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The Plant Endoplasmic Reticulum: A Cell-Wide Web

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Abbreviations used: ER, endoplasmic reticulum; RHD, reticulon homology domain; RTNLB, reticulon like; FRAP, fluorescence recovery after photobleaching; PAGFP, photoactivatable GFP; BFA, brefeldin A; ERES, ER exit sites; ARF1, ADP ribosylation factor 1; PAC, precursor accumulating; TAG, triacylglycerols; PMP, peroxisome membrane proteins.

SUMMARY

The endoplasmic reticulum (ER) in higher plants forms a pleomorphic web of membrane tubules and small cisternae that pervade the cytoplasm, but in particular form a polygonal network at the cortex of the cell which may be anchored to the plasma membrane. The network is associated with the actin cytoskeleton and demonstrates extensive mobility, which is most likely dependent on myosin motors. The ER is characterised by a number of domains which may be associated with specific functions such as protein storage, or with direct interaction with other organelles such as the Golgi apparatus, peroxisomes and plastids. Here we discuss the nature of the network, the role of shape forming molecules such as the recently described reticulon family of proteins and the function of some of the major domains within the ER network.

INTRODUCTION

The endoplasmic reticulum (ER) was first described by electron microscopists in the 1960s [1]. Subsequently, the development of vital stains, improved tissue preservation techniques and video imaging technology [2], culminating in the exploitation of fluorescent protein technology [3], has allowed the documentation of the extremely dynamic and pleomorphic nature of the ER (Fig.1, supp. movie 1, see Hepler *et. al.* and references therein [3, 4]).Traditionally the ER has been classed into two forms, rough and smooth, depending on the presence or absence of membrane bound ribosomes [5]. However, the highly dynamic nature of the organelle is exemplified by the rapid changes that can be made between cisternal and tubular forms in response to developmental [6, 7], physiological [8] or environmental cues. It is these two morphological forms that are now more commonly used to describe the organelle, even though their importance was apparent from earlier work using selective membrane staining and thick section electron microscopy [9-11]. For instance, in developing Arabidopsis roots cisternal ER is more common in meristematic and elongating cells, whereas tubular forms predominate in the more vacuolate and mature elongated cells [6]. Positionally, the ER can be described in terms of two populations, the cortical network and cytoplasmic ER which may also extend across *trans*-vacuolar strands [8]. Both forms of ER exhibit motility, although cytoplasmic ER may also get caught in cytoplasmic streams and thus show much more rapid unidirectional movement.

The ER effectively compartmentalises the cytoplasm into two fractions, a reducing cytosol and an oxidising ER lumen, bounded by the ER membrane. The outer nuclear envelope can also be considered to be a distinct domain of the ER being connected to the tubular network and, in some algae such as the yellow-green xanthophyte *Tribonema,* can function as the ER, transporting cargo directly to the *cis*-Golgi [12]. The ER has numerous and diverse functions from the generic biosynthesis of phospholipids, the synthesis, glycosylation, folding and quality control of secretory proteins [13-15], the maintenance of the calcium homeostasis of the cell [16], through to the more specialised formation of storage material such as protein and oil bodies [17-19]. Clear links between these multiple biological functions and the unique morphology and dynamics of the ER, however, have not yet been established.

In this review we will focus on recent data that have revealed the true dynamic nature of the ER network in plant cells and the functional significance of some of the more important ER domains.

THE ER AS A DYNAMIC MEMBRANE NETWORK

As previously mentioned, a typical ER network is composed of several structurally distinct domains; tubules, cisternae and the nuclear envelope. In any given static 'snapshot' of the cortical ER, the vast majority of tubules form a polygonal network underlying the plasma membrane and are interconnected by three-way junctions, with a small subset of 'open ended' tubules undergoing growth/retraction (Fig. 1, supp. movie 1). The continual extension/retraction of tubules appears to be random, although outgrowth has been observed to follow the track of Golgi bodies in tobacco epidermal cells [20]. Static nodules of ER within the dynamic network have been reported in onion bulb cells, and are also apparent in tobacco and Arabidopsis epidermal cells [21]. Recent micromanipulation studies in Arabidopsis leaf epidermal cells have indicated that these static 'islands' of ER appear to act as anchor points, presumably connected through to the plasma membrane, around which the ER remodels [22]. Drastic rearrangements of the entire network also appear to be random, apart from regions of 'fast-flowing' movement which coincide with cytoplasmic streaming. Therefore, based on these morphological observations, ER network remodelling requires factors that regulate tubule extension, network stabilisation, three-way junction and anchor point formation, and modulation of ER shape through tubulation versus cisternalisation (Fig. 2). Given the complexity of these factors and the highly dynamic nature of the ER network, efforts to model ER movement have been limited to date in any system, with static models of ER geometry [23-25] providing the foundation for future studies.

Unlike animal cells, the cortical ER network in higher plants overlies the actin cytoskeleton rather than microtubules [26-28]. Despite this fundamental difference, depolymerisation of the actin or microtubules in higher plants [21] and mammals respectively does not result in a concomitant destruction of the ER network, but does perturb tubule extension and remodelling [29]. While microtubule depolymerisation appears to have no effect on ER dynamics in mature, non-growing cells [20], it does affect the cortical network in elongating characean internodal cells [30]. ER in characean internodal cells can be split into two types; a fast cytoplasmic streaming region in the endoplasm, and the more 'sedate' cortical network above. Drug inhibition studies have shown that whilst the streaming ER is dependent on actin, cortical network remodelling is dependent on the microtubules during the early stages of cell elongation. It has also been reported that oryzalin has an effect on ER dynamics in tobacco leaf epidermal cells, Arabidopsis roots and BY-2 cells but the effects were specific to the drug rather than microtubule depolymerisation *per se* [31]. Furthermore, in mammals, long-term depolymerisation of microtubules (2 hours opposed to 15 minutes) and overexpression of full length and truncations of several microtubule-interacting proteins (CLIMP-63, tau, kinesin) results in ER network shrinkage [29] (and see review [27] and references therein). Therefore, the cytoskeleton plays an important role in tubule extension and, in mammals, network stabilisation. Interestingly, *in vitro* reconstitution studies on *Xenopus* ER microsomes have indicated that ER network formation can occur in the absence of microtubules

and thus the formation of a polygonal network may be an intrinsic property of ER membrane [32].

