Analysis of Arabidopsis Dynamin-Related Protein 1 and 2 (DRP1 and DRP2) Families

by

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Abstract

Clathrin-mediated membrane trafficking is essential for multiple stages of plant development, including the processes of cytokinesis and polarized cell expansion. One key player in clathrin-mediated trafficking in animal systems is the polymerizing GTPase dynamin. In plants, two separate families of dynamin-related proteins are involved in clathrin-mediated endocytosis: the dynamin-related-protein 2 (DRP2) family of classical dynamins as well as the DRP1 family of dynamin-related proteins. In addition to their endocytic roles, members of the DRP1 and possibly the DRP2 family also play a distinct role in formation of the cell plate during cytokinesis. Chapter 2 of this thesis is a study of the fundamental properties of the non-classical dynamin-related protein DRP1A. Bacterially expressed, GTPase active DRP1A forms large homopolymers that can interact with negatively charged liposomes. However, these homopolymers do not form regular spirals, do not undergo dynamic disassembly and do not cause liposome tubulation, suggesting that additional factors or modifications are necessary for DRP1A’s in vivo function. Chapter 3 of this thesis examines the role of the DRP2 family in plant development, as illuminated by analysis of drp2a and drp2b double insertional mutants, which display an early arrest during both male and female gametophyte development, demonstrating that the DRP2 family is independently essential for plant development. Unlike pollen from the drp1C-I mutant, arrested drp2ab gametes do not display defects in plasma membrane morphology, suggesting that the DRP2 family may function in a distinct membrane-trafficking pathway. The appendices to this thesis include preliminary work on putative DRP1A interacting proteins, the generation of a DRP2-GFP construct for live imaging, and the groundwork for an investigation of clathrin light chain localization and function.
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<th>Description</th>
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<tbody>
<tr>
<td>AICAR</td>
<td>5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside</td>
</tr>
<tr>
<td>amiRNA</td>
<td>artificial microRNA</td>
</tr>
<tr>
<td>CCP</td>
<td>clathrin-coated particle</td>
</tr>
<tr>
<td>CHC</td>
<td>clathrin heavy chain</td>
</tr>
<tr>
<td>CLC</td>
<td>clathrin light chain</td>
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<tr>
<td>CME</td>
<td>clathrin-mediated endocytosis</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DOPC</td>
<td>dioleoyl-phosphatidyl choline</td>
</tr>
<tr>
<td>DOPE</td>
<td>dioleoyl-phosphatidyl ethanolamine</td>
</tr>
<tr>
<td>DOPG</td>
<td>dioleoyl-phosphatidyl glycerol</td>
</tr>
<tr>
<td>DOPS</td>
<td>dioleoyl-phosphatidyl serine</td>
</tr>
<tr>
<td>DRP</td>
<td>Dynamin Related Protein</td>
</tr>
<tr>
<td>EM</td>
<td>electron microscopy</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FG1-7</td>
<td>female gametophyte developmental stages 1-7</td>
</tr>
<tr>
<td>FP</td>
<td>fluorescent protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GST</td>
<td>glutatione-S-transferase</td>
</tr>
<tr>
<td>LSCM</td>
<td>laser scanning confocal microscopy</td>
</tr>
<tr>
<td>MBP</td>
<td>maltose binding protein</td>
</tr>
<tr>
<td>PM</td>
<td>plasma membrane</td>
</tr>
<tr>
<td>PMM</td>
<td>plasma membrane mimetic</td>
</tr>
<tr>
<td>PH</td>
<td>pleckstrin homology</td>
</tr>
<tr>
<td>PIP</td>
<td>phosphatidylinositol phosphate</td>
</tr>
<tr>
<td>PI(4,5)P₂</td>
<td>phosphatidylinositol-4,5-bis-phosphate</td>
</tr>
<tr>
<td>PRD</td>
<td>proline rich domain</td>
</tr>
<tr>
<td>Soy PC</td>
<td>soybean phosphatidyl choline</td>
</tr>
<tr>
<td>SPT</td>
<td>single-particle tracking</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TEV</td>
<td>tobacco-etch-virus protease</td>
</tr>
<tr>
<td>TGN</td>
<td>trans-Golgi network</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>VAEM</td>
<td>variable angle epifluorescence microscopy</td>
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Chapter 1: The DRP1 and DRP2 families in plant endocytosis and development

Portions of this chapter are adapted from:

Backues, S.K. and Bednarek, S.Y. The Illuminated Plant Cell – The Dividing Cell
http://www.illuminatedcell.com/celldiv.html


Plant Cytokinesis and Cell Expansion

Two of the most fundamental processes in plant development are cytokinesis, by which new cells are formed, and cell expansion, by which existing cells grow and establish their functional morphology. Both processes require enormous amounts of membrane dynamics – targeted trafficking of membrane material to the site of growth, localized membrane fusion and proper organization of this membrane into either a new or preexisting cell boundary, and the removal of excess material as the membrane matures (Fig. 1). Formation of the cell wall occurs concomitant with membrane growth, both by deposition of preformed materials carried in the fusing vesicles as well as by on-site synthesis of carbohydrate polymers. Both processes are supported by an extensive cytoskeletal network, which both directs vesicular transport and
fusion as well as physically supporting and positioning the newly forming plasma membrane and cell wall.

Given the fundamental similarities between cytokinesis and cell expansion, it is not surprising that both processes utilize much of the same cellular machinery. Many proteins have roles in both cytokinesis and cell expansion, and many in fact appear to play essentially the same role in both places. At the same time, cytokinesis and cell expansion are not identical, and there are also other proteins and protein families that participate in only one process, or whose contribution in both is not yet clear (Backues et al., 2007; Bednarek and Falbel, 2002).

**Cytokinesis: Cell Plate Formation**

The last step in cell division, after the chromosomes have been separated during mitosis, is cytokinesis, the physical separation of the cell contents in order to form two new daughter cells. In plant cells, cytokinesis is accomplished by the *de novo* synthesis of a new plasma membrane and cell wall, termed the cell plate. The stages of somatic cell plate development include (1) creation of the phragmoplast, an array of microtubules that guides and supports the formation of the cell plate; (2) trafficking of vesicles to the division plane and their fusion to generate a tubular-vesicular network; (3) continued fusion of membrane tubules and their transformation into membrane sheets upon the deposition of callose, followed by deposition and organization of cellulose and other cell wall components; (4) recycling of excess membrane and other material from the cell plate; and (5) fusion with the parental cell wall (Otegui and Staehelin, 2000; Samuels et al., 1995; Segui-Simarro et al., 2004).

The phragmoplast is assembled from the remnants of the mitotic spindle in late anaphase, after chromosomal segregation is completed. Like the spindle, the phragmoplast is an
antiparallel array of microtubules oriented with their minus ends towards the poles of the cell and their plus ends towards the cell midline. Unlike the spindle, the microtubules of the phragmoplast do not overlap at their plus ends, but instead terminate in the phragmoplast midzone. The phragmoplast serves both as a track for the trafficking of vesicles to the phragmoplast midzone and a scaffold upon which the cell plate is assembled. These vesicles contain lipids, proteins and carbohydrates needed for the formation of a new cell boundary. In the phragmoplast midzone the vesicles undergo fusion to form a new organelle, the cell plate, which grows laterally, guided by the phragmoplast, until it fuses with the existing plasma membrane and matures into a new plasma membrane and cell wall. During this process, the phragmoplast microtubules are continually disassembled at its center, where the cell plate is more mature, and reassembled at its periphery, so that it grows outwards, ahead of the leading edge of the cell plate (Backues et al., 2007; Otegui et al., 2005; Sasabe and Machida, 2006; Segui-Simarro et al., 2004).

The first stage in the formation of the cell plate itself is the fusion of secretory vesicles at the phragmoplast midzone. Electron tomographic studies have identified the Golgi-apparatus as the source of these vesicles (Otegui et al., 2001; Segui-Simarro et al., 2004), but other studies have suggested that they contain endocytosed material as well (Baluška et al., 2005; Dhonukshe et al., 2006). They may also correspond to a recently identified trans-Golgi/endosomal membrane compartment labeled with RAB-A2/A3, which are necessary for proper cell plate formation (Chow et al., 2008). The fusion reaction itself is mediated by a machinery that includes KNOLLE, a syntaxin (Lauber et al., 1997), and its regulator KUELE (Assaad et al., 2001), as well as the additional SNARES, NSPN11 (Zheng et al., 2002) and AtSNAP33 (Heese et al., 2001).
The initial vesicle fusion events give rise to dumbbell-shaped membrane structures which have been proposed to grow by fusion with additional vesicles and each other into a tubular network (Fig. 1A). As the cell plate matures, these tubules widen and fuse laterally with each other, eventually forming a planar, fenestrated sheet (Segui-Simarro et al., 2004). Vesicle fusion and the first stages of maturation occurs initially at various points within the phragmoplast midzone, but these isolated domains subsequently fuse with each other and the entire cell plate grows outwards as it matures. The growing edges of the cell plate follow the expanding phragmoplast, with microtubules being at all times associated with areas of the cell plate rich in vesicles and tubular network, but not the flat surface of the planar fenestrated sheet (Segui-Simarro et al., 2004). As the cell plate matures, large amounts of membrane material are removed via clathrin-mediated endocytosis (Otegui et al., 2001) (Fig. 1B). Eventually, the edges of the cell plate fuse with the parental plasma membrane, often in an asymmetrical fashion (Cutler and Ehrhardt, 2002; Kang et al., 2003a), thus completing cytokinesis. The remaining fenestrae contain strands of endoplasmic reticulum passing through them, and are thought to be the precursors of plasmodesmata (Segui-Simarro et al., 2004).

The construction of the new cell wall begins within the lumen of the narrow tubules of the young cell plate. The order in which different cell wall components are deposited has been determined largely by immuno-electron microscopy (Samuels et al., 1995). The first components to arrive are pectic polysaccharides, hemicelluloses, and arabinogalactan proteins carried by the secretory vesicles that fuse to form the cell plate (Staehelin and Moore, 1995). These vesicles may carry both newly synthesized polysaccharides as well as polysaccharides derived from the mature cell wall and recycled by endocytosis (Baluška et al., 2005). The next component to be added is callose, a long-chain β-1,3-D-glucan polysaccharide that is
polymerized directly at the cell plate by callose synthases. CalS1, an Arabidopsis callose synthase, and UGT1, a UDP-glucose transferase, both localize to the cell plate when expressed in cultured tobacco cells (Hong et al., 2001; Hong et al., 2001). The highest levels of callose at the cell plate are observed as the narrow membrane tubules broaden and fuse laterally into a wide tubular network and, subsequently, a planar fenestrated sheet (Samuels et al., 1995). The force for this broadening may in fact be provided by callose polymerization, implying that the forming cell wall can act as an “internal” scaffold to stabilize the cell plate membrane (Samuels et al., 1995). As the cell plate continues to mature and fuses with the parental plasma membrane, the callose is slowly replaced with cellulose (Samuels et al., 1995), a long-chain β-1,4-D-glucan polysaccharide that is a primary component of a mature cell walls. The endo-1,4-β-glucanase KORRIGAN, an enzyme involved in cellulose synthesis, localizes to the cell plate when expressed in cultured tobacco cells, and plants lacking KORRIGAN show cytokinetic defects (Nicol et al., 1998).

**Polarized Cell Expansion**

Plant cell expansion has been historically divided into distinct categories: diffuse growth and tip-directed growth. Diffuse growth is the dominant mechanism in most cell types of the plant, and is characterized by the insertion of new membrane homogeneously around the surface of the cell. Tip-directed growth, in contrast, is characteristic of highly polarized cell types such as pollen tubes and root hairs, and involves addition of new membrane material only at the actively growing tip. Yet, despite these significant differences, both processes appear to involve some of the same machinery (Guimil and Dunand, 2007; Mathur, 2006).
Both diffuse and tip-directed cell expansion require the simultaneous modification of the cell wall, the plasma membrane, and the underlying cytoskeleton (Mathur, 2006). The cell wall provides a barrier that resists turgor pressure and prevents cell expansion, and its loosening by expansins and perhaps other cell-wall modifying enzymes (Cosgrove, 2005) is an essential part of cell growth. It has long been known that the orientation of the cellulose microfibrils that make up the cell wall is the primary determinant of the axis of cell expansion in root and hypocotyl cells, and more recently shown that expansins and xyloglucan endotransglucosylases (Baluška et al., 2000; Vissenberg et al., 2001) help determine the site of root hair initiation as well. The cytoskeleton also appears to play a mechanical role in controlling the location of cell expansion: cortical microtubules and actin bundles are found in non-growing areas, whereas a fine, dynamic actin meshwork characterizes actively growing regions of trichomes, root hairs, pollen tubes, and epidermal pavement cells (Mathur, 2006).

The upstream regulators of cytoskeletal arrangement and polarized growth are a network of signaling pathways that include Rho GTPases (Fu et al., 2005; Molendijk et al., 2001), phosphoinosittide (Böhme et al., 2004; Vincent et al., 2005) and other lipid (Ohashi et al., 2003) signals, and, in the case of tip-directed growth, a calcium gradient (Bibikova et al., 1997). Downstream of the decision of where to grow, the growth itself requires the addition of new membrane material by Rab- (Preuss et al., 2004) and exocyst- (Cole et al., 2005) mediated membrane fusion. Cell wall formation at the growing tip is primarily the result of deposition of pectin and other Golgi-synthesized polymers carried by the fusing secretory vesicles (Bernal et al., 2008; Li et al., 1994; Parre and Geitmann, 2005). In pollen tubes, subsequent desterification of the pectin by tip-localized pectin-methylesterases leads to increasing structural rigidity along
the flanks of the tube, which is essential for maintaining tip growth and preventing bursting (Bosch et al., 2005; Jiang et al., 2005; Tian et al., 2006).

**Endocytosis in Cytokinesis and Cell Expansion**

Both cytokinesis and cell expansion are fundamentally exocytic processes involving the secretion of a new cell wall and membrane components. However, the reverse process, endocytosis (Fig. 2), also plays a significant role. Electron tomographic analysis of the changes in the surface area of the cell plate during different stages of maturation has shown that ~75% of membrane delivered to the cell plate is eventually recycled (Otegui et al., 2001). Consistent with this, numerous clathrin-coated vesicles are observed budding from maturing portions of the cell plate in EM images (Otegui et al., 2001; Segui-Simarro et al., 2004), and clathrin-light-chain-GFP localizes to the mature, central portion of the growing cell plate (Fig. 1B) (Konopka et al., 2008). Similarly, a comparison of the volume of cell wall that is delivered during tip-directed growth with the area of membrane that is added, standardized to the observed size of exocytic vesicles, led to the estimate that 80% of the membrane delivered to the tips of growing pollen tubes, and 85% of that delivered in growing root hairs, is retrieved via endocytosis (Ketelaar et al., 2008). This estimate is very similar to the estimated 80% membrane retrieval in Tradescantia pollen tubes arrived at by looking at rates of secretory vesicle production from the Golgi (Picton and Steer, 1983). Clathrin-Mediated Endocytosis (CME) is clearly involved in this retrieval, as evidenced by the immunolocalization of clathrin heavy chain at the tip of growing pollen tubes (Blackbourn and Jackson, 1996) and the significant number of clathrin-coated structures that can be visualized by electron microscopy along the flanks at the subapical region (Derksen et al., 1995) (Fig. 1D). However, estimations of the volume of these clathrin-coated structures suggest
that CME is not sufficient to account for all of the membrane retrieval (Derksen et al., 1995), and recent reports suggest that smooth vesicle endocytosis at the extreme apex may play a significant role (Bove et al., 2008; Zonia and Munnik, 2008). A different investigation of endocytosis in tobacco pollen tubes using positively and negatively charged nanogold probes revealed the presence of at least two functionally distinct endocytic pathways. One, which specifically bound negatively charged nanogold, was active at the apical tip and sent its cargo to the vacuole for degradation. Another, which bound positively charged nanogold, was active primarily along the flanks of the pollen tube, just behind the growing tip, and the resulting vesicles were primarily targeted to the Golgi for another round of exocytosis (Moscatelli et al., 2007). In contrast to the reports implicating smooth vesicle endocytosis at the extreme apex, this study found that the subapical pathway accounted for the bulk of endocytosis as measured by uptake of FM4-64 (Moscatelli et al., 2007). Clearly there are multiple endocytic pathways at work in growing pollen tubes, and more study will be necessary to resolve the sites of action, protein requirements and functional significance of each.

It should not be surprising that multiple endocytic pathways are at work, because endocytosis during polarized growth must fulfill multiple functional roles. Clearly one major role is the retrieval of excess membrane material made necessary by the simple topological fact that cell wall material carried on the inside of secretory vesicles must be of sufficient volume to coat the outside of the plasma membrane once those vesicles fuse. In addition, any time membrane fusion takes place there must be selective retrieval of SNARES and other proteins involved in the fusion reaction. Furthermore, polar growth in both root hairs and pollen tubes is defined by the polar localization of a number of protein determinants, as well as chemical gradients presumably maintained by plasma-membrane localized transporters. In many other
systems, selective endocytosis is a critical aspect of the maintenance of the asymmetric localization of plasma-membrane associated polarity determinants, and endocytosis is required for polar distribution of PIN proteins in other tissues of the plant (Dhonukshe et al., 2008; Dhonukshe et al., 2007; Geldner et al., 2003; Steinmann et al., 1999). Therefore it is reasonable to assume that the maintenance of polarity is another functional role of endocytosis in cells expanding in a polarized manner (Campanoni and Blatt, 2007).

Similarly, one of the primary roles of clathrin-mediated membrane retrieval at the maturing cell plate must be the removal of excess membrane that is necessitated by the smoothing out of the cell plate as it matures (Otegui et al., 2001). At the same time, this pathway presumably also contributes to the removal of the syntaxin KNOLLE, which is targeted via multivesicular bodies to vacuoles for degradation during the later stages of cytokinesis (Reichardt et al., 2007), and other elements of the vesicle fusion machinery from the developing cell plate. More generally, the protein and lipid composition of the cell plate during the first stages of its formation is likely to be different than the composition of the mature plasma membrane, and selective removal of proteins and lipids during the course of cell wall maturation is probably a necessary part of that transition.

**Endocytosis in Plant Development**

CME has many other roles in plant development besides membrane retrieval during cytokinesis and cell expansion. Although our understanding of plant CME lags far behind our understanding of CME in yeast and animals, and it has only recently begun to be directly studied, it is clear already that it plays important roles in plant signaling and development (Geldner and Jürgens, 2006; Irani and Russinova, 2009). Only a few proteins to date have been characterized
as endocytic cargo, but their diversity is suggestive of a wide range of cargo types and endocytic routes (Geldner and Jürgens, 2006).

One protein that was shown to be regulated by endocytosis is the boron transporter, BOR1, which is internalized and degraded in the presence of excess boron (Takano et al., 2005). Ligand-mediated internalization and subsequent degradation has also been shown for FLS2, a leucine-rich-repeat receptor-kinase that activates defense signaling when stimulated by the pathogen-associated peptide flg22 (Robatzek et al., 2006). Endocytosis in this case is presumably important for attenuation of the defense response by reducing levels of the receptor; however, it is possible that it is also necessary for the initial signaling response. Another leucine-rich-repeat receptor-kinase, BRI1, is the receptor for the plant hormone brassinosteroid, which regulates photomorphogenisis as well as many other aspects of plant growth and development (Howell, 1998). BRI1 also undergoes endocytosis, but in a constitutive manner, independent of treatment with brassinosteroid. In this case endocytosis is not involved in receptor downregulation, as neither rates of endocytosis nor trafficking to the vacuole were affected by brassinosteroid treatment; instead, BRI1 localization to the endosome is necessary (and even partially sufficient) to activate downstream brassinosteroid signaling responses (Geldner et al., 2007).

Constitutive endocytosis is also essential for the proper function of the PIN auxin-efflux-carriers in auxin signaling. Auxin is perhaps the most important plant hormone, as gradients of auxin regulate plant morphogenesis and patterning beginning as early as the developing embryo sac (Pagnussat et al., 2009) and continuing through embryogenesis and into virtually every tissue of the adult plant (Howell, 1998). Auxin gradients are maintained to a large extent through the action of auxin efflux carriers of the PIN family, which localize to the plasma membrane in a
polarized manner, leading to directional auxin transport (Wisniewska et al., 2006). The establishment and maintenance of the polar localization of the PIN proteins is dependent on their constitutive endocytosis and recycling via a class of early endosomes defined by the presence of GNOM, an ARF-GTPase exchange factor. If this recycling is interfered with, either in a GNOM mutant or by treatment with the GNOM inhibitor brefeldin A, or by expression of a dominant negative form of another small GTPase, Rab5A, PIN loses its polar localization, leading to developmental defects. (Dhonukshe et al., 2008; Geldner et al., 2003; Steinmann et al., 1999). PIN internalization can be blocked by the overexpression of a dominant-negative fragment of clathrin or treatment with the drug Tyrphostin A23, confirming the requirement for CME in PIN polarization (Dhonukshe et al., 2007).

Many other plasma-membrane proteins likely also undergo clathrin-mediated internalization and recycling, although, as with the examples above, the details might be different in each case. For example, another auxin transporter, AUX1, has also been shown to be internalized and recycled, but via a different class of endosomes than PIN1, with different sensitivities to various inhibitors (Kleine-Vehn et al., 2006). In addition, a number of other plasma-membrane localized proteins, including a plasma-membrane localized water channel, PIP2, and a plasma-membrane ATPase, have been shown to undergo constitutive recycling, although in these cases the function of this recycling has not been investigated. Also, there are many plasma-membrane localized receptors in Arabidopsis, some of which contain tyrosine (Yxxφ, where φ is a bulky hydrophobic residue) or dileucine ([ED]xxxL[LI]) motifs, which act as clathrin-dependent sorting signals in animals (Irani and Russinova, 2009). Similarly, the cell-plate specific endo-1,4-β-glucanase KORRIGAN requires a dileucine motif in its cytoplasmic tail for proper localization to the cell plate during cytokinesis (Zuo et al., 2000), and the cell-
plate specific syntaxin KNOLLE requires endocytosis to restrict its localization to the cell plate (Boutté et al., 2009).

My expectation is that many, if not most, plasma-membrane localized proteins undergo endocytic recycling, with functional consequences related to localization, regulation and signaling. Furthermore, the mechanistic details of this recycling and its regulation may be different in every case, necessitating the existence of multiple distinct, though overlapping, endocytic pathways.

**Endocytic Machinery**

The production of a clathrin-coated vesicle is a multi-stage process involving large numbers of accessory and regulatory proteins (Fig. 2). Many of these proteins interact with each other via low-affinity interactions; this network of low-affinity interactions acts as a series of checkpoints, ensuring that every aspect is in place before the vesicle is released (Schmid and McMahon, 2007). The absence of any critical molecule can lead to abortive events as the vesicle fails to pass a checkpoint (Mettlen et al., 2009a), whereas the high number of interactions present in a successfully maturing vesicle – and, in particular, the “matricity” provided by the polymerization of clathrin – eventually makes the process irreversible (Schmid and McMahon, 2007).

Endocytosis has been extensively studied in mammalian and yeast systems; of these two, clathrin-mediated endocytosis in mammals, with its strict dependence on clathrin, cargo receptors and dynamin, seems more similar to what is known so far about endocytosis in plants than does yeast endocytosis, where actin appears to play a more central role.
In mammals, the central hub of CME is adapter complexes, such as AP-2, which bind to endocytic cargo, clathrin, and the majority of accessory proteins. Clathrin triskelia, each composed of three heavy chains (CHCs) and three light chains (CLCs), are a second hub, likewise interacting with many accessory proteins (Schmid and McMahon, 2007). In addition, clathrin polymerizes into a protein coat that provides a scaffold for the progression of bud formation and likely contributes to membrane deformation.

Most accessory proteins, such as the eps proteins, bind either AP-2 or clathrin or both, as well as other accessory proteins. In this way they function as “clustering proteins,” contributing to network formation (Schmid and McMahon, 2007). Many accessory proteins, such as amphiphysin, endophilin, epsin, and sorting nexin, also have the ability to induce membrane curvature, and may contribute to membrane deformation as well (Itoh and De Camilli, 2006). Some accessory proteins also bind the lipid phosphatidylinositol-4,5-bis-phosphate (PI(4,5)P$_2$), which is found in relatively high concentrations throughout the inner leaflet of the plasma membrane (Insall and Weiner, 2001) and may also be synthesized directly at endocytic sites by phosphoinositol-4-phospate-5-kinase-γ (PIP5K-γ), one isoform of which interacts with AP-2 (Nakano-Kobayashi et al., 2007). PI(4,5)P$_2$ functions in the recruitment and stabilization of many of CME accessory proteins at the plasma membrane, and thereby has a positive role in clathrin bud maturation. The destruction of PI(4,5)P$_2$ by phosphoinositol phosphatases such as synaptojanin (Slepnev and De Camilli, 2000) acts to destabilize the complex, either during bud maturation where destabilization acts as part of a checkpoint by promoting the abortion of non-productive buds (Antonescu et al., 2009), or later, after vesicle release, where destabilization aids in uncoating of the vesicle by the ATPase HSC70 (Cremona et al., 1999). Another accessory...
protein which plays both a regulatory and mechanical role in CME is dynamin, which will be discussed in more detail later.

Both dynamin as well as other accessory proteins recruit regulators of the actin cytoskeleton, and actin polymerization plays a role in vesicle release from the plasma membrane (Kaksonen et al., 2006; Merrifield et al., 2005). Although much of the actin machinery is conserved in plants, some key proteins that link endocytosis to actin polymerization in other organisms are missing, such as dynamin-binding cortactin (Cao et al., 2003; McNiven et al., 2000) and the yeast complex Pan1p/End3p/Sla1p (Tang et al., 2000; Warren et al., 2002).

The two central hubs of the CME network, AP-2 and clathrin, are both conserved in plants. AP-2 subunits have been identified in Arabidopsis by sequence homology (Holstein, 2002), and functionally by the use of the drug TyrphostinA23, which interferes with the interaction between AP-2 and its cargo, and has been shown to inhibit endocytosis in plants as it does in animals (Dhonukshe et al., 2007; Konopka et al., 2008). Both CHC and multiple CLCs have also been identified by sequence homology (Holstein, 2002; Scheele and Holstein, 2002). Antibodies raised against CHC label endocytic structures, and the overexpression of a dominant negative portion of CHC inhibits endocytosis (Dhonukshe et al., 2007). Similarly, a fluorescently-tagged CLC shows dynamic localization to sites of endocytosis as viewed by Variable Angle Epifluorescence Microscopy (VAEM) (Konopka et al., 2008).

In addition to these two central hubs, many, but not all, of the mammalian accessory proteins also appear to be conserved. Two homologues of Eps proteins have been found in Arabidopsis and shown to be involved in endocytosis (Bar et al., 2008). Similarly, three homologues of Epsin have been described (Holstein and Oliviusson, 2005), although the two investigated so far localize to the Golgi and other endomembrane compartments and appear to be
involved in trafficking to the vacuole (Lee et al., 2007; Song et al., 2006). No direct homologues of endophilin or amphiphysin have been found. However, Arabidopsis does contain three proteins, SH3P1-3, that share with endophilin and amphiphysin a C-terminal SH3 domain, which functions in protein-protein interactions. SH3P1 has been shown to colocalize with clathrin and bind auxilin, an accessory protein involved in uncoating. Unlike endophilin and amphiphysin, the SH3P proteins do not contain a membrane-deforming BAR domain at their N-terminus, but they do have a conserved alpha-helical N-terminus that may possibly serve a similar membrane-binding role; the SH3P1 N-terminus has been shown to bind phosphatidic acid, PI(4)P and PI(4,5)P₂ in spotted lipid overlay assays (Lam et al., 2001).

Resting levels of PI(4,5)P₂ in higher plants are about 10-fold lower than in animal cells, but are induced during osmotic stress (Meijer and Munnik, 2003; van Leeuwen et al., 2007), suggesting that PI(4,5)P₂ in plants may function less as a general plasma membrane marker and more as an induced signal. PI(4,5)P₂ is concentrated at the tips of growing root hairs (van Leeuwen et al., 2007; Vincent et al., 2005) and pollen tubes (Kost et al., 1999), and clearly has a function in polarized growth. Its role in growth has not been entirely deciphered, but it seems to be involved in the tip-directed calcium gradient and cytoskeletal regulation, although some of these effects may be mediated not directly by PI(4,5)P₂ but instead by inositol-trisphosphate and phosphatidic acid, which are generated by the cleavage of PI(4,5)P₂ by phospholipase C (Krichevsky et al., 2007; Meijer and Munnik, 2003). A role for PI(4,5)P₂ in plant endocytosis has not been demonstrated.
Clathrin-Mediated Trafficking at the Endosome

After an endocytic vesicle has been released from the plasma membrane and its clathrin coat removed, it fuses with the early endosome. From here vesicle contents are sorted to either be recycled back to the plasma membrane or sent to the late endosome followed by the lysosome/lytic vacuole for degradation. In animals, the early endosome appears to be a distinct compartment defined by the presence of the small GTPase Rab5 (Rink et al., 2005). In plants, the situation appears to be rather more complicated, and the nature of the early endosome has been a longstanding mystery. Recent work has suggested that three compartments described by different methodologies are actually one and the same: The early endosome, defined as the structure labeled by the endocytic tracer FM4-64 within a few minutes of its application; the partially coated reticulum, a cluster of bulbous membrane structures defined by its coated appearance in electron micrographs, and the trans-Golgi network (TGN), an organelle defined by its proximity to the trans-face of the Golgi, often as visualized at the light microscopy level with the use of various fluorescent markers (Dettmer et al., 2006; Lam et al., 2007; Lam et al., 2007; Robert et al., 2008). The presence of both early endocytic and late exocytic functions in the same physical structure suggests that a mingling of endocytic, exocytic and recycling cargos might take place. Such a mingling would be consistent with the still-controversial reports that endocytosed cell wall material, along with newly synthesized material, is integrated into the forming cell plate (Baluška et al., 2005; Dhonukshe et al., 2006).

