Journal of Microscopy

Journal of Microscopy, Vol. 280, Issue 2 2020, pp. 122–133 doi: 10.1111/jmi.12909
Received 12 March 2020; accepted 14 May 2020

The plant endoplasmic reticulum: an organized chaos of tubules and sheets with multiple functions

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Key words. Confocal microscopy, Electron microscopy, Endoplasmic reticulum, ER exit site, Fluorescent proteins, Golgi, Microscopy.

Summary

The endoplasmic reticulum is a fascinating organelle at the core of the secretory pathway. It is responsible for the synthesis of one third of the cellular proteome and, in plant cells, it produces receptors and transporters of hormones as well as the proteins responsible for the biosynthesis of critical components of a cellulosic cell wall. The endoplasmic reticulum structure resembles a spider-web network of interconnected tubules and cisternae that pervades the cell. The study of the dynamics and interaction of this organelles with other cellular structures such as the plasma membrane, the Golgi apparatus and the cytoskeleton, have been permitted by the implementation of fluorescent protein and advanced confocal imaging. In this review, we report on the findings that contributed towards the understanding of the endoplasmic reticulum morphology and function with the aid of fluorescent proteins, focusing on the contributions provided by pioneering work from the lab of the late Professor Chris Hawes.

The dynamics of the ER network

The endoplasmic reticulum (ER) has long been known to be an extremely dynamic and pleomorphic organelle. This has been investigated by video enhanced light microscopy and fluorescence microscopy using dyes such as DiOC6. (Quader & Schnepf, 1986) Such dyes implicated the actin cytoskeleton as an organizer of the ER network (Quader *et al.*, 1989; Quader & Fast, 1990), though the investigation of the true dynamic nature of the ER was greatly aided by fluorescent protein technology. (Boevink *et al.*, 1996) In *Arabidopsis thaliana* plants, fluorescent proteins were targeted to the ER and lit up the well-known fusiform bodies. (Hawes *et al.*, 2001) These ER bodies move in an actin-dependent manner but as there is no evidence of cytoskeletal elements in the ER lumen fusiform body movement must mirror the movement of the whole ER network. To investigate this, photo-activatable green fluorescent protein (paGFP) fused to the membrane spanning domain of the ER membrane chaperone, calnexin, was expressed in tobacco leaves (Runions *et al.*, 2006) using agrobacterium-mediated infiltration (Sparkes *et al.*, 2006) and indeed showed that ER membrane rapidly disperses from the activation point in a direct vector. In the absence of actin, activated membrane diffused evenly through the ER. ER movement is generally inhibited in the absence of a functional actinomyosin cytoskeleton, (Griffing *et al.*, 2014; Hawes *et al.*, 2015; Pain *et al.*, 2019) though some Brownian/disorganized motion of ER junctions can be detected even after latrunculin B treatment (Lin *et al.*, 2017; Pain *et al.*, 2019) which prevents polymerization of the actin cytoskeleton. Residual ER movement in actin-depleted cells may be due to an interaction of the ER with microtubules, as seen in *Arabidopsis*. (Hamada *et al.*, 2014)

ER movement can be divided into four classes, each applying to a different scale: ER particle dynamics, ER remodelling, bulk flow and inherent movement (for review see (Pain & Kriechbaumer, 2020)). ER network remodelling can be visualized as image overlays to provide a temporal colour-coding of the moving ER network, (Ridge *et al.*, 1999; Kriechbaumer *et al.*, 2018) which allows for a visualization of ER remodelling but lacks quantitative information. Hence, there have been recent efforts to develop computational methods to allow for quantitative dynamics analysis (for review see Pain *et al.*, 2019; Pain & Kriechbaumer, 2020). In addition, improved live cell imaging, for example the use of fluorescence recovery after photobleaching (FRAP), single molecule tracking and photoactivatable and photoconvertible fluorophores, enables researchers to study the movement of the ER not only as a total structure but also at a molecular level. (Runions *et al.*, 2006; Tolley *et al.*, 2008; Sparkes *et al.*, 2009; Tolley *et al.*, 2010; Mathur *et al.*, 2012; Griffing *et al.*, 2014; Breeze *et al.*, 2016; Holcman *et al.*, 2018).

The structure of the plant ER is composed of morphologically and dynamically discrete subdomains

Mammalian and yeast ER are distributed in 3D throughout the entirety of most cells (Friedman & Voeltz, 2011; West *et al.*, 2011) whereas most of the plant ER is functionally 2D

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and appressed between cell wall/plasma membrane and the vacuole. Here, strands of the ER can cross the vacuole and connect with the nuclear envelope. But this is only a small volume of the total ER. Electron microscopy (EM) measurements in *Arabidopsis* leaf epidermal cells (Pain *et al.*, 2019) and maize root cap cells (Stephenson & Hawes, 1986) calculated that an ER tubule is approximately 40 and 55 nm wide, respectively.

The dynamic ER structure is composed of two morphologically distinct domains: sheets (cisternae) and tubules, which are joined at three-way junctions to create a loose polygonal structure (Figure 1). Tubules are composed of highly curved surfaces and are connecting components of the plant ER (Westrate *et al.*, 2015). Cisternae were originally defined as larger ER regions forming a contiguous lumenal space between two flat ER membrane sheets (Westrate *et al.*, 2015). Recent literature in nonplant species has been suggesting though that cisternae may be composed of either densely reticulated networks of tubules, so tightly compressed that the individual tubules require super-resolution imaging in order to resolve them, (Nixon-Abell *et al.*, 2016) or are in fact perforated with holes throughout the cisternae (Schroeder *et al.*, 2019). Nixon-Abell *et al.* (2016) applied Grazing Incidence Structured Illumination Microscopy (GI-SIM) to reveal dense reticulate matrices of tubules in both Cos-7 and U-2 OS cells expressing the ER membrane marker Sec61B fused to a fluorescent protein. These dense networks appeared indeed as flat, intact cisternae when using diffraction-limited wide-field imaging. It is also suggested that these cisternal networks can be rapidly generated from tubules but due to the speed of cisternae formation and disassembly, homeotypic fusion of tubules is not required to generate cisternae. (Nixon-Abell *et al.*, 2016) STED microscopy on Cos-7 cells expressing ER lumenal markers revealed small, sub-diffraction holes throughout ER cisternae (Schroeder *et al.*, 2019); these 'nanoholes' show variable motility and are suggested to be of a similar size as nuclear pore complexes in the nuclear envelope. Mammalian reticulon 4 (RTN4), a protein required for ER tubulation, (Jozsef *et al.*, 2014) was found to localize to a subset of nanoholes that are surrounded by unstructured cisternae containing no RTN4.

Further suggested structural subdomains are puncta. At these points the plant ER connects to stable structures within the cell, most likely the plant plasma membrane (PM) (Sparkes *et al.*, 2009). Puncta are classified as being larger than a tubule with a suggested maximum size of 0.3 μm (Quader *et al.*, 1989) to allow for separation from cisternal structures (Sparkes *et al.*, 2009). Puncta are also identified by their dynamics, as they are fixed points within the network, typically persisting for at least 5–10 s (Sparkes *et al.*, 2009; Griffing, 2018). Such stable points within the network are typically ER-PM contact sites (EPCS), where the ER and PM are within 15–20 nm of each other (for review see Wang, 2020). In plants, this corresponds to 5–10% of PM area independent of cell stage or type (McFarlane *et al.*, 2017). EPCS are linked to a variety of functions, including maintaining ER stability, lipid regulation, endocytosis, (Stefano *et al.*, 2018) autophagy (Wang *et al.*, 2019) and Ca^{2+} signalling (Bayer *et al.*, 2017). Plant proteins such as NET3c might mediate the linkage between ER and plasma membrane. NET3c binds filamentous actin via C-terminal mediated dimerization and interacts with the PM via a yet-unknown mechanism (Wang *et al.*, 2014). Vesicle associated membrane protein (VAMP)-associated proteins (VAP) 27 also localizes to EPCS. VAP27-1 and VAP27-3 have been shown to interact with NET3c via a major sperm protein domain and associate with actin and microtubules at EPCS (Wang *et al.*, 2016). Other than NET3c/VAPA27 complexes, another subpopulation of EPCS are specifically labelled with Arabidopsis synaptotagmin 1 (STY1) (Sia *et al.*, 2016). SYT1 is a tether protein integral to the formation of EPCS; an *syt1* knockout results in a rescuable depletion of EPCS. SYT1 has also been shown to be involved in the transport of viral movement proteins through plasmodesmata, specialized EPCS allowing for cell–cell communication (Levy *et al.*, 2015).

The overall ER structure is mainly determined by the relative proportions of all of these subdomains. The morphology of cisternae and tubules has been related to different functional properties; although both structures are present in all eukaryotic cells, (Staehelin & Driouich, 1997) the proportion of each ER structure varies between cells and species (Shibata *et al.*, 2006) and this has been linked to the underlying cellular processes and protein secretion requirements. For example, secretory cells, such as pancreatic and salivary glands, display a higher proportion of cisternae, (Shibata *et al.*, 2006) whereas cells with a lower requirement for protein secretion, for example cortex cells, tend to have a higher proportion of tubular ER (Terasaki *et al.*, 2013). EM analysis of ER in maize root caps has shown that in peripheral secretory cells the proportion of cisternal to tubular ER is significantly higher than that in the cap meristem and statocytes. In addition, the total volume and surface area of the ER in starch cells and secretory cells was measured to be five and 10 times greater than that of a meristem cell. (Stephenson & Hawes, 1986) In *Arabidopsis*, developing root hairs show enhanced cisternal areas (Ridge *et al.*, 1999) and abscisic acid treatment of *Lepidium sativum* seeds triggers the formation of large, stacked ER cisternae (Volkmann, 1976) potentially catering for induced secretory demand.

