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Title

"The plant secretory pathway seen through the lens of the cell wall"

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Keywords

Plants; secretory pathway; cell walls; polysaccharide synthases; glycosyltransferases; conventional protein secretion; unconventional protein secretion; exocyst-positive organelle; Golgi; endoplasmic reticulum; plasma membrane; vesicles

Abbreviations

AGP- arabinogalactan-proteins; AX- arabinoxylan;; CCV- clathrin-coated vesicle; CESA- cellulose synthase A; CSLC- cellulose synthase-like C; COP- coat protein; CSC- Cellulose synthase complex; EM- electron microscopy; ER- endoplasmic reticulum; GA- Golgi apparatus; GSL- GLUCAN SYNTHASE-LIKE; GTs- glycosyltransferases; MVBs- multi-vesicular bodies; MLG- (1,3;1,4)-β-D-glucan; PM- plasma membrane; RER- rough endoplasmic reticulum; SER- smooth endoplasmic reticulum; SV- secretory vesicle; TGN- *trans*-Golgi network; XG- xyloglucan

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ABSTRACT

Secretion in plant cells is often studied by looking at well-characterised, evolutionarily-conserved membrane proteins associated with particular endomembrane compartments. Studies using live cell microscopy and fluorescent proteins have illuminated the highly dynamic nature of trafficking, and electron microscopy studies have resolved the ultrastructure of many compartments. Biochemical and molecular analyses have further informed about the function of particular proteins and endomembrane compartments. In plants there are over 40 cell types, each with highly specialised functions, and hence potential variations in cell biological processes and cell wall structure. As the primary function of secretion in plant cells is for the biosynthesis of cell wall polysaccharides and apoplastic transport complexes, it follows that utilising our knowledge of cell wall glycosyltransferases (GTs) and their polysaccharide products will inform us about secretion. Indeed this knowledge has led to novel insights into the secretory pathway, including previously unseen post-TGN secretory compartments. Conversely our knowledge of trafficking routes of secretion will inform us about polarised and localised deposition of cell walls and their constituent polysaccharides/glycoproteins. In this review, we look at what is known about cell wall biosynthesis and the secretory pathway and how the different approaches can be used in a complementary manner to study secretion and provide novel insights into these processes.

1. Introduction

The secretory pathway is crucial to cellular function during growth and development as well as in response to stresses, both biotic and abiotic. It acts as the assembly compartment for many of the proteins (rough endoplasmic reticulum (RER)), lipids (smooth ER (SER)) and polysaccharides (RER & Golgi apparatus (GA)) as well as the central sorting compartment (*trans*-Golgi network (TGN) i.e., the "post office", from which vesicles deliver cargo to the vacuole or to the plasma membrane (PM)/apoplast (cell wall). Identification of the key players has relied on morphological/anatomical studies combined with either cargo or vesicle scaffold/receptor proteins. These approaches have been particularly illuminating for the ER/GA compartments but less so for the post-TGN pathway(s) that deliver a wide range of cargos to the cell surface. Definition of the ER/GA stacks and transport (anterograde/retrograde) between these compartments is close to a consensus although whether dynamic movement between the ER and Golgi is vesicular or via interconnected tubules remains unresolved (Robinson et al., 2015). A similar conundrum is present around Golgi maturation with the cisternal progression model mostly favoured over the vesicular transport model (Glick and Nakano, 2009).

Advances in identifying the glycosylation machinery and mechanisms for cell wall polysaccharide biosynthesis, cellulosic, non-cellulosic and pectic (Doblin et al., 2010; Scheller and Ulvskov, 2010; Pauly et al., 2013; McFarlane et al., 2014), has generated new insights into the secretory pathway, including previously unseen post-TGN secretory compartments (Crowell et al., 2009; Gutierrez et al., 2009). Here, we summarise these findings and discuss how they may improve our understanding of the secretory pathway so critical to cell function. For those interested in transport to the vacuole, which is outside the scope of this review, we direct the readers to excellent reviews by Pereira et al., (2014), Viotti (2014) and Zhang et al., (2014).

2. Plant cell types and wall deposition

Plants have approximately 40 cell types and their unique shapes are determined by the functional specialisation of their cell walls. Removal of their walls, irrespective of the cell type, results in morphologically indistinguishable spherical protoplasts. The architecture and composition of cell walls is dynamic and varies depending on the type of organ, tissue and cell within the plant as well as the polysaccharides present (for a summary of wall composition and organisation see **Box. 1A-B**). During growth and through interactions with the environment, including pathogen attack, the wall is modified to accommodate changing conditions for the cell (Somerville et al., 2004). For a particular cell, there is often localised compositional heterogeneity in the wall (**Box. 1B**) suggesting that the process of deposition is highly orchestrated and connected to the secretory system, such that trafficking occurs in a specific, spatio-temporally regulated manner (Roberts, 1990; Verhertbruggen et al., 2009; Burton et al., 2010).

Model plant systems have been routinely used for investigating the cell biology of particular stages of growth, development and pathogen attack. A brief summary of the most common cell types utilised to study the cellular and molecular aspects of secretion and cell wall assembly that are discussed in this review are summarised in **Table 1**. These systems can be microscopically visualised with fluorescent proteins and live cell microscopy, high resolution spatio-temporal localisation under the electron microscope (EM) and cell processes can also be functionally assessed using pharmacological agents such as brefeldin A, concanamycin A and wortmannin (Satiatjeunemaitre and Hawes, 1992; Dettmer et al., 2006; Viotti et al., 2010). Remarkably, studies have been restricted to a few cell types, primarily due to either the difficulties associated with preserving plant cells for high resolution microscopy (Wilson and Bacic, 2012) or the limitations of live cell imaging techniques that largely focus on cells at the surfaces of organs. Nonetheless, they have provided significant insights into the secretory pathway/processes of plant cells.

3. Secretory pathways to the apoplastic space (cell wall)

3.1 Conventional protein secretion (CPS)

In CPS, cargo proteins with an N-terminal leader sequence are synthesised in the RER and following initiation of post-translational modifications, move to the GA where they are then extensively modified by either glycosylation (plants and algae) or sulfation (algae) and directed at the TGN to the PM via secretory vesicles (SVs) where they are exocytosed into the apoplast (see **Fig. 1**). Many of the proteins in the CPS pathway are highly conserved between vertebrates, yeast and plants/algae and are involved in either tethering and fusion, such as SNAPs and SNAREs (soluble N-ethylmaleimide-sensitive factor protein attachment proteins/ receptors), or in membrane curvature, such as either CLATHRIN or COAT PROTEINS I and II (COPI/II) (Sanderfoot and Raikhel, 1999; Jurgens and Geldner, 2002; Drakakaki and Dandekar, 2013).

In plants, biosynthesis and secretion of cell wall polysaccharides is one of the major roles of the CPS. Polysaccharide synthases/glycosyltransferases (GTs), that assemble the cell wall polysaccharides, are located in either the GA (the site of assembly of most non-cellulosic polysaccharides and pectins) or at the PM (the site of assembly of the mixed linkage glucans (MLGs), cellulose and callose) and have for the most part been identified as being produced in the CPS (Driouich et al., 2012; Oikawa et al., 2013; Wilson et al., 2015). For this reason, and following completion of the description of the remaining secretory pathways, the subsequent sections will primarily focus on the CPS pathway.

3.2. Unconventional protein secretion (UPS)

UPS for the most part either bypasses the conventional endomembrane compartments or is directed to multi-vesicular bodies (MVBs) after the *trans*-Golgi network (TGN) before either insertion into or translocation across the PM (see **Fig. 1**) (Ding et al., 2012; Drakakaki and Dandekar, 2013; Ding et al., 2014; Robinson et al., 2015). UPS proteins are differentiated on the basis of their lack of an N-terminal leader sequence and are referred to as leaderless secretory proteins (LSPs). While hundreds of LSPs have been putatively identified using predictive bioinformatics tools on extracellular proteomes, including cell wall proteomes (Albenne et al., 2013), only one has been verified experimentally as undergoing UPS, the Helja protein, a

mannose-specific jacalin-related lectin in sunflower seedlings (Pinedo et al., 2012; Robinson et al., 2015). Since many LSPs are thought to have a low copy number with expression restricted to certain developmental stages (Gupta and Deswal, 2012), this has made their true secretory path difficult to verify (Robinson et al., 2015). Most commonly detected are pathogen-related (PR) proteins (e.g., chitinases, glucanases and peroxidases) which also lack a signal peptide (Krause et al., 2013; Ding et al., 2014). The secretion of these PR LSPs appears to be mediated though specialised MVBs that instead of being a pre-vacuolar compartment/late endosomes, are secretory complexes that possibly fuse with the PM (Meyer et al., 2009; Ding et al., 2014). Callose, the major polysaccharide in pathogen-induced papillae, has been reported to be present in MVBs in broad bean epidermal cells penetrated by the cowpea rust fungus (Xu and Mendgen, 1994), suggesting that this is one route by which GLUCAN SYNTHASE-LIKE (GSL) proteins, the callose synthase enzymes, are targeted to the PM (**Fig. 1**). The stress-induced Ca^{2+}– dependent callose synthase POWDERY MILDEW RESISTANT (PMR) 4/GSL5 (Jacobs et al., 2003; Nishimura et al., 2003) was located in MVBs in barley epidermal cells undergoing attack, but not its cognate polysaccharide product, callose (An et al., 2006). This suggests that PMR4/GSL5 is activated once it has reached the PM region where the papilla is developing and could be used as a marker for the UPS pathway.

