Orthovanadate Induces Phytoalexin Production in Pea Suspension-Cultured Cells

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We previously reported that the addition of orthovanadate suppressed the defense responses of plant differentiated tissues induced by a fungal elicitor. In this report, the effect of orthovanadate on the defense response of pea cultured cells was examined. The activities of ATPase and PI metabolism in plasma membrane fraction, which was prepared from suspension-cultured cells, were inhibited *in vitro* by orthovanadate as well as those in plasma membranes from pea epicotyl tissues. However, orthovanadate alone induced the accumulation of a phytoalexin, pisatin in suspension-cultured cells of pea in a manner similar to $CuCl_2$. The viability of pea suspension-cultured cells was decreased by orthovanadate as well as by $CuCl_2$. These results indicated that orthovanadate acts as an abiotic elicitor to pea suspension-cultured cells as observed in those of red bean, peanut and *Petunia hybrida*.

Key words : defense response, elicitor, *Pisum sativum*, suspension-cultured cells, orthovanadate

Introduction

Plants have an ability to protect themselves against environmental stress such as invasion by phytopathogens. When attacked by pathogenic fungi, the plants are able to rapidly recognize alien substances and to establish the various defense responses. These defense responses include accumulation of phytoalexins, activation of pathogenesis-related (PR) proteins and insolubilization of cell wall-bound proline-rich glycoproteins (for review, see Dixon and Lamb[4]). It was previously reported that a pea pathogen, *Mycosphaerella pinodes*, secreted a high-molecular-weight elicitor[17,21,27] and a low-molecular-weight glycopeptide suppressor in its pycnospore germination fluid[18,21,29,22]. The elicitor from *M. pinodes* induces pea defense responses such as the production of a major phytoalexin of pea, pisatin, the activation of PR proteins such as β-1, 3 glucanase and chitinases and the formation of an as yet-unidentified infection-inhibitor[21,30,31,35]. However, the concomitant presence of the suppressor from the fungus blocked or delayed these defense responses (for review, see Shiraishi *et al.*[24]). One of the major actions of the suppressor was thought to inhibit the ATPase activity[34~36]. On the other hand, orthovanadate, an inhibitor of P-type ATPase, also blocked the active defense responses of pea tissues in a similar way to the suppressor[34~36]. Thus, inhibition of ATPase activity was thought to be important for the pathogens to avoid the active defense responses in the tissues of the host

plant. However, it was reported that vanadate induced defense responses such as phytoalexin accumulation and the activation of key enzymes in the biosynthetic pathway for phytoalexins in suspension-cultured cells of peanut[26], red bean[10] and *Petunia hybrida*[6]. In this report, therefore, we examined the effect of orthovanadate on the active defense response of suspension-cultured cells of pea.

Materials and Methods

Chemicals — Fluorescein diacetate was purchased from Sigma Chemicals Co. Ltd., St.Louis, MO. $[\gamma\text{-}^{32}P]$ ATP (6000 μCi/mmol) was obtained from Amersham (U.S.A.). Other chemicals were purchased from Wako Pure Chemical Inc., Osaka, Japan.

Cell culture — The suspension-cultured cells of pea (*Pisum sativum* L., cv. Midoriusui) used in this study had been maintained by subculturing at 7 day intervals in B5 medium containing 1.5 mg/l of 2,4-D and 0.2% casein enzymatic hydrolysate as described previously[9]. The culture was grown at 22 ℃ in 100 ml Erlenmeyer flasks containing 30 ml of the medium on a rotary shaker (100 rpm).

Preparation of elicitor from Mycosphaerella pinodes — The elicitor was prepared from the germination fluid of pycnospores of *Mycosphaerella pinodes* Vestergren, strain OMP-1 (IFO-30342, ATCC-42741) as described previously[36]. The concentration of the elicitor was determined by the method of Dubois *et al.*[5] with glucose as the standard.

Determination of pisatin accumulated in pea suspension-cultured cells — The cell culture (2.1 ml) was aliquoted into 10 ml Erlenmeyer flasks and was treated with 700 μl of test solution, which contained several concentrations of orthovanadate and $CuCl_2$ dissolved in B5 medium. After incubation at 22 ℃ for 24 h, the amount of pisatin was determined by the method of Masuda *et al.*[16]

Preparation of the plasma membrane fraction — The plasma membrane fraction was prepared from 7-day-old cultured cells or etiolated seedlings by Yoshida *et al.*[33] The protein content of the respective fraction was determined by the method of Lowry *et al.*[14] or Bradford[3] with BSA as the standard. The membrane fraction was stored at -80 ℃ until use.

