



Phragmoplastin dynamics: multiple forms, microtubule association and their roles in cell plate formation in plants

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Abstract

We have characterized 4 of the 16 members of the family of dynamin-related proteins (DRP) in *Arabidopsis*. Three members, DRP1A (previously referred as ADL1), DRP1C and DRP1E, belong to the largest group of phragmoplastin-like proteins. DRP2A (ADL6) is one of the two members that contain a pleckstrin homology (PH) domain and a proline-rich (PR) motif, characteristics of animal dynamins. All four proteins interacted in yeast two-hybrid assays with phragmoplastin, and showed different patterns of localization at the forming cell plate during cytokinesis. GFP-tagged DRP1A and DRP1C proteins were found to be associated with the cytoskeleton in G1 phase of the cell cycle. The distribution pattern of DRP1A was sensitive to propyzamid and insensitive to cytochalasin D, suggesting that DRP1A is associated with microtubules and not actin filaments. The association of DRP1A with microtubules was confirmed *in vitro* by spin-down assays. A GTPase-defective phragmoplastin acted as a dominant negative mutant, reduced transport of vesicles to the cell plate and formed dense tubule-like structures in the cell plate. We propose that DRP1 proteins may provide an anchor for Golgi-derived vesicles to attach to microtubules, which in turn direct the vesicles to the forming cell plate during cytokinesis. Whereas the DRP1 subfamily members are involved in tubulization of membranes, DRP2 may be involved in endocytosis and membrane recycling via clathrin-coated vesicles.

Abbreviations: ADL, *Arabidopsis* dynamin-like protein; C-MT, cortical microtubule; DRP, dynamin-related protein; GFP, green fluorescent protein; MT, microtubule; PH domain, pleckstrin-homology domain; Phr, phragmoplastin; Phr-MT, phragmoplast microtubule; PI, phosphatidylinositol; PI-3P, phosphatidylinositol 3-phosphate; PRD, proline-rich domain; TVN, tubulo-vesicular network; VTV, vesiculo-tubule vesicle.

Introduction

Phragmoplastin and ADL1 were the first dynamin-related proteins (DRPs) identified in plants (Dombrowski and Raikhel, 1995; Gu and Verma, 1996). During the past few years, several more DRPs from plants have been characterized. These include ADL2 (Kang *et al.*, 1998; Kim *et al.*, 2001), ADL2b (Arimura and Tsutsumi, 2002), ADL3 (Mikami *et al.*, 2000), ADL6 (Jin *et al.*, 2001; Lam *et al.*, 2002; Lee *et al.*, 2002), and ARC5 (Gao *et al.*, 2003). *Arabidopsis* DRPs have recently been classified into various

subfamilies based upon their homologies and possible functions (Hong *et al.*, 2003).

Animal dynamin was originally isolated from bovine brain extracts as a microtubule-binding protein (Shpetner and Vallee, 1989), and its GTPase activity was shown to be activated by microtubules (Shpetner and Vallee, 1992). It has been suggested that dynamin GTPase may be involved in the transport of Golgi-derived vesicles along the microtubules, from the minus end to the plus end (Fullerton *et al.*, 1998). Recently dynamin has also been found to be associated with actin and shown to play a role in actin-based

vesicle motility (Orth *et al.*, 2002; Lee and De Camilli, 2002). One of the *bona fide* dynamins from *Arabidopsis*, DRP2A (ADL6) has been shown to be associated with clathrin-coated vesicles (Lam *et al.*, 2002) and has also been localized at Golgi and implicated in vesicle trafficking (Jin *et al.*, 2001).

Golgi-derived vesicles are apparently transported along the microtubules of phragmoplast to the forming cell plate (Staehelein and Hepler, 1996; Verma, 2001; Smith, 2001; Jurgens and Geldner, 2002; Bednarek and Falbel, 2002). This is based largely on observations from electron microscopy. A recent study on the formation of syncytial-type cell plates in the endosperm revealed that vesicles that are attached to the phragmoplast microtubules have a pair of kinked, rod-shaped structures, resembling kinesin-like molecules (Otegui *et al.*, 2001). One of the possible kinesins linking vesicles to the phragmoplast microtubules is an *Arabidopsis* kinesin-like protein, AtPAKRP2, which is localized specifically to the phragmoplast and is not found associated with any other microtubule-based structures (Lee *et al.*, 2001). However, there is no *in vitro* data or immunolocalization evidence to establish a role for AtPAKRP2 in vesicle translocation (Smith, 2002). Mutations in the kinesin-like protein HINKEL cause incomplete cell walls and multinucleate cells during embryogenesis (Strompen *et al.*, 2002). Phragmoplast microtubules are essentially normal in these mutants and KNOLLE-containing vesicles are present on the cell plate (Strompen *et al.*, 2002). This suggests that HINKEL is not involved in vesicle transport. Other kinesin-related motor proteins, KCBP and TKRP125, have also been localized to the spindle poles (Smirnova *et al.*, 1998) and phragmoplast microtubules (Asada *et al.*, 1997).

In contrast to animal cells where actin microfilaments play a more prominent role in cytoskeleton functions, plant cells rely more on microtubules for various subcellular activities (Vantard *et al.*, 2000; Kutsuna and Hasezawa, 2002). Plant microtubules are organized as characteristic structures like cortical microtubules at G1 phase, preprophase band at late G2 and phragmoplast at telophase. The phragmoplast consists of two sets of microtubule arrays with their plus ends facing each other. The cell plate begins to form in the center of the phragmoplast and expands outwards by continuous fusion of Golgi-derived vesicles transported along the microtubules (see Verma, 2001). GTP is required for the translocation of microtubules in the phragmoplast (Asada *et al.*, 1991), and kinesin-related motor proteins, TKRP125 and TBK5,

have been shown to be involved in the translocation of microtubule arrays (Asada *et al.*, 1997; Matsui *et al.*, 2001). However, TKRP125 and TBK5 do not contain GTPase activity or bind GTP. Phragmoplastin, a large GTPase, is associated with the forming cell plate (Gu and Verma, 1996; 1997; Kang *et al.*, 2001; Geisler-Lee *et al.*, 2002). Disruption of microtubule-reorganization using Taxol inhibits the growth of the cell plate, presumably aborting the supply of vesicles (Gu and Verma, 1997).

We present evidence here to demonstrate that phragmoplastins and DRP1 proteins are associated with cortical microtubules at the G1 phase, cytoplasmic microtubule strands at the S phase, mitotic spindles at the anaphase and phragmoplast microtubules at the telophase of cell division. This association is sensitive to microtubule-dissociating but not actin filament-dissociating drugs. By expressing a GTPase-defective mutant of phragmoplastin, we also demonstrated that GTPase activity is required for the efficient budding and transport of phragmoplastin-containing vesicles from the Golgi bodies to the forming cell plate. Even though both DRP1 and DRP2 proteins were localized at the forming cell plate, these two groups of proteins have different functions in building the plate.

Materials and methods

Bacterial and yeast strains

Plasmid propagation was carried out in *Escherichia coli* strain Top10F' and DH5 α (Invitrogen, Carlsbad, CA). *Agrobacterium tumefaciens* strains ABI and LBA4404 were used for transformation of tobacco BY-2 cells and tobacco leaf disks, respectively, as described (Hong *et al.*, 2001a, b). *Saccharomyces cerevisiae* strain Y190 was used for screening of an *Arabidopsis* cDNA library for interaction cloning as described (Zhang *et al.*, 2000).

