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CURRENT REVIEW

Extracellular Vesicles in the Arbuscular Mycorrhizal Symbiosis: Current Understanding and Future Perspectives

Samuel Holland and Ronelle Roth†

Department of Biology, University of Oxford, Oxford OX1 3RB, U.K.

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The arbuscular mycorrhizal (AM) symbiosis is an ancient and highly conserved mutualism between plant and fungal symbionts, in which a highly specialized membrane-delimited fungal arbuscule acts as the symbiotic interface for nutrient exchange and signaling. As a ubiquitous means of biomolecule transport and intercellular communication, extracellular vesicles (EVs) are likely to play a role in this intimate cross-kingdom symbiosis, yet, there is a lack of research investigating the importance of EVs in AM symbiosis despite known roles in microbial interactions in both animal and plant pathosystems. Clarifying the current understanding of EVs in this symbiosis in light of recent ultrastructural observations is paramount to guiding future investigations in the field, and, to this end, this review summarizes recent research investigating these areas. Namely, this review discusses the available knowledge regarding biogenesis pathways and marker proteins associated with the various plant EV subclasses, EV trafficking pathways during symbiosis, and the endocytic mechanisms implicated in the uptake of these EVs.

Keywords: arbuscular mycorrhizal symbiosis, EXO70, exosome, extracellular vesicle, microvesicle, PENETRATION1, SNARE, syntaxin, tetraspanin

Background

The arbuscular mycorrhizal (AM) symbiosis is an ancient mutualistic symbiosis between fungal species within the Glomeromycotina subphylum and an estimated majority of land plants, evolving in the most recent common ancestor of the land plant lineage (Parniske 2008; Remy et al. 1994). This nutrient exchange between plant and fungal symbiont takes place in highly specialized symbiotic structures, known as arbuscules. After penetrating the plant root epidermis, AM fungal hyphae spread to the inner cortex, in which the fungus penetrates the plant cell wall but, crucially, not the plant plasma membrane (PM) and differentiates to produce the highly branched arbuscule (Parniske 2008). As the fungal arbuscule grows, large-scale remodeling of the plant PM produces the periarbuscular membrane (PAM) to which symbiotic nutrient transporters are directed (Harrison et al. 2002; Parniske 2008). Between the fungal arbuscule and the PAM is a shared matrix, derived from the apoplast, called the periarbuscular space (PAS) within which nutrients and signals are exchanged (Parniske 2008). Intimately regulated bidirectional biomolecule transport, nutrient exchange, and signaling defines AM symbiosis, yet a mechanistic understanding of the inter-organismal communication that underpins this interaction remains largely unknown; extracellular vesicles (EVs), however, appear as increasingly viable candidates as mediators of this crosstalk.

EVs are nonreplicating membrane-bound nanoparticles, naturally released from cells, that contain diverse biomolecular cargoes such as proteins, lipids, RNAs and metabolites and are implicated in numerous physiological processes (Doyle and Wang 2019; Théry et al. 2018; van Niel et al. 2018). The heterogeneity of observed EVs has hampered the characterization and manipulation of their properties and functions, although three conserved EV subclasses, differing in their biogenesis pathways (Mathieu et al. 2019; Teng and Fussenegger 2021; van Niel et al. 2018), are known in eukaryotes, namely, multivesicular body (MVB)-derived exosomes, shedding microvesicles (sometimes referred to as ectosomes), and apoptotic bodies associated with programmed cell death (Díaz-Garrido et al. 2021; Liu et al. 2021). EVs are ubiquitous in life with observations in all major eukaryotic and prokaryotic taxa (Woith et al. 2019), playing essential roles in intercellular signaling and transport within and between organisms in both animals and plants (Chen et al. 2021; Simeone et al. 2020). Initial electron microscopic observations of plant-derived EVs from the mid-1960s (Halperin and Jensen 1967) have been greatly expanded upon in the decades since, and four putative EV subclasses have been described in plants: TETRASPANIN 8/9 (TET8/9)-positive MVB-derived exosomes (Cai et al. 2018), PENETRATION 1 (PEN1)-positive EVs (Rutter and Innes 2017), exocyst-positive organelle (EXPO)-derived EVs (Wang et al. 2010), and pollensomes (Prado et al. 2014).

Despite initial observations of extracellular paramural bodies in AM symbiosis in the 1970s (Cox and Sanders 1974), the potential roles of EVs in the bidirectional biomolecule transport, nutrient exchange, and signaling that defines AM symbiosis has largely gone unresearched. As such, this review aims to compile

