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Modulation of plant plasma membrane H^+-ATPase by phytotoxic lipodepsipeptides produced by the plant pathogen *Pseudomonas fuscovaginae*

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Abstract

Pseudomonas fuscovaginae produces the lipodepsipeptides syringotoxin, fuscopeptin A and fuscopeptin B concurrently. These phytotoxins inhibit acidification of the external medium by fusicoccin-treated rice leaf sheath discs. When tested in vitro on H^+-ATPase of rice shoot plasma membranes, syringotoxin and its structural analogue syringomycin, produced by *P. syringae* pv. *syringae*, displayed a double effect. At low concentrations they stimulated the ATPase activity of native right-side-out membrane vesicles in a detergent-like manner. At higher concentrations, however, this stimulation was reversed. With membranes treated with the detergent Brij 58, inhibition of ATPase activity was observed at low concentrations of the nonapeptides. The latter effect required the presence of an intact lactone ring formed by the nonapeptide head of these molecules. In contrast, fuscopeptins A and B inhibited enzyme activity regardless of the orientation of the vesicles. These observations were confirmed using plasma membranes from a yeast strain whose own H^+-ATPase had been replaced by a single plant H^+-ATPase isoform, PMA2, from *Nicotiana plumbaginifolia*. The kinetics of inhibition induced by the most active compound fuscopeptin B, showed a non-competitive pattern, with a K_i of about 1 μM. The combination of syringotoxin (or syringomycin) with the more hydrophobic fuscopeptins, in amounts with little or no effect, resulted in strong inhibition of the enzyme activity of rice membranes, suggesting a synergistic effect for the two types of toxins.

Keywords: H^+-ATPase; Syringotoxin; Fuscopeptin A; Fuscopeptin B; (*Pseudomas fuscovaginae*)

1. Introduction

Pseudomonas fuscovaginae [1] is the causal agent of the disease known as sheath brown rot of cultivated and wild *Gramineae*. In rice, the symptoms of the disease occur at the early booting stage, and are characterized by necrosis of the flag leaf sheath and

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poor panicle exertion due to inhibition of the elongation of the upper internodes.

Recently, pathogenic strains of the bacteria were shown to concurrently produce three types of phytotoxic lipodepsipeptides involved in disease development [2,3], namely, the lipodepsinonapeptide syringotoxin (M_r 1136, structural analogue of syringomycin and syringostatin produced by strains of *P. syringae* pv. *syringae*), and two novel lipodepsipeptides, commonly named fuscopeptins: fuscopeptin A (FP-A, M_r 1817) and fuscopeptin B (FP-B, M_r 1845) (Fig. 1). The latter are structurally related to syringopeptins also produced concurrently with one of the lipodepsinonapeptides by pathogenic *P. syringae* pv. *syringae* strains [4,5].

Although the toxin mixture can reproduce the same symptoms as those observed in diseased plants, little is known about its action at the cellular level which results in the expression of the macroscopic symptoms.

Phytotoxic lipodepsipeptides are typical non-host-specific toxins. It was established several years ago that the plasma membrane of affected cells is the primary target of the toxins [6,7]. Hutchison et al. [8] showed that the haemolytic properties of syringomycin were due primary to plasma membrane pore formation. The ion channel-forming activity was non-specific and permeable to monovalent and divalent cations.

Both plant and yeast cells respond to the toxic activity of syringotoxin and syringomycin in a similar way. Physiological effects on plasma membranes include rapid efflux of K^+ accompanied by H^+ influx [9–11]. Indeed, minute quantities of syringomycin (10 nM) can induce the closure of *Vicia fava* leaf stomata [12]. On the other hand, fuscopeptin A and B induced inhibition of proton extrusion promoted by fusicoccin in maize roots [3]. An equimolar (5 μM) mixture of these toxins induced a rapid and prolonged solute efflux from rice cells [13]. Soaking rice grain in the same amount of bacterial toxins prior to sowing induced a drastic inhibition of seedling elongation [14].

The plasma membrane H^+-ATPase which acts as a primary transporter by pumping protons out of the cell, therefore creating a proton-motive force, is a key enzyme in the response of the plant cell to various internal and external stimuli. It plays an important role in cell expansion, the initiation of cell division, and the responses of the cell to light, phytohormones, nutritional status, and phytotoxins [15–18].

The effects of phytotoxic lipodepsipeptide on the plasma membrane H^+-ATPase has been investigated in some studies, almost exclusively with syringomycin, and with conflicting observations. Syringomycin stimulates H^+-ATPase in the plasma membrane of plant [19] and yeast cells [20]. On the other hand, in mung bean, stimulation was observed only with right-side-out membrane vesicles, and attributed to a detergent-like action [11]. ATPase activity was slightly inhibited by the toxin treatment of inside-out vesicles and detergent solubilised enzyme, and the authors concluded that the toxin inhibits the H^+-ATPase itself. Camoni et al. [21] showed that syringomycin and syringopeptins can permeabilize artificial phospholipid bilayer and reversibly inhibit reconstituted solubilized H^+-ATPases from plant organs, but these authors concluded that the latter effect is probably of minor importance in the biochemical effects of the toxins. Hutchison et al. [8] considered biosurfactant activity and pore formation by syringomycin as the primary physiological event leading to phytotoxicity. These authors proposed that in vivo calcium influx and the concurrent cascade of events associated with cellular signalling lead to the collapse of the pH gradient across the plasma membrane and acidification of the cytoplasm. On the other hand, possible effects of fuscopeptins A and B on the H^+-ATPase activity have not yet been studied.

