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# Plasma membrane sterol complexation, generated by filipin, triggers signaling responses in tobacco cells

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ABSTRACT

The effects of changes in plasma membrane (PM) sterol lateral organization and availability on the control of signaling pathways have been reported in various animal systems, but rarely assessed in plant cells. In the present study, the pentaene macrolide antibiotic filipin III, commonly used in animal systems as a sterol sequestrating agent, was applied to tobacco cells. We show that filipin can be used at a non-lethal concentration that still allows an homogeneous labeling of the plasma membrane and the formation of filipin-sterol complexes at the ultrastructural level. This filipin concentration triggers a rapid and transient NADPH oxidase-dependent production of reactive oxygen species, together with an increase in both medium alkalinization and conductivity. Pharmacological inhibition studies suggest that these signaling events may be regulated by phosphorylations and free calcium. By conducting FRAP experiments using the di-4-ANEPPDHQ probe and spectrofluorimetry using the Laurdan probe, we provide evidence for a filipin-induced increase in PM viscosity that is also regulated by phosphorylations. We conclude that filipin triggers ligand-independent signaling responses in plant cells. The present findings strongly suggest that changes in PM sterol availability could act as a sensor of the modifications of cell environment in plants leading to adaptive cell responses through regulated signaling processes.



## 1. Introduction

The lipidic composition of the plasma membrane (PM) of eukaryote organisms is essential for normal cell function, and alterations in the distribution or metabolism of lipid components may have serious consequences for cells and organisms [1]. In particular, several lines of evidence indicate that signaling can be controlled by changes in the lateral segregation of membrane components following alterations in the organization of PM lipids. Although there is increasing evidence that the lipid structure and the biophysical properties of membrane bilayers have significant effects on the activity of membrane proteins [2], the mechanisms by which lipid ordering affects signaling is not clear. In biological membranes, one of the most important determinants of membrane organization and of the bilayer biophysical properties is the amount of free cholesterol, the level of which is tightly regulated at the cellular and organism levels [3,4].

In recent years, the physiological importance of sterols in the cell plasma membrane has attracted attention and consequently, the use of methods allowing a manipulation of its content increased sharply. In animal cell cultures, cellular cholesterol levels are relatively easy to modify and cholesterol depletion is commonly used to test whether lipid organization and changes in membrane biophysical properties play a part in signaling pathways [3,5–15]. The polyene macrolide antibiotic filipin, the most important tool to visualize the localization of free cholesterol in cells and tissues [16], has thus been widely used as a sterol complexing drug to specifically reduce both cholesterol availability and lateral mobility at the plasma membrane of numerous animal and yeast cells. Filipin-induced changes in cholesterol organization and availability were shown to participate in the control of various receptor-mediated signaling pathways [8–13].

Instead of one major sterol—cholesterol or ergosterol—found in mammalian or fungal cells, plant cells contain a mixture of sterols that are essential components of the plasma membrane. Although their chemical structure resembles that of cholesterol, and their cellular functions are supposed to be similar, very little is known about their role in physiological responses. However, a few promising *in vitro* data report on their putative involvement in signaling processes. Both sterol concentration and molecular species were shown to modulate the PM $H^+$-ATPase activity in vesicles prepared with

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soybean phospholipids and various concentrations of individual sterols [17]. More recently, it has been established that the sterol-rich detergent resistant membranes (DRM), the counterpart of lipid rafts, are enriched in signaling proteins [18,19], thus suggesting that PM sterol organization would play an important role in cell responses.

It has been well established that filipin interacts with individual or mixed plant PM sterols [20,21] and that this interaction leads to the formation of complexes which can be observed in freeze-fractured or negatively stained membranes, as it is the case for animal and yeast cells [22–29]. However, until recently, reports about the physiological effects of filipin on living plant cells were scarce and mainly described changes in membrane permeability for various components such as potassium in etiolated stems of *Pisum sativum* [30], betacyanine in *Beta vulgaris* roots [26] or undetermined intracellular material absorbing at 260 nm in *Triticum aestivum* endosperms [31]. However, these experiments did not specifically focus on signaling processes, and most of them were performed using high concentrations of a filipin complex (a mixture of 4 pentaenes) that rapidly induced cell death, thus limiting the decrypting of the involvement of PM sterol organization in physiological responses. Filipin III was more recently reported to specifically interact with 3β-OH sterols (namely sitosterol, campesterol and stigmasterol) and used as a probe for sterol detection and fluorescence co-labeling studies in living *Arabidopsis* plants [27–29,32]. Filipin III was shown to interfere with cell polarization by inhibiting PIN protein endocytosis in *Arabidopsis* roots [29,32], and to inhibit root hair and pollen tip growth in *Arabidopsis* and *Picea* [33,34], strongly suggesting that a change in PM sterol lateral organization is a key element in controlling cell growth in plants.

The aim of the present work was to understand the determinants of such effects by analyzing the very early effects of a non-lethal concentration of purified filipin III on signaling responses in cultured tobacco cells. We report that at such concentration filipin still allows the formation of filipin-sterol complexes at the PM and leads to an early modification of PM fluidity. We also show that filipin triggers a rapid and transient production of reactive oxygen species, together with ion fluxes across the PM. Finally the regulation of all these signaling events was investigated and their link with PM lateral organization discussed.

## 2. Materials and methods

### 2.1. Materials

*Nicotiana tabacum* cv. Bright Yellow 2 (BY-2) cells and the BY-2 cell line gp3, impaired in NADPH oxidase activity [35], were grown in a modified Murashige and Skoog medium [36], according to the procedure described in [30]. Filipin III, $LaCl_3$, and staurosporine were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Filipin and staurosporine were added to cell suspension from concentrated stock solutions in DMSO. Final DMSO concentrations did not exceed 0.5% (v/v). Equivalent volumes of DMSO were added to controls. 2-Hydroxy-3-(N,N-di-methyl-N-hydroxyethyl)ammoniopropyl]-4-[β-[2-(di-n-butylamino)-6-napthyl] vinyl] pyridinium dibromide (di-4-ANEPPDHQ) was purchased from Molecular Probes Inc. (Eugene, OR, USA).

### 2.2. Filipin III treatments

Cells were harvested 7 days after subculture, filtered, resuspended (1 g/10 mL) in an incubation medium (2 mM MES buffer pH 5.9, containing 175 mM mannitol, 0.5 mM $CaCl_2$ and 0.5 mM $K_2SO_4$). After a 3 h equilibration period on a rotary shaker (140 rpm) at 25 °C, cells were treated with filipin and chemicals as indicated in the legends of the figures.

