Arch. Pharm. Res. Vol.19, No.3, pp. 219-222, 1996

Elicitor-Induced Phenylalanine-Ammonia Lyase, Cinnamic Acid 4-Hydroxylase and *p*-Coumaroyl transferase Activity in *Ephedra distachya* Cultures

Kyung-Sik Song and Yutaka Ebizuka[1]

Dept. of Agricultural Chemistry, College of Agriculture, Kyungpook National University, 1370, Sankyuk-Dong, Taegu 702-701, Korea and [1]Faculty of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan

(Received February 26, 1996)

Ephedra distachya cultures have been known to accumulate *p*-coumaroylamino acids by elicitor treatment. Based on their chemical structures, the biosynthetic pathway of *p*-coumaroylamino acids was postulated and phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (4-CH) and *p*-coumaroyl CoA: D-Ala *p*-coumaroyltransferase (*p*-CT) were supposed to be involved in the pathway. The time course inductions of these enzymes were investigated after treatment of yeast extract, yeast-derived mannan glycopeptide and D-Ala. They were detectable at only 4 hours and reached to their maximum level at 9 hours after onset of elicitor treatment. The activities of PAL and 4-CH were almost disappeared within 24 hours, however, that of *p*-CT was remained up to 48 hours irrespective of the kind of elicitors. *p*-CT showed substrate specificity to D-Ala at crude enzyme extract level.

Key Words: *Ephedra distachya*, Tissue culture, Elicitor, *p*-Coumaroylamino acids, Phenylalanine-ammonia lyase, Cinnamic acid 4-hydroxylase, *p*-Coumaroyl-transferase

INTRODUCTION

It has been demonstrated that plants induce a series of biosynthetic enzymes triggered by external factors and the signaling substances which stimulate the induction of these enzymes are termed elicitors (Müller and Börger, 1940). The production of phytoalexins, antibiotic compounds which are induced by a stimulation of elicitors, is one of the most important plant-defense mechanisms against fungal infections (Dixon, 1986; Brooks and Watson, 1984; Ayers *et al.*, 1976). The accumulation of phytoalexins has been reported in at least 17 plant families and most of them are concentrated on Angiospermae (Dixon, 1986). Only a few reports are available for gymnospermous plants. Suspension cultures of *Pinus banksiana* treated with an elicitor prepared from an ectomycorrhizal fungus, *Thelephora terrestris*, rapidly accumulated thioglycolic acid-extractable cell wall-bound phenolics with the increased level of phenylalanine-ammonia lyase (PAL) activity (Campbell and Ellis, 1992). Recently, we reported that yeast-derived mannan glycopeptide elicitors (Con A-II and its partial hydrolysate, MGP) elicited the accumulation of *p*-coumaroylamino acids in *Ephedra distachya* cultures. The produced conjugates were supposed to be phytoalexins of Ephedra (Song *et al.*, 1995). In order to understand elicitor-phytoalexin mechanism clearly, it is essential to verify subsequent behaviour of biosynthetic enzymes after elicitation. In this context, time course induction of some biosynthetic enzymes related to *p*-coumaroyl-D-Ala was investigated as a part of our serial works on 'plant-pathogen interaction mechanism of Ephedra' (Song *et al.*, 1992, 1994a, b and c; Song, 1995).

Correspondence to: Kyung-Sik Song, Dept. of Agricultural Chemistry, College of Agriculture, Kyungpook National University, 1370, Sankyuk-Dong, Taegu 702-701, Korea

MATERIALS AND METHODS

Tissue culture, elicitor purification, elicitation and bioassay

Briefly, tissue culture was performed on Murashige-Skoog's basal medium containing 30 g sucrose, 2 mg 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 mg kinetine l^{-1}. Con A-II elicitor was purified from yeast ext. by ethanol precipitation, DEAE cellulose, gel permeation and Con-A Sepharose chromatography, consecutively. Autoclaved elicitors were added to 28-day-old cultures and futher incubated for appropriate times. Bioassay of elicitor activity was measured by detemining the amount of produced *p*-coumaroy-

lamino acids with HPLC. More details are described on previous reports (Song *et al.*, 1992 and 1995).