Interestingly, the developmentally regulated and Ca^{2+}– independent callose synthase GSL1, involved in pollen tube formation in *Nicotiana alata*, is produced in the CPS (Brownfield et al., 2008) suggesting that not all GSL family members follow the same trafficking route to the PM. Using immuno-gold antibody labelling and TEM, GSL1 was found in the ER and Golgi at the early stages of pollen tube growth and in intracellular vesicles and the PM later in development (Li et al., 1999; Brownfield et al., 2008). This reinforces the need for caution in the interpretation of secretory pathway data from a single system and protein, and furthermore, that during stress-responses cells may “subvert” the “normal” secretory system in order to “fast track” secretion.

MVBs and exosomes as a pathway for secretion: Exosomes are 40-100 nm-sized membrane-bound vesicles that are secreted from the PM into the extracellular space in mammalian cells and yeast (Raposo and Stoorvogel, 2013).

They are derived from multi-vesicular endosomes and exocytosed into the extracellular space and are involved in intercellular communication carrying cargo, including microRNAs, that in mammalian tissues have been implicated in stimulating angiogenesis and nerve regeneration as well as facilitating tumour metastasis and pathogen spread (Zhang et al., 2015). Paramural bodies, which are derived from MVBs and released into the paramural space and are therefore exosomes, have been associated with the host response to powdery mildew infection in barley leaves (An et al., 2006; Meyer et al., 2009). Their cargo has yet to be identified (Drakakaki and Dandekar, 2013) so it is not yet possible to conclude if they are either a form of CPS or UPS.

EXPO as a secretion pathway: Another potential UPS route is through the specialised exocyst-positive organelle (EXPO), a double membrane-bound organelle identified by the exocyst protein EXO70E2 (Wang et al., 2010). Studies on *Arabidopsis* protoplasts and tobacco BY-2 cells showed that EXPO is not associated with CPS markers for either the GA, TGN, MVB, SVs or for the autophagosome, and was confirmed not to traffic through other endomembrane compartments by using the pharmacological inhibitors brefeldin A, wortmannin and concanamycin A, thereby suggesting it may be ER-derived. EXPO was shown to fuse with the PM releasing a single membrane-bound exosome that contained cytosolic cargo identified as the S-adenosylmethionine synthetase 2 (SAMS2) protein, one of four SAMS enzymes involved in lignin methylation in the cell wall (Shen et al., 2002; Wang et al., 2010).

Additionally, the EXPO organelle was recently proposed to have a role in *O*-glycosylation of arabinogalactan-proteins (AGPs) (**Box. 1**) (Poulsen et al., 2014; Poulsen et al., 2015), proteoglycans that form a “glycocalyx” on the PM surface by virtue of their glycosylphosphatidylinositol (GPI) membrane anchor and are also both free and cross-linked into the cell wall (Tan et al., 2012). The protein backbone of these highly glycosylated (90-98%) molecules is produced in the ER (Oka et al., 2010) with the GTs involved in their glycosylation (see **Box. 1C**) localised either to both the ER and Golgi, such as HYP *O*-GALACTOSYLTRANSFERASES (HGPT1-3) (Ogawa-Ohnishi and Matsubayashi, 2015), GALACTOSYLTRANSFERASE 2 (GALT2) and GALT5 that add the first galactose (Gal) onto hydroxyproline (Hyp) residues (Basu et al., 2013; Basu et al., 2015), or just to the Golgi as with GLUCURONOSYLTRANSFERASE 14 (GlcAT14A) (Knoch et al., 2013), GALT29A

(Dilokpimol and Geshi, 2014) and GALT31A (Qu et al., 2008; Geshi et al., 2013) that make subsequent additions to AGP glycan chains. Poulsen and colleagues (Poulsen et al., 2014) proposed that up to 80% of GALT31A was located in smaller compartments that also partially labelled with the EXO70E2 marker protein for the EXPO organelle. In the same study, GALT29A and GlcAT14A were found to co-localise mostly in the GA, but also 40% of the time with the GALT31A protein suggesting that these GTs were also transiting through the EXPO organelle. This is an intriguing observation warranting further investigation since it suggests that some cell wall-associated glycoproteins might be exported via a UPS route, even though neither their glycosylation machinery (GT proteins) nor the AGP protein backbones (Tan et al., 2012) belong to the LSP category (Robinson et al., 2015) and would therefore be expected to be part of the CPS pathway.

4. The endomembrane systems in the CPS pathway

4.1 The plant CPS pathway is highly mobile and dynamic

One of the most fascinating features about the plant secretory system is the dynamic movements that allow the targeted delivery of cargo in the otherwise stationary cell. At every stage, there is active streaming of the different secretory components relying primarily on the actomyosin cytoskeleton (Nebenführ et al., 1999; Geitmann and Nebenfuehr, 2015). The ER is a fluid organelle that associates with the actin and MT cytoskeletons and a large number of myosins have been identified as having a role in the dynamics of the ER (Avisar et al., 2009; Ueda et al., 2010). Proteins and lipids are synthesised in the ER, which has distinct subdomains, at the most basic level seen under the EM either with or without bound ribosomes (RER and SER, respectively), and as fluid tubules and cisternae with more sophisticated optical imaging techniques. These structural features are related to different metabolic and structural characteristics such as the hypothetical anchor points to the PM, which may refer to the ER-PM contact sites observed under the EM and proposed to be sites of non-vesicular lipid transfer that could be important in plant cell wall biosynthesis (Samuels and McFarlane, 2012) and ER exit sites (ERES) (Grabski et al., 1993; Boevink et al., 1996; Nebenführ et al., 1999; Hawes et al., 2015).

Proteins destined for secretion are synthesised and folded in the ER with quality control mechanisms ensuring only correctly folded proteins continue through the secretory pathway (Liu and Li, 2014). Some GTs are well characterised in the ER, such as those associated with the universally conserved *N*-linked glycan precursor assembly ($Glc_3Man_9GlcNAc_2$) (Caramelo and Parodi, 2015); however, others such as either the cellulose synthases (CESAs) (McFarlane et al., 2014) or the well characterised xyloglucan (XG) glucan backbone synthases (CSLCs; see **Box 1C** for a summary of the GTs involved in polysaccharide biosynthesis) (Chou et al., 2015) have not been directly associated with the ER. As stated in section 3.2, the protein backbones and the early stages of arabino-3,6-galactan (AG) side chain decoration assembly of AGPs occurs in the ER (Tan et al., 2012) and recently a quantitative immuno-labelling study of the MLG synthases (CSLF6 and CSLH) indicated that these proteins are also trafficked through the ER (Wilson et al., 2015).

From the ER, (glyco)proteins are secreted at ERES to the GA (Hawes, 2012; Langhans et al., 2012). In eukaryotes, transport from the ER to the Golgi (anterograde transport) involves COPII vesicles (Kang and Staehelin, 2008; Marti et al., 2010) and return of the vesicles from the Golgi to the ER (retrograde transport) involves COPI vesicles (see **Fig. 1**) (Donohoe et al., 2007; Szul and Sztul, 2011; Barlowe and Miller, 2013). Analysis of movement of ERES labelled with ERD2-YFP, which cycles between the ER and Golgi, and the COPII coat protein Sar1-GFP from *Arabidopsis* showed that the two proteins at the ER-Golgi interface moved as a unit (DaSilva et al., 2004; Runions et al., 2006). Furthermore, results using optical tweezers, where individual GA were physically held and then moved around the cell, indicated that the ER and Golgi bodies are tethered to each other (Sparkes et al., 2009). From high-resolution electron tomographic data, ribosome-excluding scaffold matrices were observed around *Arabidopsis* COPII vesicles and the Golgi (Kang and Staehelin, 2008), but no direct connections have been observed in material cryo-fixed using high pressure freezing. Tubules interconnecting the ER and GA have been observed in chemically fixed material, particularly when using either zinc iodide or potassium ferricyanide in combination with osmium tetroxide (Mollenhauer et al., 1976; Harris, 1979; Hepler, 1981). However, there is no consensus as to whether the tubules form connections between the ER and Golgi rather than vesicles (for an excellent synopsis of the prevailing views see Robinson et al., (2015)) . Regardless

of the mechanism, transport at the ERES has been shown to be highly mobile and dynamic and often associated with the Golgi (Marti et al., 2010).

4.2. The Golgi apparatus and cell wall biosynthesis

The GA is a complex organelle that transports and glycosylates proteins, synthesises and assembles most of the non-cellulosic polysaccharides and pectins as well as cycles membrane lipids (Zhang and Staehelin, 1992; Doblin et al., 2010; Driouich et al., 2012; Oikawa et al., 2013; Pauly et al., 2013). Plant cells can have hundreds of Golgi stacks that move around the cell on an actomyosin network with stop-and-go movements (Boevink et al., 1996; Nebenführ et al., 1999). During the stop movement, it has been suggested that cargo is offloaded in vesicles for secretion (Nebenführ et al., 1999; Crowell et al., 2009). From the GA, vesicles are trafficked via the TGN along different routes to the PM delivering their cargo into either the cell wall (polysaccharides and glycoproteins) or the PM (glycoproteins; see section **3.2**).

Plant GAs are differentiated from those of animal and yeast by a number of characteristics including, the dispersion of Golgi bodies throughout the cytoplasm that travel on actin filaments, active secretion of wall material during cell division for the formation of the cell plate, and not breaking down during mitosis (Drakakaki, 2015). In contrast, in animal cells Golgi complexes are is located in the perinuclear region, associated with MTs and is generally stationary throughout the cell cycle until it breaks down during mitosis (Burkhardt, 1998). Whereas in yeast, multiple Golgi compartments are found in either ordered stacks (e.g., *Pichia pastoris*) or as separated cisternae (*Saccharomyces cerevisiae*) where they mature from one type of cisterna to another during development (Papanikou and Glick, 2009).

