

Xanthomonas campestris pv. citriにおけるavrBs3遺伝子相同性領域の塩基配列の比較およびかいよう形成能との関係

誌名	日本植物病理學會報 = Annals of the Phytopathological Society of Japan
ISSN	00319473
著者名	金森, 裕之 露無, 慎二
発行元	日本植物病理學會
巻/号	64巻5号
巻号補足	
掲載ページ	p. 462-470
発行年月	1998年10月

農林水産省 農林水産技術会議事務局筑波産学連携支援センター
Tsukuba Business-Academia Cooperation Support Center, Agriculture, Forestry and Fisheries Research Council
Secretariat



Comparison of Nucleotide Sequences of Canker-forming and Non-canker-forming *pthA* Homologues in *Xanthomonas campestris* pv. *citri*

Hiroyuki KANAMORI* and Shinji TSUYUMU**

Abstract

Three DNA regions in *Xanthomonas campestris* pv. *citri* which were homologous to the *avrBs3* gene family commonly found in *Xanthomonas* spp. were cloned and their nucleotide sequences were determined. The sequences from 247 bp upstream of the translational start to 111 bp downstream from the termination site of the open reading frames in these regions were identical except in the tandem repeats of 102 bp in the middle of their coding regions. Thus, they contain the same leucine zipper, nuclear localizing signals and inverted repeats as noted by De Feyter *et al.*⁴⁾. Two of them enabled a nonpathogenic mutant missing one of these *avrBs3* homologous regions to form a canker, but the remaining one did not even 30 days after inoculation. Comparison of the tandem repeats provided us more information on its organization required for canker formation.

(Received April 30, 1998 ; Accepted July 6, 1998)

Key words : *avrBs3* family, *pthA*, citrus canker, *Xanthomonas campestris* pv. *citri*, inverted repeats, nuclear localizing signal.

INTRODUCTION

According to the Gene-for-gene theory⁵⁾, the specific interaction between an avirulence gene of the pathogen and a resistance gene of the resistant plant leads to the hypersensitive reaction, a defense reaction. Staskawicz *et al.*¹¹⁾ first cloned an avirulence gene (*avrA*) from *Pseudomonas syringae* pv. *glycinea* race 6. Since then, many *avr* genes have been isolated from different plant pathogenic bacteria. Many avirulence genes of different *Xanthomonas* species have been reported to show a high overall homology with each other (called the *avrBs3* gene family), and they share a unique structure possessing 15 to 30 tandem repeats of 102 bp in the central parts of these genes^{3,4,7)}. The analysis of the mutants with a partial deletion in these repeating units indicated that this region has a crucial role in the induction of cultivar-specific hypersensitive reaction in the incompatible plants⁶⁾.

A gene of *X. c.* pv. *citri* which shows high overall homology to *avrBs3* gene family has been reported to be responsible for canker formation on the host plant. When this gene was introduced into a weak pathogen of citrus *X. c.* pv. *citrumelo*, the transformant developed a canker. This canker-forming gene of *X. c.* pv. *citri* was named *pthA*^{12,13)}. Previously, we found three copies of

pthA-homologues on two out of three plasmids in *X. c.* pv. *citri* strain NA-1¹⁵⁾. To study the evolutionary and pathological significance of possessing three similar *pthA*-homologues, we have cloned these homologues and compared their ability to form cankers.

MATERIALS AND METHODS

Bacterial strains, plasmids and media The bacterial strains and plasmids are listed in Table 1. *Escherichia coli*, DH5 α , was the cloning host throughout this study, was cultured in Luria-Bertani (LB) medium⁸⁾ at 37°C. The strains of *X. c.* pv. *citri* were cultured in YP medium (0.5% yeast extract, 1% peptone, pH 6.8) at 27°C. For solid medium, 1.5% agar was added. The final concentrations of the antibiotics incorporated into media were 75 μ g/ml for kanamycin (Km), 50 μ g/ml for nalidixic acid (Nal) and 25 μ g/ml for tetracycline (Tet) to select resistant strains (Table 1).

Recombinant DNA techniques All DNA manipulations of DNA, including isolation of total DNA, the alkaline method for plasmid purification, DNA purification by equilibrium centrifugation in CsCl-Ethidium bromide gradient, alkaline phosphatase treatment, gel electrophoresis, size fractionation in sucrose gradient ultracentrifugation, ligation and restriction map construction followed as described by Maniatis *et al.*⁹⁾ and

* Gifu Union of Graduate Course (Shizuoka University), 836 Ohya, Shizuoka 422-8529, Japan 岐阜大学大学院連合農学研究科 (静岡大学)

** Faculty of Agriculture, Shizuoka University, 836 Ohya, Shizuoka 422-8529, Japan 静岡大学農学部

by Ausbel *et al.*²⁾. *In vitro* λ packaging and purification of the bands from the agarose gel followed the methods described by the manufacturers (Stratagene and Qiagen Co.).

For Southern blot analysis, total bacterial DNA was digested to completion with restriction endonucleases, and the fragments were separated by electrophoresis on agarose gel. DNA bands were transferred to a nylon membrane (Micron Separations) and incubated in the presence of the ³²P-labeled DNA oligomer (GGCAAGC-AGGCGCTGGAGACGGTG), RU2, as the probe. The sequence of RU2 was chosen from the conserved region within the tandem repeats of *avrBs3* gene family of *Xanthomonas* spp.^{3,4,7,12)}. Hybridization was performed at 65°C for 12–18 hr in a rotary oven as described by Maniatis *et al.*⁹⁾. The nylon membrane was wrapped in Saran™ wrap, and autoradiography was done at room temperature onto Hyperfilm-MP (Amersham) in the cassette with an X-ray intensifying screen.

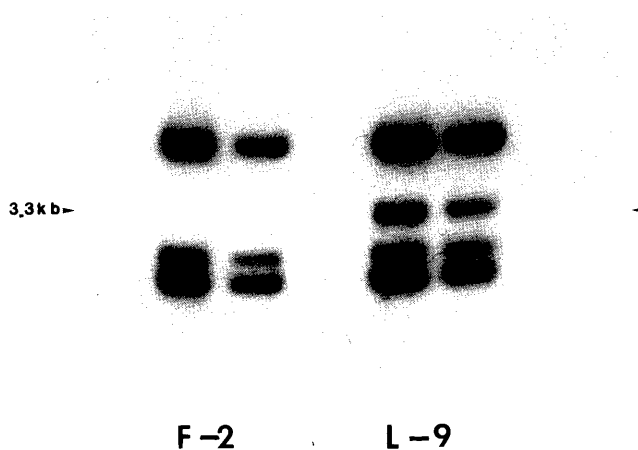


Fig. 1. Southern analysis of *Bam*HI fragments of *X. c. pv. citri* wild type strain (L-9) and nonpathogenic mutant (F-2) using RU2 as the probe. The 3.3 kb band is missing in the F-2 mutant (indicated by arrow).

