Dynamics of *Arabidopsis* Dynamin-Related Protein 1C and a Clathrin Light Chain at the Plasma Membrane

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Plant morphogenesis depends on polarized exocytic and endocytic membrane trafficking. Members of the *Arabidopsis thaliana* dynamin-related protein 1 (DRP1) subfamily are required for polarized cell expansion and cytokinesis. Using a combination of live-cell imaging techniques, we show that a functional DRP1C green fluorescent fusion protein (DRP1C-GFP) was localized at the division plane in dividing cells and to the plasma membrane in expanding interphase cells. In both tip growing root hairs and diffuse-polar expanding epidermal cells, DRP1C-GFP organized into dynamic foci at the cell cortex, which colocalized with a clathrin light chain fluorescent fusion protein (CLC-FFP), suggesting that DRP1C may participate in clathrin-mediated membrane dynamics. DRP1C-GFP and CLC-GFP foci dynamics are dependent on cytoskeleton organization, cytoplasmic streaming, and functional clathrin-mediated endocytic traffic. Our studies provide insight into DRP1 and clathrin dynamics in the plant cell cortex and indicate that the clathrin endocytic machinery in plants has both similarities and striking differences to that in mammalian cells and yeast.

**INTRODUCTION**

Dynamins and dynamin-related proteins (DRPs) constitute a structurally related, yet functionally diverse, superfamily of large GTPases (reviewed in Praefcke and McMahon, 2004; Konopka et al., 2006). Dynamins and DRPs are involved in various aspects of endomembrane and intracellular organelle dynamics, including endocytosis and post trans-Golgi network (TGN) trafficking (Damke et al., 1994; Henley et al., 1998; Jones et al., 1998; Nicoziani et al., 2000), mitochondrial fusion and fission (Moody et al., 2000; Wong et al., 2000), peroxisome inheritance (Koch et al., 2003), chloroplast division (Gao et al., 2003), and actin dynamics (McNiven et al., 2000; Schafer et al., 2002). Mammalian dynamin 1 plays critical roles in several types of endocytosis, including clathrin-mediated endocytosis (CME; Damke et al., 1994).

In mammalian cells, CME is mediated by a coordinated interplay of accessory and regulatory proteins. Invagination of the clathrin-coated bud is thought to occur through the polymerization of clathrin triskelia, which is composed of heavy chain (CHC) and light chain (CLC) subunits, the membrane remodeling activities of accessory proteins, and the force generated by actin polymerization (Conner and Schmid, 2003). Dynamin 1 subunits are recruited to and form rings and spirals around the necks of the invaginating clathrin-coated buds (Damke et al., 1994) through their lipid-interacting pleckstrin homology (PH) domain and protein-interacting Pro-rich (PR) domain (Vallis et al., 1999).

It has been proposed that there is a conformational change of the dynamin oligomer upon GTP hydrolysis (Roux et al., 2006) that along with actin polymerization (Merrifield et al., 2005) and the activity of additional membrane modifying proteins promotes the release of the clathrin-coated vesicle from the plasma membrane. Similar to mammalian cells, CME in the yeast *Saccharomyces cerevisiae* depends on a highly ordered assembly of membrane and cytoskeletal-associated proteins to initiate the recruitment of cargo proteins, budding, and release of endocytic vesicles (Kaksonen et al., 2003, 2005; Sun et al., 2006). However, a few striking differences exist, including the lack of an identified DRP in yeast CME, which is required for endocytosis in mammals (Yu and Cai, 2004; Kaksonen et al., 2005).

Clathrin-coated structures at the plasma membrane are evident in electron micrographs of several plant species (van der Valk and Fowke, 1981; Emons and Traas, 1986; Derksen et al., 1995; Robinson, 1996; Fowke et al., 1999; Dhonukshe et al., 2007). Apparent homologs for CHC, CLC, AP-2 subunits, and mammalian accessory proteins are present in the *Arabidopsis thaliana* genome (Barth and Holstein, 2004; Holstein and Oliviusson, 2005). Expression of a dominant-negative CHC inhibits uptake of the lipophilic tracer dye FM4-64 and of several plasma membrane proteins (Dhonukshe et al., 2007; Tahara et al., 2007). In addition, use of the mammalian AP-2 adaptor complex inhibitor tyrphostin A23 causes a reduction in endocytosis (Dhonukshe et al., 2007). Although the existence of CME in plants is now widely accepted, the molecular machinery responsible for the regulated uptake of membrane and endocytic cargo (Russinova et al., 2004; Robatzek et al., 2006; Sutter et al., 2007) is less well characterized.
The Arabidopsis genome contains 16 genes that are predicted to encode DRPs (Hong et al., 2003b; Gao et al., 2006), which have been assigned to six protein families based on primary sequence, predicted domain structure, and functional analysis. Members of the DRP2 family share the greatest similarity in domain structure to mammalian dynamin 1, including the PH and PR domains, and are required for Golgi-to-vacuole protein trafficking (Jin et al., 2001). DRP2A interacts with the putative Arabidopsis AP-1 subunit, γ-adaptin (Jin et al., 2001), which, in turn, interacts with clathrin and the Arabidopsis homolog of epsin (Song et al., 2006), an adaptor protein required for clathrin-dependent trafficking at the plasma membrane (Chen et al., 1998) and TGN (Duncan et al., 2003). However, the direct involvement of DRP2 in endocytosis has not been shown.

In contrast with DRP2, members of the plant-specific DRP1 protein subfamily, which lack the PH and PR domains found in dynamin 1, are required for plant cell expansion and cytokinesis (Kang et al., 2001, 2003b). The DRP1 protein subfamily consists of five members, designated A through E. In a related study, we have described the dynamics of DRP1A in comparison to DRP1C and shown that DRP1C is not fully functionally redundant with DRP1A (Konopka and Bednarek, 2008b). Limited work on DRP1C has demonstrated protein localization to the de novo plasma membrane at the cell plate and a striking enrichment in the tips of expanding root hairs. Loss-of-function drp1C mutants are male gametophytic lethal with pollen characterized by large invaginations of the plasma membrane (Kang et al., 2003a). These early data suggest that DRP1C may be involved in plasma membrane dynamics. Using a combination of confocal laser scanning microscopy (CLSM) and variable angle epifluorescence microscopy (VAEM), we show that DRP1C–green fluorescent protein (GFP) was recruited from the cytoplasm to the plasma membrane flanking the tip in rapidly growing root hairs. In epidermal cells, DRP1C-GFP was organized into discreet, dynamic foci that colocalized with and displayed similar dynamics to the plasma membrane–associated, fluorescent fusion protein of clathrin light chain (CLC–FFP). These analyses support the hypothesis that DRP1C is a component of the clathrin-associated machinery in plants.

RESULTS

DRP1C-mGFP5 Is Localized to the Division Plane, Plasma Membrane, and Cytoplasm

To determine the cellular distribution of DRP1C, a DRP1C-mGFP5 translational fusion (now referred to as DRP1C-GFP) was constructed and introduced into DRP1C/drp1C-1 plants (Kang et al., 2003a). To ensure normal expression and localization of GFP-tagged DRP1C, a genomic fragment of DRP1C including 2.9 kb of the native promoter, which is known to complement the drp1C pollen defect (Kang et al., 2003a), was positioned at the N terminus of GFP (Figure 1A). DRP1C-GFP rescued the development of drp1C-1 pollen, and drp1C-1/drp1C-1 plants transformed with DRP1C-GFP had no visual defects. Immunoblot analysis of total protein extracts from plants expressing DRP1C-GFP confirmed that the fusion protein was intact and expressed at levels similar to native DRP1C (Figure 1B). Together, these data indicated that the DRP1C-GFP fusion protein was functional in vivo.