In algae and plants, each GA is composed of a series of disc-shaped cisternae that are surrounded by a ribosome-excluding scaffold (Kang and Staehelin, 2008). The Golgi stacks are polarised with the *cis*-cisternae closest to the ER, the *medial*-cisternae in the middle of the stack and *trans*-cisternae closest to the TGN (**Fig. 1**). The number of stacks can vary depending on the species with, for example, up to nine in *Chlamydomonas* and 5-7 in *Arabidopsis* (Donohoe et al., 2013). The *cis*-cisternae appear to be assembled from COPII vesicles derived from the ER and, due to the lack of luminal contents when observed using EM techniques, are thought

to be biosynthetically inactive (Donohoe et al., 2013); however, it is likely that they are carrying cargo including wall glycoproteins such as AGPs (see section **3.2**) and extensins, which do not take up the heavy metal stains used in EM preparation techniques. The *medial*-cisternae have more background staining (Donohoe et al., 2013) and the first enzyme in the *N*-linked glycan precursor processing pathway, MANNOSIDASE 1 (Man1) (Abeijon et al., 1997), has been located to these cisternae indicating glycoprotein modification (see below). The *trans*-cisternae also have background luminal contents (Reyes and Orellana, 2008; Donohoe et al., 2013) ostensibly due to the polysaccharide synthesis machinery and polysaccharides (Driouich et al., 2012). The Golgi cisternae increase in size from *cis*- to *medial*- to *trans*-cisternae and vesicles are seen to bud from the edges of the cisternal sacs (**Fig. 1 and Table 2**). COPIa-coated vesicles recycle trafficking membrane proteins and lipids from *cis*-cisternae to the ER and COPIb-coated vesicles recycle between the TGN and the *trans*-cisternae (**Fig. 1**) (Donohoe et al., 2007). It is possible that some vesicles bud directly from the Golgi either to other compartments in the cell or to the PM (Ding et al., 2014) (**Fig. 1 and Table 2**).

Two models for maintaining the spatial segregation of polysaccharide biosynthesis and protein post-translational modification in the GA have been suggested: 1) cisternal progression (Becker et al., 1995; Bonfanti et al., 1998; Donohoe et al., 2013) and 2) vesicle cycling (Farquhar and Palade, 1981). From studies in mammals where they looked at procollagen (Bonfanti et al., 1998), yeast cisternal maturation (Papanikou and Glick, 2009), algal scales in chrysophytes (Becker et al., 1995) and studies on plant GA (Donohoe et al., 2013), the cisternal progression model, combined with retrograde vesicle cycling to return enzymes to their functional cisternae, was thought to be the likely mechanism of maintaining Golgi polarity (Luini, 2011; Donohoe et al., 2013). However, recent high resolution cryo-electron tomography (Engel et al., 2015) has shown structural connections that act like zippers between cisternae and they suggest that these connections could allow small molecules to be transferred between the cisternae with larger enzyme complexes transferred by cisternal progression. These findings are intriguing and with additional exploration could aid in deciphering the mechanisms involved in glycoprotein and polysaccharide processing within the GA sub-compartments of plants.

Sequential glycosylation within the Golgi: The spatial separation of enzymatic processes within the GA is apparent with the progression of protein *N*-linked glycosylation in mammals, yeast and plants (McCormick et al., 1999; McCormick et al., 2000; Young, 2004; Saint-Jore-Dupas et al., 2006; Strasser et al., 2006; Nilsson et al., 2009; Komis et al., 2015; Zhang et al., 2015; Dumont et al., 2016). These enzymes show positional/spatial glycosylation with monosaccharides sequentially added (from their activated nucleotide sugar donors) to the glycans (*N*- and *O*-) of the protein backbone at each progressive cisterna. In non-cellulosic polysaccharide and pectin biosynthesis, various enzymes are similarly located in different Golgi cisternae where they go through the step-wise process of adding sugars to backbone residues and then decorating these backbones with side chains (see **Box 1C and Fig.1**) (Driouich et al., 2012; Oikawa et al., 2013).

In any one Golgi cisterna, glycosylation of multiple proteins and biosynthesis of multiple polysaccharides can occur concurrently, as is the case for XG and pectin. Evidence from both biochemical GT interaction studies using either antibodies or proteomics and binary interactions, such as Forster resonance energy transfer (FRET), bimolecular fluorescence complementation (BiFC) and split ubiquitin studies in yeast, have shown that many of the enzymes involved in non-cellulosic polysaccharide and pectin biosynthesis form homo- and hetero-oligomeric complexes but with biochemical (enzyme) functional and correct localisation only occurring when these complexes are fully assembled (Oikawa et al., 2013).

One of the best-characterised polysaccharide biosynthetic pathways is that of XG, which we will describe briefly as an example (for primary references see review by Pauly et al., (2013)). During biosynthesis of XG, a (1,4)-β-glucan backbone is synthesised by the GT2 integral membrane protein CSLC4 and then decorated in a highly ordered fashion with α-D-Xyl-, β-D-Gal-(1,2)-α-D-Xyl- and fucosylated β-D-Gal-(1,2)-α-D-Xyl- by different families of type 2 Golgi-located GTs, comprising five XylTs (XXT1-XXT5), two GalTs (MUR3 and XLT2) and one FucT (FUT1), respectively (see **Box 1**). CSLC4 alone catalyses the formation of the XG glucan backbone, however, when co-expressed with XXT1 longer glucan backbone chains are formed. Further studies in *Arabidopsis* protoplasts using BiFC and fluorescence quantification by flow cytometry on the interactions between CSLC4 and XXT1, XXT2 and XXT5 concluded that these proteins form homo- and hetero-complexes,

with XXT5 most likely in the closest position to CSLC4. The XXTs themselves also form homo- and hetero-complexes. BiFC between the XXT-CSLC4 complexes and MUR3, XLT2 and FUT1 showed interactions between MUR3, XXT2/5 and CSLC4, XLT2 interacted with XXT2 and CSLC4 while FUT1 interacted with XXT2/5 and MUR3 (Chou et al., 2015). The overall picture suggests that all these GTs interact to form a multi-subunit protein complex that produces the highly decorated XG polysaccharide in the Golgi.

How to reconcile these recent molecular studies with previous immuno-EM studies that have spatially separated some of these enzymes and the sequential production of XG into different Golgi cisternae is the challenge we now face. The early work by Zhang and Staehelin (1992) utilised an antibody raised against the XG backbone and one that recognises the terminal Fuc residue of the XG chain, the CCRC-M1 antibody. They concluded that the XG glucan backbone was observed primarily in the *trans*-cisternae followed by the TGN and the Fuc-decorated XG was mostly located in the TGN but some also in the *trans*-cisternae. More recently, GFP tagged XXT1, MUR3 and FUT1 were immuno-localised in tobacco BY-2 cells with an anti-GFP antibody after high pressure freezing/freeze substitution. XXT1 was localised in the *cis*- and *medial*-cisternae and in transport vesicles associated with Golgi margins, MUR3 localised to the *medial*-cisternae and FUT1 to the *trans*-cisternae. XG was localised to the *medial*- and *trans*-cisternae, the TGN, Golgi-derived transport vesicles and the cell wall with the CCRC-M86 antibody, which recognises non-fucosylated XG.

These results suggest that there is some spatial separation of activity of the GTs involved in XG formation, which may reflect spatial separation of either the enzymes or the participation of a multi-protein complex. This appears to be somewhat of a paradox, but could be explained by understanding the read-out and the resolution of the biochemical and imaging methods employed. Antibody labelling in native tissues is the best method for correctly locating epitopes *in situ*, although we now know that “masking” of polysaccharide epitopes can occur (Marcus et al., 2008). Also, when using light microscopy, the resolution is critical as the advent of super-resolution imaging modalities (Komis et al., 2015) has revealed that some previously assumed “co-locations” based upon confocal fluorescence imaging were erroneous as the markers distributed to different vesicles (Huang et al., 2009). While

expression of proteins in heterologous systems has contributed enormously to our understanding of many processes, it has also become apparent that unless GTs are both co-expressed with their cognate complex members and at their native stoichiometric levels (i.e., using their own native promoters rather than high expression heterologous promoters that can lead to miss-folding and miss-localisation), they may not localise to the correct compartment. Furthermore, GTs and other enzyme complexes may also not be active in native tissue until they reach their final destination where they are presumably under developmental control from internal regulators that may not be present in the heterologous expression system. For example, as is the case with both chitin synthases in yeast that are located as zymogens in chitosomes that sub-tend the PM until an endogenous signal triggers transport to the PM whence they become active in the synthesis of chitin (Bartnicki-Garcia, 2006) and the developmentally regulated plant callose synthase, NaGSL1 in *Nicotiana* pollen tubes (Brownfield et al., 2008).

4.3 Cell wall biosynthesis continues in the TGN

Beyond the GA, post-Golgi trafficking to the PM, endocytosis from the PM for constitutive recycling of PM proteins including cellulose synthase complexes (CSCs), and delivery to the vacuole for degradation represents a complex network of pathways with different types of vesicles and endomembrane compartments (**Fig. 1 and Table 2**). Of these, the TGN is the most complex. It is a compartment active in both the secretory and endocytic pathways, sorting cargo to different routes similar to a “mail sorting” centre. In the secretory pathway, cargo is trafficked from the GA through the TGN to SVs that deliver material to the PM. In the endocytic pathway, the TGN acts as an early endosome and directs traffic to recycling compartments and the vacuole (Tanchak et al., 1984; Dettmer et al., 2006; Robert et al., 2008; Viotti et al., 2010; Kang, 2011; Gendre et al., 2015).

The TGN is derived from the *trans*-most cisternae of the GA in eukaryotic cells (Griffiths and Simons, 1986; Kienzle and von Blume, 2014). In plants, the TGN is often seen in close association with Golgi and termed Golgi-associated TGN (GA-TGN) (Kang and Staehelin, 2008; Kang, 2011; Gendre et al., 2015). However, TGN separated from the Golgi are called free-TGN and are independent organelles that are morphologically and functionally distinct (Kang and Staehelin, 2008; Viotti et al.,

2010; Kang et al., 2011; Uemura et al., 2014; Gendre et al., 2015). Like the Golgi and other components of the endomembrane system, the TGN are highly mobile fusing with each other and interacting closely with Golgi stacks (Viotti et al., 2010; Uemura et al., 2014).

