Cloning and Characterization of a Member of the *Xanthomonas avr/pth* Gene Family That Evades All Commercially Utilized Cotton *R* Genes in the United States

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ABSTRACT

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The highly virulent African strains of *Xanthomonas campestris* pv. *malvacearum* are quarantined pathogens in the United States and can evade or overcome all commercially utilized resistance (*R*) genes in cotton grown in the United States including the entire set of host differential lines used to distinguish 19 races of the pathogen. Nevertheless, the African strains carry multiple DNA fragments that strongly hybridize with members of the *Xanthomonas* avirulence (*avr*)/pathogenicity (*pth*) gene family. Since all previously tested members of the gene family confer avirulence against one or more *R* genes in cotton, strains carrying multiple members might not be expected to evade so many different *R* genes. The hybridizing DNA fragments were cloned from African strain

XcmN and found to confer water-soaking ability to a nearly asymptomatic mutant strain of the pathogen. Restriction mapping, Southern hybridization, and DNA sequencing of the cloned fragments from XcmN were used to identify two water-soaking genes, *pthN* and *pthN2*, as new members of the *Xanthomonas avr/pth* gene family. The complete DNA sequence of *pthN* was obtained, and it is >94% identical with all other sequenced members of the gene family. Gene fusions of *pthN* with *avrB6* (another family member) and other experiments revealed that the ability of African strain XcmN to water-soak cotton and avoid recognition by commercially used cotton *R* genes is determined by the specific repeats of multiple functional members of the *Xanthomonas avr/pth* gene family.

Additional keywords: cotton blight, gene-for-gene, *Gossypium hirsutum*, race specificity.

Strains of *Xanthomonas campestris* pv. *malvacearum* that cause bacterial blight of cotton (*Gossypium* spp.) exhibit gene-for-gene (also known as cultivar or race) specificity (8,10,16). There are at least 19 well-described races of the pathogen (2). The genetics of resistance of *Gossypium* to this pathogen is also well described, with at least 16 different resistance (*R*) genes identified (11). Different *X. campestris* pv. *malvacearum* races contain different combinations of avirulence (*avr*) genes that define the race and determine cultivar specificity. Host cultivars containing *R* genes respond to avirulent races with a hypersensitive response (HR). Ten *avr* genes were cloned and characterized from a single North American strain of *X. campestris* pv. *malvacearum*, XcmH, and most of these *avr* genes genetically "recognize" multiple *R* gene loci in an *avr* gene-for-*R* genes (plural *R* genes) manner (7,10). These 10 genes belong to a large family of *Xanthomonas avr/pth* genes that composes most of the *Xanthomonas avr* genes cloned to date (15). All of these *avr/pth* genes that have been sequenced so far are nearly identical (some of them >97% identical) in DNA sequence; not surprisingly, several individual cotton *R* gene loci genetically "recognize" multiple members of the *avr/pth* gene family in an *R* gene-for-*avr* genes (plural *avr* genes) manner (10). These *avr* genes are, therefore, less gene-for-gene specific than most others described to date.

Although most members of the *Xanthomonas avr/pth* gene family were originally cloned as *avr* genes, some members of this

gene family also confer ability to elicit host-specific, pathogenic symptoms. For example, at least seven *X. campestris* pv. *malvacearum avr/pth* genes can contribute to water-soaking symptoms on susceptible cotton plants (31,33). In addition, a pathogenicity (*pth*) gene, *pthA*, is required by *X. citri* to cause hyperplastic cancer symptoms on citrus and was originally cloned as a pathogenicity gene (28). However, all previously cloned members tested, including *pthA*, confer avirulence to *X. campestris* pv. *malvacearum* against multiple *R* genes (10,29).

Some highly virulent strains of *X. campestris* pv. *malvacearum* isolated in West Africa are virulent on all commercial cotton lines used in the United States, including the long-established, "immune" cotton line 101-102B, and all differential host lines (2,13). A new cultivar, S295, developed and used in Africa, has a single gene (*B12*) that is effective for resistance against all African strains reported to date (30). However, single genes for resistance are frequently overcome by mutation at cognate *avr* loci (18), and the *X. campestris* pv. *malvacearum avr/pth* genes undergo intergenic and intragenic recombination, generating race change variants at high frequency (10,32). Commercial use of a single effective *R* gene, such as *B12*, against a pathogen exhibiting a high level of race change variation may be unwise from a statistical point of view (21,25), unless there is some other reason to believe that the *R* gene will be longer lasting than most. Therefore, we wished to determine the molecular basis of virulence of the African strains.