DRP1C-GFP was expressed throughout pollen germination. GFP fluorescence was first observed in hydrated pollen and localized to the plasma membrane (Figure 1D) but was not detected in pollen from untransformed plants (Figure 1C). During pollen grain germination, plasma membrane–localized DRP1C-GFP converged to the region near the aperture through which the pollen tube would emerge (Figure 1D). As the pollen tube continued to grow, DRP1C-GFP remained in the distal end of the pollen tube in the cytoplasm and along the plasma membrane (Figure 1E; see Supplemental Video 1 online).

As previously observed in roots using immunofluorescence (Kang et al., 2003a), DRP1C-GFP localized to the division plane in the transition zone of roots (Figure 1F) and to the distal region of elongating root hairs (Figure 1G). In addition, live-cell imaging showed that DRP1C-GFP associated with the plasma membrane in these cell types. In aboveground tissue, DRP1C-GFP was expressed in hypocotyls (see Supplemental Figure 1A online), leaf pavement, and socket cells (Figure 1H) and in developing leaf trichomes (Figure 1H), where it localized to both the cell cortex and the cytoplasm.

DRP1C-GFP was not observed to be associated with any cytoskeletal structures or mitochondria in dividing or nondividing cells as was previously reported (Hong et al., 2003a; Jin et al., 2003). DRP1C-GFP did not colocalize with the mitochondrial tracer dye MitoTracker Orange CM-H2XRos in root epidermal cells (see Supplemental Figure 1B online) or cortical cells. To confirm the lack of mitochondrial localization, roots expressing a GFP-tagged mitochondria signal sequence fusion protein (ss-β-ATPase–GFP; Logan and Leaver, 2000) were fixed and probed with an antibody specific for DRP1C (Kang et al., 2003a). Again, α-DRP1C did not associate with the ss-β-ATPase–GFP mitochondrial marker (see Supplemental Figure 1C online).

DRP1C-GFP Is Enriched at the Lateral Tip Plasma Membrane of Growing Root Hairs

Previous studies have shown that Arabidopsis DRP1s are involved in polarized cell expansion (Kang et al., 2003a, 2003b). Root hairs are a model cell for the study of polar cell growth as they expand through membrane addition exclusively at the tip. Many signaling and membrane trafficking pathways that are required to establish and maintain this polar cell growth have been characterized (reviewed in Carol and Dolan, 2002; Šamaj et al., 2006). Thus, we analyzed the in vivo dynamics of DRP1C-GFP in these tip-growing cells. DRP1C-GFP fluorescence was not evenly distributed throughout the root hair but was primarily localized at the distal end (toward the tip) during all stages of root hair development, beginning with the appearance of the first bulge and continuing through to termination of root hair growth (Figure 2A). DRP1C-GFP fluorescence levels at the distal end of the root hair decreased as root hairs stopped growing and eventually reached levels similar to the rest of the root hair plasma membrane.

To investigate the dynamics of DRP1C-GFP during root hair growth with greater time resolution, seedlings were grown at a 30° horizontal angle through half-strength Murashige and Skoog
The lateral plasma membrane primarily at the tip apex, which is the site of membrane addition (Shaw et al., 2000), and the lateral plasma membrane within 3 h of imaging. Their growth rate oscillated over time, with rates varying between 0.4 and 1.7 \( \mu \text{m/min} \). DRP1C-GFP localized to the plasma membrane throughout the circulating cytoplasm (Figure 2B; see Supplemental Video 2 online). As the root hair elongated, the plasma membrane–associated fluorescence recovered in 47.6 \( \mu \text{m} \) of the apex (referred to as tip lateral flanks) as well as throughout the circulating cytoplasm (Figure 2C; see Supplemental Video 2 online). As the root hair elongated, the plasma membrane–associated DRP1C-GFP fluorescence intensity fluctuated both at the lateral flanks and at the tip apex throughout the 2 h of imaging (Figure 2C). The highest DRP1C-GFP fluorescence intensities at the tip apex were observed when the growth rate of the root hair was lowest (Figure 2C). At the fastest growth rates (~1.7 \( \mu \text{m/min} \)), only 6% of total membrane-associated DRP1C-GFP fluorescence was located at the tip apex. By contrast, at the slowest rate, 60% of the total plasma membrane–associated DRP1C fluorescence was present at the root hair tip apex. This analysis suggested an inverse relationship between the localization of DRP1C at the tip apex, where exocytosis occurs, and the rate of root hair growth during expansion (Figure 2C).

We performed a FRAP analysis to determine how DRP1C is recruited to the plasma membrane at the root hair tips. DRP1C could be recruited directly from the cytoplasm and/or delivered to the tip apex via an exocytic pathway and then diffuse into the root hair tip flanks during periods of rapid growth. To test these models, the diffusion of plasma membrane–associated DRP1C-GFP at the tip lateral flanks of expanding root hairs was analyzed. If DRP1C-GFP were recruited directly from the cytoplasm, the fluorescence in the entire photobleached area would recover at a uniform rate. Alternatively, if DRP1C-GFP localization depended on initial delivery to the root hair apex and subsequent diffusion, the initial fluorescence recovery would occur at the edge of the photobleached area. As a control, the fluorescence recovery rate of the cytoplasmic pool was determined, which recovered to its original intensity (corrected for photobleaching) in 17.1 ± 5.1 s (\( n = 9 \); Figures 3A, 3C, and 3D, black circles). The plasma membrane–associated fluorescence recovered in 47.6 ± 15.8 s (\( n = 18 \); Figures 3B to 3D), indicating that DRP1C-GFP did not freely diffuse at the plasma membrane. The photobleached area of the root hair tip flank was divided into two regions, one comprising the peripheral two-thirds of the photobleached area (Figures 3C and 3D, white boxes) and the other comprising the inner one-third of the bleached area (Figures 3C and 3D, black circles).
boxes). In 83% of the root hairs (n = 18), the peripheral and inner regions recovered with equal kinetics, suggesting that DRP1C-GFP was primarily recruited from the cytoplasm (Figures 3B to 3D; see DPR1C-GFP forms discreet foci at the plasma membrane below).

**Tip-Localized Dynamics of DRP1C-GFP Are Associated with Growth Rate in Inhibitor-Treated Root Hairs**

Root hair growth has been shown to require calcium gradients and reactive oxygen species as signaling modules, phosphatidylinositol metabolism, actin dynamics, and a functional secretory pathway (Carol and Dolan, 2002; Šamaj et al., 2006). To determine if these processes are also required for DRP1C-GFP polarity, DRP1C-GFP dynamics in root hairs were analyzed before, during, and after the addition of inhibitors of the above processes, including, 0.1% DMSO (control treatment; Figure 4A), 20 μM brefeldin A (secretory trafficking inhibitor; Figure 4B; Geldner et al., 2003), 30 μM cytochalasin D (F-actin inhibitor; Figure 4C; Ketelaar et al., 2003), or 30 μM tyrphostin A23 (CME inhibitor; Figure 4D; Banbury et al., 2003). Unlike the control treatment, which did not affect growth (Figure 4A), each inhibitor caused a cessation of root hair growth 10 to 30 min after inhibitor addition (Figures 4B to 4D). With the exception of tyrphostin A23
Figure 3. DRP1C-GFP is Recruited from the Cytoplasm to Sites on the Plasma Membrane.

