Dissecting sterol function during clathrin-dependent endocytosis and cytokinesis in *Arabidopsis thaliana*

Márcia Frescatada Rosa
Dissecting sterol function during clathrin-dependent endocytosis and cytokinesis in *Arabidopsis thaliana*

Márcia Frescatada-Rosa

Akademisk avhandling

Som med vederbörligt tillstånd av Rektor vid Umeå universitet för avläggande av filosofie doktorsexamen i Växters cell- och molekylärbiology, framläggs till offentligt förvar i KB3A9, KBC-huset, Fredagen den 4 Okt, kl. 10:00.

Avhandlingen kommer att förvaras på engelska.

Fakultetsopponent: Professor Wolfgang Lukowitz, Department of Plant Biology, University of Georgia, USA
Abstract
Sterols are lipid components of eukaryotic membranes. Alterations of membrane sterol composition perturb the execution of cell division, which in diverse eukaryotes can have severe consequences for development of the organism. Partitioning of the cytoplasm during cell division occurs at the final stage of cell division named cytokinesis. In somatic plant cells, cytokinesis is initiated by fusion of membrane vesicles in the plane of cell division resulting in a transient compartment termed the cell plate. Cell plate maturation relies on temporal and spatial orchestration of membrane fusion and endocytosis. Impaired vesicle fusion or defects in endocytosis result in cytokinetic defects.

In *Arabidopsis thaliana*, the KNOLLE and DYNAMIN-RELATED PROTEIN 1A (DRP1A) contribute to cytokinesis. KNOLLE mediates fusion of vesicles at the plane of cell division while DRP1A appears to be involved in cell plate maturation through its role in clathrin-mediated endocytosis.

This thesis shows that KNOLLE is specifically restricted to the cell division plane through sterol-dependent endocytosis that involves a clathrin- and DRP1A-mediated mechanism. Sterols affect internalization of KNOLLE through their role in lateral membrane organization by keeping diffusion of KNOLLE to lateral membranes in check via its endocytic removal. It is shown that the cell plate represents a high-lipid-order membrane domain that depends on the correct composition and the right concentration of sterols. Accumulation of DRP1A at the cell plate requires correct sterol concentration and composition similar to high-lipid order. Conversely, high-lipid-order at the cell plate relies on DRP1A activity suggesting a feedback between DRP1A function and lipid order establishment. Finally, it is shown that sterols are also present at the tonoplast of dividing and elongated root cells.

Taken together, the results reveal that formation of the cell plate in *Arabidopsis thaliana* depends on an intricate interplay between cytokinetic vesicle fusion, sterol-dependent lateral membrane and high-lipid-order domain organization as well as endocytic machinery function.

Keywords
*Arabidopsis*, membrane, sterols, cytokinesis, KNOLLE, endocytosis, clathrin, DRP1A
Dissecting sterol function
during clathrin-dependent endocytosis
and cytokinesis in *Arabidopsis thaliana*

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Sammanfattning


Under cytokines krävs närvaro av Arabidopsis proteinerna KNOLLE och det dynamin-relaterade proteinet 1A (DRP1A). KNOLLE är avgörande för fusion av vesiklar i celldelningsplanet medan DRP1A verkar vara involverad i cellplattans mognad, troligen genom sin roll vid clathrinmedierad endocytos.

Denna avhandling visar att KNOLLE strikt är lokalisierad till celldelningsplanet genom en sterolberoende endocytos som involverar en clathrin- och DRP1A-medierad mekanism. Steroler är involverade i regleringen av clathrin- och DRP1A-beroende endocytosis av KNOLLE genom sin roll i lateral membranorganisation. Under celldelning, tycks cellplattan bilda en domän med hög lipidordning. En liknande hög lipidordning finns i en s k ’raft’ i cellmembranen. Bildningen av en domän med hög lipidordning i cellplattan beror både på sammansättningen och koncentrationen av steroler och på DRP1A aktivitet. Dessutom visar vi att i Arabidopsisrötter är steroler också närvarande i tonoplasten i meristematiska och i sträckta celler.

Våra resultat visar att anläggningen av cellplattan sker genom ett samspel mellan en sterolberoende lateral membranorganisation, samt membranfusion och endocytotiska mekanismer.
List of Papers

I. Yohann Boutté, Márcia Frescatada-Rosa, Shuzhen Men, Cheung-Ming Chow, Kazuo Ebine, Anna Gustavsson, Lenore Johansson, Takashi Ueda, Ian Moore, Gerd Jürgens, and Markus Grebe.
Endocytosis restricts Arabidopsis KNOLLE syntaxin to the cell division plane during late cytokinesis
*The EMBO Journal, Vol. 29, 2010*

II. Márcia Frescatada-Rosa, Thomas Stanislas, Steven K. Backues, Ilka Reichardt, Shuzhen Men, Yohann Boutté, Gerd Jürgens, Thomas Moritz, Sebastian Y. Bednarek, and Markus Grebe
DYNAMIN-RELATED PROTEIN1A feeds back on high membrane lipid order during plant cytokinesis
*Manuscript*

III. Corrado Viotti, Falco Krüger, Christoph Neubert, Fabian Fink, Upendo Lupanga, Melanie Krebs, David Scheuring, Piers A. Hemsley, Yohann Boutté, Márcia Frescatada-Rosa, Susanne Wolfenstetter, Norbert Sauer, Stefan Hillmer, Markus Grebe, and Karin Schumacher
The endoplasmic reticulum is the main membrane source for biogenesis of the lytic vacuole in Arabidopsis
*Accepted for publication in The Plant Cell: 14 of August.2013*

The papers will be referred to by their Roman numbers in the text.
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**ACKNOWLEDGEMENTS**

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<th>Abbreviation</th>
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<td>TGN</td>
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<td>μg</td>
<td>micrograms</td>
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Dedicado à minha mãe
“(…) Avião de Lisboa para o mundo
Apaga-me a tristeza com as asas,
Tão nítidas no céu em que me afundo

Depois desaparece atrás das casas
E deixa-me o azul, azul profundo
E duas nuvens de razão tocadas.”

Alexandre O’Neill
INTRODUCTION

Plant cells are delimited by a cell wall and by a plasma membrane which confers them spatial identity and defines the interface between cell and the extracellular environment. The plasma membrane can be defined as a solution of membrane proteins in a lipid bilayer solvent that can interact with peripheral proteins (Simons and Sampaio, 2011). The lipid bilayer results from the self-association of the lipids hydrophobic moieties and the interaction of the hydrophilic moieties with aqueous environments. The same principle acts at the subcellular level to assemble the lipid bilayer surrounding each cellular organelle (Simons and Sampaio, 2011). Three major classes of lipids, phospholipids, sphingolipids and sterols, contribute to the vast diversity of lipids found in eukaryotic membranes. However, how these lipid components regulate the formation of a new membrane during cell division is poorly understood. In this thesis I will first focus on the role of sterols in eukaryotic membrane formation and lateral membrane organization. Next, I will introduce the de novo creation of membrane (cell plate) during cell division and finally, the endocytic process as a potential mechanism regulated by sterols and involved in the formation of the cell plate.

Sterols are structural components of membranes

Sterols derive from isoprenoids and are present in eukaryote membranes. As structural components of the lipid bilayer, sterols are involved in the regulation of membrane fluidity, permeability, dynamics, and organization (Hartmann, 1998). Whilst cholesterol and ergosterol are the major sterols in mammals and fungi, respectively (Hannich et al., 2011), higher plants displays a more complex sterol profile dominated by sitosterol, stigmasterol and campesterol (Hartmann, 1998; Schaller, 2003). Plants sterols (phytosterols) exist as free sterols, with a free β-hydroxyl group located at C-3 or as conjugated sterols (Benveniste, 2002). Conjugated sterols are divided in steryl esters (SE) and steryl glucosides (SG) depending on whether the 3-β-hydroxyl group is esterified by a fatty acid chain or linked to a sugar moiety (Hartmann, 1998; Benveniste, 2002; Schaller, 2003). Sterol conjugates have been implicated in pathogen defense responses, cellulose biosynthesis and in controlling the amount of free sterols (Silvestro et al. 2013; Kopischke et al., 2013).
Figure 1: Simplified sterol biosynthetic pathway in *Arabidopsis thaliana*. The main enzymes considered in this work are indicated. Each arrow represents one enzymatic step. Abbreviations are explained in the text. Scheme assembled based on Schaller, 2003 and Silvestro *et al.*, 2013.

Sterol biosynthesis pathway in *Arabidopsis thaliana*

The sterol biosynthesis pathway has been extensively investigated and is well characterized in *Arabidopsis thaliana* (Figure 1; Benveniste, 2002). The first step involves the condensation of acetyl-Coenzyme A (acetyl-coA) with acetoacetyl-CoA to produce 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA; Figure 1A). HMG-CoA is subsequently converted into mevalonic acid (MVA; Figure 1B). The conversion of HMG-CoA into MVA represents a rate-limiting step (Hartmann, 1998) catalyzed by the 3-hydroxy-3-methylglutaryl-CoA
reductase (HMGR; Benveniste, 2002; Suzuki and Muranaka, 2007). The *Arabidopsis* genome contains two genes encoding for HMGR, *HMG1* and *HMG2*, which are differentially regulated (Enjuto *et al.*, 1994). *HMG1* is expressed throughout the plant while *HMG2* expression is limited to young seedlings, roots and inflorescences (Benveniste, 2002). MVA is further used to produce two isoprene units: isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) (Suzuki and Muranaka, 2007). In addition, plants can synthesize IPP and DMAPP via the plastidial methylerthryiol phosphate (MEP; Lichtenthaler *et al.*, 1997). However, the MVA pathway is considered the main route involved in phytosterol biosynthesis (Suzuki and Muranaka, 2007). *hmg1* mutant plants display a dwarf phenotype, male sterility and senesce prematurely while the double mutant *hmg1;hmg2* is male gametophytic lethal (Suzuki *et al.*, 2009) highlighting the importance of the MVA pathway. Through a series of steps, IPP and DMAPP are converted into squalene (Figure 1C) marking the channeling of the isoprenoid pathway into the branch that produces sterols (Hartamann, 1998; Suzuki and Muranaka, 2007). Squalene is converted into 2,3(S)-oxidosqualene (OS) by the squalene epoxidase (SQE; Benveniste, 2002; Figure 1D). In *Arabidopsis* three gene products have shown squalene epoxidase activity DROUGHT HYPERSENSITIVE2/SQE1 (DRY2/SQE1), SQE2 and SQE3 (Rasbery *et al.*, 2007; Posé *et al.*, 2009). *DRY2/SQE1* is highly expressed in roots and during embryogenesis, *SQE2* is expressed in most tissues but at low levels and *SQE3* expression is high in leaves and very low in roots (Posé *et al.*, 2009). Knockout mutants of *SQE1* are sterile while plants carrying the weak allele *sqe1-5* displays reduced root and hypocotyls elongation, stomata and polarity defects (Rasbery *et al.*, 2007; Posé *et al.*, 2009).

The enzymatic steps that convert acetyl-CoA to squalene oxide are common in all eukaryotes but strong differences exist downstream of OS production (Benveniste, 2002). In vertebrates and fungi OS is converted to lanosterol which is then metabolized into cholesterol and ergosterol, respectively (Suzuki and Muranaka, 2007). Despite the presence of the lanosterol pathway in plants (Benveniste, 2002; Suzuki *et al.*, 2006), it only accounts for 1.5% of the sterol production in *Arabidopsis* seedlings (Ohyama *et al.*, 2009). Plants mainly convert squalene oxide into cycloartenol (Figure 1E), the first cyclic precursor
of this pathway in a reaction catalyzed by cycloartenol synthase (CAS; Hartmann, 1998; Benveniste, 2002). The Arabidopsis genome contains 13 genes encoding predicted cycloartenol synthases (Benveniste, 2002). Complete loss-of-function of CAS1 is lethal (Babiychuk et al., 2008) and cannot be compensated by overactivation of the lanosterol pathway (Ohyama et al., 2009).

Cycloartenol is further converted into cycloeucalenol through two enzymatic steps. One of these steps involves the C-24 sterol methyltransferase 1 (SMT1; Benveniste, 2002; Figure 1F). Mutants for SMT1 display numerous defects such as altered sterol composition, defective embryo morphogenesis, poor growth, and cell polarity defects (Diener et al., 2000; Schrick et al., 2002; Willemsen et al., 2003). The opening of the 9β,19-cyclopropane ring of cycloeucalenol catalyzed by the cycloeucalenol-obtusifoliol isomerase (CPI1; Figure 1G) is a step restricted to the plant kingdom (Hartmann, 1998; Lovato et al., 2000; Benveniste, 2002). CPI1 is a single copy gene in Arabidopsis (Lovato et al., 2000). The cpi1-1 mutant displays an altered sterol profile with a strong accumulation of cyclopropylsterols intermediates in addition to defects in cell polarity, cytokinesis and endocytosis (Men et al., 2008).

The conversion of obtusifoliol into 24-methylene iophenol involves two enzymatic steps. The first step is catalyzed by the C-14 reductase FACKEL/HYDRA2 (Figure 1H) and the second by the Δ8-Δ7-isomerase HYDRA1 (Figure 1I). Arabidopsis fk/hyd2 and hyd1 mutant plants exhibit several embryonic and postembryonic defects (Souter et al., 2002; Schrick et al., 2002; He et al., 2003).

