A Toxin from the Tangerine Pathotype of *Alternaria alternata* That Has Selective Toxicity to Japanese Pear*

Yasuo ITOH**, Keisuke KOHMOTO**, Norihiro SHIMOMURA**†,
Hiroshi OTANI**, Motoichiro KODAMA** and Shinich NAKATSUKA***

Abstract

An isolate of *Alternaria alternata* from citrus was found to be pathogenic to both Japanese pear (cv. Nijisseiki) and Emperor mandarin. The fungus produced a toxin that is highly toxic to the pear and less toxic to the original host, Emperor mandarin. The toxin was isolated and was designated ACT-toxin IIb. ACT-toxin IIb caused vein necrosis and loss of electrolytes from leaves of cv. Nijisseiki at concentrations more than 1×10^{-7} M. The toxin caused vein necrosis at 4×10^{-5} M, and increased losses of electrolytes from leaves of the citrus Emperor mandarin at 10^{-5} M. The toxin did not affect leaves of Japanese pear cv. Chojuro and rough lemon at 10^{-4} M. Electron microscopy showed that ACT-toxin IIb caused invaginations of the plasma membrane in leaf cells of susceptible Japanese pear cv. Nijisseiki, but did not show such effect on resistant pear cv. Chojuro. These results indicate that the mode of action of ACT-toxin IIb is similar to that of ACT-toxin Ib and AK-toxin (from the Japanese pear pathotype of *A. alternata*). ACT-toxin IIb also induced susceptibility in Japanese pear cv. Nijisseiki to a non-pathogenic isolate of *A. alternata*. The finding that the pathotype of *A. alternata* from citrus also has host-selective pathogenicity to certain Japanese pear indicates the danger of growing such susceptible pears near citrus-growing areas, and a potential problem from the introduction of the citrus pathogen into areas of Japanese pear cultivation.

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Key words: Host-specific toxin, ACT-toxin I, ACT-toxin II, Japanese pear, mandarin, *Alternaria alternata* tangerine pathotypes.

INTRODUCTION

Two pathotypes of *Alternaria alternata* (Fr.) Keissler (formerly, distinct strains of *A. citri* Ellis and Pierce) cause brown spots on young leaves and immature fruits of citrus species^{8,18,19}. One of these pathotypes is selectively pathogenic to Dancy tangerine and Emperor mandarin (*Citrus reticulata*), but has no obvious effect on rough lemon (*C. jambhiri*)^{4,18,19}. On germination, spores of this pathotype produce a toxin (ACT-toxin Ib) with the same host specificity as the fungus itself⁶. Recently, the toxin was chemically characterized; it is an ester of epoxy-decatrienoic acid and *N*-acetylvaline^{6,11}. Kono *et al.* reported toxins (ACTG-toxins) which have selective toxicity on Emperor mandarin⁹, while the structures of these toxins were different from ACT-toxin Ib. The other pathotype is selectively pathogenic to rough lemon and Rangpur lime (*C. limonia*), but not to Dancy tangerine and Emperor

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** Laboratory of Plant Pathology, Faculty of Agriculture, Tottori University, Tottori 680, Japan 鳥取大学農学部

*** Laboratory of Bioorganic Chemistry, Faculty of Agriculture, Gifu University, Gifu 501-11, Japan 岐阜大学農学部

† Present address: Tottori Mycological Institute, Tottori 689-11, Japan 現在: 日本きのこセンター菌叢研究所

mandarin^{4,19}). The rough lemon pathotype also produces a toxin (ACR-toxin¹⁰) or ACRL-toxin¹¹) with the same host specificity as the fungus.

These two pathotypes of *A. alternata* have not been found in Japan, although potential citrus hosts, such as cultivars Amanatsu, Iyo, and Kinokuni, have been cultivated for many years⁴). Kohguchi *et al.*³⁾ found in laboratory tests that the tangerine pathotype is also pathogenic to Japanese pear (*Pyrus pyrifolia*) cv. Nijisseiki. The tangerine pathotype produces at least two host-specific toxins: ACT-toxin I (formerly known as ACT-toxin A) that affects susceptible citrus and ACT-toxin II (formerly, ACT-toxin B) that is toxic to Japanese pear cv. Nijisseiki²¹). Unexpected pathogenicity of this pathotype can be explained by production of the major toxin (ACT-toxin Ib). Purified ACT-toxin Ib affected not only susceptible citrus, but also caused necrosis and induced infection by nonpathogenic *A. alternata* on leaves of Japanese pear cv. Nijisseiki, when the spores plus toxin (1×10^{-6} M) were applied⁶). However, production of other possible toxins by the tangerine pathotype had not been studied. We herein report the isolation and characterization of biological activities of ACT-toxin IIb produced by an isolate of the tangerine pathotype of *A. alternata*. ACT-toxin IIb was selectively toxic to Japanese pear cv. Nijisseiki, but had little or no effect on susceptible citrus. Abstracts of the study were published^{2,3,5)}.

