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Although there are some rare reports about the effect of styryl-based dyes on the membrane fluidity (Rodes *et al.*, 1995) and FM dyes were reported to block mechanotransduction channels (Gale *et al.*, 2001) and the activity of muscarinic and nicotinic acetylcholine receptors (for review see Gaffield and Betz, 2006) FM dyes are considered to be valuable tool for tracking of endocytosis in plant cells (Bolte *et al.*, 2004; Emans *et al.*, 2002).

Here we show that even very low concentrations of FM dyes might have some additional effects on the localization and activity of plasma membrane located proteins, namely auxin influx and efflux carriers.

Results and Conclusions

During studies of endosomal trafficking of the auxin efflux machinery components, FM styryl dyes were often used in our laboratory in tobacco BY-2 cell line. While it was very easy to reproduce already published experimental procedures describing the application of FM dyes (for review Bolte *et al.*, 2004) to cells in suspension, additional, yet uncharacterized effect of FM4-64 treatment was always observed. The application of low amount of FM4-64 (2 μM) to the BY-2 cells expressing PIN1 auxin efflux protein from *Arabidopsis thaliana* (PIN1::PIN1:GFP) (Benková *et al.*, 2003; Zažímalová *et al.*, 2007) resulted in the transient internalization of plasma membrane-located auxin efflux carrier PIN1-GFP **(Fig. 2)**. While control cells showed clear plasma membrane localization of PIN1-GFP **(Figure 2a** and Petrasek and Zazimalova 2006), already after 2 minutes of treatment with FM 4-64, remarkable increase in the endosomal pool of PIN1-GFP was observed. The timing of this phenomenon clearly preceded the endocytosis of FM 4-64 itself **(Fig. 2b)** and was observed eve when 0.2 μM FM4-64 was used (data not shown). Concentrations used in our experiments are 10-100 times lower that is reported to be non toxic for plant cells (Bolte *et al.*, 2004). There was no colocalization of vesicles containing PIN1-GFP and FM4-64 **(Fig 2c, e)**. Prolonged incubation (up to 30 min) with FM4-64 resulted in the remarkable endocytosis of FM4-64, but PIN1-GFP vesicles disappeared from cortical cytoplasm restoring control-like situation in cell chains **(Fig. 2d)** but they interestingly remained in more elongated cells even after longer treatment of 1.5h with FM4-64 **(Fig. 2f)**. This suggests cell type-specific response.

The effect of FM4-64 **(Fig. 3a)** was studied in comparison with other two endocytotic markers FM5-59 **(Fig. 3b)** and FM1-43 **(Fig. 3c)** was studied after loading cells with 2 μM FM dye on ice an subsequent observation at room temperature. Although with slower kinetics, the FM5-95, slightly less lipophilic analog of FM4-64, induced internalization of PIN1-GFP **(Fig. 3b)**. In contrast, the most lipophilic dye FM1-43 was never inducing any internalization **(Fig. 3c),** even after prolonged incubation (data not shown). From these experiments it seems that lipohilicity of the dye itself is not the only reason of induced internalization of plasma membrane-located carrier.

To follow possible mechanisms triggered by the addition of FM dyes, metabolic inhibitor sodium azide (NaN_3) was used to block all active ATP-dependent processes. 15 mM NaN_3 totaly prevented both FM4-64 endocytosis and PIN1-GFP internalization **(Fig. 4a)**. This indicates that this internalization is an active process. In contrast, 30 min treatment with 20 μM BFA, the inhibitor of anterograde vesicle trafficking, resulted in the massive formation of PIN1-GFP-containing vesicles after FM4-64 **(Fig. 4b)**. Control cells after 30 min with 20 μM BFA and without FM4-64 had no or much less BFA-induced PIN1-GFP patches formed. These results indicate that FM4-64-induced vesicles are not possibly formed from vesicles that can not fuse with plasma membrane and rather their are derivatives of plasma membrane.

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Altogether, our observations describe yet uncharacterized effects of FM endocytotic markers. These effects include both transient relocalization of some plasma membrane proteins and changes in their function. The explanation of differential effects among various FM dyes might reflect their differential lipophilicity.

Material and Methods

Plant material:
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Fig. 1:
Structure of FM dyes

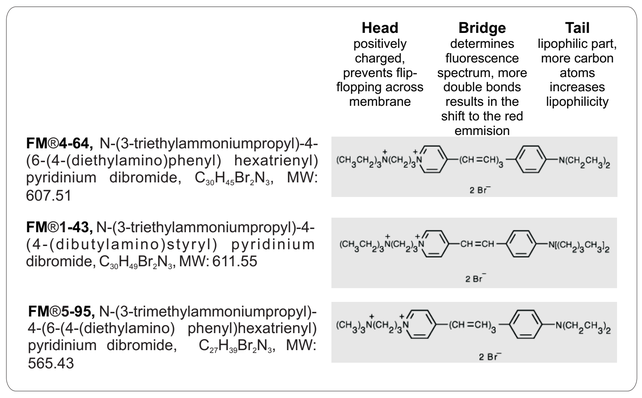

Fig. 1: The structure of FM dyes used in this study. Positively charged head region prevents flip-flopping of the dye across membrane, which fact makes these dyes optimal for tracking endocytosis. Bridge region determines fluorescence of the molecule. More double bonds in this region in FM4-64 and FM5-95 shift the emmision fluorescence to the red part of the spectrum (emmision maximum 734 nm) in contrast to FM1-43 (emmision maximum 626 nm). Tail region is responsible for the lipophilicity of the molecule. Higher amount of carbons increase the lipophilicity (Betz et al., 1996). Among tested FM dyes the most lipophilic is FM 1-43 with four carbons followed by FM4-64 and FM5-95.

