Molecular mechanisms of pathogenesis in pathovars of the rice pathogenic bacterial species *Xanthomonas oryzae*

by

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Abstract

The pathogenicity of the two closely related phytopathogens *Xanthomonas oryzae* pv. oryzae and *Xanthomonas oryzae* pv. oryzicola was studied. These bacteria cause two different diseases with distinct tissue specificities on the same host plant rice. First, *X. oryzae* pv. oryzicola, the causal agent of bacterial leaf streak of rice, was observed to interfere with resistance gene-mediated defense responses to avirulence genes from *X. oryzae* pv. oryzae in rice. The expression of two *avr* genes from *X. oryzae* pv. oryzae in *X. oryzae* pv. oryzicola failed to elicit *avr* gene-mediated plant defense-associated hypersensitive response (HR) in rice cultivars with corresponding *R* genes and failed to prevent leaf streak development. Co-inoculation of *X. oryzae* pv. oryzicola quantitatively inhibited HR of blight resistant rice to avirulent *X. oryzae* pv. oryzae and this inhibition was type III dependent. Second, differential regulation of the type III secretion system required for pathogenesis was explored as a candidate determinant of tissue specificity. Specifically, identification of upstream regulator(s) of HrpG, a regulator of type III secretion, was attempted. Type III genes of the two pathogens are activated by different environmental stimuli, yet the *hrpG* genes of vascular and non-vascular *Xanthomonas* strains functioned equivalently in genetic complementation assays. This suggests that activator(s) of HrpG rather than HrpG itself may account for differential regulation and determine the tissue specificity of *X. oryzae* pv. oryzae and *X. oryzae* pv. oryzicola. The attempt to identify HrpG activators, based on gain-of-mutagenesis, was unsuccessful but details are presented for reference for future attempts.

Chapter 1.

Molecular interactions of plants and phytopathogens: a review of literature relevant to the research presented in this thesis

LITERATURE RIVEW

The purpose of the research presented in this thesis is to improve our fundamental understanding of molecular mechanisms of plant-bacterial interaction toward the ultimate goal of advancing plant protection. This research focuses on two bacterial diseases of rice (*Oryza sativa*) caused by two closely related bacterial pathogens. These diseases include bacterial blight caused by *Xanthomonas oryzae* pv. oryzae and bacterial leaf streak caused by *X. oryzae* pv. oryzicola. Bacterial blight is a vascular disease and bacterial leaf streak is non-vascular. Together the two diseases constitute a powerful comparative model for understanding tissue specificity in bacterial interactions with plants. I address two topics in the thesis to accomplish the ultimate goal to improve plant protection: the lack of major gene resistance to bacterial streak (Chapter 2) and regulation of pathogenicities of *X. oryzae* pv. oryzae and *X. oryzae* pv. oryzicola with different tissue specificities (Chapter 3). In this chapter, I review relevant literature regarding these pathogens, their host plant, and molecular mechanisms of plant-bacterial interactions generally.

Xanthomonas oryzae

Xanthomonas oryzae (ex Ishiyama 1922) Swings et al. 1990 is a species in the gamma subdivision of the proteobacteria that is pathogenic to rice (Swings et al. 1990). The species comprises two pathovars, oryzae and oryzicola. The designation pathovar (pv.) is

used to denote subspecies distinguished by characteristic pathogenicity or host plant specificity. In the case of *X. oryzae* pv. oryzae and *X. oryzae* pv. oryzicola, both infect the same host plant, but in distinct ways, causing distinct diseases. *X. oryzae* pv. oryzae invades through hydathodes and wounds of rice leaves and travels systemically through the xylem to cause bacterial leaf blight. *X. oryzae* pv. oryzicola, by contrast, invades through stomata and colonizes the mesophyll parenchyma to cause bacterial leaf streak. Genetic identity of the two pathogens was estimated as greater than 90 % by DNA-DNA hybridization (Swings et al. 1990).

Bacterial blight of rice

Bacterial blight is one of the most important rice diseases and widely distributed in many rice growing areas such as Africa, India, East Asia, and Australia. It is currently absent from and quarantined in North and South America (Mew et al. 1993; Ou 1985; Webster and Gunnell 1992). Bacterial blight is a vascular disease, and the typical symptoms are wilting and yellowing of leaves and water-soaked stripes accompanied by lesions that develop along the leaf veins as the pathogen spreads through the xylem. Lesions eventually become opaque, whitish gray as the disease advances. Severely infected leaves tend to dry quickly. In the tropics, symptoms called kresek are observed 1 to 3 weeks after transplanting as grayish green water-soaked lesions spreading typically from the leaf tip. Kresek often results in death of seedlings. Yield losses due to bacterial blight range from 50 % on very susceptible cultivars to 10 % on moderately susceptible cultivars, and insignificant on resistant cultivars (Ou 1985). In the tropics, yield losses are more severe than in temperate regions because high humidity, rain, and warm temperatures favor the disease. The major control methods for

bacterial blight are using resistant varieties with major resistance genes, managing fertilization, especially nitrogen which increases disease in susceptible cultivars, and careful plant handling (Vera Cruz et al. 2000).

Bacterial leaf streak of rice

Rice bacterial leaf streak caused by *X. oryzae* pv. oryzicola occurs more sporadically than bacterial blight but is distributed as widely in Southeast Asian, India, and Africa (V. Verdier, personal communication, (Mew 1993; Ou 1985; Webster and Gunnell 1992)). The typical symptoms are interveinal water-soaked lesions that enlarge and coalesce to form streaks. Streaks are dark-green at first and become yellowish gray and translucent. *X. oryzae* pv. oryzicola spreads intercellularly in the leaf parenchymal tissue. Bacteria exude from stomata and form small yellow beads on the leaf surface. At the later stages of the disease, entire leaves turn brown and die. Japonica varieties are relatively less susceptible than indica varieties. The major control methods for leaf streak are resistant varieties, balancing fertilization and careful plant handling. In contrast to bacterial blight, major gene resistance to bacterial streak has not been identified. Quantitative and polygenic horizontal gene resistances are used currently (Tang et al. 2000).

Importance of rice as a staple crop and biological model

Rice is one of the most important crop plants. Rice is a staple food for over 30 countries and much of the world population. It provides 20 % of the dietary energy in the world. In Asia alone, two billion people obtain 60-70 % of their calories from rice and its products. Over 500 million tons of rice are produced every year. Rice is an important

commodity in the world, especially as an export material from developing countries. It accounts for over seven billion dollars annually in international trade (World Rice Statistics, International Rice Research Institute, http://www.irri.org/science/ricestat/index.asp). Rice fields maintain ecologically diverse habitats for both terrestrial and aquatic organisms. In many developing countries, rice cultivation provides source of income and employment (International Year of Rice 2004, http://www.fao.org/rice2004/index_en.htm).

Rice is also emerging as the leading model for cereal biology (Goff 1999). In addition to a virtually complete genomic sequence, abundant genetic resources are available for rice, including a large EST database, a growing number of mutant libraries including transposon and activation tagged mutants (Shimamoto and Kyozuka 2002). Rice is the first crop plant to be sequenced, and completion of the sequence in 2005 has been promoting functional genomics (Yu et al. 2005). Functional genomics with microarray technology is facilitating rice research on reproductive development and responses to pathogens and stress (Izawa et al. 2000; Talbot 2003). Efficient T-DNA mediated transformation using *Agrobacterium tumefaciens* promotes transgenic rice as a tool for various research (Hiei et al. 1994). The collection of rice germplasm is over 100,000 accessions at the international rice research center. This collection is an important genetic resource of biodiversity for breeding and gene functional characterization. Due to a high degree of synteny, studies in rice can be applied to additional cereal crops such as maize, wheat, and barely. Great progress in rice genomics has made rice as a model for broader comparative genomics as well (Nelson et al. 2004; Shimamoto and Kyozuka 2002).

Plant recognition of pathogens and plant defense

Plant resistance induced by recognition of pathogens can be divided into non-host specific and host-specific resistance (Nurnberger and Brunner 2002). In general, non-host specific resistance is induced by non-specific elicitors (Gomez-Gomez 2004). Non-specific pathogen elicitors include numerous microbial compounds such as lipopolysaccharides (LPS), flagellin, polypeptides and microbial cell wall derived molecules (e.g. polygalacturonides, chitosan, chitin, lipids, beta-glucans) (Dow et al. 2000; Gomez-Gomez and Boller 2002). These pathogen surface-derived molecules are referred to as pathogen-associated molecular patterns (PAMPs) (Nurnberger and Brunner 2002). PAMPs are sensed by pattern recognition receptors in plants. The *FLS2* gene in *Arabidopsis thaliana*,which encodes an LRR-type receptor kinase, is required for the perception of bacterial flagellin (Gomez-Gomez and Boller 2000). This perception activates a downstream phosphorylation cascade for defense responses (Asai et al. 2002). Hepta-β-glucoside derived from the oomycete pathogen *Phytophthora* cell wall is an elicitor of anti-microbial phytoalexin production in soybean (Mithofer et al. 2000).

Host specific resistance is generally triggered by specific pathogen elicitors and may involve any of a number of responses associated with defense (Hammond-Kosack and Jones 1996). These responses may include plant cell wall fortification involving lignin deposition and the formation of papillae by callose deposition particularly in response to microbial (e.g., fungal) penetration of plant cell walls (Hammond-Kosack and Jones 1996). Plant cell wall debris produced by microbial penetration or enzymatic activity also elicits additional cell wall fortification. Localized microbial attack can trigger systemic acquired resistance (SAR), which confers long-lasting defense against a broad range of phytopathogens. Salicylic acid (SA) is required to activate SAR (Durrant and Dong 2004). SA accumulation in activated

SAR correlates also with the expression of PR (pathogenesis-related) proteins. Sometimes PR protein induction is used as a marker of SAR. PR proteins are defined as proteins specifically induced in pathological or related situations that are thought to contribute to resistance (van Loon et al. 1994). Some known functions of PR proteins are chitinase, β-1,3-glucanase and proteinase-inhibitor with antimicrobial activities (Van Loon and Van Strien 1999). Other forms of defense include post-transcriptional gene silencing (PTGS), a targeted RNA degrading mechanism effective against some viruses that generate double-stranded RNA as an intermediate during viral replication (Voinnet 2001). PTGS is conserved in animals and fungi as RNAi (RNA interference) (Hammond et al. 2001). Most host-specific resistance responses are governed by the interaction of plant resistance genes and pathogen avirulence genes.

Major gene disease resistance in plants – gene-for-gene interactions

Flor's gene-for-gene model explains cultivar-race specific plant-microbial pathogen interactions (Flor 1971; Keen 1990). A plant carrying a dominant resistance (*R*) gene may be resistant to a specific pathogen race carrying an avirulence (*avr*) gene corresponding to the *R* gene. The interaction of *R* and *avr* genes leads to incompatibility involving recognition of the pathogen and induction of plant defense against it. In the absence of either the *R* gene or *avr* gene, the interaction is compatible and leads to disease development. The hypersensitive reaction (HR) is an early defense response associated with the incompatible interaction. It is a programmed plant cell death localized to the site of infection that may limit pathogen spreading by reducing water potential in the tissue (Klement 1982; Wright and Beattie 2004). To explain gene-for-gene interactions at the molecular level, a ligand-receptor model for the

gene products was proposed (Gabriel and Rolfe 1990). The model hypothesizes that physical interaction of Avr and R proteins is required to trigger defense responses. However, few direct Avr-R interactions have been demonstrated (e.g. AvrPto of *Pseudomonas syringe* and Pto of tomato) (Jia et al. 2000; Tang et al. 1996). Recent evidence suggests indirect interaction of Avr and R in many cases, a notion consistent with the guard model original proposed by Van der Biezen and Jones (Belkhadir et al. 2004; Marathe and Dinesh-Kumar 2003; Van der Biezen and Jones 1998). In this model, Avr proteins "attack" plant molecular targets to promote parasitism, and R proteins serve as a surveillance system to detect perturbation of these targets (Belkhadir et al. 2004; Jones and Takemoto 2004). Supportive evidence of this model is that AvrRpt2 of *Pseudomonas syringae* pv. tomato was found to modify the plant protein RIN4 which interacts with the corresponding R protein RPS2 of Arabidopsis (Axtell and Staskawicz 2003; Mackey et al. 2003). There is no evidence for direct interaction of AvrRpt2 and RPS2 like AvrPto and Pto. Either of these models, or variations of them, may account for different R-Avr interactions (Martin et al. 2003).

_R_ and _avr_ genes for bacterial blight and bacterial leaf streak

Over 40 plant *R* genes have been cloned from both monocotyledon and dicotyledon plants. R proteins fall into five major classes based on their predicted protein structures (Martin et al. 2003). Four *R* genes for bacterial blight have been cloned and were classified into two of these major classes and one unique class. Xa21 and Xa26 are receptor-like kinases consisting of an intracellular serine/threonine kinase domain, a transmembrane (TM) domain, and an extracellular leucine-rich repeat (LRR) domain (class 5 according to Martin et al. 2003) (Martin et al. 2003; Song et al. 1995; Sun et al. 2004). Xa1 is a cytoplasmic

receptor-like protein with nucleotide binding site (NBS) and LRR domains (class 2 according to Martin et al. 2003) (Yoshimura et al. 1998). The majority of known R proteins are cytoplasmic receptor-like proteins with NBS-LRR domains. The recently cloned *xa5* is in a unique class by itself. It is a recessive gene encoding a variant of the gamma subunit of transcription factor IIA (TFIIAgamma) (Iyer and McCouch 2004).

avr genes corresponding to the cloned *Xa* genes have not been isolated yet, but *avrXa7* and *avrXa10* have been cloned from *X. oryzae* pv. oryzae race2 and well characterized (Hopkins et al. 1992; Yang et al. 2000; Zhu et al. 1998; Zhu et al. 1999). *avrXa7* and *avrXa10* both are in the *avrBs3* gene family conserved in *Xanthomonas* spp. *avrXa7* is a major virulence determinant and *avrXa10* contributes less substantially in the absence of a corresponding *R* gene (Bai et al. 2000). The structure of the AvrBs3 family has nuclear localization signals (NLSs), an acidic transcription activation domain (AAD) at the C-terminus and a 102 bp repeat sequence in the middle of the gene whose number varies within the gene family (Buttner and Bonas 2002). Characterization of the *avrBs3* family suggests where these Avr proteins function and how they are recognized by corresponding R proteins (Lahaye and Bonas 2001). Deletion of NLSs in C-terminal of AvrXa7 abolishes its avirulence and virulence activities (Yang et al. 2000). The replacement of the AvrXa10 AAD with the herpes virus protein VP16 activation domain does not interfere with recognition by Xa10. Substitution of the VP16 AAD destroyed the virulence contribution, indicating that a specific protein-DNA interaction is involved in virulence, but a general interaction is sufficient for avirulence signaling (Zhu et al. 1999). But C-terminal region swapping between AvrBs3 and AvrXa7 changes interacting R protein specificities (Yang et al. 2005; Yang and White 2004). Those observations suggest that interaction with plant transcriptional system

during *X. oryzae* pv. oryzae infection is required for recognition but *R*-gene specificity involves specific differences in the C-termini of these proteins (Zhu et al. 1998; Zhu et al. 1999). The repeat domains of AvrBs3 and AvrXa7 determine *R* gene specificities (Herbers et al. 1992; Yang et al. 2005).