MATERIALS AND METHODS

Plants. Japanese pear cvs. Nijisseiki and Chojuro, Emperor mandarin and rough lemon were used for bioassaying phytotoxicity. These plants were grown in a greenhouse and young leaves were used. In addition, young leaves of many other cultivars of Japanese pear, were kindly provided from the Tottori Horticultural Experiment Station, and they were used for testing the host range of the tangerine pathotype and a toxin-sensitivity spectrum.

Fungi. Two isolates of *A. alternata* were used: a pathogenic isolate (AC-320) of the tangerine pathotype was kindly provided by Dr. J.O. Whiteside, (University of Florida, Citrus Research and Education Center, U.S.A.), and a nonpathogenic isolate (O-94). Spores were obtained from mycelial mats by the procedure described previously⁶⁾. For toxin production, the isolate AC-320 was cultivated in still culture on a modified Richards' medium⁶⁾ at 25°C for 24 days. Toxicity was determined by a leaf necrosis assay⁶⁾.

Observation of infection behavior of nonpathogenic isolate. To test pathogenicity and infection behavior, spores (5×10^5 per ml) of the nonpathogenic isolate (O-94) were suspended in a dilute solution of ACT-toxin IIb (1×10^{-7} M) and inoculated by spraying with an atomizer onto the lower surface of leaves. Control leaves were sprayed with deionized water containing spores at the same density. Fungal infection behavior was observed under a microscope⁶⁾.

High performance liquid chromatography (HPLC). Solutions containing toxin were subjected to analytical and preparative HPLC, using a Hitachi HPLC system equipped with a L-3000 photo diode array detector, L-6200 intelligent pump, and D-6000 data station. To analyze sample solutions, a Develosil ODS-5 column (4.6×250 mm) was used with a μ -Bondapak C₁₈ guard column (Waters Co. Ltd.). Preparative HPLC was with a Develosil ODS-7 column (10×250 mm) with a Develosil ODS-15/30 guard column. Develosil ODS-gels were from Nomura Chemical Co. Ltd. Sample solutions were filtered with a 0.2 μ m Millipore filter and subjected to HPLC at room temperature. Detailed conditions for HPLC are given in RESULTS.

Electron microscopy. Electron microscope observations of cells were performed as described by Kohmoto *et al.*⁶⁾

RESULTS

Isolation of ACT-toxin IIb

ACT-toxin IIb was isolated by the method similar to the isolation procedure previously described for ACT-toxin Ib⁶⁾. The method is summarized in Fig. 1. The ethyl acetate extract of toxins from culture filtrate of the isolate AC-320 was subjected to droplet countercurrent chromatography (DCC) using a

Fig. 1. Outline of the isolation procedures for ACT-toxin IIb from culture filtrates of the tangerine pathotype of *A. alternata* (isolate AC-320).

Tokyo Rikakikai model DCC-A instrument, and developed in descending mode: the solvent was carbon tetrachloride/chloroform/benzene/methanol/deionized water (3:3:6:8:2, v/v/v/v/v). Two hundred fractions (each containing 10 ml) were collected. Leaf necrosis assays showed two groups of active fractions. The first active fractions (Nos. 11 to 68) were more toxic to Japanese pear cv. Nijisseiki than they were to Emperor mandarin. The second group of active fractions (Nos. 69 to 126) was highly toxic to Emperor mandarin and slightly toxic to Japanese pear cv. Nijisseiki. Based on TLC analysis (data not shown) and bioassay, the first fractions were judged to contain ACT-toxin II and the second to contain ACT-toxin I⁶. The first fractions were pooled and evaporated. The residue was applied to Sep-pak C₁₈ cartridge column and then subjected to preparative HPLC on a Develosil ODS-7 column using acetonitrile/deionized water/acetic acid (60:40:1, v/v/v) as a mobile phase, at a flow rate of 2 ml/min. Absorbance of the effluents was monitored at 290 nm. One peak fraction (Rt 34.3-38.3 min) was highly toxic to Japanese pear cv. Nijisseiki. This fraction was evaporated and further subjected to preparative HPLC, using methanol/deionized water/acetic acid (65:35:1, v/v/v), at a flow rate of 2 ml/min. A peak fraction (Rt 35.8-38.0 min) was collected and subjected to analytic HPLC. This preparation moved as a single peak and was highly toxic to pear cv. Nijisseiki, slightly toxic to Emperor mandarin, and not toxic to pear cv. Chojuro and rough lemon.

