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PSEUDOMONAS SYRINGAE PV. *SYRINGAE* PHYTOTOXINS REVERSIBLY INHIBIT THE PLASMA MEMBRANE H^+-ATPase AND DISRUPT UNILAMELLAR LIPOSOMES

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SUMMARY: The *Pseudomonas syringae* pv. *syringae* phytotoxins syringomycin-E and syringopeptins 22-A and 25-A reversibly and noncompetitively inhibit purified H^+-ATPase solubilized from plasma membrane of maize roots. Moreover, they increase the passive permeability to protons in phosphatidylcholine/phosphatidylethanolamine liposomes. Both effects are more pronounced with syringopeptins than with syringomycin-E. Activity on phospholipid bilayers is detectable at phytotoxin concentrations not affecting H^+-ATPase activity.

Two groups of bioactive lipodepsipeptides are produced by cultures of *Pseudomonas syringae* pv. *syringae*, a plant pathogen with a very wide range of hosts [7]. One group is formed by the nonapeptides syringomycins [2, 12, 15], synthesized by strains isolated from several mono and dicots [17, 18], syringotoxins [3, 13, 15, 16], made by strains isolated from citrus plants [13], and syringostatins [14, 15, 20], made by strains isolated from lilac [14]. The other, more recently discovered group, contains metabolites called syringopeptins whose peptide moiety is composed of 22 (SP_{22}) or 25 (SP_{25}) amino acid residues [4]. Metabolites belonging to both groups also occur in cultures of a transposon generated regulatory mutant of a *Pseudomonas syringae* strain [5] proposed for the biocontrol of the Dutch elm disease [23].

SRs, STs, and SPs have been shown to display phytotoxic and antimicrobial activities, with the nonapeptides more toxic on fungi, and SPs more phytotoxic [19]. Recent studies, carried out with one member of the SR family (SR-E) and with both SP_{22} and SP_{25}, have shown that these compounds affect several membrane activities in yeast and higher plants [9, 11, 31], thus confirming in principle the results of earliest investigations [1] performed with partially purified preparations, presently known to contain both nonapeptides and SPs. In particular, it has been observed that SPs and SR inhibit H^+-translocation and

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ABBREVIATIONS: ACMA, 9-amino-6-chloro-2-methoxyacridine; BTP, 1,3-bis[tris(hydroxymethyl)-methylamino]propane; DTT, dithiothreitol; MOPS, 3-(N-morpholino)propanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; SP_{22}, syringopeptin 22-A; SP_{25}, syringopeptin 25-A; SPs, mixture of syringopeptins; SR, syringomycin E; SRs, mixture of syringomycins; STs, mixture of syringotoxins.

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ATP hydrolysis and dissipate ATP-driven H^+ gradients in plasma membrane vesicles from maize roots [11]. In order to evaluate the relative involvement of H^+-ATPase and of the phospholipid bilayer in these activities we have tested the phytotoxins on the hydrolytic activity of solubilized and HPLC purified H^+-ATPase [21], and on the permeability of liposomes with artificially generated H^+ gradients.

MATERIALS AND METHODS

Plant material. Maize seeds (*Zea mays* L., var. Logos) from Italian Dekalb (Mestre) were grown hydroponically at 27°C in the dark [24]. After seven days roots were excised and used for the preparation of plasma membranes.

Chemicals. All reagents were of the highest commercially available grade. ACMA, soybean phosphatidylcholine (type II), sheep brain phosphatidylethanolamine (type II-S), polyoxyethylene 8 myristyl ether ($C_{14}E_8$) and dodecyl-β-D-maltoside were purchased from Sigma Chemical Company.

Phytotoxins. *P. syringae* pv. *syringae* Van Hall strains B301 and B359 from the collection of the Department of Plant Pathology, University of California, Davis, USA, were used for the preparation of SR and SP_{22}, and SR and SP_{25}, respectively. Bacteria were grown at 25° C in still colture for 9-10 days in a chemically defined medium [30]. The phytotoxins were extracted and partially purified according to Bidwai *et al.* [6] and finally fractionated by reverse phase HPLC on an Aquapore RP300 column (4.6 x 250 mm, 7μm ID, Applied Biosystems) using a Beckman System Gold 126 instrument under described conditions [4]. The concentration of purified metabolites was determined by amino acid analysis after acid hydrolysis [2].

