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Research Note

Xanthomonas Avirulence/Pathogenicity Gene Family Encodes Functional Plant Nuclear Targeting Signals

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Pathogenic symptoms and host range of some *Xanthomonas* strains are determined in part by a family of avirulence/pathogenicity genes widely distributed in the genus. We identified heptad repeats similar to leucine zippers and three putative nuclear localization signals (NLSs) in the C-terminal regions of the predicted proteins of all sequenced members. The DNA coding sequences for the C-terminal regions of two members of the gene family with pathogenicity function (*avrb6* and *pthA*) were independently fused to a β-glucuronidase (GUS) reporter gene. When introduced into onion cells, both of these translational fusions were transiently expressed, and GUS activity was specifically localized in the nuclei of transformed cells. Protein extracts containing PthA elicited no symptoms when inoculated onto plants; *hrp* genes were required for *Xanthomonas* carrying *pthA* to elicit hyperplastic canker symptoms on citrus.

Additional keywords: disease resistance, gene-for-gene, nuclear localization.

Several models have been proposed to explain the genetically specific recognition between pathogen avirulence (*avr*) genes and plant resistance (*R*) genes in gene-for-gene interactions (Gabriel and Rolfe 1990). In the elicitor/receptor model, *avr* genes encode enzymes that produce low molecular weight signal molecules (elicitors) which are perceived by receptors encoded by the corresponding *R* genes and result in plant defense responses. In support of this model, *avrD* of *Pseudomonas syringae* was found to encode an enzyme involved in the synthesis of a low molecular weight glycolipid elicitor (Midland et al. 1993; Smith et al. 1993). An alternative model is the "dimer" hypothesis in which the protein product of an *avr* gene directly binds the protein product of an *R* gene or the *R* gene itself (Ellingboe 1982). In support of this model, the peptides encoded by *avr4* and *avr9* of the fungal pathogen *Cladosporium fulvum* were shown to directly induce plant defense responses (Joosten et al. 1994; van Kan et al. 1991). However, despite the fact that more than 30 bacterial *avr* genes have been reported cloned (Gabriel et al. 1993), the function of only one of them (*avrD*) is known.

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The predicted peptide sequences of several cloned *R* genes indicate a function in signal transduction (Martin et al. 1993; Bent et al. 1994; Mindrinos et al. 1994; Whitham et al. 1994). Since transcriptional activation of plant defense genes is modulated by phosphorylation (Felix et al. 1991; Yu et al. 1993), and can be blocked by inhibitors of mammalian protein kinases (Raz and Fluhr 1993), it was proposed that recognition of *avr* signals by *R* gene products triggers phosphorylation cascades, leading to plant defense responses (Lamb 1994). In mammalian signal transduction pathways (e.g., the Ras signaling pathway), extracellular signals activate receptor protein kinases, triggering phosphorylation cascades (Crews and Erikson 1993). The activated protein kinases and/or transcriptional factors enter the nucleus to induce gene expression (Kerr et al. 1992), leading to various physiological outcomes such as cell division or programmed cell death (Martin et al. 1994).

Transport of proteins into the nucleus is an active process and requires that the proteins contain suitable nuclear localization signals (NLSs; Nigg et al. 1991). There is no strict consensus NLS sequence, but most NLSs consist of either the monopartite motif K-R/K-X-R/K (usually adjacent to proline) or a bipartite motif consisting of two basic amino acids, a spacer region of any 4 to 10 amino acids and a cluster of 3 to 5 basic amino acids (Chelsky et al. 1989; Dingwall and Laskey 1991). NLSs may be located at any position as long as they are exposed on the protein surface; they may be present in multiple copies, and the effects of multiple copies are additive (Garcia-Bustos et al. 1991). Some viral pathogens encode transcriptional factors with NLSs that enter the nucleus and modulate host gene expression (Kerr et al. 1992). The bacterial plant pathogen *Agrobacterium* encodes at least two proteins with NLSs that direct the proteins to the plant cell nucleus (Citovsky and Zambryski 1993). However, NLS-containing proteins have not been described for microbial plant pathogens other than *Agrobacterium*.

