JOURNAL OF BACTERIOLOGY, Sept. 1995, p. 4963–4968
0021-9193/95/$04.00+0

Vol. 177, No. 17

Intragenic Recombination of a Single Plant Pathogen Gene Provides a Mechanism for the Evolution of New Host Specificities†

YINONG YANG‡ AND DEAN W. GABRIEL*

Plant Molecular and Cellular Biology Program and Plant Pathology Department, University of Florida, Gainesville, Florida 32611

Received 14 June 1994/Accepted 21 June 1995

Gene *pthA* is required for virulence of *Xanthomonas citri* on citrus plants and has pleiotropic pathogenicity and avirulence functions when transferred to many different xanthomonads. DNA sequencing revealed that *pthA* belongs to a family of *Xanthomonas* avirulence/pathogenicity genes characterized by nearly identical 102-bp tandem repeats in the central region. By inserting an *nptI-sac* cartridge into the tandemly repeated region of *pthA* as a selective marker, intragenic recombination among homologous repeats was observed in both *Xanthomonas* spp. and *Escherichia coli*. Intragenic recombination within *pthA* created new genes with novel host specificities and altered pathogenicity and/or avirulence phenotypes. Many *pthA* recombinants gained or lost avirulence function in pathogenicity assays on bean, citrus, and cotton cultivars. Although the ability to induce cell division (hyperplastic cankers) on citrus could be lost, this ability was not acquired on cotton or bean plants. Intragenic recombination therefore provides a genetic mechanism for the generation of multiple, different, and gratuitous avirulence genes from a single, required, host-specific pathogenicity gene.

In gene-for-gene interactions between plants and microbial plant pathogens, host plant resistance results from the genetic recognition of resistance (R) genes in the plant and avirulence (*avr*) genes in the pathogen (10, 17). It is unknown why microbial plant pathogens carry genes that function to limit virulence, since the majority of these genes are apparently dispensable (11). Numerous studies of pathogen *avr* genes have revealed spontaneous loss-of-function mutations (15, 20). Not surprisingly, these mutations can involve gene deletion (8) or transposon insertion (16). Mutational analyses also reveal spontaneous gain-of-function mutations that generate new avirulence specificities and new pathogen races at approximately the same frequency as loss-of-function mutations (28). Although presumably an *avr* gene may be reactivated by reverse mutation, there are no reports of natural mechanisms whereby new avirulence specificities, pathogen races, or *avr* genes are generated.

Xanthomonas citri is the causal agent of citrus canker disease, which occurs worldwide and is subject to eradication and quarantine regulations in many countries. A pathogenicity gene, *pthA*, is essential for the pathogen to cause hyperplastic canker symptoms on citrus (29). Furthermore, when transferred to other xanthomonads, *pthA* confers the ability to induce hyperplastic cankers on citrus and a hypersensitive response (HR) on other hosts (30). Therefore, *pthA* exhibits pleiotropic pathogenicity and avirulence functions. Southern hybridization, restriction analysis, and partial DNA sequencing have shown that *pthA* belongs to a major *avr* gene family widespread in the genus *Xanthomonas* (30). Members of this gene family include *avrBs3* and *avrBs3-2* of *Xanthomonas campestris* pv. vesicatoria (2, 3); *avrB4*, *avrb6*, *avrb7*, *avrBIn*, *avrB101*, and *avrB102* of *X. campestris* pv. malvacearum (5, 8); and *avrXa10* and *avrXa7* of *Xanthomonas oryzae* pv. oryzae (14). Among these, only *avrb6* and *pthA* are known to have pleiotropic pathogenicity functions (5, 30, 35). The most striking feature of this gene family is the presence of nearly identical 102-bp tandem repeats in the central portion of the genes. Deletion analyses have shown that the avirulence specificity of *avrBs3* is determined by the 102-bp repetitive motifs (13). By exchanging the internal repeated regions between *pthA* and *avrb6*, the 102-bp tandem repeats were found to determine both avirulence and pathogenic specificities (35).

In most organisms, repetitive regions of DNA are known to be active sites for homologous recombination (1, 22). The high frequency of such events causes them to be major genome-modifying forces in evolution. Intergenic recombination among members of the *avr-pth* gene family was therefore proposed to explain the high frequency of race change mutation found in *X. campestris* pv. malvacearum (8). In this study, we have analyzed the nucleotide sequence of *pthA* and demonstrated that intragenic recombination can also account for the creation of new *avr-pth* genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and matings. *Escherichia coli* strains DH5α (Gibco-BRL, Gaithersburg, Md.) and JM83 (37) and derivatives were grown in Luria-Bertani medium (24) at 37°C. *X. citri* 3213 (causes hyperplastic cankers on citrus) (29, 30), *X. campestris* pv. citrumelo 3048 (causes water-soaked leaf spots on citrus and common bean) (29, 30), *X. campestris* pv. malvacearum Xcm1003 (causes water-soaked leaf spots on cotton) (5), and derivatives were grown in PYGM (peptone-yeast extract-glycerol-MOPS [morpholinepropanesulfonic acid]) medium at 30°C (7). Antibiotics were used as described previously (35). Triparental matings were carried out to transfer plasmids from *E. coli* DH5α to various *Xanthomonas* strains by using pRK2013 or pRK2073 as a helper plasmid as described elsewhere (5, 29). To transfer plasmids into Xcm1003, the modifier plasmid pUFR054 carrying *Xcm*I and *Xcm*III methylase genes was used to increase the transfer frequency (6).

DNA sequence analysis. The 4.1-kb *Sal*I fragment carrying *pthA* in pZit45 (30) was recloned into PGEM 7Zf(+) vector (Promega, Madison, Wis.) to yield pUFY14.5. Sets of overlapping, unidirectional deletion subclones were generated in pUFY14.5 by using exonuclease III and mung bean nuclease as described by Promega. DNA sequencing was performed by the DNA Sequencing Core Laboratory of the Interdisciplinary Center for Biotechnology Research, University of Florida, Gainesville. Some sequencing was determined by using the dideoxy nucleotide chain termination technique with the Amersham (Arlington Heights, Ill.) multiwell microtiter plate DNA-sequencing system RPN1590. The sequence was analyzed by using the GCG package (version 7.00) from Genetics Computer Group, Inc., Madison, Wis.