The 24-methylene iophenol can be methylated in a reaction catalyzed by SMT2 or SMT3 (Carland et al., 2002) directing the biosynthetic pathway to the synthesis of free sterols (sitosterol and stigmasterol; Figure 1J). Mutants defective in SMT2 or SMT3 show patterning defects (Carland et al., 2002) and abnormal cytokinesis in floral organs (De Storme et al., 2013). A fraction of 24-methylene iophenol is used for brassinosteroids (BR) biosynthesis (Benveniste, 2002; Figure 1K). The biosynthesis of brassinosteroids involves the activity of three enzymes, Δ7-sterol-C5 desaturase, Δ5,7-sterol-Δ7 reductase, and Δ24-sterol-Δ24 reductase. In Arabidopsis, these enzymes are encoded by DWARF7, DWARF5 and DIMINUTO (DIM)/DWARF1, respectively (Benveniste, 2002; Figure 1K). Mutants for these enzymes show a similar phenotype characterized by short
stems (dwarfism), reduced fertility, prolonged life cycle, and round curled leaves (Schaller, 2003). In addition, these mutants share a modified sterol composition that is primarily characterized by depletion of the end-products sitosterol and campesterol and therefore lack of bioactive BR (Benveniste, 2002; Schaller, 2003).

All plant tissues are able to synthesize their own sterols and the majority of the enzymes involved in post-squalene sterol biosynthesis are associated with the endoplasmatic reticulum (ER; Lovato et al., 2000; Souter et al., 2002; Benveniste, 2002; Silvestro et al., 2013). However, a few sterol biosynthesis enzymes were additionally found in the plasma membrane, hence a participation of this compartment cannot be excluded (Hartmann, 1998; Schaller, 2003; Silvestro et al., 2013). Biosynthetic sterol transport occurs from the ER via the Golgi apparatus to the plasma membrane (Moreau et al., 1998). Moreover, sterols can be internalized from the plasma membrane into early endocytic compartments, from where they may be recycled back to the plasma membrane or transported to other membrane compartments (Grebe et al., 2003).

Sterol concentration and composition vary temporally and spatially during plant development (Guo et al., 1995; Schrick et al., 2002). The highest sterol content is found in tissues containing actively dividing cells like young seed stages, shoot and root meristems (Schrick et al., 2002; Schrick et al., 2011). On the other hand, different tissue types may display different sterol profiles as observed in Arabidopsis seedling and callus tissues (Schrick et al., 2002). Moreover, striking differences in sterol composition were found during embryogenesis and in different organs from Pisum sativum (Schrick et al., 2011). At the cellular level, sterol concentration increases along the biosynthetic pathway from the ER to the plasma membrane, where they primarily accumulate (Hartmann, 1998; Grebe et al., 2003; Pichler and Riezman, 2004). However, between subcellular membrane compartments, the relative amount of individual sterols remains constant (Grandmougin et al., 1989; Moreau et al. 1998).

**Cellular processes regulated by sterols**

Sterols are implicated in the regulation of several cellular processes. Plant sterols are precursors of the brassinosteroids
phytohormones which are involved in cell elongation, cell division and gene expression during post-embryonic growth (Benveniste, 2002; He et al., 2003). Additionally, sterols can also function as substrates for the synthesis of a wide range of secondary metabolites such as glycoalkaloids and saponins, which have been implicated in plant defense (Hartmann, 1998; Armqvist et al., 2003).

In Arabidopsis, mutants defective in enzymes involved in the sterol biosynthesis pathway display distinct sterol profiles and developmental phenotypes. Embryonic or post-embryonic defects in cell division and cell patterning are characteristic of mutants defective in the early biosynthetic steps. Biochemical complementation of these mutants with brassinosteroids fails to restore the wild-type phenotype (Rasbery et al., 2007; Posé et al., 2009 Diener et al., 2000; Schrick et al., 2002; Willemsen et al., 2003; He et al., 2003; O’Brien et al., 2005; Topping et al., 1997; Men et al., 2008; Schrick et al., 2004a). In contrast, mutants affected in the BR biosynthetic reactions display reduced fertility, altered leaf morphology, delayed senescence, and dwarfism but do not show embryonic defects (Clouse, 2002; Schaller, 2003). In addition, treatment with BR is able to rescue their wild-type phenotype (Schaller, 2003). These observations suggest that either the accumulation of sterol biosynthetic intermediates is toxic for the plant or, that in addition to BR, sterol intermediates can function as signaling molecules (Schrick et al., 2002; Souter et al., 2002; He et al., 2003; Schrick et al., 2011). The dry2/sqe1 mutant phenotype can be rescued by a second mutation in the SUPPRESSOR OF DRY2 DEFECTS1 (SUD1) gene which decreases HMGR activity and as a result reduces squalene accumulation (Doblas et al., 2013). These observations suggest that dry2/sqe1 development defects could result from the accumulation of an intermediate sterol (Doblas et al., 2013). In Solanum chacoense plants, the biosynthetic intermediate obtusifoliol was shown to move through the phloem and systemically induce the expression of the sterol biosynthesis enzyme CYP51 (obtusifoliol 14α-demethylase) supporting the role of sterols intermediates as mobile signaling molecules (O’Brien et al., 2005). Additionally, atypical sterols that accumulate in fk mutants are able to modulate the expression of genes involved in cell expansion and division (He et al., 2003). The role of atypical steroid molecules might be exerted by their interaction with a sterol binding domain (steroidogenic acute regulatory transfer domain - START domain). In
plants, START domains are found in homeodomain transcription factors from HD-ZIP family such as PHABULOSA, known to play a role in plant development (Clouse, 2002; Schrick et al., 2004b).

Several studies have unveiled the importance of sterol in numerous cellular processes. A specific sterol environment may be required for membrane protein folding, activity or membrane insertion (Pichler and Riezman, 2004). The activity of membrane-bound plasma membrane H^+-ATPase from corn roots was shown to be dependent on both sterol concentration and composition (Grandmougin-Ferjani et al., 1997). Low concentrations of cholesterol and stigmasterol were shown to stimulate the H^+ pumping activity of the ATPase while sitosterol and 24-methylcholesterol inhibited it (Grandmougin-Ferjani et al., 1997). In mammals, distinct endocytic pathways depend on membrane sterol composition (Pichler and Riezman, 2004; Doherty and McMahon, 2009; Baral and Dhonukshe, 2012). Similarly, the Arabidopsis cpi1-1 mutant shows defects in the internalization of the endocytic tracker FM4-64 and of the PIN-FORMED 2 auxin efflux carrier protein (Men et al., 2008).

Other processes shown to depend on correct sterol composition include plastid biogenesis (Babiychuk et al., 2008), embryonic patterning (Diener et al., 2000; Souter et al., 2002; Schrick et al., 2002), cell division and elongation (Diener et al., 2000; Schrick et al., 2002; Men et al., 2008; De Storme et al., 2013), cell polarity (Willemsen et al., 2003; Men et al., 2008; Ovecka et al., 2010), stomata and vascular patterning (Posé et al., 2009; Qian et al., 2013), and hormone signaling (Souter et al., 2002; He et al., 2003).

The function of sterols in the diversity of cellular processes they regulate might in part be exerted through their role in membrane domain organization.

**Sterols as a driving force for membrane organization**

For a long time, membranes were considered assemblies of homogenously distributed proteins and lipids as proposed by Singer and Nicolson (1972) in their classic fluid model. However, increasing evidence depict an organization of the biological membrane that is far from the proposed fluid mosaic model (Simons and Ikonen, 1997; Simons and Gerl, 2010; Cacas et al., 2012). The visualization of domains with different lipid compositions in cells such as polarized epithelial cells suggested the existence of domains within the
membrane with particular molecular, biochemical, and biophysical features (Simons & van Meer, 1998). These domains were termed as membrane rafts (Simons and Ikonen, 1997).

Figure 2: Schematic representation of the putative molecular organization of membrane rafts in the lipid bilayer. Illustration assembled based on Mongrand et al., 2010.

Membrane rafts are currently defined as highly dynamic small domains (10-200 nm) of tightly packed lipids - similar to the liquid ordered (lo) phase observed in artificial membranes - enriched in sphingolipids and sterols, depleted in unsaturated phospholipids and characterized by distinct protein content (Figure 2; Pike, 2006). The assembly of membrane rafts appears to begin at the trans-Golgi network (TGN; Klemm et al. 2009; Cacas et al., 2012). Furthermore, these small domains can cluster into larger “rafts” through lipid-lipid, protein-protein and protein-lipid interactions depending on physiological conditions or biotic and abiotic stimuli (Pike, 2006; Simon-Plas et al., 2011).

In the current model for raft formation, sphingolipids associate laterally with one another through weak interactions between aliphatic
chains. Sterols interact with sphingolipids and function as spacers, filling the voids between associated sphingolipids (Simons and Ikonen, 1997; Xu and London, 2000; Simons and Sampaio, 2011). This arrangement of lipid molecules results in a high degree of lipid packing and is the basis for the formation of membrane rafts.

Sterols play a key role in the formation of membrane domains (Xu and London, 2000; Simons and Sampaio, 2011). Several studies performed on artificial membranes have shown that insertion of sterols in a mixture of saturated lipids promotes domain formation through an increase in the degree of lipid packing (Simons & Ikonen, 1997; Xu and London, 2000; Xu et al., 2001; Henriksen et al., 2004; Beattie et al., 2005). The degree of domain formation depends on the concentration and molecular structure of the sterols present (Xu and London, 2000; Henriksen et al., 2004; Beattie et al., 2005; Bacia et al., 2005). Depletion of sterols using the cyclic oligosaccharide methyl-β-cyclodextrin (MβCD) was shown to increase the lipid acyl chain disorder in tobacco plasma membrane (Roche et al., 2008). Furthermore, sterols that do not pack well with saturated lipids are unable to promote domain formation (Xu and London, 2000; Henriksen et al., 2004; Beattie et al., 2005).

The tight packing interaction of sterols with saturated lipids, such as sphingolipids has been attributed to several factors including the planar structure of the sterols, their overall dimensions and the properties of their small polar 3-OH-group (Xu and London, 2000; Beattie et al., 2005). However, membrane domain formation can be observed in various sterol-containing membrane systems- mammals, fungi and plants (Beck et al., 2007; Xu et al., 2001) suggesting that regardless of the differences in the sterol species, lateral membrane organization is a conserved mechanism in eukaryotes.

**Membrane rafts in plants**

The high lipid packing present in membrane rafts prevents the incorporation of detergent molecules (Fiedler et al., 1993; Xu and London, 2000; Schuck et al., 2003). As a result these membrane domains are resistant to non-ionic detergent solubilization at low temperatures (Brown and Rose, 1992; Xu and London, 2000; Xu et al., 2001; Mongrand et al., 2010). This biochemical property has been extensively used to study membrane domains in different eukaryotes hence the working definition of membrane rafts is detergent-resistant.
membranes (DRMs; Magee and Parmryd, 2003; Grennan, 2007). Nevertheless, although DRM extraction is the only biochemical method available to study potential raft affinity, one must keep in mind that DRMs cannot be equated with membrane rafts (Lingwood and Simons, 2007; Mongrand et al., 2010; Owen et al., 2012a). The results obtained with the detergent solubilization method depend on several experimental factors such as the type and amount of detergent (Lingwood and Simons, 2007). Hence the results obtained from the analysis of DRMs should be supported by other approaches.

The lipid composition of DRMs extracted from different plant systems was found to be similar to those collected from animals and yeast. Plant DRMs are enriched in free sterols (sitosterol, and stigmasterol), conjugated sterols, sphingolipids, phosphatidylinositol and depleted of phospholipids (Mongrand et al., 2004; Borner et al., 2005; Laloï et al., 2007; Minami et al., 2009; Furt et al., 2010). Despite the mixture of sterols found in plants, the type and relative abundance of individual sterols present in DRMs and the membrane from where they were extracted is similar (Mongrand et al., 2004; Borner et al., 2005; Morel et al., 2006; Laloï et al., 2007).

**Biological/physiological relevance of membrane rafts**

Membrane raft domains are viewed as “membrane organizing principles” that temporally and spatially concentrate specific proteins, while excluding others, thus facilitating protein interaction and complex formation. This lateral compartmentalization of the membrane allows the coordination of different biological processes occurring in parallel (Simons & Ikonen, 1997; Cacas et al., 2012). In animals and yeast, membrane rafts have been implicated in the regulation of signal transduction, polarized intracellular sorting, cytoskeleton reorganization, endocytosis, exocytosis and entry of pathogens in living cells, (Simons and Ikonen, 1997; Bagnat and Simons, 2002a; Simons and Sampaio, 2011). Similarly, the majority of proteins identified in plant DRM fractions are involved in signal transduction and biotic and abiotic stress-responses, vesicle trafficking, cytoskeleton organization, cell polarity, and cell wall metabolism (Mongrand et al., 2004; Borner et al., 2005; Morel et al., 2006; Minami et al., 2009; Stanislas et al., 2009; Kierszniowska et al., 2009). Treatment with a defense reaction elicitor (cryptogein) was shown to induce changes in the protein composition of DRM extracted
from tobacco cells (Stanislas et al., 2009). After elicitor treatment, the authors observed a reduction in the association of components of clathrin-mediated endocytosis (CME) with DRMs and an enrichment of the signaling protein 14-3-3 (Stanislas et al., 2009). Additionally, changes in the protein content of DRMs extracted from Arabidopsis cells were observed during cold acclimation including an enrichment of CME components and the signaling protein remorin (Minami et al., 2009).