But a convincing link between structure and function of the ER has yet to be established. Loss of the GTPase RHD3 (root hair defective 3), which is involved in shaping the ER network, interferes with the unfolded protein response which may indicate a direct relationship between ER form and function (Lai *et al.*, 2014). This is also supported by the evidence that the loss of RHD3 compromises bulk-flow secretion in roots but not in leaf epidermal cells, (Zheng *et al.*, 2004; Stefano *et al.*, 2012) supporting the possibility that the link between ER structure and function may exhibit some degree of tissue specificity.

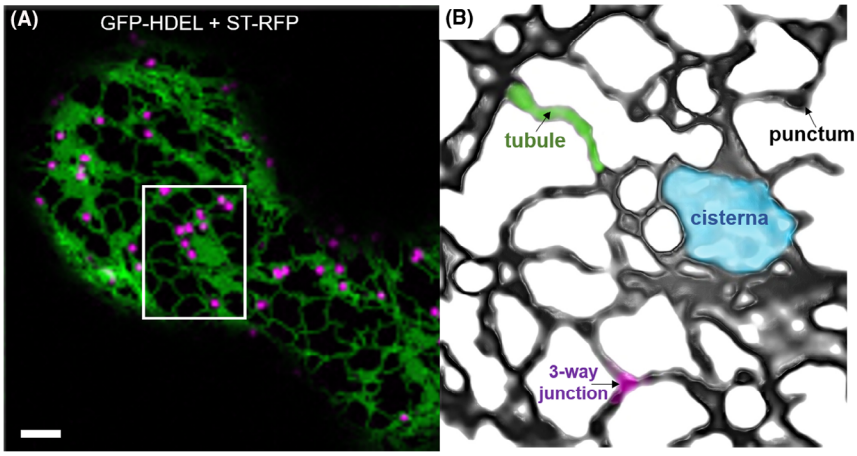

Fig. 1. The ER network structure. (A) The plant ER is labelled with the lumenal marker GFP-HDEL (green). Golgi bodies are labelled with a rat sialyltransferase membrane domain fused to RFP (ST-RFP, in magenta). Scale bar = 5 μm. (B) Diagram built on confocal image of a cortical ER region of a plant cell showing a punctum, a tubule, a cisterna and a three-way junction, which have been pseudocoloured in post-acquisition for visualization.

Building the ER network

The complex structure of the ER could theoretically be solely upheld by a balance of two types of ER-shaping proteins: R- and S-proteins (Shemesh *et al.*, 2014). Both types of proteins localize to curved ER surfaces and are able to induce membrane curvature. Along the edge of an ER tubule, R-proteins would promote straight edge formation, whilst an S-protein would induce negative membrane curvature. By amending local concentrations of these two types of ER-shaping proteins, it is theoretically possible to produce all the ER structures observed in plant and mammalian ER networks, including tubules, three-way junctions, cisternae, cisternal fenestrations and helicoidal stacks (these are not observed in plant cells) (Shemesh *et al.*, 2014). Increasing the total proportion of both R- and S-ER-shaping proteins will shift the ER structure from a more cisternal structure to unbranched tubules. Increasing the fraction of S-proteins within the total population of ER-shaping proteins increases the amount of straight ER edges and favours structures that require junctions in order to form such as three-way junctions. mLNP1, the mammalian Lunapark protein, is proposed to be an S-protein (Shemesh *et al.*, 2014) and has been suggested to stabilize three-way junctions in the ER by inducing negative membrane curvature (Chen *et al.*, 2012); reticulon proteins have been proposed as R-proteins (Shemesh *et al.*, 2014) and to stabilize straight membranes along the length of ER tubules.

ER-shaping protein families: reticulon, Lunapark and atlastin proteins

In yeast and mammalian cells a number of proteins are important for ER membrane tubulation to form the ER network: reticulon proteins and DP1/Yop1p/REEP (De Craene *et al.*, 2006; Voeltz *et al.*, 2006; Shibata *et al.*, 2008; Björk *et al.*, 2013), atlastins (Hu *et al.*, 2009; Orso *et al.*, 2009) and Rab10 (English & Voeltz, 2013) for tubule fusion, CLIMP-63, kinectin, p180 and Lunapark (LNP) proteins for cisternae (Chen *et al.*, 2012; Goyal & Blackstone, 2013; Kriechbaumer *et al.*, 2018). In plants manipulations of the ER network structure can be achieved through three families of proteins:

Reticulons: reticulon proteins (RTN) are integral ER membrane proteins found ubiquitously in eukaryotes and are associated with numerous biological functions including neurodegenerative diseases (Oertle *et al.*, 2003; Nziengui *et al.*, 2007; Yang & Strittmatter, 2007). RTNs feature a reticulon homology domain (RHD) that contains two hydrophobic regions flanking a hydrophilic loop (Nziengui & Schoefs, 2009). In plants RTNs are essential to maintain the tubular ER network (Figure 2) as they induce membrane curvature in the ER and are capable of converting cisternae into tubules (Tolley *et al.*, 2008; Sparkes *et al.*, 2009; Tolley *et al.*, 2010). In *Arabidopsis*, 21 reticulons have been identified so far and over-expression of some of these induces a constriction phenotype to ER tubules (Tolley *et al.*, 2008; Sparkes *et al.*, 2010). Reticulons are even capable of converting cisternal sheets into tubules (Tolley *et al.*, 2010). Reticulons are integral ER membrane proteins featuring two major hydrophobic helical domains with two transmembrane domains each. In this way two "V" shaped wedges joined by a cytoplasmic loop form a "W"-shaped membrane protein with C- and N-termini facing the cytoplasm. Reticulons dimerize or oligomerize and so create ER membrane tension inducing membrane curvature (Sparkes *et al.*, 2010). This topology has been shown for the Arabidopsis reticulons 1, 2, 3, 4 and 13 (Sparkes *et al.*, 2010; Tolley *et al.*, 2010). Reticulons with removed or shortened transmembrane domains lose their capability to induce ER curvature (Sparkes *et al.*, 2010; Tolley *et al.*, 2010). The transmembrane topology together with a C-terminal amphipathic helix has been proposed to be necessary for induction of membrane curva-

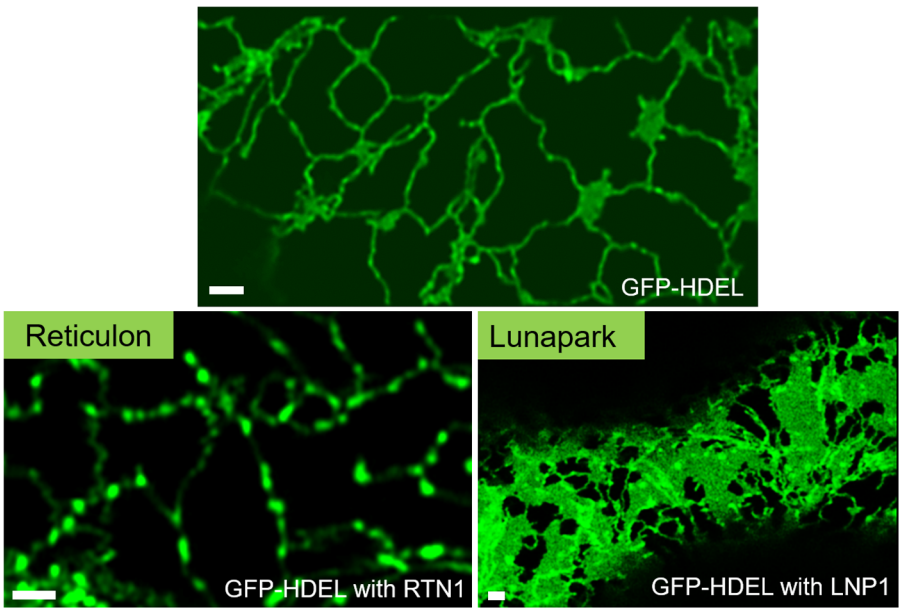

Fig. 2. Reticulon and Lunapark proteins are shaping the ER. The plant ER is labelled with the lumenal marker GFP-HDEL (green); the network structure is shown for cells only expressing GFP-HDEL or in combination with a reticulon or Lunapark protein, respectively. Overexpression of reticulon protein causes hyperconstriction of ER tubules and oppresses the lumenal marker GFP-HDEL in discrete puncta, Lunapark increases the abundance of cisternae. Scale bar = 1 μm.

ture. This is achieved through a combination of wedging of the membrane due to the transmembrane topology of the conserved reticulon homology domain and scaffolding with the RTNs forming oligomers within the membrane (Shibata *et al.*, 2006; Zimmerberg & Kozlov, 2006; Breeze *et al.*, 2016).

Arabidopsis coding for 21 reticulons in comparison to four mammalian ones raises the question about potentially varying functions. RTN3 and 6 are unique in labelling of the desmotubule within plasmodesmata. They also co-localize with viral movement proteins suggesting a role in regulating cell–cell communication and plant immunity (Knox *et al.*, 2015). Both RTN3 and 6 have also been shown to interact with a significant number of plasmodesmata proteins as well as proteins associated with ER-PM contact sites, lipid raft proteins, and proteins that interact with viral movement proteins (Kriechbaumer *et al.*, 2015). A subgroup of RTN's, RTN17-21, contain terminal extensions that might enable unique functions within the RTN family. RTN19 and 20 have been shown to be involved in sterol regulation in Arabidopsis roots via a novel mechanism, as they contain an N-terminal extension homologues to 3β-hydroxysteroid dehydrogenase (Kriechbaumer *et al.*, 2018). These 3βHSD homologues have distinct localizations on the ER; whilst RTN20 labels discrete puncta within the ER membrane, RTN19 labels the entirety of the network. Both RTN19 and 20 are not able to constrict the ER (Kriechbaumer *et al.*, 2018). A functional link of reticulons with cellular pathways has also been recently demonstrated for RTN1 and RTN2 which have been proposed to act as receptors for autophagy-mediated ER turnover (Zhang *et al.*, 2020).

