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Effect of Host-Specific AF-Toxin I Produced by the Strawberry Pathotype of *Alternaria alternata* on Protein Synthesis and Extracellular Polysaccharide Accumulation in Strawberry Protoplasts

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딸기 검은무늬병균이 생산하는 기주특이성 AF독소 I이 딸기 원형질체의 단백질 합성과 세포외 다당체 축적에 미치는 영향

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ABSTRACT : The effect of AF-toxin I produced by the strawberry pathotype of *Alternaria alternata* on the protein synthesis of susceptible strawberry protoplasts was examined by using the radiolabeled amino acids. The incorporation of the radiolabeled amino acids into newly synthesized proteins in the strawberry protoplasts was stimulated by the toxin treatment at relatively low concentrations (2.2×10^{-11} to 2.2×10^{-9} M), but not at higher concentrations (2.2×10^{-8} to 2.2×10^{-6} M). An one-dimensional SDS-polyacrylamide gel electrophoresis revealed no detectable differences in the proteins synthesized in both the toxin-treated and untreated protoplasts. The susceptible strawberry protoplasts were treated with AF-toxin I and stained with Fluostain I to detect the extracellular polysaccharides. The toxin treatment induced the accumulation of extracellular polysaccharides in a dose-dependent manner. These results indicate a transient activation of cellular metabolism in the susceptible cells by the toxin exposure.

Key words **: AF-toxin I, *Alternaria alternata* strawberry pathotype, protein synthesis, extracellular polysaccharide synthesis (EPS).**

The strawberry pathotype of *Alternaria alternata*, the causal agent of Alternaria black spot of strawberry, produces a host-specific toxin, designated as AF-toxin, during culture and spore germination (5, 11, 27). The pathogen has been reported to affect only a strawberry cultivar, Morioka-16, in the field (16), but it was also highly pathogenic to certain cultivars of Japanese pear in laboratory tests (11, 17). The host range of this pathogen is identical to the range of plants which are sensitive to AF-toxins (11). AF-toxin was characterized to consist of three related molecules, AF-toxin I, II and III (11, 13, 14). These toxins are different in host-selective toxicity to host plants: AF-toxin I causes leaf necrosis both on strawberry and Japanese pear, toxin II on pear, and toxin III strongly on strawberry and slightly on pear (11).

Physiological and cytological studies on the effects of AF-toxin on leaf tissues of a host strawberry cultivar have shown that the toxin could induce rapid dysfunction of the plasma membrane of host cells (11, 15, 19, 20, 27). An evidence of such effects is the rapid and drastic increase of K^+ efflux from toxin-treated tissues of susceptible strawberry plants but not from those of resistant ones (11, 15, 27). An electron mi-

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croscopic observation showed that many invaginations occurred on the plasma membranes of the susceptible cells within 1 to 3 hr after toxin treatment (19, 20). On the basis of the electrophysiological study, AF-toxin was presumed to affect the electrogenic proton pump (H^+-pump) in the membranes irreversibly. The toxin induced depolarization of membrane potential similarly to that of anoxia, but no change in the anoxia potential was detectable after toxin exposure (15). Although the electrogenic H^+-pump is known to be dependent on the plasma membrane ATPase (22), no significant inhibition of the ATPase activity was observed by *in vitro* toxin treatment, showing that the effect of AF-toxin on plasma membrane ATPase may be secondary event triggered by initial interaction between the toxin molecule and the unknown target site in the cells (7). Previously we observed phospholipase A_2 activation and the degradation of phospholipids by AF-toxin in the microsomes of susceptible host cells (8). However, it is not clear whether AF-toxin directly activates phospholipase A_2 or induces some other events which indirectly affect the enzyme. Thus, the mechanisms by which the toxin causes the dysfunction of the host plasma membrane and sequential events from the membrane damage to the cell death still remain to clarify.

