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Elicitors which Induce the Accumulation of *p*-Coumaroylamino Acids in *Ephedra distachya* Cultures

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Some ammonium oxalate soluble pectic fragments prepared from cultured cell wall of *Ephedra distachya* elicited the accumulation of *p*-coumaroylamino acids (*p*-CAA) in *E. distachya* cultures while water soluble and alkali soluble fractions had no activity. Partial purification of the pectic fragments fraction using DEAE-cellulose chromatography afforded two active fractions (PS-I and PS-II) which were composed of mainly uronic acids (98-99 w/w %). They elicited the accumulation of *p*-CAA in an amount of 52-60 nmol per gram fresh weight of cultures. The acidic sugar compositions of PS-I and PS-II were found to be galacturonic acid and glucuronic acid by TLC analysis. They were supposed to act as endogenous elicitors of *p*-CAA accumulation. In order to investigate the effect of ethylene on *p*-CAA accumulation, Ethrel, which is known as ethylene generator, and ACC (1-aminocyclopropane-1-carboxylic acid), a direct precursor of ethylene biosynthesis, were added to the culture. However, they did not affect on the accumulation of *p*-CAA. AVG (aminoethoxyvinylglycine), a tentative inhibitor of ethylene biosynthesis, did not affect on the elicitor activity of yeast-derived mannan glycopeptide elicitor (Con A-II), either. Consequently, no relationships between ethylene and p-CAA accumulation were recognized. Several tentative elicitors were tested for their activity. Commercial yeast glucan, $CuCl_2$, laminarin and laminariheptaose had slight activity whereas α-methylmannopyranoside and commercial yeast mannan had no elicitor activity. α-Methylmannopyranoside which has been known as a tentative inhibitor of glucan elicitor in *Glycine max* did not affect on the elicitor activity of Con A-II.

Key words: *Ephedra distachya* cultures, Endogenous elicitor, Cell wall pectic substances, Ethylene, *p*-coumaroylamino acids

INTRODUCTION

It has been known that plants induce a series of enzymes triggered by external factors and the signaling substances which stimulate the induction of enzymes are termed as elicitors (Müller *et al.*, 1940). The induction of phytoalexins, low-molecular weight antibiotic materials which are induced as a result of stimulation of elicitors, is one of the most well-studied plant-pathogen interaction mechanisms (Dixon, 1986, Brooks and Watson, 1985 and Ayers *et al.*, 1976). The accumulation of phytoalexins has been demonstrated in at least 17 plant families and most of studies has been concentrated on Angiosperms or crop plants (Dixon, 1986). Only a few reports are available for the studies on the 'elicitor-phytoalexin' mechanism of Gymnospermous plants (Cambell *et al.*, 1992a, b). Recently, we reported that the suspension cultures of *Ephedra distachya*, one of Gymnospermous medicinal plants which produce the clinically important alkaloids represented by *l*-ephedrine, accumulated the *p*-coumaroylamino acids (*p*-CAA) by the addition of yeast extract to the culture (Song *et al.*, 1992). The *p*-CAA inducing elicitor was characterized as branched α-mannan O-glycopeptide which had *ca* 4.7 kD of molecular weight (Song *et al.*, 1993a).

The concept that fragments of the host cell wall may act as elicitors of phytoalexin induction arose from the work of West's group on the elicitor activity of the polygalacturonase from *Rhizopus stolonifer* (West *et al.*, 1985), and the demonstration by Albersheim's group of similar biological activity of pectic

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Dedicated to Prof. Seung-Jo Yoo to cerebrate his glorious retire.

fragments isolated from soybean cell walls (Hahn *et al.*, 1981). This pectic fragments of plant cell wall which has elicitor activity for the accumulation of phytoalexins have been termed as 'endogenous elicitor'. So far, there was no report on endogenous elicitor in Gymnosperms.

