Keep in contact: multiple roles of endoplasmic reticulum-membrane contact sites and the organelle interaction network in plants

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Summary

Functional regulation and structural maintenance of the different organelles in plants contribute directly to plant development, reproduction and stress responses. To ensure these activities take place effectively, cells have evolved an inter-connected network amongst various subcellular compartments, regulating rapid signal transduction and the exchange of biomaterial. Many proteins that regulate membrane connections have recently been identified in plants and this is the first step in elucidating both the mechanism and function of these connections. Amongst all organelles, the endoplasmic reticulum is the key structure which likely links most of the different subcellular compartments through membrane contact sites (MCS) and the ER-PM contact sites (EPCS) have been the most intensely studied in plants. However, the molecular composition and function of plant MCS are being found to be different from other eukaryotic systems. In this article, we will summarize the most recent advances in this field, and discuss the mechanism and biological relevance of these essential links in plants.

Keywords: Endoplasmic reticulum, Membrane contact sites, Organelle interactions, Cytoskeleton, Mitochondria, Membrane trafficking, Autophagy

I. Introduction

In eukaryotic cells, membrane-bound organelles allow reactions to take place in confined compartments that confer different physiological properties (Kang *et al.*, 2022). Conventionally, the vesicle trafficking pathway is believed to ensure the specificity of cargo transport between the donor and acceptor compartments (Bassham *et al.*, 2008). With the help of modern microscopy techniques and the advances in plant genetic technology, it has become increasingly clear that the functional regulation of organelles is much more complicated. Firstly, organelles move rapidly through the activity of the cytoskeleton and its

motor proteins with such activity being extremely important for cells to respond to environmental and developmental signals (Sparkes *et al.*, 2009a; Perico *et al.*, 2021; Zhang *et al.*, 2021). Secondly, these membrane-bound structures are inter-connected through membrane contact sites (MCS) and membrane adaptor proteins, forming an integrated and physically connected network to facilitate signal and cargo exchange without vesicles (Eisenberg-Bord *et al.*, 2016; Pérez-Sancho *et al.*, 2016). The establishment of organelle structural integrity requires the direct contribution of the cytoskeleton and membrane tethering factors (Perico & Sparkes, 2018). These observations lead to several questions. For example, how are membrane contact sites established in plant cells? How are organelle interactions and membrane trafficking coordinated to fulfil the needs of cell, organ and plant development? Are there any other essential cell activities that require organelle interactions? These scientific questions are of broad interest to cell biologists but are only just beginning to be understood in plants (Figure 1a).

Following the development of green fluorescence protein (GFP) technology and confocal laser scanning microscopy, the Golgi apparatus and endoplasmic reticulum (ER) were studied in living plant cells (Boevink *et al.*, 1996; Boevink *et al.*, 1998). In these early studies, it was astonishing to find that plant Golgi bodies are closely associated with the ER network and move along the track of the ER-actin cytoskeleton, suggesting that there could be direct connections between ER and Golgi in plants (Hawes, 2005). The existence of such a connection was under much debate for many years until, that is, the successful application of optical tweezers in plants which was used to show that when a Golgi body was moved by a laser beam, the ER network always followed (Sparkes *et al.*, 2009b). This was the one of first convincing light microscopy evidence that supported the physical connection between the ER network with other organelles. Since then, a number of publications have suggested various types of possible membrane connection in plants, such as plastids-plastids, plastids (chloroplasts) - peroxisomes, ER-peroxisomes, ER-chloroplast, ER-mitochondria and ER-plasma membrane (PM) (Andersson *et al.*, 2007; Sinclair *et al.*, 2009; Schattat *et al.*, 2012; Mueller *et al.*, 2014; Wang *et al.*, 2014; Oikawa *et al.*, 2015; Greer *et al.*, 2020; Gao et al., 2016) (Figure 1b-d), with the ER network acting as the hub for the establishment of organelle-membrane contact sites, which are membranes of two organelles in close proximity (<30nm) without fusion.

II. The discovery of ER-PM contact sites in plants

Close associations between ER and PM have been seen in different plants and cell types (e.g root cells of maize and lettuce) from early TEM studies (Hawes *et al.*, 1981; Stephenson &

Hawes, 1986). Please refer to Figure 2a as an example of cortical ER at the ultrastructural level (Pain et al., 2019). It was suggested that such a close association could be involved in material transfer between two compartments (Stephenson & Hawes, 1986) and could act as anchors for cytoskeletal components and organelles providing the spatial organization for various processes, or for facilitating calcium ion influx from extracellular stores to the ER lumen (Hepler *et al.*, 1990). However, these hypothesises were only based on ultra-structural observation and were hard to prove experimentally at that time since the molecular composition of these ER structures was unknown with the corresponding genetic tools unavailable. Then, the application of time-lapse imaging and image processing tools confirmed the existence of such ER-PM association in living plant cells (Sparkes *et al.*, 2009a). Using ER persistency mapping techniques, areas of the cortical ER sub-domain were observed to remain static while the rest of the ER moved dramatically. These structures were named "ER-anchor points" and they were believed to fix the rapid remodelling network to the PM (Sparkes *et al.*, 2009a).

At this time, rapid progress in the area was being made in the yeast and mammalian cell biology fields where non-vesicular lipid trafficking pathways were being studied. For example, the interactions between the ER localised Scs2p and PM localised sterol transfer proteins, such as the Osh family, in yeast (Loewen *et al.*, 2003). The function and distribution of these lipid transfer proteins at the PM was found to be restricted by Scs2p (also known as VAP in animal and VAP27 in plants), suggesting that this protein may have an important role in forming the ER-PM connection (Loewen *et al.*, 2003). The *scs2* loss-of-function mutant exhibited reduced cortical ER coverage and was found to be defective in cell budding (Loewen *et al.*, 2007). Further studies in yeast revealed additional proteins at the ER-PM contact sites, and their function in regulating lipid transport and phospholipid metabolism was revealed (Stefan *et al.*, 2011; Manford *et al.*, 2012).

