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Extracellular vesicles as key mediators of plant–microbe interactions

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Extracellular vesicles (EVs) are lipid compartments capable of trafficking proteins, lipids, RNA and metabolites between cells. Plant cells have been shown to secrete EVs during immune responses, but virtually nothing is known about their formation, contents or ultimate function. Recently developed methods for isolating plant EVs have revealed that these EVs are enriched in stress response proteins and signaling lipids, and appear to display antifungal activity. Comparison to work on animal EVs, and the observation that host-derived small interfering RNAs and microRNAs can silence fungal genes, suggests that plant EVs may also mediate trans-kingdom RNA interference. Many fundamental questions remain, however, regarding how plant EVs are produced, how they move, and if and how they are taken up by target cells.

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Introduction

Extracellular vesicles (EVs) are small, membrane-enclosed structures released from a cell into the surrounding environment. In all three domains of life, EVs are important vehicles of intercellular communication. They serve as protective compartments for the long-distance transport of signal molecules, including proteins, nucleic acids, lipids and other metabolites. EVs are generally grouped according to how they are formed and divided into one of three classes: apoptotic bodies, microvesicles or exosomes. Apoptotic bodies are the largest and most heterogeneous of the three classes. They form when pieces of membrane bleb off of dead or dying cells. Microvesicles (MVs) bud directly from the plasma membrane, while exosomes originate within endosomal compartments known as 'multivesicular bodies' (MVBs) and are secreted from the cell when MVBs fuse with the plasma membrane [1,2].

The majority of EV research has been conducted in mammalian systems. Mammalian EVs play a crucial role in modulating immune responses and have been shown to traffic functional RNA molecules between cells. The clinical relevance of mammalian EVs combined with their ability to transport RNA have boosted research into their biology and lead to the development of EV-based therapies and diagnostic tests [1,2]. As the methods for isolating and characterizing EVs improve, researchers are beginning to explore how EVs influence physiology and environmental responses across a wide range of organisms. For example, research into bacterial and protozoan EVs has revealed that pathogens and parasites secrete vesicles containing important virulence factors [1]. Of particular interest, EVs from plant pathogenic bacteria are associated with microbe-associated molecular patterns (MAMPs), including elongation factor-thermo unstable (EF-tu), and can trigger an immune response in *Arabidopsis* [3,4]. Similarly, work with *Caenorhabditis elegans* and *Drosophila melanogaster* has revealed that EVs in both model organisms regulate development and influence mating behaviors [1].

Plant EVs

Plant cells also secrete EVs, although very little is known about their origins, composition or function. Release of EVs by plant cells was first observed in the 1960s using electron microscopy [5,6]. Observations made with both electron and light microscopy suggest that plant EVs contribute to localized immune responses. During fungal and bacterial infections, MVBs accumulate in plant cells and localize to regions of pathogen attack. In a process analogous to mammalian exosome secretion, MVBs fuse with the plasma membrane and release intraluminal vesicles (ILVs) into the apoplastic space [5,7•,8•,9]. Secreted vesicles become embedded within defensive barriers known as 'papillae', aiding their formation [10••,11]. EVs have also been observed in the extrahaustorial matrix (EHMx), a region between the plant cell membrane and an invading fungal feeding tube called a 'haustorium', which penetrates the plant cell wall and becomes enveloped in the host plasma membrane [12•]. The presence of vesicles in this region suggests that plants deliver antimicrobial agents to invading fungi. Plant EVs are known to contain antimicrobial compounds as well as defense related proteins, including the SNARE (soluble *N*-ethylmaleimide-sensitive-factor association protein receptor) protein SYNTAXIN121 (SYP121)/

PENETRATION1 (PEN1) and the ABC transporter PENETRATION3 (PEN3) [8•,10••,13]. In fact, a large percentage of defense proteins secreted in response to stress and pathogens lack canonical signal peptides and may therefore rely on unconventional secretory routes, such as EVs, in order to leave the cell [14–16].