H^+-ATPase. The H^+-ATPase was solubilized from two-phase partitioned plasma membranes of maize [24] and purified by a recent procedure [21] with minor modifications. 2 ml of membranes (4 mg/ml protein) were washed with 2 ml of 50 mM MOPS/BTP (pH 6.5), 20% glycerol, 0.5 mM ATP, 0.1 mM DTT, 0.1 mM PMSF, 1 M KCl (buffer A), containing 1.5 mg/ml of polyoxyethylene 8 myristyl ether. After 45 min centrifugation at 100,000 x g the supernatant was discarded and the pellet resuspended in buffer A containing 20 mg/ml dodecyl-β-D-maltoside. After 30 min incubation the mixture was centrifuged 45 min at 100,000 x g and the supernatant loaded onto a 5PW DEAE HPLC column (Bio-Rad) washed at 0.5 ml/min with histidine-HCl buffer (pH 7.0) containing 10% glycerol, 0.5 mM ATP, 0.1 mM DTT, 0.05 mg/ml dodecyl-β-D-maltoside (buffer B). Elution was performed in 40 min by a linear gradient from 0 to 0.5 M NaCl in buffer B. Fractions of 1 ml were assayed for enzymatic activity [26]; the H^+-ATPase eluted after 12 ml.

The inhibitory effect of the phytotoxins on the phosphohydrolytic activity of the H^+-ATPase [26] was tested at concentrations ranging from 0.1 to 35 μM. Each experiment, run at least twice with duplicate samples, was carried out with a 10 μg/ml H^+-ATPase concentration.

Liposomes. Unilamellar liposomes were prepared by sonicating to optical clarity a mixture of 25 mg each of phosphatidylcholine and phosphatidylethanolamine in 1 ml of 10 mM Tris/Mes buffer (pH 6.5) containing 1 mM DTT, 1 mM EDTA and 100 mM KCl [24].

Fluorescence measurements were carried out in a magnetically stirred cell thermostatted at 26°C, in a Perkin-Elmer LS 60 spectrofluorimeter; excitation and emission wavelengths were 430 and 500 nm, respectively.

The fluorescence of 25 μl of liposomes equilibrated in 2.5 ml of 10 mM Tris-Mes buffer (pH 6.5), 100 mM KCl, 1 mM EDTA, 1 mM DTT containing 1 μM ACMA was taken as 100%. The addition of 1 μM NaOH, required to produce a ΔpH of about 2 units between the inside of liposomes and the incubation buffer, instantaneously quenched the fluorescence of the system in consequence of ACMA accumulation inside the liposomes. The rate of proton gradient dissipation, both in absence (control) and in presence of increasing phytotoxin concentrations, was determined by monitoring the increase of fluorescence.

Analytical methods. Proteins were assayed by the method of Bradford [8] using the Pierce protein assay kit with bovine serum albumin as standard. The purified H^+-ATPase was analysed by SDS-PAGE [22].

RESULTS

Effect of the phytotoxins on solubilized H^+-ATPase

The effect of SR, SP_{22} and SP_{25} on the hydrolytic activity of the purified H^+-ATPase of maize roots has been investigated. The enzyme was purified by ion exchange HPLC according to a procedure which yields the H^+-ATPase in a non-activated form [21]. Purified preparations with a specific activity of 3-4 μmol Pi/min/mg protein, 80% inhibited by vanadate and three-fold stimulated by lysophosphatidylcholine were obtained. These features are comparable to those reported for H^+-ATPase purified by different methods from various plant tissues [25].

Fig. 1 shows that all three compounds inhibit the enzymatic activity in a concentration-dependent manner, with SPs much more effective than SR. In fact, at the highest concentration tested (35μM) SR reduced the phosphohydrolytic activity to 75% of the control, while both SP_{22} and SP_{25} brought it down to less than 10%. The IC_{50} of SPs is 10 μM, that of SR (worked out by estrapolation) is about 10 times higher.

In order to rule out the possibility that the inhibition by the phytotoxins may be artefactual, owing to the delipidated state of the soluble enzyme, the three compounds were again tested on the lysophosphatidylcholine/activated H^+-ATPase [27]; the inhibition was comparable to that reported for the delipidated enzyme (results not shown).

The inhibitory effect of the phytotoxins on the H^+-ATPase was reversible, as assessed by a dilution assay. The enzyme was incubated for 5 min with 10 μM phytotoxins, then ten-fold diluted and assayed for residual activity. As shown in Table 1, the inhibition obtained at 10 μM SPs is reduced by a ten-fold dilution to values comparable to those obtained with 1 μM concentrations. Qualitatively similar results (not shown) were obtained with SR, but its weak inhibitory activity at 10 μM concentration reduced the significance of the experimental data.

