




REVIEW



Emerging roles of tetraspanins in plant inter-cellular and inter-kingdom communication

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ABSTRACT

Inter-cellular and inter-kingdom signaling systems of various levels of complexity regulate pathogenic and mutualistic interactions between bacteria, parasites, and fungi and animal and plant hosts. Inter-kingdom interactions between mutualistic bacteria such as rhizobia and legumes during nodulation and between fungi and plants during mycorrhizal associations, are characterized by the extensive exchange of molecular signals, which allow nitrogen and phosphate assimilation, respectively. A novel aspect of this signaling exchange is the existence of specific structures, the exosomes, that carry important molecules that shape the plant–pathogen interactions. Exosomes contain a wide array of molecules, such as lipids, proteins, messenger RNA, and microRNAs, that play important roles in cell-to-cell communication in animal and plant cells by affecting gene expression and other physiological activity in distant cells within the same organism (e.g., during cancer metastases and neuron injuries). In plant cells, it has been recently reported that exosomes go beyond organism boundaries and inhibit a pathogenic interaction in plants. Plants produce and send exosomes loaded with specific small miRNA which inhibit the pathogen infection, but the pathogen can also produce exosomes carrying pro-pathogenic proteins and microRNAs. Therefore, exosomes are the important bridge regulating the signal exchange. Exosomes are small membrane-bound vesicles derived from multivesicular bodies (MVBs), which carries selected cargos from the cytoplasm (protein, lipids, and microRNAs) and under certain circumstances, they fuse with the plasma membrane, releasing the small vesicles as cargo-carrying exosomes into the extracellular space during intercellular and inter-kingdom communication. Animal and plant proteomic studies have demonstrated that tetraspanin proteins are an integral part of exosome membranes, positioning tetraspanins as essential components for endosome organization, with key roles in membrane fusion, cell trafficking, and membrane recognition. We discuss the similarities and differences between animal tetraspanins and plant tetraspanins formed during plant–microbe interactions and their potential role in mutualistic communication.

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Introduction

The multiple functions of tetraspanins

Tetraspanins constitute a diverse superfamily of transmembrane proteins, with 33 members identified in mammals and 17 in the model plant *Arabidopsis thaliana*. Although tetraspanin homologues have not yet been reported in yeast, bacteria, and archae, they are ubiquitous in eukaryotic cells and the first member of this family likely appeared around 570 million years ago.^{1,2} Several tetraspanin isoforms exist in deuterostomata, including fruitfly (*Drosophila melanogaster*), zebrafish (*Danio rerio*), and humans.^{3–5} Tetraspanins are involved in basic cell functions such as motility, fusion, and membrane and vesicular trafficking, and play key roles in diverse physiological processes including sperm–egg fusion, antigen presentation, and tissue differentiation.^{2,6–8} While knockout of the tetraspanins CD9 and CD81 in mice (*Mus musculus*) results in a clear defect in egg–sperm fusion, other tetraspanins are involved in virus and parasite interactions.^{2,6–10} More recently it has been reported that

a single point mutation, L31S substitution in the third amino acid of TM1 has been associated with field-evolved resistance of cotton bollworm to transgenic Bt cotton.¹¹ Tetraspanins also regulate cellular invasiveness or metastases through associating with peptidases, matrix metalloproteinases, and urokinase plasminogen activator surface receptors in animal cells.¹² In animals and plants, some tetraspanins have a broad tissue distribution, while others show a restricted expression; for instance, in plant cells, different tetraspanins are expressed in specialized tissues such as the reproductive tissue and meristems, during embryo development, and in specific cell niches, such as the quiescent center or the early initial cells that give rise to lateral roots.^{13–15}

Tetraspanins appear to act as molecular organizers by forming homo- or heterodimers that localize to microdomains known as tetraspanin-enriched microdomains (TEMs). As components of TEMs, both animal and plant tetraspanins maintain a network of interactions with other membrane proteins, such as integrins, and signaling molecules, such as receptors, allowing the assembly of multi-molecular signaling platforms.¹² Therefore, tetraspanins are dynamic master organizers within membranes that control the

distribution and clustering of associated partner-proteins and thereby regulate cellular functions, such as signaling and adhesion.^{14,16} Their role in vesicular trafficking and as a component of exosomes, which mediate the transport of protein, lipids, and microRNAs during intercellular and interkingdom communication, open a new avenue of research in pathogenic and mutualistic interactions in plant and animal hosts.¹⁷

Tetraspanin structure

As its name implies, tetraspanins are proteins with four membrane-spanning domains. The amino- and carboxy-terminal tails and a small loop between transmembrane regions 2 (TM2) and 3 (TM3) are localized in the cytoplasmic compartment. Two loops, a small one (ECL1) located between TM1 and TM2, and a large one (ECL2) between TM3 and TM4, are extracellular (Figure 1).^{6,18} ECL2 can be subdivided into a conserved and variable region. The conserved region mediates dimerization, whereas the variable region is required for interactions with non-tetraspanin partner molecules. The highly conserved cysteine residues in the large ECL2 are essential tetraspanin signatures, and the polar residues conserved in the TM regions stabilize the tertiary structure.¹²