In more recent studies, the protein component of plant EPCS has started to be identified (Figure 2b) and this has been achieved through the analysis of the interactions between the actin cytoskeleton, the plasma membrane and endomembrane structures (Deeks *et al.*, 2012; Wang and Hussey, 2017). To this end, a family of plant-specific actin-binding proteins (the NETWORKED family) has been identified, members of which interact with actin through their conserved N-terminal actin-binding domain, and different members associate with different membranes peripherally through their C-terminal regions, forming so-called actin-membrane contact sites (Duckney *et al.*, 2021). Among these NET proteins, NET3C was found to be

localised specifically in the region where ER membrane and PM converge. NET3C also interacts with ER localised VAP27, suggesting that the NET3C-VAP27 complex is likely to be a unique EPCS complex in plants (Wang et al., 2014). Indeed, the ER persistency analysis of VAP27-1 labelled ER network identified several immobile ER nodules that are reminiscent of the EPCS (Figure 2c). A number of additional proteins have also been shown to localise at these sites, including the plant synaptotagmin proteins (e.g SYT1, SYT3 and SYT5)(Pérez-Sancho *et al.*, 2015; Ishikawa *et al.*, 2018; Ishikawa *et al.*, 2020), the Multiple C2 domains and Transmembrane region proteins (MCTP)(Brault *et al.*, 2019), the microtubule-binding protein Kinesin-Light Chain Related proteins (KLCRs) and members of the IQ67-domain family (IQD) (Zang *et al.*, 2021a) (Figure 2d). All these proteins together with the cytoskeleton play a part in the membrane trafficking machinery and are indispensable for normal plant cell function (Wang *et al.*, 2017).

III. ER morphogenesis and dynamics

The plant ER is a highly dynamic polygonal structure composed of cisternae and tubules, which are joined at 3-way junctions and persistent nodules (Pain et al., 2019)). In Arabidopsis leaf epidermal cells and maize root cap, the width of an ER tubule was measured at approximately 40-55 nm (Stephenson & Hawes, 1986), whereas, the ER cisternae may be composed of dense ER tubules or flat ER membrane sheets with multiple nanoholes (Nixon-Abell *et al.*, 2016; Schroeder *et al.*, 2019). The structure and dynamic remodelling between tubules and cisternae are important in the normal functioning of the ER (Figure 2a). In both animals and plants, cisternae are the dominant form of ER in cells with high bio-productivity and secretory demands (Stephenson & Hawes, 1986; Shibata *et al.*, 2006; Terasaki *et al.*, 2013).

ER structural maintenance is regulated by groups of ER-shaping proteins, such as reticulon proteins (RTNs) and DP1/Yop1p/REEP (Voeltz *et al.*, 2006; Hu *et al.*, 2008;; Chen *et al.*, 2012) for tubules, atlastins/RHD3 (Hu *et al.*, 2009; Chen *et al.*, 2011; Stefano *et al.*, 2012; Lai *et al.*, 2014) and Rab10 (English & Voeltz, 2013) for tubule fusion, CLIMP-63 (Gao *et al.*, 2019), , p180 and Lunapark (LNP) proteins for cisternae (Zhao *et al.*, 2016; Kriechbaumer *et al.*, 2018a; Wang *et al.*, 2018).

In plants, RTNs induce membrane curvature in the ER by dimerisation and are capable of converting cisternae into tubules (Voeltz *et al.*, 2006; Tolley *et al.*, 2008; Sparkes *et al.*, 2010). The Arabidopsis genome encodes 21 reticulons in comparison to only 4 mammalian RTN proteins which raises the intriguing question about potentially varying functions for the

individual reticulons, such as in cell-cell communication, plant immunity, sterol regulation and autophagy (Knox *et al.*, 2015; Kriechbaumer *et al.*, 2015; Kriechbaumer *et al.*, 2018b; Zhang *et al.*, 2020; Tilsner & Kriechbaumer, 2022). LNP proteins are described as a counterpart to RTNs in plants. Arabidopsis LNP1 and 2 are capable of inducing ER sheets (Kriechbaumer *et al.*, 2018a), while mammalian and yeast LNP proteins have been described as localizing primarily to three-way junctions and to have a role in stabilizing nascent three-way junctions (Chen *et al.*, 2012; Chen *et al.*, 2015; Wang *et al.*, 2018). Atlastins are proposed to dimerize when proteins on separate ER membranes are in close proximity, to mediate the attachment of the two membranes and membrane fusion (Bian *et al.*, 2011; Moss *et al.*, 2011; Liu *et al.*, 2012). Knockdown of atlastin leads to ER fragmentation and unbranched ER tubules whilst over-expression enhances membrane fusion (Hu *et al.*, 2009; Orso *et al.*, 2009). The plant atlastin is the GTPase Root Hair Deficient 3 (RHD3)(Wang *et al.*, 1997). Overexpression of dominant negative RHD3 proteins leads to an ER network with more and longer tubular strands suggested to be a result of the inhibition of tubule fusion (Chen *et al.*, 2011; Zhang *et al.*, 2013). RHD3 proteins have also been shown to interact with reticulons in plants and mammalian systems and therefore might act synergistically to control the ER network (Lee *et al.*, 2013; Kriechbaumer *et al.*, 2015; Ueda *et al.*, 2016).

ER remodelling is a complex system but can be classified as 4 types of movement on different scales: ER particle dynamics, ER remodelling, bulk flow and inherent movement (Pain & Kriechbaumer, 2020). Bulk flow also occurs in streams, for example through the vacuole, with speeds of up to 10 µm s-1, which is similar to the rate of movement of other organelles (Ueda *et al.*, 2010; Pain *et al.*, 2019). The remodelling of the ER is mostly driven by the underlying actin cytoskeleton, together with ER-actin bridging proteins (SYP73, NET3B) and myosin motors, in particular XI class myosins (Sparkes *et al.*, 2008; Ueda *et al.*, 2010; Cao *et al.*, 2016; Wang & Hussey, 2017). Fast ER movement is inhibited in the absence of a functional actin cytoskeleton (Pain *et al.*, 2019) with some Brownian/disorganised motion of ER. Such slow ER movement may be regulated by microtubules (Hamada *et al.*, 2014; Sun *et al.*, 2020). Furthermore, the expression of ER-shaping proteins can also affect actin structure. For example, overexpression of AtLNP1 which induces ER cisternae (Kriechbaumer *et al.*, 2018a) results in a significant increase in the density of actin filaments, which are particularly dense in the region of the induced cisternae (Pain *et al.*, 2022). ER cisternae in mammalian systems have recently been associated with sub-resolution nanoholes (Nixon-Abell *et al.*, 2016; Schroeder *et al.*, 2019) and actin filaments have been implicated in supporting nanoholes in liver sinusoidal endothelial cells (Zapotoczny *et al.*, 2019). Hence, the increased actin density

linked to ER cisternae might be required to support the fenestrated cisternal structure.

Therefore, regulatory machinery and cross-talk between actin and the ER have been suggested in plants to coordinate the function of the ER-actin network during multiple subcellular processes (Pain *et al.*, 2022).

