, which includes >1- $\mu$ m large apoptotic bodies and <1- $\mu$ m small apoptotic microvesicles as products of apoptotic cell disassembly (1, 12).

Exosomes were first observed in 1983, in mammalian cells where they were reported to remove membrane and protein waste when reticulocytes mature into erythrocytes (69, 111). By the end of the 1990s, interest in exosomes had grown rapidly, due to an expanding appreciation of their wider biological functions and potential applications as therapeutics and biomarkers (40). Many studies found that exosomes contain bioactive substances, including proteins and RNA molecules, for transport from donor to recipient cells within an organism, and play important roles in processes such as regulating cell communication, fertilization, immune responses, disease processes, metastatic tumor cell growth, and tissue repair (36, 99, 146). Among the RNA molecules in animal EVs, microRNAs (miRNAs), a subclass of sRNAs, are considered key functional elements and have garnered special attention (159). Exosomal miRNAs negatively regulate the expression of genes with complementary sequences in the recipient cells. However, the molecular mechanisms and regulation of miRNA sorting into exosomes are still largely unknown (146, 151, 164, 189).

Although several studies have shown the accumulation of plant-derived vesicles at bacterial and fungal interaction sites through transmission electron microscopy (5–7, 31, 97, 169), research into the functions of EVs has developed more slowly in plants than in animal systems. Due to the limits of EV isolation protocols and detection methods in plants, our knowledge of EVs remains rudimentary in plants and plant-infecting microbes, such as bacteria, fungi, and oomycetes. Recent research showed that both plants and pathogens release EVs, which then play critical roles in host–pathogen communication (28, 30, 31, 63, 136). In this review, we highlight and discuss the current state of both plant- and microbe-derived EV research, with a special focus on the roles of EVs in plant–pathogen interactions. We discuss the experimental limitations that must be resolved in the study of EVs from both plants and pathogens and the current knowledge of the mechanisms involved in EV biogenesis and secretion. A brief introduction of the potential application of EVs in delivering crop-protecting RNA molecules and other beneficial cargos is also included.

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#### Small RNAs

**(sRNAs):** short regulatory noncoding RNAs that induce silencing of target genes at transcriptional or posttranscriptional levels

#### Extracellular vesicles (EVs):

a heterogeneous group of cell-derived membranous structures that deliver RNA, protein, and metabolite cargos and participate in many cellular processes

#### Exosomes:

cell-derived nano-sized tetraspanin-positive vesicles released into extracellular space upon fusion of the outer membrane of the multivesicular bodies with the plasma membrane

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**Tetraspanins:** a family of proteins with four membrane spans that regulate protein trafficking through membrane compartmentalization; several members are major biomarkers for exosomes

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## PLANT EXTRACELLULAR VESICLES

Plant EVs were initially observed in carrot cell cultures via electron microscopy in 1967 (58). Since then, EVs have been reported in extracellular fluids of leaves, roots, and imbibing seeds (122, 134, 135) and in media used for in vitro pollen germination and pollen tube growth (117, 118). Studies of plant immune responses provided the initial evidence for the function of plant EVs through transmission electron microscopy and confocal microscopy images, which showed MVBs underlying invasion papillae and increases in EV abundance and MVB–plasma membrane fusion events upon pathogen infection (5–7, 24, 31, 105, 106, 169). Recently, EVs were also observed in the rice periarbuscular space after inoculation with the symbiotic arbuscular mycorrhizal fungi *Rhizophagus irregularis* and *Gigaspora rosea* (134). However, the specific characteristics and the biochemical contents of plant EVs were not studied until recently. These studies have shown that plant EVs have specific protein markers and contain distinct set of RNAs, proteins, and metabolites, which are likely to have regulatory functions in recipient cells and interacting organisms (31, 135, 170).

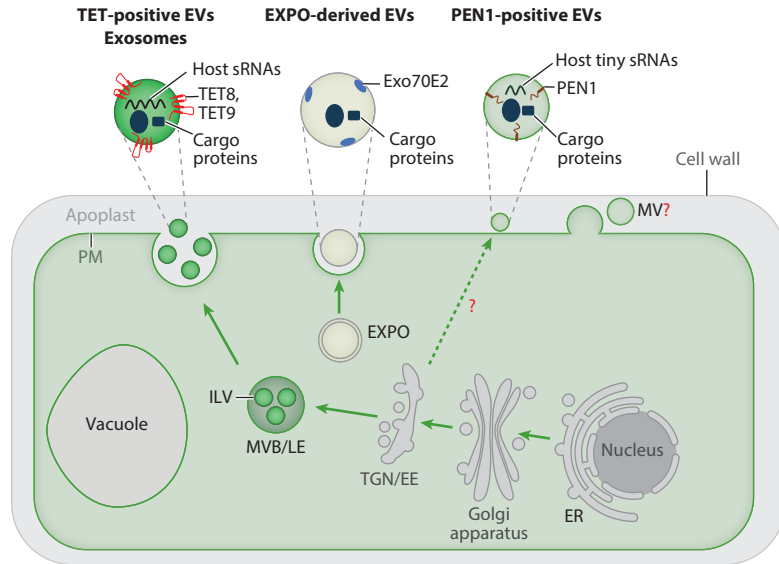
### Tetraspanin-Positive Extracellular Vesicles: Exosomes

In animal systems, proteomic analyses discovered a set of defined proteins that are highly enriched in exosomes. Among them, tetraspanin proteins, such as CD9, CD63, CD37, CD81, or CD82, are especially enriched in the membranes of exosomes and often used as exosome biomarkers (9). In plant systems, *Arabidopsis* encodes 17 members of the TETRASPANIN (TET) family. Though these *Arabidopsis* TETs share low sequence similarity with animal tetraspanins, their transmembrane structural topology is typical for tetraspanin proteins (23). Expression analysis revealed that two closely related tetraspanin genes, *TET8* and *TET9* (168), were highly induced by infection with the fungal pathogen *Botrytis cinerea* (31, 55), suggesting that they are involved in plant defense responses. Moreover, TET8 and TET9 are secreted and colocalize in EVs, which are enriched at *B. cinerea* infection sites (31) (**Figure 1a**). These tetraspanin proteins colocalize with the *Arabidopsis* MVB-marker Rab5-like GTPase ARA6 inside the cell, suggesting that TET8-positive EVs are derived from MVBs and can be considered bona fide plant exosomes (28, 31). Thus, the term plant exosome was used to describe tetraspanin-positive vesicles derived from MVBs and released into the plant extracellular space—the apoplast.