Palmitoylation of intracellular and juxta-membrane cysteines might be required for initiating tetraspanin-tetraspanin web formation. This chemical modification also protects tetraspanins from lysosomal degradation, provides a link to cholesterol and gangliosides, and promotes cell-cell contact.^{6,19} In animal cells, palmitoylation of specific integrins

also contributes to tetraspanin complex formation.²⁰ Although *bona fide* integrins have not been identified in plant cells, other proteins such as NDR1 play similar functions.²¹ In plant cells, some tetraspanins possess a tyrosine-based sorting motif (Yxx ϕ , with ϕ representing an amino acid with a bulky hydrophobic side chain) at the carboxyl end that delivers the tetraspanin to a specific intracellular compartment.^{17,22} However, internalization can also proceed through association with proteins with a sorting motif or by additional, not yet defined domains.¹² The major extracellular loop ECL2 is a key region for tetraspanin function in animal and plant cells. Disulfide bonding between conserved cysteines in ECL2 produces a sub-loop structure in this region, which shows the greatest variability between tetraspanins.^{15,23} Some tetraspanins have an additional two-to-four cysteines within this sub-loop, which may also participate in disulfide bonding. The remainder of the ECL2 region is highly conserved and contains three alpha helices.² The ECL2 loop appears to determine tetraspanin functional specificity and most specific monoclonal antibodies (mAb) bind to this region.²

Conservation of a single Cys residue at a specific point in the small loop is a unique feature of plant cell tetraspanins, suggesting that it plays a particular role that has not yet been determined. For instance, disulfide bonding between the big and small loops, which is important for tetraspanin-tetraspanin interactions, could stabilize the interaction in a redox-dependent fashion or affect the affinity for cholesterol or gangliosides present in the plasma membrane.²⁴ In contrast to animal tetraspanins, plant tetraspanins

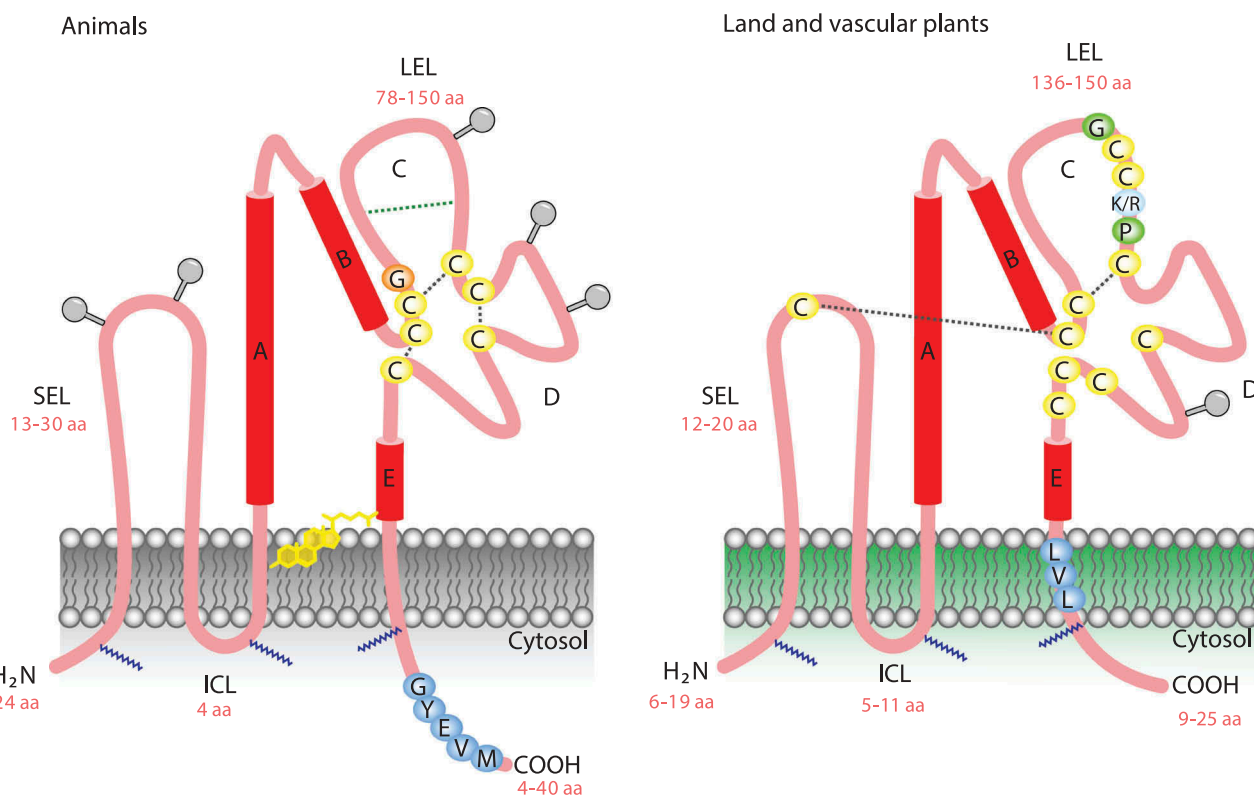


Figure 1. A general model describing the tetraspanin organization in the plasma membrane. Note the similarity and differences in the two extracellular loops. Plant tetraspanins contain a conserved cysteine that is not present in animal tetraspanin. (SEL) small extracellular loop, (LEL) large extracellular loop, (ICL) intracellular cytoplasmic loop, (GYEVM) endosome signal peptide, (GCKC/RPC) conserved sequence in plants, (LVL) putative plasmodesmata sorting.

have nine, rather than four, six, or eight, completely conserved Cys residues.¹⁴ In plants, missense mutations in these Cys residues in *trn2-2* and *trn2-3* alleles, or the exchange of an uncharged amino acid (Pro-164 in *trn2-2* and Gly-177 in *trn2-3*) with a charged amino acid (Gluc) within the conserved EC2 region containing three Cys residues, could affect folding due a defect in disulfide bridges and thereby inhibit tetraspanin function.²⁵ This is intriguing, as crystallographic data and an analysis of the molecular dynamics of tetraspanin CD81 ECL2, showed the conformational plasticity of the EC2 region under different pH values and redox environments, suggesting that it acts as a molecular sensor of environmental change.^{15,26} It is possible that plant tetraspanins could have a similar response under pH and redox changes. Little is known about the C-terminal tail of tetraspanins, a divergent and short region (4 to 40 amino acids) in animals and plants that is longer in fungi.^{14,15,27} However, the C-terminal tail has been implicated in trafficking, as discussed below.²⁸⁻³⁰