The molecular basis of the high level of virulence of the African strains could be due to any one of several possibilities. One possibility is that the African strains lack functional or active *avr/pth* genes, even though Southern hybridization with members of the gene family revealed the presence of at least four potential *Xan-*

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thomonas avr/pth homologues in each of these strains tested (10). Another possibility is suppression of phenotypic avirulence by other genes in the African strains; *avr* gene suppression has been observed at independent loci with both wheat leaf rust and flax rust pathogens (22,26). A third possibility is that the *avr/pth* homologues indicated by hybridization are functional and active pathogenicity genes, but lack avirulence activity against any of the commercially utilized *R* genes. The purpose of this investigation was to distinguish between these three different possibilities.

We report here evidence that the latter possibility is the case and that (i) the African strain XcmN carries active *avr/pth* genes that confer pathogenicity (water-soaking), but not avirulence, on commercial cotton grown in the United States; (ii) the complete DNA sequence of *pthN* and partial sequence of *pthN2* reveal that these pathogenicity genes are members of the *Xanthomonas avr/pth* gene family; and (iii) the 102-bp tandem repeat region of *pthN* determines pathogenicity without avirulence on commercial U.S. cotton, but is recognized by the *B12* gene in S295 as an *avr* gene.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture media. Strains of *Escherichia coli*, *X. campestris* pv. *malvacearum*, and plasmids used in this study are listed in Table 1. Strains of *E. coli* were grown in Luria-Bertani (LB) medium (27) at 37°C. *X. campestris* pv. *malvacearum* strains were cultured on PYGM (peptone-yeast extract-glycerol-morpholinepropanesulfonic acid) medium at 30°C (9). For culture on solid media, agar was added at 15 g/liter. Antibiotics were used at the following final concentrations: ampicillin, 30 mg/liter for low copy vectors and 100 mg/liter for high copy vectors; kanamycin, 25 mg/liter; gentamicin, 5 mg/liter; spectinomycin, 50 mg/liter; tetracycline, 15 mg/liter; rifampicin, 75 mg/liter; and chloramphenicol, 35 mg/liter.