On the other hand, many plants respond to an attack by pathogens with an enhanced ethylene production (Pegg., 1976). Exogenously applied ethylene has been found to activate or enhance biochemical defences including phytoalexin accumulation against potential pathogens in a number of cases (Chalutz *et al.*, 1969). In soybean cotyledons, induction of phytoalexin accumulation and phenylalanine ammonium-lyase (PAL) activity by cell wall elicitors from *Phytophthora megasperma* is preceded by ethylene formation (Paradies *et al.*, 1980). 5'-S-Methyl-5'-thioadenosine, a by-product of ethylene biosynthesis, has been isolated from *E. distachya* cultures and the elicited cells exhibited necrotic symtom (Song *et al.*, 1992).

These obse vations led us to examine the endogenous elicitor(s) of *E. distachya* cultures and the effect of ethylene on the *p*-CAA accumulation, as one of our serial works (Song *et al.*, 1992, 1993a and b) on 'plant-pathogen interaction mechanisms of *Ephedra*'. Several candidates which were supposed to induce the *p*-CAA or to affect on the elicitor activity of yeast-derived elicitor (Con A-II) are also discussed.

MATERIALS AND METHODS

Materials

The Ethrel (synonym of ethephon; 2-chloroethyl phosphonic acid) was purchased from Ishihara Sangyo, Co., Japan. ACC, AVG, α-methylmannopyranoside, yeast mannan, yeast glucan and laminarin were products of Sigma. Laminariheptaose was a product of Seikagaku Kogyo Co., Ltd., Tokyo, Japan. $CuCl_2$ was obtained from Wako Chemicals, Tokyo, Japan. Con A-II was prepared according to the reported method from yeast extract (Song *et al.*, 1993a).

Preparation of *E. distachya* Cell Wall (Hahn *et al.*, 1981)

30-40 day old *E. distachya* cell was harvested in a suction flask. The marc was washed with 500 ml 0.5 M sodium phosphate buffer (pH 7.0) and immediately frozen at −20℃. 168 g of frozen tissue was suspended in 1.2 l washing buffer and ground by Warring blender for 30-50 seconds with 1 minute of intervals for 3 times. The disrupted cell suspension was centrifuged at 15,000 g for 20 min. Supernatant was decanted and discarded. The pellet was washed with 1.0 l of washing buffer and centrifuged as before. The resultant pellet was washed in a similar fashion thrice with 1.0 l of deionized H_2O at 4℃. After decanted the supernatant, the remained final pellet was suspended in 600 ml ethanol. The ethanol solution was refluxed in water bath for 3 hours to remove organic soluble materials. The cell walls were collected by filtration on a coarse sintered-glass funnel. The insoluble cell walls, which were collected on sintered-glass funnel, were resuspended in an additional 500 ml ethanol and the solvent was drawn away by suction. The cell wall preparation was then washed with 2.0 l $CHCl_3$: CH_3OH (1 : 1) by repeatedly suspending the solvent and then solubilized materials away with suction. The walls were finally washed with 1.0 l acetone in the same manner. The wall preparation was dried by pulling air through it by suction for 2 hours. The preparation was then allowed to air dry for several days in a loosely covered plastic panel and finally dried with vacuum pump before weighing. Fluffy and pale yellow cell wall was obtained in an yield of 3.27 g per 168 g fresh weight of cell.

Fractionation of Cell Wall (Srisuma *et al.*, 1991)

Two gram of *E. distachya* cell wall was suspended in 100 ml water and extracted for 2 h at 80℃ with stirring. The suspension was filtered through Whatmann GF/D filter and the filtrate was lyophilized (water soluble fraction, 75 mg). The residue was suspended in 300 ml 0.5% ammonium oxalate to be extracted twice at 85℃ for an hour. After cooling at room temperature, the extract was filtered and the filtrate was dialyzed against water. The dialysate was lyophilized and stored in a desiccator until used (pectic fragments, 95 mg). The residue, which was not extractable by ammonium oxalate was extracted twice with 300 ml mixture of 1 M NaCl and $NaBH_4$ under N_2 gas at room temperature for 18 hours. The resultant suspension was filtered through Whatmann GF/D filter. Both of the residue and filtrate were dialyzed against water and the dialysate was lyophilized {designated as lignocellulose fraction (425 mg) and hemicellulose fraction (504 mg), respectively}.