Gene replacement and selection of marker-evicted strains. Gene *pthA* on pZit45 was interrupted by ligating a 3.9-kb *Bam*HI fragment containing an *nptI-sac* cartridge (24), after Klenow end filling, to *Bal*I-linearized pZit45. The

* Corresponding author. Phone: (904) 392-7239. Fax: (904) 392-6532.

† Paper R-03784 of the Florida Agricultural Experiment Station Journal Series.

‡ Present address: Waksman Institute, Piscataway, NJ 08855.

resulting plasmids were screened by restriction analyses for location of the *nptI-sac* cartridge within *pthA*. An *Sst*I fragment carrying the *pthA*::*nptI-sac* fusion from one of these plasmids (pUFY1.48) was recloned into suicide vector pUFR004 (ColE1 *mobP*$^+$ *cat*$^+$ [16]), forming pUFY10.1. Marker exchange mutant Xc1.2 (*pthA*::*nptI-sac*) was created by transferring pUFR10.1 to *X. citri* 3213 and selecting for colonies resistant to kanamycin (15 μg/ml) and sensitive to chloramphenicol (35 μg/ml) and sucrose (5%). Marker-evicted strains of *X. citri* were selected by plating Xc1.2 on PYGM medium containing 5% sucrose and selecting colonies resistant to sucrose. The colonies were then screened for sensitivity to kanamycin. Recombinant, marker-evicted derivatives of pUFY1.48 were selected in *X. campestris* pv. citrumelo 3048, *E. coli* DH5α (*recA*), and *E. coli* JM83 (*recA*$^+$) by plating on Luria-Bertani medium containing 5% sucrose. Sucrose-resistant colonies were screened for sensitivity to kanamycin.

To calculate recombination frequencies, Xc1.2, 3048/pUFY1.48, DH5α/pUFY1.48, and JM83/pUFY1.48 were grown in liquid media in the presence of appropriate antibiotics to a density of 10^9 cells per ml. Serial dilutions were then plated on media with and without 5% sucrose. Recombination frequencies were calculated by dividing the number of sucrose-resistant (also kanamycin-sensitive) colonies by the number of colonies grown on the medium without sucrose selection (34). Data shown in Results are the means and standard errors of four replicates obtained from two independent experiments.

Plant inoculations. Citrus (*Citrus paradisi* 'Duncan', grapefruit), common bean (*Phaseolus vulgaris* 'California Light Red'), and cotton (*Gossypium hirsutum* L.) plants were grown under greenhouse conditions. Cotton lines used were Acala-44 (Ac44) and its congenic resistance lines AcB1, AcB2, AcB4, AcB5, Acb6, Acb7, AcBIn, and AcBIn3 as described previously (8, 30). Plant inoculations involving *X. citri* or *pthA* or derivatives of *pthA* were carried out in BL-3P level containment (refer to Federal Register vol. 52, no. 154, 1987) at the Division of Plant Industry, Florida Department of Agriculture, Gainesville. Bacterial suspensions were standardized in sterile tap water to 10^8 CFU/ml and pressure infiltrated into the abaxial leaf surfaces of the plants. Plant inoculations were repeated at least three times on citrus and twice on bean and cotton plants.

Nucleotide sequence accession number. The nucleotide sequence of *pthA* has been submitted to GenBank and assigned accession no. U28802.

RESULTS

Sequence analysis of *pthA*. The complete DNA sequence of *pthA* was determined, and sequence comparisons revealed that this *X. citri* gene is 98% homologous to *avrBs3* and *avrBs3-2* of *X. campestris* pv. vesicatoria (2, 3), 97% homologous to *avrb6* of *X. campestris* pv. malvacearum (8), and 95% homologous to *avrXa10* of *X. oryzae* (14) along its entire length. As observed with all previously sequenced members of the gene family (8), *pthA* is flanked by nearly identical 62-bp terminal inverted repeats that precisely define the limits of homology. The predicted amino acid sequence encoded by *pthA* is presented in Fig. 1. PthA has a calculated molecular mass of 122 kDa and an isoelectric point of 7.72. PthA is a leucine-rich protein; leucine accounts for 12.3% of the total (143 of 1,163) amino acids. In the 34-amino-acid tandem repeat region, leucine accounts for 14.7% of the total (88 of 597) amino acids.

Gene replacement and selection of *pthA* recombinants in *X. citri*. Plasmid pUFY10.1 carried the *nptI-sac* cartridge inserted into repeat 10 of *pthA*. *X. citri* mutant Xc1.2 (*pthA*::*nptI-sac*) was generated from wild-type strain 3213 by marker exchange using pUFY10.1. Marker exchange was confirmed by Southern hybridization analyses using an internal fragment of *pthA* as a probe (Fig. 2, compare lanes 1 and 2) and using the *nptI-sac* cartridge as a probe (not shown). When inoculated on plants, Xc1.2 was unable to induce cankers on citrus or to elicit an HR on bean (Table 1).