Myosins and ER movement

It is well documented that organelle movement in higher plant cells is actin-dependent [26, 33-39] and thus it has been assumed that myosin motors are generating the motive forces. The role of myosins has received much attention of late, and the genetic dissection of the 17 Arabidopsis myosin genes has been carried out through overexpression of truncated variants lacking the myosin head domain [40-43]. RNAi down-regulation and T-DNA insertional mutagenesis have verified the role of some myosin isoforms in organelle movement [42-44]. The 17 myosins are split into two classes: VIII contains 4 members and XI contains 13. Class XI has been implicated in organelle movement [40-43]. Tracking algorithms can quantify the movement of discrete organelles such as Golgi bodies, peroxisomes and to some extent mitochondria. The movement characteristics of the ER network are more complicated, and algorithms to relatively easily monitor ER characteristics such as tubule growth, network remodelling, surface area versus volume are currently underway and still in their infancy [25]. Once these have been developed, a comprehensive study of any drastic or subtle effects of these myosins on ER remodelling can be undertaken. Localisation studies have indicated that of the 17 Arabidopsis myosins, class VIII myosin ATM1 is present in small puncta which were proposed to overlie the ER, although its effects on ER dynamics were not documented [45]. Biochemical co-fractionation and immunocytochemistry studies have indicated that a 175kDa heavy chain myosin is associated with the ER in tobacco BY-2 cells and therefore may be responsible for network dynamics [46]. Overexpression of two myosin tail domains (XIK and XI2) in tobacco were reported to have no effect on ER structure, although studies on ER dynamics were not presented [42].

Tubule formation, cisternae and anchoring

While the cytoskeleton provides the tracks along which ER tubule extension occurs, the 'extended' membrane is presumably composed of either 'stretched' ER membrane growing and flexing in new directions, or of *de novo* synthesised ER membrane. Such growing tubules can fuse laterally with other tubules forming the new polygons. Thus, multiple homotypic membrane fusions can be generated along one ER tubule. In mammals several genes have been shown to have an essential role in homotypic membrane fusion (see [27] and references therein). To date there are no candidate plant proteins mediating such fusion events, other than potential ER SNAREs [47].

Factors regulating three-way junction and anchor point formation are unknown (Fig. 2). An intriguing possibility is that anchor points attach the ER to the plasma membrane thus 'anchoring' it in place in the cell as suggested from the video enhanced microscopy studies of onion epidermal cells [2]. There is, however, no evidence to suggest that anchor points necessarily correspond with the position of tripartite junctions. Thus, three-way junction formation could simply be a thermodynamically favourable configuration of stretched, interconnected membrane tubules.

The biological significance of ER shape in terms of tubulation versus cisternalisation is an interesting topic which is gaining renewed interest. A shift to cisternal over tubular ER was proposed to occur due to an increased secretory load in differentiating

maize root cap cells [10] and during mobilisation of seed storage protein in germinating mung bean cotyledons [9]. Similar conclusions were drawn from several studies in mammals; Rajasekaran *et. al.* [48] showed that upon inducing secretion in rat pancreatic acinar carcinoma cells, the rough ER undergoes a structural change from tubular to cisternal form. However, this structural change was not concomitant with an overall increase in surface area, leading the authors to postulate that cisternal ER is more biosynthetically efficient. Additionally, overexpression of certain membrane proteins induces a shift to a more cisternal form of ER over tubular [49], as does a block in ER-Golgi trafficking through BFA treatment and expression of dominant negative mutants involved at the ER-Golgi interface [50]. Intriguingly, ER remodelling is drastically affected during oomycete infection of leaves and can be mimicked by mechanical stimuli [51, 52]. In both cases ER cisternae form around the infection/wound site, and are hypothesised to reflect increased protein and/or lipid production for the delivery of defence-related compounds to the site of action [51]. However, it is unclear whether this is a direct or indirect effect of the remodelling of the underlying cytoskeleton. Internal/external scaffold proteins, or regulation of internal volume by ion pumps and water flow restriction [53] have been suggested to control ER tubulation. However, the latter two models may be difficult to reconcile with the dynamic nature and permeability of the ER membrane.

Reticulons

As mentioned previously, the shape of the tubular ER does not depend on its attachment to the cytoskeleton [32], indicating the requirement for factors present within the ER membrane itself. It has recently been found that a family of membrane proteins called reticulons are enriched in tubular ER and can lead to ER tubule formation in an *in vitro* assay [54]. Reticulons are ubiquitous in higher eukaryotes. They contain a signature r̲eticulon h̲omology d̲omain (RHD) which comprises two large hydrophobic regions, possibly further subdivided into four membrane-spanning segments [55]. Each RHD transmembrane segment is longer than the typical transmembrane helices of ER-localised membrane proteins [56], and therefore is likely to be inserted into the ER membrane at an angle [57, 58]. This wedge-shaped conformation is postulated to confer curvature to the ER membrane [59, 60] (Fig. 2). The topology of plant reticulons has not yet been determined experimentally. Bioinformatic topology prediction for the Arabidopsis reticulon gene family using TOPCONS indicates that all members have a predicted 'W' topology, with N and C termini, plus the short loop between the large hydrophobic regions, exposed to the cytosol [61]. The same topology was described experimentally for mammalian Rtn4c [54]. A direct link between reticulon topology, transmembrane domain length and curvature has however not been established yet.

In the first systematic classification of reticulons [55], plant reticulon genes were denominated RTNLB (r̲eticulo̲n-l̲ike gene in plants, i.e. non-metazoan group B̲). Reticulon genes are very abundant in higher plants [55]. While only a single reticulon-like sequence was found in a search of the genome of the green alga *Chlamydomonas reinhardtii*, the moss *Physcomitrella patens* has at least 9 isoforms, and at least 5 reticulon-like proteins are encoded by the spikemoss *Selaginella moellendorffii* genome (Fig. 3). The *Arabidopsis* genome contains 21 isoforms (reported in [62]). Only 4 reticulon genes have been described in the human genome so far, but alternative splicing may account for more numerous protein products [55]. The explosion of plant RTN gene diversity is very likely to reflect the increasing

complexity and multifunctional role of the ER during higher plant evolution. It is tempting to speculate that different reticulons may underpin the variety of plant ER subdomains [63] and specialized ER functions such as the biosynthesis of oil bodies [64]. While the RHD are highly conserved and the C-termini of reticulons are in general rather short, the N-terminal regions of reticulons are highly variable both in length and in sequence. This suggests that the N-terminus may be the key region for the intrinsic reticulon biological activity and protein-protein interactions. The Arabidopsis sequences of RTNLB17 to 21 stand out for a particularly long N-terminus that likely carries enzymatic activity [62]. Indeed, RTNLB19 was first identified for its activity as a 3-β-hydroxysteroid dehydrogenase/C-4 decarboxylase [65]. RTNLB20 is also annotated as a sterol dehydrogenase [66]. The Arabidopsis genome, however, encodes several sterol dehydrogenase isoforms that do not contain a RHD [65]. This, together with the fact that the long N-terminal regions of RTNLB17, 18 and 21 share similarity with a protein of so far unknown biological function, suggests that, beside its intrinsic structural role, the reticulon homology domain may have been employed as an ER membrane-tethering domain. As enzyme-linked reticulons also exist in *P. patens* and *S. moellendorffii* (Fig. 3), it is possible that the differentiation between reticulon-like tethers and 'structural' reticulons occurred early during plant evolution.

