

Imaging of plant dynamin-related proteins and clathrin around the plasma membrane by variable incidence angle fluorescence microscopy

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Abstract Endocytosis is an essential phenomenon in eukaryotic cells. In animal cells, dynamin and clathrin play central roles in vesicle formation in the process of endocytosis, but the roles of similar proteins in plants are less well understood. Here, we observed the localization pattern and behavior of GFP-labeled *Arabidopsis* dynamin-related proteins (DRP1A and DRP2B), and clathrin light chain (AtCLC) around the plasma membrane in tobacco suspension cells by using variable incidence angle fluorescence microscopy (VIAFM). GFP fusions of DRP1A, DRP2B and AtCLC were observed as dot-like puncta 200–500 nm in diameter. The puncta moved to and away from the cell surface or also assembled and disassembled. The localization pattern and behavior of the puncta were similar to those of animal dynamin and clathrin signals reported previously. These results raise the possibility that DRP1A, DRP2B and AtCLC are involved in membrane trafficking around the plasma membrane, including endocytosis.

Key words: Dynamin-related protein, clathrin light chain, variable incidence angle fluorescence microscopy.

Endocytosis is an essential phenomenon in eukaryotic cells for engulfing external materials, for intermediating cellular signals and for regulating the abundance and distribution of plasma membrane proteins (Mellman 1996). Compared with what is known about endocytosis-related molecules in animal cells, little is known about endocytosis-related molecules in higher plants (Murphy et al. 2005). However, the *Arabidopsis* genome has possible homologues of some of the major molecules in animal endocytosis, such as dynamin and clathrin (Holstein 2002).

Dynamin, which is a large GTPase protein required for endocytosis in animal cells, assembles into a multimeric spiral at the neck of clathrin-coated pits (Sever 2002). Numerous studies have revealed that dynamin and dynamin-related proteins (DRPs) are involved in not only endocytosis but also diverse cellular membrane-remodeling events including vesicular transport, divisions of organelles and cytokinesis (Praefcke and McMahon 2004).

The *Arabidopsis* genome has 16 DRPs grouped into 6 subfamilies (DRP1-6) (Hong et al. 2003a). Members of two of these subfamilies (DRP1 and DRP2) are

candidates for plant dynamin that are involved in membrane trafficking around the plasma membrane. In *Arabidopsis*, the DRP1 family has five members, DRP1A-E (Hong et al. 2003a), which are mainly localized in the cell plate (Kang et al. 2001; Hong et al. 2003b; Kang et al. 2003a; Kang et al. 2003b). In addition, DRP1A, DRP1C and DRP1E are localized close to the plasma membrane (Kang et al. 2003a; Kang et al. 2003b). The DRP2 family has two members, DRP2A and DRP2B, which are most similar to animal dynamin in domain structure (Hong et al. 2003a). DRP2A is involved in Golgi-trafficking (Jin et al. 2001; Lam et al. 2002) and is localized to the cell plate and the region near the plasma membrane (Hong et al. 2003b). However, the detailed localization pattern and behavior of these two kinds of DRPs around the plasma membrane have not been analyzed yet.

Clathrin coats are major components of vesicle trafficking in the plasma membrane, the trans-Golgi network and endosomes in most eukaryotic cells (Brodsky et al. 2001). They are assembled at the sites of vesicle formation on the donor membrane. Clathrin coats are made up of a number of clathrin triskelions that

Abbreviations: GFP, Green Fluorescent Protein; DRP, dynamin-related protein; AtCLC, *Arabidopsis* clathrin light chain; VIAFM, variable incidence angle fluorescence microscopy; TIRFM, total internal reflection fluorescence microscopy; SRIC, surface reflective interference contrast; EF, epi fluorescence; BF, bright field

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consist of three clathrin heavy chain polypeptides and three clathrin light chain (CLC) polypeptides (Robinson 1996). The *Arabidopsis* genome has three putative genes that encode CLCs (Holstein 2002). The polypeptide encoded by one of these genes (locus number At2g40060), which shows highest homology to the mammalian CLC sequence, was shown to bind to mammalian clathrin hubs (Scheele and Holstein 2002). However, little is known about the function of this *Arabidopsis* CLC (AtCLC) around the plasma membrane.