Fig. 2:
FM4-64 stimulates transient internalization of PIN1-GFP in BY-2 cells

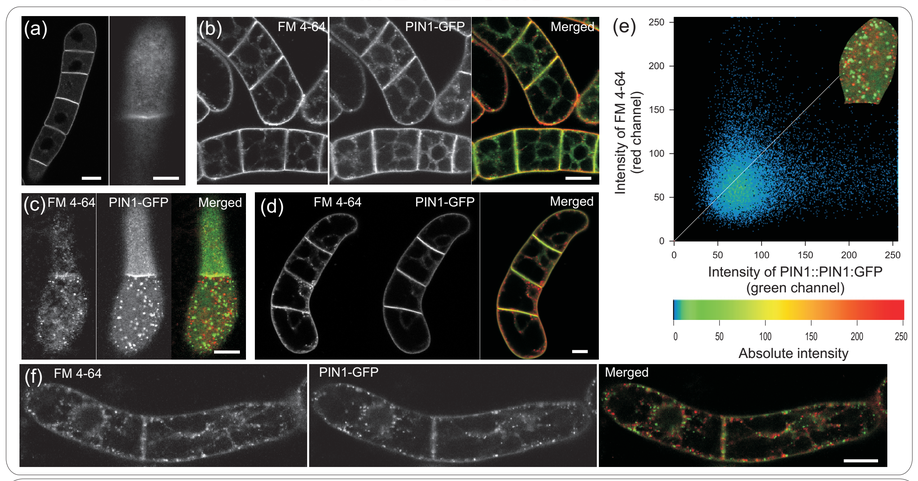

Fig. 2: FM4-64 stimulates transient internalization of plasma membrane-located PIN1-GFP auxin efflux carrier. In control exponentially growing BY-2 cells expressing AtPIN1 protein in translational fusion with GFP the PIN1-GFP was located both at plasma membranes (preferentially transversal) and in the endomembranes of cortical cytoplasm **(a)**. After very short incubation of the BY-2 suspension cells (2 to 10 min) with 2μM FM 4-64 "doting" of the GFP signal was observed **(b)**. This effect was observed before or just together with the endocytosis of FM4-64. There was no colocalization of vesicles containing PIN1-GFP and FM4-64 **(c, e)**. Prolonged incubation (up to 30 min) with FM4-64 resulted in the remarkable endocytosis of FM4-64, but PIN1-GFP vesicles disappeared from cortical cytoplasm restoring control-like situation in cell chains **(d)** but they interestingly remained in more elongated cells even after longer treatment of 1.5h with FM4-64 **(f)**. Scale bars 20 μm.

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The effect of FM4-64 **(Fig. 3a)** was studied in comparison with other two endocytotic markers FM5-59 **(Fig. 3b)** and FM1-43 **(Fig. 3c)** was studied after loading cells with 2 μM FM dye on ice an subsequent observation at room temperature. Although with slower kinetics, the FM5-95, slightly less lipophilic analog of FM4-64, induced internalization of PIN1-GFP **(Fig. 3b)**. In contrast, the most lipophilic dye FM1-43 was never inducing any internalization **(Fig. 3c),** even after prolonged incubation (data not shown). From these experiments it seems that lipohilicity of the dye itself is not the only reason of induced internalization of plasma membrane-located carrier.

To follow possible mechanisms triggered by the addition of FM dyes, metabolic inhibitor sodium azide (NaN_3) was used to block all active ATP-dependent processes. 15 mM NaN_3 totaly prevented both FM4-64 endocytosis and PIN1-GFP internalization **(Fig. 4a)**. This indicates that this internalization is an active process. In contrast, 30 min treatment with 20 μM BFA, the inhibitor of anterograde vesicle trafficking, resulted in the massive formation of PIN1-GFP-containing vesicles after FM4-64 **(Fig. 4b)**. Control cells after 30 min with 20 μM BFA and without FM4-64 had no or much less BFA-induced PIN1-GFP patches formed. These results indicate that FM4-64-induced vesicles are not possibly formed from vesicles that can not fuse with plasma membrane and rather their are derivatives of plasma membrane.