The kinetic properties of enzyme inhibition were investigated by double-reciprocal plot analysis. All three compounds behaved as non-competitive inhibitors (Fig.2).

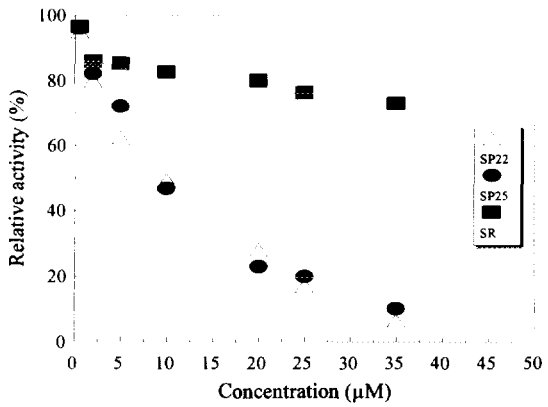

Figure 1. Inhibition of phosphohydrolytic activity of purified H^+-ATPase by SR, SP_{22} and SP_{25}. Mean values of duplicates are reported. Variations never exceeded 5 %.

Table 1. Reversibility of H^+-ATPase inhibition: dilution assay

	Percent of H^+-ATPase inhibition at different phytotoxin concentrations		
Phytotoxin	**10 μM**	**1 μM**	**10 μM/1 μM (a)**
SP_{22}	53.2 %	28.1 %	29.7 %
SP_{25}	56.6%	26.7 %	27.5 %

(a). After 5 min incubation with 10 μM phytotoxins, the samples were tenfold diluted, added with 2 mM ATP, and incubated for 30 min before phosphate assay.

Effect of the phytotoxins on the liposomes

It has been suggested that SP_{22}, SP_{25}, and SR can induce membrane perturbation in whole cells and in subcellular organelles [19, and unpublished results]. An effect of these compounds on phospholipid vesicles was investigated by determining through fluorescence quenching measurements their capability to dissipate a proton gradient artificially generated in liposomes. As shown in Fig. 3, SPs produced a detectable alteration of the proton permeability of liposomes at nanomolar concentrations, an effect which became dramatic at micromolar concentrations, when an instantaneous collapse of the gradient occurred. Here again SR resulted markedly less effective; a significant alteration of bilayer permeability was detected only at micromolar concentrations, and a complete discharge of the proton gradient was never attained.

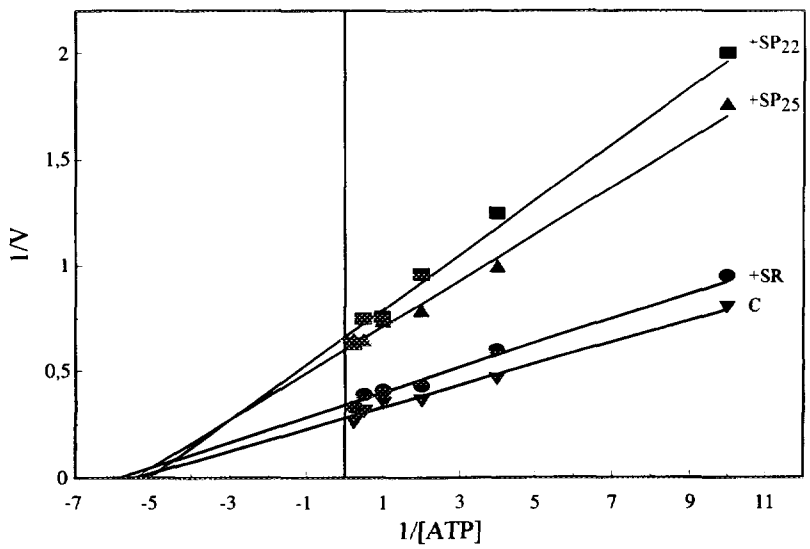

Figure 2. Double-reciprocal plot of phosphohydrolytic activity of purified H^+-ATPase in absence (c) or in presence of 10 μM SR, SP_{22} and SP_{25}.