Lunapark: Recently a novel family of LNP proteins was described as a counterpart to RTNs in plants. AtLNP1 and 2 are capable of inducing ER sheets (Kriechbaumer *et al.*, 2018) (Figure 2). The Lunapark protein family is highly conserved, with orthologues identified in plants, yeast, vertebrate and fungi (Spitz *et al.*, 2003). LNP protein family members feature two N-terminal transmembrane domains (TMDs) and a C-terminal zinc finger domain followed by the LNPARK peptide sequence which gave rise to the name Lunapark (Spitz *et al.*, 2003). The two Arabidopsis Lunapark proteins AtLNP1 and AtLNP2 display the same structure but feature a modified LNPARK peptide sequence (LNKPKH); this sequence is conserved throughout Embryophyta (Kriechbaumer *et al.*, 2018). Lunapark proteins have been implicated in the maintenance of ER structure in both yeast (Lunapark: Lnp1p) and mammalian cells (Lunapark: mLnp1) and have been described to localize primarily to three-way junctions and to having a role in stabilizing nascent three-way junctions (Chen *et al.*, 2012; Chen *et al.*, 2015; Wang *et al.*, 2016). Loss of the yeast Lnp1p causes cells with a collapsed and densely reticulated ER network (Chen *et al.*, 2012). In mammalian COS-7 cells though, a lack of mLnp1 increases the cisternal proportion within the ER network (Chen *et al.*, 2015). Arabidopsis AtLNP1 and AtLNP2 share 67% amino acid identity and localize differently on the plant ER. AtLNP1 preferentially localizes to cisternae and small puncta across the ER, whereas AtLNP2 labels the entire ER (Kriechbaumer *et al.*, 2018). Transient over-expression of AtLNP1 and AtLNP2 in tobacco leaf epidermal cells induces cisternae formation in a dose-dependent manner. *lnp1* as well

as *lnp1*/*lnp2* knockdown mutants have significantly smaller cisternal area and increased polygonal region size as well as a non-statistically significant reduction in three-way junctions per area (Kriechbaumer *et al.*, 2018). Polygonal areas are also more irregular in the mutant lines and it is suggested that this is due to the loss of cisternae, affecting network stability rather than a trend towards reduced numbers of three-way junctions (Kriechbaumer *et al.*, 2018).

Atlastins: Dynamin-related GTPases involved in the fusion of ER tubules (Liu *et al.*, 2012) are another family of proteins involved in the organization of the ER network. These are atlastin proteins in mammals and Sey1p in yeast (Hu *et al.*, 2009; Orso *et al.*, 2009; Anwar *et al.*, 2012). Atlastins are suggested to dimerize when proteins on separate membranes are in close proximity. This would result in attachment of the two membranes (Bian *et al.*, 2011; Moss *et al.*, 2011). Membrane fusion is then facilitated by a C-terminal amphipathic helical domain (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). In plants, the GTPase RHD3 (a root hair deficient mutant) (Wang *et al.*, 1997) acts as the plant ortholog of atlastin/Sey1p. Overexpression of RHD3 proteins mutated in the GTPase domain results in an ER network with more and longer tubular strands and a decrease in polygonal structures (Chen *et al.*, 2011) which might be due to inhibition of ER tubule fusion (Zhang *et al.*, 2013). RHD3 was shown to complement a yeast *sey1* mutant and to promote ER fusion in yeast (Zhang *et al.*, 2013). In contradiction to that Chen *et al.* (2011) reported that RHD3 cannot complement yeast and *Arabidopsis* mutants. It was also suggested that in Arabidopsis cotyledon cells RHD3 influences the mobility of proteins in the lipid bilayer (Stefano *et al.*, 2014). RHD3 proteins have also been shown to interact with reticulons in plants and mammalian systems and hence likely act together synergistically to control the ER network (Wang *et al.*, 1997, Lee *et al.*, 2013; Kriechbaumer *et al.*, 2015). Phosphorylation of RHD3 has been suggested to modulate the ability of this protein in shaping the ER *in vivo* (Ueda *et al.*, 2016). It will be interesting to test whether the interaction of RHD3 with other ER shapers may depend on RHD3 phosphorylation.

The ER as a biosynthetic factory

The ER is responsible for the biosynthesis and quality control of secretory proteins destined to the other organelles of the secretory pathway, the extracellular matrix, heterologous organelles (i.e. chloroplasts), or simply retained in the ER (Stefano *et al.*, 2014). The advent of fluorescent protein technology has aided the visualization of ER quality control processes and secretion. For example, using fluorescent protein fusions coupled with biochemical analyses, it has been possible to detect events of retrotranslocation of ER resident, but likely misfolded, proteins back to the cytosol (Brandizzi *et al.*, 2003), as well as to establish new paradigms on receptor-mediated retrieval of ER-resident proteins from the Golgi (Silva-Alvim *et al.*, 2018; Robinson & Aniento, 2020). Furthermore, because fluorescent protein fusions to a signal peptide are secreted very efficiently, they have been used in confocal microscopy-based screens to identify mutations on genes and gene products participating in ER protein export and bulk-flow secretion (Zheng *et al.*, 2004; Faso *et al.*, 2009).

Fluorescent protein technology has also aided the identification and functional characterization of other proteins involved in ER-Golgi protein traffic. It is generally accepted that most cargo from the ER reaches the Golgi via mechanisms dependent on the COPII machinery. For detailed insights on the COPII components in plants and their role during development and stress responses, we refer the readers to recent reviews (Brandizzi, 2018; Kurokawa & Nakano, 2019). COPII proteins are a set of functionally conserved proteins that include the ER integral membrane guanine nucleotide exchange factor (GEF) Sec12, as well as the cytosolic small GTPase Sar1 and components of the COPII coat: Sec23/24 and Sec13/31. Arabidopsis Sar1 and Sec12 were earlier shown to complement a yeast thermosensitive *sec12* mutation (d'Enfert *et al.*, 1992). Using the Sar1 and Sec12 antibodies, it was demonstrated that Sec12 is associated with the Arabidopsis microsomal fraction and that endogenous Sar1 is a microsome-peripheral protein that partitions between the cytosol and the membrane (Bar-Peled & Raikhel, 1997). Follow-up work proving that overexpression of dominant-negative mutations of Sar1 blocks protein transport from the ER to the Golgi in plant cells (Takeuchi *et al.*, 1998; Andreeva *et al.*, 2000; Phillipson *et al.*, 2001) set the stage for analyses of the behaviour of fluorescent protein (FP) fusions of the COPII proteins in vivo. (daSilva *et al.*, 2004) Using FP, functional fusions to the Arabidopsis Sec12 and Nicotiana Sar1 were made. The expression of these in tobacco leaf epidermal cells showed that, when expressed alone, a Sar1-FP was visible primarily in the cytosol with some level of enrichment in punctate structures, which were named ER export sites (ERES); however, when co-expressed with membrane cargo destined to the Golgi apparatus, the signal of Sar1-FP to the punctate structures was enhanced (daSilva *et al.*, 2004). Such a behaviour was not observed for a cytosolic FP and for soluble bulk-flow cargo (daSilva *et al.*, 2004), supporting that Sar1 recruitment to the ER membrane responds primarily to the demand of export of membrane proteins from the ER. This concept was subsequently explored by the analysis of ER export motifs on membrane cargo, such as the diacidic motif [aspartate, any amino acid, glutamate (DXE)], which was established to be functional and transplantable on cytosolic domains of membrane cargo (Hanton *et al.*, 2005). In this work, a fusion to the Arabidopsis Sec24A (FP-Sec24) was shown to be localized to the ERES; furthermore, the intensity and number of ERES were found to increase in conditions of overexpression of DXE-bearing membrane cargo but not cargo with non-functional DXE signal (Hanton *et al.*, 2007).

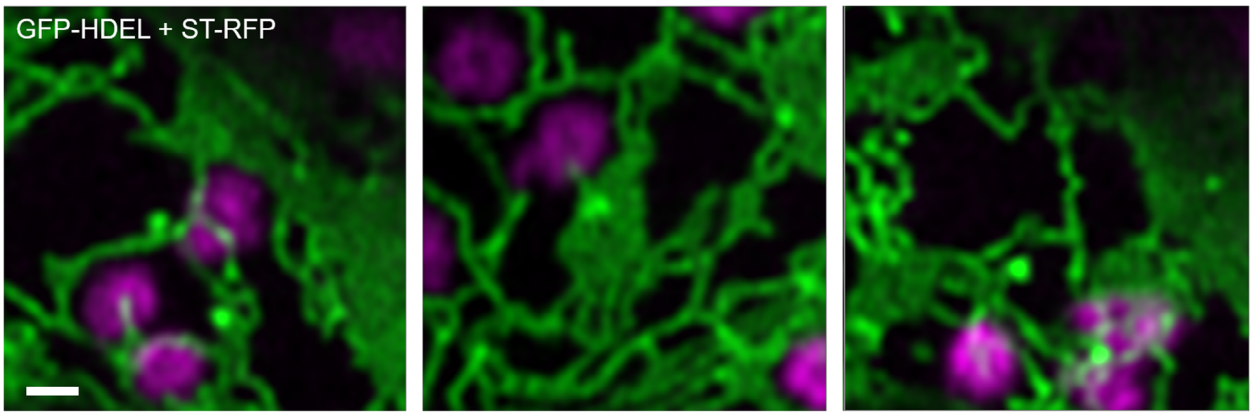

Fig. 3. Connections between ER and golgi bodies. The plant ER is labelled with the lumenal marker GFP-HDEL (green). Golgi bodies are labelled with a rat sialyltransferase membrane domain fused to RFP (ST-RFP, in magenta). Small tubules are connecting ER and Golgi bodies. Scale bar = 1 μm.