†Corresponding author: R. Roth; ronelle.roth@biology.ox.ac.uk

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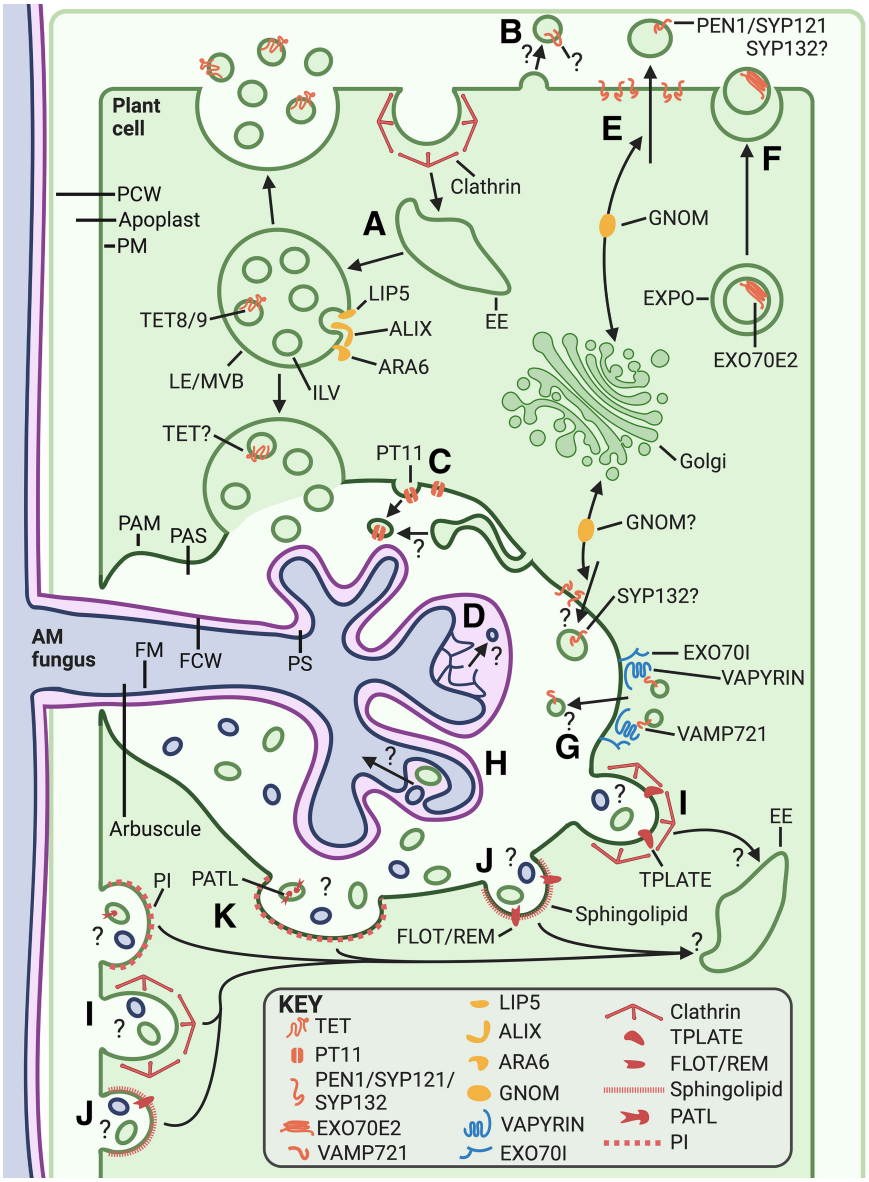
much of the understanding of the roles of the plant endomembrane system in EV biogenesis, trafficking, and uptake in relation to AM symbiosis, articulating current knowledge and avenues for future research efforts.

Exosomes

The best-characterized biogenesis pathway of an EV subclass in animals is that of exosomes derived from the endocytic pathway (Fig. 1A). Maturation of early endosomes to late endosomes and inwards budding of the late endosome membrane produces intraluminal vesicles (ILVs) within a MVB, with subsequent fusion of the MVB to the plasma membrane (PM) releasing ILVs as exosomes (Gurung et al. 2021; Mathieu et al. 2019; van Niel et al. 2018). ILV budding is driven either by the sequential action of ENDOSOMAL SORTING COMPLEX REQUIRED FOR TRANSPORT (ESCRT) proteins or through an ESCRT-independent ceramide-mediated pathway (Gurung et al. 2021; Hanson and Cashikar 2012). Plants contain orthologs of most ESCRT proteins (Paez-Valencia et al. 2016), some of which are associated with ILVs (Buono et al. 2017), but a comprehensive understanding of ESCRT-regulated EV biogenesis and release in planta remains largely unclear. Homologs of ESCRT-associated proteins have, however, been identified in lemon (*Citrus limon*)-derived EVs, including homologs of ESCRT-I subunits TUMOR SUSCEPTIBILITY GENE 101 (TSG101), VACUOLAR PROTEIN SORTING-ASSOCIATED PROTEIN 28 (VPS28), and VPS37 (Henne et al. 2011; Raimondo et al. 2015), suggesting secretion of MVB-derived exosomes. Moreover, homologs of endosomal cargo-sorting proteins were also identified in lemon EVs, including TSG101, CHARGED MULTIVESICULAR BODY PROTEIN 4 (CHMP4), and CHMP6 (Bishop and Woodman 2001; Raimondo et al. 2015; Yorikawa et al. 2005). In *Arabidopsis thaliana*, ALG-2 INTERACTING PROTEIN X (ALIX) associates with ESCRT-II subunits to package PHOSPHATE TRANSPORTER 1 (AtPHT1;1) into ILVs and is essential for correct MVB biogenesis (Cardona-López et al. 2015; Kalinowska et al. 2015); ALIX homologs have been characterized as exosome biomarkers and are also involved in MVB biogenesis in both *Homo sapiens* (Matsuo et al. 2004) and *Saccharomyces cerevisiae* (Odorizzi et al. 2003).