Variable incidence angle fluorescence microscopy (VIAFM) is a technique that uses oblique incident light to observe the fluorescence in the thin layer adjacent to the cover glass. In VIAFM, light called an "evanescent wave" is generated very near the cover glass surface when the angle of incident light is greater than the critical angle that total internal reflection occurs (Schneckenburger 2005). The microscopic technique that uses this evanescent wave for the fluorescence excitation is called total internal reflection fluorescence microscopy (TIRFM), which also allows fluorescence observations in the cellular surface layer very close to the cover glass (100–400 nm in depth, (Toomre and Manstein 2001)). In animal cells, TIRFM has been used to obtain high resolution images of the dynamics of the cytoskeleton (Krylyshkina et al. 2003) and endo/exocytosis-related molecules (Merrifield et al. 2002; Rappoport and Simon 2003; Allersma et al. 2004). To our knowledge, TIRFM has been used in only one *in vivo* plant study, in which it was used to visualize the dynamics of vesicles stained with FM4-64 (a styryl dye for endo-membranes) in pollen tubes (Wang et al. 2006).

Here, we observed the localization pattern and behavior of DRP1A, DRP2B and AtCLC just inside the surface of tobacco suspension cultured cells by using VIAFM. Our results provide further evidence that these proteins are involved in membrane trafficking around the plasma membrane, including endocytosis.

Materials and methods

Plant materials

Bright yellow-2 (BY-2) tobacco (*Nicotiana tabacum* L.) cell suspension cultures were grown in modified Murashige and Skoog medium enriched with 0.2 mg l⁻¹ 2,4-D and were maintained as described in previous study (Nagata et al. 1992). Five-day-old BY-2 cells were used in the microscopic observations.

Construction of plasmids

We obtained an EST clone of *DRP2B* (GenBank accession no. [AY528687](#)) from Kazusa DNA Research Institute (Chiba, Japan), a cDNA clone of *DRP1A* (GenBank accession no. [BT001063](#)) from the *Arabidopsis* Biological Resource Center

(Columbus, OH, USA) and a cDNA clone of *AtCLC* (GenBank accession no. [AY092995](#)) from the DNA Bank, RIKEN BioResource Center (Ibaraki, Japan) (Seki et al. 2002). Plasmids harboring *DRP2B*, *DRP1A* and *AtCLC* were constructed using Gateway cloning technology (Invitrogen). The open reading frames of *DRP2B*, *DRP1A* and *AtCLC* were amplified from the EST clone and full-length cDNA clone by PCR, using the following primer sets respectively (*DRP2B*: 5'-G G G G A C A A G T T T G T A C A A A A A A - GCAGGCTCGATGGAGGCGATCGATG-3' and 5'-G G G G A C C A C T T T G T A C A A G A A A - AGCTGGGTCTAATACCTGTAAGATGA-3', *DRP1A*: 5'-G G G G A C A A G T T T G T A C A A A A A A G C A G G - CTCGATGGAAAATCTGATCTCTC-3' and 5'-GGGG-ACCACCTTTGTACAAGAAAGCTGGGTTCACTTGGACCA AGCAACAG-3' and *AtCLC*: 5'-GGGGACAAGTTTGTAC-CAAAAAGCAGGCTCGATGTCTGCCTTTGAAGACGA-3' and 5'-GGGGACCCTTTGTACAAGAAAGCTGGGT-TTAAGCAGCAGTAACTGCC-3'). The underlined sequences are needed for subcloning the PCR product into the pDONR207 Gateway donor vector via BP recombinant reaction. The resulting entry vectors were used in LR reactions with the destination binary vector pK7WGF2 (Flanders Interuniversity Institute for Biotechnology, Belgium) to link GFP to the N-termini of DRP2B, DRP1A and AtCLC (Karimi et al. 2002).

Establishment of transgenic BY-2 cell lines expressing GFP fusions of DRP1A, DRP2B, AtCLC and tubulin.

Ti plasmids harboring *GFP-DRP2B*, *GFP-DRP1A* and *GFP-AtCLC* were individually transformed into *Agrobacterium tumefaciens* strain EHA105. A 5 ml aliquot of 3-day-old BY-2 cells was incubated with 100 µl of the overnight culture of the transformed *A. tumefaciens* as the method described previously (An 1985). After 48 h incubation at 27°C, cells were washed four times in 5 ml of modified MS medium, then plated onto solid modified MS medium containing 200 mg l⁻¹ claforan and 50 mg l⁻¹ kanamycin. After 3 weeks, selected calli were transferred onto new plates and cultured for 2 weeks additionally. As each callus reached a size of 1–2 cm in diameter, it was transferred to 95 ml of new modified MS medium with 200 mg l⁻¹ claforan and 50 mg l⁻¹ kanamycin and cultured in a rotary shaker at 130 rpm at 27°C in the dark. After 3 weeks, we selected suitable cell lines expressing GFP-fusions at the appropriate level for microscopic observation. BY-GT16 cells (BY-2 cells stably expressing GFP-tubulin fusion protein (Kumagai et al. 2001)) were kindly provided by Prof. S. Hasezawa, The University of Tokyo, Japan.