From these data and subsequent work using Sec24 and Sec13 (Hanton *et al.*, 2009), it was concluded that ERES are tunable and respond to the necessity of the cell to export signal-bearing cargo from the ER (Hanton *et al.*, 2007). Using standard confocal microscopy, not only FP-fusions to Sar1, Sec24 and Sec13 were found to be primarily concentrated at ERES that move with the Golgi apparatus (daSilva *et al.*, 2004; Hanton *et al.*, 2009), Sec23 was also demonstrated to be associated to the same structures (Stefano *et al.*, 2006). Furthermore, Sec16, which is required for COPII assembly regulation in non-plant species (Kung *et al.*, 2012; Yorimitsu & Sato, 2012), was found to interact with Sec13 and Sec31 in plant cells, supporting a conserved scaffolding role for this protein (Takagi *et al.*, 2013). The discrete localization of Sec16 in cup-shaped subdomains of the ER associated with mobile Golgi stacks and the differential dynamics of binding and release of Sec16 at ERESs compared to other COPII markers also provided support to the model that ER protein export occurs at ERES associated with motile Golgi, the so-called 'ER mobile export sites' model (Neumann *et al.*, 2003) or 'mobile secretory unit' model, (daSilva *et al.*, 2004) at least in highly vacuolated cells. In this model, the ERES are associated with the Golgi in a relatively static domain of the ER (Figure 3). Such association may facilitate the transport of cargo to and from Golgi stacks, which are largely motile in plant cells (see also reviews on ER–Golgi transport in plant cells for further discussion of this model and alternatives (Robinson *et al.*, 2015; Robinson, 2020)).The close association of the ER and the Golgi stacks at ERES finds support in experiments based on optical laser-tweezers in which individual Golgi stacks could be pulled in association with an ER tubule in actin-depolymerized cells (Sparkes *et al.*, 2009).

The ER acts as highway and platform in various physiological processes

The plant ER is distributed across the entire cell surface. Being appressed to the plant cell wall, it provides a perfect pool for important cellular signalling components including hormone receptors, signalling complexes, and calcium (Chen *et al.*, 2002; Friml & Jones, 2010; Zhang *et al.*, 2013).

The highly dynamic ER tubule and mobile membrane structure is ideal for the transport of material bound to the ER surface as well as organelles such as Golgi bodies and other structures throughout the cell; for example viral movement proteins and viral RNA are transported on the ER surface to plasmodesmata in tobacco leaf epidermal cells (Heinlein *et al.*, 1998; Schoelz *et al.*, 2011; Wu *et al.*, 2011). The ER also offers a large and dynamic surface for the location of blocks of metabolic enzymes, thus not only regulating the spatial organization of metabolic pathways but also enabling the mixing of metabolic products throughout the cytoplasm (Hawes *et al.*, 2015; Blakeslee *et al.*, 2019). Pathways can be organized into metabolons enabling highly coordinated and regulated processes. Metabolons contain sequential enzymes plus scaffolding proteins allowing for efficient channelling of metabolic intermediates from one active site directly to the next (Jørgensen *et al.*, 2005). Candidate proteins for such scaffolding are chaperones as well as membrane-anchored cytochrome P450 enzymes that can serve as nucleation points and platforms for metabolon formation (Jørgensen *et al.*, 2005). Membrane-structural proteins have also been suggested to play a role in metabolon formation, for example P450 enzymes involved in a lignin biosynthetic metabolon were co-purified with reticulon proteins (Bassard *et al.*, 2012). This increases substrate concentration and turnover rates, prevents unwanted diffusion and is key to containing labile or toxic intermediates. Hence, metabolons have great potential for the production of high value products. In plants, metabolons have been shown for the production of flavonoids (Hrazdina & Wagner, 1985) and sporopollenin in Arabidopsis (Lallemand *et al.*, 2013) and the glucoside dhurrin in *Sorghum bicolor*. (Laursen *et al.*, 2016) The dhurrin metabolon identified in sorghum enables efficient production of the defence compound through the co-operation of two p450 cytochromes anchored to the ER membrane,

co-operating with a soluble glucosyltransferase to rapidly process the dhurrin production intermediates (Laursen *et al.*, 2016). Recently a soybean isoflavonoid metabolon tethered to the ER has also been reported (Dastmalchi *et al.*, 2016).

Furthermore, an auxin metabolon has been suggested (Friml & Jones, 2010; Kriechbaumer *et al.*, 2016; Blakeslee *et al.*, 2019). The plant hormone auxin is important to nearly every aspect of plant growth and development including organ development and overall plant architecture. Auxin homeostasis is regulated by coordination of biosynthesis, transport, conjugation, storage and catabolism to optimize concentration-dependent growth responses and adaptive responses to temperature, water stress, herbivory and pathogens. Whilst much has been published on auxin transport by influx and efflux carriers, important questions remain unanswered, including how homeostasis is controlled and how auxin biosynthetic pathways are organized (Chandler, 2009). It has also been proposed that auxin metabolism is both spatially and temporally regulated (Zhao, 2014; Poulet & Kriechbaumer, 2017; Brumos *et al.*, 2018; Robert *et al.*, 2018; Yao *et al.*, 2018). The primary auxin, indole-3-acetic acid (IAA) is synthesized via the highly conserved TAA/YUC-route. In this two-step pathway, tryptophan aminotransferases (TAA, TAR1 and 2) catalyze the conversion of tryptophan to the intermediate indole-3-pyruvic acid (IPyA) (Mashiguchi *et al.*, 2011; Phillips *et al.*, 2011; Kriechbaumer *et al.*, 2012) and the YUC flavin-containing monooxygenases convert IPyA to IAA. (Tivendale *et al.*, 2014) The TAA/YUC-route is the first identified complete auxin biosynthetic pathway and is essential for most of the major developmental events in plants (Zhao, 2014). YUC enzymes were first identified via the first ever auxin biosynthetic mutants (Cheng *et al.*, 2006).

In both maize and *Arabidopsis*, a set of enzymes from the TAR and YUC group are localized to the ER membrane and more than 20% of auxin biosynthetic activity was detected in a purified 100,000 × *g* microsomal fraction containing ER membranes (Kriechbaumer *et al.*, 2015; Kriechbaumer *et al.*, 2016). Protein–protein interactions between TAA/TAR and YUC proteins using Förster Resonance Energy Transfer with Fluorescent Lifetime Imaging (FRET-FLIM) have been described (Kriechbaumer *et al.*, 2016). ER membrane-anchoring of a subset of auxin biosynthetic enzymes indicates a compartmentalization. Since many of the enzymes potentially involved in auxin biosynthesis have low substrate specificities and turnover rates, metabolic channelling in an auxin metabolon has been postulated (Müller & Weiler, 2000). Additionally, metabolon formation can contain labile or toxic intermediates; e.g. the precursor IPyA is highly unstable in water (Kriechbaumer *et al.*, 2015). The ER cannot only be considered as a surface for auxin biosynthesis but also as an auxin recycling station (Friml *et al.*, 2003). PIN and PIN-Like (PILS) auxin transporter proteins are present on the ER membrane (Mravec *et al.*, 2009; Barbez *et al.*, 2012; Dal Bosco *et al.*, 2012; Ding *et al.*, 2012; Bender *et al.*, 2013; Sawchuk *et al.*, 2013). Also, the auxin-deconjugating enzymes ILL2, IAR3 and ILR1 have been shown to reside on the ER of Arabidopsis protoplasts and thereby most likely regulate the content of active IAA by amido-IAA hydrolysis in the ER (Sanchez Carranza *et al.*, 2016). In addition, when IAA is infiltrated into tobacco leaves together with an ER marker the ER network structure appears perturbed with tubule misalignment and induction of ER cisternae. Increased cisternal area is also observed when IAA biosynthetic enzymes carrying out the first (TAA/TAR) and second step (YUC) of the biosynthesis are over-expressed together in tobacco leaf cells (Blakeslee *et al.*, 2019) indicating that auxin regulation might also be of importance for ER morphology.

The family of ethylene receptors (ETR1, ETR2, ERS1, ERS2, EIN4) is also localized at the ER, (Chen *et al.*, 2002) which is suggested to benefit signal transduction. Mostly this localization avoids for the receptor proteins having to go through the energy-consuming secretory pathway and aids a swift delivery to their site of action (Chen *et al.*, 2002). ER localization of ethylene receptors may also enable local regulation of other ER-dependent processes. For example, chitinase accumulation upon pathogen attack is prompted by ethylene signalling and is calcium dependent (Raz & Fluhr, 1992); here the localization of ethylene receptors to the ER could be key to a rapid release of calcium from the ER in response to pathogens (Chen *et al.*, 2002). In general, the ER is a calcium reservoir and releases it (Bush, 1995) to maintain cellular homeostasis and enables exocytosis, metabolism, transcription regulation, and apoptosis (Bonza *et al.*, 2013). ER-derived compartments in developing seeds also store important storage proteins such as globulins and prolamins (Galili, 2004). The ER also holds regulatory complexes such as the WAVE/SCAR (WASP family verprolin homologous/suppressor of cyclic AMP receptor) complex (Zhang *et al.*, 2013). WAVE/SCAR acts together with the Actin-Related Protein (ARP) 2/3 complex and controls actin-dependent morphogenesis in different tissues and developmental stages (Zhang *et al.*, 2013). The WAVE/SCAR subunits NAP1 and SCAR2 are located on the ER surface and the upstream regulator of the WAVE/SCAR complex, SPK1, is localized to ER-exit sites (Zhang *et al.*, 2010).

Future perspectives

Professor Chris Hawes will be remembered for the significant contributions to the plant secretory pathway, chief among these is the introduction and development of live cell imaging based on fluorescent protein technology. Around the world, the field is using tools that Chris developed and techniques that he propelled for the study of complex organelles, such as the ER.

