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Exogenous D-Ala Enhances the Accumulation of *p*-Coumaroylamino Acids in *Ephedra distachya* Cultures

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Ephedra distachya cultures have been known to accumulate two major *p*-coumaroylamino acids (*p*-coumaroylglycine and *p*-coumaroyl-D-alanine) by treatment of yeast-derived elicitors. The accumulation of these conjugates was also increased by D-Ala treatment. When D-Ala was added together with serial concentrations of yeast-derived elicitor, the accumulation of *p*-coumaroyl-D-Ala (*p*-CDA) was greatly increased in an additive manner. In feeding experiments, [1-^{14}C]-D-Ala was incorporated into *p*-CDA at a rate of 2.2% or 2.3% of added radioactivity, indicating that exogenous D-Ala served as a precursor of the conjugate. [1-^{14}C]-L-Ala was also incorporated into *p*-CDA (0.23%) in the elicitor treated cultures. This fact suggested that at least a part of *p*-CDA was produced from active conversion of L-Ala by the elicitation. In order to investigate a possible role of D-Ala as an elicitor of *p*-coumaroylamino acids (*p*-CAA), cold D-Ala was added together with labeled L-Ala. Although L-Ala seemed to be incorporated into *p*-CDA by this treatment, the incorporation ratio was too small (0.054%) to draw a clear conclusion. However, the amount of *p*-coumaroylglycine, which did not use D-Ala as a substrate, was also slightly increased by D-Ala treatment irrespective of the presence of elicitor, suggesting that exogenous D-Ala might act as an elicitor of *p*-CAA as well as a precursor substrate of *p*-CDA.

Key Words : *Ephedra distachya*, D-Ala, Elicitor, Precursor, *p*-Coumaroylamino acids, *p*-Coumaroyl-D-alanine, *p*-Coumaroylglycine

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 (Darvill and Albersheim, 1982). 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*-coumaroylglycine and *p*-coumaroyl-D-alanine (*p*-CDA) in *Ephedra distachya* cultures (Song *et al.*, 1995). The specific activities of Con A-II and MGP as elicitors were *ca* 20-fold higher than that of yeast extract. Their activities were saturated at *ca* 0.2 mg/ml medium with production of 80 nmol *p*-coumaroylamino acids (*p*-CAA)/g fresh weight cells. However, at higher concentrations (more than 0.7 mg elicitor/ml medium), the purified elicitors did not induce as much amount of *p*-CAA as yeast extract did. Two possibilities were assumed by this fact: one was the inactivation or modification of the elicitors during purification, and the other was the existence of effector(s) on MGP or Con A-II elicitor activity. The former seemed to be less likely since Con A-II elicitor was relatively stable to heat and pH. Meanwhile, the latter possibility was more likely because the co-addition of 80 S, an yeast-derived 80% ethanol soluble fraction which had a little elicitor activity (Song et al., 1995), greatly enhanced the elicitor activity of Con A-II. This 'effector' was purified from 80 S and identified as D-Ala (See results and dis-

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cussion). As a part of our series of works on 'Plant-defense mechanisms in *Ephedra*' (Song *et al.*, 1992, 1994a, b and c, 1995), the metabolic fate and possible role of exogenous D-Ala in *E. distachya* cultures was investigated.

MATERIALS AND METHODS

Elicitor purification , elicitation and bioassay

Elicitor purification, elicitation and bioassay of elicitor activity were performed according to the method described by Song *et al.*, 1995a.

Purification of Ala from yeast ext.

Ten g 80 S was suspended in 100 ml water and stored in a refrigerator. A precipitated white crystalline material was filtered through filter paper. The non-filtrate was washed with 50 ml cold acetone and MeOH, consecutively. The filtrate was combined and evaporated to dryness. Fifty ml water was added to the residue and insoluble materials were filtered off as before. The non-filtrate were combined and air dried (3.5 g). Small portion of it was dissolved in water and the solution was developed on TLC. TLC conditions were as follows: plate; Merck Art. 5715, solvent; $CHCl_3$-MeOH-H_2O (65 : 35 : 10), detection; ninhydrin and 10% sulfuric acid. Silica gel column chromatography [2.5×25 cm, Merck Art. 9385, $CHCl_3$-MeOH-H_2O (60 : 55 : 10)] of this fraction afforded Ala.