Screening of cDNA library with the two-hybrid system and β -galactosidase assay

Plasmid pAS-Phr expressing a full-length phragmoplastin (Zhang *et al.*, 2000) was used as a bait to screen an *Arabidopsis* cDNA library constructed in the λ -ACT vector (Kim *et al.*, 1997; *Arabidopsis* Biological Resource Center, Ohio State University). The cDNA library was converted into pACT-based plasmids through *in vivo* excision and the pooled plasmids

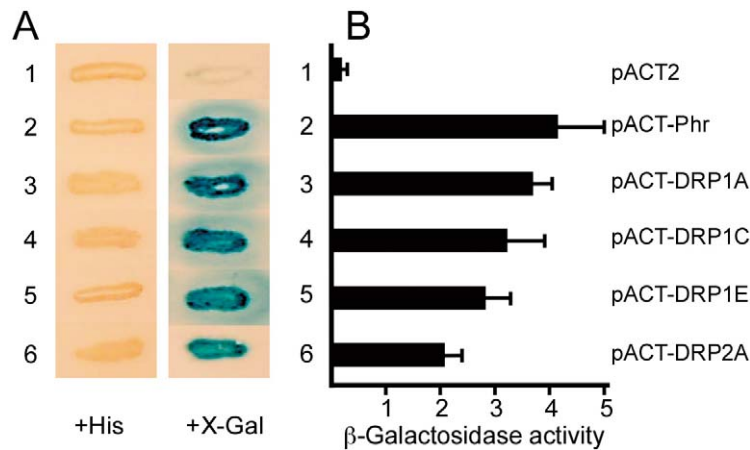


Figure 1. Interactions between phragmoplastin and DRP1/DRP2 isoforms. **A.** Phragmoplastin cDNA (Gu and Verma, 1996) cloned in pAS2 (pAS-Phr) was used to screen an *Arabidopsis* cDNA library constructed in pACT vector (Kim *et al.*, 1997). Inserts of positive clones were sequenced and named according to the nomenclature in Hong *et al.* (2003). Vector pACT2 was used as a negative control and pACT-Phr (Zhang *et al.*, 2000) as a positive control. Yeast colonies were grown in the presence of histidine (+His) and streaked on X-Gal plates. **B.** Yeast cell extracts were assayed for β -galactosidase activity, and average values from four independent assays are presented.

were used to transform yeast strain Y190 containing pAS-Phr. Positive colonies selected on SC-Trp-Leu-His medium containing 3-amino-1,2,4-triazole (3-AT, 30 mg/l) were absorbed to 3MM filter disks, permeated by liquid nitrogen treatment and stained for X-gal activity. Plasmids were isolated from the blue colonies and the inserts were sequenced. Activity of β -galactosidase was measured as described previously (Zhang *et al.*, 2000).

Isolation of full-length cDNA clones

An *Arabidopsis* FL-1 cDNA library constructed in λ ZapII (Seki *et al.*, 1998; provided by Dr K. Shinozaki, Institute of Physical and Chemical Research, Tsukuba, Japan) was screened using 32 P- α -dATP-labeled inserts from the yeast two-hybrid screening, as probes. Full-length cDNAs of DRP1A, DRP1C and DRP1E were isolated from screening of a cDNA library using partial cDNAs obtained from the yeast two-hybrid screening. The inserts were verified by DNA sequencing and the full coding region of these genes were fused with GFP under the control of the CaMV-35S promoter. Plasmids were isolated from positive phage plaques through *in vivo* excision and the inserts were sequenced.

Plasmid construction and transformation of tobacco BY-2 cells

The DRP1A, DRP1C and DRP1E inserts were subcloned at the *Hind*III or *Eco*RI site of pMON-GFP

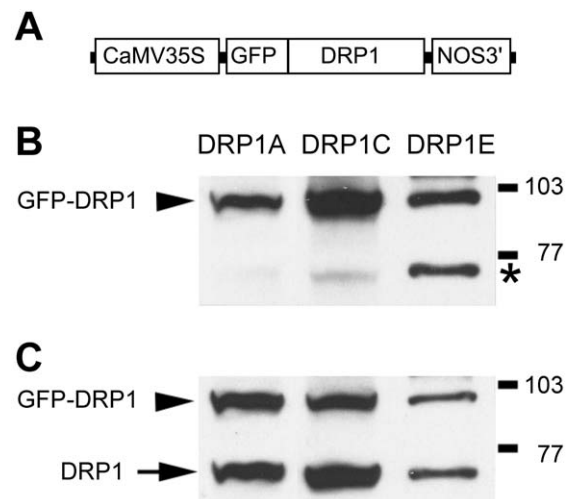


Figure 2. Expression of GFP-DRP1 fusion proteins in transgenic cells. **A.** DRP1A, DRP1C and DRP1E cDNA inserts were cloned in frame with the GFP coding region and expressed under the control of the CaMV-35S promoter. The fusion proteins were detected by antibodies against either GFP or phragmoplastin. **B.** Western blot detection of GFP-DRP1 fusion proteins in the total membrane fraction with GFP antibody. A partially degraded product of GFP-DRP1E is marked by *. It is slightly larger in molecular size than the endogenous DRP1 proteins. **C.** The same blot as in B, but detection was carried out with polyclonal antibodies against soybean phragmoplastin (Gu and Verma, 1996). The antibodies detected the GFP-DRP1 fusion proteins as well as the endogenous DRP1 proteins from the host BY-2 cells.

(Hong *et al.*, 2001, b) to fuse in frame with the GFP coding region. The entire cassette was released by *Not*I and subcloned into the same site of binary

vector pMON18342 (Pang *et al.*, 1996; Hong *et al.*, 2001a), generating pMBin-GFP-DRP1A, pMBin-GFP-DRP1C, pMBin-GFP-DRP1E and pMBin-GFP-DRP2A. Untransformed BY-2 cells, BY-2 cells with only GFP (Gu and Verma, 1997), and BY-2 cells with blank plasmid, pBI121 (Geisler-Lee *et al.*, 2002), were used as controls. About 20 independent transgenic colonies for each line were screened by measuring expression levels of the fusion protein GFP fluorescence and by western blot analysis (see below). A representative colony was chosen from each line and propagated for further observations.

Phragmoplastin cDNA (pSDL12a, Gu and Verma, 1996) was subject to site-directed mutagenesis to change the lysine (K) residue at position 47 to methionine (M), yielding pSDL12^{K47M}. The mutated sequence was confirmed by DNA sequencing. An *Sst*I fragment containing the mutated region from pSDL12^{K47M} was used to replace the same fragment of pBI121-EGP (Gu and Verma, 1997) to generate the plasmid (pBI-GFP-Phr^{K47M}).

Plant transformation

Agrobacterium tumefaciens (strain LBA4404) was transformed by electroporation with pBI-GFP-Phr^{K47M} and pBI-GFP (control). Leaf disks of tobacco (*Nicotiana tabacum* cv. Xanthi) were infected by *Agrobacterium* cells harboring the binary plasmids and transgenic plants were generated as described (Geisler-Lee *et al.*, 2002). For transformation of tobacco BY-2 cells, plasmids were transferred to *A. tumefaciens* (strain ABI) by electroporation and transgenic BY-2 colonies were selected as described (Hong *et al.*, 2001a, b).

Western blot analysis

Total proteins were extracted from BY-2 cells 5 days after inoculation (Hong *et al.*, 2001a, b). Proteins were transferred onto a nitrocellulose membrane and reacted with antibody. Two types of antibodies were used. A mouse monoclonal antibody against GFP (Clontech, CA) was used to detect the fusion protein on western blot. Polyclonal antibodies against soybean phragmoplastin has been shown to cross-react with different forms of phragmoplastin-like proteins including homologous proteins from *Arabidopsis* and tobacco (Gu and Verma, 1997). Horseradish peroxidase-conjugated goat antibodies against mouse IgG or rabbit IgG were used as a second antibody.

In vitro assay of microtubule-associated proteins

Tubulin was prepared from bovine brain (Williams and Lee, 1982). The microtubule polymerization/depolymerization cycle was repeated for 4 times and produced a crude tubulin preparation. For *in vitro* assay of microtubule-associated proteins, the total membrane fraction (see above) from BY-2 cells expressing GFP-DRP1A was dissolved in MT buffer containing 2% Triton X100 and 1 mM PMSF. The supernatant (total membrane proteins) after centrifugation at $30\,000 \times g$ for 30 min was used for co-polymerization with microtubules. The polymerization mixture contained ATP (1 mM), GTP (1 mM), MgCl₂ (4 mM), Taxol (30 μ M), glycerol (30% v/v), tubulin (2 mg/ml), membrane proteins (1 mg/ml), Triton X100 (0.5%), PMSF (1 mM) in MT buffer. The mixture was incubated for 60 min at 37 °C and centrifuged at $37\,000 \times g$ for 40 min at 28 °C. The pellet was washed with warm (28 °C) MT buffer and dissolved in 1 \times SDS loading buffer. Proteins were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane followed by western blot analysis with antibodies against soybean phragmoplastin (Gu and Verma, 1996) and antibodies against maize plasma membrane ATPase (Nagao *et al.*, 1987).