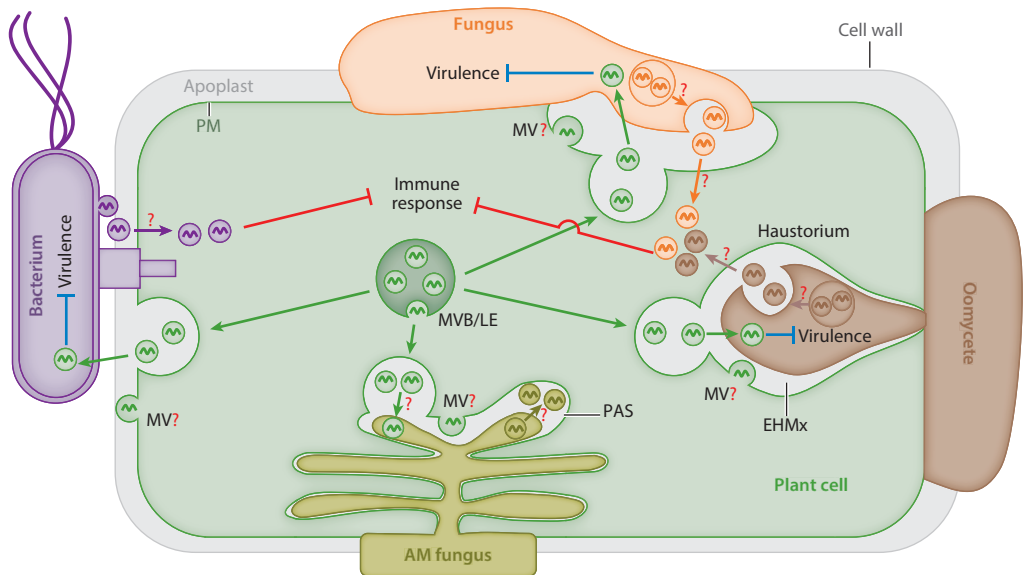
In animals, differential ultracentrifugation is the standard technique for EV isolation (154). Briefly, large EVs (e.g., apoptotic bodies) pellet at low centrifugation speeds ( $\sim 2,000 \times g$ ), medium EVs (e.g., microvesicles) pellet at intermediate speeds ( $\sim 10,000$ – $20,000 \times g$ ), and small EVs (e.g., exosomes) pellet at high speeds ( $\sim 100,000$ – $200,000 \times g$ ) (37, 66, 77, 154). Consistently, plant EVs and the TET8 marker were highly enriched in the fraction collected at  $100,000 \times g$  from leaf apoplastic fluid (31). Ultracentrifugation only allows enrichment in subtypes of EVs in the final centrifugation. Evidence has shown that other EV species of similar size could be co-isolated with exosomes at  $100,000 \times g$  (36, 78). High-speed density gradient ultracentrifugation enables further separation and purification of different EV subtypes, as different classes of vesicles tend to be enriched in different density fractions (78). For plant EV isolation, vesicles floated in a sucrose (59, 117) or OptiPrep (135) gradient facilitate the separation of different vesicle subtypes with different densities. For example, TET8- and TET9-positive exosomes are enriched in the gradient fraction of approximately  $1.12$  to  $1.19 \text{ g ml}^{-1}$  (59).

Immunoaffinity isolation is the most advanced method for the purification of a specific subclass of EVs, as it isolates the EVs by using antibodies that recognize the specific EV protein markers (such as exosome marker CD63) (77, 78, 155). This method can prevent protein or RNA contamination from cytoplasm or other classes of EVs. It is not possible to use a fused green fluorescent

**a**



**b**



- Host sRNA
- Oomycete sRNA effector
- Fungal sRNA effector
- Bacterial sRNA effector
- AM fungal sRNA effector
- Plant vesicle trafficking pathway
- Predicted plant vesicle trafficking pathway
- Fungal vesicle trafficking pathway
- Bacterial vesicle trafficking pathway
- AM fungal vesicle trafficking pathway
- Suppression of pathogen virulence genes
- Suppression of host immune response genes

*(Caption appears on following page)*

**Figure 1** (Figure appears on preceding page)

EV-mediated cross-kingdom RNAi is a communication mechanism in plant–microbe interactions. (a) Plants have at least three known EV subtypes. TET-positive exosomes are released into extracellular space by MVB fusion with the PM and the subsequent release of ILVs. The biogenesis pathway of PEN1-positive EVs remains unknown. The EXPO produces EVs by fusion with the PM to release the inner vesicles into the extracellular space. MVs may also be secreted by plant cells through outward budding directly from the PM. (b) During pathogen infection, plants secrete EVs into the extracellular space. The EV-encased sRNAs can be internalized by pathogens, where they target pathogen virulence-related genes to suppress pathogen virulence. At the same time, pathogens can deliver sRNA effectors into host plant cells to suppress host immunity. EVs have been observed in the PAS where plants and AM fungi interact. We predict that pathogens may also utilize EVs to secrete and transport sRNAs into host cells. The question mark indicates a prediction that has not yet been validated experimentally. Abbreviations: AM, arbuscular mycorrhizal; EHMx, extrahaustorial matrix; ER, endoplasmic reticulum; EV, extracellular vesicle; EXPO, exocyst-positive organelle; ILV, intraluminal vesicle; LE, late endosome; MV, microvesicle; MVB, multivesicular body; PAS, periarbuscular space; PEN1, Penetration 1; PM, plasma membrane; RNAi, RNA interference; sRNA, small RNA; TET, tetraspanin; TGN/EE, trans-Golgi network/early endosome.

protein (GFP)-tag of TETs for immunoaffinity capture of plant exosomes because both the N and C termini of the TET protein are inside the lumen of vesicles. Therefore, a native antibody that specifically recognizes the large exposed extravesicular loop, the EC2 domain of TET8, was generated to allow the successful purification of TET8-positive exosomes (59).

### Penetration 1–Positive Extracellular Vesicles

Plant EVs were also isolated at a lower ultracentrifugation speed ( $40,000 \times g$ ) from *Arabidopsis* leaf apoplastic fluid (135). Proteomic analysis indicates that these EVs contain proteins involved in biotic and abiotic stress responses, including the plant-specific Penetration 1 (PEN1) (Figure 1a). PEN1 was initially identified as a plasma membrane-associated syntaxin (79). PEN1 secretion depends on an ADP ribosylation factor–GTP exchange factor (ARF-GEF), GNOM, that mediates recycling endosome trafficking instead of the MVB pathway (105). Thus, PEN1-positive EVs likely have a different biogenesis pathway and biomarkers from exosomes.

Unlike TET8 colocalized with *Arabidopsis* MVB-marker Rab5-like GTPase ARA6 inside the plant cell, PEN1 does not colocalize with ARA6-marked MVBs, further supporting the theory that the PEN1- and TET8-positive EVs have distinct biogenesis pathways (59). Moreover, PEN1-positive EVs are enriched in the gradient fraction of 1.029 to 1.056 g ml<sup>-1</sup> (135), which are different from TET-positive EVs and may function in transporting a different class of cargos. Indeed, distinct GFP-labeled and mCherry-labeled EVs were observed when EVs were isolated from transgenic plants co-expressing two fluorescence-tagged fusion proteins, TET8-GFP and mCherry-PEN1 (59), confirming that PEN1-positive EVs and TET8-positive EVs are two distinct classes of EVs in plants.

**Exocyst-positive organelle (EXPO):** mediates cytosol-to-cell-wall exocytosis in plants and is distinct from multivesicular endosomes and autophagosomes

### Exocyst-Positive Organelle–Derived Extracellular Vesicles

Another class of plant EVs are derived from exocyst-positive organelles (EXPOs). The EXPO is a novel organelle discovered by expressing an *Arabidopsis* homolog of the exocyst protein Exo70E2 in *Arabidopsis* and tobacco (*Nicotiana tabacum*) suspension cells (Figure 1a). It has a morphology distinct from MVBs and is independent from endosomes and autophagosomes (170). Although AtExo70E2 is crucial for EXPO formation, the biogenesis of the EXPO is still unclear. Immunogold labeling revealed that EXPOs are spherical, double-membrane structures within cells. After the outer membrane of the EXPO fuses with the plasma membrane, the EXPO releases single-membrane-bound vesicles to the extracellular space (170). The size of EXPO-derived EVs can range from 200 to 500 nm in diameter, which is larger than exosomes. Thus, the plant-specific EXPO could be the origin of large plant EVs (46, 170). In tobacco, glycosyltransferases

AtGALT14A, AtGALT29A, and AtGALT31A were found to colocalize with the EXPO marker AtExo70E2, suggesting that EXPO-derived EVs may be involved in the secretion of arabinogalactan proteins in plants (116).

### Pollensomes

Olive (*Olea europaea*) pollen releases secretory nanovesicles, generically named pollensomes, during in vitro pollen germination and pollen tube growth (117). Pollensomes comprise a heterogeneous population of secretory EVs with diameters ranging from 28 to 60 nm and densities ranging from 1.24 to 1.29 g ml<sup>-1</sup> on a sucrose gradient, higher than exosomes (1.12–1.19 g ml<sup>-1</sup>), possibly because of the enrichment of polysaccharides (117). Pollensomes may play an important role in communicating with female organs for fertilization. They may also represent widespread vehicles for pollen allergens, with potential implications for allergic reactions in animals (117).