Tetraspanin interactors: what we have learned from the animal field and how little is known in plant cells

In animal cells, the most prominent tetraspanin interactors are the integrins, growth factor receptors, G-protein-coupled receptors (GPCRs), several peptidases, transmembrane proteins associated with tumor progression (CD44), epithelial cell adhesion molecule (EPCAM), immunoglobulin (Ig), protein kinase C (PKC), phosphatidylinositol 4-kinase (PI4KII), and phospholipase C γ (PLC γ).¹² By contrast, the interactors of plant tetraspanins are unknown and the literature is limited to the expression and subcellular localization of plant tetraspanins in a few cell models,^{4,14,15,31} and their role in recruiting ROS generating enzymes.³²⁻³⁴

In animal cells, TEM components have been dissociated using various detergents to examine interactions between tetraspanins and other molecules in the TEM. Different levels of interactions, ranging from strong associations to much weaker associations that are stable only in less hydrophobic detergents, such as Brij97, have been observed.²³ Three types of primary tetraspanin interactions, types I–III, exist. Type I interactions involve direct protein–protein interactions, such as those occurring in tetraspanin homodimers, homotrimers, homotetramers, and some heterointeractions.^{23,35} Most tetraspanin–integrin and tetraspanin–tetraspanin interactions are type II interactions, which are less strong. Palmitoylation of tetraspanins, and possibly of their associating proteins, is essential for this type of interaction, which may be initiated in the Golgi apparatus.³⁶ Weak type III interactions, such as those occurring between tetraspanin and several kinases, are also stabilized by palmitoylation. In addition to primary interactions, the functional activity of tetraspanins also depends on cholesterol and gangliosides, which enable higher-order tetraspanin complexes to form, which constitute the TEM.³⁷ TEMs provide a signaling platform that recruits tetraspanin and its interactors. The palmitoylation of juxta-membrane cysteine residues of tetraspanins (Figure 1) is critical for the assembly of TEMs and is necessary for tetraspanin/tetraspanin interactions. Some integrins are also palmitoylated, and this modification appears to promote TEM formation. Cholesterol embedded in the membrane of the endosome is thought to stabilize TEMs.

Although TEMs share features with lipid rafts, they are independent and distinct from these structures. For instance, lipid rafts, but not TEMs, are disrupted by Triton X-100 at 4° C and the signature molecules of classical rafts, such as the glycosylphosphatidylinositol-anchored proteins and caveolin, do not associate with tetraspanins.¹² Furthermore, TEMs can be purified in low-density sucrose gradient fractions and are enriched in certain lipids (e.g., cholesterol and ganglioside GM3), while lipid rafts have different biophysical properties, are more sensitive to cholesterol depletion, and contain different arrays of membrane proteins.^{6,7} Visualization of TEMs by fluorescence microscopy suggested that they are confined in discrete units at the nanometric scale for each class of tetraspanin. Recent single-molecule analyses of tetraspanin CD9 in animal cells shed light on tetraspanin dynamics in TEMs and the plasma membrane, redefining the tetraspanin web.^{7,38,39} These studies indicate that tetraspanins form stable interaction platforms that are distinct from lipid rafts and in permanent exchange with the rest of the cell membrane. In addition, CD9 mobility and partitioning into these platforms depends on palmitoylation and cholesterol.²

Although no direct interaction partners of tetraspanins have been identified in plant cells, the cellular distribution of tetraspanin in reproductive tissues has been described.¹⁵ Genetic data indicate that AtTET1 functions in a common pathway with TORNADO1 (TRN1), a leucine-rich-repeat protein that regulates patterning processes during *Arabidopsis thaliana* development, and double mutant analysis showed that these proteins are required for related, overlapping signaling events.¹⁴ Furthermore, AtTET1 functions together with WINDHOSE1 and 2 (WIH1/WIH2), two small peptides, to promote megasporogenesis.^{25,40,41} The short and divergent C-terminal tail of both animal and plant tetraspanins has been linked to targeting to intracellular locations and interaction with cytoskeletal or signaling molecules, including protein kinase C, integrins, and mu3A subunit AP-3.^{14,15,19} The phenotype of the *Arabidopsis* mutant *trn2-4*, which lacks 10 of 14 amino acids of the tetraspanin C-terminal tail, is as dramatic as that of the other *trn2* alleles, suggesting that this region of plant tetraspanins is as critical as that described for animal tetraspanins.²⁵ The C-terminal tail of the fungal tetraspanin Tps3 is larger than that of plant and animal tetraspanins and its specific interactors are unknown.²⁷

Tetraspanins are key players in cancer development, with multiple antagonistic effects.⁴² For example, the tetraspanin CD82 is downregulated, while tetraspanins CD151 and tetraspanin 8 are induced during metastases and support tumor progression.¹² Not all activities of tetraspanin rely on primary interactions with associating molecules; some biological phenomena can only be explained by considering the higher organization of tetraspanin complexes, which can interfere with the capacity of tetraspanins to recruit other molecules that negatively regulate a cellular event, such as the activation of a tyrosine phosphatase.⁴³ However, recruitment of specific partner molecules in TEMs such as gangliosides could contribute to CD82-mediated metastasis-inhibiting activity.¹² Tetraspanin CD9 also hampers the migration of metastasizing cells by interacting with the cytoskeleton. Indeed, overexpression of CD9 downregulates WAVE2 by a yet unknown mechanism,⁴⁴ since WAVE is part of the ARP2 and ARP3

complex in actin polymerization, it links upstream signals to activation of the ARP2 and ARP3 complex (e.g., during lamellipodium and filopodium formation). Some tetraspanins have a Yxx ϕ sorting motif, located close to the transmembrane region, that can disrupt the binding of the μ subunit of the AP2 adaptor complex, resulting in another cytoskeleton key regulatory component.^{12,17} Therefore, tetraspanins exert their function by modulating, stabilizing, or preventing the activities of their associated molecules, depending on the composition of the TEM.^{12,44,45}