Light microscopy

Ovules from transgenic and control tobacco plants were fixed in FAA (5% formalin, 5% acetic acid, 50% ethanol) mixed with phosphate-buffered saline (1:1) solution for 4 h. The tissue was dehydrated in an alcohol series and infiltrated with paraffin (Paraplast). Longitudinal sections of ovules were viewed and photographed using an Axiophot Zeiss microscope (Carl Zeiss). Root tips and shoot meristems of 4-week old plants were fixed in FAA solution for 4 h, dehydrated in alcohol series, and infiltrated with Paraffin (Paraplast). Sections (8 μ m) were stained with 1% Safranin O in 50% alcohol and 1% Fast Green in 95% ethanol. The sections were viewed and photographed.

Confocal microscopy and treatment of BY-2 cells with propyzamid

Confocal images of BY-2 cells expressing GFP-tagged proteins were acquired by means of a PCM-2000 confocal laser scanning microscope (Nikon Bioscience Confocal Systems, Melville, NY) equipped with appropriate filters for GFP. 3-D images were reconstructed from Z-series confocal data with SIMPLEPCI soft-

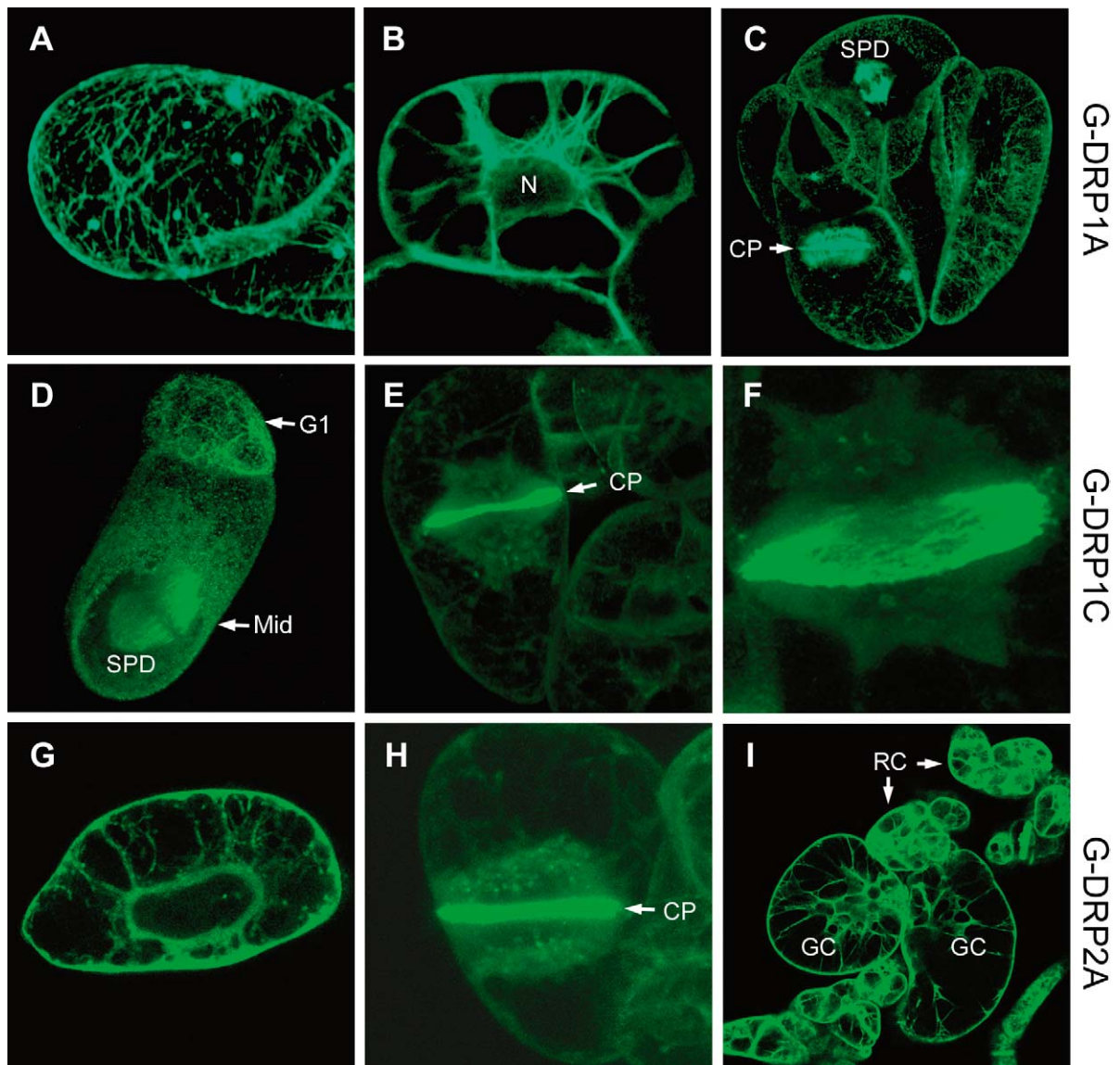


Figure 3. Localization of GFP-tagged DRP1A, DRP1C and DRP2A in BY-2 cells. A–C. Association of DRP1A with microtubules and the forming cell plate. A. In the G1-phase cell, DRP1A is associated with the strand-like structures distributed only in the cortex region of the cell. B. In the S-phase cell, the nucleus (N) is centralized and DRP1A is distributed on strand-like structures that initiate from the perinuclear region and extend along the cytoplasmic strands towards the cell cortex. C. In the metaphase cell, DRP1A is found on the spindles (SPD). From late metaphase to early anaphase, DRP1A is associated with the phragmoplast including the two sets of microtubule arrays and the forming cell plate (CP). D–F. Association of DRP1C with microtubules and the forming cell plate. In the G1 cell (D; G1), DRP1A is associated with the cortical microtubules. However, such association does not appear to be as tight as that of DRP1A (A). In the metaphase cell (D; Mid), DRP1C is associated with the mitotic spindle (SPD) and is also distributed as punctate structures throughout the cell. At anaphase (E), DRP1C is largely targeted to the forming cell plate (CP). When the 3-D confocal image in E is turned -30° around the X-axis and the phragmoplast region is amplified (F), DRP1C is largely located on the edges of the cell plate, forming a ring-like structure. G–I. Localization of DRP2A in the plasma membrane and the forming cell plate. DRP2A is found in the cytoplasm and plasma membrane in BY-2 cells (G). At cytokinesis, DRP2A is targeted to the forming cell plate (H). In the cell line expressing GFP-DRP2A, 5–10% of cells in the population continue to expand their cell size, leading to the formation of giant cells (GC). These cells can not divide and become terminal. RC, regular size cells.

ware (Compix Imaging Systems, Cranberry Township, PA). A Perkin Elmer spinning-disk confocal device attached to an inverted Nikon microscope was used to examine the effect of propyzamid and cytochalasin D on the localization of GFP-DRP1A. BY-2 cells (150 μ l) expressing GFP-DRP1A were placed in a culture well on a glass slide (Nikon Bioscience Confocal Systems, Melville, NY), and their fluorescence images were taken. Of a stock of propyzamid or cytochalasin D 2 μ l dissolved in DMSO was added to the culture well (to give a final concentration of 3 μ M for propyzamid or 100 μ M for cytochalasin D) and the same cells were photographed at different time intervals.

Electron microscopy

Root tips (2–3 mm long) of 1-week old tobacco seedlings were fixed in 2% glutaraldehyde in 75 mM phosphate buffer, pH 7.2 and were post-fixed in 2% osmium in the same buffer. Samples were dehydrated in an acetone series and embedded in Spurr's resin. Ultramicrotome sections were stained with uranyl acetate followed by lead citrate. The specimens were viewed and photographed using a Philips CM12 transmission electron microscope at 60 kV.

