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BIOLOGICAL ACTIVITIES OF PSEUDOMYCIN A, A LIPODEPSINONAPEPTIDE FROM *PSEUDOMONAS SYRINGAE* MSU 16H*

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Key Word Index—*Pseudomonas syringae*; phytotoxins; lipodepsinonapeptides; proton extrusion; stomatal movement; fusicoccin; mitochondria; plasma membrane vesicles.

Abstract—Similarly to other *Pseudomonas* lipodepsinonapeptides, pseudomycin A inhibits proton extrusion from maize roots, promotes closure of stomata in *Vicia faba*, necrosis of tobacco leaves, haemolysis of human erythrocytes, affects H^+-ATPase activity and proton translocation in plasma membrane vesicles, and stimulates succinate respiration in pea mitochondria. In general, the biological activities of pseudomycin A are lower than those of syringomycin-E, the prototype member of this family of bacterial metabolites. This difference might depend on the diverse number and distribution of charged residues in the peptide moiety of these compounds.

INTRODUCTION

The last 10 years have witnessed a conspicuous progress in the chemistry of the phytotoxins produced by strains of the pathogen *Pseudomonas syringae* pv. *syringae*, which has a wide host range. With the integrated use of FAB-mass and NMR spectrometries, and a combination of chemical and enzymatic methods, the structure of a number of lipodepsipeptides has been elucidated. Most are nonapeptides: syringomycins [1–4], produced by strains pathogenic on stone fruits, pear and grass hosts [5, 6], syringotoxin [4, 7–9], purified from cultures of strains isolated from species of *Citrus* [6, 10], syringostatins [4, 11, 12], produced by lilac pathogens [12]. More recently it has been demonstrated [13] that also the pseudomycins, metabolites present in cultures of a transposon-generated regulatory mutant of a *P. syringae* strain (pathovar not assigned) proposed for the biocontrol of Dutch elm disease [14], belong to the same class of compounds. It has also been found that syringomycins and syringotoxin can be produced by *P. syringae* pv. *atrofaciens* [15] and *P. fuscovaginae* [16], respectively. The main features common to all these metabolites are: (a) L-Ser is the *N*-terminal residue *N*-acylated by a 3-hydroxy, or sometimes 3,4-dihydroxy fatty acid, and *O*-acylated by the terminal carboxyl; (b) the *C*-terminal tripeptide is always *Z*-dehydrobutyryl-3-hydroxy-L-aspartyl-4-chloro-L-threonyl; (c) the fifth amino acid residue corresponds to a basic amino acid.

Besides the lipodepsinonapeptides, most if not all the above bacteria produce lipodepsipeptides with a larger peptide moiety, predominantly made up of hydrophobic amino acid residues. Lipodepsipeptides with 22 and 25 residues, called syringopeptins, were isolated from cultures of various strains of *P. syringae* pv. *syringae* [17, 18], of the *P. syringae* strain producing the pseudomycins [13], and of *P. syringae* pv. *atrofaciens* [15]. Two similar metabolites with 19 residues, called fuscopeptins, were produced by *P. fuscovaginae* [19]. Tolaasin and tolaasin 144, two metabolites of the pathogen on cultivated mushrooms *P. tolaasi*, belong to the same type of products; their peptide moiety is formed by 17 amino acid residues [20].

The results of studies on the chemistry of the above phytotoxic lipodepsipeptides, and in particular some peculiar molecular features, have made an assessment of their importance for plant–pathogen interaction of interest. Recent investigations on the biological activity of syringomycin E [21–24], syringostatins [22], tolaasin [25, 26], syringopeptins [21, 23, 24] and fuscopeptins [19] have suggested that these phytotoxins not only affect the plasma membrane H^+-ATPase, but primarily perturb membrane permeability. All of them have marked amphipathic character and strong

* This paper is dedicated to Professor Antonio Graniti on the occasion of his 70th birthday.