Recently developed procedures for isolating plant EVs

Procedures for isolating and purifying plant EVs have developed over the last decade. Initially, fluids collected from water-imbibed sunflower seeds and vacuum-infiltrated tomato leaves were found to contain phospholipids [17,18,19•]. The proportions of lipids in the extracellular fluids differed considerably from their tissues of origin and were altered in response to abiotic stress hormones. Extracellular lipids in both fluids could be isolated using differential centrifugation and were associated with trafficking and defense-related proteins [17,18,19•]. When Regente *et al.* [19•] used electron microscopy to examine a lipid pellet derived from sunflower seed wash, they observed numerous small vesicles ranging in size from 20 to 200 nm in diameter, each possessing a lipid bilayer. Using similar methods of differential ultracentrifugation, Prado *et al.* [20] was able to isolate vesicles from germinating olive (*Olea europaea*) pollen. These so called 'pollensomes' were also associated with trafficking and defense-related proteins, as well as known allergens. Recently, our lab found that the apoplastic wash from whole *Arabidopsis thaliana* rosettes contained lipid-bilayer vesicles, 50–300 nm in diameter [21••]. These vesicles were enriched for the known plant EV marker PEN1, as well as proteins involved in stress and defense responses. In line with these findings, we showed that *Arabidopsis* plants secrete greater quantities of EVs in response to infection with *Pseudomonas syringae* or treatment with salicylic acid [21••]. An important advance in this work was the use of multiple endosomal markers to establish that the isolated EVs were not derived from broken cells, and the use of a density gradient to obtain highly purified vesicles.

In should be noted that other studies have claimed to isolate exosome-like vesicles from different fruits and vegetables [22]. These studies are important for understanding the intestinal responses to different foods and may one day influence designs for drug delivery. However, according to the guidelines suggested by the *International Society for Extracellular Vesicles* (ISEV), the methods used to generate 'exosome-like' vesicles in these studies (i.e. grinding and juicing) are entirely too destructive to produce legitimate EVs [23]. It is more accurate to say that these studies investigated microsomal fragments.

Long-distance RNA transport

The ability to transport nucleic acids is a hallmark characteristic of EVs across all three domains of life [24–26]. The RNA content of plant EVs has not yet been examined, but it seems reasonable to predict that they also traffic RNA. Plants are capable of systemically transporting viral RNAs, mRNAs, microRNAs (miRNAs) and small interfering RNAs (siRNAs) through the phloem [27]. Loading of RNA into the phloem is thought to occur through plasmodesmata (PD) and involves RNA-binding proteins capable of increasing the PD size exclusion limit. Such proteins have been shown to mediate local RNA transport in mesophyll cells and while they have been detected in the phloem with mRNA, they have never been decisively shown to mediate the long-distance transport of RNAs [27–30]. EVs could represent an alternative pathway for loading RNAs into the phloem and may even transport RNA through the phloem or apoplast. Our proteomic data for *Arabidopsis* EVs revealed several previously identified phloem proteins that may interact with the Phloem Protein2-A1 (PP2-A1) [31]. In *Cucumis*, PP2 is thought to facilitate the long-distance transport of RNA [30]. While we did not detect AtPP2-A1 in *Arabidopsis* EVs, we did identify classes of proteins implicated in long-distance RNA transport in species of *Cucurbita* and *Cucumis*, including calcium-dependent lipid-binding proteins and lectins [21••,28–30]. In addition, we identified the RNA binding protein GLYCINE-RICH RNA BINDING PROTEIN 7 (GRP7) in Arabidopsis EVs, suggesting that RNA is packaged into EVs [21••].

Notably, EVs and MVBs have been shown to accumulate around plasmodesmata during fungal infections, and are thought to facilitate deposition of callose in and around plasmodesmata in order to block connections between living cells and cells undergoing hypersensitive cell death [7•]. The *Arabidopsis* EV proteome contains SYNAPTOTGAMIN A, which is known to interact with viral movement proteins and facilitates their movement into adjacent cells through plasmodesmata [21••,32]. Thus, EVs could potentially regulate plasmodesmata function in both negative and positive ways depending on the context.

An even more exciting possibility is that plant EVs mediate the interspecies transfer of RNAs. Plants are capable of silencing foreign transcripts through a form of RNA interference (RNAi) known as Host-Induced Gene Silencing (HIGS) [33,34]. In recent years, HIGS has been used to engineer resistance to a broad range of pests and pathogens, especially fungi. During fungal infections, double-stranded RNAs (dsRNAs) expressed in the plant are able to move from the host cell into the invading fungus where they target the expression of key housekeeping genes and virulence factors [33]. The effects of HIGS are generally not observed until after formation of haustoria, and silencing is more effective against genes that are highly expressed in haustoria than genes expressed in other cell types [34–36]. For these reasons, the transfer of RNA into pathogens is thought to

occur across haustoria or similar feeding structures. Because these structures maintain both the host and pathogen plasma membranes, plant EVs are the most likely candidate for delivering RNAs into pathogens [12•,34,36].