Results

Homo-oligomerization and hetero-oligomerization of Arabidopsis DRP1A, DRP1C, DRP1E, and DRP2A

Our previous studies demonstrated that phragmoplastin is localized at the forming cell plate in soybean (Gu and Verma, 1996; 1997), and DRP1A (ADL1) was shown to have a similar pattern of localization in *Arabidopsis* (Kang *et al.*, 2001). Our previous study has also shown that soybean phragmoplastin forms homo-oligomers *in vitro* (Zhang *et al.*, 2000) and interacts with UDP-glucose transferase (Hong *et al.*, 2001b). We used soybean phragmoplastin as bait and *Arabidopsis* cDNA as prey in order to see if any of the *Arabidopsis* DRPs were able to form hetero-oligomers with their soybean orthologue. Several DRPs showed strong interaction with soybean phragmoplastin including DRP1A, DRP1C, DRP1E, and DRP2A (Figure 1A). Interestingly, when β -galactosidase activity was quantified, the strongest interactions occurred with proteins that had higher levels of homology (Figure 1B), and all interactions were stronger than that with UDP-glucose transferase (data not shown). On the basis of these interactions, we selected DRP1A,

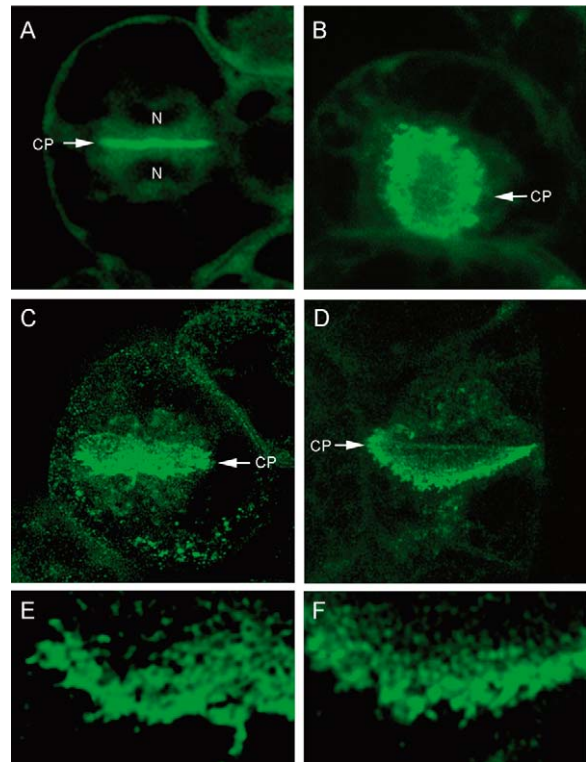


Figure 4. Localization of GFP-DRP1E fusion protein in transgenic BY-2 cells. A. Side view of the cell plate (CP) of an anaphase cell. N, daughter nuclei. B. Front view of the cell plate (CP) of a similar cell as in A. C and D. Comparison of two cytokinetic cells at different stages of cell plate (CP) formation. DRP1E is distributed as a disk-like structure at an earlier stage (C) or as a ring-like structure at later stage (D) of cell plate formation. E and F. Association of DRP1E with the tubular network. Blow-ups of the corresponding portions of the cell plate in C and D, respectively.

DRP1C, and DRP1E, as representatives of the DRP1; and DRP2A, as a representative of the DRP2 subfamily for further study.

Expression of GFP-DRP1 proteins in tobacco BY-2 cells

In order to follow their subcellular localization during the cell cycle, cDNAs for DRP1A, DRP1C, DRP1E and DRP2A were isolated and expressed as GFP-tagged proteins in BY-2 cells (Figure 2A). Expression levels of GFP-DRP1 fusion proteins were confirmed with two antibodies (Figure 2B and C and see Materials and methods). The intensities of endogenous (Figure 2C, lower band) and transgenic DRP1 proteins (Figure 2C, upper band) were roughly the same, indicating that the level of GFP-DRP1 fusion protein was similar to that of native DRP1 proteins.

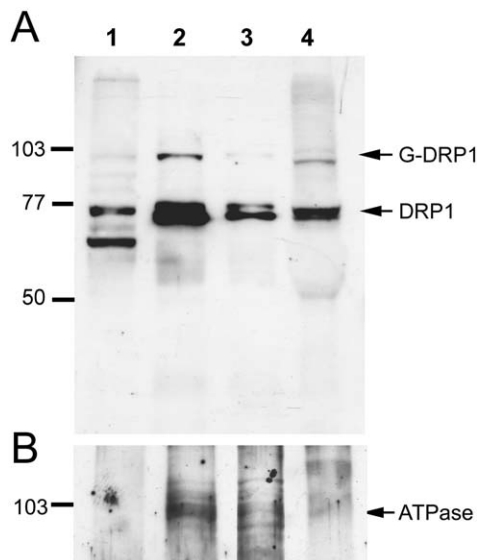


Figure 5. *In vitro* binding assay of DRP1A with microtubules. BY-2 cells expressing GFP-DRP1A was separated into soluble (lane 1) and total membrane fractions (lane 2). After polymerization of tubulins into microtubules in the presence of the plant membrane extract, the reaction mixture was separated into soluble (lane 3) and pellet (lane 4). The membrane was probed with antibody to phragmoplastin (A) or antibody to the plasma membrane ATPase (B). G-DRP1, GFP-DRP1A fusion protein. DRP1, endogenous DRP1 proteins from BY-2 cells. Molecular mass markers (kDa) are indicated.

DRP1A is associated with microtubules and targeted to the forming cell plate during cytokinesis

We used confocal microscopy to localize GFP-DRP1A in tobacco BY-2 cells and to track its subcellular localization during cell cycle. Data in Figure 4 show that GFP-DRP1A is associated with the microtubules and cell plate. BY-2 cells transformed with an empty vector (negative control) showed no fluorescence, while cells expressing GFP alone (control) exhibited fluorescence in the cytoplasm and nucleus without a specific pattern (data not shown). We followed GFP-DRP1A distribution patterns in cells at different stages of cell cycle. As expected, *Arabidopsis* DRP1A was found to be associated with the forming cell plate in cytokinetic cells. In the G1-phase cells, DRP1A is associated with the strand-like structures distributed only in the cell cortex region (Figure 3A), a pattern similar to that of cortical microtubules. In the S-phase cells, the nucleus moves to the center of the cell and DRP1A becomes distributed on strand-like structures that initiate from the perinuclear region and extend along the cytoplasmic strands towards the cell cortex (Figure 3B), a pattern identical to that of

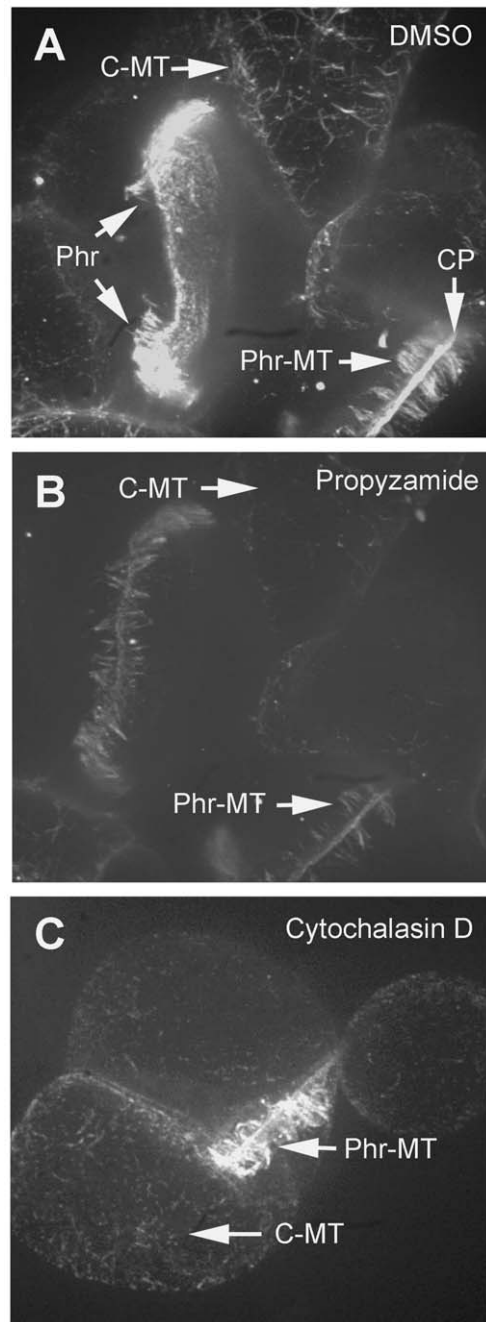


Figure 6. Effect of cytoskeleton-destabilizing agents on the localization of GFP-DRP1A. A. BY-2 cells expressing GFP-DRP1A was treated with DMSO (1%) for 15 min (mock control). B. Treatment of the same cells with propyzamide ($3 \mu\text{M}$). Note that whereas the association of DRP1A with the cortical microtubules (C-MT) is disrupted, a significant portion of DRP1A is still bound to the phragmoplast microtubules (Phr-MT), even after 15 min of treatment with propyzamide. C. Treatment with cytochalasin D ($100 \mu\text{M}$) for 10 min.