Comparative susceptibility and toxin sensitivity of Japanese pear cultivars to the tangerine pathotype of *A. alternata*

Leaves of 35 cultivars of Japanese pear were inoculated by spraying a spore suspension (1×10^6 spores per ml) of the pathogenic isolate (AC-320) with an atomizer. Ten cultivars, including cv. Nijisseiki, proved to be susceptible to the tangerine pathotype of *A. alternata*. The remaining 25 cultivars were resistant (Table 1). Sensitivity of the cultivars to ACT-toxin Ib and IIb was tested by the leaf necrosis assay method. ACT-toxin Ib was used at 1×10^{-5} and 1×10^{-4} M, and ACT-toxin IIb was used at 1×10^{-6} and 1×10^{-5} M. Dilution end points of toxicity of these toxins on cv. Nijisseiki were 1×10^{-6} M and 1×10^{-7} M, respectively. ACT-toxins Ib and IIb at each concentration caused black vein necrosis on leaves of the ten cultivars that were susceptible to the pathogen. The toxins caused no necrosis on the 25 cultivars that were resistant to the pathogen even with the higher concentrations examined. Thus, among 35 cultivars, the spectrum of sensitivity to ACT-toxins completely coincides with that of the disease susceptibility.

Biological activities of ACT-toxin IIb

A dose-response study revealed that ACT-toxin IIb induced black vein necrosis on leaves of Japanese pear cv. Nijisseiki at concentrations more than 1×10^{-7} M. This toxin also caused brown vein necrosis on leaves of Emperor mandarin at a concentration of 4×10^{-5} M (data not shown in Fig. 2). ACT-toxin IIb did not cause necrosis on leaves of resistant plants (rough lemon and Japanese pear cv.

Table 1. The correlation between susceptibility of Japanese pear cultivars to the tangerine pathotype of *Alternaria alternata* and sensitivity to its toxins (ACT-toxin Ib and IIb)

Cultivar	Inoculation ^{a)}	Treatment with ACT-toxins ^{b)}	
		Ib ^{c)}	IIb
Chojuro	—	—	—
Doitsu	+	+	+
Gion	—	—	—
Hakata-ao	+	+	+
Hakuro	+	+	+
Hokkai-wase	—	—	—
Hosui	—	—	—
Ichihara-wase	—	—	—
Imamura-aki	—	—	—
Ishii-wase	—	—	—
Kikusui	—	—	—
Kimizuka-wase	+	+	+
Kogetsu	+	+	+
Kosui	—	—	—
Kozo	—	—	—
Kumoi	—	—	—
Meigetsu	+	+	+
Niitaka	—	—	—
Nijisseiki	+	+	+
Okusankichi	—	—	—
Osa-nijisseiki	+	+	+
Seigyoku	—	—	—
Seiryu	—	—	—
Shinchi	—	—	—
Shinko	—	—	—
Shinseiki	—	—	—
Shinsui	+	+	+
Shirayuki	—	—	—
Suisei	—	—	—
Suisyu	—	—	—
Taihaku	—	—	—
Taiheiyo	—	—	—
Yakumo	—	—	—
Wase-aka	—	—	—
Wase-nijisseiki	+	+	+

a) The reactions resulting from spore inoculation (10^6 spores/ml) with an atomizer were evaluated as + (lesions formed) or — (no lesions).

b) Two concentrations of ACT-toxin Ib (10^{-6} M and 10^{-4} M) and ACT-toxin IIb (10^{-6} M and 10^{-5} M) were used. The reactions were evaluated as + (necrosis was induced from a low concentration of each toxin) or — (no necrosis even at a high concentration of each toxin).

c) ACT-toxin Ib was obtained by the methods described by Kohmoto *et al.*⁶⁾

Chojuro) even at 1×10^{-4} M. Furthermore, ACT-toxin IIb also caused a rapid increase in loss of electrolytes from leaf disks of the susceptible pear (cv. Nijisseiki) at concentrations more than 2×10^{-7} M. Emperor mandarin was affected by ACT-toxin IIb at 1×10^{-5} M with a 15% increase in electrolyte loss as compared to the control (Fig. 2B). The toxin at 1×10^{-5} M did not affect loss of electrolytes from leaf disks of cv. Chojuro and rough lemon.