Plant reticulons have so far attracted limited attention, with only three functional works published in the literature [62, 67, 68]. Three reticulons (RTNLB1, 2 and 4) were found to interact with a pilin protein of *Agrobacterium tumefaciens* in a yeast two-hybrid screen [67]. Downregulation of RTNLB1 by antisense resulted in lower rates of *Agrobacterium*-mediated transformation. GFP fusions to the coding sequences of these reticulons appeared to localise in structures reminiscent of the cortical ER in Arabidopsis roots [67]. It will be interesting to understand how these ER membrane proteins interact *in vivo* with the pilus proteins of *Agrobacterium*.

More recently, in an independent study, RTNLB2 and RTNLB4 were again fused to GFP and confirmed that these proteins localise to the ER, but also in punctate structures, in transgenic Arabidopsis or in transiently transfected protoplasts. No functional roles were established [62]. ER residence of RTNLB1 and RTNLB3 was also confirmed by recent proteome localisation data [69].

Our laboratories cloned one of the smallest reticulon isoforms, RTNLB13, which comprises an intact RHD flanked by very short N- and C-terminal regions. Upon overexpression of untagged RTNLB13 in tobacco epidermal cells, the cortical ER lost its normal reticular shape and became strikingly fragmented. However, when RTNLB13-YFP was coexpressed with the luminal marker GFP-HDEL, it was apparent that ER tubules remained intact (Fig. 4). The observed fragmentation results from a remodelling of the luminal space. FRAP analysis confirmed that overexpression of RTNLB13 results in the severely restricted diffusion of luminal ER proteins [68]. Preliminary analysis of anterograde transport by monitoring the secretory kinetics of a reporter protein under RTNLB13 overexpression indicated that, despite this severe morphological phenotype at the level of the ER, anterograde protein transport is unaffected [68]. This seems to indicate that a fully connected tubular ER network may not be necessary for a functional secretory pathway. Intriguingly, overexpression of RTNLB13 results in nodes of seemingly unrestricted ER lumen even though RTNLB13 surrounds these areas (Fig. 4). Based on the earlier discussion of ER dynamics, it is possible that lack of restriction is due to steric

hindrance from large protein complexes / scaffolds at immotile anchor points and /or potential interactions with factors required for ER network stability, perhaps with actin (Fig. 2).

16 out of the 21 Arabidopsis reticulons contain the canonic dilysine ER membrane retrieval motif KKXX [70, 71]. The addition of fluorescent proteins to the C-terminus of RTNLB2 and 4 however did not prevent the proteins form localising to the ER [62]. Similarly, C-terminal tagging of RTNLB13 with YFP did not affect ER localisation and stability [68]. It is possible that ER residence is afforded by the transmembrane topology of RTN and, more importantly, by their ability to interact with other ER-resident proteins or to homo-oligomerise [72]. The di-lysine motif could then have persisted either as an evolutionary relic or a safety valve mechanism.

Some of the interactions that guarantee ER residence are likely to be homotypic, as described for mammalian reticulons [72]. Indeed, RTNLB1-3 were found to interact with each other, as well as with AtRabE1a, in a yeast two-hybrid assay [67]. No other interactions have so far been described for plant reticulons.

Dynamics of the ER surface
ER dynamics can be split into two types; network remodelling as described above and movement of the ER membrane surface itself. Recently, Runions *et. al.* [49], using a photoactivatable GFP (PAGFP) fusion to the trans-membrane domain of calnexin (an ER resident chaperone), demonstrated that upon photoactivation the fluorescent pool of protein displayed varying velocities and migrated in a radial or vectorial manner. Upon depolymerisation of the actin cytoskeleton, only radial diffusive movement of the photoactivated pool was observed, indicating an actin-dependent vectorial movement. Such studies demonstrated that, in tobacco leaf epidermal cells, if activation of the PAGFP construct on the ER membrane is continuous, the whole of the ER network can become fluorescent in 11 minutes, indicating that the whole pool of ER-targeted protein must pass through the activation spot in that time (J. Runions, pers. comm.). Such data indicate the ER may present a mobile surface permitting movement of proteins within the cell. Certainly the ER has been implicated in the transport of viral movement proteins from the sites of synthesis in TMV infected tobacco cells to the plasmodesmata during the infection process [73, 74] and more recently in the movement of viral RNA granules [74, 75]. Further studies are required to quantify the types and the physiological significance of membrane surface movement.

FUNCTIONAL DOMAINS OF THE ENDOPLASMIC RETICULUM

It has been suggested that there are numerous functional domains within the ER network of a plant cell, ranging from areas which accumulate specific products to connections with individual organelles [63]. For instance it has been hypothesised that the junction between the outer nuclear envelope and the endoplasmic reticulum forms a gated domain which controls the exchange of protein between the two organelles [63]. However, photobleaching experiments using GFP tagged constructs of ER resident proteins have shown that there can be free diffusion of protein between the lumen of the nuclear envelope and ER [20], although some degree of control here could be expected as these connections would be the site of entry of membrane bound

proteins specific to the inner and outer nuclear envelopes [76]. Another major domain would be at the plasma membrane where the desmotubules of plasmodesmata are most likely formed from compression of cortical ER passing between neighbouring cells [77]. This extensive topic is however, outside the scope of this review.

ER exit sites

Perhaps the most dynamic and controversial domain of the endoplasmic reticulum is that which represents sites of export to the Golgi apparatus, the so called ER exit sites (ERES). This critical junction in the secretory pathway mediates the transport of both soluble and membrane cargo (proteins and lipids) and somehow involves the COPII coat protein machinery [78]. Transport between the two organelles can be bidirectional and it is thought that retrograde transport from the Golgi to the ER is mediated by COPI vesicles, as blockage of the COPI machinery either by brefeldin A (BFA) [79] or by expression of non-functional ADP ribosylation factor 1 (ARF1) results in the redistribution of Golgi membrane markers into the ER [80]. All the molecular components of ERES identified in yeast and mammalian cells exist in plants [78, 81] and most of the components, with the exception of the exit site scaffold protein Sec 16 [82] have been co-located to the Golgi using fluorescent protein constructs.