By plating Xc1.2 on a sucrose-containing medium and screening for sucrose-resistant and kanamycin-sensitive colonies, many marker-evicted strains of *X. citri* were obtained at a frequency of $(5.22 \pm 1.8) \times 10^{-5}$. To examine the recombination events, total DNAs were extracted from 24 marker-evicted derivatives of Xc1.2, digested with *Bam*HI, and probed with a ^{32}P-labelled internal *Stu*I-*Hin*cII fragment (containing the tandemly repeated region) of *pthA*. As shown in Fig. 2 (lanes 3 to 18), the derivatives of Xc1.2 each exhibited a new band, including some that were smaller than *pthA*, some that were larger than *pthA*, and some that were similar in size. In one case (lane 8, XcS20), there appeared to be a gene duplication event. There was no change in the apparent size of any of the other three fragments that hybridize with *pthA* in any derivative tested, indicating that intergenic recombination had not occurred.

```
MDPIRSRTPS PARELLPGPQ PDGVQPTADR GVSPPAGGPL DGLPARRTMS
RTRLPSPPAP SPAFSAGSFS DLLRQFDPSL FNTSLFDSLP PFGAHHTEAA
TGEWDEVQSG LRAADAPPPT MRVAVTAARP PRAKPAPRRR AAQPSDASPA
AQVDLRTLGY SQQQQEKIKP KVRSTVAQHH EALVGHGFTH AHIVALSQHP
AALGTVAVKY QDMIAALPEA THEAIVGVGK QWSGARALEA LLTVAGELRG
PPLQLDTGQL LKIAKRGGVT AVEAVHAWRN ALTGAPLN
LTPEQVVAIA SNIGGKQALE TVQRLLPVLC QAHG      1 (A)
LTPEQVVAIA SN_GGKQALE TVQRLLPVLC QAHG      2 (B) *
LTPEQVVAIA SNIGGKQALE TVQRLLPVLC QAHG      3 (A)
LTPEQVVAIA SNIGGKQALE TVQRLLPVLC QAHG      4 (A)
LTPAQVVAIA SNIGGKQALE TVQRLLPVLC QAHG      5 (C) *
LTPEQVVAIA SNGGGKQALE TVQRLLPVLC QAHG      6 (D)
LTPDQVVAIA SHDGGKQALE TVQRLLPVLC QAHG      7 (E) *
LTPQQVVAIA SNGGGKQALE TVQRLLPVLC QAHG      8 (F)
LTPEQVVAIA SHDGGKQALE TVQRLLPVLC QAHG      9 (G)
LTPEQVVAIA SNGGGKQALE TVQRLLPVLC QAHG     10 (D)
LTPEQVVAIA SNGGGKQALE TVQRLLPVLC QAHG     11 (D)
LTPEQVVAIA SNGGGKQALE TVQRLLPVLC QAHG     12 (D)
LTLDQVVAIA SNGGGKQALE TVQRLLPVLC QAHG     13 (H) *
LTPEQVVAIA SNSGGKQALE TVQRLLPVLC QAHG     14 (I)
LTPDQVVAIA SHDGGKQALE TVQRLLPVLC QAHG     15 (E) *
LTPEQVVAIA SHDGGKQALE TVQRLLPVLC QAHG     16 (G)
LTPEQVVAIA CNGGGKQALE TVQRLLPVLC QAHG     17 (J) *
LTPEQVVAIA SNGGGRPALE SIVAQLSRPD PALA     18 (K)
ALTNDHLVAL ACLGGRPALD AVKKGLPHAP ALIKRTNRRI PERTSHRVAD
HAQVVRVLGF FQCHSHPAQA FDDAMTQFGM SRHGLLQLFR RVGVTELEAR
SGTLPPASQR WDRILQASGM KRAKPSPTST QTPDQASLHA FADSLERDLD
APSPTHEGDQ RRASSRKRSR SDRAVTGPSA QQSFEVRAPE QRDALHLPLS
WRVKRPRTSI GGGLPDPGTP TAADLAASST VMREQDEDPF AGAADDFPAF
NEEELAWLME LLPQ*
```

FIG. 1. Predicted amino acid sequence of PthA. The 34-amino-acid, leucine-rich, tandem repeats are aligned and numbered for comparison. Letters A through K represent different repeat sequences. Repeat sequences unique (to date) to the PthA protein (*) and the qualifying amino acids (underlined) are indicated.

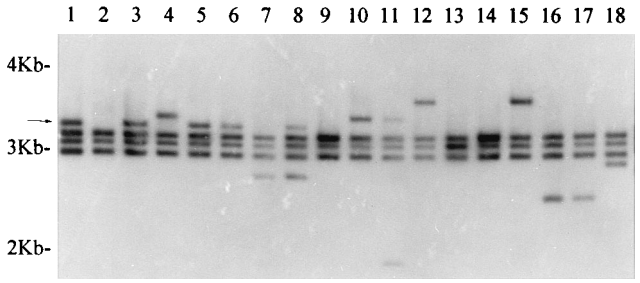


FIG. 2. Southern hybridization of *Bam*HI-digested, total DNA from *X. citri* 3213 and its *pthA* mutant derivatives. The blot was probed with the internal 2-kb *Stu*I-*Hin*cII fragment (containing the tandemly repeated region) of *pthA*. Strain 3213 carries four *Bam*HI fragments which hybridize to *pthA*; the largest fragment (arrow) encodes *pthA*. Not shown in lane 2 are two additional hybridizing fragments (1.8 and 1.6 kb) resulting from the additional *Bam*HI site in *nptI-sac*. Lane 1, 3213; lane 2, Xc1.2 (*pthA*::*nptI-sac*); lanes 3 to 18, the following *nptI-sac*-evicted derivatives of Xc1.2: XcS1, XcS5, XcS9, XcS14, XcS17, XcS20, XcS2, XcS3, XcS7, XcS8, XcS16, XcS19, XcS22, XcS13, XcS24, and XcS16, respectively.

