Plant Physiol. (1991) 97, 94–98
0032-0889/91/97/0094/05/$01.00/0

Received for publication November 27, 1990
Accepted April 22, 1991

A Lipoxygenase Pathway Is Activated in Rice after Infection with the Rice Blast Fungus *Magnaporthe grisea*

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ABSTRACT

Lipoxygenase (LOX) and lipid hydroperoxide-decomposing activity (LHDA) markedly increased in the fifth leaves of rice (*Oryza sativa* cv Aichiasahi) after infection with the rice blast fungus, *Magnaporthe grisea.* The increases in the enzyme activities were significantly higher in response to infection with an incompatible strain (race 131) compared with infection with a compatible strain (race 007) of the fungus. Using ion-exchange chromatography, we isolated three LOX activities (leaf LOX-1, -2, -3) from both uninoculated and infected leaves. The activity of leaf LOX-3, in particular, increased in the incompatible race-infected leaves. The leaf LOX-3 had a pH optimum of 5.0 and produced preferentially 13-L-hydroperoxy-9,11 (*Z,E*)-octadecadienoic acid (13-HPODD) from linoleic acid. 13-HPODD and 13-L-hydroxy-9,11 (*Z,E*)-octadecadienoic acid, one of the reaction products from 13-HPODD by LHDA, were highly inhibitory to the germination of conidia of the fungus. The present study provides correlative evidence for important roles of LOX and LHDA in the resistance response of rice against the blast fungus.

Rice (*Oryza sativa*) blast, caused by *Magnaporthe grisea,* is one of the most destructive rice diseases. Many studies have been concerned with resistance mechanisms of rice to the blast fungus, and, thus, several antifungal substances have been isolated from rice leaves (1, 4, 9–11, 14). However, the biosynthetic mechanisms of these substances in fungal-infected leaves have not been established. Therefore, it is not known whether the antifungal substances isolated from rice leaves are actually involved in the defense response of rice against the fungus.

We recently found an activity that decomposed lipid hydroperoxides (LHDA[5]) in rice seeds (17). Lipid hydroperoxides are produced from unsaturated fatty acids like linoleic and linolenic acid by LOX (EC 1.13.11.12), which is a ubiquitous enzyme in higher plants (6). In rice seeds, three LOXs were found by Ida *et al.* (8). LOX-1 and LOX-2 produce 9- and 13-HPODD in equal amounts, and LOX-3 produces preferentially 9-HPODD when linoleic acid is the substrate. LHDA subsequently catalyzes the production of antifungal monohydroxy and trihydroxy fatty acids from both 9- and 13-HPODD (17). Although physiological roles of the hydroxy fatty acids in rice seeds are still unknown, the results indicate that antifungal fatty acids are synthesized via fatty acid hydroperoxides in rice seeds. Kato *et al.* (10, 11) reported the isolation of the monohydroxy and trihydroxy fatty acids as antifungal substances from rice leaves. Thus, it is expected that both LOX and LHDA are associated with the resistance response in rice seeds and leaves to rice blast disease, if these enzymes are present in fungus-infected rice leaves. However, the biosynthetic pathway of the antifungal fatty acids has not been investigated in uninoculated or fungus-infected rice leaves. Here we report the presence of LOX and LHDA in a rice cultivar infected with *M. grisea,* rapid increase in the activities in the leaves after inoculation with an incompatible race of the blast fungus, and the partial characterization of the activities.

MATERIALS AND METHODS

Chemicals

DEAE-Toyopearl M-650 was obtained from Toyo Soda Co. (Tokyo). Sephadex G-25 was from Pharmacia Fine Chemicals (Uppsala, Sweden). Linoleic acid, linolenic acid, γ-linolenic acid, and their methyl esters were from Sigma Chemical Co. Soybean LOX was from Serva (Heidelberg).

Plant Materials

Rice (*Oryza sativa* cv Aichiasahi), at the fifth leaf stage, was inoculated with either the incompatible race 131 or the

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[5] Abbreviations: LHDA, lipid hydroperoxide-decomposing activity; 9-HODD, 9-D-hydroxy-10,12(*E,Z*)-octadecadienoic acid; 9-HPODD, 9-D-hydroperoxy-10,12(*E,Z*)-octadecadienoic acid; 13-HODD, 13-L-hydroxy-9,11(*Z,E*)-octadecadienoic acid; 13-HPODD, 13-L-hydroperoxy-9,11(*Z,E*)-octadecadienoic acid; LOX, lipoxygenase.

compatible race 007 of *Magnaporthe grisea,* the rice blast fungus. The rice seedlings and conidial suspension of the fungus were prepared as described by Peng and Shishiyama (18). The inoculated rice seedlings were kept in an incubator at 25 ± 2°C under 250 to 300 $\mu E/m^2 \cdot s$ fluorescent light with a 12-h photoperiod and 100% relative humidity. The top leaves were collected for the extraction of enzymes at prescribed intervals after inoculation.