Role of tetraspanin in cell trafficking

Although tetraspanins were first identified as cell surface markers, it is now clear that some associate with the endosomal pathway, i.e., in early and late endosomes, multivesicular bodies [MVBs], clathrin-coated vesicles, lysosomes, and various types of secretory vesicles.⁴⁶ Tetraspanins on the plasma membrane can be internalized via endocytosis and traffic to intracellular vesicles.^{47,48} Conversely, on cell stimulation, secretory vesicles containing tetraspanins fuse with the plasma membrane.⁴⁶ Late endosomes and MVBs also fuse with the plasma membrane and release vesicles containing microRNA, proteins, and lipids known as exosomes that are enriched in certain tetraspanin proteins.⁴⁹ Exosomes released from cells infected with intracellular pathogens, including mycobacteria, are potent stimulators of inflammation in uninfected cells.⁵⁰ Thirteen of the 33 mammalian tetraspanins contain potential tyrosine-based sorting motifs, which have also been described in plants. However, in plants, additional motifs have been identified in tetraspanins, and the coat protein clathrin is necessary and sufficient for the uptake of cargo into clathrin-coated vesicles (CCVs). These motifs in the tetraspanins are based on the sequences YXX ϕ [D/E]XXXL[L/I] and FXNPXY, which are recognized by a family of adaptor protein (AP) complexes that determine the cellular localization of interacting proteins.^{2,17} The intracellular trafficking of tetraspanin CD63 has been well described.^{17,22} CD63 has a conserved GYEVN motif at its C-terminus, which interacts primarily with AP-2 complexes, linking tetraspanins to clathrin-mediated endocytosis pathways, and with AP-3 complexes for trafficking from endosomes to lysosomes. Furthermore, CD63 is also targeted to lysosomes directly from the trans-Golgi network.²² Tetraspanin CD63 interacts directly through its C-terminus with syntenin, which may compete with AP-2 and AP-3 to alter the trafficking of syntenin and therefore link its spatial localization with actual function.^{17,22} It has also been recently suggested that CD63 in animal cells is internalized from the plasma membrane by endocytosis of a specialized type of lipid raft known as caveolae, linking tetraspanin to the regulation of endocytosis.^{2,17,22}

Tetraspanins as key players in pathogenic interactions in animal and plant cells

Several infectious agents evolved mechanisms to exploit tetraspanins for pathogen entry, subsequent intracellular trafficking, replication, and even exit from the host cell. Tetraspanins have been associated with or shown to mediate infection by human hepatitis C (HCV), HIV, *Plasmodium* species that

cause malaria, certain types of bacteria, and even prions.² Some viruses (e.g., HIV, human T-cell leukemia virus [HTLV]) induce cell–cell fusion in a tetraspanin-dependent manner, resulting in the formation of giant cells or viral syncytia that contribute to spread of infection.^{2,51} In some cases, tetraspanins act as pathogen receptors; however, they can also work as ‘molecular organizers’, forming TEMs by the lateral association of tetraspanins with other tetraspanin and non-tetraspanin membrane proteins.²

In T cells themselves, tetraspanins in TEMs promote actin cytoskeleton associations that may lead to more efficient signaling or contribute to the formation of the immune synapse in T cells and it has been suggested that tetraspanins facilitate antigen presentation or antigen signaling.² The localization of tetraspanins in TEMs has made it challenging to define the functions of individual family members, mainly because the antibodies that cross-link specific tetraspanins also perturb the organization and functions of interacting molecules, giving misleading information.^{23,43} However, the soluble recombinant EC2 domain is an alternative tool to investigate the role of specific tetraspanins.⁵² Recombinant EC2s appear to fold correctly and have biological activity in a number of systems.^{2,52} Nonetheless, knockouts clearly demonstrate that particular cellular functions (e.g., T-cell proliferation, sperm–egg fusion) can be affected by the modulation of a specific tetraspanin. Targeting of individual tetraspanins may provide a therapeutic means to modulate cellular processes without global detrimental effects on the organism, including plant cells.²

Plants have the capability to respond to abiotic and biotic stresses, including fungal pathogens. Fungi are responsible for major diseases of agriculturally important plants. For example, *Magnaporthe grisea* is the causative agent of the most devastating fungal disease (blast) of rice (*Oryza sativa*) worldwide, *Botrytis cinerea* is a necrotrophic pathogen affecting more than 200 different host plants, and *Colletotrichum lindemuthianum* is responsible for anthracnose diseases in a wide range of crops and ornamental plants. The ability of plant pathogenic fungi to infect their host plants depends on successful penetration into plant tissues. During the infection process, these fungi differentiate a specialized cell called the appressorium, in a process involving arrest of polarized growth, apex swelling, and cell wall reinforcement.^{27,53,54} (Figure 2). Appressorium-mediated penetration occurs through the formation of a penetration peg at the base of the appressorium lying against the plant substratum, a region called the ‘appressorium pore’. The narrow penetration peg is able to perforate the host surface, which includes the plant cuticle and cell wall, allowing the fungus to penetrate into host tissues.^{27,53,54} The penetration peg emerges at a certain point of the base of the appressorium and requires spatial and temporal coordination of cellular functions, including reorganization of cytoskeletal elements; vesicular trafficking; exocytosis, including membrane and synthesis; assembly and remodeling of the cell wall.^{53,55,56} Fungal tetraspanins have emerged as key coordinators of the infection process in several pathogenic fungi (*M. grisea*, *B. cinerea*, and *C. lindemuthianum*).^{32,54,57,58} In these phytopathogenic fungi, Pls1 is essential for appressorium-mediated penetration into the host plant, since Pls1 null mutations result in appressoria that are unable to form functional

