褐紋病菌サプレッサーによるエンドウ原形質膜ATPase再構成膜におけるプロトンポンプ活性の抑制

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著者名	天野,政史 豊田,和弘 一瀬,勇規 山田,哲治 白石,友紀
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H^+-translocating Activity in Proteoliposomes Reconstituted with Pea Plasma Membrane ATPase and Its Inhibition by Fungal Suppressor from *Mycosphaerella pinodes*

Masashi AMANO*, Kazuhiro TOYODA*, Yuki ICHINOSE*,
Tetsuji YAMADA* and Tomonori SHIRAISHI*

Abstract

Effects of the elicitor and the suppressor from a pea pathogen, *Mycosphaerella pinodes*, on the H^+-translocating activity of ATPase in pea plasma membranes were examined. The plasma membrane ATPase was solubilized with Triton X-100 and partially purified by continuous glycerol density gradient centrifugation. The ATPase fraction was reconstituted into soybean phospholipid (asolectin) liposomes by a Triton-X 100 dilution method. Almost all of resultant proteoliposome vesicles had unimembranes in size from 50 to 200 nm. The ATP-driven H^+-pumping activity was measured by quinacrine fluorescence quenching in the presence of Mg^{2+}-ATP. The activity was sensitive to orthovanadate, dicyclohexylcarbodiimide (DCCD), verapamil and neomycin but it was insensitive to azide and nitrate as well as ATP-hydrolyzing activity. Proton gradient was collapsed by NH_4Cl or a channel-forming ionophore, gramicidin D. The H^+-pumping activity in proteoliposomes was hardly affected by the elicitor. The finding suggests that a putative target molecule of or receptor for the elicitor might not associated with the solubilized ATPase or that the elicitor could not reach the target site. However, the suppressor markedly inhibited both activities in proteoliposomes, showing that the H^+-translocating ATPase might be inhibited directly *in vitro* by the suppressor as reported previously and that the fungal suppressor may disturb the regulation of pH in the host cells. Both activities were increased by the addition of phosphatidylinositolbisphosphate, even if in the presence of the suppressor. However, PIP_2 could not completely negate the effect of the suppressor. These results suggest that the H^+-pumping activity of plasma membrane ATPase is also regulated by polyphosphoinositide metabolism and that the action sites of the suppressor on the ATPase may be different from those of PIP_2.

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Key words: elicitor, H^+-translocating plasma membrane ATPase, *Mycosphaerella pinodes*, *Pisum sativum* L., proteoliposomes, suppressor.

INTRODUCTION

Mycosphaerella pinodes, a pathogen of pea, secretes both a polysaccharide elicitor and glycopeptide suppressors in its pycnospore germination fluid[21,27,28,30]. The elicitor induces several defense responses of pea, such as production of a phytoalexin, pisatin[27], and of an as yet-unidentified infection inhibitor[36] as well as activation of pathogenesis-related proteins, endochitinase and β-1,3-glucanase[28]. However, the concomitant presence of the suppressor inhibits or delays these defense responses in pea tissues[20,27,35,36] and conditions pea tissues to be susceptible to even nonpathogens[21,27].

We showed previously that the suppressor inhibited pea plasma membrane ATPase both *in vivo* and *in vitro*[26,39] and that the inhibition of the ATPase activity was highly correlated with suppression of the defense responses in pea tissues[37-39]. Recently, two suppressors from *M. pinodes*, supprescins A and B, were purified and characterized as GalNAc-*O*-Ser-Ser-Gly and Gal(β1-4)-GalNAc-*O*-Ser-Ser-Gly-Asp-Glu-Thr, respectively[28]. It was further shown that the peptide sequences of supprescins, such as Ser-Ser-Gly and Asp-Glu, might act on ATP-binding domain and phosphatase domain, respectively, in ATPase molecules[13].

Plasma membrane ATPase is known as a "master enzyme", since it is responsible for many fundamental functions in cells[25]. One of these functions was reported to translocate protons, regulating intracellular pH[4,10,19,25,33]. In regard to plant-parasite system, there were several reports that the metabolites of plant pathogens, such as syringomycins[7] and fusicoccin[24] affected ATP-dependent H^+-pumping in isolated plasma mem-

* Laboratory of Plant Pathology and Genetic Engineering, College of Agriculture, Okayama University, Okayama 700, Japan 岡山大学農学部

brane vesicles. It was also reported that fusicoccin activated H^+-pumping in proteoliposomes reconstituted with maize plasma membrane ATPase[16]. However, it has been unknown whether elicitors or suppressors from fungal pathogens affect ATP-dependent H^+-pumping activity in proteoliposomes reconstituted with plasma membrane ATPase. In this report, therefore, we reconstitute the ATPase, which is partially purified from pea plasma membranes, into liposomes by a Triton X-100 dilution method and examine the effects of the elicitor and the suppressor from *M. pinodes* on the activities of ATPase and H^+-pumping in the proteoliposomes. Furthermore, we examine whether phosphatidylinositol-bisphosphate (PIP_2), a key product in polyphosphoinositide metabolism (PI metabolism), regulates both activities in the proteoliposomes, since there is a possibility of "cross talk" between the ATPase and PI metabolism[29], which may participate in signal transduction cascade leading to defense responses in pea plants[31,32].