A large family of highly homologous avirulence (*avr*)/pathogenicity (*pth*) genes has been identified in the genus *Xanthomonas*, a major group of bacterial plant pathogens. Members include *avrBs3*, *avrBs3-2* (Bonas et al. 1989, 1993), *avrB4*, *avrb6*, *avrb7*, *avrBIn*, *avrB101*, and *avrB102* of *X. campestris* (De Feyter and Gabriel 1991; De Feyter et al.

1993), *pthA* of *X. citri* (Swarup et al. 1992), and *avrxa5*, *avrXa7*, and *avrXa10* of *X. oryzae* (Hopkins et al. 1992). This gene family comprises nearly all described *Xanthomonas avr* genes and constitutes a large portion of all *avr* genes cloned to date. Genes *pthA* and *avrb6* are particularly intriguing because of their pleiotropic, and host-specific, pathogenicity functions. In addition to conferring ability to elicit the plant-hypersensitive response (HR) on selected plant genotypes when transferred to a variety of xanthomonads, they also confer ability to elicit host-specific pathogenic symptoms (*pthA* confers ability to induce hyperplastic cankers specifically on citrus and *avrb6* confers ability to induce strong watersoaking specifically on cotton [Swarup et al. 1992; Yang et al. 1994]). Comparisons of the two genes in selected isogenic *X. campestris* background strains revealed that these two nearly identical genes (98.4% identical predicted peptide sequences; Yang et al. 1995) can determine three very different plant response phenotypes: cankers on citrus, watersoaking on cotton, or an HR on many plants (refer Fig. 1). Chimeras of *avrb6* and *pthA* revealed that the 102-bp, leucine-rich, tandem repeats in the central portion of these genes determined all three plant reaction phenotypes, and that the regions outside of the repeats are functionally interchangeable (Yang et al. 1994).

Recently, we identified a series of heptad repeats, similar to leucine zippers, and three putative nuclear localization sequences encoded by all members of this gene family sequenced to date (Fig. 1). We also examined the predicted amino acid sequences encoded by some other *avr* genes, and at least one of them, AVR4 of *Cladosporium fulvum* (Joosten et al. 1994), appears to contain a putative bipartite nuclear localization signal (KKWCDYPNLSTCPVKTPGPKPKK) in the C-terminal region. The purpose of this study was to determine if the putative NLSs in the *Xanthomonas avr/pth* gene family were functional in plant cells.

Western blot analysis revealed that the predicted 122-kDa PthA protein (Yang et al. 1995) is constitutively expressed from its native promoter in wild-type *X. citri* 3213 cells grown in rich medium (Fig. 2). The polyclonal antiserum strongly bound to three proteins, all larger than 105 kDa, in extracts of 3213. Strain 3213 contains *pthA* plus three additional putative homologues of *pthA* (Swarup et al. 1992). All three putative homologues were independently cloned, analyzed by restriction digests, Southern blots and pathogenicity assays; all appeared to be members of the *avr/pth* gene family, but unlike *pthA*, none conferred ability to elicit cankers on citrus to other xanthomonads (data not shown). Gene *pthA* was disrupted by marker exchange in 3213, using a *nptI–sac* cassette inserted at the 10th tandem repeat (Yang et al. 1995). The resulting strain, Xc1.2 (*pthA::npt-sac*), was not pathogenic to citrus and produced the expected truncated protein of 68 kDa upon translation (marked by an asterisk in lane 2, Fig. 2). Both the 122-kDa band (data not shown) and pathogenicity of Xc1.2 were restored by pZit45 (*pthA*$^+$). Identity of PthA was confirmed by comparison of *X. campestris* 3048 (wild type, causes citrus leaf spot; lane 3) with 3048/pZit45 (causes citrus canker, lane 4). Besides the protein bands that strongly bound the polyclonal antiserum, there were several smaller (less than 105 kDa), weakly reacting protein bands in all strains. Some are likely to be degraded forms of PthA (and gene family homologues in 3213 or Xc1.2) or other cross-reacting proteins, as in the case of 3048 (lane 3), which contains no members of the *avr/pth* gene family.

