

Alternaria

alternata群病原菌における宿主特異的毒素生成の制御(4)

AK毒素生成菌にのみ発現される遺伝子のクローニング

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Metabolic Regulation of Host-Specific Toxin Production in *Alternaria alternata* Pathogens (4). Molecular Cloning of mRNA in AK-toxin Producing Isolate*

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Abstract

Alternaria alternata Japanese pear pathotype produces a host-specific toxin (AK-toxin) as a determinant of host-specific pathogenicity. By employing a competitive hybridization procedure, specific transcripts were detected in a toxin producer, but not in its toxin-less avirulent mutant. Poly(A)⁺RNA fraction from the toxin producer was cloned as cDNA by *Escherichia coli* using pBR322 and screened by the competitive hybridization. The cDNA clones were obtained from transcripts which present in larger quantity in the producer than in its toxin-less mutant. Dot blot hybridization analysis by the cDNAs showed that there was a correlation between the amount of the specific RNAs and toxin productivity in several *A. alternata* isolates.

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Key words : *Alternaria alternata* Japanese pear pathotype, AK-toxin-less mutant, competitive hybridization, molecular cloning.

Introduction

In phytopathogen *Alternaria alternata*, filamentous fungus, there are now at least seven known host-parasite combinations, in which a host-specific toxin (HST) is responsible for the determination of host-specific pathogenicity^①. *A. alternata* HSTs reported so far are low molecular weight compounds that selectively affect the host plant. The chemical structures of five HSTs have been determined^{2,4-6}. Previous researches concerning HSTs of *A. alternata* pathogens disclosed their determinative role in pathogenesis⁶⁻⁸. The HSTs were shown to be released from germinating spores at the site of initial contact of pathogen with the host plant surface and to be involved in suppressing the defence activity of the host plant. The presence of HST leads to the successful colonization of the HST producer in the host plant. Thus, the infection capacity and host specificity of such pathogens are most likely governed by their ability to produce HSTs. In fact, the *A. alternata* pathogens are known to lose their pathogenicity due to the loss

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of toxin productivity^{8,10}). Consequently, the regulation of gene expression for HST production in pathogens could be highly relevant to the determination of pathogenesis.

Since the sexual stage of *A. alternata* has not been reported so far, HST productivity could not be analyzed by the classical genetic procedure. Although the chemical structures of some HSTs have already been determined, information on molecular basis of HST metabolism has been very limited. *A. alternata* Japanese pear pathotype producing AK-toxin selectively toxic to certain limited cultivars of Japanese pear including cv. Nijisseiki, is genetically unstable for toxin productivity^{8,10}). AK-toxin-less mutants were frequently derived from this pathogen.

In this paper, we employed the competitive hybridization¹¹ which was found to be a new and powerful strategy for comparing RNA species between AK-toxin producer and toxin-less mutant, and succeeded in screening out candidate cDNA of mRNA responsible for toxin production.

Materials and Methods

Fungal isolates. Isolates No. 15A and No. 15B of *A. alternata* Japanese pear pathotype causing black spot of Japanese pear were used. The former produces AK-toxin and is pathogenic to the cultivars of Japanese pear sensitive to AK-toxin. The latter is the toxin-less mutant from isolate 15A. Isolate 15B was spontaneously raised from one of stock cultures of isolate 15A. They were cultured in 200 ml potato dextrose broth in 1 liter Roux' bottles at 25 C for 7 days. Under this condition, mycelia of isolate 15A produced the highest amount of AK-toxin. Mycelial mats were harvested by sucking through Toyo No. 2 filter paper in a large Büchner funnel and washed extensively with sterile distilled water. After filtrating and drying, the weight of mycelia was measured.

The other *A. alternata* isolates, A-II-92 (Japanese pear pathotype), L-8205 (avirulent mutant of Japanese pear pathotype), AM-1 (apple pathotype causing Alternaria blotch of apple), 0-187 (strawberry pathotype causing Alternaria black spot of strawberry), AT-204 (tobacco pathotype causing brown spot of tobacco), AL-12 (tomato pathotype causing stem canker of tomato) and YA-1 (saprophyte) were also cultured, and their mycelia were harvested by the same procedure.