As mentioned earlier, no *R* gene against *X. oryzae* pv. oryzicola has been identified in rice (Rao et al. 1972; Shekhawat et al. 1972). This is particularly puzzling because *X. oryzae* pv. oryzicola, like *X. oryzae* pv. oryzae, has multiple members of the *avrBs3* family in its genome but they are not functional as virulence to complement the *X. oryzae* pv. oryzae *avrBs3* mutants (unpublished data, A. Sugio and F. White). The recently cloned *Rxo1* gene of maize mediates recognition of *X. oryzae* pv. oryzicola and HR in maize varieties expressing the gene and functions as a transgene in rice to confer resistance to *X. oryzae* pv. oryzae carrying a cloned copy of the corresponding *X. oryzae* pv. oryzicola *avrRxo1* gene (S. Hulbert, personal communication, (Zhao et al. 2004b)). *avrRxo1* is conserved in all *X. oryzae* pv. oryzicola isolates examined in the study, but it does not belong to the *avrBs3* family (Zhao et al. 2004a).

Effectors and type III secretion system

AvrXa7, AvrXa10, and AvrRxo1 are members of a large class of bacterial proteins collectively called effectors, that are involved in interactions with eukaryotic hosts and secreted through a specialized pathway known as the type III secretion system (TTSS). The TTSS of Gram-negative bacteria is conserved in both animal and plant pathogens (Hueck 1998). The TTSS forms a pilus-like structure that translocates proteins into host cells (Galan and Collmer 1999; Jin et al. 2003). In phytopathogenic bacteria the TTSS is encoded by

clustered *hrp* (Hypersensitive Response and Pathogenicity) genes and is required for HR elicitation and pathogenicity (Alfano and Collmer 1997).

Effectors include avirulence proteins and virulence proteins. In the absence of a corresponding *R* gene, many Avr proteins contribute to virulence (Bai et al. 2000; Swarup et al. 1991). This dual-functionality of avirulence and virulence led to the naming of bacterial proteins as effectors. Effectors are collectively required for pathogenicity but may individually trigger defense. Individual effectors may not always have a demonstrable role as virulence factors probably because of their redundancy and synergy.

Effector functions are also beginning to be elucidated. *xopD* in *X. campestris* pv. vesicatoria encodes a cystein protease with plant-specific SUMO (small ubiquitin-like modifier) substrate specificity (Hotson et al. 2003). AvrRpt2 of *P. syringae* eliminates RIN4 and the disappearance is required for RPS2-mediated defense response (Axtell and Staskawicz 2003). However, RIN4 modification is independent of AvrRpt2 virulence activity (Lim and Kunkel 2004). *P. syringae* effector HopPtoM is important for lesion formation (Badel et al. 2003). Another effector HopPtoA1 of *P. syringae* is required for colony formation *in planta* (Badel et al. 2002). Recently, suppression of some plant defense responses by effectors has been demonstrated (Abramovitch and Martin 2004; Espinosa and Alfano 2004). AvrPtoB in *P. syringae* inhibits host programmed cell death (Abramovitch et al. 2003). AvrPto of *P. syringae* suppresses cell wall-based defense (Hauck et al. 2003). HopPtoD2 in *P. syringae* can suppress the HR elicited by an avirulent *P. syringe* strain in *Nicotiana benthamiana* (Espinosa et al. 2003). XopX of *X. campestris* pv. vesicatoria is a virulence factor and suppresses plant cell death response in *Nicotiana benthamiana* (Metz et al. 2005). Evidence is mounting to suggest that plant defense suppression may be a common

function of effectors and a major role for the TTSS.

Recent genomic sequence projects facilitate genome-wide identification of type III effectors by bioinformatic analysis (Greenberg and Vinatzer 2003). TTSS-associated sequence patterns are useful criteria to search for candidate effectors from genomic sequence. The patterns of *hrp* regulating promoter sequences (*hrp* box in *Pseudomonas syringae* and *Erwinia* spp., PIP box in *Xanthomonas* spp. (Fenselau and Bonas 1995), and *hrp*(II) box in *Ralstonia solanacearum* (Cunnac et al. 2004) have been used to identify genes whose transcription is activated by *hrp* regulators. TTSS target sequences in the predicted N-terminus can identify the genes whose product may be translocated through the TTSS (Buttner et al. 2003; Cunnac et al. 2004; Fouts et al. 2002). Also, laboratory experiments are essential to complete and confirm the repertory of effectors.

IVET is a powerful technique to trap promoters activated *in planta* condition (Chiang et al. 1999). IVET has been used to screen the type III secretome of pathogens (Boch et al. 2002; Guttman et al. 2002). Also, an HR reporter has been used to screen for type III secreted proteins by trapping TTSS signal peptides via a fusion to a signal-less avirulence protein (Greenberg and Vinatzer 2003; Roden et al. 2004).

hrp gene regulation and the pathovars of *X. oryzae*

Necrogenic gram-negative phytopathogens can be divided into two groups based on the organization of their *hrp* gene clusters and specific regulators that activate *hrp* gene expression. Group I consists of *P. syringae* and *Erwinia* spp. and Group II comprises *Ralstonia solanacearum* and *Xanthomonas* spp. *hrp* genes in group I pathogens are regulated by HrpL, a homolog of the ECF family of alternative sigma factors, which targets the *hrp*

box sequence conserved in the promoter regions of many *hrp* and type III effector genes (Chatterjee et al. 2002; Fouts et al. 2002; Xiao and Hutcheson 1994). *hrpL* expression is dependent on the enhancer-binding-protein-like HrpR/S in *P. syringae* and two regulatory components HrpX/Y in *Erwinia* spp. (Hutcheson et al. 2001; Wei et al. 2000). Hrp regulators in group II include HrpB in *R. solanacearum* and HrpX in *Xanthomonas* spp., both members of the AraC family of transcriptional activators (Genin et al. 1992; Wengelnik and Bonas 1996). HrpB/X targets the *hrp*(II) box in *R. solanacearum* and the PIP (plant inducible promoter) box in *Xanthomonas* spp. (Cunnac et al. 2004; Fenselau and Bonas 1995; Vasse et al. 2000). In group II, the transcription of *hrpX* and *hrpB* is dependent on HrpG that belongs to the OmpR family of response regulators of two-component regulator systems conserved in most bacteria (Brito et al. 1999; Wengelnik et al. 1996b).

Regulation of the TTSS is tightly controlled. The TTSS is expressed *in planta* or in defined minimal media. Growth in defined minimal media may mimic *in planta* conditions in factors such as temperature, pH, osmolarity, and type of carbon sources (Boucher et al. 1985; Huynh et al. 1989; Rahme et al. 1992; Tsuge et al. 2002; Wei et al. 1992; Wengelnik et al. 1996a).

XOM2 is a defined minimal medium which activates *X. oryzae* pv. oryzae TTSS expression, but it does not activate TTSS in *X. oryzae* pv. oryzicola, despite near identity of their *hrp* gene clusters and known *hrp* regulatory proteins (S. Tsuge, personal communication, (Tsuge et al. 2002). Plant signal sensor genes *prhA*, *prhR*, *prhI*, and *prhJ* in *R. solanacearum* are required to express *hrpG* and *hrpB* in response to plant cell contact (Aldon et al. 2000; Brito et al. 2002; Brito et al. 1999). However, a putative sensor regulator that senses environmental change in host tissue and directly transmits signal to HrpG has yet

to be identified. Unidentified regulators upstream of HrpG may account for the differential regulation of the TTSS in *X. oryzae* pv. oryzae and *X. oryzae* pv. oryzicola. This differential regulation may be involved in the different tissue specificities of these two pathovars.

A model for understanding plant-bacterial interaction

Because the causal agents are closely related and the host is a model plant species, bacterial blight and bacterial leaf streak constitute a good comparative model to understand tissue specificity in plant-bacterial interactions. This is an important goal because understanding determinants of tissue specificity and pathogenicity promises to reveal targets for novel strategies for disease control. In this thesis, I address two questions regarding bacterial blight and bacterial leaf streak of rice. The first of these is whether *avr* genes of *X. oryzae* pv. oryzae function similarly in *X. oryzae* pv. oryzicola, toward understanding why no *X. oryzae* pv. oryzicola-specific *R* genes have been identified in rice. My results detailed in chapter 2 indicate that *X. oryzae* pv. oryzicola suppresses rice *R* gene-mediated defense and that suppression activity is type III-dependent. The second question focuses on regulation of the TTSS as a possible determinant of tissue specificity and how that regulation differs in *X. oryzae* pv. oryzae and *X. oryzae* pv. oryzicola. In the work presented in chapter 3, I demonstrated that hrpG is not the determinant of tissue specific TTSS regulation, furthermore, attempted to determine the activator(s) of *hrpG* in *X. oryzae* pv. oryzae and *X. oryzae* pv. oryzicola and the signals to which the *X. oryzae* pv. oryzicola activator might respond *in vitro*.

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Chapter 2. Inhibition of Resistance Gene Mediated Defense in Rice by *Xanthomonas oryzae* pv. *oryzicola*

A paper submitted in *Molecular Plant Microbe Interaction*

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ABSTRACT

Xanthomonas oryzae pv. *oryzae* and the closely related *X. oryzae* pv. *oryzicola* cause bacterial blight and bacterial leaf streak of rice, respectively. Although many rice resistance (*R*) genes and some corresponding avirulence (*avr*) genes have been characterized for bacterial blight, no endogenous *R/avr* gene interactions have been identified for leaf streak. Two *avr* genes from *X. oryzae* pv. *oryzae* failed to elicit the plant defense-associated hypersensitive reaction (HR) and failed to prevent development of leaf streak in rice cultivars with the corresponding *R* genes after introduction into *X. oryzae* pv. *oryzicola.* Furthermore, co-inoculation of *X. oryzae* pv. *oryzicola* quantitatively inhibited HR of blight resistant rice to avirulent *X. oryzae* pv. *oryzae*. Inhibition was effective against HR mediated by different *avr/R* gene interactions and dependent on the type III secretion system of *X. oryzae* pv. *oryzicola* . The results suggest that one or more *X. oryzae* pv. *oryzicola* type III effectors interfere with *avr/R* gene mediated recognition or signaling and subsequent defense response in the host. Inhibition of *R* gene mediated defense by *X. oryzae* pv. *oryzicola* may explain, in part, the apparent lack of major gene resistance to leaf streak.

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INTRODUCTION

The ability of plants to perceive and defend against pathogen attack is partially governed by the interaction between plant resistance (*R*) genes and pathogen avirulence (*avr*) genes. These gene-for-gene interactions underlie cultivar/race specificity in the resistance of plants to pathogenic viruses, bacteria, and fungi (Flor 1971; Keen 1990). *R* gene products directly or indirectly recognize corresponding *avr* gene products and trigger downstream signaling to activate defense responses (Martin et al. 2003). In the absence of either the *R* gene or *avr* gene, plants fail to mount sufficient defense responses, and succumb to disease. The hypersensitive reaction (HR) is an early, *R* gene mediated response to avirulence signals. HR is localized host cell death associated with limitation of pathogen spread in host plant tissues (Hammond-Kosack and Jones 1996; Klement 1982; Wright and Beattie 2004).

In phytopathogenic bacteria, most known Avr signals are proteins delivered to plant cells *via* a specialized secretion pathway called the Hrp or type III secretion system (TTSS) (Buttner and Bonas 2002; Cornelis and Van Gijsegem 2000). The TTSS is broadly conserved among Gram-negative animal and plant pathogens as a specific pathway for delivery of virulence factors into host cells (Hueck 1998). Many Avr proteins, in the absence of a corresponding *R* gene, contribute demonstrably to virulence (Bai et al. 2000; Swarup et al. 1991). The general term effector is used for all substrates of the TTSS delivered to host cells. The dual roles of some type III effectors is probably a consequence of the evolution in plants of the ability to recognize those effectors and mount resistance as mediated by *R* genes (Alfano and Collmer 2004). Recently, the functions of some type III effectors have been elucidated, and some have been shown to suppress plant defense responses including both *R*

gene-mediated and non-host HR as well as cell wall fortification and other resistance mechanisms (for reviews, see Abramovitch and Martin 2004; Chang et al. 2004)

Bacterial blight, caused by *Xanthomonas oryzae* pv. *oryzae*, is one of the most important diseases of rice (Mew et al. 1993). The disease is characterized by a high-degree of race-cultivar specificity with over thirty races of *X. oryzae* pv. *oryzae* and more than twenty-five bacterial blight *R* genes identified (Lee et al. 2003; Noda et al. 1996; Yang et al. 2003). A major class of effectors in *X. oryzae* pv. *oryzae* are members of the *avrBs3/pthA* family (Bai et al. 2000; Bonas et al.; Hopkins et al. 1992; Yang et al. 2005; Yang and White 2004; Yang et al. 2000; Zhu et al. 1998). Of these, *avrXa7* and *avrXa10* were cloned from the *X. oryzae* pv. *oryzae* race 2 strain PXO86 (Hopkins et al. 1992) and correspond to the *R* genes *Xa7* and *Xa10*, respectively. In PXO86 and KXO85, *avrXa7* contributes markedly to disease (Bai et al. 2000; Yang and White 2004). In strains lacking *avrXa7*, other members of the gene family act as major virulence factors (Yang and White 2004). The protein structures of this family share a highly conserved N-terminus, a central region of 34 aa, direct, near-perfect repeats, which vary in number, and a C-terminal region containing functional nuclear localization signals (NLSs) and an acidic transcription activation domain (AAD) (Buttner and Bonas 2002; Szurek et al. 2001; Zhu et al.). Type III secretion and delivery of AvrBs3 is directed by sequences in the N-terminus (Szurek et al. 2002), which is nearly identical to and interchangeable with the N-termini of AvrXa7 and AvrXa10 (Hopkins et al. 1992; Yang et al. 2005; Yang and White 2004; Yang et al. 2000). The NLSs are required for avirulence activity of some but not all members of the AvrBs3 family, indicating that different members are recognized differently (Ballvora et al. 2001). The AAD at the C-terminus suggests an interaction with plant transcriptional machinery during infection and determines R protein

specificity for some members (Yang et al. 2005; Yang and White 2004; Zhu et al. 1998; Zhu et al. 1999). The repeat domains of AvrBs3 and AvrXa7 also contribute to their *R* gene specificity (Herbers et al. 1992; Yang et al. 2005).