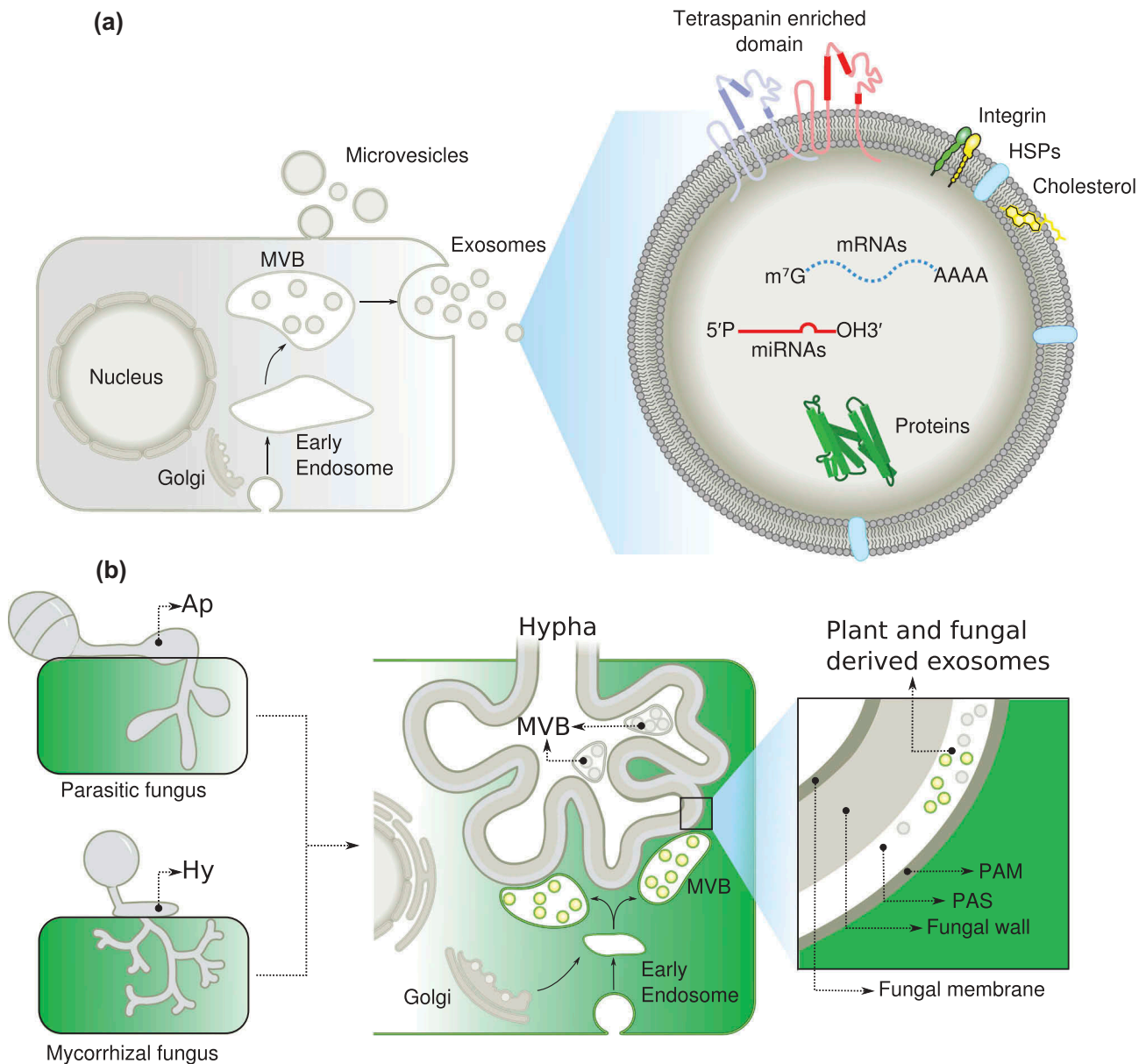


Figure 2. A model describing the role of exosomes in inter-kingdom communication. (a), Note the key role of exosomes in transporting molecules and tetraspanin in the exosome membranes as a marker. (b) Putative role of exosomes in mutualistic interactions. (MVB) multivesicular bodies, (Ap) appressorium, (Hy) hyphae, (PAM) periarbuscular membrane, (PAS) periarbuscular space, (HSPs) heat shock proteins.

penetration pegs.^{27,53,58} Using a transcriptional fusion between the *PLS1* promoter and an eGFP reporter gene, clear expression is observed during the penetration of *B. cinerea*.⁵⁴ These results suggest that processes involved in appressorium-mediated penetration are conserved among taxonomically unrelated fungal species.^{27,54} Since peg formation and penetration require the re-establishment of cell polarity at a focal point localized at the base of the appressorium, Pls1 tetraspanins might be required for the correct localization of the emergence site of the penetration peg. The finding that penetration peg was aborted or mislocalized in *C. lindemuthianum* *clpls1* null mutants supports this interpretation.⁵⁸ Therefore, functional studies of Pls1 tetraspanins in pathogenic fungi may reveal more general functions such as the generation of positional information during specific stages of fungal morphogenesis.