Separation of D- and L-Ala

The yeast ext.-derived Ala (*ca* 1 mg) was dissolved in 1 ml mobile phase and 2-10 μl solution was injected for HPLC analysis. HPLC conditions were as follows: column; TOSOH ENANTIO L1 (4.6×550 mm), column temp; 50°C, mobile phase; 0.06 mM $CuSO_4$, flow rate; 0.5 ml/min, detection; UV 254 nm.

Feeding experiment with labeled D- or L-Ala

Two μCi [1-^{14}C]-D-Ala (43.5 nmol) or -L-Ala (35.2 nmol), which were purchased from ICN Biochemicals, were added to 27 day old 10 ml culture with 2 mg D-Ala or 10 mg partially purified elicitor fraction (80 I-DI). The elicitor fraction was prepared from yeast extract according to the method described by Song *et al.*, 1995a. Control was made by adding 500 μl water instead of elicitors to the cultures. Two sets of flasks were prepared and after 48 hr incubation, cells were harvested and extracted with 10 ml MeOH by ultrasonic vibration. The extract was settled for a while in order to precipitate insoluble materials. Five ml supernatant was taken and concentrated. From one ml methanolic solution 500 μl was taken to filter through Toyo-PAK ODS (TOSOH, Japan). An aliquot (40 μl) of filtrate was injected into HPLC and the peak corresponding to *p*-CDA was fractionated to measure radioactivity. The amount of *p*-CDA was quantitated by the peak area and calibration curve (Song *et al.*, 1995a). Radioactivity was measured by scintillation counting in Toluene-TritonX100 mixture (1 : 2 v/v) containing 5.5 g 2,5-diphenyloxazol (DPO) and 0.1 g 1,4-*bis*[2-(5-phenyloxazolyl)] benzene (POPOP) per one l. All experiments were performed in duplicate.

Measurement of free Ala content

Cells (5 ml) treated with 1 mg Con A-II or 500 μl water were filtered after 12 or 24 hr of incubation. Harvested cells were extracted with 5 ml water. The volume of the filtrate and the extract were adjusted to 10 ml and 5 ml with water, respectively. Aliquots were analysed by HPLC to measure free Ala content (Song *et al.*, 1995a).

Separation of enantiomers of *p*-coumaroylalanine

This was performed as described by Song *et al.*, 1992 and 1994b.

RESULTS AND DISCUSSION

As previously mentioned, the activity of purified elicitors did not recover the complete activity of yeast ext. itself. Therefore, we tried to combine Con A-II with partially purified fractions obtained from each purification step and check the mixtures for elicitor activity. The 80 S, which was obtained as a soluble part of 80% ethanolic precipitation of yeast ext. (Song *et al.*, 1995a) was found to raise elicitor activity of Con A-II. A white crystalline material was precipitated from aqueous solution of the 80 S. The supernatant or the precipitate (2 mg/ml medium each) were added to the culture in either the presence or absence of Con A-II. The combination of Con A-II and the precipitate enhanced the accumulation of *p*-CAA (Fig. 1). TLC analysis of the precipitate, which was positive to ninhydrin reagent, revealed that it was essentially composed of free amino acids, namely Phe, Tyr, Trp, Gly and Ala. Each of authentic optical isomers (0.1 to 1.0 mg) of detected free amino acids was added to the culture with or without 1.0 mg Con A-II. L-Phe or L-Tyr was expected to show an enhanced accumulation of *p*-CAA since they might be a direct substrate of key enzymes of phenylpropanoids biosynthesis such as phenylalanine ammonia-lyase (PAL) or tyrosine ammonia-lyase (TAL) (Koukol and Conn, 1961). However, they showed no notable effect. Trp, L-Ala and Gly did not induce accumulation of *p*-CAA, either. On the contrary, regardless of the presence of Con A-II, exogenous D-Ala induced accumulation of *p*-CAA to a great extent (Fig. 2). The treatment with D-Ala (6 ng to

0.2 mg/ml medium) under the presence of 0.1 mg/ml Con A-II greatly enhanced the production of *p*-CAA compared to that of D-Ala treatment alone (the left graph of Fig. 2). Especially, when various amount of Con A-II were added under the presence of 0.1 mg/ml D-Ala, the accumulation of *p*-CAA was increased dramatically (the right of Fig. 2). The treatment with 0. 1 mg/ml of D-Ala induced *ca* 200 nmol of *p*-CAA/g fresh weight, and 0.06 mg Con A-II did only *ca* 60 nmol. Therefore, the simple mathematical sum must be 260 nmol; nevertheless, the co-addition of two substances accumulated more than 400 nmol of *p*-CAA. Above results led us to investigate the role of exogenous D-Ala in *p*-CAA production in *E. distachya* cultures. The presence of both enantiomers of Ala in 80 S was verified by silica gel column chromatography, followed by HPLC analysis equipped with a chiral column (D-Ala : L-Ala=7 : 10 by weight, Rt's were 41.5 and 43.0 min, respectively). The amount of free Ala in 80 S was determined to be 24.3 μg/mg (10.0 μg/mg as D-Ala) by HPLC.