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STRUCTURE OF PSEUDOMYCIN-A

CH_3-$(CH_2)_9$-CH(OH)-CH(OH)-CH_2-CO-L-Ser-D-Dab-L-Asp-L-Lys-L-Dab-L-*a*Thr-Z-Dhb-L-Asp(3-OH)-L-Thr(4-Cl)

STRUCTURE OF SYRINGOMYCIN-E

CH_3-$(CH_2)_8$-CH(OH)-CH_2-CO-L-Ser-D-Ser-D-Dab-L-Dab-L-Arg-L-Phe-Z-Dhb-L-Asp(3-OH)-L-Thr(4-Cl)

Dab, 2,4-diaminobutyric acid; Dhb, 2,3-dehydro-2-aminobutyric acid.

biosurfactant properties. In a variety of *in vitro* tests the lipodepsinonapeptides are several times less active than syringopeptins and fuscopeptins [19, 23, 27, 28]. The higher efficiency of the latter group of peptides might be the consequence of the long hydrophobic stretch, formed by the fatty acyl chain and most of the amino acid residues, which favours insertion into the host plasma membrane. This feature, together with the C-terminal cationic loop of the peptide moiety, is shared with other groups of membrane disrupting peptides, such as the polymyxins [29], ranalexin [30] and the type I brevinins [31].

As compared to the previously described lipodepsinonapeptides produced by *Pseudomonas syringae* pv. *syringae*, the recently isolated pseudomycins exhibit remarkable differences in the nature and/or position in the peptide moiety of the charged amino acid residues, in particular the unique occurrence of an L-Asp and an L-Lys residue [13]. Thus, it was of interest to investigate the effects of the pseudomycins on biological processes and biochemical tests affected by other lipodepsipeptides, and compare the results with those obtained with SR, the best characterized lipodepsinonapeptide.

Some preliminary data on the phytotoxicity of pseudomycin A, and on its *in vivo* and *in vitro* activities towards some processes operative in plants at the plasma membrane level have been reported [13].

RESULTS

Inhibition of H^+-extrusion in maize roots

As shown in Fig. 1(A) the strong stimulation by fusicoccin (FC) of H^+-extrusion from maize roots is reduced by 5 μM pseudomycin-A (PS), an effect not different from that observed with the same concentration of syringomycin-E (SR). In the 2–50 μM concentration range results with PS (data not reported) practically duplicate those obtained with SR [23]; namely, the inhibition by the toxins is concentration dependent and reaches a maximum at about 20 μM with both PS and SR. In the absence of FC both toxins gave pH values identical to those of the control without FC.

As previously observed for SR and syringopeptin 25A [23], the addition of PS to root segments incubated for 60 min with 0.1 mM FC is followed by the immediate inhibition of H^+-extrusion [Fig. 1(B)]. Table 1 demonstrates the irreversibility of the inhibition. Root segments were removed from the medium after 90 min incubation with 0.1 mM FC and either SR or PS (both 5 μM), washed with buffer and again incubated for 90 min with 0.1 mM FC in absence of the toxins. The acidification rate of the medium is nearly the same as that measured during the first incubation.

Inhibition of stomatal movement

Stomata present in epidermal fragments of *Vicia faba*, wide open after a 3 μM FC pretreatment [27], rapidly closed on incubation with 1 μM PS and remained closed even after a further 4 hr incubation in a 0.1 mM FC solution. The concentration dependence of the effect is identical to that established for SR [27].

Necrotic effect in detached tobacco leaves

Equal concentrations of PS and SR (5 μM) gave the same intensity of necrotic symptoms in a tobacco leaf assay [21].

Haemolytic activity

The integrity of human erythrocytes is impaired by substantially equal levels of SR and PS. Both reach a maximum of one arbitrary unit at 0.5 μM concentration.

Effect on H^+-ATPase activity of plasma membrane vesicles

The ATPase activity of right-side-out vesicles was slightly stimulated by 10 μM and more consistently