Ultrastructural changes in toxin-treated leaves

Vein necrosis was visible on leaves of susceptible Japanese pear (cv. Nijisseiki) 12 hr after treatment with drops (40 μ l) of ACT-toxin IIb solution at a concentration of 2×10^{-7} M. Since the toxic effect was first visible on the leaf veins, and later extended to neighboring mesophyll cells, further observa-

Fig. 2. Effect of ACT-toxin IIb on (A) development of vein necrosis in leaf tissues and (B) losses of electrolytes from leaf disks. Drops (40 μ l) of toxin solutions were placed on leaves of Japanese pear cv. Nijisseiki (●), cv. Chojuro (○), Emperor mandarin (■), and rough lemon (□). Areas of vein necrosis were measured 48 hr later. Electrolyte leakage was measured at hourly intervals, as described by Kohmoto *et al.*⁶⁾ and is reported as a value of relative conductance for 6 hr between toxin-treated leaves and deionized-water-treated leaves (control). The vertical bars represent the standard deviations from three experiments.

tions were focused on cells in three types of leaf tissues: vascular bundle sheaths, mesophyll adjacent to the bundle sheaths, and mesophyll remote from the bundle sheath.

The first toxin-induced ultrastructural changes appeared on plasmodesmata of leaf cells 3 hr after exposure to toxin (Plate I). ACT-toxin IIb caused invaginations of the plasma membrane at plasmodesmata; cells at the invaginated sites had membranes, vesicles, and desmotubules that extended from plasmodesmata (Plate I-C and D). Frequency of invagination at the plasmodesmata differed in the three types of tissues. At 6 hr after toxin exposure, the frequencies of invaginations were as follows: 69% in the bundle sheath, 41% in mesophyll cells adjacent to the bundle sheath, and 17% in mesophyll cells remote from the bundle sheath (Table 2). The invagination frequency increased with time. However, no effects were evident in 6 hr on chloroplasts, mitochondria, and vacuoles (Plate I-E). No ultrastructural changes were observed in leaf cells of resistant Japanese pear cv. Chojuro or rough lemon treated with 4×10^{-6} M ACT-toxin IIb (Table 2 and Plate I-F).

Effect of ACT-toxin IIb on infection by nonpathogenic *A. alternata* on susceptible and resistant leaves

Leaves of Japanese pear cv. Nijisseiki that were inoculated with nonpathogenic spores suspended in a dilute solution of ACT-toxin IIb (1×10^{-7} M) showed a significant increase in the number of infection

Table 2. Plasmodesmatal invaginations induced by ACT-toxin IIb in leaf tissues of Emperor mandarin, rough lemon and Japanese pear cvs. Nijisseiki and Chojuro

Plant and cultivar	Treatment	Time (hr)	Plasmodesmata with and without plasma membrane invagination						Mesophyll cells remote from bundle sheath		
			Vascular bundle sheath cells			Mesophyll cells adjacent to bundle sheath			Mesophyll cells remote from bundle sheath		
			No. of plasmodesmata observed	No. of plasmodesmata with invagination	No. of plasmodesmata observed	No. of plasmodesmata with invagination	No. of plasmodesmata observed	No. of plasmodesmata with invagination	No. of plasmodesmata observed	No. of plasmodesmata with invagination	No. of plasmodesmata with invagination
Japanese pear Nijisseiki	Toxin ^{a)}	1	84	0	0%	0	0	0%	108	0	0%
	Toxin ^{a)}	3	70	13	19	13	3	5	101	0	0
	Toxin ^{a)}	6	116	80	69	80	24	41	92	16	17
	Toxin ^{a)}	12	109	97	89	97	38	61	118	50	42
	Toxin ^{b)}	1	128	6	5	6	5	5	119	0	0
	Toxin ^{b)}	3	113	72	64	72	8	9	122	7	6
	Toxin ^{b)}	6	77	60	78	60	5	15	116	9	8
	Toxin ^{b)}	12	116	93	80	93	44	65	113	87	77
	Water	12	76	0	0	0	0	0	123	0	0
	Toxin ^{b)}	12	92	0	0	0	0	0	111	0	0
Chojuro	Water	12	75	0	0	0	0	0	97	0	0
Citrus											
Emperor mandarin	Toxin ^{b)}	12	108	0	0	0	0	0	74	0	0
Rough lemon	Water	12	45	0	0	0	0	0	107	0	0
	Toxin ^{b)}	12	76	0	0	0	0	0	114	0	0
	Water	12	66	0	0	0	0	0	95	0	0