Ultrastructurally, the TGN is a tubulo-vesicular mesh of membranes (Kang and Staehelin, 2008) that varies in size and morphology depending on the types of vesicles that either bud from or fuse to the membrane (Gendre et al., 2015), possibly reflecting the diverse nature of the cargo carried in different TGN cisternae. Three main types of morphologically distinct vesicles interacting with the TGN can be identified: clathrin-coated vesicles (CCVs) involved in endocytosis and protein recycling to the PM (Bandmann et al., 2012; Robinson and Pimpl, 2014), COPIb-coated vesicles for recycling trafficking machinery proteins back to the Golgi cisternae, and SVs for exocytosis at the PM (Donohoe et al., 2007; Gendre et al., 2015) (see **Fig. 1and Table 2** for a summary of their key morphological and molecular features). Depending on the size, type, and developmental stage of the cell, there are differences in the numbers and types of vesicles associated with the TGN (Kang, 2011; Uemura et al., 2014).

Vesicles then move from the TGN to the PM accumulating in large numbers at growing regions, such as the cell plate (Samuels et al., 1995), the tips of pollen tubes (Johnson and Preuss, 2002; Krichevsky et al., 2007) and root hairs (Samaj et al., 2006) and the expanding regions of cortical and epidermal cells. Vesicles also accumulate where asymmetric synthesis of secretory wall cargo is required, such as the outer epidermal periclinal face for waxy/cuticular components (Samuels et al., 2008; Kunst and Samuels, 2009), during secretion of mucilage in root cap cells (Wei et al., 2009) and transport of nutrients in transfer cells in the phloem (Offler et al., 2003). A cluster of vesicles termed secretory vesicle clusters (SVCs) (**Table 2**) has been described in tobacco BY-2 cells, *Arabidopsis* and rice plant cells (Toyooka et al., 2009). These SVCs may be unique structures but are morphologically similar in appearance to free-TGN. Alternatively, the SVCs could represent later stages of vesiculation and transport of bundled SVs that have either not separated fully or, as the authors suggest, are grouped together to transit more effectively from the TGN to the PM (Toyooka et al., 2009).

Cell wall polysaccharides and their cognate synthases have been located within the TGN, including XG and pectins (see section **4.2**) (Zhang and Staehelin, 1992; Kang et al., 2011; Driouich et al., 2012). So too are the CESAs, GSLs and CSLF6 involved in cellulose, callose and MLG synthesis, respectively, that are biosynthetically active only at the PM (Brownfield et al., 2008; Qu et al., 2008; Crowell et al., 2009; Gutierrez et al., 2009; McFarlane et al., 2014; Wilson et al., 2015). XG secretion has been linked to a RABA4b GTPase using co-immunolocalisation (Kang et al., 2011) and the sorting of XG and pectins with fidelity along the correct secretory route has been linked to the ECHIDNA (ECH) and YPT/RAB GTPase Interacting Proteins 4a and 4b (YIP4a and YIP4b) in the form of the ECH/YIP complex located on the TGN (Gendre et al., 2011; Gendre et al., 2013; McFarlane et al., 2013). ECH has been found to be required for the route of secretory traffic defined by secGFP (GFP-SYP121; secreted GFP) but not for all secretory cargos (Gendre et al., 2011). For example, localisation of both the PM-resident auxin efflux carrier PIN2 and the receptor kinase BRASSINOSTEROID INSENSITIVE 1 (BRI1) were identical in WT and *ech* mutants (Gendre et al., 2011) indicating that they are transported to the PM by a different trafficking pathway to that of secGFP.

The CESA, GSL and CSLF6 proteins are all trafficked to the PM prior to the initiation of polysaccharide synthesis (Samuels et al., 1995; Chen et al., 2009; McFarlane et al., 2014) and are activated following their insertion into the PM. Interestingly, the cellulose synthase complex (CSC) (**Fig. 1**) that comprises the CESA proteins assembles much earlier in the secretory pathway, in the Golgi (Haigler and Brown, 1986). The MLG synthase CSLF6 has been localised and biochemically enriched in its active form at the PM with 95% of MLG identified in the cell wall from quantitative EM immuno-labelling suggesting that CSLF6 is likely active primarily at the PM (Wilson et al., 2015). However, others have reported MLG synthase activity in the Golgi fraction of microsomal membrane preparations (Carpita and McCann, 2010) and location of GFP-tagged CSLF6 to the GA by immunofluorescence (Kim et al., 2015) so further studies are warranted.

4.4 Secretory vesicle traffic from endomembrane compartments and docking to the plasma membrane

Vesicle traffic to and from the PM is a highly complex and dynamic process. Many vesicles can be seen using both light microscopy and EM and often the cargo and transport carriers are unknown unless they are specifically labelled either by fluorescently-tagged proteins or by immuno-labelling. Some vesicles are identifiable under the EM by their morphology including CCVs (involved in clathrin-mediated endocytosis) and MVBs (usually involved in recycling to the vacuole as a late endosome), and to a lesser extent COPI and COPII vesicles (summarised in **Table 2**). CCVs are recognised by their hedgehog-like coat made up of the triskelions of heavy and light polypeptide chains of clathrin (Coleman et al., 1987) and are involved in endocytosis (Chen et al., 2011; Ito et al., 2012; Baisa et al., 2013) and possibly recycling from the TGN back to the PM as found in animal cells (van Dam and Stoorvogel, 2002; Gendre et al., 2011). CCVs package components from the PM and take proteins to the TGN in their role as early endosomes for either recycling or degradation via the MVB and then onto the vacuole (Reyes et al., 2011). Another mechanism for endocytosis is the clathrin-independent membrane microdomain-associated endocytosis (Fan et al., 2015) where instead micro-domain associated proteins, such as FLOTILLIN 1 (FLOT1) are involved (Li et al., 2012). Internalisation of PM proteins including PIP2;1 (Li et al., 2011) and AMT1;3 (Wang et al., 2013) follow this pathway (Fan et al., 2015). Membrane micro-domains are discussed later in this section.

SVs are trafficked from the TGN to the PM and are variable in size depending on whether they are derived from either GA-TGN (83-107 nm) or free-TGN (52-88 nm) and have either a thin or non-existent coat (Donohoe et al., 2007). Different SVs have different types of membrane cargo (Park and Juergens, 2012; Heard et al., 2015), including PM proteins such as CESAs (Crowell et al., 2009; Gutierrez et al., 2009), CSLF6 (Wilson et al., 2015) and GSLs (Brownfield et al., 2008), together with polysaccharides such as XG and pectin (Gendre et al., 2011; Drakakaki and Dandekar, 2013). The size of SVs varies depending on where they bud from (Mollenhauer et al., 1976; Donohoe et al., 2007; Wei et al., 2009; Kang et al., 2011). Budding from the TGN has been linked to the lipid kinases AtPI4Kβ1 and AtPI4Kβ2,

which are regulated upstream by RABA4b (Kang et al., 2011). Interestingly the ECH protein is also crucial to SV biogenesis from the TGN (Boutte et al., 2013).

Post-TGN trafficking routes: Successful delivery of cargo from SVs to the prescribed PM location requires transport along a cytoskeletal element, correct recognition and tethering of the vesicle to the target membrane and homotypic fusion of the vesicle facilitated via SNARE interactions (Hwang and Robinson, 2009). At this point, the membrane bound cargo is delivered to the PM and the soluble cargo is released into the apoplast. In some cases, it is possible that GTs delivered to the PM require proteolytic activation before biosynthesis of polysaccharides begins (see section **4.2**).

Most of the dynamic processes in plant cells, including vesicle trafficking, occur via interaction with actin microfilaments and associated myosins. Bundles of actin filaments are found beneath the PM in the cell cortex (Boevink et al., 1998) and precise targeting of vesicles with cargo has been associated with fanned-out arrays of individual actin filaments or thin cables (Geitmann and Emons, 2000; Geitmann and Nebenfuehr, 2015). Trafficking of CESA-YFP SVs to the PM requires actin in elongating hypocotyl cells (Gutierrez et al., 2009) and actin is involved in moving vesicles to pollen tube tips (Cheung et al., 2008). Interestingly, MTs have also been implicated in vesicle movement with MT-kinesin based vesicle delivery of some cell wall components at the phragmoplast (Lee et al., 2001) and MTs are required for the local insertion of CSCs into the PM, for acting as “rail lines” guiding the CESAs as they track backwards during cellulose biosynthesis, and for their subsequent recycling (Crowell et al., 2009; Gutierrez et al., 2009). MTs have also been implicated in directing pectin-containing vesicles (Kim and Brandizzi, 2014) to the seed coat in the *Arabidopsis* temperature-sensitive MT mutant (*mor1-1*) (McFarlane et al., 2008)

Linker proteins connecting endomembrane compartments to MTs have been identified and include the sorting nexin SNX1, which connects MTs to the TGN (Ambrose et al., 2013; Brandizzi and Wasteneys, 2013; Stierhof et al., 2013). The kinesins (MT molecular motors) have also been implicated in the stopping and/or slowing down of organelles and vesicles at specific sites (Cai and Cresti, 2012). However, from studies on actin-based chloroplast movements and positioning of the

nucleus in *Arabidopsis* and rice it has been suggested that plant kinesins have diverged in their role as a MT molecular motor and may now have evolved non-conventional functions, including actin binding (Frey et al., 2009, 2010).