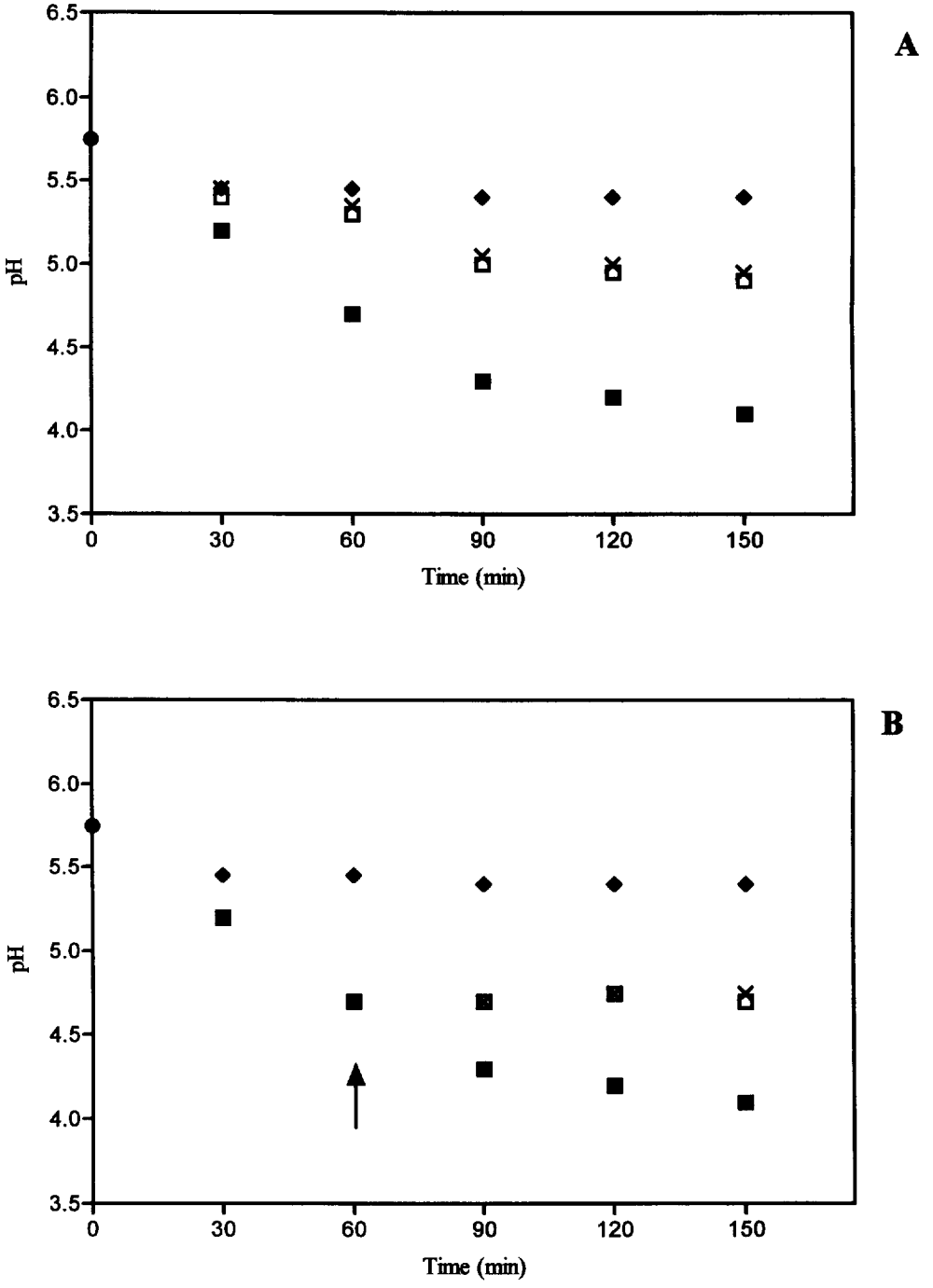

Fig. 1. A: Effect of *P. syringae* toxins on FC-stimulated proton extrusion from maize roots. Roots were incubated with 0.1 mM FC in the absence of *P. syringae* toxins (■) or in the presence of 5 μM SR (X) or 5 μM PS-A (□). ◆, control without FC. ●, initial pH value. B: Effect of *P. syringae* toxins on FC-stimulated proton extrusion from maize roots. After 1 hr incubation in the presence of 0.1 mM FC, 5 μM SR (X) or 5 μM PS-A (□) were added (arrow), ◆, control without FC; ■, FC only; ●, initial pH value. Each value is the average of two separate experiments carried out with duplicate samples: S.E. did not exceed 0.10 pH units.

by 50 μM PS; the stimulation was 10 and 40%, respectively, as compared to 30 and 40% in the case of SR [23]. As expected, both toxins inhibited the activity of inside-out vesicles; at 50 μM concentration the loss of activity was 25% with PS, and 50% with SR (Fig. 2).