### 2.3. Reactive oxygen species (ROS) production, extracellular pH modifications, medium conductivity and $K^+$ efflux

ROS production, extracellular pH, medium conductivity and extracellular $K^+$ were measured at intervals, in the incubation medium. The production of $H_2O_2$ was measured by chemiluminescence using luminol and a luminometer (BCL book, Berthold, la Garenne Colombe, France). A 250 µL aliquot of the cell suspension was added to 50 µL of 0.3 mM luminol and 300 µL of the assay buffer (175 mM mannitol, 0.5 mM $CaCl_2$, 0.5 mM $K_2SO_4$ and 50 mM MES pH 6.5). Extracellular pH modifications were monitored using a Radiometer pH meter. Medium conductivity was monitored using a B-173 conductivity meter (Horiba Instruments Ltd., Kyoto Close, Northampton, UK). The extracellular concentration of potassium was measured by flame photometry (Flame Photometer 410, Ciba Corning, Alameda, CA, USA). Samples were diluted in 15 mM LiCl, the standard was prepared by dilution of 100 mM KCl in 15 mM LiCl.

### 2.4. Cell viability

Viability of cells was tested in parallel by addition of Evans blue (0.05% final concentration), which penetrates only dead cells and leads to a blue staining of cells, and by addition of fluorescein diacetate (0.01% final concentration from a 0.5% stock solution in acetone), specific to living cells and leading to a yellow-green staining of the cytoplasm.

### 2.5. Light and transmission electron microscopy

Living cells were examined in a Zeiss Axiophot microscope (Carl Zeiss, Göttingen, Germany) equipped with differential interference contrast optics, a ×40 objective lens (NA = 0.75), and epifluorescence (filters for filipin: G365/FT395/LP420; filters for fluorescein diacetate: BP450–490/FT510/BP515–565). Images were taken with a Canon PowerShot A650IS (Canon, Tokyo, Japan). Merged images were composed using Adobe Photoshop 6.0 (Adobe systems, San Jose, CA). For transmission electron microscopy, suspension cells were fixed in 100 mM sodium phosphate buffer (pH 7.2) containing 2.5% (v/v) glutaraldehyde for 4 h at 4 °C. After fixation, cells were washed several times in the same buffer over a period of 1 h. Cells were then pelleted by low-speed centrifugation (1 min, 3000 rpm) and embedded in 2.5% (w/v) agarose. Agarose-blocks containing cells were then treated with 1% (w/v) osmium tetroxide in 100 mM sodium phosphate buffer for 1 h at 4 °C and post-fixed with 1% tannic acid in the same buffer for 30 min at room temperature in the dark. Cells were then dehydrated through a graded ethanol series and propylene oxide, and embedded in Epon (Spi-Chem, Neyco, Paris, France) according to the standard procedure for conventional transmission electron microscopy [37]. Ultra-thin sections (80 nm) were cut on a Reichert Ultracut E ultramicrotome (Leica, Rueil-Malmaison, France). They were collected on grids and counterstained with 3% (w/v) uranyle acetate in ethanol and lead citrate for conventional transmission electron microscopy. Sections were examined with a Hitachi H7500 transmission electron microscope (Hitachi Scientific Instruments Co., Tokyo, Japan) operating at 80 kV and equipped with an AMT camera driven by AMT software (AMT, Danvers, USA).

### 2.6. Fluorescence recovery after photobleaching

BY-2 cells were labeled for 60 s with 1 µL 1.5 mM di-4-ANEPPDHQ stock solution (DMSO) added in 1 mL cell suspension, then washed in incubation medium before filipin was added. A Leica TCS SP2-AOBS laser scanning confocal upright microscope (Leica Microsystems, Wetzlar, Germany) was used for all experiments. All experiments were done at room temperature (22 °C) in incubation medium after 5 min filipin treatment. The 488 nm line of an argon laser was used for

excitation and the detection bandwidth was 510–680 nm. Cells were observed using a Plan Apo 40× oil immersion (N.A. 1.25) and detection pinhole was set to the optimum value of 1 Airy unit. Image format was 512×512 pixels and scan speed was set to 800 Hz. The fluorescence intensity of two regions of interest (ROIs) of 2 μm×2 μm was measured: the photobleached area and a region within the cell that was not photobleached to check for overall photobleaching and cell position fluctuation. Background signal was found negligible by setting lower photomultiplier tube gain. After 5 prebleach scans (one scan every 800 ms) at 5% maximal laser power to determine initial fluorescence intensity, one photobleaching scan was done at 100% laser power. Post bleach fluorescence recovery was then sampled at 5% laser power for 120 s. Systematically, a second FRAP measurement was performed on exactly the same cell and bleach region. Experimental recovery curves were corrected for possible bleaching and were fitted to Eq. (**1**) by non-linear regression analysis using Levenberg–Marquadt algorithm:

$$F(t) = \left(F_0 + F\infty \times \left(t / t_{1/2}\right)\right) / \left(1 + \left(t / t_{1/2}\right)\right) \quad (1)$$

where $F_0$ is the immediate post bleach intensity, F∞ is the asymptote of fluorescence recovery and $t_{1/2}$ is the time required to recover 50% of the asymptote fluorescence. The mobile fraction $M_f$ was given by the following equation:

$$M_f = (F\infty - F_0) / (F_i - F_0) \quad (2)$$

where $F_i$ is the prebleach fluorescence intensity. Significance of statistical differences for $t_{1/2}$ and $M_f$ data were given by the non-parametric Mann–Whitney U-test (1% confidence level). For representation purposes, post bleach fluorescence intensities in FRAP recovery curves were normalized according to:

$$F(t)^{\text{norm}} = (F(t) - F_0) / (F\infty - F_0). \quad (3)$$

### 2.7. Plasma membrane isolation

Plasma membranes were isolated from BY-2 cells according to the procedures described in [38].

### 2.8. Laurdan spectrofluorimetry

Fluorescence measurements were performed on a Fluorolog-3 FL3-211 spectrometer (Jobin-Yvon, Horiba Group) in the T-format with a xenon arc lamp as light source. All fluorescence signals were recorded with emission and excitation bandwidths of 1 nm and 5 nm respectively, with integration time of 1 s, and systematically corrected from light scattering of an unlabeled sample. All data acquisition were done with the Datamax software (Jobin-Yvon/Thermo Galactic Inc.). Samples were stirred and equilibrated in a temperature-controlled chamber using a thermoelectric Peltier junction (Wavelength Electronics Inc.). Experiments were done using a 10 mm Special Optic Glass path cuvette filled with 2.5 mL of PM adjusted to a lipid concentration of 0.04 g $L^{-1}$ in buffer (10 mM Tris–MES, pH 7.5, 1 mM EDTA, 250 mM sucrose). The probe-to-lipid ratio was estimated to 1:300. PM was labeled with 1.7 μL 0.4 mM Laurdan stock solution (DMSO) added in the cuvette, and incubated at 4 °C during 15 min in the dark. All the measurements were performed at 22 °C.

## 3. Results

### 3.1. Filipin can interact with PM sterols without inducing cell death

Tobacco cells were treated with increasing concentrations of filipin III (0–20 μg/mL) for 8 h. Viability tests showed that no cell death occurred during the 8 h of the treatment for the concentrations of 1 μg/mL (Fig. 1A). On the contrary, cell death rapidly occurred between 2 and 3 h of treatment for the concentration of 20 μg/mL (Fig. 1A).