Despite constitutive expression of PthA in wild-type *Xanthomonas* cells, *hrp* (*h*ypersensitive *r*esponse and *p*atho-

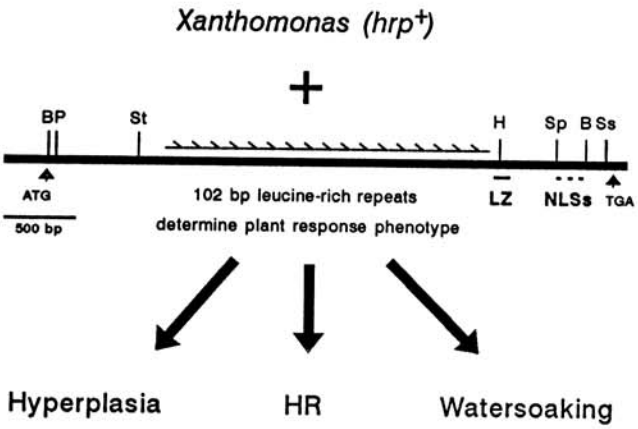

Fig. 1. General structure of members of the *Xanthomonas avr/pth* gene family. All members sequenced to date are at least 93% identical in DNA sequence, and 62-bp inverted terminal repeats mark the limits of homology. Each member carries from 14 to 23 nearly identical 102 bp tandem repeats in the central portion of each gene. These repeats are leucine-rich and determine both the plant response phenotype and plant genotype specificity when the genes are present in a number of different *Xanthomonas* (Hrp$^+$) backgrounds. Most members of the gene family were isolated as *avr* genes and confer ability to elicit the hypersensitive response (HR) on many plants. Gene *pthA* confers ability to elicit cell division (hyperplasia) of citrus and *avrb6* confers ability to elicit watersoaking of cotton. Translational start and stop codons are indicated. Highly conserved restriction enzyme sites are: B, *Bam*HI; P, *Pst*I; St, *Stu*I; H, *Hin*cII; Ss, *Sst*I. LZ, leucine zipper region; NLSs, nuclear localizing signals.

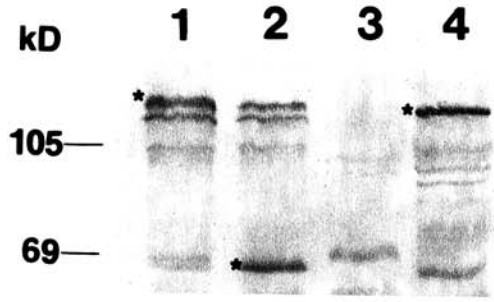

Fig. 2. Immunoblot using polyclonal anti-PthA antiserum against crude lysates of *Xanthomonas* cells grown in rich medium. Antiserum was prepared from a New Zealand rabbit by Cocalico Biologicals, Inc. (Reamstown, Penn.) using purified PthA. PthA was prepared from lysed *E. coli* strain BL21(DE3)/pLysS cells containing *pthA* cloned in pET-19b and purified by His-tag affinity chromatography (Novagen, Madison, Wisc.), followed by SDS-polyacrylamide gel electrophoresis. For Western blots, *Xanthomonas* cells were grown in PYGM (De Feyter et al. 1990) broth, harvested by centrifugation and boiled for 2 min. in sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.1% bromophenol blue). Lysates were centrifuged at 11,600 × *g* for 5 min, electrophoresed on 8% SDS-polyacrylamide gels and the separated proteins were transferred onto nitrocellulose (Towbin et al. 1979). Membranes were probed with anti-PthA antibody and visualized with goat anti-rabbit antibody conjugated with alkaline phosphatase (Sigma). Lane 1, *X. citri* strain 3213 (Gabriel et al. 1989); lane 2, marker-interruption derivative of 3213, Xc1.2 (*pthA::npt-sac*) (Yang et al. 1995); lane 3, *X. campestris* strain 3048 (Gabriel et al. 1989); lane 4, transconjugant 3048/pZit45 (PthA$^+$) (Swarup et al. 1992). Asterisks indicate PthA or its truncated derivative. The thick, top band in lane 1 is a doublet.

genicity) gene function also was required to elicit hyperplastic cankers on citrus. When a Hrp⁻ mutant derivative of 3048 (Kingsley et al. 1993) carrying pZit45 was inoculated at high concentrations (10^8 CFU/ml) into citrus leaves, no symptoms of any kind developed (results not shown). These experiments extend the work of Knoop et al. (1991), who reported that *hrp* gene function was required for *X. campestris* carrying *avrBs3* to elicit a defense response on pepper. Since most *hrp* genes appear to be part of a signal peptide-independent (type III) protein secretion system (Fenselau et al. 1992; Van Gijsegem et al. 1994) it seems likely that the proteins encoded by these *avr/pth* genes are exported from *Xanthomonas*. However, such export has not been detected to date (for example, see Brown et al. 1993). Furthermore, in experiments repeated many times, cell lysates and crude protein prepared from 3213 or 3048/pZit45 as described by He et al. (1993) failed to elicit symptoms when inoculated into leaves. This indicates that if exported, the Avr/Pth proteins are modified, protected by chaperones, and/or exported in quantities below the threshold of detection.