X. oryzae pv. *oryzicola* is closely related to *X. oryzae* pv. *oryzae*, showing more than 90% similarity by DNA-DNA hybridization (Swings et al. 1990). Unlike *X. oryzae* pv. *oryzae* , which invades the xylem to infect systemically, *X. oryzae* pv. *oryzicola* is a non-vascular pathogen (Ou 1985). Leaf streak can also cause severe yield losses, though it occurs more sporadically than blight (Mew 1993). Like *X. oryzae* pv. *oryzae*, *X. oryzae* pv. *oryzicola* harbors multiple members of the *avrBs3/pthA* family (Yang and White 2004), but no function has yet been ascribed to them. Notably, no endogenous *avr/R*-gene combination has been identified for *X. oryzae* pv. *oryzicola* and rice. We therefore investigated whether *X. oryzae* pv. *oryzicola* has the capacity to express and deliver members of the AvrBs3/PthA family using *avrXa7* and *avrXa10* and rice cultivars containing *Xa7* or *Xa10.*

RESULTS

Expression of *X. oryzae* pv. *oryzae avr* genes in *X. oryzae* pv. *oryzicola* does not cause avirulence to rice cultivars with corresponding *R* genes

To determine whether *avr* genes of *X. oryzae* pv. *oryzae* function in *X. oryzae* pv. *oryzicola* , the plasmids pHMavrXa10 and pHMavrXa7 (Table 2.1), which carry *avrXa10* and *avrXa7*, respectively, fused to the FLAG epitope tag (Brizzard et al. 1994), were transformed into *X. oryzae* pv. *oryzicola* strains BLS303 and BLS256 (Table 2.1). None of the resulting strains induced HR when infiltrated into leaves of corresponding rice cultivars IRBB10 and IRBB7, which carry resistance genes *Xa10* and *Xa7*, respectively. Instead,

transformants caused lesions that were water-soaked in appearance and typical of the parental strains (Fig. 2.1 and data not shown). Both plasmids conferred the ability to elicit HR in the respective cultivars when introduced into *X. oryzae* pv. *oryzae* strain PXO99^{A} (Fig. 2.1 and data not shown). Population growth of BLS303(pHMavrXa10) in IRBB10 was not significantly different from that of BLS303 (Fig. 2.1). Western blot analysis using anti-FLAG antibody confirmed expression of AvrXa10 in BLS303(pHMavrXa10) (data not shown). Thus, *avrXa10*, and, most likely *avrXa7* was expressed in *X. oryzae* pv. *oryzicola* but failed to render the pathogen avirulent.

X. oryzae pv. _oryzicola_ harboring _avrBs3_ elicits the HR of pepper in a _Bs3_-dependent manner

To determine if the apparent lack of avirulence function was specific for rice or, due more generally to a failure in type III secretion, the ability of *X. oryzae* pv. *oryzicola* to deliver AvrBs3 and elicit a *Bs3*-dependent response in pepper was examined. pHMavrBs3, identical to pHMavrXa10 except for the coding region of the *avr* gene (Table 1 and Materials and Methods), was transformed into *X. oryzae* pv. *oryzicola* strains BLS303 and BLS256, as well as *X. oryzae* pv. *oryzae* strain PXO99^{A}, and the resulting strains infiltrated into leaves of six-week old plants of pepper varieties ECW30R, which carries *Bs3*, and the near isogenic line ECW, which lacks the resistance gene. The parental *X. oryzae* pv. *oryzicola* and *X. oryzae* pv. *oryzae* strains did not elicit an HR in ECW30R (Fig. 2.2, top panels). However, the transformants carrying pHMavrBs3 did elicit an HR (Fig. 2.2, bottom panels). None of the strains elicited an HR on ECW (data not shown). Thus, *X. oryzae* pv. *oryzicola* effectively expresses and translocates AvrBs3 into pepper, indicating that the inability of AvrXa7 and AvrXa10 to render *X. oryzae* pv. *oryzicola* avirulent in rice is not likely due to a failure in

delivery.

Co-inoculated *X. oryzae* pv. *oryzicola* inhibits the rice HR to avirulent *X. oryzae* pv. *oryzae* in a TTSS-dependent fashion

The above results suggested that *X. oryzae* pv. *oryzicola* might specifically interfere with resistance responses to Avr proteins in rice. To test this idea, IRBB10 rice plants were co-inoculated with wild-type *X. oryzae* pv. *oryzicola* BLS303 and *X. oryzae* pv. *oryzae* PXO99^{A}(pHMavrXa10) at a 1:1 ratio. As with strains of *X. oryzae* pv. *oryzicola* inoculated alone, HR did not occur (Fig. 2.3A). The same result was observed upon co-inoculating plants of cultivar IRBB2, containing *Xa2*, with BLS303 and *X. oryzae* pv. *oryzae* strain JXO1 (Table 2.1), which is avirulent to plants containing *Xa2* (data not shown). At the same time, *X. oryzae* pv. *oryzae* PXO99^{A}(pHMavrXa10) mixed at the same ratio with the virulent parental strain PXO99^{A} and co-inoculated in the same manner to IRBB10 plants did elicit an HR (Fig. 2.3A), which was indistinguishable in extent and timing from that elicited by PXO99^{A}(pHMavrXa10) alone (Fig. 2.1B). Symptoms over time were compared among IRBB10 leaves inoculated with *X. oryzae* pv. *oryzae* PXO99^{A}(pHMavrXa10), *X. oryzae* pv. *oryzicola* BLS303, and a 1:1 mixture of the two (Fig. 2.3B). One day after inoculation, incomplete but discernible HR of infiltrated tissue was apparent for *X. oryzae* pv. *oryzae* PXO99^{A}(pHMavrXa10) only; the co-inoculated tissue and tissue inoculated with *X. oryzae* pv. *oryzicola* BLS303 alone showed no symptom. Water-soaking became visible in the tissue infiltrated with the co-inoculum and with *X. oryzae* pv. *oryzicola* BLS303 after two days and to a similar extent. The earlier appearance of HR compared to water-soaking and the lack of HR by day one following co-inoculation indicates that water-soaking is not masking the HR in this experiment. This observation provides evidence that that *X. oryzae* pv. *oryzicola*

actively interferes with rice resistance responses to the selected Avr proteins, and that the interference takes place following secretion of these proteins from the bacterial cell.

We next determined whether the inhibition of rice HR to avirulent *X. oryzae* pv. *oryzae* depends on the TTSS of *X. oryzae* pv. *oryzicola* . A *hrp* mutant of *X. oryzae* pv. *oryzicola* was generated by disrupting *hrcC*, a core component of the TTSS (Bogdanove et al. 1996), by single homologous recombination of a plasmid containing a 5' internal fragment of the gene, creating strain BLS303*hrcC*$^-$. BLS303*hrcC*$^-$, was non-pathogenic and failed to prevent an HR when co-inoculated with PXO99^A(pHMavrXa10) (Fig. 2.3A). Together, the results support a model in which *X. oryzae* pv. *oryzicola* specifically prevents or suppresses rice defense responses to avirulence signals via the plant intracellular activity of one or more endogenous type III effector proteins.

***X. oryzae* pv. *oryzicola* defense inhibition is quantitative**

Co-inoculations of IRBB10 plants were carried out using a series of different ratios of *X. oryzae* pv. *oryzae* PXO99^A(pHMavrXa10) to *X. oryzae* pv. *oryzicola* BLS303. The HR occurred at the highest ratios, indicating that HR inhibition by *X. oryzae* pv. *oryzicola* in this context is quantitative (Fig. 2.3C). The same series of co-inoculations of PXO99^A(pHMavrXa10) was also carried out with the virulent *X. oryzae* pv. *oryzae* parental strain PXO99^A. At relatively low ratios of avirulent to virulent bacteria, *X. oryzae* pv. *oryzae* also inhibited the HR (Fig. 2.3C). At the lowest ratio tested, symptoms over time were similar to those described above for co-inoculation of *X. oryzae* pv. *oryzicola* and avirulent *X. oryzae* pv. *oryzae* (data not shown). Thus *X. oryzae* pv. *oryzae* also quantitatively inhibits rice defense, but less effectively than *X. oryzae* pv. *oryzicola*.

Expression of *avrXa10* from a higher copy plasmid in *X. oryzae* pv. oryzicola leads to avirulence

Plasmid pKEB26 consists of the *avrXa10* gene with its native promoter in tandem with the *lacZ* promoter (as in pHMavrXa10) in the vector pDD62 (Table 2.1), which is a higher copy number plasmid than pHM1. We reasoned that pKEB26 would permit higher levels of *avrXa10* expression and possibly alter the balance between the secretion of AvrXa10 and the presumed inhibitor(s). Inoculation of transformants of either *X. oryzae* pv. *oryzicola* BLS303 or BLS256 carrying pKEB26 induced HR on IRBB10 plants (Fig. 2.4 and data not shown). Although this result contradicts the result with pHM1, the induction of an HR by strains carrying pKEB26 demonstrates that *X. oryzae* pv. *oryzicola* can deliver AvrXa10 into rice cells, corroborating the results with *avrBs3*. The results also provide evidence for a stoichiometric relationship between AvrXa10 and the presumed inhibitor(s) in determining the plant response. AvrXa10 expression from pKEB26 might result in a simple increase in the proportion of AvrXa10 in the plant cell, or AvrXa10 may competitively impede secretion of one or more type III-dependent inhibitor(s) of *X. oryzae* pv. *oryzicola*. While these possibilities could not be distinguished phenotypically in *X. oryzae* pv. *oryzicola* due to the lack of an HR in rice, an experiment was performed to determine if AvrXa10 expression from pKEB26 blocks *avrXa7*-mediated HR elicitation by *X. oryzae* pv. *oryzae*. pHMavrXa7 was introduced into *X. oryzae* pv. *oryzae* PXO99^{A} containing pKEB26, and the resulting strain PXO99^{A}(pKEB26, pHMavrXa7) and appropriate control strains were inoculated to IRBB7 plants and IRBB10 plants. PXO99^{A} (pKEB26, pHMavrXa7) elicited an HR on both IRBB10 and IRBB7 (Fig. 2.5). Thus, expression of *avrXa10* from pKEB26 does not block type III secretion to an extent that prevents signaling by *avrXa7*. Co-inoculation

experiments at different ratios were also conducted using PXO99^{A} carrying pKEB26 as the avirulent strain (Fig. 2.3C), and no discernible difference was observed in the ability of *X. oryzae* pv. *oryzicola* BLS303 to inhibit HR elicitation by this strain and to inhibit HR elicitation by PXO99^{A}(pHMavrXa10). Also, similar to *X. oryzae* pv. *oryzicola*, no difference was observed in the ability of *X. oryzae* pv. *oryzae* PXO99^{A} to inhibit the HR elicited by PXO99^{A}(pKEB26) or PXO99^{A}(pHMavrXa10). Thus, inhibition is overcome when pKEB26 is in *X. oryzae* pv. *oryzicola* but not when it is in a coinoculated *X. oryzae* pv. *oryzae* strain.

DISCUSSION

X. oryzae pv. *oryzae* and *X. oryzae* pv. *oryzicola* each carry multiple members of the *avrBs3/PthA* gene family (Yang et al. 2000). Defining the functions of these genes will advance our understanding of the molecular basis of bacterial blight and of bacterial leaf streak of rice and ultimately contribute to our ability to more effectively control these and other diseases caused by xanthomonads. In *X. oryzae* pv. *oryzae*, different members of the family function as major virulence determinants in different strains (Yang and White 2004). The only isolated avirulence determinants from *X. oryzae* pv. *oryzae* are members of the family, *avrXa7* and *avrXa10*, and the recently cloned *avrXa27* (Gu et al. 2005). One might expect similar functions for the *avrBs3*-like genes in *X. oryzae* pv. *oryzicola*. Evidence suggests however, that the genes in the two pathogens may function differently. First, an *avrBs3*-like gene that complements mutations in the homologous major virulence determinant genes of *X. oryzae* pv. *oryzae* could not be recovered from *X. oryzae* pv. *oryzicola* (Yang and White 2004). Second, host genes induced by *X. oryzae* pv. *oryzae* in a manner dependent on *avrBs3*-like genes in the pathogen are not induced by *X. oryzae* pv.

oryzicola (our unpublished results). Also, curiously, no endogenous *avr*/*R*-gene combination has been described for *X. oryzae* pv. *oryzicola* and rice. Experiments were therefore designed to first determine if *avr* genes of the *avrBs3*/*pthA* family from *X. oryzae* pv. *oryzae* would operate in *X. oryzae* pv. *oryzicola*. Specifically, *avrXa7* and *avrXa10* were introduced into *X. oryzae* pv. *oryzicola*, and the resulting strains tested for avirulence in rice plants with corresponding *R* genes. Neither gene conferred avirulence in either of two strains of *X. oryzae* pv. *oryzicola*. Further investigation led to the unexpected discovery that *avrXa7* and *avrXa10* failed to function because *X. oryzae* pv. *oryzicola* inhibits *R*-gene-mediated defense in rice, putatively *via* the action of one or more endogenous type III effectors.

Several lines of evidence support this conclusion. First, the lack of an effective resistance response in rice was not likely due to a failure of *X. oryzae* pv. *oryzicola* to express and deliver AvrXa7 and AvrXa10, since the same strains transformed with an equivalent *avrBs3* construct could induce a *Bs3*-dependent HR in pepper. Second, co-inoculated *X. oryzae* pv. *oryzicola* blocked HR to avirulent *X. oryzae* pv. *oryzae*, and this ability depended on a functional *X. oryzae* pv. *oryzicola* TTSS, indicating that one or more *X. oryzae* pv. *oryzicola* type III effectors likely interfere with resistance from within the plant cell. Third, inhibition of rice HR to *X. oryzae* pv. *oryzae* expressing AvrXa10 by co-inoculated *X. oryzae* pv. *oryzicola* could be overcome by increasing the proportion of the avirulent *X. oryzae* pv. *oryzae* strain, consistent with the notion that inhibition depends on the activity of a type III secreted protein or proteins that can be overwhelmed by greater amounts of the elicitor. Also, expression of *avrXa10* from a high copy number plasmid in *X. oryzae* pv. *oryzicola* resulted in HR, confirming the ability of *X. oryzae* pv. *oryzicola* to deliver this protein in rice and further suggesting a stoichiometric effect in determining the rice response.

A number of observations bear on whether inhibition involves blocking recognition of Avr signals, dampening of downstream signaling, or suppression of responses. In this study, *X. oryzae* pv. *oryzicola* prevented rice defense triggered by distinct Avr proteins, suggesting that inhibition might be targeting processes downstream of recognition. However, these effectors are structurally similar: an inhibitor might interfere similarly with recognition of either protein. In co-inoculations, *X. oryzae* pv. *oryzicola* also blocked *Xa2*-mediated HR to *X. oryzae* pv. *oryzae*, but it is not known whether the corresponding avirulence signal is like AvrXa7 and AvrXa10. Failure of *X. oryzae* pv. *oryzicola* to inhibit the AvrBs3-dependent response in pepper suggests that an inhibitor does not target the Avr proteins directly. Inhibition could be directed at shared structures in the R proteins, or occur *via* modification of a common target of the Avr proteins that is involved in recognition of the Avr proteins (Kim et al. 2005). If inhibition targets signal transduction, failure to prevent *Bs3*-mediated response in pepper might be due to a difference in the *Bs3 vs.* the *Xa7* and *Xa10* pathways, or divergence of shared signaling components between pepper and rice. If inhibition targets a component of resistance responses common to most or all *R* gene pathways, it might be expected to inhibit defense in a plant closely related to rice, such as maize. This is not the case however, as *X. oryzae* pv. *oryzicola* induces HR in maize varieties carrying the *Rxo1* gene. This induction is due to *avrRxo1*, a gene distinct from the *avrBs3/pthA* family that is conserved in all *X. oryzae* pv. *oryzicola* isolates examined (Zhao et al. 2004). Recently, transgenic rice carrying *Rxo1* was shown to be resistant to *X. oryzae* pv. *oryzicola* (S. Hulbert, personal communication), favoring the idea that *X. oryzae* pv. *oryzicola* inhibits only certain pathways. On the other hand, Rxo1 may “trump” (Abramovitch and Martin 2005) *X. oryzae* pv. *oryzicola* by suppressing defense inhibition activity in its recognition of AvrRxo1

(could AvrRxo1 be the inhibitor?). To better define the process targeted for inhibition by *X. oryzae* pv. *oryzicola*, diverse *avr-R* pairs need to be tested. Cloning of the putative inhibitor(s) and expression in avirulent *X. oryzae* pv. *oryzae* strains will best enable assays of the effect on different *R*-gene mediated responses. An intriguing possibility is that members of the *avrBs3/pthA* family themselves function in defense inhibition.