We observed cells labeled by these two concentrations of the antibiotic. At the whole cell level, filipin fluorescence was immediately observed at the periphery of the cells for both concentrations (Fig. 1B–D). The induction of plasmolysis using NaCl revealed that the labeling was homogenous and mainly localized at the plasma membrane (Fig. 1C,D). Although the fluorescence was less intense with filipin 1 μg/mL, the labeling was qualitatively identical over the plasma membrane (Fig. 1D). Moreover, no modification of the fluorescence pattern occurred during the 30 min time of incubation, suggesting (i) that no detectable plasma membrane sterol internalization occurred in tobacco BY-2 cells in the presence of filipin, and (ii) that all the further described effects of filipin on tobacco cells can be attributed to its initial binding to the plasma membrane.

At the ultrastructural level, electron microscopy pictures indicate that the plasma membrane of cells incubated for 5 min with filipin 20 μg/mL appeared corrugated with numerous 40 nm protuberances (Fig. 1E middle). Since no protuberances were observed in control cells (Fig. 1E top), these structures would correspond to filipin-sterol complexes. On the contrary, the plasma membrane of cells incubated with 1 μg/mL filipin was not significantly modified by the treatment, compared to control cells, and very scarce small protuberances were sometimes detected at the outer side of the plasma membrane (Fig. 1E bottom).

To assess the sterol-dependency of the filipin (1 μg/mL) fixation on the PM, we used methyl-β-cyclodextrin. Cyclodextrin led to a depletion of 50 (±4)% of the sterols associated to the PM (Fig. 2A). In cyclodextrin-treated membranes, a decrease of the filipin fixation, quantified by the intensity of PM-associated fluorescence, of 50 (±11)% was observed (Fig. 2B). Since our previous results demonstrated that neither the amount of phospholipids and steryl-conjugates, nor the amount of protein were affected by cyclodextrin [38], this result strongly indicates that filipin 1 μg/mL mainly interferes with PM sterols of BY-2 cells.

Having shown that filipin 1 μg/mL is able to rapidly interact with plant plasma membrane sterols without inducing the death of treated cells, we used this concentration to further study the physiological effects of this polyene antibiotic on tobacco cells.

### 3.2. Filipin induces phosphorylation and $Ca^{2+}$-regulated ion fluxes across the PM

Filipin induced a rapid increase in medium conductivity during the first 10 min of treatment reaching then a steady-state level, leading, after 30 min of treatment, to an increase of 20% compared to the initial value (Fig. 3A). Concomitantly, while extracellular $K^+$ concentrations regularly decreased in control conditions, filipin treatment not only impaired the uptake of $K^+$ by tobacco cells but also induced its regular extrusion (Fig. 3B).

Filipin also triggered a biphasic and significant alkalinization of the incubation medium (Fig. 3C). A rapid alkalinization (0.15 ± 0.02 pH unit) was observed during the first 5 min of filipin treatment, followed by a slower increase in medium alkalinization (0.15 ± 0.3 pH unit from 5 min to 30 min of filipin treatment). Medium alkalinization following filipin treatment corresponds to a maximum loss of about $2.6 \cdot 10^{-4}$ M $H^+$ equivalent.

We next examined whether the filipin-induced changes in medium conductivity and $K^+$ efflux are passive events or if they are regulated at the whole cell level. The calcium channel blocker lanthanum (50 μM) [39] and the protein kinase inhibitor staurosporine (2.5 μM) were added to the culture medium 5 min before filipin treatment. Used alone, both inhibitors did not modify the parameters analyzed during the timecourse of the experiment (Fig. S1 in the

