Research Note

Expression of a Single, Host-Specific, Bacterial Pathogenicity Gene in Plant Cells Elicits Division, Enlargement, and Cell Death

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A fundamental question about microbial pathogens is how they elicit host-specific symptoms. We report here that expression of a single gene from a plant-pathogenic bacterium in plant cells elicits host-specific symptoms diagnostic of the disease caused by the pathogen. Expression of *pthA* from *Xanthomonas citri* in citrus cells is sufficient to cause division, enlargement, and death of host cells. Since elicitation of these symptoms depends on a functional type III protein secretion system in *X. citri*, we deduce that the PthA protein is a specific plant signal, its site of action is inside the plant cell, and it is a major determinant of host range.

Additional keywords: avirulence, citrus canker, NLS, nuclear localization, virulence.

The molecular basis of disease epidemics and the identification of factors determining disease severity, symptoms, and host range are subjects of intense interest to microbial pathologists. Plant diseases are useful for such molecular studies because both host and pathogen are often genetically tractable. Plant pathogens are also particularly useful for studies involving host range, because some pathogenic genera, such as the bacterial genus *Xanthomonas*, consist of variants that can attack species of nearly all major groups of higher plants (Starr 1983). Despite the wide collective host range of the genus *Xanthomonas*, individual strains that cause serious diseases are highly restricted in host range. For example, strains of *Xanthomonas citri* cause such a severe canker disease on citrus worldwide that the pathogen is quarantined and subject to eradication in many countries. Fortunately, the only known hosts of the pathogen are in the Rutaceous (citrus) family, and the pathogen does not cause cankers on other plants, even in artificial inoculations. What accounts for its ability to attack citrus so severely and specifically?

X. citri enters its hosts naturally by rain splash through stomata and grows in the abundant intercellular space of the spongy mesophyll; large pustules or cankers are then formed on leaves, stems, and fruit as the pathogen induces cell enlargement (hypertrophy), division (hyperplasia), and, ultimately,

death (necrosis) among contacted host cells (Swarup et al. 1991; Fig. 1). Eventually, the epidermis is ruptured, allowing massive bacterial egress to the plant surface (Fig. 1), where bacteria are readily available for rain splash and repeated infection cycles. Other xanthomonads can cause mild leaf spots on citrus, but lack the ability to rupture the epidermis and form the hyperplastic cankers that are diagnostic of citrus canker disease (Gabriel et al. 1989; Swarup et al. 1991). The citrus leaf spots are much less serious diseases, and are much less efficient in dissemination and spread on citrus (Graham and Gottwald 1991). Therefore, the ability to rupture the citrus epidermis is a major factor in both the severity of the disease and in its epidemiology.

A gene for pathogenicity, *pthA*, is necessary for *X. citri* to cause citrus canker disease; insertion of *pthA* cloned on pZit45 into other xanthomonads confers the ability to rupture the citrus epidermis and elicit cankers on citrus (Swarup et al. 1991). Since (i) this ability also requires a functional type III protein secretion system, (ii) crude protein preparations containing PthA failed to elicit symptoms, and (iii) *pthA* encodes functional nuclear localization signal (NLS) sequences, it therefore appeared likely that PthA functioned inside the plant cell (Yang and Gabriel 1995a). In order to determine the direct effect(s) of expression of *pthA* on plants in the absence of the pathogen, we used two methods to transiently express the gene in plant cells. We report here that expression of *pthA* in citrus cells is sufficient to elicit the symptoms diagnostic of citrus canker disease: hypertrophy, hyperplasia, and necrosis (see Figures 2 and 3).