IV. ER-PM contact sites and the cytoskeleton

There are at least two types of ER-PM contact sites in plants, namely V-EPCS (VAP27 labelled) and S-EPCS (SYT1 labelled). Both structures are very close in proximity but do not co-localise (Siao *et al.*, 2016). As the ER network and cytoskeleton are closely associated, it is not surprising that the EPCS and cytoskeleton are also inter-connected (Hepler *et al.*, 1990; Wang *et al.*, 2016a). Although the complete disruption of the cytoskeletal network has no apparent effect on the structure of EPCS in mature cells, the turnover of EPCS proteins, namely NET3C and VAP27, has been shown to be significantly increased when the actin or microtubule networks are disrupted, respectively (Wang *et al.*, 2014). These data suggest that protein exchange between EPCS and their corresponding cytoplasmic pools require cytoskeleton-based transport and/or that the actin and microtubule networks reinforce the stability of the EPCS structure rather than being solely responsible for their structural maintenance.

4.1. Plant ER-PM contact sites and the cytoskeleton: a super-continuum of cytoskeleton-ER-PM-cell wall.

Plant cells are surrounded by cell walls which play critical roles in cell morphogenesis and protect cells against physical force and external biotic stress. The close connection between the cell wall, PM and the cytoskeleton has been observed by electron microscopy in various plant cells (Murray, 1984; Barton *et al.*, 2008; McKenna *et al.*, 2019). Plant EPCSs are likely to interact with the cell wall indirectly (Figure 3a), a feature that is so far unique to plants (Wang et al., 2016a). This was first discovered when a mannitol-induced plasmolysis assay was used to detach the PM from the cell wall. The plasmolysis resulted in a large number of EPCS and ER at the tips of the hechtian strands (Figure 3b) (Wang, *et al.*, 2016a). Moreover, in protoplasts without cell walls, the dynamics of VAP27 at ER-PM contact sites was found to be significantly increased which would indicate that the cell wall plays a critical role in regulating protein dynamics at the ER-PM contact sites (Wang, *et al.*, 2016a). Therefore, it is plausible that the EPCS is an integrated component of the “cell wall-PM-cytoskeleton” continuum, generating a “cell wall-PM-ER network-cytoskeleton” super continuum (Figure 3a).

Although the concept of the plant cell wall continuum has been proposed (Baluska *et al.*, 2003), it remains unclear which proteins mediate this association. The microtubule-associated KLCR proteins (also known as CMUs) have been found in PM fractions in proteomics studies; KLCR binds microtubules and confines their lateral movement (Liu *et al.,* 2016). However, loss of function mutants in KLCRs show that the attachment of microtubules to the PM is unaffected, indicating the existence of some other components (Bringmann *et al.*, 2012; ; Liu *et al.*, 2016). The IQ67 Domain (IQD) family of proteins also associate with both microtubules and PM (Bürstenbinder *et al.*, 2017). In particular, IQD13 regulates the planar shape of Rho GTPase domains on the PM, thereby affecting the morphology of secondary cell wall pits in metaxylem vessels (Sugiyama *et al.*, 2017). Could the ER network be part of this plant cell wall continuum by interacting with both the PM and the cytoskeleton? This hypothesis was tested in a recent study that demonstrated that both KLCRs and IQDs interact with NET3C at the ER-PM interface, forming a protein complex of VAP27-IQD-NET3C-KLCR (the VINK complex, Figure 3a, c) which was suggested to be involved in regulating the organization of the cytoskeleton and ER network together (Zang *et al.*, 2021a).

In Arabidopsis lines stably expressing VAP27-1-GFP and TUA-mCherry, the v-EPCS distribution were found to superimpose the organisational pattern of cortical microtubules (Figure 3d). This localisation was commonly found in trichomes and in transiently over expressing VAP27 *N. Benthamiana* epidermal cells (Wang et al., 2016a). Interestingly, some VAP27 proteins were shown to interact with microtubules (Wang *et al.*, 2014). High-resolution and live cell imaging further indicate that microtubules are closely attached to EPCSs (Figure 3e). Sometimes a microtubule can be seen positioned in the space between two closely positioned EPCSs. Similar behaviour was also found in cells expressing a truncated-mutant of myosin ATM1 that is associated with the PM (Bar-Sinai *et al.*, 2022), where the distribution of ATM1 tail is often observed in coordination with that of cortical microtubules. Based on these observations, EPCSs may provide structural barriers to restrict the lateral movement of microtubules and influence the organisation of the cytoskeleton. This hypothesis could be tested by analysing the arrangement of microtubules in cells with abnormal EPCS structure, for example using a plant mutant where most ER-PM tethers (the VAP27 and SYT families) been knocked out (as has been done in yeast) (Manford *et al.*, 2012), and performing comparative studies on cytoskeletal dynamics. Such a study is challenging in plants as most ER-PM tethers are from multi-gene families and there is likely to be strong functional redundancy. However, with the development of gene editing techniques, it is possible that multiple targets can be selected in one CRISPR/Cas9 vector to generate a higher order mutant for further analysis.

4.2 Actin-membrane contact sites and signal perception

The actin cytoskeleton interacts with multiple membrane compartments and regulates a plethora of subcellular responses including cell growth, cell wall deposition and organelle positioning (Kadota *et al.*, 2009; Whippo *et al.*, 2011; Lanza *et al.*, 2012; Henty-Ridilla *et al.*, 2013; Sassmann *et al.*, 2018; Wang *et al.*, 2019; Hawkins *et al.*, 2021). The actin cytoskeleton is also responsive to extracellular signals perceived at the plasma membrane. Several characterised signalling cascades have been shown to indirectly regulate actin downstream of extracellular signal perception at the plasma membrane. For example, the perception of pathogen elicitors at the membrane by FLS2 regulates actin-driven stomatal closure in a signalling cascade involving the small GTPase, RabG3B, and NET4 (Hawkins *et al.*, 2021). Recent research has elucidated novel protein complexes that mediate direct interaction between the actin cytoskeleton and plasma membrane (Duckney *et al.*, 2021), with putative roles in cell signalling. Physical sites of actin-membrane interactions would be ideally poised to transduce extracellular signals directly to the actin cytoskeleton to mediate rapid, localised actin-driven responses.

Contacts between the actin cytoskeleton and plasma membrane have been recently characterised in growing pollen tubes. These contacts are mediated by NET2 proteins (Deeks *et al.*, 2012; Duckney *et al.*, 2017), which anchor and organise actin at the plasma membrane and are important for organelle organisation and targeted delivery of secretory vesicles to the growing pollen tube tip (Duckney *et al.*, 2021). NET2A binds cortical actin cables at the pollen tube plasma membrane and interacts with integral membrane pollen-receptor-like kinase (PRK) proteins, PRK4 and PRK5, to form stable sites of actin-membrane interaction (Duckney *et al.*, 2017). PRK6 controls pollen tube chemotaxis towards the synergid cell-secreted peptide, LURE, to guide pollen tube growth to the ovule (Takeuchi & Higashiyama, 2016), and PRK5 has been characterised as the receptor for the pistil-secreted STIG-family peptide, GRIM REAPER (GRI), orthologues of which promote pollen tube growth through PRK signalling (Huang *et al.*, 2014; Wrzaczek *et al.*, 2015). As interactors of PRKs, NET2 proteins are also implicated in extracellular signal transduction during fertilisation. The *net2* mutant pollen tubes exhibit exacerbated growth defects in the pistil in comparison to pollen tubes grown in vitro (Duckney *et al.*, 2021). Therefore, NET2-mediated actin-membrane contact sites may regulate actin-dependent secretion and organelle organisation in response to extracellular signals, to control chemotactic pollen tube growth during fertilisation.