Since the time when Chris started pushing the field to new frontiers over two decades ago by demonstrating that secretory organelles can be visualized in real time in intact plant tissues, new questions have certainly arisen. For example the

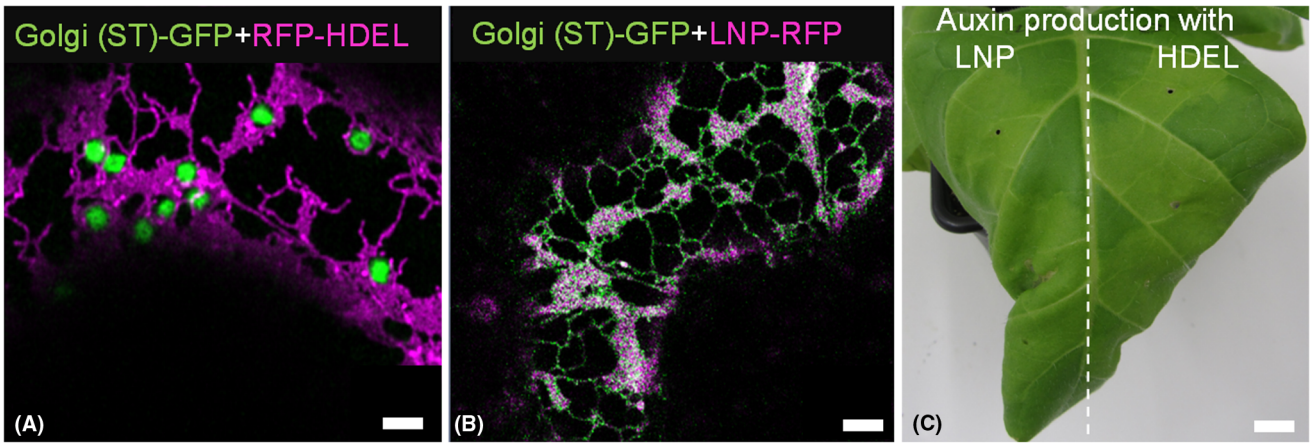

Fig. 4. Changes in ER structure can impact ER functionality. The Golgi marker ST-GFP is co-expressed with either (A) RFP-HDEL or (B) LNP1-RFP, respectively. Overexpression of LNP1-RFP results in increased cisternalization of the ER and here is seen to inhibit the transport of the *trans*-Golgi marker ST-GFP from the ER to Golgi bodies. Scale bar = 1 μm. (C) Leaf curling after transient expression of the auxin biosynthetic enzymes AtTAR2 and AtYUC5 in tobacco leaves most likely due to enhanced synthesis of the auxin indole-3-acetic acid. This curling phenotype is enhanced when the auxin biosynthetic enzymes are co-expressed with LNP1-RFP resulting in enhanced cisternae (left leaf side) in comparison to co-expression with the ER marker RFP-HDEL marker (right leaf side). Scale bar = 1 cm.

physiological relevance of the dynamic morphology of the ER is not clearly understood. The loss of RHD3 and an RHD3-isoform, RL1, is lethal, (Zhang *et al.*, 2013) supporting the idea that ER fusion is an essential process. Nonetheless, the mechanisms for ER fusion and related essential processes are not known. Whether the loss of other ER shapers such as RTNs or LNP is lethal is unknown as there is no full mutant for the 21 *Arabidopsis* RTNs and the available *lnp1lnp2* is a knock-down (Kriechbaumer *et al.*, 2018). The ER assumes a reticulated appearance in fully expanded cells but it has a more cisternal organization in non-expanded cells (Stefano *et al.*, 2014). It may be argued that in younger, still expanding cells a more cisternal ER may provide a larger organellar lumen for metabolic processes, including protein synthesis. On the other hand, a more reticulated network might be beneficial for transport mechanisms: in leaf epidermal cells, Golgi bodies are associated with ER tubules but not cisternae (Sparkes *et al.*, 2009) and yeast ERES-localized proteins have been shown to preferentially localize to high-curvature ER membranes. (Okamoto *et al.*, 2012) Figure 4 outlines examples for differing ER functionality when the ER structure is more cisternal: Preliminary data indicate that the transport between ER and Golgi might be impaired upon cisternalization of the ER (Figure 4A,B, Kriechbaumer, unpublished data). When the Golgi marker ST-GFP is co-expressed with the cisternae-inducing protein LNP1 (Kriechbaumer *et al.*, 2018), ST-GFP does not label the Golgi (Figure 4A) but the fluorophore fusion remains in the ER instead (Figure 4B), indicating a potential ER-Golgi transport impairment when the ER structure is changed in favour of cisternae Similar to the situation in expanding cells, such a modified structure might be beneficiary for metabolic processes required for growth (Figure 4C). Expression of the auxin biosynthetic enzymes AtTAR2 and AtYUC5 in tobacco leaves results in leaf curling (Kriechbaumer *et al.*, 2016) most likely due to auxin overproduction (Figure 4C, right leaf side). This curling phenotype is enhanced when the enzymes are co-expressed with LNP1 resulting in enhanced cisternae (Figure 4C, left leaf side).

It would be interesting to test whether the function of ER shapers is somehow regulated to accommodate for the different morphology of the ER in young and mature cells. Indeed, RHD3 is required for the transition between a more cisternal ER to a more tubular ER as cells expand (Stefano *et al.*, 2014). As the tubular ER contributes to the streaming of other organelles (Stefano *et al.*, 2014), an organization of a more tubular ER in mature cells may facilitate the transport of organelles to the appropriate destination. In leaf epidermal cells, for example where the central vacuole occupies 90% of the cell volume, the organelles are sandwiched between the tonoplast and the plasma membrane. Therefore, the contribution of the ER to the movement of other organelles may facilitate bypassing the steric hindrance of the vacuole in long-distance organelle positioning (Stefano *et al.*, 2014). Under this light, it will be interesting to establish whether the loss of ER structure affects the function of other organelles by facilitating their positioning in the cell. This may be indeed the case: the ER is in close contact with endosomes, and a loss of RHD3 compromises endocytosis (Stefano *et al.*, 2015). In addition, or alternatively, it is possible that a loss of ER structure may compromise the positioning of the machinery needed for other processes occurring in non-ER membranes. For example, the ER membrane-anchored VAP27 proteins that accumulate at the EPCS interact with proteins involved in endocytosis such as clathrin (Stefano *et al.*, 2018). The EPCS have also been

involved in the formation of autophagosomes (Wang *et al.*, 2019). Therefore, a loss of structure of the ER may alter the positioning of the EPCS and consequently their function in cell processes. Furthermore, the loss of RHD3 causes defects in auxin distribution in roots (Stefano *et al.*, 2015), possibly because the distribution of endosomes is altered in *rhd3* cells. Whether ER structure defects are connected with the biology of other hormones is potentially another fertile area of investigation that fluorescent protein imaging coupled with the genomics tools available for model species is poised to address.

References

Andreeva, A.V., Zheng, H., Saint-Jore, C., Kutuzov, M.A., Evans, D.E. & Hawes, C.R. (2000) Organization of transport from endoplasmic reticulum to Golgi in higher plants. *Biochem. Soc. Trans.* **28**, 505–512.

Anwar, K., Klemm, R.W., Condon, A. *et al.* (2012) The dynamin-like GTPase Sey1p mediates homotypic ER fusion in *S. cerevisiae*. *J. Cell Bio.* **197**, 209–217.

Barbez, E., Kubeš, M., Rolčík, J. *et al.* (2012) A novel putative auxin carrier family regulates intracellular auxin homeostasis in plants. *Nature* **485**, 119–122.

Bar-Peled M. & Raikhel N.V (1997) Characterization of AtSEC12 and AtSAR1. Proteins likely involved in endoplasmic reticulum and Golgi transport. *Plant Physiol.* **114**, 315–324.

Bassard, J.E., Richert, L., Geerinck, J. *et al.* (2012) Protein-protein and protein-membrane associations in the lignin pathway. *Plant Cell* **24**, 4465–4482.

Bayer, E.M., Sparkes, I., Vanneste, S. & Rosado, A. (2017) From shaping organelles to signalling platforms: the emerging functions of plant ER-PM contact sites. *Curr. Opin. Plant Biol.* **40**, 89–96.

Bender, R.L., Fekete, M.L., Klinkenberg, P.M. *et al.* (2013) PIN6 is required for nectary auxin response and short stamen development. *Plant J.* **74**, 893–904.

Bian, X., Klemm, R.W., Liu, T.Y. *et al.* (2011) Structures of the atlastin GTPase provide insight into homotypic fusion of endoplasmic reticulum membranes. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 3976–3981.

Björk, S.M., Hurt, C.M., Ho V.K. & Angelotti T. (2013) REEPs are membrane shaping adapter proteins that modulate specific g protein-coupled receptor trafficking by affecting ER cargo capacity. *PLoS One* **8**, e76366.

Blakeslee, J.J., Spatola Rossi, T. & Kriechbaumer, V. (2019) Auxin biosynthesis: spatial regulation and adaptation to stress. *J. Exp. Bot.* **70**, 5041–5049.

Boevink, P., Oparka, K., Santa Cruz, S., Martin, B., Betteridge, A. & Hawes, C. (1996) Stacks on tracks: the plant Golgi apparatus traffics on an actin/ER network. *Plant J.* **10**, 935–941.

Bonza, M.C., Loro, G., Behera, S., Wong, A., Kudla, J. & Costa, A. (2013) Analyses of Ca2+ accumulation and dynamics in the endoplasmic reticulum of Arabidopsis root cells using a genetically encoded Cameleon sensor. *Plant Physiol.* **163**, 1230–1241.

Brandizzi, F. (2018) Transport from the endoplasmic reticulum to the Golgi in plants: where are we now? *Semin. Cell Dev. Biol.* **80**, 94–105.

Brandizzi, F., Hanton, S., DaSilva, L.L., Boevink, P., Evans, D., Oparka, K., Denecke, J. & Hawes, C. (2003) ER quality control can lead to retrograde transport from the ER lumen to the cytosol and the nucleoplasm in plants. *Plant J.* **34**, 269–281.

Breeze, E., Dzimitrowicz, N., Kriechbaumer, V. *et al.* (2016) A C-terminal amphipathic helix is necessary for the in vivo tubule-shaping function of a plant reticulon. *Proc. Natl. Acad. Sci. U.S.A.* **113**, 10902–10907.

Brumos, J., Robles, L.M., Yun, J., Vu, T.C., Jackson, S., Alonso, J.M. & Stepanova, A.N. (2018) Local auxin biosynthesis is a key regulator of plant development. *Dev. Cell.* **47**, 306–318.

Bush, D.S. (1995) Calcium regulation in plant cells and its role in signaling. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **46**, 95–122.

Chandler, J.W. (2009) Local auxin production: a small contribution to a big field. *Bioessays* **31**, 60–70.