As suggested by the term “partially coated reticulum,” the plant TGN/endosomal compartment is a site of clathrin-mediated trafficking. Electron tomographic studies have shown that clathrin-coated buds are present beginning at the early TGN, and that late TGN compartments fragment into a mixture of smooth and clathrin coated vesicles (Staehelin and
Kang, 2008). Consistent with this, a native-promoter driven CLC-GFP shows prominent localization to the TGN, as well as the plasma membrane, in Arabidopsis roots (Konopka et al., 2008) (Fig. 1C). Clathrin is also present at the TGN in animal cells, where it functions in trafficking to the lysosome (Griffiths and Simons, 1986). Clathrin-mediated trafficking at TGN in animals requires some of the same accessory proteins as CME, such as dynamin-1 (Jones et al., 1998), but also involves distinct interactors, such as the AP-1 (as opposed to the AP-2) cargo-adapter complex and PI(4)P (Wang et al., 2003b). The study of two TGN-localized Arabidopsis Epsins has suggested that clathrin-mediated trafficking at the TGN in plants may be involved in sorting of cargo to the vacuole (Lee et al., 2007; Song et al., 2006), which would be analogous to the proposed role in animal cells. However, some differences are apparent, such as the involvement of PI(3)P (Lee et al., 2007) and much of the relevant machinery remains to be defined.

The Dynamin Superfamily

One of the key players in animal endocytosis is dynamin, a large, polymerizing GTPase that serves as the founding member of the dynamin superfamily (Fig. 3A). Dynamin superfamily members are found throughout eukaryotes as well as some bacterial species, and serve many functions besides endocytosis (Low and Löwe, 2006; Praefcke and McMahon, 2004). All dynamin superfamily members contain an N-terminal globular GTPase domain as well as central and usually a C-terminal coiled-coil polymerization domain. Many superfamily members also contain some sort of membrane binding domain, although the nature of this domain can vary from a full transmembrane helix, as in the case of Fuzzy Onion (FZO) (Hales and Fuller, 1997), to a membrane-inserting hydrophobic paddle in the case of bacterial dynamin-like protein (Low
and Löwe, 2006) to a phospholipid-binding PH domain as in animal dynamin. Some family members have no recognized membrane-binding domain at all. Consistent with their domain structure, all dynamins studied to date share the ability to hydrolyze GTP and to polymerize into rings and spirals, and many, although not all, bind membranes and preferentially assemble onto membrane templates (Praefcke and McMahon, 2004).

Dynamin superfamily members function in a variety of cellular roles, most of which involve in some sort of membrane remodeling reaction. For example, the membrane-tethered FZO mediates mitochondrial fusion (Hermann et al., 1998), whereas the peripheral membrane DMN1 functions in the final stages of mitochondrial division by polymerizing around the division site and promoting membrane scission (Bleazard et al., 1999; Ingerman et al., 2005). In plants, Dynamin Related Protein 5 (DRP5) and Dynamin Related Protein 3 (DRP3) mediate chloroplast and peroxisome division, respectively (Gao et al., 2003; Mano et al., 2004). In yeast, the dynamin superfamily members Vacuolar Protein Sorting 1 (VSP1) and DMN1 both function in peroxisome division (Kuravi et al., 2006), and VPS1 is also involved in both vacuolar fusion and vacuolar fission, and more generally in maintenance of vacuolar morphology, although the exact mechanisms of its action are not clear (Kuravi et al., 2006; Röthlisberger et al., 2009).

Animal dynamin is essential for many forms of endocytosis including clathrin-mediated and caveolar endocytosis (Henley et al., 1998; Oh et al., 1998). It has been primarily studied in the context of CME, where it appears to play at least two distinct roles: an early, regulatory role in bud formation and invagination, and a later, mechanical role in bud scission (Loerke et al., 2009; Mettlen et al., 2009b; Narayanan et al., 2005; Sever et al., 2000; Song et al., 2004). Dynamin undergoes a regulated cycle of action, where GTP binding stimulates polymerization, polymerization stimulates GTP hydrolysis, and GTP hydrolysis in turn stimulates disassembly of
the polymer (Warnock and Schmid, 1996). This regulated transition between soluble and polymerized forms is important for dynamin’s early regulatory role in CME, although the mechanistic details of its function at this step are unclear (Mettlen et al., 2009b). More mechanistic work has been directed at understanding dynamin’s late role in CME, where it polymerizes into rings around the neck of fully formed clathrin-coated buds and promotes membrane scission (Macia et al., 2006; Takei et al., 1995). Dynamin polymerizes around and thereby tubulates liposomes in vitro (Chen et al., 2004; Danino et al., 2004; Roux et al., 2006; Stowell et al., 1999; Zhang and Hinshaw, 2001), and, upon addition of GTP, undergoes a conformational change (Stowell et al., 1999; Sweitzer and Hinshaw, 1998b; Zhang and Hinshaw, 2001) which can cause membrane scission (Danino et al., 2004; Sweitzer and Hinshaw, 1998b). How exactly dynamin couples GTP hydrolysis to membrane scission is still an area of ongoing research; the most recent studies suggest that membrane scission is not the result of a concerted conformational change as much as it is a stochastic process of collapse of the narrow membrane tubule upon repeated rounds of dynamin assembly, constriction and disassembly (Bashkirov et al., 2008; Pucadyil and Schmid, 2008).

Animal dynamin is a “classical” dynamin, as defined by the presence of two domains in addition to the conserved GTPase and self-assembly domains: a membrane-binding Pleckstrin Homology (PH) domain, and a C-terminal Proline Rich Domain (PRD). Dynamin’s PH domain binds PI(4,5)P₂, which is essential for its localization to the plasma membrane (Achiriloaie et al., 1999; Salim et al., 1996), and also inserts a variable loop into the membrane bilayer, which is necessary for dynamin’s liposome tubulation activity (Ramachandran et al., 2009). Dynamin’s PRD interacts with a variety of SH3-containing CME-accessory proteins, and is therefore presumably important for network formation during CME (Mettlen et al., 2009b; Schmid and
McMahon, 2007). Interestingly, although both the PH domain and PRD are essential to the function animal dynamin in CME, nonclassical dynamins lacking both the PH domain and the PRD have been shown to be involved in CME in both Tetrahymena (Elde et al., 2005) and Dictyostelium (Wienke et al., 1999).

**Dynamins in Arabidopsis**

There are six major families of Dynamin Related Proteins (DRPs) in Arabidopsis, most of which have had at least a preliminary functional characterization (Fig. 3B). Like other organisms, Arabidopsis has a small family of dynamins, DRP3A and 3B, involved in mitochondrial and peroxisomal division (Arimura et al., 2004; Arimura and Tsutsumi, 2002; Hong et al., 2003; Logan et al., 2004; Mano et al., 2004). DRP5B is required for chloroplast division (Gao et al., 2003), probably functioning in a manner analogous to DRP3, whereas DRP5A was recently shown to have a novel function in cytokinesis. The mechanistic details of DRP5A’s function remain to be determined, but it is expressed in a cell cycle-dependent manner and initially appears during prophase as bright spots around the nucleus, which subsequently become localized to the division plane during cytokinesis (Miyagishima et al., 2008), and *drp5a* null mutants display defects in cell plate maturation in root cells, although only at elevated temperatures. The DRP4 family has four members (although three of these may be pseudogenes) and shares homology with mammalian Mx proteins (Hong et al., 2003). Mammalian MxA has antiviral activities (Haller and Kochs, 2002), while MxB functions in nuclear import (King et al., 2004), but to date there is no published study of DRP4. Arabidopsis also contains a Fuzzy Onion homologue, but instead of mitochondrial fusion, it appears to be involved in maintenance of chloroplast morphology (Gao et al., 2006).
Two separate families of Dynamin Related Proteins play a role in clathrin mediated trafficking in Arabidopsis: the DRP2 family of classical dynamins and the DRP1 family of plant-specific dynamins. The DRP2 family has two members, DRP2A and DRP2B, which are 93% identical at the amino acid level and are both evenly expressed throughout development, suggesting redundant function. Although the DRP2s share only ~20% amino acid identity with mammalian dynamin 1, they are similar in domain structure and organization, containing both a PH domain and a C-terminal SH3 domain, and are therefore categorized as classical dynamins, with a presumptive role in clathrin mediated trafficking. In contrast, the DRP1 family contains only the three protein domains conserved in all dynamin superfamily members, and no recognized membrane- or protein-binding domains (Fig. 3B). However, recent studies have demonstrated that it too is involved in clathrin-mediated endocytosis (Collings et al., 2008; Konopka and Bednarek, 2008; Konopka et al., 2008). The DRP1 family contains 5 members, which share 79% amino acid sequence identity throughout their length. Each DRP1 family member shows distinct expression profiles throughout development, and two of the DRP single mutants, drp1a-2 and drp1c-1, display distinct phenotypes (Kang et al., 2001; Kang et al., 2003a; Kang et al., 2003b). Moreover, DRP1C expressed under the DRP1A promoter cannot rescue the drp1a-2 phenotype, suggesting that these two family members have distinct functions at the protein level (Konopka and Bednarek, 2008). Therefore, there are at least three functionally distinct classes of DRPs putatively involved in CME: The DRP2 family, DRP1A/1E, and DRP1C.
DRP1 Proteins in CME

Mammalian dynamin is critically dependent on its membrane-binding PH domain and protein-interacting PRD for its localization to and appropriate function at sites of CME. Therefore we were surprised to discover that the DRP1 family, which lacks both the PH domain and PRD and has no other recognized membrane or protein interaction domains in their place, was also involved in CME.

Early hints for the DRP1s’ endocytic role came from studies of single DRP null mutants. The *drp1c-1* mutant is male gametophytic lethal, with mutant pollen grains showing a striking proliferation of PM sheets after pollen mitosis II, and then collapsing upon desiccation (Kang et al., 2003b). This severe defect in plasma membrane morphology implicated DRP1C in maintenance of the PM, possibly via some sort of membrane trafficking pathway. Similarly, the *drp1a-2* mutant, under some growth conditions, displays an early seedling arrest which may be related to defects in cell expansion. Under other growth conditions (the inclusion of 1% sucrose in the growth media or growth on 1% instead of 0.6% agar), the *drp1a-2* mutant shows relatively normal overall plant morphology, but has specific defects in two highly polarized cell types: trichomes, which are normally tribranched, are predominantly bibranched in the mutant, and stigmatic papillae cells, which are normally elongated, instead expand isotropically, leading to a severe fertility defect (Kang et al., 2001; Kang et al., 2003a). Isotropically expanded *drp1a-2* stigmatic papillae, similar to *drp1c-1* mutant pollen, have a proliferation of plasma membrane, again suggesting a defect in plasma membrane maintenance (Kang et al., 2003a). The case for an endocytic role of DRP1A was strengthened by the isolation of another *DRP1A* mutant allele, *drp1a*<sup>rsw9</sup>. Like *drp1a-2*, *drp1a*<sup>rsw9</sup> is a null allele, but it is in the Columbia ecotype instead of the Wassilewskijia ecotype, and shows a polarized cell expansion (root swelling) phenotype that is
distinct from, although likely related to, the seedling arrest seen in \textit{drp1a-2}. Relative to WT, \textit{drp1a}^\textit{rsw9} seedlings have cell walls with reduced cellulose content and decreased endocytic uptake of the lipophilic sterol dye, FM4-64 (Collings et al., 2008).

The strongest evidence for DRP1A and -1C’s role in CME comes from analysis of fully functional, native promoter-driven DRP1A and 1C- fluorescent proteins (FPs). DRP1-FPs localize to the cell plate, cytoplasm and plasma membrane, and in the case of DRP1C-GFP, the plasma membrane localization is strongest in areas thought to be experiencing high rates of endocytosis, such tip of pollen tubes and the flanks, just behind the tip, of actively growing root hairs (Konopka and Bednarek, 2008) (Fig. 1D). At the PM, DRP-FPs form dynamic foci that appear and disappear and resemble sites of endocytosis labeled by dynamin-1 in mammalian cells. Clathrin-Light-Chain 2-FPs (CLC2-FPs) form PM foci with a similar appearance, and ~95% and ~80% of these foci colocalize with DRP1C-FP and DRP1A-FP, respectively. Moreover, the dynamics of DRP1A, -1C and CLC-FP foci are halted by treatment with the AP2 inhibitor tyrphostin A23, verifying that these foci represent sites of CME (Konopka and Bednarek, 2008; Konopka et al., 2008). Interestingly, while these studies were being performed, it was reported that a Tetrahymena dynamin-related-protein, DRP1 (not directly homologous to Arabidopsis DRP1) was likewise involved in CME, even though it also lacked a PH domain and PRD (Elde et al., 2005). The authors of this paper suggested that dynamin had been independently recruited to a role in CME multiple times during evolutionary history, a hypothesis that is supported by our finding of a CME function for the DRP1 proteins in Arabidopsis. However, questions still remain regarding how DRP1 is targeted to sites of CME, given that is has neither a recognized membrane binding domain nor any known interactions with other CME players. Also, dynamin plays both early regulatory and late mechanical roles in
mammalian CME, as well as participating in a variety of protein-protein interactions at the forming bud and acting as a recruiter of the actin polymerization machinery. Do DRP1s play all of these same roles, or only a subset thereof? These questions await further analysis.

**DRP1 in Cell Plate Formation**

In addition to defects in plasma membrane maintenance and cell expansion, DRP1 mutants also show prominent defects in cytokinesis. Curved cell plates resulting in incomplete or misplaced cell walls are seen in \textit{drp1a} \textsuperscript{rsw9} mutants (Collings et al., 2008), and \textit{drp1a-2/drplc} double mutants have an embryo lethal phenotype, with multiple cytokinetic defects including incomplete cell walls and multinucleate cells (Kang et al., 2003a).

CME is important for cell plate formation because \textasciitilde75\% of membrane delivered to the cell plate is recycled by clathrin-mediated trafficking during cell plate maturation (Otegui et al., 2001). In addition, DRP1A-mediated endocytosis at the plasma membrane during late stages of cytokinesis is important for restricting the localization of the cell-plate specific SNARE, KNOLLE, to the cell plate (Boutté et al., 2009). The same may also be true for the endo-1,4-\(\beta\)-glucanase KORRIGAN, which contains endocytic motifs essential for its cell plate localization (Zuo et al., 2000), and perhaps other cell-plate specific proteins. Therefore, the cytokinetic defects seen in \textit{drpl} mutants may be related to DRP1s role in CME. However, other evidence points to a distinct role of the DRP1 family in cell plate formation. In particular, the localization of DRP1A and -1C at the cell plate is not consistent with an exclusive role in endocytic recycling. Recycling takes place at the central, maturing regions of the cell plate, and, consistent with this, CLC2-FPs localize to the central region of the cell plate (Konopka and Bednarek, 2008) (Fig. 1B). DRP1-FPs localize with CLC2-FP to the cell plate center, but show an even
stronger presence at the leading edges of the cell plate, where CLC2-FPs are not present, and are also recruited to the cell plate earlier during its formation than CLC2-FPs (Kang et al., 2003a) (Fig. 1A). Electron tomographic studies of cell plate formation during endosperm cellularization found electron dense rings and spirals encircling constricted membrane tubules during the tubular-vesicular network phase of cell plate formation. These rings, which were not associated with clathrin-coated buds, labeled positive for DRP1A by immuno-EM (Otegui et al., 2001). During somatic cytokinesis, similar rings and spirals were seen both during the tubular-vesicular network phase and also on the dumbbell-shaped intermediates formed by the very first rounds of vesicle fusion at the forming cell plate, although the presence of DRP1A on the dumbbell-shaped intermediates was not verified (Segui-Simarro et al., 2004).

If DRP1s have a function at the cell plate that does not involve clathrin, what might it be? One suggestion is that the DRP1s might have a role in molding the membrane, not promoting scission but maintaining the tubular nature of the membrane or helping to stretch the products of vesicle fusion into their characteristic dumbbell shape (Segui-Simarro et al., 2004). Alternatively, DRP1 could be acting to localize other cell-plate-associated enzymes, either by direct interactions (e.g. GLS6; see section on DRP1 interactions below) or by restricting diffusion between different membrane domains.

The existence of these DRP1 containing rings raises not only functional but also mechanical questions: for example, how is DRP1 recruited to the membrane, since it has no recognized membrane-binding domain? Is its membrane association via interaction with other membrane-associated protein partners, or through a novel, uncharacterized membrane-binding domain? More generally, how is DRP1 recruited to the cell plate (and how does this compare to its recruitment to sites of CME at the PM) and how is its activity regulated? Finally, why do the
DRP1-containing rings not appear to be mediating any sort of membrane scission, unlike what has been observed for other dynamin superfamily members – is this the result of the presence or absence of specific interacting proteins, or a fundamental characteristic of DRP1A itself?

**DRP1-interacting proteins**

To date, only three DRP1-interacting proteins have been described, two of which are enzymes involved in callose synthesis at the cell plate. Callose, a β-1,3-glucan polymer, is a major polysaccharide component of the developing cell plate whose deposition precedes the synthesis of cellulose (Samuels et al., 1995). It has been suggested that callose deposition within the developing cell plate provides a spreading force that widens the tubules and converts the network into a fenestrated sheet (Samuels et al., 1995; Staehelin and Hepler, 1996). Two callose synthases, GSL6 and GSL8 (Chen et al., 2009; Hong et al., 2001), have been found to be associated with the cell plate and defects in GSL8 results in cytokinetic abnormalities (Chen et al., 2009). The soybean homologue of DRP1, GmDRP1, interacts with GSL6 and with a cell plate-specific UDP-glucose transferase (Hong et al., 2001; Hong et al., 2001). Thus DRP1 may help to localize enzymes required for callose biosynthesis at the cell plate through their direct interaction with membrane-associated DRP1. Alternatively, DRP1 may function in the recycling of these enzymes via endocytosis.

The other reported DRP1A interactor is VAN3, an ARF-GAP that likely regulates membrane trafficking at the TGN, and was found to interact with DRP1A via yeast-2-hybrid and co-immunoprecipitation assays (Koizumi et al., 2005; Sawa et al., 2005). Both loss-of-function van3 (Koizumi et al., 2005) and drp1a-2 mutants (Sawa et al., 2005) display defects in vascularization. The van3/drp1a-2 double mutants either fail to germinate or show enhanced
defects in vascular structure relative to the single mutants, indicating a genetic interaction between \textit{DRP1A} and \textit{VAN3} (Sawa et al., 2005). Consistent with a role of DRP1A in ARF-dependent trafficking, \textit{DRP1A} was shown to genetically interact with \textit{GNOM} (Sawa et al., 2005), which encodes an ARF-GEF involved in membrane recycling between the PM and endosomes (Geldner et al., 2003). Sawa and colleagues postulate based on transient expression analysis of DRP1A- and VAN3-FPs in protoplasts that these proteins function coordinately in vesicle trafficking at the TGN. However, stably expressed DRP1A-FPs under their native promoter in \textit{Arabidopsis} roots do not show significant TGN localization, leaving it unclear whether DRP1A’s interaction with VAN3 at the TGN is only transient, or whether DRP1A and VAN3 are actually functioning together at a different subcellular compartment.

**DRP2 Subcellular localization**

DRP2A, like animal dynamin, has a PH domain, which may play a role in its subcellular targeting. This domain in isolation binds PI(4,5)P\textsubscript{2} as well as PI(3)P and PI(4)P (Lam et al., 2002; Lee et al., 2002), but full length DRP2A shows a preference for PI(3)P, with less binding to PI(4)P and no binding to PI(4,5)P\textsubscript{2} in lipid-overlay assays. The binding to PI(3)P and PI(4)P was verified by liposome sedimentation (Lee et al., 2002). PI(3)P is reported to localize to the endosomes (Vermeer et al., 2006), while PI(4)P is found at the Golgi, the PM and the newly formed cell plate (Vermeer et al., 2008). A DRP2A-interacting protein, \textit{AtSeh1}, binds to the C-terminus of DRP2A thereby blocking its interaction with PI(3)P (Lee et al., 2006), suggesting that DRP2A’s \textit{in vivo} localization and/or function may be regulated in part by controlling its membrane association.
The first report of the subcellular localization of DRP2 used antibodies generated against the C-terminal half of DRP2A for immunolocalization in cryosections of liquid-grown root tips, as well as overexpression of DRP2A-GFP under a viral promoter in Arabidopsis protoplasts. Under these conditions, DRP2A was seen at cytosolic puncta that colocalized with Golgi markers (Jin et al., 2001) (Fig. 1C). This would be somewhat consistent with the interaction of DRP2A with PI(4)P, but less so with PI(3)P, unless the puncta reported as Golgi in this paper also represent TGN/endosomes. More recent reports using an overexpressed DRP2B-GFP in Arabidopsis roots (Fujimoto et al., 2008) as well as exogenously expressed DRP2A-GFP (Hong et al., 2003) and DRP2B-GFP (Fujimoto et al., 2007) in tobacco suspension-cultured cells have seen DRP2 primarily at the plasma membrane and the forming cell plate, with perhaps also some localization to the endosomes. Like DRP1A and -1C-FPs, DRP2B-GFP formed dynamic foci at the plasma membrane reminiscent of sites of endocytosis (Fujimoto et al., 2007). Interestingly, DRP2B-FPs do not localize exclusively to the center of the maturing cell plate like CLC-FPs, but instead to the entire plate, especially the leading edges, more similar to the DRP1-FPs (Fig. 1A). In fact, despite their completely different targeting domains, DRP2B-GFP and DRP1A-RFP show complete temporal colocalization at the forming cell plate when exogenously expressed in tobacco suspension-cultured cells (Fujimoto et al., 2008). Moreover, both DRP2-GFP and DRP1-GFP show significant localization to the TGN/endosomes in Arabidopsis protoplasts (Jin et al., 2001; Sawa et al., 2005), but not in whole roots (Fujimoto et al., 2008; Kang et al., 2003a; Konopka and Bednarek, 2008). This suggests that the localization of both dynamin-families is affected by the enzymatic removal of the cell wall, which is not surprising for proteins putatively involved in endocytic trafficking. However, these results leave it unclear whether the DRP1s or DRP2s function primarily at the TGN/endosomes or at the plasma membrane, or an equal
mixture of both, and whether or not these two protein families show any real differences in their localizations/sites of function.

Still, the overall picture that emerges from DRP2-GFP localization studies is that DRP2 is found at the plasma membrane, cell plate, Golgi and TGN/endosomes. This is consistent with immuno-EM localization of DRP2 in developing pollen grains, which likewise showed it to be associated with the plasma membrane, Golgi, and TGN/endosomes, and to colocalize with clathrin at all of these localizations (Lam et al., 2002). However, the details of DRP2-FP localization differ between studies (Fujimoto et al., 2008; Hong et al., 2003; Jin et al., 2001), and the variety of constructs, tissue types and even organisms used makes it unclear what is responsible for this variation. Moreover, none of the DRP-FPs yet tested are known to be functional. Ultimately the only way to resolve these quandaries will be with the use of native-promoter-driven, full length FP-tagged constructs stably expressed in whole Arabidopsis plants, whose functionality has been verified by complementation analysis.

**DRP2A-interacting CME-accessory proteins**

In addition to interacting with PI(4,5)P$_2$ via its PH domain, dynamin also interacts with various CME-accessory proteins, many of which contain SH3-domains that bind dynamin’s C-terminal PRD (Schmid and McMahon, 2007). Three closely related proteins with SH3 domains are found in Arabidopsis, and one of these, ArSH3P3, binds DRP2A’s C-terminal PRD. In addition, DRP2A also binds γ-adaptin, a subunit of the AP-1 complex involved in clathrin-mediated trafficking at the Golgi in animals, through another PxxP motif found in DRP2A’s GED (Lam et al., 2002).
**DRP2 Cellular Function?**

Overall, the research to date suggests that DRP2 is probably involved in clathrin-mediated trafficking, as expected from its domain structure, and may function at the plasma membrane, the Golgi-TGN/endosomes and the developing cell plate. However, there is little direct evidence regarding DRP2’s role in the cell, and nothing at all relating to the part it plays in the larger process of plant development. The only evidence of DRP2 function is that overexpression of a presumed dominant-negative form of DRP2A in Arabidopsis protoplasts blocks trafficking of soluble secretory cargo to the central vacuole, but not to the plasma membrane (Lam et al., 2001). Under these conditions DRP2A localized only to the Golgi, whereas other studies have shown other localizations as well, raising questions about the relevance of these results to whole plants.

Questions of DRP2’s function, both at the cellular and whole-plant developmental level are made more interesting by the observation that the DRP1 family also functions in CME, and that these two protein families, although divergent in domain structure, have very similar subcellular localizations. Do DRP1s and DRP2s play redundant roles in clathrin-mediated trafficking? If not, what separate functions are played by each? Further complicating this question is the possibility that these dynamin-related proteins, known to homopolymerize, may also form heteropolymers. *GmDRP1* has been reported to interact in directed yeast-2-hybrid with not only Arabidopsis DRP1s but also DRP2A (Hong et al., 2003). The formation of mixed DRP1-DRP2 polymers would be surprising, given the different sizes and domain structures of these two protein families, and the fact that heteropolymerization has not been reported for any other dynamin. As of yet, there is no *in vivo* evidence for any sort of DRP heteropolymerization,
leaving this as a rather speculative possibility. However, it underscores the importance of understanding the cellular and developmental roles of both the DRP1 and the DRP2 family.

**Thesis Outline**

The research presented in this thesis focuses on two basic sets of questions regarding the molecular and developmental function of the DRP1 and DRP2 families of proteins, and their roles in cytokinesis and cell expansion.

1) **How is the DRP1 family targeted and regulated?**
   
a. How is DRP1A associated with cellular membranes,

b. What protein interactors does DRP1A have,

c. What are DRP1A’s fundamental biochemical characteristics, and

d. Are these characteristics or DRP1A’s *in vivo* localization modulated by its protein interactors?

Chapter 2 of this thesis addresses questions (a) and (c), while Appendix A includes a preliminary report on potential DRP1A interacting proteins.

2) **What is the cellular and developmental role of the DRP2 family?**

   a. Are the DRP2 proteins functionally redundant with the DRP1 proteins, or are they independently essential for plant development?

   b. If independently essential, what is their functional role?

   c. Are DRP2 proteins found in the same structures as DRP1 proteins, and, if so, are their functions at these sites identical or distinct?
Chapter 3 of this thesis, an analysis of DRP2 null mutants, addresses questions (a) and (b), while Appendix B is a report on progress towards generation of a functional DRP2-GFP to address question (c).

In addition, Appendix C is a progress report on a collaborative project focused on understanding the role of CLC in plant development, which is complementary to the study of Arabidopsis DRPs.

Together, these studies are part of a larger goal of fully understanding the role of Arabidopsis dynamin related proteins, and membrane trafficking in general, during cytokinesis and cell expansion.
**Figures**

**Figure 1: Membrane trafficking pathways in cytokinesis and cell expansion**

The sites of action of Clathrin Light Chain (CLC) and the Dynamin Related Protein (DRP) 1 and 2 families during cell plate maturation (A-B), clathrin-mediated Golgi-vacuole trafficking (C) and clathrin-mediated endocytosis (D) are indicated.
Figure 2: Steps and players of animal endocytosis

The major steps in animal clathrin mediated endocytosis, from site initiation to vesicle uncoating, are outlined along with some of the major players in each step. Proteins in bold have apparent homologues in Arabidopsis, while those in italics do not. Proteins in parentheses are Arabidopsis proteins that, while not direct homologues, may function similarly to some of the animal endocytic players that are not present in the Arabidopsis genome. Figure adapted with permission from Catherine Konopka.
Figure 3: The dynamin superfamily

Selected members of dynamin superfamily (A) and dynamin-related proteins (DRPs) in plants (B), with domain structure and proposed function of each. GTPase = GTP hydrolyzing domain. Middle = Coiled-coil polymerization domain. GED = GTPase effector domain (also coiled coil and involved in polymerization). PRD = Proline Rich Domain (binds SH3 domain-containing protein interactors). PH = Pleckstrin Homology (binds phospholipid headgroups). HP = Hydrophobic Paddle (membrane-inserting) TM = Transmembrane. Insert B = Uncharacterized hydrophobic region. TP = Transit Peptide (chloroplast targeting). All putative membrane-interacting domains are color-coded yellow.
A

**Dynamin Superfamily Members**

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B

**Arabidopsis Dynamin-Related Proteins**

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Chapter 2: Arabidopsis Dynamin-Related Protein 1A polymers bind, but do not tubulate, liposomes.