(A) and (B) Time course of photobleaching and recovery of DRP1C-GFP pools in the cytoplasm (A) and lateral tip plasma membrane (B) in growing root hairs. Numbers represent time in seconds from the bleach. White shapes indicate bleached areas. Bars = 10 μm.

(C) Enlarged confocal images of root hairs at time 0 in (A) and (B). The photobleached region indicated in (B) is subdivided into peripheral and middle areas (indicated by dashed and solid squares, respectively) used for the graph in (D).

(D) GFP fluorescence intensity was measured for regions shown in (C) and plotted versus time. The images and graphs shown are representative of 18 separate FRAP experiments.
polarized localization of DRP1C-GFP at the apical and lateral plasma membrane was abolished within 15 min of growth cessation, resulting in a nonpolar distribution of DRP1C-GFP throughout the root hair (Figures 4B and 4C). These results suggested that the known requirements for active root hair tip growth are most likely not directly required for DRP1C-GFP recruitment or dynamics at the plasma membrane but that DRP1C-GFP localization at the tip of root hairs is intimately tied with active growth. This is supported by the result that DRP1C-GFP was not mislocalized in several root hair mutants, including the tip growth expansion mutants rhd2, rhd4, cow1, tip1, cob-1, cob-2, and erh1 (see Supplemental Figure 2 online).

tyrA23 inhibits mammalian AP-2 binding of endocytic cargo and has been shown to block CME in mammalian cells (Banbury et al., 2003) and endocytic traffic in plants (Dhonukshe et al., 2007). Although CME has not yet been shown to be required for tip growth in Arabidopsis, clathrin-coated structures have been observed at the flanks of root hair tips (Emons and Traas, 1986). We sought to determine if disrupting CME would alter the dynamics of DRP1C-GFP in root hairs. Within ~15 min of tyrA23 application, root hair growth slowed and stopped. (Figure 4D). In contrast with the other inhibitor treatments, DRP1C-GFP fluorescence at the apical or lateral plasma membrane decreased but never completely disappeared upon growth cessation. Instead, DRP1C-GFP remained localized at the tip up to 30 min after growth of the root hair had ceased. A further analysis of the effects of tyrA23 on DRP1C-GFP dynamics is presented below.

DRP1C-GFP Forms Discreet Foci at the Plasma Membrane

To examine DRP1C-GFP dynamics at the plasma membrane of transformed plants, VAEM was used, which allows for imaging of the plant cell cortex with a high signal-to-noise ratio (Konopka and Bednarek, 2008a). The distribution of DRP1C-GFP was visualized using VAEM in root hair tips and flanks (Figures 5A and 5B), expanding epidermal cells of the differentiation and elongation zone of the root (Figures 5E and 5F; see Supplemental Video 3 online), mature atrichoblasts in the root, leaf pavement cells, hypocotyl epidermal cells, and guard cells (see Supplemental...
Figure 3 online). In all cell types, DRP1C-GFP was prominently organized into discreet cortical-associated foci (Figures 5A to 5C; see Supplemental Video 3 online). To eliminate any differences that may occur in different cell types, DRP1C-GFP foci were initially analyzed in expanding root epidermal cells. In addition, only foci that had lifetimes (defined as the length of time that a focus was visible in the imaging plane) of >2 s were included in the analysis. The average lifetime of the DRP1C-GFP foci was 17.7 ± 8.8 s (n = 175; Figure 5.

Figure 5. DRP1C-GFP Forms Discreet, Dynamic Foci at the Plasma Membrane.

DRP1C-GFP root hairs ([A] and [B]) and expanding root epidermal cells ([E] to [G]) imaged with VAEM. (B) Time series of boxed area in (A). Numbers indicate elapsed time from start of imaging in seconds. (C) Fluorescence intensity profile of two foci indicated in (B) with arrow (red) and arrowhead (green). (D) Average normalized fluorescence (black line) and SD (gray lines) of 22 foci from one cell over time. The peak fluorescence was centered at time 0. (E) and (F) Images from a time series, acquired at 0.5-s intervals. Blue arrow indicates the same focus in (E) and (F). A focus that split off from the original is indicated by the yellow caret in (E). (G) Images from a time series, acquired at 0.5-s intervals. The focus indicated with an orange caret merges with the focus indicated by the green arrow. (H) The average density (±SD) of foci for four expanding (gray bars) and four nonexpanding (white bars) cells averaged from five different time points in two locations for each cell. Expanding cells were root epidermal cells just distal (toward the root tip) to the first root hair bulge. Nonexpanding cells were atrichoblasts (non-hair cells) in regions of the root with mature root hairs. (I) and (J) Roots expressing DRP1C-GFP were incubated in 50 μM tyrA23 for the time indicated and imaged in the presence of the inhibitor. The same root was imaged for the 1- and 3-min time points. Numbers indicate time elapsed from addition of inhibitor. Bars = 1 μm in (A), (B), (E) to (G), and (I) and 10 μm in (J).
After 3 min of treatment with tyrA23, endocytic traffic in cultured plant cells (Dhonukshe et al., 2007). To test the hypothesis that DRP1C plays a role in endocytosis, we compared the density and behavior of foci in expanding and nonexpanding epidermal root cells, the average lifetime of the DRP1C-GFP foci did not vary widely between cell types. The average lifetime was 17.7 ± 8.8 s in expanding root epidermal cells and 24.2 ± 12.4 s in nonexpanding root epidermal cells. By contrast, the density of DRP1C-GFP foci varied between cell types, with the highest density being measured in actively expanding cells (Figure 5H). In addition, more foci exhibited splitting and fusion in expanding cells (45%, n = 182) than in nonexpanding cells (28%, n = 182).

Inhibiting CME Disrupts DRP1C-GFP Foci Dynamics

To test the hypothesis that DRP1C plays a role in endocytosis, drp1C-1/drp1C-1 seedlings transformed with DRP1C-GFP were treated with 50 μM tyrA23, which was previously shown to inhibit endocytic traffic in cultured plant cells (Dhonukshe et al., 2007). After 3 min of treatment with tyrA23, ~15% of the cortical-associated foci imaged with VAEM began to increase in size and fluorescence intensity (Figure 5I) and the cytoplasmic pools of DRP1C-GFP began to amass into immobile structures with high GFP fluorescence as viewed by time-lapse CLSM (Figure 5J; see Supplemental Video 4 online). Thirty min after the addition of tyrA23, the cortical and cytoplasmic-associated DRP1C-GFP fluorescent signal was observed in immobile cortical-association foci and cytoplasmic structures, respectively (Figures 5I to 5J). After 30 min of tyrA23 treatment, <1% (n = 600) of the cortical foci entered or disappeared from the cell cortex during 2 min of imaging. By contrast, the phosphotyrosine analog tyrphostin A51, which does not inhibit mammalian clathrin/AP2–cargo interaction (Crump et al., 1998; Banbury et al., 2003), had no effect on localization or behavior of DRP1C-GFP (see Supplemental Figure 4A online). The effect of tyrA23 was rapidly reversible, with a complete disappearance of immobile cytoplasmic DRP1C-GFP structures within 5 min of washout (see Supplemental Figure 4B online). tyrA23 most likely does not inhibit the GTPase activity of DRP1C, as tyrA23 had no inhibitory effect on the GTPase activity of Escherichia coli expressed DRP1A (Supplemental Figure 4C online), which is 88% identical to DRP1C in its GTPase domain.