In leaves, live cell imaging of epidermal cells expressing a range of exit site markers such as the small GTPase Sar1p [50], COPII coat components Sec23p/Sec24p, Sec 13 [80, 83], ER and *cis*-Golgi SNARES [84] in combination with Golgi membrane markers [26] resulted in the development of the 'motile export site' hypothesis. This proposes that Golgi bodies and the ER exit site exist as a tight unit embedded into the ER membrane and are motile over the ER membrane [49, 50, 78]. Such a concept has been challenged in BY2 cells where Yang *et. al.* [85] suggested there was only transient association of Golgi stacks with ERES. However, more recently it has been confirmed that in Arabidopsis leaves, tobacco leaves and BY2 cells, COPII exit site proteins Sec24 and Sec13 maintain a constant association with Golgi stacks [83]. Thus, unless new Golgi stacks are being formed, exit sites and Golgi bodies are never found separate from each other. It has however been shown that the ER has the capacity to form new exit sites, and thus new Golgi, in response to the expression of membrane cargo such as the ERD2 protein, but not in response to over-expression of soluble secretory cargo such as secreted GFP [86]. Also the ER has the capacity to produce new Golgi stacks after the dissolution of the Golgi with Brefeldin A [79]. A study on tobacco BY2 cells showed that the first reformation event was the appearance at the ER surface of buds and clusters of vesicles which appeared to fuse together to form mini-stacks that subsequently differentiated into large Golgi stacks prior to fission into two stacks [87]. To date there is no evidence that this process requires the formation of free COPII vesicles at the ERES.

The exact physical nature of ER exit sites is a matter of controversy. In leaves, hypocotyls and suspension culture cells (i.e. vacuolate cells) it is clear that Golgi bodies are intimately associated with the ER [26, 78, 88]. However the structure of the ER-Golgi interface is still a matter of speculation. We have proposed that due to the closeness of the two organelles, cargo transfer could easily be mediated by direct membrane connections or tubules [89]. This would require the COPII scaffold to form in order to initiate membrane curvature and maybe concentrate putative cargo receptors, but would not require the formation of independent COPII vesicles. Thus,

the Golgi itself could be considered to be a specialised domain of the ER with ERES initiating the biogenesis of a new Golgi stack depending on the physiological requirements of the cell. Direct connections between the plant Golgi and the ER have long been reported in the ultrastructural literature using conventional fixation and selective staining techniques [20,90-93]. In contrast, there have been no reports of COPII vesicles between the ER and Golgi in the majority of plant tissues studied by live cell imaging of ER and Golgi. In rapidly frozen freeze-substituted root and suspension culture material, however, tomographic analysis reveals vesicles which were assumed to be COPII [94, 95]. These data were used to support the 'stop-and-go' hypothesis of Golgi function, whereby rapidly moving free Golgi are captured by tethering proteins such as P115 (see below) at the ERES, where cargo exchange takes place via COPII vesicles. When replete, Golgi bodies would be released back into the cytosol. However, until the necessary live cell imaging experiments can be successfully carried out on such cytoplasmically dense cells, there will be no firm evidence for one population of Golgi stacks that exist free of the ER and another population that is permanently attached to ER exit sites, but can occasionally break free from their tethers [89]. However, it is clear that in meristems Golgi movement appears restricted compared with that in more vacuolate cells and as such it is possible that there may be populations of Golgi stacks with different ER associations [78].

The functional connection between the ER and Golgi body is mirrored by the close association of the two compartments; live cell imaging has indicated that Golgi body movement appears to mirror the underlying ER (supp. movie 2), and on occasion ER tubule formation appears to follow the path of Golgi bodies [20, 26, 49]. Therefore, based on these observations, the question as to whether the movement of these compartments are (in) dependent of one another was posed [89]. Recently, using laser trapping technology we have shown that it is possible to capture and manipulate individual Golgi bodies in Arabidopsis leaf epidermal cells co-expressing fluorescent ER and Golgi markers [22]. If the actin cytoskeleton was depolymerised to inhibit Golgi movement, Golgi bodies associated with the cortical ER could be captured in the focussed laser beam and any lateral movement of the beam resulted not only in lateral displacement of the Golgi stack but also in the extension or growth of the associated ER tubule (Fig. 5, supp. movie 3). Thus, Golgi bodies do appear to have an attachment to the ER. On occasions when Golgi bodies could be pulled free from ER tubules it was possible to recapture the ER simply by docking a Golgi body onto the tip of the tubule, which resulted in attachment being re-established. This supports the contention that there must be a system of tethering factors or peripheral matrix proteins that can freely attach to the ER maintaining the cohesiveness of the export site/Golgi complex [94]. These results do not formally prove that in an unperturbed system Golgi body movement induces ER remodelling directly. Therefore, the question remains as to whether Golgi body movement and ER remodelling are interdependent processes which utilise the same set of molecular motors/tethering factors, or whether actin polymerisation directly affects ER tubule growth through tethering factors (Fig. 2).

Although there is an extensive literature on ER-Golgi tethering factors in mammalian and yeast cells (see [96, 97]) only recently have peripheral membrane proteins been identified in plant Golgi which may have a tethering role [98, 99]. Some of these, the homologues of P115, CASP and Golgin 84 appear to be located towards the *cis*-face of the Golgi and could be candidates for ER tethering factors [94, 98]. Sinka *et. al.*.

[100] have recently proposed a model for the Golgi whereby the organelle is surrounded by a mass of tentacular molecules of tethering protein that, via Rab binding sites, capture Rab containing membranes such as ER to Golgi carriers. Some plant *trans*-Golgi associated proteins have also been shown to bind small ATPases such as ARL1 [101-103] and AtRabH1[b/c] [102]. As yet, Rab binding has not been reported on *cis*-Golgi proteins but such a model could explain the ability of individual laser-trapped Golgi to re-capture ER membranes.

PAC vesicles and ER-to-vacuole transport

Several proteins can be targeted to the vacuole directly from the ER, in a route that does not involve the Golgi apparatus. Hara-Nishimura [104] reported that pumpkin storage protein precursors exit the ER in large precursor- accumulating (PAC) vesicles which eventually fuse with protein storage vacuoles. The PAC vesicles seem to acquire proteins carrying Golgi-modified N-glycans, which are seen by EM at the periphery of the ER-derived protein core [104]. In addition to storage proteins, a class of cysteine proteases which carry the ER retention signal H/KDEL, have also been observed to travel to the vacuole, where the ER retention signal is removed [105]. A small proportion of some recombinant proteins bearing H/KDEL have also been shown to reach the vacuole, in a route that may [106] or may not [107] require transport through the Golgi apparatus. The molecular details of these ER-to-vacuole transport routes are at present unclear.