This chapter is adapted from:


Abstract

The Arabidopsis Dynamin-Related-Protein 1A (AtDRP1A) is involved in endocytosis and cell plate maturation in Arabidopsis. Unlike dynamin, AtDRP1A does not have any recognized membrane binding or protein-protein interaction domains. We report that GTPase active AtDRP1A purified from E. coli as a fusion to maltose binding protein forms homopolymers visible by negative staining electron microscopy. These polymers interact with liposomes whose lipid composition mimics that of the inner leaflet of the Arabidopsis plasma membrane, suggesting that lipid binding may play a role in AtDRP1A function. However, AtDRP1A polymers do not appear to assemble and disassemble in a dynamic fashion and do not have the ability to tubulate liposomes in vitro, suggesting that additional factors or modifications are necessary for AtDRP1A’s in vivo function.
**Introduction**

The *Arabidopsis* Dynamin-Related-Protein 1A (*At*DRP1A) is a member of the dynamin superfamily of GTPases that plays a critical role in *Arabidopsis* development (Collings et al., 2008; Kang et al., 2001; Kang et al., 2003a; Konopka and Bednarek, 2008; Sawa et al., 2005). It is essential for proper maturation of the cell plate during cytokinesis (Kang et al., 2001; Kang et al., 2003a; Otegui et al., 2001), and recent studies have also suggested that it functions like dynamin in clathrin-mediated endocytosis (CME) (Konopka and Bednarek, 2008). Dynamin is the founding and best characterized member of the dynamin superfamily, and plays both early regulatory and late mechanical roles in the formation and severing of clathrin-coated vesicles from the plasma membrane (PM) (Mettlen et al., 2009b). During CME, dynamin activity at endocytic buds is regulated by a combination of its pleckstrin-homology (PH) domain, which binds the signaling phospholipid PI(4,5)P$_2$, and its proline-rich-domain (PRD), which binds other endocytic proteins. Neither of these domains, nor any other recognized lipid- or protein-binding domains, are present in *At*DRP1A, raising the question of how *At*DRP1A is targeted and regulated during endocytosis and cytokinesis.

Subcellular fractionation studies have revealed that *At*DRP1A is a peripheral membrane protein that is predominantly present as a high molecular weight protein complex (Kang et al., 2001; Park et al., 1997). However, it has not been determined whether *At*DRP1A binds directly to membranes or indirectly via other protein partners, and whether the high molecular weight complexes are homopolymers of *At*DRP1A or multi-protein complexes. The soybean homologue of *At*DRP1A, *Gm*DRP1 (Phragmoplastin), was reported to form a homopolymer when purified from *E. coli* as a glutathione-S-transferase (GST) fusion protein, and two self-interaction domains were identified by yeast-two-hybrid and *in vitro* binding studies (Zhang et
However, GST-\textit{Gm}DRP1 was purified under denaturing conditions, and was not demonstrated to have GTPase activity, limiting its utility for biochemical characterization.

Here we present the \textit{in vitro} characterization of GTPase active, \textit{E. coli}-expressed, \textit{At}DRP1A, including evidence of its inherent self-interaction and lipid-binding ability. Significantly, purified \textit{At}DRP1A behaves very differently than purified dynamin, and in ways that are difficult to reconcile with what is known of its \textit{in vivo} activity, suggesting that additional factors or modifications are needed for \textit{At}DRP1A to function.

**Materials and Methods**

**General Reagents:**

All reagents were purchased through Fisher Scientific (Pittsburg, PA) unless otherwise noted. SDS-PAGE and immunoblotting conditions and \(\alpha\)-DRP1A antibodies are described in (Kang et al., 2001) with the exception that Supersignal West Pico (Pierce, Rockford IL) was used as the chemiluminescence substrate for detection of the HRP-labeled secondary antibodies. Rabbit \(\alpha\)-MBP antibodies were purchased from Immunology Consultants Laboratories (Newberg, OR). Spotted lipid assays were performed as described by Dowler et al. (Dowler et al., 2002). All oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA) and PCR amplification was performed with PfuUltra (Stratagene, La Jolla, CA).

**Generation of the His\textsubscript{8}-MBP-\textit{At}DRP1A expression clone:**

The \textit{At}DRP1A coding sequence (full length, including stop codon) was PCR amplified using primers 5’-ggggacaagtttgacaaaaagcaggctcaatggaaaatctgatctctcttgttaa-3’ (forward) and 5’-ggggaccactttgtacaagactggtctactggcgaacagcaacagcatcgatctcg-3’ (reverse), which
introduced attB1/B2 recombination sites at the ends of the gene. The PCR product was inserted into the plasmid pGEM-T-EASY (Promega, Madison WI) by TA cloning and then recombined into pDONR201 (Invitrogen, Carlsbad, CA) using standard Gateway® cloning procedures (Invitrogen, Carlsbad, CA). A DNA sequence encoding the Tobacco-Etch Virus (TEV) cleavage site was inserted in frame with the first codon of DRP1A by site-directed mutagenesis (New England Biolabs, 2008) using the 5’ phosphorylated primers 5’-gagaacctctattccagggcagaaatctgctctgtttgtaac-3’ and 5’-tgagctgtttttgttacaaag-3’, which annealed to AtDRP1A and pDONR201, respectively. The AtDRP1A coding sequence was verified by sequencing, and then recombined into the destination vector pVP16 (Blommel and Fox, 2007) to create pVP16-TEV-DRP1A. pVP16 contains His₈-Maltose Binding Protein (His₈-MBP) in frame with the N-terminus of the gene inserted into the recombination site (e.g. TEV-DRP1A).

pVP16-DRP2A was generated using similar procedures but without the insertion of a TEV cleavage site; the primers for initial amplification of the DRP2A coding sequence from seedling cDNA were 5’-ggggcacaaggtaaaaagcaggctattaatatgtgagcagctgtgtg-3’ and 5’-gggggacacattgtacaagaaagctgaggaatctctaagaacc-3’.

**Expression and Purification of DRP1A:**

pVP16-TEV-DRP1A in *E. coli* strain B834pRARE2 was used to inoculate 50 mLs of LB (Luria Broth + carbenicillin₅₀µg/ml/chloramphenicol₃₄µg/ml) which was grown to saturation 16 hours with shaking at 37°C. This culture was diluted 1:20 into 1 L LB and immediately induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). The 1 L culture was grown an
additional 16 hours with shaking at 18°C. The bacteria expressing His$_8$-MBP-TEV-DRP1A were harvested by centrifugation (10 min at 2,000g) and resuspended in 10 mls H(0.15)NG buffer (25 mM HEPES pH 7.5, 0.15 M NaCl, 5% v/v glycerol, 10 mM β-ME) with protease inhibitors and 1mg/ml lysozyme prior to lysis by French Press (2 passes, 1900 PSI). The lysis mixture was cleared twice by centrifugation at 2,000g and TX-100 was added to the supernatant to a final concentration of 2% (v/v) before incubation with 1 ml bed volume amylose resin (New England Biolabs, Ipswich, MA) in a 10 ml disposable poly-prep column (Biorad, Hercules, CA) at 4°C with rotation for ≥ 30 minutes. The unbound was drained by gravity flow and the resin washed with 10 mls H(0.15)NG+1 mM ATP+2% TX-100 and then 50 mls cold H(0.15)NG prior to elution with H(0.15)NG+10 mM maltose. The concentration of purified His$_8$-MBP-TEV-DRP1A was measured with Biorad protein assay reagent (Biorad, Hurcules, CA) and diluted to 0.5 μg/ml (5 μM). Purified His$_6$-TEV protease (Blommel and Fox, 2007) was added to a concentration of 0.25 μg/ml and the cleavage reaction was incubated with rotation at 22°C for 24-48 hours. Purity of the preparation and completeness of the cleavage reaction was assayed by SDS-PAGE. Cleaved AtDRP1A was frozen in liquid nitrogen and stored at -80°C. His$_8$-MBP-DRP2A was expressed and purified using identical procedures with the exception that all purification steps were performed in a cold room at 4°C.

GTPase assays:

Colorimetric GTPase assays for determination of kCAT and kM were performed essentially as described by Leonard et al. (Leonard et al., 2005). In brief, purified AtDRP1A (0.1 μM final) was mixed with GTP (50-500 μM final) in reaction buffer (20 mM HEPES, pH 7.5,
150 mM NaCl, 2 mM MgCl₂) and incubated at 22°C; 0, 2, 5, 10 and 15 minute timepoints were taken and concentrations of released phosphate determined by addition of the color reagent (1 M HCl, 0.1% w/v Malachite Green, 1% w/v Ammonium Molybdate Tetrahydrate) and measurement of absorbance at 660 nm on a plate reader (Bio-Tek instruments EL311).

**Fractionation:**

Purified His₈-MBP-AtDRP1A or AtDRP1A was diluted to 200 nM in H(0.15)NG+2 mM MgCl₂ with or without 1 mM GTP and incubated 5 minutes 22°C. After 5 minutes, an additional 1 mM GTP was added to the +GTP sample, and 200 µl of each sample was transferred to a TLA100.1 tube and pelleted for 30 minutes at 150,000g in a Beckman (Fullerton, CA) tabletop ultracentrifuge. The load and upper 80 µl of the reaction volume were analyzed by SDS-PAGE and immunoblotting against AtDRP1A and MBP. For sucrose gradient fractionation, 200 µl of 1.25 µM AtDRP1A was loaded on top of a 4.8 ml 5-50% (w/v) sucrose gradient in H(0.075)N (25 mM HEPES pH 7.5, 0.075 M NaCl, 2 mM MgCl₂, and 10 mM β-ME) poured on an Autodensiflow gradient maker (Labconco, Kansas City, Kansas). Gradients were centrifuged 18 hours at 4°C in a SW50.1 rotor at 150,000g. 200 µl fractions were collected using a gradient collector (model 640, Isco Inc., Lincoln, NE). Fractionation standards (75 µg BSA, 75 µg Catalase, 50 µg AtCDC48 (Rancour et al., 2002)), were loaded on an identical gradient and centrifuged and fractionated in parallel. The fractions were analyzed by SDS PAGE followed by Coomassie staining (fractionation standards) or immunoblotting using α-AtDRP1A antibodies, and refractive index was used to compare fractions between gradients.
Liposome Generation:

DOPC, DOPS, DOPE, Soy PC and PI(4,5)P₂ were purchased from Avanti Polar Lipids (Alabaster, AL). PI(3)P, PI(4)P and PI(5)P were from Cayman Chemical (Ann Arbor, MI), and β-sitosterol was from Calbiochem (San Diego, CA). Dried lipids were resuspended in chloroform or 1:1 chloroform:methanol and mixed by vortexing in a 12x75mm glass test tube. The lipid mixture was spiked with³H-DOPC (Perkin-Elmer) to 6 µCi/ml and dried under a gentle stream of Argon until visibly dry (10-15 min), and then placed under house vacuum for an additional 30 minutes. The resulting film was resuspended to 330 mM total lipid in H(0.15NG) buffer and allowed to hydrate 15 minutes at RT before being vortexed for 5 minutes. The mixture was then subjected to 5 freeze-thaw cycles (liquid nitrogen-37°C water bath) before being extruded through a 50 nm polycarbonate membrane (Avanti Polar Lipids, Alabaster, AL) and stored at -80°C under argon until use.

Liposome Flotation Assays:

Liposome flotation assays were performed generally as described in (Tucker et al., 2004). Purified ArDRP1A protein (200 nM to 1 µM final concentration) was mixed with 50 nm liposomes (44 mM final concentration) and buffer H(0.15)NG to a final volume of 75 µl in a sialinized 0.65 ml ultracentrifuge tube and incubated 30 minutes at 22°C with occasional mixing. The binding reaction was diluted with an equal volume of ice-cold 80% (w/v) Accudenz (Accurate Chemical and Scientific Corporation, Westbury, NY) in H(0.015)NG, transferred to the bottom of a 5x41mm Ultra-Clear centrifuge tube (Beckman-Coulter), and overlayed with 300 µl 30% (w/v) Accudenz followed by 100 µl H(0.15)NG then centrifuged 1 hour or more at
243,000g at 4°C in an SW50.1 rotor with tube adapters. Fractions (80 µl) were collected from the top and analyzed for lipid content by scintillation counting and protein content by SDS-PAGE followed by Coomassie staining or immunoblotting.

**Negative Staining Electron Microscopy:**

All electron microscopy was performed at the UW Madison Medical School EM Facility on a Phillips CM120 STEM. For visualization of AtDRP1A, purified protein was diluted to 1µM in buffer H(0.075)N with or without 1 mM GTP, dried onto a pioloform (Ted Pella, Redding, CA) coated copper grid, and stained with Nano-W® (Nanoprobes, Yaphank, NY), an organo-tungstate stain. Liposomes were diluted to 100 mM lipid in H(0.075)N, mixed with an equal volume 1% OsO₄, then dried onto pioloform coated grids and stained with Nano-W®. For visualization of AtDRP1A bound to liposomes, purified AtDRP1A was mixed with liposomes to a final concentration of 1 µM AtDPR1A and 100 mM lipid in H(0.075)N and incubated for 30 minutes 22°C with occasional mixing. The mixture was then stained with OsO₄ and Nano-W® as for liposomes.
Results and Discussion

Purification of GTPase active AtDRP1A

GTPase active AtDRP1A was expressed in E. coli as a translational fusion to a His$_8$-MBP tag, purified using amylose affinity chromatography and treated with His$_6$-TEV protease to remove the His$_8$-MBP tag (Fig. 1A). The GTPase activity of the purified protein increased approximately two-fold upon cleavage of the His$_8$-MBP tag (Fig. 1B). In contrast, expression of other affinity tagged forms of AtDRP1A, including GST-AtDRP1A in E. coli and S. cerevisae and AtDRP1A-His$_6$ in S. cerevisae, did not yield GTPase-active protein (Supplemental Table 1).

His$_8$-MBP and His$_6$-TEV protease could not be removed from the mixture by immobilized nickel affinity chromatography due to non-specific binding of AtDRP1A to the nickel resin. Therefore, in all subsequent experiments, AtDRP1A was assayed in the presence of cleaved His$_8$-MBP and His$_6$-TEV, with a mixture of His$_6$-TEV and purified His$_8$-MBP serving as a negative control.

The kCat and kM of GTP hydrolysis by AtDRP1A was measured by a colorimetric GTPase assay (Leonard et al., 2005) in the presence of varying concentrations of GTP (Fig. 1C). k$_{\text{cat}}$ and k$_{\text{M}}$ values varied between preparations, as has been observed for dynamin (Marks et al., 2001). The average k$_{\text{cat}}$ value was 28±5.34/min, which falls between the k$_{\text{cat}}$ values reported for lipid-tubule stimulated (105±47/min) and unstimulated dynamin (2.6±0.98/min) (Song et al., 2004). The average k$_{\text{M}}$ was 99±59 µM, which is higher than that reported for stimulated (37±18 µM), but similar to that of unstimulated (102±35 µM) dynamin (Song et al., 2004).
Purified *At*DRP1A is polymeric

As shown in Fig. 1A, His$_8$-MBP-DRP1A, *At*DRP1A, and His$_8$-MBP migrated as 110 kD, 65 kD and 45 kD polypeptides, respectively, when analyzed by SDS-PAGE. However, when the *At*DRP1A cleavage mixture, containing *At*DRP1A, His$_8$-MBP and His$_6$-TEV, was subjected to centrifugation at 150,000g, *At*DRP1A, but not His$_8$-MBP, was fully depleted from the supernatant (Fig. 2A). His$_8$-MBP-*At*DRP1A likewise pelleted at 150,000g, indicating that both His$_8$-MBP tagged and tag-free *At*DRP1A form large homopolymers in the presence of 150 mM NaCl. This sedimentation behavior was not altered by incubation with 1 mM GTP (Fig. 2A). When subjected to velocity sedimentation gradient analysis in the presence of 75 mM NaCl, *At*DRP1A sedimented beyond the 17 S / 550 kDa protein standard, CDC48 (Rancour et al., 2002), further demonstrating the polymeric nature of *E. coli* expressed *At*DRP1A (Fig 2B). This behavior is distinctly different than that of purified dynamin, which is found both in polymeric and soluble, dimeric/tetrameric forms, with the soluble form favored in the presence of ≥25 mM NaCl or upon addition of GTP (Eccleston et al., 2002; Ramachandran et al., 2007; Warnock et al., 1996).

When purified dynamin is induced to polymerize by dilution into ≤25 mM NaCl buffers or by addition of GDP-BeF$_2$, it forms rings and spirals with a constant diameter ((Carr and Hinshaw, 1997; Hinshaw and Schmid, 1995). In contrast, the size and shape of tag-free *At*DRP1A polymers visualized by negative staining electron microscopy (EM) was found to be highly heterogeneous, with no discernible regularity in structure (Fig. 2C). Addition of GTP to purified *At*DRP1A did not result in a visible change in polymer size or structure (Supplemental Fig. 1).
Similar to animal dynamin (Warnock et al., 1996) GST-GmDRP1 was reported to exist predominantly as monomers and dimers in the presence of 150 mM NaCl, only forming large polymers with a helical nature at 15 mM NaCl (Zhang et al., 2000). However, these results were based solely on EM analysis, and were not verified through other analytical methods. The smallest AtDRP1A particles we observed by EM (Fig. 2C, arrowheads) were similar in appearance to those interpreted as 68 kDa monomers or dimers of GST-GmDRP1 by Zhang et al., (Zhang et al., 2000); however by analytical sedimentation analysis (Fig. 2B) we estimate these structures to be comprised of more than 10 subunits (i.e. ≥ 680 kDa). The larger AtDRP1A structures we observed at 75 mM NaCl (Fig. 2C, arrows) are similar in appearance to the helical arrays of GST-GmDRP1 imaged by Zhang et al. (Zhang et al., 2000) at 15 mM NaCl. However, the AtDRP1A structures (Fig 2C) are heterogenous in size and curvature, and resemble neither the regular polymers formed by purified dynamin (Warnock et al., 1996) nor the 45 nm diameter AtDRP1A-containing rings observed encircling cell plate membrane tubules during syncytial endosperm cellularization (Otegui et al., 2001).

**AtDRP1A interacts with PM-mimetic liposomes**

In interphase Arabidopsis cells, AtDRP1A-GFP localizes to endocytic sites at the PM (Konopka and Bednarek, 2008), and fractionation studies of cell extracts have similarly shown AtDRP1A to be primarily associated with microsomal membranes (Kang et al., 2001; Park et al., 1997). Previous studies have demonstrated that dynamin assembles onto PI(4,5)P₂-containing liposomes via specific interactions between the PI(4,5)P₂ headgroup and dynamin’s PH domain, and that this interaction is essential for dynamin’s function in CME (Achiriloaie et al., 1999; Salim et al., 1996). However, the AtDRP1A amino acid sequence does not contain any predicted
lipid binding domains (SMART server http://smart.embl-heidelberg.de/ (Letunic et al. 2009). Therefore, we examined whether or not AtDRP1A polymers had any intrinsic affinity for PM phospholipids through binding studies to protein-free PM-mimetic (PMM) liposomes, whose lipid composition closely resembled that of the cytosolic face of the plant PM bilayer. Previous studies have determined the total lipid composition of the Arabidopsis PM (Uemura et al., 1995) and have shown that PS is restricted to the inner leaflet of plant cell PMs (O'Brien et al., 1997). PMM liposomes were generated from a mixture of 40 mol% \(\beta\)-sitosterol, 25 mol% Soy PC, 20 mol% DOPE, 10 mol% DOPS and 5 mol% DOPG with trace amounts of H\(^3\)-DOPC. Binding was assayed by liposome flotation followed by scintillation counting and immunoblotting.

AtDRP1A, but not His\(_8\)-MBP, showed robust binding to PMM liposomes (Fig. 3A).

The PMM liposomes have a net negative charge due to the presence of DOPS and DOPG, suggesting that the interaction with polymers of AtDRP1A, which is predicted to have a net positive charge (PI=8.5), might be based on charge-charge interactions. Consistent with this, AtDRP1A did not show binding to uncharged liposomes lacking DOPS and DOPG (40 mol% \(\beta\)-sitosterol, 40 mol% Soy PC, 20 mol% DOPE) (Fig. 3B).

Interestingly, in spotted lipid overlay assays AtDRP1A did not show binding to DOPS, but instead showed specific binding to PI(3)P and PI(5)P, with less binding to PI(4)P (Supplemental Fig. 2A). However, in liposome flotation assays AtDRP1A showed similar binding to DOPC-based liposomes containing 20% DOPS or 10% PI(3)P, PI(4)P or PI(5)P, as well as liposomes containing as little as 2% DOPS (Supplemental Fig. 2B-C).

As a control, we expressed and purified His\(_8\)-MBP-AtDRP2A (Supplemental Fig. 3A), which contains a PH domain and has previously been reported to bind preferentially to PI(3)P, with weaker binding to PI(4)P (Lee et al., 2002), and assayed its lipid binding specificity in
spotted lipid overlay and liposome flotation assays. Like AtDRP1A, His$_8$-MBP-AtDRP2A showed specific binding to PI(3)P and PI(5)P in spotted lipid overlay assays, with less binding to PI(4)P (Supplemental Fig. 3B), but showed relatively equal binding to PI(3)P, PI(4)P, PI(5)P and PS in liposome flotation assays (Supplemental Fig. 3C). Therefore, the discrepancy between the spotted lipid overlay and liposome flotation assays appears not to be specific to AtDRP1A, but may be a more general feature of these two assays.

**AtDRP1A-induced liposome clustering**

Both dynamin and the yeast dynamin-related-protein ScDMN1 (involved in mitochondrial fission) have been shown to assemble onto the outer surface of liposomes *in vitro*, and cause the deformation of those liposomes into tubules (Chen et al., 2004; Danino et al., 2004; Ingerman et al., 2005; Stowell et al., 1999; Sweitzer and Hinshaw, 1998a; Zhang and Hinshaw, 2001). To determine whether AtDRP1A polymers similarly affect liposome structure, protein free PMM liposomes (Fig. 3C) and PMM liposomes preincubated with AtDRP1A (Fig. 3D) were stained and visualized by EM. Liposomes bound to AtDRP1A appeared as darkly staining clusters, which were not observed in protein-free liposome samples. The addition of GTP to these AtDRP1A-liposome complexes resulted in no discernible change in structure (Supplemental Fig. 4). This clustering of liposomes onto AtDRP1A polymers is very different than what has been observed for dynamin or ScDMN1 *in vitro*, and is also distinct from the AtDRP1A-containing rings observed encircling cell plate membrane tubules *in vivo* during syncytial endosperm cellularization (Otegui et al., 2001).
Conclusions

Our analysis of the in vitro structure and membrane lipid interaction of purified, bacterially-expressed AtDRP1A suggest that the plant-specific DRP1 family has distinct characteristics from animal dynamin, even though previous studies have demonstrated that AtDRP1A, like dynamin, functions in CME (Konopka and Bednarek, 2008). Likewise, the propensity of purified AtDRP1A to form stable, GTP-insensitive, heterogeneous polymers that promote liposome clustering contrasts with the in vivo observation that AtDRP1A-GFP exists in a cytoplasmic (presumably soluble) pool (Kang et al., 2003a), and that AtDRP1A can polymerize around membrane tubules during cell plate formation (Otegui et al., 2001). This suggests that E. coli expressed AtDRP1A, while GTPase active, is lacking one or more in vivo factors necessary for modulating the polymeric state of individual AtDRP1A subunits, and thereby polymerizes inappropriately into a form that does not retain full functionality.

One possibility is that the activity and polymeric structure of AtDRP1A is regulated by post-translational modification, such as phosphorylation. Park et al. (Park et al., 1997) found approximately 10% of cellular AtDRP1A to be soluble upon cell disruption, and reported that this soluble form migrated slightly slower on SDS-PAGE gels. This slower migration could be reversed by alkaline-phosphatase treatment, suggesting that the soluble form of AtDRP1A is phosphorylated. These results, together with our observations that E. coli expressed AtDRP1A, which lacks phosphorylation, forms stable polymers, point to the need for further study of native AtDRP1A, in particular the identification of post-translational modifications and/or relevant interacting proteins. The ability of these putative modifications or interacting proteins to modulate the polymeric and membrane binding characteristics of purified AtDRP1A will be a key step in understanding the targeting and regulation of the plant-specific DRP1 family.
**Figures**

**Figure 1: Production of GTPase active AtDRP1A**

(A) SDS-PAGE gel analysis of amylose-resin purified, *E. coli* expressed His$_8$-MBP-AtDRP1A and AtDRP1A prior to (lane 1) and after (lane 2) cleavage of MBP with TEV protease. Proteins were detected by Coomassie staining. M= Molecular Mass Standards

(B) The GTPase activity of purified AtDRP1A, His$_8$-MBP-AtDRP1A and a mixture of His$_8$-MBP and His$_6$-TEV (negative control) was assayed with a colorimetric assay to measure released phosphate.

(C) The activity of TEV-cleaved AtDRP1A at various concentrations of GTP was used to calculate the kCat and kM for AtDRP1A.
**Figure 2: AtDRP1A forms large polymers in vitro**

(A) Purified MBP-AtDRP1A and TEV-cleaved AtDRP1A (200 nM) was centrifuged at 150,000g in a pH 7.5 HEPES buffer containing 150 mM NaCl and 2 mM MgCl₂. The load and supernatant (S150) were analyzed by immunoblotting with antibodies against AtDRP1A and MBP. Immunoblot was overexposed to demonstrate absence of AtDRP1A in the S150.

(B) AtDRP1A (50 nM) was separated on a 5-50% (w/v) sucrose gradient by centrifugation at 150,000g for 18 hours and gradient fractions were analyzed by immunoblotting with α-AtDRP1A antibodies. Black and gray bars represent the peak and range, respectively, for three molecular weight markers (BSA 4.4S, Catalase 11.4S and AtCDC48 17S) run in parallel on a separate gradient and detected by SDS-PAGE gel analysis and Coomassie staining. Equivalent fractions were determined by measurement of refractive index.

(C) AtDRP1A (1 µM) visualized by electron microscopy after negative staining with NanoW®. Right panels are higher magnification views. Arrowheads indicate smaller AtDRP1A particles; arrows indicate larger AtDRP1A structures. Scale bars = 100 nm.
Figure 3: *At*DRP1A binds and clusters protein-free liposomes

(A-B) Liposome flotation assay.  (A) Arabidopsis Plasma Membrane Mimetic liposomes (“PMM”: 40 mol% β-sisterol, 25 mol% Soy PC, 20 mol% DOPE, 10 mol% DOPS and 5mol% DOPG) or (B) neutrally charged liposomes (“Neutral”: 40 mol% β-sisterol, 40 mol% Soy PC, 20 mol% DOPE) spiked with trace H\textsuperscript{3}-DOPC were generated by extrusion through a 50nm membrane.  These liposomes (44 mM) were incubated with purified *At*DRP1A (250 nM) and separated from the load by flotation centrifugation on a 40%-30%-0% (w/v) Accudenz step-gradient.  The two top (1, 2) and the two bottom (6, 7) fractions of the gradient were analyzed by scintillation counting and SDS-PAGE followed by immunoblotting with α-DRP1A and α-MBP antibodies.

(C) PMM liposomes were stained with OsO4 followed by NanoW® and visualized by electron microscopy.

(D) PMM liposomes were incubated with *At*DRP1A then stained and visualized as in (C). Arrows indicate clusters of liposomes induced by binding of *At*DRP1A.

Scale bars in C-D = 100 nm
### Supplemental Table 1: DRP1A expression constructs

<table>
<thead>
<tr>
<th>Construct</th>
<th>Vector</th>
<th>Expression system</th>
<th>Expression conditions</th>
<th>Observations</th>
<th>GTPase active?</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST(^Y)-AtDRP1A</td>
<td>pET29b</td>
<td><em>E. coli</em> Rosetta</td>
<td>2 hours 37°C 0.2-1mM IPTG</td>
<td>No expression</td>
<td>N/A</td>
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<tr>
<td>GST(^Y)-AtDRP1A-His(_6)</td>
<td>pYES-DEST52</td>
<td><em>S. cerevisiae</em> inv. suc.</td>
<td>16 hours 28°C 2% galactose</td>
<td>Poor elution from glutathione resin. Frequent precipitation.</td>
<td>No</td>
</tr>
<tr>
<td>AtDRP1A-His(_6)</td>
<td>pYES-DEST52</td>
<td><em>S. cerevisiae</em> inv. suc.</td>
<td>16 hours 28°C 2% galactose</td>
<td>Persistent contamination with yeast proteins</td>
<td>No</td>
</tr>
<tr>
<td>His(_8)-MBP-T-AtDRP1A</td>
<td>pVP16</td>
<td><em>E. coli</em> B834pRARE2</td>
<td>14 hours 18°C 1mM IPTG</td>
<td>Well behaved</td>
<td>Yes</td>
</tr>
</tbody>
</table>

### Supplemental Methods

**Generation of expression clones:**

GST was amplified from pGEX (GE Healthcare, Little Chalfont, UK) using the forwards primer 5’-caattaatcagtaacagttatactgtctcatactagg-3’, which modified the end of the 5’UTR and the first two codons to better match the *S. cerevisiae* consensus initiator sequence (GST\(^Y\)), and then inserted into pET29b containing the full length AtDRP1A coding sequence, to create an N-terminal translational fusion (GST\(^Y\)-AtDRP1A). This entire construct was then subcloned into pENTR2B (Invitrogen, Carlsbad, CA) and subsequently recombined into pYES-DEST52, which encodes a C-terminal His\(_6\) tag, for expression of GST\(^Y\)-AtDRP1A-His\(_6\) in *S. cerevisiae*. The full length length AtDRP1A coding sequence was cloned into pENTR2B (Invitrogen, Carlsbad, CA) and subsequently recombined into pYES-DEST52 to create AtDRP1A-His\(_6\). For generation of His\(_8\)-MBP-T-DRP1A see main text.
**Purification of yeast-expressed GST^Y-AtDRP1A-His_6:**

An overnight culture of *S. cerevisiae inv. suc.* containing the expression construct in was diluted into 1 L of -ura synthetic drop-out media (BD biosciences, San Jose, CA) with 2% galactose to an optical density of 0.4, then grown a further 16 hours at 28°C before harvesting by centrifugation for 5 minutes at 1,000g. The pellet from 250 mls of culture was resuspended in 2 mls of buffer TNG (50 mM Tris, pH 7.4, 1 M NaCl, 5% glycerol, 1 mM DTT) plus protease inhibitors, and transferred to a 30 ml corex tube. One ml 0.5 mm acid washed glass beads were added, and cells were lysed by 10 30 sec pulses of vortexing, with 30 sec on ice between each pulse. The lysis mixture was cleared twice by centrifugation at 2,000g and TX-100 added to the supernatant to a final concentration of 2% before incubation with 250 µl Glutathione Sepharose 6-fast-flow (GE Healthcare, Little Chalfont, UK) for 2 hours 4°C, with rotation. The resin was drained by gravity flow and was washed once with 10 mls TNG+0.1%TX-100+1 mM ATP, then three times with 10 mls TNG followed by step elution with 15 mM glutathione, pH 8.3 in TNG. Only partial (< 50%) elution was observed; longer incubation times the elution buffer did not improve recovery, but agitation of the resin by pipetting up and down prior to elution did, although complete recovery was not obtained.