Tetraspanins as regulators of localized ROS generation by NADPH oxidases (NOX)

The wide distribution of tetraspanins in eukaryotes and their notable absence in unicellular fungi suggest that tetraspanins co-emerged with multi-cellular organisms during evolution, and are thus associated with multicellularity.⁴ It is largely unknown how tetraspanins engage in multi-faceted functions at the cellular level. However, there is an emerging co-occurrence between tetraspanins and the mechanisms of ROS generation.³³ In the nematode *C. elegans*, the rigid and flexible cuticular exoskeleton, which is composed of collagen, protects the internal tissues. Once secreted, this collagen is tyrosine cross-linked in a ROS-dependent manner, in a process assisted by BLI-3, a DUOX NADPH oxidase.⁵⁹ This process also requires the participation

of tetraspanin TSP-15, which recruits the NADPH oxidase.^{33,34,60} Inactivation of this tetraspanin or of BLI-3 produces similar phenotypes. This finding suggests a connection between the mechanism generating ROS and the tetraspanins. In fungi, tetraspanins have been related to NOX function based on the fact that mutants lacking either of these proteins show the same phenotypes. Furthermore, during plant infection with the pathogenic fungus *M. grisea* or *B. cinerea*, the tetraspanin PLS1 specify the infection sites, and the tetraspanin and ROS generated by NADPH oxidase coordinate ROS production at the infection site. Furthermore, in the pathogenic *C. lindemuthianum*, a tetraspanin is required in the appressorium, which is a swollen not growing structure, but in order to progress the fungal infection a re-initiation of the polarity is required.^{32–34,58,61} Furthermore, in *Claviceps purpurea*, Nox2 and Pls1 are also important for host–pathogen interaction.⁶²

These data point to a strong connection between tetraspanins and the ROS generating machinery, both in animal and plant cells, suggesting that tetraspanins regulate localized ROS production.^{33,34,60,63} Localized ROS production is also required during Casparian strip lignification in plants, where CASP, a protein that recruits NADPH oxidase, plays a role similar to tetraspanin, by bringing together NADPH oxidase and peroxidase and ensuring the localized activation of the oxidase.⁶⁴ Tetraspanins have also been found at the tip of growing pollen tubes, a region that requires NADPH oxidase activity for the generation of ROS, key players in the regulation of polar growth.^{65,66} Moreover, tetraspanins accumulate at the site of female gametophyte differentiation, suggesting an active role during pollen fertilization, a well-described ROS-dependent process that regulates programmed cell death.¹⁵ It is tempting to speculate that tetraspanins recruit the ROS-generating machinery in a site-localized manner.

Exosomes as signaling shuttles, from inter-cellular communication to inter-kingdom communication

Inter-cellular and inter-kingdom signaling require specific molecules that can diffuse and be recognized by a different cell. Exosomes have emerged as a ubiquitous mechanism for transferring information between cells and organisms across all three kingdoms of life.^{67,68} Initially described in animal cells as a general signaling pathway that allows communication between tumor and distal tissue during metastases, exosomes have also been shown to function in immune surveillance in plant cells during host–pathogen interactions,^{69–72} and we propose that exosomes could also contribute to the regulation of mutualistic interactions.

Exosomes containing proteins, lipids, and RNAs produced by fungal pathogens can promote plant infection by transmitting pathogen-related molecules that could escape the host immune system or inhibit immune responses by favoring immune cell apoptosis. Furthermore, exosomes secreted by the plant host can have anti-infection roles by inducing immune responses that inhibit pathogen proliferation or infection. Therefore, exosomes might act as regulatory “bridges” during plant–pathogen interactions.⁷⁰

How are exosomes produced and how are they related to tetraspanins? The general view is that exosomes are derived

from multivesicular bodies (MVBs), which form when the endosomal membrane undergoes invagination, taking up proteins, lipids, inhibitors of apoptosis, and microRNAs from the cytosol.^{73,74} The resulting vesicles inside the endosomes are termed MVBs. MVB either fuse with lysosomes or the plasma membrane, releasing their intraluminal vesicles as exosomes. There are other similar structures such as the ectosomes and microvesicles, which are assembled and released directly from the plasma membrane and therefore represent a different size and origin. The molecular composition of exosomes reflects their origin from intraluminal vesicles and includes several tetraspanins both in animals and plants.⁷¹ It is important to note that exosomal proteins maintain their activity. Another notable feature is phosphatidylserine content on the outer membrane leaflet of the exosome in animal cells, which facilitates or triggers exosome uptake by phosphatidylserine-binding proteins (such as scavenger receptors, integrins, and complement receptors). The finding that exosomes can contain mRNAs and microRNAs that can be transferred to target cells where they can be translated or mediate RNA silencing, is a critical aspect of intercellular communication during the immune response, cell-to-cell spread of infectious agents, and tumor progression.^{75–79} This process is specific to the target cell, such that RNA is transcribed in one cell, but travels long distances to affect another cell. Notably, the relative abundance of proteins, mRNA, and microRNA differs between exosomes and the cytoplasm of donor cells, suggesting a certain degree of specificity in the segregation of these components and therefore in the specificity of this type of communication. This implies that cargo is actively sorted into multivesicular bodies, through mechanisms such as monoubiquitylation, localization in cholesterol-rich membrane microdomains, or higher-order oligomerization. Although the contribution of tetraspanins to the composition of exosomes is well established, their impact on the functions of exosomes is unknown. However, it seems likely that tetraspanins are involved in the cross-talk between distant cells.¹² Specific tetraspanins and their interacting proteins, might contribute to exosome function and define the target cell.⁸⁰ Future studies providing the molecular mechanism to select the cargo and target cells by the exosomes will provide important information to manipulate the formation and targeting of exosomes.^{2,81,82}

Exosomes in inter-kingdom communication during plant–pathogen interactions and future directions in mutualistic interactions