Construction of a genomic library Total DNA from NA-1 strain of *X. c. pv. citri* was partially digested with *Sau*3AI. Treated DNA was fractionated on a sucrose gradient to obtain fragments larger than 25 kb. After treatment with alkaline phosphatase and subsequent ligation to the *Bam*HI site on the cosmid vector pLAFR3¹⁰⁾, the DNA was packaged *in vitro* with a Gigapack Plus packaging kit (Stratagene). The pack-

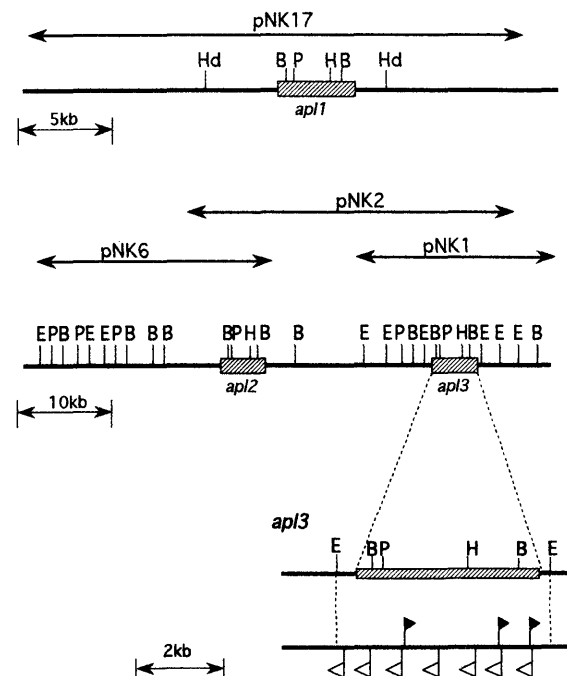


Fig. 2. Restriction maps of the regions containing *apl* genes. The sites for the insertion of Tn3-Spice in APL3 are indicated with triangle flags. The direction of the insertions from 5' to 3' (over the line) and reverse (under the line) are shown. Positive (▲) and negative (△) ice-nucleation activity as a result of these insertions are also shown. B, *Bam*HI; E, *Eco*RI; H, *Hinc*II; Hd, *Hind*III; P, *Pst*I.

Table 1. Bacterial strains and plasmids used in this study

Designation	Relevant characteristic	Reference of source
<i>Xanthomonas campestris</i> pv. <i>citri</i>		
L-9	Nal ^r	This study
NA-1	wild type	This study
F-2	Tn5 inserted pathogenicity deficient mutant of L-9	Tsuyumu <i>et al.</i> ¹⁵⁾
<i>Escherichia coli</i>		
DH5 α	<i>F</i> -, <i>endA</i> 1, <i>hsdR</i> 17(<i>rk</i> + <i>mk</i> -), <i>supE</i> 44, <i>thi</i> -1, <i>recA</i> 1, <i>gyrA</i> 96, <i>relA</i> 1, ϕ 80 <i>dlacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169	BRL Co.
Plasmids		
pLAFR3	<i>IncP</i> , <i>cos</i> , <i>rlx</i> , <i>tet</i> ^r , cosmid vector	Staskawicz <i>et al.</i> ¹¹⁾
pHSG298	pMB1, <i>lacZ</i> , Km ^r	Takara Co.
Spice-Tn3	<i>inaZ</i> , Ap ^r , Sp ^r , Sm ^r	Lindgren <i>et al.</i> ⁸⁾
pShee	<i>tnpA</i> , pACYC replicon, Cm ^r	Lindgren <i>et al.</i> ⁸⁾
pNK1	pLAFR3 clone of 17 kb DNA fragment containing <i>apl3</i> from NA-1	This study
pNK2	pLAFR3 clone of 27 kb DNA fragment containing <i>apl2</i> and <i>apl3</i> from NA-1	This study
pNK6	pLAFR3 clone of 20 kb DNA fragment containing <i>apl2</i> from NA-1	This study
pNK17	pLAFR3 clone of 30 kb DNA fragment containing <i>apl1</i> from NA-1	This study

aged phage particles were used to infect *E. coli* DH5 α . The transformants were selected by their resistance to Tet (25 μ g/ml) and formation of white colonies on an X-gal plate.

Presence of tandem repeats Subclone of 3.1 kb,

3.3 kb and the 3.9 kb RU2-homologous *Bam*HI fragments in the vector pHSG298 were used to check the existence of tandem repeats commonly found in the *avrBs3/pthA* gene family of *Xanthomonas* spp. Because all of the tested *avrBs3* gene family contains single *Bal*I