To test how FM dyes interfere with the action of plasma membrane-located auxin influx and efflux carriers, radioactively labeled synthetic auxins 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthalene-1-acetic acid (NAA) were used as markers, respectively (Petrasek *et al.*, 2006). The active auxin influx was decreased immediately after the addition of 2 μM FM1-43 and to lesser extent after 2 μM FM4-64 as indicated by significantly decreased auxin accumulation in 2-day-old BY-2 cells **(Fig. 5a).** The active auxin efflux was also influenced by FM1-43, but FM4-64 had no effect **(Fig. 5b)**. These results clearly document that not only the localization of auxin efflux carrier PIN1 is changed after FM4-64, but also the activity of influx carrier might be directly influenced by the most lipophilic FM dye, FM 1-43. Since we have not studied the localization of influx carriers, possible interference with their localization can not be ruled ut.

Altogether, our observations describe yet uncharacterized effects of FM endocytotic markers. These effects include both transient relocalization of some plasma membrane proteins and changes in their function. The explanation of differential effects among various FM dyes might reflect their differential lipophilicity.

Material and Methods

Plant material:
Tobacco BY-2 (*Nicotiana tabacum* L. cv. Bright Yellow 2; Nagata *et al.* 1992) cells transformed with *Arabidopsis thaliana PIN1::PIN1:GFP* (Benková *et al.*, 2003; Zažímalová *et al.*, 2007) were cultivated in darkness at 25°C on orbital incubator (IKA KS501, IKA Labortechnik, Stufen, Germany; 120 rpm) in liquid medium (3% sucrose, 4.3 $g.l^{-1}$ Murashige and Skoog salts, 100 $mg.l^{-1}$ inositol, 1 $mg.l^{-1}$ thiamin, 0.2 $mg.l^{-1}$ 2,4-dichlorophenoxyacetic acid, and 200 $mg.l^{-1}$ KH_2PO_4, pH 5.8) supplemented with 100 $\mu g\ ml^{-1}$ kanamycin and 100 $\mu g\ ml^{-1}$ cefotaxim and subcultured weekly. Calli were maintained on the same media solidified with 0.6% w/v agar.

Inhibitors and Fluorescent dyes:
FM dyes 4-64, 5-95, 1-43 (Molecular Probes cat. n. T13320, T23360, T3163) and brefeldin A (Sigma-Aldrich) were kept as a 20 mM stock solutions in DMSO at -20°C, respectively. Sodium azide (Sigma-Aldrich) was added from water stock solution.

Application of FM dyes and inhibitors to BY-2 cells:
2μM (or 6μM) FM dyes were added to 1 ml of 2-3-day-old BY-2 cells shaken in the multi-well plate.
Alternatively, protocol of Emans *et al.* (2002) was used. 1 ml of 2-3-day-old BY2 culture was placed on ice for 15 min and then supplemented with 2μM FM dye. After 15 min of incubation on ice, cells were transferred to an orbital shaker at 26°C and scanned at indicated time.
Brefeldin A and sodium azide were added directly to the cultivation media in the final concentration 20 μM and 15 mM respectively. Appropriate amount of the solvent was added to controls.

Microscopy:
For all *in vivo* observation Zeiss LSM510-DUO confocal microscope with a 40x C-Apochromat objective (NA=1.2W) was used. Fluorescence signals of GFP (excitation 488 nm, emission 505-550nm), FM 4-64 and FM 5-59 (both excitation 561 nm, emission >575 nm) were detected. Sequential scanning was used to avoid any cross-talk of fluorescence channels.
To separate FM1-43 and GFP fluorescence, spectral fluorescence detection and subsequent linear unmixing was applied. Lambda series (excitation 488 nm and 561 nm) were collected with Zeiss META system. Nineteen channels (10.7 nm wide) of the META detector were used to span an emission range of 497-700 nm. Linear unmixing was performed using the Zeiss LSM Image Examiner software. Reference spectra of GFP and FM1-43 were acquired from single labeled specimens.

Auxin accumulation:
^{3}H-NAA and ^{3}H-2,4-D accumulation into the cells was measured in 0.5 ml cell suspension aliquots according as described in Petrášek *et al* (2003) and Petrášek *et al.* (2006). FM dyes were added at the beginning of the accumulation assay.

Fig. 1:
Structure of FM dyes

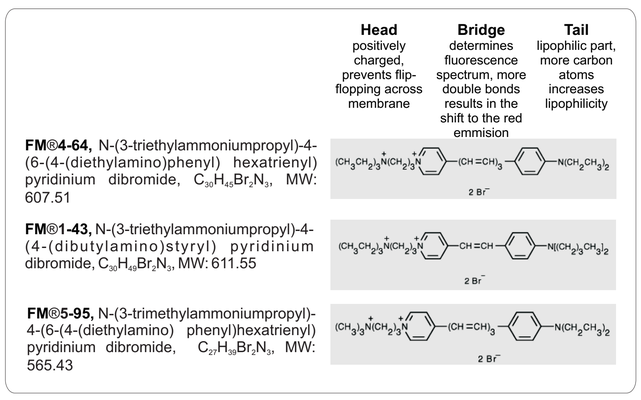

Fig. 1: The structure of FM dyes used in this study. Positively charged head region prevents flip-flopping of the dye across membrane, which fact makes these dyes optimal for tracking endocytosis. Bridge region determines fluorescence of the molecule. More double bonds in this region in FM4-64 and FM5-95 shift the emmision fluorescence to the red part of the spectrum (emmision maximum 734 nm) in contrast to FM1-43 (emmision maximum 626 nm). Tail region is responsible for the lipophilicity of the molecule. Higher amount of carbons increase the lipophilicity (Betz et al., 1996). Among tested FM dyes the most lipophilic is FM 1-43 with four carbons followed by FM4-64 and FM5-95.

