Uptake of a Fluorescent Marker in Plant Cells Is Sensitive to Brefeldin A and Wortmannin

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We assessed FM1-43 [N-(3-triethylammoniumpropyl)-4-(4-[dibutylamino]styryl)pyridinium dibromide] as a fluorescent endocytosis marker in intact, walled plant cells. At 4°C, FM1-43 stained the plasma membrane, and after 30 to 120 min of incubation at 26°C, FM1-43 labeled cytoplasmic vesicles and then the vacuole. Fluorimetric quantitation demonstrated dye uptake temperature sensitivity (≈65% reduction at 16°C, >90% at 4°C). FM1-43 uptake in suspension cells was stimulated more than twofold by brefeldin A and inhibited ≈0.4-fold by wortmannin. FM1-43 delivery to the vacuole was largely inhibited by brefeldin A, although overall uptake was stimulated, and brefeldin A treatment caused the accumulation of large prevacuolar endosomal vesicles heavily labeled with FM1-43. Three-dimensional time lapse imaging revealed that FM1-43-labeled vacuoles and vesicles are highly dynamic. Thus, FM1-43 serves as a fluorescent marker for imaging and quantifying membrane endocytosis in intact plant cells.

INTRODUCTION

Endocytosis in animal cells begins with the budding of invaginated coated pits from the plasma membrane into coated vesicles, which then uncoat and fuse with the endocytic organelles (Gruenberg et al., 1989; Gruenberg and Maxfield, 1995). By this action, both membrane proteins and fluid phase molecules are sequestered from the cell exterior into intracellular organelles, from where they can recycle to the plasma membrane or be transported to other destinations, such as the lysosomes. Thus, endocytosis serves to balance membrane secretion and to remove, transport, or degrade receptor ligand complexes from the cell surface (Goldstein et al., 1985). Although clathrin–dependent endocytosis is involved in receptor internalization, clathrin-independent endocytosis pathways also contribute to internalization. These include uptake via non-clathrin-coated caveolae (Anderson, 1998), macropinocytosis (Swanson, 1989; Hewlett et al., 1994), and phagocytosis (Aderem and Underhill, 1999).

Our understanding of animal cell endocytosis was advanced by the use of fluorescent and electron-opaque markers that can be internalized (Salzman and Maxfield, 1988, 1989; Griffiths et al., 1989). The use of fluid phase markers has permitted the development of assays that reconstitute endocytic vesicle fusion (Braess, 1987, 1992; Gruenberg et al., 1989; Emans et al., 1995; Emans and Verkman, 1996) and led to the identification of many proteins that catalyze and control fusion, such as the small GTPase rab5 (Gorvel et al., 1991) and its effector proteins (Christoforidis et al., 1999a, 1999b; McBride et al., 1999).

In plants, there is evidence that endocytosis is likely to occur (Low and Chandra, 1994; for review, see Battey et al., 1999; Marcote et al., 2000). Endocytosis is postulated to counterbalance membrane secretion (Samuels and Bialputra, 1990) and permit cell volume to respond to changes in osmolality (Thiel et al., 1998; Kubitscheck et al., 2000). However, the study of plant cell endocytosis has been hampered by the scarcity of markers that can be used in the presence of the plant cell wall (Low and Chandra, 1994; Buchanan et al., 2000).

The cell wall acts as a semipermeable barrier to the use of tracer molecules that could be used to label the fluid phase of the plant endocytic pathway, essentially excluding proteins >25 kD (Carpita et al., 1979). As a result, the majority of the evidence for endocytosis in plants has come from the use of plant cell protoplasts (Fowke et al., 1991), in which the cell wall is removed enzymatically together with some cell surface proteins (Fowke and Gamborg, 1980), and there are uncertainties regarding how closely protoplast membrane traffic may resemble that of intact cells.

Plants express much of the endocytic machinery identified in animal cells, such as coated vesicles (Robinson et al., 1998; Pimpl et al., 2000) and clathrin heavy chains (Coleman...
et al., 1987; Blackbourn and Jackson, 1996), \(\beta\)-adaptins (Holstein et al., 1994), and small ras-like rab family GTPases, which include homologs of rab5, which is known to be involved in animal cell endocytosis (Anantalaahochai et al., 1991; Terry et al., 1992; Borg et al., 1997). Arguments have been put forward that the general machinery used in endocytosis is conserved from animals to plants, although specific endocytic proteins may have different functions from one kingdom to another (Marcote et al., 2000).

Using protoplasts and electron microscopy, clathrin-coated pits and clathrin-coated vesicles similar to those found in animals have been observed (Emons and Traas, 1986; Galway et al., 1993), and their number increases with cell growth (Samuels and Blaputra, 1990). Endocytosed markers are proposed to enter plant cells via coated pit invagination and budding into the cytoplasm (Marcote et al., 2000). Electrophysiological studies indicate that early endocytic vesicles have a diameter between 70 and 100 nm (Thiel et al., 1998), and ultrastructural analysis has shown that the endocytic pathway, as in animal and yeast cells, consists of a series of structurally distinct organelles (Galway et al., 1993).

The markers used to follow plant endocytosis have included heavy metal salts, lucifer yellow, and insoluble membrane markers (reviewed by Fowke et al., 1991; Oparka et al., 1991). The disadvantages are that these dyes may not be entirely cell impermeant and may enter by nonendocytic routes (O’Drisscoll et al., 1991), as has been shown for fluorescein isothiocyanate (Cole et al., 1990) and lucifer yellow (Wright and Oparka, 1989; Roszak and Rambour, 1997), or that the marker can be observed only by electron microscopy in fixed protoplasts, such as for cationized ferritin (Fowke et al., 1991; Galway et al., 1993).