The ER as a storage compartment: Oil bodies, grass storage proteins, fusiform bodies.

Whether or not they can be classified as specific domains, the ER in many tissue types has the capacity to store material in so called "ER bodies" [19]. Such material can either remain in the ER or be exported from the ER and exist as discrete organelles such as oil bodies or protein bodies [19, 63].

Oil bodies are essential storage organelles in seeds and are formed from the endoplasmic reticulum by insertion of triacylglycerols (TAGs) within the lipid bilayer of the ER (see [108] for review). TAGs are synthesised by diacylglyceride transferases (DGAT) which are located to distinct sub-domains of the ER [109]. Being hydrophobic, TAGs accumulate between the lipid bilayer and form a bud that enlarges into an oil body, which can eventually break free from the ER. The lipid monolayer of the oil body is characterised by small proteins of the oleosin family which cover the surface of the oil bodies. These proteins are synthesised on the ER membrane and are transported on the ER to sites of synthesis of oil bodies [110]. This again demonstrates the capacity of the ER surface to act as a dynamic surface for transport of macromolecules (see above).

In dicotyledonous plants, most of the protein that is destined for storage in specialised vacuoles during seed maturation is passed through the Golgi apparatus and deposited in a storage vacuole [111, 112]. However, in many grasses and cereals storage proteins such as the prolamins are sequestered in the ER as an insoluble matrix and form distinct protein bodies that may remain in the ER or be delivered directly to the vacuole for storage [15, 17, 19, 113].

Perhaps one of the most striking examples of "ER bodies" are the fusiform bodies commonly found in the lumen of the ER of Arabidopsis and highlighted by many GFP fusions [18, 114, 115]. These can be large, 1 micron in diameter and up to 10 microns long and predominantly contain a β-glucosidase (PYK10) with the C-terminal ER retrieval KDEL motif. Similar bodies containing an inducible β-glucosidase (BGL1) have been identified in wounded cotyledons and rosette leaves. Remarkably, these fusiform bodies move at considerable speed in the cytosol and as it is assumed that the ER has no luminal cytoskeleton, the motive force must come from movement of the whole of the ER, thus reflecting the motile nature of this organelle, as revealed by the photoactivation experiments [49].

Peroxisome biogenesis and the role of the ER

Until recently, the potential role of the ER in peroxisome biogenesis has been hotly debated. The cortical ER is far reaching throughout the cell, and observations indicating an intimate association between the ER and other classes of organelle can be frequently found. This is compounded by the variable morphology of peroxisomes, which in some cases are spherical but can even have long tail-like protrusions called peroxules [37, 116], which apparently co-align with the ER [117]. Such observations, and the occasional apparent direct membrane continuities between peroxisomes and the ER seemed to indicate that peroxisomes arose from the ER [118]. However, the development of molecular and genetic tools proved that peroxisome matrix proteins were synthesised on free polyribosomes and inserted directly into peroxisomes via interaction with cytosolic receptors (PEX5 and PEX7). The 'multiplication-by-division' model whereby peroxisomes arise from growth and division of pre-existing peroxisomes was thus proposed [119]. However, this model could not reconcile how peroxisomes were able to be synthesised *de novo* in certain yeast and mammalian cell line mutants, and how peroxisome membrane proteins (PMPs) and lipids could be synthesised and transported to the organelle.

Several targeting studies and chemical (BFA) perturbation at the ER-Golgi interface have indicated that certain plant PMPs (APX, PEX10, PEX16) are located to the ER [120-126]. However, transient expression studies in tobacco leaf epidermal cells indicated that both PEX2 and PEX10 do not localise to the ER upon BFA treatment or through genetic perturbation (Sar1-GTP locked mutant) at the ER-Golgi interface [127]. Studies of Tomato Bushy Stunt Virus (TBSV) replication protein, p33, have shown that it targets to the peroxisomes and traffics to the ER in vesicular carriers containing PMPs, but it is unclear whether this retrograde pathway occurs in uninfected cells [128]. Similar studies of PMPs in yeast and mammalian cell cultures have been performed, and are detailed in several reviews [129, 130]. The development of new fluorescent protein tools allowing the visualisation of pools of protein through photoactivation, have shown that Pex16p, a PMP in mammals, is present in the ER and subsequently traffics to the peroxisomes [131].

The current model for peroxisome biogenesis therefore appears to be an interplay between the autonomous 'multiplication-by-division' and the ER vesiculation model, whereby peroxisome precursors containing early PMPs bud from the ER into which additional matrix and late PMPs are post-translationally inserted to allow for growth and division.

ER-plastid interactions

For many years there have been regular reports of connections between ER and plastid envelopes (see [132] and [63] for reviews) which have mainly been from ultrastructural studies [133]. This has led to much speculation as to the function of such connections and whether they also facilitate direct transfer of macromolecules between the two organelles.

It is well established that chloroplast development requires lipid precursors, such as diacylglycerols, that are synthesised in the ER membrane [132, 134]. Therefore there has been much speculation on the possible routes of transfer of lipid precursors from the ER to the plastid membrane, including protein-mediated transfer, vesicle trafficking or direct transfer via contact sites between the two organelles [132]. The validity of these contact sites has been tested in protoplasts expressing GFP targeted to the lumen of the ER. On rupturing protoplasts, ER fragments remained attached to chloroplasts [134] and optical trapping and displacement of such chloroplasts resulted in stretching out of the ER fragments [135]. This does not however preclude the possibility that the ER in such a disrupted system is "sticky" and experiments need to be performed *in vivo* to confirm these results. Recently an Arabidopsis gene (*TGD4)* has been described which encodes an ER membrane protein which is proposed to be a component of the machinery mediating lipid transfer to the chloroplast membrane as a mutant prevents the availability of ER diacylglycerol for chloroplast galactoglycerolipid synthesis [134]. The authors proposed one model where TGD4 could be active in mediating lipid transfer at ER chloroplast contact sites. However, it has also been reported that there may be a trafficking pathway from the ER to plastids via the Golgi apparatus. Several chloroplasts proteins including an carbonic anhydrase [136] and a nucleotide pyrophosphatase/phosphodiesterase [137] have been shown to be N-glycosylated and their transport BFA sensitive indicating transport through the Golgi. If it is assumed that the transport vector from the Golgi to the chloroplast is membrane bounded then this could also be a pathway for the trafficking of plastid lipids or lipid precursors.

Whilst it is not impossible that such ER plastid membrane contact sites are involved in lipid transfer between the organelles it must be appreciated that in many cells there is limited free cytosolic space. This may be restricted by large central vacuoles or even by the sheer number of chloroplasts themselves. Therefore, in the context of the presence of a highly mobile ER phase within the cell it is hardly surprising that it frequently makes and breaks contact with the surface of other organelles.