Chen, J., Stefano, G., Brandizzi, F. & Zheng, H. (2011) Arabidopsis RHD3 mediates the generation of the tubular ER network and is required for Golgi distribution and motility in plant cells. *J. Cell Sci.* **124**, 2241–2252.

Chen, S., Desai, T., McNew, J.A., Gerard, P., Novick, P.J. & Ferro-Novick, S. (2015) Lunapark stabilizes nascent three-way junctions in the endoplasmic reticulum. *Proc. Natl. Acad. Sci. U.S.A.* **112**, 418–423.

Chen, S., Novick, P. & Ferro-Novick, S. (2012), ER network formation requires a balance of the dynamin-like GTPase Sey1p and the Lunapark family member Lnp1p. *Nat. Cell Biol.* **14**, 707–716.

Chen, Y.F., Randlett, M.D., Findell, J.L. & Schaller, G.E. (2002) Localization of the ethylene receptor ETR1 to the endoplasmic reticulum of Arabidopsis. *J. Biol. Chem.* **277**, 861–866.

Cheng, Y., Dai, X. & Zhao, Y. (2006) Auxin biosynthesis by the YUCCA flavin monooxygenases controls the formation of floral organs and vascular tissues in Arabidopsis. *Genes Dev.* **20**, 1790–1799.

Dal Bosco, C.D., Dovzhenko, A. *et al.* (2012) The endoplasmic reticulum localized PIN8 is a pollen-specific auxin carrier involved in intracellular auxin homeostasis. *Plant J.* **71**, 860–870.

daSilva, L.L., Snapp, E.L., Denecke, J., Lippincott-Schwartz, J., Hawes, C. & Brandizzi, F. (2004) Endoplasmic reticulum export sites and Golgi bodies behave as single mobile secretory units in plant cells. *Plant Cell* **16**, 1753–1771.

Dastmalchi, M., Bernards, M.A. & Dhaubhadel, S. (2016) Twin anchors of the soybean isoflavonoid metabolon: evidence for tethering of the complex to the endoplasmic reticulum by IFS and C4H. *Plant J.* **85**, 689–706.

De Craene, J.O., Coleman, J., Estrada deMartin, P., Pypaert, M., Anderson, S., Yates, J.R., Ferro-Novick, S. & Novick, P. (2006) Rtn1p is involved in structuring the cortical endoplasmic reticulum. *Mol. Biol. Cell* **17**, 3009–3020.

d'Enfert, C., Gensse, M. & Gaillardin, C. (1992) Fission yeast and a plant have functional homologues of the Sar1 and Sec12 proteins involved in ER to Golgi traffic in budding yeast., *EMBO J.* **11**, 4205–4211.

Ding, Z., Wang, B., Moreno, I. *et al.* (2012) ER-localized auxin transporter PIN8 regulates auxin homeostasis and male gametophyte development in Arabidopsis. *Nat. Comm.* **3**, 941.

English, A.R. & Voeltz, G.K. (2013) Rab10 GTPase regulates ER dynamics and morphology. *Nat. Cell. Biol.* **15**, 169–178.

Faso, C., Chen, Y.N., Tamura, K. *et al.* (2009) A missense mutation in the Arabidopsis COPII coat protein Sec24A induces the formation of clusters of the endoplasmic reticulum and Golgi apparatus. *Plant Cell* **21**, 3655–3671.

Friedman, J.R. & Voeltz, G.K. (2011) The ER in 3D: a multifunctional dynamic membrane network. *Trends Cell Biol.* **21**, 709–717.

Friml J., Vieten, A., Sauer, M., Weijers, D., Schwarz, H., Hamann, T., Offringa, R. & Jürgens, G.l. (2003) Efflux-dependent auxin gradients establish the apical-basal axis of Arabidopsis. *Nature* 13;**426**, 147–153.

Friml, J.J. & Jones, A.R. (2010) Endoplasmic reticulum: the rising compartment in auxin biology. *Plant Physiol.* **154**, 458–462.

Galili, G. (2004) ER-derived compartments are formed by highly regulated processes and have special functions in plants. *Plant Physiol.* **136**, 3411–3413.

Goyal, U. & Blackstone, C. (2013). Untangling the web: mechanisms underlying ER network formation. *Biochim. Biophys. Acta* **1833**, 2492–2498.

Griffing, L.R. (2018) Dancing with the stars: using image analysis to study the choreography of the endoplasmic reticulum and its partners and of movement within its tubules. *The Plant Endoplasmic Reticulum* (ed. by C. Hawes & V. Kriechbaumer), Vol. **1691**, pp. 75–102. Berlin: Springer.

Griffing, L.R., Gao, H.T. & Sparkes, I. (2014) ER network dynamics are differentially controlled by myosins XI-K, XI-C, XI-E, XI-I, XI-1, and XI-2. *Front. Plant Sci.* **5**, 1–12.

Hamada, T., Ueda, H., Kawase, T. & Hara-Nishimura, I. (2014) Microtubules contribute to tubule elongation and anchoring of endoplasmic reticulum, resulting in high network complexity in Arabidopsis. *Plant Physiol.* **166**, 1869–1876.

Hanton, S.L., Chatre, L., Renna, L., Matheson, L.A. & Brandizzi, F. (2007) De novo formation of plant endoplasmic reticulum export sites is membrane cargo induced and signal mediated. *Plant Physiol.* **143**, 1640–1650.

Hanton, S.L., Matheson, L.A., Chatre, L. & Brandizzi, F. (2009) Dynamic organization of COPII coat proteins at endoplasmic reticulum export sites in plant cells. *Plant J.* **57**, 963–974.

Hanton, S.L., Renna, L., Bortolotti, L.E., Chatre, L., Stefano, G. & Brandizzi, F. (2005) Diacidic motifs influence the export of transmembrane proteins from the endoplasmic reticulum in plant cells. *Plant Cell* **17**, 3081–3093.

Hawes, C., Kiviniemi, P. & Kriechbaumer, V. (2015) The endoplasmic reticulum: a dynamic and well-connected organelle. *J. Integr. Plant Biol.* **57**, 50–62.

Hawes, C., Saint-Jore, C., Martin, B. & Zheng, H.Q. (2001) ER confirmed as the location of mystery organelles in Arabidopsis plants expressing GFP! *Trends Plant Sci.* **6**, 245–246.

Heinlein, M., Padgett, H.S., Gens, J.S., Pickard, B.G., Casper, S.J., Epel, B.L. & Beachy, R.N. (1998) Changing patterns of localization of the tobacco mosaic virus movement protein and replicase to the endoplasmic reticulum and microtubules during infection. *Plant Cell* **10**, 1107–1120.

Holcman, D., Parutto, P., Chambers, J.E. *et al.* (2018) Single particle trajectories reveal active endoplasmic reticulum luminal flow. *Nat. Cell Biol.* **20**, 1118–1125.

Hrazdina, G. & Wagner, G.J. (1985) Metabolic pathways as enzyme complexes: evidence for the synthesis of phenylpropanoids and flavonoids on membrane associated enzyme complexes. *Arch. Biochem. Biophys.* **237**, 88–100.

Hu, J., Shibata, Y., Zhu, P.P., Voss, C., Rismanchi, N., Prinz, W.A., Rapoport, T.A. & Blackstone, C. (2009) A class of dynamin-like GTPases involved in the generation of the tubular ER network. *Cell* **138**, 549–561.

Jørgensen, K., Rasmussen, A.V., Morant, M., Nielsen, A.H., Bjarnholt, N., Zagrobelny, M., Bak, S. & Møller, B.L. (2005) Metabolon formation and metabolic channeling in the biosynthesis of plant natural products. *Curr. Opin. Plant Biol.* **8**, 280–229.

Jozsef, L., Tashiro, K., Kuo, A. *et al.* (2014) Reticulon 4 is necessary for endoplasmic reticulum tubulation, STIM1-Orai1 coupling, and store-operated calcium entry. *J. Biol. Chem.* **289**, 9380–9395.

Knox, K., Wang, P., Kriechbaumer, V., Tilsner, J., Frigerio, L., Sparkes, I., Hawes, C. & Oparka, K. (2015). Putting the squeeze on plasmodesmata: a role for reticulons. *Plant Physiol.* **168**, 1563–1572.

Kriechbaumer, V., Botchway, S.W. & Hawes, C. (2016) Localization and interactions between Arabidopsis auxin biosynthetic enzymes in the TAA/YUC-dependent pathway. *J. Exp. Bot.* **67**, 4195–4207.

Kriechbaumer, V., Botchway, S.W., Slade, S.E., Knox, K., Frigerio, L., Oparka, K. & Hawes, C. (2015) Reticulomics: protein-protein interaction studies with two plasmodesmata-localized reticulon family proteins identify binding partners enriched at plasmodesmata, endoplasmic reticulum, and the plasma membrane. *Plant Physiol.* **169**, 1933–1945.

Kriechbaumer, V., Breeze, E., Pain, C., Tolmie, F., Frigerio, L. & Hawes, C. (2018) Arabidopsis Lunapark proteins are involved in ER cisternae formation. *New Phytol.* **219**, 990–1004.

Kriechbaumer, V., Maneta-Peyret, L., Fouillen, L. *et al.* (2018) The odd one out: Arabidopsis reticulon 20 does not bend ER membranes but has a role in lipid regulation. *Sci. Rep.* **8**, 2310.

Kriechbaumer, V., Seo, H., Park, W.J. & Hawes, C. (2015) Endoplasmic reticulum localization and activity of maize auxin biosynthetic enzymes. *J. Exp. Bot.* **66**, 6009–6020.

Kriechbaumer, V., Wang, P., Hawes, C. & Abell, B.M. (2012) Alternative splicing of the auxin biosynthesis gene YUCCA4 determines its subcellular compartmentation. *Plant J.* **70**, 292–302.

Kung, L.F., Pagant, S., Futai, E. *et al.* (2012) Sec24p and Sec16p cooperate to regulate the GTP cycle of the COPII coat. *EMBO J.* **15**, 1014–1027.

