Dependence on Ethylene of the Induction of Phenylalanine Ammonia-Lyase Activity in Rice Leaf Infected with Blast Fungus[†]

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The application of ethephon (ETP), an ethylene generating chemical, to press-injured rice leaves induced PAL (EC 4.3.1.6) activity. This induction disappeared when the concentration was low. A Scatchard plot for ligand binding gave a linear relationship between the PAL reaction rate at the time for the maximum rate of induction (t_m) along the sigmoidal progress and the ratio of the PAL reaction rate *versus* ETP in a specific range of concentration. A similar application of glutamate (Glu) induced PAL activity, and the application of 1-aminocyclopropane-1-carboxylic acid (ACC) also induced PAL activity. The induction of rice leaf PAL with Glu was inhibited in the presence of tiron (TR), a superoxide scavenger, was weakly inhibited with aminooxyacetic acid (AOA), but was not inhibited with abscisic acid (ABA). The induction with ACC was also inhibited in the presence of TR, was weakly inhibited with AOA, and further was weakly inhibited with ABA. The inoculation of blast fungus conidia or application of fungal proteoglucomannan (RIF) to press-injured rice leaves induced PAL activity. The induction proceeded along sigmoidal curve accompanying the distinct auto-inactivation phase. Each approximated t_m (midpoint of an individual sigmoidal curve) was determined; $t_m = 2.4$ hr with an incompatible race, $t_m = 5.3$ hr with a compatible race, and $t_m = 11.0$ hr with RIF stimulation. The 3 inhibition profiles of TR, AOA and ABA upon rice leaf PAL induction either with inoculation or with stimulation were quite similar to the inhibition profiles upon PAL induction with Glu.

The inductions of PAL and peroxidase in rice leaves infected with blast fungus have been reported by Matsuyama and Kozaka.¹⁾ A high-

er rate of ethylene evolution at an early stage of rice blast infection on a resistant cultivar has been also observed by Kozaka and

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Abbreviations: PAL, phenylalanine ammonia-lyase; Cin, cinnamate; RIF, resistant-reaction inducing factor (stimulant, blast fungus hyphal component or proteoglucomannan in previous papers); TR, tiron (1,2-dihydroxybenzene-3,5-disulfonic acid disodium salt monohydrate); AOA, amino-oxyacetic acid (carboxy-methoxylamine hemihydrochloride); CY, cycloheximide; ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; Glu, glutamate monosodium salt; α -KGA, α -ketoglutarate disodium salt; ETP, ethephon (2-chloroethyl phosphonic acid); IAA, β -indoleacetic acid.

Teraoka.²⁾ The dependence of peroxidase induction on ethylene in sweet potato tuberous root has been demonstrated by Imaseki.³⁾ Stahmann *et al.* have proposed an attractive hypothesis that endogenous ethylene induces resistant characteristics in sweet potato tuberous root concomitantly with the peroxidase induction.⁴⁾ In this paper, to elucidate the dependence of PAL induction on ethylene in rice leaves infected with blast fungus, time course surveys of the induction of rice leaf PAL with press-injured application of either ETP, an ethylene generating chemical, Glu and ACC were made. The inhibition spectra of the induction of rice leaf PAL either with Glu or ACC were determined using TR (a superoxide scavenger^{5,6)}), AOA to (an inhibitor to the pyridoxal enzyme⁷⁾), and ABA (an inhibitor to the induction of ACC synthase⁸⁾). Since the dynamic behavior of superoxide generation at a very early stage by rice leaves infected with blast fungus has already been elucidated,⁹⁾ and the conversion of ACC to ethylene in the methionine pathway, which is ubiquitous in higher plants, has been reported to be dependent on endogenous superoxide,^{5,6)} TR was used in this study to determine the dependence of ethylene generation on the superoxide anion. Time course surveys were also made of the induction of rice leaf PAL with press-injured inoculation of either an incompatible or compatible blast fungus conidia, and with press-injured stimulation of RIF. After the determination of t_m (the time for the maximum rate of induction after inoculation or stimulation, corresponding to the midpoint of an individual sigmoidal curve), the inhibition spectra of the induction of rice leaf PAL with inoculation of either the incompatible or compatible conidia, or with stimulation by RIF, were determined using TR, AOA, ABA or CY, and were compared with the inhibition spectra obtained with Glu or ACC, which are precursors for ethylene biosynthesis. All experimental data were collectively evaluated to discuss the dependence upon ethylene of PAL induction in rice leaves infected with blast fungus.