Another possible function of the cortical ER network is a role in graviperception of statocytes. A number of years ago it was suggested that graviperception may be sense by the sedimentation of amyloplasts (statoliths) onto cortical ER in root cap statocytes which would generate a signal to the root growth zone [138]. This model however was subsequently dismissed [139], but has recently been revisited [140]. In Arabidopsis root cells it was shown that sedimenting statoliths can cause deformation of cortical ER, as they are induced to sediment by reorientation of the root. It was proposed that this interaction is a mode of mechanosensing that could induce the gravity perceiving response.

OUTSTANDING QUESTIONS

Although the ER is one of the major organelles in the cell occupying a major portion of the cytosol, and much is known about its functions in terms of protein synthesis, folding, glycosylation and quality control, there are still many questions to be answered regarding its structure and relationships to other organelles. For instance, why does the ER maintain such an energetically unfavourable shape in being tubular with some cisternae, while alterations to its shape do not seem to severely affect anterograde secretory traffic? Is there a function for the movement of the ER network in terms of moving proteins and even other organelles around the cytoplasm? Is there direct exchange of lipids and protein between the ER and other organelles such as mitochondria and plastids and - most intriguingly - what proteins are involved in anchoring the cortical ER network to the plasma membrane? Is there direct molecular exchange with the plasma membrane and is the cortical microtubule network interacting in any way with the ER? Such questions will undoubtedly be addressed in the near future.

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Figure legends

Figure 1. Morphology and dynamics of higher plant ER.
Electron micrograph of an osmium impregnated maize root tip meristem cell (a) and confocal image of tobacco epidermal leaf cells expressing an ER lumenal marker (GFP-HDEL) (b) clearly showing the two structural domains, tubules and cisternal elements. Consecutive images were taken of GFP-HDEL in tobacco leaf epidermal cells, false coloured and overlaid to generate a single plane image. The dynamic nature of cortical ER remodelling is apparent as the 3 images were taken 6.5 seconds apart where white indicates GFP-HDEL fluorescence at all three time points (c). Scale bar 500nm (a) and 5μm (b, c).

Figure 2. Schematic representation of higher plant ER dynamics.
ER tubule growth / retraction may be (in)dependent on Golgi body (G) movement; (1) Golgi body movement, via myosins (blue) processing along actin (blue arrowheads) remodels the attached / tethered ER (yellow spheres), or (2) Golgi body movement is a direct result of association with the ER. Actin polymerisation may also remodel the ER through interaction with actin associated factors (3, purple circles). Potential factors involved in three way junctions (red sphere) and anchor points (blue star) formation are unknown. ER tubulation appears to be due to reticulons (W), and their speculated hetero/homoligomerisation. Factors required for reticulon association / movement within the ER membrane are unknown (green circle).

Figure 3.   Evolutionary relationships of plant reticulon homology domains
The RHD sequences from the indicated reticulon proteins were aligned with ClustalW. The tree was produced with MEGA4.1 using the minimum evolution method with 1000 bootstrap repetitions. Yeast Rtn1p and Rtn2p were used as the outgroup. Bootstrap test results are shown where higher than 50. *P. patens*, *M. moellendorffii* and *C. reinhardtii* reticulons are here defined as PpRTNLB, SmRTNLB and CrRTNLB, respectively. For their full accession numbers refer to supplementary figure S1.

Figure 4. Overexpression of AtRTNLB13 induces constrictions in the tubular ER.
Tobacco epidermal cells were agroinfiltrated with constructs encoding RTNLB13-YFP (green) and the luminal ER marker RFP-HDEL (red). Note that while RTNLB13 labels the tubular ER, the luminal marker is constricted into discrete sections of the network. Scale bar, 5 μm.

Figure 5. Golgi micromanipulation affects ER remodelling.
A Golgi body (magenta, white arrow), in an *Arabidopsis thaliana* leaf epidermal cell treated with latrunculin b, was trapped and subsequent movement resulted in the remodelling of the associated ER (green). Sequential images from a movie sequence are shown and times are indicated. Scale bar 2μm.

Supplementary material

Supplementary figure 1. Reticulon homology domains sequences used for phylogenetic analysis.
The sequences are shown in FASTA format. The accession numbers for each individual sequence are listed after the RTNLB numbers.

Supplementary movie 1. Movement of cortical ER in a tobacco leaf epidermal cell.
A time series of tobacco leaf epidermal cells expressing an ER marker, GFP-HDEL, was generated. Continual ER remodelling through tubule growth / retraction, three way junction formation, transitions between tubular and cisternal forms of ER, static regions or so called anchor points which the ER remodels around are observed. Some anchor points (blue circles) are highlighted, as is an area of cortical ER undergoing constant remodelling and transition from a tubular to cisternal form (white ellipse). Scale bar 5μm.

Supplementary movie 2. Movement of cortical ER and Golgi bodies in an Arabidopsis epidermal leaf cell.
Golgi body (red) movement seemingly follows the cortical ER network (green). Scale bar 2μm.