In this work, we have therefore measured the activity of the rice shoot plasma membrane H^+-ATPase in the presence of individual or mixed toxins. As kinetics data cannot be unambiguously interpreted with membranes or solubilized enzymes prepared from whole plant organs which probably express various H^+-ATPase isoforms, we also investigated the effects of these phytotoxins on plasma membranes from a yeast strain expressing a single plant H^+-ATPase isoform. The overall effects of the toxins on plant cell H^+-ATPase are reviewed and discussed in relation to the macroscopic symptoms induced on whole plants.

2. Materials and methods

2.1. Toxin preparation

All experiments were carried out with strain UPB 264 of *Pseudomonas fuscovaginae* [22], and all solvents were HPLC grade. Cultures were initiated in the modified minimal medium of Ayer, Rupp and Johnson according to [23], and grown for 48 h at 28°C. The bacteria-free culture filtrate was obtained by centrifugation (Sorvall, GSA) for 20 min at $5000 \times g$, and filtration through a 0.22-μm membrane (Millipore Corporation, USA). The filtrate was concentrated tenfold in vacuo at 50°C with a rotary evaporator. This crude extract retained toxic activity in rice plants and was fungitoxic on *Geotrichum candidum* and *Saccharomyces cerevisiae*. The pH was adjusted to 3 (HCl) and submitted to 3 *n*-butanol (2:3 v/v ratio) extractions. The butanol phases were pooled and evaporated until dry. To get rid of residual butanol, ultra pure water (HPLC grade) was added twice to form the butanol-water azeotrope and evaporated until dry. The final residue was dissolved in water. A primary fractionation of this crude extract was achieved by reverse phase low pressure chromatography, using a preparative C_{18} bulk packing material of pore size 125 Å and bead size 55–105 μm (Millipore Corporation, USA), in a column of 16.5×2.5 cm. The column was made stable with degassed 50% (v/v) methanol and 0.1% TFA (in water) before use. The toxins were fractionated by a stepwise elution using 0.1% TFA in acetonitrile, at a flow rate of 1.5 ml min^{-1}. Syringotoxin was eluted at 42% acetonitrile, fuscopeptin A at 60%, and fuscopeptin B at 80% (v/v). Individual compound fractions were dried and dissolved in 0.1% TFA, then subjected to HPLC purification. This was achieved with a 3-ml Resource TM RPC column (Pharmacia Biotech Benelux), fitted to a 100-μl sample loop. The elution was kept steady for 25 min by mixing solvent A (0.09% TFA in water) with solvent B (acetonitrile/isopropanol, 4:1, v/v, plus 0.04% TFA) in a linear gradient (40 to 92% B), at a flow rate of 1 ml min^{-1} and a specific detection wavelength of 214 nm. Single peaks corresponding to the respective phytotoxins (retention time 5.5 min for ST, 13 min for FP-A and 15 min for FP-B) were collected and quantified using the appropriate standard. Aliquots were dried (plus two subsequent washings) in vacuo at 60°C, then stored at −20°C until used.

Pure syringomycin E was kindly provided by Dr J.Y. Takemoto, Utah State University, USA.

2.2. Effects of the phytotoxins on fusicoccin-induced H^+-extrusion

Rice flag leaf sheath discs of 0.5 cm diameter, punched at the early booting stage were used. The effects of the phytotoxins were assessed as in Höllander-Czytko and Weiler [24] with some modifications. After being made stable as described by the authors, the discs were layered on top of 100 μl of the stable buffer solution (pH 5.8), containing 5 μM fusicoccin in addition to 5 μM of each phytotoxin alone or in combination, and incubated for 8 h. The stable buffer contains bromocresol purple (0.175 mM), an acid-base indicator that is purple at a pH higher than 5.8 and yellow at a pH lower than 5.2 [24]. In the presence of fusicoccin, the plant material acidified the medium to the extent that the indicator transition interval was reached ($pH \leq 5.2$) [24].

2.3. Homogenization and preparation of rice plasma membranes

Rice boots (cultivar Ambalalava) were sampled randomly on greenhouse growing plants at their early booting, the stage during which rice plant is highly susceptible to *P. fuscovaginae* disease [25]. The lamina and panicle were discarded, the remaining leaf sheath and culm (100 g fresh weight) cut into pieces and washed in two litres of distilled water plus a drop of Tween 20, then rinsed twice. All subsequent steps were at 0–4°C.