Park *et al.* (21) observed that area-ratio of rough endoplasmic reticular and Golgi regions to cells increased in AF-toxin-treated strawberry cells before cell death. Such increase might involve the functional activation of the organelles: the enhancement of protein, lipid and polysaccharide synthesis. The host-specific AK-toxin, which is produced by the Japanese pear pathotype of *A. alternata*, is known to cause the same type of plasma membrane dysfunction in susceptible pear cells as AF-toxin does (4, 18). It was shown that pretreatment of susceptible pear tissues with protein synthesis inhibitors, such as blasticidin S and cycloheximide, inhibit necrosis development by AK-toxin (4). Victorin, a host-specific toxin produced by *Cochliobolus victoriae*, also induces rapid dysfunction of plasma membrane in sensitive oat cells (3, 23). Walton and Earle (24) reported that victorin causes sensitive oat protoplasts to produce an extracellular polysaccharide (EPS). In the present paper, we document the stimulation of biosynthetic processes, production of protein and EPS, by AF-toxin in susceptible strawberry protoplasts.

MATERIALS AND METHODS

Plants. Strawberry (*Fragaria grandiflora* Fhrh.) cultivar Morioka-16, susceptible to *A. alternata* strawberry pathotype, was used in this study. The susceptible cultivar Morioka-16 carries the disease-susceptible heterozygous allele which is semidominantly inherited as a single locus (26). The clone M16-aa of the self-pollinated F1 progeny of Morioka-16 was also used. Clone M16-aa which lacks the susceptible allele is resistant to the pathogen and the toxins (9, 26). Plants were grown in pots in a greenhouse at 18 to 24°C.

Preparation of cell suspension cultures and their protoplasts. Suspension-cultured cells and protoplasts of strawberries were prepared by the method described previously (9). The protoplasts were obtained from 4-day-old cultured cells. The viability of strawberry cultured cells and protoplasts was determined by a MTT-colorimetric assay, as described previously (9).

Preparation of AF-toxin I. Highly virulent strain T-32 of *A. alternata* strawberry pathotype, which is a stock culture in Plant Pathology Laboratory, School of Agricultural Sciences, Nagoya University, was used for AF-toxin I preparation. The strain was still-cultured at 25°C for 14 days in 1 liter Roux bottles, each containing 300 ml of potato dextrose broth. AF-toxin I was isolated from the culture filtrate as described previously (13, 14).

Incorporation of radioactive amino acids into proteins in protoplasts. Protoplast suspension (100 μl) in Linsmaier and Skoog medium (10) containing 0.5 M mannitol (LM) was incubated with AF-toxin I and 740 KBq of [^{35}S]methionine / [^{35}S]cystein (8 : 2) mixture (44.7 TBq / mmol, New England Nuclear) at 25°C for 1 hr. Then, the protoplasts were collected by centrifugation at $125 \times g$ for 5 min and resuspended in 100 μl of LM. The incorporation of the radioactive amino acids into hot acid-insoluble materials was determined as described by Mans and Novelli (12) with some modifications. Aliquots (5 μl) of protoplast suspension were spotted on the filter paper (Whatman 3 MM filter), and the filters dropped into ice-cold 10% trichloroacetic acid (TCA). After incubation for 15 min, the filters were washed with 5% TCA at room temperature and heated to a gentle boil in 5% TCA for 15 min. The filters were washed sequentially with 5% TCA, 95% ethanol and diethyl ether at room temperature for 5 min, respectively. The filters were dried and placed into 5 ml of liquid scintillation cocktail (Scintisol EX-H, Dojin). Measurement of the ra-

dioactivity was made on a liquid scintillation spectrometer (Beckman LSC 5800), and counts were corrected to disintegrations per minute (dpm).