Assay of LOX and LHD Activities

LOX activity was assayed with a Hansatech oxygen electrode unit as described in Ohta *et al.* (15). The reaction mixtures (1 mL) contained 7.5 mM linoleic acid and 0.1% (w/v) Tween 20 in 0.1 M sodium acetate buffer, pH 6.0. One unit of LOX was defined as the amount of the enzyme that causes the disappearance of 1 μmol O_2 min^{-1} at 30°C.

LHDA was measured as the decrease in the amount of conjugated dienes determined by spectrophotometry at 234 nm with 13-HPODD as substrate, according to the conditions described previously (17). 13-HPODD was prepared by the action of commercial soybean LOX on linoleic acid (17). One unit of LHDA was expressed as the amount that causes disappearance of 1 μmol of HPODD min^{-1} at 25°C.

Extraction of LOX and LHD Activities

The infected rice leaves (2 g) were macerated with a mortar and pestle, and the enzymes were extracted at 4°C for 1 h in 10 mL of 0.1 M sodium phosphate buffer, pH 6.5, containing 1% Tween 20. The slurry was centrifuged at 13,000*g* for 15 min at 4°C, and the supernatant was used for the assay of LOX and LHDA.

Separation of LOX Components

LOXs in rice leaves were separated by the procedures described for LOXs in germinating seedlings (15). Thus, the crude extracts of rice leaves were passed through a column of Sephadex G-25 equilibrated with 25 mM Tris-HCl buffer, pH 7.5, containing 10% (v/v) glycerol (buffer A). The enzyme extract was then loaded on a column of DEAE-Toyopearl (1 cm i.d. × 13 cm) equilibrated with buffer A at a flow rate of 130 $mL/cm^2 \cdot h$. The column was washed with buffer A and then eluted with a 100-mL linear gradient of 0.0 to 0.3 M NaCl in buffer A. The fractions with LOX activity were pooled. The separation steps were performed at 4°C. Protein was determined by the dye binding assay of Bradford (3).

Identification of the Reaction Products

The reaction products of LOX with linoleic acid were identified by HPLC as follows. Approximately one unit of the enzyme from extracts of the race 131-infected leaves after DEAE-Toyopearl chromatography was incubated with 5 mL of 2 mM linoleate solution that contained 0.1% Tween 20 in 0.2 M sodium acetate, pH 6.0, at 30°C for 30 min. The reaction products were extracted by Bligh and Dyer's method (2) and fractionated with a Shimadzu LC-5A system on a YMC A-012 SIL column (6 mm i.d. × 150 mm; Yamamura Chemical Laboratories Co, Kyoto, Japan) eluted with a solvent system of *n*-hexane with 2.5% (v/v) isopropanol and 0.2% (v/v) acetic acid at a flow rate of 1 mL/min. The area under each peak was determined with a Shimadzu Integrator Chromatopac C-R3A.

Bioassay of Antifungal Activity

Antifungal activities of 13-HPODD and 13-HODD were tested against *M. grisea* race 131 as reported (17). For each treatment, three replicates were examined. Inhibitory effects of the fatty acids are expressed as percentages relative to control values.

RESULTS

Induction of LOX Activity in Fungus-Infected Rice Leaves

LOX activity rapidly increased in the leaves infected with the incompatible race 131, beginning 24 to 36 h after inoculation, as compared with LOX activity in the healthy leaves (Fig. 1). Although the activity increased in the leaves infected with the compatible race 007, the increase in the leaves of the incompatible interaction was much greater than that in the leaves of the compatible interaction.