Schuck and Simons (2004) proposed that clustering of individual rafts can be viewed as a mechanism for selective recruitment of proteins and lipids with affinity for membrane rafts. In plants, several developmental and physiological processes require the strict spatio-temporal positioning of proteins in the membrane (Martin et al., 2005). For example, the different polar localization of PIN proteins at the cell membrane regulates the direction of the auxin flux. The polarized distribution of PIN1, PIN2 and PIN3 is known to be disrupted in Arabidopsis sterol-deficient mutants (Souter et al., 2002; Willemsen et al., 2003; Men et al., 2008) and PIN1 was identified in Arabidopsis DRM fractions (Titapiwatanakun et al., 2009). These observations suggest a potential role for sterol-dependent membrane domains in the polarized localization of PIN proteins.

The association of proteins to a lipid-enriched environment may be accompanied by conformational changes that modify protein activity and/or their capacity to recruit specific proteins (Lingwood and Simons, 2010; Cacas et al., 2012). The auxin transporter ABCB19, present in DRMs (Borner et al., 2005; Morel et al., 2006), is required to stabilize PIN1 localization in membrane microdomains and the combined action of PIN1 and ABCB19 at the membrane enhances the rate and specificity of auxin efflux (Titapiwatanakun et al., 2009). In Picea meyeri, the production of reactive oxygen species (ROS) by the NADPH oxidase (NOX) is essential for the apical growth of the pollen tube (Liu et al., 2009). NOX enzymatic activity at the tip of the growing pollen tube was shown to be microdomain-associated and sterol-dependent (Liu et al., 2009).

The polar distribution of sterol-enriched/membrane rafts within the plasma membrane has been implicated in polarized cellular events such as tip growth and cell division in animals, fungi and plants. Sterols were shown to accumulate at the tip of growing root hairs in Arabidopsis (Ovecka et al., 2010) and clustering of membrane
domains were observed at the tip of the mating projection of budding yeast (Bagnat and Simons, 2002b), the growing hyphae of Candida albicans (Martin and Konopka, 2004) and the growing pollen tube in Picea meyeri (Liu et al., 2009). Furthermore, fission yeast accumulates sterols (Wachtler et al., 2003) and echinoids display a high membrane lipid order (Ng et al., 2005) at the medial site of cell division.

In brief, sterols are components of cellular membranes that are crucial for lateral membrane organization hence formation of membrane domains. The establishment of distinct domains within the membrane appears to be essential for the regulation of diverse processes such as cell polarity, endocytosis and cell division.

**Cell division**

Cell division is the process by which a cell produces two daughter cells. This cellular event has played an essential role in the history of eukaryotes namely in the emergence and during the reproduction of multicellular organisms. Cell division can be divided into two major events the duplication of nuclear DNA termed karyokinesis or mitosis (subdivided in four steps prophase, metaphase, anaphase and telophase) and the partitioning of cytoplasm and organelles named cytokinesis (Jürgens, 2005). Cytokinesis can vary extensively in between eukaryotes regarding both the supportive sub-cellular structures as well as the underlying processes (Jürgens, 2005; Baluška et al., 2006).

In plants, partitioning of the cytosol and organelles of a dividing cell is achieved by the de novo creation of a plasma membrane and cell wall between daughter nuclei (Jürgens, 2005; Backues et al., 2007). Higher plants exhibit cell type-specific modes of cytokinesis. The most common type occurs in somatic cells where nuclear division and cytokinesis are coupled (Jürgens, 2005). In contrast, in endosperm cellularization nuclear divisions are not immediately followed by cytokinesis (Otegui et al., 2001). In this section I will focus on the predominant mode of somatic cytokinesis in plants.
**Somatic cytokinesis**

Due to the presence of a cell wall, the timing and positioning of the new plasma membrane/cell wall is extremely important for correct morphogenesis and proper architecture of the plant body (Hong and Verma, 2007). The plane of cell division is predetermined before the onset of mitosis by the appearance of the preprophase band (PPB) (Karahara *et al.*, 2010; McMichael and Bednarek, 2013). The PPB is an array of actin and cortical microtubules (MT) placed just beneath the plasma membrane around the nuclei (Karahara *et al.*, 2010; Figure 3A). This cytoskeleton structure disappears at the end of prophase, leaving behind an imprint that defines the cortical division zone (CDZ; Karahara *et al.*, 2010; McMichael and Bednarek, 2013).

![Diagram of cytokinesis](image)

**Figure 3:** Organization of the cytoskeleton, Golgi apparatus, TGN/EE and coated vesicles from (A) late prophase to (B and C) cytokinesis in plants. N, nucleus; CP, cell plate; CDS, cortical division site; other abbreviations are explained in the text. Image assembled based on McMichael and Bednarek, 2013.

During late anaphase, remnants of the mitotic spindle assemble into the phragmoplast (Jürgens, 2005; McMichael and Bednarek, 2013; Figure 3B). The phragmoplast is a cytoskeleton-array composed of antiparallel MT and actin microfilaments (MFs) arranged with their plus ends towards the plane of cell division (Samuels *et al.*, 1995; Otegui *et al.*, 2001; Seguí-Simarro *et al.*, 2004). This structure directs TGN-derived secretory (Seguí-Simarro *et al.*, 2004; Reichardt *et al.*, 2007) and probably endocytic vesicles (Dhonukshe *et al.*, 2006; Baluška *et al.*, 2006) to the plane of division where they fuse to form a transient membrane compartment – the cell plate (Samuels *et al.*, 1995; Otegui *et al.*, 2001; Seguí-Simarro *et al.*, 2004; Figure 3A and B).

Cell plate formation can be divided into three stages: homotypic vesicle fusion (merging of membranes of the same type),
cell plate expansion and maturation (Hong and Verma, 2007). The initial stage involves the fusion of vesicles or membrane tubules giving rise to a tubular-vesicular network (TVN; Samuels et al., 1995; Otegui et al., 2001; Seguí-Simarro et al., 2004). As the cell plate expands, the phragmoplast disassembles from the central region and new MT and MFs polymerize at the growing edges (Figure 3B). This reorientation of the phragmoplast facilitates the centrifugal expansion of the cell plate by directing arriving vesicles to the growing margins. Callose is deposited in the TVN lumen and is thought to provide the spreading force to convert the TVN into a planar fenestrated sheet (Samuels et al., 1995; Otegui et al., 2001; Seguí-Simarro et al., 2004). While the outer regions of the cell plate expand, the central region enters the maturation phase which involves removal of excessive membrane by endocytosis (Samuels et al., 1995; Otegui et al., 2001; Seguí-Simarro et al., 2004). Ultimately, the cell plate fuses with the parental membrane at the site that was earlier marked by the PPB (Jürgens, 2005; McMichael and Bednarek, 2013). This process is accompanied by the replacement of callose by cellulose thus forming a mature cell wall that physically separates the two daughter cells (McMichael and Bednarek, 2013). Interestingly, the expansion of the cell plate often appears to be asymmetric with one side fusing with the parental membrane earlier than the other (Cuttler and Ehrhardt, 2002; Jürgens, 2005).

During cytokinesis, vesicle trafficking towards the plane cell of division appears to be the default pathway (Bednarek and Falbel, 2002; Jürgens, 2005; Esseling-Ozdoba et al., 2008; Hehnly and Doxsey, 2011). This targeted vesicle trafficking is facilitated by the phragmoplast and likely increases the efficiency and timing of cell plate formation. The localization of known plasma membrane proteins such as PIN1 (Geldner et al., 2001), the syntaxin PENETRATION RESISTANCE 1 (PEN1; Müller et al., 2003) and PM-H+-ATPase at the cell plate further support the existence of this default trafficking pathway during cytokinesis (Jürgens, 2005; McMichael and Bednarek, 2013).

In brief, the *de novo* creation of a membrane at the plane of cell division results from a temporal and spatial orchestration of membrane fusion and endocytosis (Hong and Verma, 2007). Impaired
vesicle fusion (Lukowitz et al., 1996; Lauber et al., 1997) or defects in endocytosis (Collings et al., 2008), result in cytokinetic defects.

**Membrane fusion during somatic cytokinesis**

The docking of transport vesicles to the appropriate membrane is coordinated by Rab/Ypt proteins from the Ras family of small GTPases that interact with tethering protein complexes present on the target membrane (Fujimoto and Ueda, 2012). During cytokinesis, *Arabidopsis* RABA2 and RABA3 isoforms localize to the TGN/EE and to the growing edges of the cell plate (Chow et al., 2008). Dominant-negative interference with RABA2 function results in the appearance of multinucleated cells and incomplete cell walls which suggests a role in the transport of vesicles from the TGN to the cell plate (Chow et al., 2008). Vesicle fusion at the division plane is further aided by the exocyst complex that associates with both the leading edge and the maturing regions of the cell plate (McMichael and Bednarek, 2013). However, membrane fusion is ultimately driven by the assembly of specific soluble N-ethylmaleimide-sensitive factor adaptor protein receptor (SNARE) complexes (*trans*-SNARE complex) between transport vesicles and target membranes (Jürgens, 2004; McMichael and Bednarek, 2013).

The *trans*-SNARE complex consists of a four α-helix bundle. Three of the helices are derived from SNARE proteins that are present in the target membrane (Sanderfoot et al., 2000): one helix from a t(arget)-SNARE and two from either a single N-ethylmaleimide-sensitive factor adaptor protein 25 (SNAP25)-type SNARE or two individual t-SNAREs (Uemura et al., 2004, Figure 4A). The fourth helix is derived from a v(esicle)-SNARE present in the vesicle membrane (Sanderfoot et al., 2000; Uemura et al., 2004; Figure 4A). Following fusion, SNARE proteins become part of the same membrane and the four α-helix bundle exists as a *cis*-SNARE complex (Figure 4C). This complex is disassembled by the ATPase activity of N-ethylmaleimide-sensitive factors (NSF) that releases the individual SNAREs for further fusion events (Sanderfoot et al., 2000).
Figure 4: Assembly of SNARE complexes. Four SNARE helices are required to form the *trans*-SNARE complex (labeled a-, b-, c-, and d- helices as described in Scales *et al.*, 2000). The a-helix is contributed by a syntaxin, (A, top) helices b and c are provided by either a SNAP25-type of SNARE or (A, bottom) two distinct t-SNAREs. When a vesicle docks on the target membrane, the v-SNARE present on the vesicle assembles with the t-SNARE complex on the target membrane to form a (B) *trans*-SNARE complex. After fusion, all SNAREs are present in the same membrane forming a (C) *cis*-SNARE complex. Image assembled based on Sanderfoot *et al.*, 2000.

Distinct SNARE complexes regulate membrane fusion in different trafficking pathways (Uemura *et al.*, 2004). In *Arabidopsis*, the t-SNARE KNOLLE is specifically involved in the homotypic fusion of vesicles during cytokinesis (Lukowitz *et al.*, 1996; Lauber *et al.*, 1997). Mutations in the *KNOLLE* gene result in accumulation of unfused vesicles in the plane of cell division (Lauber *et al.*, 1997). KNOLLE is exclusively expressed in dividing cells (Lukowitz *et al.*, 1996; Lauber *et al.*, 1997) and is a plant-specific member of the SYNTAXIN-OF-PLANTS 1 (SYP1) family (Sanderfoot *et al.*, 2000). During early mitosis, KNOLLE localizes to intracellular patches likely representing the TGN (Reichardt *et al.*, 2007). From telophase onwards, KNOLLE accumulates at the forming cell plate until the end of cytokinesis (Lauber *et al.*, 1997), when it is targeted for degradation (Lauber *et al.*, 1997; Reichardt *et al.*, 2007).

KNOLLE forms two SNARE complexes that are required for vesicle fusion during cell plate formation. The first of these is a trimeric plasma-membrane type complex formed by the interaction of KNOLLE with SNAP33 (Heese *et al.*, 2001) and the v-SNARE
VESICLE-ASSOCIATED MEMBRANE PROTEIN (VAMP) 721, 722 (El Kasmi et al., 2013). The second complex is a tetrameric complex that includes the NOVEL PLANT-SPECIFIC SNARE 11 (NPSN11; Zheng et al., 2002), SYP71 and VAMP721, 722 (El Kasmi et al., 2013).

The inability to form one or the other KNOLLE-complex has minor effects on cytokinesis as shown by the double mutants npsn1;syp71 (El Kasmi et al., 2013) and vamp721;vamp722 (Zhang et al., 2011). In contrast, mutations that affect both KNOLLE-complexes resemble the knolle mutant (Lukowitz et al., 1996; Lauber et al., 1997; El Kasmi et al., 2013). Whether these SNARE-complexes reside in the same vesicle or in two distinct vesicles, one that may originate from the secretory pathway and another that may be provided by the endocytic pathway is unknown. However, there is some evidence that plasma membrane material may also contribute to cell plate formation (Dhonukshe et al., 2006). Vesicles that are formed from the plasma membrane most likely differ in their composition from those originating from the secretory pathway. Hence, existence of two different KNOLLE-complexes could ensure homotypic fusion of vesicles of different origins at the plane of cell division.