Furthermore, actin-membrane contacts may also integrate regulation of the cytoskeleton

with calcium signalling at the plasma membrane, a process that may require contributions from the ER network. The NET3C-KLCR1-IQD2 complex connects the ER, cytoskeleton and plasma membrane, and is implicated as a calcium signalling effector through the interaction of IQD proteins with calmodulin (Levy *et al.*, 2005; Bürstenbinder *et al.*, 2017; Zang *et al.*, 2021a). Calcium influx across the plasma membrane through membrane-localised calcium channels occurs rapidly in response to extracellular stimuli to regulate calcium-dependent responses (Xu *et al.*, 2022), and the NET3C-KLRC1-IQD2 complex may be regulated by such calcium signals. KLRC1 and IQD2 regulate cell growth and morphology, whilst ER-PM contact sites regulate exocytosis, endocytosis, lipid transfer and autophagy (discussed later)(Zang *et al.*, 2021a). These processes may be coordinated by calcium at cytoskeleton-ER-plasma membrane contact sites in response to extracellular signals.

Taken together, we have suggested that the ER-PM contact sites, as part of the super continuum of cell wall-PM-ER-cytoskeleton, provide another level of regulation of cytoskeleton arrangement and dynamics.

V. The biological relevance of plant ER-PM contact sites

With the help of Arabidopsis genetics, the functions of some ER-PM tethering proteins have been characterised. The double mutant, *net3c/net3b,* was found to be defective in pollen development and seed production (Wang *et al.*, 2014). The synaptotagmin 1 loss of function mutant (*syt1)* causes a reduction in cell viability under salinity stress (Schapire *et al.*, 2008), and inhibits cell-to-cell movement of plant viruses (Uchiyama *et al.*, 2014). Over-expression of the VAP27 homologue in poplar improves biomass production (Gandla *et al.*, 2021), while over-expressing VAP27 genes of wheat promote plant drought tolerance (Singh *et al.*, 2018). But, how do these phenotypes link to the activities of ER-membrane contact sites at the subcellular level? Moreover, what happens at the cellular, organ and whole plant level when two organelles fail to connect? Here, we will use EPCS as an example to discuss the possible roles of this link in plants.

5.1 Plant ER-PM contact sites in lipid homeostasis and signalling

Lipid transport between ER and other intracellular membranes depends on lipid transfer proteins, which target various ER-membrane contact sites (Levine & Loewen, 2006; Holthuis & Menon, 2014; Saheki *et al.*, 2016). In animal cells, ER-localized VAPA and VAPB function as the major players in directing lipid trafficking within cells; they interact with oxysterol-binding protein (OSBP) and its homologues and are involved in the transportation of oxysterols,

cholesterol and glycerophospholipids (Kentala *et al.*, 2015). In the absence of VAPA and VAPB, the function of OSBP/OPRs is affected leading to the re-distribution of PI4P (Dong *et al.*, 2016). In yeasts, oxysterol-binding homology 3 (Osh3) has been shown to be recruited to ER-PM contacts by Scs2/Scs22 in response to changes in PM PI4P (phosphatidylinositol 4-phosphate) levels. The loss of function of Scs2/Scs22 impaired Osh3 targeting to the ER, and PI4P levels in the PM were greatly elevated (Stefan *et al.*, 2011). Other Osh family membranes, such as Osh6 and Osh7 have also been reported to target the ER-PM contact sites in order to maintain phospholipid metabolism (Wong *et al.*, 2021).

It is known that the phosphoinositide lipid composition of the PM can determine its properties and can act as the key regulator of membrane trafficking (Platre *et al.*, 2019). The changes in phospholipid polarity as the result of impaired ER-PM interaction can generate pleiotropic developmental defects. This idea was supported by yeast studies (Stefan *et al.*, 2011; Omnus *et al.*, 2020), but is also likely to be true in plants (Platre *et al.*, 2019; Wang & Hussey, 2019). Similarly, plant ER-PM contact sites have also been reported to be important in lipid metabolism and homeostasis. SYT proteins are composed of a lipid transfer domain, which plays a role in lipid transfer between the ER and the PM (Qian *et al.*, 2022). The localization of plant SYTs relies on PM PI4P and is regulated by abiotic stress (Lee *et al.*, 2019). Lack of SYT1 and SYT3 increases the accumulation of diacylglycerol during cold stress, indicating an important role of SYTs in lipid metabolism and PM integrity (Ruiz-Lopez *et al.*, 2021).

In addition, the function of EPCS in lipid homeostasis and transport may be important in plant immune responses and symbiosis. For example, during powdery mildew infection, a feeding structure called a haustorium is formed within the host cell. Each haustorium is enveloped by a highly modified extrahaustorial membrane (EHM), which derives from the host PM (Kwaaitaal *et al.*, 2017; Qin & Wei, 2021). At the powdery mildew infection sites, one of the major PM phosphoinositides, PI(4,5)P2, is significantly up-regulated and targets the EHM through an actin-dependent trafficking pathway. The depletion of PI(4,5)P2 impairs fungal development, thus increasing plant disease resistance (Qin *et al.*, 2020). As EPCS are well-known for their involvement in regulating phospholipid signalling and PM phospholipid composition, and that the ER membrane is closely associated with the EHM (Kwaaitaal *et al.*, 2017; Breeze *et al.*, 2020), it is expected that plants with altered ER-PM interaction may have a strong impact on the response to fungal pathogen invasion. In addition, lipid transport between ER and PM may also contribute to plant symbiosis, since fatty acids that are synthesized by host plants are preferably utilised as the carbon source by arbuscular

mycorrhiza fungi (Jiang *et al.*, 2017; Luginbuehl *et al.*, 2017). Arguably, the most effective route for lipid molecules (that are made in the ER) to be exported from host cells is going to be through a non-vesicular trafficking pathway at the EPCS, where lipid transfer proteins can be actively recruited. Evidence from an electron microscopy study supports this idea, where synaptotagmins are found at the interface of the symbiotic membrane of Medicago root nodules (Gavrin *et al.*, 2017), and the Arabidopsis *syt1* loss-of-function mutant exhibits enhanced disease resistance to fungal infection (Kim *et al.*, 2016). Taken together, it is plausible that plant ER-PM contact sites as well as some of the resident proteins form an important part of the plant-fungal cross-talk by maintaining homeostasis of the lipid profiles in the PM, as well as regulating lipid transport.