microtubules (Kutsuna and Hasezawa, 2002). In a metaphase cell (Figure 3C, upper right cell), DRP1A is associated with the spindle. During late anaphase, DRP1A is localized to the phragmoplast microtubule arrays and the forming cell plate (Figure 3C, lower left cell).

DRP1C is preferentially localized to the growing edges of the cell plate

Like DRP1A, GFP-tagged DRP1C is also associated with the microtubules and the forming cell plate (Figure 3D–F). However, the association between DRP1C and microtubules is apparently not as tight as that of DRP1A (compare Figure 3D with Figure 3B). In the mitotic cells (Figure 3D), DRP1C is localized with mitotic spindle and is also distributed as punctate structures throughout the cell. At cytokinesis (Figure 3E), DRP1C is largely targeted to the forming cell plate, especially to the edges of the plate (Figure 3F). Thus, the distribution of DRP1C is distinct from that of DRP1A, suggesting that it has a different role in cell plate formation.

DRP1E is localized to the tubulo-vesicular network of the growing cell plate

DRP1E, tagged with GFP, was also localized at the forming cell plate at the late stages of telophase (Figure 4A and B). The ring-like structure in Figures 4B (front view) and Figure 4D (side view) indicates that DRP1E is redistributed from the center of the cell plate to its growing edges. As compared with the localization of DRP1A and DRP1C at the cell plate, DRP1E appeared to be associated with the tubular network (Figure 4C–F). These results are interesting because they may provide a clue for the functional differences between the DRP1E and other DRP1 proteins. It is possible that DRP1A and DRP1C are involved in the formation of tubulo-vesicular structures, whereas DRP1E may play a role in the formation/consolidation of the tubular network. That different phragmoplastins play different roles in cell plate formation is consistent with subtle differences in their localization patterns during different stages of the cell division.

DRP2A is also targeted to the cell plate at cytokinesis

DRP2A and DRP2B are two ‘true’ DRPs and are highly homologous to each other, sharing 92% sequence identity (Hong *et al.*, 2003). However, they have only 23% homology with DRP1A, thus seem

to be unlikely candidates for phragmoplastin orthologues, yet DRP2A shows strong interaction with phragmoplastin. We expressed DRP2A as a GFP-tagged protein in BY-2 cells and examined the localization of this protein during cell cycle. DRP2A was present as punctate structures in the cytoplasm (Figure 3G), possibly in the Golgi network as suggested recently (Jin *et al.*, 2001; Lam *et al.*, 2002). However, DRP2A was also concentrated at the forming cell plate and only slightly in the phragmoplast microtubules (Figure 3H). In the cell line expressing GFP-DRP2A, 5–10% of the cells continue to expand in cell size (Figure 3I), leading to the formation of giant cells (GC). It is possible that this mutant phenotype is the result of expansion of vacuoles caused by excessive endocytosis of plasma membrane due to over-expression of DRP2A. These transgenic ‘giant cells’ do not divide and become terminal. Based on this phenotype, the subcellular localization, and the low homology to DRP1A, we propose that DRP2A does not have the same function as DRP1A. Rather, it suggests that DRP2 proteins may be more like their animal counterparts and involved in membrane recycling (endocytosis) at the plasma membrane (Battey *et al.*, 1999), a phenomenon that is very active during cell plate formation. Further studies are needed to elucidate the molecular nature of this phenomenon.

Cytoskeletal association of DRP1A; binding to microtubules in vitro

In order to confirm the relationship between microtubules and DRP1A, we carried out a spin-down assay to verify if DRP1A binds to microtubules *in vitro* (Figure 5A). Total membrane proteins were prepared from BY-2 cells expressing GFP-DRP1A. The membrane proteins dissolved in Triton X100 were mixed with purified tubulins, and polymerization of microtubules was carried out in the presence of GTP, Mg²⁺ and Taxol (a microtubule-stabilizing agent) at 37 °C. The polymerization mixture was separated by centrifugation into soluble and pellet (microtubule) fraction. Western blot analysis with polyclonal antibodies against soybean phragmoplastin was performed to detect if DRP1A is pulled down together with microtubules. As mentioned above (see Materials and methods), phragmoplastin antibodies reacted with both the tagged protein (GFP-DRP1A) and the endogenous DRP1 from BY-2 cells. As expected, both the tagged protein and the endogenous proteins were detected in the microtubule pellet fraction (Figure 5A,