Determination of ATPase activity in vitro — The activity of ATPase was determined by the method of Perlin and Spanswick[19]. Assay of ATPase was carried out at 37 ℃ for 60 min in 80 mM Tris-MES buffer (pH 6.5) that contained 1 mM Mg-ATP in the absence or presence of several concentrations of Na_3VO_4.

Phosphorylation of lipid in vitro — Determination of lipid phosphorylation *in vitro* was carried out according to the method as described previously[28,29]. In brief, the reaction mixture was adjusted to 50 μl containing 20 mM Tris-MES (pH 6.5), 15 mM $MgSO_4$, 100 mM GTP, 20 nM $[\gamma\text{-}^{32}P]$ ATP, and 12.5 μg of plasma membrane fraction in the presence or absence of the elicitor (100 μg/ml , glucose equiv.) and Na_3VO_4 at the concentration of 1 mM or 0.1 mM. The reaction was initiated by the addition of the plasma membrane fraction. After incubation at 0 ℃ for 20 min, the reaction was terminated by the addition of 200 μl of chilled chloroform/methanol (v/v = 1/2).

Extraction and analysis of phosphorylated lipids — Phospholipids were extracted by the method of Bligh and Dyer[2] with slight modifications. The extract with chloroform/methanol (v/v = 1/2) was centrifuged at 7,000 x g for 15 min to remove the denatured proteins. The supernatant was transferred to an Eppendolf tube and 60 μl of chloroform and 1% (w/v) potassium chloride solution were added. After vigorous mixing, the lower phase was collected and washed with methanol/water (v/v = 10/9). The resultant lower phase containing lipids was recollected and dried in vacuo. The residue was then

dissolved in 40 μl of chloroform/methanol (v/v = 2/1) and applied onto a silica gel TLC plate (Whatman, K5) that had been presoaked in 1% (w/v) potassium oxalate for 75 s and then dried at 110 ℃ for 6 h prior to use. The development was carried out using chloroform/methanol/32% ammonia/water (86/76/6/18, by vol.). After development, the plate was exposed to 0.05% (w/v) primuline solution in 80% (v/v) acetone to visualize the lipid compounds. The incorporation of radioactivity from $[\gamma^{32}\text{-}P]$ ATP into phosphatidylinositol-monophosphate (PIP) and phosphatidylinositol 4, 5- bisphosphate (PIP_2) was determined with a Bio-imaging scanner system (Bas 2000 system, Fujix, Tokyo, Japan).

Measuring of viability of cultured cells by fluorescein diacetate-staining - Viability of cell culture was determined by fluorescein diacetate (FDA)-staining. Three hundred μl of cultured cells were aliquoted into 10 ml Erlenmeyer flasks and incubated at 22 ℃ for 24 h in B5 medium in the absence or presence of several concentrations of Na_3VO_4 and $CuCl_2$. After incubation, 300 ml of fluorescein diacetate solution (0.1 mg/ml water containing 2% acetone) was added and incubated at 22 ℃ for 20 min. The number of living cells was measured under a fluorescence microscope[33].

Results and Discussion

In our previous papers, orthovanadate, which is well known as an inhibitor of plasma membrane ATPases, was found to inhibit polyphosphoinositide (PI) metabolism and the active defense responses of pea tissues[28,29,34,35,36]. From these results, we suggested that the plasma membrane ATPase may cross-talk with PI metabolism and may play an important role in signal transduction cascade leading to the active defense responses of pea plants[24,29]. As described in the introduction, however, orthovanadate was reported to mimic the effects of fungal elicitors in plant cultured cells[6,10,26]. As a first step, there-

fore, the *in vitro* effect of orthovanadate on ATPase activity and PI metabolism in plasma membrane fraction, which was prepared from pea suspension-cultured cells, was examined. As shown in Fig. 1, orthovanadate inhibited the ATPase activity in plasma membrane fraction from pea suspension-cultured cells in a dose-dependent manner as well as that from pea epicotyl tissues. One mM of orthovanadate completely negated the activity of plasma membrane ATPase of the cultured cells.