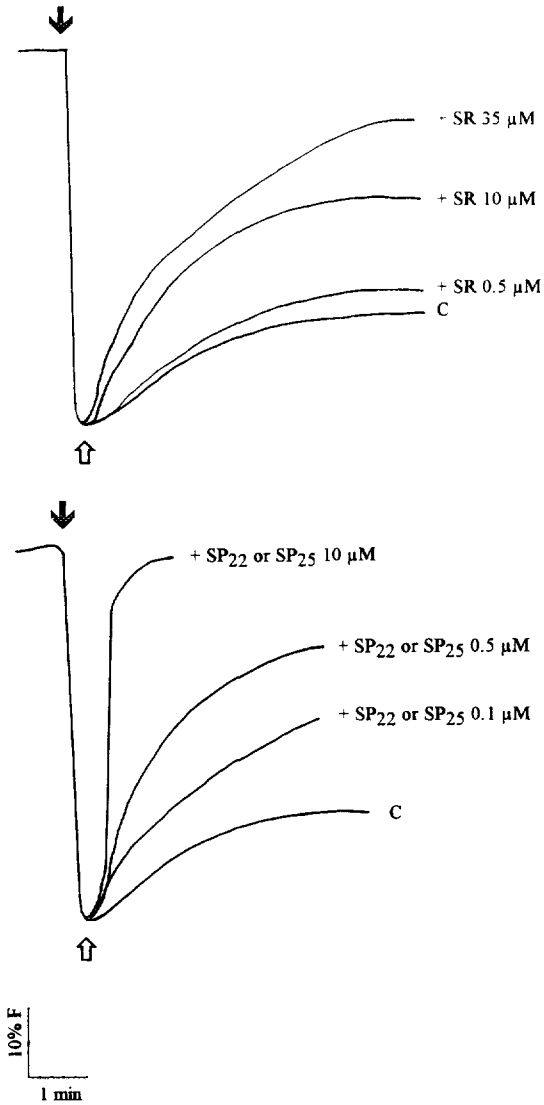

Figure 3. Dissipation of pre-formed proton gradient in liposomes by SR, SP_{22} and SP_{25}. The solid arrow indicates the addition of 1 μM NaOH, the open arrow the addition of the phytotoxins. Traces refer to fluorescence of 1 μM ACMA.

DISCUSSION

The results of the present paper confirm the dual role of *P. syringae* pv. *syringae* phytotoxins suggested by some studies on their mode of action [9, 11]: inhibition of the plasma membrane H^+-ATPase and alteration of membrane permeability. The first role has been proved by testing SR and SPs on the phosphohydrolytic activity of the solubilized enzyme (Fig.1), namely under conditions independent of the protein localization in microsomal vesicles and of the involvement of hypothetical binding sites on the membrane. The inhibitory effect does not result from an irreversible damage to the enzyme, as proven by the restoration of the catalytic activity when the concentration of the phytotoxins in the inhibited sample was decreased by dilution(Table 1),nor from competition with ATP at the binding site, as shown by kinetic

analysis (Fig. 2). At variance with other well known non competitive inhibitors of plasmalemma H^+-ATPase, like vanadate and diethylstilbestrol, the *P. syringae* pv. *syringae* phytotoxins lack specificity, since they also inhibit the plant mitochondrial and vacuolar ATPases [unpublished results].

The demonstration of a permeabilizing effect on the phospholipid bilayer has emerged from experiments carried out with phosphatidylcholine/phosphatidyethanolamine liposomes, a model system again free of nonessential components. In both tests the active concentration of SP_{22} was identical to that of SP_{25}, and always several times higher than that of SR. This different efficiency reflects structural differences; in fact the peptide moiety is nearly identical in both SPs, but markedly different in size and polarity from that of SR. The long hydrophobic region, contributed not only by the fatty acyl chain but also by most of the amino acid residues, and the C-terminal cationic loop of the peptide with two positively charged residues in subterminal positions, confers to SPs an overall character not dissimilar from that of two other groups of membrane disrupting peptides, the polymyxins [29] and the type 1 brevinins [10, 28].

Data so far accumulated during the studies on bioactive *P. syringae* pv. *syringae* lipodepsipeptides support the assumption that the primary action of these phytotoxins consists in a damage to membrane permeability. The reversible inhibition of the plasma membrane H^+-ATPase is probably of minor importance. In fact, at equal doses the activity of this enzyme is much less sensitive to the toxins than the phospholipid bilayer. Furthermore some effects of the toxins on physiological plant processes, such as H^+-extrusion from root segments [11] and movement of leaf stomata [unpublished], are not reversed by removing the toxins, and can be better accounted for by the irreversible cell damage consequent to the disruption of membrane integrity.

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