ALIX is also implicated in the ceramide-mediated pathway through associations with flotillins and ceramides, the latter produced from sphingomyelin by a neutral sphingomyelinase, in lipid raft microdomains of the endosome membrane (Trajkovic et al. 2008). The relevance of the ceramide-mediated ILV bud-



ding pathway in plant exosome biogenesis remains unclear, although glycosylinositolphosphoceramides (GIPCs), a class of sphingolipids that are major constituents of plant lipid rafts (Gronnier et al. 2016), are enriched in *Arabidopsis* rosette leaf EVs (Liu et al. 2020). Interestingly, GIPCs were recently implicated in mutualistic symbioses. In *Medicago truncatula*, expression of the GIPC glycosylase GLUCOSAMINE INOSITOL PHOSPHORYLCERAMIDE TRANSFERASE1 (GINT1) is upregulated in symbiotic tissues, with silencing of *GINT1* promoting early arbuscule senescence (Moore et al. 2021).

In AM symbiosis, an increased accumulation of cytoskeletal components, endoplasmic reticulum, and MVBs is observed during the development of the plant prepenetration apparatus and arbuscules (Genre et al. 2008), suggesting MVB biogenesis, and possibly exosome secretion, is also responsive to symbiotic signaling. Compelling evidence for exosome biogenesis and secretion in AM symbiosis came from a recent ultrastructural study in rice (*Oryza sativa*) in which transmission electron microscopy (TEM) of the PAM and PAS showed MVB fusion to the PAM and release of exosome-like EVs to the PAS (Fig. 1A) (Roth et al. 2019). Future work to isolate these EVs and to identify and characterize their cargos will provide greater understanding of symbiotic crosstalk in AM symbiosis and is likely to provide novel mechanistic insights into arbuscule formation, function, and collapse.

MVB biogenesis (An et al. 2006a, b; Wang et al. 2014) and EV secretion (Rutter and Innes 2017) increases in response to plant pathogens; inoculation of *Arabidopsis* with *Pseudomonas syringae* increased MVB biogenesis, which was attributed to the activity and upregulation of LYST-INTERACTING PROTEIN 5 (LIP5) (Wang et al. 2014). LIP5 plays an essential role in MVB biogenesis by interacting with the AAA ATPase SUPPRESSOR OF K^+ TRANSPORT GROWTH DEFECT 1 (SKD1) to regulate the ATP-dependent disassembly and dissociation of ESCRT proteins from MVB membranes (Haas et al. 2007). Accumulation of MVBs and exosome-like paramural vesicles was abolished in *lip5* mutants, which showed increased susceptibility to *P. syringae* despite flg22- and salicylic acid-dependent immune signaling being unaffected, suggesting MVB-derived exosomes are necessary for basal immunity to *P. syringae* (Wang et al. 2014).

Tetraspanins, a highly conserved membrane protein superfamily implicated in numerous cellular processes, are essential for exosome biogenesis through an ESCRT-independent pathway and are used as marker proteins in animal studies due to their enrichment in exosomal membranes, although not without controversy (Andreu and Yáñez-Mó 2014). Mammalian tetraspanins show broad or tissue-specific distributions suggesting functional diversification within the protein family (Andreu and Yáñez-Mó 2014), which has been supported by expression analysis of the *Arabidopsis* tetraspanin family (Reimann et al. 2017). CD63, a well-characterized human exosome biomarker with broad tissue distribution (Andreu and Yáñez-Mó 2014), shows structural homology to the *Arabidopsis* TETRASPANIN 8 (TET8) (Cai et al. 2018). TET8 has been proposed as a plant exosome biomarker, following fluorescent imaging that demonstrated co-localization with ARA6 (Cai et al. 2018), a Rab5-like GTPase previously established as an MVB marker (Ebine et al. 2011). *Arabidopsis* TET8 has also been implicated in exosome biogenesis, with *tet8* mutants secreting fewer EVs relative to wild-type plants (Liu et al. 2020). Interestingly, application of GIPCs was able to partially rescue this phenotype (Liu et al. 2020), suggesting interplay between a TET8-dependent and ceramide/GIPC-dependent pathway for exosome or MVB biogenesis in *Arabidopsis*. TET8 has also been implicated in transcriptional responses to plant-microbe interactions in *Arabidopsis* and common bean (*Phaseolus vulgaris*), with *AtTET8* transcription induced upon *Botrytis cinerea* infection (Cai et al. 2018) and *PvTET8* induced in response to pathogen elicitors, Nod-factors, or *Rhizobium tropici* inoculation (Jimenez-Jimenez et al. 2019). Interestingly, *TET8* transcription was not increased in *Phaseolus vulgaris* inoculated with AM fungi *Rhizophagus*