Transient expression of *pthA* in citrus leaves induced small, raised cankers on sweet orange leaf surfaces that first became visible beginning 3 days after particle bombardment and 7 days after *Agrobacterium tumefaciens* inoculation. Control bombardments and *A. tumefaciens* inoculations with the expression vector alone showed no evidence of cankers or canker-associated symptoms. For particle bombardment, leaves were surface sterilized and bombarded with tungsten particles coated with DNA as described by Gray et al. (1994). The treated leaves were placed on Murashige and Skoog medium (Gibco BRL, Grand Island, NY) and incubated at 22°C under 16 h of light. For *A. tumefaciens* delivery, clones were mobilized into strain GV2260 with triparental matings and inocu-

lated into citrus leaves as described (Kapila et al. 1997). Semi-thin sections of resin-embedded tissue were stained with toluidine blue. Cell division and cell enlargement were caused when *pthA* was delivered to citrus cells by either method, and the symptoms elicited were qualitatively typical of the symptoms induced by control *X. citri* inoculations, although *X. citri* induced much larger lesions (compare Figures 1 and 2). These quantitative differences are likely due to relatively inefficient gene transfer and/or gene expression, as well as non-uniform gene transfer to cells in a target area in the transient expression methods. It is also possible that additional pathogenicity factors from *X. citri* are needed to enhance the symptoms triggered by the action of *pthA*.

No cankers were elicited on citrus in transient expression assays in which another member of the gene family, *avrb6*, was substituted for *pthA* on the same vectors (Table 1; Fig. 3). *avrb6* is 97% identical to *pthA* in DNA sequence and was cloned from *X. campestris* pv. *malvacearum* (the cotton blight pathogen; Yang and Gabriel 1995b). Expression of *avrb6::uidA* in citrus cells was confirmed by β -glucuronidase (GUS) assays with pYD12.22. (Translational fusion of *pthA* with *uidA* on pYD12.9 showed GUS expression levels similar to those on pYD12.22 [data not shown], and did not affect the canker phenotype, which was comparable to pYD12.5.)

Progressive development of necrotic tissue was also observed in these transient expression assays in sweet orange, similar to control inoculations with *X. citri* but delayed and greatly reduced (Fig. 2B, D). Transiently expressed *pthA* caused cell death and necrotic tissue development as lesions aged, with necrosis becoming apparent 2 weeks following particle bombardment and 3 weeks following *A. tumefaciens* delivery. *X. citri* caused necrotic tissue development beginning after 8 days on sweet orange. Since the necrotic tissue developed following particle bombardment (in the absence of any bacteria) and was not observed in control bombardments, the necrosis was induced by expression of *pthA* and not by wounding or the action of bacterial enzymes or other inducers. These data strongly indicate that *pthA* causes programmed cell death (PCD) in citrus, in addition to cell division and cell enlargement. Inducing cell death in compatible interactions is thought to be important in providing nutrients for microbial pathogens, particularly after release onto the leaf surface (Greenberg 1997). Inducing PCD may be essential for high

levels of growth of *X. citri*, since mutations of *pthA* in *X. citri* significantly reduce pathogen growth in planta (Swarup et al. 1991). Since *pthA* is citrus specific (see below) and causes cell death, if *pthA* thereby obtains enough nutrients to support high levels of growth in citrus, then *pthA* is a primary determinant of host range on citrus.

Finally, rupture of the citrus epidermis was observed when *pthA* was delivered by *A. tumefaciens*, although it was greatly reduced, compared with the *X. citri* controls (Figs. 1 and 2B, D). This suggests that the eruption force may come from the growth and physical expansion of bacterial population in planta and, in the case of *X. citri*, additional expansion may be caused by the hydration of xanthan gum produced by these bacteria. The proteases, lipases, and pectinases that are produced by *X. citri* (Gabriel et al. 1989) may also contribute to the symptoms by degrading cell walls and other cellular components.

The pathogenic symptoms directly elicited by *pthA* expression in citrus cells were host specific. In transient expression assays of *pthA* in tobacco, bean, and cotton, no canker phenotype was observed. Instead, a rapid necrotic reaction known as a hypersensitive response (HR) phenotype was usually observed (see for example Figure 4); however, in both cotton and bean there was evidence of cultivar-specific differences in the strength and timing of the necrotic reactions, some of which appeared quite weak (data not shown). These observations are consistent with published results of transferring *pthA* into xanthomonads that are pathogenic to plant hosts other than citrus; when placed in bean-specific or cotton-specific xanthomonads that are then inoculated on their respective hosts, *pthA* causes a reduction of symptoms on all susceptible cultivars and also a cultivar-specific HR (Swarup et al. 1992). Pathogen genes that elicit an HR are called avirulence (*avr*) genes. *pthA* is therefore a citrus-specific pathogenicity (*pth*) gene as well as an *avr* gene in plants other than citrus. Since the hypertrophy and hyperplasia caused by *pthA* that lead to epidermal rupture are host specific and epidemiologically significant, *pthA* contributes to host range by causing hypertrophy and hyperplasia, in addition to its possible involvement in increasing nutrient availability through cell death.