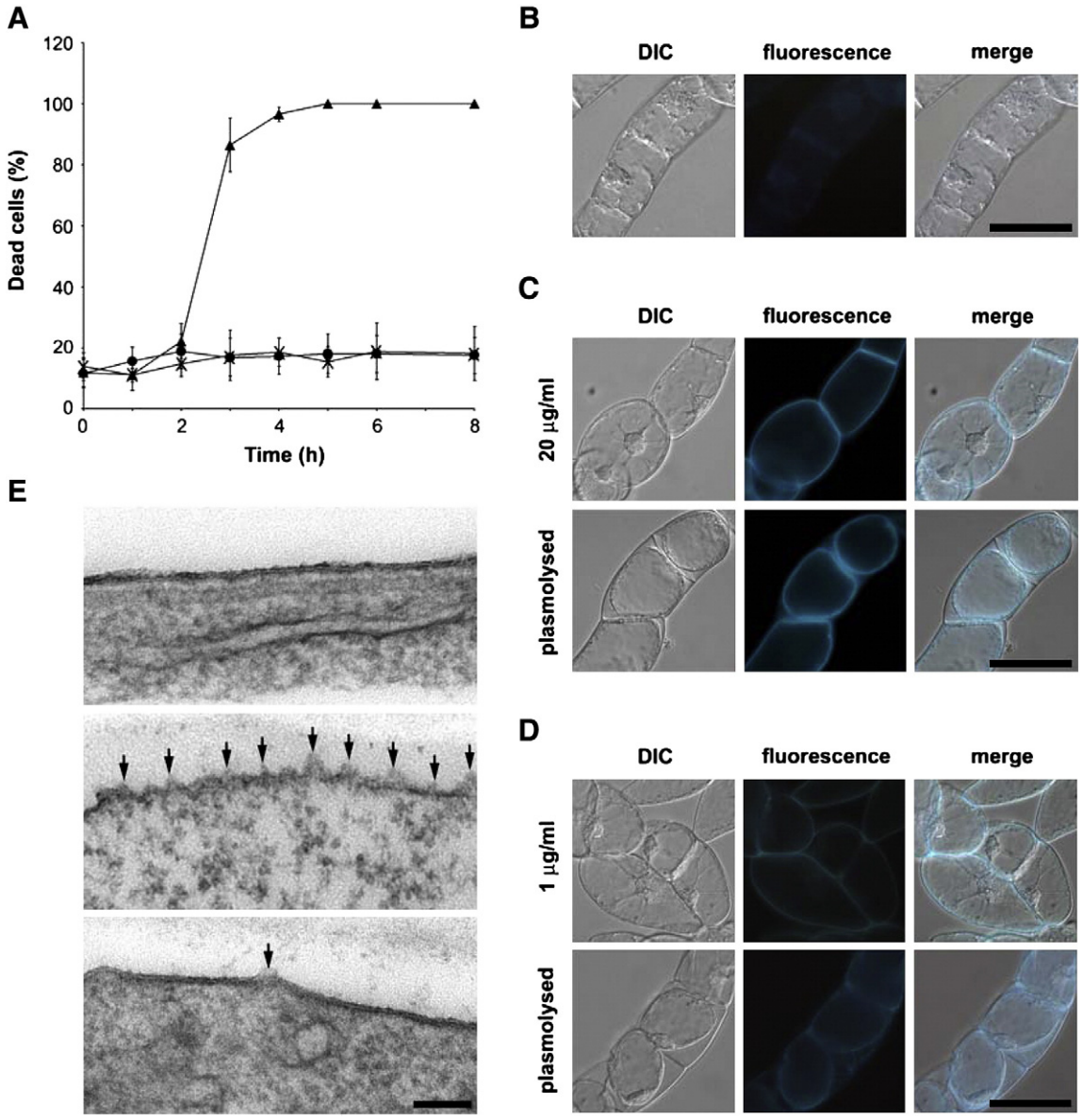


**Fig. 1.** Labeling of tobacco BY-2 cell plasma membrane by filipin III. A. Effects of filipin on cell viability; (▲) cells treated with filipin 20 µg/mL; (●) cells treated with filipin 1 µg/mL; (×) control cells. The values show the average ± SE of three replicate experiments. B–D. Localization of filipin fluorescence at the PM. Cells were incubated for 5 min with filipin 1 µg/mL or 20 µg/mL. B. Control cells. C. Cells treated with filipin 20 µg/mL. D. Cells treated with filipin 1 µg/mL. For C and D, the addition to the culture medium of NaCl that induces plasmolysis clearly shows that the labeling corresponds to the plasma membrane. The scale bars represent 50 µm. E. Ultrastructural effects of filipin on the plasma membrane of BY-2 cells. Control cells (top); cells treated with filipin 20 µg/mL (medium); cells treated with filipin 1 µg/mL (bottom). Arrows show the filipin-sterol complexes. The scale bar represents 100 nm.

supplementary material) but showed different effects on filipin-induced responses. Following the addition of filipin, the increase in medium conductivity was completely abolished in the presence of staurosporine (Fig. 3A), strongly suggesting that protein phosphorylations are crucial regulators of filipin response. The inhibition by lanthanum was much less pronounced and did not affect the initial response (Fig. 3A).

Both staurosporine and lanthanum also significantly reduced the filipin-induced $K^+$ efflux (Fig. 3B). Interestingly, although $K^+$ efflux was reduced by 70% in the presence of lanthanum, the increase of medium conductivity was reduced by only 30%, suggesting that $K^+$ efflux is only partly responsible for the filipin-induced increase in medium conductivity.

The filipin-induced increase of extracellular pH was rapidly and strongly inhibited by staurosporine (80% after the first 5 min of filipin treatment) (Fig. 3C), suggesting that phosphorylations are required for the induction of the filipin-induced medium alkalinization. On the contrary, no significant effect of lanthanum was observed during the first 5 min of filipin treatment, suggesting that calcium channels are not involved in the triggering of medium alkalinization. After the first 5 min of filipin treatment, the filipin-induced medium alkalinization was then inhibited by lanthanum. The similarity of both the evolution of extra cellular conductivity and pH, and their sensitivity to inhibitors, suggest that proton exchanges could represent a major component of the ion fluxes triggered by filipin. These data also indicated that phosphorylation events are required for the initiation of such processes whereas profile calcium fluxes could be needed for the second phase of medium alkalinization. When added 15 min after filipin treatment, staurosporine only blocked medium alkalinization whereas lanthanum triggered a return of the medium pH to its initial value after 60 min (Fig. 3D). These results suggest that the initiation of ion fluxes across the membrane triggered by filipin, is dependent on protein kinase-mediated phosphorylations, whereas maintaining these fluxes requires functional $Ca^{2+}$ channels. As a control, we

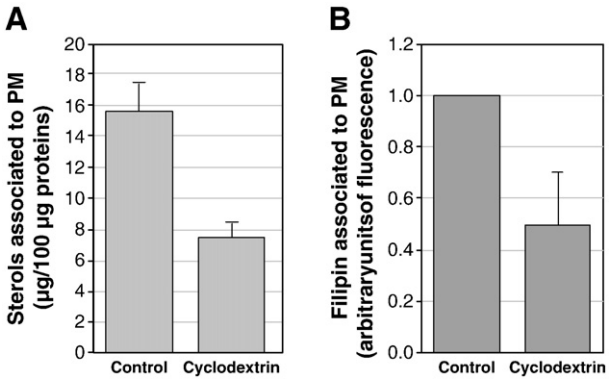


**Fig. 2.** Filipin ability to bind to tobacco plasma membrane is dependent on the sterol content of the membrane. Plasma membrane from tobacco BY-2 cells was treated or not with the sterol chelating agent methyl-β-cyclodextrin for 30 min according to [38]. A. Sterols from purified PM fractions were quantified by gas chromatography. B. PM fractions were incubated for 5 min with filipin at room temperature. After washing and ultracentrifugation (50,000 *g* for 40 min at 4 °C), control and methyl-β-cyclodextrin-treated membranes were spotted on a polyvinylidene fluoride membrane and the filipin associated to PM was quantified by measuring fluorescence remaining in the PM using Quantity One software. The values show the average ±SE of three replicate experiments.

checked the effects of a permeabilizing agent, Triton X-100 0.5%. It resulted in a significant increase in both medium conductivity and leakage of $K^+$ that were not lanthanum or staurosporine-sensitive (Fig. S2 in the supplementary material).

As these results suggested a protein-kinase-mediated regulation of ion fluxes triggered by filipin, we compared the profiles of total phosphorylated proteins from control and filipin-treated cells. The corresponding autoradiography shows that filipin induced the labeling of at least two polypeptides of 99 kDa and 34 kDa (Fig. 4). These filipin-induced phosphorylated polypeptides almost disappeared in the presence of staurosporine.

Altogether, these results clearly indicate that the filipin-induced changes in medium composition are not purely passive and resulting from plasma membrane destabilization following interaction with membrane sterols, but are the consequence of a regulated signaling process involving calcium influx and phosphorylated proteins.

### *3.3. Filipin induces a phosphorylation and $Ca^{2+}$-controlled production of active oxygen species*

The production of reactive oxygen species (ROS) is a very common feature of signaling events in response to various stresses. Filipin triggered a rapid, intense and transient production of ROS revealed by luminol chemiluminescence (Fig. 5). The production of ROS was

