重金属イオンによるイネ葉におけるフィトアレキシン類の誘導

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Note

Induction of Phytoalexins with Heavy Metal Ions in Rice Leaves

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Abstract: Droplets of heavy metal salts (1 mM) were applied to holes punctured by a glass capillary tube on the surfaces of detached rice leaves, and the accumulation of phytoalexins in tissues surrounding the holes and in droplets were quantitated with gas chromatography-mass spectrometry-selected ion monitoring (GC-MS-SIM). This analytical method was highly sensitive and accurate, minimizing material and time requirements. Copper ion treatment induced two diterpenoid phytoalexin groups of rice plants, *i.e.*, oryzalexins A, B, C, and D, and momilactones A and B. These phytoalexins differed in diffusion rates from leaves into droplets. Among the momilactones, momilactone A showed a marked induction. Among the oryzalexins, oryzalexin B was induced to the greatest extent. The accumulation of momilactone A was first noted 12 hr after application of copper ion, reaching maximum after 72 hr. As these phytoalexins accumulated brown spots appeared in areas surrounding the punctured holes. Iron and mercury ions made up approximately 37% and 20% of the elicitor activity of copper ion, respectively. Manganese and cobalt ions hardly showed any elicitor activity.

INTRODUCTION

The accumulation of phytoalexins in response to microbial invasion is considered one of the defense mechanisms in higher plants.[1] Compounds presently known as phytoalexins of rice plants include oryzalexins A, B, C, D, and momilactones A and B.[2-7]

The production of phytoalexins is triggered not only by microbial invasion, but also by various substances, such as cell walls isolated from various fungi, heavy metals, detergents and ultraviolet light.[8]

Elicitors of rice phytoalexins have not previously been studied because of a difficulty in simultaneous determination of trace amounts of different rice phytoalexins. This limitation, however, has been overcome with the use of gas chromatography-mass spectrometry-selected ion monitoring (GC-MS-SIM), which enables to determine picogram quantities of phytoalexins in rice leaves.

The present paper describes the elicitor activity of several heavy metals, including copper, mercury, iron, cobalt and manganese ions, elucidated with GC-MS-SIM.

MATERIALS AND METHODS

1. *Plant Material*

Rice plants (*Oryza sativa* L. Nihonbare) were cultivated in a greenhouse. At the 6th leaf stage, 5th stage leaves were detached and used in an assay of elicitor activity.

2. *Chemicals*

Oryzalexins A, B and C were synthesized by Drs. K. Mori and M. Waku (The University of Tokyo).[9] Oryzalexin D was synthesized from 3-hydroxy-(+)-sandaracopimaradiene according to the method described in our previous paper.[6] Momilactone A was isolated from ultraviolet-irradiated rice leaves.[10] Momilactone B was kindly supplied by Dr. Y. Hayashi (Forestry and Forest Products Research Institute). Copper chloride, iron chloride, mercury chloride, manganese chloride and cobalt chloride were used for the measurement of elicitor activity of phytoalexin in rice leaves.

3. *Analysis with GC-MS-SIM*

Analyses were performed with a Shimadzu QP-1000 gas chromatograph-mass spectrometer. A bonded phase-fused silica capillary column (25 m × 0.2 mm, 0.33 μm film thickness, Hewlett-Packard) was inserted directly into the source of the mass spectrometer. Samples were injected by using the solventless technique into the column at 250°C. The column was then programmed to reach 300°C at a rate of 10°C/min and was held at 300°C for 3 min. The injection port and the ion source were maintained at 320 and 250°C, respectively. The electron beam energy of electron impact (EI) was 70 eV. Helium was used as the GC carrier gas at a flow rate of 1 ml/min.

4. Assay of Elicitor Activity

Droplets of elicitor solution (1 mM, 25 μl) were applied to holes punctured by a glass capillary tube (1 mm diam.) on the surface of a rice leaf. Treated leaves were kept in a plastic box at 30°C under high humidity. After an appropriate time, the droplets were collected with a micropipette and the leaf tissues surrounding the holes were excised with a corkborer (6 mm diam.). The excised leaf tissues were boiled in 70% methanol for 3 min, and the methanol extract was concentrated under nitrogen and partitioned three times with diethyl ether. The droplets were also partitioned with diethyl ether. Both diethyl ether layers were separately evaporated under nitrogen and dissolved in methanol to give a final volume of 1 ml per 10 holes, which was subjected to GC-MS. A sample for momilactone B was converted to its TMS derivative by incubating in 100 μl of *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) at 65°C for 30 min.

RESULTS

Table 1 shows the accumulation of rice phytoalexins induced by 1 mM copper chloride in the leaf tissues surrounding the holes and in the droplets. Copper ion elicited momilactone A to the greatest extent, giving a total quantity of 103.2 ng/hole. The accumulation of momilactone B induced by copper chloride was only 5.5 ng/hole. The diffusion rates of momilactones A and B from the leaf into the droplet were 30% and 47%, respectively. Among the oryzalexins, oryzalexin B was elicited most strongly, with the total accumulation of 40.4 ng/hole. The oryzalexins differed in diffusion rates; oryzalexin D diffused into droplets at a much higher rate than the other oryzalexins.