Dynamics of Cortical-Associated CLC

Cortical-associated DRP1C-GFP displayed characteristics that were reminiscent of the dynamics of dynamin 1 during CME (Merrifield et al., 2002). Based on this and our finding that tyrA23 inhibits DRP1C-GFP dynamics, CLC fluorescent fusion proteins were created and expressed in planta for real-time imaging of clathrin dynamics at the plasma membrane. Fluorescent fusions of CLC have been successfully used for illuminating clathrin dynamics in yeast (Sun et al., 2007) and mammalian cells (Merrifield et al., 2002). C-terminal fusions to mGFP5 (Konopka and Bednarek, 2008a), mOrange, or enhanced cyan fluorescent protein (ECFP) were used. All three CLC fusion proteins exhibited similar localizations (Figures 6A to 6F): to intracellular organelles that colocalized with the TGN marker, N-sialyl transferase-YFP (Figures 6D to 6F; Batoko et al., 2000), within the cytoplasm, and at the plasma membrane of root epidermal cells (Figures 6A to 6C) and root hairs (Figure 6G). Plasma membrane-associated CLC-GFP exhibited a similar distribution as DRP1C-GFP in growing root hairs. In time-lapse images (see Supplemental Video 5 online), CLC-GFP was localized to the tip flanks in growing root hairs and redistributed to the tip-most region of the plasma membrane as growth ceased.

CLC-GFP localization at the plasma membrane was examined using VAEM. Similar to DRP1C-GFP, plasma membrane-associated CLC-FFPs were organized into dynamic, discreet foci (Figures 6H to 6J; see Supplemental Video 6 online). CLC-GFP foci had a similar lifetime distribution (Figure 6J) as DRP1C-GFP in expanding root epidermal cells, with the average lifetime of 19.7 ± 6.8 s. The density of CLC-GFP foci was also similar in expanding root epidermal cells (3.48 ± 0.55 foci/μm²) to that of DRP1C-GFP (3.54 ± 0.62 foci/μm²).

DRP1C and CLC-FFP Localization in Expanding Root Epidermal Cells Were Perturbed upon Cytoskeleton and Sterol Disruption

Actin dynamics and membrane sterols are required for efficient CME in mammals and yeast (Lamaze et al., 1997; Rodal et al., 1999; Engvist-Goldstein and Drubin, 2003; Toshima et al., 2006). To determine cellular components that are required for DRP1C-GFP and CLC-FFP dynamics, seedlings were treated with molecular inhibitors of cytoskeletal dynamics, sterol synthesis, and membrane and protein trafficking and then imaged with VAEM. Specifically, 1 and 50 μM latrunculin B (latB; F-actin inhibitor), 10 μM oryzalin (microtubule inhibitor; Kandasamy and Meagher, 1999), 50 mM 2,3-butanedione monoxime (BDM;
myosin inhibitor; Paves and Truve, 2007), 10 μg/mL fenpropimorph (sterol synthesis inhibitor; He et al., 2003), 50 μM wortmannin (Jaillais et al., 2006), and 50 μM brefeldin A (Jaillais et al., 2006) were assessed and their effects were compared with mock treatment with 0.1% DMSO (except for fenpropimorph, which was compared with half-strength MS). Inhibitors were used at concentrations previously shown to effectively inhibit their corresponding cellular processes, and where possible, inhibition was visually confirmed as described below. All inhibitors, except wortmannin and brefeldin A, had significant effects on foci dynamics (Figures 7A to 7C).

Seedling roots were treated with low (1 μM) and high (50 μM) concentrations of latB for 20 min to inhibit cytoplasmic streaming independent and dependent processes, respectively. F-actin depolymerization was verified using the actin probe GFP-ABD2 (Sheahan et al., 2004). DRP1C-GFP foci became immobile. Foci that were present at the beginning of imaging rarely (<1%) disappeared from the cell cortex. DRP1C-GFP foci became immobile. Foci that were present at the beginning of imaging rarely (<1%) disappeared from the cell cortex. DRP1C-GFP foci increased in size and fluorescence intensity, similar to those observed after tyrA23 treatment (Figure 7C).

Microtubules are the major cytoskeletal component of the cortex of plant cells (Ehrhardt and Shaw, 2006). Thus, microtubule organization may be important for regulating processes at
the plasma membrane. To examine the role of the microtubule cytoskeleton on cortical-associated DRP1C and CLC dynamics, expanding epidermal root cells expressing DRP1C-GFP or CLC-GFP were imaged after 20 min incubation with 10 μM oryzalin, which caused complete depolymerization of microtubules, as assessed by a microtubule binding domain–GFP reporter (Granger and Cyr, 2001). Both DRP1C-GFP and CLC-GFP foci had a wider lifetime distribution than with 0.1% DMSO treatment (Figures 7A and 7B, white bars), and the average focus lifetime of DRP1C-GFP and CLC-GFP with oryzalin treatment was 2.6 and 1.6 times, respectively, that of mock-treated roots. In addition, DRP1C-GFP and CLC-GFP had a greater mobility within the plane of the cell cortex after treatment with oryzalin. Twice as many cortical-associated DRP1C-GFP foci were observed to split and/or merge, and more than twice as many CLC-GFP foci moved laterally in the cell cortex compared with mock-treated roots. Interestingly, foci dynamics were greatly inhibited in the absence of both the actin and microtubule cytoskeletons (Figures 7A and 7B, gray bars). In seedlings incubated with 10 μM oryzalin and 1 μM latB for 20 min, DRP1C and CLC foci displayed an average focus lifetime of 135.2 ± 71.3 s and 46.2 ± 35.5 s (n = 106), respectively (as opposed to 17.7 ± 8.8 s and 19.7 ± 6.8 s in mock-treated cells, respectively). In addition, the mobile DRP1C-GFP and CLC-GFP foci that were present in mock-treated seedlings or with either drug alone were not apparent in seedlings treated with both oryzalin and latB, with <2% of DRP1C and CLC foci being mobile within the cell cortex.

Plant cell expansion and division are greatly affected by sterols present in the plasma membrane (He et al., 2003; Schrick et al., 2004). CME in mammalian cells also requires the presence of sterols in the plasma membrane (Rodal et al., 1999). Fenpropimorph has been used as a sterol synthesis inhibitor to probe the

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**Figure 7.** DRP1C-GFP and CLC-GFP Foci Dynamics Are Inhibited by Cytoskeleton Disruption.