Targeting of fungal genes by host-derived RNAs appears to be an important component of the plant immune system. The highly conserved and expressed plant microRNAs miR159 and miR166 were recently shown to target genes in the fungal pathogen *Verticillium dahliae*, reducing its virulence on cotton [37••]. Importantly, this work showed that mutating the target genes in *V. dahliae* to make them insensitive to these miRNAs increased the virulence of *V. dahliae*. Again, EVs are attractive candidates for mediating the transfer of miRNAs from host to pathogen.

The transport of RNA between plants and fungi appears to be bidirectional. Small RNAs from the fungal pathogen *Botrytis cinerea* have been shown to target host defense genes in Arabidopsis and tomato [38]. Furthermore, using HIGS to silence *B. cineria Dicer-like 1* and *Dicer-like 2* genes dramatically reduces *B. cinerea* virulence, suggesting that small RNAs play an important role in fungal infection [39].

Long distant transport of defense compounds

Plant EVs may mediate the transport of important defense compounds, including glucosinolates (GSLs). GSLs are nitrogen and sulfur-containing secondary metabolites found mainly in Brassicaceae plants. Enzymes known as myrosinases hydrolyze GSLs to produce bioactive compounds, many of which are toxic to invading pests [40]. The presence of several GSL transporter proteins in the EV proteome suggests that EVs may mediate GSL transport [21••]. For example, the abundant EV protein PEN3 is believed to transport GSLs, and functions in conjunction with the peroxisome-localized myrosinase PEN2 [41]. During fungal or oomycete infections, PEN2 hydrolyzes specific tryptophan derived indole GSLs to produce antifungal compounds [42,43]. Plasma membrane-localized PEN3 is thought to secrete these compounds into the apoplast at sites of pathogen contact. However, the presence of PEN3 in EVs suggests that EVs themselves may be loaded with toxic molecules that PEN3 unloads into papillae, the EHMx or perhaps even pathogens [13]. In support of this idea, a recent report from Regente *et al.* [44••] suggests that EVs from sunflower seedlings are taken up by *Sclerotinia sclerotiorum* spores and negatively affect their growth [44••].

Plants can also transport GSLs bi-directionally through the phloem, allowing them to store cytotoxic compounds safely away from where they were synthesized and to reallocate defensive compounds in response to stress or attack. The mechanisms behind GSL long distance transport remain largely unknown, although GLUCOSINOLATE TRANSPORTER-1 (GTR1) and GLUCOSINOLATE TRANSPORTER-2 (GTR2) are involved in GSL movement into and out of the phloem [45]. *Arabidopsis* EVs contain GTR1 as well as other proteins involved in the GSL metabolism, including the myrosinase EPITHIOSPECIFIC MODIFIER1, suggesting that EVs may be involved in some aspect of GSL transport or metabolism [21••].