One mM of orthovanadate also inhibited above 50% of the incorporation of radioactivity from ^{32}P-ATP into PIP and PIP_2, as compared to the water control in spite of the presence or absence of the elicitor from *M. pinodes* (Fig. 2). These results showed that orthovanadate interfered with enzymes such as phosphatidylinositol kinase (PI-kinase) and phosphatidylinositol-4-mono-
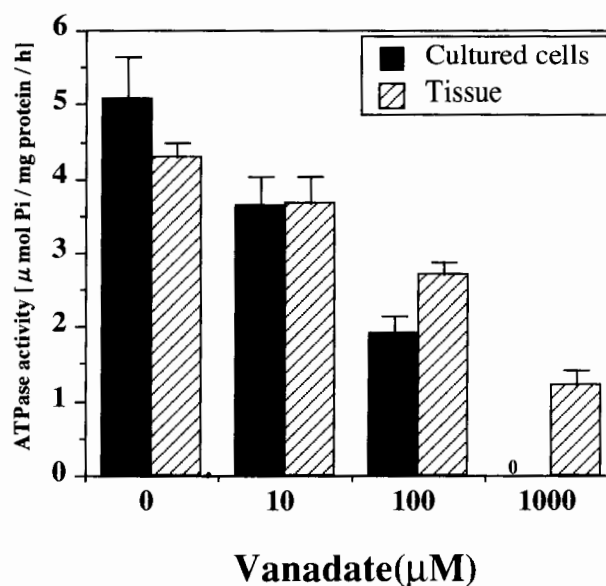

Fig. 1   Effect of orthovanadate on the activity of ATPase of plasma membrane prepared from suspension-cultured cell or etiolated epicotyl tissues of pea plants. The assay was carried out at 37 ℃ for 60 min in 80 mM Tris-MES (pH 6.5) containing 1 mM Mg-ATP in the absence (O) or presence of Na_3VO_4 at the concentration of 10, 100 and 1000 μM, respectively. Each value represents the mean with standard deviation (SD) of the results from triplicate experiments.
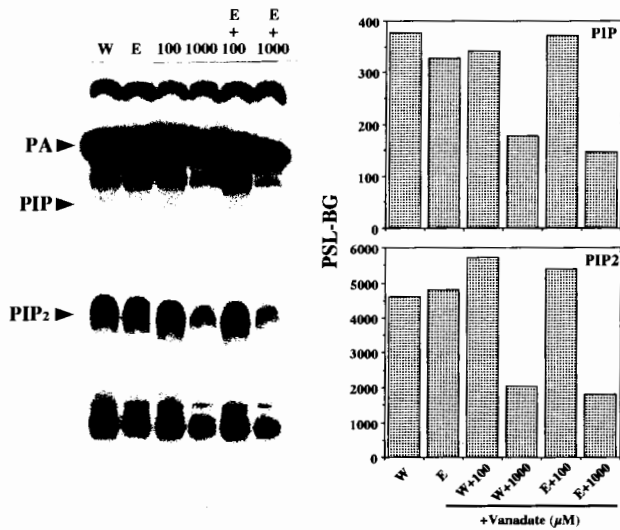
Fig. 2 Effect of orthovanadate on incorporation of radioactivity from γ-^{32}P-ATP into PIP and PIP$_2$ in plasma membrane fraction isolated from pea suspension-cultured cells. The assay was carried out at 0 °C for 20 min in 20 mM Tris-MES (pH 6.5) containing 15 mM MgSO4, 100 μM GTP and 20 nM [γ-^{32}P] ATP in the absence (W) or presence of 100 μg/ml (glucose equiv.) elicitor from *M. pinodes* (E), 100 or 1000 μM Na$_3$VO$_4$, and 100 μg/ml of the elicitor plus Na$_3$VO$_4$ as described previously[28]. The left column showed silicagel thin-layer chromatogram of ^{32}P-labeled phospholipids extracted from plasma membrane fraction isolated from pea suspension-cultured cells; the right columns represented the extent of incorporation of radioactivity into PIP and PIP$_2$, respectively, that were determined by photo-stimulated fluorography with a Bio-imaging analyzer (Bas 2000). The data was calculated as PSL unit of the spot of PIP or PIP$_2$ − PSL unit of the background).

