Suppression of Pisatin Accumulation by an Inhibitor of Protein Kinase*

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Phytoalexins (PAs) are believed to play important roles in defense mechanisms of plant against the attack by phytopathogenic microorganisms. It was recently demonstrated that the production of pisatin, a major PA of pea plant, was regulated at the level of gene expression of two key-enzymes in flavonoid synthetic pathway, phenylalanine ammonia-lyase and chalcone synthase when the tissues were treated with elicitor and/or suppressor from *Mycosphaerella pinodes*¹⁾. It is, however, not clear how the elicitor is recognized by pea cells, and what is the second messenger in the process of signal transduction for the defense reaction. In carrot suspension-cultured cells, Ca^{2+} and cAMP seem to play as the second messengers for the production of 6-methoxymellein, a PA of carrot²⁾. The increase of Ca^{2+} in soybean cultured cells was believed to enhance the enzyme activities in glyceollin biosynthetic pathway and its accumulation³⁾. However, it was reported that fluctuation of Ca^{2+} content did not correlate to the chitosan- or *Fusarium solani*-induced pisatin production in pea pod tissue⁴⁾. In this paper, we report the effect of some putative inhibitors of signal transduction on pisatin accumulation and discuss the regulatory mechanisms in the early process of pisatin production in pea epicotyls.

Inhibitors used in this experiment were lanthanum chloride <LaCl₃> (Wako), EGTA (Dojin), 1,2-bis(*o*-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid <BAPTA> (Dojin), verapamil hydrochloride <Ver> (Dojin), TMB-8 (Aldrich), chlorpromazine hydrochloride <Chl> (Dojin), amitriptyline <Ami> (Sigma), quinacrine dihydrochloride <Qui> (Sigma), caffeic acid (Sigma), and K-252a (Kyowa).

Pea seeds (*Pisum sativum* L. cv. Midoriusui) were sown in vermiculite in a plastic container, and were grown in a growth chamber at 22 ± 2 C for 6 days in the dark. Pea epicotyls were detached from seedlings, and cut into 1.5-cm length. Epicotyl sections were divided longitudinally into two parts and were floated on 50 μl of aqueous solution containing each inhibitor (the final conc. is shown in Table 1) as cut surfaces were perfectly contacted with the solution. All incubations described below were carried out at 22 ± 2 C in the dark. After 3 hr incubation, the solution was replaced with a mixture of the same conc. of inhibitor and elicitor (500 $\mu\text{g/ml}$, glucose equiv.) prepared from *M. pinodes* as described previously⁵⁾. The treatment with

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elicitor or water alone was used as a positive or negative control, respectively. The amount of pisatin accumulated in the tissue was determined 18 hr after the replacement with mixture by the method of Masuda *et al.*⁶⁾ Three to six epicotyls per each treatment were used for one experiment, and the experiment was repeated at least 2 times. The effect of application time with Ver or K-252a was also examined at the final conc. of 250 μ M or 100 μ M, respectively. That is, pea epicotyls were pre-treated with 50 μ l of the inhibitor solution 3, 2, or 1 hr before elicitor-treatment, respectively, and the solution was replaced with the mixture of inhibitor and elicitor. To determine the effect of Ver or K-252a after the elicitor-treatment, epicotyls were pre-treated with water followed by the replacement with elicitor alone or the mixture of inhibitor and elicitor. The former solution (elicitor alone) was replaced with the mixture 3 or 6 hr after the elicitor-treatment, respectively, and pisatin accumulation was determined 18 hr after the elicitor treatment as described above.

As shown in Table 1, very little effect was observed by the treatment with LaCl_3 , EGTA, Qui, caffeic acid, or TMB-8 on pisatin accumulation in pea epicotyls induced by elicitor. However, the mixture of BAPTA, Ami, or Chl and elicitor enhanced pisatin accumulation compared with the elicitor-treatment alone. Calmodulin inhibitors, such as Ami or Chl showed elicitor-activity since they induced pisatin accumulation in the absence of elicitor (data not shown). However, the addition of Ver and/or K-252a markedly suppressed pisatin accumulation. Further experiment clearly showed that the addition of Ver suppressed pisatin accumulation at any time before or after the elicitor-treatment, whereas K-252a inhibited the accumulation when it was administered only before the elicitor treatment (Fig. 1A and B).

Although Ver is known as a Ca^{2+} channel blocker, it is unlikely that Ca^{2+} channelling is directly concerned with the signal transduction for pisatin production because Ver suppressed pisatin accumulation to the same extent, even if, it was administered 6 hr after the elicitor-treatment when pisatin biosynthetic pathway has already been activated⁵⁾. Kendra and Hadwiger⁴⁾ reported that only Ver among 6 calcium channel antagonists tested suppressed pisatin accumulation in immature pea pods induced by chitosan, and they suggested that calcium and calmodulin might not be involved in the pisatin production. Together with the results that LaCl_3 and EGTA were unable to inhibit pisatin accumulation, it can hardly be considered that Ca^{2+} plays as the second messenger for signalling pisatin production in pea tissues induced by the elicitor.