**Purification of yeast-expressed AtDRP1A-His_6:**

Expression and lysis conditions were the same as for GST^Y-AtDRP1A except that the lysis buffer and wash buffers were HNG (25mM HEPES pH 7.5, 0.15 M NaCl, 5% v/v glycerol, 10 mM β-ME) +20 mM imidazole, the affinity resin was Ni-NTA-agarose (GE Healthcare, Little Chalfont, UK), and the elution was in HNG +500 mM imidazole. The presence of contaminating
yeast proteins was not affected by changing the concentration of imidazole in the binding buffer; the *S. cerevisiae* genome encodes 14 proteins with a stretch of 6 or more contiguous histidines.

**Supplemental Figure 1: AtDRP1A polymers are unaffected by GTP**

(A) AtDRP1A (1 µM) visualized by electron microscopy after 5 minutes of incubation with 1 mM GTP. Arrows indicate large AtDRP1A polymers; arrowheads indicate smaller polymers. Scale bars = 100nm
Supplemental Figure 2: Spotted lipid and liposome flotation assays give differing results regarding AtDRP1A’s lipid binding specificity.

(A) Normalized results of independent spotted lipid overlay assays. The indicated phospholipids (500 or 100 pmols) were probed with AtDRP1A and detected with α-AtDRP1A antibodies. Spot intensity was measured by scanning densitometry, and normalized to the darkest spot on the blot. Mean and standard deviation of N ≥ 3 independent experiments is plotted.

(B-C) Liposome flotation assay: 1 µM (B) or 250 nm (C) AtDRP1A was incubated with liposomes (44 mM) containing DOPC plus the indicated percentages of specific phospholipids and spiked with trace H₃-DOPC. Liposomes were floated on a 40%-30%-0% (w/v) Accudenz step-gradient. Flotation was verified by scintillation counting (data not shown) and the two top and the two bottom fractions of the gradient were analyzed by SDS-PAGE and Coomassie staining (B) or immunoblotting with α-DRP1A antibodies (C).
Supplemental Figure 3: AtDRP2A behaves like AtDRP1A in lipid-binding assays

(A) Purified His$_8$-MBP-AtDRP2A visualized by SDS-PAGE and Coomassie staining.

(B) Spotted lipid overlay assay using the indicated amounts of each phospholipid probed with purified His$_8$-MBP-AtDRP2A and detected with $\alpha$-MBP antibodies.

(C) Liposome flotation assay: His$_8$-MBP-AtDRP2A was incubated with liposomes containing DOPC plus the indicated percentages of specific phospholipids and spiked with trace $^3$H$_2$-DOPC. Liposomes were floated on a 40%-30%-0% (w/v) Accudenz step-gradient. Flotation was verified by scintillation counting (data not shown) and the two top and the two bottom fractions of the gradient were analyzed by SDS-PAGE and Coomassie staining.
Supplemental Figure 4: GTP has no effect on the structure of AtDRP1A-bound liposomes.

AtDRP1A preincubated with PMM liposomes (as in Figure 3C), then incubated 5 minutes with 1mM GTP, then visualized by electron microscopy. Arrows indicate clusters of liposomes induced by binding of AtDRP1A.
Chapter 3: The Arabidopsis Dynamin-Related-Protein 2 (DRP2) family plays an essential role in gametophyte development

Abstract

Clathrin-mediated membrane trafficking is critical for multiple stages of plant growth and development. One key component of clathrin-mediated trafficking in animal systems is the polymerizing GTPase dynamin, which plays both regulatory and mechanical roles. Similarly, other organisms utilize dynamin-related proteins in clathrin-mediated trafficking. Plants are unique in the apparent involvement of both a family of classical dynamins (DRP2) as well as a family of dynamin related proteins (DRP1) in clathrin-mediated endocytosis. Our analysis of drp2 insertional mutants demonstrates that the DRP2 family, like the DRP1 family, is essential for Arabidopsis development. Gametes lacking both DRP2A and DRP2B were inviable, arresting prior to the first mitotic division in both male and female gametogenesis. Mutant pollen displayed a variety of defects including ectopic callose deposition, altered Golgi morphology and branched or irregular cell plates, consistent with a role of the DRP2 family in membrane trafficking. However, drp2 mutants underwent an earlier arrest than drp1 mutants, and, unlike drp1 mutants, did not display accumulation of excess plasma membrane or other gross defects in plasma membrane morphology. This suggests that the DRP2 family plays a distinct role in gametophytic development, possibly reflecting the existence of multiple non-redundant endocytic pathways in Arabidopsis that utilize different classes of dynamin related proteins. We also discuss the utility of aniline blue staining for detection of mutants with defects in pollen membrane trafficking.
**Introduction**

Dynamins and dynamin related proteins (DRPs) are a large superfamily of polymerizing GTPases involved in a variety of membrane-remodeling processes (Praefcke and McMahon, 2004). Animal dynamin, the founding and best characterized member of the dynamin superfamily, plays both early regulatory and late mechanical roles in clathrin-mediated endocytosis (CME) at the plasma membrane (PM) (Pucadyil and Schmid, 2008), as well as less well-characterized roles in caveolar endocytosis (Henley et al., 1998; Oh et al., 1998; Yao et al., 2005), clathrin-mediated membrane trafficking at the Golgi (Jones et al., 1998), actin dynamics (Orth and McNiven, 2003), and cytokinesis (Konopka et al., 2006). In plants, two separate families of dynamin-related proteins, the DRP1 family and the DRP2 family, appear to function in clathrin-mediated membrane trafficking.

The DRP2 family consists of two members, DRP2A and -2B, that share 93% amino acid sequence identity. These have a domain structure and organization similar to that of animal dynamin, including a membrane binding pleckstrin homology (PH) and a protein-interacting proline-rich domain (PRD), and as such represent the “classical” dynamins in plants (Fig 1A). Evidence for the function of the DRP2 family in CME includes the interaction of DRP2s with putative CME accessory proteins via their PRDs (Lam et al., 2002), immuno-EM localization of DRP2B to PM-associated clathrin-coated structures (Lam et al., 2002), and the localization of DRP2B-GFP to dynamic PM foci that resemble CLC PM-associated foci (Fujimoto et al., 2007). DRP2 has also been reported to localize to the trans-Golgi-network (TGN) by immuno-EM and when transiently expressed in protoplasts, and in protoplasts has been suggested to function in trafficking to the vacuole (Jin et al., 2001).
The DRP1 family contains five members, DRP1A-E, all of which lack the PH and PRD domains. Nevertheless, recent analyses of DRP1 null mutants (Collings et al., 2008; Kang et al., 2003a; Kang et al., 2003b) as well as the localization and dynamics of DRP1-fluorescent fusion proteins at the PM, suggest that the DRP1 family also functions in CME (Konopka and Bednarek, 2008; Konopka et al., 2008). In addition, both DRP1 and DRP2 localize to the forming cell plate (Fujimoto et al., 2008; Kang et al., 2001; Konopka and Bednarek, 2008), and DRP1A and E play a role in cell plate formation (Collings et al., 2008; Kang et al., 2003a).

Clathrin-mediated endocytosis plays a number of critical roles in plant development, including retrieval of excess membrane material during tip-directed growth and cell plate maturation, (Blackbourn and Jackson, 1996; Derksen et al., 1995; Otegui et al., 2001; Segui-Simarro et al., 2004) maintaining the polar localization of auxin transporters necessary for proper establishment of auxin gradients (Dhonukshe et al., 2007), and regulating the signaling of cell-surface hormone and pathogen-defense receptors (Geldner et al., 2007; Robatzek et al., 2006). Consistent with this, \textit{drp1} null mutants show strong developmental defects, up to and including embryonic and gametophytic lethality. \textit{drp1a} mutants have defects in cell expansion and cell wall deposition in various tissues, most significantly the stigmatic papillae cells, which fail to elongate and accumulate excess PM, consistent with a defect in endocytosis (Collings et al., 2008; Kang et al., 2001; Kang et al., 2003a). \textit{drp1a, drp1e} double mutants are embryonic lethal and display cytokinetic defects (Kang et al., 2003a). \textit{drp1c-I} mutants have male gametophytic lethality that manifests late in pollen development as an accumulation of and disorganization of PM and internal membranes, and eventually leads to shriveled, inviable pollen (Kang et al., 2003b).
Similar to \textit{drp1c-1} mutants, \textit{drp2a, drp2b} double mutants displayed male gametophytic lethality, but unlike any of the \textit{drp1} mutants, they also displayed female gametophytic lethality. Both \textit{drp2ab} male and female gametophytes arrested very early during gametophyte development, prior to the first mitotic division, and did not show the plasma membrane morphology defects seen in the \textit{drp1} mutants. Therefore, although both the DRP1 and the DRP2 families are involved in endocytosis, they do not play redundant roles. Instead, the DRP2 family is independently essential for both male and female gametophyte development, and the phenotype of the mutant gametophytes suggests that the DRP2 family of classical dynamins may function in a distinct endocytic or membrane-trafficking pathway from the DRP1 family of dynamin-related proteins.

\section*{Methods}

\textbf{General Methods:}

Unless otherwise noted, all materials and reagents were purchased from Fisher Scientific (Pittsburg, PA). All oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA).

\textbf{Plant Growth Conditions:}

Seeds were sterilized with 70\% Ethanol + 1\% v/v TX-100 for 5 minutes followed by 1 minute in 95\% ethanol, and plated on solid media containing 0.5X MS salts (Caisson Labs, North Logan, UT) and 0.6\% agar (Sigma, St. Louis, MO). Plates were stratified 3 days at 4°C.
before germination for 5-7 days in continuous light. Seedlings were transplanted to Metro Mix 360 potting soil (Sun Gro, Vancouver BC) and grown under 16 hours light/8 hours dark conditions at 24°C.

**Insertional Alleles and Genotyping**

Insertional alleles were acquired from the Arabidopsis Biological Resource Center (ABRC, Columbus, Ohio), and verified by PCR-based genotyping. The T-DNA insert was detected by amplification using a T-DNA primer (774 for line *drp2b-4*, 926 for all other lines) in combination with the left primer indicated in Table 1; the absence of the insert (to distinguish heterozygous from homozygous plants) was detected by amplification using the left and right primers indicated in Table 1. The exact position of the insert was determined by direct sequencing of the genotyping PCR reaction.

Genomic DNA extraction for genotyping was performed in a 96-well format, modified from Michaels and Amasino, 2001 (Michaels and Amasino, 2001). Tissue samples were placed in Costar Cluster Tubes in the provided racks, and one 1/8”x1/2” steel dowel pin (Small Parts Inc.), 500 µl of extraction buffer (200 mM Tris pH 8.0, 240 mM NaCl, 25 mM EDTA, 1% SDS, autoclaved) and 50 µl chloroform was added to each tube. One rectangular block with dimensions slightly larger than the tube rack was placed beneath the rack and another was placed on a paper towel directly on top of the lids of the tubes. Hand-pressure was applied to the blocks and the tubes were shaken vigorously until the tissue was broken and the buffer was visibly green, followed by incubation at 55°C for 10 minutes and 10 minutes centrifugation at 2,500g to pellet debris. 75 µl isopropanol was dispensed into each well of a 200 µl conical bottom 96-well plate, and 75 µl of the supernatant from each tube was added to the isopropanol and mixed by
pipetting up and down several times. The plate was sealed with sealing tape and incubated 10 minutes room temperature for DNA precipitation before centrifugation 10 minutes 2,500g to pellet DNA. Supernatant was removed by quickly inverting plate over sink, then quickly blotting the inverted plate on paper towels (a fresh stack of 2-3 towels per blot) and shaking firmly to remove all liquid. The plate was allowed to dry 10-15 minutes in hood to remove all traces of isopropanol, and 100 µl TE (10 mM Tris pH 8.0, 1 mM EDTA) was added. The samples were incubated at 4°C overnight, and mixed by pipetting prior to using 1.2 µl per 15 µl PCR reaction.

PCR was performed using Paq5000 polymerase (Stratagene, La Jolla, CA) for 40 cycles with the primers and annealing temperatures indicated in Table 1.

**RT-PCR**

RNA extraction was performed with TRIZol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol, and reverse transcription was performed using a MLV-RT kit from Promega (Promega, Madison WI). Equal loading of cDNA was verified using primers 747 and 748 which recognize UBIQUITIN 10. Gene-specific transcripts were detected using a primer pair flanking the insertional site (or, for *drp2a-4*, immediately downstream of the insertional site). Primers 970 and 859 were used for all *drp2a* alleles, primers 964 and 1056 were used for *drp2b-3* and primers 962 and 1055 were used for *drp2b-2* and 2b-4 (Table 2).

**Immunoblotting conditions:**

Seven day old seedlings were ground in liquid N₂, and resuspended in a minimum volume of 2x homogenization buffer (50 mM HEPES, pH 7.5, 300 mM NaCl, 10% glycerol).
Protein concentrations were measured using Biorad protein assay reagent (Biorad, Hurcules, CA) and 37 µg total protein was loaded per lane on a 7.5% acrylamide gel. Proteins were separated by SDS-PAGE and transferred (67 volts, 2.25 hours in Towbin buffer (Towbin et al., 1979)) onto 0.45 µm Hybond-ECL nitrocellulose (GE Healthcare, Little Chalfont, UK). Antibodies crossreacting with both DRP2A and DRP2B were a generous gift from Antje Heese, and were used at 1:500 for immunoblotting. α-DRP1A antibodies were described in (Kang et al., 2001), and were used at 1:1000. HRP-conjugated Donkey-α-Rabbit secondary antibodies were from GE Healthcare (Little Chalfont, UK), and Roche Lumilight Plus (Roche, Basel, Switzerland) was used as the detection reagent.

**Light Microscopy and staining**

Dissected siliques were viewed with a Leica M165 FC dissecting microscope (Leica Microsystems, Bannockburn, IL). Pollen, anthers and all semithin sections were viewed with a Zeiss Axioskop epifluorescence microscope (Carl Zeiss AG, Oberkochen, Germany). Color images were acquired with a Leica DFC 480 camera (Leica Microsystems, Bannockburn, IL), and single color fluorescence images were acquired with a Micro Max camera (Princeton Instruments, Trenton NJ). Images were processed using Adobe Photoshop and Illustrator CS2 (Adobe Systems, San Jose, CA).

For determination of female gametophyte developmental stage, whole floral rosettes were infiltrated under vacuum with FAA fixative (45% v/v ethanol, 5% v/v acetic acid, 3.7% v/v formaldehyde) until the tissue sank, incubated in a new change of FAA for 2 hours 22°C and then 12 hours at 4°C. The tissue was rinsed twice with 50% ethanol, then incubated 30 minutes 22°C in 50% v/v ethanol, then 30 minutes 22°C in 70% v/v ethanol. New 70% ethanol was
added and the tissue was incubated at 4°C until it was no longer visibly green (~1 week). After clearing was complete, the tissue was rehydrated in a 60% v/v, 40% v/v, 20% v/v ethanol series (30 minutes per step). The pistils were dissected out of the flowers and slit open with an 18g1½ needle then stained with 0.1 mg/ml propidium iodide in ddH₂O for 8 hours at 4°C. Individual ovules were picked out with an 18g1½ needle under a Leica Stereozoom 6 Plus dissecting microscope (Leica Microsystems, Bannockburn, IL) and placed on a glass slide. ddH₂O and a coverslip were added, and the ovules observed on a Nikon Elipse TE2000-U Laser Scanning Confocal Microscope (Nikon, Tokyo, Japan).

Pollen was isolated by vortexing 4 to 6 open flowers in 500 µl pollen isolation buffer (100 mM NaPO₄, pH 7.5, 1 mM EDTA, 0.1% v/v TX-100) followed by centrifugation for 30 seconds at 1,500g. The pollen pellet was transferred to a Bright-Line haemocytometer (Hausser Scientific, Horsham, PA) for counting and imaging.

For determination of pollen developmental stage and viability, anthers were dissected from unopened floral buds and squashed beneath a coverslip on a glass slide in either a solution containing 3 µg/ml 4',6-diamidino-2-phenylindole (DAPI) in pollen isolation buffer or in Alexander Stain (Bonhomme et al., 1999).

For detection of callose deposition, anthers were dissected from unopened floral buds and squashed beneath a coverslip on a glass slide in a solution containing 3 µg/ml DAPI and 0.1% w/v aniline blue in pollen isolation buffer at pH 8.5. Pollen was stained for 30 minutes in the dark prior to visualization on a Olympus BX60 epifluorescence microscope (Olympus Corporation, Tokyo, Japan) under UV excitation (Olympus U-MWU cube: 330-385 nm excitation, 400nm dichroic mirror, 420 nm long pass emission) and imaging with an Olympus
DP70 color camera at the Plant Imaging Center (Dept. of Botany, University of Wisconsin, Madison).

**High Pressure Freezing and Transmission Electron Microscopy**

Pistils from four consecutive unopened buds in each of six WT and six DRP2A/drp2a-1; DRP2B/drp2b-2 plants were dissected and cut in 100 mM sucrose, then transferred to a Type B freezing hat (Ted Pella, Redding, CA) in 100 mM sucrose and frozen using a HPM 010 High Pressure Freezer (ABRA Fluid, Widnau, Switzerland). Anthers from five consecutive unopened buds in each of two WT and two DRP2A/drp2a-1; DRP2B/drp2b-2 plants were dissected and placed in 100% hexadecene before being transferred to a Type B freezing hat in 100 mM sucrose and high pressure frozen.

All samples were freeze-substituted with 2% w/v OsO₄ (Ted Pella, Redding, CA) in EM grade acetone (Electron Microscopy Sciences, Hatfield, PA) for 5 days on dry ice, followed by 24 hours at -20°C, 4 hours at 4°C, and 1 hour at 22°C. Samples were washed 3 times with EM grade acetone, the infiltrated with gradually increasing amounts of Epon resin mix (50% w/w EMbed-18, 22% w/w NMA, 28% w/w DDSA (Electron Microscopy Sciences, Hatfield PA) in EM grade acetone. Incubation was at 22°C for at least 8 hours in each change of resin; 3 changes of 100% Epon and 2 changes of 100% Epon plus accelerator (1.6% v/v DMP-30) were completed before polymerization at 60°C for 24 hours.

Semithin (2.5 µm and 1.0 µm) sections were cut using glass knives on a Reichert Om U3 microtome and stained with 0.01% Toluidine blue. Ultrathin (90 nm) sections were cut using a Microstar diamond knife (Microstar Tech, Huntsville, TX) on a Leica Ultracut E microtome (Leica Microsystems, Bannockburn, IL), stained with 8% uranyl acetate in 50% v/v ethanol
(saturated) followed by Reynold’s lead citrate and observed with a Phillips CM120 STEM (FEI, Hillsboro, OR) at the University of Wisconsin Medical School Electron Microscopy Facility.

**Results**

**drp2AaBb double mutants display male and female transmission defects**

To analyze the function of DRP2A and DRP2B we isolated and characterized several independent T-DNA insertional lines for **DRP2A** and **DRP2B**. The position of the T-DNA in each line was verified by PCR amplification using gene specific and T-DNA specific primers followed by DNA sequencing of the PCR product (Fig. 1B). Expression of **DRP2A** and **DRP2B** in wild-type (**WT**) and each mutant was examined by reverse-transcriptase-PCR and protein levels were assayed by immunoblotting with polyclonal α-DRP2 antibodies that cross-react with both DRP2A and DRP2B (Fig. 1C-F). All **drp2a** and **drp2b** alleles except **drp2b-3**, which was not further analyzed, were shown to be transcript nulls and have substantially reduced levels of total DRP protein, consistent with an absence of either DRP2A or DRP2B in the **drp2a** and **drp2b** lines, respectively. No developmental defects were observed in any single mutant.

To examine the genetic interaction between **DRP2A** and **DRP2B**, crosses between homozygous **drp2a** and **drp2b** plants were generated. F1 progeny from the crosses were allowed to self-fertilize and the genotype of F2 progeny was determined by PCR analysis. No homozygous **drp2aabb** plants, nor any plants heterozygous for one allele and homozygous for the other (**drp2Aabb** or **drp2aaBb**), were recovered. The same results were obtained for all combinations of **drp2a** and **drp2b** null alleles tested (Table 3), indicating a fully penetrant, synthetic transmission defect of **drp2a** and **drp2b** through both the male and the female gametes.

To verify the observed transmission defects we performed reciprocal crosses between **DRP2A/drp2a-1; DRP2B/drp2b-2 (drp2AaBb)** plants and **WT** plants. When **WT** pollen was used
to fertilize \textit{drp2AaBb} ovaries, no progeny (0/120) with the \textit{drp2AaBb} genotype were recovered. Similarly, when \textit{drp2AaBb} pollen was used to fertilize WT ovaries, only one \textit{drp2AaBb} plant out of 198 progeny was recovered (Table 4). The one \textit{drp2AaBb} plant recovered may be the result of a very low level of partial penetrance of the pollen phenotype, or may be due to experimental error. Together, these data demonstrate a near-complete defect in the simultaneous transmission of both mutant alleles through both the male and the female gametes, suggesting that \textit{drp2ab} mutant gametes are inviable.

**Early developmental arrest of \textit{drp2ab} ovules**

To determine whether the female transmission defect in the \textit{drp2AaBb} double mutant was associated with a defect in seed development, morphological analysis of maturing siliques from \textit{DRP2A/drp2a-1; DRP2B/drp2b-2} plants was performed. The double mutant was found to have ~25% empty spaces in its siliques, as compared to less than 5% empty spaces in the \textit{WT} or either of the single \textit{drp2} mutants. Small white stubs were observed in these empty spaces, suggesting an early abortion of the mutant ovules (Fig. 2A-B). This aborted ovule phenotype was observed in all heterozygous combinations of \textit{drp2a} and \textit{drp2b} null alleles tested (Table 5), consistent with a failure in female gametophyte (embryo sac) development.

In order to determine the stage in embryo sac development at which \textit{drp2ab} gametophytes deviated from normal development, we performed Laser Scanning Confocal Microscopic analysis on chemically fixed and propidium iodide stained pistils from \textit{WT} and \textit{DRP2A/drp2a-1; DRP2B/drp2b-2} (\textit{drp2AaBb}) plants at various stages of development. Developing embryo sacs were identified by the large size of their cells and nuclei and their position at the center of the ovule, surrounded by two layers of integuments. The number and
arrangement of the nuclei in the embryo sac allowed the determination of its Female Gametophyte (FG) developmental stage, FG1-FG7, based upon the nomenclature of Christensen et al (1997) (Christensen et al., 1997). Development of the embryo sac in WT pistils was relatively synchronous, with only one or two developmental stages being represented within a single pistil, consistent with previous reports (Christensen et al., 1997). In every *drp2AaBb* pistil examined, a class of ovules was observed that contained only a small, single-celled embryo sac with a single prominent nucleus, morphologically identical to WT embryo sacs at the FG1 stage of development (Fig. 3A-F). FG1-arrested embryo sacs were seen in heterozygous *drp2AaBb* pistils at all stages of development up to and including early post-fertilization, after which the entire ovule appeared to degenerate. Of the 55 embryo sacs that appeared to deviate from normal development in *drp2AaBb* pistils of various ages, 100% were arrested at the FG1 stage; no other phenotypes were observed. Light microscopy of toluidine-blue stained semi-thin sections of resin-embedded *drpAaBb* pistils verified the presence of FG1-arrested gametophytes (Fig. 3G,H), demonstrating that gametogenesis of *drp2ab* embryo sacs is quantitatively arrested at the single-celled FG1 stage of embryo sac development. Ovules containing *drp2ab* embryo sacs eventually degenerated, leading to the appearance of the 25% empty spaces observed in mature *drp2AaBb* siliques.

**Arrested *drp2ab* embryo sacs show no defects in membrane or cellular morphology**

*drp1a* and *drp1c* null mutants show defects in plasma membrane morphology (Kang et al., 2003a; Kang et al., 2003b) and cell wall deposition (Collings et al., 2008), consistent with the proposed role of the DRP1 family in endocytosis. To determine whether arrested *drp2ab* embryo sacs showed similar membrane defects, or other defects in subcellular morphology,
pistils from WT and drp2AaBb plants were prepared for transmission electron microscopy (TEM) by high-pressure freezing followed by freeze-substitution with OsO₄ and embedding in resin. Pistils were staged based on embryo sac morphology in semi-thin sections observed by light microscopy, and drp2ab embryo sacs were identified as those embryo sacs arrested at stage FG1 in a drp2AaBb pistil where the majority of embryo sacs were at stage FG3 or later (Fig. 3G,H).

TEM analysis of FG1 arrested embryo sacs revealed no defects in plasma membrane structure, nor any other visible defects in subcellular morphology (N = 7). Instead, the drp2ab embryo sacs arrested at FG1 were very similar in appearance to WT embryo sacs at the FG1 stage of development (Fig. 4).

drp2AaBb plants produce shriveled pollen

Consistent with a defect in male gametophyte development in the drp2AaBb double mutant, ~20% of released pollen grains from drp2AaBb anthers were visibly small and shriveled (Fig. 5B). Identical phenotypes were observed in all combinations of drp2a and drp2b null alleles tested (Table 5). In contrast, less than 2% shriveled pollen was seen in either WT or single homozygous drp2 null alleles (Fig 5A, Table 5). Alexander staining of mature, released pollen from DRP2A/drp2a-1; DRP2B/drp2b-2 anthers verified that the shriveled pollen grains were inviable (Fig 5C-D). The percentage of pollen that was visibly shriveled varied from plant to plant, ranging from ~10% to ~25%, suggesting that environmental conditions may play a role in the manifestation of this phenotype. To confirm that the defective pollen phenotype was the result of abnormal gametophytic, not a sporophytic development, the drp2a-1 and drp2b-2 alleles were introgressed into the quartet mutant, in which the four products of a single pollen meiosis remain associated as a tetrad throughout pollen development (Preuss et al., 1994). As
expected for a gametophytic mutation, tetrads from anthers of a *DRP2A/drp2a-1; DRP2B/drp2b-2; qrt/qrt* plants displayed either zero, one or two shriveled pollen grains (Fig. 5 E-G), supporting the conclusion that the shriveled grains represented *drp2ab* gametes.

**Development of *drp2ab* pollen is arrested prior to pollen mitosis I**

Pollen development proceeds through well defined stages (Borg et al., 2009; Owen and Makaroff, 1995; Twell et al., 2006) that can be easily visualized by DAPI staining to follow the number, shape and position of nuclei. DAPI stained pollen from *DRP2A/drp2a-1; DRP2B/drp2b-2* (*drp2AaBb*) anthers appeared normal during the tetrad (Fig 6A) and released microspore (Fig 6B) stages of development. Microspore polarization also proceeded without visible defect (Fig 6C). However, by the early bicellular stage, some pollen grains were observed that were slightly smaller and displayed either aberrant or, more commonly, no DAPI staining (Fig 6D). Pollen grains with aberrant DAPI staining were not seen in *WT* anthers (Supplemental Fig. 1). By the later bicellular stage of development, the difference in size between the normal and mutant grains was readily distinguishable, and defects began to be seen in the overall integrity of the pollen grain (Fig. 6E) which were even more severely manifested in later stages of pollen development including tricellular (Fig. 6F) and mature pollen (Fig. 6G).

To examine the subcellular structure of developing and arrested *drp2ab* pollen, *drp2AaBb* anthers at the polarized microspore, bicellular and early tricellular stages were processed by high-pressure-freezing/freeze-substitution and embedded in resin. No defects were observed in toluidine-blue stained sections of *WT* or *drp2AaBb* anthers at the polarized microspore stage (Fig. 6H and 6L) or in grains undergoing pollen mitosis I (Fig. 6I and 6M). In *drp2AaBb* anthers at the early bicellular stage, however, we observed grains that had not undergone pollen mitosis I
but were instead arrested at the polarized microspore stage, with no evidence of vacuole division or mitotic entry (Fig. 6N). Some of these grains were beginning to show signs of cytoplasmic collapse, such as a darkening of the cytoplasm and its detachment from the pollen coat. Other grains had undergone complete cytoplasmic collapse. Both arrested and collapsed pollen were likewise observed in anthers at the tricellular stage, although the proportion of collapsed vs arrested pollen was higher in older anthers, suggesting a progressive collapse of the arrested grains (Fig. 6O). The total amount of arrested and collapsed pollen together accounted for ~30-35% of all pollen grains at all stages post pollen mitosis I, close to the expected 25% *drp2ab* gametes. Neither arrested nor collapsed pollen were observed at any stage in parallel-processed anthers from *WT* plants (Fig. 6H-K).

**Defective cell plates are observed in *drp2AaBb* anthers**

The cell plate formed during pollen mitosis I has a characteristic hemispherical shape, forming a cage around the generative nucleus (Brown and Lemmon, 1991; Heslop-Harrison, 1968; Park and Twell, 2001), which appears as a smooth semicircle in toluidine blue stained 1 μm sections (Fig. 7A-B). The majority of dividing pollen grains in *drp2AaBb* anthers had morphologically normal cell plates, but some plates were aberrantly shaped, showing irregular contours (17 out of 139, Fig. 7C-D) or even possessing branched structures (6 out of 139, Fig. 7E-F) Electron microscopy (EM) verified the presence of abnormal cell plates in *drp2AaBb* anthers (Fig. 7G-L). No cells with persistent cell plates or aberrantly shaped generative nuclei were observed in older, bicellular, *drp2AaBb* anthers.