Fig. 3 Effects of orthovanadate, CuCl$_2$ and the elicitor from *M. pinodes* on pisatin accumulation in pea suspension-cultured cells. The amount of pisatin was determined 24 h after incubation in the absence (the water control; 0) or presence of 100 μg/ml of the elicitor from *M. pinodes* (E), Na$_3$VO$_4$ or CuCl$_2$ by the method of Masuda et al.[16]

phosphate kinase (PIP-kinase) in PI metabolism. This result coincides with our previous data that orthovanadate inhibited the ATPase, PI-kinase and PIP-kinase in the plasma membranes prepared from pea epicotyl tissues[28,36]. Thus, when taken together with our previous findings, it was shown that there was no difference between plasma membranes from suspension-cultured cells and those from epicotyl tissues in the sensitivity of these enzymes to orthovanadate. In other words, the *in vitro* effect of orthovanadate on the fundamental functions in plasma membranes of the cultured cells is as same as that of
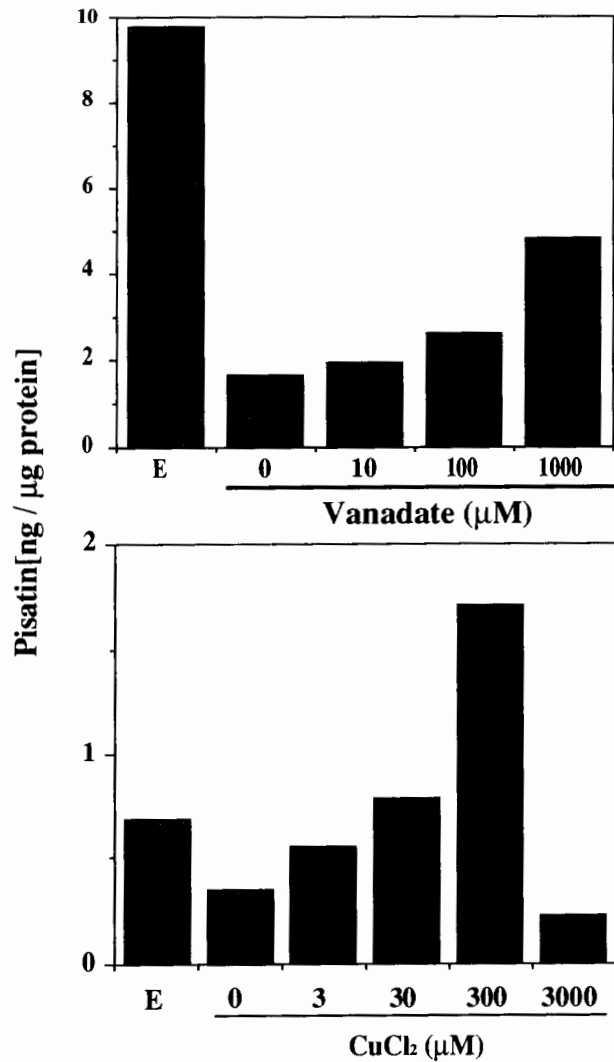
the tissues.

In the next step, the *in vivo* action of orthovanadate on the defense response in pea suspension-cultured cells was examined. As shown in Fig. 3, the elicitor from *M. pinodes* induced the accumulation of pea phytoalexin, pisatin, in the cultured cells as observed in pea epicotyl tissues. Copper(II) chloride, well known as an abiotic elicitor, also induced pisatin accumulation in a dose-dependent manner, while

3 mM of CuCl$_2$ did not. Orthovanadate also induced the accumulation of pisatin in a dose-dependent manner. These results indicate that orthovanadate acts as an abiotic elicitor on the suspension-cultured cells of pea as well as those of peanut and red bean[26,10]. Thus, the effect of orthovanadate seems to be the same on the suspension-cultured cells of broad plant species. In addition, orthovanadate was reported to act as a non-specific suppressor for one of defense responses, the activation of endochitinase and β-1,3-glucanase, in the tissues of the four leguminous plant species tested[35].