MATERIALS AND METHODS

Chemicals. TR was obtained from Fisher Scientific Co., Fair Lawn, NJ. AOA, ABA, CY, ACC and α -KGA were obtained from Sigma Chemical Co., St. Louis, MO. Phenylalanine and Glu were obtained from Wako Pure Chemical Industries Ltd., Tokyo, and ETP was a product of Nissan Chemical Industries Ltd., Tokyo.

Blast fungus races and preparation of conidia. Blast fungus (*Pyricularia oryzae* CAVARA) H-373 (race No. 007) and TH 68-85 (race No. 031) were used as previously described.⁹⁾ The conidia were also prepared as previously described.⁹⁾

Rice seedlings and their inoculation with blast fungus conidia or stimulation with RIF. The rice plant used (cultivar: Jukkoku), its genetic characteristics, the culture conditions and the inoculation with blast fungus conidia or stimulation with RIF were the same as previously described.⁹⁾ The rice leaves used for preparing the PAL source were the 4th leaves, which were completely developed.

Preparation of PAL source. One hundred mg of press-injured 4th leaves that had been treated with chemicals and/or inoculated were cut into pieces and homogenized in a Biotron (BT 10/20 ~ 3500, Switzerland) in 3 ml of 0.1 M potassium dihydrogen phosphate buffer at pH 6.5 and below 5°C containing 20 mM dithiothreitol, 2% (v/v) glycerol and 0.01% Triton X-100. The homogenate was centrifuged at 23,000 $\times g$ and 0°C for 20 min. The supernatant obtained was used for the crude enzyme source of PAL.¹⁰⁾

Determination of PAL activity. The reaction mixture contained 20 μ mol phenylalanine in 100 μ mol sodium borate buffer at pH 8.8 to a total volume of 3.9 ml.¹⁰⁾ The reaction was started at 40°C by adding 0.1 ml of the crude enzyme source already described. The reaction was continued for 5 to 10 min within a 5% decrease of phenylalanine. The reaction was stopped by the addition of 0.5 ml of 5 N HCl. The absorbance at 225 nm and 270 nm was determined with a Shimadzu UV-260 recording spectrophotometer in conjunction with a Shimadzu HCC-260 temperature-controlled cell holder and MC-303 multistirrer. For the blank run, a reaction mixture without phenylalanine was used at the reference position after adding 0.5 ml of 5 N HCl. Using the absorption coefficients of phenylalanine (0.0824 mm⁻¹ cm⁻¹ at 255 nm and 0.0129 mm⁻¹ cm⁻¹ at 270 nm) and of *trans*-cinnamic acid (0.0057 mm⁻¹ cm⁻¹ at 255 nm and 0.0079 mm⁻¹ cm⁻¹ at 270 nm), the concentration of phenylalanine or *trans*-cinnamic acid was calculated by the usual simultaneous equations.

Approximation of t_m (time to the maximum rate of rice leaf PAL induction after inoculation or stimulation). As the induction of PAL progressed along a sigmoidal curve for the time axis, the time for the maximum rate of induction coincided with the midpoint of the sigmoidal curve. The midpoint of a given sigmoidal curve was mathematically approximated as previously described.¹¹⁾

RESULTS

Induction of rice leaf PAL with ETP and the Scatchard plot for ligand binding

On a 4th rice leaf that had just developed, press-injured spots (2 mm in diameter) were made in 2 progressions along both sides of the leaf central rib. One progression had 12 press-injured spots at intervals of 1 cm. Five μ l of ETP aqueous solution was applied to each press-injured spot, and the time-course determination of the induction of rice leaf PAL was started.