Fig. 1. Potential biogenesis and uptake pathways of extracellular vesicles (EVs) in arbuscular mycorrhizal (AM) symbiosis. **A,** Exosome biosynthesis involves budding of the multivesicular body (MVB) membrane to produce intraluminal vesicles (ILVs) and subsequent fusion of an MVB with the plasma membrane (PM) releasing the ILVs as exosomes. MVBs have been observed fusing to the periarbuscular membrane (PAM), supporting the release of exosomes into the periarbuscular space (PAS) during AM symbiosis. ALIX (ALG2-INTERACTING PROTEIN X), ARA6, and LIP5 (LYST-INTERACTING PROTEIN 5) have been implicated in correct MVB biogenesis and ILV budding in *Arabidopsis thaliana*, and the plant tetraspanins TET8 and TET9 have been suggested as plant exosome marker proteins. Further investigation is needed to identify whether any TET proteins act as biomarkers for MVB-derived EVs observed in the PAS. **B,** Microvesicles typically result from outward budding of the PM, although, to date, limited evidence supports PM-derived microvesicles in planta and, thus, no marker proteins have been proposed for PM-derived plant microvesicles. **C,** Observations that EVs in the PAS contain the PAM-specific membrane transporter PT11 (PHOSPHATE TRANSPORTER 11) in *Oryza sativa* provides strong evidence for microvesicle secretion from PAM budding. Membrane tubules (memtubs) have also been observed in the PAS, which contain vesicular regions separated from the tubule by constrictions that have been suggested to be microvesicles in the process of excision. **D,** Paramural memtubs have also been observed in AM fungi, potentially suggesting fungal EVs formed from budding memtubs are also released to the PAS during AM symbiosis. **E,** The biogenesis pathway of PENETRATION 1 (PEN1)-positive EVs has not yet been elucidated, although the ARF-GTP exchange factor GNOM is implicated in PEN1 recycling between the PM and the *trans*-Golgi network. PEN1/SYNTAXIN OF PLANTS 121 (SYP121) acts as a marker protein for this EV subclass. It is unknown if SYP132 also acts as a marker for PEN1/SYP132-positive EVs secreted to the apoplast or whether GNOM also facilitates secretion of SYP132-positive EVs to the PAS. **F,** EXPO-derived EVs are released following fusion of an EXPO with the PM. EXO70E2 (EXOCYST SUBUNIT EXO70 FAMILY PROTEIN E2) has been identified as a marker protein for exocyst-positive organelle (EXPO)-derived EVs. **G,** The plant-specific VAPYRIN protein interacts with both EXO70I and members of the VESICLE-ASSOCIATED MEMBRANE PROTEIN 721 (VAMP721) clade during AM symbiosis, with these proteins localizing either at or adjacent to the PAM. Arbuscule development is impaired in both *vapyrin* and *exo70i* mutants, suggesting a role for these proteins in inter-symbiont communication. Determining whether an EXO70-VAPYRIN-VAMP721 complex acts in AM symbiosis to secrete VAMP721-containing EVs to the PAS requires further investigation. **H,** Observations that the fungal pathogen *Sclerotinia sclerotiorum* internalizes sunflower (*Helianthus annuus*)-derived EVs through an unidentified endocytic pathway, suggesting AM fungi may also internalize EVs from the PAS through endocytosis. **I,** Clathrin-mediated endocytosis may internalize EVs from the PAS or apoplast into the plant cell, as the major plant endocytic pathway. Subunits of the TPLATE complex, which is involved in clathrin-mediated endocytosis, are found at the PAM. Like other endocytic pathways shown, the fate of any internalized EVs is unknown, but they may enter the endosomal system through early endosomes (EEs). **J,** Lipid raft–mediated endocytosis may also internalize EVs from the PAS or apoplast, in which membrane microdomains enriched in sphingolipids and containing flotillin and remorin protein complexes act as the site of endocytosis. Flotillin and remorin proteins have been associated with symbiotic membranes and have been identified in plant-derived EVs. **K,** Phosphatidylinositols (PIs) may internalize EVs from the PAS or apoplast, having been associated with endocytosis of ectomycorrhizal effectors. Patellins, a protein family that has PI-binding activity, have been identified in plant-derived EVs, providing a basis for this hypothesis that requires future investigation. FCW = fungal cell wall, FM = fungal membrane, FMT = fungal memtub, FLOT/REM = flotillin/remorin, LE = late endosome, PCW = plant cell wall, PATL = patellin, PS = paramural space. Protein and lipid color denotes function: blue = proteins associated with exocytic trafficking during AM symbiosis, orange = EV biomarkers, red = endocytosis-associated proteins that may be involved in EV uptake, and yellow = EV biogenesis-associated proteins. Created with Biorender.com.

irregularis (Jimenez-Jimenez et al. 2019), suggesting that increased TET8 expression is a specific response within root nodulation symbiosis (RNS) or a highly dynamic and transient response during AM symbiosis. Although it is still unclear if *TET8* transcription directly correlates with exosome secretion, confirmation of this would provide support for increased secretion of TET8-positive exosomes in mutualistic plant-microbe interactions. AtTET8-positive EVs derived from *Arabidopsis* during infection by *B. cinerea* were previously found to carry small RNAs, with 32 identified putative targets in *B. cinerea* functioning in biosynthetic pathway, fungal metabolism, and vesicle trafficking (Cai et al. 2018). These results support a role for TET8-positive EVs in cross-kingdom RNA interference (RNAi) in plant-microbe interactions, offering an exciting line of enquiry for the role of EVs and small RNAs in modulating AM symbiosis if TET8 is identified as an EV biomarker in symbiotic EVs.