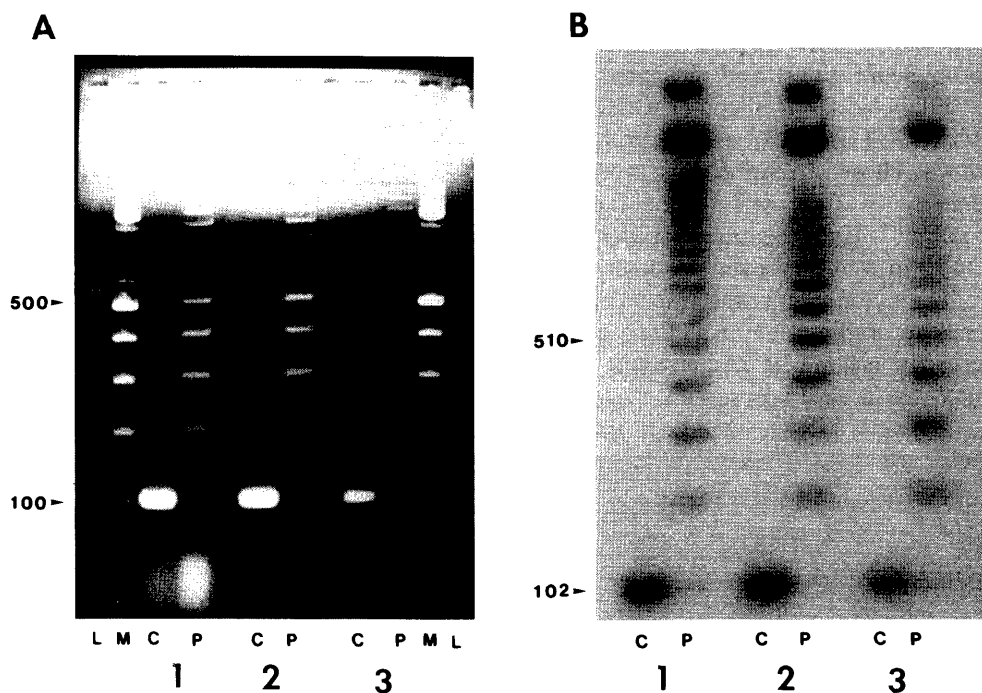


Fig. 3. Ladder formations after the partial digestion of three *avrBs3*-homologous regions with *Bal*I. Electrophoretic (A) and hybridization patterns using RU2 as the probe (B) are shown. Fragments of both ends are lambda-*Hind*III (L). Second and next to the last lanes are 100 bp-ladder marker (M). Digestion with *Bal*I was done either completely (C) or partially (P). The tested regions were *apl1* (1), *apl2* (2) and *apl3* (3). The numbers on the left show length of fragments (bp).

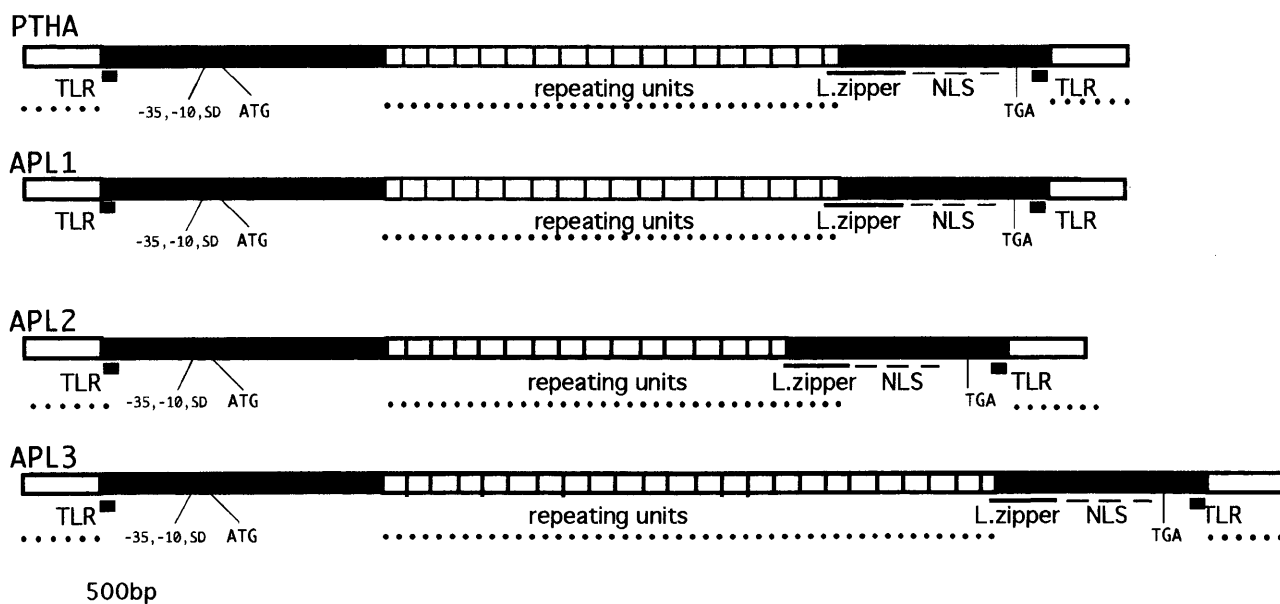


Fig. 4. Comparison of characteristic regions in PTHA, APL1, APL2 and APL3. A difference was observed only in the tandem repeating regions (dotted lines). Each single box in tandem repeating regions indicates one repeating unit (34 amino acids). TLR (terminal long repeat), -35 and -10 region of σ 70-type promoter, SD (Shine-Dalgarno sequence), ATG (translational starting site), tandem repeats region, L. zipper (leucine zipper), NLS (nuclear localizing signal) and TGA (translational terminating site) are shown.

sites in each repeat, these clones were partially digested with *Bal*I at 37°C for 1 hr. The fragments were separated on a 2% agarose gel followed by Southern analysis using RU2 as the probe.

DNA sequencing A deletion series of the RU2-homologous *Bam*HI fragments and the subclones from these fragments were used to determine the primary structure of the DNA. The dideoxy sequencing method of Sanger *et al.*¹⁰⁾ was performed with Sequitherm Cycle-sequencing Kit (Epicenter Technology) using an automatic DNA sequencer (LI-COR). Base image IR software Ver. 2.10 (LI-COR) and GENETYX (Software

Developing Co.) were used to analyze the sequences. Homology and other searches were done via DDBJ (DNA data base of Japan).

Electroporation *X. c. pv. citri* cells were harvested when the absorbance at 600 nm reached 0.6. The cells were then washed four times by centrifugation in sterilized distilled water and resuspended in 30% glycerol at a cellular density of about 2×10^9 cfu/ml. Plasmid DNA (3 μ g) was added to a 40 μ l suspension of the competent cells. Electroporation was performed using an Electro square porator (BTX, T820FE.) with 10 pulses of 99 μ sec at 3 kV. The treated cells were added to fresh YP-medi-