We analyzed the predicted amino acid sequences of PthA, Avrb6, AvrBs3, and AvrXa10, and identified three clusters of basic amino acids, characteristic of monopartite NLSs. All three putative NLSs were located at the carboxyl-termini of gene family members (at positions 1020 to 1024 [K-R-A-K-P], 1065 to 1069 [R-K-R-S-R], and 1101 to 1106 [R-V-K-R-P-R] of PthA; refer Fig. 1). These sequences are very similar to the adenovirus E1a sequence (K-R-P-R-P). In addition, three putative casein kinase II sites were found in close proximity to these three putative NLSs (at positions 994 to 997 [TELE], 1068 to 1071 [SRSD], 1120 to 1123 [TAAD] of PthA). Many nuclear localized proteins have casein kinase II sites (S/T-X-X-D/E) at a distance of 10 to 30 amino acids from the NLSs (Rihs et al. 1991).

To determine if these NLSs are functional in plant cells, the DNA coding sequences for the C-terminal regions (191 amino acids) of PthA and Avrb6 were translationally fused with the 5′ end of a β-glucuronidase (GUS) reporter gene. The resulting constructs were introduced into onion epidermal cells by microprojectile bombardment. Intracellular localization of the GUS fusion proteins (approximately 90 kDa), containing the C-terminal region of PthA and Avrb6, was determined histochemically. In three independent transformation tests, GUS activity was localized specifically to the nuclei of transformed plant cells (Fig. 3A). More than 100 nuclei were observed to be GUS positive. Some diffuse GUS activity was observed several times within the cytoplasm. The location of nuclei was confirmed by staining with the nucleus-specific dye 4′,6-diamidino-2-phenylindole (DAPI) and observed under fluorescence microscope (Fig. 3B). In control experiments, the 68-kDa GUS protein alone was not observed to be localized. GUS is a useful marker for these purposes because it is not known to localize to the nucleus (Restrepo et al. 1990; Varagona et al. 1992). These results demonstrate that the *Xanthomonas avr/pth* gene fragments encode functional NLSs.

It remains to be determined if functional NLSs are required for any or all three of the plant reaction phenotypes determined by *avrb6* and *pthA*. One member of the *avr/pth* gene family, *avrBs3-2*, does not require NLSs for activity, since it confers avirulence activity to *X. campestris* pv. vesicatoria on all tomato cultivars tested, with up to 2/3 of its coding region (including the NLS region) deleted (Bonas et al. 1993). However, this report is exceptional. Activity assays using detailed deletion analyses and transposon mutagenesis of at least 10 other members of this *avr/pth* gene family (including *avrb6* and *pthA)* demonstrate that the regions outside of the repeats (including the NLS regions) are required for activity (Bonas et al. 1993; De Feyter et al. 1993; Hopkins et al. 1992; Swarup et al. 1991 1992). If NLSs are required for Avr/Pth activity, it would provide additional indirect evidence to support the proposal by Fenselau et al. (1992) that *Xanthomonas*