Isolation of DNA and RNA from mycelia. Mycelia (50 g fresh weight) were partially ground in a mortar by a pestle with liquid nitrogen. The ground mycelia were suspended in 100 ml of SDS-lysis buffer containing 0.2 M Na₂B₄O₇, 30 mM ethylene glycol-bis(β -aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA) and 1% SDS (w/v) which was preheated at 65 C. The suspension was well mixed and filtrated with 8 layers of sterilized guaze. The filtrate was extracted with water-saturated phenol. The phenol extraction achieved by shaking and centrifugation was repeated three to five times until denatured proteins at the interface became invisible. Then, the aqueous phase was extracted twice with chloroform and once with diethyl ether. The double volume of ethanol was added to the aqueous phase and kept at -20 C for more than 2 hr. The precipitate was collected by centrifugation at 13,000 $\times g$ for 10 min, dissolved in 10 ml of TE buffer containing 10 mM Tris (pH 7.0) and 1 mM EDTA, and incubated

at 4°C for 30 min. Insoluble substances were removed by centrifugation at $13,000\times g$ for 10 min. The supernatant was added with two volumes of ethanol, and the precipitate was suspended in 2 M LiCl solution. After incubating overnight at 4°C, the suspension was centrifuged at $13,000\times g$ for 10 min. The precipitate was resuspended in 2 M LiCl solution and re-precipitated. The final precipitate was dissolved in 1 ml of sterile distilled water and used as RNA preparation. The supernatant from LiCl precipitation was dialyzed against TE buffer. The dialyzed solution in dialysis tube was added with two volumes of ethanol, and resulting precipitate was dissolved in 1 ml of TE buffer. The solution was applied to Sephacryl S-1000 column chromatography (1.5×20 cm) for fractionation. Each fraction (1 ml) was subjected to measurement of absorbance at 260 nm and to electrophoresis in a 0.5% agarose gel. The fractions containing DNA were extracted once with phenol-chloroform (1:1, v/v) and with diethyl ether. DNA was precipitated with two volumes of ethanol. The precipitate was dissolved in 100 μ l of TE buffer and used as DNA preparation.

Competitive hybridization analysis of DNA with RNA. Analysis of the levels of RNAs from isolates 15A and 15B and screening of positive cDNA clones were carried out by the competitive hybridization method¹¹. *Bam*HI-digested DNA prepared from isolates 15A and 15B were electrophoresed in a 0.8% agarose gel and transferred to GeneScreen membrane (New England Nuclear) by the method of Southern¹⁰. The RNAs of isolates 15A and 15B were used for the hybridization on the filter. The filter (10 μ g DNA in a lane) was prehybridized with unlabeled RNA (100 μ g) and then hybridized with ³²P-labeled RNA (2×10^6 dpm). The ³²P-labeled probe RNA with a specific activity in excess of 10^7 dpm/ μ g was prepared by incubating 2 μ g of hydrolyzed RNA with T4 polynucleotide kinase and 50 μ Ci of [γ -³²P] ATP (4,000 Ci/mmol). After hybridization, the filter was washed and subjected to autoradiography.

Tetracycline-resistant colonies were transferred to replica nitrocellulose membranes (50 mm in diameter) for colony hybridization. For screening the cDNA of mRNA which is detected only in isolate 15A, competitive colony hybridization was performed with RNAs of isolates 15A and 15B. Both membranes were hybridized with unlabeled RNA of isolate 15B (200 μ g). Subsequently, one of them was hybridized with ³²P-labeled RNA (2×10^6 dpm) of isolate 15A and the other was with that (2×10^6 dpm) of isolate 15B as a control. Positive colonies could be detected only on membrane hybridized with labeled RNA of isolate 15A.

Construction of a cDNA library of poly(A)⁺RNA from isolate 15A. Poly(A)⁺RNA was prepared by chromatography with oligo(dT)-cellulose from RNA preparation of isolate 15A³¹. The first strand of cDNA was synthesized by reverse transcriptase with a oligo(dT) primer and the second strand was produced by using RNase H and *Escherichia coli* DNA polymerase I. Homopolymer tracts of dC were added to the 3' ends of this cDNA with terminal deoxynucleotidyl transferase³¹. The oligo(dC)-tailed cDNA and oligo(dG)-tailed pBR322 at *Pst*I site were annealed³¹, and used to transform *E. coli* RR1³¹. Colonies grown on tetracycline-containing agar were subjected to the competitive hybridization as mentioned above. For confirming the presence of the specific mRNA in the poly(A)⁺RNA fraction, the radioactive first strands of cDNA synthe-

sized by reverse transcriptase with [α - ^{32}P]dCTP were employed for competitive hybridization against DNA of isolate 15A. The restricted DNA fragments (10 μg) on filter were hybridized with unlabeled RNA (100 μg) of isolate 15B and then hybridized with ^{32}P -labeled cDNA (10⁶ dpm).