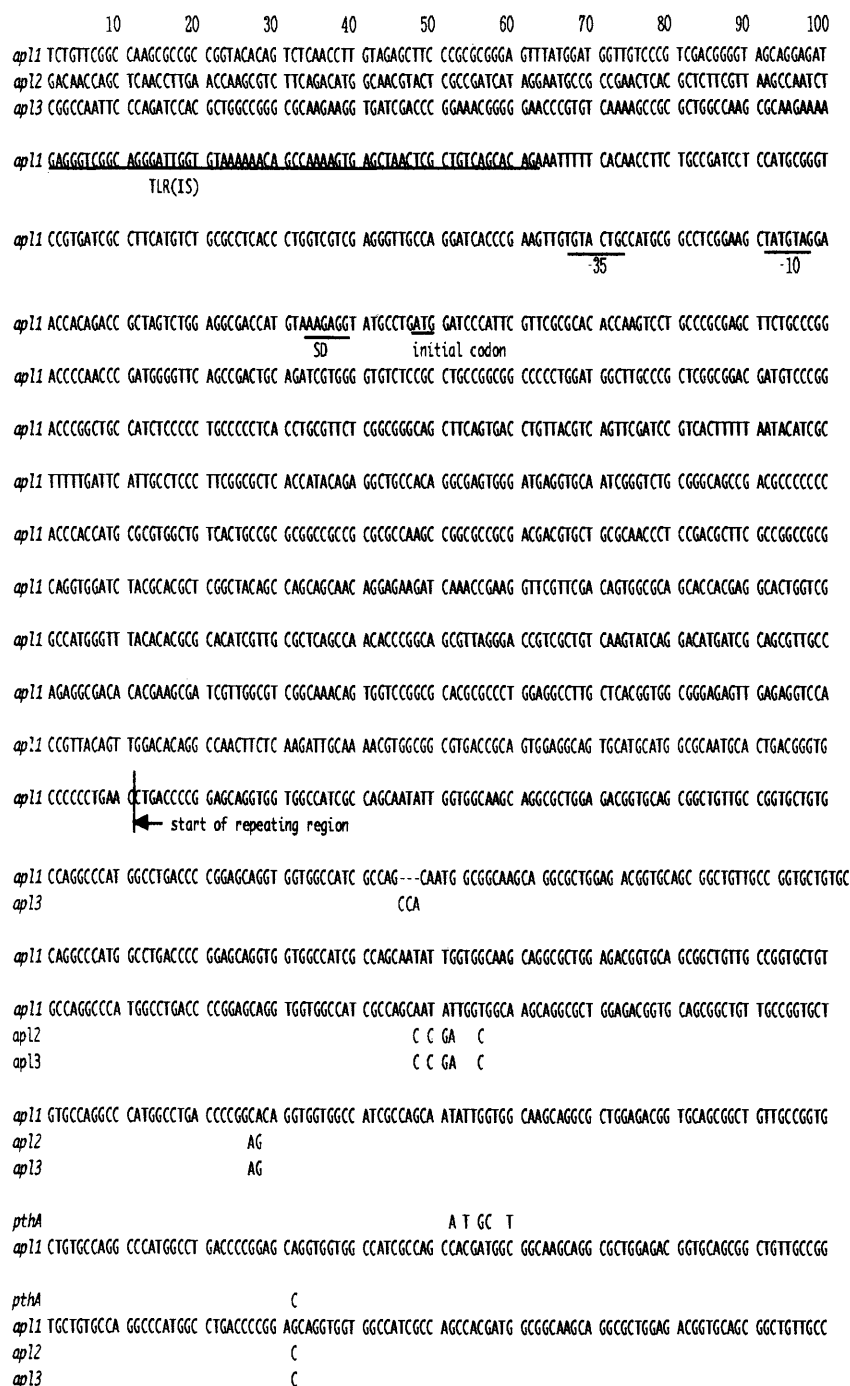


Fig. 5 (1)

um and incubated at 27°C for more than 1 hr. Transformants were selected on YP-agar plates containing Tet and Km at 27°C.

Inoculation tests Peeled seeds of *Citrus natsudaidai* HAYATA were planted in a 15 cm diameter pot and cultivated in a growth chamber for more than 14 days, then in a greenhouse. A suspension of bacteria (*ca.* 1.0×10^8 cfu/ml) in sterile distilled water was infiltrated into

the intercellular spaces of the leaves of about 2-month-old seedlings using a sterile syringe and needle (20 G). To prevent the loss of transformed cosmids, 25 µg/ml Tet was added to the bacterial suspension. The inoculated leaves were wrapped with aluminum foil to prevent Tet being degraded by light. After inoculation, plants were incubated in growth chambers at 27°C, and the symptoms were checked periodically.

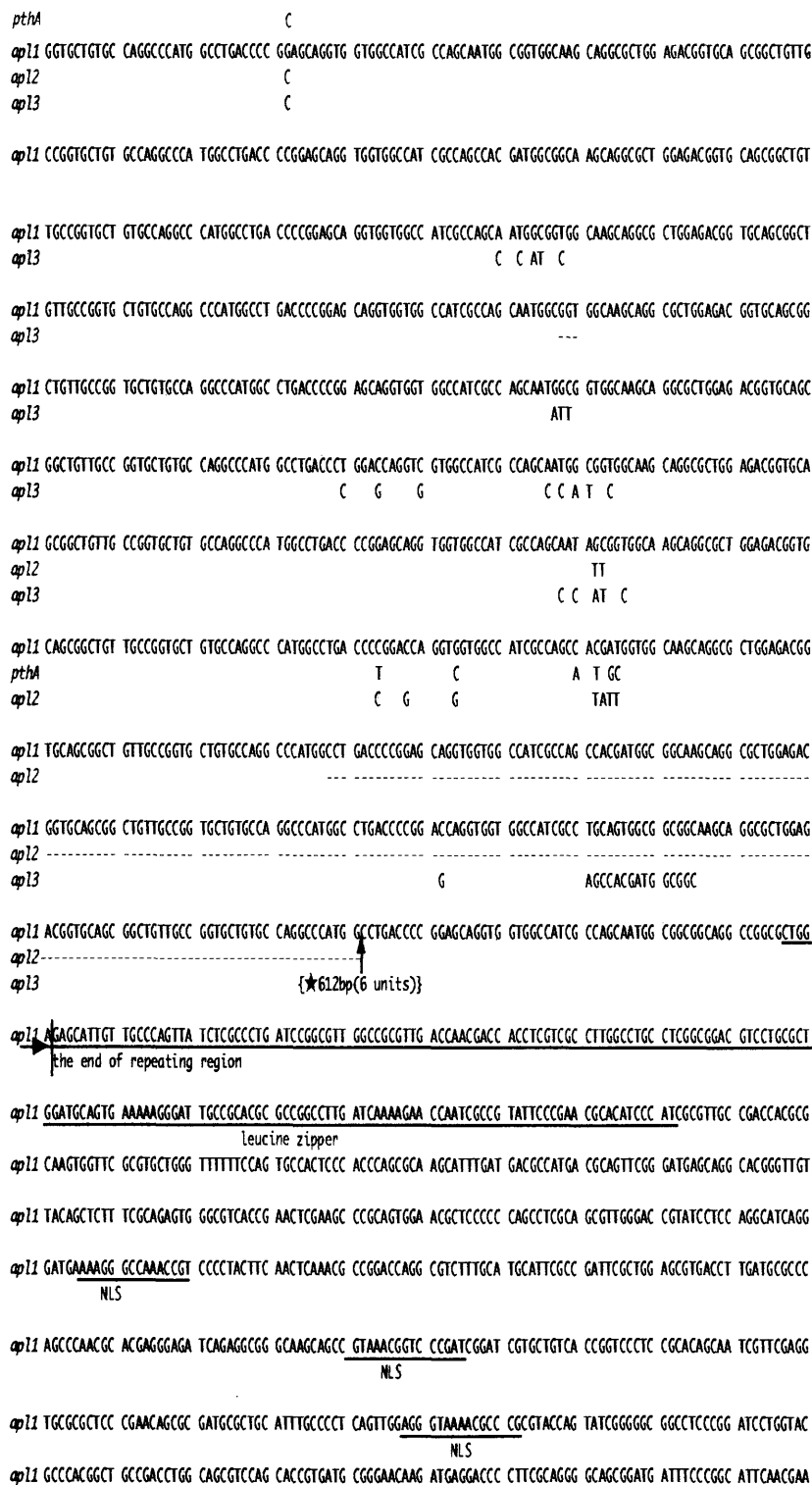


Fig. 5 (2)