It was reported that an endogenous elicitor was released from the kidney bean (*Phaseolus vulgaris*) cells which were partially damaged by infection, freezing or treatment with abiotic chemicals such as HgCl$_2$ and chloroform[1,7,8]. Such an endogenous elicitor was thought to induce phytoalexin biosynthesis in the surrounding cells. We, therefore, examined the effects of orthovanadate, CuCl$_2$ and the elicitor from *M. pinodes* on the viability of pea suspension-cultured cells. As shown in Fig.4, the elicitor (500 µg/ml, glucose equiv.) from *M. pinodes* scarcely affected the viability of the cultured cells. On the other hand, viability of the cultured cells was decreased by above 100 µM of orthovanadate and above 30 µM of CuCl$_2$ in a dose-dependent manner. About 50% and 90% of the cells were damaged by treatment with 1 mM orthovanadate and 3 mM CuCl$_2$, respectively. These results suggested that the mode of action of these chemicals on pea suspension-cultured cells may be considerably different from that of the elicitor from *M. pinodes*. Thus, the induction of pisatin accumulation by orthovanadate and CuCl$_2$ seems to be due to their toxicity and it is thought that the damaged cells by treatment with these chemicals might release certain elicitor molecule(s) to induce phytoalexin biosynthesis in the living cultured cells. This concept seems to be probable, because the production of pisatin looked like
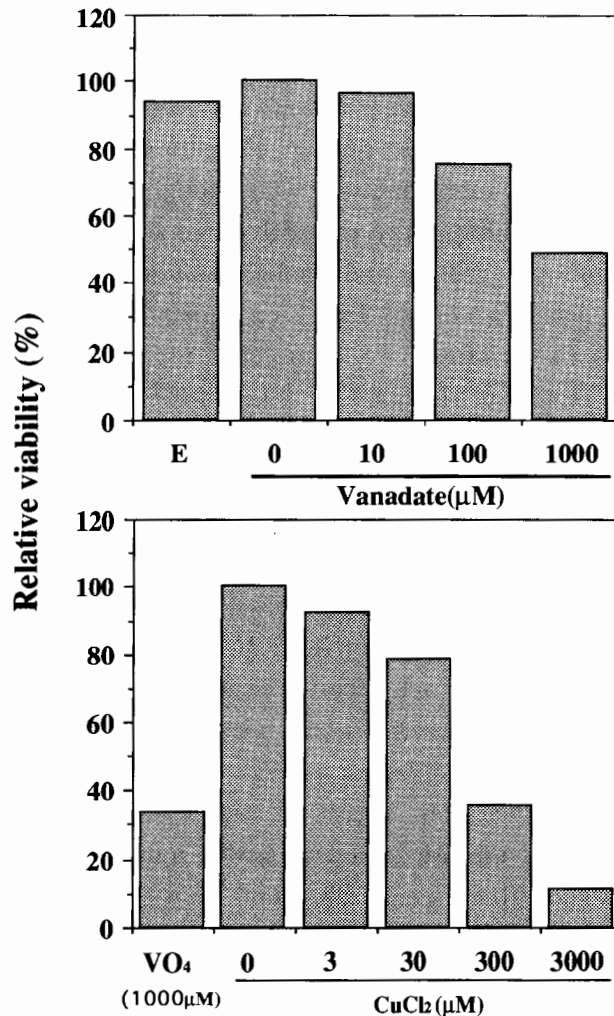

Fig. 4   Effects of orthovanadate and CuCl$_2$ on the viability of pea suspension-cultured cells. The cultured cells were incubated at 22 °C for 24 h in B5 medium in the absence (the water control; O) or presence of the elicitor from *M. pinodes* (500 µg/ml), Na$_3$VO$_4$ or CuCl$_2$, and were stained at 22 °C for 20 min with 0.1 mg/ml of fluorescein diacetate. The living cells were measured under a fluorescence microscope.

inversely proportional to the viability of the cells and also a highly toxic concentration of CuCl$_2$ could not apparently induce phytoalexin accumulation (Figs. 3 and 4).

In this connection, fluorescein diacetate has been used to detect *in vivo* either the plasma membrane's damage or the cytoplasmic pH[25,33]. Yoshida *et al.*[33] reported that the fluorescence in the cultured cells of mung bean was markedly reduced after chilling for 24 h, suggesting acidification of the cytoplasms. A decrease in