Isolation of plasmid DNA. Plasmids of positive clones selected by the competitive hybridization were extracted by using an alkaline lysis procedure³⁾ and purified by CsCl/ethidium bromide density gradient centrifugation.

Analysis of inserted cDNA. Plasmids isolated from positive clones were treated with *Pst*I restriction endonuclease, and the digested fragments were electrophoresed in a 1.5% agarose gel for determining size of inserted cDNA.

Hybridization probes were also made from the plasmids. The ^{32}P -labeled probe with a specific activity of more than 10⁷ dpm/ μg was prepared by subjecting 1.0 μg of plasmid DNA to nick translation reaction with 30 μCi of [α - ^{32}P] dCTP (4,000 Ci/mmol). The filter used once in the competitive hybridization analysis of RNA was reused after washing with rehybridization solution according to instruction manual from New England Nuclear and subsequently drying at room temperature. The filter was hybridized with the ^{32}P -labeled plasmid DNA for detecting DNA fragments homologous to the cDNA.

RNA dot blot hybridization. RNAs of various *A. alternata* isolates were denatured with glyoxal, dot-blotted on GeneScreen membrane and hybridized with ^{32}P -labeled plasmid DNA. After autoradiography, mRNA homologous to cDNA in the plasmid was quantified by scanning with a densitometer.

Results

Comparative analysis of RNAs from isolates 15A and 15B

DNA-RNA-competitive hybridization was used to find the regions of genome containing genes which were expressed only in a pathogen or a mutant. In Fig. 1, radioactive bands were clearly found in lanes 1 and 5. These filters containing DNA fragments of isolates 15A (lane 1) and 15B (lane 5) were prehybridized with RNA of isolate 15B and then hybridized with ^{32}P -labeled RNA of isolate 15A. In other combinations, positive bands were not observed. This result suggests the possibility that the unique RNAs were transcribed in isolate 15A but not in isolate 15B, and that such RNA would be responsible for AK-toxin production.

Screening of cDNA clones from isolate 15A

Mycelia of isolate 15A cultured for 7 days showed the highest potential for toxin production. Therefore, poly(A)⁺RNA from such mycelia is expected to contain mRNA for AK-toxin production and to be useful for producing cDNA of the mRNA. The cDNA of RNA present only in isolate 15A was screened by competitive hybridization from a cDNA library.

For checking whether the abundant RNA species in isolate 15A is poly(A)⁺RNA, the first strand labeled by reverse transcriptase was employed for competitive hybridization using filter containing restricted DNA fragments of isolate 15A (Fig. 2). The filter

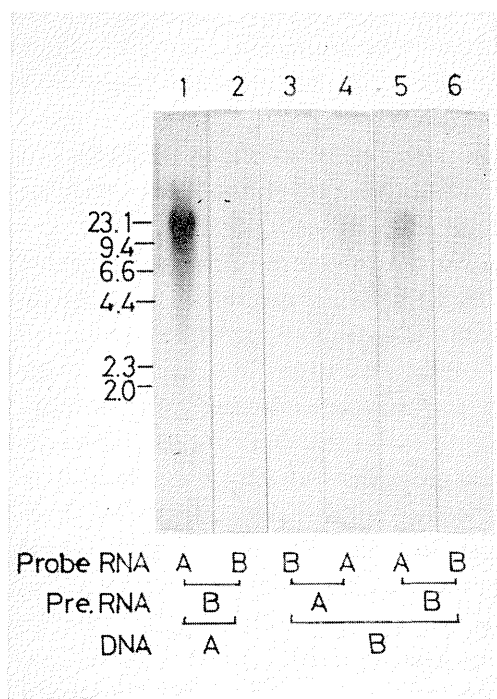


Fig. 1. Competitive hybridization of RNA from mycelia of isolates 15A (A) and 15B (B) with DNA from both mycelia. DNA from each isolate was digested with *Bam*HI, electrophoresed in a 0.8% agarose gel and transferred to GeneScreen membrane. DNA on each lane was prehybridized with unlabeled RNA (Pre. RNA) and then hybridized with 32 P-labeled RNA (Probe RNA) as indicated in the figure. The membrane was washed for removing free probe RNA and subjected to autoradiography. The sizes of molecular weight standards (*Hind*III-digested λ DNA) are given in kilobases.