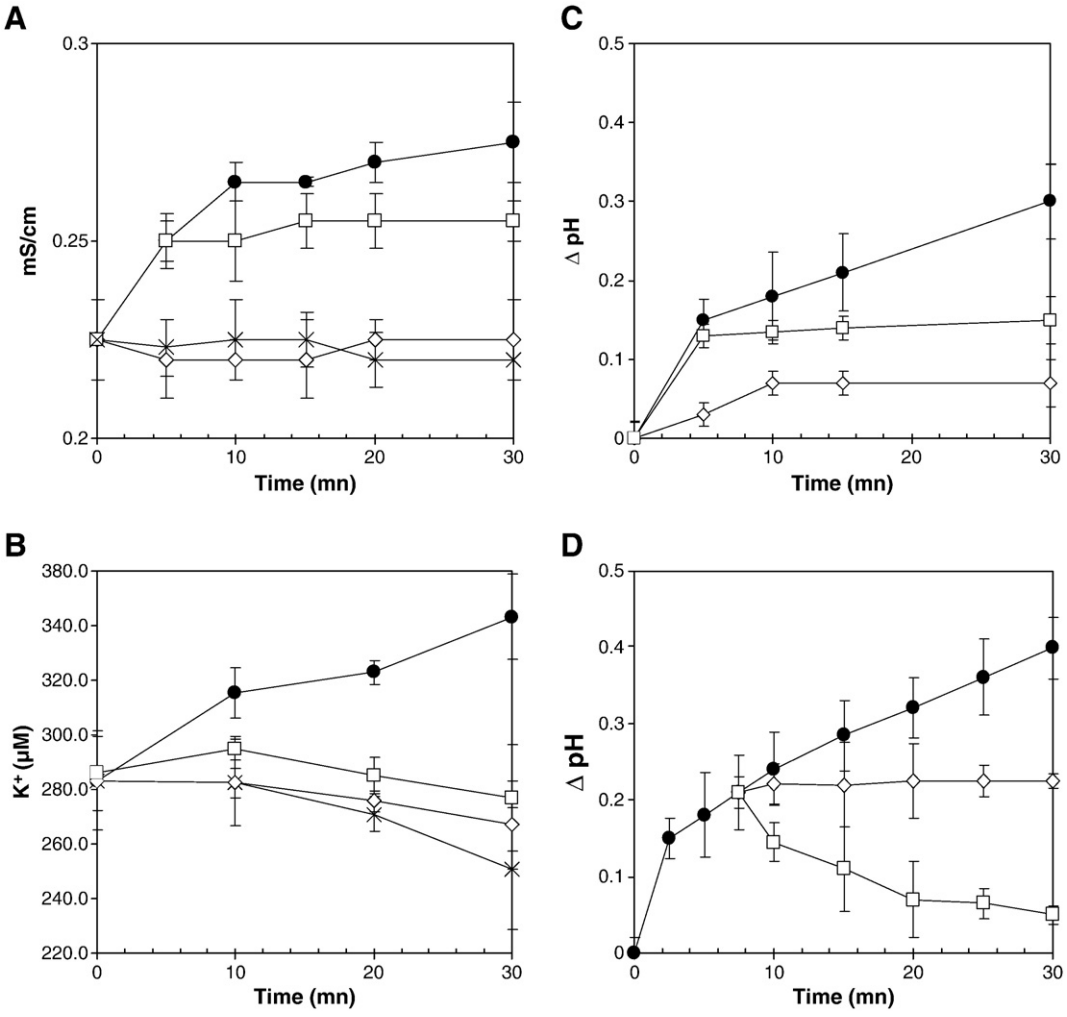


**Fig. 3.** Characterization of the filipin-induced increase in medium conductivity, extracellular $K^+$ and medium alkalinization. A. Effects of staurosporine and lanthanum on filipin-induced increase in medium conductivity. B. Effects of staurosporine and lanthanum on filipin-induced increase in extracellular $K^+$. C–D Effects of staurosporine and lanthanum on filipin-induced increase in extracellular pH. For A, B and C, staurosporine (2.5 µM) or lanthanum (50 µM) was added to cell suspension 5 min before the addition of filipin (1 µg/mL) at t0. For D, staurosporine (2.5 µM) or lanthanum (50 µM) was added to cell suspension 15 min after filipin (1 µg/mL) treatment. (●) cells treated with filipin alone; (◇) cells treated with filipin and staurosporine; (□) cells treated with filipin and lanthanum; (×) control cells. The values show the average ±SE of three replicate experiments.

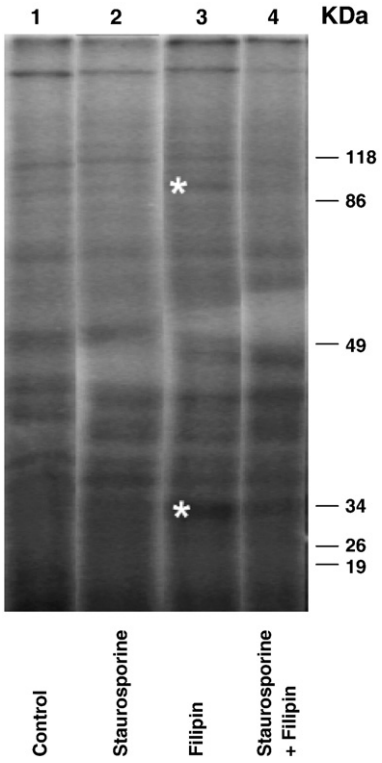


**Fig. 4.** Filipin induces specific protein phosphorylations. Tobacco BY-2 cells were preincubated for 15 min with carrier-free [$^{32}$P]Pi (50 μCi/10 mL incubation medium). Cells were then incubated for 5 min with staurosporine (2.25 μM) or with the same volume of water (control) before filipin (1 μg/ml) was added. After a 5 min incubation period, cells were withdrawn by filtration, washed with 5 mL of cold incubation medium, and immediately frozen in liquid nitrogen. After grinding and TCA precipitation, total protein extracts were subjected to SDS-PAGE followed by autoradiography of the dried gel. Lane 1, control; lane 2, staurosporine (2.5 μM); lane 3, filipin (1 μg/mL); lane 4, staurosporine (2. 5 μM) + filipin (1 μg/mL). The molecular mass (in kDa) is indicated on the right-hand side of the gel. Asterisks indicate phosphoproteins of 99 and 34 kDa.

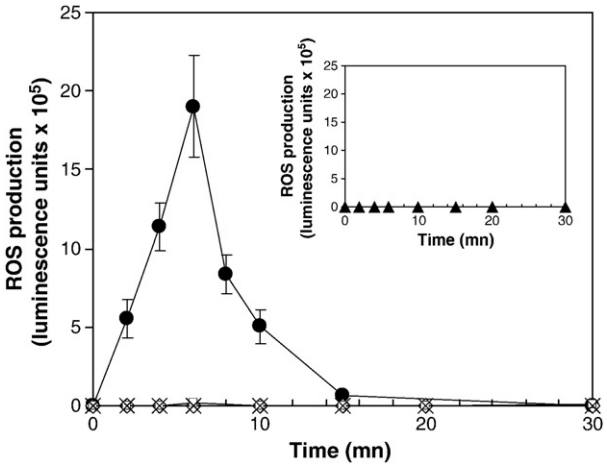


**Fig. 5.** Effects of filipin on the induction of ROS production. Filipin 1 μg/mL was added to BY-2 cell suspension at t0 of the experiment. ROS production was assessed by chemiluminescence as indicated in the "Materials and methods section". When indicated, staurosporine (2.5 μM) or lanthanum (50 μM) were added to tobacco BY-2 cell suspension 5 min before addition of filipin (1 μg/mL). (●) cells treated with filipin alone; (◇) cells treated with filipin and staurosporine; (□) cells treated with filipin and lanthanum; (×) control cells. The values show the average ± SE of three replicate experiments.

detected 2 min after the addition of filipin, reached a maximum at 6 min and returned to the control values 15 min after the beginning of the treatment. When added 5 min before filipin treatment, both staurosporine and lanthanum completely inhibited the production of ROS induced by the antibiotic (Fig. 5). These results show that filipin-induced ROS production is also highly controlled by phosphorylations and calcium fluxes. Although several mechanisms might operate to generate ROS, it has been previously reported that the plasma membrane oxidase NtrbohD is responsible for ROS production in elicited tobacco cells [35]. When tobacco cells transformed with an antisense construct of NtrbohD (gp3 cells) [35] treated with filipin, no ROS production was observed while extracellular medium alkalinization was the same as in wild type cells (Fig. 5, inset). These results show that the plasma membrane NADPH oxidase NtrbohD is responsible for the phosphorylation and $Ca^{2+}$-regulated filipin-induced ROS production in tobacco cells.