MATERIALS AND METHODS

Chemicals. Bovine serum albumin (BSA; fatty acid free) was obtained from Nacalai Tesque Inc., Kyoto, Japan. Tris(hydroxymethyl)aminomethane, ATP, gramicidin D, neomycin, quinacrine, phosphatidylinositol-4,5,-bisphosphate (PIP_2) and soybean phospholipid (type II-S) were purchased from Sigma Co. Ltd. A column (Econopac 10 Disposable SM-2) for exclusion of Triton X-100 was obtained from Bio-Rad laboratories, Richmond, CA. Other chemicals were purchased from Wako Pure Chemical Inc., Osaka, Japan.

Plant material. Seeds of pea (*Pisum sativum* L., cv. Midoriusui) were sown on moistened vermiculite in a plastic container, and were grown in darkness at 22±2°C for 9 to 12 days.

Preparation of elicitor and suppressor from M. pinodes. The elicitor and suppressor were prepared from the germination fluid of pycnospores of *M. pinodes* (Berk. et Blox.) Vestergren, strain OMP-1 (IFO-30342, ATCC-42741) as described previously[9,39]. The concentrations of stock solutions of the elicitor and the suppressor were adjusted to 1 mg/ml (glucose equiv.) and 1 mg/ml (BSA equiv.) by the methods of Dubois *et al.*[6] and Lowry *et al.*[15], respectively. In this experiment, we used the partially purified suppressors, which contained supprescins A and B, for measuring overall effect of the suppressor on the activities of ATPase and H^+-pumping.

Preparation of plasma membrane fraction. The fraction of plasma membranes was prepared from 9 to 12-day-old pea seedlings as described previously[39]. The isolated plasma membrane fraction was stored at −80°C until usage of it.

Solubilization and partial purification of pea plasma membrane ATPase. The ATPase was solubilized from the plasma membrane fraction with 0.5% (v/v) Triton X-100 by 5 times of sonication for 30 sec at 0°C in a bath-type sonicator (Branson 3200, Yamato, Tokyo, Japan). The solubilized fraction was, then, centrifuged at 100,000×*g* for 30 min. The supernatant was collected and overlayed on 23-43% (v/v) continuous glycerol gradient containing 5 mM Tris-HCl (pH 7.6), 1 mM DTT, 1 mM PMSF, 0.005% (w/v) Zwittergent 3-14, 1 μg/ml antipain and 1 μg/ml leupeptin (total volume was 33 ml). After centrifuging for 21 hr at 100,600×*g*, each 1 ml of sample was collected from top of the gradient into an Eppendolf tube. Two fractions (Nos. 9 and 10; see Fig. 1) were subsequently used for reconstitution into liposomes.

Reconstitution of ATPase into liposomes. Reconstitution of ATPase was carried out by the method described by Kasamo[10] and Kasamo and Yamanishi[12] with a slight modification. Ten mg of phospholipid (from soybean) were suspended in 1 ml of 0.5% Triton X-100 in 20 mM Tris-MES (pH 7.2) by sonication, and then, the phospholipid emersion was poured into 1 ml of ATPase fraction (*ca.* 100 μg protein, BSA equiv.) in 10 mM Tris-MES (pH 7.2) containing 100 mM KCl and 1 mM EDTA (buffer A). After the mixture was stirred for 15 sec and incubated on ice for 15 min, it was applied to a SM-2 column, which had been equilibrated with buffer A. After SM-2 column was washed with 5 ml of buffer A, the eluate was diluted about 5 fold with buffer A and centrifuged at 100,000×*g* for 30 min to precipitate the proteoliposomes. The pellet was suspended with 0.15 to 0.2 ml of 25 mM Tris-MES (pH 7.5) containing 45% (v/v) glycerol, 0.3 M KCl and 2 mM EDTA, and was stored at −20°C until usage for assays of ATPase and H^+-pumping. The content of protein in each fraction was determined by the method of Bradford[3] with BSA as the standard.