A fluorescent dye that can be used to quantify endocytosis in intact, walled living cells would have several advantages. It would permit the imaging of the organelles involved in plant endocytosis in vivo. Additionally, it would permit characterization of the rate and extent of plant endocytosis in both single cells and a cell suspension. Furthermore, pharmacological agents could be screened for effects on both endocytosis and the destination of a marker. Such a fluorescent marker would aid in the characterization of the molecular machinery involved in plant cell endocytosis (Marcote et al., 2000).

The use of the styryl FM dyes as markers for neuronal endocytosis and exocytosis (Betz and Bewick, 1992; Betz et al., 1996; Henkel et al., 1996; Cochilla et al., 1999) and endocytosis in yeast (Vida and Emr, 1995), amoebae (Heuser et al., 1993), fungi (Fischer-Parton et al., 2000), and Fucus zygotes (Belanger and Quatrano, 2000a, 2000b) prompted us to evaluate using the amphipathic styryl dye FM1-43 [N-(3-

\[\text{triethylammoniumpropyl})-4-(4-\text{dibutylamino)}\text{styryl)pyridinium dibromide}\] to follow endocytosis in walled plant cells. The rationale was that the dye is sufficiently small to cross the cell wall (<1 kD) and integrate into the plasma membrane or be internalized directly. If labeled fluorescent plasma membrane were internalized into cells, it could be identified within cells after the marker remaining at the plasma membrane was removed by washing. To date, studies on the uptake of FM dyes in plants have used maize protoplasts (Carroll et al., 1998), marine algal cells (Battey et al., 1999; Belanger and Quatrano, 2000a, 2000b), and Vicia faba guard cell protoplasts (Kubitscheck et al., 2000), but there are few data on the use of FM1-43 as a marker to analyze the morphology of the endocytic organelles, the temperature dependence of its uptake, and the dynamics of endocytosis in intact, walled plant cells.

Here, we demonstrate that FM1-43 was internalized into intact, walled tobacco Bright Yellow 2 (BY-2) suspension cells, from which it was transported in a time- and temperature-dependent manner to internal vesicles and then to the vacuole. Endocytic compartments, including the vacuole, could be imaged in living cells, and dye uptake could be quantified by cuvette fluorometry in a large cell population. The use of FM1-43 as a marker for intact plant cell endocytosis revealed that brefeldin A (BFA) stimulated endocytosis but wortmannin inhibited endocytosis. FM1-43 also was suitable for imaging vacuole and vacuolar strand dynamics in real time by time lapse and four-dimensional (4D) imaging.

**RESULTS**

**FM1-43 Plasma Membrane Labeling and Internalization**

Our intent was to evaluate fluorescent markers suitable for following endocytosis in real time in intact, walled plant cells. The prerequisites we selected for a marker for endocytosis in plant cells were that it would be water soluble, readily cross the cell wall, have a high quantum yield, be cell impermeable, be insensitive to pH, and be readily imaged with conventional or confocal microscopy. After screening a number of possible fluorophores and fluorophore conjugates, such as low molecular weight polar dyes or rhodamine dextran conjugates (<6 kD) theoretically capable of crossing the cell wall, we identified FM1-43 as a suitable marker for plasma membrane labeling. Other styryl dyes (FM4-64 and FM3-25) were found to be less suitable because of their low brightness in cuvette studies compared with that of FM1-43 (FM4-64) and because of difficulties in either comprehensively excluding that they were delivered to the mitochondria (FM4-64) or with dye removal after labeling (FM3-25).

FM1-43 is a styryl chromophore that is readily soluble in water but essentially nonfluorescent until bound to membranes. It then undergoes a 50- to 100-fold enhancement in fluorescence quantum yield as the hydrophobic portion of the dye dissolves into the outer leaflet of the membrane (Schote and Seelig, 1998). However, two positively charged

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quaternary ammonium groups prevent the dye from crossing the bilayer, and it remains as an outer leaflet membrane-impermeant marker (Betz and Bewick, 1992; Schote and Seelig, 1998; Cochilla et al., 1999). The ready solubility of FM1-43 in physiological buffers permits its use as a marker of endocytosis in neurons and animal cells, in which endocytosed FM1-43 can be distinguished from plasma membrane labeling that can be washed out readily with excess marker-free buffer (Betz et al., 1992).

We selected the tobacco BY-2 suspension line as a model plant cell line because of its rapid growth under controlled conditions, simple morphology, and very low autofluorescence. Tobacco BY-2 cells possess simple plastids but do not develop chloroplasts when grown on a simple sugar-rich medium in the dark (Nagata et al., 1992). To assess FM1-43 as a marker for BY-2 cell internalization, BY-2 cells were incubated at 4°C with 20 μM FM1-43 for 15 min and imaged by confocal sectioning, which revealed that the dye was restricted to the plasma membrane (Figure 1A).

We confirmed that FM1-43 was membrane bound and not intercalated into the cell wall matrix by imaging and fluorescence. FM1-43 staining was observed only on the surface of living cells (Figure 1A) and never in cell wall fragments or cell wall ghosts, and it was seen only on the periphery of BY-2 cells, where the cytoplasm had detached from the cell wall, but not on the cell wall itself (data not shown). The low binding to the plant cell wall was to be expected given that the fluorophore is enhanced in fluorescence only by binding to membranes and is unlikely to be fluorescent when bound to the cell wall matrix. Membrane incorporation was confirmed by fluorimetry; the emission spectra of FM1-43 showed an ~60-nm blue shift when added to plant cells compared with the emission spectra in solution, as expected for membrane binding (Molecular Probes, Eugene, OR) (Betz and Bewick, 1992; Betz et al., 1992; Schote and Seelig, 1998) (Figure 2B).