Microvesicles

Much less is known concerning the biogenesis pathway preceding the secretion of microvesicles in plants (Fig. 1B), however animal-based studies indicate microvesicles are produced by outward budding of the PM (Yáñez-Mó et al. 2015) in response to changes in membrane dynamics, fluctuations in ion homeostasis, and intracellular signaling cascades (Clancy et al. 2021). Microvesicles have been found to transport bioactive molecules between cells (Lv et al. 2019), which subsequently act in cell-cell signaling (Loyer et al. 2014) and have been characterized in HeLa cells through identification of the tetraspanin CD9 marker protein (Mathieu et al. 2021). EVs isolated from the apoplastic fluid of *Nicotiana benthamiana* were reported to contain TRANSMEMBRANE 9 SUPERFAMILY MEMBER 22, AP-COMPLEX subunits, and MEMBRANE STEROID-BINDING PROTEIN 2, suggesting a subpopulation of these isolated vesicles originated directly from the plasma membrane (Woith et al. 2021).

Limited evidence from plant-pathogen interactions supports the secretion of microvesicles, potentially due to the lack of subclass-specific marker proteins for these EVs. However, compelling evidence for the secretion of microvesicles during AM symbiosis (Fig. 1C) has been provided from TEM tomography and three-dimensional reconstruction of the PAS in rice and *M. truncatula*, which revealed an evagination of the PAM that showed constriction between a vesicular part and the PAM evagination, which is likely a microvesicle in the process of membrane excision (Ivanov et al. 2019; Roth et al. 2019). Additionally, immunogold labeling and TEM in transgenic rice expressing fluorescently tagged rice PHOSPHATE TRANSPORTER 11 (OsPT11), a PAM marker, indicated EV-like structures in the PAS contained OsPT11 suggesting a fraction of EVs at the symbiotic interface are derived from the PAM, as expected for microvesicles (Roth et al. 2019). Moreover, in *M. truncatula,* two classes of vesicular and tubular compartments, named intramatrix compartments (IMCs), assembled in the PAS (Ivanov et al. 2019). The vesiculo-tubular structures categorized as IMC-Is were still connected to the periarbuscular membrane and cytosol, although their morphology was observed to change throughout symbiosis and resembled microvesicles that were not excised from the membrane (Ivanov et al. 2019). IMC-IIs were linearly tubular and some were found as discrete compartments in the PAS, although there was no evidence for IMC-II docking to either plant or fungal membranes (Ivanov et al. 2019). Thus, it is unclear if either IMC-Is or IMC-IIs are, indeed, related to microvesicles or, indeed, any EV subclass or play any functional role in AM symbiosis. Building on initial observations of fungal paramural bodies in AM symbiosis (Moore and McAlear 1961; Scannerini and Bonfante-Fasolo 1983), elongated membrane tubules (memtubs) have been observed in the fungal paramural space during symbiosis with both rice and *M. truncatula* (Fig. 1D) (Ivanov et al. 2019; Roth et al. 2019). Although vesicle excision from these memtubs has not been directly observed, it is possible that fungi also secrete EVs to the PAS during symbiosis. These observations, alongside the previously discussed MVB fusion to the PAM (Roth et al. 2019), emphasize the heterogeneity of EVs present in the PAS during AM symbiosis but also raise questions as to their respective functions. Should these EV subclasses be isolated, identification of any commonly or differentially couriered cargos would provide exciting insight into the myriad roles of plant endo- and exocytic pathways in AM symbiosis; subsequent functional characterization of these cargos may even support EV subclass-specific roles during symbiosis, providing further understanding of the complex regulatory networks underpinning AM symbiosis.

PEN1-Positive EVs

The biogenesis pathway that produces PEN1-positive EVs (Fig. 1E) has not yet been established, however PEN1 has been identified from analysis of EVs secreted by both *Arabidopsis* and *N. benthamiana* and plays a key role in plant nonhost immunity (Collins et al. 2003; Rutter and Innes 2017; Zhang et al. 2020). Proteomic analysis of AtPEN1-postive EVs identified receptor-like kinases and other defense-associated proteins (Rutter and Innes 2017), suggesting a functional role for this EV subclass in plant-microbe interactions. AtPEN1, also called SYNTAXIN OF PLANTS 121 (AtSYP121), is a member of the t-SNARE (target soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) protein superfamily and interacts with the t-SNARE AtSNAP33 and the exocytotic vesicle associated v-SNARE VESICLE ASSOCIATED MEMBRANE PROTEIN 721 (AtVAMP721) to mediate membrane fusion (Collins et al. 2003; Nielsen and Thordal-Christensen 2012).

Interestingly, in animals, t-SNAREs were shown to interact with MVB-associated v-SNAREs to facilitate MVB docking and exosome release (Kreitzer et al. 2003; Martinez-Arca et al. 2003). Additionally, use of the tetraspanin-based pH-sensitive optical reporter CD63-pHluorin and live-cell and correlative light-electron microscopy imaging in HeLa cells showed the t-SNAREs SNAP23 and SYNTAXIN-4 were required for MVB-PM fusion and CD63-enriched EV release distinct from general transport vesicles fusing with the PM (Verweij et al. 2019). In *Arabidopsis*, however, imaging of fluorescently labeled apoplastic TET8 and PEN1 identified distinct EVs due to a lack of colocalization, suggesting PEN1 does not function in exosome release and, thus, these act as biomarkers for distinct plant EV subclasses (He et al. 2021). ARA6, previously found to mark MVBs involved in exosome biogenesis (Ebine et al. 2011) and to co-localize with TET8 (Cai et al. 2018), also did not co-localize with PEN1 (He et al. 2021), further supported this distinction and suggested PEN1-positive EVs arise from a different biogenesis pathway to TET8-positive exosomes (Cai et al. 2018). The rapid delivery of cargo from PEN1-positive EVs at pathogen contact sites during pre-invasive nonhost immunity is facilitated by the ARF-GTP exchange factor GNOM, which recycles PEN1 between the PM and *trans*-Golgi network (Nielsen et al. 2012), however any involvement of the MVB pathway in this process remains unresolved. Efforts to elucidate the biogenesis pathway of PEN1-positive EVs would be of benefit to the field moving forward, allowing further investigation into subclass-specific responses within symbioses.