Assays of ATPase and H^+-pumping. The ATPase activity was determined by the method of Perlin and Spanswick[23]. The assay was carried out at 37°C for 30 min using 0.3 to 0.8 μg protein per assay in 30 mM Tris-MES (pH 6.5) containing 3 mM ATP-$MgSO_4$. The H^+-pumping activity was determined by quinacrine fluorescence quenching with a spectrofluorometer (Model F-2000; Hitachi, Tokyo, Japan), with the wavelengths of excitation and emission at 425 and 500 nm, respectively, as described by Kasamo[10]. Typically, proteoliposomes containing *ca.* 5 μg protein were diluted with 600 μl of 30 mM Tris-MES (pH 6.8) containing 0.25 M sucrose, 50 mM KCl and 4 μM quinacrine in the presence or absence of 1 mM Na_3VO_4, 500 μM DCCD, 250 μM neomycin, 0.1 mM NaN_3, 50 mM $NaNO_3$, 100 μg/ml of the elicitor, 100 μg/ml of the suppressor or 20 μg/ml PIP_2. After preincubation at 25°C for 10 min, the reaction was started by the addition of 3 mM ATP-$MgSO_4$. The assay was carried out at 25°C for 10 min and amount of formed protons was determined from the results of fluorescence change as described by Vianello *et al.*[34]

RESULTS

Distribution of ATPase activity in a glycerol density gradient

After solubilized pea plasma membranes were ultracentrifuged, the protein contents and ATPase activities in the supernatant and pellet were determined. About 75% of proteins in plasma membrane fraction were recovered in the supernatant fraction, which contained about 70% of total ATPase activity after solubilization. The specific activities of ATPase in plasma membranes and the solubilized fraction were 5.58±0.55 and 6.92±0.45 μmol P_i/mg protein/hr, respectively. Figure 1 showed the distributions of protein and ATPase activity in continuous glycerol density gradient. The major peak of ATPase activity was separated from that of proteins. Two fractions of the major peak of ATPase, Nos. 9 and 10, were used for reconstitution into liposomes. The specific activity of ATPase in the mixture of Nos. 9 and 10 fraction was 8.20±0.31 μmol P_i/mg protein/hr. Thus,

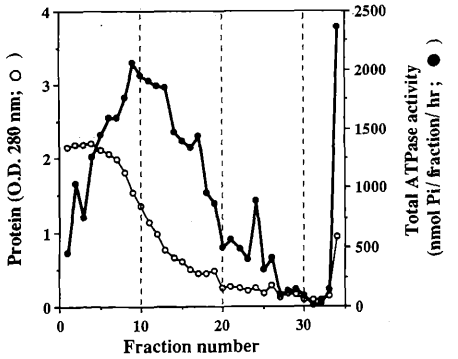

Fig. 1. Distribution of ATPase activity (●) and protein (○) after a liner 23 to 43% glycerol density gradient centrifugation of the Triton X-100 solubilized plasma membrane fraction. The ATPase activity was measured in the presence of 3 mM ATP-$MgSO_4$ and 30 mM Tris-MES (pH 6.5) as described in the text.

Table 1. Effects of several inhibitors and the elicitor and the suppressor from *M. pinodes* on the activity of ATPase in proteoliposomes

Treatment (final concentration)	ATPase activity[a] (μmol P_i/mg protein/hr)	Mean value as % of control
Control	6.34±0.20	100
Control (+0.01% Triton X-100)	8.78±0.23	138.5
Vanadate (1 mM)	1.64±0.01	25.9
Neomycin (0.25 mM)	1.70±0.09	26.8
Verapamil (0.25 mM)	1.65±0.10	26.0
Azide (0.1 mM)	6.07±0.05	95.7
Nitrate (50 mM)	5.79±0.05	91.3
Elicitor (100 μg/ml)	6.06±0.16	95.6
Suppressor (100 μg/ml)	1.27±0.01	20.0

a) The assay for ATPase activity was carried out in 30 mM Tris-MES (pH 6.5) that contained 3 mM $MgSO_4$ and 3 mM ATP at 37°C for 30 min by the method described by Perlin and Spanswick (1981).