After insertion of FM1-43 into the plasma membrane at 4°C, labeled BY-2 cells were incubated at 26°C, the optimal growth temperature (Nagata et al., 1992), for 30, 60, or 120 min. At each time point, the cells were washed in ice-cold marker-free growth medium, which efficiently removed any plasma membrane FM1-43 from control cells kept at 4°C (data not shown). After 30 min of incubation at 26°C, the FM1-43 distribution changed to label internal punctate vesicles (Figure 1B, arrows). This was striking when series of confocal sections were used to construct a pseudo three-dimensional (3D) projection image (Figure 1B, right). After incubation for 60 to 120 min, the pattern of staining shifted from cytoplasmic vesicles toward one that resembled the vacuole (Figure 1D, arrows). Projection images showed that FM1-43 was delivered to the vacuole and that the morphology of the labeled compartments changed relative to the time of dye internalization (Figures 1A to 1D, right). Therefore, FM1-43 satisfied the prerequisites for an internalized marker in that it crossed the cell wall, was internalized into cells, and revealed endocytic organelle morphology with stable, bright fluorescence at relatively low concentrations.

**FM1-43 Internalization Is Temperature Dependent**

An ideal fluorescent marker for endocytosis would be useful for quantitating uptake in a large population of cells by cuvette fluorimetry. This would allow the temperature dependence of uptake and the effects of pharmacological agents, known to affect endocytosis in animal cells or postulated to act in plant cells, to be measured in a large population of intact, walled plant cells.

To determine if FM1-43 would be suitable as a fluorimetric marker and to analyze the temperature dependence of FM1-43 uptake, a time-course endocytosis experiment was performed. BY-2 cells were labeled with 20 μM FM1-43 at 4, 16, or 26°C, and at 50, 60, or 120 min after incubation began, an aliquot of cells was withdrawn and washed extensively to remove plasma membrane dye. Dye internalization then was quantitated by cuvette fluorimetry. Cell-associated fluorescence was detected readily in a dilute cell suspension (~5 × 10⁵ cells/mL) and gave a robust signal (Figures 2A and 2B), whereas cell autofluorescence was negligible (~0.1% of the labeled control). Internalization of FM1-43 was maximal over the range of temperatures studied, at 26°C, and cell-associated fluorescence increased with a single exponential time course (Figure 2). Dye uptake was strongly temperature dependent and was inhibited by ~65% at 16°C and by >90% at 4°C after a 120-min incubation (Figure 2A). Therefore, FM1-43 uptake satisfied the prerequisite that it should show temperature dependence, a hallmark of endocytosis in animal cells.

**FM1-43–Labeled Vesicular Staining Can Be Chased into the Vacuole**

A prerequisite for identifying endocytosis is that it is mediated by vesicle traffic (Gruenberg and Maxfield, 1995). This predicts that the morphology of fluorescent labeling with an endocytic marker will change as the marker transits from one compartment to another through membrane traffic. Therefore, we determined if the marker FM1-43 could be internalized into a vesicle population and if the marker then could be chased into the vacuole.

After a 30-min pulse of internalization, FM1-43 was found in cytoplasmic vesicles (Figure 3A). When these cells were washed free of FM1-43 and further incubated in marker-free medium at 26°C, FM1-43 staining shifted to the vacuole and cytoplasmic vesicular staining diminished within 60 min (Figure 3B). This is in agreement with the observed time course of dye internalization (Figures 1 and 2). Interestingly, vesicular profiles were stained after longer chase times (~120 min), which may indicate that the
Figure 1. Internalization of FM1-43 into Plant Suspension Cells.

Confocal sections of living BY-2 cells labeled with FM1-43 at 4°C ([A], arrows indicate plasma membrane) and after incubation at 26°C for 30 min ([B], arrows indicate vesicles), 60 min (C), or 120 min ([D], arrows indicate vacuolar membrane).

(A) Left, FM1-43 confocal section; middle, transmission image; right, overlay image.
(B) to (D) Left, FM1-43 confocal section; middle, overlay with transmission image; right, projection image of an ~30-μm confocal Z series traversing a BY-2 cell.

n, nucleus; v, vacuole. Bar = 20 μm.
marker being delivered to a compartment connected to the vacuole by vesicle traffic (Figure 3C, arrow).

**FM1-43 Colocalizes with a Fluid Phase Vacuolar Marker**

We found that the highly polar dye Alexa 568 hydrazide was delivered to the vacuole over time at 26°C in intact walled tobacco BY-2 cells. This occurs presumably either by endocytosis, because it is a polar charged fluorophore and staining was not visible in the cytoplasm after prolonged uptake (Figure 4A, left), or by a nonendocytic transport mechanism similar to that observed for other small soluble fluorophores, such as fluorescein isothiocyanate (Cole et al., 1990) and lucifer yellow (Wright and Oparka, 1989; Roszak and Rambour, 1997).

To determine if FM1-43 was delivered to the same compartment as was the fluid phase dye, cells were labeled with the Alexa 568 dye and then washed and labeled with FM1-43 as described for Figure 1. There was little colocalization between the dyes when the FM1-43 was restricted to the plasma membrane (Figure 4B), but as FM1-43 uptake progressed, FM1-43 colocalized with the vacuolar Alexa dye (Figures 4C and 4D).

**FM1-43 Uptake Is Stimulated by BFA and Inhibited by Wortmannin**

FM1-43 is a stable fluorophore with a high quantum yield and molecular extinction coefficient compared with many other fluorophores. It can be detected easily in low dilutions of cells by cuvette fluorimetry after internalization, with a high signal-to-noise ratio (Figure 2). As shown in Figure 5, we took advantage of these properties to investigate the effects of two pharmacological agents, Brefeldin A (BFA) and wortmannin, on endocytosis. BFA is known to affect membrane traffic in the animal cell endocytic and secretory pathways (Lippincott-Schwartz et al., 1989, 1991) and to affect the plant Golgi (Satiat-Jeunemaitre and Hawes, 1992), acting in part through its actions on adenosine diphosphate ribosylation factor (ARF) nucleotide exchange (Orci et al., 1991; Donaldson et al., 1992; Morinaga et al., 1996). Wortmannin is a specific inhibitor of the phosphatidylinositol (PI) 3-kinase that has been shown to block endocytosis in baby hamster kidney cells (Clague et al., 1995). Wortmannin also has been shown to be active in plant cells, where it inhibits protein sorting to the vacuole at micromolar concentrations through action on both the PI 3- and PI 4-kinases (Matsuoka et al., 1995).