Supplementary movie 3. Movement of laser trapped Golgi bodies remodels the cortical ER.
The actin cytoskeleton in Arabidopsis leaf epidermal cells was depolymerised, and movement of trapped Golgi bodies (magenta) resulted in remodelling of the underlying ER network (green). Scale bar 5μm.
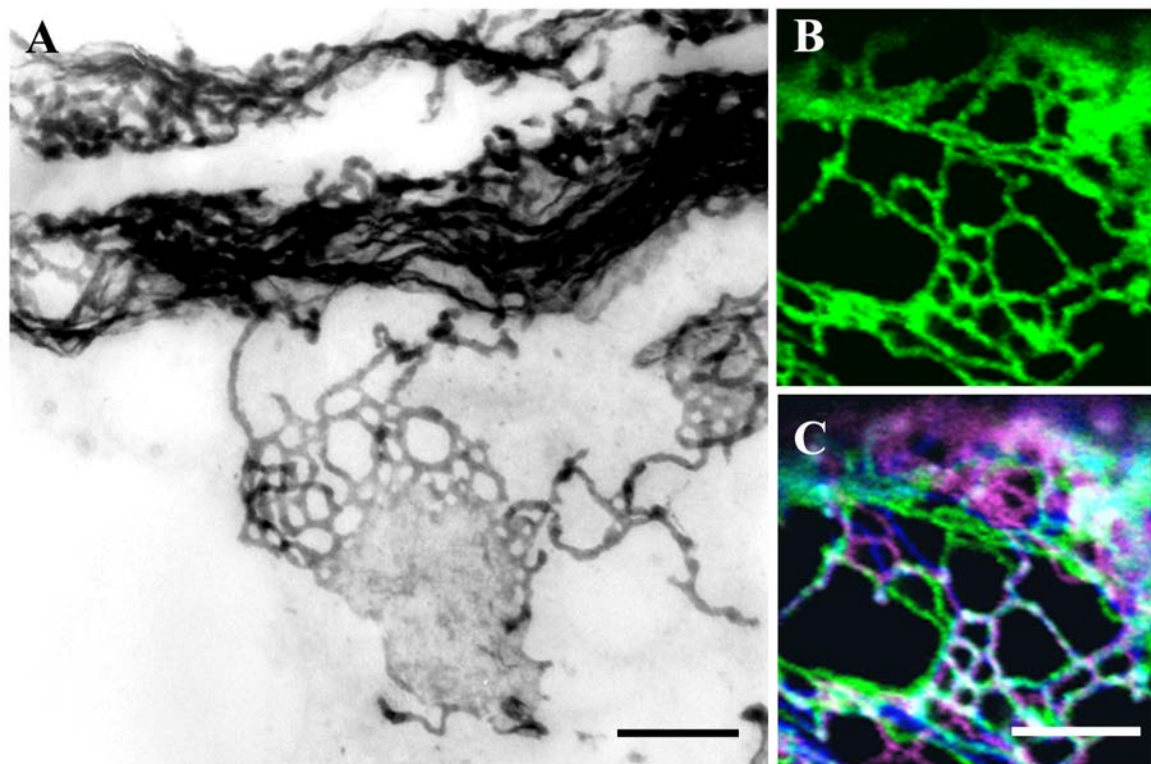
Figure 1. Sparkes *et al*.

ER tubule growth / retraction

ER stability, 3 way junctons & anchor points
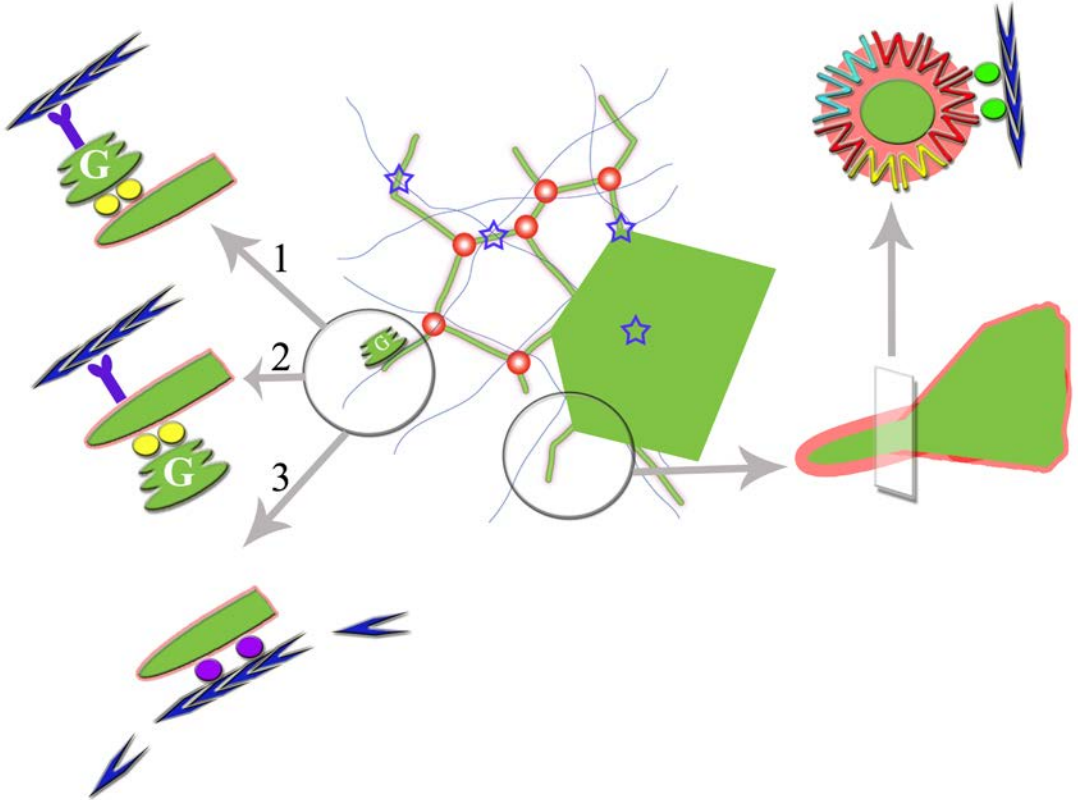
ER tubulation versus cisternalisation
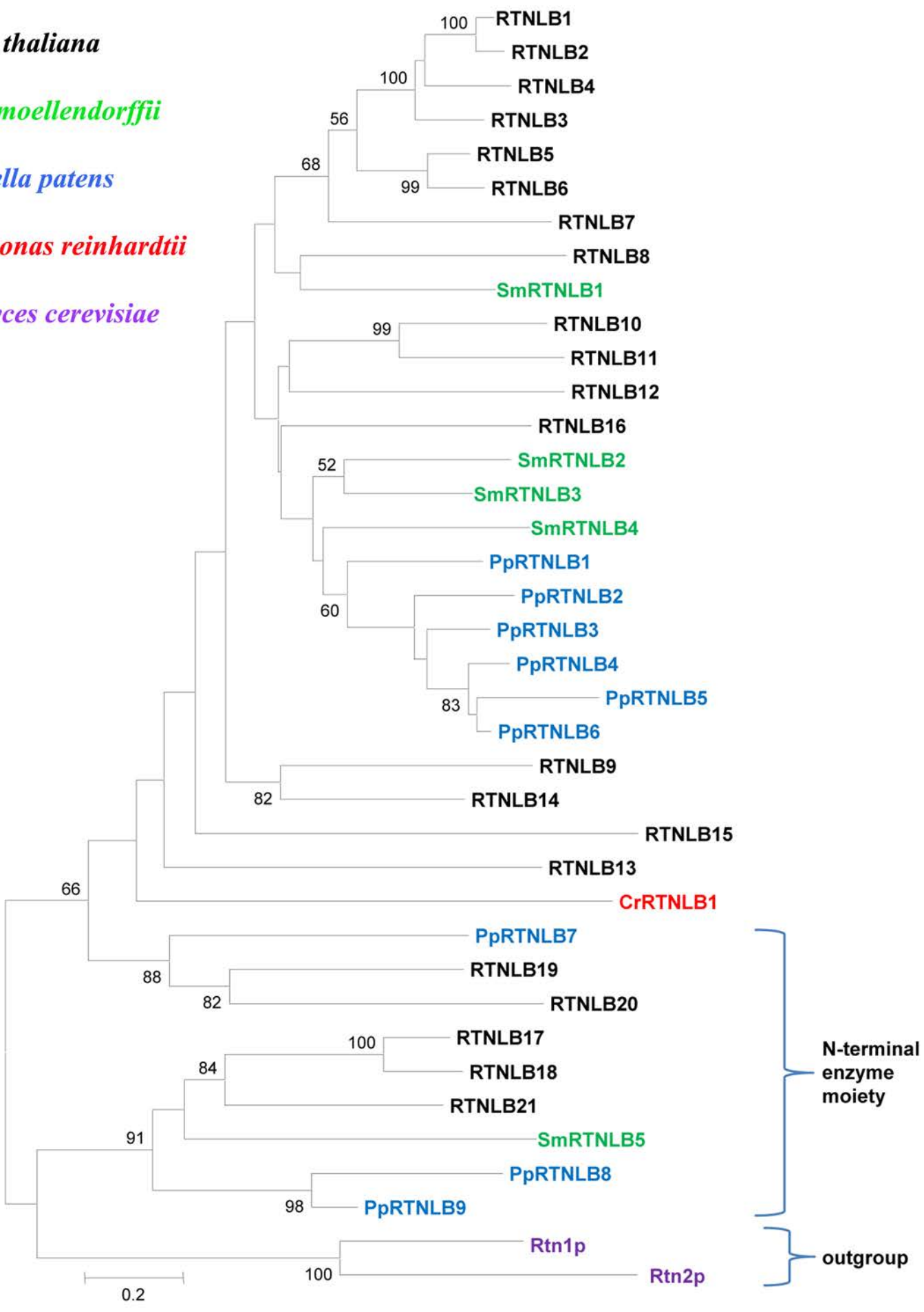