A difference in the effect on defense inhibition of expressing *avrXa10* from a high *vs.* low copy plasmid in *X. oryzae* pv. *oryzicola* and expressing it in a high *vs.* low copy plasmid in *X. oryzae* pv. *oryzae* co-inoculated with *X. oryzae* pv. *oryzicola* suggested that there is no significant difference in the amount of secretion of *avrXa10* due to the different plasmids, but that expression of *avrXa10* from the high-copy plasmid overcame inhibition in *X. oryzae* pv. *oryzicola* due to an effect on delivery of the putative inhibitor(s) from the bacterium. The hypothesis that type III secretion is limiting and that overexpression of an effector can compromise delivery of other effectors remains to be tested conclusively, but it is consistent with observations in *X. oryzae* pv. *oryzae* that complementation of a mutation in a virulence determinant of the *avrBs3/pthA* family using a plasmid-borne clone often fails to fully restore virulence in quantitative assays (Bai et al. 2000; Yang and White 2004; Yang et al. 2000): this failure could be due to a reduction in secretion of other virulence factors. pKEB26 did not prevent HR mediated by pHMavrXa7 in *X. oryzae* pv. *oryzae*, but the fact that *avrXa7* itself was on a multi-, albeit low-copy plasmid, may have contributed to this result. The HpaB protein, required for secretion of AvrBs3 and several other effectors (Buttner et al. 2004), provides an example of a possible rate-limiting factor for type III secretion.

Recent evidence suggests that diverse type III effectors contribute to virulence by inhibiting plant defense responses (reviewed in Abramovitch and Martin 2004; see also

DebRoy et al. 2004; Metz et al. 2005). Our demonstration that *X. oryzae* pv. *oryzicola* inhibits R-gene mediated defense in its host plant in a TTSS-dependent manner supports the notion that defense inhibition is a general mechanism in bacterial pathogenesis of plants and an important function of the TTSS. Further, our results demonstrate that one pathogen can inhibit host defense against another otherwise avirulent pathogen in the same plant.

The quantitative nature of defense inhibition by *X. oryzae* pv. *oryzicola* suggests an intriguing question: aside from disabling basal immunity and crippling host resistance responses mediated by *R* genes, might there be a role for defense inhibitors in modulation of cell death during disease development? HR is a rapid form of programmed cell death (PCD) associated with successful defense against pathogens. Yet some evidence suggests that necrogenic plant pathogens such as *X. oryzae* pv. *oryzicola*, *X. oryzae* pv. *oryzae*, *X. campestris* pv. *vesicatoria*, *P. syringae* pv. *tomato*, and others may rely on controlled elicitation of plant PCD to colonize plant tissues, or to generate lesions to aid in pathogen dispersal (Brunings and Gabriel 2003; Greenberg and Yao 2004). If manipulation of PCD is involved in disease development, a role for some bacterial effectors might be to dampen PCD so that it occurs only to an extent that promotes pathogen exploitation of host tissues, and survival. Whether this is the case for *X. oryzae* pv. *oryzicola*, *X. oryzae* pv. *oryzae*, and other necrogenic pathogens remains to be explored.

The ability of *X. oryzae* pv. *oryzicola* to inhibit *R*-gene mediated defense in rice provides a plausible explanation for the lack of known major gene resistance to bacterial leaf streak in this crop. Our results provide the first demonstration that *X. oryzae* pv. *oryzicola* depends on the TTSS for pathogenesis and suggest that the many *avrBs3/pthA* family members it contains are in fact expressed and their products secreted. The roles of these

genes in pathogen-host interaction remain an important target for research. In addition to virulence functions, one might predict that avirulence functions might be revealed by heterologous expression of the genes in *X. oryzae* pv. *oryzae* inoculated to differential rice cultivars. Also to be considered, the ability of *X. oryzae* pv. *oryzicola* to block elicitation of the HR by co-inoculated avirulent *X. oryzae* pv. *oryzae* implies that *R* genes to bacterial blight and perhaps other diseases may not be effective when *X. oryzae* pv. *oryzicola* is infecting the plant. Further elucidation of rice defense inhibition by *X. oryzae* pv. *oryzicola* is therefore a priority as well. Such elucidation should lead to a better understanding of the role of plant defense inhibition in pathogenesis generally, and could contribute to the development of new strategies for disease control that target the defense inhibition mechanism and in the case of bacterial leaf streak, uncover useful gene-for gene interactions.

MATERIALS AND METHODS

DNA manipulation

Standard methods of DNA manipulation were carried out as described (Sambrook et al. 1989). Plasmids were isolated using the Wizard miniprep (Promega, Madison, Wisconsin) and the HiSpeed Midiprep (Qiagen, Valencia, California) kits. *X. oryzae pv. oryzicola* genomic DNA for PCR was extracted using the DNeasy kit (Qiagen, Valencia, California).

Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. pHMavrXa10, pHMavrXa7, and pHMavrBs3 have been described in detail previously (Yang et al. 2000; Zhu et al. 1998). Briefly, pHMavrXa10 contains *avrXa10* cloned in pBluescript KS (+) inserted into pHM1 such that *avrXa10* is downstream of and oriented with the *lacZ*

promoter of each plasmid, which is expressed constitutively in *Xanthomonas* spp. (Soby and Daniels 1996). A 24 bp sequence encoding the FLAG epitope (Brizzard et al. 1994) is inserted near the 3' end of the open reading frame of *avrXa10*. Replication in *Xanthomonas* is directed by the origin of pHM1. pHMavrXa7 and pHMavrBs3 are identical to pHMavrXa10 except for the gene-specific sequence between the conserved *Sph*I sites in the *avr* genes. pKEB26 was constructed as follows. The *Bam*HI to *Xho*I fragment of the multiple cloning site (MCS) of pBluescript II KS(+) (Table 1) was inserted between these sites in pDD62 (Table 2.1), creating pKEB27, with unique sites *Bam*HI, *Eco*RI, *Eco*RV, *Hind*III, and *Xho*I in the MCS. The 4.8 kb *Eco*RI to *Hind*III fragment of pCH43 (Table 2.1) containing the *avrXa10* open reading frame and 252 bp upstream was then inserted between these sites in pKEB27, resulting in pKEB26. In pKEB26, *avrXa10* is downstream of and oriented with the *lacZ* promoter of pKEB27. To construct pTOPO*hrcC*174-781, a 608 bp internal fragment (bp 174 to 781) of the *hrcC* open reading frame was amplified from *X. oryzae* pv. *oryzicola* BLS256 genomic DNA by PCR using primers SM20 (forward primer; 5'-CACCTTGACCCTGTGCGGCATT-3') and SM21 (reverse primer; 5'-TTTGTCCGCCAGCCAGTCCATC-3') designed based on the *hrcC* sequence of *X. oryzae* pv. *oryzae* (GenBank ID AB115081). The PCR product was cloned in pCRBluntII by the TOPO cloning reaction (Invitrogen, Carlsbad, CA). *Escherichia coli* was cultured in Luria-Bertani medium (Sambrook et al. 1989) at 37 °C. *X. oryzae* strains were grown in glucose yeast extract (GYE; glucose 20 g and yeast extract 10 g per liter) medium or nutrient broth yeast extract (NBY, Vidaver 1967) medium at 28 °C . Ampicillin (100 μg/ml), kanamycin (25 μg/ml), and spectinomycin (100 μg/ml) were added to growth media as appropriate for plasmid selection.

Plasmid transformation

Plasmids were introduced by electroporation into *E. coli* cells supplied with pCRBluntII according to the manufacturer's protocol (Invitrogen, Carlsbad, CA), and into *Xanthomonas* cells as described previously (Tsuge et al. 2001). Transformation of *Xanthomonas* strains was confirmed by reisolation and transfer of plasmids to *E. coli*.

Plant materials and inoculations

Rice plants of the indica variety IR24 and its near isogenic lines IRBB2 containing *Xa2*, IRBB7 containing *Xa7*, and IRBB10 containing *Xa10* were grown in LC-1 soil mixture (SunGRO, Bellevue, Washington, USA) in a growth chamber under cycles of 12 hr light at 28 °C and 12 hr dark at 25 °C. Fertilizer (Peters Professional, Saint Louis, Missouri) and iron chelate micronutrient (Becker Underwood, Ames, Iowa) were applied with watering every two days at rates of 0.25 g/L and 4.5 g/L respectively. Plants were inoculated at four weeks after sowing by infiltration of leaves with bacterial suspension using a needleless syringe (Schaad et al. 1996). Pepper plants of cv. ECW30R carrying the *Bs3* resistance gene were grown in a standard potting mix in a greenhouse and inoculated by syringe infiltration at six weeks. For inoculations, overnight bacterial cultures were washed twice and resuspended with sterile H_2O. Unless otherwise indicated, strains inoculated individually were suspended to an $O.D._{600} = 0.5$, and strains inoculated together were suspended to an $O.D._{600} = 1.0$ before mixing. Following inoculation, plants were incubated at room temperature under constant fluorescent light in the laboratory.

Bacterial population growth assay

Infiltrated areas of rice leaves were removed with a #1 cork borer and ground in liquid nitrogen in 1.5 ml microcentrifuge tubes by using a plastic pestle (Kontes pellet pestle;

Kontes, Vineland, New Jersey, USA). Samples were mixed with 0.5 ml sterile water, then serial dilutions were made and spotted (10 μl per spot) in triplicate on GYE agar plates with appropriate antibiotics. Plates were incubated at 28°C until single colonies could be counted. The number of colony forming units per cm^2 leaf area was then estimated and standard deviation was calculated using colony counts from the three triplicate spots from each of three samples per time point per inoculum. Experiments were repeated at least three times.

Construction of *hrcC⁻* knockout mutant

pTOPO*hrcC*174-781, which is not replicated in *Xanthomonas*, was introduced into *X. oryzae pv. oryzicola* strain BLS303. Recombinants were selected on GYE plates containing kanamycin. Disruption of *hrcC* by single homologous recombination was assayed by PCR using the M13forward(-20) primer corresponding to the vector sequence upstream of the insert in pTOPO*hrcC*174-781 and primer SM25 (5'-ATGGCTCCTGCCTGTACCA-3') which corresponds to the 3' end of *hrcC*. Each of five isolates showing disruption of *hrcC* was inoculated to rice and confirmed to be non-pathogenic. One was chosen arbitrarily for co-inoculation experiments and designated as BLS303*hrcC⁻*.

Immunodetection of AvrXa10

X. oryzae pv. oryzicola BLS303(pHMavrXa10), alongside *X. oryzae* pv. *oryzae* $PXO99^A$ (pHMavrXa10) and *X. oryzae pv. oryzicola* BLS303 as positive and negative controls, respectively, were grown in NBY to $O.D._{600} = 0.7$. As described above, AvrXa10 encoded by pHMavrXa10 contains the FLAG epitope (Brizzard et al. 1994). For each culture, cells in 2 ml were resuspended in 75 ml 1 X Laemmli buffer (Laemmli 1970) and heated to 100 °C for 3 min to obtain total cellular proteins. Polyacrylamide (10%) gel electrophoresis and western blotting of samples was performed using a standard protocol

(Bowen et al. 1980). Mouse monoclonal anti-FLAG antibody (Sigma, St.Louis, MO) followed by rabbit anti-mouse peroxidase-conjugated antibody (Pierce, Rockford, IL) and the ECL plus Western Blotting Detection System (Amersham, Piscataway, NJ) with Blue Sensitive Autoradiography film (Midwest Scientific, St. Louis, MO) were used according to manufacturer protocols to detect AvrXa10.

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Table 2.1. Bacterial strains and plasmids used in this study.

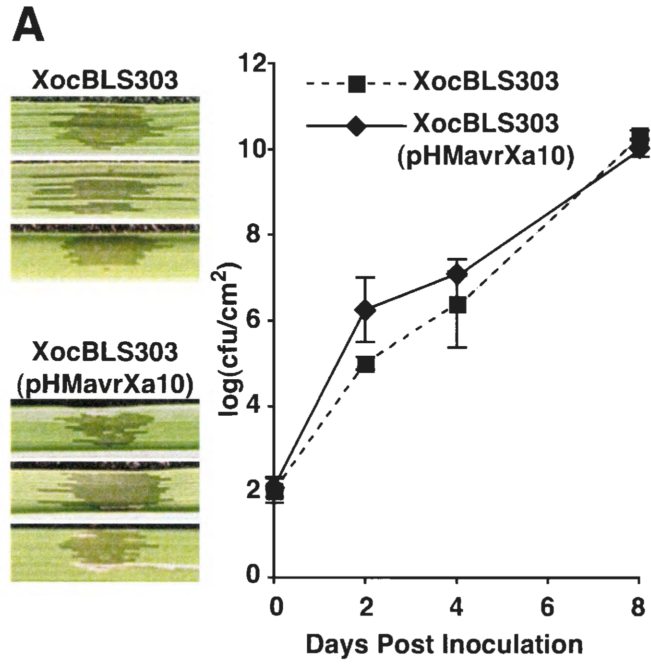
Strain or plasmid	Relevant characteristics	Source or reference
Escherichia coli		
TOP10	F$^-$ *mcrA* Δ(*mrr-hsd*RMS-mcrBC) ϕ80*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*Δ139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen, Carlsbad, CA
Xanthomonas oryzae pv. *oryzae*		
PXO99^A	Azacytidine resistant derivative of Philippine race 6 strain PXO99, virulent to rice cultivars IR24, IRBB7, and IRBB10	(Choi and Leach 1994)
JXO1	Japanese race 1 strain. Avirulent to IRBB2	(Yang and White 2004)
Xanthomonas oryzae pv. *oryzicola*		
BLS256	Philippines isolate	J. Leach
BLS303	Philippines isolate	J. Leach
BLS303*hrcC*$^-$	BLS303 *hrcC*:pTOPO*hrcC*MT, Km^r	This study
Plasmids		
pBluescript II KS(+)	Cloning vector, Ap^r	Stratagene, La Jolla, CA
pCH43	pUC118 carrying 4.79 kb fragment of *X. oryzae* pv. *oryzae* PXO86 genome containing *avrXa10* and flanking DNA, Ap^r	(Hopkins et al. 1992)
pCRBluntII	TOPO cloning vector, Km^r	Invitrogen, Carlsbad, CA
pDD62	Derivative of pVSP1 (Van Der Bij et al. 1996), broad host range, high copy, Km^r	(Mudgett et al. 2000), B.Staskawicz
pHM1	Derivative of pRI40 (Innes et al.) containing multiple cloning site of pUC19, broad host range, low copy, Sp^r	R. Innes
pHMavrXa10	pHM1 carrying *avrXa10* with FLAG epitope tag near 3' end, fused with *lacZ* promoter, Sp^r	(Zhu et al. 1998)
pHMavrXa7	pHM1 carrying *avrXa7* with FLAG epitope tag near 3' end, fused with *lacZ* promoter, Sp^r	(Yang et al. 2000)
pKEB26	pDD62 carrying *avrXa10* with native promoter, downstream of and oriented with *lacZ* promoter of pDD62, Km^r	This study