Initially, suspension cells were pretreated with 100 μg/mL BFA or 33 μM wortmannin for 30 min, FM1-43 was internalized for 60 min at 26°C, and cell-associated fluorescence was quantitated after extensive washing by centrifugation. As shown in Figure 5, pretreatment of cells with 100 μg/mL BFA significantly stimulated endocytosis compared with control cells incubated at 26°C. BFA did not permeabilize the cells directly, because cells pretreated with BFA at 26°C and held on ice during FM1-43 labeling had similar cell-associated fluorescence to control cells held at 4°C (Figure 5A). After subtraction of the background 4°C signal, the average stimulation of endocytosis by 100 μg/mL BFA over the control was ~136% (four experiments; n = 13) or 2.4-fold. Pretreatment of the cells with 33 μM wortmannin inhibited endocytosis by ~40% (two experiments; n = 8) (Figure 5A).
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Figure 3. FM1-43 Labeling Can Be Chased to the Vacuole.
Confocal sections of BY-2 cells labeled with FM1-43 using a pulse chase protocol. 
(A) BY-2 cells labeled for 30 min at 26°C with FM1-43 and washed. Left, FM1-43 image; right, projection image of a series of confocal Z sections. 
(B) BY-2 cells labeled for 30 min at 26°C with FM1-43, washed, and chased for 60 min in marker-free medium. Left, FM1-43 image; right, projection image. 
(C) BY-2 cells labeled for 30 min at 26°C with FM1-43, washed, and chased for 120 min in marker-free medium. Left, FM1-43 image; right, projection image. The arrow indicates a vesicle. Bar = 20 μm.

or 0.4-fold over the untreated control incubated at 26°C. Wortmannin pretreatment did not permeabilize the cells directly, because cells pretreated with 33 μM wortmannin and then held on ice during FM1-43 labeling and endocytosis did not have appreciably greater cell-associated fluorescence than did untreated control cells held on ice (Figure 5A). Investigation of the concentration dependence of BFA stimulation revealed that a significant effect was not detected at 10 μg/mL (Figure 5B).

Quantitative confocal imaging of cells labeled with FM1-43 across a time course demonstrated that cell-associated fluorescence was increased significantly in the presence of BFA (Figure 5C, right) compared with that in untreated control cells (Figure 5C, left), as expected from the fluorimetric measurements (Figures 5A and 5B). However, the morphology of the endocytic pathway was altered dramatically by BFA treatment. After 60 min of uptake, the majority of FM1-43 labeling in BFA-treated cells was found in an accumulation of large vesicles (Figure 5C), which were observed only rarely in control cells (Figure 1C). These vesicles presumably are prevacuolar because they were labeled before FM1-43 reached the vacuole. Furthermore, after 120 min of uptake under BFA treatment, prevacuolar vesicle labeling increased, but there was an inhibition of vacuolar delivery of FM1-43. In control cells, vacuoles were labeled clearly after 120 min (Figure 5C, left), but under BFA treatment, vacuolar labeling was minor and the majority of FM1-43 was accumulated in prevacuolar vesicles. Thus, although BFA treatment increased the overall uptake of FM1-43, dye delivery to the vacuole was inhibited and labeling accumulated in large prevacuolar compartments.

In contrast, BFA had no significant effect on plastids labeled with stroma-targeted green fluorescent protein in suspension cells or on plastid dynamics (data not shown). This indicated that it did not disturb the structure of an unrelated compartment (plastids) under our experimental conditions. BFA is known to affect the secretory pathway in plants (Satia-Jeunemaitre and Hawes, 1992; Driouch et al., 1993) and the secretory and endocytic pathways in animal cells (Lippincott-Schwartz et al., 1989, 1991). Therefore, the effect we observed on the endocytic pathway is unlikely to be nonspecific toxicity; rather, its effect on endocytosis is analogous to effects on the secretory pathway (i.e., disruption of vesicle traffic).

FM1-43–Labeled Vacuoles Are Highly Dynamic

We used the stable fluorescent signal of FM1-43 in the vacuole to observe vacuolar dynamics using time lapse confocal microscopy and 4D imaging (3D time lapse imaging). BY-2 cells were pulse labeled with FM1-43 for 120 min at 26°C, washed on ice to remove plasma membrane staining, and then chased in marker-free medium.

Imaging the cells using a relatively deep confocal Z section showed that FM1-43 staining lined the vacuolar membrane, presumably after delivery via endocytic vesicle fusion/membrane traffic (Figures 4 and 6B). We observed that the vacuole was strikingly dynamic and that vesicle movement through transvacuolar strands could be observed in real time in living cells by time lapse confocal imaging (Figure 6A). We observed a collection of FM1-43–labeled vesicles moving through strands as a bolus that arose from the pe-
Figure 4. FM1-43 Is Internalized into Vacuoles Labeled with a Fluid Phase Marker.

Confocal sections of cells incubated with 100 μM fluid phase Alexa 568 dye for 4 hr at 26°C, labeled on ice with 20 μM FM1-43 for 15 min at 4°C, and incubated for 30 to 120 min at 26°C.

(A) Control cells labeled with Alexa 568 for 4 hr at 26°C (left) or FM1-43 at 4°C (right).

(B) Alexa 568–labeled cells after FM1-43 labeling at 4°C.

(C) and (D) Alexa 568–labeled cells after FM1-43 labeling and a 60-min incubation at 26°C (C) or a 120-min incubation at 26°C (D).

In (B) to (D), at left, FM1-43 images; middle, Alexa 568 images; right, overlay images with FM1-43 fluorescence shown as green and Alexa 568 fluorescence shown as red. Bar = 20 μm.
Figure 5. FM Dye Uptake Is Stimulated by BFA and Inhibited by Wortmannin.