Recombinant DNA techniques. Plasmids were isolated from *E. coli* by alkaline lysis, and standard recombinant DNA procedures were as described (27). Restriction enzyme digestion, alkaline phosphatase treatment, DNA ligation, Southern blots on nylon membranes, and random priming reactions were performed as recommended by the manufacturers.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Reference
<i>E. coli</i>		
DH5	F ⁻ , <i>endA1</i> , <i>hsdR17</i> (r _k ⁻ m _k ⁺), <i>supE44</i> , <i>thi-1</i> , <i>recA1</i> , <i>gyrA</i> , <i>relA1</i> , <i>80dlacZ M15</i> , (<i>lacZYA-argF</i>)U169	Gibco BRL ^b
DH5αMCR	DH5 <i>mcrA mcrBC mrr</i>	Gibco BRL ^b
<i>Xanthomonas campestris</i> pv. <i>malvacearum</i>		
XcmN	Natural isolates from cotton from Upper Volta, Africa; virulent on all cotton differential lines including Stoneville 2B-S9, Stoneville 20, Mebane B-1, 1-10B, 20-3, 101-102B, Gregg, Empire B4, and DPxP4, but avirulent on S295	7, 16, 23, and this study
Xcm1003	Spontaneous Spe ^r Rif ^r derivative of XcmN	7
XcmH	Natural race 4 isolate from cotton; carries 10 <i>avr/pth</i> genes	16
XcmH1005	Spontaneous Rif ^r derivative of XcmH	31
HM2.2S	<i>avrB4</i> ⁻ , <i>avrB5</i> ⁻ , <i>avrB6</i> ⁻ , <i>avrBn</i> ⁻ , <i>avrBlm</i> ⁻ , <i>avrB101</i> ⁻ , <i>avr102</i> ⁻ , and marker-evicted mutant derivative of XcmH1005	33
<i>X. campestris</i> pv. <i>alfalfae</i>		
KX-1	Spontaneous Spe ^r derivative of KS, isolated from alfalfa	24
Plasmids		
pBluescript II KS	ColE1, Ap ^r , <i>lacZ</i> ⁺ , high copy vector	Stratagene ^c
pRK2013	ColE1, Km ^r , Tra ⁺ , conjugation helper plasmid	12
pUFR042	IncW, Nm ^r , Gm ^r , Mob ⁺ , <i>mob</i> (P), <i>lacZ</i> ⁺ , Par ⁺ , <i>cos</i> , shuttle vector	7
pUFR043	IncW, Mob ⁺ , <i>lacZ</i> ⁺ , Gm ^r , Nm ^r , <i>cos</i> , shuttle vector	7
pUFR047	IncW, Gm ^r , Ap ^r , Mob ⁺ , <i>lacZ</i> ⁺ , Par ⁺ , shuttle vector	10
pUFR053	IncW, Gm ^r , Cm ^r , Mob ⁺ , <i>lacZ</i> ⁺ , Par ⁺ , shuttle vector	Yuan and Gabriel ^d
pUFR054	IncP, Tc ^r , Mob ⁺ , containing methylases <i>XmaI</i> and <i>XmaIII</i>	8
pUFR135	2.9- + 0.5-kb <i>Bam</i> HI fragments from XcmH containing intact <i>avrB6</i> in pUFR042; <i>lacZ</i> :: <i>avrB6</i>	10
pQY104.3	3.4-kb internal <i>Bam</i> HI fragment of <i>pthA</i> cloned in pUC19	Yuan and Gabriel ^d
pPKC5	Cosmid clone containing 36.7-kb insert from XcmN genome carrying <i>pthN</i> and <i>pthN2</i> in pUFR043	This study
pPKC5.1	14-kb <i>Eco</i> RI fragment from pPKC5 in pUFR047	This study
pPKC5.2	2.6-kb <i>Eco</i> RI fragment from pPKC5 in pUFR047	This study
pPKC5.3	8.7-kb <i>Eco</i> RI fragment from pPKC5 carrying <i>pthN</i> and <i>pthN2</i> in pUFR047	This study
pPKC5.5	8.7-kb <i>Eco</i> RI fragment from pPKC5, opposite in orientation from pPKC5.3	This study
pPKC5.30	0.7-kb <i>Eco</i> RI fragment from pPKC5 in pUFR047	This study
pPKC6.1	Partial subclone of pPKC5 containing 10.7-kb fragment only	This study
pPKC6.10	Partial subclone of pPKC5 containing 10.7- and 8.7-kb fragments	This study
pPKC6.19	Partial subclone of pPKC5 containing 14-, 10.7-, 8.7-, and 2.6-kb fragments	This study
pPKC6.58	Partial subclone of pPKC5 containing 10.7-, 8.7-, and 2.6-kb fragments	This study
pPKC8.37	pPKC5.3 religated following restriction digestion with <i>Sst</i> II; contains 3.0-kb <i>pthN</i> fragment, truncated 1.0-kb at the 5' end	This study
pPKC9.1	5.4-kb <i>Sst</i> II/ <i>Hind</i> III fragment of pPKC5.3 containing <i>pthN2</i> in pBluescript KS	This study
pPKC10.1	5.4-kb <i>Sst</i> II/ <i>Hind</i> III fragment containing <i>pthN2</i> from pPKC9.1 in pUFR053	This study
pPKC13.12	3.5-kb <i>Pvu</i> II fragment from pPKC5.3 containing <i>pthN</i> truncated 500 bp at the 3' end, in <i>Sma</i> I site of pBluescript KS	This study
pPKC14.2	3.5-kb <i>Sst</i> II/ <i>Hind</i> III fragment containing <i>pthN</i> from pPKC13.12 in pUFR047	This study
pPKC15.2	3.0-kb <i>Sst</i> II/ <i>Eco</i> RI fragment from pPKC8.37, containing <i>pthN</i> truncated 1.0-kb at the 5' end in pBluescript KS	This study
pPKC18.5	4.0-kb <i>Sst</i> II/ <i>Hind</i> III fragment containing intact <i>pthN</i> in pBluescript KS	This study
pPKC19.4	4.0-kb <i>Sst</i> II/ <i>Hind</i> III fragment containing intact <i>pthN</i> in pUFR047	This study
pPKC22.6	Chimeric gene carrying 1.6-kb <i>Stu</i> I/ <i>Aat</i> II fragment of <i>avrB6</i> from pUFR135, replacing 102-bp <i>pthN</i> repeats in pPKC18.5	This study
pPKC24.1	4.0-kb <i>Sst</i> II/ <i>Hind</i> III fragment containing chimeric gene from pPKC22.6 in pUFR047	This study

^a Abbreviations: Ap = ampicillin, Cm = chloramphenicol, Gm = gentamicin, Km = kanamycin, Mob⁺ = mobilizable, Nm = neomycin, Par⁺ = partition proficient, Rif = rifampicin, Spc = spectinomycin, Tc = tetracycline, and Tra⁺ = self-transmissible.

^b Gibco BRL, Gaithersburg, MD.

^c Stratagene Inc., La Jolla, CA.

^d Q. Yuan and D. W. Gabriel, unpublished data.

Construction of the DNA library. Total DNA of XcmN was prepared as described (17) and purified by cesium chloride-ethidium bromide density centrifugation. The genomic DNA was partially digested by *Sau3A* and size-fractionated using sucrose density gradient centrifugation. DNA fragments in the size range of 35 to 40 kb were used for the construction of the library in pUFR043, as described (6). The recombinant linear fragments were packaged into phage heads with Gigapack Gold Packaging Mix (Stratagene Inc., La Jolla, CA) and transduced into *E. coli* strain DH5 α MCR, following manufacturer's instructions. Individual clones in the library were selected on LB agar medium containing kanamycin and stored in LB broth containing 14% glycerol in microtiter dishes at -70°C .