Unlike KNOLLE itself, SNAP33, NSP11, SYP71 and VAMP721/722 are expressed in dividing and non-dividing cells (Heese et al., 2001; Zheng et al., 2002; El Kasmi et al., 2013; Zhang et al., 2011). This indicates that specificity of cytokinetic SNARE complexes is determined by KNOLLE. Three factors contribute to this specificity: first, a high-level of KNOLLE expression during M phase (Lukowitz et al., 1996; Lauber et al., 1997; Völker et al., 2001; Reichardt et al., 2011), second, targeting to the cell plate likely due to the absence of a sorting sequence (Völker et al., 2001; Touihri et al., 2011) and third, membrane fusion activity at the cell plate, which depends on the interaction with the regulatory Sec1/Munc18 (SM) protein KEULE to promote the trans-SNARE complex formation. (Waizenegger et al., 2000; Assaad et al., 2001; Park et al., 2012).

After membrane fusion, the KNOLLE-SNARE complexes are likely disassembled by the AAA-ATPase NSF, which is known to interact with KNOLLE (Rancour et al., 2002).
Endocytosis during somatic cytokinesis

Endocytosis is an energy-dependent mechanism conserved among eukaryotes where different types of molecules become internalized from the cell surface (Mellman, 1996; Chen et al., 2011). The internalization of membrane, along with resident lipids and proteins, contributes to the regulation of membrane homeostasis (Doherty and McMahon, 2009; Baral and Dhonukshe, 2012). Following internalization, a succession of steps involving carriage of the cargo molecules through endosomal compartments ends in either the degradation of the cargo in the lytic compartment or in the recycling to the plasma membrane/cell wall/cell plate (Robinson et al., 2008; Contento and Bassham, 2012). Endocytosis has been implicated in several biological processes such as signaling, pathogen entry and defense, cell polarity, mitosis, cell wall morphogenesis and cytokinesis (Dhonukshe et al., 2007; Doherty and McMahon, 2009; Chen et al., 2011; Baral and Dhonukshe, 2012; Adam et al., 2012).

Endocytosis is thought to play a major role in cytokinesis by assisting the establishment of PPB “memory” cues (Karahara et al., 2009), providing material for the construction of the cell plate (Dhonukshe et al., 2006) and recycling proteins and lipids during expansion, maturation and fusion of the cell plate with the parental membrane (Otegui et al., 2001; Seguí-Simarro et al., 2004; Van Damme et al., 2011; McMichael and Bednarek, 2013). The endocytic traffic doubles during cytokinesis and genetic and pharmacological interference with endocytosis results in cytokinetic defects (Dhonukshe et al., 2006; Karahara et al., 2009).

In eukaryotes different endocytic pathways exist that vary in the mode of vesicle formation and the molecular players involved. Animal cells display six known endocytic mechanisms clathrin-, claveolin-, flotillin-associated, CLIC/GEEC (clathrin-independent carriers/GPI- enriched early endosomal compartments), phagocytosis, and macropinocytosis (Doherty and McMahon, 2009; Baral and Dhonukshe, 2012). To date, only clathrin-, flotillin-associated and phagocytosis-like internalization have been demonstrated in plants (Baral and Dhonukshe, 2012). Of these pathways, the most significant in plants appears to be the clathrin-dependent (Dhonukshe et al., 2007). The CME has been implicated in somatic cytokinesis in plants (Seguí-Simarro et al., 2004). The CDZ of Arabidopsis and onion cells is enriched in clathrin-coated vesicles (CCVs; Karahara et al., 2009;
Van Damme et al., 2011) likely involved in localized changes of the PPB-associated plasma membrane/cell wall domain (McMichael and Bednarek, 2013). CCVs have also been detected in the phragmoplast (Dhonukshe et al., 2006; Reichardt et al., 2007) and budding from maturing regions of the cell plate in Arabidopsis and tobacco cells (Samuels et al., 1995; Otegui et al., 2001; Seguí-Simarro et al., 2004). Altogether, these observations suggest a role for clathrin-dependent protein internalization in cytokinesis.

**Clathrin-mediated endocytosis**

CME is the mechanism by which membrane cargo-proteins are recruited into developing clathrin-coated pits (CCP) that undergo progressive invagination from the membrane until they pinch off and form a CCV (Doherty and McMahon, 2009; Chen et al., 2011). This endocytic pathway is brought about by a dynamic network of protein-protein and protein-lipid interactions and is involved in multiple steps of the post-Golgi trafficking in eukaryotes (Doherty and McMahon, 2009).

--- Recruitment --- Maturation --- Scission

![Diagram](image-url)

**Figure 5:** Schematic representation of CCV formation during clathrin-dependent endocytosis. Abbreviations are explained in the text. Image assembled based on Chen et al., 2011.
Membrane curvature is an important prerequisite for vesicle formation during endocytosis (Ovecka and Lichtscheidl, 2005; Doherty and McMahon, 2009). The lipid characteristics of a membrane are known to influence its vesicle budding competence (Bacia et al., 2005) but on their own they are not sufficient to drive vesicle formation (Kirchhausen, 2012). Peripheral membrane proteins with an epsin N-terminal homology (ENTH) domain have been proposed as a membrane curvature inducing, sensor or stabilizing agents (Horvath et al., 2007; Kirchhausen, 2012). In addition, these proteins may bind cargo and membrane lipids to clathrin and adaptor protein (AP) complexes (Ford et al., 2002; Horvath et al., 2007). AP complexes and possibly accessory proteins like ADP-RIBOSYLATION FACTOR 1 (ARF1; Teh and Moore, 2007), coordinate the sorting of cargo and recruitment of clathrin at the membrane site destined to internalization (Doherty and McMahon, 2009; Fujimoto and Ueda, 2012; Figure 5). Different adaptor and accessory proteins may control the internalization of distinct cargoes either in the same membrane or in different endomembrane compartments (Doherty and McMahon, 2009). The nucleation of clathrin at the site of internalization promotes polymerization of the clathrin-coat. The basic structural unit of the clathrin coat is the triskelion, a three-legged molecule, in which each leg consists of a clathrin heavy chain (CHC) and a light chain (CLC; Figure 5). The assembly of the clathrin-coat into lattices-like structures reinforces and stabilizes the deformation of the membrane shaping the CCP (Doherty and McMahon, 2009; Fujimoto and Ueda, 2012). As the CCP matures, dynamin or DYNAMIN-RELATED PROTEINs (DRPs) are recruited to the neck of the CCP and assemble into a ring-like polymer. Constriction of this polymer mediated by GTP hydrolysis, together with the activity of other membrane modifying proteins, releases the vesicle from the membrane (Doherty and McMahon, 2009; Figure 5). Once in the cytoplasm, ATPases such as the HEAT SHOCK COGNATE 70 (HSC70) protein and its co-factor auxilin remove the clathrin coat (Eisenberg and Greene, 2007; Figure 5). The uncoated vesicles eventually fuse with early endosomes (EE) and vesicle cargo is further sorted (Doherty and McMahon, 2009; Chen et al., 2011) either for degradation or rerouted to its original location (Pichler and Riezman, 2004; Robinson et al., 2008; Chen et al., 2011).
Orthologues of various components of the CME machinery present in animals are found in plant genomes and have been implicated in different steps of cytokinesis. For example, the *Arabidopsis* EPSIN-LIKE CLATHRIN ADAPTOR 1 (AtECA1) accumulates at the cell plate in dividing cells and has been shown to interact *in vitro* with CHC and co-localizes with CLC at the cell plate (Song *et al.*, 2012). Additional components of the CME present in plants are the AP complexes. The *Arabidopsis* genome encodes five AP complexes (AP-1 to 5), each composed of four subunits (Teh *et al.*, 2013). AP1M2 corresponds to the µ-adaptin subunit of the TGN-localized AP-1 complex. The *ap1m2* mutant displays endocytic and cytokinetic defects with the presence of unfused vesicles and incomplete cell walls in plane of cell division (Teh *et al.*, 2013). Furthermore, KNOLLE is mis-localized to intracellular aggregates around the plane of division in *ap1m2*, suggesting that AP-1-clathrin complexes are involved in the transport of KNOLLE from the TGN to the cell plate (Teh *et al.*, 2013). In addition, plants have a unique adaptor-like protein named T-PLATE (Van Damme *et al.*, 2011). *tplate* mutants show curved cell walls and cell plates unanchored from the parental membrane (Van Damme *et al.*, 2006). This adaptor protein interacts with clathrin and localizes to the expanding cell plate and the cortical division zone (Van Damme *et al.*, 2006; Van Damme *et al.*, 2011). The core components of the clathrin-coat are also conserved in plants. The *Arabidopsis* genome contains two CHC genes (*CHC1* and *CHC2*; Kitakura *et al.*, 2011) and three CLC genes (*CLC1*, *CLC2* and *CLC3*; Wang *et al.*, 2013). CLC is known to localize to the cell plate of *Arabidopsis* root cells (Konopka *et al.*, 2008; Ito *et al.*, 2011; Mravec *et al.*, 2011). *chc2* and *clc2;clc3* double mutant display internalization defects (Wang *et al.*, 2013; Kitakura *et al.*, 2011).

Six subfamilies of DRPs (DRP1-DRP6) are found in *Arabidopsis* (Hong *et al.*, 2003). Live cell imaging of dividing cells has revealed the presence of DRP1A, DRP1C, DRP1E, DRP2A and DRP2B at the cell plate in *Arabidopsis* and tobacco cells (Kang *et al.*, 2003; Hong *et al.*, 2003; Konopka *et al.*, 2008; Konopka and Bednarek, 2008a; Fujimoto *et al.*, 2008; Fujimoto *et al.*, 2010). Mutation of the *DRP1A* gene results in several endocytic and cytokinetic defects (Collings *et al.*, 2008). Moreover embryos defective in both *DRP1A* and *DRP1E* show perturbed cell plate
formation and fail to germinate (Kang et al., 2003). drp1c mutants are male gametophytic lethal and a cross between drp1a and the weak and viable allele drp1c2 yields no double homozygous seedlings though, no defect in cytokinesis was observed in drp1a;drp1c2 embryos (Mravec et al., 2011).

The role of dynamin-related proteins in somatic cytokinesis

Dynamin and DRPs belong to a superfamilly of structurally related but functionally diverse large GTPases that play a central role in membrane biogenesis by regulating membrane fission and tubulation during cell expansion and cytokinesis (Konopka et al., 2008; Fujimoto and Ueda, 2012). A common feature of dynamin and DRPs is their ability to homo-oligomerize around lipid bilayers and to deform membranes (Praefcke and McMahon, 2004). Animal dynamin is characterized by five distinct domains: the N-terminal GTPase domain, a middle domain that is crucial for self-assembly, a GTPase-effector domain (GED), which stimulates the GTPase activity, a pleckstrin homology (PH) domain that confers binding to the membrane phosphoinositides, and a proline-rich domain (PRD) required for interaction with other CME components via Src homology 3 (SH3) domains (Heymann and Hinshaw, 2009; Fujimoto and Ueda, 2012). Of the six subfamilies of DRPs (DRP1-DRP6) present in Arabidopsis (Hong et al., 2003) only DRP1 and DRP2 isoforms have been implicated in cell plate formation and CME (Hong et al., 2003; Kang et al., 2003; Collings et al., 2008; Konopka et al., 2008; Fujimoto et al., 2008; Fujimoto et al., 2010; Backues et al., 2010; Taylor et al., 2011). DRP1 isoforms are plant-specific and lack any recognized lipid (PH) or protein interaction domain (PRD; Backues and Bednarek, 2010). In contrast, members of the DRP2 family are considered “classical” dynamins harboring all five domains of mammalian dynamin (Konopka et al., 2006; Bednarek and Backues 2010; Heymann and Hinshaw, 2009). Interestingly, the GTPase domain of animal dynamin has higher similarity to the GTPase domain of DRP1 isoforms than that of DRP2 members (Fujimoto and Ueda, 2012).

In Arabidopsis the DRP1 subfamily consists of five members (DRP1A-DRP1E) of which DRP1A, DRP1C and DRP1E are widely expressed in plant tissues (Kang et al., 2003; Collings et al., 2008). DRP1A, DRP1C and DRP1E have previously been shown to localize
to the cell plate and intracellular compartments in dividing cells (Lauber et al., 1997; Kang et al., 2001; Kang et al., 2003; Konopka et al., 2008; Collings et al., 2008). However, so far only DRP1A and DRP1E have been shown to play a role in somatic cytokinesis (Kang et al., 2003; Collings et al., 2008). Absence of DRP1E per se has little effect on plant development but embryonic cells of drp1a;drp1e mutants display several cytokinetic defects and drp1a;drp1e double mutant seeds fail to germinate suggesting functional redundancy between DRP1A and DRP1E (Kang et al., 2003). Electron tomographic analysis of Arabidopsis cells revealed ring-like spirals constricting the tubular network and surrounding vesicles in both somatic and endosperm cell plates (Otegui et al., 2001; Seguí-Simarro et al., 2004). These spiral structures were shown to contain DRP1A and/or DRP1E homo- or heteropolymers (Otegui et al., 2001). Indeed, Arabidopsis DRP1A is present in membrane fractions as a high molecular mass complex of 400-600 KDa and as a monomer in soluble fractions (Park et al., 1997). Moreover, yeast-two-hybrid (Y2H) interaction and in vitro-binding assays demonstrated that Arabidopsis DRP1A has the ability to self-interact and polymerize, respectively (Fujimoto et al., 2010; Backues and Bednarek, 2011). Little information is available regarding DRP1E but Y2H assays showed that it can interact with the soybean DRP1A homologue phragmoplastin, suggesting that DRP1E has the ability to form heteropolymers (Hong et al., 2003).