No previous studies have reported that microbial pathogenicity genes that are normally expressed in the microbe can elicit pathogenic symptoms when expressed inside plant cells.

Fig. 1. Phenotype elicited on *Citrus sinensis* (sweet orange) by *Xanthomonas citri* infection, 35 days after inoculation. To the left: an adaxial surface view under low level magnification. To the right: a thin section of the same leaf tissue pictured on the left (bars are 100 μ m). Arrow labeled "N" indicates necrotic tissue; arrow labeled "B" indicates *X. citri* bacteria oozing from ruptured epidermis.

Fig. 2. Phenotypes elicited on *Citrus sinensis* (sweet orange) by *pthA* delivered into leaf cells and transiently expressed. Left column: surface views of the leaves with low level magnification. Right column: thin sections of the same leaf tissue pictured on the left (bars are 100 μ m in all photos). **A**, Control leaf tissue 3 weeks after biolistic bombardment with pBI221. **B**, Effect of biolistic delivery of *pthA*, 3 weeks after bombardment with pYD12.5. Shown are the increase in number of cells (hyperplasia), loss of free extracellular space (apoplast) in the spongy mesophyll, raised lesion areas, and necrotic epidermal layer of cells, compared with **A**. **C**, Control leaf tissue 45 days after inoculation with *Agrobacterium tumefaciens* GV2260. **D**, Effect of delivery of *pthA* via *A. tumefaciens* GV2260/ pYD 40.1, 45 days after inoculation. There are similarities with **B**, and also an increase in volume of many mesophyll cells (hypertrophy), compared with **C**, along with the hyperplasia and more confluent symptoms. Arrows labeled “N” indicate necrotic tissues. There are similarities with Figure 1, but the symptoms are quantitatively less severe and the epidermal layer is barely ruptured.

However, several microbial *avr* genes can elicit an HR when expressed inside plant cells carrying specific resistance (*R*) genes (Gopalan et al. 1996; Tang et al. 1996; Scofield et al. 1996; Van den Ackerveken et al. 1996; de Feyter et al. 1998; Stevens et al. 1998). Both *avrB* and *avrRpt2* cause cell death

Fig. 3. DNA constructions used in the transient expression assays. *pthA* was cloned from pZit45 into pUC118 with a uracil DNA glycosylase (UDG) polymerase chain reaction (PCR) technique (Nisson et al. 1991) that allowed creation of multiple cloning sites at strategic locations. The resulting clone pYD9.3 had *EcoRI/XbaI* and *HindIII/BglII* sites immediately flanking the *pthA* coding region. For transient expression in plants, the *pthA* and *avrb6* coding regions were recombined into pBI221 (CLONETECH Laboratories, Palo Alto, CA) such that they would be expressed from the 35S promoter, and also creating translational fusions with *uidA* with the *XbaI* and *BglII* restriction sites (pYD12.9 and pYD12.22, respectively) and transcriptional fusions with *uidA* with the *XbaI* and *SmaI* sites (pYD12.5 and pYD12.2, respectively). These genes were also cloned into the *Agrobacterium tumefaciens* binary vector pB48.212 (Li et al. 1994), forming pYD40.1 and pGZ6.4. Left and right border sequences of T-DNA: represented by T_L and T_R. Nos, nopaline synthase. A, translational enhancer region from tobacco mosaic virus.