Cell-associated FM1-43 fluorescence was quantitated after a 60-min internalization of the dye and normalized to control cell labeling at 4°C. 
(A) Cells were pretreated with BFA (100 μg/mL), wortmannin (33 μM), or solvent. 
(B) Cells were pretreated with 100, 50, or 10 μg/mL BFA or solvent. 
(C) Confocal images of BY-2 cells labeled with FM1-43 at 4°C and then incubated at 26°C for 30, 60, and 120 min. Left, control; right, plus 100 μM BFA. 4°C panels are of a single confocal slice, and 26°C panels are projection images of an ~30-μm confocal Z series traversing a BY-2 cell. All image acquisition parameters were kept constant for imaging control and BFA-treated cells at each time point. 
In (A) and (B), data shown are means ±SD and are representative of at least two experiments, each containing four individual measurements. Bars = 15 μm.
ripheral cytoplasm. The bolus of vesicles expanded the strand as it passed through at a velocity of \( \sim 0.5 \ \mu m/sec \) (Figure 6A, arrows). To be certain that discrete strands passing through the vacuolar space were imaged, transvacuolar strands were identified by serial Z sectioning before time lapse imaging was initiated. This enabled strands to be distinguished in a confocal section from invaginations of the vacuolar wall into the vacuolar space or large bridges of cytoplasm extending from the nucleus to the wall. The transvacuolar strands were mobile, and the stalk of the strands often migrated along the wall of the vacuole. The strands remained rigid during movement, and we also observed both ends of the strands moving at similar velocities across the vacuole in concert with vesicle movement through the strand (data not shown).

It was challenging to image the movement of transvacuolar strands, because they were rarely orientated so that they could be imaged in a single confocal Z section over time. Therefore, we selected 4D imaging to observe vacuolar dynamics because it captured movement within a large 3D volume of a cell. For 4D imaging, a broad lateral section through a cell was defined and then imaged in four to eight \( \sim 1-\mu m \)-deep consecutive confocal Z sections. The sections were then used to create a 3D projection image on image processing (Figure 6B, 1). To create a 4D imaging series, the 3D series was acquired every 30 or 60 sec (Figure 6B, 1 to 9). Each Z series was image processed to create a projection image.

The 4D imaging series shown in Figure 6B shows a reconstructed \( \sim 7-\mu m \) section through the middle of a chain of BY-2 cells, which was acquired as a series of consecutive Z sections. Each panel shows a 3D projection image of the time lapse series of Z sections, but in contrast to the projection images shown above (Figure 1, right), the projection image does not show the entire cell but an \( \sim 7-\mu m \) lateral section, and images were captured over time. The nucleus is located in the middle of the image and is surrounded by a halo of labeled inner vacuolar membrane, vesicles, broad bridges of cytoplasm (Figure 6B, 1, closed arrows), and thin transvacuolar strands (Figure 6B, 1, arrowheads). 4D imaging revealed that strands fuse laterally to create broader strands (Figure 6B, 2 to 5, arrowheads) and that vesicles, presumably endocytic because they were labeled with FM1-43, moved in three dimensions within the transvacuolar strands (Figure 6B, 4 to 9, open arrows). The fusion of two strands did not appear to involve lateral movement of the strands, but vacuolar membrane moved along both strands from between the nuclear base of the strands toward the side of the vacuole, so that the strands were converted into a sheet of membrane stretching from the nucleus to the vacuolar wall (Figure 6B, 2 to 5, arrowheads).

Vesicles moved along and within strands, and this was seen most readily in 4D imaging movies of vacuolar dynamics (Figure 6B). Vesicles at the base of the fused vacuolar strands described above appeared to move in three dimensions, and this was independent of strand movement (Figure 6B, 4 to 9, open arrows).

**DISCUSSION**

In this study, the unique features of an amphiphatic styryl dye, FM1-43, were exploited as a noninvasive marker to reveal the morphology, uptake kinetics, and ultrastructural dynamics of the endocytic pathway in living plant cells.

Here, we have characterized FM1-43 as a fluorescent marker for membrane uptake in intact, walled plant cells. The scarcity of such a marker has hampered investigations into plant cell endocytosis (Buchanan et al., 2000). Our intent was to determine whether FM1-43 satisfied the prerequisites of a marker for endocytosis. Although the literature on the use of FM1-43 in neurobiology is extensive (Betz et al., 1996; Cochilla et al., 1999), there have been few studies on its use in plants and, to our knowledge, no examination of its use in intact plant suspension cells. The uptake of FM dyes was shown in *V. faba* guard cell protoplasts (Kubitscheck et al., 2000), maize root cap protoplasts (Carroll et al., 1998), and zygotes from marine alga (Battey et al., 1999; Belanger and Quatrano, 2000a). These studies prompted us to examine whether FM1-43 was suitable for tobacco suspension cells, which are walled, in contrast to protoplasts, and can be cultured easily as a large, synchronized population (Nagata et al., 1992).

The prerequisites defined for a fluorescent marker for membrane endocytosis in plant cells were similar to those for animal cell endocytosis (Salzman and Maxfield, 1988, 1989): that the probe be nontoxic, demonstrate a robust fluorescence in the membrane, is able to cross the plant cell wall, and is internalized into cells by a route that is strongly temperature dependent. Our results indicate that FM1-43 fulfills many of these prerequisites.

When cells were incubated at 4°C with FM1-43, confocal imaging demonstrated that staining was restricted to a pattern consistent with the plasma membrane. Plasma membrane incorporation of the dye was likely because no fluorescent labeling was observed in the cell walls or cell wall fragments. Incubation of the cells at 26°C, the optimal culture temperature for BY-2 cells, gave a cytoplasmic vesicle staining pattern after 30 min. Incubation at 26°C for 60 or 120 min shifted the dye localization to a pattern consistent with vacuolar labeling. Cationized ferritin is delivered to the vacuole after 60 min of exposure (Fowke et al., 1991), which is consistent with the data presented here. Ferritin delivery is mediated by multivesicular bodies, which are similar to those observed in animal cells that mediate early to late endosome transport along microtubules (Gruenberg et al., 1989) and which fuse with the vacuole. It is possible that FM1-43 is delivered by a similar vesicular carrier. To confirm this speculation, further analysis is necessary using techniques, such as photooxidation, that permit FM1-43 localization by electron microscopy (Nishikawa and Sasaki, 1996).