During cytokinesis two roles have been assigned to DRP1A and likely to DRP1E, given the localization and sequence similarity between the two proteins (Kang et al., 2001; Ito et al., 2012; McMichael and Bednarek, 2013). First, they are involved in membrane-remodeling by constricting hourglass-shaped vesicles into the dumb-bells that, with addition of further vesicles, build the TVN (Otegui et al., 2001; Seguí-Simarro et al., 2004). Indeed, DRP1A is an early marker of the cell plate appearing prior to TVN formation (Hong et al., 2003; Kang et al., 2003) and apparently to CLC appearance (Ito et al., 2012), which suggests an additional role independent of clathrin during cytokinesis. Secondly, as part of the CME machinery these DRPs may play a role in removing excess membrane material from the forming cell plate, thus contributing to cell plate maturation. Arabidopsis DRP1A forms dynamic foci in the cell cortex that partially overlap with CLC and react to tyrphostin A23 (tyr A23), a
drug known to inhibit cargo recruitment into CCP suggesting a role for DRP1A after CCP formation (Konopka and Bednarek, 2008a).

Despite the lack of a “classical” lipid-binding domain, *E. coli*-expressed, recombinant DRP1A has been shown to bind to liposomes that had a lipid composition resembling the plant plasma membrane (Backues and Bednarek, 2011). This purified DRP1A showed GTPase activity and formed polymers however, these were heterogeneous and neither responded to GTP addition nor showed membrane deformation activity (Backues and Bednarek, 2011). These observations suggest that specific protein modification (such as phosphorylation) and/or *in vivo* factors are required to modulate DRP1A oligomerization and functionality (Backues and Bednarek, 2011). The *in vivo* factors necessary for DRP1A function may be other DRP1 isoforms or DRP2 family members.

The *Arabidopsis* DRP2 gene family is composed of two members (DRP2A and DRP2B) that share 93% amino acid sequence identity (Bednarek and Backues, 2010; Taylor, 2011). Both isoforms are expressed throughout development and localize to the TGN, plasma membrane and cell plate (Jin et al., 2001; Hong et al., 2003; Fujimoto et al., 2007; Fujimoto et al., 2008 Bednarek and Backues, 2010; Taylor, 2011). *Arabidopsis drp2* single mutants display a wild-type phenotype and normal expression levels of the respective other DRP2 gene (Backues et al., 2010; Taylor, 2011). However, plants lacking both isoforms undergo early developmental arrest prior to the first mitotic cell division during female and male gametogenesis which suggests they are functionally redundant (Backues et al., 2010; Taylor, 2011).

Expression of an inducible dominant-negative version of DRP2A inhibited the internalization of FM4-64 in root hair cells (Taylor, 2011). DRP2A has previously been shown to associate with clathrin-coated structures in *Arabidopsis* pollen grains (Lam et al., 2002). In addition, analysis of foci dynamics at the cell cortex showed that DRP2B assembles and disassembles together with DRP1A at CLC foci (Fujimoto et al., 2010). Taken together these observations suggest that both DRP2 isoforms participate in CME.

Cells of *drp2a;drp2b* anthers show abnormally shaped cell plates and both DRP2 isoforms localize to the plane of division during somatic cytokinesis, implying a role for DRP2A and DRP2B in cell plate formation (Backues et al., 2010).
The DRP2B isoform was shown to co-localize with DRP1A at the plane of division during cytokinesis (Fujimoto et al., 2008). Moreover, Y2H assays revealed that DRP2A can interact with phragmoplastin (Hong et al., 2003) and DRP2B can interact with DRP1A (Fujimoto et al., 2010). These findings suggest that DRP1A and DRP2 isoforms work together during cell plate formation. Interestingly, in contrast with DRP1A, DRP2B did not self-interact in Y2H experiments (Fujimoto et al., 2010) and its presence at plasma membrane was unaffected by inhibition of cargo recruitment into CCP (Fujimoto et al., 2010). These observations suggest that DRP1A and DRP2B may not function in the same way though it’s conceivable to assume their functions may be complementary (Fujimoto et al., 2010).

Membrane sterols are required for the establishment of distinct domains within the membrane, which appears to be essential for the regulation of diverse processes such as cell polarity and cell division. The de novo creation of a membrane during cell division results from a temporal and spatial orchestration of membrane fusion and endocytosis. Defects in vesicle fusion or endocytosis result in cytokinesis defects. Interestingly, in mammals and plants, cytokinesis defects have been related to membrane sterols. However, the precise role of sterols in cell plate formation and ultimately, in the physical separation of two daughter cells still remains unknown.

AIM

The aim of this work was to understand how sterols affect de novo creation of a membrane during cytokinesis. To achieve this goal the following questions were addressed:

1. During cytokinesis, do sterols specifically affect proteins involved in cell plate formation?
2. Are proteins involved in cell plate formation associated with sterols in DRMs and potentially in “membrane rafts”?
3. Is there a functional requirement of sterol concentration and/or composition for proteins associated with cell plate “membrane rafts”?
RESULTS AND DISCUSSION

Cytokinesis defects can severely impact the normal development of an organism. In mammals, incomplete cytokinesis produces multinucleated cells that may develop into tumors (Norman and King, 2010). In plants, a strict spatial regulation of cytokinesis and consequently cell wall positioning is crucial for plant body architecture (Hong and Verma, 2007).

In various eukaryotes, cytokinesis defects have been related to membrane sterols. In sea urchin and zebrafish cells, cholesterol depletion interrupts cytokinesis (Ng et al., 2005, Atilla-Gokcumen et al., 2010). Furthermore, several Arabidopsis sterol biosynthesis mutants exhibit cytokinesis defects (Souter et al., 2002; Schrick et al., 2002; He et al., 2003; De Storme et al., 2013). However, the exact role of sterols in the physical separation of two daughter cells still remains unknown.

In this section I start by describing the tools used to modulate the sterols environment. Later, I will discuss the results obtained during this work and integrate them in the context of the present knowledge in the field.

Genetic and pharmacological tools used to modulate the composition and concentration of sterols in Arabidopsis

In Arabidopsis several sterol-biosynthesis mutants display an altered sterol profile. To address how membrane sterols affect cytokinesis we used the sterol biosynthesis mutant cpi1-1 (Figure 1G) as a genetic tool. This mutant has an altered sterol composition characterized by the accumulation of 9β,19-cyclopropylsterols intermediates (99% of the total sterol content; Men et al., 2008). Similar to other sterol-defective mutants (Souter et al., 2002; Schrick et al., 2002; He et al., 2003; De Storme et al., 2013) cpi1-1 shows numerous cytokinetic defects such as multinucleated cells and incomplete cell walls (Men et al., 2008).

Besides sterol-defective mutants, sterol biosynthesis inhibitors have been widely used to study sterols function (Hartmann, 1998). The effect of an inhibitor on the sterol profile depends on the target enzyme(s). If the target enzyme is part of the general isoprenoid pathway, its inhibition may cause a decrease in the total amount of free sterols and other isoprenoids. In contrast, inhibitors that act
downstream of cycloartenol (Figure 1) induce a replacement of the naturally occurring \( \Delta^5 \)-sterols (campesterol, sitosterol and stigmasterol) by biosynthetic intermediates. Two commonly used sterol biosynthesis inhibitors are fenpropimorph and lovastatin. Fenpropimorph is a broad-range inhibitor that primarily targets CPI1 but also affects the enzymes FACKEL, HYDRA1 and \( \Delta^7 \)-reductase (Schrick et al., 2004; Figure 1). As a result, seedlings treated with fenpropimorph show a decrease in the amount of \( \Delta^5 \)-sterols and predominantly accumulate 9\( \beta \),19-cyclopropylsterols (He et al., 2003; Men et al., 2008). Alternatively, lovastatin affects the activity of HMGR (Figure 1B) and for that reason it is used to induce a net reduction of the total sterol amount (Laule et al., 2003).

**Altered sterol composition affects KNOLLE localization during late cytokinesis**

In *Arabidopsis*, cell plate formation requires the fusion of vesicles in the plane cell of division, which is mediated by the syntaxin KNOLLE (Lukowitz et al., 1996; Lauber et al., 1997). In wild type, KNOLLE localization is restricted to the cell plate and to intracellular compartments of dividing cells. Conversion of the sterol profile induced either by *cpi1-1* mutation or treatment with fenpropimorph (fen; 200 \( \mu \)g/mL) induced a mis-localization of KNOLLE to the lateral plasma membrane (Paper I, Figure 1B and D). Together with the cytokinetic defects previously described for *cpi1-1* (Men et al., 2008), these results suggest that membrane sterols may influence cytokinesis by affecting KNOLLE localization in the plane of cell division.

**Diffusion of KNOLLE along the plane of cell division is not affected by sterol composition**

In *cpi1-1* and fen-treated cells, KNOLLE mis-localization is only observed when the cell plate has fused to at least one side of the plasma membrane. This implies that mis-localization of KNOLLE induced by changes in sterol composition, is unlikely to be a result of mistarget trafficking of vesicles carrying this syntaxin to the plasma membrane. In this scenario, KNOLLE should be observed at the plasma membrane at any stage starting from prophase, when KNOLLE is first expressed in dividing cells (Lauber et al., 1997). Sterols affect membrane fluidity and possibly the diffusion of proteins
within the lipid bilayer (Owen et al., 2012a). Thus, the sterol-induced lateral mis-localization of KNOLLE may result from an increased diffusion rate. Fluorescence-recovery-after-photo-bleaching (FRAP) experiments showed that KNOLLE can indeed diffuse from the cell plate to the plasma membrane through the cell plate-plasma membrane contact site (Paper I, Figure 5D-F). However, the lateral mobility of KNOLLE in cpi1-1 is similar to wild type (Paper I, Figure 5E-H), which suggests that altered sterol composition does not affect KNOLLE diffusion in the plane of cell division.

**KNOLLE endocytosis is affected by changes in membrane sterol composition**

Endocytosis has previously been implicated in the formation of the cell plate. During cytokinesis, a major portion of the membrane incorporated in the cell plate is removed probably, through endocytosis (Samuels et al., 1995; Otegui et al., 2001, Segui-Simarro et al., 2004). This material is then either targeted for degradation or, most likely, recycled back to the cell plate to ensure timely cell division.

The cpi1-1 mutant has previously been shown to be defective in the internalization of FM4-64 and PIN2 from the plasma membrane and dividing cells (Men et al., 2008). Interestingly, blocking of energy-dependent processes induced a lateral mis-localization of KNOLLE that resembled the cpi1-1 mutant phenotype (Paper I, Figure 5Q and Figure S5C). Such an energy-dependent process involved in the restriction of KNOLLE to the cell plate may be endocytosis. To address whether changes in sterol composition affect KNOLLE endocytosis we used brefeldin A (BFA). In Arabidopsis roots, BFA inhibits endocytosis to same extent (Naramoto et al., 2010) but mostly induces accumulation of internalized material in intracellular agglomerations termed BFA-compartments. These BFA-compartments represent accumulations of TGN, early and recycling endosomal compartments (Geldner et al., 2001; Geldner et al., 2003; Grebe et al., 2003; Demeter et al., 2006). Endocytosis is estimated by quantifying the amount of protein trapped at the cell plate and accumulated in the BFA compartments. Wild type and cpi1-1 seedlings were treated with BFA or the solvent control for 0, 60 and 90 minutes (min), in the presence of the protein biosynthesis inhibitor cycloheximide (CHX). For both genotypes, we observed very few cell
plates labeled with KNOLLE after 90 min treatment and therefore excluded this time point from the analysis. However, after 60 min, more KNOLLE label was retained in the plane of cell division in the cpi1-1 mutant than in the wild type (Paper I, Figure 6J-N) suggesting that KNOLLE internalization is affected by changes in sterol composition.

KNOLLE can diffuse laterally along the plane of cell division but it is rarely observed in the plasma membrane before the end of cytokinesis in the wild type. Altogether, these findings strongly suggest that KNOLLE lateral diffusion is counterbalanced by a sterol-dependent endocytosis from the plane of cell division.

Modifications of the amount and/or the structure of free sterols have previously been shown to induce endocytic defects in mammalian and yeast cells (Heese-Peck et al., 2002; Pichler and Riezman, 2004). Our findings suggest that in Arabidopsis membrane sterols affect cytokinesis by modulating KNOLLE internalization from the plane of cell division. During late cytokinesis, the whole circumference of the cell plate usually does not fuse evenly with the plasma membrane. At a point when the cell plate has just attached to one place of the plasma membrane, internalization of KNOLLE from mature regions and recycle to areas where membrane fusion is still necessary may be critical to complete fusion of the whole cell plate with the parental membrane.

Membrane sterol composition differentially affects the localization of cell plate-proteins

Interestingly, not all cell-plate-localized-proteins are affected by energy-dependent processes or changes in sterol composition. For example, the GREEN FLUORESCENCE PROTEIN (GFP)-DRP1A fusion protein does not display the strong ectopic lateral plasma membrane localization observed for KNOLLE in cpi1-1 (Paper I, Figure 1G-L). Moreover, in contrast to KNOLLE localization, blocking of protein biosynthesis and of energy-dependent processes had no visible effect on DRP1A-GFP and PIN2 localization at the cell plate or at the plasma membrane (Paper I, Supplementary figure 5A-L). Collectively, these findings suggest that a sterol-dependent endocytosis specifies KNOLLE localization to the cell plate during late cytokinesis.
Altered sterol composition and interference with components of the cytoskeleton affect the localization of KNOLLE in a different manner

During cytokinesis, membrane trafficking is directed towards the plane of division, along the phragmoplast (McMichael and Bednarek, 2013). Cytokinesis ends when the whole cell plate fuses with the plasma membrane at the site formerly defined by the PPB (Karahara et al., 2010). Given the importance of the cytoskeleton during cell division, we investigated the involvement of cytoskeleton components, microtubules and actin filaments, in the localization of KNOLLE.