PAL activity measurement

PAL activity was determined by published methods (Zuker, 1965; Zimmermann and Hahlbrock, 1975) with slight modifications. Briefly, 30 mg yeast ext., 2 mg Con A-II and 2 mg D-Ala were added to each of 10 ml 27 day old *E. distachya* cultures, separately. Water (500 µl) treated culture was used as a control. Elicited cells were harvested after 2, 4, 6, 9, 12, 24 and 48 h of further incubation. One gram cells were immediately frozen by liquid N_2 and homogenized with mortar and pestle. The homogenates were suspended in 2 ml 0.1 M Tris buffer (pH 7.6) and centrifuged at 4°C, 10,000 g for 20 min. The supernatant was used as an enzyme source. Two hundred µl enzyme ext. (120 µg protein by Lowry's method) was diluted to 500 µl with 0.5 M sodium borate buffer (pH 8.8) and the mixture was added to an equal volume of the same buffer containing 0.02 M L-Phe. Control was made by excluding L-Phe from the mixture. After an h of incubation at 30°C, the absorbance of the mixture was measured at UV 290 nm against control. The amount of product was calculated from the increase in absorbance by means of an extinction coefficient for cinnamate of 10,000/cm/M.

4-CH activity measurement

4-CH activity was detemined by published method (Hyodo and Yang, 1971) with some modifications. One g elicited cells were homogenized as before. The powdered cells were suspended in 2 ml 0.2 M postassium phosphate buffer (pH 7.5) containing 2 mM mercaptoethanol and 50 mg polyclar AT. After centrifuged as before, supernatant was served as an enzyme source. 4-CH activity was measured by incubating 200 µl enzyme ext. (120 µg as protein) with 800 µl 0.2 M potassium phosphate buffer (pH 7.5) containing 14.1 nmol [ring U-^{14}C]-cinnamic acid (1.11×10^5 dpm; purchased from ICN Biochemicals, Inc., USA), 5 µmol NADPH 4Na (purchased from Oriental Yeast Co., Ltd., Japan) and 2 µmol mercaptoethanol at 30°C. Control was made by removing NADPH from the reaction mixture. After one h of incubation, the reaction was stopped by adding 50 µl acetic acid containing 500 µg each of cinnamic acid and *p*-coumaric acid. Two ml EtOAc was added to the mixture and one ml of organic layer was taken out for evaporation. The EtOAc solution was chromatographed on TLC plate [Merck, Art. 5715; C_6H_6-HOAc (9 : 1)] and bands corresponding to *p*-coumaric acid were scraped off to extract them with one ml EtOH. Radioactivity of the ext. was measured by scintillation counting as before.

p-CT activity measurement

Crude enzyme ext. was prepared by the exactly same method as that of 4-CH. The typical measurement of *p*-CT activity was carried out as follows: one hundred µl enzyme ext. (60 µg as protein) was added to 400 µl 0.2 M potassium phosphate buffer (pH 7.5) containing 12.5 nmol *p*-coumaroyl-CoA and 84.4 µmol [1-^{14}C]-D-Ala (2.2×10^5 dpm). The reaction mixture was incubated for 30 min at 30°C. The mixture without *p*-coumaroyl-CoA was served as a control. After the reaction was stopped by 100 µl 0.1 M HCl, 2 ml EtOAc was added and aliquots (1.5 ml) of the organic layer were taken out to measure radioactivity. Since preliminary experiment showed that the radioactivity of EtOAc extractable fraction almost corresponded to that of *p*-coumaroyl-D-Ala, dpm of the EtOAc layer was directly considered as that of *p*-coumaroyl-D-Ala.

Substrate specificity of *p*-CT

Enzyme ext. (400 µl) was added to 600 µl 0.2 M potassium phosphate buffer (pH 7.5) containing 2 mM mercaptoethanol, 250 nmol *p*-coumaroyl-CoA and 1 µmol amino acids. The mixture was incubated for 30 min at 30°C. The reaction was stopped by adding 100 µl 0.1 M HCl and extracted with 2 ml EtOAc. The organic layer (1.5 ml) was evaporated to dryness and 40 µl of MeOH solution was injected for HPLC analysis. The identification and quantitation of produced *p*-coumaroylamino acids were made by comparing their retention times and peak areas with those of synthetic standards. HPLC analysis and synthesis of standard materials were performed by reported method (Song *et al.*, 1992).