Colony hybridization and bacterial conjugation. Each of the 846 clones in the DNA library was transferred to nylon membranes using a sterile, 46-pronged colony replicator. Colonies were lysed on the membrane and the DNA bound to the membrane exactly as described (27). Colony hybridization was done using ^{32}P -labeled pQY104.3. Phenotypic activities of *E. coli* library clones were tested by mobilizing the clones into Xcm1003 or mutant strain HM2.2S (33) of *X. campestris* pv. *malvacearum*. Transfer of DNA was accomplished by triparental mating using pRK2013 as a transfer helper and pUFR054 to methylate the DNA as described (8). Transconjugants in *X. campestris* pv. *malvacearum* were selected on PYGM agar containing appropriate antibiotics.

Plant inoculations. The cotton (*G. hirsutum*) line used for water-soaking activity assays was Acala 44 (Ac44); its congenic resistant lines Acb6 and AcBIn3 were used for avirulence activity assays (assessed visually as a macroscopic HR). A set of cotton differential lines including AcB1, AcB2, AcB4, AcB6, AcB7, AcBIn, AcBIn3; the standard set of cotton differential lines (8,11,31) composed of Stoneville 2B-S9, Stoneville 20, Mebane B-1, 1-10B, 20-3, 101-102B, Gregg, Empire B4, and DPxP4; and S295 were also used for avirulence activity assays. For heterologous system assays, *X. campestris* pv. *malvacearum* genes were transferred into *X. campestris* pv. *alfalfae* strain KX-1 and inoculated onto common bean

(*Phaseolus vulgaris*) cultivar California Light Red Kidney (Sacramento Valley Milling, Inc., Ordbend, CA). Inoculations were as described (10): essentially, bacterial suspensions of *Xanthomonas* (10^8 CFU/ml) in sterile tap water were pressure-infiltrated into leaves of 4- to 5-week-old cotton and bean plants. Plants were grown in the greenhouse, transferred to growth chambers before inoculation, and maintained as described (7). Pathogenic symptoms were observed daily and results recorded 2 to 6 days after inoculation.

Construction of subclones and chimeric genes. To isolate and clone *pthN*, pPKC5.3 was first digested with *PvuII*, and the 3.5-kb *PvuII* fragment carrying most of *pthN* was cloned into the *SmaI* site of pBluescript, forming pPKC13.12. A 3.0-kb *SstII/EcoRI* fragment from pPKC8.37, containing the remainder of *pthN*, was directionally cloned into pBluescript, forming pPKC15.2. Intact *pthN* was then restored by replacing the 0.5-kb *AatII/HindIII* fragment in pPKC13.12 with the 1.2-kb *AatII/HindIII* fragment from pPKC15.2, forming pPKC18.5. The 4.0-kb fragment containing reconstituted *pthN* in pPKC18.5 was digested with *SstI* and *HindIII* and recloned into pUFR047, forming pPKC19.4.

To isolate and clone *pthN2*, subclone pPKC5.5 was digested with *SstII* and *HindIII*, generating a 5.7-kb fragment. The fragment was recloned directionally into pBluescript KS to generate pPKC9.1. Activity of *pthN2* was assayed by recloning it into pUFR053, forming pPKC10.1.

To test the influence, if any, of the regions of *pthN* flanking the 102-bp repeats on the specific phenotype conferred by the gene, the tandemly arranged repeats of *pthN* were replaced as a unit by the repeats from *avrB6*. pPKC18.5 (*pthN*) and pUFR135 (*avrB6*) were double-digested with *StuI* and *AatII*, and the 1.6-kb *StuI/AatII* fragment containing the 102-bp repeats of *avrB6* and the 5.4-kb *StuI/AatII* fragment containing the 5' and 3' ends of *pthN* were recovered from an agarose gel and purified by the phenol-freeze-fracture method (1). The repeat region of *avrB6* was used to replace the repeat region of *pthN*, resulting in the chimeric *avr/pth* gene on pPKC22.6. pPKC22.6 was digested with *SstI* and *HindIII*, and the 4.0-kb fragment containing the chimeric gene was recloned into pUFR047, forming pPKC24.1. The activity of the chimeric gene was tested by conjugating into strains HM2.2S and Xcm1003 of *X. campestris* pv. *malvacearum* and inoculating on cotton.

DNA sequencing and analyses. For sequencing the regions of *pthN* outside of the repeats, *pthN* was directionally cloned into the

Fig. 1. Water-soaking activity assays of cosmid clones from the XcmN library selected on the basis of hybridization to an internal *Bam*HI fragment of *pthA* (avirulence [*avr*]/pathogenicity [*pth*] family member). The clones were conjugated into *Xanthomonas campestris* pv. *malvacearum* strain HM2.2S and inoculated on Acala 44 cotton at high levels (10^8 CFU/ml; HM2.2S is asymptomatic at 10^6 CFU/ml): a, HM2.2S/pUFR043 (vector); b, HM2.2S/pPKC2; c, HM2.2S/pPKC4; d, HM2.2S/pPKC5 (*pthN*, *pthN2*); e, HM2.2S/pPKC9; f, HM2.2S/pPKC12; g, HM2.2S/pPKC15; h, HM2.2S/pPKC16; and i, HM2.2S/pPKC18. Note the water-soaking conferred by pPKC5, pPKC16, and pPKC18. Photo taken 5 days after inoculation.