Roots expressing CLC-GFP (A) or DRP1C-GFP (B and C) were incubated with inhibitors for 20 min (A and B) or time indicated (C, white numbers), transferred to a slide, and imaged in the presence of the inhibitor with VAEM. Roots were incubated with 0.1% DMSO (A and B, black), 10 μM oryzalin (A and B, white), 1 μM latB (A, striped), 10 μM oryzalin + 1 μM latB (A and B, gray), or 50 μM BDM (C). The foci dynamics were analyzed for 20 to 30 foci in each of 6 to 10 cells from four different roots and the lifetime distribution was plotted (A and B). Seedlings expressing CLC-GFP (D) or DRP1C-GFP (E) were germinated and grown on either half-strength MS (black bars) or half-strength MS + 10 μg/mL fenpropimorph (white bars), then transferred to a slide and imaged by VAEM in half-strength MS media. The foci dynamics were analyzed for 10 to 20 foci in each of 7 to 10 cells from two to seven different roots, and the lifetime distribution was plotted (D and E). Bars = 1 μm.
effects of altered membrane sterol profiles on plant morphology (He et al., 2003; Schrick et al., 2004). Seedlings germinated and grown on half-strength MS + 10 μg/mL fenpropimorph had altered DRP1C-GFP and CLC-GFP foci dynamics (Figures 7D and 7E). The average DRP1C-GFP foci lifetime from seedlings grown on fenpropimorph was 39.3 ± 20.9 s (n = 200), which was 1.9 times that of seedlings grown on 0.5× MS media (Figure 7D). Likewise, the average lifetime of CLC-GFP foci when seedlings were grown on fenpropimorph was 34.0 ± 23.2 s (n = 90), which was 1.7 times that of seedlings grown on half-strength MS (Figure 7E). Together, these inhibitor studies indicated that the cortical environment was critical for efficient DRP1C-GFP and CLC-GFP foci dynamics.

**DRP1C-GFP and CLC-GFP Foci Colocalize at the Plasma Membrane**

As cortical-associated DRP1C-GFP and CLC-GFP foci displayed very similar dynamics, we decided to test whether these foci colocalize in the cell cortex. Plants expressing both DRP1C-GFP and CLC-mOrange were imaged using dual fluorescence color VAEM imaging (Figure 8A; see Supplemental Video 7 online). As demonstrated by single fluorophore imaging, both DRP1C and CLC fusion proteins formed discrete foci in the cell cortex (Figure 8A, yellow arrowheads). CLC-mOrange was also found in larger structures that represent TGNs (Figure 8A, blue arrowhead, and Figures 6D to 6F). We found that 48.8% (n = 3 cells) of pixels that had green fluorescence above an intensity threshold that included all fluorescence within the cell boundaries also contained mOrange fluorescence above the intensity threshold. Conversely, only 15.3% (n = 3 cells) of pixels that contained orange fluorescence above threshold intensity also contained green fluorescence above the threshold intensity. This was most likely due to the TGN-associated CLC-mOrange fluorescence signal. On the other hand, when only foci were analyzed for the presence of green or orange fluorescence, 95.1% of all CLC-mOrange foci analyzed (n = 1044) overlapped with DRP1C-GFP foci, while 94.6% of all CLC-GFP foci analyzed (n = 1049) overlapped with CLC-mOrange foci. The total percentage of overlapping foci was 90.4% (n = 1100). To eliminate the possibility that the high percentage of colocalization was due to random overlap of the highly dense foci in the cell cortex of elongating epidermal cells, the red channel image from different cells was rotated 180° with respect to the green channel, an analysis technique that has been used previously to show nonrandom colocalization (Delcroix et al., 2003; Dedek et al., 2006). The average peak distance for the original image (3.29 pixels; n = 256) was significantly different from that of the rotated images (6.43 pixels; n = 253; P < 0.000001), indicating that the colocalization was statistically significant.

The dynamics of DRP1C-GFP and CLC-mOrange were analyzed for 88 foci from expanding epidermal root cells in 10 individual roots that displayed overlapping DRP1C-GFP and CLC-mOrange localization. In 85% of these foci, the DRP1C-GFP and CLC-mOrange fluorescence decreased within 1 s of each other (Figures 8B and 8C), suggesting that DRP1C-GFP and CLC-mOrange resided on the same structure in the cell cortex. Of those that displayed simultaneous disappearance, 65% of foci (n = 68) had simultaneous rise in fluorescence (Figure 8B), indicating that the fluorophores were recruited concurrently, while 28% of foci were characterized by a peak in CLC-mOrange fluorescence before DRP1C-GFP (Figure 8C), suggesting a stepwise recruitment. Furthermore, 9% of all foci had concurrent recruitment of the DRP1C-GFP and CLC-mOrange but did not have simultaneous disappearance (Figure 8D). The time difference between the disappearances of the two foci ranged from 2.5 to 7 s. Finally, 6% of foci did not display coordinated dynamics of DRP1C-GFP and CLC-mOrange (Figure 8E).

**DISCUSSION**

Members of the DRP1 family are required for cell plate and plasma membrane dynamics during cytokinesis and cell expansion, respectively (Otegui et al., 2001; Kang et al., 2003a, 2003b). However, the specific processes in which DRP1 isoforms function and their interaction with the molecular machinery required for membrane trafficking are largely uncharacterized (Backues et al., 2007). In this study, we used live-cell imaging of a functional DRP1C-GFP to examine its localization and dynamics at the plasma membrane of various plant cell types. DRP1C-GFP was found to be associated with dynamic foci containing CLC. The coordinated dynamics of DRP1C and CLC in the cell cortex suggest that they may function together in CME in plant cells. Similarly, recent studies have suggested a role for DRP1A in CME in Arabidopsis (Collings et al., 2008; Konopka and Bednarek, 2008b). Interestingly, DRP1A, CLC, and DRP2B, a DRP that contains PH and PR domains, were recently shown to form cortical foci when heterologously expressed in tobacco Bright Yellow 2 cells (Fujimoto et al., 2007). However, only short-term (<6 s), single color imaging was performed, so it is not clear whether the dynamics of DRP1A and DRP2B are similar.

**DRP1C and CLC Colocalize into Foci at Regions of Active Membrane Trafficking**

Recent studies have begun to elucidate the endocytic molecular machinery in plants. The auxin efflux carrier, PIN1, was shown to be constitutively endocytosed in a clathrin-dependent manner (Dhonukshe et al., 2007), while the endocytic pathway for the hormone receptor BRI1 (Russinova et al., 2004) and plant defense receptor FLS2 (Robatzek et al., 2006) has not been identified. The central importance of clathrin-dependent trafficking in plant cells was demonstrated by the expression of a dominant-negative CHC, which disrupted internalization of several cargos (Dhonukshe et al., 2007). Here, we demonstrated that CLC-GFP was associated with the distal plasma membrane in expanding root hairs (Figure 6G), and at the cell plate in dividing root cells (Figures 6A to 6C), in agreement with previous morphological studies (Emons and Traas, 1986; Fowke et al., 1999; Otegui et al., 2001; Segui-Simarro et al., 2004; Segui-Simarro and Staehelin, 2006).

Components of the endocytic machinery in yeast and mammals have been shown to colocalize as punctate structures at the plasma membrane (Merrick et al., 2002, 2005; Kaksonen et al., 2003, 2005; Elde et al., 2005; Le Clainche et al., 2007) by total internal reflection fluorescence microscopy. Using VAEM, which
Figure 8. DRP1C-GFP and CLC-mOrange Foci Colocalize at the Plasma Membrane.