Microscopic observations

Transgenic BY-2 cells expressing GFP fusion proteins were subjected to vital imaging by using a fluorescence microscope (Nikon Eclipse TE2000-E and a CFI Apo TIRF 100×H/1.49 numerical aperture objective) with a Nikon TIRF2 system (Nikon, Tokyo, Japan). 40 µl of BY-2 cultured medium was mounted onto a slide glass (76×26 mm, Matsunami, Osaka,

Japan), and 0.12–0.17 mm thickness cover glass (24×60 mm, Matsunami, Osaka, Japan) was placed on slide glass. No centrifugal and decompression treatment to samples between slide and cover glass was not done before microscopic observations. To bring the BY-2 cells more close to the cover glass by gravity, samples were observed by inverted fluorescence microscope. GFP fusion proteins were excited with a 488 nm laser. All images were acquired with a Cool SNAP HQ2 CCD camera (Roper Scientific, Trenton, NJ, USA) controlled by Metamorph software (Universal Imaging, West Chester, PA, USA). In VIAFM and epifluorescence images, each frame was exposed for 100 milliseconds (with intervals of about 9.5 milliseconds between frames in movies). Before observation by VIAFM, we found the cell surface attached to the cover glass by using surface reflective interference contrast (SRIC) microscopy (Izzard and Lochner 1976), in which the area in the cell that adheres to the cover glass appears slightly darker than the surroundings. For SRIC, a filter cube containing excitation filter 510–560 nm, dichroic mirror 505 nm and emission filter 510–560 nm is used. Acquired images were prepared and analyzed with Photoshop 7.0 (Adobe Systems, Mountain View, CA, USA) and Image pro plus 4.0 (Media Cybernetics, Silver Springs, MD, USA).

Results

VIAFM can be used to visualize the fluorescence just inside the surface of a tobacco suspension cell.

To confirm that the fluorescent signals of molecules fused with GFP in tobacco suspension cultured cells (BY-2 cells) are visible by VIAFM, we observed the

localization pattern of cortical microtubules by using transgenic BY-2 cells expressing GFP-tagged tobacco α -tubulin (Kumagai *et al.* 2001). Plant microtubules are located very near the plasma membrane in interphase and are arranged transverse to the elongation axis of the cell (Hashimoto and Kato 2006). A VIAFM image (Figure 1A) and an epi fluorescence image (Figure 1B) show fibrous structures of cortical microtubules. The VIAFM image is clearer and sharper than the epi fluorescence image. These results indicate that VIAFM could be used to see the fine structure of the fluorescent of GFP fusion proteins around the plasma membrane of BY-2 cells.

Surface reflective interference contrast (SRIC) images are generated by the interference of light reflected at the cover glass-medium interface and the medium-cell surface interface (Izzard and Lochner 1976). Therefore, the cell surface that adheres to the cover glass appears darker than the surroundings in the SRIC image of a cell (Figure 1C). The same area is shown in the bright field image (Figure 1D) in which the focus was changed to show the outline of the cell. A comparison of the two images reveals that the attached area is very limited. Moreover, the dark stripes in the SRIC image (Figure 1C) were displayed at vertical intervals of one-half wavelength of illumination light (about 255–280 nm in our SRIC study) on the cell surface. The region shown in red in the SRIC image (Figure 1C) is the area where GFP fluorescence was observed in the VIAFM image (Figure 1A). A comparison of the region shown in red and the region surrounded with the innermost stripe in the SRIC