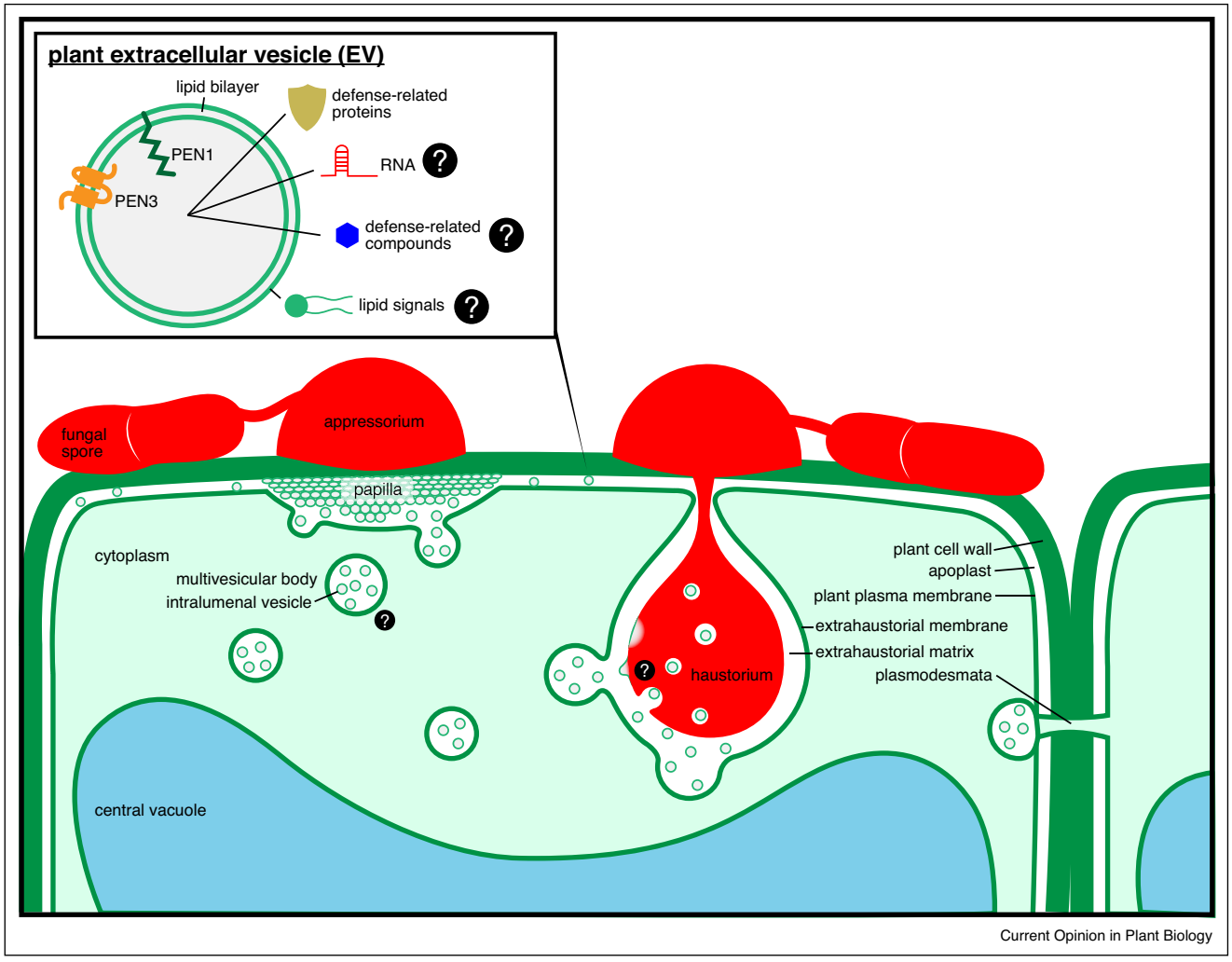
EVs as lipid signals

It is possible that the lipids comprising plant EVs may themselves serve as signal molecules. Extracellular fluids from cultured tomato cells and water-imbibed sunflower seeds contain high amounts of phosphatidic acid (PA) and phosphatidylinositol-4-phosphate (PI4P) [17,18,46]. Both phospholipids accumulate in response to pathogen infection, elicitor treatment or stress hormones and can modulate defensive signaling [18,47,48]. PA functions upstream of important immune responses and positively regulates immunity by activating mitogen-activated protein kinase (MAPK) signaling, triggering the production of reactive oxygen species (ROS) and affecting the activities of ion channels [47,48]. Similarly, exogenous application of PI4P can stimulate immune responses, including the production of ROS, defense gene transcription and initiation of the hypersensitive response [46]. PA and PI4P incorporated into the EV membrane may allow secreted vesicles to regulate plant immune responses. The lipid content of *Arabidopsis* EVs has not yet been analyzed, but the proteome includes phospholipase C and phospholipase D, which contribute to separate pathways for PA synthesis. This suggests that *Arabidopsis* EVs at least have the ability to generate PA [21••].

Traveling beyond the cell wall

The idea that plant EVs could mediate intercellular communication has been questioned on the grounds that the cell wall should prevent anything as large as a vesicle from passing through it. If this were true, however, we should not be able to isolate vesicles from intercellular wash fluid. Somehow, plant EVs are making it past the wall. This puzzling scenario is not unique to plants. Other cell-walled organisms, including fungi, Gram-positive bacteria and mycobacteria, secrete EVs in spite of their surrounding barriers [49,50]. How these organisms accomplish EV secretion is just as mysterious, although there are possible explanations. Mechanical forces, such as turgor pressure, may force the EVs through the wall. Alternatively, organisms may release EVs by regulating cell wall thickness, pore size or integrity. A third possibility is that EVs are associated with enzymes capable of modifying the cell wall, in which case they may stimulate cell wall remodeling [49,50]. To understand how plant EVs move through cell walls, we need a deeper understanding of the cell wall as a dynamic, living structure. More information on the biophysical properties of plant EVs is also needed. As lipid structures, it is possible that EVs can compress as they move through pores in the cell wall.