The plant material was homogenised using a blender, in 400 ml of a medium containing 50 mM Tris-HCl (pH 8.0), 250 mM sorbitol, 2 mM EDTA, 0.6% (w/v) insoluble polyvinylpyrrolidone, 1 mM phenylmethylsulfonyl fluoride and 5 mM DTT. The homogenate was filtered through 4 layers of miracloth, centrifuged for 10 min at $7000 \times g$ (Beckman JA-14). The pellet was discarded and the supernatant centrifuged for 30 min at $100\,000 \times g$ (Beckman A641). The pellet (microsomal fraction) was

suspended in 10 ml of a medium containing 5 mM KH_2PO_4 (pH 7.8), 330 mM sucrose and 3 mM KCl.

Plasma membrane vesicles were purified from the microsomal fraction in an aqueous polymer two phase system essentially as described in [26]. The purified plasma membrane preparation, consisting of predominantly right-side-out vesicles, was suspended in 10 mM Tris-MES (pH 6.5), 330 mM sucrose and 2 mM DTT. Aliquots of 100 μl were frozen in liquid N_2 and stored at −80°C until used.

2.4. Preparation of Saccharomyces cerevisae membranes

The preparation of plasma membranes from the yeast expressing only the H^+-ATPase PMA2 isoform of *N. plumbaginifolia*, was as in [27]. This yeast strain had been deleted from its own two H^+-ATPase genes, and contains a plasmid bearing the *pma2* gene from *N. plumbaginifolia*.

2.5. Protein assay

The amount of the membrane proteins was determined by the method of [28], using BSA as standard.

2.6. Enzyme assay

With rice membranes, the vanadate-sensitive ATPase assay was performed at 30°C, in a medium containing 6 mM Na_2ATP, 9 mM $MgSO_4$, 50 mM MES-Tris (pH 6.5), 10 mM sodium azide (mitochondrial and chloroplastic ATPase inhibitor), 50 mM KNO_3 (vacuolar ATPase inhibitor), 0.1 mM sodium molybdate (acid phosphatase inhibitor) [27,29]. The phytotoxin solubilised in HPLC-grade water was added to the assay medium containing 70 μg membrane proteins, and the mixture was preincubated for 10 min at room temperature. When indicated, 0.02% Brij 58 (polyoxyethylene-20-cetyl ether) [40,41] was added in the medium during preincubation. The reaction (final volume of 700 μl) was started by the addition of MgATP. After 10, 20, 30, 40, 50, and 60 min, successive 100-μl aliquots were mixed with 1% SDS to stop the reaction. ATPase activity was determined spectrophotometrically (700 nm, Beckman DU 640) after reaction of total phosphate with 1,2,4-aminonaphtosulfonic acid dissolved in 3% (w/v) $Na_2S_2O_5$ [30].

With yeast membranes, ATPase assays were performed as above except that 60 μg membrane proteins were used, and aliquots (100 μl) were taken after 4, 8, 12, and 16 min. For kinetic studies, the concentration of $(MgATP)^{2-}$ varied from 1 to 6 mM and an ATP regeneration system was used [27]. The amount of $MgSO_4$ and Na_2ATP to be added to the reaction mixture to produce the required final concentrations of free Mg^{2+} and $(MgATP)^{2-}$ was calculated [31]. The ATP concentration of the solution was estimated from the absorbance at 259 nm ($e = 1.54 \times 10^4\ M^{-1} \times cm^{-1}$).

Each specific activity was calculated by linear regression from the slope of phosphate released vs. time. We checked that the ATPase activity was linear with protein concentration.

2.7. Statistics

Results for rice are from two independent membrane preparations and those for yeast from 3 different batch cultures. All values are given as means ± S.E. of at least 3 replications.

3. Results

3.1. Toxin purification and bioassays

P. fuscovaginae toxins were purified at a semi preparative scale by RP-HPLC. Production varied from 2.8 to 4 mg syringotoxin, and 2.3 to 3.3 mg for each fuscopeptin per litre of culture. As both purified syringotoxin (from *P. fuscovaginae*) and syringomycin (from *P. syringae* pv. *syringae*) have been reported to be toxic for some fungi, we checked that both inhibited the growth of *Geotrichum candidum* in standard plate assays [32]. The *Saccharomyces cerevisiae* wild type strain YPS14-4 [33] was also sensitive to the two lipodepsinonapeptides (data not shown). For both species fungitoxic activity was alkaline-labile. Indeed, alkali treatment (10 min in 1 M KOH followed by neutralisation with HCl 1 N) induces the opening of the lactone ring formed by the nonapeptide moiety, and among other features, the loss of the chlorine