RESULTS

Isolation of cosmid clones containing RU2-homologous regions

Restriction enzyme analysis of 30 randomly selected clones from a pLAFR3 library of *X. c. pv. citri* NA-1 DNA revealed insertion of random DNA fragments with an average DNA insert size of 25 kb (data not shown). Fifteen clones of 2100 transformants contained regions that hybridized with the probe RU2.

Presence of tandem repeats in RU2-homologous regions

Southern analysis of DNA from NA-1 strain showed that three *Bam*HI fragments (3.1 kb, 3.3 kb, 3.9 kb) hybridized with RU2 (Fig. 1). The cosmid clone pNK2 contained *Bam*HI fragments of 3.1 kb and 3.9 kb, and another clone pNK17 contained a *Bam*HI fragment of 3.3 kb. Restriction maps of the area covered with these clones are shown in Fig. 2. The presence of a single *Bal*I cleavage site in the repeating units has been reported in the *Xanthomonas avrBs3* genes^{4,7)}.

The presence of similar tandem repeats in each RU2 homolog was confirmed by observing of ladder formation of *ca.* 100 bp DNA fragments on agarose gel after partial digestion with *Bal*I and by Southern blot hybridization using RU2 as the probe (Fig. 3). Thus, all of the 3.3, 3.1 and 3.9 kb *Bam*HI fragments were considered to contain the *avrBs3/pthA* gene family of *Xanthomonas* spp. These fragments will be called APL (*avr/pth*-like) 1, APL2 and APL3, respectively (Figs. 1-3).

Table 2. Comparison of the deduced amino acid sequences downstream of the tandem repeats in PthA, Apl1, Apl2 and Apl3

No.	var. block1				var. block2			
	PthA	Apl1	Apl2	Apl3	PthA	Apl1	Apl2	Apl3
1	PE	PE	PE	PE	SNI	SNI	SNI	SNI
2	PE	PE	PE	PE	SN-	SN-	SN-	SHG
3	PE	PE	PE	PE	SNI	SNI	SNI	SNI
4	PE	PE	PE	PE	SNI	SNI	SHD	SHD
5	PA	PA	PE	PE	SNI	SNI	SNI	SHD
6	PE	PE	PE	PE	SNG	SHD	SHD	SHD
7	PD	PE	PD	PD	SHD	SHD	SHD	SHD
8	PQ	PE	PQ	PQ	SNG	SNG	SNG	SNG
9	PE	PE	PE	PE	SHD	SHD	SHD	SHD
10	PE	PE	PE	PE	SNG	SNG	SNG	SHD
11	PE	PE	PE	PE	SNG	SNG	SNG	SN-
12	PE	PE	PE	PA	SNG	SNG	SNG	SNI
13	LD	LD	LD	PE	SNG	SNG	SNG	SHD
14	PE	PE	PE	PE	SNS	SNS	SNI	SHD
15	PD	PD	PE	PE	SHD	SHD	SHD	SNI
16	PE	PE	PE	PE	SHD	SHD	SNG	SHD
17	PE	PE		PD	CNG	CNG		SHD
18	PE	PE		PE	SNG	SNG		SNG
19				PE				SHG
20				PE				SNG
21				PE				SNI
22				PE				SHG
23				PE				CNG
24				PE				SNG

a) No : position of repeat unit.

b) Variable block1 : 3rd and 4th amino acids in each repeating unit expressed with single letter.

c) Variable block2 : 11th, 12th and 13th amino acid.

apl1 GAGGAGCTCG CATGTTGAT GGAGTATTG CCTAGTGAG GCTCAGTCGG TGACTACCTG AGCGTCGGCA GGGATTGGTG TAAGTAACCT TTACTGACAG
Terminal codon

apl1 CGAGTTAGCC CACTTTTGGC TGTTTTTAC ACAATCCCT GCCTCCCTCC ACCTGGGCA CAGTCCCTC GGTGCGCATC AATCGATGCA CCGATGGCGA
LTR(1S)

apl2 GGGG TTCGGGGAGC AATGGAACAG GGAAGTCAGT TAAGCCTTCA CCAGTGCCGG TCTGTACCTT CCATGCGGGC GTGGCTCTCT
apl3 TCTCCTCTCT GCGGGCTAGC AGTAGCAATG GAACAGGGAA GTACAGTAGG

apl1 CTGCAACGTC ACCAGATCGG TAGATCC

apl2 GAGAAACAGT GCGGTCATC CGACCG

apl3 ATAAACCTT TCATTGCCAT AATGAGC

★612bp(6 units)

CTGACCCCGG AGCAGGTGGT GGCCATCGCC AGCAATGGCG GTGGCAAGCA GGCGCTGGAG ACGGTGCAGC GGCTGTTGCC GGTGCTGTGC CAGGCCCATG
GCTGACCCC GGAGCAGGTG GTGGCATCG CCAGCCACGA TGGCGGCAAG CAGGCGCTGG AGACGCTGCA GCGGCTGTTG CCGGTGCTGT GCCAGGCCA
TGGCTGTACC CCGGAGCAGG TGGTGGCCAT CGCCAGCAAT GGCCTGGCA AGCAGGCGCT GGAGACGGTG CAGCGGCTGT TGGCGGTGCT GTGCCAGGC
CATGGCCTGA CCGCGAGCA GGTGGTGGCC ATCGCCAGCA ATATTGGTGG CAAGCAGGCG CTGGAGACGG TGCAGCGGCT GTTGCCTGTG CTGTGCCAGG
CCATGGCCT GACCCCGGAG CAGTGTGTGG CCATGCCAG CCACGATGGC GGCAAGCAGG CGCTGGAGAC GGTGCAGCGG CTGTGCGCGG TGCTGTGCCA
GGCCCATGGC CTGACCCCGG AGCAGGTGGT GGCCATCGCC TGCAATGGCG GTGGCAAGCA GGCGCTGGAG ACGGTGCAGC GGCTGTTGCC GGTGCTGTGC
CAGGCCCATG GC

Fig. 5 (3)

Fig. 5. Comparison of nucleotide sequences of *apl1*, *apl2*, *apl3* and *pthA*. The whole sequences around *apl1* are shown, whereas those of *apl2*, *apl3* and *pthA* are shown only where a difference was observed in comparison to those in *apl1*. The number of the repeats in *apl2* was two units shorter than that of *apl1*. The one in *apl3* was 6 units longer than in *apl1*, and the sequence of these six repeats is shown in the last part of that figure (*). The TLR, -35 and -10 region of σ 70-type promoter, ATG, tandem repeats region, L. zipper, NLS and TGA are indicated.