Table 1 Accumulation of rice phytoalexins in leaf tissues surrounding holes and in droplets after treatment with 1 mM $CuCl_2$.

Phytoalexin	Content (ng/hole)		Total content (ng/hole)	Diffusion rate (%)
	Leaf	Droplet		
Momilactone A	72.2	31.0	103.2	30
Momilactone B	2.9	2.6	5.5	47
Oryzalexin A	23.3	2.5	25.8	10
Oryzalexin B	33.5	6.9	40.4	17
Oryzalexin C	21.9	1.8	23.7	8
Oryzalexin D	15.0	4.9	19.9	25

The amounts of phytoalexins were measured 72 hr after application of copper solution. The diffusion rate is expressed as [(content of droplet)/(total content)] × 100 (%).

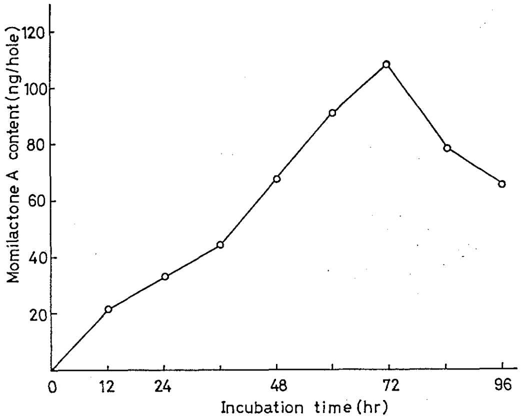

Fig. 1 Time course of momilactone A accumulation after application of 1 mM $CuCl_2$.

Momilactone A content was expressed as total content of momilactone A in both the leaf tissue around the hole and the droplet.

Table 2 Accumulation of momilactone A with several heavy metal ions.

Metal (1 mM)	Total content of momilactone A (ng/hole)
$CuCl_2$	103.2
$FeCl_2$	38.2
$HgCl_2$	20.4
$CoCl_2$	7.5
$MnCl_2$	6.9
Control (distilled water)	4.9

The amount of momilactone A was measured 72 hr after application of elicitor solution.

The time-course elicitor activity of copper ion for momilactone A is shown in Fig. 1. Momilactone A was first detected 12 hr after application, and the content increased in proportion to incubation time. Maximum accumulation was observed 3 days after application. Areas surrounding the punctured holes turned brown and the color got darker in relation to increase in momilactone content. Application of distilled water did not affect the color.

Several metal ions were examined for elicitor activity (Table 2). In addition to copper ion tested, iron and mercury ions also showed elicitor activity, while other tested metal ions, such as manganese and cobalt, hardly showed any elicitor activity.

DISCUSSION

Since copper ion strongly induced rice phytoalexins, it is considered to be a typical abiotic elicitor. Other phytoalexins such as wyerone, ipomeamarone, rishitin, pisatin, glyceollin, phaseollin and kievitone are also known to be produced by treatment with heavy metals.[8,11] Due to relatively low solubility in distilled water, the phytoalexins hardly diffused from leaves into droplet solution (Table 1), while pisatin from detached pea pods diffused into droplets at a rate of approximately 50%.[12] The drop-diffusate technique has been used for the detection of rice phytoalexins, but our result that diterpenoid phytoalexins hardly diffused into droplets suggests that the droplet technique cannot be the sole experimental source to detect diterpenoid phytoalexins. Rice phytoalexins differ in diffusion rates. Therefore, both droplets and leaf tissues around holes should be used as experimental materials for the determination of rice phytoalexins.

A minimum of 10 holes per leaf and 25 μl of elicitor solution per hole are sufficient to measure the content of rice phytoalexins by GS-MS-SIM. This method to analyze elicitor activity is highly sensitive and requires only a short period of time.

In the control experiment, application of distilled water resulted in the accumulation of approximately 5 ng of momilactone A in extracts. This was probably due to the holes punctured by a glass capillary tube, because low levels of phytoalexins are known to accumulate in plants responding to a wound.

The phytoalexin content gradually increased as brown spots around the holes increased, as so was in the case of ultraviolet-irradiated rice leaves. There is the correlation between the appearance of brown spots and the accumulation of phytoalexins in rice leaves.

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要約

重金属イオンによるイネ葉におけるフィトアレキシン類の誘導

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イネ葉片上にキャピラリーガラスを用いて穿孔し，数種の1 mM 重金属溶液を穿孔部に滴下後，滴下液および穿孔部周辺に集積するフィトアレキシン類をガスクロマトグラフ質量分析計を用いて分析した．本法は迅速・簡便に高感度でフィトアレキシンの定量が可能であり，その結果，銅イオンによりオリザレキシンA, B, C, Dおよびモミラクトン A, Bの生成を確認し，オリザレキシン類ではB，モミラクトン類ではAが最も多量に検出された．穿孔部周辺から滴下液へのフィトアレキシン類の移行率は8～47%であり，フィトアレキシンの種類によって著しく異なった．モミラクトンAの蓄積は銅溶液添加後，3日目で最大に達し以後減少し，フィトアレキシンの生成蓄積に伴って穿孔部周辺の褐変化が認められた．鉄および水銀イオンのエリシター活性は銅イオンの37%および20%を示したが，コバルトおよびマンガンイオンのエリシター活性はほとんど認められなかった．