5.2 Is the ER-PM interaction involved in ER stress and ER-autophagy?

Autophagosome biogenesis is likely to be controlled by ER-MCS regulated lipid homeostasis as the formation of nascent phagophore assembly sites (PAS) requires a direct contribution from the ER membrane coupled with a specific phospholipid enrichment (Gomez et al., 2022). Autophagy is an evolutionarily conserved degradation mechanism for recycling proteins or impaired organelles in the vacuole (Zhuang *et al.*, 2018). This process is regulated by conserved AUTOPHAGY-RELATED (ATG) proteins, which mediate autophagosome formation and cargo selection (Wang *et al.*, 2020). In eukaryotes, many environmental stresses (such as phosphate starvation, salt stress, and heat stress) cause the accumulation of misfolded proteins in the ER and induce ER stress (Zeng *et al.*, 2019). To overcome these unfavourable conditions, cells activate the Unfolded Protein Response (UPR), ER-Associated Degradation pathway (ERAD) and selective autophagy of bulk ER membrane (ER-phagy) to relieve the stress (Brandizzi, 2021). It has been suggested that the ERAD pathway is preferred in mild stress conditions, whilst ER-phagy is required in more severe conditions where there will be a high demand for ER-related degradation (Chen *et al.*, 2020; Pastor-Cantizano *et al.*, 2020). Several ER-phagy receptors have been identified in plants, including AtSec62 (Hu *et al.*, 2020), AtC53 (Stephani *et al.*, 2020), AtRHD3 (Sun *et al.*, 2022), AtATI1 and AtATI2 (Wu *et al.*, 2021) in Arabidopsis, and RTN1 & 2 (Zhang *et al.*, 2020) in maize. These receptors regulate the degradation of ER membrane similarly; they interact with ATG8 through the conserved AIM (ATG8-interaction motif) domain and with the ER membrane. When ER stress is induced (e.g through tunicamycin treatment), unwanted ER membrane containing misfolded protein is recruited to autophagosomes through receptor-ATG8 interactions (Stephani *et al.*, 2020).

As well as the ER intrinsic regulators, ER-related autophagy requires contributions from

regulators of the actin cytoskeleton. The SCAR/WAVE complex for example that can activate the ARP2/3 complex and promote actin nucleation (Deeks *et al.*, 2004). NAP1 as a component of the SCAR/WAVE complex that is recruited to the ER surface upon autophagy activation (e.g mechanical stress), and recruits ATG8 as autophagosomes mature (Wang, *et al.*, 2016b). Interestingly, some NAP1 labelled foci are found to be v-EPCS associated, suggesting a potential link between ER-PM interaction and actin nucleator-regulated autophagy (Wang *et al.*, 2016b). Recently, more evidence has become available to further confirm that ER-PM contact sites are required for autophagosome assembly when ER-phagy is induced (Nascimbeni *et al.*, 2017; Nthiga *et al.*, 2020; Zhao *et al.*, 2020). In Arabidopsis, an endocytosis regulator AtEH1/Pan1 is required for autophagosome formation through its interaction with actin and VAP27-1 at the ER-PM contact sites (Wang *et al.*, 2019), possibly regulating the turnover of endocytic cargoes. The overexpression of AtEH1/Pan1 promotes autophagic activity in *N. benthamiana* leaf epidermal cells, while reduced expression of either AtEH1 or VAP27-1 blocks autophagy and the resulting mutant plants are more sensitive to starvation (Wang *et al.*, 2019). A similar study has been conducted recently in yeast, where it has been demonstrated that the inhibition of Pan1-End3 (homologues of AtEH1/Pan1) or the Arp2/3 complex reduces autophagy activity following treatment with rapamycin and show that the actin assembly at endocytic sites plays an important role in ER-phagy (Liu *et al.*, 2022). Meanwhile, Scs2 contributes to the formation of ER-containing autophagosomes and physically interacts with Atg40 at ER sheets or tubules after ER-phagy is induced (Liu *et al.*, 2022). As the process of selective autophagy is precisely regulated, the ER-PM contact sites may be indispensable for certain types of autophagy, such as the selective degradation of endocytic and exocytic cargos that are normally close to the PM (Figure 4a)(Zhao *et al.*, 2020).

Based on these findings that actin assembly is necessary for the formation of autophagosomes at the phagophore assembly site (PAS) and proteins at the ER-PM contact sites are important for autophagy (Figure 1a, d)(Wang *et al.*, 2016b; Wang *et al.*, 2019; Wang *et al.*, 2020; Liu *et al.*, 2022), it is possible that it is the VAP27 proteins that are the core component involved in the recruitment of the ER-phagy receptor and other autophagy regulating factors under stress (Figure 4b, Nthiga *et al.* 2020, Zhao *et al.* 2020, Liu *et al.* 2022, Ye et al., 2022). In Arabidopsis, the abundance of ER-PM contact sites increases dramatically under nitrogen-starvation and salt stress, both of which induce autophagy, suggesting that the formation of EPCS is correlated with elevated autophagy activity. Therefore, it is worth exploring in the future (1) whether ER-phagy activity is attenuated when ER-PM contact sites are disrupted, and (2) whether ER-phagy receptors can be recruited to ER-membrane contact sites through their

interaction with putative tethering proteins (e.g. VAP27 or SYT1) in plants.

So, what advantage do plants have in using the ER-MCS for autophagy? It is well-known that the ER membrane is the main membrane donor for autophagosome formation (Zhuang *et al.*, 2018); having the PAS, autophagic cargos (e.g vesicles or organelles) and ER membrane closely attached is certainly going to be more effective for fast turnover. Furthermore, autophagosome biogenesis requires the cooperative effort of the membrane-trafficking pathway, the cytoskeleton and the establishment and maintenance of local lipid composition. Clearly the ER-PM contact sites have all the necessary machinery in place to service autophagosome formation.

5.3 Membrane trafficking, endocytosis and exocytosis

Since EPCS regulates phospholipid homeostasis which is essential for cell polarity and membrane identity, EPCS may work as a hub where endocytosis and exocytosis take place (Lewis & Lazarowitz, 2010; Jahn & Fasshauer, 2012; Stefano *et al.*, 2018). It is well known that synaptotagmin proteins participate in regulating both exocytosis and endocytosis in both animal and plant cells (Min *et al.*, 2007; Jean *et al.*, 2010; Lewis & Lazarowitz, 2010). Arabidopsis synaptotagmin 1 (SYT1) has been found to negatively regulate the secretory pathway in response to fungal infection (Kim *et al.*, 2016). Moreover, proteins at the EPCS are also involved in endocytosis. Arabidopsis SYT1 partially localizes with endosomes and the expression of a dominant-negative mutant of SYT1 inhibited the formation of endosomes (Lewis & Lazarowitz, 2010). Furthermore, VAP27 proteins have been reported to be associated with endosomes; VAP27-1 and VAP27-3 bind to clathrin and lipids that are enriched in the endocytic membranes (Stefano *et al.*, 2018). Knocking-out both VAP27-1 and VAP27-3 results in the disruption of endocytosis and impaired plant growth possibly caused by an aberrant homeostasis of endosome membranes and the ER network (Stefano *et al.*, 2015; Stefano *et al.*, 2018).