(A) Expanding root epidermal cells expressing CLC-mOrange and DRP1C-GFP imaged with VAEM. Three foci that had both mOrange and GFP fluorescence are indicated (yellow arrowheads). A larger internal structure that only had mOrange fluorescence is indicated (blue arrowhead). Bars = 1 μm.

(B) to (E) Intensity profiles of GFP (green) and mOrange (red) fluorescence in overlapping foci. Corresponding mOrange (top), GFP (middle), and merged (bottom) images for which the intensities were measured are below each time point indicated in the graph. White circles in the first frames indicate measured regions for fluorescence intensity.
gives a comparable signal to noise ratio in plant cells as total internal reflection fluorescence microscopy (Konopka and Bednarek, 2008a), we similarly observed that DRP1C-GFP and CLC-FFPs organized into discreet dynamic foci at the cell cortex. Both DRP1C-GFP and CLC-FFP dynamics in these foci were altered by compounds that affect cytoskeletal dynamics (Figures 7A and 7B), membrane sterol content (Figures 7C and 7D), and tyrA23, further supporting the model that DRP1C and CLC are part of the same machinery. DRP1C-GFP also formed foci at the root hair tip flanks, where clathrin-coated structures are abundant (Emons and Traas, 1986). Although the density of DRP1C-GFP foci at the root hair tip prevented kinetic imaging of individual foci, FRAP studies suggested that DRP1C-GFP was recruited to the flanks of growing root hairs in the same manner as in expanding root epidermal cells.

**Arabidopsis DRP1C and CLC Display Distinct Dynamics from Dynamin 1 and Clathrin in Mammals**

DRP1C-GFP and CLC-GFP dynamics were suggestive of gradual accumulation of DRP1C and clathrin network formation at plasma membrane sites (Figures 5B to 5D, 6I, and 6J). Unlike the recruitment of dynamin 1, which is thought to require an established clathrin network (Merrifield et al., 2002; 2005; Conner and Schmid, 2003), DRP1C-GFP and CLC-mOrange were recruited concurrently in more than half of the foci where the FFPs colocalize (Figure 8B). Only 30% of foci exhibited the stepwise recruitment (Figure 8C) previously described for dynamin 1 (Merrifield et al., 2002).

The rapid disappearance of CLC-GFP foci most likely corresponds to vesicle release from the plasma membrane (Merrifield et al., 2002, 2005; Kaksonen et al., 2005). In mammalian cells, dynamin 1 foci disappear concomitantly with vesicle fission (Merrifield et al., 2002) as a result of dynamin 1 release from the vesicle membrane. DRP1C-GFP disappearance could likewise be due to its release from the vesicle. Alternatively, DRP1C-GFP disappearance may represent movement of the oligomer away from the plasma membrane, possibly on the clathrin-coated structure. In support of the latter model, both CLC-GFP and DRP1C-GFP foci moved laterally out of the plane of focus upon disappearance from the cell cortex. Cortalysm streaming did not permit long-term imaging of the internalized structures and so their subsequent fate is unknown. DRP1C-GFP also displayed additional behaviors, including foci splitting and fusion, not reported for dynamin 1 (Merrifield et al., 2002), but which have been reported for CLC foci in mammalian cultured cells (Yarar et al., 2005).

Besides differences in the dynamics of CLC in plants and animals, the regulation of CME in plants also differs, especially regarding the role of the cytoskeleton. ARP2/3-dependent actin polymerization is thought to provide the force needed to drive the clathrin-coated vesicle away from the plasma membrane in both yeast (Kaksonen et al., 2005; Sun et al., 2006) and mammalian cells (Merrifield et al., 2005; Yarar et al., 2005). However, directly coupled actin polymerization, if required, is most likely not ARP2/3 dependent in plants. Arabidopsis mutants in the ARP2/3 complex (crl, dis1 arp3, wrm arp2, and dis2) or its nucleators (dis3 scar2, grl, pir, sra1, itb1, and brk1) do not display dramatic morphological defects (Deeks and Hussey, 2003), which would be expected if ARP2/3-dependent actin polymerization were required for endocytosis. Interestingly, high concentrations of latB (50 μM) were needed to completely disrupt DRP1C-GFP dynamics in epidermal cells. It is possible that vesicle fission and movement away from the plasma membrane does not need to be directly coupled to actin polymerization via an activator of polymerization like cortactin but instead uses the force generated by cytoplasmic streaming.

Unlike in mammals and yeast cells, in which there is little evidence of microtubule involvement in CME, microtubules were required for efficient DRP1C and CLC dynamics. This was surprising because neither DRP1C-GFP nor CLC-GFP foci organized in filamentous-like arrays or moved in a linear fashion, similar to other microtubule-associated proteins (Paredes et al., 2006; Konopka and Bednarek, 2008a). However, clathrin-coated structures clustered around cortical microtubules have been observed in micrographs (Fowke et al., 1999). It is plausible that cortical microtubules act as a trellis for stabilization of the endocytic protein network or as a diffusion barrier similar to the cortical actin network in mammalian cells (Giner et al., 2007).

The local environment, including protein, membranes, and physical forces is likely very different in an epidermal root cell than in mammalian cultured cells; most striking is the presence of turgor pressure and the force of cytoplasmic streaming. Therefore, it should not be surprising that the proteins facilitating or regulating CME in plants are also different. In fact, two important interactors of mammalian dynamin, amphiphysin, which can remodel membranes, and cortactin, an activator of actin nucleation, are not evident in plants. This is consistent with our finding that CME in plants involves members of the DRP1 subfamily, which do not contain the same identifiable lipid or protein binding domains as mammalian dynamin. Together, these observations point to significant differences in CME between plants and animals. DRP1A also forms foci that colocalize with CLC and DRP1C, albeit with lower frequency and different dynamics (Konopka and Bednarek, 2008b). Other recent studies have shown that DRP2B also forms foci at the plasma membrane, although its dynamics have not been studied (Fujimoto et al., 2007). This suggests the possibility that multiple CME pathways may exist in plants that use different dynamin isoforms, although further research is needed in this area. It may be that the need for multiple dynamin isoforms in plant CME is due to different cortical environments that a cell encounters as it differentiates or the wide variety of intracellular and extracellular stimuli involved in plant signaling.

The morphological characterization of clathrin-coated structures suggests a structural role for clathrin in plant endocytosis (van der Valk and Fowke, 1981; Emons and Traas, 1986; Derksen et al., 1995; Fowke et al., 1999; Dhouksh et al., 2007). If DRP1C functions similarly to dynamin 1 in CME in mammals, questions about the nature of the regulatory network arise. DPR1C lacks the PH and PR domains required for the recruitment and function of dynamin 1 during endocytosis. Also, direct homologs of many of the dynamin 1–associated proteins (cortactin and amphiphsin) have not been identified in the Arabidopsis genome, although proteins with similar domains, such as ANTH, BAR, and SH3, have been identified (Holstein and Oliviusson, 2005; Koizumi
et al., 2005; Jaillais et al., 2006). If and how these proteins interact with DRP1C or clathrin is unknown. Identification and characterization of the putative CME core machinery and endocytic membrane cargo, together with CLC and DRP1C dynamics, will help further advance the understanding of CME and its regulation in plants.