Electrophoretic analysis of proteins. Protoplasts (100 μl) were incubated in LM containing a mixture of AF-toxin I and 370 KBq of [^{3}H]leucine (5735 GBq/mmol, New England Nuclear) at 25°C for 1.5 hr. Then, the protoplasts were centrifuged at 125×*g* for 5 min and added with 45 μl of gel sample buffer [25 mM Tris-HCl (pH 6.8), 200 mM dithiothreitol, 5 mM EDTA, 5% SDS, 0.05% bromophenol blue and 10% glycerol]. The samples were heated at 90°C for 7 min and subjected to the electrophoresis in 4.5% SDS-polyacrylamide gels in the buffer system of Laemmli (6). Proteins were stained with Coomassie brilliant blue R-250 and then subjected to fluorography with EN ^{3}HANCE (New England Nuclear) according to the manufacturer's recommendations. Dried gels were exposed to Kodak X-OMAT AR film at −80°C for 15 days.

Microscopic observation of extracellular polysaccharides. Protoplasts in LM were incubated with AF-toxin I at 25°C for various periods, and an aliquot of the suspension was stained with 0.3% Fluostain I (Dojin). The ratio of stained and unstained protoplasts were determined by observation with an Olympus fluorescence microscope (exiter filter; BG3, barrier filter; Y475).

RESULTS AND DISCUSSION

Effect of AF-toxin I on incorporation of radiolabeled amino acids into proteins. Effect of AF-toxin I on protein synthesis was examined using protoplasts. Protoplasts isolated from suspension-cultured cells of susceptible cultivar Morioka-16 and resistant clone M16-aa were incubated with AF-toxin I at different concentrations (2.2×10^{-11} to 2.2×10^{-6} M) for 1 hr, and then the incorporation of ^{35}S-labeled amino acids into protein during the toxin exposure was measured. When the protoplasts were treated with AF-toxin I at relatively low concentrations (2.2×10^{-11} to 2.2×10^{-9} M), incorporation of radiolabeled amino acids into newly synthesized protein was apparently enhanced (Fig. 1). The incorporation was stimulated to the level of about 60% above the control at 2.2×10^{-10} M of AF-toxin I (Fig. 1). However, the toxin treatment at higher concentrations had no effect on the incorporation (Fig. 1). This result supported electron microscopic observation

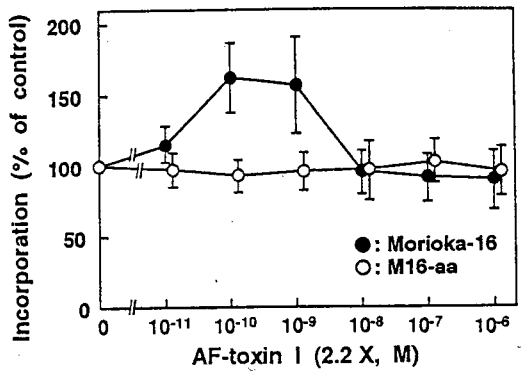

Fig. 1. Effect of AF-toxin I on incorporation of ^{35}S-labeled amino acids into protein in strawberry protoplasts. Protoplasts were isolated from suspension- cultured cells of susceptible cultivar Morioka-16 (●) and resistant clone M16-aa (○). Protoplast suspensions were incubated with a mixture of the radiolabeled amino acids and AF-toxin I at 25°C for 1 hr, and the amino acid incorporation into protein was determined. Each value represents the percentage of incorporation relative to the untreated control (the mean of four separate experiments with standard deviation).

(7, 21) that indicated temporary activation of cellular metabolism in the susceptible leaf cells by AF-toxin treatment.

Host-specific Victorin produced by *C. victoriae* and PC-toxin by *Periconia circinata* are known to change the characteristics of plasma membrane of susceptible cells of oat and sorghum, respectively (23). Gardner *et al.* (1, 2) showed that pretreatment of susceptible oat and sorghum tissues with cycloheximide protected the cells from Victorin and PC-toxin action, respectively. Since such protective effect was canceled after removal of the inhibitor from the tissues, they concluded that toxin sensitive site is a protein constantly turning over. Thus, involvement of *de novo* protein synthesis in process of symptom development appears to be a common feature in membrane-related toxin action.