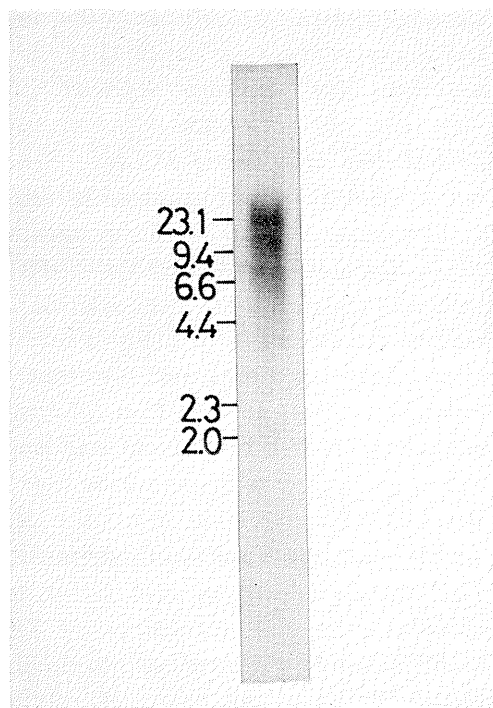


Fig. 2. Competitive hybridization of RNA of isolate 15B (Prehybridization) and labeled first strand (Probe DNA) synthesized by reverse transcriptase with poly(A)⁺RNA and [α - 32 P] dCTP with DNA of isolate 15A. DNA of isolate 15A was digested with *Bam*HI, electrophoresed in a 0.8% agarose gel and transferred to GeneScreen membrane. The membrane was washed for removing free probe DNA and subjected to autoradiography. The sizes of molecular weight standards (*Hind*III-digested λ DNA) are given in kilobases.

was prehybridized with unlabeled RNA of isolate 15B and then hybridized with the labeled first strand. The same pattern of autoradiogram as observed in lane 1 in Fig. 1 was obtained. Thus, it was evident that poly(A)⁺RNA fraction contained the unique mRNA present only in isolate 15A but not in isolate 15B.

E. coli RR1 cells were transformed by recombinant plasmids including cDNA. Two sets of replica nitrocellulose filters containing lysed colonies were prehybridized with unlabeled RNA of isolate 15B. One set was subsequently hybridized with 32 P-labeled RNA of isolate 15A and the other was done with that of isolate 15B as a control. Among 586 tetracycline-resistant transformants, eight colonies showed positive signals by the competitive hybridization (Fig. 3). They seemed to be transformed with plasmids containing 15A-specific cDNA, which were named pABP series of plasmid. Four colonies