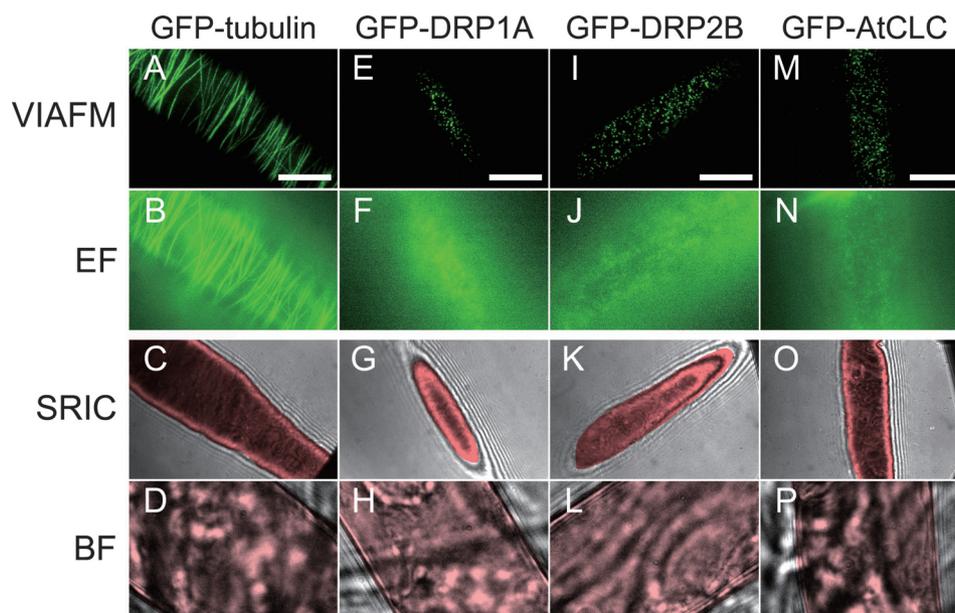


Figure 1. Localization patterns of GFP fusions of tubulin, DRP1A, DRP2B and AtCLC in the surface area of transgenic tobacco BY-2 cells. Each column of panels shows a portion of a single cell expressing GFP fusions of tubulin, DRP1A, DRP2B and AtCLC. Each row shows a different imaging method. VIAFM, variable incidence angle fluorescence microscopy; EF, epi fluorescence; SRIC, surface reflective interference contrast, BF, bright field. Bar, 10 μ m. Red region in the SRIC images indicates the area illuminated by the 488 nm excitation laser light in the VIAFM images. Red region in the BF images indicates the portion of the cell that is being observed.

image (Figure 1C) shows that these two regions highly overlap. These results suggest that the intracellular GFP molecules within the thin layer near the cover glass were excited in BY-2 cells.

The practical depth of illumination by VIAFM in our study is estimated at about 450–500 nm from the cell surface attached to the cover glass. This value was calculated from the distance between the cover glass and the cell surface just beneath the edge of the region where GFP fluorescence was observed (shown in red in Figure 1C), and the thickness of the BY-2 cell wall which is about 200 nm (Follet-Gueye et al. 2003). The distances between the cell surface and the cover glass are calculated from the intervals of dark stripes in the SIRC image (Figure 1C). According to the data of the limit depth of illumination and the thickness of cell wall, the illumination depth from the plasma membrane nearest the cover glass in our VIAFM study would be about 250–300 nm.

Localization patterns of DRP1A, DRP2B and AtCLC around the plasma membrane.

DRP1A, DRP2B and AtCLC fused with GFP were observed by VIAFM. In animal cells, it is confirmed that dynamin and clathrin fused with fluorescent tags retain their intracellular functionalities (Cao et al. 1998; Gaidarov et al. 1999). However, it is unknown whether fluorescent fusions of *Arabidopsis* DRP1A, DRP2B and CLC are properly functional. In the VIAFM images, the fluorescence of GFP-DRP1A (Figure 1E), GFP-DRP2B (Figure 1I) and GFP-AtCLC (Figure 1M) appears as small dot-like puncta, which are much clearer than the signals in the epi fluorescence images (Figure 1F, J, N). The diameters of most these puncta are about 200–500 nm. The size of the AtCLC puncta in the VIAFM images was larger than the size of clathrin coated vesicles (70–90 nm in diameter) in electron microscopic images (Barth and Holstein 2004), probably because the fluorescence signals emitted from the puncta make them look larger. The size of animal clathrin light chain puncta in TIRFM images (200–500 nm, (Merrifield et al. 2002; Rappoport and Simon 2003; Bellve et al. 2006)) was also larger than the actual size (120 nm in average (Conne and Schmid 2003)).