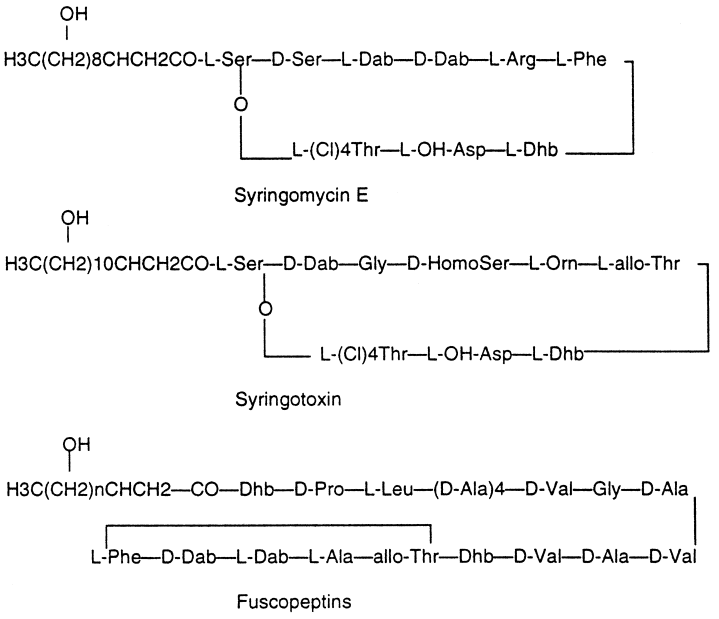


Fig. 1. Structure of the phytotoxic lipodepsipeptides used in this study. Abbreviations are Dab, 1,4-diaminobutyric acid, and Dhb, 2,3-dehydroaminobutyric acid; the amino acids are identified using standard three-letter biochemical notation; syringomycin was purified from *P. syringae* pv. *syringae* broth culture, syringotoxin and the fuscopeptins are *P. fuscovaginae* products; the hydroxyl fatty acid moiety is a 3-hydroxyoctanoate ($n=4$) in fuscopeptin A and a 3-hydroxydecanoate ($n=6$) in fuscopeptin B.

atom of the C-terminal threonine (Fig. 1), which is said to be implicated in biocidal activity [34,35].

On the other hand, the two fuscopeptins showed no fungitoxic activity at the concentration range tested (10–90 μM). However, each of the lipodepsipeptides (30 μM) induced significant necrosis in the detached rice leaf sheath bioassay, after 72 h of incubation (data not shown). This effect was not altered after alkali treatment.

3.2. Effects of the phytotoxins on fusicoccin-mediated H^+-extrusion

Fusicoccin causes auxin-like responses in plant tissues [36,37]. It promotes growth and affects ion flux across the plasma membrane. These responses are generally ascribed to stimulation of the plasma membrane H^+-ATPase [38,39]. When incubated with fusicoccin, control rice sheath discs responded by acidifying the incubation medium (Table 1). This effect was altered in the presence of the lipodepsipeptides in all combinations tested, apart only from syringotoxin (ST). This discrepancy can be explained by the fact that by itself, the amount of toxin used (5 μM) was unable to collapse the low pH induced by fusicoccin up to the transition point of the acid indicator used. In accordance with this, 10 μM of ST resulted in the same response as for the other lipodepsipeptides. Therefore, we concluded that in vivo, the phytotoxins alone, or in combination, dissipate the pH gradient promoted by the plasma membrane H^+-ATPase of rice cells.

3.3. Effects of syringotoxin and syringomycin on plant H^+-ATPase activity

Plasma membranes prepared by two-phase partitioning are predominantly sealed right-side-out (apoplastic side out, native orientation) [26,40]. As the ATP binding site of the plasma membrane H^+-ATPase is located in the cytoplasmic side of the cell, and thus within the vesicles, the substrate has to be made accessible to the enzyme. We tested the efficiency of Brij 58, shown to be a detergent that does not permeabilise the membranes but inverts the vesicles into the inside-out orientation [41,42]. Optimal conditions were obtained with 0.02% Brij 58, that increased H^+-ATPase activity from 0.578 to 2.548 μmol Pi min^{-1} mg^{-1}.

We investigated the modulation of ATPase activity

by syringotoxin and syringomycin using enriched plasma membrane fractions from rice boots. When native (right-side-out) rice membranes were used, ATPase activity was enhanced in the presence of the phytotoxins (Fig. 2). Optimum stimulation was observed for both toxins at 80 μM. Higher stimulation was observed for syringotoxin (2-fold, compared to syringomycin, 1.5-fold) (Fig. 2). Under these experimental conditions in which most membrane vesicles are right-side-out, the active sites of the enzyme are normally masked and the observed stimulation can be due to permeabilization of the vesicles to the substrate. We could equally consider a stimulation per se of the enzyme. To discriminate between these two possibilities, Brij 58 treatment was used. If the toxins stimulated the activity of the enzyme, this effect had to be additive to that of the detergent. Data presented in Fig. 2 show that, on the contrary, the toxin treatments inhibited enzyme activity (45% inhibition at 160 μM).

At the organ level, plant plasma membranes express several isoforms of H^+-ATPase with distinct kinetics [43]. Our data therefore, cannot be easily interpreted. However, it has been shown that the plasma membrane H^+-ATPase from the yeast *S. cerevisiae* could be functionally replaced by single H^+-ATPase isoforms from *N. plumbaginifolia* ([27], Luo et al., in preparation), thus allowing kinetic characterization of single plant H^+-ATPase isoforms. In addition, the yeast plasma membranes used are not sealed, thus circumventing the need for inverting the vesicles with Brij 58. Indeed, Brij 58 showed no effect on ATPase activity of the yeast membrane preparation. We thus tested toxin effects on plasma membranes from yeast cells exclusively expressing the PMA2 H^+-ATPase from *N. plumbaginifolia*. Syringotoxin and syringomycin inhibited yeast-expressed plant H^+-ATPase activity (Fig. 3), to a larger extent than that of rice membranes (Fig. 2).