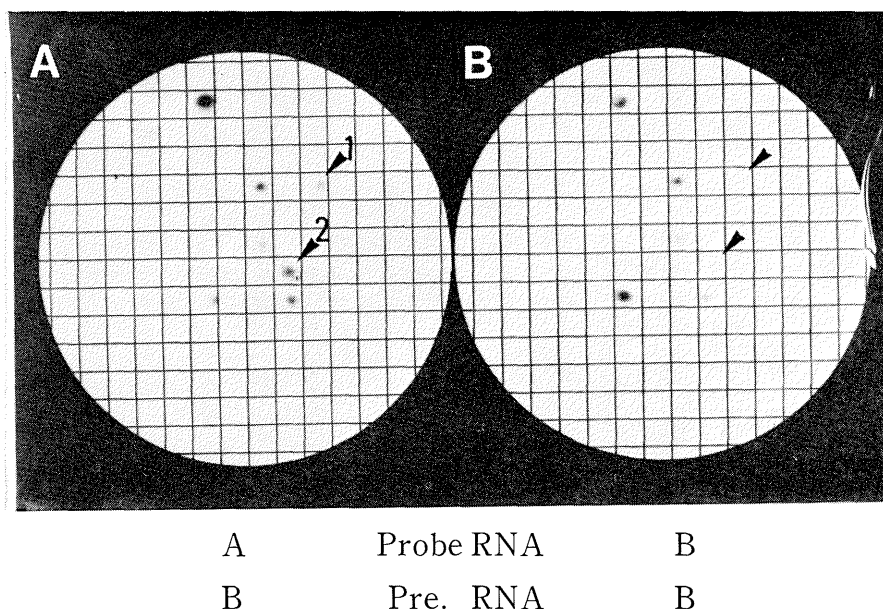


Fig. 3. Competitive colony hybridization of RNAs from isolates 15A and 15B with cDNA cloned in *E. coli*. *E. coli* RR1 cells were transformed by recombinant plasmids composed of cDNA and pBR322. Colonies resistant to tetracycline were transferred to two replica nitrocellulose membranes and lysed with NaOH. Both membranes were prehybridized with unlabeled RNA (Pre. RNA) of isolate 15B. Subsequently, left membrane (A) was hybridized with ^{32}P -labeled RNA (Probe RNA) of isolate 15A, and right one (B) with that of isolate 15B as a control. The membranes were washed for removing free probe RNA and subjected to autoradiography. Arrows indicated the positive clones (1, pABP1; 2, pABP2) hybridized only with labeled RNA of isolate 15A but not of isolate 15B. Only a pair of filters containing 100 colonies each are shown as an example.

of them were subjected to further analyses.

Analysis of cDNA

Plasmids were prepared from four positive clones, and the lengths of the cloned inserts were determined by electrophoresis after digesting the plasmid DNAs by *Pst*I. All of them contained cDNAs of approximately 300 base pairs.

Plasmid DNAs were labeled by nick translation with [α - ^{32}P]dCTP and subjected to hybridization with the filter containing electrophoretically separated restricted DNA fragments of isolate 15A. Labeled pBR322 was also employed as a control. As shown in Fig. 4, cDNAs were hybridized with one or two of the multiple bands observed in lane 1 of Fig. 1. It was suggested that the cDNAs were generated from a group of objective mRNAs which seemed to be transcribed only in isolate 15A.

The level of mRNA homologous to each cDNA in mycelia of several *A. alternata* isolates was determined by dot blot hybridization (Fig. 5). RNA was separately prepared from AK-toxin producers, AK-toxin-less mutants, other host-specific toxin producers and a saprophyte. The mRNA level of each isolate was quantified by densitometrically

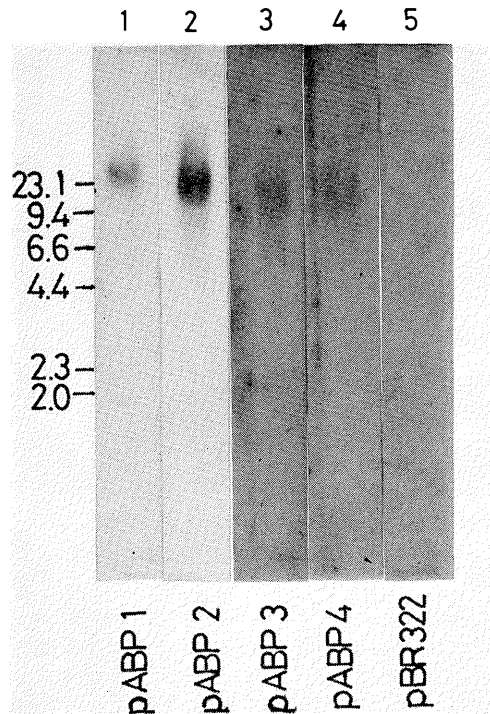


Fig. 4. Hybridization of ^{32}P -labeled plasmids (pABP 1, 2, 3 and 4, and pBR322) with DNA of isolate 15A. The DNA was digested with *Bam*HI, electrophoresed in a 0.8% agarose gel and transferred to GeneScreen membrane. DNA in each lane was hybridized with ^{32}P -labeled plasmids. The membrane was washed for removing free probe DNA and subjected to autoradiography. The sizes of molecular weight standards (*Hind*III-digested λ DNA) are given in kilobases.

scanning. In the case of mRNA homologous to pABP1, isolate 15B possessed approximately half of the mRNA in isolate 15A. The other isolates tested similarly had the mRNA less than 10% of isolate 15A. Isolates A-II-92 was evident to produce AK-toxin, but the amount of the mRNA was only 10% of isolate 15A.

Quantitative analysis of mRNA homologous to pABP2 indicated that all isolates except for two AK-toxin-less mutants, isolates 15B and L-8205, possessed the mRNA more than 70% of isolate 15A. In contrast, the amounts of mRNA in these AK-toxin-less mutants were approximately 10% of isolate 15A.