Because the FM1-43 crossed the cell wall and plasma membrane–associated FM1-43 could be removed by washing, the intracellular sequestration of the dye could be followed by
BY-2 cells were labeled with 20 μM FM1-43 for 120 min at 26°C and then washed and imaged. 

(A) Time lapse imaging of a single confocal section (~1 μm) showing vesicle movement through a transvacuolar strand. Time scale is shown in seconds. Arrows indicate vesicles and membrane boluses. Bar = 20 μm.

(B) 4D imaging of labeled cells showing strand and vesicle movement. Z series (~7 μm deep) of approximately six confocal sections were acquired at 1-min intervals. 3D images were reconstructed for each time point and then used to create a 4D imaging movie. The montage shows nine images taken at 1-min intervals. Closed arrows, bridge of cytoplasm; arrowheads, transvacuolar strand; open arrows, vesicles within a strand. Bar = 20 μm.
measuring cell-associated fluorescence. Dye sequestration was time and temperature dependent: it was reduced by >65% by a temperature shift from 26 to 16°C and by >90% at 4°C. Uptake at 26°C followed a simple exponential time course, and the fluorescence emission spectra of sequestered FM1-43 showed a blue shift (peak emission λ, 560 nm) compared with that of the dye in solution in water (peak emission λ, 620 nm), consistent with the intercalation of the dye into the membrane (Schote and Seelig, 1998). Therefore, FM1-43 was internalized into intact cells by means of a time- and temperature-dependent mechanism by which a significant fraction of the dye remained bound to membranes.

We speculate that FM1-43 is taken up by endocytic vesicles at the plasma membrane of tobacco suspension cells because dye uptake experiments show temperature-dependent traffic of the dye from the plasma membrane through endomembrane compartments to the vacuole. This is supported by the evidence that FM dyes can be used to study endocytosis in many experimental organisms, including yeast, Dictyostelium, invertebrates, rat hippocampal and frog motor neurons, starfish oocytes, and transgenic mouse-derived hippocampal cell lines (reviewed by Betz et al., 1996; Cochilla et al., 1999).

However, alternative pathways may contribute to plant cell labeling; for example, the direct internalization of lipids from the plasma membrane to the endoplasmic reticulum (ER) has been shown for soybean cells (Grabski et al., 1993), but the extent of this activity for specific lipids or fluorescent lipid analogs is unclear. We observed little evidence of the direct delivery of FM1-43 from the plasma membrane to the ER, based on the morphology and dynamics of the labeled compartments and the temperature sensitivity of dye uptake. Even after long-term chase after cell labeling (16 to 48 hr), FM1-43 was found only in the vacuole, and there was no evidence of ER staining in intact cells. Although it has been demonstrated that fluorescent analogs of phosphatidylcholine or phosphatidylethanolamine are transported from the yeast plasma membrane by a nonendocytic transbilayer mechanism to internal organelles, FM4-64, a dye closely related to FM1-43, enters yeast by an endocytic mechanism requiring increased temperature (Grant et al., 2001). This indicates that members of the FM family of dyes are not taken up by a nonendocytic pathway and so are not available for delivery to the ER from the plasma membrane via the cytoplasm. In addition, FM1-43 has a positive electric charge that prevents it from crossing the membrane, so it remains partitioned in the outer lipid leaflet of the animal cell plasma membrane (Sulzer and Holtzman, 1989; Schote and Seelig, 1998). Thus, we speculate that FM1-43 does not enter plant cells via the nonendocytic pathway observed for the phosphatidylcholine analog (Grabski et al., 1993) but serves as a marker for temperature-dependent membrane internalization from the plasma membrane via vesicle traffic, as is well established for FM4-64 in yeast (Vida and Emr, 1995).

Recently, a biochemical marker for receptor-mediated endocytosis was developed for rice suspension cells based on the specific binding and internalization of biotin-labeled proteins (Bahaj et al., 2001). FM1-43 has some advantages over protein markers in that it is a membrane marker whose uptake can be both imaged and easily quantified in living intact cells. We propose that FM1-43 will complement studies on receptor-mediated endocytosis by providing a marker for the default pathway of membrane endocytosis.

Interestingly, Belanger and Quatrano (2000a) analyzed the distribution and uptake of FM4-64 in fertilized Fucus zygotes. It was demonstrated that FM4-64 can be delivered to perinuclear endomembrane organelles that may include the vacuole, Golgi, or ER, and then to the newly forming cell plate. They proposed that the initial uptake of FM4-64 is mediated by endocytosis at the plasma membrane, from which label is trafficked to the perinuclear region. We believe that this finding supports our proposal that FM1-43 is endocytosed into plant cells under our conditions, but we speculate that the membrane traffic pathways in BY-2 cells are dissimilar to those in Fucus zygotes and that membrane trafficking is specialized along the developmental pathways required for zygote development.

A feature of FM1-43 as an endocytosis marker is that it can be used with intact plant cells, in which it crosses the cell wall and implants into the plasma membrane. This permits analysis and imaging of the effects of pharmacological agents and plant signaling compounds on a defined stage of membrane traffic. BFA strongly stimulated temperature-dependent endocytosis of the marker, whereas wortmannin acted as an inhibitor. BFA stimulated endocytosis in BY-2 cells at 50 to 100 μg/mL, a concentration that is within the range used for plants and plant suspension cells (Satiat-Jeunemaitre and Hawes, 1992; Gomez and Chrispeels, 1993; Boevink et al., 1998; Wee et al., 1998; Mullen et al., 1999). Interestingly, Belanger and Quatrano (2000a) demonstrated that although BFA inhibited the asymmetric growth of Fucus zygotes and the formation of the cell plate, uptake of FM4-64 continued but labeled membrane was not delivered to the perinuclear region.