**METHODS**

**Construction and Transformation of Fluorescent-Tagged DRP1C and CLC**

The DRP1C-GFP plant expression vector was constructed as follows: A SalI/HpaI DRP1C genomic construct from pBK03K (Kang et al., 2003a), which included 1.8 kb of 5′ untranslated (UTR) and promoter elements, and 3′ UTR of DRP1C were subcloned into pZP211 (Hajdukiewicz et al., 1994) containing mGFP5 and the nopaline synthase (NOS) terminator (Kang et al., 2003b) using Smal and SalI sites (pSB29). The native 3′ UTR was removed from pSB29 by restriction digestion using KpnI and SacI sites and a DRP1C coding DNA fragment amplified with primers 5′-AGAGATGCTATGATGTTCTGCAATGGAGAATACATGC-3′ and 5′-CTAGAGCTCCTTCAACGCGACTGATGATGC-3′ was inserted after digestion with KpnI and SacI (pSB31).

The CLC-mOrange plant expression vector was constructed as follows: The coding sequence for mOrange (Shaner et al., 2004) was PCR amplified from pRSET-B mOrange (a gift from R. Tsien) using primers 5′-AGGCTCTTACCTATGATGTTCTGCAATGGAGAATACATGC-3′ and 5′-AGGCTCTTACCTATGATGTTCTGCAATGGAGAATACATGC-3′, subcloned as a SalI fragment into a pZP221-B vector (Kang et al., 2001), resulting in pZP221B-mo-NOS. A genomic fragment of Atg240600 (CLC) was PCR amplified from BAC T28M21 (ABRC) with primers 5′-AGGCTCGAGTGCGAGATGATTAGATGATGC-3′ (CLC for) and 5′-AGGCTCGAGTCGTAACCTGCGACTGATGATGC-3′ (CLC rev) and subcloned with PstI and SacI into pZP221B-mo-NOS.

CLC-ECFP was constructed as follows: The coding sequence for ECFP was PCR amplified from pECFP-C1 (Clontech) using primers 5′-AGGCTCGAGTGCGAGATGATTAGATGATGC-3′ and 5′-AGGCTCTTACCTATGATGTTCTGCAATGGAGAATACATGC-3′, subcloned as a SacI fragment into a modified pZP221-B vector (Kang et al., 2001) resulting in pZP221B-ECFP-NOS. A genomic fragment of Atg240600 (CLC) was PCR amplified from BAC T28M12 (ABRC) with primers CLC for and CLC rev and subcloned as a PstI-SacI fragment into pZP221B-ECFP-NOS, resulting in pZP221B-CLC-ECFP. The DNA sequence of all constructs was verified.

*Arabidopsis thaliana* ecotype Wassilewskija wild-type or DRP1C/DRP1C-1 (Kang et al., 2003a) plants were transformed with the constructs for CLC-FPP or DRP1C-GFP, respectively, using the *Agrobacterium tumefaciens*-mediated floral dip method (Clough and Bent, 1998). Transgenic plants were selected either on solid medium (0.6% phytagar), half-strength MS medium (Caisson Labs) containing 100 μg/mL kanamycin (DRP1C-GFP) or on soil, sprayed once with 20 μg/mL ammonium glusofinata (CLC-ECFP, Liberty).

**Immunoblot Analysis**

To determine expression level of the transgenic DRP1C-GFP construct, total protein extracts were prepared from wild-type, wild-type transformed with DRP1C-GFP, DRP1C/drp1C-1, and drp1C-1/drp1C-1 transformed with DRP1C-GFP seedlings grown vertically on half-strength MS + 1% phytagar. Seven-day-old seedlings were homogenized in SDS-PAGE sample buffer (Laemmli, 1970), incubated at 65°C for 15 min, and insoluble debris was cleared by centrifugation at 16,000 g for 10 min at room temperature. The supernatant was separated on a 12.5% (w/v) SDS-polyacrylamide gel and analyzed by immunoblotting as described (Kang et al., 2001) using anti-DRP1C (Kang et al., 2003a) and biotin-conjugated anti-GFP antibodies (Rockland Immunochemicals). HRP-conjugated anti-rabbit secondary antibodies (GE Healthcare) and HRP-conjugated streptavidin (Rockland Immunochemicals) were used to detect the primary antibodies, and anti-DRP1C and anti-GFP, respectively.

**Immunofluorescence and Live-Cell Confocal Microscopy**

For indirect immunolocalization of DRP1C in plants expressing ss-β-ATPase-GFP (Logan and Leaver, 2000), 5-d-old seedlings that were grown vertically on half-strength MS + 0.6% phytagar were fixed in 4% (w/v) formaldehyde (Ted Pella) in PME (55 mM PIPES-KOH, 2.5 mM MgSO4, and 1 mM EGTA, pH 6.9) for 1 h under vacuum. All subsequent incubations were conducted at room temperature in a humid chamber unless otherwise noted. Fixed seedlings were washed in PME, placed on Probe-On-Plus slides ( Fisher Scientific), dried on a slide warmer at 55°C, incubated in permeabilization buffer (0.1% Nonidet P-40 in PME) for 10 min, and treated with 0.1% (w/v) pectolyase and 0.5% (w/v) maceroenzyme in PME for 20 min. The roots were then washed in 0.1% Triton X-100 in PME and then in PME. For immunolabeling, the roots were blocked with 3% BSA (w/v) in PME for 1 h and probed with anti-DRP1C (Kang et al., 2003a) in PME + 3% BSA overnight at 4°C. Roots were then washed in PME + 3% BSA and incubated with Cy3-conjugated anti-rabbit antibodies (Jackson Immunoresearch) in PME + 3% BSA for 1 h. Afterward, roots were washed with PME + 3% BSA, covered with Vectashield (Vector Laboratories), topped with a cover slip, and sealed with fingernail polish.

To examine the association of DRP1C with mitochondria, 5-d-old drp1C-1/drp1C-1 seedlings transformed with DRP1C-GFP were incubated with 5 μM MitoTracker Orange CM-H2TMRos (Molecular Probes) in half-strength MS for 30 min at room temperature and rinsed for 2 min in half-strength MS before imaging.

All CLSM images, except for those of the FRAP experiments, were captured using a Nikon TE2000-U inverted confocal laser scanning microscope fitted with a ×60/1.4 numerical aperture (NA) PlanApo VC objective lens and EZ-C1 acquisition software (Nikon). For colocalization studies in epidermal and cortical cells, DRP1C-GFP and β-ATPase-ss-GFP were excited at 488 nm (Melles Griot) while MitoTracker Orange and Cy3 (anti-DRP1C) were excited at 543 nm (Melles Griot). CLC-FPs were detected using 488-nm light (GFP), 543-nm light (mOrange), or 480-nm light (ECFP, Melles Griot). All dual-color imaging was performed using sequential scans to prevent bleed through fluorescence.

For imaging of trichomes expressing DRP1C-GFP, seedlings with one to two pairs of true leaves were selected and the roots were removed. Seedlings were inverted onto a cover slip within water and covered by a glass slide.

Pollen was allowed to germinate in vitro as described previously (Kang et al., 2003a) on a glass slide for 0 to 12 h, covered with a cover slip, and imaged with CLSM as described above, except a ×100/1.4 NA PlanApo lens was used.

For time-lapse imaging of growing root hairs, seedlings were grown on half-strength MS + 0.5% phytagar covered cover slips as described (Konopka and Bednarek, 2008a) and imaged by CLSM with either the ×60 or ×100 objective lens. One hundred and fifty microfilaments of half-strength MS + 1% (w/v) sucrose was pipetted on top of the agar and covered with parafilm to prevent desiccation during imaging.