Behaviors of GFP-DRP2B, GFP-DRP1A and GFP-AtCLC puncta around the plasma membrane

The time-course behavior of GFP-DRP2B puncta around the plasma membrane was examined by VIAFM in two regions shown in Figure 2A. Some puncta remained in a static state (Figure 2B, spot 1), some disappeared (Figure 2B, spot 2), some appeared (Figure 2C, spot 1) and some were short-lived (rapid appearance and disappearance) (Figure 2C, spot 2). An additional movie is shown in supplemental movie A, which consists of 50 frames

covering a period of 5.475 s. These patterns are shown graphically in Figure 2D. Similar patterns were observed in cells expressing, GFP-DRP1A and GFP-AtCLC (see supplemental movies B and C, respectively). Analysis of the three movies indicated that for each construct, most of the puncta were static and about a quarter of them were dynamic during 5.475 s in our observation (Figures 2E, F, G).

Discussion

Our VIAFM images clearly show that fluorescent fusions of DRP1A, DRP2B and AtCLC near the plasma membrane are localized in dot-like puncta and that the behaviors of their puncta showed several patterns: static, disappearing, appearing and short-lived. These fluorescent transition patterns of puncta are thought to indicate the vertical movement of fluorescent fusion proteins into/out of the area illuminated by the excitation light generated near the cover glass (Steyer and Almers 1999; Merrifield et al. 2002) or the assembly/disassembly of fluorescent fusion molecules (Merrifield et al. 2002; Rappoport and Simon 2003). Figure 3 shows a schematic model of the movement patterns. In TIRFM images of animal cells, fluorescent fusion proteins of dynamin and clathrin appeared as puncta with sizes (about 200–500 nm in diameter) and fluorescent transition patterns similar to those observed in our plant cells (Merrifield et al. 2002; Rappoport and Simon 2003; Bellve et al. 2006). These results support the idea that DRP1A, DRP2B and AtCLC that are localized around the plasma membrane are involved in plant endocytosis, as has been shown to be the case for dynamin and clathrin in animals.

Unlike animal cytokinesis, plant cytokinesis involves the transport of massive amounts of membrane into the division plane to construct a large membranous organelle called the cell plate, during which exo/endocytosis and membrane remodeling are highly active (Jürgens 2005). DRP1, DRP2 and clathrin have been localized to the cell plate (Otegui et al. 2001; Hong et al. 2003b; Kang et al. 2003a; Kang et al. 2003b) where they are thought to be involved in membrane trafficking (Jürgens 2005). We propose that DRP1, DRP2 and AtCLC are also involved in membrane trafficking around the plasma membrane as well as in construction of the cell plate.

In this study, we investigated the localization pattern and behaviors of DRP2B around the plasma membrane in tobacco BY-2 suspension cultured cells. Another *Arabidopsis* DRP2, DRP2A, which is 93% identical and 96% similar at the amino acid level to DRP2B, associates with the Golgi apparatus in *Arabidopsis* protoplasts (Jin et al. 2001). Previous confocal microscopic observations revealed that GFP-tagged DRP2A (GFP-DRP2A) also localized around the plasma