## EXTRACELLULAR VESICLES IN CROSS-KINGDOM SMALL RNA TRAFFICKING AND PLANT IMMUNITY

Plant EV secretion is increased by pathogen infection, suggesting that EVs play important roles in plant immunity (5–7, 31, 97, 128, 169). Because of the material transport nature of EVs, plants and many interacting microbes have evolved to utilize EVs for the exchange of functional molecules, including RNAs, proteins and metabolites, between host cells and interacting organisms.

### Cross-Kingdom Small RNA Trafficking from Plants to Pathogens by Extracellular Vesicles

sRNA-mediated RNA interference (RNAi) is a conserved gene-silencing mechanism in eukaryotes to regulate endogenous and exogenous gene expression. In plants, which lack an immune system analogous to that of mammals (i.e., based on lymphocytes and antibodies), RNAi is one of the primary adaptive defense mechanisms that regulates plant immune responses against viral, bacterial, fungal, and oomycete pathogens (29, 63, 179). RNAi has been co-opted via genetic modification for resistance to a broad range of crop pests and pathogens (107, 174, 175). Recent studies have found that sRNAs can move across the cellular boundaries between plant and animal hosts and their interacting pathogens and parasites, thus triggering gene silencing in *trans*, a process termed cross-kingdom RNAi (29, 63, 73, 143). *Arabidopsis* delivers a selective set of miRNAs and small interfering RNAs (siRNAs), including phased secondary siRNAs (phasiRNAs), into interacting *B. cinerea* cells, inducing the silencing of fungal genes that are involved in pathogenicity (31).

It has been demonstrated that plant endogenous sRNAs are secreted in EVs (31). The EV-enriched sRNAs are also enriched in the same gradient fractions of TET-positive exosomes (59). The ribonuclease treatment of isolated EVs could effectively remove contaminating RNAs that may attach to EV surfaces or co-sediment with EVs. Plant EV-enriched sRNAs were protected from micrococcal nuclease digestion unless Triton X-100 was added to rupture the EVs (31), confirming that these sRNAs are encased inside the EVs. As immunoaffinity capture is the most powerful tool to isolate pure, specific subclasses of EVs, He et al. (59) generated a TET8-specific antibody and specifically purified the TET8-positive exosomes by immunoaffinity pull-down. EV-encased sRNAs were highly enriched in immunoaffinity-purified TET8-positive exosomes (59). *Arabidopsis* double mutants of exosome markers *TET8* and *TET9* displayed decreased secretion and transport of host sRNAs into fungal cells (31). These results indicate that the TET8-positive exosomes are the major class of EVs that transport sRNAs.

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#### Plant immunity:

plant innate system that includes two forms of defense against invading pathogens: preformed (constitutive) and pathogen-induced defense responses

#### Cross-kingdom RNA interference

##### (cross-kingdom

##### RNAi): sRNAs travel

across organismal boundaries between hosts and microbes and silence genes in *trans* in interacting organisms

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The sRNA-containing exosomes can be efficiently taken up by fungal cells (31) (**Figure 1b**). More than 70% of plant sRNAs found in purified *B. cinerea* protoplasts isolated from infected plants were also present in plant EVs (31), suggesting that EV-mediated transport is a major plant sRNA delivery pathway. Double mutants of *tet8 tet9* display enhanced susceptibility to fungal infection (31). Moreover, fungal cells isolated from the double mutants contained a remarkably reduced amount of plant sRNAs (31). These EV-transferred sRNAs target genes involved in fungal virulence because the mutant fungal strains lacking each of those targets were less virulent than the wild-type (WT) strains (31). The identification of fungal target genes of plant sRNAs is an effective way to identify new virulence-related genes, such as *vacuolar protein sorting 51* (*Bc-Vps51*), the large subunit of the dynactin complex (*Bc-DCTN1*) and a suppressor of actin-like phosphoinositide phosphatase, all of which are involved in vesicle trafficking and play important roles in *B. cinerea* pathogenicity (31). It is worth noting that Vps51 protein is also necessary for the full virulence of the human fungal pathogen *Candida albicans* (84). This study offered a powerful perspective on EV-mediated sRNA translocation into fungal cells, supporting the involvement of a protective shuttle mechanism in cross-kingdom communication. This finding was reinforced by the recent discovery that *Arabidopsis* also transports secondary phasiRNAs into an oomycete pathogen, *Phytophthora capsici*, which go on to silence target genes in the pathogen (62) (**Figure 1b**).

The *TET8* knockout mutant had a lower amount of cellular glycosyl inositol phospho ceramides (GIPCs) (~22% of the WT level) and secreted fewer EVs (~60% of the WT level) (83). This suggests that TET8 mediates exosome production in association with GIPCs and provides an important impetus for studying the roles of TETs and TET-positive EVs in transporting functional molecules. Recently, PEN1-positive EVs were reported to carry a class of 10–17-nt tiny RNAs (17), although it is not clear if these RNAs have any biological function. Plant sRNA-induced silencing of pathogen genes was also observed in crop plants, such as cotton and wheat, which deliver miRNAs, likely also by EVs, that target *Verticillium dahliae* and *Fusarium graminearum* virulence genes, respectively, reducing the virulence of these fungal pathogens (68, 190).

### **Plant RNA-Binding Proteins Contribute to Small RNA Selective Loading and Stabilization in Extracellular Vesicles**

One important observation from EV-sRNA profiling analysis is that the profile of EV-associated sRNAs is distinct from that of total sRNAs (31), suggesting a regulatory process for selective loading of sRNAs into EVs. To understand the underlying molecular mechanism, He et al. (59) performed proteomic analysis on *Arabidopsis* EVs isolated at 100,000 × g and identified a list of RNA-binding proteins, including RNAi pathway protein AGO1, DEAD-box RNA helicases (RH11, RH37) and Annexins (ANN1, ANN2). All of these RNA-binding proteins are enriched in the same sucrose gradient fractions as sRNAs and TET8 (approximately 1.12 to 1.19 g ml<sup>-1</sup>), as well as in the immunoaffinity-purified TET8-positive exosomes (59). Moreover, these RNA-binding proteins were still detectable in exosomes after trypsin treatment. These results indicate that these RNA-binding proteins are secreted mainly by exosomes (59).

He et al. (59) have further demonstrated that *Arabidopsis* AGO1, RH11, and RH37 selectively bind to the sRNAs that are transported by exosomes but not to those that are not secreted by EVs. The *Arabidopsis* genome encodes ten AGO proteins and each binds with a distinct set of sRNAs. Only AGO1 was secreted by EVs, and AGO2, AGO4, and AGO5 were not detected in the EV fractions, nor were their associated sRNAs (59). These results suggest that AGO1, RH11, and RH37 contribute to the selective loading of sRNAs into EVs, mainly the TET-positive exosomes



(59). On the contrary, Annexins bind to sRNAs nonspecifically but play a role in stabilizing sRNAs in EVs because the accumulation of sRNAs in EVs was reduced in the *ann1ann2* mutants (59).

### Cross-Kingdom Small RNA Trafficking from Pathogens to Hosts

Plant pathogen-derived sRNAs can also move into host cells to suppress host immunity (63, 174, 177–180) (**Figure 1b**). The fungal pathogen *B. cinerea* delivers a suite of sRNAs into *Arabidopsis* and tomato cells to suppress host immunity by using the host RNAi machinery (180). These fungal sRNAs are loaded into the *Arabidopsis* AGO1 protein, leading to the silencing of host immune response genes, including MAPKs, cell wall-associated kinases, and genes involved in the accumulation of reactive oxygen species (180). Subsequently, other microbes were identified that also transport sRNAs into host plant cells to induce cross-kingdom RNAi, including the fungal pathogens *V. dahliae* (175) and *Puccinia striiformis* (167), the oomycete pathogen *Hyaloperonospora arabidopsidis* (49), and even the prokaryotic symbiotic bacterium *Bradyrhizobium japonicum* (126).