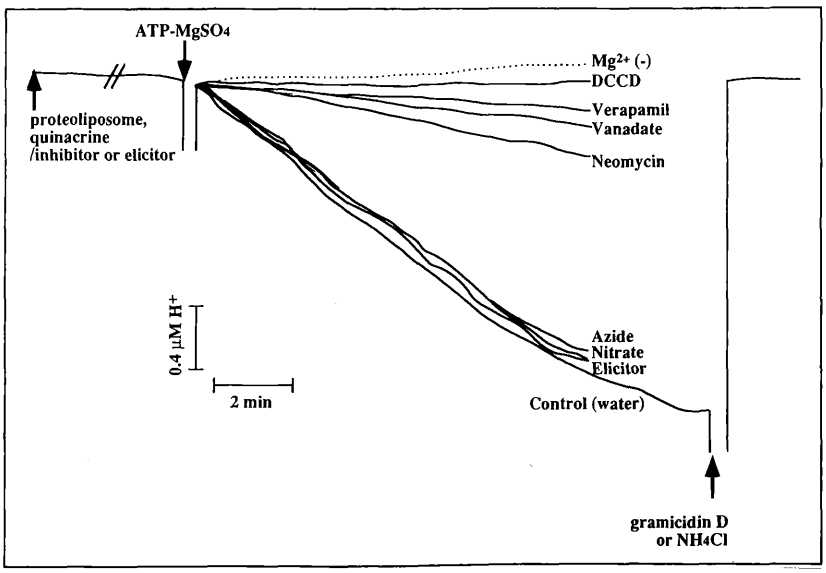

Fig. 2. ATP-dependent H^+-pumping activity in proteoliposomes, as measured by monitoring quinacrine quenching fluorescence. The reaction was started by the addition of 3 mM ATP-$MgSO_4$ (middle allow). List of tested compounds was as follows: Na_3VO_4 (1 mM), neomycin (0.25 mM), verapamil (0.25 mM), NaN_3 (0.1 mM), $NaNO_3$ (50 mM) and the elicitor (100 μg/ml; glucose equiv.), respectively. Note that the absence of Mg^{2+} (dotted line) showed no H^+-pumping activity and that the addition of 90 mM NH_4Cl or 2.5 μM gramicidin D (right allow) collapsed the proton gradient.

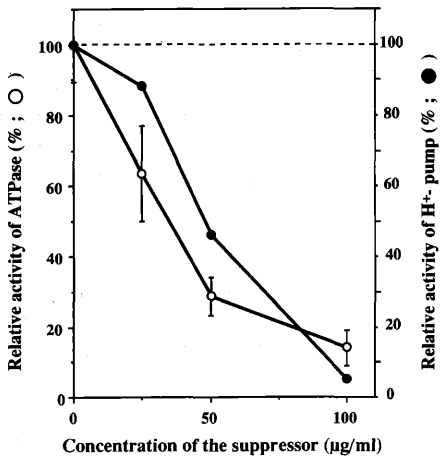

Fig. 3. Effect of the suppressor from *M. pinodes* on the activities of ATPase (○) and H^+-pumping (●) in proteoliposomes. The concentration of suppressor was determined with BSA as the standard as described in the text. The relative activities were given as the percentages of the activities of ATPase (7.86±0.07 μmol P_i/mg protein/hr) and H^+-pumping (65.6 μmol H^+/mg protein/min) in the absence of the suppressor.

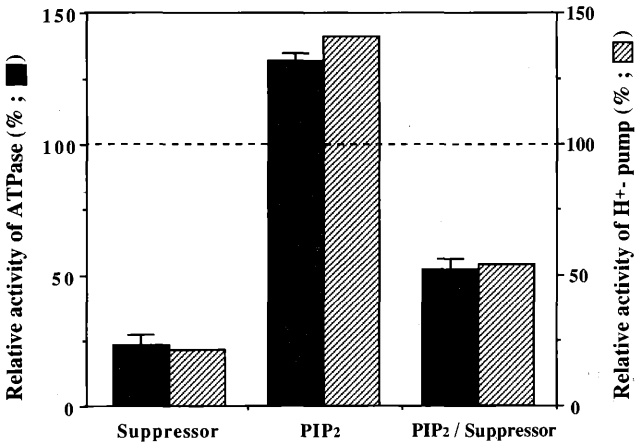

Fig. 4. Effect of the suppressor and PIP_2 on the activities of ATPase (■) and H^+-pumping (▨) in proteoliposomes. The concentrations of PIP_2 and suppressor were 20 μg/ml and 100 μg/ml (BSA equiv.), respectively. The relative activities were given as the percentage of those of ATPase (9.26±0.73 μmol P_i/mg protein/hr) and H^+-pumping (67.0 μmol H^+/mg protein/min) in the absence of both substances (the control, which was presented by the broken line).

no marked increase of the specific activity of ATPase was observed by these handlings, as reported by Vara and Serrano[33] with oat root plasma membranes. In addition, the ATPase activity in these fractions was inhibited by orthovanadate, verapamil and the suppressor from *M. pinodes* but not by azide and nitrate (data not shown).

ATPase activity in proteoliposomes

A transmission electronmicroscopic observation with 1% phosphotangustenic acid in 0.1 M phosphate buffer (pH 7.2) showed that almost all of proteoliposomes had the size from 50 nm to 200 nm (data not shown) as reported by Kasamo[10]. This result showed that the Triton X-100 dilution method with a SM-2 column was also available to make proteoliposomes. Table 1 showed the effects of orthovanadate, azide, nitrate, verapamil, neomycin, the elicitor and the suppressor from *M. pinodes* on ATPase activity in proteoliposomes. The ATPase activity in proteoliposomes was inhibited by orthovanadate, verapamil, neomycin and the suppressor from *M. pinodes* but was hardly affected by azide, nitrate and the elicitor from the fungus. In addition, the ATPase activity in proteoliposomes was increased approximately 1.4 fold in the presence of 0.01% (v/v) Triton X-100 compared to that in the absence of the detergent (Table 1). The ATPase activity in the detergent-treated proteoliposomes was also inhibited by orthovanadate and the suppressor (data not shown).