RESULTS AND DISCUSSION

Based on their chemical structures, the biosynthetic pathway of *p*-coumaroylamino acids was postulated as Fig. 1. Biosynthetic enzymes such as PAL (phenylalanine ammonia lyase), 4-CH (cinnamic acid 4-hydroxylase) and *p*-CT (*p*-coumaroyl-CoA: D-Ala coumaroyltransferase) were supposed to be involved in this pathway. After elicitation, the time course activities of these enzymes were investigated. Elicitation was made by adding Con A-II (yeast-derived mannan glycopeptide, Song *et al.*, 1995), yeast ext. and D-Ala to the culture, separately. Induced PAL activity was detectable after 4 h of elicitation and markedly increased after 9 h, regardless of the kinds of elicitor. They showed relatively sharp peaks around 6 to 12 h and decreased after 24 h. 4-CH activity also exhibited sharp increase after 9 to 12 h of elicitation and almost disappeared after 24 h. In UV-irradiated or Pmg elicitor-treated suspension cultures of *Petrosel-*

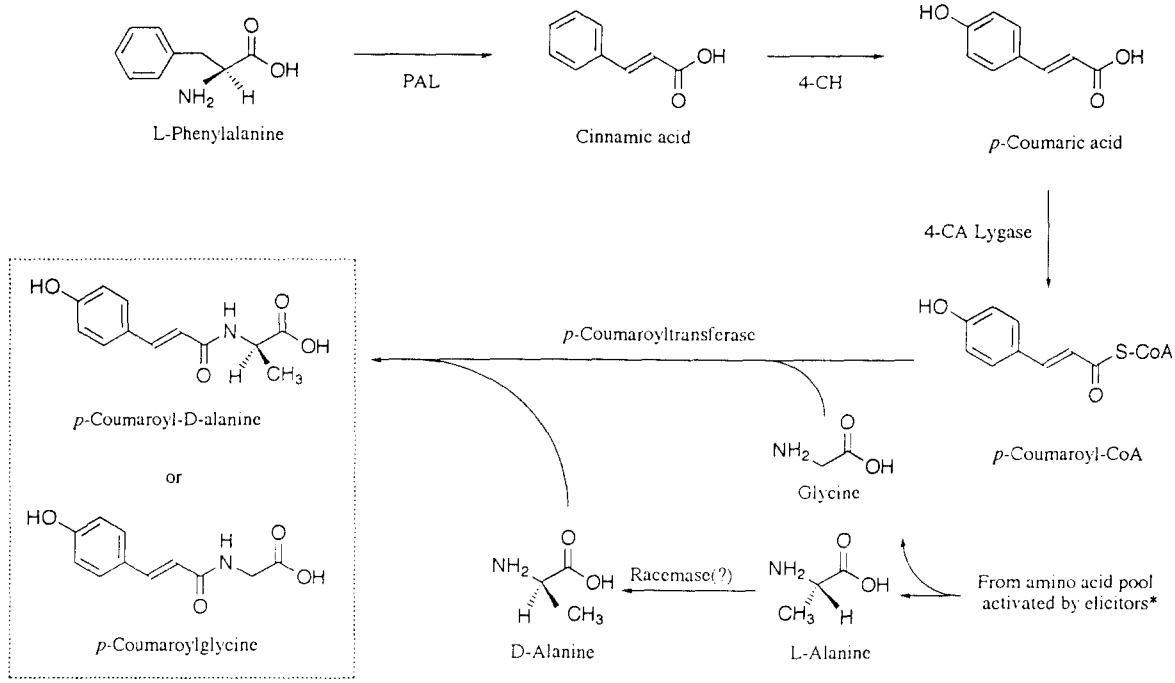

Fig. 1. Tentative biosynthetic pathway of *p*-coumaroylamino acids. *This was supposed by the fact that the amino acid contents of elicited cultures were higher than those of control. Refer to Song, 1995.