Non-clathrin-dependent endocytosis (NCE) is another route for cells to uptake substrate from outside the cell. This process, although not fully characterised in plants, also relies on EPCS and Ca2+ signalling. The ER-resident protein reticulon 3 (RTN3) colocalizes with E-Syt1 and promotes the formation of EPCS that are required for NCE invagination (Caldieri *et al.*, 2017). The association of RTN3 and EPCS may also exist in plants, as the interaction between Arabidopsis RTN3, VAP27 and SYT1 has been reported in a protein interaction study (Kriechbaumer *et al.*, 2015). As VAP27 and SYT1 have been reported as regulating endocytosis

in plants, this suggests that both types of EPCS may be involved in the membrane trafficking pathway by interacting with different partners. However, whether the EPCS-dependent endocytosis has any cargo specificity is not clear. A study showed that VAP27-1 physically interacts with PM intrinsic proteins (PIPs) in both maize and Arabidopsis (Fox *et al.*, 2020). ZmVAP27-1 and ZmPIP2;5 colocalize close to EPCS and endocytic structures when exposed to salt stress suggesting that VAP27 may mediate the endocytosis of the aquaporins (Fox *et al.*, 2020). Certainly, it would be of great interest to identify the protein cargoes that are specifically transported at EPCS, and how EPCS, actin and phospholipids coordinate this process.

5.4 Manipulating the level of ER-PM connections has dramatic effects on cell activity

It is evident that the contact between the ER network and PM not only acts as a structural element within the cell but this contact also plays other essential roles by recruiting different proteins or alternating the strength of ER-PM interaction (Figure 5). In animal cells, EPCS is used as a 'gatekeeper' of different ion channels. The most well-known example is calcium transport from the extracellular space through a PM localised Ca^{2+} channel, Orai1, and the ER localised STIM1 protein (Jing *et al.*, 2015; Chang *et al.*, 2018). Upon depletion of the ER calcium store, STIM1 oligomerizes at the ER-PM junction (with the help of STIM2) and triggers the interaction and activation of Orai1 to allow Ca2+ entry across the PM (Son *et al.*, 2020). This is a precisely regulated process which requires a constant distance between ER-PM; cells with artificially enlarged cortical ER and enhanced ER-PM interaction are less sensitive to agonist-induced Ca2+ release (Henry *et al.*, 2022). Conversely, EPCS formation may also negatively regulate ion transport, for example, potassium. In neuronal cells, a potassium channel, Kv2.1, interacts with the cortical ER through the VAP proteins to form a connection between ER and PM (Johnson *et al.*, 2018; Kirmiz *et al.*, 2018). In the resting state, VAP interacts with Kv2.1 protein clusters which is regulated by phosphorylation and blocks the channel; the ER is dis-associated from the PM when K^+ influx is required (Figure 5a). As the EPCS occupies a large portion of the cell surface (10%>), its regulation of ion channel activity is essential in neuronal physiology (Johnson *et al.*, 2018). Recent proteomic and forward genetic screens revealed that a PM-localised mechanosensitive (MS) ion channel, MSL10 interacts with VAP27 in Arabidopsis (Codjoe et al, 2022), suggesting a similar mechanism may exist in plants. It is highly possible that in certain cell types, for example guard cells, additional regulations of ion channels is important. This is because such cells require rapid ion exchange to maintain their electrophysical property for rapid signalling events; stomata would be a

prime example as these open and close in response to calcium and potassium signals working in concert with the cytoskeleton (Jiang *et al.*, 2012). Another example has come from the study of exocytosis in fission yeast (Ng *et al.*, 2018). To ensure polarized exocytosis, EPCS form in the non-growing region to prevent exocytic vesicle tethering at the PM (Figure 5). Conversely, active exocytosis at the tip growing region prohibits local EPCS formation (Ng *et al.*, 2018). When EPCS formation is massively enhanced by over-expressing an artificial ER-PM tether, cell polarity and secretion are strongly affected (Figure 5b). A similar phenomenon may also be found in plant tip growing cells (Figure 5c). In Arabidopsis, the ER distributes in the subapical zone of growing root hairs, with Rab-A2a-labelled secretory vesicles accumulating at the apical dome (Qi *et al.*, 2016), which may suggest that the role played by EPCS in regulating polarized secretion is common in yeast and plants. However, this hypothesis has not been tested as we do not have an effective tool to prevent ER-PM connections in plants. But, by over-expressing EPCS tethering proteins, such as NET3C, it is possible to do the opposite and promote EPCS formation (Wang *et al.*, 2016a; Zang *et al.*, 2021a). Studying cell polarity and physiology when ER-PM connections are enhanced may also provide useful ideas for future functional studies.

5.5. ER-PM contact sites and the plasmodesmata

PDs are pores between neighbouring cells that transverse cell walls and the central desmotubules are a continuous part of the ER network (Hepler, 1982; Fitzgibbon *et al.*, 2010). Within the symplastic pathway, PDs serve as channels for directional transport of biomolecules, such as phytohormones or proteins, between neighbouring cells (Christensen *et al.*, 2009). During virus infection, SYT1 is recruited to PDs by viral movement proteins to alter viral cell-to-cell movement (Levy *et al.*, 2015; Ishikawa *et al.*, 2020). Another great example of EPCS and PD interaction is from a study of grafting using correlative light electron microscopy (Chambaud *et al.*, 2022). Here, it was found that the formation of PD between the scion and rootstock require ER–PM tethering at the place where cell walls are thinner.

In addition, actin is linked to PDs via formins or NET proteins (e.g NET1)(Deeks *et al.*, 2012), and the size exclusion limit of PDs is increased in mutants defective in formin FH2 (Diao *et al.*, 2018). Depolymerization of actin filaments increases the permeability of tobacco mesophyll PDs (Ding *et al.*, 1996; Su *et al.*, 2010). However, the exact molecular machinery involved in mediating ER-cytoskeleton cross-talk and its connection with PDs remains elusive. Could the protein complex containing cytoskeletal regulators and membrane tethering factors at the EPCS contribute to PD function? The VINK complex, which localises at the super continuum

of the cell wall-PM-ER-cytoskeleton (Zang *et al.*, 2021a; Zang *et al.*, 2021b), may emerge as a promising candidate to fulfil the roles required for PD function. Such protein complexes may act as a gating system by mediating ER-PM interaction around PD by potentially detaching the ER from the PM upon a local activation of actin polymerisation (a mechanism that has been described in animal cells)(van Vliet *et al.*, 2017). Alternatively, EPCS close to the PD may recruit cytoskeletal components that regulate cytoskeletal organization and move cargo towards the PD along the ER-cytoskeleton track.