H^+-pumping activity in proteoliposomes

As shown in Fig. 2, intraliposomal acidification was started by the addition of ATP-$MgSO_4$ but not in the absence of $MgSO_4$. In the absence of ATP, the activity was also undetected (data not shown). The ATP-driven H^+-pumping activity in proteoliposomes was collapsed immediately by the addition of 90 mM NH_4Cl or 2.5 μM gramicidin D. The H^+-pumping activity was inhibited by orthovanadate, DCCD, neomycin and verapamil but was hardly affected by azide, nitrate and the elicitor as well as the ATPase activity (Table 1). In addition, the activity was increased about 1.5 fold by 200 μM fusicoccin (a kind gift from Prof. A. Graniti, Bari University, Bari, Italy), compared to the control experiment (data not shown). Figure 3 showed the effect of the suppressor from *M. pinodes* on the activities of ATPase and H^+-pumping in proteoliposomes. Both activities were dose-dependently inhibited by the suppressor.

Effects of the suppressor and PIP_2 on the activities of ATPase and H^+-pumping in proteoliposomes

The effects of the suppressor and PIP_2 on the activities of ATPase and H^+-pumping in proteoliposomes were shown in Fig. 4. Both activities were enhanced about 1.3 fold by the addition of 20 μg/ml of PIP_2, compared to the control (neither addition of PIP_2 nor the suppressor; presented by the broken line in Fig. 4). On the other hand, the suppressor decreased about 75% of both activities of the control. Thus, the change of H^+-pumping activity, that was induced by these substances, was quite similar to that of ATPase activity.

Interestingly, the inhibition of both activities by the suppressor was scarcely affected by the concomitant presence of PIP_2 as well as the enhancement of them by PIP_2 was not negated by the concomitant presence of the suppressor. For example, the suppressor decreased 6.90±0.27 μmol P_i/mg protein/hr of the ATPase activity in the absence of PIP_2. On the other hand, in the

presence of PIP_2, 7.20±0.29 μmol P_i/mg protein/hr of the ATPase activity was inhibited by the suppressor. Thus, in the presence and absence of PIP_2, the reduction rates of the ATPase activities by the suppressor were 74.5 and 77.8% of the control (9.26±0.73 μmol P_i/mg protein/hr), respectively. It was also shown that, in spite of the presence and absence of the suppressor, PIP_2 increased 2.62±0.53 and 2.92±0.55 μmol P_i/mg protein/hr of the ATPase activity (28.3 and 31.5% of the control), respectively.

DISCUSSION

In this report, we investigated the effects of the elicitor and the suppressor from *M. pinodes* on H^+-pumping activity in proteoliposomes reconstituted with the ATPase that was partially purified from pea plasma membranes. The activity of H^+-pumping in proteoliposomes was dependent on ATP and Mg^{2+}, and was inhibited by orthovanadate, DCCD, verapamil and neomycin that inhibited pea plasma membrane ATPase (Table 1, Fig. 2). However, the H^+-pumping activity was hardly affected by azide and nitrate as well as the ATPase activity (Table 1, Fig. 2). These results show that pea plasma membrane ATPase also carries the H^+-pumping activity as observed on those of tomato[1], radish[4], mung bean[10] and oat[33].

Low and Heinstein[14] reported that change of pH was rapidly induced by the elicitor from *Verticillium dahliae* in cultured cells of cotton, tobacco or soybean. They thought that such changes might be involved in the initial events of the elicitor-signal transduction. It was also reported that rapid alkalinization in the extracellular fluid was induced by the elicitor or inoculation with an incompatible pathogen and such a change was thought to be dependent on plasma membrane H^+-ATPase[2,5,8]. However, in our present study, both activities of ATPase and H^+-pumping in proteoliposomes were not affected by the polysaccharide elicitor from *M. pinodes* (Table 1, Fig. 2). Our results suggest that the receptor for the elicitor might not be associated with the solubilized ATPase in proteoliposomes or that the elicitor could not reach the target sites on ATPase if the sites existed in the inside of proteoliposomes.