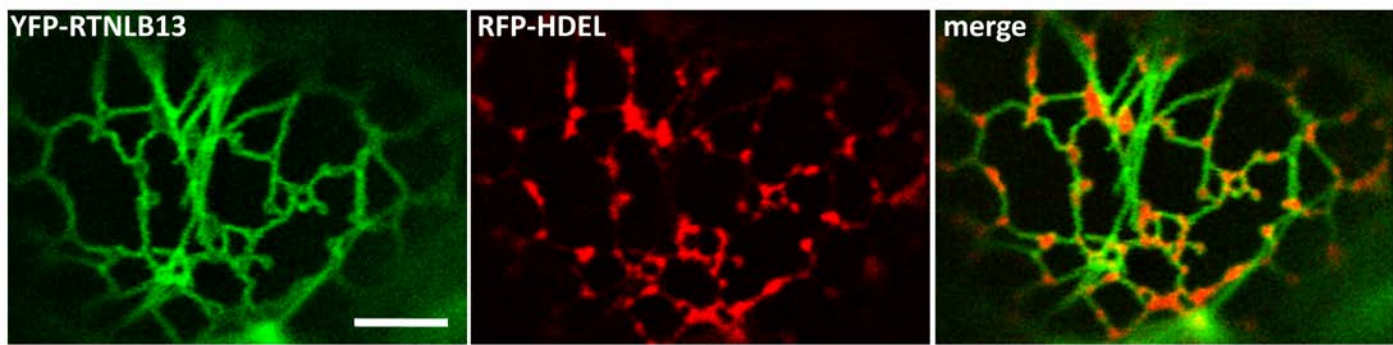**Figure 2. Sparkes** *et al.*

Figure 3. Sparkes *et al.*
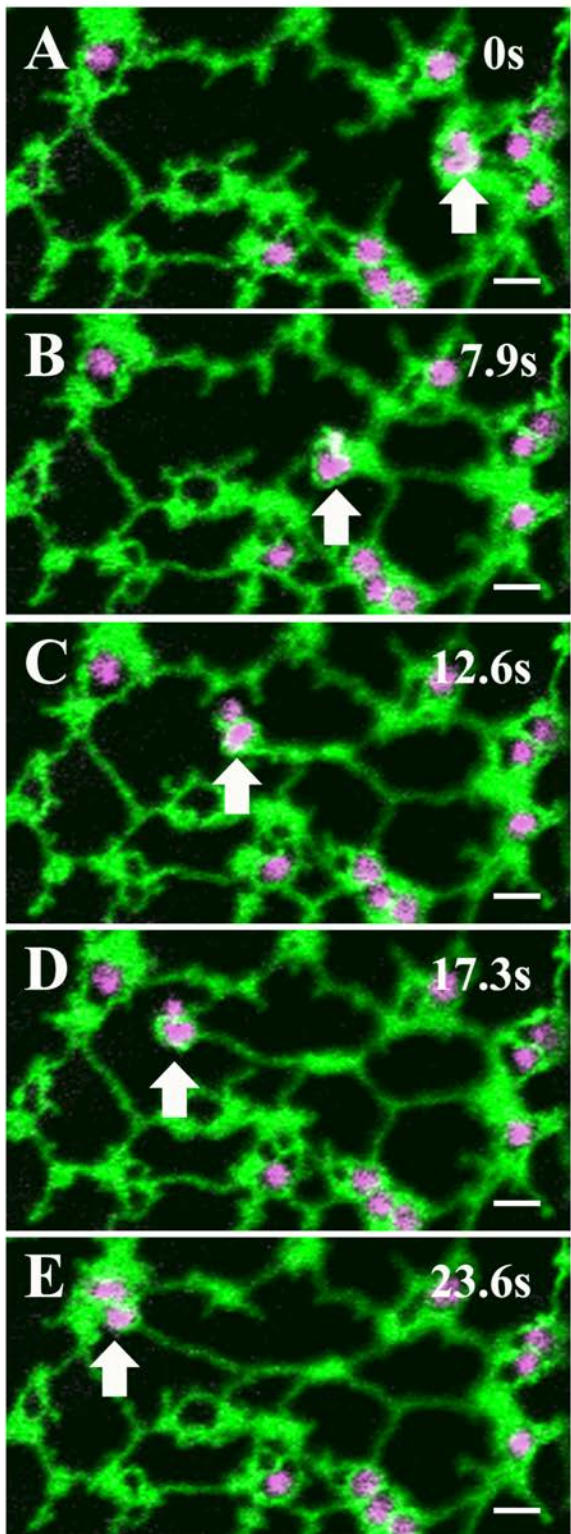
Figure 4. Sparkes *et al.*

Figure 5. Sparkes *et al.*

Supplementary figure 1. Sparkes *et al.*

Arabidopsis thaliana

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Selaginella moellendorffii

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Physcomitrella patens

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Saccharomyces cerevisiae

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