Kurokawa K. & Nakano, A. (2019) The ER exit sites are specialized ER zones for the transport of cargo proteins from the ER to the Golgi apparatus. *J. Biochem.* **165**, 109–114.

Lai, Y.S., Stefano, G. & Brandizzi, F. (2014) ER stress signaling requires RHD3, a functionally conserved ER-shaping GTPase. *J. Cell Sci.* **127**, 3227–3232.

Lallemand, B., Erhardt, M., Heitz, T. & Legrand M. (2013) Sporopollenin biosynthetic enzymes interact and constitute a metabolon localized to the endoplasmic reticulum of tapetum cells. *Plant Physiol.* **162**, 616–625.

Laursen, T., Borch, J., Knudsen, C. *et al.* (2016) Characterization of a dynamic metabolon producing the defense compound dhurrin in sorghum. *Science* **354**, 890–893.

Lee, H., Sparkes, I., Gattolin, S., Dzimitrowicz, N., Roberts, L.M., Hawes, C. & Frigerio, L. (2013) An Arabidopsis reticulon and the atlastin homologue RHD3-like2 act together in shaping the tubular endoplasmic reticulum. *New Phytol.* **197**, 481–489.

Levy, A., Zheng, J. & Lazarowitz, S. (2015) Synpatotagmin SYTA forms ER-plasma membrance junctions that are recruited to plasmodesmata for plant virus movement. *Curr. Biol.* **25**, 2018–2025.

Lin, C., White, R.R., Sparkes, I.A. & Ashwin, P. (2017) Modeling endoplasmic reticulum network maintenance in a plant cells. *Biophys. J.* **113**, 214–222.

Liu, T.Y., Bian, X., Sun, S., Hu, X., Klemm, R.W., Prinz, W.A., Rapoport, T.A. & Hu, J. (2012) Lipid interaction of the C terminus and association of the transmembrane segments facilitate atlastin-mediated homotypic endoplasmic reticulum fusion. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 2146–2154.

Mashiguchi, K., Tanaka, K., Sakai, T. *et al.* (2011) The main auxin biosynthesis pathway in Arabidopsis. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 18512–18517.

Mathur J., Mammone, A. & Barton, K. A. (2012) Organelle extensions in plant cells. *J. Integr. Plant Biol.* **54**, 851–867.

McFarlane, H.E., Kyoung-Lee, E., Van-Bezouwen, L., Ross, B., Rosado, A. & Samuels, A. (2017) multiscale structural analysis of the plant ER-PM contact sites. *Plant Cell Physiol.* **58**, 478–484.

Moss, T.J., Andreazza, C., Verma, A., Daga, A. & McNew, J.A. (2011) Membrane fusion by the GTPase atlastin requires a conserved C-terminal cytoplasmic tail and dimerization through the middle domain. *Proc. Natl. Acad. Sci. U.S.A* **108**, 11133–11138.

Mravec, J., Skůpa, P., Bailly, A. *et al.* (2009) Subcellular homeostasis of phytohormone auxin is mediated by the ER-localized PIN5 transporter. *Nature* **459**, 1136–1140.

Müller, A. & Weiler, E. W. (2000) IAA-synthase, an enzyme complex from Arabidopsis thaliana catalyzing the formation of indole-3-acetic acid from (S)-tryptophan. *Biol. Chem.* **381**, 679–686.

Neumann, U., Brandizzi, F. & Hawes, C. (2003) Protein transport in plant cells: in and out of the Golgi. *Ann. Bot.* **92**, 167–180.

Nixon-Abell, J., Obara, C.J., Weigel, A.V. *et al.* (2016) Increased spatiotemporal resolution reveals highly dynamic dense tubular matrices in the peripheral ER. *Science* **354**, 433–445.

Nziengui, H., Bouhidel, K., Pillon, D., Der, C., Marty, F. & Schoefs, B. (2007) Reticulon-like proteins in *Arabidopsis thaliana*: structural organization and ER localization. *FEBS Lett.* **581**, 3356–3362.

Nziengui, H. & Schoefs, B. (2009) Functions of reticulons in plants: What we can learn from animals and yeasts. *Cell. Mol. Life Sci.* **66**, 584–595.

Oertle, T., Klinger, M., Stuermer, C.A.O. & Schwab, M.E. (2003) A reticular rhapsody: phylogenic evolution and nomenclature of the RTN/Nogo gene family. *FASEB J.* **17**, 1238–1247.

Okamoto, M., Kurokawa, K., Matsuura-Tokita, K., Saito, C., Hirata, R. & Nakano, A. (2012) High-curvature domains of the ER are important for the organization of ER exit sites in *Saccharomyces cerevisiae*. *J. Cell Sci.* **125**, 3412–3420.

Orso, G., Pendin, D., Liu, S. *et al.* (2009) Homotypic fusion of ER membranes requires the dynamin-like GTPase Atlastin. *Nature* **460**, 978–983.

Pain, C. & Kriechbaumer, V. (2020) Defining the dance: quantification and classification of endoplasmic reticulum dynamics. *J. Exp. Bot.* **71**, 1757–1762.

Pain, C., Kriechbaumer, V., Kittelmann, M., Hawes, C. & Fricker, M. (2019) Quantitative analysis of plant ER architecture and dynamics. *Nat. Comm.* **10**, 1–15.

Phillips, K.A., Skirpan, A.L., Liu, X. *et al.* (2011) vanishing tassel2 encodes a grass-specific tryptophan aminotransferase required for vegetative and reproductive development in maize. *Plant Cell* **23**, 550–566.

Phillipson, B.A., Pimpl, P., daSilva, L.L., Crofts, A.J., Taylor, J.P., Movafeghi, A., Robinson, D.G. & Denecke, J. (2001) Secretory bulk flow of soluble proteins is efficient and COPII dependent. *Plant Cell* **13**, 2005–2020.

Poulet A. & Kriechbaumer V. (2017) Bioinformatics analysis of phylogeny and transcription of TAA/YUC auxin biosynthetic genes. *Int. J. Mol. Sci.* 18, **18**(8), 1791.

Quader, H. & Fast, H. (1990) Influence of cytosolic pH changes on the organisation of the endoplasmic reticulum in epidermal cells of onion bulb scales: Acidification by loading with weak organic acids. *Protoplasma* **157**, 216–224.

Quader, H., Hofmann, A. & Schnepf, E. (1989) Reorganization of the endoplasmic reticulum in epidermal cells of onion bulb scales after cold stress: Involvement of cytoskeletal elements. *Planta* **177**, 273–280.

Quader H. & Schnepf E. (1986) Endoplasmic reticulum and cytoplasmic streaming: Fluorescence microscopical observations in adaxial epidermis cells of onion bulb scales. *Protoplasma* **131**, 250–252.

Raz, V. & Fluhr, R. (1992) Calcium requirement for ethylene-dependent responses. *Plant Cell* **4**, 1123–1130.

Ridge, R.W., Uozumi, Y., Plazinski, J., Hurley, U.A. & Williamson, R.E. (1999) Developmental transitions and dynamics of the cortical ER of Arabidopsis cells seen with green fluorescent protein. *Plant Cell Physiol.* **40**, 1253–1261.

Robert, H.S., Park, C., Gutièrrez, C.L. *et al.* (2018) Maternal auxin supply contributes to early embryo patterning in Arabidopsis. *Nat. Plants* **4**, 548–553.

Robinson, D.G. (2020) Plant Golgi ultrastructure. *J. Microsc.* https://doi.org/10.1111/jmi.12899.

Robinson D.G. & Aniento F. (2020) A model for ERD2 function in higher plants. *Front. Plant Sci.* **25**, 343.

Robinson, D.G., Brandizzi, F., Hawes, C. & Nakano, A. (2015) Vesicles versus tubes: is endoplasmic reticulum-Golgi transport in plants fundamentally different from other eukaryotes? *Plant Physiol.* **168**, 393–406.

Runions, J., Brach, T., Kühner, S. & Hawes, C. (2006) Photoactivation of GFP reveals protein dynamics within the endoplasmic reticulum membrane. *J. Exp. Bot.* **57**, 43–50.

Sanchez Carranza, A.P., Singh, A., Steinberger, K., Panigrahi, K., Palme, K., Dovzhenko, A. & Dal Bosco, C. (2016) Hydrolases of the ILR1-like family of Arabidopsis thaliana modulate auxin response by regulating auxin homeostasis in the endoplasmic reticulum. *Sci. Rep.* 11 **6**, 24212.

Sawchuk, M.G., Edgar, A. & Scarpella, E. (2013) Patterning of leaf vein networks by convergent auxin transport pathways. *PLos Genet.* **9**, e1003294.

Schoelz, J.E., Harries, P.A. & Nelson R.S. (2011) Intracellular transport of plant viruses: finding the door out of the cell. *Mol. Plant* **4**, 813–831.

Schroeder, L.K., Barentine, A.E., Merta, H., Schweighofer, S., Zhang, Y., Baddeley, D., Bewersdorf, J. & Bahmanyar, S. (2019) Dynamic nanoscale morphology of the ER surveyed by STED microscopy. *J. Cell Biol.* **218**, 83–96.

Shemesh, T., Klemm, R.W., Romano, F.B. *et al.* (2014) A model for the generation and interconversion of ER morphologies. *Proc. Natl Acad. Sci.* **111**, 5243–5251.

Shibata, Y., Voeltz, G.K. & Rapoport, T.A. (2006) Rough sheets and smooth tubules. *Cell* **126**, 435–439.

Shibata, Y., Voss, C., Rist, J.M., Hu, J., Rapoport, T.A., Prinz, W.A. & Voeltz, G.K. (2008) The reticulon and DP1/Yop1p proteins form immobile oligomers in the tubular endoplasmic reticulum. *J. Biol. Chem.* **283**, 18892–18904.

Sia, W., Wang, P., Voigt, B., Hussey, P.J. & Baluska, F. (2016) Arabidopsis SYT1 maintains stability of cortical endoplasmic reticulum networks and VAP27-1-enriched endoplasmic reticulum-plasma membrane contact sites. *J. Exp. Bot.* **67**, 6161–6171.