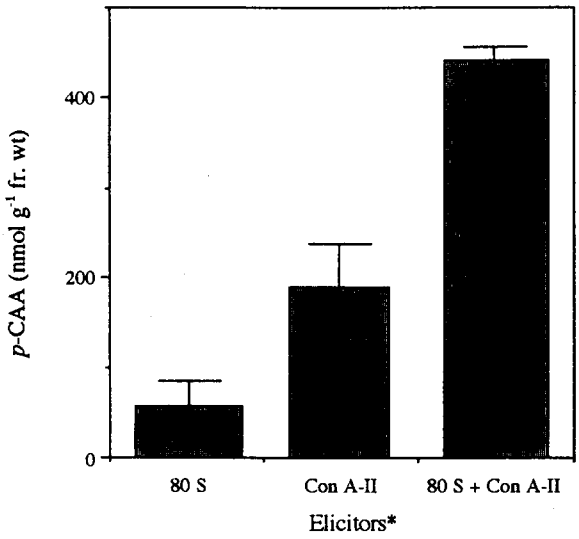

Fig. 1. Co-addition effect of 80 S on the elicitor activity of Con A-II. *Each two mg of Con A-II or 80 S was added to the culture separately or in combination with each other. The 80 S means eighty % ethanol-soluble fraction prepared from yeast extract.

It was supposed that exogenous D-Ala act as a precursor of *p*-CDA by its chemical structure. To investigate the metabolic fate of D-Ala, [1-^{14}C]-D-Ala was fed into *E. distachya* cultures. By elicitation with 80 I-DI [partially purified elicitor fraction prepared from yeast extract (Song *et al.*, 1995a)], labeled D-Ala was incorporated into *p*-CDA at a rate of 2.2% of added radioactivity (Table 1). This clearly indicated that exogenous D-Ala was served as a substrate of *p*-CDA. It has been known that some exogenous D-amino acids were metabolized to their conjugated form, possibly as a result of detoxification pathway of D-amino acids in plants (Robinson, 1976). However, in our case, [1-^{14}C]-L-Ala was also incorporated into *p*-coumaroylalanine (0.23%) by 80 I-DI elicitation, and the produced conjugate was identified as a pure D-isomer by HPLC. Since the radioactive enantiomers of Ala employed in these experiments had more than 99% of optical purity (confirmed by HPLC which could separate less than 1% of optical isomer) it was doubtless that at least a part of elicitor-induced *p*-CDA was biosynthesized from L-Ala. Such a conversion of L-Ala into *p*-CDA (not *p*-coumaroyl-L-Ala) might be explained by an active mechanism including some important physiological purposes like plant-defense mechanism rather than a detoxification mechanism. It seemed to be strange that there were no differences in incorporation rates between control and elicitor treat-