VI. The VAP27 proteins lead the dance at multiple MCS

VAP27 and its homologues in animals and yeast (VAP-A/B, Scs2 respectively) have been identified at multiple membrane interfaces and interact with different proteins. In plants, VAP27-1 may interact with the nuclear pore complex at the nuclear envelop (Tang *et al.*, 2020); interacts with SEIPIN proteins at ER-lipid droplet (LD) junctions to regulate LD biogenesis (Greer *et al.*, 2020) and interacts with TraB family proteins at the ER-mitochondria contact site (Li *et al*., 2022). The VAP27 proteins, as a major component of different MCS may therefore confer a different functionality on the ensuing complex.

For example, the formation of the ER-mitochondrial contact sites (EMCS) that are involved in mitochondrial dynamics and function, also require the contribution of VAP27 proteins. In animal cells, VAP-B and PTPIP51 (a mitochondrial outer membrane protein) are found to interact with the ER-mitochondrial interface; knocking down either reduces the level of contact between the two organelles (Stoica *et al.*, 2014). In plants, MELL1 (mitochondria-ER-localized LEA-related LysM domain protein 1) and Miro 2 (Mitochondrial Rho GTPase 2) are two potential candidates for plant EMCS components that could be involved in the maintenance of mitochondrial morphology, mitochondrial dynamics and ER-mitochondrial tethering (Mueller & Reski, 2015; Yamaoka & Hara-Nishimura, 2014; White *et al.*, 2020). A recent functional study in Arabidopsis identified a novel component of eukaryotic EMCS, comprising the VAP27 family and an outer mitochondrial membrane protein from the TraB-family, TRB1 (Li *et al*., 2022). The interaction between VAP27 and TRB1 was found not only to be involved in maintaining ER-mitochondrial connections but also to regulate the turnover of damaged mitochondria through the autophagy pathway. In this process, TRB1 acts as a mitophagy receptor and interacts with ATG8, indicating that the establishment of ER-mitochondrial connections and the initiation of mitophagy are two interrelated processes.

It is well known that ER is an important organelle for protein modification and folding and

mitochondria are responsible for energy metabolism. Both structures are very sensitive to stress conditions that will induce the ER or mitochondrial unfolded protein response (Ngoh *et al.*, 2012; Lebeau *et al.*, 2018;; Zhou *et al.*, 2020). In animal cells treated with TM or DTT, the number of EMCS changes significantly, implying that ER stress can affect its interaction with mitochondria in order to maintain the homeostasis of both organelles (Bravo *et al.*, 2011; Sanchez-Alvarez *et al.*, 2017). Although the current studies in this area are mostly in animal systems, it can be postulated that some homologous proteins in plants are likely to have the same function. For example, mitochondrial respiratory activity and retrograde signalling are essential for plants to overcome ER reductive stress, indicating the existence of inter-organelle crosstalk in plants (Fuchs *et al.*, 2022). Arabidopsis mutants with dysfunctional EMCS complex, such as the TRB1 or VAP27 loss-of-function mutant, are both sensitive to mitochondrial stress (2,4-Dinitrophenol, treatment) because of impaired mitophagy activity. This finding further indicates that EMCS along with VAP27-TraB1 participates in mitophagy, which in turn maintains healthy mitochondrial function and energy homeostasis (Li *et al*., 2022).

So far, the protein composition of other plants' unique ER-MCSs have not been identified; the ER-chloroplasts/plastids and the ER-vacuole contact sites for example, but early studies have provided some preliminary results that suggest the involvement of VAP27 proteins. In a study of membrane proteins that transport directly from ER to storage vacuoles, VAP27 was found to localise to pre-vacuolar or vacuole-like organelles (Oufattole *et al.*, 2005). Also, during viral infection, VAP27 was found to interact with viral proteins that are enriched at the chloroplast envelope (Wei *et al.*, 2013). Whether VAP27 is involved in the formation of these MCS will be the subject of further in-depth studies.

VII. Interpreting protein function at ER-MCS: the caveats and a suggestion.

Since the identification of ER-MCS and MCS resident proteins, the molecular machinery of many subcellular activities has been re-visited. However, there are some caveats in interpreting protein interaction and localisation to ER-MCS. Most organelles may move along the same actin filament in a densely packed cytoplasm, resulting in a close proximity with the ER network that found in the same confined region. Therefore, complementary techniques are required to provide a clear indication of physical association between two organelles.

According to the principle of the bimolecular fluorescence complementation (BiFC) assay, a set of artificial MCS reporters have been designed to study protein location at different ER-membrane contact sites (Yang *et al.*, 2018; Tao *et al.*, 2019). To do this, the ER membrane and

another organelle membrane are each labelled with half of the super-folder GFP (spGFP), spGFP1-10 and spGFP11, respectively; signal can be detected at the interface of the two organelles if they are in close contact (at a distance less than 30nm). However, it should be noted that over-expression of these MCS reporters can induce the formation of MCS artificially. This problem is especially prominent when the split-YFP derived reporter is used (Tao *et al.*, 2019). When studying protein interactions between the ER and PM, non-specific dimerization between YFP-derived BiFC pairs may induce false interactions and create signals that are very similar to known EPCS proteins. These data highlight the risks of using such reporters to study membrane protein interactions at putative MCS (Tao *et al.*, 2019). Another type of ER-MCS reporter has been designed using protein domains that target different membrane compartments. The EPCS reporter, MAPER-GFP is an example, which contains a transmembrane domain targeted to the ER and a lipid-binding motif that can interact with PM (Lee *et al.*, 2019). At low levels of expression, MAPER-GFP localises to the ER network and is preferably enriched at the ER-PM junction. However, when its expression is high, this reporter inevitably induces additional ER-PM interaction by enhancing ER-PM interaction and therefore affecting ER morphology (Henry *et al.*, 2022). So, when studying ER-membrane interactions with any of the reporters that are currently available, it is essential that the impact of the level of reporter expression is considered.