Effect on H^+*-translocation in plasma membrane vesicles*

Figure 3(A) reports the effect of 10 min pre-incubation of the vesicles with 10 μM toxins on the

Table 1. Irreversible effect of Pss toxins on FC-stimulated proton extrusion from maize roots; after a first 90′ incubation with 5 μM SR or PS in the presence of 0.1 mM FC, roots were washed and submitted to a second 90′ incubation with 0.1 mM FC in the absence of SR or PS. The initial pH for both steps was 5.75. Each value is the average of two separate experiments, carried out with duplicate samples; S.E. did not exceed 0.15 pH units

	pH after first incubation	pH after second incubation
No additions	5.55	5.70
FC	4.50	4.40
SR	5.55	5.70
PS	5.55	5.70
SR + FC	5.20	5.30
PS + FC	5.15	5.25

formation of the pH gradient and shows that PS has a weaker inhibitory activity than SR. As shown in Fig. 3(B), the pH gradient formed by the addition of ATP to Triton X-100-activated plasma membrane vesicles was affected by 20 μM PS slightly more than 0.2 mM vanadate, a specific inhibitor of the plasma membrane ATPase, while it was collapsed markedly by 10 μM, and completely by 20 μM SR at a rate comparable to that observed with 20 mM of the freely permeant ammonium sulphate.

Effects on mitochondria

Stimulation of succinate respiration by pea mitochondria (Fig. 4) is less pronounced in the presence of PS than SR. The effect of 5 μM SR is equalled by 20 μM PS. The small inhibition of the transmembrane potential produced in pea mitochondria by 20 μM SR [28] is not observed with the same amount of PS. The ATPase activity of pea mitochondria is virtually insensitive to both SR and PS, up to 50 μM concentrations.

DISCUSSION

These results show that the two lipodepsinonapeptides SR and PS have a very similar activity in a number of *in vivo* and *in vitro* biological tests. Quantitatively, PS is in general less active than SR, perhaps in consequence of the different number and distribution of charged residues in the peptide moiety. These differences might influence the ability to form channels, a property recently ascribed to SR [32, 33], but likely shared with the phytotoxic lipodepsinonapeptides structurally related to it. This group of bacterial metabolites, pseudomycins included, are toxic to a broad range of plant pathogenic fungi [34, and unpublished data]. PS has also been tested on fungi responsible for systemic mycoses in humans and, in consequence of its high activity, has been claimed as a potentially interesting antimycotic drug [14]. Data on the activity of SR against human fungal pathogens, at present not available in the scientific literature, are highly desirable in order to compare them with those of PS and decide if, in case of commercial applications, the production of SR by *Pseudomonas syringae* pv. *syringae* B359, a strain from millet (collection of the Department of Plant Pathology of the University of California at Davis), is preferable to