Complementation of nonpathogenic mutant

A mutant, F-2, which lost its pathogenicity to *C. natsudaidai*, was found out to have also lost the 3.3 kb *avrBs3* homologous *Bam*HI fragment (Fig. 1). An F-2 transformant with pNK17 (containing only APL1) was reverted to show pathogenicity on *C. natsudaidai* in 7 days as in the case of inoculation with the wild type. F-2 transformants with pNK6 (containing only APL2) and with pNK2 (containing both APL2 and APL3) developed the canker symptom but only after 14–26 days. On the other hand, the F-2 transformant with pNK1 (containing

only APL3) never had a canker symptom even 30 days after inoculation. Thus, APL1 complemented completely and APL2 only partially complemented the F-2 mutant, but APL3 did not (Plate I).

Nucleotide sequences of APL regions

The nucleotide sequences of APL1, APL2 and APL3 were determined in both directions using deletion series and subclones from pNK2, pNK6, pNK1 and pNK17. Major open reading frames containing 1163 nt (*apl1*) in APL1, 1095 nt (*apl2*) in APL2 and 1367nt (*apl3*) in APL3 were found (Fig. 6). Long terminal repeats (LTR) which

<i>pthA</i>	249	TCTGTTTCGGCCAAGCGCCGCCGTACACAGTCTCAACCTTGTAGAGCTTC
<i>apl1</i>	1	TCTGTTTCGGCCAAGCGCCGCCGTACACAGTCTCAACCTTGTAGAGCTTC
<i>apl2</i>	1	GACAACCAGTCAACCTTGAACCAAGCGTCTTCAGACATGGCAACGTACT
<i>apl3</i>	1	CGGCCAATTCACAGATCCACGCTGGCCGGCGCAAGAAGGTGATCGACCC
<i>pthA</i>	298	CCGCGCGGGAGTTTATGGATGGTGTCCCGTCGACGGGTAGCAGGAGAT
<i>apl1</i>	51	CCGCGCGGGAGTTTATGGATGGTGTCCCGTCGACGGGTAGCAGGAGAT
<i>apl2</i>	51	CGCCGATCATAGGAATGCKGCCGAACACAGCTCTTCGTTAAGCCAATCT
<i>apl3</i>	51	GGAAACGGGGGAACCCGTGTCAAAAGCCGCGCTGGCCAAGCGCAAGAAAA
<i>pthA</i>	349	GAGGGTCGGCAGGGATTGGTGTAATAAACAGCCAAAAGTGAGCTAACTCG
<i>apl1</i>	101	GAGGGTCGGCAGGGATTGGTGTAATAAACAGCCAAAAGTGAGCTAACTCG
<i>apl2</i>	101	GAGGGTCGGCAGGGATTGGTGTAATAAACAGCCAAAAGTGAGCTAACTCG
<i>apl3</i>	101	GAGGGTCGGCAGGGATTGGTGTAATAAACAGCCAAAAGTGAGCTAACTCG
<i>pthA</i>	399	CTGTCAGCACAGAAATTTTTCACAACCTTCTGCCGATCCTCCATGCGGGT
<i>apl1</i>	151	CTGTCAGCACAGAAATTTTTCACAACCTTCTGCCGATCCTCCATGCGGGT
<i>apl2</i>	151	CTGTCAGCACAGAAATTTTTCACAACCTTCTGCCGATCCTCCATGCGGGT
<i>apl3</i>	151	CTGTCAGCACAGAAATTTTTCACAACCTTCTGCCGATCCTCCATGCGGGT
<i>pthA</i>	4076	TTGCCTCAGTGAGGCTCAGTCGGTGACTACCTGAGCGTCGGCAGGGATTG
<i>apl1</i>	3828	TTGCCTCAGTGAGGCTCAGTCGGTGACTACCTGAGCGTCGGCAGGGATTG
<i>apl2</i>	3624	TTGCCTCAGTGAGGCTCAGTCGGTGACTACCTGAGCGTCGGCAGGGATTG
<i>apl3</i>	4440	TTGCCTCAGTGAGGCTCAGTCGGTGACTACCTGAGCGTCGGCAGGGATTG
<i>pthA</i>	4126	GTGTAAGTAACCTTTACTGACAGCGAGTTAGCCACTTTTGGCTGTTTTT
<i>apl1</i>	3878	GTGTAAGTAACCTTTACTGACAGCGAGTTAGCCACTTTTGGCTGTTTTT
<i>apl2</i>	3674	GTGTAAGTAACCTTTACTGACAGCGAGTTAGCCACTTTGGGGTTCGGGG
<i>apl3</i>	4490	GTGTAAGTAACCTTTACTGACAGCGAGTTAGCCACTTTTGGCTGTTTTT
<i>pthA</i>	4176	TACACAAATCCCTGCCTCCCTC CCCTGGGCACGCAGTCCCTCGGTGCGC
<i>apl1</i>	3928	TACACAAATCCCTGCCTCCCTC CCCTGGGCACGCAGTCCCTCGGTGCGC
<i>apl2</i>	3724	AGCAATGGAACAGGGAAGTCAGT TAAGCCTTCACCAGTGCCTGCTGTAC
<i>apl3</i>	4540	TACACAAATCCCTGCCTCCCTC CTCTGCTCTGCGGGCTACGAGTAGCA
<i>pthA</i>	4226	ATCAATCGATGCACCGATGGCGACTGCAACGTCACCAGATCGGTAGATCC
<i>apl1</i>	3978	ATCAATCGATGCACCGATGGCGACTGCAACGTCACCAGATCGGTAGATCC
<i>apl2</i>	3774	CTTCATGCGGGCGTGGCTCTCTGAGAAACAGTGGCGGTATCGGCACCG
<i>apl3</i>	4590	ATGGAACAGGGAAGTCAGTAGGCATAAAACCTTTCATTGCCATAATGAGC