Fig. 2:
FM4-64 stimulates transient internalization of PIN1-GFP in BY-2 cells

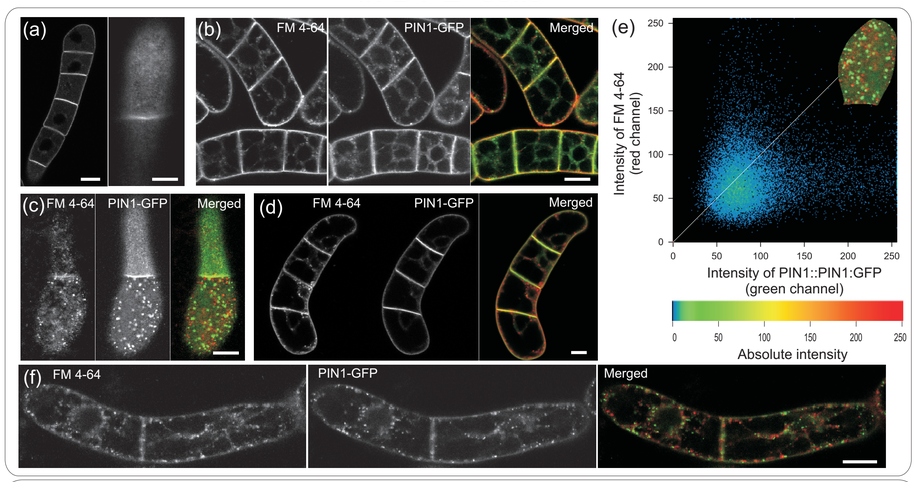

Fig. 2: FM4-64 stimulates transient internalization of plasma membrane-located PIN1-GFP auxin efflux carrier. In control exponentially growing BY-2 cells expressing AtPIN1 protein in translational fusion with GFP the PIN1-GFP was located both at plasma membranes (preferentially transversal) and in the endomembranes of cortical cytoplasm **(a)**. After very short incubation of the BY-2 suspension cells (2 to 10 min) with 2μM FM 4-64 "doting" of the GFP signal was observed **(b)**. This effect was observed before or just together with the endocytosis of FM4-64. There was no colocalization of vesicles containing PIN1-GFP and FM4-64 **(c, e)**. Prolonged incubation (up to 30 min) with FM4-64 resulted in the remarkable endocytosis of FM4-64, but PIN1-GFP vesicles disappeared from cortical cytoplasm restoring control-like situation in cell chains **(d)** but they interestingly remained in more elongated cells even after longer treatment of 1.5h with FM4-64 **(f)**. Scale bars 20 μm.

Fig. 3:
FM1-43 in contrast to FM4-64 and FM5-95 has no effect on the internalization of PIN1-GFP