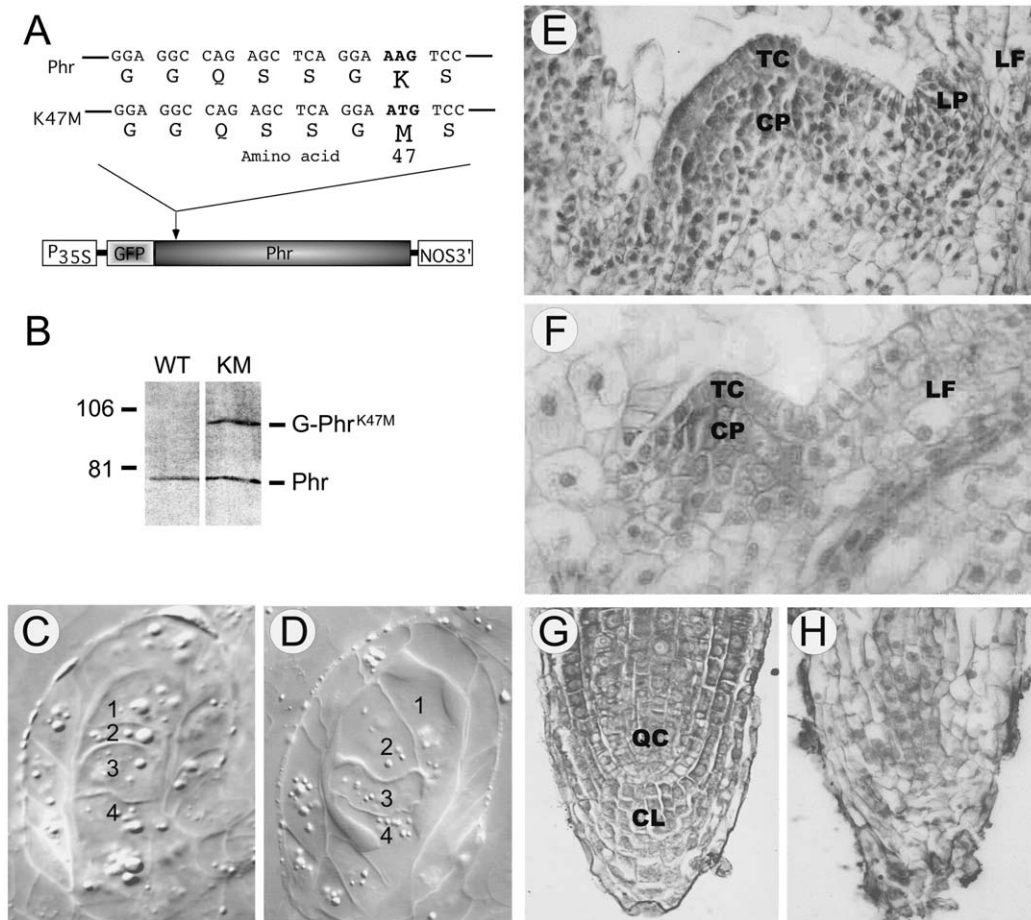


Figure 7. Phenotypes of female gametophyte, shoot and root apical meristems of tobacco plants expressing Phr^{K47M} . **A.** The lysine residue (K) at position 47 of phragmoplastin was replaced with a methionine (M) using site-directed mutagenesis, fused in-frame with GFP under the control of the CaMV 35S promoter. Transgenic plants were generated with this construct. **B.** Western blot of the total membrane fraction from wild-type control (WT) and plants expressing Phr^{K47M} . G-Phr, GFP-Phr fusion protein. Phr, endogenous phragmoplastin from BY-2 cells. Molecular mass markers (kDa) are indicated. **C** and **D.** Longitudinal sections through ovules of control (**C**) and transgenic plants (**D**). Successive meiotic cell divisions I and II of the megagametophyte initial produced four cells as indicated by numbers 1–4 with cell 1 being the megaspore cell. Cell plate was laid often in an oblique orientation (between cells 1 and 2 in **D**), especially during meiotic cell division II in the mutant megagametophytes. **E** and **F.** Shoot apical meristem of control (**E**) and transgenic plants (**F**). Note that transgenic plants expressing Phr^{K47M} lost the majority of their tunica (TC), corpus (CP) and leaf primordia (LP). LF, leaf. **G** and **H.** Root apical meristem of control (**G**) and transgenic plants (**H**). Note that the transgenic plants lost their quiescent center (QC) and the neighboring cells enlarged and became differentiated. CL, columella cells.

lane 4), suggesting that DRP1A binds to microtubules *in vitro*. The same membrane was also reacted with polyclonal antibodies to the plasma membrane ATPase (Nagao *et al.*, 1987). The ATPase was detected in the membrane fraction (Figure 5B, lanes 2 and 3) but was not pulled down with microtubules (Figure 5B, lane 4), suggesting that this microtubule-binding assay is specific. Microtubule-binding activity appears to be one of the properties shared by members of the DRP family, as VPS1/SPO15, a dynamin homologue from yeast, also binds to microtubules *in vitro* (Yeh *et al.*,

1991) while animal dynamin was originally isolated as a microtubule-binding protein (Shpetner and Vallee, 1989).

DRP1A is not associated with actin microfilaments

To further test if DRP1A is associated with microtubules or actin *in vivo*, we examined the changes in localization pattern in live BY-2 cells treated with propyzamid (a microtubule-destabilizing agent) and cytochalasin D (an actin microfilament-dissociating

drug). Because both chemicals were dissolved in DMSO, cells treated with DMSO alone were used as controls. Treatment with DMSO did not change the localization pattern of DRP1A even after an extended period (Figure 6A). In contrast, incubation with propyzamid (3 μ M) diminished the association of DRP1A with the cortical microtubules (Figure 6B). It is interesting to note that the intensity of fluorescence associated with the phragmoplast (including phragmoplast-microtubules and the cell plate) was also reduced by propyzamid treatment. However, the fluorescence in the phragmoplast could not be eliminated completely, even after an extended time period. This suggests that vesicles containing DRP1A may have started to fuse to each other into tubule-like structures in the phragmoplast region before reaching to the cell plate (see also Otegui *et al.*, 2001). Tubule-like structures are more stable than vesicles. When microtubules are dissociated, vesicles may fall apart whereas tubule-like structures may stay in place, as indicated in Figure 6B. To exclude the possibility of association between DRP1A and actin microfilaments, we also employed cytochalasin D (100 μ M) to treat BY-2 cells, but did not observe any changes in the pattern of cortical distribution of DRP1A (Figure 6C).

Expression of Phr^{K47M}, a GTPase-defective phragmoplastin, disrupts the orientation of cell plate during megasporogenesis in transgenic tobacco plants

To study the role of GTPase of phragmoplastin in its localization and function, we expressed a phragmoplastin mutant defective in its GTPase activity in transgenic tobacco plants. The lysine (K) residue at position 47 of soybean phragmoplastin was changed to methionine (M) via site-directed mutagenesis (Figure 7A). This lysine residue is absolutely conserved in the tripartite GTP-binding motif (GXXXSGKS/T, DXXG and T/NKXD) in all GTP-binding proteins. Mutation in this residue has been shown to abolish GTP binding and GTPase activity in many GTP-binding proteins including dynamin (Marks *et al.*, 2001). One representative transgenic plant line (No. 3) was chosen for further study as it displayed a phenotype typical of that observed in all 20 independent lines studied. This line contained a single-copy insertion as confirmed by genomic Southern blot analysis (data not shown), and expressed the GFP-Phr^{K47M} mutant protein (94 kDa) as detected by Western blot analysis (Figure 7B). The expression level of the

mutant protein was about the same as the endogenous phragmoplastin (68 kDa). We analyzed the pattern of cell plate formation during early female gametogenesis in transgenic plants expressing mutated phragmoplastin. Longitudinal sections of ovules at early stages of megaspore development (Figure 7D) showed that at the 4-cell stage, the orientation of the cell wall was altered in transgenic plants as compared to that of control plants (Figure 7C). The cell wall between megaspores 2 and 3 was twisted, and the one between megaspores 1 and 2 was completely disoriented (Figure 7D). This phenotype is similar to that observed in BY-2 cells and transgenic tobacco plants overexpressing phragmoplastin (Gu and Verma, 1997; Geisler-Lee *et al.*, 2002).

Severe growth retardation caused by the expression of Phr^{K47M}

In the T₁ population of transgenic Phr^{K47M} plants, 94% of the seedlings were not able to grow beyond the stage of first or second pair of true leaves and died. The remaining 6% of the plants survived and were able to produce a limited number of seeds. The plant size and seed numbers were reduced significantly. Progeny from the surviving plants continue to exhibit severe (93–94%) and mild (6–7%) mutant phenotypes. The apical dome of the shoot meristem of the Phr^{K47M} plants decreased in size and lost the majority of their tunica, corpus and leaf primordia (Figure 7F). Phr^{K47M} seedlings developed normal green cotyledons but produced dysfunctional first pair of true leaves (Figure 8A). Chlorosis was found in the entire leaf (Figure 8B) or in the enlarged central vein (Figure 8D). Leaf epidermal cells of the surviving plants lacked the normal shape of neck-lobe pattern and were elongated (Figure 8C). The mutant leaves were smaller and contained much less cells as compared to the control plants.

Radicles (embryonic root) emerged from the transgenic Phr^{K47M} seeds on the third day after germination, but root growth was arrested shortly after germination. Phr^{K47M} seedlings had no lateral roots, but did possess aborted adventitious root primordia emerging from the hypocotyl. Arrested roots of 4-week old Phr^{K47M} seedlings lacked distinct cell files and appeared as a disorganized cell mass (Figure 7H). No quiescent center or columella could be recognized as compared to the controls (Figure 7G). The enlarged and vacuolated cells in the Phr^{K47M} root meristem may be attributed to the loss of undifferentiated status of the