a) Concentration of the toxin was 2×10^{-7} M.b) Concentration of the toxin was 4×10^{-6} M.

Table 3. Effect of ACT-toxin IIb on colonization of leaf tissues by a non-pathogenic isolate (O-94) of *Alternaria alternata*

Plant and cultivar ^{a)}	Inoculation	Spore germination (%)	No. of germ tubes per spore	Appressoria per germ tube (%)	Infection hyphae per appressorium (%)
Japanese pear					
Nijisseiki	O-94 + Toxin ^{b)}	92.3 ± 1.7 ^{c)}	1.2 ± 0.1	43.9 ± 14.0	30.4 ± 8.2
	O-94	91.2 ± 0.1	1.3 ± 0.1	38.7 ± 4.1	6.9 ± 3.5
Chojuro	O-94 + Toxin	91.6 ± 1.0	1.4 ± 0.1	26.0 ± 3.2	11.4 ± 0.4
	O-94	90.8 ± 1.3	1.3 ± 0.1	44.4 ± 11.2	11.9 ± 5.1
Citrus					
Emperor	O-94 + Toxin	91.4 ± 5.9	1.3 ± 0.1	39.6 ± 8.7	14.1 ± 3.1
mandarin	O-94	91.1 ± 2.2	1.3 ± 0.1	39.1 ± 9.2	11.9 ± 4.0
Rough	O-94 + Toxin	90.9 ± 4.1	1.3 ± 0.1	43.1 ± 3.4	9.8 ± 3.1
lemon	O-94	89.5 ± 2.6	1.2 ± 0.1	47.8 ± 5.4	10.8 ± 3.0

a) Japanese pear cv. Nijisseiki is susceptible to both the Japanese pear pathotype and to this isolate of the tangerine pathotype of *A. alternata*. The Emperor mandarin is susceptible to the tangerine pathotype and rough lemon is resistant.

b) Spore suspension (5×10^5 spores/ml) was sprayed on the lower surface of leaves. 10^{-7} M toxin was used.

c) Values are the means $\pm$ S.D. from three experiments with observation of 100 spores.

hyphae produced. With the same treatment, leaves of Japanese pear cv. Chojuro, Emperor mandarin, and Rough lemon had no increase in the rate of formation of infection hyphae (Table 3). ACT-toxin IIb alone had no obvious effect on leaves of these Japanese pear and citrus types under the condition.

Release of ACT-toxin IIb during spore germination

The presence of ACT-toxin IIb in spore germination fluids was quantified by a method similar to that used for ACT-toxin Ib⁹⁾. The germination rate of spores of the pathogenic isolate AC-320 reached about 80% in 6 hr incubation. ACT-toxin IIb was first detected after 18 hr of incubation; the amount was estimated at 0.024 ± 0.016 pg per germinated spore. After 24 hr, there was an average of 0.027 ± 0.007 pg of ACT-toxin IIb per spore.

DISCUSSION

The major host-specific toxin (ACT-toxin Ib) of the tangerine pathotype of *A. alternata* was confirmed for the pathogenic isolate AC-320⁹⁾. In addition, we have isolated from the identical cultures of the isolate AC-320 a new toxin that is highly active against Japanese pear cv. Nijisseiki, but is less toxic to susceptible tangerines. The toxin was designated ACT-toxin IIb, and characterized by Nakatsuka *et al.* as 8-(*N*-2'',3'',4''-trihydroxy-4'',6''-dimethyl-6''-octenoylvalyl)-9,10-epoxy-9-methyl-decatricenoic acid (Fig. 3)¹²⁾. It contains a central-*cis* decatricenoic acid moiety that is the same stereoisomer found in AK-toxins I and II¹⁶⁾ from the Japanese pear pathotype of *A. alternata*. It is also identical to the b type isomers of AF-toxins produced by the strawberry pathotype of *A. alternata*¹⁴⁾. The determination of structural details of ACT-toxin IIb will be published by Nakatsuka elsewhere.