LOX Components in Fungus-Infected Rice Leaves

We isolated the three LOXs (leaf LOX-1, -2, -3) from both race 131-infected and uninoculated leaves by DEAE-Toyopearl chromatography (Fig. 2). The results indicated that the increase in LOX activity was attributed mainly to the most acidic component, designated as leaf LOX-3. The activity of the leaf LOX-3 was 10 times higher than that in the healthy

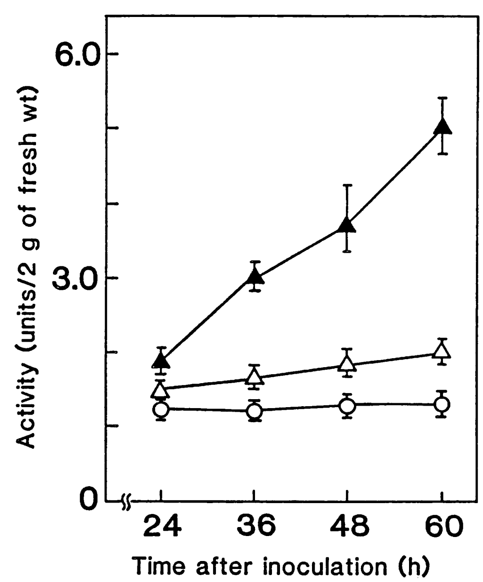

Figure 1. Changes in LOX activities after inoculation. The top leaves of rice seedlings at the fifth leaf stage were collected at indicated times after inoculation. LOX activities were measured as described in "Materials and Methods." Each value is expressed as the average of three independent experiments. SD was expressed as ± value. —○—, healthy leaves; —▲—, race 131-infected leaves (incompatible); —△—, race 007-infected leaves (compatible).

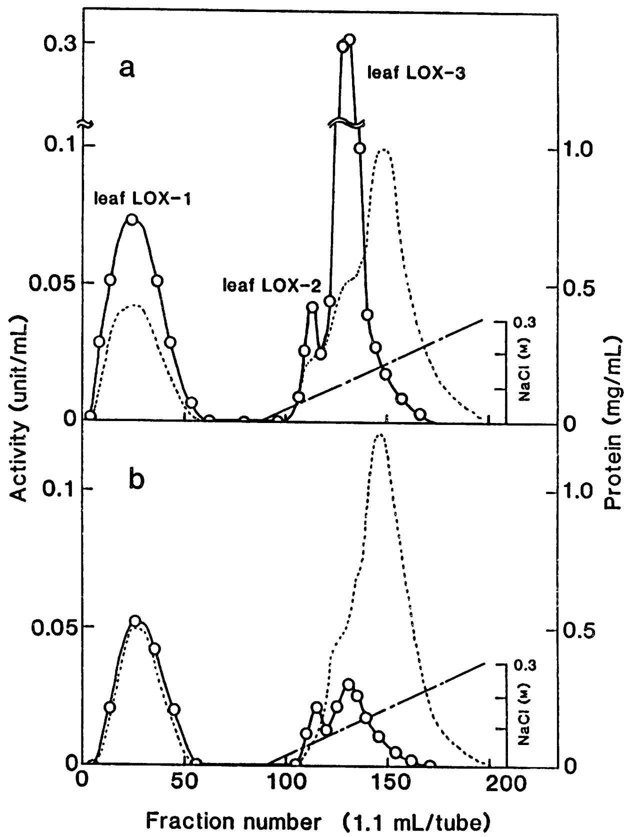

Figure 2. Elution profiles of LOX components from rice leaves on a DEAE-Toyopearl column. Rice leaves (8.9 g) were macerated with a mortar and pestle, and the enzymes were extracted at 4°C for 1 h in 45 mL of 0.1 M sodium phosphate buffer, pH 6.5, containing 1% Tween 20 at 4°C for 1 h. The slurry was centrifuged at 13,000*g* for 15 min at 4°C, and the supernatant was used for the separation of LOX components as described in "Materials and Methods."—, LOX activity; - - - -, protein. a, race 131-infected leaves; b, healthy leaves.

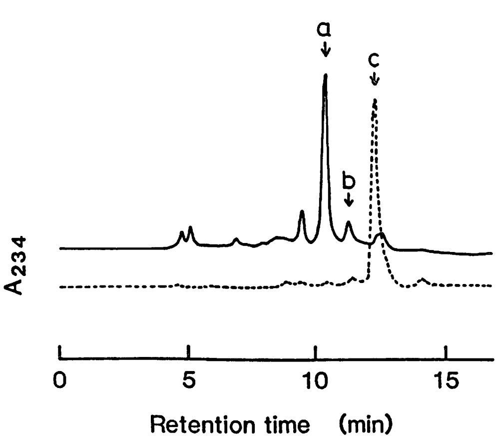

Figure 3. HPLC analysis of reaction products of leaf LOX-3 with linoleic acid as a substrate. a, 13-(*Z,E*)-HPODD; b, 13-(*E,E*)-HPODD; c, 9-(*E,Z*)- and 9-(*E,E*)-HPODD. —, leaf LOX-3; - - -, seed LOX-3.