The results are summarized in Fig. 1. The approximated t_m values were 11.6 hr for 50 ppm, 6.6 hr for 100 ppm, 7.5 hr for 250 ppm and 8.3 hr for 500 ppm. These results indicate that exogenous ethylene induced rice leaf PAL. From classic Michaelis-Menten types of

FIG. 1. Dynamic Behavior of Rice Leaf PAL Induction with ETP.

Ordinate: rate of PAL activity.

Abscissa: time after the press-injured application of ETP. ○—○, application of 50 ppm (0.35 mm) ETP, t_m approximated to 11.6 hr as indicated with the dotted vertical line; △—△, application of 100 ppm (0.69 mm) ETP, t_m approximated to 6.6 hr; □—□, application of 250 ppm (1.74 mm) ETP, t_m approximated to 6.9 hr; ●—●, application of 500 ppm (3.46 mm) ETP, t_m approximated to 8.3 hr.

PAL activity in the merely press-injured run was subtracted from the corresponding runs as a blank.

plots of the application of a lower concentration of ETP than 50 ppm (0.35 mm), the repeated preliminary experiments indicated that the application of a concentration lower than 16.2 ppm (0.11 mm) did not induce PAL. After subtracting 0.11 mm from exogenously applied concentrations of ETP, an effective dose, $[\text{He}]_{\text{ef}}$, was obtained, and the relationship between the initial rate of PAL activity at t_m , V_{PAL} on the abscissa, and the ratio of $V_{\text{PAL}}/[\text{He}]_{\text{ef}}$ on the ordinate were plotted to obtain a Scatchard plot¹²⁾ for ligand binding. The result is shown in Fig. 2. The Scatchard plot for ligand binding gave a linear relationship in a specific range of ETP concentration. The result indicates that the induction of rice leaf PAL activity by the application of ETP was a phenomenon of "saturation" in a specific range of ETP concentration, like the formation of an enzyme-substrate complex. The apparent dissociation constant (K_s) calculated was 2.35×10^{-7} M, when the putative binding sites had been tentatively set at 1 binding site per functional proteinic unit.

Induction of rice leaf PAL with Glu or ACC and the inhibition experiment with TR, AOA and ABA

With similar press-injured application to

FIG. 2. Scatchard Plot for Ligand Binding between $[\text{He}]_{\text{ef}}$ and V_{PAL} at t_m .

Ordinate: $V_{\text{PAL}}/[\text{He}]_{\text{ef}}$, where V_{PAL} was the initial rate of PAL activity at t_m and $[\text{He}]_{\text{ef}}$ was the effective dose of ETP. Abscissa: V_{PAL} .

$[\text{He}]_{\text{ef}}$ of ETP was 0.06, 0.13, 0.17, 0.24, 0.33, 0.58, 1.62, 2.25 or 3.35 mm from the left-hand side.

that for ETP already described, it was observed that the application of a 5 μ l solution containing 1 mM Glu induced rice leaf PAL activity, and that the application of a 5 μ l solution containing 1 mM ACC also induced rice leaf PAL activity. In the rice leaf system

FIG. 3. Dynamic Behavior of Rice Leaf PAL Induction with Glu or ACC.

Ordinate: rate of PAL activity.

Abscissa: time after the application of a 5 μ l solution of 1 mM Glu or ACC on a press-injured spot (2 mm in diameter).

The application procedure was similar to that for ETP, see the text. The dotted vertical lines show the individual midpoint approximated for the sigmoidal progress of PAL induction.

□—□, Glu; ○—○, ACC; ■—■ α -KGA.