**TEM analysis of arrested *drp2ab* pollen**
The ultrastructure of WT and arrested *drp2ab* pollen was examined by TEM to determine whether the early arrest was associated with defects in PM or Golgi morphology or other subcellular defects. The polarized microspore stage of WT pollen development could be subdivided into two substages based on the appearance of the intine and of the endoplasmic reticulum (ER). The early polarized microspore stage was characterized by a relatively thin and smooth intine and the presence of thin, darkly staining ER profiles (Fig. 8A,E). In contrast, the later polarized microspore stage, just before pollen mitosis I, was characterized by a much thicker and very convoluted intine, full of cytoplasmic inclusions, and rounder, lighter staining ER profiles (Fig. 8B,F). Development of pollen in WT anthers was synchronized so that early and late polarized microspores were not found in the same anther. However, in *drp2AaBb* anthers at the late polarized microspore stage, 7/29 microspores appeared to be arrested at the early polarized microspore stage based on intine morphology. Three of these arrested grains also contained thin ER profiles characteristic of the early polarized microspore stage. Similarly, in anthers at pollen mitosis I, (Fig. 8C,G), 43/147 grains appeared to be arrested at the early polarized microspore stage, displaying mostly or completely smooth intines; 22 of these arrested grains also contained thin ER profiles.

As seen by brightfield microscopy, some arrested pollen persisted into the bicellular stage of development (Fig. 8I-J,O-P), while others collapsed (Fig. 8K). About half of these persistent arrested grains (7/17) still displayed thin ER profiles, but 2/17 contained plastids with starch granules (characteristic of the bicellular stage) (Fig. 8L) and 16/17 had more convoluted intine structure than the arrested grains observed in earlier stages (Fig. 8P). However, this intine was often more heterogenous in appearance than in WT grains, and in 7/17 cases contained lightly-staining material reminiscent of callose deposits (Fig. 8M-N).
Golgi stacks in the persistent arrested microspores in bicellular anthers contained on average slightly more cisternae than in WT grains at the polarized microspore stage, and were more variable in morphology (Fig. 8Q-T). Even when stacks with the same number of cisternae were compared, the Golgi in persistent arrested grains were significantly longer and narrower than in WT (Fig. 8U), reflecting differences in the dimensions and spacing of the individual cisternae. The same Golgi phenotype, but less severe, was also observed in arrested grains in anthers undergoing pollen mitosis I. No defects in plasma membrane morphology, or accumulation of excess plasma membrane or internal membranes was observed in the arrested pollen grains.

**_drp2ab_ and _drp1c-1_ pollen display ectopic callose deposition.**

Pollen from _WT_, _DRP2A/drp2a-1; DRP2B/drp2b-2 (drp2AaBb)_ and _DRP1C/drp1c-1_ anthers at the bicellular and tricellular stage was stained with aniline blue to detect callose deposition. _WT_ pollen at these stages showed very little callose staining, consistent with previous reports (Johnson and McCormick, 2001) (Fig. 9A-B). In contrast, ~15% of pollen from _drp2AaBb_ anthers at the late bicellular stage showed bright callose staining on the surface of the grain (Fig. 9C-D). The intensity of the staining was variable, and was sometimes found in speckles or concentrated at the three apertures of the grain. Brightly staining grains were likewise seen in anthers from _DRP2A/drp2a-4, DRP2B/drp2b-4_ plants, but not in anthers from single homozygous _drp2a-1, drp2a-4, drp2b-2_ or _drp2b-4_ plants. Interestingly, this staining was brightest in anthers at the late bicellular and early tricellular stages, but progressively dimmer in the later tricellular stage and almost absent in mature, released pollen. Anthers from _DRP1C/drp1c-1_ plants similarly contained grains with bright callose staining at the surface, in
speckles and sometimes in large inclusions in the grain (Fig. 9E-F). In these plants staining was first observed at the mid-tricellular stage, and persisted into mature, released pollen.

To determine whether ectopic callose deposition was a general feature of pollen-lethal mutants, we stained bicellular and tricellular anthers from F1 crosses between WT Ler plants and seven independent Salk insertional mutants with putative reciprocal translocations (Katie Clark and Patrick Krysan, personal communication). In these F1 plants, incorrect homologous pairing causes 50% of the pollen to have large chromosomal deletions (“subhaploid gametophytes”) that lead to pollen lethality (Curtis et al., 2009). All seven of these lines displayed visibly shriveled pollen, but only one out of the seven showed significant levels of callose staining at any stage (Supplemental Fig. 2), demonstrating that ectopic callose deposition is not a general feature of all developmentally defective pollen.

Discussion

**DRP2A and DRP2B play functionally redundant roles in plant development**

DRP2A and DRP2B are 93% identical at the amino acid level, and are both expressed throughout plant development, suggesting that they might serve redundant functions in plant morphogenesis. Consistent with this, no morphological or developmental defects were observed in single homozygous *drp2a* or *drp2b* mutants. In contrast, Abe et al. (1998) reported an aerial rosette phenotype in 50-90% of plants from homozygous *drp2a* (*drp2a-1, drp2a-2, drp2a-3*) and *drp2b* (*drp2b-2, drp2b-3*) single mutant lines, as compared to only 10% aerial rosettes in WT plants. However, this phenotype was not observed in either WT or any *drp2a* or *drp2b* mutant plants analyzed in this study.
The *drp2* family is essential for plant development

Both the *DRP1* and *DRP2* families have been implicated in endocytosis (Collings et al., 2008; Fujimoto et al., 2007; Jin et al., 2001; Kang et al., 2003a; Kang et al., 2003b; Konopka and Bednarek, 2008; Konopka et al., 2008; Lam et al., 2002) raising the question of whether they play distinct or redundant roles in Arabidopsis development. Previous work has shown that members of the *DRP1* family are essential for normal PM dynamics and cell plate biogenesis at various stages of growth (Collings et al., 2008; Kang et al., 2003a; Kang et al., 2003b; Konopka and Bednarek, 2008; Konopka et al., 2008). Here we show that members of the DRP2 family of dynamin-related proteins are likewise essential for plant development, and that both male and female gametes require at least one functional DRP2 family member in order to progress beyond the single-celled stage of development.

*drp2* pollen phenotypes suggest roles in cell plate formation and membrane trafficking

~30% of pollen grains in *drp2AaBb* anthers at pollen mitosis I are arrested at the early polarized microspore stage, consistent with the ~33% arrested or collapsed grains seen in bicellular *drp2AaBb* anthers. This class of arrested grains likely represents the *drp2ab* gametes, known from segregation analyses to be inviable. In addition, a range of cell plate formation defects are also observed in pollen grains undergoing pollen mitosis I in *drp2AaBb* anthers. The branched cell plates in particular are reminiscent of defects seen in cell-plate defective pollen mutants such as *gemini pollen I* (Park and Twell, 2001) and *gsl10-1* (Toller et al., 2008), and suggest a role for DRP2 in cell maintenance of proper cell plate morphology, consistent with its previously reported cell plate localization (Fujimoto et al., 2008). However, unlike in *gemini pollen I* or *gls10-1*, persistent defective cell plates or ectopic cell walls are not visible at later
stages of development, suggesting that they do not hinder completion of cytokinesis. Also, the large proportion (17%) of cell plates in a \textit{drp2AaBb} anther that have dips or branches, in addition to the 30% of grains that are arrested at the polarized microspore stage and never enter pollen mitosis I, suggests that these cell plate defects may be characteristic of singly deficient \textit{drp2Ab} or \textit{drp2aB} pollen grains, although further analysis of pollen development in \textit{drp2} single mutants would be necessary to verify this hypothesis.

The polarized-microspore arrested \textit{drp2ab} gametes show some phenotypes suggestive of a defect in membrane trafficking, such as altered Golgi morphology and ectopic callose deposition. Ectopic callose deposition is also seen in defective pollen in the \textit{drp1c-1} mutant, and Van Damme et al. (Van Damme et al., 2006) reported the formation of large ectopic callose deposits in pollen of the \textit{tplate} mutant, which also has a shriveled pollen phenotype. \textsc{Tplate} has domains similar to those of coat proteins, and, like the DRPs, is putatively involved in membrane trafficking during cytokinesis and cell expansion. In contrast, only one out of seven mutants that show pollen lethality due to large chromosomal deletions had significant amounts of callose staining, demonstrating that ectopic callose deposition is not a general feature of disrupted pollen development, but instead a phenotype specific to a subclass of pollen mutants, and may be indicative of mutants with defects in membrane trafficking pathways.

An aniline blue staining screen for pollen mutants with ectopic callose deposition has been previously carried out by Johnson and McCormick (Johnson and McCormick, 2001). In addition to pollen with a precocious germination phenotype, they found mutants with intense spots of callose (\textit{polka dot pollen}) and diffuse callose staining over the surface of the grain (\textit{emotionally fragile pollen}). Based on the similar staining patterns seen in \textit{tplate}, \textit{drp1c-1} and
*drp2ab* pollen, we speculate that these as-of-yet uncharacterized mutants may also prove to have defects in membrane-trafficking or cell wall deposition pathways.

**drp2ab and drp1 mutants show distinct membrane defects**

Although arrested *drp2ab* pollen show phenotypes consistent with the presumed membrane-trafficking role of the DRP2 family, these phenotypes are distinct from those observed in the *drp1* mutants. While both *drp2ab* and *drp1c-1* pollen display developmental defects, the defect in *drp1c-1* pollen does not manifest itself until the bicellular stage, and is characterized by a profound disorganization and accumulation of PM, as is also seen in failed stigmatic papillae cells in the *drp1a-2* mutant (Kang et al., 2003a; Kang et al., 2003b). In contrast, PM morphology defects are not seen in either arrested *drp2ab* pollen grains or arrested *drp2ab* embryo sacs.

One explanation for this could be that the DRP1 family’s primary role is in CME at the plasma membrane, as suggested by the analysis of DRP1A-GFP in Arabidopsis roots (Konopka and Bednarek, 2008), whereas the DRP2 family’s primary role is at the TGN, or plays more equal roles at the PM and TGN and therefore its absence does not cause PM accumulation. This would be consistent with the defects in Golgi morphology seen in the arrested *drp2ab* pollen grains, as well as the report that DRP2A-GFP localizes to the Golgi in Arabidopsis protoplasts and plays a role in clathrin-mediated trafficking to the vacuole (Jin et al., 2001). However, DRP2B-GFP in whole Arabidopsis roots localizes primarily to the plasma membrane (Fujimoto et al., 2008), whereas DRP1A-RFP in Arabidopsis protoplasts localizes in part to the TGN (Sawa et al., 2005), suggesting that the apparent differences in localization between DRP2 and DRP1 may actually be a function of the tissue in which the experiments were performed; differential
localization of DRP1 and DRP2 has not yet been demonstrated using the same technique in the same tissue.

Also, the defects in Golgi morphology seen in the arrested *drp2ab* pollen, although distinctive, seem much less severe than the PM defects seen in *drp1c-1* pollen, and only become apparent long after the *drp2ab* grains have begun to deviate from normal development. The earliest phenotype seen in both *drp2ab* pollen and *drp2ab* embryo sacs is a developmental arrest, prior to the initiation of the first mitotic division, unaccompanied by any discernable defects in subcellular morphology. Therefore, another interpretation of this data is that DRP2, but perhaps not DRP1, is essential for a very specialized membrane trafficking pathway, operating either at the PM or the TGN, whose cargo is crucial for the forwards progression of gametophyte development. This interpretation does not rule out the possibility that DRP2 is also involved in bulk membrane trafficking at either the PM or TGN, but suggests that the early developmental arrest seen in the *drp2ab* mutants is not the result of a general block in clathrin-mediated trafficking, but instead a specific block in a pathway of particular developmental importance. This specific block, by causing developmental arrest, may in turn largely mask DRP2’s contribution to bulk membrane trafficking, leaving only more subtle hints such as the ectopic callose deposition and defects in Golgi morphology seen in the persistent arrested *drp2ab* pollen grains long after the initial arrest.

**Role of the DRP2 family in gametophyte development**

What might be the molecular cause of this specific developmental block seen in *drp2ab* gametophytes? Given that both *drp2ab* pollen and *drp2ab* embryo sacs arrest prior to pollen mitosis I, one possibility is that loss of DRP2 function may adversely affect cell cycle
progression. Other cell cycle control mutants with gametophytic defects have been described, but these show a somewhat more variable arrest than *drp2ab* gametes. For example, in homozygous RING-type E3 ligase *rhf1a rhf2a* double mutants, 60% of embryo sacs arrest at stage FG1, but occasional embryo sac arrests at other stages were also seen. Similarly, 30-40% of pollen grains in *rhf1a rhf2a* plants failed to go through pollen mitosis I, and collapsed thereafter, but 15-20% of pollen grains also arrested during the bicellular stage (Liu et al., 2008). In contrast to the *rhf1a rhf2a* mutants, the *drp2ab* gametophytic defect is fully penetrant in both the male and the female, and the embryo sacs quantitatively arrest prior to the first mitotic division, suggesting that if DRP2 does play a role in cell cycle control, it is a very strong modulator of that pathway. Alternatively, DRP2 could be causing a pre-mitotic arrest through some other pathway. Interestingly, *agl23*, which encodes a MADS-Box transcription factor, shows a selective FG1 arrest. Although only 16% of female gametophytes in an *AGL23/agl23-1* plant arrest, the arrest is always at FG1, never at any other developmental stage (Colombo et al., 2008).

How might DRP2, a classical dynamin with putative roles in clathrin-mediated endocytosis and/or post-Golgi trafficking, cause an FG1 arrest? One possibility is that DRP2-mediated membrane trafficking functions in the perception of an extracellular signal necessary for progression of gametophytic development. Membrane trafficking is known to be involved in perception of some plant hormones, such as brassinosteroids, (Geldner et al., 2007), and may prove to be involved in the perception of others as well. In addition, membrane trafficking is necessary for the maintenance of auxin gradients in many tissues of the plant (Dhonukshe et al., 2008; Dhonukshe et al., 2007; Geldner et al., 2003; Steinmann et al., 1999).
The female gametophyte is in intimate contact with the surrounding maternal tissue of the ovule throughout its development, and may be dependent on signals from that tissue for its development. Although very little is known about what signaling pathways might function in gametophyte development, there are some hints that signaling is taking place. Mutants in a putative sensor histidine kinase CKI1, whose ligand is unknown, show female gametophytic defects beginning at developmental stage FG4 (Hejatko et al., 2003; Pischke et al., 2002); other pathways could be acting earlier in development. Auxin has been shown to play a role in cell type specification during female gametophyte development, in addition, 26/399 embryo sacs in a tir1, afb1, afb2, afb3 quadruple auxin perception mutant arrested at the FG1 stage of development (Pagnussat et al., 2009).

Male gametophyte development may be less dependent on non-cell autonomous signals, because pollen from many plant species can undergo normal development in vitro without the use of exogenous hormones beginning at the late polarized microspore or early bicellular stage, although pollen thus cultured is prone to altered developmental fates, such as the generation of haploid embryos (Touraev et al., 1997). Signaling from the parental tissue may play a role in very early pollen development, or signals may be passed between pollen grains. Auxin activity in developing pollen peaks at the microspore stage, and pollen from the tir1, afb1, afb2, afb3 quadruple auxin perception mutant actually shows accelerated development (Cecchetti et al., 2008), consistent with the idea that auxin plays tissue-type specific roles in cell cycle control (del Pozo et al., 2005).

Although a role for DRP2 in perception of an extracellular signal necessary for female gametophyte development is speculative, it is consistent with the observed phenotype of the drp2ab embryo sacs: a selective FG1 arrest (in contrast to the somewhat later and more
variable arrest seen in female gametophyte mutants defective in basic cellular functions (Pagnussat et al., 2009)) with no visible defects in cell morphology. However, further studies will be necessary to fully determine the role that DRP2 is playing in gametophyte development, and whether its function is specific to the gametophyte or, as its broad transcription profile would suggest, a general function of plant cell growth. Moreover, additional comparison of the DRP2 and DRP1 families in membrane trafficking, especially using comparable techniques such as visualization of native-promoter driven, fully functional FP fusions in whole Arabidopsis roots, will be necessary to unravel the contributions made by each of these protein families to clathrin mediated trafficking and to help understand why clathrin mediated trafficking in plants, unique among organisms, requires the involvement of both a family of classical dynamins (DRP2) as well as a family of non-classical dynamin-related proteins (DRP1).
**Tables**

**Table 1: *drp2* insertional alleles**

The T-DNA insert was detected by PCR amplification of total genomic DNA preps using a T-DNA primer (774 for line *drp2b*-4, 926 for all other lines) in combination with the left primer at the indicated annealing temperature (TM); the absence of the insert (to distinguish heterozygous from homozygous plants) was detected by amplification using the left and right primers at the indicated annealing temperature (TM). The exact position of the insert was determined by direct sequencing of the genotyping PCR reaction. Primers sequences are listed in Table 2.

<table>
<thead>
<tr>
<th>line</th>
<th>position</th>
<th>left primer</th>
<th>right primer</th>
<th>TM (°C)</th>
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<tbody>
<tr>
<td><em>drp2a</em>-1</td>
<td>SALK_071036</td>
<td>2246</td>
<td>1164</td>
<td>1165</td>
</tr>
<tr>
<td><em>drp2a</em>-3</td>
<td>SALK_011319</td>
<td>3979</td>
<td>1171</td>
<td>1172</td>
</tr>
<tr>
<td><em>drp2a</em>-4</td>
<td>SALK_018859</td>
<td>1374</td>
<td>858</td>
<td>1165</td>
</tr>
<tr>
<td><em>drp2b</em>-2</td>
<td>SALK_134887</td>
<td>1120</td>
<td>1161</td>
<td>1160</td>
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<tr>
<td><em>drp2b</em>-3</td>
<td>SALK_124686</td>
<td>6396</td>
<td>861</td>
<td>865</td>
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<tr>
<td><em>drp2b</em>-4</td>
<td>WISCDSLOX_256E05</td>
<td>255</td>
<td>1162</td>
<td>1163</td>
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Table 2: Oligonucleotides used in this study

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</tr>
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<td>748</td>
<td>cgactgtgcattagaagaagaagat</td>
</tr>
<tr>
<td>774</td>
<td>aacgtccgcaatggatttaagtttc</td>
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<tr>
<td>858</td>
<td>taacctgttacgttttaacctttacattgcagct</td>
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<td>859</td>
<td>atccactgttctgctgacactacatctctca</td>
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<tr>
<td>861</td>
<td>actgttctctgtaacggtctcttcgcttt</td>
</tr>
<tr>
<td>865</td>
<td>tccatgagacaagactttcagaagct</td>
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<tr>
<td>926</td>
<td>ggtgtaggttacgtagtgggcctagc</td>
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<td>962</td>
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<td>964</td>
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<td>968</td>
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<td>1056</td>
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<td>1160</td>
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Table 3: *drp2* segregation distortion

Heterozygous *drp2AaBb* mutant plants were allowed to self, and the genotype of their progeny was determined by PCR using allele-specific primers. Percentage of offspring recovered from genotypic class is indicated, and compared to the percentage that would be expected if there were no defects in transmission or viability.

<table>
<thead>
<tr>
<th>genotype</th>
<th>WT</th>
<th><em>drp2AABB</em></th>
<th><em>drp2AaBb</em></th>
<th><em>drp2AAbb</em></th>
<th><em>drp2AaBb</em></th>
<th><em>drp2Aabb</em></th>
<th><em>drp2aabb</em></th>
<th>N=</th>
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<tbody>
<tr>
<td>expected %</td>
<td>6</td>
<td>25</td>
<td>25</td>
<td>12.5</td>
<td>25</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>DRP2A/drp2a-1, DRP2B/drp2b-2</em></td>
<td>4</td>
<td>18</td>
<td>34</td>
<td>44</td>
<td>0</td>
<td>0</td>
<td>140</td>
<td></td>
</tr>
<tr>
<td><em>DRP2A/drp2a-1, DRP2B/drp2b-4</em></td>
<td>14</td>
<td>29</td>
<td>30</td>
<td>27</td>
<td>0</td>
<td>0</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td><em>DRP2A/drp2a-3, DRP2B/drp2b-2</em></td>
<td>6</td>
<td>39</td>
<td>29</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>85</td>
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</table>

Table 4: Analysis of progeny from *drp2AaBb/WT* reciprocal crosses

The genotype of F1 progeny from reciprocal crosses between *DRP2A/drp2a-1; DRP2B/drp2b-2* and *WT* plants was determined by PCR amplification using allele-specific primers (Table 1). Percentage of offspring recovered with each genotype is indicated, and compared to the percentage that would be expected if there were no transmission defects.

<table>
<thead>
<tr>
<th>genotype</th>
<th>WT</th>
<th><em>drp2AABB</em></th>
<th><em>drp2AABb</em></th>
<th><em>drp2AAbb</em></th>
<th><em>drp2AaBb</em></th>
<th><em>drp2aabb</em></th>
<th><em>drp2aabb</em></th>
<th>N=</th>
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<tbody>
<tr>
<td>expected %</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><em>WT ♀ X drp2AaBb ♂</em></td>
<td>19</td>
<td>46</td>
<td>34</td>
<td>0.5</td>
<td>198</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>drp2AaBb ♀ X WT ♂</em></td>
<td>18</td>
<td>43</td>
<td>39</td>
<td>0</td>
<td>120</td>
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</table>
Table 5: *drp2AaBb* plants have shriveled pollen and failed ovules

Mature pollen from open flowers was collected and normal and shriveled grains counted by light microscopy. Maturing siliques were dissected and viewed under a dissecting scope. While 1-2 failed ovules were common even in *WT* siliques, siliques from *drp2AaBb* plants typically had ≥ 5 failed ovules per silique.

<table>
<thead>
<tr>
<th>genotype</th>
<th>% shriveled</th>
<th>N</th>
<th>≥ 5 failed ovules?</th>
</tr>
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<tbody>
<tr>
<td><em>WT</em></td>
<td>0.7</td>
<td>2165</td>
<td>No</td>
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<tr>
<td><em>drp2a-1/2a-1</em></td>
<td>1.4</td>
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<tr>
<td><em>drp2a-3/2a-3</em></td>
<td>0.2</td>
<td>1334</td>
<td>No</td>
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<tr>
<td><em>drp2a-4/2a-4</em></td>
<td>0.1</td>
<td>716</td>
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<td><em>drp2b-2/2b-2</em></td>
<td>0.1</td>
<td>1153</td>
<td>No</td>
</tr>
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<td><em>drp2b-4/2b-4</em></td>
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<td>870</td>
<td>No</td>
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<td>2A/2a-1, 2B/2b-2</td>
<td>21.6</td>
<td>3841</td>
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<tr>
<td>2A/2a-1, 2B/2b-4</td>
<td>15.9</td>
<td>2222</td>
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<tr>
<td>2A/2a-3, 2B/2b-2</td>
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<td>2A/2a-3, 2B/2b-4</td>
<td>16.1</td>
<td>2739</td>
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**Figures**

**Figure 1: drp2 insertional alleles**

(A) Diagram of DRP2 protein domains. PH = pleckstrin homology (membrane binding), GED = GTPase effector domain, PRD = proline rich domain (binds SH3 domain-containing proteins). The middle and GED are coiled-coil domains presumably involved in polymerization.

(B) Position of insertional alleles for DRP2A and DRP2B relative to exons (black bars), introns (black lines) and UTRs (gray bars).

(C-D) Reverse-transcription/PCR demonstrating that all alleles except drp2b-3 are transcript nulls. RNA was extracted from seedling tissue of the indicated homozygous genotype (drp2a-1, drp2a-3, drp2a-4, drp2b-2, drp2b-3, drp2b-4), reverse transcribed, and amplified with primers to detect DRP2A (C) or DRP2B (D) transcript. Control primers against UBIQUITIN 10 (UBQ) were used to verify equal loading of all samples.

(E-F) Immunoblot verifying that all alleles except drp2b-3 have reduced total levels of DRP2 protein, consistent with a complete loss of DRP2A or DRP2B. Total cellular extracts from seedlings homozygous for the indicated genotype (drp2a-1, drp2a-3, drp2a-4, drp2b-2, drp2b-3, drp2b-4) were probed with antibodies raised against a peptide common to both DRP2A and DRP2B as well as either DRP1A or MPK6 to demonstrate equal loading.
Figure 2: *drp2AaBb* siliques have ~25% aborted ovules

(A) Immature siliques from WT and *DRP2A/drp2a-1; DRP2B/drp2b-2* (*drp2AaBb*) plants. Arrows indicate empty spaces with aborted ovules. Scale bar = 1 mm

Figure 3: *drp2ab* embryo sacs arrest at developmental stage FG1 (single nucleate).

(A-F) Propidium iodide staining of fixed, dissected ovules from WT (A-C) and *DRP2A/drp2a-1; DRP2B/drp2b-2* (drp2AaBb) (D-F) plants at developmental stages FG1 (A,D), FG6 (B,E) and shortly after fertilization (C,F). Dashed line indicates the border of the embryo sac.

(G) Semi-thin section through a *drp2AaBb* pistil at stage FG3. Black arrow indicates an ovule with an arrested embryo sac, as identified by the lack of the large central vacuole (V) found in normally developing gametophytes at stage FG3 and later. Scale bars A-G = 20 µm

(H) Higher magnification view of the arrested ovule indicated in (G). Arrow indicates the boundary of the FG1-arrested embryo sac. Scale bar = 5 µm
Figure 4: *drp2ab* FG1-arrested embryo sacs show no defects in membrane morphology

*WT* embryo sacs at stage FG1 (A-B) were visualized by TEM and compared to FG1-arrested embryo sacs in *DRP2A/drp2a-1; DRP2B/drp2b-2 (drp2AaBb)* pistils at stage FG3-FG5 (C-D). All samples were high-pressure frozen, freeze-substituted with OsO$_4$ and embedded in Epon. Arrows indicate the boundary of the FG1 embryo sac. $N =$ Nucleus, $D =$ degenerating non-functional megaspores. No defects in membrane structure or cellular organization were visible in the FG1-arrested mutant embryo sacs ($N =$ 7 arrested embryo sacs). Scale bars = 1 $\mu$m in (A,C) and 200 nm in (B,D).
Figure 5: *drp2ab* pollen grains are shriveled and inviable

Mature pollen from a WT (A,C) or *DRP2A/drp2a-1; DRP2B/drp2b-2* (B,D) anthers visualized by light microscopy (A,B) or Alexander staining (C,D). Arrows indicate shriveled, inviable grains. (E-G) Representative images of pollen quartets from a *DRP2A/drp2a-1; DRP2B/drp2b-2; qrt/qrt* mutant. Zero (E), one (F) or two (G) shriveled grains were observed per quartet, consistent with a gametophytic defect. Scale bars = 20 µm
Figure 6: *drp2ab* pollen grains arrest or collapse at pollen mitosis I

(A-G) Pollen from *DRP2A/drp2a-1; DRP2B/drp2b-2 (drp2AaBb)* anthers stained with DAPI to label nuclei and visualized under fluorescent or brightfield optics. Aberrantly staining or nonstaining grains (arrows) were first observed at the early bicellular stage (D), and began showing structural defects by the late bicellular stage, (E), eventually giving rise to completely shriveled pollen (G).