Because membrane traffic generally is conserved from yeast to animals, we speculate that BFA exerts its action in plants by mechanisms analogous to those in animal cells. In animal cells, one of the major effects of BFA is the breakdown of the Golgi through its inhibitory action on the guanine nucleotide exchange factor (GEF) for ARF1 (reviewed by Chavrier and Goud, 1999) and the restriction of vesicle budding events from organelles. Several ARF GEFs in animals and yeast are BFA sensitive, including yeast Sec7p; however, the BFA target in plant cells is uncharacterized. Whether an ARF is associated with the plant endocytic pathway also is unclear, but in animal and yeast cells there is clear involvement of ARFs in endocytosis. Mutation of ARF1 in yeast inhibits endocytic transport to the vacuole (Gaynor et al., 1998), and expression of ARF6 mutants in nonpolarized animal cells, in which the wild-type protein is localized to endosomes and the plasma membrane, disrupts endocytosis (D’Souza-Schorey et al., 1998). Furthermore,
ARF6 is found on the apical surface of polarized Madin-Darby canine kidney cells, in which overexpression of the ARF6-Q67L mutant, predicted to be locked in the GTP-bound form, stimulates apical endocytosis (Altschuler et al., 1999). The Arabidopsis protein GNOM is a membrane-associated ARF GEF involved in the polar localization of the auxin efflux carrier PIN1 (Steinmann et al., 1999). The GEF activities of GNOM and PIN1 localization are BFA sensitive. It is attractive to speculate that GNOM or similar plant ARF GEFs regulate vesicle trafficking at the plant cell plasma membrane and that the stimulation of dye uptake by BFA is through its action on an ARF GEF involved in endocytosis.

We believe that the stimulation of FM1-43 uptake upon BFA treatment is based on an increase in membrane internalization that may be coupled to decreased recycling and an inhibition of transport to the vacuole. Prevacuolar endosomal vesicles accumulate and appear to enlarge under BFA treatment (Figure 5C), and this may be related to BFA disrupting budding from an endosomal compartment and hence dye recycling to the plasma membrane, or to a fusion/sorting step in which these vesicles would be consumed. Furthermore, if budding is reduced along the recycling endocytic pathway, it would lead to a net increase in internalized FM1-43 by affecting a step that would lead to dye loss from the cell (i.e., production of membrane destined for recycling).

A BFA-sensitive step is likely to be found between the prevacuolar endosomal compartment and the vacuole. Confocal imaging revealed that the majority of FM1-43 labeling was found in the large prevacuolar endosomes and that there was an inhibition of dye delivery to the vacuole upon BFA treatment (Figure 5C). Therefore, BFA may act at a transport step from this prevacuolar compartment to the vacuole, and the prevacuolar compartment may accumulate as a consequence of inhibited transport.

BFA affects ER-to-Golgi transport in plants, in which it selectively blocks anterograde but not retrograde transport (Boevink et al., 1998). Transport and sorting of proteins to the vacuole also show differential sensitivity to BFA, phytohemagglutinin transport via the Golgi to the vacuole is blocked, and transport of tonoplast intrinsic protein is unaffected (Gomez and Chrispeels, 1993). Importantly, BFA prevents coat protein (AtArf1p and coatamer) recruitment to the Golgi membrane in vitro, a fundamental stage in non-clathrin-coated vesicle budding (Pimpl et al., 2000). Thus, the effects we observed in endocytosis may relate to coat protein recruitment at a stage of endocytosis responsible for dye loss from the cell—other steps of endocytosis are unaffected because they do not possess a BFA-sensitive component—by analogy with retrograde Golgi-to-ER transport and vacuolar delivery of tonoplast intrinsic protein.

Wortmannin is a PI 3-kinase inhibitor that inhibits endocytosis (Clague et al., 1995; Li et al., 1995) and receptor trafficking in animal cells (Shpetner et al., 1996). Although the concentrations used here are higher than those used in animal cells, they are in the range known to affect protein sorting to the vacuole in tobacco BY-2 cells, in which it inhibits PI 3-phosphate, PI 4-phosphate, and phospholipid synthesis (Matsuoka et al., 1995). Our data provide evidence that endocytosis in BY-2 cells has a wortmannin-sensitive component, but further investigation is required to characterize the mechanism by which it acts.

The robust fluorescence of FM1-43 in the vacuole permitted imaging of endocytic vesicle traffic through transvacuolar strands and the dynamics of the strands themselves. We often observed vesicles moving as a bolus through vacuolar strands and the movement and reorganization of the strands, as has been observed with green fluorescent protein fusion proteins targeted to the vacuole (Cutler et al., 2000). 4D imaging of living FM1-43 cells gave dramatic images of the vacuole and vacuolar strand dynamics. Strands could be observed to fuse laterally along their length, like a zipper closing, and move through three dimensions.

We have demonstrated the utility of FM1-43 as a fluorescent marker for resolving the kinetics of endocytosis morphology and the dynamics of the endocytic pathway in plant cells. Using FM1-43 as a marker for plant endocytosis in intact plant cells revealed BFA and wortmannin sensitivity. Further work will focus on identifying the protein targets that are affected by BFA and wortmannin treatment and that may regulate plant cell endocytosis.

METHODS

Tobacco Suspension Cultures

Tobacco (Nicotiana tabacum cv Bright Yellow 2 [BY-2]) cells were cultured in Murashige and Skoog (1962) basal salt with minimal organics (BY-2 medium: 0.47% [w/v] Murashige and Skoog salts, 0.15 μg/mL thiamine, 0.02 μg/mL, 2.4-D, 2 mg/mL KH2PO4, and 3% [w/v] sucrose; pH 5.2) in an orbital shaker (New Brunswick Scientific, Nürtingen, Germany) at 180 rpm and 26°C in the dark. All experiments with BY-2 cells were performed using cells after a 5% (v/v) dilution and 3 days of subcultivation.