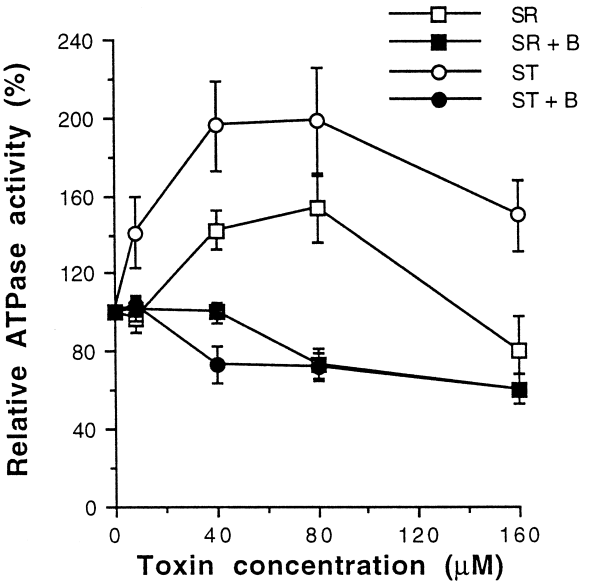


Fig. 2. Effects of syringotoxin (ST) and syringomycin (SR) on H^+-ATPase activity in rice shoot plasma membrane, in the absence or presence (+B) of 0.02% Brij 58. Values are given as a percentage of the specific activity of the control (0.578 ± 0.086 μmol Pi min^{-1} mg^{-1} or 2.548 ± 0.098 μmol Pi min^{-1} mg^{-1}, in the absence or presence of Brij 58, respectively). ATP hydrolysis was measured as described in Section 2.

Table 1
Effects of *Pseudomonas fuscovaginae* toxins and syringomycin on fusicoccin-mediated acidification of the external medium by rice flag leaf sheath

Treatment	pH[a]	
	Without fusicoccin	With fusicoccin
Control	P	Y
ST	P	Y
ST (10 μM)	P	P
SR	P	P
FP-A	P	P
FP-B	P	P
ST+FP-A	P	P
ST+FP-B	P	P
ST+FP-A+FP-B	P	P

[a]Unless otherwise stated, toxins were used in all combinations at a concentration of 5 μM; ST: syringotoxin; SR: syringomycin; FP-A: fuscopeptin A; FP-B: fuscopeptin B; incubation was as described in Section 2 and results are from 5 replicates. The pH of the incubation was evaluated using the acid-base indicator bromocresol purple which is purple (P) at a pH higher than 5.8 and yellow (Y) at a pH lower than 5.2 [24].

Furthermore, when linearised toxins with no biological activity were used, they showed no effect on ATPase activity with yeast membranes or with Brij-treated rice membranes (Fig. 4). However, stimulation of the activity by syringotoxin (Fig. 4) or syringomycin (data not shown) still occurred, though to a lesser extent, with native rice membranes.

These results suggest that phytotoxins exhibit a double effect. The cyclic and linearised forms can permeabilise biological membranes to ATP [11],

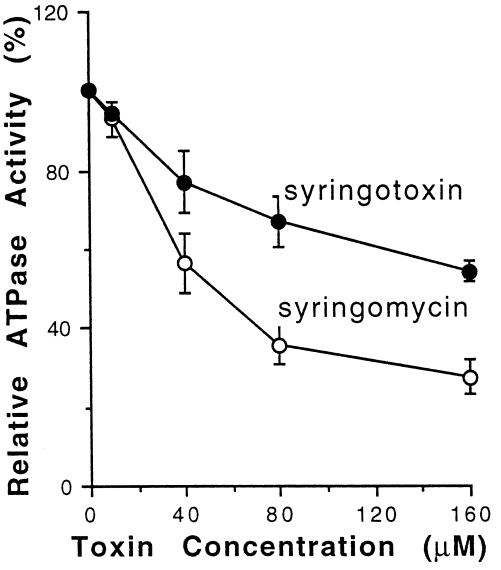


Fig. 3. Effects of syringotoxin and syringomycin on the activity of yeast expressed PMA2 H^+-ATPase. Relative values are given as a percentage of the specific activity of the control (0.208 ± 0.037 μmol Pi min^{-1} mg^{-1}).