### 3.4. Filipin induces regulated changes in plasma membrane fluidity

Modifications in plasma membrane viscosity can be deduced from changes in the lateral mobility of amphiphile fluorescent styryl probes [40,41]. To study the effects of filipin on the fluidity and organization of the plasma membrane of BY-2 cells, we examined the diffusional mobility of the styryl fluorophore, di-4-ANEPPDHQ, following filipin III treatments by conducting FRAP experiments. We preliminarily checked that the relative fluorescence intensity of di-4-ANEPPDHQ is not quenched by the presence of filipin (Fig. S3 in the supplementary material). Averaged normalized fluorescence recovery plots, half-time recovery ($t_{1/2}$) and mobile fraction (Mf) of di-4-ANEPPDHQ in BY-2 plasma membrane for filipin treatments are presented in Fig. 6. As a control, we first analyzed untreated BY-2 cells and found $t_{1/2} = 45 \pm 3$ s (Fig. 6A–B) and $M_f = 82 \pm 4\%$ for the first bleach and $94 \pm 2\%$ for rebleach (Fig. 6A–C). Such high mobile fraction values are consistent with a rather free diffusion of di-4-ANEPPDHQ in the membrane, with only ~10% of the molecules immobilized. It must be mentioned here that $t_{1/2}$ values were equal when bleach and rebleach were performed consecutively on exactly the same cell and region. We next analyzed how the kinetics of fluorescence recovery of di-4-ANEPPDHQ in BY-2 plasma membrane was affected by filipin. As seen from Fig. 6A, the recovery of fluorescence was significantly slowed in response to filipin 1 μg/mL treatment. We found the half-time of recovery $t_{1/2} = 73 \pm 3$ s, noticeably larger than for control experiments (Fig. 6B). The slowing of di-4-ANEPPDHQ lateral diffusion was not accompanied by a change in mobile fraction, since $M_f$ is $82 \pm 4\%$ and $92 \pm 4\%$ for the first and second bleaches, respectively (Fig. 6C). This slowed diffusional mobility in filipin 1 μg/mL treated BY-2 cells suggests an increase of plasma membrane viscosity.

Since staurosporine have been shown to be the most potent inhibitor of filipin-induced signaling events described above, we also considered its influence on changes in membrane fluidity. We must first note that the presence of staurosporine by itself did not significantly affect the kinetics of fluorescence recovery compared to control experiments (Fig. S4 in the supplementary material). Treatment by staurosporine 2.5 μM was done 5 min prior to the addition of filipin. Fig. 6A compares the fluorescence recovery curve for cells treated with both staurosporine and filipin, with fluorescence recovery curves for control and filipin-treated cells. Clearly, staurosporine pretreatment in the presence of filipin restored kinetics of recovery identical to the control experiment. Analysis of the recovery plots resulted in a half-time of recovery of $48 \pm 4$ s equal to that of control and significantly different from filipin alone (Fig. 6B). Mobile fraction was not significantly modified with values of $89 \pm 4\%$ and $98 \pm 1\%$ for bleach and rebleach, respectively (Fig. 6C). This restored diffusional mobility in staurosporine/filipin-treated cells clearly suggests that filipin affects membrane fluidity via a phosphorylation-regulated signaling mechanism.

Filipin-induced increase in PM viscosity was also assessed by studying Laurdan fluorescence. Steady-state Laurdan fluorescence spectroscopy was performed using plasma membrane (PM) isolated

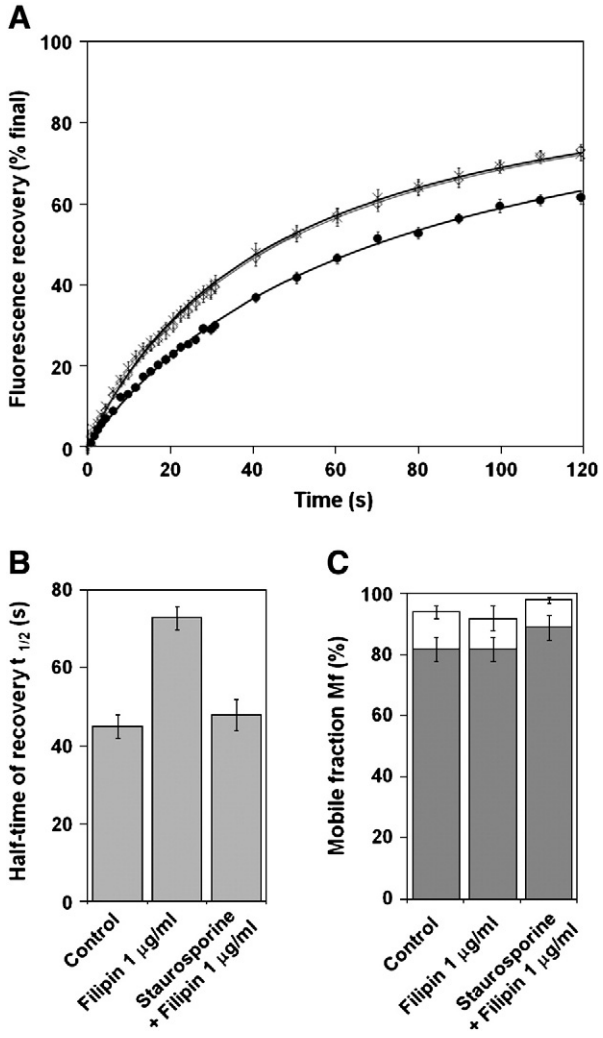


**Fig. 6.** FRAP experiments at the plasma membrane of filipin-treated tobacco BY-2 cells, with or without staurosporine pretreatment. Tobacco BY-2 cells were labeled for 60 s at 22 °C with 1.5 µM di-4-ANEPPDHQ in incubation medium, then washed twice in the same medium before filipin (1 µg/mL) was added for 5 min. A. Normalized fluorescence recovery plot for filipin-treated cells (●) differs from that of control cells (×) but not from cells incubated for 5 min with staurosporine 2.5 µM (◇). Solid lines represent results of fit to Eq. (1). Error bars indicate ± S.E. Note that "normalized plot" means that it is only the mobile fraction that is represented (final percentage: 100%) and that the fluorescence level is not normalized to the initial fluorescence. B. Filipin 1 µg/mL induces an increase in time to half-maximal recovery compared with control and staurosporine pretreatment experiments. C. Mobile fraction is not affected by filipin 1 µg/mL, with or without staurosporine, for the two consecutive bleach and rebleach. For B–C, values are means ± S.E., $p<0.001$. N = 35 cells for control; 34 for filipin 1 µg/mL; 17 with staurosporine pretreatment.