Some known EPCS proteins, such as VAP27-1 and SYT1 linked to a fluorescent protein (FP), are also used as EPCS markers. However, these contain a single transmembrane domain that is targeted to the entire ER membrane, with only a portion enriched at the EPCS (Figure 6a, b). This is very different from other commonly used FP markers that label one specific subcellular structure (e.g. GFP-HDEL for the ER) . Therefore, when a protein interacts or is co-localized with VAP27 or SYT1, the first question to ask is where this interaction will take place. Does this interaction have a biological relevance at the ER-MCS specifically or the ER network in general? For example, it is known that VAP27 labelled stationary puncta interacts with the actin-binding protein, NET3C, and confocal images indicate that this interaction takes place at the EPCS only as no co-localization was detected on any other part of the ER (Figure 6c, d)(Wang *et al.*, 2014). On the other hand, the ER shaping protein, reticulon 3 (RTN3) has also been identified as an interacting partner of VAP27 (Kriechbaumer *et al.*, 2015). When VAP27-1-YFP and RTN3-RFP are co-expressed in *N. Benthamiana* epidermal cells, the two proteins only co-localize on the ER network (Figure 6e, f). The VAP27-1-YFP labelled EPCS is separate from the RFP signal indicating that the interaction may not take place at the EPCS. Therefore, for studying protein activities at EPCS, showing that there is an interaction/co-localisation

between VAP27 and another candidate protein is not sufficient to demonstrate that their activities are related to the EPCS because most of the VAP27 is found on the ER network. We suggest that further confirmation of the relationship between the two proteins of interest at the EPCS is essential. Further evaluations, such as ER persistency analysis (Figure 2b), high-magnification images on the EPCS structure (Figure 3e; Figure 6c, d) and possibly some additional ultrastructural studies would be necessary (Wang *et al.*, 2018).

VIII. Conclusions

In 2014, we identified the first protein complex at the plant ER-PM contact sites (Wang *et al.*, 2014) and proposed that such a connection could be involved in cytoskeleton organisation, biotic stress responses and various membrane trafficking pathways (Wang *et al.,* 2017). Eight years later, with the development of advanced microscopy techniques, proteomic and genomic tools, some of these proposed functions are starting to be elucidated by an increasing group of plant cell biologists (Pérez-Sancho *et al.*, 2015; Ishikawa *et al.*, 2018; Brault *et al.*, 2019; Ishikawa *et al.*, 2020; Zang *et al.*, 2021a). It is becoming clear that most organelles' interactions are essential for life. Like human beings living in a globalised society, organelles also need to be kept in contact.

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Author contributions

P.W. and T.Z edited the manuscript with contributions from all authors.

Data availability

Data available on request from the authors.

Figure legends

Figure 1. An overview of various ER-organelle interactions and membrane contact sites in plants. (a) An illustration of ER-membrane contact sites that have been reported in plants includes ER-PM contacts sites, plasmodesmata, ER-mitochondrial contact sites, ER-Golgi

interface, ER-chloroplast interaction and ER-autophagosome interaction. VAP27 is likely to participate in most of these connections according to the literature in mammalian systems and plants. **(b-d)** A few examples of ER-organelle interactions at the light microscopy level, such as ER (GFP-HDEL) and Golgi bodies (ST-RFP; scale bar = 1 μm) (McGinness et al., 2022); ER (GFP-HDEL) and mitochondria (Mito-mCherry; scale bar = 2 μm) (Li et al., 2022); ER (RFP-HDEL) and autophagosomes (NAP1-GFP; scale bar = 2 μm) (Wang et al., 2016b). All images were taken from tobacco leaf epidermal cells.

Figure 2. The ER-PM contacts and their protein composition. (a) Zinc-iodine-osmium (ZIO) stained cortical ER from an Arabidopsis leaf epidermal cell, images captured using SBF-SEM and 3D reconstruction. Green boxes and arrows, tubular ER; magenta boxes and arrows, cisternal ER (Scale bar = 1 μm). (Pain et al., 2019). **(b)** The protein interaction network of VAP27, solid lines indicate confirmed interaction, and dashed line indicates predicted interaction (Image modified from Zang et al., 2021b). **(c)** Persistency analysis of VAP27-1-YFP that are transiently expressed in *N.Benthamiana* leaves. Images were coloured red (0s), green (15s) and cyan (30s), and overlaid; the resulting magenta colour represents persistent signals over 30 seconds (Wang et al., 2014). **(d)** Identified proteins that localise to the ER-PM contact sites in plants.

Figure 3. The super continuum of cell wall-plasma membrane-ER network-cytoskeleton. (a) The VINK complex contains the VAP27, IQD NET3C and KLCR proteins, localised at the ER-PM interface and bridge in-between the actin cytoskeleton and microtubules. **(b)** In a leaf epidermal cell, V-EPCS (VAP27-1-YFP) and the ER membrane are found at the tips of the hechtian strands (PM derived, PIP2-CFP) after plasmolysis (scale bar = 10 μm). (Wang et al., 2016a) **(c)** The co-expression of GFP-IQD2, VAP27-YFP and RFP-NET3C induces the formation of ER-actin-microtubule hybrid structures (scale bar = 10 μm). (Zang et al., 2021a). **(d)** In Arabidopsis trichomes, the localisation of VAP27-1-GFP labelled EPCSs were found to follow the organisational pattern of cortical microtubules (TUA-mCherry; scale bar = 10 μm) (Wang et al., 2016a). **(e)** Super-resolution microscopy (HIS-SIM) of root cells expressing VAP27-1-GFP and TUA-mCherry (Wang et al., 2016a) reveals that V-EPCS are often attached to the cortical microtubules (scale bar = 10 μm). Interestingly, some microtubule filaments are found passing through two adjacent V-EPCSs (zoomed images).

Figure 4. VAP27 regulated autophagy in plants. (a) At the ER-PM contact sites, VAP27 interacts with the endocytic machinery to regulate endocytosis and autophagy (Wang et al., 2019). **(b)** VAP27 likely interact with autophagy receptors to regulate autophagosomes

formation at the ER surface.

Figure 5. The level of ER-PM interaction determines polarised secretion and ion transport. **(a)** In neuronal cells, the VAP interact with PM localised ion channels (Kv2.1) and regulate potassium influx. Such process is also regulated by phosphorylation. **(b)** In fission yeasts, enhanced ER-PM association restricts the direction of exocytosis and contributes to cell polarity. **(c)** In plants, tip growth cells may control exocytosis and directional growth through regulating the level of ER-PM interactions.

Figure 6. The localisation of VAP27 and SYT1 in respect to the ER network and its interacting patterners. (a-b) VAP27-1 and SYT1 localise to the entire ER network and enrich at the ER-PM contact sites (Wang et al., 2016a; Siao et al., 2016). **(c-d)** GFP-NET3C localisation and interaction with VAP27-1-YFP at the EPCS specifically; little co-localization was identified in any other part of the cell (Wang et al., 2016a). **(e-f)** VAP27-1-YFP co-localized and interact with the RTN3-RFP on the ER network, but not at the EPCS (Kriechbaumer et al., 2015) (Scale bar = 10 μm).

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