The potential effect of AF-toxin on the synthesis of specific proteins was observed by electrophoretic analysis of proteins synthesized *in vivo* in the presence of [^{3}H]leucine. Protoplasts of the susceptible strawberry were incubated with or without AF-toxin I (2.2×10^{-10} M) in the presence of [^{3}H]leucine at 25°C for 1.5 hr, and the proteins were subjected to one-dimensional gel electrophoresis. The one-dimensional gel revealed no detectable differences in the proteins synthesized in toxin-treated and untreated protoplasts (Fig. 2).

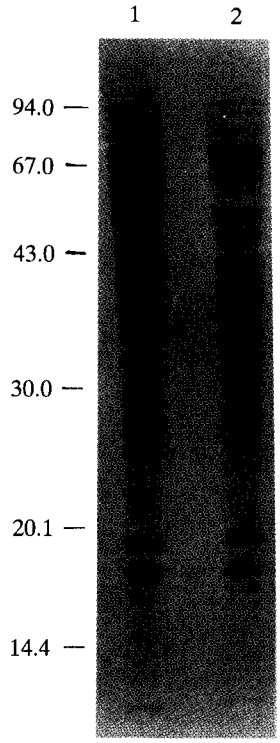

Fig. 2. Fluorograph of SDS-polyacrylamide gel of proteins synthesized *in vivo* by strawberry protoplasts during AF-toxin I exposure for 1.5 hr. Protoplasts isolated from suspension-cultured cells of susceptible cultivar Morioka-16 were incubated with [^{3}H]leucine (370 KBq) in the absence (lane 1) or presence (lane 2) of AF-toxin I (2.2×10^{-10} M) at 25°C for 1.5 hr. Protoplast extracts carrying the same radioactivity were loaded in each lane. Molecular size standards (in kDa) are given to the left of the photograph.

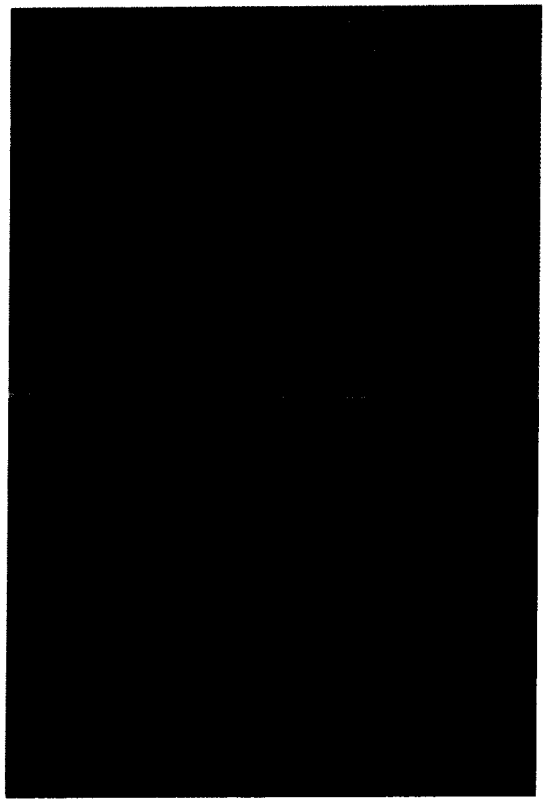

Fig. 3. Extracellular polysaccharide accumulation in strawberry protoplasts treated with AF-toxin I. Protoplasts isolated from suspension-cultured cells of susceptible cultivar Morioka-16 were incubated with AF-toxin I (2.2×10^{-6} M) at 25°C for 12 hr. The protoplasts were stained with 0.3% Fluostain I and photographed under visible (A) and ultraviolet (B) lights.

Walpert and Dunkle (25) reported that a 16-kDa protein was selectively synthesized in sorghum roots after PC-toxin treatment and might be involved in the development of cellular damage. However, any specific proteins synthesized in AF-toxin-treated strawberry cells could not be identified by the one-dimensional gel electrophoresis. Comparison of proteins in toxin-treated and untreated cells by using two-dimensional gel electrophoresis might make it possible to detect such specific proteins.