Fig. 6. Comparison of the sequences in upstream and downstream regions of *pthA*, *apl1*, *apl2* and *apl3*. The sequence of the reported inverted repeats found in *X. c. pv. citri* is shown by a single underline. The region homologous to the 5' end of the reported ISXc5 of *X. c. pv. citri* is shown by double underlines. A bold broken line indicates the border line of the inverted repeat.

were first noted in both upstream and downstream regions of *pthA* by De Feyter *et al.*⁴⁾ were also seen in the 5' upstream and 3' downstream regions of *apl1*, *apl2* and *apl3* (Figs. 4, 5 and 6), except the downstream region of *apl1* was interrupted with an insertion sequence previously reported in *X. c. pv. citri* (ISXc5)¹³⁾ at the 27th nt from the 3' end of the LTR. The regions containing *apl1*, *apl2*, *apl3* and *pthA* were otherwise identical except in the tandem repeats. Thus, they contained the same promoter, SD sequences, leucine zipper (LZ), nuclear localization signals (NLSs) and the downstream transcriptional terminators (Figs. 4 and 5). The total number of repeats in both Apl1 and PthA was 17.5, as opposed to 15.5 and 23.5 in Apl2 and Apl3, respectively. The amino acid sequences deduced from the 102 bp tandem repeats within *apl1*, *apl2*, *apl3* and *pthA* were variable only at the 3rd, 4th, 12th and 13th amino acids within each repeat, as pointed out by Yang *et al.*¹⁶⁾ A comparison of the amino acid sequences in each tandem repeat is shown in Table 2. The amino acids in these variable regions were identical to those of *pthA* except in the third repeating unit those of *apl2* and of *apl3* were considerably different from those of *pthA* (Figs. 4-6, Table 2).

DISCUSSION

Previously, we isolated 10 mutants deficient in pathogenicity on *C. Natsudaidai* by mutagenesis with Tn5¹⁵⁾. However, many of them, including F-2, were later shown to be missing the APL1 region (data not shown). Now, these mutants are considered to have lost their pathogenicity because of the missing the APL1 rather than because of the Tn5 insertion. If this is the case, *apl1* is mandatory for canker formation. Because we do not have mutants deficient in either *apl2* or *apl3*, the roles of *apl2* and *apl3* are unclear. However, *E. coli* containing pHIR11, the *hrp* cluster of *Pseudomonas syringae* pv. *syringae*, together with *apl1* could cause the canker symptom on *C. natsudaidai*, but those contained *apl2* or *apl3* could not (to be published elsewhere). Considering these data together with the delayed disease development after inoculation with the transformant of F-2 mutant with *apl2* (Plate I), *apl2* may have only partial canker-inducing ability but *apl3* may have no such activity. Thus, the repeating units common to both *pthA* and *apl1*, and yet distinguishable from those of *apl2* and of *apl3* can be considered to be important for the canker symptom.

The region other than the tandem repeat, including LRR and NLS of PthA, Apl1, Apl2 and Apl3 were completely identical. It has been shown that NLS of *avrBs3* and *pthA* has, in fact, been shown to be responsible for the translocation of their translational products into plant nuclei^{1,17)}. Because NLS identical to those in PthA were found in Apl1, Apl2, Apl3, all of them may be expected to be translocated into plant nuclei. However, the possibility remains that alternation in the tandem

repeats may result in a failure in translocation.

Identical sequences extended 247 bp upstream from their translational starts and 111 bp downstream from the termination codons. Both ends of these regions were identical and formed the inverted repeats as first noted in *pthA* by De Feyter *et al.*⁴⁾. Many of the Tn5-induced mutants deficient in the pathogenicity lost Apl1 might be due to the presence of these inverted repeats. Similar inverted repeats were found in *avrBs3*³⁾ and *avrB6*⁴⁾. The presence of multiple copies of the *avrBs3* gene family in several pathovars of *X. campestris* and *X. oryzae* pv. *oryzae*^{3,4,7,12)} may be explained by such mechanism. Also, such inverted repeats at the ends of this regions should be functional for in the horizontal transfer of these gene family on transmissible plasmids in various *Xanthomonas* spp.

We do not know the significance of the ISXc5 insertion after the 28th base of the downstream inverted repeat of *apl1*. This element was first observed in a plasmid of *X. c. pv. citri* by Tu *et al.*¹⁴⁾. This element may be considered to be mobile in *X. c. pv. citri*.

We thank N.T. Keen for critically reading a rough draft of this manuscript and for sending pLAFR3 and pHIR11, and J. Leach for suggestions on the transformation of *Xanthomonas* species. This work was supported in part by Grants-in-Aid for Scientific Research (Nos. 04660045, 09460026) from the Ministry of Education, Science, Sports and Culture of Japan and by a grant for "Plant Biotechnology" from the Ministry of Agriculture, Forestries and Fishery to S.T.

Literature cited

1. Ackerveken, G.V., Marois, E. and Bonas, U. (1996). Recognition of the bacterial avirulence protein *AvrBs3* occurs inside the host plant cell. *Cell* 87: 1307-1316.
2. Ausbel, F.M., Brend, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1987). *Current Protocols in Molecular Biology*, John Wiley & Sons, New York.
3. Bonas, U., Stall, P.E. and Staskawicz, B. (1989). Genetic and structural characterization of the avirulence gene *avrBs3* from *Xanthomonas campestris* pv. *vesicatoria*. *Mol. Gen. Genet.* 218: 127-136.
4. De Feyter, R., Yang, Y. and Gabriel, D.W. (1993). Gene-for-gene interaction between cotton R genes and *Xanthomonas campestris* pv. *malvacearum* *avr* genes. *Mol. Plant-Microbe Interact.* 2: 225-237.
5. Flor, H. (1955). Host-parasite interaction in flux rust. *J. Bacteriol.* 45: 680-685.
6. Herbers, K., Strauch, J.C. and Bonas, U. (1992). Race-specificity of plant resistance to bacterial spot disease determined by repetitive motif in a bacterial avirulence protein. *Nature* 356: 172-174.
7. Hopkins, C.M., White, F.F., Choi, S.H., Guo, A. and Leach, J.E. (1992). Identification of a family of avirulence genes from *Xanthomonas oryzae* pv. *oryzae*. *Mol. Plant-Microbe Interact.* 6: 451-459.
8. Lindgren, P.B., Frederick, R., Govindarajan, A.G., Panopoulos, N.J., Staskawicz, B.J. and Lindow, S.E. (1987).