DEAE-cellulose Chromatography

The pectic fragments fraction (60 mg) was applied on DEAE-cellulose column (Whatman DE 52, 2.5×20 cm), which has been equilibrated with 0.1 M potassium phosphate buffer (pH 7.7). After the column was washed with 200 ml of equilibration buffer, 0.1-0.5 M NaCl solution prepared in the same buffer was poured into the column with gradual increase of NaCl concentration (total elution volume was 200 ml). Finally, the column was washed with 200 ml of equilibration buffer containing 1.0 M NaCl. Every five ml of

eluent was collected to be monitored by anthrone-H_2SO_4 and Lowry's method.

Colorimetric Analysis

m-Hydroxybiphenyl method (Blumenkrantz and Asboe-Hansen, 1973) for the determination of uronic acid, anthrone-H_2SO_4 method for total carbohydrate (Dische, 1953) and Lowry's method for protein (Lowry *et al.*, 1951) were carried out according to reported methods using galacturonic acid, glucose and BSA (bovine serum albumin) as standards, respectively.

Elicitation

Appropriate amount of test samples were suspended in 500 µl of distilled water and autoclaved at 121℃ for 20 min. The autoclaved solution was added to the 28-29 day old *E. distachya* cultures and the cultures were subsequently incubated for 48 hours. The elicited cells were extracted with methanol by ultrasonic vibration. The produced amount of *p*-CAA in each elicited cells was analyzed by HPLC. HPLC analysis and the evaluation of elicitor activity of test samples were carried out by means of the reported method (Song *et al.*, 1993a).

TLC Analysis of Active Fraction

One milligram of each PS-I and PS-II was hydrolyzed with 2 ml 95% TFA in a sealed tube at 121℃ for 1 hour. The hydrolysate was filtered through cotton and the filtrate was evaporated to dryness. To the residue, 500 µl of distilled water was added and the solutions were analyzed by TLC. TLC conditions were as follows: plate; Abicell SF cellulose plate, solvents; pyridine : ethyl acetate : acetic acid : water=36 : 36 : 7 : 21.

RESULTS AND DISCUSSION

Endogenous Elicitor

The cell wall preparations of *E. distchya* cultures were classified into four fractions (water soluble, pectic fragments, hemicellulose and lignocellulose) according to their solubility, which is determined by types of bonds and bonding strength. Each one or ten mg cell wall fractions was added to the culture to test their *p*-CAA inducing elicitor activity. As presented in Table I, pectic fragments fraction accumulated *p*-CAA in an amount of 48-56 nmol per gram fresh weight while the other fractions did not showed any significant activity. About 98-99 w/w % of the active fraction was determined to be uronic acids by *m*-hydroxybiphenyl method. This fraction was applied to DEAE-cellulose column. Neither anthrone-H_2SO_4 positive nor Lowry positive material was detected from the equilibration buffer-eluted fraction. 0.1-0.5 M NaCl eluted fraction (PS-I, 15 mg) and 1.0 M NaCl washed fraction (PS-II, 30mg) were obtained as anthrone positive but Lowry negative substances whose acidic sugar composition was found to be galacuronic acid and glucuronic acid by comparing their Rf values on TLC plates with authentic samples (Rf values were 0.25 and 0.30, respectively). 1.0 or 5.0 mg of each PS-I and PS-II was added to the culture to evaluate their elicitor activity. Both of them were elicitor active but did not exhibit any significant differences in their activities as shown in Table I. Further purification of PS-I and PS-II were not carried out since the amount of these fractions were too small.