Eukaryotic regulatory small RNAs (sRNAs) that induce RNA interference (RNAi) are involved in a plethora of biological processes, including host immunity and pathogen virulence for successful infection.^{83–85} In plants, diverse classes of sRNAs contribute to the regulation of host innate immunity. These immune-regulatory sRNAs operate through distinct RNAi pathways that trigger transcriptional or post-transcriptional gene silencing. Remarkably, the influence of regulatory sRNAs is not limited to the individual organism in which they are generated and it can sometimes extend to interacting species from different kingdoms.^{71,86} Similarly, many pathogen-derived sRNAs regulate pathogen virulence, where they trigger gene silencing in the interacting organism, a phenomenon called cross-kingdom RNAi.^{85,86} Aggressive

fungal pathogens such as *Botrytis* and *Verticillium* spp. that cause severe crop losses worldwide secrete effector proteins that suppress host immune responses.⁸⁶ Notably, the plant fungal pathogen *Botrytis cinerea* delivers and translocates small RNA effectors into host cells, where they hijack the plant RNAi machinery and suppress host immunity.^{85,86} Upon *B. cinerea* infection, expression of the microRNA Bc-siR37 is specifically induced in the fungus, downregulating at least eight predicted Arabidopsis target genes and thereby enhancing disease susceptibility to *B. cinerea*.⁸⁶ Furthermore, knockout of the target genes of Bc-siR37 enhances disease susceptibility, further indicating that these genes promote plant defense against *B. cinerea*.⁸⁶ In summary, by regulating the expression of specific target genes in the plant host, it is possible to control multiple fungal diseases simultaneously. Therefore, cross-kingdom RNAi opens up a vastly unexplored area of research on mobile sRNAs in the host-pathogen interactions.^{71,78,85} The big question is how is cross-kingdom communication carried out? Exosomes are produced in all domains of life, but little is known about exosomes in plants despite their implication in plant defense.^{71,87-89} This work lays the foundation for the concept of cell-to-cell communication through exosomes in plants.⁷⁹ This idea is also supported in sunflower (*Helianthus annuus*), where exosomes in extracellular fluids of seedlings are enriched in cell wall remodeling enzymes and defense proteins which can be taken up by the phytopathogenic fungus *Sclerotinia sclerotiorum*, and affect fungal growth and even killing fungal cells.⁸⁸

In animal cells, sRNAs can be transported by extracellular vesicles, specific transmembrane proteins, high-density lipoprotein complexes, or gap junctions.⁹⁰ The gastrointestinal parasite *Heligmosomoides polygyrus* also secretes exosomes, thereby transporting microRNAs (miRNAs) into mammalian cells and suppressing host immunity.⁷⁶ By contrast, the mechanism by which sRNAs are transported from hosts to interacting pathogens is unknown. In plant cells, it is well known that sRNAs travel through plasmodesmata, and thus systemically through the vasculature; however, cross-kingdom/organism RNA interference (RNAi) mediated by exosomes opens up a new area of research.⁷¹ Because exosomes transfer miRNAs between animal cells as part of a long-distance signaling system and also in parasites interacting with mammalian host cells, the recent finding that plants and pathogenic fungi employ a similar mechanism involving exosome-mediated sRNA transfer indicates that exosomes constitute a general mechanism for intercellular and inter-kingdom signaling. This is a bi-directional communication with exosomes generated in the host to control the pathogen, but also with exosomes generated in the pathogen to promote the infection. Thus, studies should examine how exosomes travel across the boundaries between organisms of different taxonomic kingdoms.⁷¹ In Arabidopsis, the cuticle is an important barrier against pathogens. However, in cuticle mutants, higher ROS production is able to sustain constitutive resistance against *B. cinerea* due to an increase in the cuticle permeability, which facilitates the diffusion and recognition of the cuticle monomers that induce the innate response. However, increased cuticle permeability could also facilitate exosome transport and diffusion in the permeable cuticle, allowing for neutralization of the pathogen through the exchange of miRNAs.⁹¹⁻⁹³

Mammalian tetraspanins, such as CD63, are specific exosome markers.⁷⁷ Arabidopsis has 17 *TETRASPANIN* (*TET*)-like genes, but only closely related *TET8* and *TET9*, which are structurally similar to mammalian CD63, are induced by *B. cinerea* infection, at the fungal infection sites and as part of the exosomes.^{15,94} Since mammalian exosomes are derived from MVBs, it is not surprising that the *Arabidopsis thaliana* MVB marker Rab5-like GTPase ARA6⁹⁵ is also enriched at infection sites, and is partially co-localized with *TET8*, suggesting that *TET8*-associated vesicles are likely derived from MVBs. Electron microscopy studies revealed in Arabidopsis that MVBs fuse with the plasma membrane and release extracellular vesicles at infection sites. Furthermore, *TET8*-associated vesicles have been visualized with *TET8-GFP*, but not ARA6, because ARA6 localizes to MVB outer membranes that fused to and remained on plasma membranes.⁷¹ Thus, *TET8*-labeled extracellular vesicles can be considered as a specific marker for plant exosomes. The observations that *TET8-GFP*-labeled exosomes can be taken up by fungal cells under *in vitro* conditions⁷¹ and that exosome-carried sRNAs can be translocated to fungal cells supports the involvement of a shuttle mechanism in inter-kingdom communication.

Animal tetraspanin proteins can induce the formation of specific TEMs; therefore, the finding that *TET8-CFP* and *TET9-YFP* are co-localized at fungal infection sites suggests that a particular TEM exists in those regions where heterotetraspanin organization is required. Such a TEM could play a key role in exosome biogenesis and translocation since the *tet8* T-DNA knock-out mutant and two independent *tet9* CRISPR frame-shift lines resulted in a weak, but consistently enhanced susceptibility to *B. cinerea*, as compared to the wild type.⁷¹ Moreover, *tet8tet9* double mutants showed pronounced enhanced susceptibility, suggesting that *TET8* and *TET9* have partially redundant functions, and supporting the idea that *TET8*- and *TET9*-associated exosomes contribute to plant immunity against fungal infection by transferring host sRNAs into fungal cells.⁷¹ The transferred sRNAs are thought to suppress fungal pathogenicity by targeting fungal virulence genes. Most Arabidopsis sRNAs transferred to *B. cinerea* have predicted target genes, such as those involved in trafficking pathways, that are downregulated after infection. When such target genes were mutated in *B. cinerea*, the resulting strains showed reduced virulence on Arabidopsis, supporting the idea that vesicle trafficking pathways are important for fungal virulence.^{71,86} Furthermore, transgenic Arabidopsis lines overexpressing those sRNAs displayed a reduced susceptibility to *B. cinerea* and the pathogen showed reduced expression of the target genes.⁷¹ Therefore, exosomes play an essential role in cross-kingdom sRNA trafficking between Arabidopsis and the fungal pathogen *B. cinerea*.