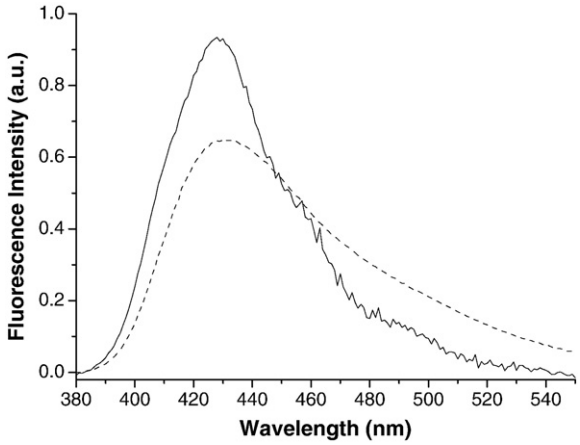


**Fig. 7.** Filipin treatment rigidifies the plasma membrane of tobacco BY-2 cells. Steady-state Laurdan fluorescence spectroscopy was performed on PM isolated from BY-2 cells treated for 5 min with filipin or with the corresponding volume of DMSO for control experiments, before PM preparation. The normalized emission spectra of Laurdan in control PM (dash lines) exhibited a maximum at 430 nm and a broad shoulder is observed at higher wavelength. This shoulder is due to the contribution of an additional band at 490 nm. Such spectral features denote a membrane in the liquid-state. For filipin-treated PM (solid lines), emission spectra of Laurdan were obtained by subtracting filipin spectral contribution (acquired just before Laurdan labeling) to the recorded raw data. Resulting spectra indicated a strong decrease in the ratio of intensities from 490 to 430 nm.

from BY-2 cells previously treated either with 1 µg/mL filipin III or with the corresponding volume of DMSO. Working with PM fractions rather than whole cells ensured to avoid pitfalls, such as Laurdan internalization or autofluorescence. The normalized emission spectra of Laurdan in non-treated PM exhibited a maximum at 430 nm and a broad shoulder is observed at larger wavelength due to the contribution of an additional band at 490 nm (Fig. 7). Such spectral features denote a membrane in the liquid-state. For filipin-treated PM, emission spectra of Laurdan were obtained by subtracting filipin spectral contribution (acquired just before Laurdan labeling) to the recorded raw data. Resulting spectra indicated a strong decrease in the ratio of intensities at 490 to 430 nm (Fig. 7). According to previous data on BY-2 isolated PM [38], this is similar to spectra obtained at low temperature (4 °C). Undoubtedly, filipin treatment strongly rigidifies the plasma membrane of BY-2 cells at 22 °C and probably promotes the predominance of a highly ordered state.

The increase in PM viscosity is unlikely to result from an enrichment of sterols at the PM since no significant differences in sterol levels was observed in membranes isolated from control and filipin-treated cells (Fig. 8A). Since filipin-induced signaling effects could also be the result of sterol redistribution rather than sterol level alteration, we checked the effects of filipin on DRM isolation. DRM fractions were purified according to [38]. Our data revealed that the amount of DRM isolated from control and filipin-treated cells is not significantly modified (Fig. 8B).

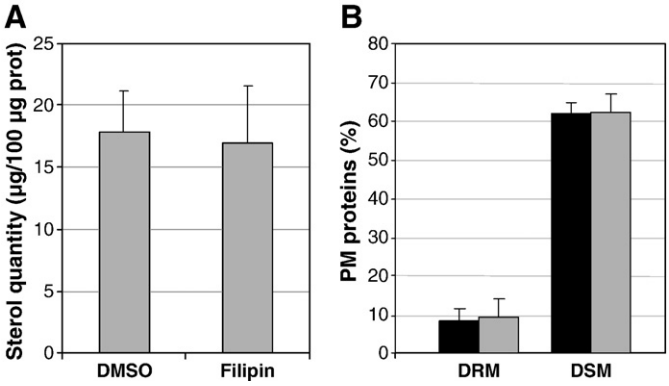


**Fig. 8.** Filipin treatment does not induce significant changes in PM sterol content. A. Tobacco BY-2 cells in the incubation medium were treated with 1 µg/mL filipin for 5 min or with DMSO alone (control). PM purification and PM sterol quantification by gas chromatography were performed according to [38]. The values show the average ± SE of three replicate experiments. No significant differences in PM sterol content were observed between control and filipin-treated membranes. B. Highly enriched PM fractions were purified from cells treated with 1 µg/ml of filipin (gray columns) or with DMSO alone (black columns). After solubilization by Triton X-100 1%, protein fractions were separated through a density gradient and each fraction was precipitated with trichloroacetic acid before quantification. Distribution of proteins between detergent-soluble fraction (DSM) and fraction resistant to solubilization (DRM) is presented as average of three replicate experiments ± SE.

## 4. Discussion

### 4.1. Formation of stable filipin-sterol complexes at the plasma membrane of BY-2 cells

Filipin has been widely adopted for both sterol localization and sequestration in numerous animal and fungi systems and provides an essential tool for those purposes in plants. Whatever the filipin concentrations tested in this study, a continuous labeling was mainly observed at the PM after 5 min of incubation. These results are consistent with those of Grebe et al. [27] who reported the fluorescent detection of PM sterols with filipin in a living plant system. For both concentrations tested, filipin induced the formation of protuberances of about 40 nm mean diameter at the PM of tobacco cells in agreement with previous observations performed in *Arabidopsis* or red beet cells that were treated with 20 or 40 μg/mL, respectively [25,27], and in the range of protrusions from 19 to 94 nm reported in planar phospholipid bilayers containing cholesterol following treatment with 196 μg/mL filipin [42].

Although the observation of such protuberances has been clearly related to the presence of sterols within the membrane and classically interpreted as the visualization of filipin-sterols complexes, the definitive demonstration of this conclusion has not been provided.