The distribution of mRNAs homologous to pABP3 and pABP4 was rather similar to pABP1. There was no significant relationship between the mRNA level and AK-toxin productivity in the other isolates.

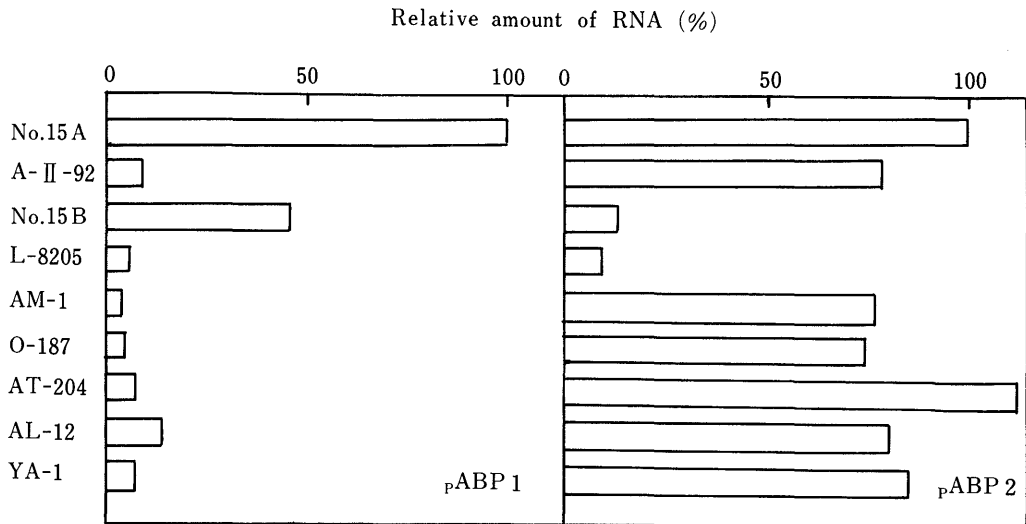


Fig. 5. Relative amount of RNA homologous to pABP1 and pABP2 in nine isolates of *A. alternata*. RNA was isolated from six isolates of five pathotypes, two avirulent mutants of Japanese pear pathotype and one saprophyte of *A. alternata*. Each RNA (10 μ g) was denatured and dot-blotted on GeneScreen membrane after a series of dilution and hybridized with 32 P-labeled pABP1 or pABP2. The membrane was washed for removing free probe DNA and subjected to autoradiography. The densities of dots on developed X-ray film were measured with a densitometer. Relative amount of the RNA is represented as the percentage of isolate 15A.

Discussion

AK-toxin-less mutant, isolate 15B, is similar to the parental isolate 15A in many features, *e. g.*, the color, shape, growth and conidiation of mycelia cultured on agar or in liquid medium, and the color, shape, germination and appressorium formation of spores. They can be distinguished only in AK-toxin productivity and pathogenicity directed by AK-toxin production. Thus, the mutation from isolate 15A to isolate 15B seems to have occurred only in a site of genome for AK-toxin production, although the mutation in other sites may not completely be eliminated. In addition, certain transcripts from DNA sequences were detected only in isolate 15A. The transcripts seemed to hybridize with DNA of not only isolate 15A but also isolate 15B. These results suggested that undetectable change of DNA sequences occurred in a gene of isolate 15A and caused AK-toxin-less mutation.

Consequently, the construction of cDNAs from mRNAs in a AK-toxin producer is the first step for revealing genes responsible for the toxin production. The cDNA, which was synthesized from mRNA of isolate 15A and screened by competitive colony hybridization, was expected to hybridize with RNA only from AK-toxin producers in dot blot hybridization experiment. However, mRNAs homologous to the cDNAs existed in all the used isolates with quantitative differences. The amounts of mRNAs homologous to the cDNAs were considerably less in isolate 15B than in isolate 15A. The mRNAs homologous to pABP1, 3 and 4 were not considered to play important role in AK-toxin

production, because isolate A-II-92, another AK-toxin producer, contained mRNAs homologous to those cDNAs at lower amount than isolate 15B. pABP2 was different from the other. The level of mRNA homologous to pABP2 was significantly low only in AK-toxin-less mutants. This result suggests that our AK-toxin-less mutants might lose their toxin productivity by considerable decrease of a certain enzyme involved in toxin production.