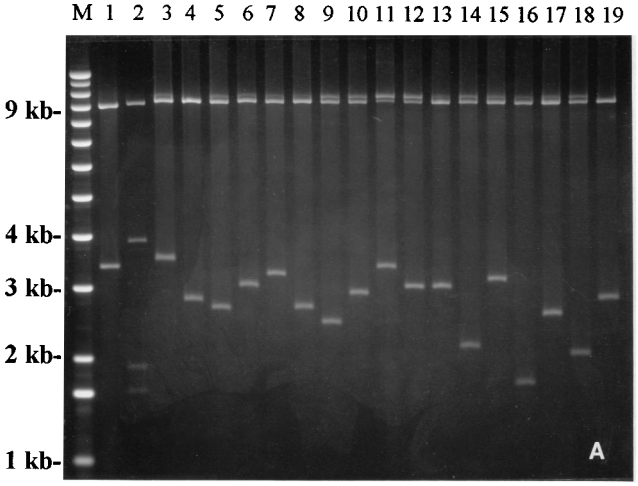
The 24 marker-evicted derivatives of Xc1.2 were inoculated into citrus and bean plants. They exhibited different plant reaction phenotypes, including some entirely new phenotypes, distinct from that of the wild-type 3213 strain (Table 1). Five derivatives reverted to the parental 3213 phenotype, inducing strong canker lesions on citrus (XcS1, XcS9, XcS14, and XcS15). Two derivatives caused weak cankers (XcS17 and XcS20). Six derivatives elicited a previously unreported HR on grapefruit (XcS6, XcS7, XcS11, XcS12, XcS13, and XcS24). Eleven derivatives caused no pathogenic symptoms. In contrast to wild-type strain 3213, XcS5 retained the ability to elicit hyperplastic cankers on citrus plants but lost the ability to elicit an HR on bean plants, indicating that these two pleiotropic functions are separately encoded on *pthA*.

Intragenic recombination of *pthA* in *X. campestris* and *E. coli*. Although there was no evidence of recombination between *pthA* and the other three DNA fragments that hybridize with *pthA* in Xc1.2, the presence of the other three fragments raised the possibility of intergenic recombination in this strain. To avoid this possibility, plasmid pUFY1.48 was introduced into three bacterial strains which carry no DNA that hybridizes with *pthA*: *X. campestris* pv. citrumelo 3048, *E. coli* DH5α (*recA*), and *E. coli* JM83 (*recA*$^+$). Transconjugants 3048/pUFY1.48, DH5α/pUFY1.48, and JM83/pUFY1.48 were plated on sucrose-containing medium and screened for marker eviction. Again, many marker-evicted derivatives were obtained. The frequencies of marker eviction of pUFY1.48 in strains 3048, DH5α, and JM83 were $(4.1 \pm 0.4) \times 10^{-4}$, $(1.1 \pm 0.1) \times 10^{-2}$, and $(1.0 \pm 0.1) \times 10^{-2}$, respectively.

TABLE 1. Phenotypes of *X. citri* marker-evicted strains on citrus and bean plants

Strain(s)	Phenotype on the indicated host[a]	
	Citrus	Bean
Xc3213	C	HR
Xc1.2	0	0
XcS1, XcS9, XcS14, and XcS15	C	HR
XcS5	C	0
XcS17 and XcS20	wC	wHR
XcS12, XcS13, and XcS24	HR	wHR
XcS7	wHR	wHR
XcS6 and XcS11	wHR	0
XcS2[b]	0	0

[a] C, canker lesions; wC, weak canker lesions; wHR, weak HR; 0, no symptoms.
[b] Plus an additional 11 strains: XcS3, XcS4, XcS8, XcS10, XcS16, XcS18, XcS19, XcS20, XcS21, XcS22, and XcS23.



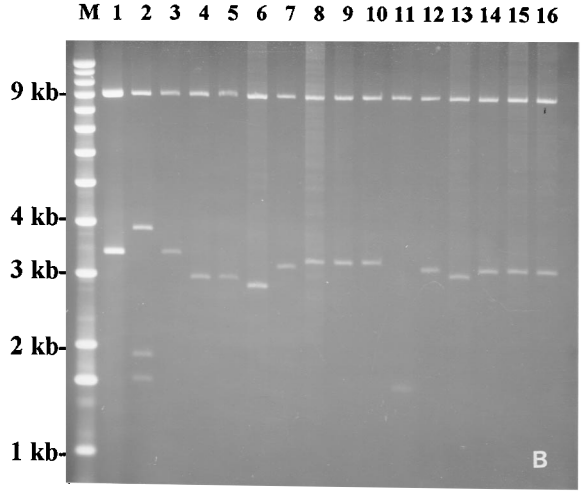


FIG. 3. (A) Intragenic recombinants of *pthA* generated in *X. campestris* pv. citrumelo 3048. Plasmid DNAs were digested with *Bam*HI and fractionated in a 0.7% agarose gel. Lane 1, pZit45 (*pthA*); lane 2, pUFY1.48 (*pthA*::*nptI-sac*); lanes 3 to 19, pCS2, pCS16, pCS13, pCS14, pCS5, pCS6, pCS1, pCS17, pCS23, pCS7, pCS11, pCS12, pCS15, pCS19, pCS21, pCS18, and pCS22, respectively. (B) Intragenic recombinants of *pthA* generated in *E. coli* DH5α. Plasmid DNAs were digested with *Bam*HI and fractionated in a 0.7% agarose gel. Lane 1, pZit45; lane 2, pUFY1.48; lanes 3 to 16, pDS2, pDS5, pDS4, pDS13, pDS1, pDS10, pDS14, pDS9, pDS3, pDS6, pDS7, pDS8, pDS11, and pDS12, respectively. Lanes M, molecular size markers.

To examine the recombination events, plasmid DNAs from 24 marker-evicted strains from 3048 and 14 strains from DH5α were analyzed. As shown in Fig. 3, different-size *Bam*HI fragments were observed, demonstrating the generation of new members of the *avr-pth* family from *pthA* by intragenic recombination.