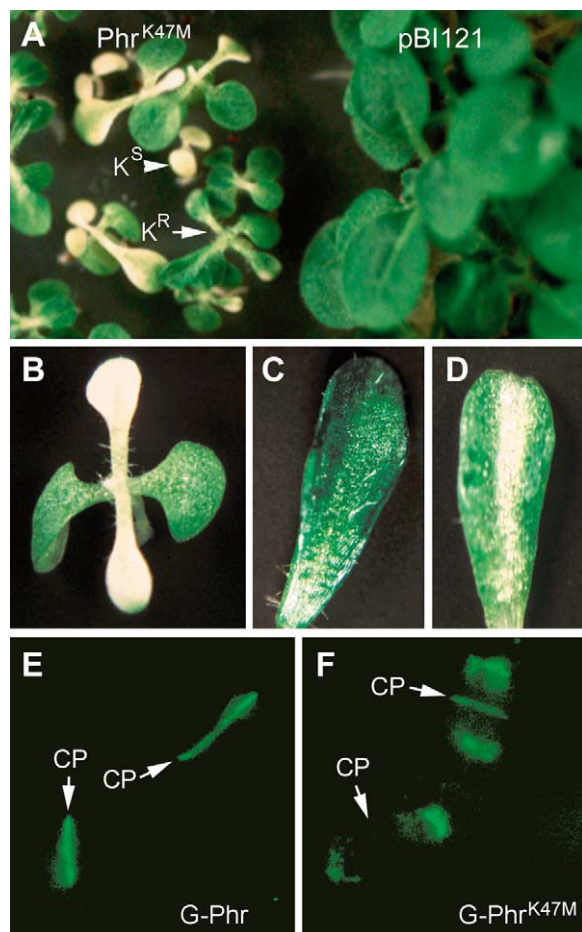


Figure 8. Phenotype of tobacco seedlings expressing Phr^{K47M} and localization of the mutant protein. **A.** Seedlings of the mutant plants (Phr^{K47M}) and control expressing the vector (pBI121) were grown on MS medium containing kanamycin. Mutant seedlings segregated into kanamycin-resistant (K^{R}) and kanamycin-sensitive (K^{S}) at a 3:1 ratio. **B–D.** Mutant seedling phenotypes. The cotyledons were green and looked normal. Chloroplast development in the true leaves was apparently retarded, leading to chlorosis of the entire leaf (**B**) or the central vein (**D**). The leaf epidermis cells lost the normal shape of neck-lobe pattern and the leaves became elongated (**C**). Most of the mutant plants did not develop beyond the first pair of true leaves. **E** and **F.** Tobacco BY-2 cells expressing GFP- Phr^{K47M} (G- Phr^{K47M}). As compared to the control (G-Phr) where the fusion protein was located entirely to the cell plate (CP), the mutant (Phr^{K47M}) protein was retained in the distal ends of the phragmoplast. A very limited amount of the mutant protein was transferred to the cell plate as compared to control.

meristematic initials, which are normally cytoplasmically dense (Figure 7G). In addition the irregular cubic shape of these cells indicated isodiametric expansion of these cells.

GTPase mutation retards the delivery of phragmoplastin-containing vesicles to the cell plate

Cells expressing Phr^{K47M} were smaller and grew at a slower rate as compared to the cells expressing GFP alone. At cytokinesis, Phr^{K47M} remained at the distal ends (Golgi-rich regions) of the cell and only a very small amount of the protein was targeted to the cell plate (Figure 8F). In contrast, GFP-phragmoplastin was found only at the cell plate (Figure 8E). This result suggests that GTPase activity of phragmoplastin is required for budding of vesicles from the Golgi bodies and for their transport to the cell plate. In fact, the Golgi stacks from Phr^{K47M} plants were narrower, and the vesicles near the trans-cisterna were more electron-dense, as compared to that in the control cells (data not shown). It is possible that Phr^{K47M} forms heterodimers with DRP2 in the Golgi stacks and thus inhibits the budding of the vesicles.

Accumulation of tubule-like structures in the cell plate caused by the expression of Phr^{K47M}

Cell plate formation is affected by the expression of Phr^{K47M} . Electron microscopy showed that entire cell plate in transgenic plants was filled with intertwined tubule-like structures (Figure 9B). These structures are similar to the tubules formed by dynamin in the presence of GTP- γ -S (Takei *et al.*, 1995) and the vesiculo-tubule-vesicle (VTV) structures observed at the forming cell plate (Samuels *et al.*, 1995; Staehelin and Hepler, 1996). In control plants, the tubule-like structures are usually observed only at the growing edges of the cell plate (Figure 9C; Samuels *et al.*, 1995), while in the center of the cell plate, the tubular network is consolidated into fenestrated sheet (Figure 9A; Samuels *et al.*, 1995). The latter step was apparently delayed and/or blocked in transgenic Phr^{K47M} plants. These tubule-like structures were filled with uncharacterized electron-dense material.

Discussion

We have recently defined six functional groups for *Arabidopsis* DRPs according to their phylogenetic relationships and the presence of conserved motifs

(Hong *et al.*, 2003). This grouping is further supported by the subcellular localization and possible functional differences among members of this family. Three cloned members of the phragmoplastin-like subfamily (DRP1A, DRP1C and DRP1E) and one from the dynamin-related subfamily (DRP2A) encode proteins that interact with phragmoplastin and are targeted to the forming cell plate. The remaining two DRP1 members, DRP1B and DRP1E are apparently expressed only in suspension culture (Kang *et al.*, 2003b).

Multiple Arabidopsis DRPs function at the forming cell plate

Multiple DRP1 proteins are targeted to the cell plate (Figures 3 and 4) and can form heterodimers (Figure 1). These proteins are not functionally redundant because homozygous seedlings of a knockout of *DRP1A* by T-DNA insertion are severely retarded in growth (Kang *et al.*, 2001). This suggests that DRP1A has a unique function which can not be replaced by other members of the DRP1 subfamily. Surprisingly, the severe growth retardation phenotype of the homozygous DRP1A knockout line can be rescued by addition of sucrose (Kang *et al.*, 2001). Sucrose may affect the supply of UDP-glucose and it has been shown that phragmoplastin interacts with UDP-glucose transferase (Hong *et al.*, 2001b). The sucrose-rescued *adl1A* mutant plants are infertile and display defects in the development of leaf trichomes (Kang *et al.*, 2003a). Plants with gene knockout in DRP1E (*adl1E*) show no discernible phenotypes and are completely fertile. *DRP1A* and *DRP1E* double mutants (*adl1A-2 adl1E-1*) develop defective embryos (Kang *et al.*, 2003a). Differences in the localization pattern of several phragmoplastin isoforms further suggest specific functions of these proteins in cell plate formation. First, all DRP1s are not expressed exactly in the same subcellular location at the same time. Second, the localization pattern of DRP1A, DRP1C and DRP1E appears different at the cell plate. DRP1A is tightly associated with the microtubules at all phases of cell cycle (Figure 3A–C), while DRP1C is loosely associated with the cortical microtubules and the mitotic spindle (Figure 3E), but not with the phragmoplast microtubules (Figure 3E). It is not known if DRP1E is also associated with the microtubules; the localization pattern of DRP1E at the cell plate is very unique, marking the tubule-like structures (Figure 4C–F). According to the current model of cell plate formation (Samuels *et al.*, 1995; Staehelin and Hepler, 1996;

Verma, 2001), Golgi-derived vesicles are fused to each other to form vesicle-tubule-vesicle (VTV) structures. VTV are then fused to each other (Verma, 2001) to generate the tubulo-vesicular network (TVN). It is possible that DRP1A functions at the step of VTV formation as proposed (Verma, 2001), whereas DRP1E plays a role in the formation of TVN. Third, since DRP1 proteins can form homo- and heteropolymers (Figure 1), a change in the ratio of one DRP1 to the others could alter the property of the heteropolymer. The presence of multiple DRP1s in the same cell could have the potential to produce a wide spectrum of DRP1 heteropolymers that may finely regulate the process of membrane fusion at different levels during cell plate formation.

A close association of Golgi-derived vesicles and microtubules suggests that these vesicles slide along the microtubules (Staehelin and Hepler, 1996; Verma, 2001; Smith, 2001; Otegui *et al.*, 2001; Jurgens and Geldner, 2002; Bednarek and Falbel, 2002). Several kinesin-related proteins in *Arabidopsis* including AtPAKRP2, Hinkel, KCBP and TKRP125 have been suggested to play a role in microtubule-based translocation activities (Asada *et al.*, 1997; Smirnova *et al.*, 1998; Lee *et al.*, 2001; Strompen *et al.*, 2002). Dynamin has been suggested to interact with the motor protein kinesin (Kretzer *et al.*, 2000). Here we presented molecular evidence to suggest that DRP1A may be one of the proteins that participate in vesicle transport along the microtubules. Data presented in Figure 3A–C demonstrate that DRP1A-containing vesicles are tightly bound to the cortical microtubules, cytoplasmic microtubule strands, spindle microtubules and phragmoplast microtubules, in addition to the forming cell plate. We further demonstrated that DRP1A is associated with microtubules *in vitro* (Figure 5), and the *in vivo* localization pattern of DRP1A is altered by microtubule-destabilizing agent (Figure 6).