cytoplasmic pH was also induced by treatment of cultured cells with diverse biotic and abiotic elicitors[6,14]. In *Petunia hybrida* cultured cells, the primary processes during elicitation of the phenylpropanoid pathway was thought to be a change (inhibition) in the activity of the plasma membrane ATPase and a subsequent decrease in the proton gradient[6]. In this case, orthovanadate was also an effective elicitor. Thus, together with the reports of Hattori and Ohta[10] and Steffens *et al.*[26], there is the possibility that the cytoplasmic acidification, which is induced by broad environ-mental stresses, switches on the signal transduc-tion cascade leading to defense responses. Pres-ently, it is unknown whether CuCl₂, ortho-vanadate or the elicitor from *M. pinodes* induces the cytoplasmic acidification in pea cultured cells and which plays a crucial role in phytoalexin production, cytoplasmic acidification or certain programmed cell death. However, since the elicitor from *M. pinodes* hardly induced any damage (fluorescence decrease) in pea cultured cells (Fig. 4), rapid cytoplasmic acidification might be more important for induction of defense responses in the cultured cells.

As described, the *in vivo* effect of ortho-vanadate on pea cultured cells is quite different from that on pea differentiated tissues. That is, orthovanadate acts as an elicitor on the cultured cells but as a suppressor on the differentiated tissues, while the chemical inhibited *in vitro* the ATPase and PI metabolism in plasma membrane fractions isolated from both cultured cells and tissues (Figs. 1 and 2; Toyoda *et al.*[28], Yoshioka *et al.*[36]). Therefore, the action of orthovanadate only on the plasma membrane functions may fail to explain the mechanism of its apparently con-tradictory effect. We previously reported that plant cell wall may play important roles in recog-nition of pathogenic signals, determination of host-parasite specificity and regulation of defense responses. In fact, the generation of active oxygen species dependent on certain

peroxidase(s) and the activity of ATPase in cell walls were non-specifically enhanced by the elicitor from *M. pinodes* and were inhibited by the suppressor in a species-specific manner[11~13]. The suppressor inhibited *in vitro* the ATPase activities in plasma membranes isolated from both the host and non-host plants of *M. pinodes*, while the ATPase activities of non-host cells were never inhibited *in vivo* by the suppressor, neither were those of cell walls isolated from non-hosts *in vitro*[12,13,20]. Furthermore, it was found that orthovanadate and the suppressor, which were placed on the pea leaf surface, severe-ly inhibited the ATPase activities associated with all membrane systems in pea epidermal cells[20]. It is likely, therefore, that the cell wall (-bound enzymes) might affect or regulate *in vivo* the function of other organella such as plasma membrane and vacuole. If so, the difference between the action of orthovanadate on the cul-tured cells and that on differentiated tissues seems to have resulted from the action of orth-ovanadate on the cell walls. Further experiments are needed to elucidate the mechanism of such a reversible effect of orthovanadate, however, we emphasize that the results and conclusions obtained from these experiments with cultured cells are not always applicable to the differentiat-ed tissues of plants that are actually living in a real stressful environment.

References

1) Bailey, J.A. and Berthier, M. : Phytoalexin accumula-tion in chloroform-treated cotyledons of *Phaseolus vulgaris*. Phytochemistry, **20**, 187-188 (1981)

2) Bligh, E.G. and Dyer, W.J. : A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol., **37**, 911-917 (1959)

3) Bradford, M.M. : A rapid and sensitive method for quantitation of microgram quantities of protein utiliz-ing the principle of protein-dye binding. Anal. Bio-chem., **72**, 248-254 (1976)

4) Dixon, R.A. and Lamb, C.J. : Molecular communica-tion in interactions between plants and microbial

pathogens. Annu. Rev. Plant Physiol. Plant Mol. Biol., **41**, 339-367 (1990)

5) Dubois, S.M., Gilles, K.A., Halmiton, J.K., Rebers, P. A. and Smith, F. : Colorimetric method for determination of sugars and related substance. Anal. Chem., **28**, 350-356 (1956)

6) Hagendoorn, M., Poortinga, A., Wong Fong, Sang, H., van der Plas, L. and Walraven, H. : Effect of elicitors on the plasma membrane of *Petunia hybrida* cell suspensions. Plant Physiol., **96**, 1261-1267 (1991)

7) Hargreaves, J.A. : Investigations into the mechanism of mercuric chloride stimulated phyoalexin accumulation in *Phaseolus vulgaris* and *Pisum sativum*. Physiol. Plant Pathol., **15**, 279-287 (1979)

8) Hargreaves, J.A. and Bailey, J.A. : Phytoalexin production by hypocotyls of *Phaseolus vulgaris* in response to constitutive metabolites released by damaged bean cells. Physiol. Plant Pathol., **13**, 89-100 (1978)