Table 1. Effect of putative inhibitors on pisatin accumulation in pea epicotyls induced by the elicitor

Treatment with a)	Pisatin accumulation (μ g/g fresh weight) b)
Distilled water	0.84 $\pm$ 0.47
Distilled water + Elicitor	5.89 $\pm$ 2.52
LaCl_3 (500 μ M) + Elicitor	5.25 $\pm$ 1.26
Quinacrine (500 μ M) + Elicitor	6.84 $\pm$ 1.62
Chlorpromazine (500 μ M) + Elicitor	21.45 $\pm$ 8.63
BAPTA (500 μ M) + Elicitor	11.21 $\pm$ 0.21
Amityryptiline (500 μ M) + Elicitor	20.84 $\pm$ 10.28
TMB-8 (500 μ M) + Elicitor	5.71 $\pm$ 0.31
Verapamil (500 μ M) + Elicitor	0.23 $\pm$ 0.22
Caffeic acid (500 μ M) + Elicitor	6.01 $\pm$ 2.76
EGTA (1 mM) + Elicitor	6.01 $\pm$ 0.86
K252a (200 μ M) + Elicitor	0.0 $\pm$ 0.00

a) Pea epicotyls were pre-treated for 3 hr with water or inhibitor (the final concentration was shown in a parenthesis), and then, were replaced by the mixture of the same conc. of inhibitor and elicitor (final 500 μ g/ml glucose equiv.) from *Mycosphaerella pinodes*.

b) Pisatin accumulation was determined 18 hr after the second treatment (see text).

Fig. 1. Effect of verapamil (A) or K-252a (B) with respect to the addition time after elicitor treatment on pisatin accumulation in pea epicotyls. E and W show elicitor- and water-control, respectively. Numbers represent the time of treatment with verapamil or K-252a. Zero time defines the time of the elicitor-treatment. Bars represent a standard deviation. The experimental procedure is described in the text.

On the other hand, K-252a, known as a strong inhibitor ($K_i=17-25$ nM) of A-, G- and C-protein kinase⁷⁾, inhibited pisatin accumulation when it was applied to pea epicotyls only before the elicitor-treatment. This fact suggests that the signal transduction occurs very rapidly after the elicitor-treatment and that the process is inhibited as far as K-252a has reached to the action site in pea cells before the elicitor is recognized by the receptor. In addition, protein kinase seems to play a key role in signal transduction for pisatin production since K-252a (20 μ M) markedly inhibited *in vitro* phosphorylation of pea plasma membrane proteins incorporated with γ -³²P-ATP (unpublished data).

It was recently demonstrated that tomato plasma membrane proteins, especially 29- and 34-kd proteins, were phosphorylated *in vitro* by the treatment with an endogenous elicitor which induced the production of proteinase inhibitor of tomato⁸⁾. The binding activity of a putative repressor protein of CHS-gene was weakened by phosphorylation in elicited bean cultured cells (Lamb, C., personal communication). These facts together with our data strongly suggest that protein phosphorylation is one of the indispensable process in signal transduction for plant defense reaction induced by the elicitor as well as in that of animal cells induced by hormones. Namely, the process of phytoalexin production may be summarized as follows; 1) the fungal signal (elicitor) binds to the receptor site on the plasma membrane^{9,10)}, 2) protein kinase is activated directly or indirectly by an unknown second messenger, 3) the transcription and translation of defense genes, *e.g.*, PAL- and CHS-gene, are activated by protein phosphorylation, and 4) biochemical barriers against fungi are produced by the gene products.

Further investigation on the identification of protein kinase is under way.

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和 文 摘 要

白石友紀・堀 直裕・山田哲治・奥 八郎：数種の情報伝達系阻害剤によるピサチン蓄積の抑制について

情報伝達系への関与が予想される阻害剤のピサチン蓄積にたいする影響について、エンドウ上胚軸を用いて調べた結果、 LaCl_3 , EGTA BAPTA, amitriptyline, chlorpromazine, quinacrine, TMB-8 および caffeic acid は抑制効果を示さなかった。一方、カルシウムチャンネル阻害剤として知られる verapamil とプロテインキナーゼの強力な阻害剤である K-252a はエリシターで誘導されるピサチンの蓄積を顕著に阻害した。verapamil はピサチン生合成系活性化後に添加した場合にも顕著な効果を示すことから、情報伝達系以外への作用が予想された。一方、K-252a はエリシター処理前に投与される場合に限り顕著な抑制効果を示した。本結果は、エリシター認識からピサチン蓄積に至る初期過程にプロテインキナーゼの関与することを強く示唆する。

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