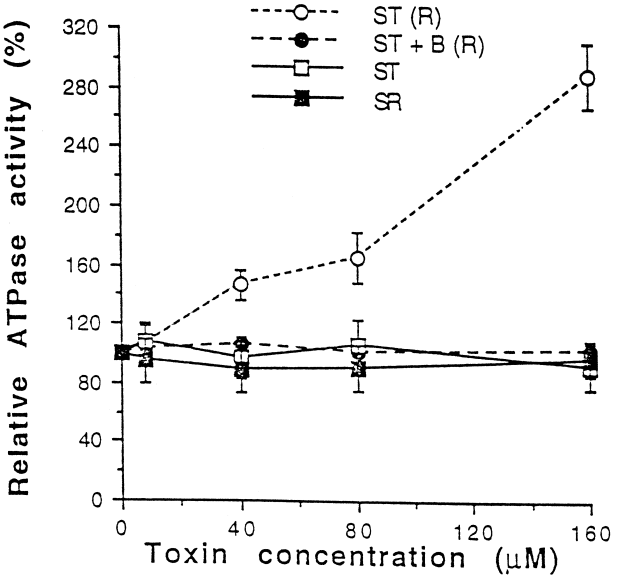
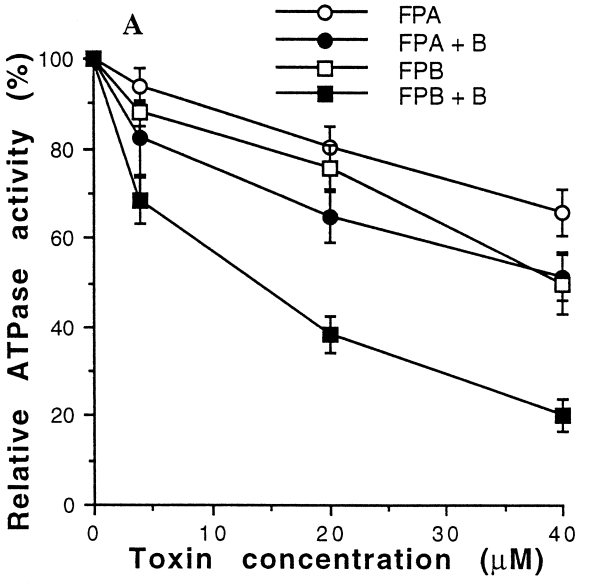


Fig. 4. Effects of linearized syringotoxin (ST) and syringomycin (SR) on plant plasma membrane H^+-ATPase activity. Linearization was performed by treating a stock preparation of the respective toxins with 1 N KOH for 10 min followed by neutralization with 1 N HCl. ATP hydrolysis for rice membranes (R) or PMA2-expressing yeast membranes (Y) was assayed in the absence or presence (+B) of 0.02% Brij 58 as described in Section 2. In addition, control assays contained the appropriate amount of KCl with respect to the toxin concentration used.



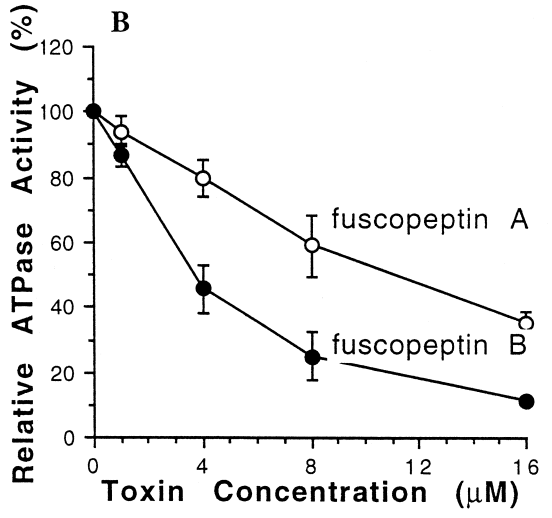


Fig. 5. Effects of fuscopeptin A (FP-A) and fuscopeptin B (FP-B) on H^+-ATPase activity of rice shoot plasma membrane (A) and PMA2-expressing yeast membranes (B). A: Relative values are given as a percentage of the specific activity of the control (0.578 ± 0.086 μmol Pi min^{-1} mg^{-1} or 2.346 ± 0.147 μmol Pi min^{-1} mg^{-1}, in the absence or presence of Brij 58, respectively. ATP hydrolysis was assayed as in Fig. 2. B: Relative values are given as a percentage of the specific activity of the control (0.238 ± 0.047 μmol Pi min^{-1} mg^{-1}); ATP hydrolysis was assayed as in Fig. 3.

far below their critical micellar concentration (1 mM for syringomycin, [8]; 1.02 mM for syringotoxin, this study). The inhibition of H^+-ATPase