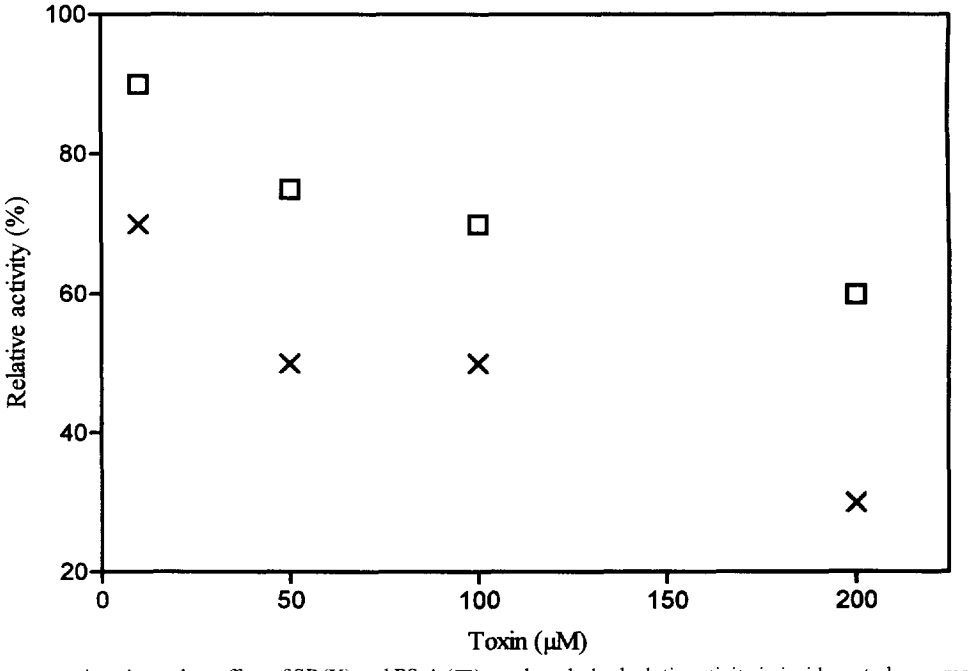

Fig. 2. Concentration-dependent effect of SR(X) and PS-A (□) on phosphohydrolytic activity in inside-out plasma membrane vesicles from maize roots. The experiments were repeated three times with duplicate samples: S.E. did not exceed 10%.