The vesicles that traffic between the TGN and the PM vary in size and also in their cargo shown to carry either mixed classes of complex polysaccharides (see **Table 2**) (Sherrier and Vandenbosch, 1994) or both polysaccharides and glycoproteins (Driouich et al., 1993). Correct spatial targeting of cargo, in this case cell wall material, requires vesicles to be transported to the correct region(s) of the PM for docking and exocytosis of their contents into the apoplast. Some trafficking routes have been identified by different membrane proteins that are either involved in the trafficking process itself, such as secretory carrier membrane protein 2 (SCAMP2) (Toyooka et al., 2009), syntaxin of plants 61 (SYP61) (Drakakaki et al., 2012), the RAB GTPase ARA6 (Ebine et al., 2011), vesicle-associated markers such as v-SNARE VAMP72 (Sanderfoot, 2007) and PM proteins destined for delivery.

SmaCCs/MASCs: Interestingly, CESA-YFP complexes (Paredez et al., 2006) imaged using live cell techniques have identified a novel pathway followed by the CSCs. CESA-YFP co-localises with the vacuolar-type H^+-ATPase-a1 (VHA-a1) endosomal TGN marker (Crowell et al., 2009; McFarlane et al., 2014; Gendre et al., 2015), but was also identified in SVs that do not label with known secretory pathway markers indicating that these were novel trafficking compartments (Crowell et al., 2009; Gutierrez et al., 2009). These compartments have been termed either microtubule-associated cellulose synthase compartments (MASCs) (Crowell et al., 2009) and/or small CESA compartments (SmaCCs) (Gutierrez et al., 2009). These MASCs/SmaCCs were shown to interact with the cytoskeleton and were partially localised with the TGN. Constitutive cycling, where the CSCs are endocytosed from the PM, maintained in the cell cortex for a period of time and then again cycled back to the PM is thought to be involved in the regulation of cellulose synthesis (Crowell et al., 2009; Gutierrez et al., 2009). For other cell wall synthase complexes and their polysaccharide products, vesicle trafficking and exocytosis has not been well characterised. It would be interesting to investigate whether they follow either the same or alternative pathways to the CSC/CESAs.

Further dissection of the divergent pathways of the different types of SVs to the PM to understand the delivery of cell wall material to the apoplast is also being investigated using proteomic techniques. An analysis of the *Arabidopsis* SYP61 proteome detected many associated partner proteins (Drakakaki et al., 2012). While many of the associated proteins and complexes were identified as being involved in vesicle fusion with the TGN and the endocytic pathway, SYP61 was co-localised with the primary wall CESAs (CESA1, CESA6 and CESA3; Drakakaki et al., 2012) and also GSLs (Drakakaki, 2015) as cargo suggesting a role in trafficking CESAs and GSLs to the PM. Since SYP61 has been found to co-localise with SmaCCs/MASCs (Crowell et al., 2009) it could be involved in either endocytosis to the vacuole for degradation or constitutive recycling of CESAs to the PM, similar to the cycling of the $KAT1K^+$ channel at the PM (Sutter et al., 2007). Interestingly, SYP61 interacts with SYP121 and was suggested to be involved with facilitating the secretion of cell wall components to the papillae induced in response to pathogen attack (Drakakaki et al., 2012). Further analysis of proteomes associated with GTs could shed light on potential targets for proteins involved in unknown trafficking routes but these would need to be verified by microscopic immuno-labelling as the proteomics sample preparations are notoriously prone to “contamination”.

Vesicle docking and fusion sites: SNARE complexes are involved in vesicle fusion across the eukaryotic kingdom (reviewed in Grefen and Blatt, 2008) and are vital for successful membrane fusion and exocytosis at the PM. To function, SNARES require two complementary SNARE subsets, the vesicle membrane (v-SNARE) subset that is comprised of three components, such as the SYP41/SYP61/VTI12 SNARE complex (Sanderfoot et al., 2001; Zouhar et al., 2009), and the target membrane (t-SNARE) subset with one component, such as SYP132 (Uemura et al., 2004). These interactions can be either specific or more promiscuous with multiple pairings for specific SNAREs. When the v-SNARE and t-SNARE complexes are brought together, the action of the molecules brings specific vesicle and target membranes together for connection and homotypic fusion (Grefen and Blatt, 2008).

Specific SNAREs have been implicated in cell wall formation, including SYP61 (see above), SYP111/KNOLLE, SYP112, SYP121 and SYP122. For example, SYP111/KNOLLE is involved in cell plate formation with mutants having incomplete

cross walls with only SYP112 able to rescue the phenotype; SYP121 and SYP122 can regulate exocytosis (Tyrrell et al., 2007; Rehman et al., 2008), are required for development of papillae in response to pathogen attack (Collins et al., 2003; Nuhse et al., 2003; Assaad et al., 2004; Zhang et al., 2008) and also interact with the SNARE complex SNAP33, which is more unrestricted in its binding specificity interacting with different partner SNAREs in different locations throughout the cell (Grefen and Blatt, 2008).

Targeting of PM proteins and cell wall polysaccharides to specific surface locations may not only involve SNAREs. It may also depend on either distinct lipid species or different types of membrane domains that contain receptors for “universal for single or multi-pass” TMD-containing proteins, as shown for the sucrose transporter 1 (SUT1) from *Solanum tuberosum* (Kruegel et al., 2008) and the aquaporin PIP2;1 (Li et al., 2011). Alternatively, spatially located receptors for particular trafficking signals could reel in SVs with the appropriate cargo. It has been found that some PM proteins have specific spatial locations; for example PIN1, which is involved in auxin delivery, has been found at the poles of the cell (Lau et al., 2012) and the Casparian strip domain proteins (CASPs) which are found at the longitudinal circumference of cells (Roppolo et al., 2011). This specificity in location could be applicable to other types of membrane proteins involved in cell wall deposition. Interestingly, the PM sites where plasmodesmata form lack labelling for H^+-ATPase (Fleurat-Lessard et al., 1995) and are associated with regions of lower lipid diffusion (Grabski et al., 1993).

However, most proteins are located in punctate membrane domains isotropically distributed over the entire PM (Konrad and Ott, 2015) representing an essentially homogeneous distribution over the surface of the PM. These punctate membrane domains could represent PM micro-domains (size >100 nm) that are increasingly recognised as discrete regions where there may be connections between the cytoskeleton, PM and the cell wall and could be sites targeted for vesicle release of particular polysaccharides as well as for incorporation of proteins into the PM (Jarsch et al., 2014; McKenna et al., 2014; Konrad and Ott, 2015; Tapken and Murphy, 2015). The initial biochemical characterisation of membrane micro-domains was from lipid rafts isolated from microsomal membranes by non-ionic detergents and called detergent-resistant membranes (DRMs) (size < 40 nm) (Borner et al., 2005).

It is now generally agreed that DRMs are isolation artefacts as membrane sterols have a biophysical tendency to aggregate into large sheets (Zurzolo et al., 2003; Tanner et al., 2011; Malinsky et al., 2013). Micro-domains are now being microscopically observed *in situ* using FRAP (fluorescence recovery after photobleaching) techniques that delve into the mobility of different proteins and lipids (Martiniere et al., 2011). Membrane micro-domains may be formed by aggregations of similar lipids that particular proteins preferentially associate with. Some of these micro-domains may either interact with the cytoskeleton as anchoring points or have proteins that interact with the cell wall creating localised regions for cell wall interactions with the PM or both, such as FORMIN1 (FH1) (Martiniere et al., 2011). Wall proteins implicated in PM-cell wall attachment include AGPs, fasciclin-like AGPs (FLAs), FORMIN 1 (FH1) and WALL ASSOCIATED KINASES (WAKs) (Doblin et al., 2010; Kohorn and Kohorn, 2012; Wolf et al., 2012). In some cases, for example the WAKs, their association (covalent and/or non-covalent) with specific wall molecules, such as glycine-rich proteins (GRPs) and pectins; Kohorn and Kohorn (2012)), may also determine their spatio-temporal distribution (and hence targeting) in the PM.

The exocyst protein complex, an evolutionarily conserved octameric complex comprised of the subunits SEC3, SEC5, SEC6, SEC8, SEC10, SEC15, EXO84 and EXO70, is believed to function as an exocytic vesicle tether at the PM prior to SNARE complex formation (Zhang et al., 2010). While most of the subunit proteins are encoded by 1-3 genes, EXO70 has 23 paralogues in *Arabidopsis* and poplar and 41 in rice (Chong et al., 2010). This diversity of EXO70 genes suggests that there may be multiple functions of the exocyst complexes in secretion (Zarsky et al., 2013), including EXO70E2 described earlier with the EXPO organelle (section 3.2). Within single cells, the exocyst has been linked to defective secretion in seed coat pectin (Kulich et al., 2010), pollen tubes (Hala et al., 2008), root hairs (Synek et al., 2006) and hypocotyls (Hala et al., 2008) and is believed to be involved in polarised, spatio-temporal secretion (Synek et al., 2006; Zhang et al., 2010). Variable angle epi-fluorescence microscopy (VAEM) of labelled exocyst subunits SEC6, SEC8, EXO70A1 and EXO84b indicates that they are PM located and co-localise with the vesicle marker VAMP721 approximately 20% of the time in *Arabidopsis* roots (Fendrych et al., 2013). Although caution should be exercised in the interpretation of

the following data from Chong et al., (2010) due to poor fixation they have concluded that a number of fluorescently-tagged EXO70 family proteins have been found throughout the cytosol, within the nucleus in tobacco BY-2 cells (Chong et al., 2010), and in puncta similar to those observed with Golgi-specific labelling in *Arabidopsis* protoplasts (Elias et al., 2003); whereas EXO70E2 and EXO70G1 have been localised to the TGN and the late endosome/pre-vacuolar compartment (Chong et al., 2010). While these data suggest that the exocyst is likely to be involved in spatio-temporal secretion by tethering vesicles, no conclusive data for spatial deposition of either particular vesicle types or cell wall components is as yet defined.