- An ice nucleation reporter gene system: identification of inducible pathogenicity genes in *Pseudomonas syringae* pv. *phaseolicola*. EMBO J. 8: 1291-1301.
9. Maniatis, T., Sambrook, J. and Fritsch, E.F. (1989). Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 10. Sanger, F., Nicklens, S. and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 71: 1342-1346.
 11. Staskawicz, B., Daulbeck, D., Keen, N.T. and Napoli, C. (1987). Molecular characterization of cloned avirulence genes from race 0 and race1 of *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. 169: 5789-5794.
 12. Swarup, S., De Feyter, R., Bransky, R.H. and Gabriel, D.W. (1991). A pathogenicity locus from *Xanthomonas citri* enable strains from several pathovars of elicit cancer-like lesion on citrus. Phytopathology 81: 802-809.
 13. Swarup, S., Yang, Y., Kingsley, M.T. and Gabriel, D.W. (1992). An *Xanthomonas citri* pathogenicity gene, *pthA*, pleiotropically encodes gratuitous avirulence on nonhost. Mol. Plant-Microbe Interact. 3: 204-213.
 14. Tu, J., Wang, H.R., Chang, S.F., Chang, Y.C., Lurz, R., Dobrinski, B. and Wu, W.C. (1989). Transposable elements of *Xanthomonas campestris* pv. *citri* originating from indigenous plasmids. Mol. Gen. Genet. 217: 505-510.
 15. Tsuyumu, S., Takikawa, Y., Furutani, N., Tanaka, K., Kanamori, H., Kondou, Y. and Nagai, H. (1996). Factors involved in the pathogenicity of *Xanthomonas campestris* pv. *citri*. In Molecular Aspects of Pathogenicity and Resistance (Mills, D. et al. eds.), APS Press, Minnesota, pp. 105-114.
 16. Yang, Y. and Gabriel, D.W. (1995). Intragenic recombination of a single plant pathogen gene provides a mechanism for the evolution of new host specificities. J. Bacteriol. 177: 4963-4968.
 17. Yang, Y. and Gabriel, D.W. (1995). *Xanthomonas* avirulence/pathogenicity gene family encodes functional plant nuclear targeting signals. Mol. Plant-Microbe Interact. 8: 627-631.

和文摘要

金森裕之・露無慎二： *Xanthomonas campestris* pv. *citri* における *avrBs3* 遺伝子相同性領域の塩基配列の比較およびかいよう形成能との関係

カンキツかいよう病菌において、*Xanthomonas* 属菌の *avrBs3* 遺伝子ファミリー特有の構造をもつ3つの領域をクローニングし、塩基配列を決定した。これら領域のうち2つは病徴発現までの日数は異なるもののカンキツ葉におけるかいよう形成能をもつが、1つにはなかった。また、これら3つの *avrBs3* 相同領域間および既報の *pthA* との違いは構造領域内の 102 bp を1単位とした繰返し配列のみに存在した。したがって、カンキツ葉でのかいよう形成に必要とされる本領域の繰返し配列における情報が得られた。また、上記3領域は、開始コドンの上流 247 bp から、終止コドンの下流 111 bp までにおよんでいた。すなわち、これら領域は De Feyter らによって報告されている LZ (Leucine zipper), NLS (Nuclear Localization Signals), 逆方向反復配列 (LTR) を共通してもっていた。前述の3領域の塩基配列は、これら領域が細胞内で容易に移動可能であることを示唆した。

Plate I

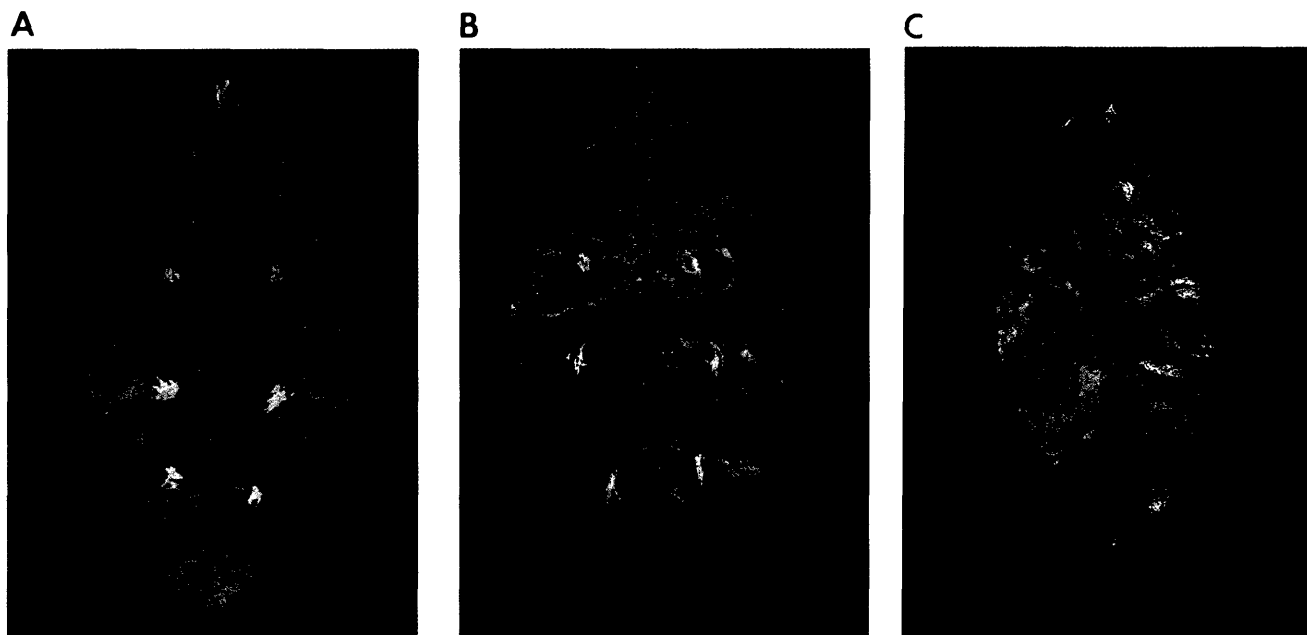


Plate I. Symptoms on *Citrus natsudaidai* after inoculation with wild type and the transformants of F-2 with each APLs. The symptoms appeared 5 days, 7 days and 21 days after inoculation with wild type, transformants of pNK17(*apl1*)(C) and of pNK6(*apl2*)(B), respectively. No symptoms appeared even 30 days after inoculation with the transformants of pNK1(*apl3*)(A). Photographs were taken when symptoms appeared. (A) and (B), top: wild type (L-9); middle and bottom: right-mutant (F-2), left-transformant. (C) top: wild type (L-9); middle and bottom: right-transformant, left-mutant (F-2).