The microtubule-associated protein 65-3 (MAP65-3; PLEIADE) is involved in the interaction of antiparallel microtubules at the phragmoplast (Müller et al., 2004). pleiade mutants (ple1 and ple N52166) displayed a localization of KNOLLE similar to the wild type (Figure 6A-C). However, although we did not quantify KNOLLE fluorescence, we consistently observed less KNOLLE accumulation at the cell plate in the two ple mutant alleles analyzed (Figure 6B-C) when compared to the wild type (Figure 6A). These observations are consistent with the proposed role of phragmoplast microtubules in the transport of vesicles to the plane of cell division. We further examined mutants defective in the CLIP-associated protein (CLASP), a regulator of microtubule dynamics that is involved in PPB formation and microtubule stability (Ambrose et al., 2007; Kirk et al., 2007). clasp-1 mutant displayed KNOLLE at the cell plate and endomembrane compartments resembling the wild type (Figure 6A and D). As both ple and clasp-1 mutants are defective in specific aspects of microtubule organization, we further tested how a more general defect in microtubule organization would influence KNOLLE localization. To this end, four-day-old wild type seedlings were treated with the microtubule destabilizing drug oryzalin (Sigma, Sweden; 1 μM) for 24 h. Oryzalin treatment severely affected both chromosome arrangement and cell division (Figure 6F). In contrast to control treatment with the solvent (DMSO; Figure 6E), KNOLLE was only observed in endomembrane compartments in oryzalin-treated seedlings (Figure 6F) similar to the observations made by Geldner and co-workers (Geldner et al., 2001). Collectively, these findings reveal that disturbance of the microtubule cytoskeleton affects the
localization of KNOLLE in a manner different from interference with sterol composition.

**Figure 6:** Interference with components of the cytoskeleton do not cause ectopic localization of KNOLLE to the lateral plasma membrane. (A-H) KNOLLE whole-mount immunofluorescence localization (red) in cytokinetic cells from five-day-old *Arabidopsis* seedlings of (A) wild-type Columbia-0 (Col-0), (B) ple-1, (C) ple Salk_N522169, (D) clasp-1. (E-G) four-day-old seedlings of (E) wild-type Col-0 treated for 24 h with 0.1% DMSO, (F) 1μM oryzalin, (G) 10μM Latrunculin B. (H) five-day-old act7-4 mutants seedlings. All mutants used were in Col-0 background. The ple-1 mutant allele was kindly provided by Prof. Marie-Theres Hauser (Müller et al., 2002). DNA stained with DAPI (blue). Scale bars are 5 μm.

The actin cytoskeleton has previously been implicated in endocytosis. In animals, actin is thought to be involved in the regulation of membrane curvature and to provide the pulling force that keeps the vesicle neck under tension, hence promoting dynamin fission activity (Doherty and McMahon, 2009). In plants, the role of actin in endocytosis is still unclear (Baral and Dhonukshe, 2012). However, actin has been implicated in the internalization of cell wall pectins in maize (Baluška et al., 2002) and in the internalization of PIN1 in *Arabidopsis* (Nagawa et al., 2012).

To address whether defects in the organization of the actin cytoskeleton affect KNOLLE localization, we treated four-day-old wild type seedlings with the actin depolymerizing drug latrunculin B
(Lat B; Sigma, Sweden; 10 μM) for 24 h. In Lat B-treated cells KNOLLE was observed at the cell plate and in intracellular aggregates that seemed to decorate the plasma membrane (Figure 6G). The KNOLLE signal detected at the cell plate of Lat B-treated cells was consistently weaker (Figure 6G) compared to control cells (Figure 6E) but nevertheless, no lateral mis-localization of KNOLLE was observed.

Since the ACTIN7 (ACT7) isoform had previously been implicated in cell division (Kandasamy et al., 2009), we studied KNOLLE localization in the act7-4 mutant. In act7-4 cells, we observed KNOLLE at the cell plate and in large cytoplasmic compartments but no lateral plasma membrane localization was observed (Figure 6H).

Our results show that genetic or pharmacological interference with actin microfilaments does not cause ectopic localization of KNOLLE at the plasma membrane during cytokinesis. The weaker KNOLLE signal found in the cell plate and its accumulation in intracellular compartments either decorating the plasma membrane (lat B-treatment) or close to the plane of division (act7-4) suggests a role for actin in: the delivery of KNOLLE to the plane of cell division; the recycling of KNOLLE from the plasma membrane to the plane of cell division or the recycling of KNOLLE at the cell plate.

Altogether, neither genetic nor pharmacological interference with the actin or the microtubule cytoskeleton caused defects in KNOLLE localization that resembled the lateral mis-localization observed upon interference with sterol biosynthesis.

**Interference with CME induces mis-localization of KNOLLE similar to cpi1-1 mutation**

Currently, the major endocytic pathway present in plants is the CME (Dhonukshe et al., 2007). Therefore we tested if KNOLLE is also internalized through this pathway. Indeed, pharmacological interference with cargo recruitment into CCP by employing the inhibitor tyr A23, induced a lateral mis-localization of KNOLLE that resembled the one observed in cpi1-1 (Paper I, Figure 7M-P). Moreover, endocytosis-defective drp1a mutants also showed KNOLLE ectopically localized to the plasma membrane in late cytokinetic cells (Paper I, Figure 7Q-S). These findings suggest that
KNOLLE is internalized through a clathrin- and DRP1A-dependent mechanism.

Analysis of drp1a;cpil1 double mutant revealed a synergistic interaction between DRP1A and sterols (Paper I, Figure 8) implying that sterols and CME may converge during cell plate formation before or during KNOLLE internalization. Interestingly, cholesterol-depletion in human cells was shown to reduce dynamin-2-dependent internalization of the amyloid-β precursor protein (APP; Cossec et al., 2010). Moreover, cholesterol reduction in chinese hamster ovary cells, reduced transferrin endocytosis by increasing the residence time of clathrin-coated pits at the cell membrane (Subtil et al., 1999). Together with our findings, these results suggest that membrane sterol content can modulate the localization of membrane proteins by affecting their clathrin-dependent internalization.

In brief, during late cytokinesis the syntaxin KNOLLE is specifically restricted to the cell division plane through a sterol-dependent endocytosis that involves a clathrin- and DRP1A-mediated mechanism. However, the link between membrane sterols and this clathrin- and DRP1A-dependent mechanism remains unclear.

In *Arabidopsis* the cell plate is a high-lipid order membrane domain

To further understand how membrane sterols influence cell plate development we decided to study the formation of membrane domains during cytokinesis. Sterols play a key role in the organization of membrane rafts (Xu and London, 2000; Simons and Sampaio, 2011). Membrane rafts are dynamic domains enriched in sterols and sphingolipids, biophysically characterized by a high membrane order/lipid packing (Xu and London, 2000; Pike 2006). Some raft-associated features have been found in the cell division plane of distinct eukaryotes such as fission yeast (Wachtler et al., 2003), echinoids (Ng et al., 2005) and mammals (Skop et al., 2004). However, membrane order during cytokinesis has not been addressed by employing high-lipid-order-sensitive probes. Therefore, we decided to study the importance of membrane organization during cytokinesis in *Arabidopsis* by measuring membrane order in dividing cells.
The degree of lipid packing/membrane order can be quantified using order-sensitive dyes such as di-4-ANEPPDHQ (Owen et al., 2012b). di-4-ANEPPDHQ is a membrane order fluorescence probe that exhibits a blue shift in emission for membranes in liquid-order (lo) phase relative to membranes in liquid-disordered (ld) phase, regardless of the size of the individual domains or the presence of proteins (Dinic et al., 2011; Owen et al., 2012b). Membrane order is quantified by calculating the generalized polarization (GP) value, which is a ratiometric measurement of the fluorescence intensity recorded in the detection channels 560 nm (green) for the lo phase and 620 nm (red) for the ld phase (Owen et al., 2012b). GP values closer to 1 indicate a higher membrane order (Owen et al., 2012b). Using this technique in dividing wild type cells of Arabidopsis, we observed a higher GP mean value for the cell plate compared to the plasma membrane (Paper II, Figure 1B left and D). Hence, our results identify the plane of cell division in Arabidopsis as a domain of high membrane lipid order.

High lipid-order of cell plate membranes in Arabidopsis is sterol-dependent

To further evaluate our observations that the cell plate represents a high lipid order membrane domain we labeled cpi1-1 mutant as well as seedlings treated with either fen or lovastatin (lov) with di-4-ANEPPDHQ. In contrast to the wild type and the control (DMSO), cpi1-1 and fen-treated cells showed similar GP mean values for the cell plate and the plasma membrane (Paper II, Figure 1B-D), suggesting a similar degree of lipid packing. In lov-treated cells, the cell plate displayed reduced degree of lipid packing in comparison to the control (Paper II, Figure 1D) while still showing higher lipid order than the plasma membrane. Interestingly, comparison between cell plate and plasma membrane GP values revealed that changes in sterol composition (cp1l-1 or fen-treatment; Paper II, Figure 1B left and D) or reduction in sterol concentration (lov-treatment; Paper II, Figure 1D) primarily affected the cell plate lipid order. Collectively, these findings strongly suggest that establishment of the cell plate as a high-lipid-order membrane domain is sterol-dependent.

The presence of high-lipid-order membrane domains has been associated with developmental processes that require a tight coordination between exocytosis and endocytosis such as pollen tube
growth (Liu et al., 2009) or formation of the mating projection in budding yeast (Bagnat and Simons, 2002b). Cytokinesis requires delivery of exocytic vesicles to the plane of cell division and removal of excess membrane through endocytosis during a short period of time (Otegui et al., 2001, Segui-Simarro et al., 2004). The lateral compartmentalization provided by the presence of high-lipid-order membrane domains might facilitate the synchronization and the efficiency of the different biological events involved in cell plate formation.

**Components of the CME machinery accumulate in DRMs and at the sterol-containing cell plate**

Membrane rafts are thought to act as platforms where specific proteins come together through cooperative interactions between proteins, sterols and sphingolipids (Cacas et al., 2012). It has previously been shown that the CME components DRP1A-, DRP2B- and CLC are not uniformly distributed but rather appear as mobile, dot-like foci in the plasma membrane of Arabidopsis root epidermal and tobacco suspension cells (Fujimoto et al., 2007; Konopka and Bednarek, 2008a; Fujimoto et al., 2010). Additionally, proteomic analysis has revealed the presence of CHC, CLC, DRP1A, DRP2A, and DRP2B in DRMs obtained from tobacco and Arabidopsis leaf or cell culture plasma membranes (Mongrand et al., 2004; Morel et al., 2006; Minami et al., 2009; Stanislas et al., 2009). To determine if components of the CME are preferentially associated with sterol-enriched domains we studied the protein composition of DRMs extracted from Arabidopsis wild type and cpi1-1 mutant.

The results obtained from the detergent solubilization method used in DRM extraction depend on several experimental factors such as the type and amount of detergent, detergent-to-lipid ratio, duration of the extraction, and temperature (London and Brown, 2000; Schuck et al., 2003; Simons and Gerl, 2010). Furthermore, the efficiency of detergent solubilization can be influenced by membrane lipid composition which varies according to the tissue type, developmental stage and environmental conditions (Schuck et al., 2003; Grennan, 2007; Simons and Gerl, 2010). Despite these drawbacks, analysis of DRM fractions provides a biochemical tool to estimate as to whether proteins can associate with sterol-rich membranes a feature common to high-lipid-order domains and membrane rafts. To reduce the
number of possible complications of tissue type and developmental phase we used undifferentiated cells from wild type and cpi1-1 mutant root callus cultures.

In our experiments, the CME components DRP1A, CLC, CHC, and ARF1 were found preferentially associated with DRMs in both wild type and cpi1-1 root callus cultures (Paper II, Figure 2A and C). These results reveal that components of the CME machinery are enriched in DRMs from root cells and likely have more affinity towards sterol-enriched domains in vivo.

Since sterols are key players in membrane lateral organization, mutations in the synthesis of these lipids may disrupt membrane raft formation (Grennan 2007). The ability to extract DRM fractions from cpi1-1 suggests that, despite its altered sterol profile, sterol-enriched domains are still formed in this mutant. Nevertheless, membrane rafts displaying abnormal sterol composition may be affected in their ability to recruit or to modulate protein conformation/function. However, no difference was observed for the localization of the tested CME components between wild type and cpi1-1 DRMs (Paper II, Figure 2A and C). This may in part be explained by the fact that the sterol composition profile of cpi1-1 root callus is affected to a lesser extent than that of cpi1-1 mutant roots (Paper II, Supplementary figure 2; Men et al., 2008). Nevertheless, our observations suggest that components of CME preferentially associate with DRMs and potentially with membrane rafts in Arabidopsis root cells.