5 Conclusions and future research directions

The merging of modern molecular and cell biological techniques with advances in both our understanding of the biosynthetic machinery for cell wall polysaccharides/glycoproteins and imaging modalities has contributed enormously to our understanding of the cells secretory pathway. For example, new proteins have been identified, such as ECHIDNA that plays an important role in sorting at the TGN, alternative trafficking routes have being elucidated and new organelles, such as EXPO, identified and new roles for known organelles, such as the MVB, revealed. However, there remain many unresolved questions, such as:

- the molecular mechanisms of sorting cargo and identifying vesicle route(s)?
- how spatio-temporal sorting and secretion to selected regions of the cell wall is both initiated and executed (i.e., do all polysaccharides use the same trafficking pathway?)
- are all Golgi simultaneously performing the same function? Unravelling this conundrum would contribute to answering whether different Golgi make different cargo or do all Golgi make the same cargo and sorting occurs at the post-TGN; i.e., is there a central “mail box” or a series of distributed “mail boxes”?
- how are the dynamic cytoskeletal-based movements of the endomembrane system related to secretion and cell wall deposition?

To address these questions, multi-pronged approaches with innovative and evolving techniques and technologies are needed. For example, in the past decade the

applications of live cell microscopy using TIRF, spinning disk confocal or super-resolution microscopy (Komis et al., 2015) combined with computational/software advances to de-convolute images, fluorescent proteins, molecular genetics and biochemical dissection have seen significant advances in our understanding of the CSC and its interaction with MTs using a stable and well defined system, the *Arabidopsis* hypocotyl (McFarlane et al., 2014); although there is still much to unravel to fully understand even this one example! The molecular and biochemical characterisation of complexes involved in the synthesis of non-cellulosic polysaccharide biosynthesis (see **Box 1C**) will enable similar approaches to be employed to track these complexes in the GA and their polysaccharide products on route to the cell wall.

We are witnessing incredible advances in imaging techniques and modalities, including correlative light and EM which combines fluorescence microscopy with EM for dynamic imaging and high resolution morphological analysis; serial section block face imaging where larger samples, such as whole organs, are sectioned automatically providing visualisation of spatial interactions in three dimensions; electron tomography increases Z-resolution on the nanometer scale and together with the development of molecular probes, such as APEX2 (Lam et al., 2015) for EM, the visualisation and identification of supramolecular complexes *in situ* will become a reality. New atomic force microscopy modalities that can correlate localised physical properties and variations in spatial organisation of plant cell walls *in situ* will enable us to relate structure with function (Zhang et al., 2015).

In parallel, we are seeing the development of new probes for live cell imaging and EM that when used in combination with biochemical approaches will greatly contribute to our understanding of trafficking pathways and secretion:

- for polysaccharides this includes small oligosaccharide probes (Mravec et al., 2014) generated by click chemistry (Anderson et al., 2012, fluorescently-tagged precursors for *in muro* labelling (Dumont et al., 2016), and lipophilic dyes (Stocker and Timmer, 2013)
- for proteins this includes tuneable fluorescent probes that are pH-sensitive (Martiniere et al., 2013), responsive to protein maturation (mKaede) (Brown et

al., 2010) or photoactivatable (PA-FP) (Henderson et al., 2007) and useful for super-resolution imaging (Mishin et al., 2015).

Thus, the next decade will undoubtedly lead to exciting new developments in our understanding of the morphology and the molecular mechanisms defining synthesis and transport through the secretory pathway.

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Figure legends

Box 1. Organisation of the plant primary cell wall (A/B) and the constituent polysaccharides/proteoglycans and the known biosynthetic enzymes (B).

(A) Cell walls provide structural support, are a physical barrier to pathogens, and house a range of signalling molecules to perceive and respond to developmental and environmental cues. Walls are complex, diverse and dynamic, changing throughout the processes of cell division, growth and differentiation. Walls begin to be formed in the cell plate during cell division which becomes the middle lamella between adjacent cells. As cells expand during growth the cells lay down a primary wall that can then be substantially thickened when cells cease to expand and undergo differentiation; this latter wall is termed the secondary wall which is often lignified. The types of polysaccharides present in the wall vary depending on the plant species, cell type and location, developmental stage and history of environmental stresses. Primary walls are composed predominantly of a complex array of polysaccharides (~90%) and some proteins (~10%). In all cell-types, rigid cellulose microfibrils are embedded in a gel-like matrix of non-cellulosic polysaccharides and pectins. In general, walls differ mostly in the composition and abundance of matrix polysaccharides and their intermolecular associations, both covalent and non-covalent. In the primary walls of dicots, gymnosperms and non-commelinoid

monocots (often referred to as Type I walls) xyloglucans (XGs) and pectic polysaccharides are most abundant whereas in the Poales and related commelinoid monocots (often referred to as Type II walls), glucuronoarabinoxylans (GAXs) and (1,3;1,4)-β-D-glucans predominate and pectin levels are relatively low. In reality, a continuum of structures between the Type I and Type II walls exist across the plant kingdom. Image taken from (Yokoyama and Nishitani, 2004).

(B) Spatial distribution of wall materials varies depending on cell type and polysaccharide. In this example, the distribution of a pectic arabinan can be seen in an *Arabidopsis* stem using the LM13 and LM16 antibodies that recognise different arabinan epitopes. LM13 recognises longer α-(1,5)-arabinan epitopes, found at the junctions between three cells. LM16 binds to branched arabinans, found in the walls shared between two cells, i.e., away from the junction zones. Images from (Verhertbruggen et al., 2009).

The depicted polysaccharide structures are stylised and vary between the major plant groups. For detailed structures, see (Mohnen, 2008; Scheller and Ulvskov, 2010; Pauly et al., 2013; Basu et al., 2015). Glycosidic linkages are shown in their anomeric configuration (α/β) and linkage position (e.g., β4 indicates the sugar is in a β-(1,4)- linkage). The known GTs are indicated by their protein abbreviation. Where the GTs are part of biosynthetic complexes, they are not enclosed in brackets.

Figure 1. Diagram of trafficking pathways in plant cells.

The diagram is based on an electron tomogram model of a barley root *(Hordeum vulgare)* Golgi stack near the plasma membrane (PM) of the secretory pathways in plant cells showing different endomembrane compartments, transport vesicles and trafficking routes to the PM. The vacuole, ER, Golgi, COPII vesicles, SVs and CCVs were present in the original tomogram and are drawn to scale. The cell wall, MVB, EXPO organelle, exosome and SmaCC/MASCs were added to the diagram to complete the known secretory pathway components with sizes of the vesicle structures based on the data in Figure 2. Some extra CCVs and SVs were added for clarity to depict the pathways followed and the location of GTs was derived from what is known in the literature and described in the text. The conventional protein secretory pathway (CPS-black arrows) plays the major role in the assembly of the cell wall matrix phase polysaccharides. The unconventional protein secretory

pathway (UPS-white arrows) appears to be less commonly employed for normal cellular processes but may become more significant during pathogen attack when elements of the defence machinery for the construction of papillae, such as callose synthases, are transported (MVBs) to localised areas of the PM. CSC complexes move from the GA to the PM in MASCs/SmaCCs and may also be cycled at the PM. CCVs are involved in endocytosis, recycling of PM components and are thought to be trafficked between the TGN and PM. Representations of GTs include CSLF6 (red), GSL1 (yellow), GSL5 (orange) and the XG biosynthesis enzymes XT1, MUR3 and FUT1 (blue); see **Box 1** for a full description of these acronyms. Some polysaccharides are known to be trafficked in the endomembrane system including XGs and pectins. These have not been shown in the diagram.

Table 1. Examples of plant cell types discussed in this review.

The cell types listed are only a few of the estimated 40 different types in plants. Many of the cell types are observed using either *Arabidopsis* or *Nicotiana* plant tissue as these have been well characterised and are model systems with molecular-genetic tools available.

Table 2. Vesicle morphology in plant cells described from EM approaches.

The SV, COP11, COP1a, COP1b and CCV images are from electron tomogram slices (taken from Donohoe et al., 2007). The SV and CCV images are representative of EM images in the higher plant literature and identification was made by morphological characteristics. COP11 and COP1 vesicles were identified by morphology, location near the Golgi and with antibody labelling for COP11 (anti-*At*SAR1) and COP1 (anti-*A*t-γ-COP). The COP1a and COP1b distinction is attributed to slight differences in the coat of the vesicles and on the location of the vesicle as discussed in Donohoe et al., 2007. The CESA-vesicle image is a freeze fracture replica (Giddings et al., 1980) showing the CSC rosettes (arrows) within the vesicle and is probably similar to the SmaCCs/MASCs described recently from fluorescence images. Currently there are no clear EM images published of SmaCCs/MASCs. The SVC (Toyooka et al., 2010) in the centre of the image, made up of 7 vesicles, could be equivalent to the free-TGN described by Kang (2011). MVBs are distinctive organelles that contain small vesicles within the MVB lumen

(Segui-Simarro and Staehelin, 2006). MVBs have been described in many plant cells after either endocytosis or in relation to pathogen response (An et al., 2006). The EXPO organelle was described by Wang et al., (2010) and is part of the UPS pathway. The EXPO organelle fuses with the PM and releases an exosome.

Bars=50 nm for SV, COPII, COPIa,b, and CCV; Bars=100 nm for CESA-vesicle, SVC, MVB and EXPO.

† Sizes measured from references cited using ImageJ, approximation only.