ACT-toxin IIb is the 5'-deoxy derivative of ACT-toxin Ib (Fig. 4). This reduction, however, made ACT-toxin IIb ten-fold more toxic to pear cv. Nijisseiki but 2,000 fold less toxic to Emperor mandarin, as compared with ACT-toxin Ib. Hence, a hydroxy group at the 5'-site appears to be important for selective toxicity to tangerines and related citrus. Reversed-phase HPLC analysis suggests a considerable difference in polarity between ACT-toxin Ib and IIb. ACT-toxin IIb had an approximately two times longer retention time on reversed-phase column than ACT-toxin Ib had (data not shown). This difference may result from a modification of the three-dimensional structure that also alters toxicity against sensitive plants. The modification may relate to an association with a putative binding site in sensitive cells.

Determination of the structure-activity relationships should contribute to the basis for a molecular

Fig. 3. Molecular structures of ACT-toxin Ib, Ic, and IIb.

study of toxic action against host cells. Comparative studies on HV-toxin derivatives, produced by *Cochliobolus victoriae*, showed that the latent aldehyde group of HV-toxin is bound covalently to a receptor protein in plant cells²⁰. The case with host-specific toxins from *A. alternata* is more complex; fourteen related toxins contain the decatrienoic acid in their molecules and are toxic to Japanese pear cv. Nijisseiki^{6,14}. These 14 toxins are AF-toxin I, II, III, (each contains three stereoisomers: a, b and c), AK-toxin I and II; ACT-toxins Ib, Ic, and IIb. The relationship between structure and toxicity of the new ACT-toxin IIb to cv. Nijisseiki supports the hypothesis that an amide NH at the position 8 has an important role in specificity and toxicity of these decatrienoic acids¹⁴. However, the functional group that directly interacts with sites of action in cells has not been pinpointed to date.

ACT-toxin Ib and IIb were toxic only to Japanese pear cultivars that are susceptible to the tangerine pathotype of *A. alternata* (Table 1), in addition to its original host of citrus plants. Thus, they are clearly host-specific toxins. Besides, the tangerine pathotype had the same host range among Japanese pear cultivars as did the Japanese pear pathotype of *A. alternata*¹⁶. It is clear that ACT-toxin Ib and IIb can reproduce the same symptoms and have the same host-specificity as does the pathogen. The physiological effects of ACT-toxin Ib and IIb on pear cv. Nijisseiki were qualitatively indistinguishable in the three assay methods (induction of necrosis, increase in loss of electrolytes, and infection by nonpathogenic *A. alternata*)⁶. The only difference was that ACT-toxin IIb required a 10-fold lower concentration than a dose of ACT-toxin Ib for toxic effects, though ACT-toxin IIb was first detected by HPLC analysis from germinating spores 6 hr later than ACT-toxin Ib was. The selective pathogenicity of the tangerine pathotype of *A. alternata* to Japanese pear, and to its original citrus host, could be based on the production of ACT-toxin IIb, besides ACT-toxin Ib.

There was a close relationship between necrosis and dysfunction of plasma membranes in toxin-treated susceptible leaf tissues, as indicated by loss of electrolytes from the tissues. This was evident in a dose-response relationship with ACT-toxin IIb, where necrosis was correlated electrolyte losses (mainly, K⁺) (Fig. 2). In addition, electron microscopy of leaves of Japanese pear cv. Nijisseiki treated with ACT-toxin IIb showed that invaginations of the plasma membrane occurred at the plasmodesmata. The invaginations were first observed 3 hr after exposure to the toxin; they increased rapidly during the following 3 hr. This alteration is similar to the ultrastructural changes that occur in cells of Japanese

pear cv. Nijisseiki following exposure to AK-toxin¹⁷⁾ or ACT-toxin Ib⁹⁾. Finally, several metabolic inhibitors that counteracted on AK-toxin action¹⁵⁾, also suppressed action of ACT-toxin Ib and IIb (unpublished data). From these results, it is likely that the gene for susceptibility to the Japanese pear pathotype of *A. alternata* and to AK-toxins also confers Japanese pear's susceptibility and sensitivity to the tangerine pathotype of *A. alternata* and to ACT-toxins Ib and IIb. We postulate that the primary sites of action in Japanese pears are the same for these three toxins.