An interesting parallel has been reported in humans: cross-kingdom trafficking of human miRNAs into the parasite *Plasmodium falciparum* inhibits expression of its pathogenicity genes, which explains why sickle cell anemia patients, who have elevated levels of these transferred miRNAs, are more resistant to malaria.^{71,96} It is unclear whether human exosomes are also responsible for sRNA delivery, and how ubiquitous such sRNA trafficking-mediated defense mechanisms are in animals and plants. Functional studies of host-transferred sRNAs may reveal

important virulence genes in pathogens and pests. Knowledge of tetraspanin activity during exosome biogenesis, cargo loading, and cross-kingdom RNAi may suggest a strategy to develop effective delivery methods of pathogen-targeting artificial RNAs with the goal of controlling plant diseases during pre- and post-harvesting of crops.

MVBs proliferate in susceptible and resistant barley (*Hordeum vulgare*) leaves in response to infection by the biotrophic powdery mildew fungus *Blumeria graminis* f. sp. *hordei*, and this response is associated with the cell wall defense response.^{97,98} This response is also exacerbated in the cytoplasm of haustorium-containing epidermal cells, where MVBs proliferate in the vicinity of the cell wall-associated oxidative microburst.⁹⁹ Since MVBs are related to exosome biogenesis, this could explain the exosome production observed at infection sites.

The successful establishment of the arbuscular mycorrhizal (AM) symbiosis relies on the formation of arbuscules in the inner cortex of the root, which mediates a nutrient exchange between the plant and fungus (Figure 2). A variety of transporter proteins on the peri-arbuscular membrane facilitate nutrient exchange and communication. Arbuscule formation depends on fungal recognition by the host plant, which requires the tight coordination of several cellular processes ranging from the formation of the pre-penetration apparatus to initial fungal colonization and development of arbuscules. During the early stages of infection, mycorrhizal associations also require cross-talk between the host and the beneficial fungus, and this signaling can occur before the hyphae have come into contact with the host. Plants secrete strigolactones, which induce hyphal branching, and stimulate the production and secretion of Myc factors, which allow the colonization process.^{100,101} It would be interesting to determine if MVBs proliferate at the arbuscule region and to determine at which stages of the mutualistic interaction exosomes are produced. So far, there is clear evidence that the secretory pathway of the plant host plays a critical role in the arbuscule formation, but it is unclear how this pathway is redirected during arbuscule development. It is attractive to speculate that exosomes coordinate the exchange of proteins, lipids, and sRNA during the establishment of AM symbiosis. Transcription factors are known to function in the transcriptional reprogramming of cortical cells during arbuscule development, and it is possible that they are modulated by exosomes released from fungi and the plant host. Studies involving the silencing and overexpression of key proteins involved in exosome biogenesis and function, such as the tetraspanins, will provide further insight into exosome biogenesis and the possible reprogramming of hyphal polarity in the hyphopodium structure which requires a further re-initiation of polarity to infect the host cells and generates the arbuscule formation.

Proteomic analyses of exosome cargo components formed during the AM symbiosis will be instrumental in determining their composition relative to those reported in pathogenic interactions.^{71,102} Recently, H₂O₂ has been described as an important component of axonal regeneration after acute injury.¹⁰³ However, the injured neurons do not express the NADPH oxidase required for ROS generation. Instead, macrophages recruited to the vicinity secrete exosomes

carrying NOX enzymes, which are taken up by the injured neuron via endocytosis and promote axonal growth in a ROS-dependent manner.^{103–105} This is the first report describing the role of exosomes in NADPH oxidase translocation in injured neurons. This is an interesting finding since NOX enzymes also function in the injury response of the fungus *Trichoderma atroviride*.¹⁰⁶ It remains to be determined whether an exosome shuttle system carrying ROS generating enzymes contributes to localized ROS production in mutualistic and pathogenic interactions, at the infection thread or during arbuscule formation. Since the arbuscules and the infected cells require an intense vesicular trafficking, it will be important to decipher the role of tetraspanins and the exosomes in the exchange of key proteins, miRNA and mRNA, required for the mutualistic interaction (Figure 2). It could be involved in hyphal colonization during mycorrhizal association and arbuscular formation. During the legume–rhizobia interaction, the infection thread allows migration of the rhizobium through the root hairs until they reach the cortical cells. It is not clear how root hairs are inverted during infection thread formation, but this process clearly involves a complete change in root hair polarity. Given the involvement of tetraspanins in membrane fusion and endocytosis, we anticipate that tetraspanins might be key players in the regulation of root hair or fungal hypha polarity and in the exocytosis and endocytosis occurring at the tip of migrating infection threads. It is clear that the exosome role is just emerging in the plant biology field, but the parallelism between the pathogenesis and mutualism, and the different strategies used by the host, pathogen or symbiont to modulate the interaction, point out to the exosomes as a key player for tuning the interaction by exchanging important components required for a successful interaction. In this scenery, specific tetraspanin such as AtTET8, which are specific markers for exosomes, will open the door for the manipulating the number of exosomes and understand the role of exosomes in pathogenic and mutualistic interaction.

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