Silva-Alvim, F.A., An, J., Alvim, J.C. *et al.* (2018) Predominant Golgi residency of the plant K/HDEL receptor is essential for its function in mediating ER retention. *Plant Cell* **30**, 2174–2196.

Sparkes, I. A., Frigerio, L., Tolley N. & Hawes, C. (2009) The plant endoplasmic reticulum: a cell-wide web. *Biochem. J.* **423**, 145–155.

Sparkes, I., Runions, J., Hawes, C. & Griffing, L. (2009) Movement and remodeling of the endoplasmic reticulum in nondividing cells of tobacco leaves. *Plant Cell* **21**, 3937–3949.

Sparkes, I., Tolley, N., Aller, I. *et al.* (2010) Five Arabidopsis reticulon isoforms share endoplasmic reticulum location, topology, and membrane-shaping properties. *Plant Cell* **22**, 1333–1343.

Sparkes, I.A., Ketelaar, T., De Ruijter, N.C.A. & Hawes, C. (2009) Grab a Golgi: laser trapping of Golgi bodies reveals in vivo interactions with the endoplasmic reticulum. *Traffic* **10**, 567–571.

Sparkes, I.A., Runions, J., Kearns, A. & Hawes, C. (2006) Rapid, transient expression of fluorescent fusion proteins in tobacco plants and generation of stably transformed plants. *Nat. Prot.* **1**, 2019–2025.

Spitz, F., Gonzalez, F. & Duboule, D. (2003) A global control region defines a chromosomal regulatory landscape containing the HoxD cluster. *Cell* **113**, 405–417.

Staehelin, L.A. & Driouich, A. (1997) Brefeldin A effects in plants' are different Golgi responses caused by different sites of action? *Plant Physiol.* **114**, 401–403.

Stefano, G., Hawes C. & Brandizzi F. (2014) ER - the key to the highway. *Curr. Opin. Plant Biol.* **220**, 30–38.

Stefano, G., Renna, L., Chatre, L., Hanton, S.L., Moreau, P., Hawes, C. & Brandizzi, F. (2006) In tobacco leaf epidermal cells, the integrity of protein export from the endoplasmic reticulum and of ER export sites depends on active COPI machinery. *Plant J.* **46**, 95–110.

Stefano, G., Renna, L., Lai, Y., Slabaugh, E., Mannino, N., Buono, R.A., Otegui, M.S. & Brandizzi, F. (2015) ER network homeostasis is critical for plant endosome streaming and endocytosis. *Cell Discov.* 17;**1**, 15033.

Stefano, G., Renna, L., Moss, T., McNew, J.A. & Brandizzi, F. (2012) In Arabidopsis, the spatial and dynamic organization of the endoplasmic reticulum and Golgi apparatus is influenced by the integrity of the C-terminal domain of RHD3, a non-essential GTPase. *Plant J.* **69**, 957–966.

Stefano, G., Renna, L., Wormsbaecher, C., Gamble, J., Zienkiewicz, K. & Brandizzi, F. (2018) Plant endocytosis requires the ER membrane-anchored proteins VAP27-1 and VAP27-3. *Cell Rep.* **23**, 2299–2307.

Stephenson, J.L.M. & Hawes, C.R. (1986) Stereology and stereometry of endoplasmic reticulum during differentiation in the maize root cap. *Protoplasma* **131**, 32–46.

Takagi, J., Renna, L., Takahashi, H. *et al.* (2013) MAIGO5 functions in protein export from Golgi-associated endoplasmic reticulum exit sites in Arabidopsis. *Plant Cell* **25**, 4658–4675.

Takeuchi, M., Tada, M., Saito, C., Yashiroda, H. & Nakano, A. (1998) Isolation of a tobacco cDNA encoding Sar1 GTPase and analysis of its dominant mutations in vesicular traffic using a yeast complementation system. *Plant Cell Physiol.* **39**, 590–599.

Terasaki, M., Shemesh, T., Kasthuri, N. *et al.* (2013) Stacked endoplasmic reticulum sheets are connected by helicoidal membrane motifs. *Cell* **154**, 285–296.

Tivendale, N.D., Ross, J.J. & Cohen, J.D. (2014) The shifting paradigms of auxin biosynthesis. *Trends Plant Sci.* **19**, 44–51.

Tolley, N., Sparkes, I., Craddock, C.P., Eastmond, P.J., Runions, J., Hawes, C. & Frigerio, L. (2010) Transmembrane domain length is responsible for the ability of a plant reticulon to shape endoplasmic reticulum tubules in vivo. *Plant J.* **64**, 411–418.

Tolley, N., Sparkes, I., Hunter, P.R. *et al.* (2008) Overexpression of a plant reticulon remodels the lumen of the cortical endoplasmic reticulum but does not perturb protein transport. *Traffic* **9**, 94–102.

Ueda, H., Yokota, E., Kuwata, K. *et al.* (2016). Phosphorylation of the C-terminus of RHD3 has a critical rRole in homotypic ER membrane fusion in Arabidopsis. *Plant Physiol.* **170**, 867–880.

Voeltz, G.K., Prinz, W.A., Shibata, Y., Rist, J.M. & Rapoport, T.A. (2006) A class of membrane proteins shaping the tubular endoplasmic reticulum. *Cell* **124**, 573–586.

Volkmann, D. (1976) In cress roots (*Lepidium sativum L.*), abscisic acid prevents the development of the central cap cells into statocytes. *Planta* **130**, 89–91.

Wang, H., Lockwood, S.K., Hoeltzel, M.F. & Schiefelbein, J.W. (1997) The ROOT HAIR DEFECTIVE3 gene encodes an evolutionarily conserved protein with GTP-binding motifs and is required for regulated cell enlargement in Arabidopsis. *Genes Dev.* **11**, 799–811.

Wang, P., Hawkins, T., Richardson, C., Cummins, I., Deeks, M., Sparkes, I., Hawes, C. & Hussey, P.J. (2014) The plant cytoskeleton, NET3c and VAP27 mediate the link between the plasma membrane and endoplasmic reticulum. *Curr. Biol.* **24**, 1397–1405.

Wang, P., Pleskot, R., Zang, J. *et al.* (2019) Plant AtEH/Pan1 proteins drive autophagosome formation at ER-PM contact sites with actin and endocytic machinery. *Nat. Commun.* **10**, 5132.

Wang, P., Richardson, C., Hawkins, T.J., Sparkes, I., Hawes, C. & Hussey, P.J. (2016) Plant VAP27 proteins: domain characterization, intracellular localization and role in plant development. *New Phytol.* **210**, 1311–1326.

Wang, S., Tukachinsky, H., Romano, F.B. & Rapoport, T.A. (2016) Cooperation of the ER-shaping proteins atlastin, lunapark, and reticulons to generate a tubular membrane network. *Elife* **13**, e18605.

West, M., Zurek, N., Hoenger, A. & Voeltz, G.K. (2011) A 3D analysis of yeast ER structure reveals how ER domains are organized by membrane curvature. *J. Cell Bio.* **193**, 333–346.

Westrate, L., Lee, J., Prinz, W. & Voeltz, G. (2015) Form follows function: the importance of endoplasmic reticulum shape. *Annu. Rev. Biochem.* **84**, 791–811.

Wu, C.H., Lee, S.C. & Wang, C.W. (2011) Viral protein targeting to the cortical endoplasmic reticulum is required for cell-cell spreading in plants. *J. Cell Biol.* **193**, 521–535.

Yang, Y.S. & Strittmatter, S.M. (2007) The reticulons: a family of proteins with diverse functions. *Genome Biol.* **8**, 234–244.

Yao, X., Tian, L., Yang, J., Zhao, Y.N., Zhu, Y.X., Dai, X., Zhao, Y. & Yang, ZN. (2018) Auxin production in diploid microsporocytes is necessary and sufficient for early stages of pollen development. *PLoS Genet.* **14**, e1007397

Yorimitsu, T. & Sato, K. (2012) Insights into structural and regulatory roles of Sec16 in COPII vesicle formation at ER exit sites. *Mol. Biol. Cell.* **23**, 2930–2942.

Zang, J., Zhang, T., Hussey, P. & Wang, P. (2020) Light microscopy of the endoplasmic reticulum-membrane contact sites in plants. *J. Microsc.* **280**, 134–139.

Zhang, C., Kotchoni, S.O., Samuels, A.L. & Szymanski, D.B. (2010) SPIKE1 signals originate from and assemble specialized domains of the endoplasmic reticulum. *Curr. Biol.* **20**, 2144–2149.

Zhang, C., Mallery, E., Reagan, S., Boyko, V.P., Kotchoni, S.O. & Szymanski, D.B. (2013) The endoplasmic reticulum is a reservoir for WAVE/SCAR regulatory complex signaling in the Arabidopsis leaf. *Plant Physiol.* **162**, 689–706.

Zhang, M., Wu, F., Shi, J., Zhu, Y., Zhu, Z., Gong, Q. & Hu, J. (2013) ROOT HAIR DEFECTIVE3 family of dynamin-like GTPases mediates homotypic endoplasmic reticulum fusion and is essential for Arabidopsis development. *Plant Physiol.* **163**, 713–720.

Zhang, X., Ding, X., Marshall, R.S., Paez-Valencia, J., Lacey, P., Vierstra, R.D. & Otegui, M.S. (2020) Reticulon proteins modulate autophagy of the endoplasmic reticulum in maize endosperm. *eLife* **9**, e51918.

Zhao, Y. (2014) Auxin Biosynthesis. *Arabidopsis Book* **12**, e0173.

Zheng, H., Kunst, L., Hawes, C. & Moore, I. (2004) A GFP-based assay reveals a role for RHD3 in transport between the endoplasmic reticulum and Golgi apparatus. *Plant J.* **37**, 398–414.

Zimmerberg, J. & Kozlov, M.M. (2006) How proteins produce cellular membrane curvature. *Nat. Rev. Mol. Cell Biol.* **7**, 9–19.