Fig. 2. Southern hybridization of three XcmN clones conferring water-soaking activity to *Xanthomonas campestris* pv. *malvacearum* strain HM2.2S. The blot was probed with a ^{32}P -labeled, 3.4-kb internal *Bam*HI fragment of *pthA*. Each plasmid was digested with the following enzymes and loaded in order: *EcoRI*, *Bam*HI, *EcoRI/Bam*HI, *KpnI*, *EcoRI/KpnI*, *SstI*, and *EcoRI/SstI*: lanes a to g, pPKC4; lanes h to n, pPKC5; and lanes o to u, pPKC16. Intensely hybridizing bands represent doublets.

*Sst*I/*Hind*III sites of pBluescript, forming pPKC18.5. DNA was extracted and purified using the Qiagen (Qiagen Inc., Chatsworth, CA) protocol for midi-prep preparation. For sequencing the repeat region of *pthN*, clone pPKC18.5 was digested with *Sst*I and *Stu*I to generate a sticky end with a 3' overhang (resistant to Exonuclease III [Promega Corp., Madison, WI]) and a blunt end (susceptible to Exonuclease III), respectively. Exonuclease III was then used to generate a series of nested deletions in the resulting linear fragments. Samples were removed at timed intervals to tubes containing S1 nuclease to remove the single-stranded tails remaining after Exonuclease III digestion. After filling in the overhanging termini using the Klenow fragment (DNA polymerase), these deletion derivatives were diluted and self-ligated to circularize the plasmids. The ligation mixtures for each time interval were used to transform competent cells. A number of subclones from each time point were then screened to select for appropriately sized deletions.

Sequencing was performed by the ICBR DNA Sequencing Core (University of Florida, Gainesville), using vector-based T7 or T3 promoter primers or custom-synthesized (ICBR DNA Synthesis Core) oligonucleotide primers. Overlapping DNA sequences were assembled using GCG version 7 sequence analysis software package by Genetic Computer Group, Inc. (Madison, WI).

RESULTS

X. campestris pv. *malvacearum* genomic library, colony hybridization, and identification of cloned pathogenicity genes.

A genomic library of strain XcmN composed of 846 clones was constructed using cosmid vector pUFR043 and maintained in *E. coli* strain DH5 α MCR. Based on DNA restriction profiles of plasmids from 18 arbitrarily chosen clones, the XcmN genomic library appeared to contain random inserts with an average size of 36.7 kb. Colony hybridization using pQY104.3 as a probe, which carries a 3.4-kb internal fragment from a member of the *Xanthomonas* *avr/pth* gene family, detected 18 hybridizing clones in the library. Eight cosmid clones were selected for activity assays after an examination of their restriction digestion patterns indicated that they likely carried full-length members of the gene family. Three of these eight clones (pPKC5, pPKC16, and pPKC18) conferred strong water-soaking activity to strain HM2.2S of *X. campestris* pv. *malvacearum* on cotton line Ac44 (Fig. 1). Cosmid clone pPKC4 conferred very weak water-soaking activity, and the remaining four clones had no evident phenotypic effect on the pathogenicity of HM2.2S on Ac44.

Observations of the restriction digestion profiles of the clones restoring water-soaking ability showed that they possessed some DNA fragments in common (Fig. 2; some data not shown). South-

ern hybridization of restriction digests of clones conferring strong water-soaking revealed the presence of three *Eco*RI and three *Bam*HI fragments of >3.0-kb each that strongly hybridized to pQY104.3, indicating that each of these clones might carry up to three *avr/pth* genes. In contrast, only one *Eco*RI and one *Bam*HI fragment from pPKC4 hybridized to pQY104.3 (Fig. 2; some data not shown).

Molecular characterization of pPKC5 and subcloning. The three hybridizing *Eco*RI fragments from pPKC5 were subcloned into pUFR047. In addition, subclones with more than one of the five total *Eco*RI fragments were also obtained by partially digesting pPKC5 with *Eco*RI and religating to recircularize the plasmids. A restriction map of pPKC5 (36.7 kb), its subclones, and their relative water-soaking activities in HM2.2S on Ac44 cotton is shown in Figure 3. pPKC5 conferred excellent water-soaking ability to HM2.2S, at levels not distinguished from the wild-type strain XcmN. Most of the activity of pPKC5 was limited to an 8.7-kb *Eco*RI fragment present in pPKC5.3. This fragment alone conferred most, but not all, of the activity of pPKC5. Both the 14.0- and 10.7-kb fragments hybridized with pQY104.3; neither fragment alone conferred water-soaking activity to HM2.2S, and these fragments were not investigated further.