By contrast, the suppressor from *M. pinodes* markedly inhibited both activities of ATPase and H^+-pumping in proteoliposomes in a dose-dependent manner (Figs. 3 and 4). This result did not conflict with our previous reports in regard to ATPase activity in pea cells and plasma membranes treated with the suppressor[26,39]. Kato *et al.*[13] suggested that the suppressor might directly inhibit the ATPase activity in pea plasma membranes *in vitro* by affecting ATP-binding domain and phosphatase domain in the ATPase molecules. Therefore, the inhibition of both activities in proteoliposomes by the suppressor is thought to be directly evoked, since these domains may be arranged on the outside of proteoliposomes. Thus, together with our previous reports, the present data clearly show that one of the action sites of the *M. pinodes*-suppressor is H^+-translocating plasma membrane ATPase.

Figure 4 shows that PIP_2, a key product in PI metabolism, enhances about 1.3 fold both activities of the ATPase and H^+-pumping in the proteoliposomes, compared to the control. It was reported that ATPase activity in plasma membranes was regulated by the circumferential lipid components[11]. Memon *et al.*[18] reported that inositol phospholipids activated plasma membrane ATPase in cultured cells of sunflower and carrot. Furthermore, Memon and Boss[17] demonstrated that inositol phospholipids in plasma membranes were closely associated with vanadate-sensitive ATPase activity, suggesting that changes in the content of inositol phospholipids in plasma membranes might severely affect the ATPase activity. It was also reported that a phospholipid, lysophosphatidylcholine, stimulated ATP-dependent H^+-pumping in isolated oat root plasma membrane vesicles[22]. We previously showed that the suppressor also affected PI metabolism in pea plasma membranes[31,32] and that PIP_2 enhanced the ATPase activity in pea plasma membranes about 1.8 fold at a concentration of 40 μM (our unpublished data)[29]. Neomycin, however, was reported to inhibit PI-metabolism and ATPase in pea plasma membranes[29,31]. In the present study, H^+-pumping activity in proteoliposomes were also inhibited by the addition of neomycin, a trapper of PIP_2 (Table 2, Fig. 2). Together with previous reports, our present data indicate that PI metabolism, which is in the signal transduction cascade leading to defense responses, regulates H^+-pumping activity of pea plasma membrane ATPase and that there is a close functional association (cross talk) between ATPase and PI metabolism in pea plasma membranes.

As described in the Results, the values of both activities of ATPase and H^+-pumping, that were enhanced by PIP_2 or were reduced by the suppressor, seem to be constant in spite of the concomitant presence of respective substance (Fig. 4). In other words, the respective effects of the suppressor and PIP_2 on pea H^+-translocating ATPase may not be influenced by mutual presence. Such a finding suggests that the target sites of the suppressor on pea plasma membrane ATPase may be different from those of PIP_2 and that each substance may act independently on H^+-translocating ATPase.

In conclusion, we propose that the suppressor inhibits H^+-pumping activity in plasma membrane ATPase, thereby disturbing host homeostasis (regulation of intracellular pH) and interfering host defense responses, ultimately enabling *M. pinodes* establishing its infection on the host plant. However, it has been obscure how the suppressor inhibits the H^+-translocating ATPase species-specifically *in vivo*. Further experiments are needed for elucidation of the mechanism in determination of host-parasite specificity. The system of reconstituted proteoliposomes may contribute to such pur-

pose.