Separation of Pth and Avr activities and confirmation of homologous recombination. The plasmids derived from the 24 3048 (pCS series) and 14 DH5α (pDS series) marker-evicted strains were individually introduced into *X. citri* B21.2 (a non-

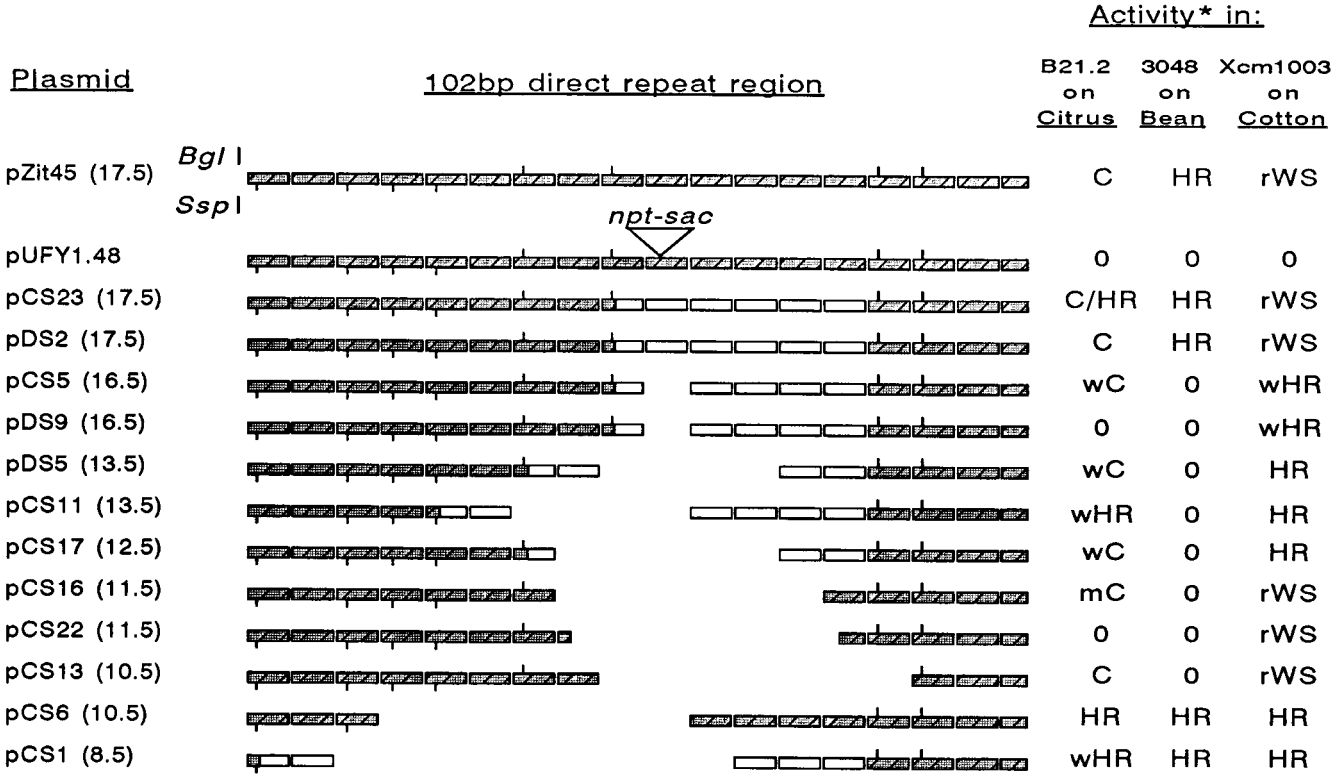


FIG. 4. Biological activity of *pthA* and intragenic recombinants. Only the 102-bp tandem repeat region of the genes on the indicated plasmids are shown; each repeat unit known or assumed present is represented by a solid block. For pCS6, pCS13, pCS16, and pCS22, internal deletions (blank areas) of precise multiples of 102 bp affecting the indicated repeats were determined by DNA sequencing. For the remaining recombinants, restriction enzyme analyses (see text) were used to determine the number of repeats deleted (assumed to be precise multiples of 102 bp); open blocks represent repeats present but not identified. *Bgl*I and *Ssp*I sites are indicated by short vertical lines above and below the blocks. Activity conferred by the plasmid to the indicated strain when inoculated at a high (10^8 CFU/ml) level on the indicated host plant is shown to the right of the figure. (When inoculated without the indicated plasmids, *X. citri* B21.2 [*pthA*::Tn*5-gus*] is asymptomatic on citrus, *X. campestris* pv. citrumelo 3048 elicits water-soaking symptoms on bean, and *X. campestris* pv. malvacearum Xcm1003 elicits water-soaking symptoms on cotton.) 0, no change in plant reaction phenotype; C, wild-type hyperplastic cankers; mC, moderate cankers, smaller than the wild type; wC, very small canker lesions; wHR, weak HR; rWS, reduced water-soaking of Xcm1003 on cotton.

pathogenic, *pthA*::Tn*5-gusA* derivative of 3213 which is virulent only on citrus), *X. campestris* pv. citrumelo 3048 (virulent on citrus and bean), and *X. campestris* pv. malvacearum Xcm1003 (virulent only on cotton). The resulting transconjugants were inoculated onto citrus, bean, and cotton plants (Fig. 4). Similar to the results observed with derivatives of Xc1.2, these *pthA* recombinants exhibited altered phenotypes and/or host specificities. For example, pCS23 conferred to B21.2 and 3048 a stronger ability to cause canker lesions on citrus than that conferred by pZit45 (*pthA*), and a weak HR often preceded the canker symptoms. Unlike *pthA* on pZit45, however, the recombinant gene on pCS13 did not confer the ability to elicit an HR on bean plants to strain 3048. These results demonstrate that the abilities to confer hyperplastic cankers on citrus plants and the HR on bean plants are independently encoded on *pthA*.

Plasmids pCS1 and pCS6 conferred the ability to elicit an HR on citrus and bean plants to B21.2 and 3048, respectively, and the ability to elicit an HR on nine tested cotton lines (Ac44 and eight resistant lines with different resistance genes) to Xcm1003. All *pthA* derivatives tested that conferred to Xcm1003 the ability to induce an HR on susceptible cotton line Ac44 also elicited an HR on all tested cotton resistance lines. The *pthA* derivatives that reduced water-soaking of Xcm1003 on Ac44 also conferred the ability to elicit an HR on one or more of the resistant cotton lines. All *pthA* derivatives tested conferred to Xcm1003 the ability to induce an HR on one or more resistant cotton lines, indicating that all *pthA* derivatives tested were functional genes.