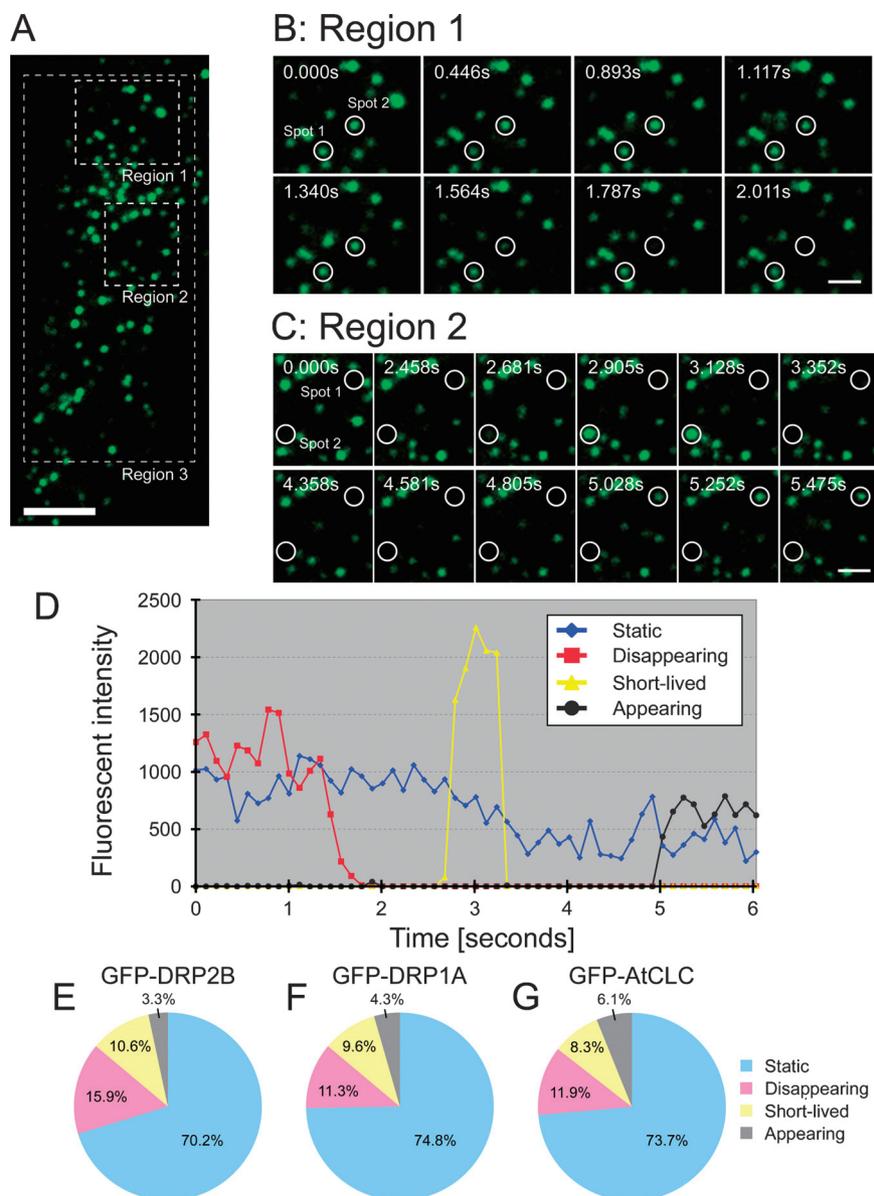


Figure 2. Analysis of GFP-DRP2B puncta behaviors around the plasma membrane. (A) VIAFM images of GFP-DRP2B fluorescence. Bar, $3\ \mu\text{m}$. (B) Eight frames from a movie of region 1 showing a static punctum (spot 1) and a disappearing punctum (spot 2). Bar, $1\ \mu\text{m}$. Exposure time per frame is 100 ms. (C) Twelve frames of a movie of region 2 showing an appearing punctum (spot 1) and a short-lived punctum (spot 2). Bar, $1\ \mu\text{m}$. (D) Line chart representing the time-course transition of fluorescent intensity in each pattern of GFP-DRP2B puncta. (E–G) Pie chart showing the percentage of the each pattern of GFP-DRP2B (E), GFP-DRP1A (F) and GFP-AtCLC (G) dynamics during 5.475 s.

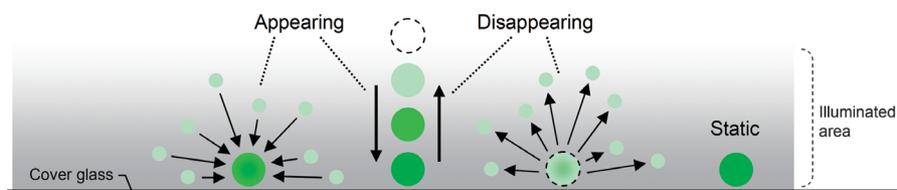


Figure 3. Schematic model of putative movement patterns of GFP fluorescent puncta in VIAFM. In the VIAFM images, appearing puncta are GFP fusion molecules moving into the *illuminated area* and/or assembling, and disappearing puncta are GFP fusion molecules moving out of the *illuminated area* and/or disassembling. Static state puncta do not move or disassemble.

membrane, and in the cytoplasm, possibly in the Golgi network of BY-2 cells (Hong et al. 2003b). In our confocal laser microscopic observations, the intracellular localization pattern of GFP-DRP2B (data not shown) showed strong similarity to the localization pattern of GFP-DRP2A described in these previous studies. The similarity in intracellular localization pattern and amino acid sequence between DRP2A and DRP2B raises the possibility that DRP2B and DRP2A are involved in membrane trafficking not only around the plasma membrane but also around the Golgi network.

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