Since KNOLLE is mis-localized in cpi1-1 late cytokinetic cells we decided to study its localization in DRMs extracted from cpi1-1. The KNOLLE syntaxin is specifically expressed during cell division. Hence, the KNOLLE protein observed in membrane fractions including DRMs is specifically derived from dividing cells, mostly from membranes harboring cell plate-forming material, recycled material from the PM and from the cell plate itself. KNOLLE was not found enriched in DRM fractions derived from wild type cell suspension cultures (Paper I, Figure 4). However, when we analyzed DRMs derived from root callus cultures of the wild type and the cpi1-1 mutant, KNOLLE accumulated in non-DRMs and appeared to be deprived from the DRM fraction in the mutant (Figure 7A). This observation might reflect the low membrane order found in the cell plate of cpi1-1 cells compared to the wild type (Paper II, Figure 1B-D). Hence, both approaches used for the study of membrane order,
Figure 7: Depletion of KNOLLE protein from DRMs in cpi1-1 mutant root callus cultures. (A) Western-blot analysis of DRM fractions extracted from three-week-old Arabidopsis callus cultures of wild-type Landsberg erecta (Ler) and cpi1-1 mutant (in Ler background). Equal amounts of membrane protein (5-7 µg) were loaded from the control fraction extracted at Triton X-100 detergent/protein (w/w) ratio 0 (Non-DRM) and the DRM fraction extracted at ratio 8. Immunoblot probed with antibodies against KNOLLE and the DRM-depleted marker protein SMT1. (B) Coomassie staining used as a loading control. Similar results were observed in the two independent biological experiments performed. Note, the western-blot and the Coomassie gel correspond to the one shown in Paper II, Figure 2A.

biochemical (DRM extraction) and biophysical (assessment of membrane order in live cells) suggest that conversion of the sterol profile negatively affects high-lipid order at the cell plate.

To study the association of CME components with sterol-rich membranes during cytokinesis, we labeled seedlings expressing DRP1A-GFP, CLC-GFP or DRP2B-GFP with filipin III. Filipin III is a polyene that forms fluorescence complexes with cholesterol or related sterols bearing a free 3’-OH group thus allowing the visualization of membrane sterols by fluorescence microscopy (Gimpl and Gehrig-Burger 2010; Boutté et al., 2011). Filipin III labeling revealed that sterols co-localize with DRP1A-GFP (Paper II, Figure 2E-G), DRP2B-GFP (Paper II, Figure 2H-J), and CLC-GFP (Paper II, Figure 2K-M) at the cell plate. Additionally, CLC-GFP co-localized with sterols in intercellular compartments (Paper II, Figure 2L), likely representing TGN/EE compartments (Ito et al., 2012).
Together with our biochemical analysis, these results suggest that components of the CME machinery preferentially associate with DRMs and localize to sterol-containing membranes during cytokinesis. In mammals, membrane rafts have been proposed as platforms for the assembly of CME machinery and development of CCP (Simons and Sampaio, 2011). Our results experimentally support such a role for a high-lipid-order membrane domain during plant cytokinesis.

Loss of individual CHC or DRP2 gene function does not affect KNOLLE localization or CPI-1 loss-of function

Our observations revealed that a clathrin- and DRP1A-mediated endocytic mechanism restricts KNOLLE to the cell division plane during cytokinesis. Therefore we further addressed whether loss of function of the two individual DRP2 genes (drp2a and drp2b; Backues et al., 2010) or the two individual CHC (chc1 and chc2; Kitakura et al., 2011) genes would also affect KNOLLE localization. However, the single mutants drp2a, drp2b, chc1 and chc2 displayed KNOLLE at the cell plate and endomembrane compartments similar to the wild type (Paper II, Supplementary figure 3A-F). Additionally, reciprocal crosses between drp2 or chc single mutants and cpi1-1/+ heterozygous plants revealed no seedling progeny in the F4 generation with a phenotype that deviated from the parental lines (Table I). Hence, in contrast to drp1a rsw9; cpi1-1 (Paper I, Figure 8), no obvious

<table>
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<th>cpi1-1-phenotype</th>
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<tr>
<td>expected (%)</td>
<td>observed (%)</td>
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<tr>
<td>Col-0</td>
<td>0</td>
</tr>
<tr>
<td>cpi1-1/+ (Col-0)</td>
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</tr>
<tr>
<td>chc1-2; cpi1-1/+</td>
<td>25</td>
</tr>
<tr>
<td>chc2-1; cpi1-1/+</td>
<td>25</td>
</tr>
<tr>
<td>chc2-2; cpi1-1/+</td>
<td>25</td>
</tr>
<tr>
<td>drp2a-1; cpi1-1/+</td>
<td>25</td>
</tr>
<tr>
<td>drp2b-2; cpi1-1/+</td>
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genetic interaction was observed between CPI1 and the individual
DRP2 or individual CHC genes. These findings can be explained by
the previously described redundancy within these two gene families as
shown by early lethality of the respective double mutants (Backues et al., 2010; Kitakura et al., 2011).

**DRP1A accumulation at the cell plate is sensitive to membrane sterol concentration and composition**

Pharmacological interference with sterol composition has
previously been shown to increase the residence time of DRP1A and
CLC foci at the cell cortex in Arabidopsis (Konopka and Bednarek,
2008b). Thus, we investigated whether altered sterol composition
would affect the localization of components of the CME machinery
during cytokinesis. In wild type, DRP1A mainly localized to the edges
of the forming cell plate but it was also present in endomembrane
compartments and at the plasma membrane (Paper II, Figure 3A). In
comparison to the wild type, more DRP1A accumulated at the cell
plate of cpi1-1 (Paper II, Figure 3B). Quantitative analysis of DRP1A
immunofluorescence at the plane of cell division corroborated these
observations (Paper II, Figure 3C). In contrast, the cell plate-localized
proteins DRP2B-GFP and CLC-GFP showed wild-type localization in
cytokinetic cells of the cpi1-1 mutant (Paper II, Supplementary figure
3J-R).

Genetic and pharmacological interference with membrane sterol composition induced lateral mis-localization of KNOLLE
(Paper I, Figure 1B and 1D). We obtained similar results for seedlings
treated with lov (Paper II, Figure 3E-H) or with a lower fen
centration (50 μg/mL; Paper II, Figure 3F-I) than previously used
(200 μg/mL; Paper I; Figure 1D). Thus, conditions that interfered with
cell plate membrane order induced lateral mis-localization of
KNOLLE, which prompted us to investigate DRP1A under similar
conditions. Compared to cells of the DMSO-treated control seedlings
(Paper II, Figure 3J and Supplementary figure 3S), more DRP1A
immunolabel and DRP1A-GFP signal accumulated at the cell plate of
fen-treated seedlings (Paper II, Figure 3K, Supplementary figure 3T).
In contrast, reduction in the total amount of sterols strongly decreased
anti-DRP1A or DRP1A-GFP fluorescence (Paper II, Figure 3L and
Supplementary figure 3U). Quantification of DRP1A fluorescence
intensity at the cell plate further supported our observations (Paper II,
Figure 3M and Supplementary figure 3V). However, although DRP1A-GFP seedlings treated with fen displayed a higher accumulation of DRP1A-GFP compared to the control this was not statistically significant. This increase in intensity was consistent with but not as pronounced as the significant difference observed for the endogenous protein upon fen treatment or in the cpi1-1 mutant background. This may reflect subtle differences between DRP1A-GFP and the native, endogenous protein.

Altogether, these findings imply a differential effect of sterol composition and concentration on the localization of DRP1A. A specific sterol environment such as the one found in membrane rafts, may be required for correct protein folding, activity or ability to interact with other factors (Pichler and Riezman, 2004; Cacas et al., 2012). For example, the activity of corn plasma membrane H⁺-ATPase (Grandmougin-Ferjani et al., 1997) and the Arabidopsis thaliana NOX (Liu et al., 2009) were shown to be dependent on membrane sterols. Our findings suggest that changes in sterol composition affect the release of DRP1A from membranes and likely DRP1A function during CME.

In mammals and plants, the concentration of sterols increases along the secretory pathway (Moreau et al., 1998; Simons and Sampaio, 2011; Paper I, Figure 2P). Theoretically, a reduction in the net sterol content may affect the establishment of such gradient and subsequently disturb the sorting of lipids and proteins at the TGN (Simons and van Meer, 1988; Simons and Sampaio, 2011). This interpretation is consistent with the decrease in lipid membrane order and in DRP1A signal observed at the cell plate of lov-treated cells (Paper II, Figure 1D and 3L). Alternatively a reduction in sterol content might affect post-translational modifications of DRP1A that may be required for membrane binding. Human dynamin 1 and DRP1 are known to undergo reversible phosphorylation (Heymann and Hinshaw, 2009) and in the case of dynamin1 this modification determines its cellular distribution (Liu et al., 1994a; Liu et al., 1994b). In Arabidopsis, the soluble form of DRP1A has previously been shown to be phosphorylated (Park et al., 1997). Additionally, changes in the membrane phosphoproteome have been reported for the hmgr1-1 mutant defective in the sterol biosynthesis enzyme targeted by lovastatin (Heintz et al., 2011). While DRP1A was not examined in this study (Heintz et al., 2011) similarly the
phosphorylation state of DRP1A may be affected in a low-sterol-content environment and potentially DRP1A subcellular localization.

$drp1a^{rsw9};knolle^{X37-2}$ double mutant analysis shows a synergistic interaction between DRP1A and KNOLLE

In *Arabidopsis*, the distribution of DRP1A at the cell plate (Lauber *et al.*, 1997; Kang *et al.*, 2003) resembles that of KNOLLE (Lukowitz *et al.*, 1996; Figure 8A-C). DRP1A is involved in the internalization of KNOLLE (Paper I, Figure 7R-S) and PIN2 proteins (Mravec *et al.*, 2011) from the cell plate and has previously been shown to interact with PIN2 at the cell plate. We therefore generated $drp1a^{rsw9};knolle^{X37-2}/+$ plants to study the potential genetic interaction between *DRP1A* and *KNOLLE* among their progeny.

**Figure 8:** Cell plate co-localization of DRP1A and YFP-KNOLLE and synergistic interaction between DRP1A and KNOLLE. (A-C) Cytokinetic cells of five-day-old seedlings. (A) anti-DRP1A (red) immunolocalization in roots of seedlings expressing (B) YELLOW FLUORESCENCE PROTEIN (YFP)-KNOLLE (green). Merged image of A and B (C). DNA staining by
DAPI (blue). Scale bars are 5 μm. Phenotypes of eight-day-old seedlings of (D) knolle<sup>x37-2</sup> and (E) drp1a<sup>rsw9</sup>;knolle<sup>x37-2/+</sup> F4 progeny.

 drp1a<sup>rsw9</sup> homozygous mutant seedlings, in the Col-0 background, are characterized by stunted and swollen roots (Collings et al., 2008). On the other hand, homozygous knolle<sup>x37-2</sup> seedlings (in Ler background) display a rather severe mutant phenotype that ranges from white tuber (German: Knote)-shaped (Figure 8D) to small, green, elongated individuals that develop root hairs (Lukowitz et al., 1996). Unexpectedly, analysis of the progeny of self-pollinated drp1a<sup>rsw9</sup>;knolle<sup>x37-2/+</sup> F3 plants revealed no individual with the characteristic knolle<sup>x37-2</sup> phenotype (Figure 8E; Table II). This observation was confirmed by PCR-based genotyping and dCAPS marker analysis, which revealed no double mutant among 205 seedlings (143/205 were heterozygous for the knolle<sup>x37-2</sup>mutant allele). Thus, the presence of two mutated copies of the DRP1A gene enhanced the knolle phenotype strongly suggesting a genetic interaction between DRP1A and KNOLLE genes.

Table II. Phenotypic analysis of F4 progeny derived from drp1a<sup>rsw9</sup>;knolle<sup>x37-2/+</sup> F3 parental plant

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<th>knolle&lt;sup&gt;x37-2&lt;/sup&gt;-phenotype</th>
<th>Number of seedlings</th>
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<tr>
<td></td>
<td>Expected (%)</td>
<td>Observed (%)</td>
</tr>
<tr>
<td>Col-0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ler</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>drp1a&lt;sup&gt;rsw9&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>knolle&lt;sup&gt;x37-2/+&lt;/sup&gt;</td>
<td>25</td>
<td>8.9</td>
</tr>
<tr>
<td>drp1a&lt;sup&gt;rsw9&lt;/sup&gt;;knolle&lt;sup&gt;x37-2/+&lt;/sup&gt;</td>
<td>25</td>
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The seedling-lethal phenotype of knolle<sup>x37-2</sup> homozygous mutants results from defective cell divisions observed already during embryogenesis (Lukowitz et al., 1996). Additionally, knolle;keule double mutants display even earlier, embryonic lethality with obvious cytokinetic defects from the two-cell stage (Waizenegger et al., 2000). Therefore, we traced back progeny of drp1a<sup>rsw9</sup>;knolle<sup>x37-2</sup> plants to the octant stage of embryogenesis to see whether they would exhibit...
early embryonic cytokinetic defects as observed for \textit{knolle;keule} double mutants (Waizenegger \textit{et al.}, 2000).

Figure 9: Absence of phenotypically \textit{knolle}^{X37-2} embryos in F4 progeny from \textit{drp1a}^{rs9};\textit{knolle}^{X37-2}/+ F3 plants suggests synergistic genetic interaction between DRP1A and KNOLLE. (A-R) Nomarski images of embryo whole-mount preparations as described by Llavata-Peris \textit{et al.}, 2013. (A-F) octant or dermatogen stage, (G-L) globular stage, (M-R) heart stage embryos of (A, G, M) wild-type Col-0, (B, H, N) Wild-type Ler, (C, I, O) \textit{drp1a}^{rs9} in Col-0, (D, J, P) \textit{drp1a}^{rs9} in Ler, (E, K, Q) \textit{knolle}^{X37-2} (\textit{kn}^{X37-2}), and (F, L, R) \textit{drp1a}^{rs9};\textit{knolle}^{X37-2}/+ (\textit{drp1a}^{rs9};\textit{kn}^{X37-2}) are depicted. Scale bars are 20 µm.