Much of the variation within the predicted 34-amino-acid repeat sequence of *pthA* occurred at positions 12 and 13, and most repeats encoded either asparagine-glycine, asparagine-isoleucine, or histidine-aspartate at these positions. The latter two amino acid combinations in the *pthA* repeats could be detected at the DNA level by the presence of *Ssp*I or *Bgl*I restriction sites, respectively. The *Ssp*I restriction enzyme cut specifically after the 36th nucleotide of the 1st, 3rd, 4th, and 5th repeats of *pthA*, while the *Bgl*I enzyme cut specifically after the 38th nucleotide of the 7th, 9th, 15th, and 16th repeats. By comparing the *Bgl*I and *Ssp*I fragments of intragenic recombinants with those of *pthA*, the specific repeats deleted or affected in 12 recombinants were tentatively determined (Fig. 4). In order to establish the nature of the recombination events and to precisely determine which repeats were affected, the complete DNA sequences of pCS13, pCS16, pCS6, and pCS22 were determined. In all four cases, loss of the *nptI-sac* cartridge involved a precise deletion of a multiple of 102 bp that preserved the 102-bp direct repeat structure characteristic of the gene family. No frameshifts, inversions, or additions were found; these results demonstrated that the recombination events producing the four genes examined were homologous.

Deletions affecting the first eight repeats always reduced or abolished the canker-eliciting ability of *pthA* (e.g., the deletions

in pCS1, pCS6, pCS11, pCS16, and pCS22). However, deletions or recombinations that did not evidently affect the first eight repeats could also reduce or abolish canker (e.g., those in pCS5 and pCS9). This indicated either that the first eight repeats play a role in proper spacing of a canker-active region or that several regions in the repeats play essential roles or both. Deletion of repeats 9 to 15 did not affect the ability to elicit cankers (e.g., pCS13), demonstrating that these repeats do not play an active role in canker elicitation, except perhaps in spacing. Recombination events evidently affecting only repeats 9 to 14 and deleting one repeat resulted in clones with little or no canker-eliciting activity (e.g., pDS9 and pCS5). Interestingly, pCS16 and pCS22, each with 11.5 repeats and varying only in the sequence of a recombinant repeat in position 8, gave two different phenotypes (moderate cankers and no cankers, respectively).

DISCUSSION

The complete DNA sequence of *pthA* revealed that *pthA* is highly similar to *avrb6*, *avrBs3*, *avrBs3-2*, and *avrXa10* along its entire length. Western blot (immunoblot) analyses using anti-PthA serum revealed that the predicted 122-kDa PthA protein was constitutively expressed in wild-type *X. citri* and in *E. coli* and *X. campestris* pv. citrumelo cells carrying pZit45 (*pthA*) (36). The primary region which differentiates *pthA* from other members of the *Xanthomonas avr*/*pth* gene family is the 102-bp direct repeat region. Experiments using chimeric fusions of *pthA* and *avrb6* have demonstrated that the DNA coding regions outside the repeats are isofunctional and that the host-specific pathogenicity and any avirulence phenotypes are determined by the repeats (35). Despite the very few differences in the predicted amino acid sequences of all members of the *avr*/*pth* gene family, *pthA* is the only gene reported to date to induce cell divisions in any plant, in addition to its pleiotropic ability to confer avirulence to pathogens of cotton and bean plants (30).

As shown in Fig. 1, the *pthA* repeats may be classified into 11 types (A to K), depending on slight differences in sequence. Among them, repeat types B, C, E, H, E, and J have been found only in PthA and not in the predicted peptide sequences of the four other published members of the *avr*/*pth* gene family sequenced to date (*avrb6*, *avrBs3*, *avrBs3-2*, and *avrXa10*). In addition to the presence of unique repeats, PthA also differs from its homologs in the arrangement of repeat types that are found in other members of the gene family within the repeat region. An analysis of 12 intragenic recombinants using *Bgl*I and *Ssp*I restriction enzyme digestion and DNA sequencing of four of the recombinants did not allow localization of a specific, canker-active repeat or block of repeats. These findings are consistent with and extend the results of two studies of *avrBs3* and *avrBs3-2* deletion derivatives (generated by restriction digests) which also failed to localize HR activity (2, 13). Furthermore, mutations affecting *pthA* activity on citrus plants (cankers or an HR) did not necessarily affect activity on bean or cotton plants (HR); host specificity and phenotypic plant responses appeared to be determined by different repeat regions. The data are consistent with the interpretation that several regions in the repeats play essential roles in both host specificity and phenotype elicitation.

Since the production of levan sucrase encoded by *sac* is lethal to gram-negative bacteria in the presence of sucrose (24), an *nptI-sac* cartridge was inserted into the middle of the tandemly repeated region of *pthA* to provide selection for recombination events within the repeat region. Homologous intragenic recombinants of *pthA* were generated by single crossover events between intragenic repeats on the same plasmid (*cis* recombination), resulting in deletion of the *nptI-sac* marker and restoration of a smaller functional gene, or by double crossovers between plasmids carrying identical copies of the same gene (*trans* recombination). All of the *E. coli* and most of the *X. campestris* pv. citrumelo intragenic recombinant plasmids could have been generated by single *cis* crossover events (Fig. 3). Double recombination events in *trans* can result in functional genes carrying a smaller or larger number of repeats than the original gene or the same number of repeats. Presumably, *trans* recombination was responsible for generating pCS2, pCS23, and pDS2 (Fig. 3A) and also many of the *X. citri* recombinants (i.e., XcS14, XcS3, XcS8, and XcS22; Fig. 2).