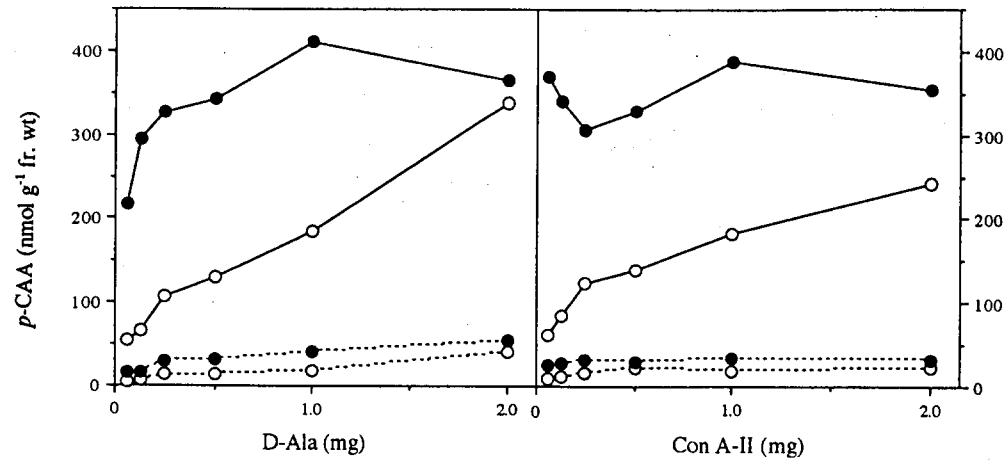

Fig. 2. Co-addition effect of D-Ala and Con A-II on the accumulation of *p*-coumaroylamino acids. Broken lines indicate the induced amount of *p*-coumaroylglycine and solid lines *p*-coumaroyl-D-Ala. Left; in the presence (●) or absence (○) of 1.0 mg Con A-II, serial concentration of D-Ala were added. Right; under the presence (●) or absence (○) of 1.0 mg D-Ala, serial concentrations of Con A-II were added.

Table 1. Metabolic fate of [^{14}C-1]-D- and -L-Ala in *E. distachya* cultures

Labeled Ala	Added with	*p*-CDA (%)[a]	*p*-CDA (nmol)[b]
[^{14}C-1]-L-Ala	D-Ala	2,360 (0.054)	76.8
	80 I-DI[c]	9,950 (0.23)	32.6
	Control	7,860 (0.18)	3.9
[^{14}C-1]-D-Ala	D-Ala	15,100 (0.34)	88.6
	80 I-DI[c]	98,300 (2.2)	56.7
	Control	100,200 (2.3)	6.5

Either 2 μCi of [^{14}C-1]-D- (43.5 nmol) or -L-Ala (35.2 nmol) was added to 10ml of culture (approximately equivalent to 1 g fr. wt. of cells) together with elicitors. After further 48 hr of incubation, radioactivity of *p*-coumaroyl-D-alanine was measured.

[a] Dpm of *p*-coumaroyl-D-alanine g^{-1} fr. wt. of cells. Parentheses mean incorporation % (incorporated dpm per 2 μCi).

[b] Amount (nmol) of *p*-coumaroyl-D-alanine g^{-1} fr. wt. cells.

[c] Eighty % ethanol-insoluble dialysate fraction prepared form yeast extract (See text).

ed cells. This can be explained by the activated alanine pool in elicited cells and subsequent dilution of radioactivity. Actually, free Ala contents were 12.5 times higher in 12 hr-elicited cells (30.0 μmol/g fr. wt.) than that of control (2.4 μmol).

It was very interesting that the production of *p*-coumaroylglycine, which did not use D-Ala as a substrate, was also increased by the addition of D-Ala. In combination with the additive increase in *p*-CDA amount shown in Fig. 2, this fact encouraged us to investigate the role of D-Ala as an elicitor of *p*-CAA accumulation. If D-Ala acted as an elicitor, labeled L-Ala might be converted into *p*-CDA with a convincing incorporation rate. Labeled L-Ala seemed to be incorporated into *p*-CDA by an addition of cold D-Ala as shown in Table 1. However, the rate was lower than that of control which produce a small amount of *p*-CDA. Such diluted incorporation rate was supposed to be originated from the high concentration of cold D-Ala which was employed for elicitation. Also, the incorporation rates of rdioactive L-Ala-treated cells were lower than those of labeled D-Ala-treated ones. This might be caused by the higher possibility of L-Ala to serve as a precursor of various proteins. In fact, the incorporation rate of labeled L-Ala into non-acetone extractable fraction was *ca* 350% higher than that of labeled D-Ala. Due to these diluting factors, it was difficult to draw a clear conclusion by means of feeding experiment. Nevertheless, *p*-coumaroylglycine which did not use D-Ala as a substrate was also induced by D-Ala treatment (Fig. 1). In addition, the time course induction of phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (4-CH) and coumaroyl CoA: D-Ala coumaroyltransferase (CT), which were supposed to be related to the biosynthesis of *p*-CAA in *E. distachya* cultures, showed their maximum activity around 9 hr after onset of yeast ext. or Con A-II treatment, irrespective of the kind of elicitors. D-Ala also induced above three enzymes in a very similar time course pattern (Unpublished data).

Many microorganisms have been known to produce D-amino acids and D-Ala occupies the major part of them (Robinson Burge, 1976). In combination with the above observations, our present results suggest that D-Ala might work as a signaling compound (elicitor) of *p*-CAA in *E. distachya*.

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