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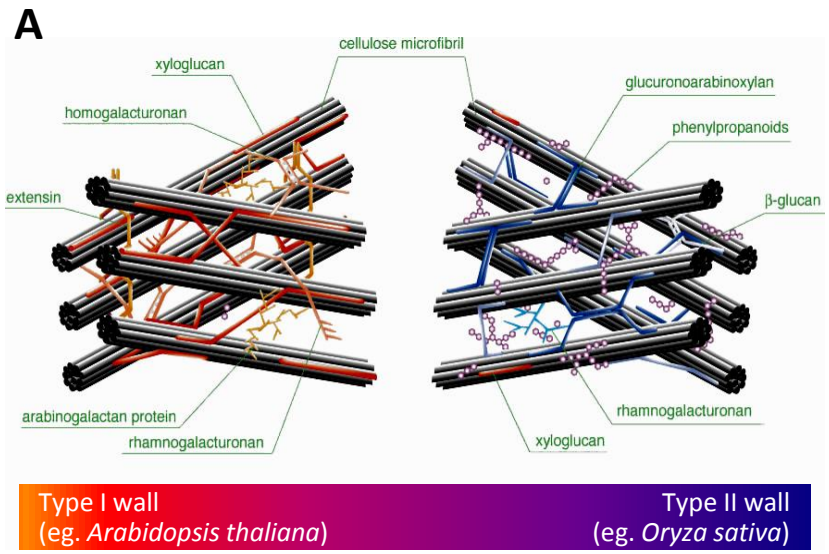
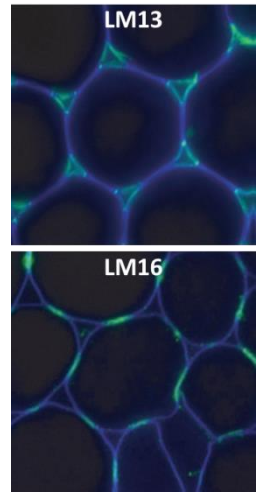
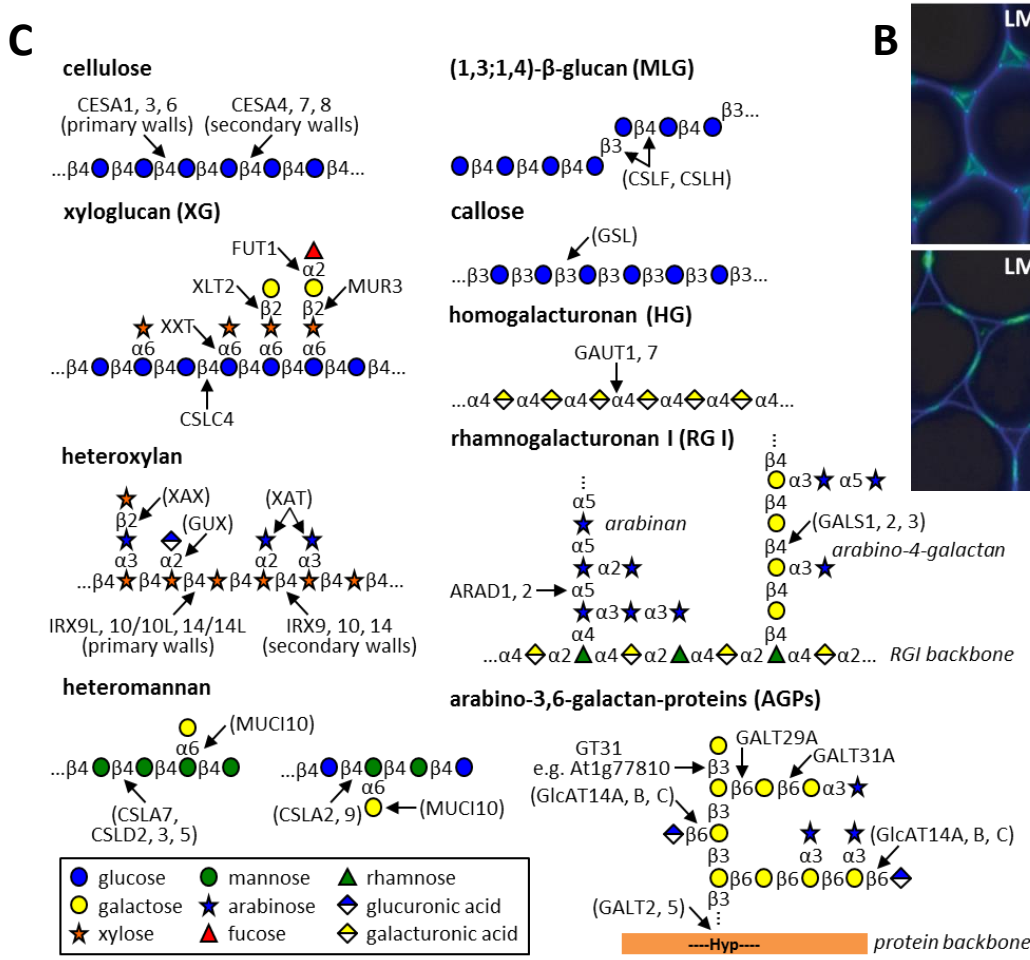
Box 1. Organisation of the plant primary cell wall (A/B) and the constituent polysaccharides/proteoglycans and the known biosynthetic enzymes (B).



(A) Cell walls provide structural support, are a physical barrier to pathogens, and house a range of signalling molecules to perceive and respond to developmental and environmental cues. Walls are complex, diverse and dynamic, changing throughout the processes of cell division, growth and differentiation. Walls begin to be formed in the cell plate during cell division which becomes the middle lamella between adjacent cells. As cells expand during growth the cells lay down a primary wall that can then be substantially thickened when cells cease to expand and undergo differentiation; this latter wall is termed the secondary wall which is often lignified. The types of polysaccharides present in the wall vary depending on the plant species, cell type and location, developmental stage and history of environmental stresses. Primary walls are composed predominantly of a complex array of polysaccharides (~90%) and some proteins (~10%). In all cell-types, rigid cellulose microfibrils are embedded in a gel-like matrix of non-cellulosic polysaccharides and pectins. In general, walls differ mostly in the composition and abundance of matrix polysaccharides and their intermolecular associations, both covalent and non-covalent. In the primary walls of dicots, gymnosperms and non-commelinoid monocots (often referred to as Type I walls) xyloglucans (XGs) and pectic polysaccharides are most abundant whereas in the Poales and related commelinoid monocots (often referred to as Type II walls), glucuronoarabinoxylans (GAXs) and (1,3;1,4)-β-D-glucans predominate and pectin levels are relatively low. In reality, a continuum of structures between the Type I and Type II walls exist across the plant kingdom. Image taken from Yokoyama & Nishitani (2004).

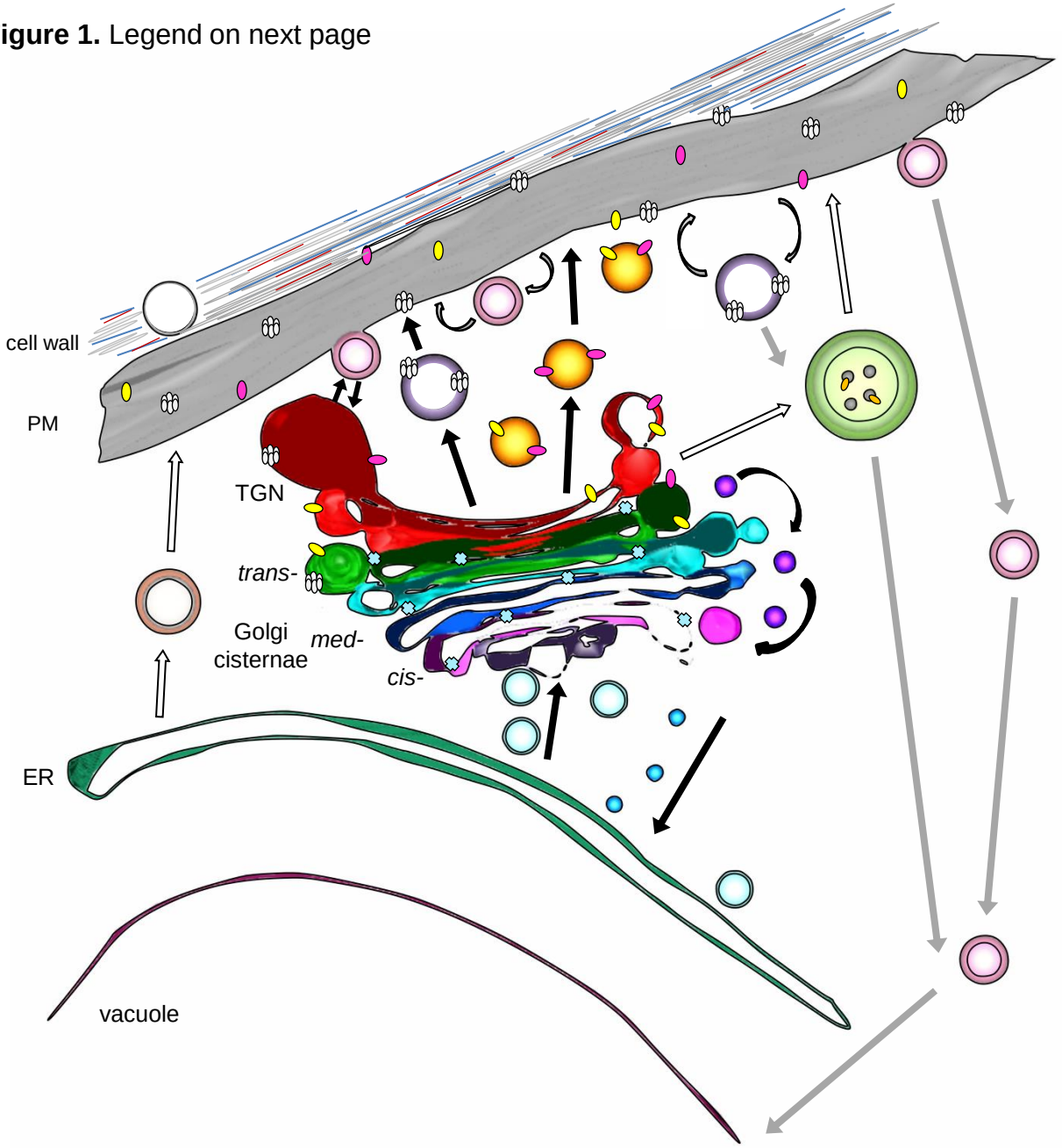
(B) Spatial distribution of wall materials varies depending on cell type and polysaccharide. In this example, the distribution of a pectic arabinan can be seen in an *Arabidopsis* stem using the LM13 and LM16 antibodies that recognise different arabinan epitopes. LM13 recognises longer α-(1,5)-arabinan epitopes, found at the junctions between three cells. LM16 binds to branched arabinans, found in the walls shared between two cells, i.e., away from the junction zones. Images from Verhertbruggen et al., (2009).