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Literature cited

1. Anthon, G.E. and Spanswick, R.M. (1986). Purification and properties of the H^+-translocating ATPase from the plasma membrane of tomato roots. Plant Physiol. 81 : 1080-1085.
2. Atkinson, M.M. and Baker, C.J. (1989). Role of the plasmalemma H^+-ATPase in *Pseudomonas syringae*-induced K^+/H^+ exchange in suspension-cultured tobacco cells. Plant Physiol. 91 : 298-303.
3. Bradford, M.H. (1976). Rapid and sensitive method for the quantitative microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72 : 248-254.
4. Cocucci, M.C., Michelis, M.I.D., Pugliarello, M.C. and Rasi-Caldogno, F. (1985). Reconstitution of proton pumping activity of plasma membrane ATPase purified from radish. Plant Sci. Lett. 37 : 189-193.
5. Conrath, U., Jeblick, W. and Kauss, H. (1991). The protein kinase inhibitor, K-252a, decreases elicitor-induced Ca^{2+} uptake and K^+ release, and increases coumarin synthesis in parsley cells. FEBS Lett. 279 : 141-144.
6. Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956). Colorimetric method for determination of sugars and related substances. Anal. Chem. 28 : 350-356.
7. Giorgio, D.D., Camoni, L. and Ballio, A. (1994). Toxins of *Pseudomonas syringae* pv. *syringae* affect H^+-transport across the plasma membrane of maize. Physiol. Plant. 91 : 741-746.
8. Hagendoorn, M.J.M., Poortinga, A.M., Wong Fong Sang, H.W., van der Plas, L.H.W. and van Walraven, H.S. (1991). Effect of elicitors on the plasmamembrane of *Petunia hybrida* cell suspensions: role of ΔpH in signal transduction. Plant Physiol. 96 : 1261-1267.
9. Hiramatsu, M., Ichinose, Y., Shiraishi, T., Oku, H. and Ouchi, S. (1986). Regulation of pisatin biosynthesis in pea leaves by elicitor and suppressor produced by *Mycosphaerella pinodes*. Ann. Phytopathol. Soc. Jpn. 52 : 53-58.
10. Kasamo, K. (1987). Reconstitution and characterization of H^+-translocating ATPase from the plasma membrane of *Phaseolus mungo* L. roots. Plant Cell Physiol. 28 : 19-28.
11. Kasamo, K. and Nouchi, I. (1987). The role of phospholipids in plasma membrane ATPase activity in *Vigna radiata* L. (mung bean) roots and hypocotyls. Plant Physiol. 83 : 323-328.
12. Kasamo, K. and Yamanishi, H. (1991). Functional reconstitution of plasma membrane H^+-ATPase from mung bean (*Vigna radiata* L.) hypocotyls in liposomes prepared with various molecular species of phospholipids. Plant Cell Physiol. 32 : 1219-1225.
13. Kato, T., Shiraishi, T., Toyoda, K., Saitoh, K., Satoh, Y., Tahara, M., Yamada, T. and Oku, H. (1993). Inhibition of ATPase activity in pea plasma membranes by fungal suppressors from *Mycosphaerella pinodes* and their peptide moieties. Plant Cell Physiol. 34 : 439-445.
14. Low, P.S. and Heinstein, P.F. (1986). Elicitor stimulation of the defense response in cultured plant cells monitored by fluorescent dyes. Arch. Biochem. Biophys. 249 : 472-479.
15. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193 : 265-275.
16. Marra, M., Ballio, A., Fullone, M.R. and Aducci, P. (1992). Some properties of a functional reconstituted plasmalemma H^+-ATPase activated by fusicoccin. Plant Physiol. 98 : 1029-1034.
17. Memon, A.R. and Boss, W.F. (1990). Rapid light-induced changes in phosphoinositide kinases and H^+-ATPase in plasma membrane of sunflower hypocotyls. J. Biol. Chem. 265 : 14817-14821.
18. Memon, A.R., Chen, Q. and Boss, W.F. (1989). Inositol phospholipids activate plasma membrane ATPase in plants. Biochem. Biophys. Res. Commun. 162 : 1295-1301.
19. Nelson, N. (1988). Structure, function, and evolution of proton-ATPases. Plant Physiol. 86 : 1-3.
20. Oku, H., Shiraishi, T. and Ouchi, S. (1977). Suppression of induction of phytoalexin, pisatin, by low-molecular-weight substances from spore germination fluid of pea pathogen, *Mycosphaerella pinodes*. Naturwissenschaften 64 :643.
21. Oku, H., Shiraishi, T., Ouchi, S., Ishiura, M. and Matsueda, R. (1980). A new determinant of pathogenicity in plant disease. Naturwissenschaften 67 : 310.
22. Palmgren, M.G. and Sommarin, M. (1989). Lysophosphatidylcholine stimulates ATP dependent proton accumulation in isolated oat root plasma membrane vesicles. Plant Physiol. 90 : 1009-1014.
23. Perlin, D.S. and Spanswick, R.M. (1981). Characterization of ATPase activity associated with corn leaf plasma membranes. Plant Physiol. 68 : 521-526.
24. Rasi-Caldogno, F., Michelis, M.I.D., Pugliarello, M.C. and Marré, E. (1986). H^+-pumping driven by the plasma membrane ATPase in membrane vesicles from radish : Stimulation by fusicoccin. Plant Physiol. 82 : 121-125.
25. Serrano, R. (1989). Structure and function of plasma membrane ATPase. Annu. Rev. Plant Physiol. Plant Mol. Biol. 40 : 61-94.
26. Shiraishi, T., Araki, M., Yoshioka, H., Kobayashi, I., Yamada, T., Ichinose, Y., Kunoh, H. and Oku, H. (1991). Inhibition of ATPase activity in pea plasma membranes *in situ* by a suppressor from a pea pathogen, *Mycosphaerella pinodes*. Plant Cell Physiol. 32 : 1067-1075.
27. Shiraishi, T., Oku, H., Yamashita, M. and Ouchi, S. (1978). Elicitor and suppressor of pisatin induction in spore germination fluid of pea pathogen, *Mycosphaerella pinodes*. Ann. Phytopathol. Soc. Jpn. 44 : 659-665.
28. Shiraishi, T., Saitoh, K., Kim, H.M., Kato, T., Tahara, M., Oku, H., Yamada, T. and Ichinose, Y. (1992). Two