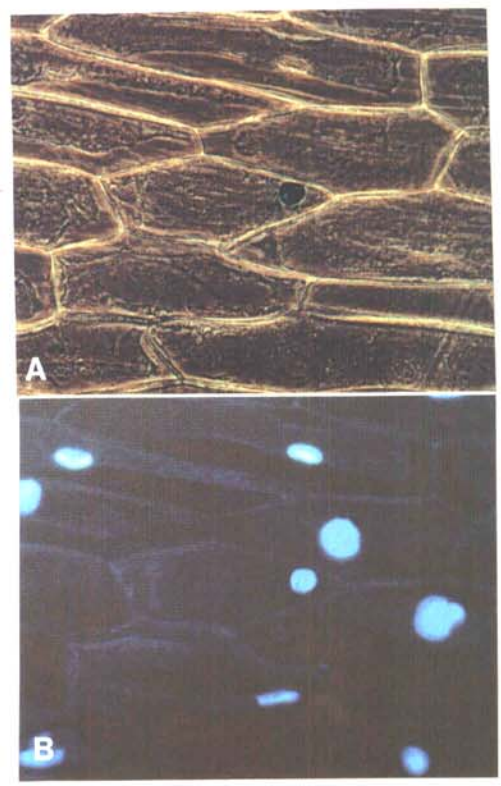

Fig. 3. Nuclear localization of β-glucuronidase fusion proteins containing the C-terminal region of PthA in transformed onion epidermal cells. The DNA coding sequences for the C-terminal 191 amino acids of PthA and Avrb6 were amplified from pZit45 (Swarup et al. 1992) and from pUFR127 (De Feyter and Gabriel 1991), respectively, using the polymerase chain reaction (PCR) with primers 5′-CTCTAGAGCCATGACGCAGTTC and 5′-CAGATCTCTGAGGCAATAGCTC. *Xba*I and *Bgl*II restriction sites were incorporated into the primers, allowing the amplified fragments to be recloned in frame with a Gus reporter gene on pBI221 (Clonetech, Palo Alto, CA). This resulted in placement of the fragments between the 35S promoter and the β-glucuronidase (GUS) reporter gene on pBI221, and created translational gene fusions with GUS. The Helium Biolistic gene transformation system (Du Pont) was used to transform the epidermal cell layers of white onion as described (Varagona et al. 1992). DNA samples were prepared as described (Taylor and Vasil 1991). After microprojectile bombardment at a pressure of 1,100 psi, petri dishes were sealed with Parafilm and incubated overnight at 28°C in the dark. The histochemical GUS assay was used to determine the location of GUS fusion proteins in onion cells as described previously (Jefferson 1987; Varagona et al. 1992). Cellular location of the blue indigo dye produced by oxidative dimerization of the GUS product was determined under bright-field optics using a Zeiss Axiophot microscope and compared with the location of DAPI-stained nuclei observed under fluorescence optics. **A,** Onion tissue sample analyzed using X-glucuronide. **B,** The same tissue sample then was stained with the nuclear stain DAPI.

secretes AvrBs3 protein directly into the intercellular spaces of plants colonized by the bacteria. It would also support our hypothesis that the Avr/Pth proteins (or processed fragments) are imported into the plant cell, perhaps by receptor-mediated endocytosis (Horn et al. 1989), and that the proteins can be translocated to the plant nucleus. This idea is being tested.

In prokaryotic and eukaryotic proteins, repetitive domains often function as binding sites and determine molecular specificity (Wren 1991; McConkey et al. 1990). The predicted amino acid sequences of PthA, Avrb6, AvrBs3, and AvrXa10 contain 34 amino acid tandem repeats that are leucine rich. Further analysis of their amino acid sequences revealed the presence of leucine zipperlike heptad repeats (LESIVAQ LSRPDPA LAALTNDH LVALAC LGGRPA LDAVKKG LPHAPAL IKRTNRR IPERTSH, at positions 884 to 945 of PthA) closely linked to the 34 amino acid tandem repeats of all these proteins (Fig. 1). Recently, sequence analyses of several cloned plant resistance genes, such as *RPS2* of *Arabidopsis*, revealed the presence of leucine-rich repeats in their encoded proteins (Mindrinos et al. 1994). Leucine zippers and leucine-rich repeats serve as the sites of protein-protein binding; leucine zippers may directly interact with DNA (Johnson and McKnight 1989). It is possible that leucine-rich repeats mediate direct interactions between pathogen Avr/Pth and plant Resistance/Susceptibility proteins to form dimers or multimers. In at least some cases, and perhaps only in the absence of specific *R* genes (a requirement for watersoaking or canker phenotypes), the NLS signals may direct transport to the plant nucleus. After entering the nucleus, Avr/Pth proteins or protein complexes may act on nuclear transcriptional factors, leading to different physiological outcomes such as hyperplasia (cell division) of citrus, watersoaking of cotton, or the HR (cell death).

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