Table I. *Positional Specificity of Leaf LOX-3*

Products of the reaction of leaf LOX-3 with linoleic acid separated by HPLC as described in "Materials and Methods" and quantified by integration of peak areas.

	Proportion of Product as		
	13-(*Z,E*)-HPODD	13-(*E,E*)-HPODD	9-(*E,Z*)- and 9-(*E,E*)-HPODD
		%	
Leaf LOX-3	71.0	14.3	14.7
Seed LOX-3[a]	0.4	1.5	98.1

[a] Ohta *et al.* (16).

leaves after inoculation with the incompatible fungus. Activities of the other two LOXs (leaf LOX-1, leaf LOX-2) also increased slightly.

Characterization of the Induced LOX

The leaf LOX-3 produced preferentially 13-HPODD from linoleic acid (Fig. 3, Table I) in contrast with the LOX-3 derived from seed (16). Most LOXs from plants have pH optima between pH 5.5 and 7.0 (6). However, the leaf LOX-3 showed a more acidic pH optimum around pH 5.0 (Fig. 4). Other leaf LOXs (leaf LOX-1, -2) also showed pH optima around pH 5, indicating that LOX components in leaves differed from LOXs in seeds or germinating seedlings (8, 15). In contrast with the other rice LOXs reported (8, 15, 16), the leaf LOX-3 was active against some unsaturated fatty acids and their methyl esters in addition to linoleic acid (Table II).

Induction of LHD Activity in Fungus-Infected Rice Leaves

LHDA also markedly increased in the incompatible race-infected leaves, in agreement with the increase in LOX activity

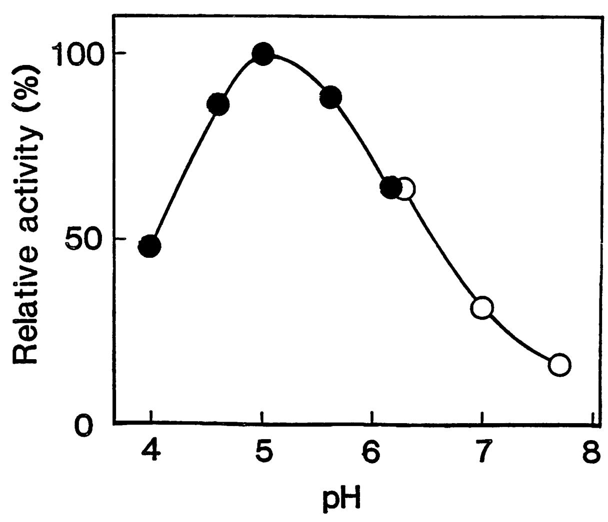

Figure 4. pH activity profile of leaf LOX-3. The activity of the leaf LOX-3 was measured in each pH region. The activity was expressed relative to the maximum. The buffers used (ionic strength = 1.2) were: 0.1 M sodium acetate for pH 4.0 to 6.0 (●); 0.1 M sodium phosphate for pH 6.0 to 8.0 (○).

Table II. *Substrate Specificity of Leaf LOX-3*

LOX activity was quantified by oxygen consumption measured with an oxygen electrode unit as described in "Materials and Methods." Each activity is expressed as percentage relative to the activity for linoleic acid as substrate.

Substrate (7.5 mM)	Relative Activity
	%
Linoleic acid	100
Linolenic acid	77
γ-Linolenic acid	54
Methyl linoleate	20
Methyl linolenate	12

(Fig. 5). In contrast, LHDA in leaves inoculated with the compatible race increased only slightly.

Antifungal Activities of Hydroperoxy and Hydroxy Fatty Acids

We examined the antifungal activities of 13-HPODD and 13-HODD, the major products of the LOX pathway in fungus-infected leaves, against race 131. Table III shows that both 13-HPODD and 13-HODD inhibited conidial germination of the fungus at the concentration of 50 μg/mL. In the rare exceptions where a few spores germinated in 13-HPODD, germ tube elongation was substantially inhibited. Appressoria were not formed from germ tubes of spores incubated in either fatty acid.