Effect of AF-toxin I on EPS accumulation. Protoplasts isolated from susceptible cultivar Morioka-16 were incubated with different concentrations (2.2×10^{-11} to 2.2×10^{-7} M) of AF-toxin I, and the EPS accumulation outside the protoplasts was examined by staining with Fluostain I, which is specific for β-glucans (Fig. 3). EPS accumulation was stimulated in the strawberry protoplasts by toxin treatment in parallel with a decline of cell viability (Fig. 4).

The AF-toxin caused accumulation of polysaccharide-components in the spaces between the cell walls and the invaginated plasma membranes (19). Furthermore, electron microscopic morphometry showed that the area of Golgi regions and the number of exocytotic Golgi vesicles increased in the toxin-treated susceptible cells (7, 21). Golgi vesicles, which contain materials such as polysaccharides and membranous sources, were observed to fuse frequently with invaginated plasma membrane. When protoplasts of the susceptible strawberry were treated with AF-toxin I, polysaccharides appeared outside the protoplasts in a dose-de-

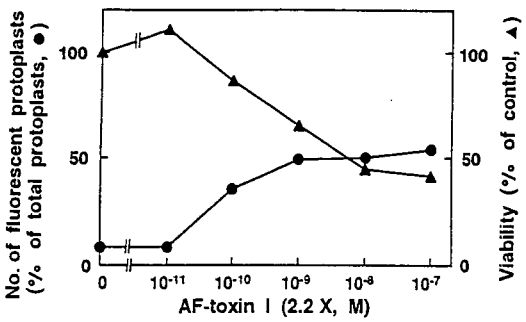

Fig. 4. Effect of AF-toxin I on extracellular polysaccharide accumulation in strawberry protoplasts. Protoplasts isolated from suspension-cultured cells of susceptible cultivar Morioka-16 were incubated with AF-toxin I at 25°C for 12 hr. The protoplasts were stained with Fluostain I, and fluorescent protoplasts were counted. Each value represents the percentage of fluorescent protoplasts. Cell viability was measured by MTT assay (9) and expressed as the percentage of control untreated with the toxin. Data are from a single experiment since similar results were obtained in the duplicate experiments.

pendent manner. Thus, polysaccharides accumulated in the invaginated spaces are now considered to be provided from the fused Golgi vesicles. These data suggested that the plasma membrane invaginations resulted from frequent fusion of Golgi vesicles with the damaged plasma membrane. The fusion might be involved in a process for repairing the membrane damaged by the toxin. The similar phenomenon was reported in oat protoplasts treated with host-specific HV-toxin produced by *C. victoriae* (3, 24).

요 약

딸기 검은무늬병균(*Alternaria alternata* strawberry pathotype)이 생산하는 AF독소 I이 감수성 딸기세포의 단백질합성에 미치는 영향을 방사선표식 아미노산을 이용하여 조사하였다. 낮은 농도 (2.2×10^{-11}에서 2.2×10^{-9} M)의 독소를 감수성 protoplast에 처리한 경우 새롭게 합성된 단백질내의 방사선표식 아미노산의 incorporation이 증가한 반면, 높은 농도에서는 아무런 변화가 없었다. 그러나, 독소처리구와 대조구를 SDS-polyacrylamide gel로 전기영동하여 비교한 결과 독소처리에 의해 특이적으로 합성되는 단백질은 발견할 수 없었다. 또한, 감수성 딸기 protoplast에 AF독소 I를 처리한 후 Fluostain I로 염색하여 세포외 polysaccharide의 축적에 대하여 조사하였다. 그 결과, 처리한 독소 농도가 증가함에 따라 세포외 polysaccharide의 축적이 증가하는 것으로 나타났다. 이러한 결과는 세포내 대사가 독소에 의해 일시적으로 증가하는 것을 시사하는 것이다.

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