It is exciting to find that sRNAs from both plant and animal fungal pathogens, as well as prokaryotic bacterium, use the same mechanism by loading into host AGO1 to silence host genes (49, 126, 175, 180). The mosquito fungal pathogen *Beauveria bassiana* transfers an miRNA to the host cells and hijacks mosquito AGO1 to silence host immunity gene *Toll receptor ligand Spätzle 4* (38). Although prokaryotes do not have the RNAi machinery, rhizobial tRNAs give rise to sRNA fragments, which can move into plant hosts and bind with host AGO1 to silence host genes (126).

In mammalian systems, EVs have been shown to carry sRNAs in body fluids, a likely mode of sRNA trafficking between cells within an organism. It is not surprising that diverse parasites have evolved to exploit these natural cell-to-cell communication pathways to interact with host cells and tissues (35, 141). EVs secreted by the gastrointestinal nematode, or helminth, *Heligmosomoides polygyrus*, deliver miRNAs to mouse host cells and suppress inflammation and innate immune responses during infection (26). Future research is needed to determine whether plant pathogens also utilize EVs to deliver sRNAs to their hosts.

EVs have also been observed at the interface of plants and symbiotic arbuscular mycorrhizal fungus. Plasma membrane-derived microvesicles have been observed in the periarbuscular interface during rice root interaction with *R. irregularis* (134). These microvesicles may also carry proteins and RNA molecules involved in the interorganismic signal exchange throughout the arbuscule lifespan.

### Extracellular Vesicles in Plant Defense

Infection with fungal pathogen *B. cinerea* increases both EV and sRNA abundance in *Arabidopsis* extracellular fluids (31). Infection with bacterial pathogen *P. syringae* pv. tomato DC3000 or treatment with defense hormone SA also increases EV secretion by *Arabidopsis* cells (135). As indicated above, plant EVs can be effectively taken up by fungal pathogens, including *B. cinerea* and *Sclerotinia sclerotiorum* (31, 123). Spores of *S. sclerotiorum* treated with sunflower-derived EVs showed growth inhibition, morphological changes, and cell death (123). These findings support the hypothesis that EVs play an important role in immune responses.

Besides RNA cargo, *Arabidopsis* EVs contain proteins involved in defense responses (135). The presence of various proteins involved in immune signaling, i.e., BAK1-interacting receptor-like kinase 2, Glycine-rich protein 7, RPM1-interacting protein 4, and Suppressor of BIR1-1 in plant EVs, suggests that EVs may modulate pathogen recognition by promoting the extracellular trafficking of key signaling proteins that are involved in immune signaling (135). Antimicrobial defense-related proteins, including members of the myrosinase-glucosinolate system, such as glucosinolate transporters PEN 3 and Glucosinolate transporter 1, as well as the myrosinase,

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**Outer membrane vesicles (OMVs):**

Gram-negative bacteria-derived EVs that are produced during cell wall turnover by the pinching off of the bacterial outer membrane

**Cytoplasmic membrane vesicles (CMVs):**

EVs produced by Gram-positive bacteria created by membrane blebbing

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Epithiospecifier modifier 1 (135), are associated with innate immunity in response to diverse pathogens and pests (61, 138, 185). The EV proteome was also enriched for proteins involved in the polarized immune response pathway, i.e., PEN1 (Syntaxin-121), Syntaxin-122, and Syntaxin-132 (135), indicating that plant EVs may also be involved in protein transport during immune signaling.

Beyond their functioning in defense, plant exosomes also contribute to systemic viral infection in plants. A recent study showed MVBs fusing with the plasma membrane and releasing numerous 60–150-nm EVs in *Nicotiana benthamiana* by transmission electron microscopy during Turnip mosaic virus (TuMV) infection (101). TuMV components (RNA and proteins) were detected in MVBs and EVs, suggesting that TuMV may be released into the extracellular space by exosomes (101). This study showed, using focused ion beam extremely high-resolution scanning electron microscopy, that EVs move within the cell wall (101), although the mechanism for cross-cell-wall transfer of plant EVs remains unclear.

Direct uptake of EVs by fungal cells has been observed (31, 123). But even now, the exact mechanism of EV uptake in plant and fungal cells is still unclear. In mammalian systems, various mechanisms for EV uptake have been proposed, including phagocytosis, macropinocytosis, clathrin-mediated and/or caveolin-mediated endocytosis, or direct fusion at the target cell plasma membrane to deliver their protein, messenger RNA (mRNA), and miRNA cargos (102). Two different endocytosis pathways, clathrin-mediated and clathrin-independent, have been discovered in plants and fungi (53, 86, 119). One or both may be involved in the uptake of EVs in plant and fungal cells, and further studies are needed to determine the precise uptake mechanisms.

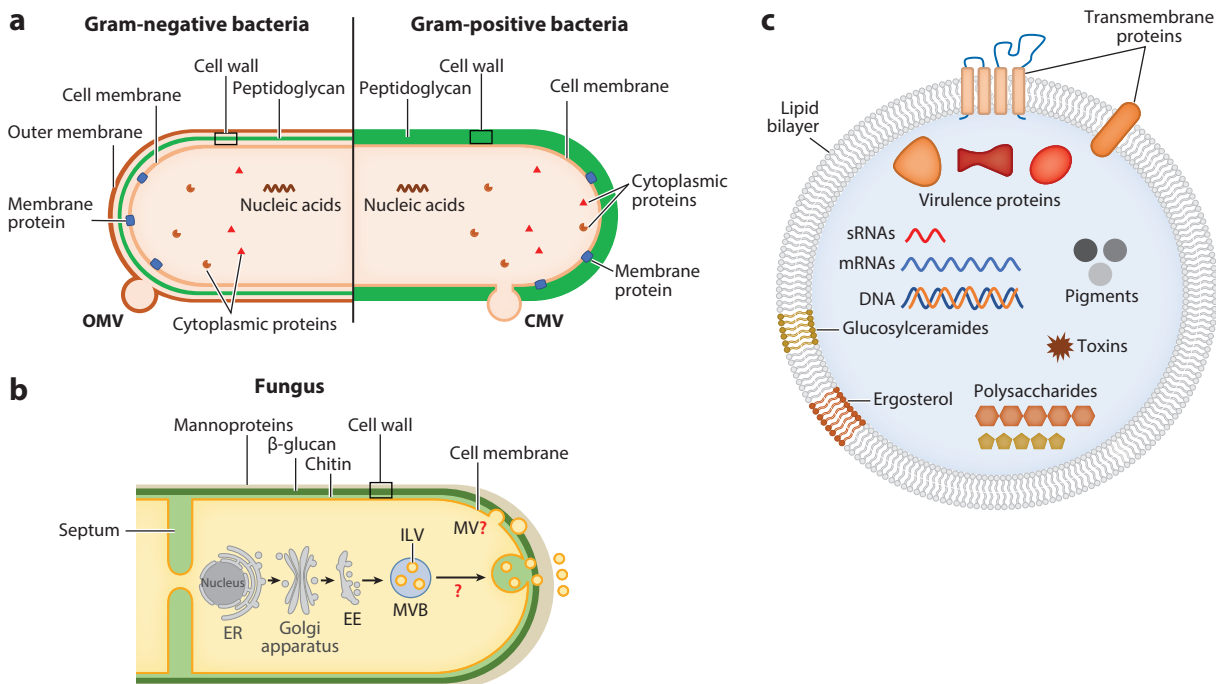
## EXTRACELLULAR VESICLES IN PLANT-INTERACTING MICROBES

The first study of microbial EVs was in the Gram-negative bacterium *Escherichia coli* in the 1960s (20), while, due to the thickness of most microbial cell walls and a lack of outer membrane structures, EVs in other microbes, such as Gram-positive bacteria, archaea, and fungi, were largely overlooked during the subsequent two decades. The increasing importance of EVs to the biology of a wide variety of organisms drove the rapid discovery of EVs produced by fungi (129), mycobacteria (90), Gram-positive bacteria (81), and archaea (51) (**Figure 2**). The existence of EVs across most eukaryotes and prokaryotes suggests that cell-to-cell vesicular transport is a universal phenomenon.

