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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