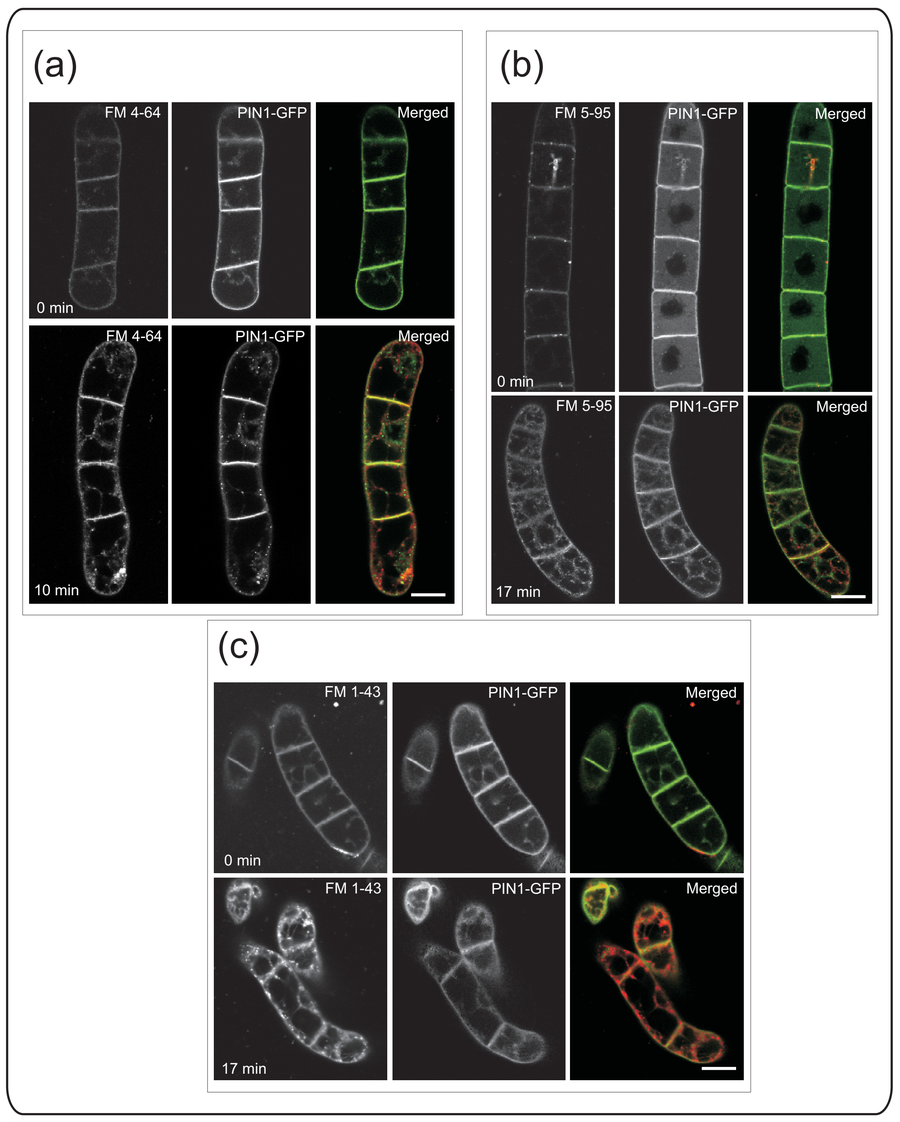

Fig. 3: FM5-95 and FM1-43 have contrasting effects on the internalization of PIN1-GFP. The comparison of the effect of FM4-64 **(a)** and other two endocytotic markers FM5-59 **(b)** and FM1-43 **(c)** after loading cells with 2 μM FM dye on ice an subsequent observation at room temperature. Although with slower kinetics, the FM5-95, slightly less lipophilic analog of FM4-64, induced internalization of PIN1-GFP **(b)**. In contrast, the most lipophilic dye FM1-43 was never inducing any internalization **(c),** even after prolonged incubation (data not shown). Scale bars 20 μm

Fig. 4:
Internalization of PIN1-GFP after FM4-64 is blocked by sodium azide, but not by BFA

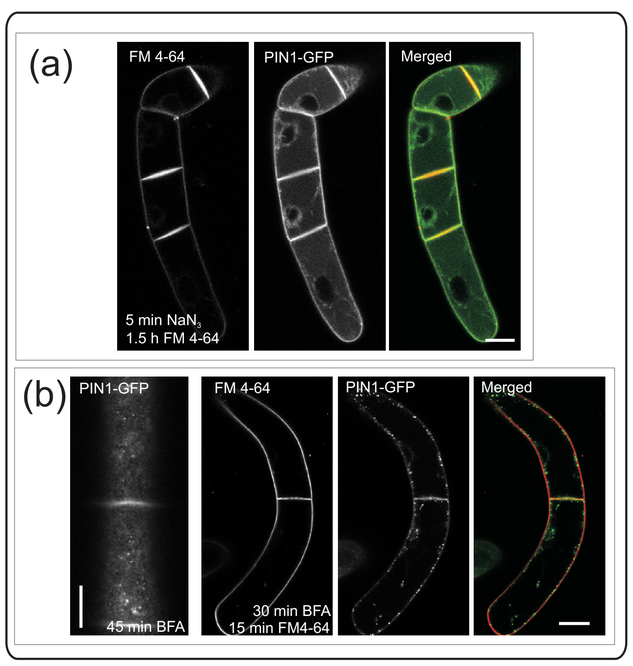

Fig. 4: Internalization of PIN1-GFP after FM4-64 is an active process. Treatment of BY-2 cels with 15 mM sodium azide (NaN_3) totaly prevented both FM4-64 endocytosis and PIN1-GFP internalization **(a)**. This indicates that this internalization is an active process. In contrast, 30 min treatment with 20 μM BFA, the inhibitor of anterograde vesicle trafficking, resulted in the massive formation of PIN1-GFP-containing vesicles after FM4-64 **(b)**. Control cells after 30 min with 20 μM BFA and without FM4-64 had no or much less BFA-induced PIN1-GFP patches formed. Scale bars 20 μm.

Fig. 5:
The activity of auxin influx and efflux carriers is differentially influenced by FM dyes