(H-O) Semi-thin sections through WT (H-K) and *drp2AaBb* (L-O) anthers. Collapsing pollen (black arrows) and pollen arrested at the polarized microspore stage (black arrowheads) were observed in mutant anthers by the early bicellular stage (N). White arrows in I and M indicate cell plates. Scale bars (A-O) = 20 µm.
Figure 7: Cell plate defects are seen in $drp2AaBb$ anthers at pollen mitosis I

(A-F) Semi-thin sections of pollen from $drp2AaBb$ anthers at pollen mitosis I, showing the range of observed cell plate phenotypes. (G-L) TEM images of pollen from WT (G,J) and $drp2AaBb$ (H,I,K,L) anthers at pollen mitosis I. GN = Generative Nucleus. White dotted lines in (G-I) indicate cell plate. Scale bars = 1 µm.
Figure 8: *drp2ab* pollen phenotypes

Pollen in WT and *drp2AaBb* anthers visualized by TEM.  (A,E) WT grain at the early polarized microspore stage.  (B,F) WT grain at the late polarized microspore stage.  (C,G) A normally developing grain from a *drp2AaBb* anther at pollen mitosis I.  (D,H) A grain arrested at the early polarized microspore stage from a *drp2AaBb* anther at pollen mitosis I.  (I,O) A normally developing grain from a *drp2AaBb* anther at the bicellular stage (J,P) An arrested but not collapsed grain in a *drp2AaBb* anther at the bicellular stage.  (K) An arrested grain from *drp2AaBb* anther at the bicellular stage undergoing cytoplasmic collapse.  (L) A plastid with starch granules (arrows) from an arrested grain in a *drp2AaBb* anther at the bicellular stage of development.  (M,N) Abnormal cell wall deposits in arrested grains from a *drp2AaBb* anther at the bicellular stage.  (Q) Golgi stack from a WT pollen grain at the polarized microspore stage.  (R-S) Golgi stacks from arrested grains in a *drp2AaBb* anther at the bicellular stage.  (T) Graph of the number of visible cisternae for Golgi stacks in WT pollen at the polarized microspore stage and arrested pollen grains in *drp2AaBb* anthers at the bicellular stage.  (U) The average ratio of the maximum width of each Golgi stack vs the length of the entire stack for five-stack Golgi in WT pollen at the polarized microspore stage and arrested pollen grains in *drp2AaBb* anthers at the bicellular stage.  Mean ± SEM for N = 9 stacks is plotted.  Asterisk indicates P < 0.5 (student t-test).  Scale bars = 1µm in (A-D,I-J,M) and 200nm in (E-H,K-L, N-T).  V = vacuole N = Nucleus GC = Generative Cell GN = Generative Nucleus VN = Vegetative Nucleus.  Black arrows in (A,D) indicate thin, dark ER profiles.  White arrowheads in (E-H,O-P) indicate the intine.  White arrowheads in (M,N) indicate abnormal cell wall deposits.
Figure 9: Ectopic callose deposition

(A-F) Pollen from developing WT (A-B), *drp1c-1* (C-D) and *drp2AaBb* (E-F) anthers stained with DAPI to label the nuclei (blue) and aniline blue to detect callose deposits (yellow) and visualized under fluorescent optics. Scale bars = 100 µm in (A,C,E) and 20 µm in (B,D,F).
Supplemental Figure 1: WT pollen development

Pollen from DRP2A/drp2a-1; DRP2B/drp2b-2 (drp2AaBb) anthers stained with DAPI to visualize the nucleus and visualized under fluorescent or brightfield optics. Scale bars = 20 µm
Supplemental Figure 2: Aniline blue staining of pollen defective mutants

Developing pollen at the bicellular and tricellular stage of development from the F1 progeny of a cross between WT Ler and WT Col (A-B) or a Salk insertional allele with a large chromosomal translocation (C-P) stained with DAPI to label the nuclei (blue) and aniline blue to detect callose deposits (yellow) and visualized under fluorescent optics. Scale bars = 100 µm in (A,C,E,G,I,K,M,O) and 20 µm in (B,D,F,G,H,J,L,N,P).
Chapter 4: Future Directions

The work presented in this thesis has answered some questions regarding the function of the DRP1 and DRP2 families in cytokinesis and cell expansion, but, characteristic of scientific research, has also raised many new questions that need to be addressed, and which could lead in a number of different directions. Questions still remain about the mechanistic details of DRP1A’s function, as well as the exact role of the DRP2 family in gametophyte development. Looking further, the different phenotypes of the DRP1 and DRP2 families of endocytic dynamins raises the possibility that there are multiple endocytic pathways operating in Arabidopsis, and so another possible route of research is the molecular investigation of plant endocytic pathways. Finally, the gametophytic arrest of the \textit{drp2ab} double mutants raises questions about the control of gametophyte development, in particular of the female gametophyte, about which very little is known.

In this chapter I will discuss possible experimental approaches for moving forwards into each of these four research questions:

1) How are DRP1A’s intrinsic characteristics modulated \textit{in vivo} to allow its cellular function?

2) What is the function of the DRP2 family in gametophyte development?

3) What are the different endocytic pathways in Arabidopsis - what machinery does each utilize, and which cargos does each traffic?

4) How is early female gametophyte development controlled?
Modulation of DRP1A’s intrinsic properties

Our analysis of the *in vitro* structure and lipid interaction of purified, bacterially-expressed *At*DRP1A (Chapter 2) suggest that the plant-specific DRP1 family has distinct characteristics from animal dynamin, even though previous studies have demonstrated that *At*DRP1A, like dynamin, functions in CME (Konopka and Bednarek, 2008). Likewise, the propensity of purified *At*DRP1A to form stable, GTP-insensitive, heterogeneous polymers that promote liposome clustering contrasts with the *in vivo* observation that *At*DRP1A-GFP exists in a cytoplasmic (presumably soluble) pool (Kang et al., 2003a), and that *At*DRP1A can polymerize around membrane tubules during cell plate formation (Otegui et al., 2001). This suggests that *E. coli* expressed *At*DRP1A, while GTPase active, is lacking one or more *in vivo* factors necessary for modulating the polymeric state of individual *At*DRP1A subunits, and thereby polymerizes inappropriately into a form that does not retain full functionality.

The two leading candidates for an *in vivo* modulator of DRP1A are 1) post-translational modifications, such as phosphorylation, the presence of which has been previously hypothesized (Park et al., 1997), and 2) interacting proteins. In addition to a potential role in modulating DRP1A’s activity, DRP1A interacting proteins may also be part of the network of clathrin-mediated endocytic players, or involved in DRP1A’s role at the cell plate. Efforts to date towards identifying DRP1A interacting proteins, including the isolation of a few candidate interactors, as well as future experiments for testing these interactions and examining DRP1A post-translational modifications, are all described in Appendix A.
Role of the DRP2 family in non-gametophytic development

Analysis of \textit{drp2AaBb} double mutants demonstrated a role for the DRP2 protein family in gametophyte development, as both male and female \textit{drp2ab} gametes arrest before undergoing a single mitotic division (Chapter 3). However, published microarray analyses show that both \textit{DRP2A} and \textit{DRP2B} are expressed at consistent levels throughout the entire life-cycle of the plant, suggesting that they also play a role in sporophytic growth and development (http://jsp.weigelworld.org/expviz/expviz.jsp (Schmid et al., 2005)). Three hypotheses can be advanced regarding the role of the DRP2 family in sporophytic development, as compared to its gametophytic role. 1) The DRP2 family is essential only in the gametophyte, and plays a lesser, nonessential role (or no role at all) in the sporophyte. 2) The DRP2 family plays important but distinct roles in both the gametophyte and the sporophyte. These roles could be closely related (e.g. DRP2 could function in perception of extracellular signals during both gametophytic and sporophytic development) or entirely separate. 3) The DRP2 family is essential for viability at the cellular level, regardless of cell type or context. The fully penetrant gametophytic defects in \textit{drp2AaBb} double mutants prevented us from generating adult plants deficient in both \textit{DRP2A} and \textit{DRP2B} to help test these hypotheses. Therefore, we must turn to other approaches to determine the role of the DRP2 family in non-gametophytic tissues.

A commonly used technique for reducing the level of target genes in plants is artificial microRNAs (amiRNAs). MicroRNAs are naturally occurring small, non-coding RNAs that suppress expression of target genes with which they share sequence homology. Endogenous microRNAs often play key roles in developmental regulation in plants. Online tools exist to modify the sequence of known microRNAs in order to target a gene of interest (Schwab et al.,
Stable transformants harboring these targeted microRNAs under an inducible promoter (e.g. a glucocorticoid inducible AlcR-GR promoter available in our lab) can be generated from Arabidopsis plants or suspension-cultured cells (T87 cells) using Agrobacterium-mediated transformation. Reduction of DRP2 levels upon induction of amiRNAs directed against DRP2A or DRP2B could be monitored by RT-PCR and/or immunoblotting with α-DRP2 antibodies.

The induction of amiRNAs in sporophytic tissue (e.g. whole Arabidopsis seedlings) should allow us to distinguish hypothesis 1 from hypotheses 2 and 3, and, depending on the mutant phenotype, may give insight into DRP’s sporophytic function. We could use either amiRNAs against one DRP2 family member in a background null for the other member (e.g. drp2a amiRNA in homozygous drp2b-2 plants) or amiRNAs targeted simultaneously to both family members (drp2ab amiRNA), with the expectation that all of these combinations would give similar phenotypes. The phenotype could be analyzed in a tractable tissue, such as seedling roots, by standard microscopy techniques, including the use of fluorescent organelle markers, endocytic tracers such as FM4-64, and high-pressure freezing transmission electron microscopy (HPF-TEM).

In order to distinguish hypothesis 3 from hypotheses 1 and 2 we would use amiRNA knockdown of both DRP2 family members in T87 cells. Suspension cultured cells are free living diploid cells and, unlike either the gametophyte or any sporophytic cell, are not organized into a tissue and are probably less dependent on cell-to-cell signaling. Therefore, if reduction in DRP2 levels causes arrest or lethality in T87 cells, this will suggest that DRP2 is essential for cellular viability, regardless of the developmental context. Conversely, if no defects are seen in drp2ab amiRNA cells despite significant reductions in DRP2 expression levels, this will suggest that DRP2’s primary role is in cell-to-cell communication or some other aspect of multicellular life.
If growth defects are seen in \textit{drp2ab}^{amiRNA} T87 cells, these should prove a particularly tractable system for phenotypic analysis, especially HPF-TEM, to complement the analysis of \textit{drp2ab} mutant gametophytes described in Chapter 3.

**Molecular dissection of DRP-dependent endocytic networks**

Unique among organisms to date, plants utilize at least two distinct dynamin related protein families in clathrin-mediated endocytosis (CME). Moreover, our overall knowledge of the mechanisms of CME in plants is rudimentary, especially as compared to our understanding of the complex network of proteins and lipids required for CME in yeast and mammalian cells (Schmid and McMahon, 2007). Plants have homologs of the core CME components, including AP2, clathrin and dynamins, but some of the accessory CME factors common to yeast and mammalian cells (e.g. Sla1p, cortactin, and amphiphysin) are absent. The study of CME in plants by traditional biochemical and reverse genetic approaches is complicated both by gene redundancy and because some CME components function in multiple membrane trafficking pathways (e.g. at the trans-Golgi network as well as the PM). Recently, dynamic imaging of PM-associated clathrin-coated particles (CCPs) coupled with siRNA knockdown and/or overexpression of the CME machinery has yielded a more comprehensive view of the temporal and spatial complexity of CME events in animal cells (Jaqaman et al., 2008; Loerke et al., 2009; Mettlen et al., 2009a), and these techniques should be similarly informative in plants.

One central question that can be addressed by dynamic imaging is whether the DRP1 and DRP2 families function independently or cooperatively in CME. Secondly, this imaging could help in the development of a temporal model of the protein-interaction network involved in the initiation and maturation of CCPs at the PM. This imaging would be performed using Variable
Angle Epifluorescence Microscopy (VAEM) coupled with Single-Particle-Tracking (SPT) of endocytic cargo and known or putative endocytic machinery. Analogous to total internal reflectance microscopy (TIRF-M), VAEM allows for imaging of dynamic events at the PM of individual cells in whole plant tissues or suspension-cultured cells in real-time with high-sensitivity and high signal-to-noise (Konopka and Bednarek, 2007).

Previous lifetime and colocalization measurements of PM-associated DRP1 and clathrin light chain 2 (CLC2) foci (Konopka and Bednarek, 2008; Konopka et al., 2008) were performed manually. Automated SPT algorithms (e.g. (Jaqaman et al., 2008)) can overcome this partially subjective and rate limiting step for data analysis and permit medium-to-high throughput processing of multiple samples and experiments. Implementation and validation of existing (Jaqaman et al., 2008) and/or development of new SPT methods would be done in collaboration with the UW-Madison Laboratory for Optical and Computational Instrumentation.

VAEM/SPT analysis of CCP dynamics in cell overexpressing or deficient in various putative CME players could determine the contribution of each part of the CME machinery. For this we could make use of the *drp1a*<sup>rsw9</sup> null mutant (Collings et al., 2008), clc null mutants (see Appendix 3 for current progress on characterization of clc insertional alleles) as well as the *drp2*<sup>amiRNA</sup> seedlings proposed above. In addition, the use of T87 cell lines transformed with inducible overexpression or amiRNA constructs would allow high-throughput analysis of candidate CME accessory proteins. Use of these lines would not only will speed the analysis of candidate proteins but also help overcome likely problems with analysis in whole plants including gene family redundancy and mutant lethality, as seen for example with *drp2*. Direct comparison of CCP dynamics in T87 cells expressing *drp1a*<sup>amiRNA</sup> with those in *drp1a*<sup>rsw9</sup> mutant
seedlings will allow us to determine whether the same effects are seen in the T87s as in whole plants.

Previous studies using whole intact cells versus protoplasts have yielded conflicting results with regard to the localization, function and trafficking of proteins within the plant endomembrane system and PM. Therefore, we would conduct these studies in intact plant cells with fluorescent protein (FP) tagged proteins expressed at or near endogenous levels (as over- or underexpression may also perturb CCP formation). Where possible, we will use constructs whose functionality has been verified by complementation analysis of the corresponding mutant, as has been done for DRP1-FPs (Kang et al., 2003a; Konopka et al., 2008). We believe this will yield the most physiologically relevant data.

**Role of DRP1 vs DRP2 in CCP formation:**

Like DRP1, DRP2A and 2B localize to the PM, and cell plate, and DRP2B-GFP forms foci at the PM similar to those observed with DRP1 ((Fujimoto et al., 2007) and Appendix 2). Moreover, directed yeast-2-hybrid experiments have suggested that DRP1 and DRP2 family members may heteropolymerize (Hong et al., 2003). However, the analysis of *drp1* and *drp2* mutants indicates that these two DRP families are not functionally redundant and result in distinct mutant phenotypes (see Chapter 3). To examine the role of DRP2 in CME we would like to follow the dynamics of DRP2-FP foci at the PM by VAE and SPT, as has been done for DRP1A and 1C and CLC-FPs (Konopka and Bednarek, 2008; Konopka et al., 2008). To this end we have generated DRP2-GFP constructs and are testing their ability to complement the *drp2* mutant phenotype (see Appendix 2). To directly determine whether DRP1 and DRP2 are found on the same or different structures, the extent of DRP1 and DRP2s colocalization in PM-
associated foci could be examined by VAEM imaging of complementary FP-tagged fusion proteins.

If DRP1 and DRP2 are found in the same structures at the plasma membrane, one potential model is that DRP1A and 1C, which lack any discernible protein targeting motifs, are recruited into CCPs via their interaction with DRP2s. A prediction of this model is that depletion of DRP2 would affect DRP1 recruitment to CCPs whereas the association of DRP2 with CCPs should be independent of DRP1. To test this, DRP1-FPs could be imaged in \( drp2^{amiRNA} \) and overexpression plants and cell lines. Finally, the role of both DRP1 and DRP2 in CCP formation could be assessed by analysis of other CCP makers, such as CLC-FPs and σ2-A-FPs (see below) in \( drp1 \) and \( drp2 \) deficient and overexpressing plants and cell lines.

**Functional analysis of CLC in CCP formation:**

Clathrin Light Chain (CLC) is a CME accessory protein conserved through eukaryotes, but its functions seem to vary between organisms (see introduction to Appendix 3). In Arabidopsis there are three CLCs, which share ~55% amino acid identity with each other and only ~30% identity with mammalian CLC. Our lab has previously used CLC2 as a marker for CCPs (Konopka and Bednarek, 2008; Konopka et al., 2008), but has not further examined its functional role. Appendix 3 describes progress to date in the investigation of CLC function, focusing on A) determining if CLC1- and CLC3-FPs show identical localization to CLC2-FPs, and B) characterizing null mutants for each CLC (and combinations thereof), to address the question of functional redundancy between CLC1-3 and determine the overall role of CLC in plant development. In addition to this work, we could also use any CLC-FP that shows a different localization from CLC2-FP as an additional marker of CCPs. Also, we could directly
analyze the role of CLCs in CCP formation and maturation by VAEM/SPT imaging of the dynamics (i.e. lifetime and mobility) of DRP1/2s- and σ2-A-FPs (see below) at the PM of clc null mutant plants and/or CLC overexpressing or clc^amiRNA T87 cell lines.

**AP2 as a marker of CCP**

The AP2 adapter complex is one of the core pieces of CME machinery, linking cargo recognition to assembly of a clathrin coated bud. Of the four subunits that comprise each of the AP1 (Golgi) and AP2 (PM) complexes, two of them, α- and σ-adaptin, are unique to AP2. The α subunit mediates cargo recognition, which is affected by FP fusions to the N- and C-termini, but the σ2-A subunit appears to stabilize AP2 and studies in animal systems have relied of the use of C-terminal FP-tagged σ2-A as a reporter of AP2 function and localization (Ehrlich et al., 2004; Mettlen et al., 2009a). In Arabidopsis the AP2 σ2-A subunit is encoded by a single gene, At1g47830, and so generation of functional σ2-A-FPs should not be technically challenging. These σ2-A-FPs could then be used as an additional marker of CCPs for colocalization and mutant analyses.

**Putative plant CME accessory factors in CCP formation:**

We could extend the above studies on DRPs and CLCs in order to address the function of a wider range of putative accessory factors in plant CME. As described in chapter 1, homologs of some, but not all, of the accessory factors that function in yeast and mammalian endocytosis have been identified in plants, but the functions of many of these proteins have not been characterized. Using VAEM/SPT imaging of CLC-, DRP1/2s- or σ2-A-FFP foci at the PM we could (1) determine if FP-tagged candidate proteins colocalize with CCPs and if so, (2) analyze
the dynamics of CCP initiation and maturation in candidate protein overexpressor and amiRNA T87 cell lines.

Based on current models of the protein machinery involved in mammalian and yeast endocytosis (Schmid and McMahon, 2007), we would focus on the following putative plant CME factors in CCP formation: AP2 and EHDs (Bar et al., 2008) (initiation), EPSINs (Holstein and Oliviusson, 2005; Lee et al., 2007; Song et al., 2006) and SH3Ps (Lam et al., 2001) (invagination), and ARP2/3 (Kotchoni et al., 2009) (release). In addition we would analyze whether AtSeh1, which binds to the C-terminus of DRP2A thereby blocking its interaction with PI(3)P, (Lee et al., 2006) potentially regulates CCP formation in vivo.

This high-throughput study would require a streamlined infrastructure in order to be feasible; in particular, robust and reproducible multichannel VAEM protocols optimized for T87 cells and the development of SPT methods would be critical. The ability to freeze cell lines for storage would also be very helpful, as the generation of cell lines is time consuming and the continual maintenance of multiple lines as calli on plates could prove burdensome.

**Cargo-selective CME pathways?**

If the above studies demonstrated the existence of multiple CME pathways with differing CCP dynamics or distinct requirements for DRPs or other accessory proteins, the next step would be to determine which cargo molecules use each pathway. Some endocytic cargos have already been identified, such as the pathogen-defense receptor FLS2 (Robatzek et al., 2006) and the brassinosteroid receptor Bri1 (Geldner et al., 2007), and many more are likely to be discovered in coming years. Once the cell lines described above have been established and tested to determine which most clearly define each endocytic pathway, it would be relatively
simple to generate or acquire cargo-FPs and determine which endocytic pathways each uses by testing their colocalization with accessory protein FPs and their dynamics in accessory protein overexpressing or amiRNA lines. In this way we could define both the machinery and the relevant cargo of the endocytic pathways operating at the PM in Arabidopsis, which would be a significant contribution to the fields of plant cell biology and development.

**Mapping of FG1-arrested female gametophytic mutants**

Female gametophyte (embryo sac) development is a fascinating process of both developmental and evolutionary interest, as the gametophyte is encased within the maternal tissue of the ovule. Unfortunately, this encasing also makes the embryo sac particularly difficult to study, as the relevant structures are buried inside layers of maternal tissue. Therefore, while embryo sac development has received thorough morphological characterization, the understanding of the molecular events underlying this morphology is extremely limited, especially as compared to what is known of development of sporophytic tissues such as roots and flowers.

Of particular interest is the question of signaling between the maternal, diploid ovule and the haploid gametophyte. Auxin gradients have been shown to play a role in cell-fate determination within the female gametophyte, and may also possibly have a role in progression of gametophyte development, as 26 out of 399 embryo sacs in an auxin-perception quadruple mutant failed to progress beyond the single-nucleate FG1 stage of development (Pagnussat et al., 2009). Also, mutants in a putative sensor histidine kinase CKI1, whose ligand is unknown, show morphological defects beginning at stage FG4 (Hejatko et al., 2003; Pischke et al., 2002). I
think it is likely, given the intimate association of the gametophyte with the surrounding maternal tissue, that additional signaling pathways are operating in gametophyte development.

Due to the difficulties associated with the study of the female gametophyte, the most useful approaches to date have been the isolation of mutants defective in embryo sac development. In particular, a recent large-scale transposon screen identified 130 mutants with defects in female gametophyte function, many of which had morphologically defective embryo sacs. This screen identified both genes implicated in basic cellular metabolism and other housekeeping genes, as well as a variety of putative transcription factors, protein degradation components (perhaps involved in cell cycle regulation) and potential signal transduction components (Pagnussat et al., 2005). However, this screen identified only one mutant that arrested at stage FG1, the first fully haploid step of gametophyte development. In this mutant the transposon site is in a gene of unknown function with multiple transmembrane domains, eda8. In contrast to the results of this screen, previous genetic screens did identify multiple mutants whose sole defect was an FG1 arrest. These include four T-DNA insertional mutants, fem2, fem3, gfa4, and gfa5, as well as one x-ray mutant, gf (Christensen et al., 1997; Christensen et al., 1998; Feldmann et al., 1997; Redei, 1965).

All five of these FG1-arresting mutants have been roughly mapped to a portion of the genome, but the affected gene has not been determined. All of these mutants are publicly available, and modern technology should enable the affected gene to be identified relatively quickly. For T-DNA mutants, Thermal Assymetric Interlaced PCR (TAIL-PCR) can be used to directly give an insertion site (Liu et al., 1995), which can be checked against the rough mapping data to verify that the insertion site is linked to the causative mutation. If the site is not linked, or if complexities such as multiple insertions make TAIL-PCR unfeasible, the mutants could be
mapped using deep sequencing of a single mapping cross (Schneeberger et al., 2009) which, while expensive, can be completed much more quickly than traditional mapping, and would also be suitable for mapping of $gf$.

If progression of gametophyte development is indeed under control of maternally-generated signals, these mutants, which arrest only at FG1, could reveal aspects of that signaling pathway. Alternatively, the mutants might be involved in cell-autonomous developmental pathways, and might encode transcription factors like AGL23, which shows selective FG1 arrest, or factors involved in cell-cycle control. Curtis et al. (Curtis et al., 2009) recently described an FG1 arrest in a mutant with chromosomal rearrangements, and suggested that a chromosomal integrity checkpoint might be operating during stage FG1. If so, these FG1-arresting mutants might likewise harbor chromosomal rearrangements, or encode some of the machinery involved in this checkpoint. A final possibility is that these mutants might encode proteins necessary for the most basic cellular functions, such as DNA synthesis or metabolism. However, this seems less likely given that the transposon mutants identified by Pagnussat et al. (Pagnussat et al., 2009) with defects in basic cellular functions all showed a somewhat later and often variable developmental arrest. Regardless of the outcome, determining the causes of selective FG1 arrest should both help in understanding the phenotype of the $drp2$ double mutants as well as shedding light on the earliest, most poorly understood stage of female gametophyte development.
Appendix A: The Search for DRP1A interactors

Introduction

Animal dynamin directly interacts with a variety of clathrin accessory proteins and the actin regulator cortactin (Lanzetti, 2007; Schmid and McMahon, 2007), and these interactions are essential for its role in endocytosis. Most of these interactions are via dynamin’s proline rich domain (PRD), which is not conserved in the DRP1 family of dynamin-related proteins. It seems likely that DRP1A nevertheless does participate in protein-protein interactions during endocytosis – in fact, it would befit a regulatory GTPase to have many protein-protein interactions through which to exert its regulatory effects. Moreover, I would expect the targeting of DRP1A to sites of endocytosis and the regulation of its polymerization to involve protein interactors, as is the case for animal dynamin. But to date, only a few DRP1-interacting proteins have been found, two of which are involved in cell wall synthesis at the forming cell plate while the third, Van3, is reported to localize to the trans-Golgi-network (TGN). No interacting proteins have been found to date that appear to be related to DRP1’s role in clathrin-mediated endocytosis.

In this appendix I describe two methods potentially applicable to the detection or verification of DRP1-interacting proteins. The first method, immunoprecipitation of DRP1A from suspension cultured cell extracts, was not utilized to search for interactors, but could potentially serve this purpose, or else prove a useful technique for verification of interactions detected by other means. The second method, a split-ubiquitin screen, did yield potential DRP1 interacting proteins, some of which remain possible targets for future work. These candidates and their initial characterization are described.
Materials and Methods

Immunoprecipitation

Protoplasts were generated from 500 µl packed 4-day old Arabidopsis suspension cultured (T87) cells as described in (Kang et al., 2001) and incubated in a 6-well plate with gentle shaking at 24°C for 30 minutes with a total of 75 µCi of $^{35}$S-labeled methionine and cysteine (EasyTag® EXPRE$^{35}$S$^{35}$S mix, Perkin-Elmer, Waltham, MA) dissolved in 2 mls of protoplast isolation buffer (23.2 g/L Gamborg’s salts, 0.45 M sucrose, 1.3 mM KH$_2$PO$_4$, 0.25 µg/ml 2,4-dichlorophenoxyacetate, pH 5.7). The labeled protoplasts were transferred to a 15 ml centrifuge tube and collected by centrifugation (5 minutes at 50g), washed twice with 12 mls protoplast wash buffer (0.4 M betaine, 10 mM CaCl$_2$, 3 mM MES-KOH pH 5.7), resuspended in 2 mls denaturing homogenization buffer, and disrupted by 7 passes through a 25 gauge needle. The lysate was cleared by centrifugation for 5 minutes at 2,000g, and 100 µl of the supernatant was incubated with 50 µl 20% vol/vol proteinA-sepharose (GE Healthcare, Little Chalfont, United Kingdom), 10 µl affinity purified α-DRP1A antibodies (Kang et al., 2001) and 840 µl IP dilution buffer (56 mM Tris, pH7.4, 168 mM NaCl, 5.6 mM EDTA, 1.12% vol/vol TX-100) with or without a 5-fold molar excess of DRP1A- or DRP1C-specific peptide. The immunoprecipitations were incubated 14 hours with rotation at 4°C, washed four times in IP buffer (50 mM Tris, pH7.4, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% TX-100), washed once in IP buffer without any detergent, resuspended in 15 µl SDS-PAGE sample buffer and separated by SDS-PAGE. The gel was dried on a gel drier (model 583, Biorad, Hercules, CA), exposed to a phosphoimaging screen for 78 hours, and imaged on a Typhoon® gel scanner (GE Healthcare).
General DNA Manipulation Methods

PCR amplification was performed with Pfu-Ultra (Stratagene, La Jolla, CA). Site-directed mutagenesis was performed using Phusion polymerase (New England Biolabs, Ipswich, MA), according to the protocol from New England Biolabs (New England Biolabs, 2008). All restriction enzymes and DNA modifying enzymes were purchased from New England Biolabs.

Split-Ubiquitin Screen

Yeast strain NMY51 and all vectors used for the split-ubiquitin screen were purchased from Dualsystems Biotech (Schlieren, Switzerland). Yeast media stocks were purchased from BD Biosciences (Franklin Lakes, NJ). The coding sequence of DRP1A (no stop codon) was amplified with primers 5’-ggccattacggccatggaaaatctgatctctcttg-3’ and 5’-ggccgaggcggccttcttggaccaagcaacagcatcgat-3’, which include directional Sfi1 sites. The PCR product was inserted into the plasmid pGEM-T-EASY (Promega, Madison WI) by TA cloning, then subcloned into the Sfi1 site of bait vectors pBT3-N, pBT3-C and pBT3-STE and the prey vector pPR3-N.

A library of Arabidopsis cDNAs from 6-day old light- and dark-grown seedlings in the bait vector pDSL-NX was purchased from Dualsystems and reamplified by transformation of 4 ng of the library into 80 µl of MegaX H10BT1R Electrocompetent cells (Invitrogen, Carlsbad, CA). Serial dilutions verified that the transformation generated >3x10^7 colony forming units. The entire transformation was divided equally between 15 1 L bottles of 37°C 2x Luria Broth (LB) containing 0.135% Seaplaque agarose (BioWhittaker Molecular Applications, Rockland, ME) with 50 µg/ml carbenicillin, mixed 1-2 minutes with a stirbar, then chilled 1 hour in an ice-water bath to stabilize the suspension. Each bottle was gently transferred to a 30°C incubator and
incubated for 16 hours. Colonies were harvested by centrifugation for 30 minutes at 6000g, resuspended in LB, pelleted again 10 minutes 6000g, and resuspended again with thorough mixing in 300 mls 2xLB with 12.5% glycerol before snap freezing and storage at -80°C. Four 1 L cultures of LB were inoculated with 10 mls each of the reamplified library freezer stock, grown with shaking 16 hours at 22°C, and DNA was isolated by Gigaprep (Qiagen, Germantown, MD), yielding 1.6 mg of purified reamplified library.

The split-ubiquitin screen was performed according to the protocol from Dualsystems. pBT3-STE-DRP1A in yeast NMY51 was transformed with 28 µg reamplified library and plated on leu/trp/his/ade drop-out plates containing 2.5 mM 3-amino-1,2,4-triazol (3-AT). A second screen used 89.2 µg reamplified library and was plated on leu/trp/his/ade drop-out plates containing only 1 mM 3-AT for reduced stringency. All verification and sequencing was also performed according to protocols from the Dualsystems manual.

Interactor Follow-up

An expression construct for CXE12 in the vector pET24d, which attaches a C-terminal His<sub>6</sub> tag, and a cxe12 insertional null mutant were described in (Gershater et al., 2007), and were obtained from the Robert Edwards lab (Durham University, Durham, UK). pET24d-CXE12 was transformed into E. coli (Rosetta strain: EMD Chemicals, Darmstadt, Germany) and expressed and purified by nickel affinity chromatography as described in (Gershater et al., 2007).

A construct for expression of full length HSP81.3 with an N-terminal GST tag was created by Catherine Konopka. HSP81.3 was amplified from seedling cDNA using primers 5’-ggatccaatggcggagctgacctttgcttc-3’ and 5’-ggatccggctcaatctctcatcttgctaccttcagcatg-3’, which include flanking BamH1 sites. The PCR product was inserted into the plasmid pGEM-T-
EASY (Promega, Madison WI) by TA cloning. This construct and the modified expression vector pET41B-TEV, which encodes an N-terminal GST tag, were both digested with NotI, then filled in with T4 polymerase to create a blunt site, digested with BamHI and ligated to form pET41B-TEV-HSP81.3.