AtSYP122, the closest homology of AtPEN1/AtSYP121, is localized to the PM and similarly interacts with AtSNAP33 and AtVAMP721; AtSYP122 does not, however, colocalize with At-

PEN1 and does not show similar accumulation at pathogen penetration sites (Nielsen et al. 2012), suggesting plant syntaxins display a similar functional diversification to that seen in plant tetraspanins. Indeed, analysis of the protein cargoes secreted by *syp121* and *syp122* mutant *Arabidopsis* seedlings indicated that these homologous SNARE proteins were associated with distinct cargo subsets, and it was proposed this differential protein secretion was the cause of observed phenotypic differences in the mutants (Waghmare et al. 2018). Expansion of the SNARE protein family in plants has therefore been suggested to permit context-specific secretion and EV release (Fujimoto and Ueda 2012), and thus, the release of EVs in AM symbiosis may involve symbiosis-specific SNARE proteins. In *M. truncatula*, the symbiosis-specific SYP132 has been implicated in symbiotic interactions, with MtSYP132 RNAi knockdown lines impaired for both AM and RNS (Huisman et al. 2016). More recently in rice, phenotypic characterization of a *syp132*/*syp131b* double mutant found significantly reduced AM fungal colonization and arbuscule abundance (Liu et al. 2022). It has been previously observed that alternative splicing of MtSYP132 in *M. truncatula* was shifted towards the MtSYP132α splice form in arbusculated cells and root nodules (Huisman et al. 2016) and that the MtSYP132α splice form was specifically localized to the symbiosome membrane and was necessary for symbiosome maturation (Pan et al. 2016); although both MtSYP132 isoforms were found to equally label the PAM and PM, these experiments bypassed the native alternative splicing, suggesting the MtSYP132α splice form may also specifically label the PAM (Huisman et al. 2016). However, evidence from *M. truncatula* suggests functional redundancy between symbiotic and nonsymbiotic SNARE proteins can rescue single mutant phenotypes to permit symbiosis (Huisman et al. 2020). Expression of the nonsymbiotic homolog MtPEN1/MtSYP121 in the *syp132* mutant background partially restored AM symbiosis, suggesting a possible role for one or both PEN1-like and SYP132-derived EVs in AM symbiosis. Efforts to explore the specificity of previously identified 'symbiotic SNAREs' in *M. truncatula* found that these SNAREs did not selectively interact with one another but that their complexes were more rapidly degraded (Huisman et al. 2020). One hypothesis presented to explain this evidence suggests the release of exosomes using these SNAREs is restricted to functional arbuscules, with the rapid turnover of the SNARE protein complexes linked to the ephemeral nature of the fungal arbuscule (Huisman et al. 2020). Alternatively, while the results presented suggest the secretion of exosomes for symbiotic engagement likely does not involve specific SNARE interactions, it has been suggested that this specialized function is, instead, achieved through differential expression patterns (Huisman et al. 2020; Wang et al. 2022). Interestingly, while the MtSYP132α splice form was clearly the most highly expressed syntaxin in arbuscule-containing cells, the nonsymbiotic SYP12 clade member MtPEN1/SYP121 was also expressed (Huisman et al. 2020). Recent evidence has suggested that differentially expressed long noncoding RNAs (lncRNAs) regulate the expression of SYP132 in *M. truncatula* during RNS (Yu et al. 2022), and, with initial work identifying lncRNAs in *Arabidopsis* extracellular fluid (Karimi et al. 2022) and mammalian exosomes (Huang et al. 2013), investigations into the roles of lncRNAs and into SNARE proteins themselves in regulating EV release may provide further insight into the dynamics and functions of SNARE proteins during symbioses.

EXPO-Derived EVs

Although the secretion of EXPO-derived EVs (Fig. 1F) has been partially elucidated, with fusion of a double membrane–bound EXPO to the PM releasing a single vesicle to the apoplast (Wang et al. 2010), the biogenesis pathway preceding membrane fusion is still unknown. EXPOs were initially identified in *Arabidopsis* and tobacco (*Nicotiana tabacum*) through fluorescent and immunogold labeling of EXOCYST SUBUNIT EXO70 FAMILY PROTEIN E2 (EXO70E2), and further evidence has since supported the role of EXO70E2 in recruiting other exocyst complex subunits to an EXPO (Ding et al. 2014; Wang et al. 2010). Use of EXO70E2 as a marker protein has supported an EXPO-derived EV biogenesis pathway distinct from the exosome biogenesis pathway; immunogold labeling of MVBs and EXPOs showed significantly different morphologies, and MVBs, unlike EXPOs, identified through this analysis were not labeled with antibodies for EXO70E2 (Wang et al. 2010). EXPO-derived EVs were found to be induced in response to the bacterial pathogen *P. syringae*, with the immune signaling protein RPM1-INTERACTING PROTEIN 4 (RIN4) identified in *Arabidopsis*-derived EVs (Rutter and Innes 2017) found to colocalize with EXO70E2 at the PM and promote the secretion of EVs, with an increased abundance of EXO70E2 observed in the apoplast (Wu et al. 2021). Whether EXPO-derived EVs are also secreted in AM symbiosis is unknown but may be an area of interest to future investigations.