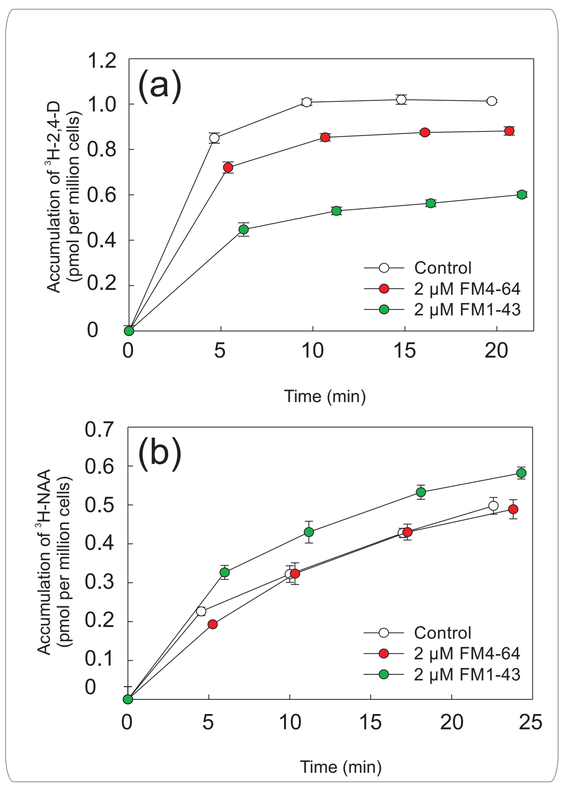

Fig. 5: The activity of plasma membrane-located auxin influx and efflux proteins is influenced by both FM1-43 and FM4-64. To test the auxin influx and efflux through carriers, radioactively labeled synthetic auxins 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthalene-1-acetic acid (NAA) were used as markers, respectively (Petrasek *et al.*, 2006). The active auxin influx was decreased immediately after the addition of 2 μM FM1-43 and to lesser extent after 2 μM FM4-64 as indicated by significantly decreased auxin accumulation in 2-day-old BY-2 cells **(a).** The active auxin efflux was also influenced by FM1-43, but FM4-64 had no effect **(b)**. Error bars represent SEs of the mean (*n=4*).

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Probing endocytosis with FM dyes in plants: Tracking or dragging?

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Introduction

Amphiphilic styryl dyes of FM family, originally developed to stain synaptic vesicles *in vivo* (Betz and Bewick, 1992; Gaffield and Betz, 2006), are very often used in tracking endocytosis in plants (for review see Bolte et al., 2004). Thanks to the character of their molecules **(Fig. 1)**, the application to plant tissues or cell cultures results in their insertion into plasma membrane, where they start to be fluorescent after incorporation into lipid bilayer. Depending on cell type, they are quickly internalized into cells by active processes of endocytosis followed by their incorporation into endomembrane system including tonoplast and the whole plasma membrane recycling machinery.

Although there are some rare reports about the effect of styryl-based dyes on the membrane fluidity (Rodes *et al.*, 1995) and FM dyes were reported to block mechanotransduction channels (Gale *et al.*, 2001) and the activity of muscarinic and nicotinic acetylcholine receptors (for review see Gaffield and Betz, 2006) FM dyes are considered to be valuable tool for tracking of endocytosis in plant cells (Bolte *et al.*, 2004; Emans *et al.*, 2002).

Here we show that even very low concentrations of FM dyes might have some additional effects on the localization and activity of plasma membrane located proteins, namely auxin influx and efflux carriers.

Results and Conclusions

During studies of endosomal trafficking of the auxin efflux machinery components, FM styryl dyes were often used in our laboratory in tobacco BY-2 cell line. While it was very easy to reproduce already published experimental procedures describing the application of FM dyes (for review Bolte *et al.*, 2004) to cells in suspension, additional, yet uncharacterized effect of FM4-64 treatment was always observed. The application of low amount of FM4-64 (2 μM) to the BY-2 cells expressing PIN1 auxin efflux protein from *Arabidopsis thaliana* (PIN1::PIN1:GFP) (Benková *et al.*, 2003; Zažímalová *et al.*, 2007) resulted in the transient internalization of plasma membrane-located auxin efflux carrier PIN1-GFP **(Fig. 2)**. While control cells showed clear plasma membrane localization of PIN1-GFP **(Figure 2a** and Petrasek and Zazimalova 2006), already after 2 minutes of treatment with FM 4-64, remarkable increase in the endosomal pool of PIN1-GFP was observed. The timing of this phenomenon clearly preceded the endocytosis of FM 4-64 itself **(Fig. 2b)** and was observed eve when 0.2 μM FM4-64 was used (data not shown). Concentrations used in our experiments are 10-100 times lower that is reported to be non toxic for plant cells (Bolte *et al.*, 2004). There was no colocalization of vesicles containing PIN1-GFP and FM4-64 **(Fig 2c, e)**. Prolonged incubation (up to 30 min) with FM4-64 resulted in the remarkable endocytosis of FM4-64, but PIN1-GFP vesicles disappeared from cortical cytoplasm restoring control-like situation in cell chains **(Fig. 2d)** but they interestingly remained in more elongated cells even after longer treatment of 1.5h with FM4-64 **(Fig. 2f)**. This suggests cell type-specific response.

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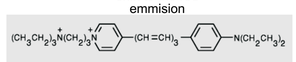
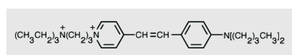
Fig. 1:
Structure of FM dyes

	Head	Bridge	Tail
	positively charged, prevents flip-flopping across membrane	determines fluorescence spectrum, more double bonds results in the shift to the red emmision	lipophilic part, more carbon atoms increases lipophilicity

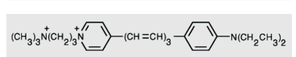
FM®4-64, N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl) hexatrienyl) pyridinium dibromide, $C_{35}H_{45}Br_2N_3$, MW: 607.51



FM®1-43, N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl) pyridinium dibromide, $C_{36}H_{49}Br_2N_3$, MW: 611.55