9) Hashimoto, T., Yamada, T., Tada, A., Kawamata, S., Tanaka, Y., Sriprasertsak, P., Ichinose, Y., Kato, H., Izutsu, S., Shiraishi, T., Oku, H. and Ohtsuki, Y. : Transient expression in electroporated pea protoplasts: elicitor responsiveness of a phenylalanine ammonia-lyase promoter. Plant Cell Rep., **11**, 183-187 (1992)

10) Hattori, T. and Ohta, Y. : Induction of phenylalanine ammonia-lyase activation and isoflavone glucoside accumulation in suspension-cultured cells of red bean, *Vigna angularis*, by phytoalexin elicitors, vanadate, and elevation of medium pH. Plant Cell Physiol., **26**, 1101-1110 (1985)

11) Kiba, A., Miyake, C., Toyoda, K., Ichinose, Y., Yamada, T. and Shiraishi, T. : Superoxide generation in cell wall during a plant-pathogen interaction. Plant Cell Physiol., **37**, s127 (supplement) (1996)

12) Kiba, A., Toyoda, K., Ichinose, Y., Yamada, T. and Shiraishi, T. : Specific response of partially purified cell wall-bound ATPases to fungal suppressor. Plant Cell Phyisiol., **37**, 207-214 (1996)

13) Kiba, A., Toyoda, K., Yamada, T., Ichinose, Y. and Shiraishi, T. : Specific inhibition of cell wall-bound ATPases by fungal suppressor from *Mycosphaerella pinodes*. Plant Cell Physiol., **36**, 809-817 (1995)

14) Low, P. and Heinstein, P. : Elicitor stimulation of the defense response in cultured plant cells monitored by fluorescent dyes. Arch. Biochem. Biophys., **249**, 472-479 (1986)

15) Lowry, O.H., Rosebrough, N.J., Farr, A. and Randall, R.J. : Protein measurement with the Folin reagent. J. Biol. Chem., **193**, 265-275 (1951)

16) Masuda, Y., Shiraishi, T., Ouchi, S. and Oku, H. : Rapid and accurate analysis of isoflavonoid phytoalexins by high-performance liquid chromatography. Ann. Phytopathol. Soc. Jpn., **49**, 558-560 (1983)

17) Matsubara, M. and Kuroda, H. : The structure and physiological activity of glycoprotein secreted from conidia of *Mycosphaerella pinodes* II. Chem. Pharm. Bull., **35**, 249-255 (1987)

18) Oku, H., Shiraishi, T. and Ouchi, S. : Suppression of induction of phytoalexin, pisatin by low-molecular-weight substances from spore germination fluid of pea pathogen, *Mycosphaerella pinodes*. Naturwissenschaften, **64**, 643 (1977)

19) Perlin, D.S and Spanswick, R.M. : Characterization of ATPase activity associated with corn leaf plasma membranes. Plant Physiol., **68**, 521-526 (1981)

20) Shiraishi, T., Araki, M., Yoshioka, H., Kobayashi, I., Yamada, T., Ichinose, Y., Kunoh, H. and Oku, H. : Inhibition of ATPase activity in plasma membranes in situ by a suppressor from a pea pathogen, *Mycosphaerella pinodes*. Plant Cell Physiol., **32**, 1067-1075 (1991)

21) Shiraishi, T., Oku, H., Yamashita, M. and Ouchi, S. : Elicitor and suppressor of pisatin induction in spore germination fluid of pea pathogen, *Mycosphaerella pinodes*. Ann. Phytopathol. Soc. Jpn., **44**, 659-665 (1978)

22) Shiraishi, T., Saitoh, K., Kim, H. M., Kato, T., Tahara, M., Oku, H., Yamada, T. and Ichinose, Y. : Two suppressors, Supprescins A and B, secreted by a pea pathogen, *Mycosphaerella pinodes*. Plant Cell Physiol., **33**, 663-667 (1992)

23) Shiraishi, T., Yamada, T., Oku, H. and Yoshioka, H. : Suppressor production as a key factor for fungal pathogenesis. In Molecular Strategies of Pathogens and Host Plants, Edited by Patil, S. S. *et al.*, Springer-Verlag, New York, pp. 151-162 (1991)