Albersheim and other groups have demonstrated that the active principle of endogenous elicitor of *Gly-*

Table I. Elicitor activity of *E. distachya* cell wall fractions

Elicitor (mg)		Products[a] (nmol/g fr.wt.)
Cell wall	1.0	2.0
	10.0	2.1
Water soluble fr.	1.0	1.6
	10.0	1.8
Lignocellulose fr.	1.0	1.2
	10.0	2.8
Hemicellulose fr.	1.0	1.3
	10.0	4.3
Pectic fragments	1.0	48.3
	10.0	56.2
PS-I	1.0	52.1
	5.0	60.8
PS-II	1.0	55.3
	5.0	65.4

[a]Amount of elicitor-induced *p*-coumaroylamino acids

Table II. Effect of ethrel, ACC, and AVG on the elicitor activity of Con A-II

Elicitor (ml or mg)		Products[a] (nmol/g fr.wt.)
Con A-II	1.00	68.5
Ethrel	0.01	3.0
	0.10	ND[b]
Ethrel	0.01	
+Con A-II	1.00	65.3
ACC[c]	0.1	3.5
	1.0	4.8
ACC	1.0	
+Con A-II	1.0	63.2
AVG[c]	0.5	5.1
	1.0	5.5
AVG	1.0	
+Con A-II	1.0	64.5

a Amlount of elicitor-induced *p*-coumaroylamino acids
[b]Not detected
[c]For abbreviations, see text

Table III. Elicitors of *p*-CAA accumulation in *E. distachya* cultures

Elicitor (mg)		Products[a] (nmol/g fr.wt.)
Yeast glucan	1.0	12.5
	5.0	18.2
Yeast mannan	1.0	3.0
	5.0	8.1
$CuCl_2$	0.1	20.5
	1.0	ND[b]
MMP[c]	1.0	3.0
MMP	1.0	
+Con A-II	1.0	64.8
Laminarin	1.0	11.5
	5.0	18.3
Laminariheptaose	1.0	13.5
	5.0	22.1

[a] Amount of elicitor-induced *p*-coumaroylamino acids
[b] Not detected
[c] α-Methylmannopyranoside

cine max was an α-galacturonide (Nothnagel *et al.*, 1981). Considering the acidic sugar composition, PS-I and PS-II were also supposed to be a kind of galacuronides containing glucuronic acid moiety. Although it is not clear which moiety is responsible for the elicitor activity, this is the first report on the endogenous elicitors of Gymnosperms.

The Effect of Ethylene on *p*-CAA accumulation

The Ethrel, known as an ethylene generator (Sisler *et al.*, 1984), was tested for its effect on *p*-CAA accumulation. 0.01 or 0.1 ml (as 10% solution) Ethrel was administrated to the 10 ml of culture in the presence or absence of Con A-II. When more than 0.1 ml of Ethrel was added, cell showed serious disruption. Neither Ethrel itself nor co-addition with Con A-II affected on the accumulation of *p*-CAA (Table II). ACC, a precursor of ethylene biosynthesis (Lieberman, 1979) or AVG a tentative inhibitor of ethylene biosynthesis (Lieberman, 1979) also did not affect on the production of *p*-CAA regardless of the presence of Con A-II as presented in Table II. The necrotic symptom of cells caused by elicitation was not changed significantly by the addition of ACC or AVG, either. Consequently, no notable relationships were recognized between ethylene and the accumulation of *p*-CAA in *E. distachya* cultures.

Other Materials which Elicit the Accumulation of *p*-CAA

Very diverse materials have been found to act as elicitors of phytoalexins. Several tentative elicitors were tested for their *p*-CAA inducing activity. Commercial yeast mannan, commercial yeast glucan, laminarin, laminari-7-ose and $CuCl_2$ were tested for their elicitor activity. Although commercial yeast glucan, $CuCl_2$, laminarin and laminariheptaose had slight activity, none of them were as effective as Con A-II (Table III). Commercial yeast mannan had no elicitor activity. α-Methylmannopyranoside, a tentative inhibitor of the binding site of β-glucan elicitors (Marcan, 1979), had no elicitor activity nor inhibitory effect on the elicitor activity of Con A-II.

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