Other EXO70 exocyst subunits have been implicated in AM symbiosis, however, particularly for the development of the symbiotic interface. EXO70I specifically localized to hyphal tips in arbusculated cells and showed partial colocalization and interaction with VAPYRIN; phenotypic characterization of *exo70i* mutants also showed impaired arbuscule branching and limited incorporation of the STUNTED ARBUSCULE 1 and 2 heterodimer in the PAM, suggesting reduced lipid export into the PAS (Zhang et al. 2015). EXO70 subunits are also implicated in the mutualistic root nodulation symbiosis. Although nodulation is not impaired in *exo70i* mutants (Zhang et al. 2015), the exocyst subunit EXO70H4 is necessary for nodulation (Liu et al. 2019), being specifically localized to the tip of the rhizobial infection thread, and was required for initiation and maintenance of polar infection thread growth (Liu et al. 2019). Interestingly, *exo70h4* mutants phenocopied vapyrin mutants and EXO70H4 was also shown to interact with VAPYRIN (Liu et al. 2019). The interaction of multiple EXO70 subunits with VAPYRIN, a plant-specific protein essential for both nodulation and AM symbiosis (Murray et al. 2011), is particularly interesting given VAPYRIN also associates with VAMP721m (Bapaume et al. 2019). Phylogenetic analysis of VAMP72 proteins in multiple plant species clustered VAMP721m with VAMP721d/e, producing a clade which does not contain a homolog from the nonsymbiotic species *Arabidopsis* (Bapaume et al. 2019). Partial silencing of the v-SNARE VAMP721d in soybean (*Glycine max*) impaired bacterial release during nodulation, which was suggested to be the result of reduced delivery of NODULATION PECTATE LYASE 1 (NPL1) to the infection thread pectin matrix (Gavrin et al. 2016). In *M. truncatula*, green fluorescent protein (GFP)-tagged NPL showed accumulation at the infection thread tip and vesicle-like punctate patterning (Liu et al. 2019), suggesting a role for VAMP721d and EVs in the delivery of pectate lyases during nodulation. Given GFP-tagged VAMP721d localizes to the growing hyphal tip in arbusculated cells (Genre et al. 2012), it has been suggested an EXO70-VAPYRIN-VAMP721 complex acts at this distinct PAM domain; any functional characterization of this complex would be of great interest, given the potential role for VAMP721 in directing nodulation-specific EV secretion. Given the characterization of EXO70E2 as an EV biomarker (Wang et al. 2010), further investigation into the functions and interactions of EXO70 subunits, VAPYRIN, and VAMPs may provide further insight into the role of EVs in mutualistic symbioses. Indeed, additional uncharacterized EXO70 subunits identified as one

or both expressed and upregulated in AM and RNS (Liu et al. 2019; Zhang et al. 2015) may warrant particular interest.

Uptake of EVs by Recipient Cells

Delivery of EV cargos to recipient cells requires trafficking at the extracellular PM face. Mechanisms to incorporate EVs in animal cells have been well-characterized (Mathieu et al. 2019; Prada and Meldolesi 2016), although recent evidence suggests few mammalian EVs actually fuse to recipient cells and deliver their cargo (Albanese et al. 2021). Evidence for the uptake of plant-derived EVs by pathogenic fungi in different pathosystems (Cai et al. 2018; Regente et al. 2017) suggests symbiotic microbes are able to incorporate plant-derived EVs, but confirmation of this is of particular interest in light of present uncertainties in the animal EV field.

EVs derived from sunflower (*Helianthus annuus*) appear to be internalized by the fungal pathogen *Sclerotinia sclerotiorum* through an endocytic mechanism and not through direct fusion to the fungal membrane (Fig. 1G) (Regente et al. 2017). Several endocytic mechanisms have been characterized in eukaryotes (Thottacherry et al. 2019), with clathrin-mediated endocytosis and lipid raft–mediated endocytosis of particular note. Clathrin-mediated endocytosis (Fig. 1H) is the dominant endocytic mechanism in plants (Narasimhan et al. 2020) and has been associated with EV uptake in mammals (Costa Verdera et al. 2017). Subunits of the TPLATE complex involved in clathrin-mediated endocytosis have been identified from *Arabidopsis*-derived EVs (Movahed et al. 2019) and at the PAM in carrot (*Daucus carota*) and *M. truncatula* (Russo et al. 2019). Although this localization of TPLATE complex proteins was suggested for a role in PAM biogenesis (Russo et al. 2019), clathrin-mediated endocytosis may also act throughout the symbiosis to enable uptake of symbiont-derived EVs.