24) Shiraishi, T., Yamada, T., Saitoh, K., Kato, T., Toyoda, K., Yoshioka, H., Kim, H. M., Ichinose, Y., Tahara, M. and Oku, H. : Suppressor : Determinants of specificity produced by plant pathogens. Plant Cell Physiol., **35**, 1107-1119 (1994)

25) Slavik, J. : Intracellular pH topography : determination by fluorecsent probe. FEBS Lett., **156,** 227-230 (1983)

26) Steffens, M., Ettl, F., Kranz, D. and Kindl, H. : Vanadate mimics of fungal cell wall in eliciting gene activation in plant cell cultures. Planta, **177,** 160-168 (1989)

27) Thanutong, P., Oku, H., Shiraishi, T. and Ouchi, S. : 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 (1982)

28) Toyoda, K., Shiraishi, T., Yoshioka, H., Yamada., T, Ichinose, Y and Oku, H. : Regulation of polyphosphoinositide metabolism in pea plasma membrane by elicitor and suppressor from a pea pathogen, *Mycosphaerella pinodes*. Plant Cell Physiol., **33,** 445-452 (1992)

29) Toyoda, K., Shiraishi, T., Yoshioka, T, Ichinose, Y and Oku, H. : Rapid changes in polyphosphoinositide metabolism in pea in response to fungal signals. Plant Cell Physiol., **34,** 729-735 (1993)

30) Yamada, T., Hashimoto, H., Shiraishi, T. and Oku, H. : Suppression of pisatin, phenylalanine ammonia-lyase mRNA, and chalcone synthase mRNA by putative pathogenicity factor from the fungus *Mycosphaerella pinodes*. Mol. Plant-Microbe Interact., **2,** 256-261 (1989)

31) Yamamoto, Y., Oku, H., Shiraishi, T., Ouchi, S. and Koshizawa, K. : Non-specific induction of pisatin and local resistance in pea leaves by elicitor from *Myco-*

sphaerella pinodes, *M. melonis* and *M. ligulicola* and effect of suppressor from *M. pinodes*. J. Phytopathol., **117,** 136-143 (1986)

32) Yoshida, S., Kawata, T., Uemura, M. and Niki, T. : Properties of plasma membrane isolated from chilling-sensitive etiolated seedlings of *Vigna radiata* L. Plant Physiol., **80,** 152-160 (1986)

33) Yoshida, S., Matsuura, C. and Maeshima, M. : Chilling injury and tonoplast proton pumps. In Plant Water Regulations and Growth under Stress (Proceedings of the Yamada Conference XXII, Osaka, Japan) pp. 83-90 (1989)

34) Yoshioka, H., Shiraishi, T., Kawamata, S., Nasu, K., Yamada, T., Ichinose, Y. and Oku, H. : Orthovanadate suppresses accumulation of phenylalanine ammonialyase mRNA and chalcone synthase mRNA in pea epicotyls induced by elicitor from *Mycosphaerella pinodes*. Plant Cell Physiol., **33,** 201-204 (1992)

35) Yoshioka, H., Shiraishi, T., Nasu, K., Yamada, T., Ichinose, Y. and Oku, H. : Suppression of 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 (1992)

36) Yoshioka, H., Shiraishi, T., Yamada, T., Ichinose, Y. and Oku, H. : 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 (1990)

バナジン酸によるエンドウ培養細胞における
フィトアレキシン生産の誘導

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　バナジン酸は，エンドウ組織に褐紋病菌エリシターの処理で誘導される一連の防御応答を抑制することが報告されている．そこで，本研究ではバナジン酸のエンドウ培養細胞に対する影響を調べた．バナジン酸は培養細胞から分離した原形質膜画分のポリホスホイノシチド代謝系関連酵素やATPaseの活性を濃度依存的に阻害した．次に，褐紋病菌エリシター，塩化銅（非生物的エリシターの一種），およびバナジン酸の *in vivo* での影響を調べたところ，いずれの単独処理においてもエンドウ培養細胞のピサチン生産を誘導した．褐紋病菌エリシターはエンドウ培養細胞の細胞死（FDAの染色性喪失）を誘導しなかったが，塩化銅，バナジン酸は明らかな毒性を示した．以上の結果とこれまでの知見に基づいて，エンドウ培養細胞に対しては非生物的エリシターとして作用するバナジン酸の作用機構を考察した．