Increasing evidence indicates that EVs derived from microbes and parasites play a prominent role in modulating host immunity at various levels (26, 121, 136, 187). Pathogen EVs carry a plethora of proteins, nucleic acids, and lipid cargos, which may be involved in multiple signaling pathways, provoking prototypic pattern-triggered immune responses and supporting infection (136). Over the past decade, studies of microbial EVs have mainly focused on those microorganisms that infect mammals (21, 42, 57, 108), whereas reports focused on plant-interacting microbes have so far been limited.

### Bacterial Extracellular Vesicles

In Gram-negative bacteria, 50- to 300-nm EVs are produced in *in vitro* conditions during bacterial growth on solid or in liquid media and, additionally, during intracellular interactions with humans (103, 157). Gram-negative bacteria-derived EVs are produced during cell wall turnover by the pinching off of the bacterial outer membrane and are called outer membrane vesicles (OMVs) (65). EVs produced by Gram-positive bacteria are also created by membrane blebbing and are called cytoplasmic membrane vesicles (CMVs) (**Figure 2a**). EVs derived from bacteria carry varied cargos, enriched with communication compounds, virulence factors, toxins, adhesins, DNA,



**Figure 2**

Microbial EV formation and secretion pathways. (a) OMVs are secreted by Gram-negative bacteria from blebbing of the outer membrane. CMVs are produced by Gram-positive bacteria from blebbing of the cytoplasmic membrane. (b) Fungi can produce EVs to pass through the outer thick cell wall, although the mechanisms of fungal EV release are largely unknown. (c) Microbial EVs contain functional components including microbe-derived RNA, lipids, proteins, nucleic acids, and metabolites. The question mark represents a prediction that has not yet been validated experimentally. Abbreviations: CMV, cytoplasmic membrane vesicle; EE, early endosome; ER, endoplasmic reticulum; EV, extracellular vesicle; ILV, intraluminal vesicle; mRNA, messenger RNA; MV, microvesicle; MVB, multivesicular body; OMV, outer membrane vesicle; sRNA, small RNA.

and RNA. By inter- and intraspecies delivery of molecules, EVs are involved in biofilm formation, interspecific competition, bacterial adherence to the host, and innate bacterial defense by adsorption of antimicrobial peptides and bacteriophages (89).

By contrast, little is known about EVs from plant-interacting bacteria, despite the observance of EVs in the cultured, plant-interacting microbe that causes fire blight, *Erwinia amylovora*, as early as the 1980s (80). Some studies showed plant-interacting bacteria release OMVs that participate in biofilm formation, virulence, and modulation of plant immunity (71). Several Gram-negative hemibiotrophic pathogens of the *Xanthomonadaceae* family release EVs in culture or during plant infection, as determined by biochemical purification and electron microscopy, such as *Xanthomonas campestris* (causes black rot and bacterial leaf spot), *Xanthomonas oryzae* (causes blight of rice), *Xanthomonas citri* (causes citrus canker), and *Xylella fastidiosa* (causes Pierce's disease in grapes and variegated chlorosis in citrus) (15, 64, 91, 94, 104, 140, 147, 149). *X. fastidiosa*, which colonizes the xylem of important crop plants, releases OMVs as an extracellular antiadhesive factor that may have alternative roles in modulating movement and biofilm formation (64). Those OMVs are also enriched with virulence factors and signaling molecules which may contribute to host-pathogen interactions (54). The plant Gram-negative bacterial pathogen *P. syringae*, which causes bacterial speck disease in *Arabidopsis* and tomato, was also reported to release EVs (34). These observations

suggest that EVs may be involved in cross-kingdom communication between bacteria and plants. Whether pathogen-derived EVs are taken up by the host cells remains to be investigated.

### Fungal Extracellular Vesicles

Fungal EVs are often thought to be derived from MVBs or budding directly from the plasma membrane (43) (**Figure 2b**). Proteomic profiling has been used to address the functions and biogenesis of fungal EVs. An enrichment of the endosomal sorting complexes required for transport (ESCRT) machinery proteins was found in EVs of the yeast *Saccharomyces cerevisiae*. Mutation of ESCRT machinery components reduced EV production and altered the morphology and size of EVs, as observed by transmission electron microscopy (191). Although this study shows that ESCRT machinery participates in vesicle production, the precise biogenesis mechanism has yet to be described. One study in *Cryptococcus neoformans*, a causal agent of cryptococcosis in humans, revealed that the production of EVs containing major virulence determinants was decreased when the expression of the exocyst component Sec6 was suppressed by RNAi (112). The exocyst is involved in the fusion of vesicles to the plasma membrane, and so its involvement in EV production is no surprise.

Like Gram-positive bacteria, fungi have a thick wall outside of the cellular membrane, which hindered the search for EVs because of the assumption that membrane-derived vesicles could not traverse such barriers. Fungal EV production was observed in *C. albicans* via transmission and scanning electron microscopy in the 1990s (8). Although the mechanisms of EV release are still unknown, recent results confirmed that fungal vesicles could reach the extracellular space through the cell wall (163). It has been suggested that EVs may transit through channels in the fungal cell wall (25). Experimental evidence has been provided that liposomes of approximately 80 nm in diameter can traverse fungal cell walls intact (166). This demonstrates that the viscoelastic properties of fungal cell walls may allow EV transit to and from the fungal cell membrane. It has also been postulated that EVs may modify the cell wall, as many fungal and bacterial EV proteomes have revealed an enrichment of cell wall-modifying enzymes (25). Indeed, a recent study of EV secretion in *S. cerevisiae* revealed that cell wall-remodeling enzymes, glucan synthase subunit Fks1 and chitin synthase Chs3, were enriched in the EV proteome. Fks1 and Chs3 were essential for the release of EVs, suggesting that they play a role in cell wall remodeling (191). However, the precise mechanisms by which EVs traverse cell surface barriers remains to be determined.

Electron microscopy showed that numerous EVs are present at the plant cell-haustorium interface during interaction with the fungus *Golovinomyces orontii*, although the EVs remain of unknown origin (97). Since abundant MVBs were observed in haustoria, it is plausible that plant-interacting oomycetes and fungi could release exosome-like EVs during the infection process. Recently, EVs have been isolated from the plant beneficial fungus *Trichoderma reesei* (42) and from the major cotton pathogen *Fusarium oxysporum* (22).

### Composition and Function of Microbial Extracellular Vesicles

There are few universal markers associated with fungal EVs that are also shared with EVs from mammalian and plant cells (41). The conserved tetraspanin homologs, for example, have not yet been identified in fungal EVs. However, a recent report described a range of proteins consistently enriched in *C. albicans* EVs that included the tetra-transmembrane claudin-like Sur7 family (41).

EVs produced by fungi can interfere with the immunomodulatory activity of host cells. Several studies in recent years have shown that fungal EVs, purified from cultured supernatants, contain proteins, nucleic acids, lipids, polysaccharides, and pigments. EVs isolated from the nonpathogenic model yeast *S. cerevisiae* contained 400 cargo proteins (109) (**Figure 2c**).

In addition to the set of proteins involved in biological processes and cellular metabolism, there are many molecules found in pathogen EVs that are specifically involved in virulence (21). For example, EVs from the Gram-positive bacterium *Bacillus anthracis* deliver the cytolytic toxin anthrolysin to human host cells (127). Fungal pathogen *C. neoformans* secretes EVs that carry the polysaccharide glucuronoxylomannan, which is essential for virulence (130, 160). EVs also expose surface carbohydrate pathogen-associated molecular patterns that are recognized by host innate immune receptors, in addition to the immunogenic  $\alpha$ -linked galactopyranosyl epitopes, present both on the surface of and within fungal EVs (39, 160). A polyketide synthase involved in the biosynthesis of melanin and other pigments was found in the EV cargo of the fungus *Alternaria infectoria*, a pathogen of wheat and an opportunistic pathogen of humans (148). Packaging of pigments into EVs may represent a common process across fungi by which these compounds are secreted. Recently, a striking observation in the plant-pathogenic fungus *F. oxysporum* was the deep purple color of the EVs, derived from the pigment naphthoquinone. Two polyketide synthases present within EV cargo suggested that the biosynthesis of this pigment occurred in EVs (22). *F. oxysporum* has been shown to release EVs containing fusarubin cluster-esterase, which is involved in the biosynthesis of toxins that are important for infection (22). Our understanding of whether, and how, phytopathogens may utilize EVs to deliver virulence proteins to plant cells is limited.