FRAP experiments were performed on a Zeiss 510 Meta confocal microscope (Carl Zeiss) equipped with a ×63/1.4 NA PlanApo Chromat objective. Circular or rectangular areas were photobleached using 30 iterations of the 488-nm line from a 200-mW argon laser operating at 100% laser power. Fluorescence recovery was monitored at 1-s intervals.
VAEM

Cortical-associated DRP1C-GFP and CLC-FFP dynamics were imaged using variable angle fluorescence microscopy as described (Konopka and Bednarek, 2008a). Briefly, seedlings were transferred from vertically growing plates to a glass slide with 150 μL of half-strength MS and covered with a cover slip. Plants were imaged with a Nikon Eclipse TE2000-U fitted with the Nikon T-FL-TIRF attachment and a Nikon ×100/NA 1.45 CFI Plan Apo TIRF objective. For double fluorescence imaging, GFP and mOrange were excited with a 488- and 543-nm laser, respectively, and the fluorescence emission spectra were separated with a 540LP dichroic mirror and filtered through either a 515/30 (GFP) or 585/65 (mOrange) filter in a Dual View filter system (Photometrics).

Image Analysis

For trichomes and root hairs, z-series were recombinated using the average projection command on Image J 1.36b (National Institutes of Health; http://rsb.info.nih.gov/ij/). Root hair length was calculated from XYZ coordinates of the tip and base of the root hair. For growing root hairs, time series images were captured every 8 to 10 s and compiled using Image J. Growth rate was determined using MetaMorph’s (Molecular Devices) track points application. An intensity threshold value was assigned to each root hair so that the maximum number of pixels in the plasma membrane was included, and the number of pixels in the cytoplasm was limited. Threshold area was calculated using the region measurements application in Metamorph (Molecular Devices). Analysis of FRAP, DRP1C-GFP, CLC-GFP, and CLC-mOrange foci dynamics was performed using Image J.

Fluorescence recovery in the FRAP experiments was corrected for photobleaching as follows. The percentage of inherent photobleaching (PIP) for each frame during recovery was determined by measuring the change in fluorescence intensity of a region that was not initially photobleached. This fluorescence intensity in the initial bleached regions was multiplied by (1 + PIP) for each frame.

A focus was identified by a local increase in intensity above a designated threshold assigned to each time-lapse image for >2 s. Foci dynamics were analyzed for plants that were grown on half-strength MS solid medium and imaged in half-strength MS liquid medium. Lifetime was calculated from the first frame the focus appeared to the frame that it disappeared from the imaging planes. The intensity profiles in Figures 5, 6, and 8 were generated using Image J’s Region of Interest (ROI) Multi Measure Plugin. Circular ROIs included all pixels of the focus, and a mean intensity for the ROI was recorded. To determine if colocalization was random, the green channel of dual-color images was rotated 180° relative to the red image using Adobe Photoshop CS2 (Adobe Systems). The distance from each focus in the green channel to the nearest focus in the red channel was measured using MetaMorph. All images for figures were processed in Adobe Photoshop CS2.

Inhibitor Studies

tyrA23, tyrphostin A51, cytochalasin D, wortmannin, brefeldin A, and latB were purchased from EMD Biosciences; oryzalin was purchased from Restek; lanthanum chloride and BDM were purchased from Sigma-Aldrich. Stock solutions of lanthanum chloride and BDM were prepared in deionized water. All other inhibitors were dissolved initially in 100% DMSO for a stock solution. Inhibitors were diluted in half-strength MS (for VAEM imaging of expanding root epidermal cells and confocal imaging of the root tip) or half-strength MS + 1% sucrose (for confocal imaging of root hairs). The final DMSO concentration was 0.1% (v/v) or less in all working solutions. For VAEM analysis, inhibitor treatment was for 20 min unless otherwise stated. Foci lifetime analysis for inhibitors was compared with a mock treatment in 0.1% DMSO, except for fenpropimorph.

For treatment of growing root hairs, inhibitors were pipetted on top of agar and allowed to diffuse into the agar to reach the root hairs. Time series imaging was initiated prior to the addition of the inhibitor to confirm the growth status of the root hair. For treatment of root tips with inhibitors, 5- to 7-d-old vertically grown seedlings were transferred from 1% agar plates to 3 mL of final working concentration in half-strength MS in a 12-well culture plate. After the indicated time, seedlings were transferred to a glass slide with 150 μL of inhibitor solution, covered with a glass cover slip, the excess liquid was wicked away, and they were imaged as described above. For tyrA23 washout experiments, seedlings were incubated with 50 μM tyrA23 in half-strength MS for 20 min, transferred to media without the drug for 5 min, and subsequently imaged in half-strength MS.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative database under accession numbers At2g40060 (CLC) and At1g14830 (DRP1C).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. DRP1C Localizes to the Plasma Membrane in Hypocotyls and Does Not Associate with Mitochondria. Supplemental Figure 2. DRP1C-GFP Retains Its Tip Localization in Several Root Hair Expansion Mutants. Supplemental Figure 3. DRP1C-GFP Forms Cortical Foci in Various Epidermal Cells. Supplemental Figure 4. tyrA23, but Not tyrA51, Reversibly Inhibits DRP1C Dynamics in Vivo but Not DRP1 GTPase Activity in Vitro. Supplemental Video 1. Time-Lapse Images of a Growing Pollen Tube Expressing DRP1C-GFP. Supplemental Video 2. Time-Lapse Images of Growing Root Hair Expressing DRP1C-GFP. Supplemental Video 3. Time-Lapse Images of Expanding Root Epidermal Cell Expressing DRP1C-GFP Imaged with VAEM. Supplemental Video 4. Time-Lapse Images of Seedling Root Tip Expressing DRP1C-GFP Treated with 50 μM tyrA23. Supplemental Video 5. Growing Root Hair from Root Expressing CLC-GFP. Supplemental Video 6. Time-Lapse Images of Expanding Root Epidermal Cell Expressing CLC-GFP Imaged with VAEM. Supplemental Video 7. Time-lapse VAEM Images of Expanding Root Epidermal Cell Expressing DRP1C-GFP (Middle Panel) and CLC-mOrange (Left Panel), and Merged (Right Panel).

ACKNOWLEDGMENTS

We thank T. Martin and members of his lab for extensive use of their epifluorescence-TIRF microscope. We thank R. Tsien for his generous gift of the mOrange fluorescent protein construct. The University of Wisconsin-Madison Plant Imaging Facility was partially supported by funding from the National Science Foundation Grant DBI-0421266. We thank members of our lab, especially D. Rancour, S. Park, and C. McMichael, for critical reading of the manuscript and helpful discussions. This research was supported by funding to S.Y.B. from the USDA National Research Initiative Competitive Grants Program (Project...
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Dynamics of Arabidopsis Dynamin-Related Protein 1C and a Clathrin Light Chain at the Plasma Membrane
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PLANT CELL 2008;20;1363-1380; originally published online May 23, 2008;
DOI: 10.1105/tpc.108.059428

This information is current as of May 18, 2010

Supplemental Data http://www.plantcell.org/cgi/content/full/tpc.108.059428/DC1
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