Competitive hybridization method is useful in comparative analysis of RNA and screening cDNA, even if enzymes as the products of genes for toxin production were not purified as those for AK-toxin. HST has been considered to be a secondary metabolite in plant pathogenic filamentous fungi. For detecting transcripts for all enzymes involved in the metabolism, it is essential to make a larger size of cDNA library. The achievement of this cDNA library of mRNAs in *A. alternata* will provide considerable progress in studies on genes for toxin production and its expression.

Literature cited

1. Bogorad, L., Gubbins, E. J., Krebbers, N., Larrinua, I. M., Mulligan, B. J., Muskavitch, K. M. T., Orr, E. A., Rodermeil, S. R., Schantz, R., Steinmetz, A. A., Vos, G. D. and Ye, Y. K. (1983). *Methods Enzymol.* 97 : 524-554.
2. Gardner, J. M., Kono, Y., Tatum, J. H., Suzuki, Y. and Takeuchi, S. (1985). *Phytochemistry* 24 : 2861-2867.
3. Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982). *In Molecular Cloning*, Cold Spring-Harbour Laboratory, New York.
4. Nakatsuka, S., Goto, T., Kohmoto, K. and Nishimura, S. (1986). *In Natural Products and Biological Activities*, A Naito Foundation Symposium (Imura, H., Goto, T., Murachi, T. and Nakajima, T. eds.). University of Tokyo Press, Elsevier Science Publishers. pp. 11-18.
5. Nakatsuka, S., Ueda, K., Goto, T., Yamamoto, M., Nishimura, S. and Kohmoto, K. (1986). *Tetrahedron Lett.* 27 : 2753-2756.
6. Nishimura, S. and Kohmoto, K. (1983). *Ann. Rev. Phytopathol.* 21 : 87-116.
7. Nishimura, S. and Kohmoto, K. (1983). *In Toxin and Plant Pathogenesis* (Daly, J. M. and Deverall, B. J. eds.). Academic Press Australia, Sydney, pp. 137-157.
8. Nishimura, S., Kohmoto, K., Otani, H., Ramachandran, P. and Tamura, F. (1982). *In Plant Infection: The Physiological and Biochemical Basis* (Asada, Y., Bushnell, W. R., Ouchi, S. and Vance, C. P. eds.). Japan Scientific Societies Press, Tokyo. pp. 199-213.
9. Southern, E. M. (1975). *J. Mol. Biol.* 98 : 503-517.
10. Tsuge, T., Hayashi, N. and Nishimura, S. (1986). *Ann. Phytopath. Soc. Japan* 52 : 488-491.

和 文 摘 要

柘植尚志・小林裕和・西村正陽 : *Alternaria alternata* 群病原菌における宿主特異的毒素生成の制御(4)
AK毒素生成菌にのみ発現される遺伝子のクローニング

宿主特異的毒素(以下 HST)依存の病原性を示す *A. alternata* 群病原菌では、完全世代が発見されておらず、病原性を支配する HST 生成の遺伝解析はなされていない。そこで、ナシ黒斑病菌(AK毒素生成菌) No. 15A 菌株とその毒素生成能失活株 No. 15B 菌株を用い、遺伝子レベルでの比較検討を行なった。両株菌体より DNA および RNA を調製し、それらの相同性を利用したコンペティティブハイブリダイゼーションを行なった結果、15A 菌株のみに特定の RNA が多量に存在することが明らかとなった。なお、この RNA は、15B 菌株の DNA に対しても相補性を持つ mRNA であることを確認した。そこで、15A 菌株の cDNA クローニングを試みた。その全 RNA からポリ(A)+RNA を調製し、cDNA を作成した。これを pBR322 の *Pst*I 部位に挿入して、大腸菌 RR 1 菌株を形質転換した。得られた586の形質転換体から、15A 菌株および15B 菌株の RNA によるコンペティティブコロニーハイブリダイゼーションにより、8個のポジティブコロ

ーンを選抜した。各種 *A. alternata* 菌株を用いて、プラスミドに組み込まれた cDNA と相補性を持つ mRNA 量を、そのうち 4 クローンについて検定した。その結果、AK 毒素生成菌株、その他の HST 生成菌株および腐生的菌株に比べ、AK 毒素生成能失活菌株においてのみ、著しく転写量の少ない mRNA が見いだされた。