Akin to plant and mammal EVs, RNA-seq analysis identified a variety of RNA species, including sRNA, noncoding RNA, transfer RNA, and mRNA, in fungal EVs (114). A study of the fatal human pathogen *Cryptococcus gattii* demonstrated that RNA cargos are protected by intact fungal EVs and are delivered to host cells as virulence factors (19). Although DNA packaged within microbial EVs has been reported in many microbes (47), DNA cargo in fungal EVs seems to be less important to virulence, as the removal of DNA from EVs showed no effect on host cells (19). While its purpose remains to be revealed, possible functions of such export include genetic exchange between compatible organisms.

Studies of RNA in EVs derived from plant pathogens or interacting microbes have not been reported. Based on studies of other organisms, we assume RNAs transported by EVs should play a pivotal role in microbe development and infection (**Figure 2c**). Indeed, as indicated above, the eukaryotic fungal pathogens *B. cinerea* and *V. dahliae*, the oomycete pathogen *H. arabidopsidis*, and the prokaryotic symbiont *Rhizobium B. japonicum* have recently been shown to deliver sRNAs into plant host cells, where they utilize the plant RNAi machinery to target the expression of immune-associated genes (49, 175, 180). While the mechanism of delivery for these sRNAs has not been demonstrated, their packaging into EVs is a highly likely possibility to explore in the future.

## **ROLE OF EXTRACELLULAR VESICLES IN DELIVERING CROP-PROTECTING RNA INTERFERENCE AND OTHER BENEFICIAL CARGO**

RNAi-based engineered resistance is produced by integrating a pest or pathogen gene sequence into the host genome for the expression of pathogen-specific double-stranded RNA (dsRNA) or sRNAs, the key trigger molecules of RNAi. The plant RNAi machinery processes the dsRNA into siRNA duplexes through the actions of Dicer enzymes (18). Subsequently, siRNA is incorporated into the AGO proteins to direct degradation of a homologous transcript (18). Pests feeding on the host, or fungi and oomycete pathogens infecting the host, take up either dsRNA or siRNAs, which silence the critical target gene that the pests or pathogens need to multiply and to infect the plants, thereby rendering the genetically modified (GM) host resistance. This modification of plants to express pest- or pathogen-specific dsRNA is also referred to as host-induced gene

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**Host-induced gene silencing (HIGS):**

occurs when transgenic plants express dsRNAs or sRNAs that target pathogen or insect virulence-related genes to combat plant diseases

**Spray-induced gene silencing (SIGS):**

spray application of pathogen gene-targeting dsRNAs and sRNAs to control fungal and potentially other pathogens

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silencing (HIGS). HIGS has been commercially successful at providing protection against pests and pathogens of crops, ranging from insects such as western corn rootworm (14) to viruses including Papaya ringspot virus (67). However, the adoption of HIGS is limited due to the technical difficulties of engineering many crops and the issues of acceptance and regulation of genetically modified crops across different jurisdictions.

A promising alternative to HIGS that has emerged in recent years is spray-induced gene silencing (SIGS). It involves the topical or exogenous application of dsRNA to protect plants from pests and pathogens instead of expressing the dsRNA by integrating it into the host genome (173). In 2001, Tenllado & Díaz-Ruíz (152) showed that resistance to Pepper mild mottle virus, Alfalfa mosaic virus, and Tobacco etch virus was evident following foliar dsRNA application. SIGS-based resistance against chewing insects has been demonstrated in coleopterans such as Colorado potato beetles, which are susceptible to dietary dsRNA (27). More recently, SIGS-based resistance to fungal pathogens, including *B. cinerea*, *F. graminearum*, and *S. sclerotiorum*, has been demonstrated (74, 93, 175). Both HIGS and SIGS have been extensively reviewed due to their significance in crop protection (29, 74, 75, 107, 174, 175, 188). Importantly, SIGS is considered non-GM in jurisdictions such as Australia, circumventing the regulatory issues facing HIGS (56).

The underlying mechanisms of environmental RNA uptake vary in different species. In the model worm, *Caenorhabditis elegans*, several *systemic RNA interference defective (SID)* genes are responsible for RNA transport and environmental RNAi (70). However, no homologs of these proteins are found in plants and fungi (174). Eukaryotic cells take up extracellular materials, including RNAs, mainly through endocytosis. For example, the suppression of four key genes of clathrin-dependent endocytosis in the red flour beetle (*Tribolium castaneum*) significantly impairs cellular uptake of dsRNA in *T. castaneum* (184). The genetic screening of S2 cells in *Drosophila* identified components of the endocytic pathway, including genes for clathrin heavy chain and its adaptor, which indicates the involvement of clathrin-mediated endocytosis for *Drosophila* in dsRNA uptake (137). In the fungal phytopathogen *S. sclerotiorum*, clathrin-mediated endocytosis has also been proven to function in exogenous dsRNA uptake (183). Endocytic inhibitor treatment and RNAi-mediated knockdown of several clathrin-mediated endocytic gene transcripts confirmed the involvement of clathrin-mediated endocytosis in facilitating dsRNA uptake in *S. sclerotiorum* (183). These studies suggest that endocytosis is a fundamental cellular process that mediates the uptake of extracellular RNAs.

The efficiency of RNA uptake varies among different types of eukaryotic microbes and in different cell types. Many aggressive fungal pathogens of plants, such as *B. cinerea*, *S. sclerotiorum*, *Rhizoctonia solani*, *Aspergillus niger*, and *V. dahliae*, can efficiently take up environmental dsRNAs (120). However, no RNA uptake was observed in *Colletotrichum gloeosporioides* and only weak uptake was seen in the nonpathogenic fungus *Trichoderma virens* (120). The nonfungal eukaryotic oomycete pathogen *Phytophthora infestans* also showed rather limited uptake of environmental dsRNAs (120). Consistent with RNA uptake efficiency, the application of dsRNAs targeting virulence-related pathogen genes has been shown to suppress disease caused by pathogens that have high RNA uptake efficiency, whereas the application of dsRNA in pathogens with low RNA uptake efficiency did not significantly suppress pathogen infection (120). Accordingly, efficient dsRNA uptake in pathogens is essential for success of SIGS in crop protection.

For SIGS to be effective, the challenges of enhancing dsRNA uptake and movement to maximize dosage are formidable. Given the importance of EVs in cross-kingdom RNAi and plant defense, it is reasonable to expect that EVs and comparable artificial nanovesicles can be utilized for RNAi-mediated crop protection. Importantly, the use of nanovesicles as RNAi delivery agents in mammalian systems provides valuable insight and lessons for enacting similar approaches in plants.