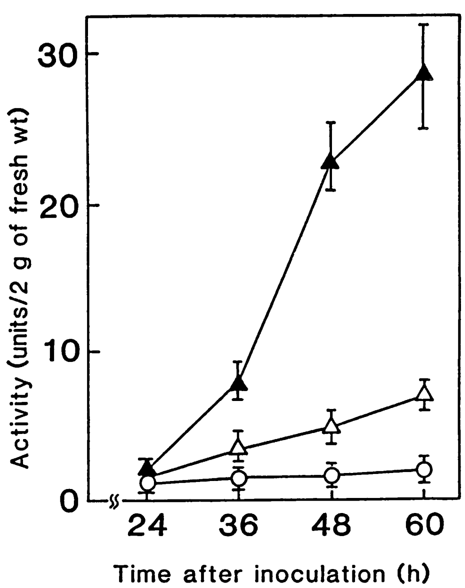

Figure 5. Change in LHDA after inoculation. The top leaves of rice seedlings at the fifth leaf stage were collected at the indicated times after inoculation, and then the decomposing activities for 13-HPODD were measured as described in "Materials and Methods." Each value was expressed as the average of three independent experiments. SD was expressed as ± value. —○—, healthy leaves; —▲—, race 131-infected leaves; —△—, race 007-infected leaves.

Table III. *Antifungal Activities of 13-HPODD and 13-HODD (50 μg/mL) against M. grisea*[a]

	Inhibition[b]	
	13-HPODD	13-HODD
	%	
Conidial germination	98	100
Germ tube growth	98	100
Appressorium formation	—[c]	—[c]

[a] Race 131 was used as the test fungus. [b] A suspension of conidia in 10% ethanol was used as a control. [c] No formation of appressoria.

DISCUSSION

This study showed that LOX and LHD activities in leaves of rice cv Aichiasahi were rapidly activated by infection with the incompatible race 131 but not with compatible race 007 of the rice blast *M. grisea*. The time when the activities were markedly induced (36 h after inoculation) was coincident with the growth inhibition of infection hyphae in the incompatible combination (18). Furthermore, *in vitro* products of both LOX and LHDA from race 131-infected leaves strongly inhibited conidial germination of the fungus. These results indicated that the rapid increases in LOX and LHDA are closely correlated with the resistance response of rice cultivars to infection by the blast fungus.

Our previous work showed that an activity that decomposed lipid hydroperoxides (LHDA) was present in rice seeds (17). The results indicated that the activity converted 13-HPODD (9-HPODD) to 13-HODD (9-HODD) and 9,10,13-trihydroxyoctadec-11-enoic acid (9,12,13-trihydroxyoctadec-10-enoic acid), which had antifungal activity against the rice blast fungus (10, 11, 17). We observed that LHDA in rice leaves also produced the hydroxy fatty acids, but it is possible that other enzymes that convert lipid hydroperoxides to their special products are present in the fungus-infected leaves. When linoleic acid was used as substrate, 13-HPODD, from which 13-HODD was generated by LHDA, was a major product of leaf LOX-3 predominantly induced in the fungus-infected leaves. The lack of substrate specificity by leaf LOX-3 suggests that other lipid hydroperoxides and their hydroxides, particularly those from linolenic acid, may be produced in infected leaves (see Table II).

Separation of LOX components clearly demonstrated that leaf LOX-3, in particular, was activated upon infection by the incompatible race of the fungus. Substrate specificity, positional specificity of oxygenation, and pH optimum of the enzyme were quite different from other rice LOXs reported (8, 15). Specific induction of LOX components is of interest, not only to elucidate regulation mechanism of the resistance expression to the blast fungus, but also to determine the physiological significance of the presence of multiple LOXs in many plants. We have cloned a cDNA of LOX-2 in germinating seedlings (unpublished data) and are screening a leaf LOX-3 clone to clarify the dynamic behavior of gene expression influenced by fungus-infection.

In spite of the widespread distribution of LOX in plants, the physiological roles of the enzyme have not been extensively evaluated (7). Some recent studies showed that LOX activity increased in resistant tissues in several host-pathogen combinations (*e.g.* oats/*Puccinia coronata avenae* (20), *Cucumis sativa*/*Pseudomonas syringae* pv *pisi* (12, 13), *Phaseolus vulgaris* (L.)/*P. syringae* pv *phaseolicola* (5), *et al.*). Preisig and Kuć (19) suggested that LOX plays a role in the hypersensitive reaction of potato to *Phytophthora infestans* induced by arachidonic acid. The present study provides correlative evidence for an important role of LOX in plant disease resistance.

ACKNOWLEDGMENTS

We are indebted to Dr. R. Yoshino and H. Koga, Hokuriku National Agricultural Experiment Station, Joetsu, 943-01 Japan, for supplying the rice cultivars and the pathogen isolates. We also thank Dr. D. Shibata for a critical review of the manuscript.

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