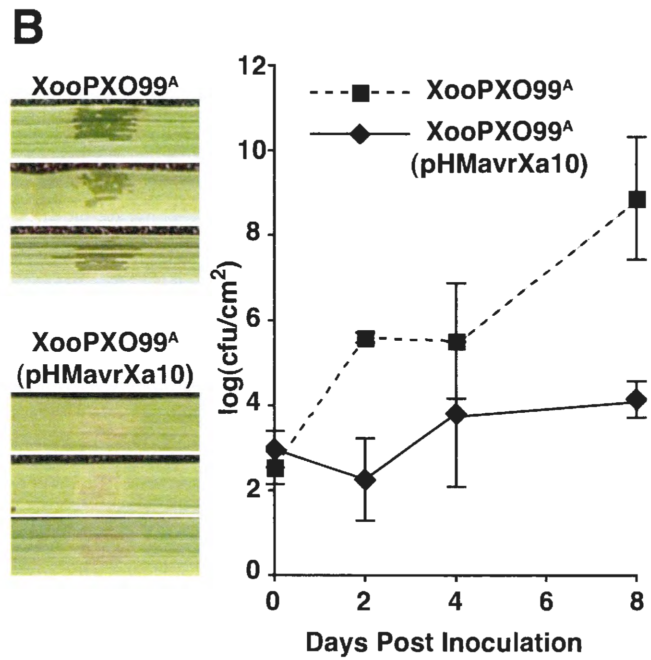
Table 3.1. (continued)

Plasmid	Relevant characteristics	Source or reference
pKEB27	pDD62 containing the 30 bp *BamHI* to *XhoI* fragment of pBluescript II KS(+), Km^r	This study
pTOPO*hrcC*174-781	pCRBluntII containing bp 174 to 781 (608 bp) of the *hrcC* open reading frame of Xoc BLS256, Km^r	This study
pHMavrBs3	pHM1 carrying *avrBs3* with FLAG epitope tag near 3' end, fused with *lacZ* promoter, Sp^r	(Zhu et al. 1998)

Ap: ampicillin, Km: kanamycin, Sp: spectinomycin

Figure 2.1. *avrXa10* in plasmid pHM1 does not render *X. oryzae* pv. oryzicola avirulent to rice plants containing *Xa10*. Shown are symptoms at two days and plots of bacterial population growth over eight days for (**A**) *X. oryzae pv. oryzicola* (Xoc) BLS303 and BLS303(pHMavrXa10), compared to (**B**) *X. oryzae* pv. oryzae (Xoo) $PXO99^A$ and PXO99A(pHMavrXa10), inoculated to leaves of rice cv. IRBB10 by syringe infiltration. Virulent strains cause dark lesions water-soaked in appearance. Avirulent strains induce the HR, visible as lighter brown collapsed tissue. For population growth assays, vertical bars represent standard deviation of values (cfu/cm^2, colony-forming units per cm^2 of leaf) from three samples. Experiments were repeated three times with similar results.


A
XocBLS303
XocBLS303
(pHMavrXa10)
XocBLS303
XocBLS303
(pHMavrXa10)
log(cfu/cm²)
0
2
4
6
8
10
12
Days Post Inoculation


B
XooPXO99A
XooPXO99A
(pHMavrXa10)
XooPXO99A
XooPXO99A
(pHMavrXa10)
log(cfu/cm²)
0
2
4
6
8
10
12
Days Post Inoculation

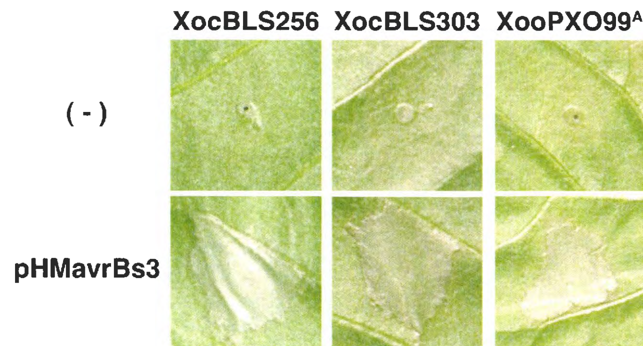
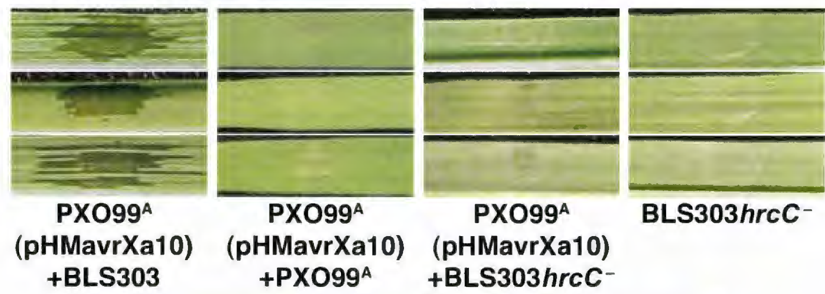


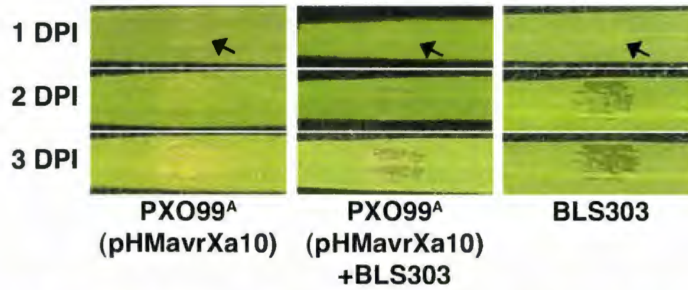
Figure 2.2. *X. oryzae* pv. oryzicola (Xoc) strains BLS256 and BLS303 and *X. oryzae* pv. oryzae (Xoo) strain PXO99^A transformed with *avrBs3* induce the HR of pepper plants carrying the *Bs3* gene. Leaves of pepper cv. ECW30R were infiltrated using a syringe with the indicated strains carrying no plasmid (-) or pHMavrBs3, encoding AvrBs3. Photographs were taken two days after inoculation. HR is apparent as light brown collapsed tissue.

Figure 2.3. *X. oryzae* pv. oryzicola (Xoc) inhibits HR of rice cv. IRBB10 to *X. oryzae* pv. oryzae (Xoo) carrying *avrXa10* in a type III secretion dependent and quantitative manner unaffected by copy number of the *avrXa10* construct, and inhibition activity is weakly conserved in *X. oryzae* pv. oryzae. (**A**) Leaves are shown two days following inoculation with the indicated strains suspended individually to an $O.D._{600} = 1.0$ and infiltrated together at a 1:1 ratio or suspended to an $O.D._{600} = 0.5$ and infiltrated separately. (**B**) Leaves are shown one, two, and three days post-inoculation (DPI) as in (A) with the indicated strains. Black arrows point to infiltrated areas. (**C**) Leaves are shown three days after inoculation with the indicated strains, suspended individually to $O.D._{600} = 0.5$ and mixed at the indicated ratios before infiltration. An asterisk indicates that the corresponding strain suspension was replaced with an equivalent volume of water. Dark lesions appearing water-soaked are typical for virulent *X. oryzae* pv. oryzicola and *X. oryzae* pv. oryzae strains. Light brown collapsed tissue indicates the HR. The tan colored ring visible at one DPI in the leaf inoculated with *X. oryzae* pv. oryzicola BLS303 in (B) is the result of wounding with the syringe. Note that at the minimum concentration tested in co-inoculations, the avirulent *X. oryzae* pv. oryzae strain by itself elicited a robust HR.

A



B



C

XooPXO99^A (pHMavrXa10) / XocBLS303

XooPXO99^A (pKEB26) / XocBLS303

XooPXO99A (pHMavrXa10) / XooPXO99^A

XooPXO99^A (pKEB26) / XooPXO99^A

Ratio: 1/9*, 1/9, 1/4, 3/7, 2/3, 1/1, 3/2, 7/3, 4/1, 9/1, 9/1*

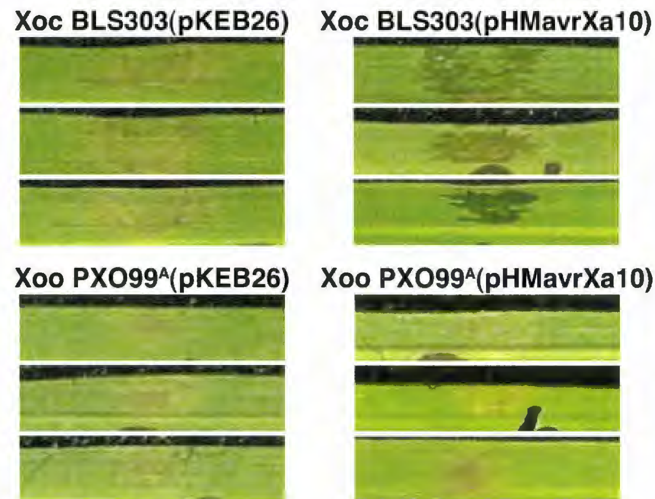


Figure 2.4. Expression of *avrXa10* from the high copy number plasmid pKEB26 renders *X. oryzae* pv. oryzicola avirulent to rice plants containing *Xa10*. Leaves of rice cv. IRBB10 were inoculated by syringe infiltration with *X. oryzae* pv. oryzicola (Xoc) BLS303(pKEB26), BLS303(pHMavrXa10), *X. oryzae* pv. oryzae (Xoo) PXO99A(pKEB26), or *X. oryzae* pv. oryzae $PXO99^A$(pHMavrXa10), and photographed after two days. Virulent strains cause dark lesions water-soaked in appearance. Avirulent strains induce the lighter colored HR.

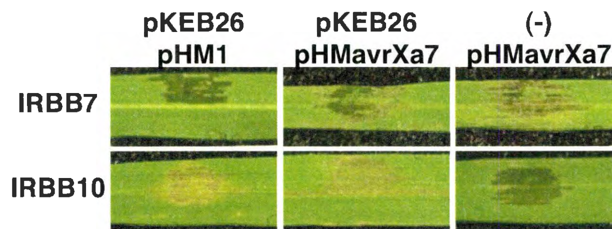


Figure 2.5. Expression of *avrXa10* from the high copy number plasmid pKEB26 does not block signaling mediated by *avrXa7*. Leaves of rice cv. IRBB7, and cv. IRBB10 as a control, were inoculated by syringe infiltration with *X. oryzae* pv. oryzae PXO99^{A} containing the indicated plasmids. Leaves were photographed four days following inoculation. Dark, water-soaked appearance indicates lack of *avr/R* gene mediated response. HR, apparent as lighter, localized tissue collapse, indicates *avr/R* gene interaction. HR of IRBB7 indicates delivery of *avrXa7*-dependent signal.

Chapter 3.

Defining the determinant(s) of differential *hrp* gene regulation in *Xanthomonas oryzae* pathovars toward understanding their role in tissue specificity

ABSTRACT

Xanthomonas oryzae pathovars oryzae and oryzicola have distinct tissue specificity and cause different diseases on the same host, rice. *X. oryzae* pv. oryzae causes bacterial blight of rice by invading the xylem. *X. oryzae* pv. oryzicola colonizes the parenchymal apoplast of rice to cause bacterial leaf streak. A defined *hrp* gene inducing minimal medium for *X. oryzae* pv. oryzae failed to activate the type III secretion system (TTSS) encoding *hrp* genes of *X. oryzae* pv. oryzicola suggesting that differential bacterial responses to environmental cues in different plant tissues may account for the tissue specificity of the diseases. However, swapping of HrpG, a regulatory protein of the TTSS and a member of the OmpR family of response regulators of bacterial two-component regulatory systems, among vascular and non-vascular *Xanthomonas* pathogens showed that HrpG is functionally conserved and therefore not a determinant of tissue specific type III regulation. Important regulatory differences likely lie upstream of HrpG. To identify activators of HrpG, a gain-of-function mutagenesis screen was attempted, but proved unsuccessful. This attempt proposes modifications to the screen. Identification of upstream regulators may shed light on the role of activation of the TTSS in tissue specificity.

INTRODUCTION

The type III secretion system (TTSS) conserved in both animal and plant bacterial pathogens delivers effector proteins into host cells that collectively are indispensable for pathogenesis (Hueck 1998). In plant pathogens, the TTSS is encoded in the clustered *hrp* genes so named because mutations in these genes disrupt both the ability of the pathogen to elicit the plant defense associated *h*ypersensitive *r*esponse (HR) in resistant plants and its *p*athogenicity in susceptible plants (Alfano and Collmer 1997).

The virulence functions of phytopathogenic effector proteins in host plant cells are beginning to be understood (Greenberg and Vinatzer 2003; Lahaye and Bonas 2001). AvrPtoB in *Pseudomonas syringae* pv. tomato DC3000 inhibits the HR in the non-host plant *Nicotiana benthamiana* (Abramovitch et al. 2003). The effectors in the *avrBs3* family of *Xanthomonas* spp. have functional nuclear localization signals and may modulate plant transcription directly in the plant nucleus (Lahaye and Bonas 2001; Szurek et al. 2001; Szurek et al. 2002; Yang et al. 2000). *xopD* of *Xanthomonas campestris* pv. vesicatoria encodes a cysteine protease with plant-specific SUMO (small ubiquitin-like modifier) substrate specificity (Hotson et al. 2003).