PAL activity in the merely press-injured run was subtracted from the corresponding runs as a blank.

TABLE I. INHIBITORY EFFECT OF TR, AOA AND ABA ON THE INDUCTION OF RICE LEAF PAL WITH PRESS-INJURED APPLICATION OF Glu OR ACC

Substrate for PAL induction	Inhibition % at t_m		
	TR	AOA	ABA
Glu	83.0	33.6	0
ACC	97.0	32.5	36.0

Application procedure for the inhibitors: (1) 5 μ l of 500 ppm TR, 500 ppm AOA or 50 ppm ABA was respectively mounted on the press-injured spots (2 mm in diameter), (2) and after 4 hr standing, 5 μ l of 1 mM Glu or ACC containing TR (100 ppm), AOA (100 ppm) or ABA (50 ppm) was mounted, and (3) the homogenization medium also contained TR (100 ppm), AOA (100 ppm) or ABA (50 ppm).

used for this study, the application of a similar concentration of α -KGA did not induce rice leaf PAL, these results being shown in Fig. 3. The approximated t_m for the induction of PAL with Glu was 7.5 hr, and that for the induction of PAL with ACC was 6.6 hr.

When 5 μ l of a solution of TR (500 ppm), AOA (500 ppm) or ABA (50 ppm) was respectively mounted on the press-injured spots (2 mm in diameter), and after 4 hr of standing, 5 μ l of a 1 mM Glu or ACC solution containing TR (100 ppm), AOA (100 ppm) or ABA (50 ppm) was applied with a microsyringe. The homogenization medium also contained TR (100 ppm), AOA (100 ppm) or ABA (50 ppm) to prevent a rebound effect, and the inhibition percentage given by each inhibitor is summarized in Table I. It is to be noted that (1) TR, a superoxide scavenger, markedly inhibited the induction of rice leaf PAL either with Glu or ACC, (2) ABA did not inhibit PAL induction with Glu, but weakly inhibited this with ACC, and (3) AOA weakly inhibited this either with Glu or ACC.

FIG. 4. Dynamic Behavior of Rice Leaf PAL Induced by Inoculating with an Incompatible or Compatible Blast Fungus Conidia or with the Stimulation by RIF.

Ordinate: rate of PAL activity.

Abscissa: time after press-injured inoculation or stimulation. Number of conidia in the inoculum: 2.0×10^5 conidia/ml water. Concentration of RIF applied: 10 mg of partially purified RIF/ml water.

△—△, incompatible inoculation; ▲—▲, compatible inoculation; ●—●, RIF stimulation; ○—○, merely press-injured.

FIG. 5. Inhibitory Effect of TR, AOA, ABA and CY on the Induction of Rice Leaf PAL with Press-injured Inoculation of an Incompatible or Compatible Blast Fungus Conidia or with Press-injured RIF Stimulation.

Ordinate: rate of PAL activity.

Abscissa: time after press-injured inoculation or stimulation.

A: $\triangle$ — $\triangle$, incompatible inoculation; B: $\blacktriangle$ — $\blacktriangle$, compatible inoculation; C: $\bullet$ — $\bullet$, RIF stimulation; $\blacktriangledown$ — $\blacktriangledown$, TR inhibition; $\circ$ — $\circ$, AOA inhibition; $\square$ — $\square$, ABA inhibition; $\blacksquare$ — $\blacksquare$, CY.

PAL activity in merely the press-injured run with or without an individual inhibitor was subtracted from the corresponding runs as a blank.

TABLE II. INHIBITORY EFFECT OF TR, AOA, ABA OR CY ON THE INDUCTION OF RICE LEAF PAL WITH PRESS-INJURED INOCULATION OF AN INCOMPATIBLE OR COMPATIBLE BLAST FUNGUS CONIDIA, OR WITH PRESS-INJURED RIF STIMULATION

Inoculation or stimulation	Inhibition % at t_m			
	TR	AOA	ABA	CY
Incompatible	69.8	35.7	0	NT*
Compatible	60.9	42.3	0	NT
RIF	52.5	38.9	0	100

* NT, not tested; the number of conidia in the inoculum and the concentration of RIF applied were same as those Fig. 4.