FM®5-95, N-(3-trimethylammoniumpropyl)-4-(6-(4-(diethylamino) phenyl)hexatrienyl) pyridinium dibromide, $C_{27}H_{39}Br_2N_3$, MW: 565.43



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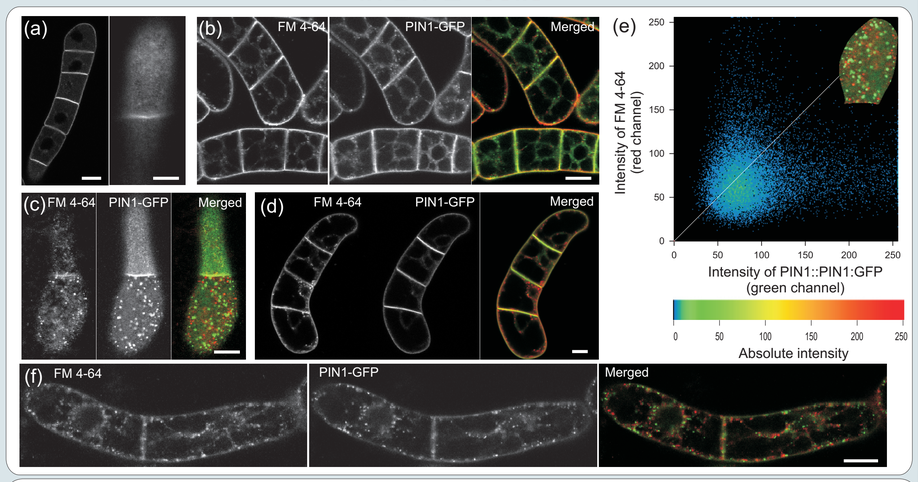

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FM1-43 in contrast to FM4-64 and FM5-95 has no effect on the internalization of PIN1-GFP

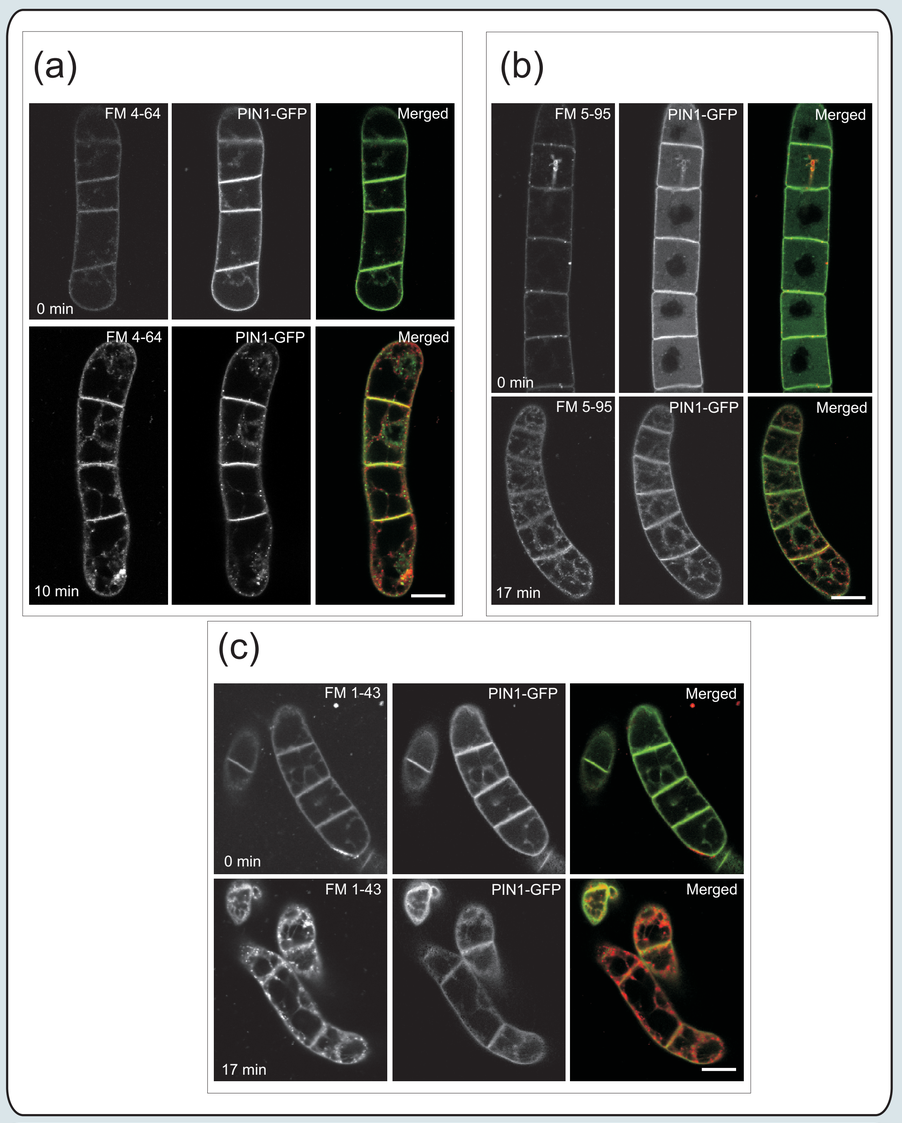

Fig. 3: FM5-95 and FM1-43 have contrasting effects on the internalization of PIN1-GFP. The comparison of the effect of FM4-64 **(a)** and other two endocytotic markers FM5-59 **(b)** and FM1-43 **(c)** after loading cells with 2 μM FM dye on ice an subsequent observation at room temperature. Although with slower kinetics, the FM5-95, slightly less lipophilic analog of FM4-64, induced internalization of PIN1-GFP **(b)**. In contrast, the most lipophilic dye FM1-43 was never inducing any internalization **(c),** even after prolonged incubation (data not shown). Scale bars 20 μm