The frequency of *pthA* recombination in *E. coli* was observed to be *recA* independent. Strain DH5α (*recA*) yielded recombinants at nearly the same frequency as strain JM83 (*recA*$^+$). Since recombination involving small repeated sequences (<1 kb) is affected only slightly by *recA* mutations (22), recombination of *pthA* was expected to be *recA* independent. The frequency of *pthA* recombination in *E. coli* was much higher than that observed in *X. campestris* pv. citrumelo and *X. citri*. This may be due to differences in the copy numbers of plasmids in which intragenic recombination occurred. Recombination of *pthA* in *X. citri* Xc1.2 involved a native plasmid, while recombination in *E. coli* and *X. campestris* pv. citrumelo involved pUFY1.48, which appeared (on the basis of our plasmid DNA extractions) to be present at a higher copy number in *E. coli* than in *X. citri* or *X. campestris*. However, since we did not determine plasmid copy numbers, these results may be due to different genetic backgrounds in each strain.

The role of the 34-amino-acid, leucine-rich, tandem repeats in determining pathogenic or avirulence specificity is striking, but it is not known how the repeats determine these functions. Leucine-rich repeats are often involved in protein-protein interactions, and one of the important biological functions for repetitive domains in prokaryotic and eukaryotic proteins is ligand binding. In a family of clostridial and streptococcal ligand-binding proteins, for example, conserved C-terminal repeat sequences function as binding sites (33). Similarly, 34-amino-acid repetitive motifs called tetratricopeptides found in proteins encoded by many mitotic genes (e.g., CDC16, CDC23, and nuc2+ of yeasts and BimA of *Aspergillus nidulans*) are implicated to pair with WD-40 repeats found in many proteins, including the β-subunit of G-proteins and some transcriptional factors (12, 26, 32). Recently, a family of cloned plant resistance genes was shown to encode proteins containing leucine-rich repeats (4, 27). It is possible that the repetitive domain of Pth or Avr proteins may also function as a ligand binding site, specifically interacting with the leucine-rich repeats encoded by the corresponding plant resistance genes.

The evolutionary success of many plant pathogens may rely on their ability to avoid host recognition. Tandemly repeated motifs in *pthA* and other members of the gene family provide hot spots for recombination and thus may accelerate evolution of this gene family. Intragenic recombination between inexact repeats not only introduces genetic variation, but also has the potential to amplify mutant repeats. As shown in Results, homologous recombination can lead to either duplication or deletion of repeat blocks. Such a mechanism for generating genetic variation at a high frequency may, however, also have the negative side effect of generating new *avr* genes.

Previously reported natural mechanisms for microbial race change included deletions (8), point mutations (15, 20), and transposon inactivation (16) of *avr* genes and always resulted in a loss of avirulence on a specific host plant and not gains of specific avirulence. In this study, we demonstrated that intra-

genic recombination of a bacterial gene not only caused the loss of pathogenic or avirulence phenotypes on a specific host, but in some cases resulted in the gain of new pathogenic phenotypes and avirulence specificities. Some intragenic recombinants lost the ability to confer cankers on citrus and instead elicited an HR on citrus, indicating that normosensitive (canker) reactions and HRs may share a very similar signal transduction pathway, as initially suggested by Klement (18).

The gene-for-gene concept is a major model for research on the reciprocal evolution between plants and parasites (31). In the pathogen, avirulence genes may mutate and evolve to overcome resistance by evading host recognition. In the plant, resistance genes may also evolve to recognize new avirulence genes, resulting in new resistance genes. Recent genetic mapping data have shown that disease resistance loci (especially those corresponding to different races of the same pathogen) are often clustered within small genetic intervals, suggesting that intralocus recombination may be responsible for generating resistance specific for new races of plant pathogens (9, 21, 23). Intragenic recombination of *pthA* demonstrated in this report provides compelling evidence that intragenic and intralocus recombination may play an important role during the reciprocal evolution of gene-for-gene interactions.

ACKNOWLEDGMENTS

We thank Gary Marlow for excellent technical assistance and Robert De Feyter for critically reviewing the manuscript.