Regulatory proteins that control the expression of the TTSS in phytopathogenic bacteria also have been characterized. Necrogenic Gram-negative phytopathogens can be classified into two groups based on the type of regulators they use, as well as the order and orientation of their *hrp* genes: group I consists of *P. syringae* pathovars and *Erwinia* spp., and group II comprises *Ralstonia solanacearum* and *Xanthomonas* spp. (Alfano and Collmer 1997). HrpL, one of the *hrp* regulators in group I is homologous to the ECF family of alternative sigma factors. TTSS genes activated by HrpL have a conserved sequence known

as the Hrp box in their promoter regions (Xiao and Hutcheson 1994). The transcription of *hrpL* is activated by HrpR and HrpS in *P. syringae* and HrpX and HrpY in *Erwinia amylovora* (Hutcheson et al. 2001; Wei et al. 2000). In group II, HrpB in *R. solanacearum* and HrpX in *Xanthomonas* spp. activate *hrp* gene expression. HrpB and HrpX are members of the AraC family of transcriptional activators (Genin et al. 1992; Vasse et al. 2000; Wengelnik and Bonas 1996). TTSS genes in group II also share a specific promoter sequence targeted by HrpB or HrpX. It is called the *hrp*(II) box in *R. solanacearum* and the PIP (plant inducible promoter) box in *Xanthomonas* spp. (Buttner and Bonas 2002; Cunnac et al. 2004). The transcription of HrpB and HrpX is dependent on HrpG, a member of the OmpR family of response regulators of bacterial two-component regulatory systems (Vasse et al. 2000; Wengelnik et al. 1996b).

Two-component regulatory systems, comprising a sensor and a response regulator, are conserved in many Gram-negative bacteria, and typically function in bacterial response to environmental signals (Hoch 2000). Generally, sensor regulators have two domains: a signal recognition domain (input domain) that senses signals from outside of cells or environmental changes such as osmolarity, and a kinase domain that transduces signals to response regulators. Response regulators also typically have two domains: a domain phosphorylated by a sensor and an output domain such as a transcription activator or DNA binding domain. GacS and A (Global ACtivator) are the sensor and response regulator of a two-component system conserved in *P. syringae* pathovars that activates expression of *hrp* regulators, *hrpR* and *hrpS*, as well as three alternate sigma factors including HrpL (Chatterjee et al. 2003; Heeb and Haas 2001).

In *R. solanacearum*, plant signal sensors PrhA, PrhR, PrhI and PrhJ are required to express *hrpB* in response to plant cell contact (Aldon et al. 2000; Brito et al. 2002; Brito et al. 1999; Marenda et al. 1998). PrhA has a transmembrane domain and is thought to be a sensor, though a specific plant signal to which it responds has yet to be identified.

A sensor partner for HrpG also is yet to be found. It is predicted that a sensor would recognize plant signals or environmental cues during infection and activate HrpG to turn on expression of the TTSS genes. Loss-of-function mutagenesis, such as transposon mutagenesis screening, in previous studies has failed to identify a HrpG activator, suggesting that disruption of the activator may be pleiotropic, or lethal, or that there may be distinct, redundant activators.

The TTSS is activated *in planta* or in some cases during incubation in defined minimal media. These media have enabled characterization of factors, such as temperature, pH, osmolarity, and carbon source, that may be involved in induction of the TTSS during interactions with different plants and plant tissues (Boucher et al. 1985; Huynh et al. 1989; Rahme et al. 1992; Tsuge et al. 2002; Wei et al. 1992; Wengelnik et al. 1996a).

Whether differential *hrp* gene regulation plays a role in defining host specificity or tissue specificity is still an open question, however. Two pathogens of rice offer a powerful model to address this question. *Xanthomonas oryzae* pathovars oryzae and oryzicola infect the same host plant, rice, but cause distinct diseases by colonizing different tissues (Mew 1993; Swings et al. 1990). *X. oryzae* pv. oryzae invades through hydathodes and wounds of rice leaves and travels systemically through the xylem to cause bacterial leaf blight. *X. oryzae* pv. oryzicola, by contrast, invades through stomata and colonizes the mesophyll parenchyma to cause bacterial leaf streak.

The minimal medium XOM2 activates the TTSS in *X. oryzae* pv. oryzae but not in *X. oryzae* pv. oryzicola (S. Tsuge, personal communication and (Tsuge et al. 2002), indicating that the *hrp* gene expression is indeed differentially regulated in *X. oryzae* pv. oryzae and *X. oryzae* pv. oryzicola. Nonetheless, reciprocal heterologous expression of the *hrpX* gene among *hrpX* mutants of *X. oryzae* pv. oryzae, *X. campestris* pv. campestris, and *X. campestris* pv. armoraciae demonstrated that HrpX functions equivalently among vascular and non-vascular xanthomonads (Kamdar et al. 1993). Thus the differential regulators must function upstream of HrpX.

In the work presented here, I confirmed that the synthetic *hrp* medium for *X. oryzae* pv. oryzae does not activate *hrp* regulation of *X. oryzae* pv. oryzicola and show that HrpG of *X. oryzae* pv. oryzae and HrpG of *X. oryzae* pv. oryzicola are functionally equivalent. Based on these results, I attempted to identify upstream activators of HrpG in *X. oryzae* pv. oryzae. My approach was gain-of-function mutagenesis with the chemical mutagen ethylmethane sulfonate (EMS), using a strain with a selectable marker cloned downstream of a *hrp* promoter including a PIP box to identify *hrp* activated mutants. EMS generates G:C to A:T single nucleotide transitions (Sega 1984). Single amino acid mutations can constitutively activate sensor and response regulators in two-component signal transduction systems (Martinez-Hackert et al. 1996; Smith et al. 2004). Also, as mentioned previously, loss-of-function mutagenesis has thus far failed to reveal activators of HrpG, suggesting the rationale for the approach described here is valid. Unfortunately, the screen failed to isolate any bona fide regulatory mutants. Nevertheless, details from the study will be useful in optimizing conditions for a future attempt.

I also attempted to define an *hrp* gene-inducing minimal medium for *X. oryzae* pv. oryzicola. This project was also unsuccessful but the details should be useful as a reference for future attempts.

RESULTS

Activation of *X. oryzae* pv. oryzicola *hrp* genes *in vitro* and *in planta*

XOM2 medium, modified XVM2 for *X. campestris* pv. vesicatoria, can induce *X. oryzae* pv. oryzae type III secretion *in vitro* but it cannot induce *X. oryzae* pv. oryzicola type III secretion (Tsuge et al. 2002, S. Tsuge, personal communication and data below). This observation supports the idea that different tissue specificities of *X. oryzae* pv. oryzae and *X. oryzae* pv. oryzicola may involeve differential regulation of type III secretion. pHMPIPGUS2 was used for a *hrp* activation reporter. It has the promoterless *uidA* gene fused with the promoter region of the *hrcU* gene containing the PIP box from *X. oryzae* pv. oryzae strain T7174 in pHM1 (Tsuge et al. 2002). pHMPIPGUS2 was introduced into *X. oryzae* pv. oryzicola strain BLS256 and *X. oryzae* pv. oryzae strain PXO99^{A} by electroporation, generating BLS256(pHMPIPGUS2) and PXO99^{A}(pHMPIPGUS2) respectively. No GUS activity in BLS256(pHMPIPGUS2) was observed in XOM2 while PXO99^{A}(pHMPIPGUS2) showed activity in XOM2, as well as other minimal media, XVM2 and XVM1 (Table 3.2). To test whether the reporter construct is functional in *X. oryzae* pv. oryzicola, I attempted to use the PIP box from *X. oryzae* pv. oryzicola BLS256 fused to a promoterless *uidA* gene in pHM1, designated as pHM:PIPXoc:GUS, and a fragment containing a promoterless *uidA* gene fused to the PIP box from pHMPIPGUS2 in another broad host-range plasmid pUFRO47, designated as pUFRO47:PIPGUS. Both *hrp* reporter

constructs in *X. oryzae* pv. oryzicola BLS256 had no GUS activity in XOM2 (data not shown). Next, to test whether the reporter construct functions in *X. oryzae* pv. oryzicola *in planta*, GUS activity in rice tissue inoculated with *X. oryzae* pv. oryzicola strain BLS256(pHMPIPGUS2) was measured. No GUS activity was observed for BLS256(pHMPIPGUS2) (Table 3.3). These data suggest that the PIP box from the *hrcU* promoter region is not activated even *in planta*.

Additionally, I attempted to create a *hrp* gene inducing minimal medium for *X. oryzae* pv. oryzicola. Various media based on media effective for other phytopathogenic bacteria were tested for inducing GUS activity of BLS256(pHMPIPGUS2). No GUS activity was observed in those modified minimal media (Table 3.2).

HrpG of vascular and non-vascular *Xanthomonas oryzae* functions equivalently in X. campestris pv. vesicatoria*ΔhrpG*

To test whether *hrpG* of the vascular pathogen *X. oryzae* pv. oryzae and the non-vascular pathogen *X. oryzae* pv. oryzicola function equivalently for type III regulation, the *hrpG* genes were expressed individually in *X. campestris* pv. vesicatoria 85-10*ΔhrpG*, a strain of the non-vascular pathogen *X. campestris* pv. vesicatoria lacking *hrpG* (courtesy of Dr. U. Bonas). The *hrpG* expression vectors pSM1 ($hrpG_{Xoo}$) and pSM2 ($hrpG_{Xoc}$) were electrotransformed to *X. campestris pv.* vesicatoria 85-10*ΔhrpG* resulting in 85-10*ΔhrpG*(pSM1) and 85-10*ΔhrpG*(pSM2). HR induction was used as a visible marker of *hrpG* complementation because *X. campestris* pv. vesicatoria wildtype strain 85-10 can elicit HR on tobacco (*Nicotiana tabacum* cv. Xanthi) (Fig. 3.1.A). Both 85-10*ΔhrpG*(pSM1) and 85-10*ΔhrpG*(pSM2) induced HR but 85-10*ΔhrpG* with the empty vector did not (Fig. 3.1.A).

Therefore, HrpG of *X. oryzae* pv. oryzae and *X. oryzae* pv. oryzicola both function equivalently in the non-vascular pathogen *X. campestris* pv. vesicatoria.

For reciprocal *hrpG* expression in *X. oryzae* pathovars, construction of *ΔhrpG* strains of *X. oryzae* pv. oryzae and *X. oryzae* pv. oryzicola was attempted. Only *X. oryzae* pv. oryzicola BLS256*ΔhrpG* was obtained. BLS256*ΔhrpG* was electrotransformed with pSM1 and pSM2 resulting in BLS256*ΔhrpG*(pSM1) and BLS256*ΔhrpG*(pSM2). Pathogenicity on the susceptible rice cultivar IR24 was used for a visible marker of *hrpG* complementation. BLS256*ΔhrpG*(pSM1) and BLS256*ΔhrpG*(pSM2) restored pathogenicity as indicated by water soaking of inoculated tissue (Fig. 3.1.B). Thus, at least *hrpG* of *X. oryzae* pv. oryzae functions equivalently to the HrpG in *X. oryzae* pv. oryzicola.

Gain-of-function EMS mutagenesis

The results above support the idea that components upstream of HrpG may activate theTTSS differentially between *X. oryzae* pv. oryzae and *X. oryzae* pv. oryzicola. To identify a HrpG activator, I conducted a gain-of-function EMS mutagenesis.

Optimization of EMS mutagenesis

To generate single nucleotide mutations which create amino acid substitutions and conformational changes in protein structures, alkylating agents that add an ethyl group to numerous position were chosen for the chemical mutagen. The alkylating agents EMS (ethylmethane sulfonate) and MNNG (N-Methyl-N'-Nitro-N-Nitrosoguanidine) generate G:C to A:T single nucleotide transition (Miller 1992; Sega 1984). To optimize the mutagen exposure time for mutagenesis, lethality and rate of mutation to rifampicin resistance were measured as described by Miller (Tanksley et al. 1992) (Fig. 3.2). Fifteen minutes of exposure to EMS caused 40 % lethality and 0.78 X 10^{-6} frequency of rifampicin resistance,

and was chosen for further experiments. MNNG was too strong to obtain moderate mutagenesis since even five minutes of exposure to MNNG killed most of *X. oryzae* pv. oryzae PXO99^A, so it was not used further.

Gain-of-function mutagenesis

Reporters for the gain-of-function mutagenesis containing a *hrp* promoter fused to a chloramphenicol reisistance gene were constructed as described in materials and methods. Transformants of PXO99^A of *X. oryzae* pv. oryzae containing reporters, PXO99^A(pSM7) and PXO99^A(pSM8), were exposed to EMS for fifteen minutes. Nineteen colonies from *X. oryzae* pv. oryzae PXO99^A(pSM7) were recovered on GYE plates containing spectinomycin and chloramphenicol. No colony was recovered from the PXO99^A(pSM8) strain. Most of the mutants grew much slower than the wildtype on GYE agar plates and none of the mutants could be cultured in liquid media with antibiotics (data not shown). To avoid mutations in the reporter gene conferring chloramphenicol resistance, pUFRO47:PIPGUS was transformed to the mutants to measure the type III autoactivation ability. However, only one of the EMS mutants was transformed with the GUS reporter, and it did not show GUS activity *in vitro* (data not shown).

DISCUSSION

Kamdar et al. (Kamdar et al. 1993) demonstrated that HrpX is functionally equivalent in *X. oryzae* pv. oryzae, *X. campestris* pv. campestris, and *X. campestris* pv. armoraciae. Here, I demonstrated the functional equivalence of HrpG in *X. oryzae* pv. oryzae and *X. oryzae* pv. oryzicola. The observation that XOM2 medium induces *hrp* gene expression in *X. oryzae* pv. oryzae but not *X. oryzae* pv. oryzicola (S. Tsuge, personal commnication and my results) suggests that *X. oryzae* pv. oryzae and *X. oryzae* pv. oryzicola *hrp* genes are

differentially regulated. Differential regulation of *hrp* genes may contribute to tissue specificity. Toward identifying possible signals that control *hrp* gene regulation in *X. oryzae* pv. oryzicola, I sought to define a synthetic medium that can activate *hrp* genes in this pathovar. Based on the hypothesis that differential *hrp* gene regulation plays a role in defining the different tissue specificities of *X. oryzae* pv. oryzae and *X. oryzae* pv. oryzicola, I next sought to isolate HrpG activators, as candidate controllers of differential *hrp* gene regulation. I attempted a gain-of-function mutagenesis to accomplish this goal, using a reporter construct of the promoterless chloramphenicol acetyl transferase gene fused to a *X. oryzae* pv. oryzae *hrp* promoter as a selectable marker for *hrp* gene activated mutants.

Heterologously expressed *hrpG* of *X. oryzae* pv. oryzae or *X. oryzae* pv. oryzicola complemented a *ΔhrpG* strain of *X. campestris* pv. vesicatoria for HR elicitation in tobacco. *hrpG* of *X. oryzae* pv. oryzae restored pathogenicity to a *ΔhrpG* strain of *X. oryzae* pv. oryzicola in rice. These results indicate that *hrpG* genes of vascular and non-vascular *Xanthomonas* pathogens function equivalently and that HrpG is not a determinant of differential type III activation or of different tissue specificities. The amino acid sequences of HrpG proteins from *X. oryzae* pv. oryzae, *X. oryzae* pv. oryzicola, *X. campestris* pv. vesicatoria, *X. campestris* pv. campestris (a vascular pathogen of crucifers) and *X. axonopodis* pv. citri, a non-vascular pathogen of citrus) show more than 75 % identifies. The C-terminal region shows a slight difference between HrpG of vascular and non-vascular pathogens. The HrpG sequence of the non-vascular pathogens *X. oryzae* pv. oryzicola, *X. axonopodis* pv. citri, and *X. campestris* pv. vesicatoria contains a proline residue at 252 and those of the vascular pathogens, *X. oryzae* pv. oryzae and *X. campestris* pv. campestris contains leucine and serine residues respectively. However, this residue is located outside of

the predicted helix-loop-helix (HLH)-DNA binding domains and in light of the HrpG swapping experiments clearly does not represent a functionally significant difference.