Activity assays of *pthN* and *pthN2*. More extensive restriction mapping of the 8.7-kb *Eco*RI fragment in subclone pPKC5.3 suggested the presence of two members of the *Xanthomonas* *avr/pth* gene family, based on similarities with other family members. These genes were named *pthN* and *pthN2*. Gene *pthN2*, when mobilized into HM2.2S on pPKC10.1 and inoculated on Ac44 cotton, caused weak water-soaking symptoms (Fig. 4). Gene *pthN*, when mobilized into HM2.2S on pPKC19.4, was found to confer a high level of water-soaking activity on Ac44, almost to that of pPKC5.3 (Fig. 4). When either *pthN* or *pthN2* was mobilized into *X. campestris* pv. *alfalfae* strain KX-1, the transconjugants failed to elicit an HR on *P. vulgaris* (as is typical of other tested members of the *Xanthomonas* *avr/pth* gene family [29; D. W. Gabriel, unpublished data]). No phenotypic changes were detected that distinguished the transconjugants from the KX-1 controls (without *pthN* or *pthN2*).

Strain XcmN and its derivative Xcm1003 were avirulent on cotton cultivar S295. Transconjugants of Xcm1003 carrying *pthN* on pPKC19.4 (therefore, carrying additional copies of *pthN*) elicited a stronger (more intense and appearing 12 h earlier) HR on S295 than Xcm1003 alone, indicating that *pthN* was reacting with the resistance gene *B12* in S295. Transconjugants of Xcm1003 carrying *pthN2* on pPKC10.1 (therefore, carrying additional copies of *pthN2*) elicited an HR on S295 that was not distinguished from that of Xcm1003 alone. Transconjugants of Xcm1003 carrying either *pthN* or *pthN2* were virulent on AcB1, AcB2, AcB4, AcB6,

Fig. 3. Restriction map of pPKC5 and localization of *pthN* and *pthN2*. Subclones and their biological activities on cotton are shown below the partial restriction map of pPKC5. Arrows indicate the direction of transcription. Restriction sites are abbreviated as follows: A, *Aat*II; B, *Bam*HI; E, *Eco*RI; Hc, *Hinc*II; H, *Hind*III; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*II; S, *Sst*II; and St, *Stu*I.

Ac67, AcBIn, AcBIn3, Stoneville 2B-S9, Stoneville 20, Mebane B-1, 1-10B, 20-3, 101-102B, Gregg, Empire B4, and DPxP4, reacting similarly to Xcm1003.

Sequence analysis. Gene *pthN* was sequenced completely and is available as GenBank accession number AF016221. The gene was found to be 3.6 kb in size with a single open reading frame. The central region of this gene was characterized by 13.5 nearly perfect, tandemly arranged, 102-bp direct repeats. As with all other sequenced members of the gene family, the ends of the gene were defined by 62-bp terminal inverted repeats, and the predicted PthN protein carried 13.5 34-amino acid, leucine-rich repeats, a series of leucine zipper-like heptad repeats, and three putative nuclear localization signals (Fig. 5). Little variation was seen in comparisons of the predicted amino acid sequences of different repeats of PthN, except at the twelfth and thirteenth amino acid positions within each repeat (Fig. 5). The amino acid in the twelfth position was always a histidine or asparagine, while that in the thirteenth position was always an isoleucine, aspartate, or glycine.

Activity assays of chimeric *pthN* with *avrB6* repeats in Xcm1003. When the chimeric gene formed by replacing the *pthN* repeats with those from *avrB6* on pPKC24.1 was mobilized into Xcm1003 and inoculated on cotton line Ac66, a strong HR was observed, similar to that elicited by *avrB6* in Xcm1003. When pPKC24.1 was moved into HM2.2S and inoculated on susceptible Ac44 cotton, strong water-soaking, typical of HM2.2S transconjugants carrying *avrB6*, was observed. The flanking regions of *pthN* outside the repeats were, therefore, isofunctional with the equivalent regions of *avrB6*, and the repeat regions of *pthN* and *avrB6* appeared to confer equivalent levels of water soaking to HM2.2S.

DISCUSSION

We report here the isolation and characterization of two new members of the *Xanthomonas avr/pth* gene family that function for pathogenicity (water-soaking) in *X. campestris* pv. *malvacearum*, but that are not recognized as *avr* genes by any of the *R*

genes currently in commercial use in North America. These genes were cloned from a highly virulent *X. campestris* pv. *malvacearum* strain, XcmN, that is representative of a group of African strains that evades or defeats all but one gene for resistance against this pathogen in cotton worldwide. This group of strains is even more virulent than the strains belonging to race 18 (previously the most virulent described race) and has been proposed to be categorized as a new race (2,13). Since this strain overcomes the resistance of the previously "immune" line 101-102B, work is underway to find new sources of resistance to the pathogen (2).