## Extracellular Vesicles and RNA Interference in Mammalian Systems

The majority of RNA-based drugs used in the clinic or currently in development are based on antisense oligonucleotides (ASOs) or siRNAs (16). Antisense RNA molecules can be used as a therapeutic strategy to treat neurological disorders (133). However, there are still significant barriers to efficient RNA-based treatment strategies. For example, even when treatment is administered to the target region, mammals possess multiple and redundant barriers to exogenous RNA uptake: dsRNA-degrading RNases are present in blood, and RNA is cleared from the bloodstream via the kidneys (131). Naked dsRNAs do not readily diffuse through mammalian cell membranes due to their size and negative charge, instead relying on endocytosis for internalization, giving rise to the possibility of endosomal capture (131, 171). Once in the cell, longer dsRNAs elicit a nonspecific immune response, necessitating the use of smaller siRNAs and siRNA analogs for induction of gene knockdown (96). To overcome barriers to uptake, the use of nanoparticles for therapeutic delivery has been an active area of research for more than 50 years (158). This approach aims to deliver the appropriate payload quantity to the correct location while reducing dosage frequency and avoiding toxicity (161). Liposomes, which consist of a lipid bilayer enclosing an aqueous volume, are a prominent example of this tactic (142). Though synthetic, they offer good biocompatibility and, in a manner analogous to EVs, can be functionalized with ligands to target specific cell types (161). The first approvals for a lipid nanoparticle–delivered siRNA for human therapeutics were granted in 2018, with the siRNA targeting transthyretin, the cause of transthyretin-induced amyloidosis (2). Liposomes have also been extensively researched for delivery of anticancer siRNAs (110). The protection and delivery of cargos have recently come to prominence due to the accelerated search for a coronavirus disease 2019 (COVID-19) vaccine (124, 144). Indeed, two promising vaccines just authorized for use in the United States are the modified mRNA-1273 vaccine from Moderna, Inc. and the nucleoside-modified RNA BNT162b2 of Pfizer–BioNTech; both are lipid nanoparticle–encapsulated mRNA-based vaccines encoding the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike protein (115, 153).

While similar in structure to liposomes, naturally occurring EVs such as exosomes offer several advantages as RNAi delivery agents, including superior biocompatibility and reduced clearing by phagocytes, as well as improved membrane permeability (85). As EVs have evolved to transport RNA from cell to cell, delivery of dsRNAs by EVs can be highly efficient relative to synthetic liposomes (85). There are, however, drawbacks to EVs; payload loading can be suboptimal, difficult, and laborious, often relying on the use of cultured cells and complex purification protocols, and EVs possess existing cargos that may have unintended impacts (11, 50). Interestingly, a source of EV-like vesicles increasingly researched for delivery of therapeutic drugs are juices of plant products such as grape, grapefruit, and ginger (132, 176). Plant-derived nanoparticles possess many of the therapeutic benefits of their mammalian cell culture counterparts, with components such as phosphatidic acid and phosphatidyl choline aiding in their effectiveness (132). These plant-derived nanoparticles have been shown to deliver a range of payloads, including siRNA into mammalian cells *in vitro* (176, 192). Further breakthroughs in purification and loading, such as selective loading of siRNAs, will likely bring EVs, including plant-derived nanoparticles, to the forefront of therapeutic delivery platform development over the coming years (3).

## Extracellular Vesicles and Nanoparticles as Carriers of RNA Interference for Crop Protection

Given the importance of maximizing the location- and time-specific abundance of RNAi trigger molecules for RNAi-mediated crop protection, strategies that protect the dsRNA at its point of application as well as enhance uptake and transport to local and distal tissues are highly desirable.

Due to their successes in therapeutics, various synthetic and natural nanocarriers are becoming the focus of researchers seeking solutions to the delivery problem (30). A successful example of exploiting inorganic carrier nanoparticles for SIGS-mediated plant protection is BioClay, a complex of layered double hydroxide (LDH) particles and target-specific dsRNA (98, 182). Following foliar application, BioClay has been shown to extend the window of protection against viruses from days to weeks. The LDH component of the complex dissolves under acidic conditions, controlling the rate of release of dsRNA. A factor evident for BioClay and other SIGS approaches is the induction of systemic resistance following exogenous RNAi application without the need for genetic modification (74, 98, 182). Besides BioClay, researchers have found that carbon quantum dots are efficient carriers of dsRNAs for inducing systemic RNAi when used to treat rice striped stem borer larvae (172). Recently, a newly developed nanotube-based delivery platform for direct delivery of siRNA showed high silencing efficiency of endogenous genes in intact plant cells, owing to the effective intracellular delivery and protection of siRNA from nuclease degradation (45). These studies have established new methods for overcoming RNA application limitations and could enable a variety of plant biotechnology applications based on RNAi (45).

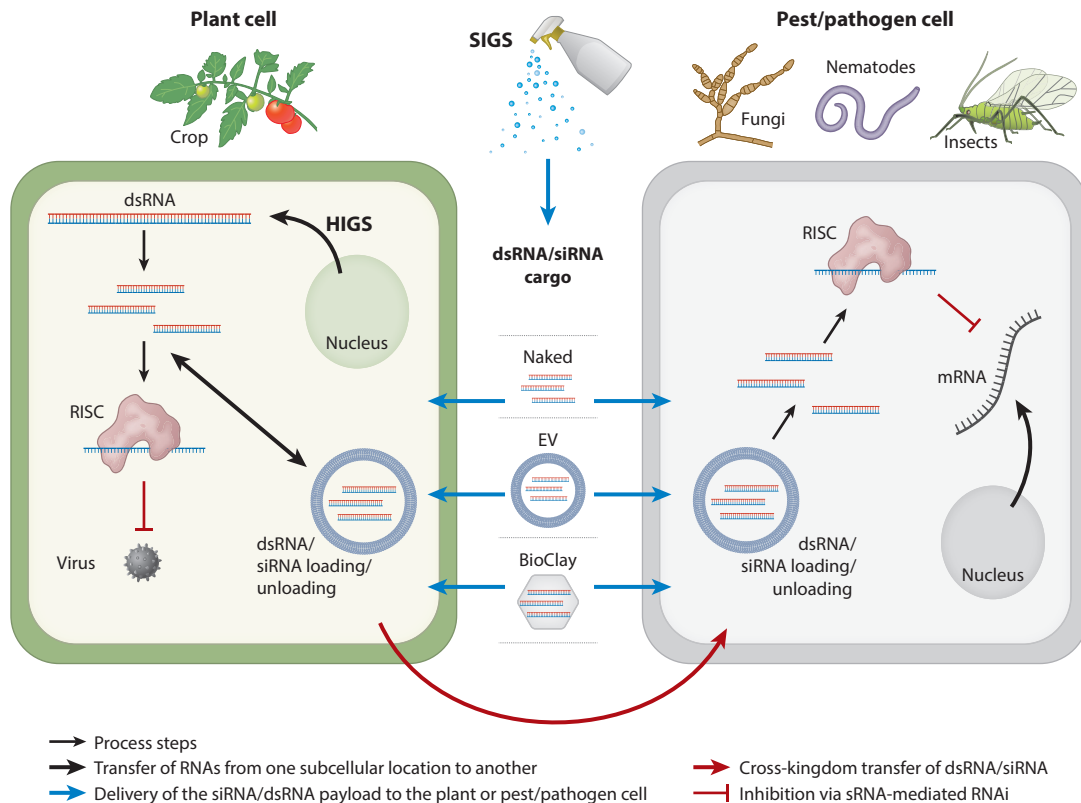
The wealth of research and development carried out on liposomes and EVs for delivery of RNAi in mammalian systems has begun to inform the crop protection field. Indeed, liposomes have been recently used with success to protect and deliver dsRNA to the Neotropical stink bug *Euschistus heros* (32), as well as to the Queensland fruit fly *Bactrocera tryoni* (150). This raises the question, could EVs or organic nanovesicles be adopted for SIGS to further improve RNAi efficiency against target pests and pathogens? As indicated in previous sections, siRNAs derived from fungal *F. graminearum* targeting dsRNAs generated via HIGS, but not SIGS, are present in *Arabidopsis* EVs (76), suggesting that dsRNA or siRNA loading onto EVs prior to exogenous application could offer protective and delivery benefits that the plant itself does not provide (Figure 3).

For protection against plant viruses where the desire is for cellular dsRNA uptake rather than transfer to a pest or pathogen, the benefits of EVs could also be significant. The presence of functional RNA in EVs indicates that even though the purported plant cell wall pore size is approximately 5 nm and EVs are somewhat larger, efficient delivery takes place. As indicated above for fungal EVs, plant EVs may demonstrate sufficient structural plasticity to fit through cell wall pores, or plant cell walls themselves may be flexible to allow EVs and their contents through (181). Synthetic nucleic acid delivery agents such as carbon nanotubes avoid pore size exclusion through a high aspect ratio (44), though their persistence in the environment limits their suitability for crop protection (113). Unlike carbon nanotubes, EVs would be expected to rapidly degrade, which, along with biocompatibility, is a key consideration for regulatory approval as a crop protection product (87).