Various defined minimal media for type III activation of phytopathogens failed to activate the TTSS of *X. oryzae* pv. oryzicola *in vitro*. The fact that XOM2 medium for *X. oryzae* pv. oryzae did not activate the *X. oryzae* pv. oryzicola TTSS suggests that activation of the TTSS of *X. oryzae* pv. oryzicola *in planta* may require different environmental signals from that of *X. oryzae* pv. oryzae. The TTSS of *X. oryzae* pv. oryzae can be weakly activated in XVM2 and XVM1 media, which induce *hrp* gene expression in *X. campestris* pv. vesicatoria, a non-vascular pathogen. Therefore, signals involved in activation *in vitro* may not correlate directly with signals involved in activation *in planta*, or regulation in *X. oryzae* pv. oryzae may be relatively less stringent. Puzzlingly, a GUS reporter assay failed to detect activation of the TTSS in *X. oryzae* pv. oryzicola *in planta*, despite my observation that *hrpG* is required for pathogenesis. The GUS reporter uses a *hrp* gene promoter from *X. oryzae* pv. oryzae. The sequence of the promoter is nearly identical to the corresponding promoter in *X. oryzae* pv. oryzicola. Though the reporter functions well in *X. oryzae* pv. oryzae ((Tsuge et al. 2002) and this study), I cannot rule out the possibility that for some unknown reason, the reporter is not functioning in *X. oryzae* pv. oryzicola and fails to detect *hrp* gene activation.

The *hrp* promoter used in the reporter construct originates from the *hrcU* gene and contains a typical PIP box (TTCGC-N_{15}-TTCGC, (Fenselau and Bonas 1995). The product of *hrcU* of *X. oryzae* pv. oryzicola is a core component of the TTSS predicted to localize in the inner membrane (Van Gijsegem et al. 2002). A non-polar mutation of *hrcU* of *R. solanacearum* disrupts HR induction on the non-host tobacco, pathogenicity on the host tomato, and translocation of the PopA effector (Van Gijsegem et al. 2002). Furthermore,

hrcU in *R. solanacearum* and *Xanthomonas* spp. resides in the multicistronic *hrpC* operon with other required genes, including *hrcV*, encoding the major outer membrane protein of the type III apparatus (Bonas et al. 1992). It is therefore highly unlikely that *hrcU* in *X. oryzae* pv. oryzicola is not required for the TTSS, so apparent failure of the promoter sequence to function in the reporter construct used is perplexing. It is possible that a different reporter, a different vector, or a different experimental approach altogether must be used to effectively assay activation of the *X. oryzae* pv. oryzicola TTSS. For example, northern hybridization may be used to confirm the expression of *hrp* genes.

To identify HrpG activators in *X. oryzae* pv. oryzae, I mutagenized the *X. oryzae* pv. oryzae strain carrying a selectable marker fused with an *hrp* promoter by EMS, which can cause nucleotide substitutions. Although nineteen colonies were recovered after EMS treatment, none of them could be cultured in liquid media with antibiotics, or be confirmed as type III autoactivation mutants using the *hrp* promoter-driven GUS reporter construct. EMS treatment may have affected expression of the selectable marker directly in some way. Also, it is possible that the construct is somewhat leaky on solid media resulting in false positives. Increasing the concentration of antibiotics for the selection of EMS mutants may help to reduce the latter problem.

Sensor kinases and response regulators in two-component systems can be activated by conformational changes brought about by single amino acid substitutions (Smith et al. 2004). For example, several point mutations in OmpR, which is a response kinase in osmotic change sensing in *E. coli*, have been shown to activate the protein without signal activation from EnvZ, the corresponding sensor kinase (Brissette et al. 1991). An amino acid substitution in PhoQ, which is a sensor kinase that controls virulence of *Salmonella enterica*

serovar typhimurium, affects the amount of the signal phosphate transfer to its response regulator PhoP (Gunn et al. 1996; Sanowar et al. 2003). Thus, the gain-of-function mutagenesis approach to isolating *hrpG* activators is reasonable. Using a visible marker rather than selection, or conducting a larger screen may be necessary for success however.

Most two-component systems rely on phosphorylation for signaling. Conformational changes of sensor histidine kinases are brought about by environmental stimuli and typically involve autophosphorylation. Activated sensor kinases then typically transduce signal by phosphorylating a response regulator, which in turn induces activating conformational changes of the response regulator (West and Stock 2001).

Based on the mechanism of signal transmission in two-component systems, a yeast two-hybrid (Y2H) approach might also be effective to identify candidates for *hrpG* activators, which might be expected to physically interact with the response regulator. A total genomic cDNA search of protein interactors with HrpG in *X. axonopodis* pv. citri identified one histidine kinase related to two-component systems. The clone was isolated only once in the screen (Alegria et al. 2004). The number of clones isolated can reflect the level of gene expression. Currently Y2H screening for *hrpG* interactors among predicted sensor components found in the *X. oryzae* pv. oryzae genome sequence is ongoing (G. Dancik, H. Ochiai, H. Kaku, S. Makino, and A. Bogdanove, unpublished).

The identification of HrpG activators will shed light on the relationship between the regulation of the TTSS and tissue specificity. Also, the TTSS activators can be useful targets for further research toward better disease control. Understanding of the signals that activate the TTSS may enable manipulation of the plant or the environment to forestall or repress the activation of this pathway essential to pathogenesis.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The bacterial strains and plasmid used in this study are listed on Table 3.1. *Escherichia coli* cells were grown in Luria-Bertani medium (Sambrook et al. 1989) at 37 ^{o}C. *Xanthomonas oryzae* pv. oryzae and pv. oryzicola were grown in Glucose Yeast Extract (GYE; glucose 20 g and yeast extract 10 g per liter) medium, nutrient broth yeast extract (Vidaver 1967) medium, or synthetic minimal medium XOM2 (Tsuge et al. 2002) at 28 ^{o}C. Appropriate antibiotics were added to growth media for plasmid or mutant selection at the following concentrations: ampicillin (100 μg/ml), chloramphenicol (20 μg/ml), kanamycin (25 μg/ml), gentamicin (25 μg/ml), rifampicin (75 μg/ml), spectinomycin (100 μg/ml).

Plant materials and inoculation test

Rice plants of the indica variety IR24 were grown in LC-1 soil mixture (SunGRO, Bellevue, Washington, USA) in the growth chamber in cycles of 28 ^{o}C daylight for 12 hr and 25 ^{o}C night for 12 hr. Fertilizer (Peters Professional, Saint Louis, Missouri) and iron chelate micronutrient (Becker Underwood, Ames, Iowa) were applied with watering every two days at 0.25 g/L and 4.5 g/L respectively. Four week old rice plants were used for bacterial inoculation. *Nicotiana tabacum* cv. Xanthi (tobacco) was grown in a standard potting mix in a greenhouse and inoculated at six weeks. For inoculation, overnight cultures of bacterial strains were washed with sterile H_2O twice and adjusted to 1 x 10^8 cfu/ml (cfu; colony forming unit, $O.D._{600} = 0.5$) with a spectrophotometer. Cell suspensions were infiltrated to plant leaves with needleless syringes.

DNA manipulations

Standard recombinant DNA manipulations were performed as described previously by Sambrook and Maniatis (Sambrook et al. 1989). Plasmid preparations were performed with the Wizard miniprep (Promega, Madison, WI) and the HiSpeed Midiprep (Qiagen, Valencia, CA). Genomic DNA was extracted with the Dnasy kit (Qiagen, Valencia, CA). Restriction enzymes, Klenow fragment and DNA ligase were used according to manufacturers' instructions.

Plasmid transformation

Plasmids were introduced by electroporation into *E. coli* cells supplied with pCRBluntII according to the manufacturer's protocol (Invitrogen, Carlsbad, CA), and into *Xanthomonas* cells as described previously (Tsuge et al. 2001).

Construction of *hrp* activation GUS reporters

The 214 bp sequence immediately preceeding the *hrcU* start codon and containing the PIP box (Plant Inducible Promoter, TTCGC-N_{15}-TTCGC) was amplified by PCR from genomic DNA of *X. oryzae* pv. oryzicola strain BLS256 with the primers MS83 (forward primer), 5'- ACTGCACCTCGTTTTTATGG –3' and MS84 (reverse primer), 5'-TCCCCCGGGTGCCTTATCCTTCCGCG –3'. The amplified fragment was cloned into the pCRBluntII TOPO cloning vector (Invitrogen, Carlsbad, CA), generating pCRBluntII:PIPXochrcU. The *BamHI* fragment containing the open reading frame of *uidA* gene from pHMPIPGUS2 was ligated to *BamHI* site of pCRBluntII:PIPXochrcU, resulting in pHM:PIPXoc:GUS. pUFRO47:PIPGUS was constructed by ligating a *EcoRI-SacI* fragment, blunted at the *EcoRI* site by fill-in, containing the PIP box and *uidA* gene from pHMPIPGUS2 into *SacI* and blunt *SalI* sites of pUFRO47.

Beta-glucuronidase assay

Bacterial strains were grown in XOM2 medium to exponential phase ($O.D._{600} = 0.6$). 200 μl of cell cultures were spun down and resuspended with 250 μl GUS extraction buffer (50 mM $NaPO_4$, 10 mM β-Mercaptoethanol, 10 mM Na_2EDTA, 0.1 % sodium lauryl sarcosine, 0.1 % Triton X-100) and mixed with 250 μl GUS assay buffer (2 mM 4-methylumbelliferyl b-D-glucuronide (MUG) in GUS extraction buffer (Research Product International Corporation, Mt. Prospect, IL) and incubated at 37 °C. At each time point 100 μl of samples were removed to 1900 μl stop buffer (0.2 M Ma_2Co_3). Enzymatic activity was measured by Hoefer fluorimeter. The obtained values were used to calculate rate of accumulation of 4-methylumbelliferone (nanomole/minute) per bacterial cell (Jefferson 1987).

Hrp expression activity test in minimal media

Overnight cultures of bacterial strains were washed with minimal media twice and adjusted to $O.D._{600} = 0.3$ with a spectrophotometer. Cell suspensions were incubated for 6 hrs at 28 °C and used for a time course GUS assay as described above.

***In planta hrp* expression activity test**

Seven days after inoculation of bacterial suspension to four week old rice plants of cultivar nipponbare by needleless syringe at $O.D._{600} = 0.5$, inoculated leaf tissue was excised by a #1 cork borer and ground with a plastic pestle (Kontes pellet pestle; Kontes, Vineland, NJ) with liquid nitrogen. Ground leaf tissue was resuspended in 500 μl sterile H_2O and 250 μl of the homogenate was mixed with 250 μl of 2 X GUS extraction buffer. A time course GUS assay was performed as described above.

Construction of *ΔhrpG* mutant by double recombination

The 2785 bp fragment including 759 bp upstream of the start codon of *hrpG*, the 792 bp *hrpG* open reading frame, and 1234 bp downstream was amplified by PCR from genomic DNA of *X. oryzae* pv. oryzicola BLS256 with primers MS81 (forward primer), 5'-GCCGGTCTCTCTCTTGGG-3' and SM11 (reverse primer), 5'-CCGGTCGGCTCTCACTG-3'. The PCR product was cloned in pCRBluntII by TOPO cloning reaction (Invitrogen, Carlsbad, CA). The internal 1154 bp region including the *hrpG* ORF was deleted by *Mlu*I digestion and ligation by T-4 ligase. The resulting plasmid, pTOPO*ΔhrpG*, was electroporated into *X. oryzae* pv. oryzicola strain BLS256. The first recombination was selected on kanamycin GYE agar plates. Kanamycin resistant colonies were cultured in GYE liquid medium without kanamycin for about four weeks with subculturing every two days in fresh GYE. The second recombination was isolated by screening for kanamycin sensitivity. The kanamycin-sensitive isolates wee tested for pathogenicity on IR24 and were non-pathogenic. One was chosen arbitrarily as BLS256*ΔhrpG*. Complementation of *hrpG* of BLS256*ΔhrpG* by pSM2 restored pathogenicity (see results).

Construction of *hrpG* expression vectors

The *hrpG* open reading frame and 759 bp upstream, containing the putative promoter region was amplified by PCR from genomic DNA of *X. oryzae* pv. oryzae strain PXO86 and *X. oryzae* pv. oryzicola strain BLS256 with primers MS81 (forward primer) and MS82 (reverse primer), 5'- TCGACTCAGCAGGCGGCTGTGC-3'. The PCR products were cloned into the broad host range plasmid pDD62, and designated as pSM1 and pSM2 respectively.

Construction of gain-of-function mutagenesis reporter plasmids

The 214 bp sequence immediately preceeding the *hrcU* start codon and containing the PIP box (Plant Inducible Promoter, TTCGC-N_{15}-TTCGC) was amplified by PCR from genomic DNA of *X. oryzae* pv. oryzae strain PXO86 and *X. oryzae* pv. oryzicola strain BLS256 with primers MS83 (forward primer) and MS84 (reverse primer). The amplified fragments were cloned into pCRBluntII TOPO (Invitrogen, Carlsbad, CA), generating pCRBluntII:PIPXoohrcU and pCRBluntII:PIPXochrcU respectively. The chloramphenicol acetyl transferase (CAT) gene was amplified by PCR from pBC SK- (Stratagene, La Jolla, CA) with primers SM3 (forward primer), 5'- CTGCAGTAAGGAAGCTAAAATGG –3' and SM4 (reverse primer), 5'- GCGGCCGCTGCCTTAAAAAAATTA –3' and cloned into pBCBluntII TOPO. The *PstI-NotI* fragment containing the CAT gene was subsequently subcloned into each pCRBluntII:PIP. The *Sac*I-*Sal*I fragments of the PIPXoohrcU:CAT and PIPXochrcU:CAT fragments were subcloned into the broad-host range plasmid pHM1, and designated as pSM7 and pSM8, respectively.

EMS mutagenesis

EMS (ethylmethane sulfonate, Sigma chemical, St. Louis, MO) mutagenesis of *X. oryzae* pv. oryzae was performed as described for *E. coli* by Miller (Miller 1992). One ml of an overnight culture of *X. oryzae* pv. oryzae $PXO99^A$(pSM7) or *X. oryzae* pv. oryzae $PXO99^A$(pSM8) at $O.D._{600}$ = 0.5 was washed twice with Minimal A buffer, and resuspended in 400 μl Minimal A buffer. The cell suspension was added to 6 μl of EMS and incubated for 30 minutes at 28 °C with moderate shaking. After incubation, the suspension was washed with Minimal A buffer twice and resuspended in 400 μl Minimal A buffer. The cell

suspension was plated on GYE agar plates with spectinomycin and chloramphenicol and incubated at 28 °C.

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Table 3.1. Bacterial strains and plasmids used in this study.