Genes *pthN* and *pthN2* belong to a large family of *Xanthomonas avr/pth* genes that includes *pthA* of *X. citri* (29); *avrB6*, *avrB4*, *avrB7*, *avrBln*, *avrB101*, *avrB102*, *avrBn*, *avrB5*, *avrB103*, and *avrB104* of *X. campestris* pv. *malvacearum* (7,10,33); *avrBs3* and *avrBs3-2* of *X. campestris* pv. *vesicatoria* (4,5); and *avrXa5*, *avrXa7*, and *avrXa10* of *X. oryzae* (20). Although most members were originally cloned as avirulence genes, *pthA* of *X. citri* and *pthN* and *pthN2* of *X. campestris* pv. *malvacearum* were cloned as pathogenicity genes. Many members of the gene family from *X. campestris* pv. *malvacearum*, originally cloned as *avr* genes, have subsequently been found to pleiotropically encode pathogenicity (water-soaking) in compatible interactions on host plants (31,33).

With the exception of *pthN* and *pthN2*, all previously cloned *avr/pth* genes from *X. campestris* pv. *malvacearum* are known to encode multiple avirulence specificities in the source strain on differential host cultivars, and when transferred to other xanthomonads and inoculated on the recipient's differential hosts. Although we have tested *pthN* and *pthN2* only in *X. campestris* pv. *malvacearum* on cotton and in *X. campestris* pv. *alfalfae* on bean, the only resistance gene that we have reason to believe "recognizes" *pthN* as an *avr* gene is cotton gene *B12* in S295. We know of no *X. campestris* pv. *malvacearum* strains that are virulent on S295 and, therefore, we could not test *pthN* and *pthN2* for avirulence activity on S295 in the usual manner. However, transconjugants of Xcm1003 carrying additional copies of *pthN*, but not *pthN2*, on a plasmid vector elicited an HR that was more intense and occurred earlier than that elicited by Xcm1003 alone. Both *pthN* and *pthN2* confer water-soaking ability to Xcm1003 and, therefore, both genes are expressed in Xcm1003. This indicates, but does not prove, that the *B12* resistance gene in S295 "recognizes" *pthN* (possibly among other *avr* genes) as an *avr* gene, and provides evidence that there was no suppression of *pthN* avirulence activity in Xcm1003 on cotton cultivar S295.

Strain XcmN and its derivative, Xcm1003, are avirulent on S295, but both are virulent on all other cotton lines tested including all standard differentials. Although we knew from using Xcm1003 as a recipient strain in avirulence gene assays on these other cotton lines that it does not suppress the avirulence conferred by genes cloned from other xanthomonads (29,31; D. W. Gabriel, unpublished data), we wanted to test the possibility that some region of *pthN* outside of its repeats was able to suppress phenotypic avirulence that would otherwise be conferred by the repeats on other cotton lines. A chimeric construction with the flanking region of *pthN* and repeats of *avrB6*, when moved into Xcm1003 and inoculated on cotton Ac66, conferred the *avrB6* HR phenotype. We conclude that the leucine-rich repeats of PthN confer water-soaking without eliciting an HR on all known cotton *R* genes except *B12* in S295 and that the high virulence exhibited by the African strains of *X. campestris* pv. *malvacearum* is not due to some special property of these strains suppressing avirulence. Rather, the high virulence of the African strains may be achieved because the African strains simply carry fewer *avr/pth* gene family members than North American strains (about half as many), reducing the chances for *avr* gene recognition by *R* genes, and those *avr/pth* members that are carried by the African strains

Fig. 4. Water-soaking symptoms caused by *pthN* and *pthN2*. Subclones were conjugated into *Xanthomonas campestris* pv. *malvacearum* strain HM2.2S and inoculated on Acala 44 cotton at high levels (10^8 CFU/ml; HM2.2S is asymptomatic at 10^6 CFU/ml): a, HM2.2S/pUFR047 (vector); b, HM2.2S/pPKC10.1 (*pthN2*); c, pPKC19.4 (*pthN*); d, pPKC5 (*pthN*, *pthN2*); and e, pPKC5.3 (*pthN*, *pthN2*). Photo taken 5 days after inoculation.

units to be released to the leaf surface, where they are available for rain splash (33). Southern hybridization of the *Bam*HI and *Eco*RI single and double digests showed that the clones restoring full water-soaking contained at least three strongly hybridizing *Bam*HI fragments, whereas the clone causing partial water-soaking contained a single hybridizing *Bam*HI fragment. This is consistent with previous observations that members of the *avr/pth* gene family in *X. campestris* pv. *malvacearum* encode weak, but synergistically additive, water-soaking ability, with some members encoding stronger water-soaking than others (33).