Table 1. Symptoms elicited by *pthA* and *avrb6* in *Xanthomonas citri* and when delivered directly into sweet orange leaf cells^a

Strain and/or clone	Sweet orange host
<i>X. citri</i>	
3213 (wild type, <i>pthA</i> ⁺)	C+++++
B21.2 (<i>pthA</i> ::Tn5- <i>uidA</i>)	0
B21.2/pUFR047(vector)	0
B21.2/pZit 45 (<i>pthA</i>)	C+++++
<i>A. tumefaciens</i> delivery	
GV2260	0
GV2260/pYD40.2 (vector)	0
GV2260/pGZ6.4 (<i>avrb6</i>)	0
GV2260/pYD40.1 (<i>pthA</i>)	C++
Particle bombardment	
pBI221 (vector)	0
pYD12.22 (<i>avrb6</i> :: <i>uidA</i>)	0
pYD12.9 (<i>pthA</i> :: <i>uidA</i>)	C+
pYD12.2 (<i>avrb6</i>)	0
pYD12.5 (<i>pthA</i>)	C+
pYD40.2 (Δ <i>pthA</i>)	0
pYD40.1 (<i>pthA</i>)	C+

^a *X. citri* strain 3213^T is the species type strain (Gabriel et al. 1989). B21.2 is a nonpathogenic mutant derivative of 3213^T that is marker-interrupted at *pthA* (Swarup et al. 1991). pZit45 carries *pthA* cloned in vector pUFR047 (Swarup et al. 1992). All other plasmids except pYD40.2 are new plasmids described in Figure 3; pYD40.2 is a *pthA* deletion derivative of pYD40.1 (the 3.3-kb internal *BamHI* fragment of *pthA* was removed). C: macroscopically visible, hyperplastic cankers; +: subjective, estimated degree of symptom severity (C++++ is about 5× stronger than C+); 0: no symptoms beyond damage caused by the delivery method.

and necrosis in plants evidently lacking cognate *R* genes, thus suggesting a possible role for these *avr* genes in pathogenicity (Gopalan et al. 1996; McNellis et al. 1998). Gene *pthA* belongs to a large avirulence/pathogenicity family—the *avrBs3/pthA* gene family (Leach and White 1996)—that is widespread in the genus *Xanthomonas*. Members are conserved among all strains of some species and/or pathovars, including all strains causing cotton blight, rice blight, and common bean blight (Gabriel 1997). A causal role in pathogenicity for members of the gene family has also been demonstrated with cotton blight (Yang et al. 1996) and is strongly suggested for rice blight (Leach and White 1996).

For either pathogenicity or avirulence phenotypes to be elicited by most bacterial plant pathogens, including *X. citri*, a functional type III protein secretion system encoded by *hrp* (hypersensitive response and pathogenicity) genes is required (He 1998; Alfano and Collmer 1997). Function of *pthA* in *X. citri* for either pathogenicity or avirulence is *hrp* dependent (Yang and Gabriel 1995a). Type III protein secretion systems are essential for pathogenicity of many bacteria, including *Yersinia pestis* (black plague) and enteropathogenic *Yersinia* spp., *Shigella* spp., and *Escherichia coli* (Hueck 1998). The type III secretion system is a host cell contact-dependent, toxic protein injection device in mammalian pathogens (Silhavy 1997). The system appears to work similarly in plant pathogens, but fewer genes are known to be involved and there may be several important differences (He 1998). After PthA was found to encode functional NLS sequences, the protein was proposed as a candidate effector signal secreted by the *X. citri* *hrp* system directly or indirectly into the plant cell (Yang and Gabriel 1995a; Gabriel 1997). Taken together, these results provide strong indirect evidence that PthA is secreted from *X. citri* and functions inside the plant cell. Although *A. tumefaciens* causes crown gall disease by transfer of a piece of DNA into plant cells, this represents the first report of a bacterial pathogen that causes disease by transfer of a protein signal into the plant cell.

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Fig. 4. Hypersensitive response (HR) elicited by *pthA* in *Phaseolus vulgaris* (common bean) cv. California Light Red leaf after inoculation with *Agrobacterium tumefaciens* GV2260/pYD40.1.

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