A truncation encoding only the C-terminal half of HSP81.3, beginning with amino acid 358, identical to that present in the split-ubiquitin clone, was generated from pET41B-TEV-HSP81.3 by site directed mutagenesis using the 5’ phosphorylated primers 5’-ccgatcccgatccatgggactcctg-3’ and 5’-ggtattggtgactctgaagatcttcctc-3’. Full length and C-term GST-HSP81.3 were expressed in *E. coli* (Rosetta strain) for 16 hours at 18°C and purified by glutathione affinity chromatography.

Purified full length GST-HSP81.3 (3 µg), C-term GST-HSP81.3 (6 µg) and CXE12 (6.3 µg) were incubated with amylose resin-bound MBP-T-DRP1A (Chapter 2) for 1 hour at 4°C with rotation in H(0.15)NG buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 5% v/v glycerol) with 10 mM β-ME and 0.1% v/v NP-40. The resin was separated from the unbound by centrifugation and both were analyzed by SDS-PAGE.

5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) and geldanamycin was purchased from Enzo Life Sciences (Plymouth Meeting, PA). DRP1A-GFP was described in (Kang et al., 2003a), and all imaging was performed as described in (Konopka et al., 2008).

**Results and Discussion**

**Immunoprecipitation of DRP1A from Arabidopsis suspension-cultured cell extracts**

Immunoprecipitation of a protein of interest has been widely used to detect or verify protein-protein interactions. In addition, immunoprecipitation can also be used to answer
questions about the in vivo status of a target protein, such as its turnover rate and whether or not the protein of interest is post-translationally modified. Therefore, I optimized a protocol for the immunoprecipitation of DRP1A using isoform-specific peptide antibodies (Kang et al., 2001) from T87 protoplasts labeled with $^{35}$S cysteine/methionine. This protocol isolated a single band that migrated at the expected MW for DRP1A, and which could be abrogated by the competitive inhibition of the antibodies by the inclusion of a 5-fold molar excess of the peptide against which the antibodies were generated (Fig. 1).

The use of denaturing conditions (2% SDS) during lysis was necessary for the successful immunoprecipitation of DRP1A, indicating that native DRP1A is in a conformation that may limit or block access of the antibody to antigenic region. This is consistent with other reports that DRP1A in cell extracts is found in a large protein complex (see Chapter 2) and the fact that the antigenic region lies between the middle domain and the GED, an area which, based on the structure of animal dynamin polymers is expected to be on the inner surface of the polymer spiral. This requirement for a denaturing lysis means that protein crosslinking would have to be performed in order to use immunoprecipitation to isolate DRP1A interacting proteins, and this is the reason that this technique was not pursued as a general method for finding DRP1A interactors. However, crosslinking-immunoprecipitation followed by immunoblot against target proteins might be a useful way to verify interactions detected by other methods.

**Split-Ubiquitin Screen**

The split-ubiquitin system is a modified yeast-2-hybrid interaction assay designed for the identification of bait proteins that are membrane bound or otherwise unable to translocate into the nucleus (Johnsson and Varshavsky, 1994; Stagljar et al., 1998). For the identification of
DRP1A interactors I utilized a commercially available Arabidopsis cDNA library (Dualsystems Biotech, Schlieren, Switzerland) in which the bait is N-terminally or C-terminally translationally fused to half of ubiquitin and the entire LexA-VP16 transcription factor. The prey is fused to the other half of ubiquitin, with a point mutation to weaken the self-interaction of the two halves of ubiquitin.

Yeast expressing DRP1A in pBT3-N, pBT3-C and pBT3-STE were transformed with the Dualsystems-provided positive and negative prey controls as well as pPR3-DRP1A as an additional positive prey control to verify expression and lack of self-activation of the reporters. None of the bait constructs showed self-activation; pBT3-STE-DRP1A showed the strongest interaction with both positive prey controls. pBT3-STE includes a small non-targeting leader sequence derived from the \( S. \ ceravisiae \) Ste2 protein which can improve protein expression. pBT3-STE-DRP1A still showed only ~1/3 as many colonies with the positive prey control as the Dualsystems-provided positive bait control, and expression of DRP1A could not be detected from any of the bait constructs by immunoblotting against DRP1A, suggesting that low expression was a limiting factor. However, pBT3-STE-DRP1A likely had somewhat higher expression than the other prey constructs (although still not detectable by immunoblot), and so it was selected as the prey for the library screen.

Two library screens were performed. The first was at slightly higher stringency and yielded 10 colonies; the second, using more library and a slightly lower stringency, yielded 127 colonies. All colonies picked also tested positive using a \( \beta \)-galactosidase assay. DNA was isolated from all clones and retested by direct transformation into yeast expressing pBT3-STE-DRP1A or a negative bait control to test for spurious interaction. On retest, 5 and 69 of the colonies from the first and second screen, respectively, showed no interaction; 1 and 35 showed
interaction with the negative control bait, and 4 and 18 were confirmed as positive interactions. The strength of the interaction varied greatly, sometimes being only barely above background. By far the strongest interactor was CXE12, which was pulled out 3 times in the first screen (accounting for 75% of all confirmed interactors from that higher stringency screen) and 5 times in the second. See Table 1 for a complete list of the putative interactors that were identified. Of these, CXE12, HSP81.3 and GRF9 were chosen for follow-up work.

**CXE12**

CXE12 is a soluble carboxylesterase, described by Gershater et al. (Gershater et al., 2007) as the primary enzyme responsible for bioactivation of the proherbicide methyl-2,4-dichlorophenoxyacetate (2,4D-methyl). It is hypothesized to normally function as part of the xenobiotic detoxifying system. There is no *a priori* reason to believe that CXE12 is involved in clathrin-mediated trafficking or any other DRP1A-dependent process; however, a very strong interaction was shown between CXE12 and DRP1A in the split-ubiquitin assay.

A His tagged version of CXE12 generated by the Roberts lab was expressed and purified and incubated with purified resin-bound His₈-MBP-T-DRP1A (Chapter 2) to assay its ability to directly bind DRP1A *in vitro*. However, no binding was detected (Fig. 2A). This may be because purified His₈-MBP-T-DRP1A, which does not appear to be fully functional, is not in the proper conformation for CXE12 binding, or the binding may require other plant factors not present in this *in vitro* system. Alternatively, it may be that CXE12 does not interact with DRP1A in Arabidopsis, and that the interaction detected by the split-ubiquitin screen was spurious or bridged by a yeast protein without homology in Arabidopsis.
The *cxe12* null mutant was crossed with the *drp1A-2* null mutant. However, the progeny have not been screened to determine whether or not there is any sort of synthetic phenotype, either in cytokinesis/cell expansion or in bioactivation of 2,4D-methyl.

**HSP81.3**

HSP81.3 is one of four members of the HSP90 family in Arabidopsis (Sangster et al., 2007), two of which (HSP1.2 and HSP1.3) were identified in the split-ubiquitin screen. HSP90 is a chaperone that binds to and maintains the functionality of a wide variety of protein clients (Pearl and Prodromou, 2006). In Arabidopsis, the HSP90 family has been shown to act as a “capacitor” of phenotypic variation, and its inhibition either by RNAi or by the drug geldanamycin makes plants much more susceptible to a variety of stresses and causes pleiotropic phenotypes (Queitsch et al., 2002; Sangster et al., 2007).

*E. coli* expressed, purified DRP1A forms a large, irregular polymeric complex that is stable in the presence of GTP and unable to tubulate membrane bilayers (Chapter 2). In contrast, DRP1A in living plant cells, like dynamins in other systems, is believed to polymerize into regular rings and spirals encircling membrane templates (Otegui et al., 2001) and to cycle between the polymerized and depolymerized state. One possible explanation for this discrepancy is that a protein interactor (e.g. a protein chaperone) is responsible for maintaining DRP1A in a state that only polymerizes into regular rings and spirals that are competent for regulated disassembly. Therefore, the interaction with HSP83.1, if biologically relevant, might have important implications for the understanding of DRP1A function.

The c-terminal half of HSP81.3 contained in the clone identified in the split-ubiquitin screen includes the majority of the cargo-binding domain, suggesting that DRP1A may be a
client of HSP81.3. However, the relatively low strength of the detected interaction and the fact that the interacting clone contained only a partial, not a full length, protein, make this a very tentative conclusion. Given that this region of HSP81.3 is likely involved in a wide variety of protein-protein interactions, it may have characteristics that make it likely to bind to most any protein when out of its biological context, and so this may not represent a genuine DRP1A-interacting protein.

GST-tagged full length and C-terminal HSP83.1 were incubated with purified resin-bound His$_8$-MBP-T-DRP1A (Chapter 2) to assay their ability to directly bind DRP1A \textit{in vitro}. However, no binding was detected (Fig. 2B-C). This may be because purified His$_8$-MBP-T-DRP1A is not in the proper conformation for HSP83.1 binding, or the binding may require cochaperones or other plant factors not present in this \textit{in vitro} system. Alternatively, it may be that HSP83.1 does not interact with DRP1A in Arabidopsis, and that the interaction detected by the split-ubiquitin screen was simply spurious. When seedlings expressing DRP1A-GFP were treated with 50 µM geldanamycin, an HSP90 inhibitor known to function in Arabidopsis (Queitsch et al., 2002), no change in the localization of DRP1A-GFP was visible by confocal microscopy. This is further evidence that the HSP81.3 – DRP1A interaction detected in the split-ubiquitin assay is not biologically relevant.

\textbf{GRF2 and GRF9}

GRF2, also known as GF14ω, and GRF9, aka GF14µ, are both members of the 13-member Arabidopsis 14-3-3 protein family. 14-3-3 proteins are found throughout eukaryotes and serve a variety of regulatory roles. They bind to specific sites on client proteins, often when those sites have been phosphorylated, and modify the client protein’s function; this often plays a
crucial role in a signal transduction pathway (Ferl et al., 2002). DRP1A does not have a canonical Rxx(S/T)xP phosphorylation/14-3-3 binding consensus sequence (Sehnke et al., 2001), but it does have an RxxSxL sequence that may possibly serve in its place. Also, some 14-3-3 proteins have been reported to bind to alternate sequences, and even to non-phosphorylated sequences (Ferl et al., 2002).

GRF9 is found primarily in chloroplast starch granules, where it functions in regulation of starch accumulation, e.g. during phosphate starvation, primarily by binding starch synthase III (Cao et al., 2007; Sehnke et al., 2001). GRF9 also binds the flowering regulator CONSTANS, and a GRF9 insertional mutant with reduced protein levels shows defects in flowering time and phytochrome responses (Mayfield et al., 2007). A function for GRF2 has not been described, but it cycles between the nucleus and the cytoplasm in a cell-cycle regulated manner.

The data accumulated to date about GRF9 do not show any link to cytokinesis or clathrin-mediated trafficking, arguing against (although not ruling out) the hypothesis that GRF9 regulates DRP1A function. GRF2 seems a more likely candidate; alternatively, a different 14-3-3 family member may be the more relevant interactor, as there can be some redundancy in 14-3-3-client interactions and the specific family members identified in this split ubiquitin screen may simply represent those contained in the cDNA library, not necessarily those with the highest affinity for DRP1A (Robert Ferl, personal communication).

The in vitro DRP1A binding assay used for CXE12 and HSP81.3 is unlikely to give a meaningful result in the case of GRF2 because 14-3-3 proteins typically bind to phosphorylated sequences and *E. coli*-expressed DRP1A is not expected to be appropriately phosphorylated. As a first step in examining the possible interaction between DRP1A and GRF family members I used a general 14-3-3 inhibitor, AICAR, to see if 14-3-3 inhibition had an effect on DRP1A
localization. AICAR interferes with the binding of 14-3-3s to their client proteins, is known to penetrate cell walls and membranes, and has been shown to disrupt the localization of GRF proteins in Arabidopsis (Paul et al., 2005).

Seedlings expressing DRP1A-GFP were treated with 10mM AICAR and their roots were visualized by confocal microscopy to see if the localization of DRP1A was altered. In two separate experiments, AICAR treatment appeared to cause a temporary delocalization of DRP1A from the plasma membrane into the cytoplasm (Fig. 3B). In the first experiment, the delocalization began at 60 minutes of treatment, and normal localization was restored by 90 minutes. In the second experiment, the delocalization began after 75 minutes of treatment, and normal localization was restored by 150 minutes. No delocalization was seen at any time with roots treated with buffer alone (Fig 3A).

This data is suggestive of a possible role for a 14-3-3 protein, such as GRF2 or GRF9, in DRP1A localization. However, it is very preliminary and should be repeated before any conclusions are drawn. It would be useful (although more challenging) to verify the delocalization using time-lapse imaging of a single root over the course of treatment, as opposed to separate roots treated for a given time period as was done in this experiment. Alternatively, the imaging of many more roots (perhaps 6-8, as opposed to the 1-2 imaged in this experiment) could also overcome the issue of variability in DRP1A-GFP localization between seedlings and give convincing evidence of an effect. It might also be helpful to image the treated roots by Variable Angle Epifluoresence Microscopy, perhaps at shorter treatment times, to determine if 14-3-3 inhibition by AICAR has any effect on foci dynamics.

**Conclusions and Future Directions**
Of the potential DRP1A interactors detected by the split-ubiquitin screen, the most promising candidate is the GRF family of 14-3-3 proteins. The HSP81.3 interaction, weak to start with, now seems more likely not to actually occur in Arabidopsis, and the CXE12 interaction, although strong by split-ubiquitin, could not be replicated using purified proteins. It may still be worth assaying the cxe12 drp1A-2 double mutants for genetic interaction; however, the interaction with the GRFs seems a more promising lead to follow, particularly given that these proteins are well suited to play a regulatory role in DRP1A function.

The first step in verifying a potential GRF-DRP1A interaction will be repeating the experiments of AICAR treatment on DRP1A-GFP localization in order to verify the delocalization that was seen and define optimal treatment lengths and concentrations. Should this delocalization be verified, a good next step might be using the split-ubiquitin system to do a directed interaction assay between DRP1A and all 13 members of the GRF family, in order to determine which GRFs have the highest affinity for DRP1A and are therefore likely the most important regulators of DRP1A function. Further work could then focus on verifying this interaction by other methods such as bimolecular-fluorescence complementation, as well as investigating the role of the relevant GRF(s) in DRP1A regulation by isolating grf insertional alleles and crossing these with the drp1A-2 or drp1A^rsw9 (Collings et al., 2008) lines or transforming them with DRP1A-GFP.

14-3-3 proteins typically, though not always, interact with phosphorylated client proteins. Therefore, the interaction of GRF2 and 9 with DRP1A detected by split-ubiquitin suggests that DRP1A may be phosphorylated when expressed in yeast, and is perhaps also phosphorylated when expressed in Arabidopsis. A role for phosphorylation in the regulation of DRP1A (as is known for mammalian dynamin) has been previously suggested based on mobility shifts of
DRP1A in cell extracts (Park et al., 1997). In addition, Dobrowloska (unpublished data) has found that the Arabidopsis osmotic stress response kinase ASK1 can phosphorylate *E. coli* expressed His$_8$-MBP-T-DRP1A *in vitro*, with one of the phosphorylation sites being a conserved serine in the P-loop of the GTPase domain that is presumably critical for GTP hydrolysis. Therefore, an investigation of the phosphorylation status of DRP1A under various conditions is a high priority.

The first step in an investigation of DRP1A phosphorylation could be immunoprecipitation of DRP1A from T87 cell extract using the denaturing conditions described, followed by mass-spectrometry to identify which residues are phosphorylated. This would probably be best done in non-protoplasted T87 cells, as protoplasting (enzymatic digestion of the cell wall) might be expected to disturb endocytosis and lead to differential regulation of DRP1A. The protoplasting described in the immunoprecipitation protocol was primarily to facilitate the $^{35}$S labeling of the cells, and should not be necessary for the immunoprecipitation itself, provided alternative cell disruption methods (e.g. grinding in liquid N$_2$) are used to generate the cell lysate. It might also be possible to extend the immunoprecipitation methods to use other tissues such as seedling roots.

Subsequent to establishing a baseline phosphorylation pattern for DRP1A, I would next determine DRP1A phosphorylation in cells treated with salt or other osmotic stress, both because of the potential phosphorylation of DRP1A by ASK1 as well as reports that salt stress modulates endocytosis (Leshem et al., 2007). DRP1A phosphorylation in cells treated with direct inhibitors of endocytosis such as TyrphostinA23 would also be of interest.

Finally, once phosphorylation sites of interest were determined, the function of these modifications could be determined by introducing non-phosphorylatable and phosphomimetic
mutations into DRP1A-GFP, to determine the effect on localization and endocytic foci dynamics, as well as *E. coli* expressed His$_8$-MBP-T-DRP1A, to determine the effect on DRP1A’s biochemical properties.
Table 1: Putative DRP1A interactors from a split-ubiquitin screen

Eight independent colonies were identified as CXE12, and two were identified as Rubisco small chain 1B; all other putative interactors were identified only once.

<table>
<thead>
<tr>
<th>Gene number</th>
<th>Name</th>
<th>Interaction strength</th>
</tr>
</thead>
<tbody>
<tr>
<td>At3g48690</td>
<td>CXE12</td>
<td>Strong</td>
</tr>
<tr>
<td>At5g23530</td>
<td>CXE18</td>
<td>Strong</td>
</tr>
<tr>
<td>At1g78300</td>
<td>GRF2</td>
<td>Medium</td>
</tr>
<tr>
<td>At2g42590</td>
<td>GRF9</td>
<td>Medium</td>
</tr>
<tr>
<td>At5g56010</td>
<td>HSP81.3</td>
<td>Medium</td>
</tr>
<tr>
<td>(c-terminal half)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>At5g56030</td>
<td>HSP81.2</td>
<td>Weak</td>
</tr>
<tr>
<td>(c-terminal half)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>At4g10840</td>
<td>Kinesin light chain related</td>
<td>Medium</td>
</tr>
<tr>
<td>(c-terminal 2/3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>At5g38430</td>
<td>Rubisco small chain 1B</td>
<td>Medium</td>
</tr>
<tr>
<td>At5g45550</td>
<td>mob1/phocein family protein</td>
<td>Weak</td>
</tr>
<tr>
<td>At1g37130</td>
<td>Nitrate reductase 2</td>
<td>Weak</td>
</tr>
<tr>
<td>At3g16400</td>
<td>(NOT IN FRAME)</td>
<td>N/A</td>
</tr>
</tbody>
</table>

(NOT IN FRAME)
Figure 1: Immunoprecipitation of DRP1A from cell lysates

DRP1A-specific peptide antibodies were used to immunoprecipitate DRP1A from denatured cell lysates of T87 protoplasts labeled with $^{35}$S cysteine/methionine. Competition of the immunoprecipitation with a five-fold molar excess of the peptide against which the antibodies were raised (1A), but not a non-specific peptide (ns), eliminated the recovery of DRP1A, demonstrating the specificity of the reaction.
Figure 2: CXE12 and HSP83.1 do not bind to DRP1A in vitro

His\textsubscript{8}-MBP-T-DRP1A prebound to amylose resin was incubated with purified CXE12-His\textsubscript{6} (A), GST-HSP81.3 (B) and GST-HSP81.3 C-terminal half (C). Resin-bound His\textsubscript{8}-MBP-T-DRP1A was isolated out of the reaction mixture by centrifugation, and the supernatant (Unbound, U) and resin (Bound, B) was analyzed by SDS-PAGE/Coomassie staining. No band of the appropriate size for CXE12, HSP81.3 or HSP81.3C was detected in the bound fraction, indicating that there was no binding of any of these proteins to His\textsubscript{8}-MBP-T-DRP1A.
Figure 3: Delocalization of DRP1A-GFP upon AICAR treatment

Arabidopsis seedlings expressing DRP1A-GFP were incubated in 0.5X MS media (A) or 0.5X MS Media containing 10mM AICAR for 75 minutes prior to imaging by confocal microscopy. Scale bars = 20 µm.
Appendix B: Generation and Partial Characterization of DRP2-GFPs

Introduction

In plants, two distinct families of dynamin-related proteins are thought to function in clathrin-mediated trafficking, including clathrin mediated endocytosis (CME): the DRP1 family and the DRP2 family. The DRP2 family contains two members, DRP2A and DRP2B, which share 93% amino acid similarity to each other. Unlike members of the DRP1 family, but like animal dynamin, DRP2A and B are “classical dynamins” containing a pleckstrin homology and proline rich domain, and there is evidence that these domains play a role in regulating their function (Lam et al., 2002; Lee et al., 2006; Lee et al., 2002). Genetic evidence has demonstrated that the DRP2 family, like the DRP1 family, is essential for Arabidopsis development, suggesting that it plays a unique role in clathrin-mediated trafficking, and that in fact there may be multiple non-redundant endocytic pathways operating in plants (Chapter 3). However, nothing is known about how DRP2-dependent endocytosis compares to DRP1-dependent endocytosis.

One key tool for the study of endocytosis in a variety of systems is fluorescent protein (FP) fusions of the protein of interest. This not only allows the visualization of a protein’s subcellular localization, but also of its dynamics, and can allow tracking and investigation of the structures (e.g. clathrin coated particles) with which that protein associates. Functional FP fusions of DRP1A and -1C were instrumental in demonstrating the endocytic role of those two proteins, and shed light on the overall process of plant endocytosis (Konopka and Bednarek,
Functional DRP2-FPs would likewise be an invaluable tool for studying the role of the DRP2 family and the mechanisms of clathrin-mediated trafficking in plants.

The most useful FP fusions are those that A) are expressed under the control of their native regulatory elements at near-native expression levels, and B) have been demonstrated to be functional by their ability to complement the defects seen in the corresponding null mutant. C-terminal FP fusions to DRP1A and DRP1C have been demonstrated to be fully functional, although in the case of DRP1C this required the use of a full length genomic sequence including 1.8 kB of upstream sequence, 5’UTR, and all introns (although not the 3’UTR).

To date, the only DRP2-FP constructs that have been described in the literature have used the 35S viral promoter combined with the coding sequence of DRP2A or 2B, and somewhat conflicting results for the localization of DRP2 to the Golgi and/or plasma membrane have been obtained with these constructs in various expression systems (Fujimoto et al., 2008; Hong et al., 2003; Jin et al., 2001). Also, because our work (Chapter 3) is the first report of a DRP2 null mutant phenotype, none of these constructs have been assayed for functionality. Therefore, I began generating and imaging DRP2-GFP fusions and testing their ability to rescue the gametophytic defects seen in drp2AaBb double mutants.

**Materials and Methods**

**Generation of DRP2-GFP constructs:**

All PCR amplification of coding sequences and promoters was performed with Pfu-Ultra polymerase (Stratagene, La Jolla, CA). All full length genomic PCR amplification and site directed
mutagenesis was performed with Phusion polymerase (New England Biolabs, Ipswich, MA). All restriction enzymes, phosphatases and ligases were likewise from New England Biolabs (Ipswich, MA). All primers were purchased from Integrated DNA Technologies (Coralville, IA). All plasmid isolation was performed with miniprep kits from Dennville Scientific (Metuchen, NJ).

The coding sequence of DRP2A was amplified using primers 974 and 985 at 45°C from cDNA generated from 7-day old seedlings using an Accuscript cDNA synthesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. The coding sequence of DRP2B was amplified using primers 973 and 985 at 45°C from a DRP2B cDNA clone in pUNI (Clone U13279) acquired from the Arabidopsis Biological Resource Center (ABRC, Ohio State University, Columbus, OH). Primers 985 and 973/4 contain AttB1 and AttB2 sites, respectively, and primer 985 also contains a Swa1 site between the AttB1 site and the start codon. The PCR product was purified by PEG precipitation and recombined into pDONRzeo (Invitrogen, Carlsbad, CA) using standard Gateway® cloning procedures. The construct was verified by sequencing then recombined into pMDC83 to generate 35S:2A-GFP and 35S:2B-GFP. The DRP2B 1.5kb promoter was amplified from BAC F14N23 (acquired from ABRC) using primers 1236 and 1237, which include a Swa1 site, at 52°C. The resulting PCR product and pDONRzeo-DRP2B were both digested with Swa1, and the DRP2B promoter was inserted by blunt-end ligation. The resulting construct was verified by sequencing and then recombined into pMDC107 to generate 2B-prom-CDs-GFP.

The full length genomic sequence of DRP2A, including 1.7 kb of upstream sequence (promoter), was amplified from BAC F14N23 (acquired from ABRC) using primers 1244 and 974 at 51°C. The full length genomic sequence of DRP2B, including 1.5 kb of upstream sequence (promoter), was amplified from BAC T30E16 using primers 1245 and 973 at 51°C.
The PCR products were precipitated with sodium acetate/ethanol (PEG precipitation caused a dramatic mobility shift in the product, presumably due to PEGylation, and thus could not be used) and recombined into pDONRzeo. The resulting constructs were verified by sequencing then recombined into pMDC107 to generate 2Agen-GFP and 2Bgen-GFP and pMDC99 and 123 to generate 2Agen and 2Bgen.

Full length DRP2A and promoter in pDONRzeo was altered by two rounds of site-directed mutagenesis (New England Biolabs, 2008) using phosphorylated primers, each time followed by sequencing. The first round inserted a SwaI site directly after the final codon of DRP2A using primers 1283 and 1284 at 67°C. The second round inserted an AgeI site followed by the sequence for a 10-alanine linker immediately before the ATG of DRP2A using primers 1285 and 1286 at 60°C. EGFP in pGEM (SB1016, generated by D. Rancour) was digested with AgeI and BspE1 and inserted into the introduced AgeI site of DRP2Agen-pDONRzeo. A pair of complementary phosphorylated primers contain stop codons in every reading frame in both directions (1294 and 1295) were mixed, heated to 95°C for 10 min then slowly (0.1°C/sec) cooled to 10°C. Ten picomols of this mixture was blunt ligated into the introduced SwaI site of DRP2Agen-pDONRzeo. All junctions were verified by sequencing and the final construct was recombined into pMDC99 to generate GFP-2Agen.

All final expression constructs were transformed into WT Columbia (2B-GFP) or drp2A-1 and drp2B-2 (all other constructs) using Agrobacterium strain GV3101. Plants were selected on 0.6% agar 0.5XMS plates containing 25 µg/ml hygromycin B (MP Biomedical, Solon, OH) or, for pMDC123, 15 µg/ml glufosinate (AgrEvo, Berlin, Germany). General plant growth conditions were as in Chapter 3. Prior to imaging, seedlings were grown vertically for 4-5 days on 1% agar 0.5XMS plates containing 25 µg/ml hygromycin B. Plants were screened for
fluorescence using a Leica M165 FC dissecting microscope (Leica Microsystems, Bannockburn, IL). Laser Scanning Confocal Microscopy (LSCM) and Variable Angle Epifluorescence Microscopy (VAEM) was performed as described in (Konopka et al., 2008). DRP1A-mOrange was described in (Konopka and Bednarek, 2008), and CLC2-mOrange was described in (Konopka et al., 2008).

**Results and Discussion**

**DRP2-GFP constructs**

A variety of GFP-tagged DRP2 constructs were generated and introduced into the *drp2A-1* and *drp2B-2* mutant backgrounds (Table 2, Fig. 1). 35S promoter-driven DRP2B-GFP (35S-2B-GFP) and native promoter driven, full length DRP2A genomic and DRP2B genomic -GFPs (2Agen-GFP and 2Bgen-GFP) all gave rise to viable lines with sufficient fluorescence for LSCM imaging, and all showed a consistent localization (see below). A construct in which the DRP2B promoter was used to drive expression of the DRP2B coding sequence fused to GFP (2Bpro-CDs-GFP) was generated but not imaged.

In contrast, an N-terminal GFP-DRP2A construct (2Apro-GFP-2Agen) only gave rise to lines with very dim fluorescence. This construct uses the same promoter and intron elements as 2Agen-GFP, which has bright fluorescence, suggesting that location of GFP (N-terminal vs C-terminal) affects the folding or stability of the fusion protein.

**DRP2-GFPs localize to the cell plate and plasma membrane like DRP1-GFPs**

Native promoter driven, full length genomic 2Agen-GFP and 2Bgen-GFP both localized to the cell plate, plasma membrane and (more faintly) to the cytoplasm in Arabidopsis roots as
imaged by Laser Scanning Confocal Microscopy (LSCM) (Fig. 2A and B). No difference in localization between 2Agen-GFP and 2Bgen-GFP was evident, and identical localization was also seen with a 35S:DRP2B-GFP (Fig. 2C). The localization of all DRP2-GFPs was very similar to that previously described for DRP1A-GFP (Kang et al., 2003a), as was verified by the colocalization of 35S:2B-GFP with DRP1A-mOrange (Fig. 2D). This localization was generally similar to that reported for a 35S:2B-GFP by Fujimoto and colleagues (Fujimoto et al., 2007; Fujimoto et al., 2008); however, very few cytoplasmic foci were evident in my hands. The failure of 35S:2B-GFP to colocalize with the TGN marker CLC2-mOrange further supported the conclusion that, contrary to previous reports, DRP2-GFP did not show significant localization to the TGN under the conditions tested.