Three out of eight cosmid clones from XcmN, identified by hybridization to pQY104.3, conferred strong water-soaking ability to the mutant strain HM2.2S of *X. campestris* pv. *malvacearum*. Strain HM2.2S was derived from a North American race 4 isolate (XcmH) by repeated marker-eviction (deletion) mutations of members of the *Xanthomonas avr/pth* gene family in that strain (33). At low levels of inoculum ($<10^6$ CFU/ml), the strain is completely asymptomatic, but HM2.2S grows within susceptible cotton plants at the same rate and to the same levels as the wild type; the water-soaking phenotype allows at least 1,600 times more colony forming

Fig. 5. Predicted amino acid sequence of PthN (GenBank accession number AF016221) (bottom) aligned with Avr6 (10) (top). The predicted amino acid sequences of these proteins are 96% similar and 92% identical. The 34-amino acid, leucine-rich repeats are found in the central portion of the protein and are arranged here in pairs. The leucine zipper-like region and nuclear localization signals are underlined by single and double lines, respectively. The arrows indicate the location of the fusion sites for the predicted chimeric protein (expressed from pPKC24.1) with the repeat region of PthN replacing that of Avr6. The *StuI* and *AarI* sites used to make the fusions are located at nucleotides 1,281 to 1,286 and 2,901 to 2,907 on the published *avr6* sequence (GenBank accession number L06634.)

gene family including *pthA* of *X. citri* (29), *avrb6* of *X. campestris* pv. *malvacearum* (31), *avrXa10* of *X. oryzae* (20), and *avrBs3* and *avrBs3-2* of *X. campestris* pv. *vesicatoria* (3,4,5). Like all other members of this gene family, *pthN* had 62-bp terminal inverted repeats similar to those of the Tn3 family (10) and 102-bp tandem, direct repeats in the central region of the gene. Also, like all other members of the gene family, the 102-bp repeats encoded predicted 34-amino acid, leucine-rich repeats. Although it is known that the 102-bp repeats of members of this gene family determine the host-specific pathogenicity and avirulence functions of members of this gene family (19,31), the mechanism is unknown.

Gene *pthN* is functionally similar to *avrb6* in terms of the high degree of water-soaking it confers to *X. campestris* pv. *malvacearum* on cotton. It also carries the same number of repeats (13.5) as *avrb6*, which is the lowest number of repeats found in any naturally occurring member of the *Xanthomonas avr/pth* gene family. Perhaps surprisingly, the predicted sequence of PthN was no more related in overall identity to Avr6 than to most other members (all except AvrXa10 are in the 96.1 to 96.5% identity range). This was true even when the repeat regions alone were compared. However, in comparisons of the predicted peptide sequences of the repeats of these two water-soaking genes with the predicted peptide sequences of the repeats of all other members sequenced to date, a number of unusual similarities emerged. For example, repeat 6 of PthN is identical to that of Avr6, and repeats 3, 5, 7, 9, 10, and 12 are nearly identical. In addition, in repeats 5 to 9 of both PthN and Avr6, but in no other sequenced members, there are alternating polar versus nonpolar amino acids in position 13 of each repeat. Position 13 is the most variable position found in the repeat regions of all members of the gene family sequenced so far. These data are consistent with the findings of Yang and Gabriel (32), who found that deletions in *pthA* affecting the first eight repeats always affected the ability of *pthA* to elicit cankers on citrus, but deletions affecting repeats 9 through 15 sometimes did not. Their data strongly indicate that the location, spacing, and perhaps order of the repeats clustered in positions 1 to 9 may be critical to the phenotype conferred. We are currently constructing chimeric genes containing synthetic repeats to test the hypothesis that repeats 5 to 9 determine the pathogenicity phenotype conferred.

There is no reason to believe that the single *B12* gene found in S295 will not be evaded by some recombination event in an African strain, which can occur at high frequency in *X. campestris* pv. *malvacearum* (10). Extra copies of *pthN2* cloned in Xcm1003 did not confer a stronger HR on S295 than Xcm1003 alone, but extra copies of *pthN* did confer a stronger HR. Although this evidence is weak, this may indicate that Xcm1003 is avirulent on S295 because of *pthN*, but not because of *pthN2* and perhaps the other *avr/pth* genes found in Xcm1003. Now that it is known that the protein products of the *Xanthomonas avr/pth* genes function in the plant cell, the genes can be tested by directly expressing them in the plant cell (14), obviating the need for finding a strain virulent on S295. If even one of the *avr/pth* genes found in XcmN can evade recognition by the *B12* gene and still confer water-soaking on cotton, it is likely that a new strain will evolve that can also defeat the *B12* gene. This possibility is now under investigation.

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