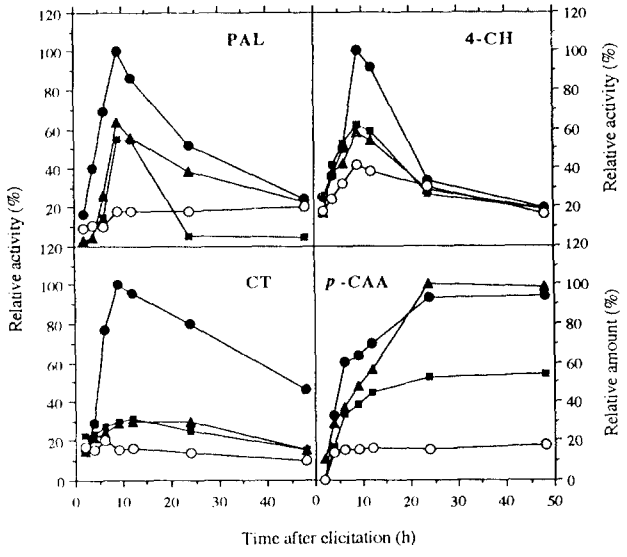

Fig. 2. Time course activitires of PAL, 4-CH and CT. Thirty mg yeast extract (—●—), 2 mg D-Ala (—■—) and 2 mg Con A-II (—▲—) was added to the cultures as elicitors. Water (—○—) was used as a control. A hundred % of activity means 188 kal/Kg, 5.3 kal/Kg and 37.5 kal/Kg for PAL, 4-CH and *p*-CT, respectively.

inum hortense, the induction of PAL and 4-CH occurred within 4 to 12 h after onset of elicitation (Hahlbrock *et al.*, 1981; Ragg *et al.*, 1981). Our results were very similar to those of above reports which made angiospermous plants a subject of study. The *p*-CT, which transfers *p*-coumaroyl moiety from *p*-coumaroyl-Co-A to amino acids, showed its maximum at 9 to 12 h after elicitation as in the cases of PAL and 4-CH but the activity was remained after 48 h to a some extent. *p*-CT activity was the highest in yeast extract-elicited cells and this supported the previous result described by Song, 1995. In this paper, he claimed that yeast extract accumulated much more *p*-coumaroylamino acids than D-Ala or Con A-II did. The reason was found to be synergic effect of Con A-II and D-Ala, and both were constituents of yeast extract. Although elicitor-induced *p*-CT activity has rarely been investigated, it was supposed to play an important role in plant-pathogen interaction. The partially purified tyramine hydroxycinnamoyl transferase was induced by the treatment of chitosan in *Nicotiana glutinosa* (Villegas and Brodelius, 1990). The role of formed tyramine conjugate was supposed to be a phytoalexin. Avenanthramide A, a conjugate of hydroxyanthranilate and *p*-coumaric acid, has been known as a phytoalexin of *Avena sativa* (Crombie and Mistry, 1990) and it was supposed to be synthesized by the action of *p*-coumaroyl-transferase. Although purification must be preceded to clarify the substrate specificity of a certain enzyme, it was carried out at a crude enzyme extract level in order to obtain more informations about *p*-CT . Among tested amino acids, only D-Ala was successfully conjugated with *p*-coumaroyl-CoA (Table I). Zenk *et al.* (1965) reported that acetyl transferase purified from yeast showed specificity to D-Ala as a substrate. In our best knowledge, the presence of D-Ala-specific *p*-CT has never been reported.

Table I. Substrate specificity of crude *p*-coumaroyltransferase

Substrate	Product (nmol)
D-Ala	17.5
L-Ala	3.9
D-Thr	ND*
L-Thr	ND
D-Ser	ND
L-Ser	ND
D-Val	ND
L-Val	ND

*Not detected. Enzyme ext. (400 µl) was added to 600 µl 0.2 M potassium phosphate buffer (pH 7.5) containing 2 mM mercaptoethanol, 250 nmol *p*-coumaroyl-CoA and 1 mol amino acids. The mixture was incubated for 30 min at 30°C. The reaction was extracted with 2 ml EtOAc and the organic layer (1.5 ml) was evaporated to dryness for HPLC analysis. The identification and quantitation of produced *p*-coumaroylamino acids were made by comparing their retention times and peak areas with those of synthetic standards (Song *et al.*, 1992).