suppressors, Supprescins A and B, secreted by a pea pathogen *Mycosphaerella pinodes*. Plant Cell Physiol. 33 : 663-667.
29. Shiraishi, T., Yamada, T., Saitoh, K., Kato, T., Toyoda, K., Yoshioka, H., Kim, H.M., Ichinose, Y., Tahara, M. and Oku, H. (1994). Suppressors : Determinants of specificity produced by plant pathogens. Plant Cell Physiol. 35 : 1107-1119.
30. Thanutong, P., Oku, H., Shiraishi, T. and Ouchi, S. (1982). Isolation and partial characterization of an elicitor of pisatin production from spore germination fluid of pea pathogen, *Mycosphaerella pinodes*. Sci. Rep. Fac. Agric. Okayama Univ. 59 : 1-9.
31. Toyoda, K., Shiraishi, T., Yamada, T., Ichinose, Y. and Oku, H. (1993). Rapid changes in polyphosphoinositide metabolism in pea in response to fungal signals. Plant Cell Physiol. 34 : 729-735.
32. Toyoda, K., Shiraishi, T., Yoshioka, H., Yamada, T., Ichinose, Y. and Oku, H. (1992). Regulation of polyphosphoinositide metabolism in pea plasma membranes by elicitor and suppressor from a pea pathogen, *Mycosphaerella pinodes*. Plant Cell Physiol. 33 : 445-452.
33. Vara, F. and Serrano, R. (1982). Partial purification and properties of the proton-translocating ATPase of plant plasma membranes. J. Biol. Chem. 257 : 12826-12830.
34. Vianello, A., Dell'antone, P. and Macri, F. (1982). ATP-dependent and ionophore-induced proton translocation in pea stem microsomal vesicles. Biochim. Biophys. Acta 689 : 89-96.
35. Yamada, T., Hashimoto, H., Shiraishi, T. and Oku, H. (1989). Suppression of pisatin, phenylalanine ammonia-lyase mRNA, and chalcone synthase mRNA by a putative pathogenicity factor from the fungus *Mycosphaerella pinodes*. Mol. Plant-Microbe Interact. 2 : 256-261.
36. Yamamoto, Y., Oku, H., Shiraishi, T., Ouchi, S. and Koshizawa, K. (1986). Non-specific induction of pisatin and local resistance in pea leaves by elicitors from *Mycosphaerella pinodes, M. melonis* and *M. ligulicola* and the effect of suppressor from *M. pinodes*. J. Phytopathol. 117 : 136-143.
37. Yoshioka, H., Shiraishi, T., Kawamata, S., Nasu, K., Yamada, T., Ichinose, Y. and Oku, H. (1992). Orthovanadate suppresses accumulation of phenylalanine ammonia-lyase mRNA and chalcone synthase mRNA in pea epicotyls induced by elicitor from *Mycosphaerella pinodes*. Plant Cell Physiol. 33 : 201-204.
38. Yoshioka, H., Shiraishi, T., Nasu, K., Yamada, T., Ichinose, Y. and Oku, H. (1992). Suppression of the activation of chitinase and β-1,3-glucanase in pea epicotyls by orthovanadate and suppressor from *Mycosphaerella pinodes*. Ann. Phytopathol. Soc. Jpn. 58 : 405-410.
39. Yoshioka, H., Shiraishi, T., Yamada, T., Ichinose, Y. and Oku, H. (1990). Suppression of pisatin production and ATPase activity in pea plasma membranes by orthovanadate, verapamil and a suppressor from *Mycosphaerella pinodes*. Plant Cell Physiol. 31 : 1139-1146.

和 文 摘 要

天野政史・豊田和弘・一瀬勇規・山田哲治・白石友紀：褐紋病菌サプレッサーによるエンドウ原形質膜 ATPase 再構成膜におけるプロトンポンプ活性の抑制

エンドウ褐紋病菌のサプレッサーは宿主エンドウの原形質膜 ATPase 活性を抑制する。本報告では，可溶化・部分精製したエンドウ原形質膜 ATPase をリポソームに再構成し，ATPase とプロトンポンプ活性に対するエリシターとサプレッサーの影響について調べた。再構成膜におけるプロトンポンプ活性は ATP と Mg^{2+} に依存し，ATPase の阻害剤で抑制されることから ATPase 依存性であることが推察できた。また，プロトンの勾配はプロトンイオノフォアや NH_4Cl の添加で打ち消された。本活性に対するエリシターとサプレッサーの影響を調べたところ，エリシターは全く影響を及ぼさなかったが，サプレッサーは濃度依存的に活性を抑制した。さらに，ホスファチジルイノシトール２リン酸は本活性を上昇させたが，その作用点はサプレッサーの作用点とは異なることが示唆された。これらの結果から，エンドウ褐紋病菌はサプレッサーを分泌し，エンドウ細胞におけるホメオスタシスを攪乱することにより抵抗反応を抑制し，感染に成功するものと考察した。