The application procedure for the inhibitors was the same as that in Table I; CY (20 ppm) was applied, after 4 hr standing, CY (10 ppm) was mixed with RIF solution and applied. The homogenization medium also contained CY (10 ppm).

TABLE III. INHIBITORY EFFECT OF TR, AOA AND ABA ON RICE LEAF PAL INDUCED WITH PRESS-INJURED RIF STIMULATION

Inhibitor	Concentration (ppm in reaction mixture)	Inhibition % (with initial velocity)
TR	100	6.9
	200	—3.5
AOA	100	—6.7
	200	4.6
ABA	10	—3.9
	20	6.2
CY	5	—3.9
	10	6.2

PAL induction in the merely press-injured run with or without an individual inhibitor was subtracted from the corresponded runs.

Induction of rice leaf PAL by inoculating with an incompatible or compatible blast fungus conidia, by stimulating with RIF, and by the inhibition experiments with TR, AOA, ABA and CY.

With similar press-injured application to that for ETP already described, it was observed that the inoculation with a 5 μ l aqueous suspension of an incompatible or compatible

blast fungus conidia, or stimulation with RIF induced the rice leaf PAL. These results are shown in Fig. 4. The approximated t_m for the induction of PAL with incompatible inoculation was 2.4 hr, that with compatible inoculation was 5.3 hr, and that with RIF stimulation was 11.0 hr. This orderliness in the incompatible and compatible inoculations and the RIF stimulation coincided with rice leaf

peroxidase induction,¹³⁾ but the induction of rice leaf PAL was generally determined to be an earlier phenomenon when compared with the induction of peroxidase. The orderliness within the incompatible and compatible inoculation also coincided with superoxide generation (incompatible t_m , 0.5 hr; compatible t_m , 2.9 hr).⁹⁾

The inhibitory effects of TR, AOA, ABA and CY on the induction of rice leaf PAL with press-injured inoculation of incompatible or compatible blast fungus conidia, and with press-injured RIF stimulation were determined with a similar procedure to that already described, the results being summarized in Table II and Fig. 5. It is to be noted that the inhibition spectra comprising TR, AOA and ABA were quite similar to that for rice leaf PAL induction with Glu that has already been described, and that PAL induction with RIF stimulation was completely inhibited with CY when 20 ppm of CY was applied 4 hr before stimulation, and when 10 ppm was applied with RIF stimulation. The latter indicates that PAL induction was operated and regulated by *de novo* synthesis. The pretreatment of rice seedlings with TR, AOA or ABA did not interfere with the infection of blast fungus.

Direct inhibitory effects of TR, AOA and ABA on rice leaf PAL activity

The direct inhibitory effects of TR, AOA, ABA and CY on the activity of rice leaf PAL was determined near the maximum rate of PAL activity, when rice leaf PAL was induced with press-injured RIF stimulation. The results are summarized in Table III, and indicate that the enzymic activity of PAL itself was inhibited neither by TR, AOA, ABA nor CY. This revealed that TR, AOA, ABA and CY inhibited the process of rice leaf PAL induction. The concentration of individual inhibitor in the reaction mixture, which was introduced with the PAL source (Tables I and II), was estimated as being a few ppm, because of extensive dilution of the PAL source.