(C) The depicted polysaccharide structures are stylised and vary between the major plant groups. For detailed structures, see Pauly et al. (2013), Scheller & Ulvskov (2010), Mohnen (2008) and Basu et al. (2015). Glycosidic linkages are shown in their anomeric configuration (α/β) and linkage position (e.g., β4 indicates the sugar is in a β-(1,4)- linkage). The known GTs are indicated by their protein abbreviation. Where the GTs are part of biosynthetic complexes, they are not enclosed in brackets.

Figure 1. Legend on next page



Pathways depicted

CPS

UPS

Endocytosis/ vacuolar traffic

Constitutive cycling/ PM protein recycling

Endomembrane pathway vesicles/vesicle-like structures

CSC complex with vesicle (MASC/ SmaCC)

CCV

SV

COPII

COPIa

COPIb

MVB

EXPO

exosome

Representations of GTs

CSC

CSLF6

GSL1

GSL5

XT1/MUR3/FUT1

Figure 1. Diagram of trafficking pathways in plant cells.
The diagram is based on an electron tomogram model of a barley root *(Hordeum vulgare)* Golgi stack near the plasma membrane (PM) of the secretory pathways in plant cells showing different endomembrane compartments, transport vesicles and trafficking routes to the PM. The vacuole, ER, Golgi, COPII vesicles, SVs and CCVs were present in the original tomogram and are drawn to scale. The cell wall, MVB, EXPO organelle, exosome and SmaCC/MASCs were added to the diagram to complete the known secretory pathway components with sizes of the vesicle structures based on the data in Figure 2. Some extra CCVs and SVs were added for clarity to depict the pathways followed and the location of GTs was derived from what is known in the literature and described in the text. The conventional protein secretory pathway (CPS-black arrows) plays the major role in the assembly of the cell wall matrix phase polysaccharides. The unconventional protein secretory pathway (UPS-white arrows) appears to be less commonly employed for normal cellular processes but may become more significant during pathogen attack when elements of the defence machinery for the construction of papillae, such as callose synthases, are transported (MVBs) to localised areas of the PM. CSC complexes move from the GA to the PM in MASCs/SmaCCs and may also be cycled at the PM. CCVs are involved in endocytosis, recycling of PM components and are thought to be trafficked between the TGN and PM. Representations of GTs include CSLF6 (magenta), GSL1 (yellow), GSL5 (orange) and the XG biosynthesis enzymes XT1, MUR3 and FUT1 (blue); see **Box 1** for a full description of these acronyms. Some polysaccharides are known to be trafficked in the endomembrane system including XGs and pectins. These have not been shown in the diagram.

Table 1. Examples of plant cell types discussed in this review.

The cell types listed are only a few of the estimated 40 different types in plants. Many of the cell types are observed using either *Arabidopsis* or *Nicotiana* plant tissue as these have been well characterised and are model systems with molecular-genetic tools available.

Cell stage	Cell process	Tissue Commonly Used	Comments
Division	Cell plate formation	Root meristem, shoot apical meristem	*Arabidopsis* roots fit easily under the microscope for live cell microscopy and fix well for EM.
Expansion (isotropy)	Deposition of polysaccharides around the periphery	Root expansion zone	After division, plant cells grow by expansion under turgor pressure and require cell wall deposition to maintain the thickness, strength and integrity of the wall.
Elongation (anisotropy)	Expansion in one direction to elongate cells.	Root elongation zone and the hypocotyl/epicotyl	These tissues show differential stages of growth along their length with early elongation occurring closer to the meristematic zone and later elongation stages and slower growth rates further away near the root hair zone or the base of the hypocotyl/epicotyl.
Asymmetric deposition	One-sided deposition of specialised cell surface components	Cuticle and wax secretion in epidermal cells, Casparian strip in endodermal cells; Root epidermal cells secreting mucilage	Analysis of secretion on one side of the cell reveals differences in trafficking.
Polarised growth	Tip growing cells	Root hairs, pollen tubes	Apical vesicular trafficking and cell wall deposition to accommodate rapid growth.
Plasmodesmata	Interconnect cells to form the symplast within the plant	Observed in epidermal cells	Intercellular trafficking can be studied using live-cell imaging and spatial deposition of GSL proteins and callose rather than in response to pathogens.
Suspension cultured cells	General cell biology	*Arabidopsis* protoplasts, tobacco BY-2 cells	BY-2 cells can be synchronised. Both can be grown in bulk for biochemistry and can be visualised under the microscope.
Transfer cells	Plant cells with secondary wall ingrowths	Phloem, seed transfer cells in culture, cotyledons	Involved in nutrient transport, can study secondary cell wall growth.
Seeds and mucilage	Mucilage secretion	Seeds	Used to study the mechanisms of mucilage secretion and wall mechanics.
Infection structures	Structures developed by the plant in response to pathogen attack	Callose-rich papillae to prevent infection; extra-haustorial matrix that allows biotrophic fungal infection to proceed	These processes are most often observed on leaf epidermal cells as they can be visualised under the microscope. Similar structures are created in roots in the soil during pathogen attack.

Table 2. Legend on next page

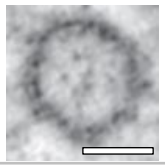
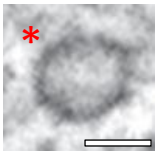
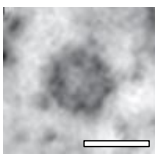
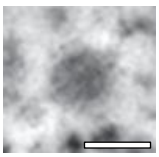
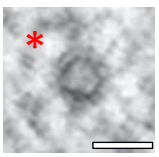
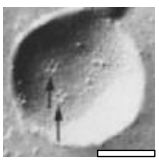
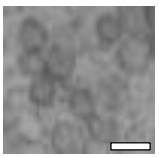
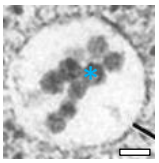
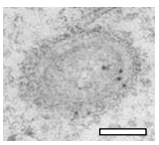
Vesicle	Example image	Pathway	Size	Membrane and coat	Contents	Ref
SV (secretory vesicle)		Secretion from TGN to PM	~80 nm range of 52-107 nm	Staining varies, most are lightly stained	Most are lightly stained, variable.	Donohoe et al., 2007.
COPII		Transport from the ER to Golgi (ERES)	~60 nm	Indistinct, globular dark layer, outer coat (*)	Lightly stained	Donohoe et al., 2007.
COPIa (Coat protein 1a)		Recycling from cis-Golgi to ER	~45 nm	Limiting membrane, irregular, not well defined	More lightly stained than COPIb	Donohoe et al., 2007.
COPIb		Recycling between TGN & cisternae	~45 nm	Two layer coat, dark outer layer, distinct geometry	More darkly stained	Donohoe et al., 2007.
CCV (clathrin coated vesicles)		Endocytosis & PM protein recycling	~35 nm	Hedgehog-like outer coat (*), membrane for vesicle.	Electron lucent	Donohoe et al., 2007; Robinson & Pimpl, 2014
CESA-vesicle (MASC/ SmaCC?)		CESA secretion, constitutive cycling and endocytosis	~200 nm†	unknown	unknown	Giddings et al., 1980.
SVC (SV cluster)		Secretion from TGN to PM	Clusters of 5-12 vesicles of 50-100 nm	Outer membrane	Lightly stained	Toyooka et al., 2010.
MVB (multi-vesicular body)		Endocytosis/ UPS	~360 nm†	Outer membrane	Lightly stained with intraluminal vesicles (*)	Segui-Simarro et al., 2005.
EXPO (exocyst-positive organelle)		UPS	~260 nm†	Double membrane	Lightly stained	Wang et al., 2010.

Table 2. Vesicle morphology in plant cells described from EM approaches.

The SV, COP11, COP1a, COP1b and CCV images are from electron tomogram slices (taken from Donohoe et al., 2007). The SV and CCV images are representative of EM images in the higher plant literature and identification was made by morphological characteristics. COP11 and COP1 vesicles were identified by morphology, location near the Golgi and with antibody labelling for COP11 (anti-*At*SAR1) and COP1 (anti-*A*t-γ-COP). The COP1a and COP1b distinction is attributed to slight differences in the coat of the vesicles and on the location of the vesicle as discussed in Donohoe et al., 2007. The CESA-vesicle image is a freeze fracture replica (taken from Giddings et al., 1980) showing the CSC rosettes (arrows) within the vesicle and is probably similar to the SmaCCs/MASCs described recently from fluorescence images. Currently there are no clear EM images published of SmaCCs/MASCs. The SVC (Toyooka et al., 2010) in the centre of the image, made up of 7 vesicles, could be equivalent to the free-TGN described by Kang (2011). MVBs are distinctive organelles that contain small vesicles within the MVB lumen (image taken from Segui-Simarro et al., 2005). MVBs have been described in many plant cells after either endocytosis or in relation to pathogen response (An et al., 2006). The EXPO organelle was described by Wang et al., (2010) and is part of the UPS pathway. The EXPO organelle fuses with the PM and releases an exosome.
Bars=50 nm for SV, COPII, COPIa,b, and CCV; Bars=100 nm for CESA-vesicle, SVC, MVB and EXPO.
† Sizes measured from references cited using ImageJ, approximation only.