The ability of functionalized liposomes or naturally occurring EVs to direct payloads to specific cell types is particularly important for therapeutic uses and could also prove beneficial in a crop protection context. Plant viral pathogens can exhibit tissue tropism and infect specific cell and tissue types, such as certain begomoviruses that are restricted to phloem cells (100). The ability to direct dsRNA delivery to these cells could, for example, increase the effectiveness of a begomovirus-targeting dsRNA. Similarly, for insect pests that feed exclusively on phloem sap or for fungi that target specific tissues such as roots, localization of dsRNA could lead to increased uptake and improved efficacy.

As SIGS approaches to crop protection move forward, additional protective and delivery advantages of EV-mediated RNAi approaches will likely become apparent. Marrying the target specificity of RNAi with safe delivery agents represents a paradigm shift from past practices to





**Figure 3**

RNAi and roles for EVs in crop protection. HIGS and SIGS approaches are used to deliver dsRNA/siRNA trigger molecules to crop pests and pathogens including viruses, fungi, nematodes, and insects. Following cellular internalization, RNAi trigger molecules suppress target gene expression and generate host resistance. The HIGS approach produces dsRNA in plant cells via genetic modification, with export, transport, and uptake in pest and pathogen cells likely involving EVs. For SIGS approaches where dsRNA is applied exogenously, nanocarriers such as clays, liposomes, and EVs protect the RNAi cargo and are proposed to enhance uptake. Abbreviations: dsRNA, double-stranded RNA; EV, extracellular vesicle; HIGS, host-induced gene silencing; mRNA, messenger RNA; RISC, RNA-induced silencing complex; RNAi, RNA interference; SIGS, spray-induced gene silencing; siRNA, small interfering RNA.

more sustainable non-GM production systems in the future. Regulatory considerations do, however, exist regarding the environmental deployment of EVs. The ability for EVs to promote unintended impacts to nontarget species by enhancing delivery and cellular uptake of dsRNAs and siRNAs warrants further investigation on a case-by-case basis.

### Extracellular Vesicles as Carriers of Other Beneficial Cargo

Though highly effective for the delivery of nucleic acids, EVs and synthetic vesicles also possess the potential to carry many other beneficial molecules. For therapeutic uses, EV cargos range from anticancer plant-derived bioactives such as celastrol and curcumin to gold nanoparticles for improved imaging (10, 95). The advantages EVs offer for these payloads could provide protection of the cargo from environmental stresses such as digestion in the case of oral delivery as well as transport to target tissues and cells (3, 125). An additional advantage for using EVs in this manner is the reduction in opportunities for adverse cargo interaction with other pharmaceuticals (125). In

contrast to liposomes, EVs are able to cross the blood–brain barrier, which can be desirable for the delivery of drugs to sites such as the central nervous system (158). EV payload types can include hydrophilic molecules in the aqueous center as well as encapsulated hydrophobic molecules in the lipid bilayer (161). Methods for loading different cargos as well as the efficiency of their loading vary greatly and depend on the source of EVs and the type of cargo (52, 161). Understanding and using the endogenous cellular selective sorting and packaging capabilities of EVs have been proposed to improve both loading and delivery of active compounds (52).

The advantages of EVs likely extend to other beneficial cargo for improving plant health, including nutrient delivery and targeted distribution of conventional pesticides. Delivery agents such as gold (13) and silica (82) nanoparticles have been used for these purposes, but, as with their therapeutic use, EVs may offer delivery efficiency and cell-type targeting benefits. Similar to multiple drug loading in liposomes (4), combining dsRNA and other beneficial payloads in a single delivery vehicle may be feasible, further reducing the environmental impacts associated with field application to broadacre crops.

## CONCLUSION

As we have highlighted, both plants and their infecting pathogens release EVs, mediating cross-kingdom communication between plants and microbes. Plants load and release defensive cargos into EVs in response to infection. Specifically, plant EVs deliver sRNAs into pathogens, thereby mediating cross-kingdom RNAi. In addition to sRNAs, mRNA and long noncoding RNA (lncRNA) have been found in mammalian EVs involved in cell-to-cell communication (48, 186). It will be interesting to find out if mRNAs and lncRNAs are also trafficked between plant and pathogen cells.

To support the infection, pathogens may utilize EVs to deliver virulence proteins or RNAs to plant cells. Compared with host cells (e.g., mammalian and plant cells), there exist few studies of EVs in diverse microbial pathogens. In most studies, microbial EVs are isolated from cultured media *in vitro*. As many RNAs and proteins are induced during infection, it would be interesting and important to investigate microbial EVs purified from extracellular fluids of infected plants in order to obtain more details about the contribution of EVs to plant–pathogen interactions.

In animal systems, recent studies showed that different RNA-binding proteins are involved in different types of miRNA sorting in distinct EV subtypes (33, 92, 139, 145, 151, 165). In plants, several RNA-binding proteins, including AGO1 and RNA helicases, also play an important role in selective sRNA loading into EVs (59). Different families of molecules or independent pathways contribute to the formation of exosomes and their subsequent secretion, suggesting that, even among exosomes, different subtypes may exist (78). The heterogeneity of EVs introduces an extra level of complexity in EV research. Recent advances in immunoaffinity isolation have resulted in the development of novel multiplex bead-based immunoaffinity approaches, allowing the precise capture of specific subtypes of EVs. Beads have been coated with antibodies capable of recognizing specific EV protein markers, including tetraspanin CD9, CD81, or CD63 (154). There is a need to develop a more comprehensive understanding of the protein markers and the basic biology of both plant and microbial EVs. Immunoaffinity isolation can be a powerful tool for purifying specific classes of EVs directly from the interaction interface. For example, antibodies that recognize tetraspanins could be utilized for direct capture of plant exosomes, which may then be followed by systematic analysis of plant exosomal RNA, protein, and metabolite cargos. The development of more accurate methods of isolation of plant and microbe EVs will contribute not only to a better understanding of EV origin and function but also to the development of novel EV-based strategies in delivering crop protective measures, such as RNAs.

## SUMMARY POINTS

1. Small RNAs (sRNAs) travel across organismal boundaries between hosts and microbes and silence genes in *trans*, a mechanism called cross-kingdom RNA interference (RNAi). Cross-kingdom RNAi plays an important role in host immunity and microbial virulence.
2. Plant extracellular vesicles (EVs) function as a defense system by delivering sRNAs into pathogens, thereby mediating cross-species and cross-kingdom RNAi to silence virulence-related genes.
3. Both plant and microbial EVs play important roles in cross-kingdom molecular exchange between hosts and interacting microbes to modulate host immunity and pathogen virulence.
4. Host-induced gene silencing (HIGS) and spray-induced gene silencing (SIGS) have the potential to become important eco-friendly disease-control tools and to confer efficient crop protection.
5. For SIGS approaches, the development of novel delivery methods for double-stranded RNAs (dsRNAs) or sRNAs using artificial EVs or nanocarriers will increase the efficiency and longevity of the new generation of RNA-based fungicides.

## FUTURE ISSUES

There are many questions remaining to be answered in the plant EV field:

1. What are the precise biogenesis and secretion pathways for plant and microbial EVs?
2. How are RNAs, proteins, and metabolites selectively loaded into EVs?
3. Are other classes of RNAs transported between plants and pathogens?
4. Do pathogen EVs contain effector molecules that are functional within host plant cells?
5. How can EVs cross the plant–pathogen interface and cell walls and then enter specific recipient cells?
6. How are EVs taken up by the recipient cells?
7. How are different cargos released from EVs into recipient cells?
8. What are the other communication molecules carried by EVs?

## DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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175. This is the first study that demonstrated fungal RNA uptake from environment.

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