Lipid rafts (also referred to as PM microdomains) are sphingolipid- and sterol-enriched regions associated with membrane microdomain-associated proteins and are linked to lipid raft–mediated endocytosis (Fig. 1I) (Qiao and Libault 2017; Zhang et al. 2019). Remorins, plant-specific membrane microdomain-associated proteins, were enriched in tomato (*Solanum lycopersicum*) sphingolipid-enriched membranes (Raffaele et al. 2009), interact with known symbiotic receptors (Lefebvre et al. 2010), and have been implicated in the uptake of bacterial EVs by *Arabidopsis* (Tran et al. 2022). In *M. truncatula*, the membrane microdomain-associated proteins FLOTILLIN2 (FLOT2) and FLOT4 are essential for nodulation (Haney and Long 2009), with FLOT4-directed localization of the remorin SYMREM1 to symbiotic membrane microdomains necessary for rhizobial infection (Liang et al. 2018). SYMREM1 stabilizes the infection thread and symbiosome membranes during nodulation, and the related remorin REM2.1 is suggested to stabilize the PAM (Su et al. 2023), suggesting a wider role for remorins and potentially lipid raft–mediated endocytosis in symbioses. The relationship between lipid rafts, their constituent lipids, and their associated proteins remains unclear but may offer mechanistic insights into EV uptake during AM symbiosis.

In a similar manner to lipid raft–mediated endocytosis, phosphoinositide lipids may contribute to EV uptake (Fig. 1J). An endocytic mechanism, conserved in animals and plants, involves the phosphoinositide phosphatidylinositol 3-phosphate (PI3P) and was shown to internalize the protein MYCORRHIZAL-INDUCED SMALL SECRETED PROTEIN 7 released by the ectomycorrhizal fungus *Laccaria bicolor* in *Populus trichocarpa* root cells (Plett et al. 2011). In *Phaseolus vulgaris*, loss-of-function PHOSPHATIDYLINOSITOL 3-KINASE mutants impaired both arbuscule and nodule development, suggesting PI3P is essential for both AM and RNS (Estrada-Navarrete et al. 2016), although a specific function was not established. Proteomic analyses of plant-derived EVs have identified PATELLIN 1 (PATL1) and PATL2 (Rutter and Innes 2017), with both capable of binding PI3P and PI4P (Peterman et al. 2004; Suzuki et al. 2016). PI4P is also associated with plant endocytosis, with local accumulation at the PM producing PI4P-enriched early endosomes (Rodriguez-Furlan et al. 2019). Interestingly, both PI4P and PI(4,5)P_2 (phosphatidylinositol-4,5-bisphosphate) accumulate at distinct regions of the PAM in *M. truncatula* (Ivanov and Harrison 2019), and SYMREM1 was shown to bind to both PI(3)P and PI(4,5)P_2 (Su et al. 2023). Further studies are needed to determine the role of phosphoinositides in AM symbiosis and if they are involved in endocytic uptake of EVs.

Future Perspectives

With initial TEM observations of EVs and memtubs in the PAS during AM symbiosis, it is likely that EVs play some functional importance in these interactions. In pathogenic plant-microbe interactions, plant- and fungal-derived EVs have been implicated in immunity and cross-kingdom RNAi (Cai et al. 2018; De Palma et al. 2020; Hou et al. 2019; Kwon et al. 2021; Regente et al. 2017). With putative small RNA targets identified in AM fungi for AM host plant-derived small RNAs (Mewalal et al. 2019) and in AM host plants for AM fungal-derived small RNAs (Silvestri et al. 2019), whether EVs mediate transport of small RNAs for symbiotic cross-kingdom RNAi presents an exciting opportunity for future investigation. Moreover, with evidence that a group of small secreted proteins are found only in AM host plants (Hu et al. 2022) and that small secreted protein secretion follows trafficking through MVBs or EXPOs (Hu et al. 2021), any confirmation of EVs as functional protein couriers between symbionts would provide exciting insight into the mechanisms of inter-organismal communication in AM symbiosis. Evidence from the root nodule symbiosis has suggested a potential role for EVs in the delivery of cell-wall remodeling enzymes that permit symbiosis to the symbiotic interface (Gavrin et al. 2016). The VAMP721 proteins implicated in this targeted delivery are also associated with essential developmental-related proteins in AM symbiosis; clarifying whether EVs are involved in the development of arbuscules or the PAM would provide valuable insight into the large-scale developmental remodeling that defines symbiosis.

The identification that EVs in mutualistic symbioses have differing electron densities and appear to arise from multiple biogenesis pathways suggests EVs in AM symbiosis are a highly heterogeneous population (Ivanov et al. 2019; Roth et al. 2019). Whether these EV subpopulations therefore show functional diversification or differential spatiotemporal secretion, reflecting the highly dynamic nature of AM symbiosis, will be a key avenue for future research. As the field grows out of its infancy, further investigations are likely to provide greater insight into how the biogenesis, trafficking, and uptake of EVs during symbiosis is necessary for symbiotic engagement. Whether symbiotic EVs are differentially enriched in certain cargos and the means through which this is achieved may provide further insight into outstanding questions concerning how plants enter into symbiosis while avoiding pathogen infection (Thoms et al. 2021). And finally, the actual recipients of EVs in AM symbiosis, whether arbusculated or neighboring plant cells or the fungal symbiont, is still unknown and would add valuable understanding to the role of EVs during symbiosis.

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