DRP2A is a *bona fide* dynamin from *Arabidopsis* and is also targeted to the forming cell plate at cytokinesis (Figure 3H). A GTPase-defective mutation of dynamin blocks endocytosis in the plasma membrane at the early stage of coated vesicle formation (McNiven *et al.*, 2000). The role of DRP2A in cell plate formation appears to be different from that of DRP1s, although both types of proteins are present at the same location. While DRP1s may be involved in vesicle delivery from the Golgi to the cell plate and membrane tubule formation, the DRP2A may function in membrane recycling from the plasma membrane and cell plate, a process involving endocytosis (Bat-

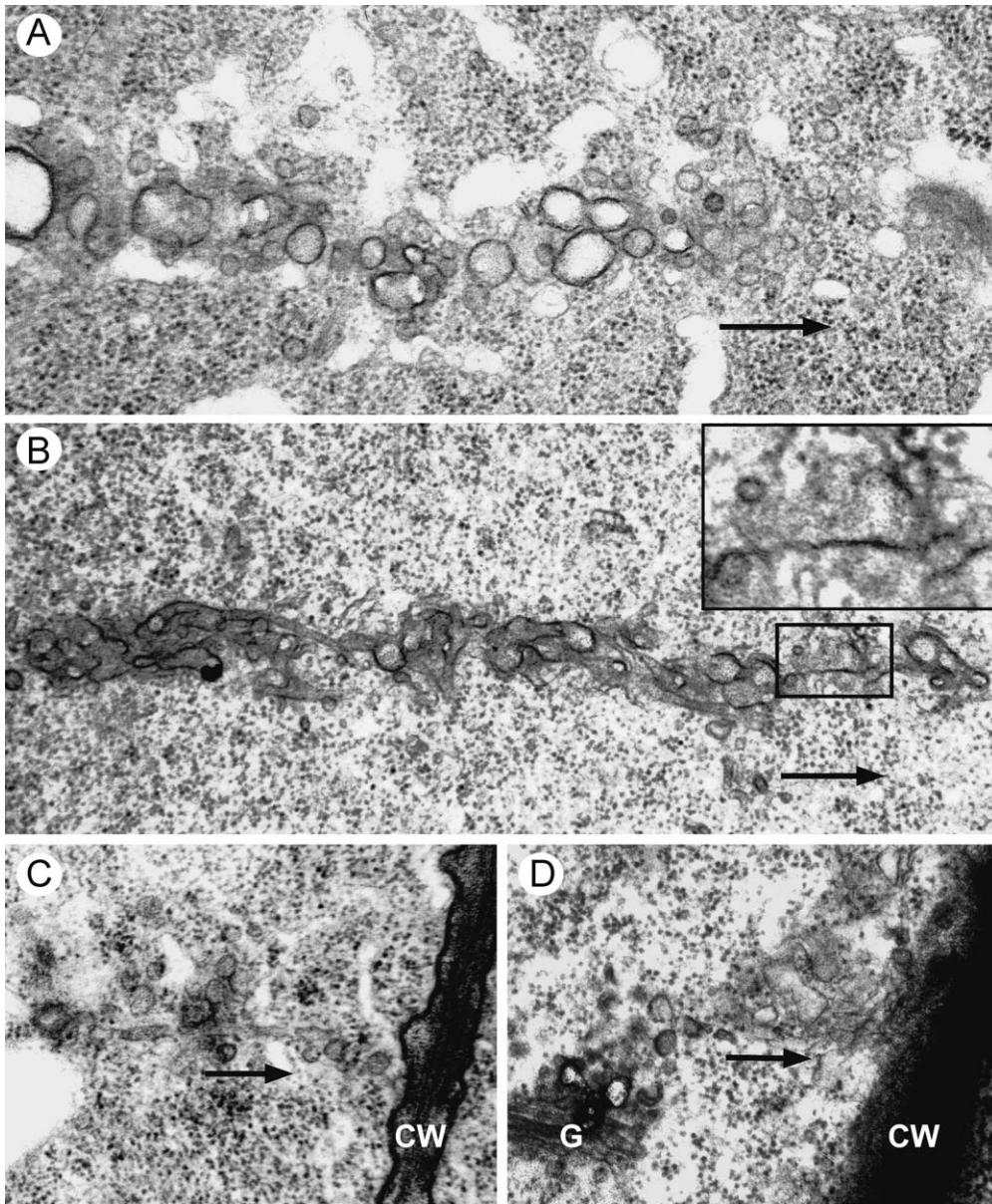


Figure 9. Ultrastructure of the cell plate in tobacco plants expressing Phr^{K47M}. A and B. Cell plate of control (A) and the Phr^{K47M} mutant root cells (B). The cell plate of the mutant plant is composed of intertwined vesicle-tubule structures, whereas only fenestrated sheets is found in the control cell plate. The inset shows magnified tubule structures with a striped band of phragmoplastin which is similar to the structures observed with dynamin in the presence of GTP γ -S in animal cells (Takei *et al.*, 1995). C and D. The 'T' junction region between the growing cell plate and the cell wall (CW) of the mother cell in the control plant (C) and the mutant (D). Tubule-like structures are found only in the mutant cell plate. Arrows indicate the direction of cell plate growth. G, Golgi stacks.

tey *et al.*, 1999). DRP2A (formerly ADL6) has been demonstrated to be associated with clathrin-coated vesicles (Lam *et al.*, 2002) known to be involved in membrane recycling. DRP2A has also been localized in the Golgi apparatus and has been suggested to be involved in cargo delivery from the Golgi to the vacuole (Jin *et al.*, 2001). If DRP2A behaves like dynamin, it is expected that DRP2A is associated with the plasma membrane, and coated vesicles as shown in Figure 3G and by Lam *et al.* (2002). Vesicles originating from the plasma membrane could be transported to the Golgi (membrane recycling) or to the vacuole (receptor internalization). Cell plate is completed in a limited time window, and both processes of vesicle fusion and membrane recycling have to occur simultaneously.

GTPase-defective phragmoplastin acts as a dominant negative mutant

Because phragmoplastin forms homopolymers as well as heteropolymers with other DRPs (Figure 1; Zhang *et al.*, 2000), expression of a mutant Phr^{K47M} could affect the function of not only phragmoplastin itself, but also other isoforms of DRP1 and DRP2 proteins, generating a dominant-negative effect. In BY-2 cells, Phr^{K47M} was retained in the Golgi and only a small amount of the protein reached at the forming cell plate (Figure 8E and F). This result suggests that GTPase activity of phragmoplastin is required for the vesicle budding from the trans-Golgi network and their transport to the cell plate. Apparently the small amount of the mutant protein that reaches to the cell plate is enough to disturb the function of DRP1 and DRP2 proteins by forming functionally-defective heteropolymers. The effect of such a dominant-negative mutation is very deleterious as the cell plate development is arrested at the TVN step. The pleiotropic effects of Phr^{K47M} expression in tobacco plants range from the retardation of root, leaf and shoot development, to the misplacement of the cell plate in female gametophyte (Figure 8). Cell plate formed during meiosis in Phr^{K47M} plants is also disoriented (Figure 7), suggesting that cell plate formation in both mitosis and meiosis is affected by this mutation. These results are consistent with recent observations with a micro-induction system that allows to control the expression level of phragmoplastin in the shoot apical meristem (Wyrzykowska and Fleming, 2003). It is worth noting that the null mutation in *VPS1/SPO15*, a phragmoplastin homologue from yeast, inhibits the separation of spindle poles during meiosis (Yeh *et al.*, 1991) and

disrupts protein sorting to the vacuole (Vater *et al.*, 1992).

The dynamics of the DRPs in plants suggests that this group of GTPases have unique functions in cell biology involving tubule formation of membranes, recycling of membranes and translocation of vesicles via microtubule cytoskeleton (Hong *et al.* 2003). Furthermore, differences in the localization pattern of various subfamily members suggests that the expression of these proteins is tightly regulated to control various membrane biogenesis activities.

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