E. distachya cultures exhibited a serious senescence (cell browning) after elicitor treatment. In addition, the amount of thioglycolic acid-extractable polyphenols was significantly increased within 9 h after elicitor treatment (unpublished result). Therefore, the elicitor-induced PAL and 4-CH activities might not entirely be responsible for the biosynthesis of *p*-coumaroylamino acids but participate in the biosynthesis of lignin, which has been known as one of the most rapid defensive mechanism of plants (Dixon, 1986; Hahlbrock *et al.*, 1981).

REFERENCES CITED

Ayers, A. R., Ebel, J., Valent, B. and Albersheim, P., Host-pathogen interactions. *Plant Physiol.*, 57, 760-765 (1976).

Brooks, C. J. W. and Watson, D. G., Phytoalexins. *Nat. Product Rep.*, 427-459 (1984).

Campbell, M. M. and Ellis, B. E., Fungal elicitor-mediated responses in pine cell cultures. *Planta*, 186, 409-417 (1992).

Crombie, L. and Mistry, J., The phytoalexins of oat leaves: 4H-3,1-benzoxazin-4-ones or amides? *Tetrahedron Lett.*, 31, 2647-8 (1990).

Dixon, R., The phytoalexin response. *Biol. Rev.*, 61, 239-291 (1986).

Hahlbrock, K., Lamb, C. J., Purwin, C., Ebel, J., Fautz, E. and Sch fer, E., Rapid response of suspension-cultured parseley cells to the elicitor from *Phytophthora megasperma* var. *sojae*. *Plant Physiol.*, 67, 768-773 (1981).

Hyodo, H. and Yang, S. F., Ethylene-enhanced formation of cinnamic acid 4-hydroxylase in excised pea epicotyl tissue. *Arch. Biochem. Biophysics.*, 143, 338-339 (1971).

Müller, K. O. and B rger, H., *Arbeiten aus der Biologischen Bundesanstalt für Land-und Forstwirtschaft* (Berlin), 23, 189 (1940).

Ragg, H., Kuhn, D. N. and Hahlbrock, K., Coordinate regulation of 4-coumarate:CoA ligase and phenylalanine ammonia-lyase mRNAs in cultured plant cells. *J. Biol. Chem.*, 256, 10061-10065 (1981).

Song, K. S., Exogenous D-Ala enhances the accumulation of *p*-coumaroylamino acids in *Ephedra distachya cultures*. *Arch. Pharm. Res.*, 18, 336-339 (1995).

Song, K. S., Ishikawa, Y., Kobayashi, S., Sankawa, U. and Ebizuka, Y., *N*-Acylamino acids from *Ephedra distachya cultures*. *Phytochemistry*, 31, 823-826 (1992).

Song, K. S., Sankawa, U. and Ebizuka, Y., Elicitors which induce the accumulation of *p*-coumaroylamino acids in *Ephedra distachya* cultures. *Arch. Pharm. Res.*, 17, 26-30 (1994a); *p*-Coumaroylamino acids from yeast-elicited *Ephedra distachya* cultures. *Arch. Pharm. Res.*, 17, 51-53 (1994b); A novel benzoic acid derivative from yeast-elicited *Ephedra distachya cultures*. *Arch. Pharm. Res.*, 17, 54-55 (1994c).

Song, K. S., Sankawa, U. and Ebizuka, Y., Mannan glycopeptide elicits *p*-coumaroylamino acids in *Ephedra distachya* cultures. *Phytochemistry*, 38, 95-102 (1995).

Villegas, M and Brodelius, P. E., Elicitor-induced hydroxycinnamoyl-CoA:tyramine hydroxycinnamoyltransferase in plant cell suspension cultures. *Physiologia Plantarum*, 78, 414-420 (1990).

Zenk, M. H. and Schmitt, J. H., Reinigung und Eigenschaften von Acetyl-CoA: D-Aminosäure-α-N-Acetyltransferase aus Hefe. Biochem. Zeit., 342, 54-56 (1965).

Zimmermann, A. and Hahlbrock, K., Light-induced changes of enzyme activities in parseley cell suspension cultures 4-hydroxylase in excised pea epicotyl tissue. *Arch. Biochem. Biophysics*, 166, 54-62 (1975).

Zuker, M., Induction of phenylalanine deaminase by light and its relation to chlorogenic acid synthesis in potato tuber tissue. *Plant Physiol.*, 40, 779-784 (1965).