REFERENCES

1. **Albertini, A. M., M. Hofer, M. P. Calos, and J. H. Miller.** 1982. On the formation of spontaneous deletions: the importance of short sequence homologies in the generation of large deletions. Cell **29:**319–328.
2. **Bonas, U., J. Conrads Strauch, and I. Balbo.** 1993. Resistance in tomato to *Xanthomonas campestris* pv. vesicatoria is determined by alleles of the pepper-specific avirulence gene *avrBs3*. Mol. Gen. Genet. **238:**261–269.
3. **Bonas, U., R. E. Stall, and B. Staskawicz.** 1989. Genetic and structural characterization of the avirulence gene *avrBs3* from *Xanthomonas campestris* pv. *vesicatoria*. Mol. Gen. Genet. **218:**127–136.
4. **Dangl, J. L.** 1995. Piece de resistance: novel classes of plant disease resistance genes. Cell **80:**363–366.
5. **De Feyter, R., and D. W. Gabriel.** 1991. At least six avirulence genes are clustered on a 90-kilobase plasmid in *Xanthomonas campestris* pv. *malvacearum*. Mol. Plant-Microbe Interact. **4:**423–432.
6. **De Feyter, R., and D. W. Gabriel.** 1991. Use of cloned DNA methylase genes to increase the transfer frequency of foreign genes into *Xanthomonas campestris* pv. malvacearum. J. Bacteriol. **173:**6421–6427.
7. **De Feyter, R., C. I. Kado, and D. W. Gabriel.** 1990. Small, stable shuttle vectors for use in *Xanthomonas*. Gene **88:**65–72.
8. **De Feyter, R., Y. Yang, and D. W. Gabriel.** 1993. Gene-for-genes interactions between cotton *R* genes and *Xanthomonas campestris* pv. *malvacearum avr* genes. Mol. Plant-Microbe Interact. **6:**225–237.
9. **Dickinson, M. J., D. A. Jones, and J. D. G. Jones.** 1993. Close linkage between the *Cf-2*/*Cf-5* and *Mi* resistance loci in tomato. Mol. Plant-Microbe Interact. **6:**341–347.
10. **Gabriel, D. W., M. T. Kingsley, Y. Yang, J. Chen, and P. Roberts.** 1993. Host-specific virulence genes of *Xanthomonas*, p. 141–158. *In* C. I. Kado and J. Crosa (ed.), Molecular mechanisms of bacterial virulence. Kluwer Academic Publishers, Dordrecht, The Netherlands.
11. **Gabriel, D. W., and B. G. Rolfe.** 1990. Working models of specific recognition in plant-microbe interactions. Annu. Rev. Phytopathol. **28:**365–391.
12. **Goebl, M., and M. Yanagida.** 1991. The TPR snap helix: a novel protein repeat motif from mitosis to transcription. Trends Biochem. Sci. **16:**173–177.
13. **Herbers, K., J. Conrads-Strauch, and U. Bonas.** 1992. Race-specificity of plant resistance to bacterial spot disease determined by repetitive motifs in a bacterial avirulence protein. Nature (London) **356:**172–174.
14. **Hopkins, C. M., F. F. White, S.-H. Choi, A. Guo, and J. E. Leach.** 1992. Identification of a family of avirulence genes from *Xanthomonas oryzae* pv. oryzae. Mol. Plant-Microbe Interact. **5:**451–459.
15. **Joosten, M. H. A. J., T. J. Cozijnsen, and P. J. G. M. de Wit.** 1994. Host resistance to a fungal tomato pathogen lost by a single base-pair change in an avirulence gene. Nature (London) **367:**384–386.
16. **Kearney, B., P. C. Ronald, D. Dahlbeck, and B. J. Staskawicz.** 1988. Molecular basis for evasion of plant host defence in bacterial spot disease of pepper. Nature (London) **332:**541–543.
17. **Keen, N. T.** 1990. Gene-for-gene complementary in plant-pathogen interactions. Annu. Rev. Genet. **24:**447–463.
18. **Klement, Z.** 1982. Hypersensitivity, p. 149–177. *In* M. S. Mount and G. H. Lacy (ed.), Phytopathogenic prokaryotes, vol. II. Academic Press, New York.
19. **Knoop, V., B. Staskawicz, and U. Bonas.** 1991. Expression of the avirulence gene *avrBs3* from *Xanthomonas campestris* pv. vesicatoria is not under the control of *hrp* genes and is independent of plant factors. J. Bacteriol. **173:** 7142–7150.
20. **Kobayashi, D. Y., S. J. Tamaki, D. J. Trollinger, S. Gold, and N. T. Keen.** 1990. A gene from *Pseudomonas syringae* pv. glycinea with homology to avirulence gene D from P. s. pv. tomato but devoid of the avirulence phenotype. Mol. Plant-Microbe Interact. **3:**103–111.
21. **Martin, G. B., S. H. Brommonschenkel, J. Chunwongse, A. Frary, M. W. Ganal, R. Spivey, T. Wu, E. D. Earle, and S. D. Tanksley.** 1993. Map-based cloning of a protein kinase gene conferring disease resistance in tomato. Science **262:**1432–1436.
22. **Petes, T. D., and C. W. Hill.** 1988. Recombination between repeated genes in microorganisms. Annu. Rev. Genet. **22:**147–168.
23. **Pryor, T.** 1987. The origin and structure of fungal disease resistance genes in plants. Trends Genet. **3:**157–161.
24. **Ried, J. L., and A. Collmer.** 1987. An *nptI-sacB-sacR* cartridge for constructing directed, unmarked mutations in Gram-negative bacteria by marker exchange-eviction mutagenesis. Gene **57:**239–246.
25. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
26. **Sikorski, R. S., M. S. Boguski, M. Goebl, and P. Hieter.** 1990. A repeating amino acid motif in CDC23 defines a family of proteins and a new relationship among genes required for mitosis and RNA synthesis. Cell **60:**307–317.
27. **Staskawicz, B. J., F. M. Ausubel, B. J. Baker, J. G. Ellis, and J. D. G. Jones.** 1995. Molecular genetics of plant disease resistance. Science **268:**661–667.
28. **Statler, G. D.** 1985. Mutations to virulence and avirulence in *Melampsora lini*. Phytopathology **75:**771–773.
29. **Swarup, S., R. De Feyter, R. H. Brlansky, and D. W. Gabriel.** 1991. A pathogenicity locus from *Xanthomonas citri* enables strains from several pathovars of *X. campestris* to elicit cankerlike lesions on citrus. Phytopathology **81:**802–809.
30. **Swarup, S., Y. Yang, M. T. Kingsley, and D. W. Gabriel.** 1992. A *Xanthomonas citri* pathogenicity gene, *pthA*, pleiotropically encodes gratuitous avirulence on nonhosts. Mol. Plant-Microbe Interact. **5:**204–213.
31. **Thompson, J. N., and J. J. Burdon.** 1992. Gene-for-gene coevolution between plants and parasites. Nature (London) **360:**121–125.
32. **van der Voorn, L., and H. L. Ploegh.** 1992. The WD-40 repeat. FEBS Lett. **307:**131–134.
33. **Wren, B. W.** 1991. A family of clostridial and streptococcal ligand-binding proteins with conserved C-terminal repeat sequences. Mol. Microbiol. **5:**797–803.
34. **Xu, B., C. Paszty, and P. F. Lurquin.** 1988. A plasmid-based method to quantitate homologous recombination frequencies in gram-negative bacteria. BioTechniques **6:**752–759.
35. **Yang, Y., R. De Feyter, and D. W. Gabriel.** 1994. Host-specific symptoms and increased release of *Xanthomonas citri* and *X. campestris* pv. malvacearum from leaves are determined by the 102-bp tandem repeats of *pthA* and *avrb6*, respectively. Mol. Plant-Microbe Interact. **7:**345–355.
36. **Yang, Y., and D. W. Gabriel.** *Xanthomonas* avirulence/pathogenicity gene family encodes functional plant nuclear targeting signals. Mol. Plant-Microbe Interact., in press.
37. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.