DISCUSSION

Since Stahmann *et al.*⁴⁾ proposed an attractive hypothesis for the induction of resistant reactions in sweet potato tuberous root against black rot disease (causal organism, *Ceratocystis fimbriata*), in which ethylene may have been the trigger to result in the induction of resistant reactions that inhibited further spread of the pathogen, there have been many reports describing that ethylene might participate in the induction of resistant reactions.^{14~17)} On the contrary, there are opposing opinions that ethylene may merely relate to the formation of disease symptoms, and that ethylene may rather relate to the establishment of infections.^{18~22)} Researches in this respect on the rice plant have been originated by an unexpected finding that the efficacy of probenazol, a non-fungicidal protectant, against rice blast is clearly counteracted by pretreating with ABA.²³⁾ At that time, Yoshii and Imaseki⁸⁾ have already demonstrated that ABA clearly counteracted the induction of ACC synthase (pyridoxal enzyme) activity with IAA. Matsumoto *et al.*²⁴⁾ and Iwata *et al.*²⁵⁾ have further demonstrated that the suppression of rice blast incidence by IAA or ethephon was strongly counteracted by the application of ABA prior to inoculation, and that the negative correlation coefficient between peroxidase induction and the decrease of blast incidence was -82% .

In the present report, it was first experimentally demonstrated that the induction of rice leaf PAL activity clearly depended on exogenous ETP, an ethylene generating chemical, indicating linear relationships in the Scatchard plot for ligand binding in a specific range of exogenous ETP concentration (Figs. 1 and 2). It was also demonstrated that rice leaf PAL induction was accomplished with exogenous Glu or ACC but not with α -KGA (Fig. 3). Originally, the glutamate pathway for ethylene biosynthesis was operable within such microorganisms as *Penicillium digitatum*,²⁶⁾ *Pseudomonas syringae* pv. *phaseolicola*²⁷⁾ and *Pseudomonas syringae* pv.

glycinea,²⁸⁾ but recently, Hyodo and Uritani²⁹⁾ have suggested that the pathway for ethylene biosynthesis in infected sweet potato root tissue may differ from the methionine pathway in which ACC serves as an intermediate in the adjacent healthy tissue; they emphasized that the possibility of a glutamate pathway in the infected tissue should be investigated by future study. In the present study, the inhibition spectrum obtained by the application of TR, AOA and ABA on the induction of rice leaf PAL with Glu was different from that obtained with ACC, and the induction of PAL with Glu was not inhibited by the application of ABA (Table I). It seems likely that about 36% of the PAL induction with ACC might depend on the inductive formation of ACC synthase (Table I and Fig. 3). The inhibition spectra obtained by the application of TR, AOA and ABA on the induction of rice leaf PAL activity with incompatible or compatible inoculation or with RIF stimulation were quite similar to that with Glu (Figs. 4, 5 and Table II). The determination of t_m for rice leaf PAL induction with an incompatible or compatible fungus race, or with RIF stimulation, indicated that PAL induction was quite an early phenomenon when compared with those t_m values for rice leaf peroxidase induction.¹³⁾ These observations bear out the suggestion that the glutamate pathway may operate for the initiation reaction of endogenous ethylene generation^{29~31)} in infected rice leaf tissue. On the other hand, the marked inhibition of rice leaf PAL induction with TR indicated the involvement of a superoxide anion and/or the derived active oxygen species in the ethylene biosynthesis both with the glutamate and methionine pathway^{5,6,9,13,32)} (Tables I, II, III and Fig. 5). The explanation for weak inhibition given by the application of AOA on PAL induction with Glu is uncertain at present, but it might be related to aminotransferases (pyridoxal enzymes) partially concerned with the conversion of α -KGA to Glu. It is generally accepted that (1) both PAL and peroxidase play indispensable roles in operat-

ing the hydroxycinnamate pathway leading to lignin for the formation of a physical barrier against phytopathogenes, and (2) PAL functions to feed cinnamate to the pathway and peroxidase functions to polymerize oxidatively the resultant hydroxycinnamates.³⁰⁾ The ABA sensitive characteristics for the induction of rice leaf peroxidase will be reported relevant to the operation of a hydroxycinnamate pathway in the forthcoming paper.

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