Fig. 4:
Internalization of PIN1-GFP after FM4-64 is blocked by sodium azide, but not by BFA

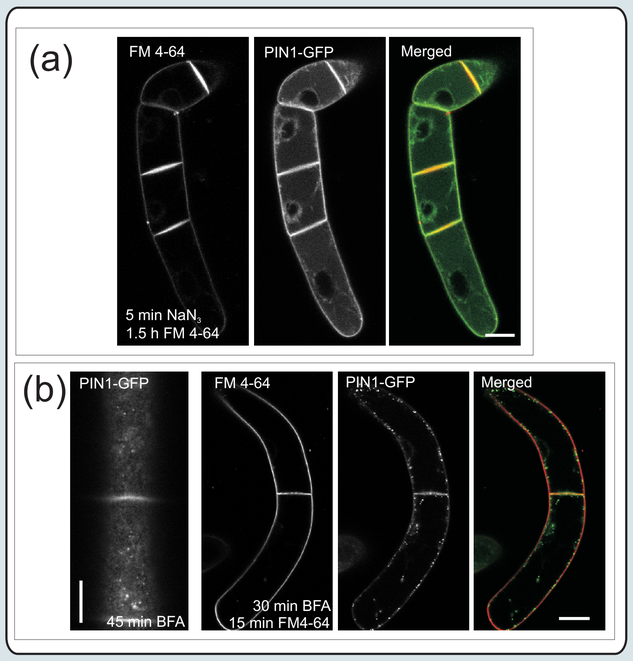

Fig. 4: Internalization of PIN1-GFP after FM4-64 is an active process. Treatment of BY-2 cels with 15 mM sodium azide (NaN_3) totaly prevented both FM4-64 endocytosis and PIN1-GFP internalization **(a)**. This indicates that this internalization is an active process. In contrast, 30 min treatment with 20 μM BFA, the inhibitor of anterograde vesicle trafficking, resulted in the massive formation of PIN1-GFP-containing vesicles after FM4-64 **(b)**. Control cells after 30 min with 20 μM BFA and without FM4-64 had no or much less BFA-induced PIN1-GFP patches formed. Scale bars 20 μm.

Fig. 5:
The activity of auxin influx and efflux carriers is differentially influenced by FM dyes

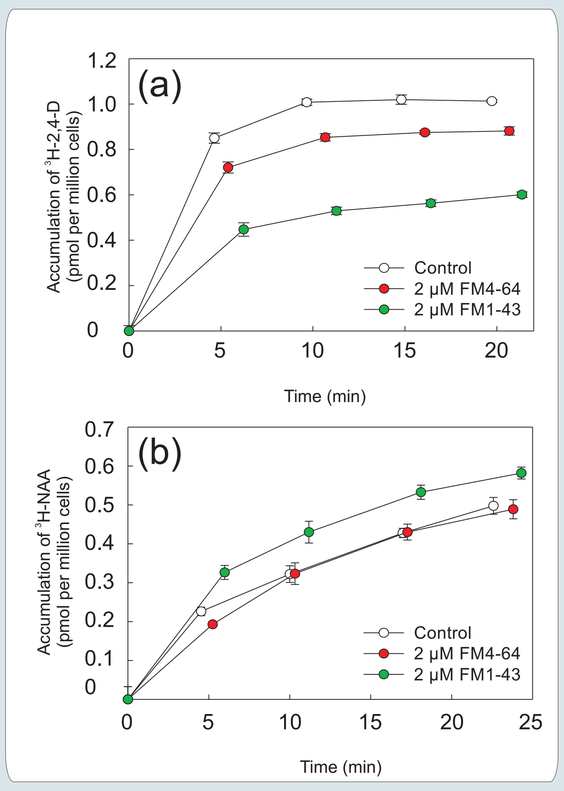

Fig. 5: The activity of plasma membrane-located auxin influx and efflux proteins is influenced by both FM1-43 and FM4-64. To test the auxin influx and efflux through carriers, radioactively labeled synthetic auxins 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthalene-1-acetic acid (NAA) were used as markers, respectively (Petrasek *et al.*, 2006). The active auxin influx was decreased immediately after the addition of 2 μM FM1-43 and to lesser extent after 2 μM FM4-64 as indicated by significantly decreased auxin accumulation in 2-day-old BY-2 cells **(a).** The active auxin efflux was also influenced by FM1-43, but FM4-64 had no effect **(b)**. Error bars represent SEs of the mean (*n=4*).

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