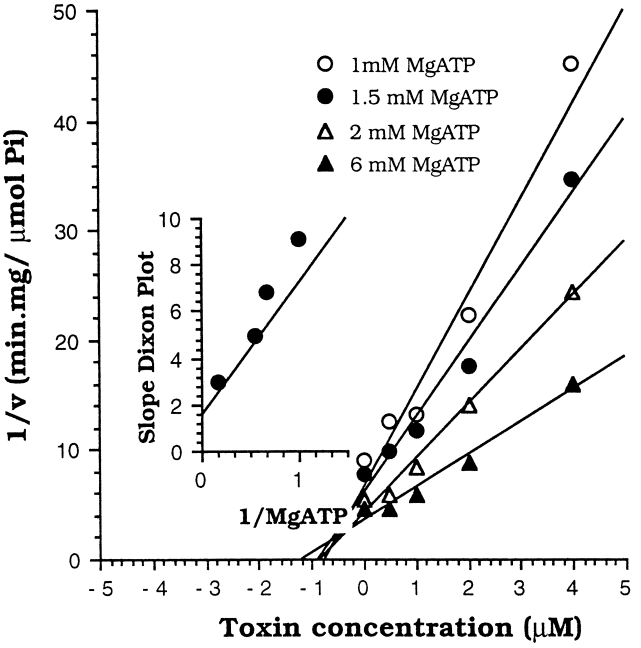


Fig. 6. Kinetics of the inhibition by fuscopeptin B of yeast-expressed plant PMA2 H^+-ATPase. The main graph is the Dixon plot obtained (r^2 0.98) with MgATP varying from 1 to 6 mM and 3 mM free Mg^{2+}; the inset shows the replot of the slope of the linear regressions vs. 1/MgATP; the exact measured concentrations of $MgATP^{2-}$ in the reaction medium at pH 6.5, were 0.99, 1.48, 1.90 and 5.86 mM, respectively.

activity was only observed for their biologically active cyclic form when they came into contact with the enzyme.

3.4. *Effects of the fuscopeptins on H^+-ATPase activity*

The two fuscopeptins are less hydrophilic than syringotoxin and syringomycin. Their modulation of H^+-ATPase activity was also investigated. Fuscopeptin A and fuscopeptin B inhibited ATPase activity of rice membranes (Fig. 5A). Little difference was observed between the inhibition rate induced by the two compounds with predominantly right-side-out vesicles. This inhibition was enhanced for both toxins in the presence of Brij 58 and the potency of fuscopeptin B was higher than that of fuscopeptin A (Fig. 5A). At 40 µM for example, fuscopeptin B induced 80% inhibition compared to about 45% for fuscopeptin A. Similar data were obtained with yeast membranes expressing plant PMA2, although lower quantities of the bacterial toxins were needed in this case to achieve inhibition, with fuscopeptin B again being more efficient than fuscopeptin A (Fig. 5B). We used fuscopeptin B and yeast membranes to study the kinetics of the inhibition. The Dixon plot depicted in Fig. 6 shows a pattern of non-competitive inhibition with a K_i (inhibition constant) of about 1 µM.

Since the toxins (syringotoxin and the fuscopeptins) are produced concurrently by the bacterium, we looked for a possible synergistic effect between the two types of lipodepsipeptides on the activity of the enzyme. Syringotoxin or syringomycin (20 µM) was combined with 4 µM of fuscopeptin A or fuscopeptin B for the treatment of rice membranes in the presence or absence of Brij 58, and to 0.5 or 1 µM of the fuscopeptins for yeast membranes. With rice membranes, the result of the mixture treatment was a drastic inhibition of the activity of the enzyme (Fig. 7). These results suggest that there might be a synergistic effect between the two types of molecules. The combination of amounts which, alone, have little or no effect resulted in strong inhibition. Almost 90% inhibition was recorded in all combinations containing fuscopeptin B. On the contrary, these combinations showed no striking effect with the yeast membranes (data not shown).

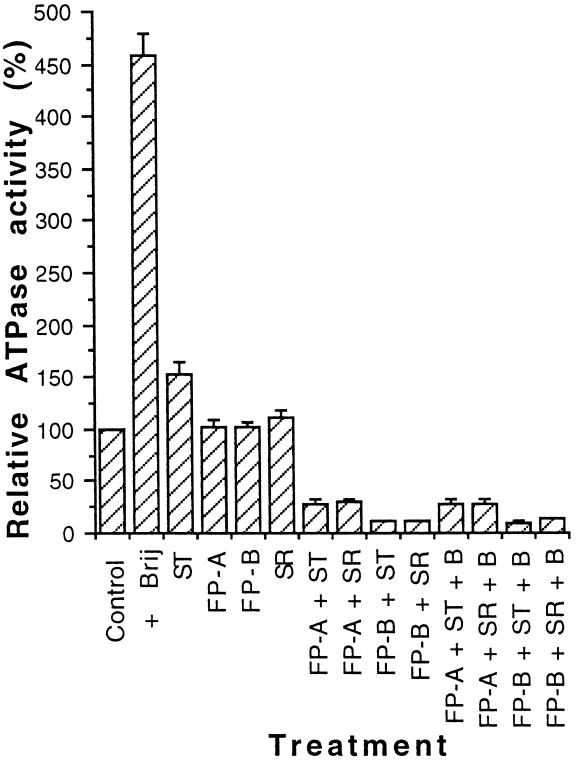


Fig. 7. The combined effects of syringotoxin or syringomycin with the fuscopeptins on rice shoot plasma membrane H^+-ATPase activity. Twenty μM syringotoxin (ST) or syringomycin (SR) were combined with 4 μM of fuscopeptin A (FP-A) or fuscopeptin B (FP-B). ATP hydrolysis was measured as in Fig. 2. Relative values are given as a percentage of the specific activity of the control (0.623 ± 0.045 μmol Pi min^{-1} mg^{-1}).