**DRP2-GFP forms foci at the plasma membrane**

When the plasma-membrane-associated pool of 35S:2B-GFP was observed by Variable Angle Epifluorescence Microscopy (VAEM), it formed dynamic foci similar to those seen with CLC2- and DRP1A- and DRP1C-FPs (Fig. 2E) (Konopka and Bednarek, 2008; Konopka et al., 2008). This is consistent with the report of Fujimoto and colleagues that such foci were formed by a 35S-DRP2B-GFP construct expressed in tobacco suspension cultured cells (BY2) (Fujimoto et al., 2007). Surprisingly, these foci did not show significant colocalization with CLC2-mOrange foci (Fig. 2F), raising questions about their nature. More VAEM imaging will be necessary to verify this result, preferably once the functionality of the DRP2-GFP construct has been demonstrated.
Uncovering of a hygromycin-dependent morphology defect in the *drp2a-1* background

The 35S:2B-GFP, 2Agen-GFP and 2Bgen-GFP constructs all confer resistance to hygromycin. When *drp2a-1*, but not *WT* or *drp2b-2*, lines transformed with each of these constructs were germinated on plates containing 25 μg/ml hygromycin B, a large proportion of plants displayed meristematic defects. These manifested first as an asymmetry in the emergence of the first true leaves, followed after transfer to soil by misshapen leaves and the presence of multiple shoot apical meristems, eventually leading multiple primary inflorescences (Fig. 3). The multiple meristem phenotype was seen in 395/574 (69%), 94/216 (44%) and 73/166 (44%) of homozygous *drp2a-1* plants containing 35S:2B-GFP, 2Agen-GFP and 2Bgen-GFP, respectively. These phenotypes were not seen in *drp2a-1* plants germinated on plates without hygromycin, regardless of whether or not a transgene was present, and were also not seen in *drp2b-2* plants containing any of these transgenes even when germinated on plates containing hygromycin. Therefore, this defect appears to be the result of a particular sensitivity of *drp2a-1* plants to hygromycin, and may represent a partial silencing of the hygromycin resistance in the *drp2a-1* mutant background. Whether this phenotype is the result of the disruption of *DRP2A* or some other, unrelated feature of this particular insertional mutant is not clear; this can be tested by the introduction of the transgenes into the *drp2a-3* or *drp2a-4* mutant lines. If this phenotype is also seen in these lines, it might be specific to *DRP2A* and should be pursued further; if not, the use of the *drp2a-1* allele should simply be avoided when generating drug-resistant transgenic lines.
Rescue of \textit{drp2AaBb} gametophytic defects

The ultimate test of the functionality of any protein-FP fusion is its ability to rescue the defects seen in the corresponding null mutants. \textit{drp2aa} and \textit{drp2bb} single mutants have no morphological defects but \textit{drp2AaBb} double mutants fail in both male and female gametophyte development, which manifests itself in the form of transmission defects as well as giving rise to \(~12\text{-}20\%\) shriveled pollen in \textit{drp2AaBb} anthers and \(~25\%\) empty spaces in \textit{drp2AaBb} siliques (see Chapter 3).

The ability of the 35S:2B-GFP construct to rescue this phenotype was not tested because the 35S promoter has been previously reported to have no activity in Arabidopsis pollen (Wilkinson et al., 1997), and no information is available about its activity during embryo sac development. Therefore, we focused on the native promoter-driven construct.

We screened hygromycin-selected F1 progeny of a cross between a \textit{drp2a-1} line and a \textit{drp2b-2} line, each of which had been independently transformed with 2Agen-GFP, for shriveled pollen to determine the ability of 2Agen-GFP to rescue the pollen phenotype. These hygromycin-selected F1 plants may have one or two independent copies of 2Agen-GFP, both present in a heterozygous state, which would segregate in the pollen and the embryo sacs and decrease the proportion of failed gametes observed. Assuming that 2Agen-GFP was present in a heterozygous state in the \textit{drp2a-1} and \textit{drp2b-2} parents, a full rescue would give rise to as 2/3 of \textit{drp2AaBb} F1 plants with 12.5\% inviable gametes and 1/3 of the \textit{drp2AaBb} plants with 6\% inviable gametes. If the transgene did not rescue, 100\% of the \textit{drp2AaBb} F1 plants would be expected to have 25\% inviable gametes. Eight \textit{drp2AaBb} F1 plants expressing 2Agen-GFP all had between 11\% and 17\% visibly shriveled pollen, similar to what is observed in \textit{drp2AaBb}
plants without any transgene, suggesting that the 2Agen-GFP construct does not rescue the pollen defect. These plants show faint but clear GFP fluorescence in maturing pollen, brightest at approximately the bicellular-tricellular stage then fading to background levels at pollen maturity. This suggests that the construct is expressed; however, expression levels or timing may not be native. Also, the GFP signal appears diffuse throughout the pollen grain, suggesting that the membrane targeting of 2Agen-GFP in pollen may be disrupted. Either of these could explain the apparent failure of this construct to rescue the male gametophytic defect.

Aborted ovules were also seen in \textit{drp2AaBb} F1 plants expressing 2Agen-GFP, however, the proportion of the shriveled ovules was not determined, leaving it unclear whether or not this construct is able to rescue the female gametophytic defect. F1 \textit{drp2AaBb} plants expressing 2Bgen-GFP similarly had both shriveled pollen and shriveled ovules, but again the proportions of the failed gametes was not determined. Ten \textit{drp2AaBb} F1 plants expressing 2Apro-GFP-2Agen had between 10\% and 25\% visibly shriveled pollen, suggesting that the 2Apro-GFP-2Agen construct likewise does not rescue the pollen defect.

**Conclusions and Future Directions**

The DRP2-GFP constructs described in this appendix have already provided some intriguing results, such as the lack of localization of DRP2 to TGN/endosomes in whole Arabidopsis roots and the possibility that, unlike DRP1-FGs, DRP2-GFP might not be present in the same PM foci as CLC2-mOrange. However, this latter result is very preliminary, and extensive VAEM imaging of native promoter driven DRP2A and -2B -GFPs, alone and in combination with CLC2-mOrange, will be necessary to define the function of DRP2-containing PM foci.
This imaging would be best done in a homozygous *drp2aabb* line in order to eliminate possible interference from endogenous DRP2s; this would require the ability of the DRP2-GFPs to rescue the lethality seen in *drp2ab* gametes. Initial assays of the ability of 2Agen-GFP and 2Apro-GFP-2Agen to rescue the pollen lethality have not been promising; however, it may still be possible to recover homozygous rescued plants if there is a partial pollen rescue, or if the constructs rescue the embryo sac phenotype. Therefore, it may be worth using PCR-based genotyping to screen for the existence of *drp2Aabb*, *drp2aaBb* or *drp2aabb* plants expressing 2Agen-GFP or 2Bgen-GFP F2 generation. Given A) the potential complexities of having two independently segregating copies of the DRP2-GFP transgene, and B) the presumably spurious hygromycin-dependent vegetative phenotypes seen in the *drp2a-1* background, I suggest crossing brightly fluorescent lines containing 2Agen-GFP or in the *drp2a-3* background or 2Bgen-GFP in the *drp2b-2* background with non-transgenic *drp2b-2* or *drp2a-3* plants, respectively, and using these offspring for testing of rescue and subsequent imaging.

Even if the DRP2Agen-GFP or DRP2Bgen-GFP constructs are not able to rescue either gametophytic defect, they may still be useful for fluorescent imaging, especially if this failure to rescue can be traced to a lack of proper expression in the gamete. A useful experiment to determine whether or not the promoter elements included in these constructs are sufficient would be to test the ability of the 2Agen construct, which differs from 2Agen-GFP only by the absence of the GFP, to rescue the gametophytic lethality. If 2Agen also fails to rescue, it will suggest that some other part of the genomic sequence, such as more upstream material or the 3’ UTR is necessary for proper expression of DRP2A in the pollen, and further experiments might be able to determine exactly what is needed for proper expression. If, in contrast, 2Agen is able to rescue, this will suggest that the presence of the GFP is causing a defect in the function of the
DRP2A protein in the gametophyte, and this would recommend caution when using GFP-tagged DRP2A or DRP2B to study the localization or dynamics of this protein in any tissue.
## Tables

### Table 1: Oligonucleotides used in the this study

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### Table 2: DRP2 expression constructs

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<td>DRP2A CDs</td>
<td>C-term</td>
<td>Not in plants – transformants infertile.</td>
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<td>pMDC83</td>
<td>35S</td>
<td>DRP2B CDs</td>
<td>C-term</td>
<td>Cell plate, PM foci. Not expected to rescue (35S promoter).</td>
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<td>DRP2B CDs</td>
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<td>C-term</td>
<td>Cell plate, PM (no VAEM yet). Rescue not yet clear.</td>
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<td>DRP2A genomic</td>
<td>N-term</td>
<td>Faintly cell plate, but too dim to be useful. Does not seem to rescue.</td>
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Figures

Figure 1: DRP2 expression constructs

Schematic of DRP2 expression constructs discussed in this appendix, including vector backbone, promoter elements, start and stop codons and other translated and untranslated sequences. Upstream region includes all material 1.6 kB upstream of the endogenous start codon, including the native 5’UTR.

- **R** - Recombination sequence
- **A** - 10 alanine linker

- **Coding sequence**
- **Genomic sequence**
- **~1.6 kb upstream sequence**
- **Start Codon**
- **Stop Codon**
**Figure 2: DRP2-GFPs localize to the forming cell plate and the plasma membrane.**

Native promoter driven, full length genomic DRP2A (A) and DRP2B (B), as well as the 35S promoter driven DRP2B CDs (C-G) show strongest localization to the forming cell plate, with weaker localization to the plasma membrane and a faint cytoplasmic background when visualized by LSCM in *Arabidopsis* roots (A-D). This localization is indistinguishable from that shown by native promoter driven, fully functional DRP1A and DRP1C-FPs, and 35S:2B-GFP colocalizes with DRP1-GFP by LSCM (D). In contrast to previous reports, these DRP2-GFPs do not show significant localization to the TGN. Although occasional bright foci are seen, these do not colocalize with CLC2-mOrange (E). Scale bars A-D = 20 μm.

As previously reported, 35S:2B-GFP does form dynamic foci at the plasma membrane reminiscent of sites of endocytosis as viewed by VAEM (F,G) but these sites do not show significant colocalization with bona fide clathrin-mediated-endocytic sites labeled with CLC2-mOrange (G). Scale bars F-G = 1 μm.
Figure 3: *drp2a-1* hygromycin sensitivity

Comparison of *drp2a-1/2a-1* : 35S-2B-GFP plants germinated on no drug plates (A) with those germinated on plates containing 25 µg/ml hygromycin B (B-D). (C) Higher magnification view of a plant from (B). Arrows indicate multiple meristems. (D) The same plant as in (C), after the transition to flowering. Arrows indicate multiple primary inflorescences. Scale bars = 1 mm
Appendix C: Characterization of the Function and Localization of

*Arabidopsis* Clathrin Light Chains

The work described in this appendix has been a collaborative effort among a number of workers in the Bednarek lab. The project was initiated by Catherine Konopka, who generated the CLC2-GFP construct (Konopka et al., 2008) and began the analysis of the insertional alleles, which was continued by the undergraduate researchers Nihdi Doshi and Matt Rammer, under Catherine’s and subsequently my direction. The production of CLC2 for antibody generation was performed by Nidhi Doshi, the characterization of the antisera by western blot was performed by Matt Rammer, and the production and visualization of CLC1-GFP was performed by Matt Rammer. The colocalization of CLC2-mOrange with DRP1A- and DRP2B-GFPs was performed by me.

Introduction

Clathrin Light Chains (CLCs) are conserved elements of the clathrin-mediated endocytic machinery, found in all eukaryotes. CLCs contain multiple protein-binding motifs that allow them to interact with clathrin heavy chain and other elements of the clathrin-mediated endocytic machinery (Schmid and McMahon, 2007). *In vitro* data suggests that CLC plays an inhibitory role in Clathrin Heavy Chain (CHC) trimerization (Pishvae et al., 1997; Ybe et al., 2003; Ybe et al., 1998), however, the in *vivo* relevance of this has recently been debated based on the 5-fold lower abundance of CLC than CHC in mammalian cells (Poupon et al., 2008). *S. cerevisiae clc* deletion mutants display a slow growth phenotype similar to (although not identical with) the
phenotype observed in loss-of-function \textit{chc} mutants (Silveira et al., 1990). Similarly, Dictyostelium \textit{clc} null mutants grow more slowly than wild-type and show cytokinetic and osmoregulatory defects. In these cells, clathrin-coated particles still form; however, a greater proportion of CHC is cytoplasmic, suggesting that CLC might promote membrane association of CHC (Wang et al., 2003a). In mammals, where CLC has been shown to link clathrin-coated particles to the actin cytoskeleton, perhaps via its interaction with the actin-regulating protein Hip1R (Poupon et al., 2008), there are two distinct isoforms of CLC, CLCa and CLCb, and their depletion by RNAi leads to distinct effects on clathrin-coated pit lifetimes and maturation frequencies (Mettlen et al., 2009a). However, these effects are very subtle, and do not result in measurable defects in uptake of various endocytic cargo or morphological alteration of the clathrin coated pits as viewed by electron microscopy (Huang et al., 2004; Poupon et al., 2008). Instead, the primary phenotype seen in mammalian CLC-depleted cells are defects in clathrin-mediated trafficking at the Trans Golgi Network (TGN) (Poupon et al., 2008).

Although CLC is conserved through eukaryotes, it is not clear that it has an identical function in each organism. Moreover, in Arabidopsis there are three CLCs, which share \textasciitilde 55\% amino acid identity with each other and only \textasciitilde 30\% identity with either mammalian CLCa or CLCb. These three CLCs may serve redundant roles in clathrin-mediated trafficking, or, like mammalian CLCa and b, they may have distinct functions.

Purified Arabidopsis CLC2 has been demonstrated to interact with bovine CHC (Scheele and Holstein, 2002), and CLC2-FPs localize to TGN and dynamic plasma-membrane associated foci, consistent with the known locations of clathrin-mediated trafficking in plants (Konopka et al., 2008). However, while this \textit{in vitro} data and localization support the presumptive role of CLC2 in clathrin-mediated trafficking, the actual function of CLC1-3, or their role in the larger
process of plant development, are unknown. The aims of this project are to A) determine and compare the subcellular distribution of CLC1-3–FPs, and to B) characterize null mutants for each CLC (and combinations thereof), to address the question of functional redundancy between CLC1-3 and their the overall role in plant development. This appendix is an update on the current status of this project.

**Materials and Methods**

**Genes Described in This Study:**

CLC1 = At2g20760, CLC2 = At2g40060, CLC3 = At3g51890

**Production of CLC1-GFP**

General PCR and DNA manipulation conditions were as described in Appendix A. All restriction enzymes were from New England Biolabs (Ipswich, MA).

The full length genomic sequence of CLC1, including 1.5 kb of upstream sequence, was amplified from the BAC F5H14 using primers 1264 and 1266, which anneal to the upstream region and final codon of CLC1 and contain a Nsi1 and a Sac1 restriction site, respectively. The PCR product was inserted into the plasmid pGEM-T-EASY (Promega, Madison WI) by TA cloning and verified by sequencing. The 1.5 kb region upstream of CLC1 contains a portion of another unrelated gene, At2g20770, which is transcribed in the opposite direction. To avoid potential issues related to expression of this partial gene fragment we performed site-directed mutagenesis (New England Biolabs, 2008) using the 5’ phosphorylated primers 1267 and 1268 to delete the A of the start codon (ATG) of At2g20770. The product of this mutagenesis was verified again by sequencing, then digested with Nsi1 and Sac1 and cloned into Pst1/Sac1
digested pZP211-GFP (Kang et al., 2003a). The final construct was transformed into both Columbia (Col) and Wassilewskija (WS) ecotype using Agrobacterium strain GV3101. Seedlings were selected on Kanamycin prior to imaging. All fluorescent microscopy was performed as described in (Konopka et al., 2008)

**Expression and Purification of CLC2**

The coding sequence of Clathrin Light Chain 2 was amplified using primers 1280 and 1281, which introduce attB1 and aatB2 recombination sites at the 5’ and 3’ ends of the gene, respectively. Primer 1280 also contains a sequence coding for the Tobacco Etch Virus (TEV) protease recognition site, between the attB1 recombination site and the translational start site of CLC2. Amplification was performed using Phusion polymerase (New England Biolabs, Ipswich, MA) at an annealing temperature of 61°C. The PCR product was PEG purified and recombined into pDONR221 and subsequently pDEST15 (Invitrogen, Carlsbad, CA) and pVP16 (Blommel and Fox, 2007) according to the Gateway® protocol. pDEST 15 and pVP16 encode Glutathione-S-Transferase (GST) or His-tagged Maltose Binding Protein (His8-MBP), respectively, in frame with the N-terminus of the insert. pDEST15–CLC2 was transformed into the Rosetta strain of E. coli (EMD Biosciences, Darmstadt, Germany) for protein expression, and pVP16-CLC2 was transformed into E. coli strain B834 pRare2 (CESG, UW Madison).

Cultures of E. coli expressing pVP16-CLC2 were grown to saturation, diluted 1:10 in LB (Luria Broth + carbenicillin\(^{50\mu g/ml}\)/chloramphenicol\(^{34\mu g/ml}\)), grown for 2 hours at 37°C, induced with 0.5mM Isopropyl β-D-1-thiogalactopyranoside (IPTG), and grown an additional 2 hours at 22°C. The 250 ml cultures were harvested by centrifugation (10 min at 5000g), and the pellet resuspended in 20 mls lysis buffer (25 mM HEPES, 100 mM KCl, 5 mM EDTA, 10 mM β-
mercaptoethanol, 2 mg/ml lysozyme, 0.25 mg/ml DNAse I (2500 U/mg), protease inhibitors) prior to lysis by sonication (3 x 20 sec). The lysis mixture was cleared by centrifugation for 25 min at 12,000g and the supernatant incubated with 1 ml bed volume amylose resin (New England Biolabs, Ipswich, MA) for 1 hour at 4°C. The resin was washed thrice with 50 mls PBS (3mM NaPO$_4$, 1.5 mM KPO$_4$, 137 mM NaCl, 2.7 mM KCl, pH 7.3, 10 mM β-mercaptoethanol) and eluted in 0.5 ml steps with PBS containing 10 mM maltose. The concentration of the eluted protein was measured with Pierce 660 nm detection Reagent (Fisher Scientific, Pittsburgh, PA), and the protein was treated with His$_8$-TEV protease (Blommel and Fox, 2007) at a 1:20 w/w ratio for 16 hours at 4 °C. The cleavage mixture was incubated with 0.2 ml bed volume Nickel-Sepharose 6-Fast Flow (GE Healthcare, Little Chalfont, UK) in the presence of 10 mM imidazole for 1 hour at 4 °C to remove His$_8$-TEV and His$_8$-MBP. The unbound was collected, concentrated to 1.2 mg/ml on a 4000 MW cutoff Amicon centrifugal filter unit (Millipore, Billerica, MA), and analyzed for purity by SDS-PAGE followed by Coomassie staining.

Rabbit Anti- CLC2 antibodies were generated against the purified protein (Covance Research Products, Denver, PA) and immunoblot analysis of the crude sera were performed as described in (Kang et al., 2001) on purified CLC2 protein and total seedling extracts (WT and clc2-2, 0.8 g frozen cell powder from N$_2$(l) grinding per 1 ml 2x SDS-PAGE sample buffer).

**Isolation and characterization of clc insertional alleles**

Plant growth conditions, genomic DNA extraction and RT-PCR were as described in Chapter 3. Primers for PCR-based genotyping are identified in Table 1, and primers for RT-PCR are identified in Table 2. Salk Insertional alleles were obtained from the Arabidopsis Biological Resource Center (Columbus, OH), and GABI-KAT insertional alleles were obtained from the Genomanalyse im biologischen System Pflanze (GABI) at Bielefeld University, Germany.
Results and Discussion

Localization of CLC1-GFP

Roots of WT seedlings of both the Columbia (Col) and Wassilewskija (WS) ecotype expressing CLC1-GFP (native promoter driven, full length genomic construct) were imaged by Laser Scanning Confocal Microscopy (LSCM) to determine the subcellular localization of CLC1. The brightest fluorescence was seen in mobile puncta in the cytoplasm which resembled TGN/endosomes. Significant fluorescence was also seen at the cell cortex and at the central regions of maturing cell plates (Fig. 1A). Overall, the distribution of CLC1-GFP in the cell was indistinguishable from that seen with CLC2-GFP (Fig. 1B, (Konopka et al., 2008))

The cell-cortex-associated pool of CLC1-GFP was imaged by Variable Angle Epifluorescence Microscopy (VAEM). CLC1-GFP formed foci at the cell cortex (Fig. 1C) that appeared and disappeared and resembled those observed for CLC2-GFP (Fig. 1D, (Konopka et al., 2008)).

Colocalization of CLC2 with DRP1 and DRP2 at the cell plate

LSCM imaging of CLC2-GFP at the forming cell plate suggested that it localized primarily to the central, maturing regions of the cell plate (Fig 1B, (Konopka et al., 2008)). This is in contrast to DRP1A, (Kang et al., 2003a), -1C, (Konopka et al., 2008), -2A and -2B (Appendix B, (Fujimoto et al., 2008)), which localize to the entire cell plate, are brightest at the leading edges, and are very early markers of cell plate formation. To verify that the DRPs had distinct spatial and temporal localization as compared to CLC-FPs, plants expressing DRP1A-GFP (Kang et al., 2003a) and 35S:DRP2B-GFP (Appendix B) were crossed with plants expressing CLC2-mOrange (Konopka et al., 2008), and forming cell plates were visualized by LSCM. Both
DRP2B (Fig. 2A) and DRP1A (Fig. 2B-C) could clearly be seen to localize to the edges of the cell plate, which were not labeled by CLC-mOrange, and to be present on very young cell plates where CLC-mOrange labeling was not yet visible (Fig. 2A, arrow and Fig. 2B).

**CLC insertional alleles**

According to publicly available microarray data (Schmid et al., 2005), all three CLC genes are expressed at similar levels in all tissues except for pollen, which has little to no expression of *CLC2* (Fig. 3). Therefore, if all three CLC family members are functionally redundant at a protein level, we would expect to see no defects (or only very subtle defects) in single null mutants, necessitating the generation of double and/or triple mutants via crossing of the single null alleles. To date, single null alleles have been identified and RT-PCR verified for *CLC2 (clc2-1 and clc2-2)* and *CLC3 (clc3-2)* (Fig. 4, Table 2). *Clc2-2* has been also verified as a protein null with α-clc antisera (see below). A potential *CLC1* allele, *clc1-1*, proved not to have reduced transcript levels (Fig. 4B). Three additional *clc1* alleles have been obtained, of which *clc1-3* and *clc1-4* appear the most promising based on insert location (Fig. 4A); isolation of homozygote lines for these alleles is ongoing.

**CLC2 antisera**

Purified, *E. coli* expressed CLC2 (Fig. 5, lane 1) was used for production of rabbit α-CLC2 antisera. This antisera was tested by immunoblotting against purified CLC2 as well as WT and *clc2-2* seedling extract to verify immunoreactivity. Two prominent immunoreactive bands were detected in both purified CLC2 and WT which were absent in *clc2-2* (Fig. 5, arrows). The larger of these two bands, migrating at approximately 40 kDa, likely represents full length
CLC2. CLC2 is only 29 kDa in length, but mammalian CLCs have been previously reported to migrate at a significantly larger size than would be expected from their molecular weight (Scarmato and Kirchhausen, 1990), although this was not reported previously for purified GST-CLC2 (Scheele and Holstein, 2002). The lower band may be a prominent breakdown product of CLC2. Three fainter immunoreactive bands can be detected in both WT and clc2-2. The largest of these, at ~70kDa, is also seen in the purified CLC2 lane, and may represent a crossreacting contaminant in the preparation. The band at ~35 kDa (Fig. 5, asterisks) may represent CLC3, which has a predicted size of 27 kDa but may likewise be migrating at a larger size, and whose high level of sequence similarity to CLC2 make it likely to also be recognized by this antisera. The lowest band, at ~25 kDa, is likely a nonspecific crossreacting band. The putative identification of CLC3 should be verified by the use of the clc3-2 mutant; also, affinity purification of the antisera against alternatively tagged CLC2 may help to reduce background and remove nonspecific bands.

**Conclusion and Future Directions**

The localization of CLC1-GFP to the TGN and plasma membrane-associated foci suggests that it is functioning at the same cellular localizations as CLC2, although co-labeling with CLC1-mOrange will be necessary to verify this. The lack of apparent phenotypic defects in verified clc2 and clc3 null mutants suggests that functional redundancy may exist within the Arabidopsis CLC family; alternatively, the CLC family as a whole may not be critical for plant development. Isolation of homozygous clc1 null mutants is now the highest priority for this project, followed by the generation of double and triple mutants to determine whether or not Arabidopsis CLC is required for plant development and/or endocytosis.
Tables

Table 1: *clc* insertional alleles.

Plants were genotyped by PCR using the T-DNA primer and the left gene specific primer at the annealing temperature indicated. “Position” is counted in the genomic sequence, from the translational start site, and was verified by sequencing of the PCR product generated from genotyping. “ND” indicates that we have not independently verified the position of the T-DNA. “Homs” indicates whether or not homozygous lines of that allele have been identified. Sequence of primers is given in Table 3.

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<td>Exon 3</td>
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<td>932</td>
<td>56</td>
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Table 2: RT-PCR of clc insertional alleles.

TM was 55°C for all primer sets except 1089+1090, for which it was 58°C. Sequence of primers is given in Table 3.

<table>
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<tr>
<th>Name</th>
<th>Location</th>
<th>Upstream primers</th>
<th>Flanking Primers</th>
<th>Downstream primers</th>
<th>Result</th>
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<tbody>
<tr>
<td>clc1-1</td>
<td>Intron 1</td>
<td>1062+1063</td>
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<td>1088+876</td>
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<tr>
<td>clc2-3</td>
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<td>1089+1090</td>
<td>Knockdown or possible null</td>
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<td>929+931</td>
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<td>929+931</td>
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<tr>
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<td>3’ UTR</td>
<td>1131+1132</td>
<td>929+931</td>
<td>Not a null</td>
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Table 3: Oligonucleotides used in the this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ &gt; 3’)</th>
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<td>842</td>
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<td>ggaagcaggaacaaaccaatgttcatatttg</td>
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</table>
Figures

Figure 1: CLC1-GFP, like CLC2-GFP, localizes to TGN, the cell plate, and endocytic foci.

(A,B) LSCM image of roots expressing CLC1-GFP (A) or CLC2-GFP (B). Arrows indicate cell plates, arrowheads indicate mobile TGN. Scale bar = 20 µm

(C,D) VAEM image of epidermal cells of roots expressing CLC1-GFP (C) or CLC2-GFP (D). Large, bright, and out-of-focus foci are mobile TGN (arrowheads), while the smaller foci (e.g. circled area) represent cortical clathrin-mediated-endocytic particles. Scale bar = 1 µm
**Figure 2: DRP1A and DRP2B are recruited to the cell plate ahead of CLC2**

Cell plates in seedling roots expressing CLC2-mOrange together with 35S:DRP2B-GFP (A) or DRP1A:GFP (B) were imaged by LSCM.

(A) In this root tip, a younger cell plate (arrow) is labeled only with 35S:DRP2B-GFP, whereas an older cell plate (arrowhead) shows both 35S:DRP2B-GFP and CLC2-mOrange labeling.

Scale bar = 20 µm

(B-C) Two images from a time lapse series of a forming cell plate. At time 0, the cell plate is labeled only with DRP1A-GFP, whereas after 3 minutes it is labeled with both DRP1A-GFP and CLC2-mOrange, although the CLC2-mOrange does not extend all the way to the edges of the cell plate (white lines). Scale bar B-C = 5 µm
Figure 3: Expression of CLC family members through development

Expression data for CLC1-3 was obtained from the AtgenExpress Visualization Tool (AVT; http://jsp.weigelworld.org/expviz/expviz.jsp (Schmid et al., 2005)). Data for representative tissues (e.g. roots, flowers) and tissues for which strong changes in relative expression levels were observed (e.g. senescing and cauline leaves, developing seeds) were selected and graphed.
Figure 4: CLC insertional mutants

(A) Schematic of the three CLC genes in Arabidopsis, indicating untranslated regions (thick gray bars), exons (thick black bars), and introns (thin black lines). The position of each insertional allele is indicated with an arrow.

(B) RT-PCR of insertional alleles for which homozygous lines have been isolated, using primers either upstream, flanking, or downstream of the insertional site, as appropriate.
A

**CLC1**

- $clc1-3$
- $clc1-4$
- $clc1-1$
- $clc1-2$

**CLC2**

- $clc2-1$
- $clc2-2$
- $clc2-3$

**CLC3**

- $clc3-1$
- $clc3-2$
- $clc3-3$

B

**CLC1**

- Genotype: $WT$  $clc1-1$
- Primer set: flanking $clc1-1$

**CLC2**

- Genotype: $WT$  $clc2-1$  $WT$  $clc2-2$  $WT$  $clc2-2$  $WT$  $clc2-2$  $WT$  $clc2-3$  $WT$  $clc2-3$
- Primer set: flanking $clc2-1$  flanking $clc2-2$  downstream $clc2-2$  upstream $clc2-3$  flanking $clc2-3$

**CLC3**

- Genotype: $clc2-3$  $clc3-1$  $clc3-2$  $clc3-3$
- Primer set: flanking $clc3-1$, upstream $clc3-2$ and 3-3  downstream $clc3-1$, flanking $clc3-2$ and 3-3
Figure 5: α-CLC2 antibodies

Purified CLC2 (lane 1, Coomassie staining) was used to generate rabbit antisera against CLC2. This antisera recognized 2 bands in purified CLC2 and WT seedling extract which were absent in extract from clc2-2 homozygous seedlings (arrows). The lower band may be a breakdown product. In addition, the antisera reacted more weakly with bands found in both clc2-2 and WT extract, one of which (*) might be CLC3, if it, like CLC2, is migrating ~10 kDa higher than its true molecular weight.
References