Chemicals, Media, and Fluorophores

All fluorophores were purchased from Molecular Probes. All other fine chemicals were supplied by Sigma-Aldrich (Deisenhofen, Germany). FM1-43 [N-(3-triethylammoniumpropyl)-4-(4-[dibutylamino]styryl)pyridinium dibromide] was kept as a 20 mM stock solution in DMSO. Alexa 568 hydrazide was a 50 mM stock in sterile water. Brefeldin A (BFA) was kept as a 50 mg/mL stock in methanol, and wortmannin was kept as a 10 mM stock in DMSO at −20°C.

Plant Cell Confocal Imaging

BY-2 cells were imaged with a TCS-SP spectral confocal microscope equipped with argon ion, krypton, and helium-neon lasers.
(Leica, Heidelberg, Germany). Images were acquired with a 1.2 numerical aperture ×63 oil immersion PLAN-APO objective. FM1-43 was excited with the 488-nm argon laser line, and confocal sections were collected using a 510- to 580-nm emission setting. Alexa 568 was excited with the 568-nm krypton laser line, and confocal images were collected using 600- to 620-nm emission.

**Plant Cell FM1-43 Labeling**

Four milliliters of a 3-day-old BY-2 culture was placed on ice for 15 min and then supplemented with 20 μM FM1-43. After 15 min of incubation on ice, the cells were transferred to an orbital shaker at 26°C and 180 rpm. After 30, 60, and 120 min at 26°C, a 1-mL aliquot was removed and washed three times by centrifugation (at 1600 g, 4°C, for 5 min) in 15 mL of marker-free ice-cold BY-2 medium. The pellet was resuspended in 1 mL of BY-2 medium, and the cells were imaged.

**FM1-43 and Alexa 568 Double Labeling**

Four milliliters of a BY-2 culture was placed on ice for 15 min and then supplemented with 100 μM Alexa 568 hydrazide. The cells were transferred to a 26°C orbital shaker for 4 hr. BY-2 cells were chilled by the addition of ice-cold BY-2 medium and washed three times by centrifugation (at 1600g, 4°C, for 5 min) in 50 mL of ice-cold BY-2 medium. The cell pellet was resuspended in 4 mL of ice-cold BY-2 medium, and an aliquot was imaged. The Alexa 568-labeled cell suspension was supplemented with 20 μM FM1-43 and incubated for 15 min on ice and then imaged. Cells then were transferred to a 26°C orbital shaker incubator. After 30, 60, and 120 min at 26°C, a 1-mL aliquot was removed and washed three times by centrifugation (at 1600g, 4°C, for 5 min) in 15 mL of marker-free ice-cold BY-2 medium. The cells were resuspended in 1 mL of BY-2 medium and imaged. Confocal FM1-43 and Alexa 568 images were acquired serially and overlaid using Metamorph (Universal Imaging, West Chester, PA).

**Plant Cell Fluorimetry Measurements**

Four milliliters of a 3-day-old BY-2 culture was placed on ice for 15 min and then supplemented with 20 μM FM1-43. After 15 min of incubation on ice, the cells were transferred to a 26°C orbital shaker incubator at 180 rpm. After 30, 60, and 120 min at 26°C, a 1-mL aliquot was removed and washed three times by centrifugation (at 1600g, 4°C, for 5 min) in marker-free ice-cold BY-2 medium. Cell-associated fluorescence was quantified by fluorimetry using an SLM Aminco Bowman AB-2 fluorospectrometer (Polyecl, Waldbronn, Germany) and a 1-cm path length polymethylacrylate cuvette. FM1-43 was excited at 486 nm (16-nm bandpass), and emission was detected at 580 nm (16-nm bandpass). For each time course, the cell-associated fluorescence was normalized to the average fluorescent signal of the cells at the start of the incubation. In single time point measurements, cell-associated fluorescence was normalized to the cell-associated fluorescence of control cells incubated at 4°C. Excitation and emission spectra were acquired using a 5-nm/sec scan speed, and the results of three measurements were averaged (4-nm bandpass).

**Time Lapse Imaging**

Four milliliters of a BY-2 cell culture was labeled for 120 min with 20 μM FM1-43 at 26°C, washed three times by centrifugation in ice-cold BY-2 medium (at 1600g, 4°C, for 5 min), and then resuspended in 4 mL of marker-free BY-2 medium. BY-2 cells were incubated at 26°C in a shaker incubator at 180 rpm and imaged. Confocal XY sections (~2 μm in Z) were acquired at four frames per minute for 10 min.

**Four-Dimensional Imaging**

Cells were labeled as for time lapse imaging and imaged. A lateral confocal Z series, typically 6 to 8 μm, was defined by Z sectioning. This Z series then was acquired automatically every 30 or 60 sec for up to 25 min. The sequential series then was image processed using custom journals to generate a series.

**Software and Image Processing**

Images were acquired as eight-bit multi-image TIF files using the Leica spectral confocal TCSNT software, version 1.6.58, running under Windows NT on a 450-mHz Pentium II personal computer equipped with 384 megabytes of random access memory. Image overlay was achieved either using the host Leica software after parallel confocal image acquisition or with the Universal Imaging Metamorph 4.5 software after serial image acquisition. Multi-image TIF files of confocal time lapse images were montaged and labeled in Metamorph before export as *.TIF files. Image files were archived on CD-ROMs media. For presentation, individual files were imported into Adobe Photoshop (Mountain View, CA) running under Windows NT and saved as 24-bit CMYK files. For four-dimensional (4D) imaging, custom Metamorph journals were used to automatically extract each series from the multi-image TIF confocal files and generate individual projection images using Metamorph.

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# Uptake of a Fluorescent Marker in Plant Cells Is Sensitive to Brefeldin A and Wortmannin

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