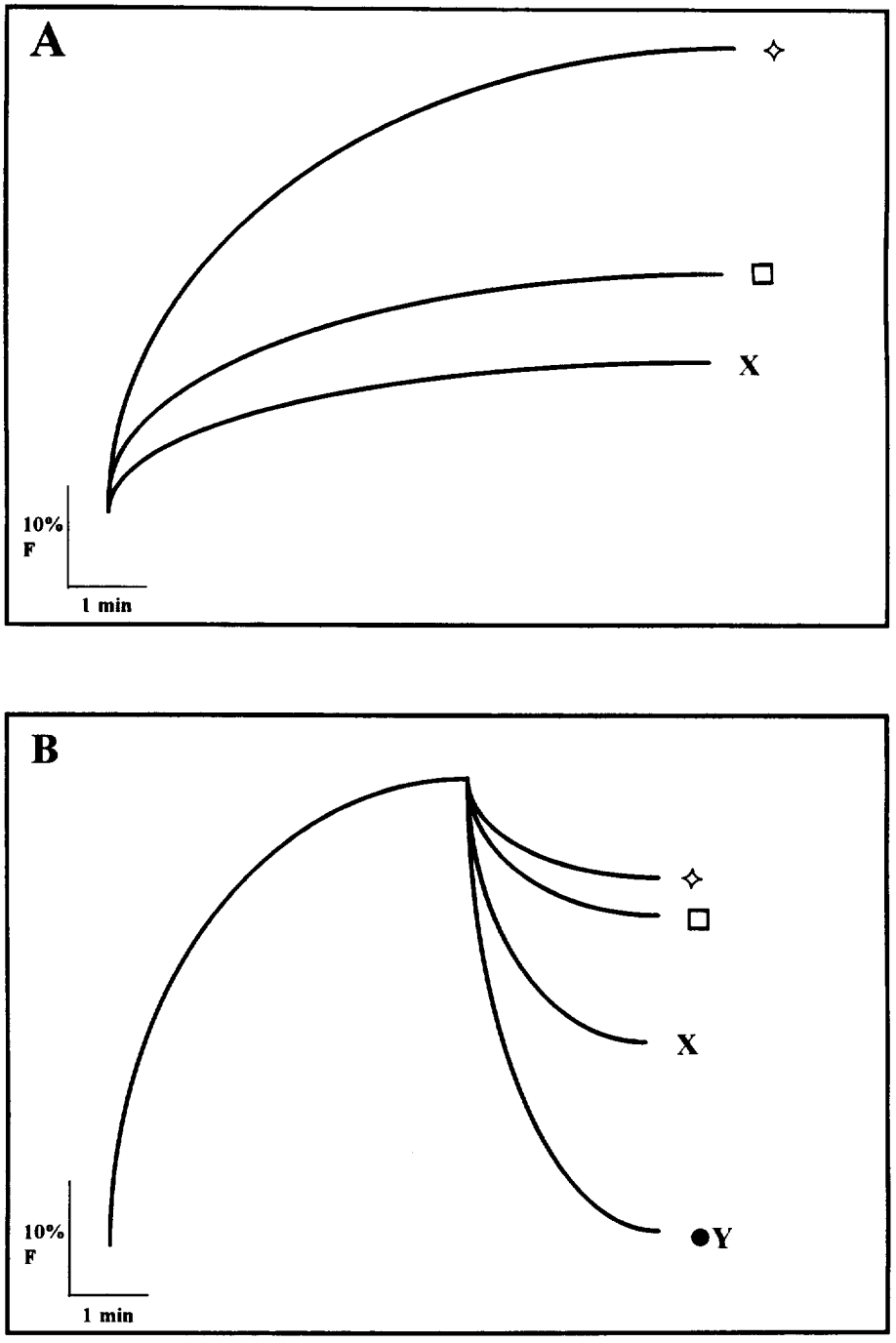

Fig. 3. A: Proton translocation in plasma membrane vesicles preincubated with 10 μM SR (X) or 10 μM PS-A (□); (◇), control. B: Dissipation of the pH gradient in plasma membrane vesicles: X, 10 μM SR; Y, 20 μM SR; □, 20 μM PS-A; (◇), 0.2 mM vanadate; ●, 20 mM ammonium sulphate. The experiments were carried out five times, with similar results.

that of PS by the engineered *P. syringae* MSU 16H. We have observed [unpublished data] that the yield of active metabolites is more or less the same for the two organisms, while the amount of pseudomycins made by the wild-type *P. syringae* MSU 174 is at least 10 times less than that obtained with the transposon-generated mutant.

EXPERIMENTAL

Phytotoxins. SR and PS were obtained as described in refs [2] and [13], respectively. FC was a recrystallized sample of a batch produced as in ref. [35].

Biological tests. Movement of stomata, prepd from epidermal strips of *Vicia faba*, was measured under

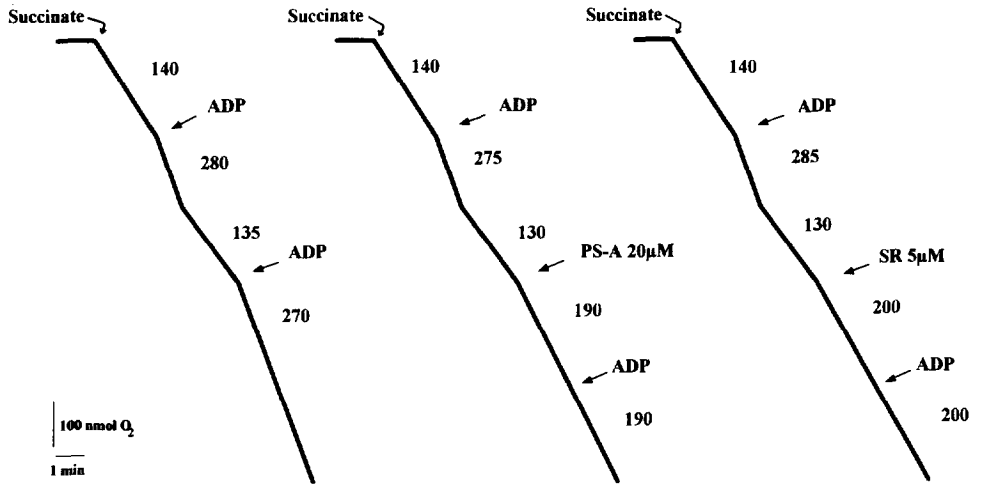

Fig. 4. Typical experiments showing the effect of PS-A and SR on pea mitochondrial respiration. The experiments were carried out five times, with similar results.

conditions described in ref. [27]. Proton extrusion from maize roots, ATP-dependent proton translocation in plasma membrane vesicles prepd from maize roots, phosphohydrolytic activity of H^+-ATPase before and after enzyme solubilization from the same vesicles, were measured by methods described in refs [23] and [24]. Pea mitochondria, prepd as in ref. [8], were used for respiration experiments on NADH and succinate, and for ATPase and $\Delta\Psi$ determinations as in ref. [28]. The necrosis assay on tobacco leaves was carried out as in ref. [21]. Haemolysis experiments with human erytrocytes were carried out as in ref. [36].

Chemical assays. Proteins were assayed by the method of Bradford [37] using Bio-Rad reagent and Bio-Rad bovine gammaglobulin as standard (Richmond, CA, U.S.A.).

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