4. Discussion

The toxin syringotoxin and the structurally analogous syringomycin showed similar effects. They stimulated H^+-ATPase activity of rice native membranes. This effect was not observed in the presence of Brij 58, suggesting that the toxins permeate the membranes to ATP [11] or, like Brij 58, invert the vesicles [43]. This interpretation was confirmed by the observation that, in the presence or absence of Brij 58, the toxins did not stimulate H^+-ATPase activity of non-sealed plasma membranes from a yeast strain expressing the plant PMA2 isoform. This may be the most plausible explanation for the conflicting results obtained by [19] and [20] on the one hand, and by [11] on the other. The first authors observed stimulation by syringomycin, which was reduced after thawing their membrane preparation, a process which normally results in partly reverting the right-side-out vesicles [48]. The latter authors observed inhibition by syringomycin in inside-out vesicles.

At higher concentrations, syringotoxin and syringomycin partly inhibited plant H^+-ATPase activity in both rice and yeast membranes. These data thus extend those of Camoni et al. [21] to a new plant species and, more important, show that a single plant H^+-ATPase isoform expressed in another environment is sensitive to the toxins. Moreover, we showed that this effect is linked to the presence of an intact lactone ring formed by the nonapeptide moiety. Two explanations, not necessarily mutually exclusive, may account for this: proteolipids surrounding the enzyme, the so-called annulus lipids [49] are substituted, on the cytoplasmic side, by the lipophilic toxins, or else the nonapeptide head interacts directly with the enzyme, resulting in either case in reduced activity.

Is there any relationship between ATPase inhibition as observed in vitro and in vivo inhibition of external medium acidification? It might be argued that, for syringomycin and syringotoxin, the effective concentration in the latter is lower than in the former. Caution is required when making this comparison because in vitro inhibition was observed easily within minutes while the in vivo effect on the external medium acidification was recorded after an 8-h period, thus allowing possible cumulative effects. In addition, as these drugs are both hydrophobic and positively charged, they might well be accumulated within the plant cells, thus leading to a higher internal concentration. Nevertheless, the major in vivo effect might well be related to the permeation effect. The phytotoxic lipodepsipeptides produced by *P. fuscovaginae* are typical amphiphilic molecules with a polar peptide head and a hydrophobic 3-hydroxyl fatty acid tail. Various toxic amphiphilic compounds act predominantly by decreasing membrane integrity, resulting in a dissipation of the pH gradient across the cytoplasmic membranes [11]. The inhibition by these toxins of external acidification mediated by fusicoccin-treated rice sheath discs can be interpreted as a result of the increased passive flux of protons across the cell membrane (predominantly inward current). An unspecific pore forming capacity has been shown for syringomycin and syringopeptins with artificial lipid bilayers [8,50], probably using a colloid

osmotic mechanism as proposed by [52]. Since in a membrane-like environment lipodepsipeptides can form amphiphilic α-helices [44,45], and probably pores [46,47], this organisation might possibly be considered for rice membranes even though no experimental data support this at the present time. Another possibility would be to consider a direct uncoupling between ATPase activity and proton pumping.

The situation is different with the fuscopeptins. Although these compounds can be considered to be related to syringopeptins, their chemical structure is different and since they are produced by the pathogen, their potential effect had to be evaluated. They did not show any stimulation of H^+-ATPase activity in native sealed membranes. We can suggest that their more hydrophobic nature does not allow them to mimic the inverting or permeation effect of syringotoxin and syringomycin. However, both fuscopeptin A and fuscopeptin B were much stronger inhibitors of plant H^+-ATPase than syringotoxin or syringomycin. In the presence of a single plant isoform of the enzyme (in yeast membranes), the enzyme was more sensitive to the fuscopeptins than in a heterogeneous situation (several isoforms with possibly distinct toxin sensitivity) as is probably the case in rice membranes. The kinetics of the inhibition induced by fuscopeptin B showed a non-competitive pattern (decreased V_{max}, same K_m). This suggests that the toxin can combine with the enzyme, inducing a sufficient distortion to prevent the proper positioning of the catalytic centre of the enzyme, giving a non-productive complex. Contrary to the other two drugs, fuscopeptins A and B were both effective in vitro and in vivo in the same range of concentration. In this case, their in vivo effect might well be directly related to the ATPase inhibition.

More interesting is the synergistic effect on inhibition of the enzyme for plant membranes, obtained by combining syringotoxin or syringomycin to the fuscopeptins. These data indicate possible interactions, at the enzyme level, between the two toxins and the less hydrophilic fuscopeptins concurrently produced by *P. fuscovaginae*. One possible explanation is that the two types of toxins act at different sites on the enzyme, resulting in a drastic inhibition of its activity. Syringotoxin may modify the enzyme without greatly inhibiting activity, but rendering it more accessible to the fuscopeptins. This cannot be linked to the membrane permeation properties of syringotoxin or syringomycin since, in combination, the inhibition rate was higher than that recorded with an individual toxin in the presence of Brij 58. The fact that for yeast membranes containing a single plant isoform of the enzyme no synergistic effect was observed for the two types of toxins indicates either that the lipid environment, which differs in plant and yeast membranes, has a direct effect on toxicity [53,54] or that the lipodepsipeptide effects are more complex with membranes expressing different H^+-ATPase isoforms [50,51].

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