Similar to the wild type (Figure 9A, G and M), no embryonic defects were observed in the parental \textit{drp1a}^{rs9} line in Col-0
background (Figure 9 C, I and O) consistent with results obtained for the \textit{drp1a\textsubscript{adi1a}} allele in Wassilewskija (Ws) background (Kang \textit{et al}., 2001). Since all \textit{drp1a\textsubscript{rs}w9};\textit{knolle\textsubscript{X37-2}/+} plants had a Ler appearance and the phenotype resulting from loss of \textit{DRP1A} appears to be ecotype-dependent (Kang \textit{et al}., 2001; Collings \textit{et al}., 2008), we also examined embryos from plants carrying the \textit{drp1a\textsubscript{rs}w9} allele in Ler background. Similar to \textit{drp1a\textsubscript{rs}w9} in Col-0 background (Figure 9 C, I and O) and to wild type Ler embryos (Figure 9B, H and N), \textit{drp1a\textsubscript{rs}w9} mutant embryos in the Ler background showed normal development for the embryonic stages analyzed (Figure 9D, J and P). As previously reported (Lukowitz \textit{et al}., 1996), the proportion of mutant embryos among the progeny of \textit{knolle\textsubscript{X37-2}/+} plants was about 25\% (26.8\%; 41/153; Figure 9E, K and Q). Surprisingly, analyses of F4 embryos derived from F3 \textit{drp1a\textsubscript{rs}w9};\textit{knolle\textsubscript{X37-2}/+} plants did not revealed any \textit{knolle}-like embryo or enhanced embryonic defect (0/142; Figure 9F, L and R) suggesting that \textit{drp1a\textsubscript{rs}w9};\textit{knolle\textsubscript{X37-2}/+} double mutants are already impaired prior to octant stage or are gametophytic lethal.

These results further suggest a functional interaction between \textit{DRP1A} and \textit{KNOLLE}. Such interaction may be likely to facilitate \textit{KNOLLE} internalization from the plane of cell division.

Ultrastructural analysis of \textit{knolle} mutant embryos revealed unfused vesicles along the plane of cell division, which implies that vesicle traffic to the plane of division is not affected in this mutant (Lauber \textit{et al}., 1997). Indeed, Lauber \textit{et al}. (1997) showed that \textit{DRP1A} is normally localized to the cell plate in \textit{knolle} embryos. Conversely, we have shown that \textit{KNOLLE} is present at the cell plate in \textit{drp1a} cells in addition to its lateral mis-localization (Paper I, Figure 7Q-S). Together, these findings suggest that \textit{DRP1A} and \textit{KNOLLE} do not require each other to be targeted to the cell plate. Thus, if \textit{DRP1A} and \textit{KNOLLE} would interact directly this interaction would most likely occur at the cell plate, similar to observations on \textit{DRP1A} and PIN2 (Marvec \textit{et al}., 2011) and \textit{KNOLLE} and KEULE interaction (Park \textit{et al}., 2012).

**\textbf{DRP1A is involved in the lipid organization of the cell plate}**

Both \textit{drp1a} and \textit{cpi1-1} display endocytosis and cell division defects (Collings \textit{et al}., 2008; Men \textit{et al}., 2008) in addition to the lateral mis-localization of \textit{KNOLLE} observed during late cytokinesis (Paper I, Figure 1B and 7Q-S). Modifications of the sterol
environment that induced the lateral mis-localization of KNOLLE also affected the lipid order of the cell plate membrane and the localization of DRP1A during cytokinesis. Therefore, we decided to investigate whether DRP1A itself maybe involved in establishment of membrane lipid order using the order-sensitive dye di-4-ANEPPDHQ. In contrast to the wild type, the cell plate and plasma membrane showed similar GP mean values in *drp1a* mutants (Paper II, Figure 4B), implying a comparable degree of lipid packing. These results suggest that DRP1A function is required for high-lipid-order of cell plate membranes.

Despite some similarities with other eukaryotes there is no visible accumulation of sterols at the plane of cell division in *Arabidopsis*. This suggests that other factors besides sterols may be required to establish high-lipid-order at the cell plate. Endocytosis is known to regulate the plasma membrane protein and lipid composition (Pérez-Gomez and Moore, 2007) and membrane rafts have been speculated to be turned over constantly through endocytosis and membrane flow (Ovecka and Lichtscheidl, 2005). Mukherjee and Maxfield (2000) have proposed that each time a vesicle buds from a membrane some sorting of the lipid components occurs. Accordingly, a reduction of DRP1A accumulation at the cell plate by lov-treatment or accumulation of DRP1A caused by the *cpl1-1* mutation and fen-treatment decrease the cell plate lipid order. This may most likely result from defective endocytosis at the plane of cell division as demonstrated by the lateral mis-localization of KNOLLE observed for the three conditions. Thus, our findings suggest that DRP1A is involved in the establishment or maintenance of the cell plate membrane order through its endocytic activity.

Taken together, in *Arabidopsis* the cell plate functions as a high-lipid-order domain. Clustering of membrane sub-domains at the cell plate may facilitate the coordination between the different cellular processes necessary to physically divide the two daughter cells in a short period of time. Establishment of the cell plate as a high-lipid-order membrane seems to depend on sterols and DRP1A endocytic function.

**Presence of sterols at the vacuolar membrane, tonoplast**

In animals and plants, sterol concentration increases along the secretory pathway from the ER to the plasma membrane where they
primarily accumulate (Hartmann, 1998; Pichler and Riezman, 2004). Although biosynthetic cargo such as the vacuolar H\(^+\)-adenosinetriphosphatase (V-ATPase) is also delivered to the tonoplast (Reyes et al., 2011), the presence of sterols at the vacuolar membrane is poorly studied.

*Arabidopsis* V-ATPase is a multisubunit proton-pump whose localization at the tonoplast depends on the isoform of VHA-a, which is assembled into the multisubunit enzyme complex in the ER (Neubert et al., 2008). The VHA-a3 isoform targets the V-ATPase to the tonoplast. Interestingly, VHA-a3 has been detected in detergent resistant membranes (Keinath et al., 2010). Analyses of *Arabidopsis* roots labeled with filipin III showed a partial co-localization between filipin III-sterols and VHAa3-GFP at the tonoplast in both meristematic (Paper III, Figure 3A) and elongated cells (Paper III, Figure 3B). Furthermore, transmission electron microscopy (TEM) imaging revealed membrane deformations characteristic of filipin III-sterol complexes at the plasma membrane, TGN, multivesicular bodies and the tonoplast of *Arabidopsis* wild type root cells (Paper III, Figure 3C). Collectively, these findings show that sterols are also present in the tonoplast of *Arabidopsis* cells where they co-localize with the DRM-associated protein VHA-a3.

In budding yeast, imbalances in the sterol profile result in vacuolar fragmentation suggesting that vacuole fusion requires correct sterol composition (Pichler and Riezman, 2004). The yeast DRP homolog Vps1p has previously been shown to interact with the t-SNARE Vamp3p on vacuolar membranes and this interaction appears to be necessary for tonoplast homeostasis (Peters et al, 2004). Together with our results, these findings suggest that in *Arabidopsis* the tonoplast is a sterol-rich membrane likely displaying lateral organization. Hence, it will be interesting to investigate if this lateral organization exists and whether it may contribute to the regulation of vacuolar membrane fusion and/or fission similar to the situation at the cell plate.
CONCLUSIONS AND FUTURE PERSPECTIVES

The de novo creation of a membrane during cytokinesis results from the temporal and spatial orchestration of membrane fusion and endocytosis (Hong and Verma, 2007). Interestingly, both membrane fusion and endocytosis appear to be modulated by sterol components of membranes. This works showed that a correct sterol composition and concentration are required for the lateral organization of the cell plate as a high-lipid-order domain (Paper II). This domain likely functions as a platform for the assembly of components of the clathrin-dependent endocytosis. Thus, by regulating the assembly of components of CME and/or their activity, sterols modulate the localization of proteins, such as KNOLLE that are internalized by clathrin- and DRP1A-dependent mechanisms (Paper I).

The syntaxin KNOLLE is essential for homotypic vesicle fusion in the plane of cell division which ultimately leads to formation of the cell plate. A defect in the internalization of KNOLLE possibly affects the removal of this syntaxin from regions where fusion has already occurred. This may influence the pool of KNOLLE available at and/or recycled back to the plane of division for the further rounds of fusion necessary for the complete physical separation of the two daughter nuclei. Further experiments could be performed to strengthen the relation between membrane sterols, lipid order and endocytosis during cytokinesis. Several sterol-defective mutants display cytokinetic defects and hence could be studied with regards to the cell plate membrane order and KNOLLE internalization. For example, the *Arabidopsis smt1* mutants also show lateral mis-localization of KNOLLE in late cytokinetic cells (Paper I).

DRP1A endocytic activity appears to be essential for KNOLLE internalization (Paper I) and the establishment or maintenance of the cell plate membrane order (Paper II). Indeed, endocytosis has been proposed to regulate membrane lipid and protein composition. In the future, it would be interesting to study cell plate membrane order in other endocytosis-defective mutants and how that affects KNOLLE internalization from the plane of cell division.

Despite the lack of a “classical” lipid-binding domain, DRP1A is able to bind membrane lipids (Backues and Bednarek, 2011). Using sterol biosynthesis inhibitors we observed a differential response of DRP1A to sterol composition and concentration (Paper II). However,
how sterols affect the function or localization of DRP1A at a molecular level still remains unresolved. Our findings suggest that a correct sterol composition is required to release DRP1A from the cell plate (Paper II). Future experiments may be performed to assess whether, for example, a specific sterol composition is required for DRP1A oligomerization or GTPase activity.

We observed a strong genetic interaction between DRP1A and KNOLLE that resulted in synthetic lethality of drp1a:knolle double mutants prior to early embryogenesis (Paper II). However it is unclear whether DRP1A and KNOLLE proteins interact directly or if DRP1A is just part of the “endocytic-complex” that internalizes KNOLLE. Co-immunoprecipitation studies may be performed to address whether DRP1A and KNOLLE interact in a proteins complex. In addition GST pull-downs or Y2H assays may address whether the two proteins are able to interact directly. Additionally, it would be interesting to assess the effect of membrane sterols composition in the establishment of DRP1A and KNOLLE interaction. Fluorescence resonance energy transfer (FRET) or bimolecular fluorescence complementation (BiFC) experiments may reveal as to whether DRP1A and KNOLLE associate at the cell plate and whether this interaction is affected in a sterol-defective background such as the cpi1-1 mutant.

In yeast, tonoplast homeostasis appears to depend on sterols as well as on the coordinated activities of the yeast DRP homolog Vps1p and a t-SNARE Vamp3p (Peters et al., 2004). This work showed that in Arabidopsis, sterols are also found in the tonoplast (Paper III). Moreover, the tonoplast marker protein VHA-a3 has previously been shown to associate with DRMs, suggesting that lateral membrane organization may also be present at the vacuolar membrane. Hence, it would be interesting to address if in Arabidopsis, tonoplast homeostasis depends on membrane sterols.

This work shows that in Arabidopsis, cell plate formation results from the interplay between sterol-dependent lateral membrane organization, membrane fusion and endocytic machinery.

In plants, the development of the cell plate can be used as a system to study in vivo the mechanisms involved in membrane formation. In the future it would be interesting to investigate if the processes involved in cell plate formation are similar to those required
for the homeostasis of the lipid bilayer surrounding each organelle. If so, organelle cellular membranes could be used as a model to study \textit{in vivo} membrane formation in other eukaryotes.
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The contribution of the author Márcia Frescatada-Rosa (MFR) to the papers included in this thesis was as follow:


-MFR performed the practical work and analyzed the results required for Figure 1J-N and Supplementary figure 2A-L.

II. Márcia Frescatada-Rosa, Thomas Stanislas, Steven K. Backues, Ilka Reichardt, Shuzhen Men, Yohann Boutté, Gerd Jürgens, Thomas Moritz, Sebastian Y. Bednarek, and Markus Grebe. DYNAMIN-RELATED PROTEIN1A feeds back on high membrane lipid order during plant cytokinesis. Manuscript

-MFR performed the practical work and analyzed the results required for Figure 2A-M, Figure 3A-M and Supplementary figure 3A-V. MFR also wrote the manuscript together with MG.

III. Corrado Viotti, Falco Krüger, Christoph Neubert, Fabian Fink, Upendo Lupanga, Melanie Krebs, David Scheuring, Piers A. Hemsley, Yohann Boutté, Márcia Frescatada-Rosa, Susanne Wolfenstetter, Norbert Sauer, Stefan Hillmer, Markus Grebe, and Karin Schumacher. The endoplasmic reticulum is the main membrane source for biogenesis of the lytic vacuole in Arabidopsis. Accepted for publication in The Plant Cell

-MFR performed the filipin labeling experiments for Figure 3A-C including confocal laser scanning microscopy (CLSM) and TEM image acquiring and analysis.

Additionally, the author MFR performed all the practical work and analysis required for the figures presented in the results and discussion section of this thesis.