Figure 1



Summary of current plant EV knowledge and research questions for the future. For decades, plant EVs have been shown to accumulate in infected cells, underneath sites of pathogen attack and papillae formation. These EVs are thought to contribute to the development of defensive barriers. Plant EVs form inside of multivesicular bodies (MVBs), but questions remain about whether a subset of these compartments are specialized for EV secretion. Plant cells may also secrete different populations of EVs through a variety of mechanisms. EVs have also been observed around fungal haustoria and inside the extrahaustorial matrix. Plant EVs containing antimicrobial compounds or RNAs may deliver their cargo into fungal haustoria either by fusing with the fungal membrane or being taken up through endocytosis. Some evidence already suggests that germinating fungal spores can take up plant EVs, although the process by which uptake occurs is unknown [44••]. Finally, plant EVs accumulate around plasmodesmata in infected cells and are thought to block adjacent cells from HR-stimulating signals. Isolated plant EVs are bilipid structures enriched for proteins involved in stress and defense responses. Similar to mammalian EVs, plant EVs may also contain RNAs, although the RNA content of plant EVs has not yet been examined. Proteins involved in glucosinolate metabolism also suggest the plant EVs contain antimicrobial compounds. Furthermore, a lipid analysis of extracellular plant fluids suggests that plant EVs may be composed of important defense-related lipid signals.

The biogenesis of plant EVs

It is assumed that plant EVs are analogous to mammalian exosomes, but in truth, very little is known about the biogenesis of EVs in plants. Transmission electron microscopy studies have shown that plant EVs are released from structures similar to MVBs, but these compartments look different from MVBs imaged in other tissues. In roots, MVBs are uniform in structure, ranging in size from 200 to 500 nm in diameter with small intraluminal vesicles ~35 nm in diameter [51]. Comparatively, MVBs that accumulate underneath papillae are often irregular in shape, range in size from 300 nm to 3 μm and the internal membranous compartments are a diverse set of vesicles and tubules [8•]. Late endosomes do localize to regions of EV secretion, as evidenced by the accumulation of MVB-associated Rab5 GTPases ARA6 and ARA7 at sites of pathogen attack. Furthermore, ARA6 has been shown to regulate the formation of PEN1

secretory complexes at the plasma membrane, and the activation of Rab GTPases by GTP exchange factors (GEF) is required for the secretion of PEN1 [11,52,53]. However, it seems as though multiple endosomal pathways contribute to EV secretion at different stages of infection. For example, ARA6 and ARA7 are largely dispensable for the secretion of PEN1 into papillae but affect its accumulation in haustorial encasements [52]. The evidence suggests that endosomal compartments involved in EV secretion differ from MVBs responsible for delivering materials to the vacuole, and there may be specialized classes of endosomes for secreting EVs under different circumstances.

In mammalian systems, EVs actually represent a cocktail of secreted vesicles with different origins and functions. It is entirely possible that there are several independent mechanisms for producing EVs in plants, and these may include the secretion of vesicles by the exocyst-positive organelle (EXPO) or fusion of the vacuole with the plasma membrane [54,55]. Another possibility is the existence of secretory autophagosomes. In yeasts and mammals, autophagosomes contribute to the unconventional secretion of proteins. How exactly this is accomplished is a matter of debate. It is thought to involve fusion events between autophagosomes and MVBs and may lead to the release of EVs [56–58]. A similar process may occur in plants, which could account for the unusual morphology of MVBs underneath papillae.

Conclusions

Plant EVs were first observed in the 1960s, roughly fifteen years before the discovery of mammalian exosomes. Despite a sizable head start, research into plant EVs has languished for over half a century. In contrast, mammalian EV research rapidly expanded, mushrooming into a large and varied field with promising medical applications. The ability to isolate vesicles from biological fluids and conditioned media undoubtedly contributed to the meteoric rise of mammalian EV research. Plant biologists, on the other hand, have had to be content with observing EVs squashed into the margins of cells and wondering what role they might play. Emerging techniques for isolating plant EVs promise to finally elevate this field of research. New discoveries are slowly verifying the role of plant EVs in immunity and hint that these external organelles mediate signaling and communication throughout plants or even into invading pathogens (Figure 1). These findings will impact our understanding of plant immunity and challenge the traditional view of signaling in plants. Although this review focuses on the involvement of plant EVs in defense responses, it is probable that EVs contribute to several aspects of plant physiology including reproduction and symbiotic relationships. The universal nature of EVs further suggests that any discoveries in plants could advance our understanding of mammalian EVs. Plant EVs may even find a place in emerging industries as therapeutic tools in human health. At the moment, the field is new and ripe with unanswered questions. It is time for a harvest.

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