The interaction between membrane associated cholesterol of animal cells and filipin has been extensively described [24,43–45]. Concerning the ability of the drug to complex plant sterols, it has been previously established using UV absorption and circular dichroism that filipin interacts with sterols in model and natural membranes containing individual or mixed plant sterols, and induces the formation of filipin-sterol complexes [20]. This is confirmed by our results indicating that the binding of the antibiotic to isolated PM is correlated with their sterol content. None of the well known macrolide antibiotics were demonstrated to associate with something else other than lipids and, filipin interaction with membranes is not dependent on the protein environment [20]. However, in order to test, in our model, the putative involvement of membrane proteins in the binding of filipin, we examined the influence of trypsine incubation of the membrane to the amount of antibiotic associated to the membrane. No significant decrease in filipin fluorescence was observed in treated membranes compared to control membranes (data not shown). Thus, although we cannot definitely exclude that filipin might be associated with a very low abundant proteic receptor, our data strongly suggest that the effects triggered by filipin on tobacco cells are mediated by its interaction with membrane sterols.

### 4.2. Low concentrations of filipin induce signaling events in BY-2 cells without triggering cell death

Filipin treatment has been frequently reported to be linked to rapid cell lysis [24]. We showed here that a filipin concentration of 20 μg/mL rapidly led to cell death. On the contrary, a concentration of 1 μg/mL is not lethal for BY-2 cells. Our results are consistent with those of Kotler-Brajburg et al. who showed that below a concentration of 2 μg/mL, no deleterious effect of the drug was observed for Hela cells, while cell viability fell and lysis occurred for 20 μg/mL [46]. Furthermore, we also report that, at this non-lethal concentration, filipin induced a rapid increase in ionic fluxes across the PM. Cellular leakage or cell permeabilization promoted by filipin have been reported sometime ago using various animal and plant cells or tissues, but mainly for very high filipin concentrations (up to 100 μg/mL) [25,26,30,31,47,48]. Although it has been reported that filipin induces microscopic defects in the bilayer [42,43], we have shown in the current report that both the filipin-induced alkalinization and the increase in medium conductivity and $K^+$ concentration, are highly regulated by the protein kinase inhibitor staurosporine and by the calcium channel blocker lanthanum. These results clearly show that induced ionic fluxes are unlikely to only result from PM lesions. It has been suggested that the filipin-induced loss of $K^+$ would be a consequence of the increase in proton permeability [49]. We cannot exclude that a specific exchange $H^+/K^+$ occurs following filipin treatment. However, this exchange infers a 1:1 $H^+$:$K^+$ stoichiometry that was not found in our system since $H^+$ loss was about 4-times higher that $K^+$ extrusion.

We also report that filipin is able to induce a rapid NADPH oxidase-dependent production of ROS by tobacco BY-2 cells. Moreover, we also show that ROS production is highly regulated by lanthanum and staurosporine, strengthening the fact that the sterol complexing drug filipin triggers ligand-independent signaling responses. Interestingly, it has already been reported that changes in PM cholesterol availability using pharmacological agents can also trigger ligand-independent signaling pathways in various animal systems. For instance, the use of different agents interacting with sterols including filipin, was also reported to induce a ligand-independent activation of the death receptor Fas followed by activation of caspase-8 and apoptosis in keratinocytes [12]. In a recent paper, Mierch et al. clearly demonstrated that modulation of plasma membrane cholesterol in fibroblasts can greatly modulate signaling responses such as nitric oxide signaling [14].

Taken together, these results show that plant plasma membrane sterol availability is a key element in the triggering of signaling responses.

### 4.3. Link between filipin-induced membrane rigidification and cell signaling

Various kinds of environmental events, such as chemical agents, may cause alterations in membrane fluidity of living animal cells and microorganisms [50–52]. Consistently, the current study revealed, using FRAP experiments, that filipin led to a PM rigidification. It can be hypothesized that the simple presence of filipin molecules that intercalated between lipids affected membrane packing, thus membrane fluidity. Although we cannot exclude an effect of the random insertion of filipin modifying the packing of phospholipids and the triggering of fluidity changes, in our conditions, this is probably not, in particular due to the very low amount of filipin used, the predominant mechanisms leading to the responses observed. The fact that the modifications of fluidity induced by filipin are staurosporine-sensitive confirms that they are unlikely to result from a mechanical effect but rather from a signal transduction process.

It has been well established that changes in the levels of plasma membrane cholesterol induce modifications in membrane physical properties such as fluidity. For instance, plasma membrane fluidity has been shown to be modified by either addition of free cholesterol in liposomes or red blood cells [53,54], or following cholesterol depletion using methyl-β-cyclodextrin in various cell systems such as lysosomes [55], or CHO cells [56]. Interestingly, in close agreement with our current results, plasma membrane fluidity was also reported to decrease when isolated membranes or cells that were treated with the cholesterol-binding compounds saponins [57–59] or deoxycholic acid, proved to cause a marked rearrangement of sterols within the plasma membrane of human cells [60].

The rapid filipin-induced rigidification of the PM could also be triggered by changes in PM lateral organization following raft disruption. Since DRM isolation is not affected by the filipin treatment in our system, our results strongly suggest that raft disruption is however unlikely to be responsible for the increase in PM viscosity.

It could be speculated that filipin-induced signaling effects in tobacco cells might result from changes in the biophysical properties of the membrane. By using sterol modulators in keratinocyte isolated membranes, it has been previously reported that cholesterol can modulate receptor function and signaling by changes in membrane fluidity [61]. In plants, it has also been reported that alterations in

membrane fluidity induce signaling events such as calcium influx leading to the activation of specific genes in response to changes in ambient temperature [62,63]. For instance, it would be tempting to suggest that a change in membrane properties is the initial step that may stimulate NADPH oxidase causing local ROS production. In favor of this hypothesis, it has been very recently established that rapid changes in membrane fluidity by either elevated temperatures or benzyl alcohol in tobacco BY-2 cells also trigger the induction of a $H_2O_2$ burst [64]. The link between the change in membrane fluidity and enzyme activation is not well understood, however it can be hypothesized that it could involve a mechanotransduction process. Indeed, mechanical stretches have been reported to induce ROS production involving NADPH oxidases in vascular smooth muscle [65] and in *Picea meyeri* [34].

However, a striking point of our study resides in the fact that filipin-induced changes in PM fluidity are also actively controlled by phosphorylations, since they are abolished by staurosporine. This clearly demonstrates that PM physical properties are not only directly affected in a mechanical way by sterol complexation, but that this sterol complexation triggers the onset of a signaling process, which results in a decrease in membrane fluidity. Our results on the effects of staurosporine are in agreement with the findings of Chakraborty et al. [66] that in macrophages, both membrane viscosity and oxidative burst are significantly lowered by the protein kinase inhibitor staurosporine [66]. However, the striking effects of staurosporine in the control of PM fluidity remain to be elucidated.

In summary, our results show that a very low filipin concentration, modifying sterol availability of the PM, triggers a controlled signaling process involving complex regulated activity of plant membrane systems, and in particular of an NADPH oxidase. Moreover, the modification of PM biophysical properties (here fluidity) is demonstrated as part of this signaling cascade. The present findings strongly suggest that changes in PM sterol organization and availability could act as sensors of the modifications of cell environment in plants. Future studies will have to elucidate the molecular actors responsible for the activation of this signaling process.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamem.2010.07.026.

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