The host-pathogen interaction of the tangerine pathotype appears to be similar to that of the Japanese pear pathotype of *A. alternata*. This includes the events in initial colonization⁷⁾. The indications are that the dysfunction of plasma membranes by ACT-toxin Ib and IIb is related to inhibition of general defense mechanisms in host tissues, which in turn allows for hyphal penetration and colonization of cells and tissues (Table 3). Thus, ACT-toxins Ib and IIb are considered to be host recognition factors for the pathogen similar to the situation with other host-specific toxins from *Alternaria* and *Bipolaris*^{13,21)}.

Two facts indicate that the tangerine pathotype of *A. alternata* is a potential pathogen of Japanese pear cv. Nijisseiki. First, the tangerine pathotype produced on germination a toxin that selectively affects Japanese pear cv. Nijisseiki. Second, inoculation experiments with spores of the tangerine pathotype resulted in colonization of tissues and symptom development in pear cv. Nijisseiki. This emphasizes possible problems for plant quarantine. It is probable that the tangerine pathotype of *A. alternata* would become a problem if susceptible Japanese pears are introduced into citrus-growing areas of U.S.A. and Australia. Also the introduction of the tangerine pathotype into Japan may add to disease problems with both citrus and pears. This is the first example of the use of host-specific toxin to predict a new problem for agriculture. Studies of toxin-producing fungi obviously have unexpected implication.

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和 文 摘 要

伊藤靖夫・甲元啓介・霜村典宏・尾谷 浩・児玉基一朗・中塚進一：タンゼリンの brown spot 病菌の生産するニホンナシに選択毒性を示す毒素

タンゼリンの brown spot 病菌は日本で未発生であるにもかかわらず、二十世紀など特定のニホンナシ品種に対しナシ黒斑病菌と同様の病原性を示す。本病原菌は、宿主特異的毒素 ACT-toxin Ib の他に、ニホンナシに活性を示す毒素を培養液および孢子発芽液に生産する。これを単離して ACT-toxin IIb と命名した。本毒素は二十世紀ナシ葉に対し 1×10^{-7} M, 宿主のタンゼリンやマンダリン葉に対し 4×10^{-5} M 以上で壊死を誘起するが、非宿主カンキツ葉や長十郎ナシ葉には 1×10^{-4} M でも作用しなかった。毒素処理二十世紀ナシ葉では電解質の異常漏出と原形質膜の陥入現象が見られた。非病原性アルタナリア孢子に微量の毒素を添加して噴霧接種すると、二十世紀ナシ葉だけに感染が成立した。これらのことから、ACT-toxin IIb も宿主特異的毒素の属性を有すると判断された。タンゼリンの brown spot 病菌はニホンナシにとって潜在的病原菌であり、植物検疫上重要であると考えられる。

Explanation of Plate**Plate I**

Electron micrographs of Japanese pear and citrus leaf tissues treated with ACT-toxin IIb and with deionized water. Ultrastructure of vascular bundle sheath cells of the susceptible Japanese pear leaf (cv. Nijisseiki) treated with (A) deionized water for 12 hr ($\times 6,100$) and (B) 2×10^{-7} M ACT-toxin IIb for 6 hr. The toxin treated cells show many invaginations of plasma membrane (arrows) ($\times 6,100$).

- C. Invagination of plasma membranes in the susceptible Japanese pear leaf (cv. Nijisseiki) treated with 2×10^{-7} M ACT-toxin IIb for 6 hr. Invaginated portions, extended desmotubules (ED), tubular structure (TS), vesicles (V), and membrane structure (MS) were found ($\times 12,800$).
- D. Typical plasma membrane invagination in the susceptible Japanese pear leaf (cv. Nijisseiki) treated with 2×10^{-7} M ACT-toxin IIb for 6 hr. The space between invaginated plasma membrane and cell wall included densely stained membrane structure, vesicles and tubular structure ($\times 36,700$).
- E. Mesophyll cells of the susceptible Japanese pear leaf (cv. Nijisseiki) treated with 2×10^{-7} M ACT-toxin IIb for 6 hr; there are invaginations of plasma membrane. No structural changes of chloroplasts (CHL), mitochondria (M), and vacuoles (VA) were observed ($\times 9,100$).
- F. Vascular bundle sheath cells of the resistant Japanese pear (cv. Chojuro) treated with 4×10^{-6} M ACT-toxin IIb for 12 hr; there are no ultrastructural changes ($\times 6,100$).

Plate I