Strain	Relevant characteristics	Source or reference
Escherichia coli		
DH12S	φ80d*lac*ZM15 *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) *ara*D139 Δ(*ara*, *leu*)7697 Δ*lac*X74 *gal*U *gal*K *rps*L (Str^R) *nup*G *rec*A1/F' *pro*AB$^+$ *lac*IqZΔM15	Invitrogen, Carlsbad, CA
Xanthomonas campestris pv. vesicatoria		
85-10	tomato and pepper race 2, Rif^r	U. Bonas
85-10*ΔhrpG*	85-10 *hrpG* deletion mutant, Rif^r	Wengelnik et al. 1996
Xanthomonas oryzae pv. oryzae		
$PXO99^A$	Azacytidine resistant derivative of Philippine race 6 strain PXO99, virulent to rice cultivars IR24	Hopkins et al. 1992
Xanthomonas oryzae pv. oryzicola		
BLS256	Philippines isolate	J. Leach
BLS256*ΔhrpG*	BLS256 *hrpG* deletion mutant	This study

Plasmid	Relevant characteristics	Source or reference
pBC SK-	Cloning vector, Cm^r	Stratagene, La Jolla, CA
pCRBluntII	TOPO cloning vector, Km^r	Invitrogen, Carlsbad, CA
pDD62	Broad host range plasmid, Km^r	B.Staskawicz
pHM1	Broad host range plasmid, Sp^r	R. Innes
pHMPIPGUS2	pHM1 carrying promoterless *uidA* fused with PIP box, Sp^r	Tsuge et al. 2002
pHM:PIPXoc:GUS	pHM1 carrying promoterless *uidA* fused with PIPXochrcU, Sp^r	This study
pSM1	pDD62 carrying $hrpG_{Xoo}$ with native promoter, Km^r	This study
pSM2	pDD62 carrying $hrpG_{Xoc}$ with native promoter, Km^r	This study
pSM7	pHM1 carrying PIPXoohrcU:CAT, Sp^r	This study
pSM8	pHM1 carrying PIPXochrcU:CAT, Sp^r	This study

Table 3.1. (continued)

Plasmid	Relevant characteristics	Source or reference
pTOPO*ΔhrpG*	*ΔhrpG* suicide vector, Km^r	This study
pUFRO47	Broad host range plasmid, Ap^r, Gm^r	D. Gabriel
pUFRO47:PIPGUS	pUFRO47 carrying PIPGUS fragment of pHMPIPGUS2, Ap^r, Gm^r	This study

Ap: ampicillin, Gm: gentamicin, Km: kanamycin, Rif: rifampicin, Sp: spectinomycin

Table 3.2. GUS activity in minimal media.

		XocBLS256 (pHMPIPGUS2)		XooPXO99A (pHMPIPGUS2)		
Medium	Carbon source[a]	GUS activity	Growth[c]	GUS activity	Growth	Reference
XOM2 (pH 6.7)	D-Xylose (12mM)	-	-	++	++	Tsuge et al. 2002
	L-Arabinose	-	-/+	ND	ND	
	D-Galactose	-	++	ND	ND	
	Glucose	-	-	ND[b]	ND	
	Glycerol	-	-	ND	ND	
	Mannitol	-	-/+	ND	ND	
	Myo-inositol		-	ND	ND	
	Raffinose	-	-/+	ND	ND	
XVM1 (pH 6.5)	Sucrose (20mM) Methionine(13mM)	-	-	+	-	Schulte et al. 1992
*hrp*MM (pH 5.7)	Fructose	-	-	ND	ND	Huynh et al, 1989
	Glutamate	-	-	ND	ND	
	Sucrose	-	-	ND	ND	
	D-Xylose	-	-	ND	ND	

a Concentrations of carbon sources are 10mM unless otherwise noted.

b ND: not determined.

c Growth was determined by O.D. values after 6 h incubation in minimal media started from O.D. = 0.3. ++ stands for more than twice O.D. value. -/+ stands for approximately 1.5 times O.D. value.

Table 3.3. *In planta* GUS activity assay.

	XocBLS256 (pHMPIPGUS2)	XocBLS256 (pHM1)	XooPXO99A (pHMPIPGUS2)	XooPXO99A (pHM1)
GUS activity (units/10^8cfu)	31,250	471,429	3,592,500	18

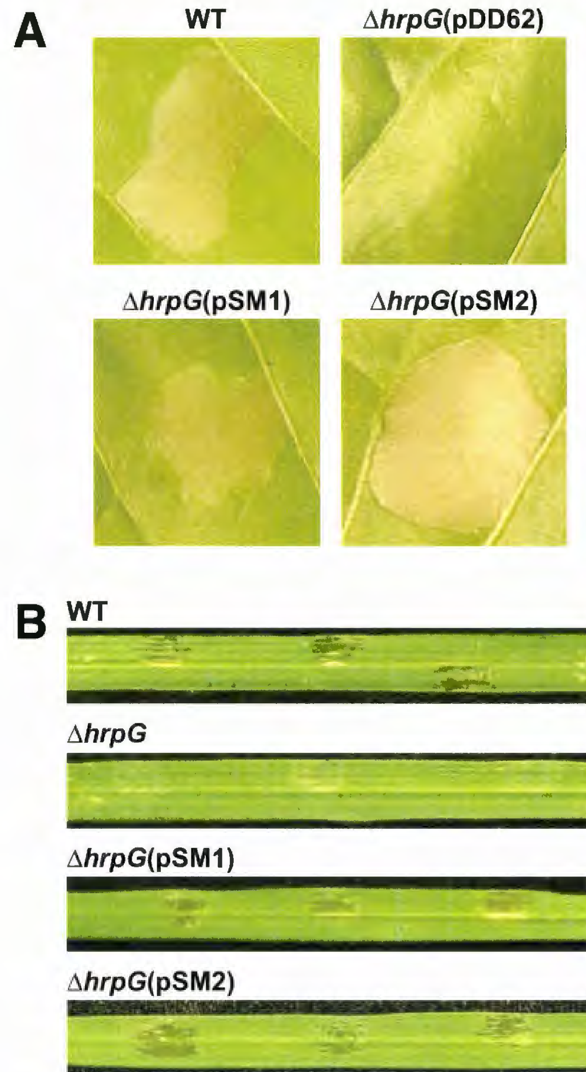

Figure 3.1. HrpG of vascular and non-vascular *X. oryzae* pathovars functions equivalently in *X. oryzae* pv. oryzicola*ΔhrpG* and *X. campestris* pv. vesicatoria*ΔhrpG*. (**A**) *X. campestris* pv. vesicatoria strains 85-10*ΔhrpG*(pSM1) and 85-10*ΔhrpG*(pSM2) restored HR induction in tobacco. Pictures were taken 24 hrs after inoculations. (**B**) *X. oryzae* pv. oryzicola strains BLS256*ΔhrpG*(pSM1) and BLS256*ΔhrpG*(pSM2) restored development of water-soaking lesion in rice. Pictures were taken two days after inoculations.

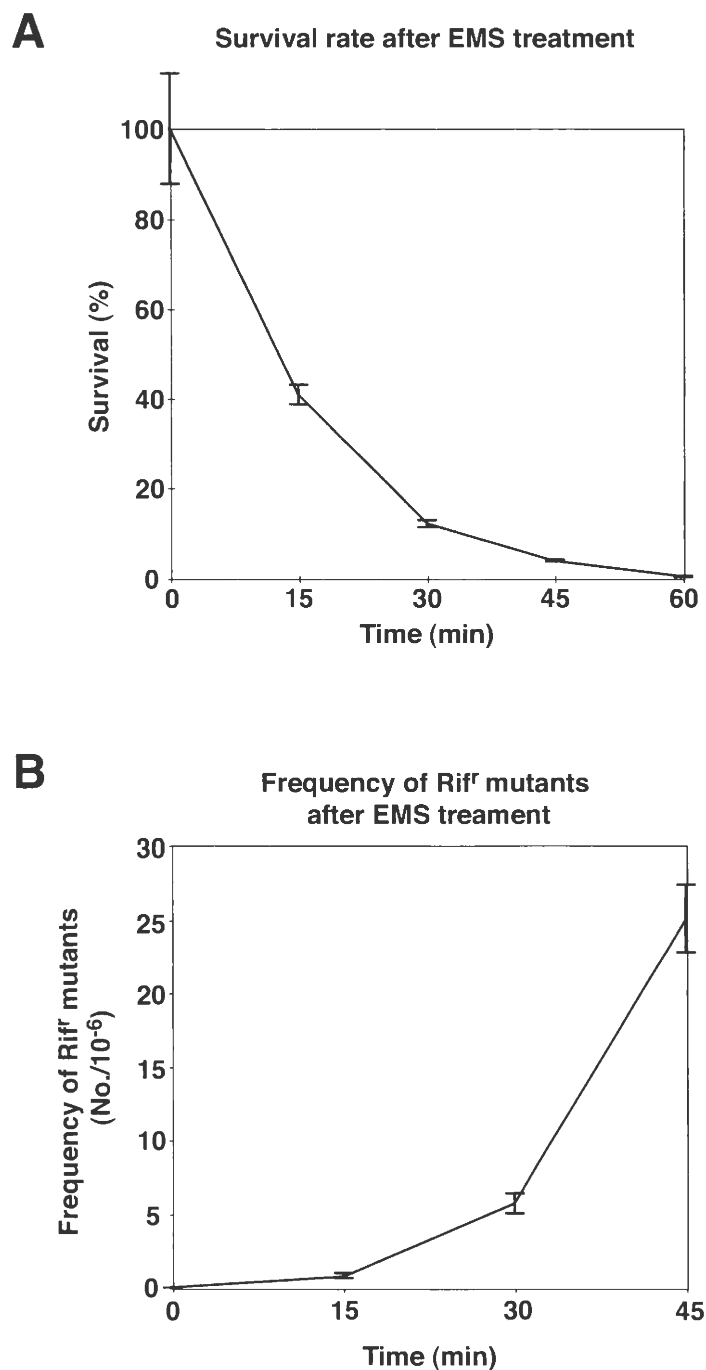

Figure 3.2. Lethality and mutation rates on EMS exposure time. (**A**) Survival rate after EMS treatment. (**B**) Frequency of Rif^r mutants after EMS treatment.

Chapter 4. Future perspective

The purpose of this study was to improve understanding of the molecular interaction of plants and phytopathogenic bacteria. Two closely related rice bacterial pathogens of rice, *Xanthomonas oryzae* pv. oryzae and pv. oryzicola were examined in two studies. The first study showed that *X. oryzae* pv. oryzicola inhibits rice defense responses, which may explain the absense of major gene resistance to bacterial leaf streak, the disease it causes. The results suggested that *X. oryzae* pv. oryzicola qualitatively inhibits *R* gene-mediated defense responses in a type III secretion-dependent fashion. The putative type III-secreted defense inhibitor and its point of action are important targets for future research. A first step will be to determine whether inhibition is effective against diverse *avr-R* interactions. In the second study, identification of the determinant(s) of differential type III secretion (*hrp*) gene regulation in the two *Xanthomonas* oryzae pathovars was attempted. In the defined minimal medium XOM2, *hrp* activation was observed for *X. oryzae* pv. oryzae but not *X. oryzae* pv. oryzicola indicating differential type III regulation that might play a role in tissue specificity. Swapping of the *hrpG* gene, a type III secretion gene activator, between non-vascular and vascular *Xanthomonas* pathogens indicated that HrpG, the highest known *hrp* regulator, is not a determinant of *hrp* regulation in tissue specificity and suggests that HrpG activator(s) could be the determinant. The identification of HrpG in these pathogens is functionally equivalent and therefore is not a determinant of tissue specificity. An attempt to identify HrpG activating genes using a gain-of-function mutagenesis approach failed. This remains an important goal however, and slight modification to this approach, e.g., use of a screening marker rather than a selectable marker as reporter, could lead to success.

Appendix A.

Screens for the defense inhibiting effector(s) in *Xanthomonas oryzae* pv. oryzicola

To identify the effector(s) involved in defense inhibition by *X. oryzae* pv. oryzicola, two screens are in progress. The first is a screen of transposon-induced mutants of *X. oryzae* pv. oryzicola BLS303(pHMavrXa10) for strains able to elicit the HR on IRBB10 plants. This screen is likely to succeed only if the inhibitor is single copy. HR-eliciting strains would be expected to have insertions in the defense inhibitor gene. Approximately 10,000 mutants are required for an insertion on average every 500 bp in the genome of *X. oryzae* pv. oryzicola, estimated to be roughly 5 Mbp based on the sizes of sequenced *Xanthomonas* genomes (Da Silva et al. 2002; Lee et al. 2005). Approximately 6,000 mutants have been screened so far. Seven reduced virulence and five non-pathogenic mutants have been isolated, but an HR-eliciting mutant remains to be found (Fig. A.1). In *X. oryzae* pv. oryzae, several *avrBs3* homologs are identified as virulence determinants for bacterial blight (Yang et al. 2005; Yang and White 2004). Knockouts of the virulence genes remarkably reduce virulence. Conserved *avrBs3* homologs in *X. oryzae* pv. oryzicola may function as virulence factors in *X. oryzae* pv. oryzicola as well. Cloning of genes disrupted by transposon insertion is a next step to determine virulence factors in *X. oryzae* pv. oryzicola.

The other screen is for transformants of *X. oryzae* pv. oryzae strain PXO86RA, the strain from which *avrXa10* and *avrXa7* were isolated, carrying cosmid clones of *X. oryzae* pv. oryzicola BLS303 DNA, that no longer elicit HR on IRBB10 plants. Approximately 700 transformants are required for 5 X coverage of the *X. oryzae* pv. oryzicola genome. To date, roughly 100 have been tested with no positives.

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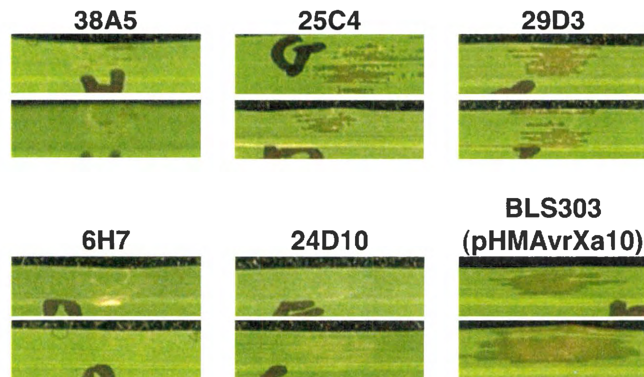

Figure A.1. Representative reduced water-soaking and non-pathogenic transposon induced mutants of *X. oryzae* pv. oryzicola BLS303(pHMavrXa10) inoculated to IRBB10 plants. 38A5, 25C4 and 29D3 are reduced water-soaking mutants. 6H7 and 24D10 are non-pathogenic mutants. *X. oryzae* pv. oryzicola BLS303(pHMavrXa10) was used as a reference. Four week olds plants were inoculated with bacterial suspensions at $O.D._{600} = 0.5$ with needleless syringes. Pictures were taken four days after inoculation.

Appendix B.

Quantitative CyaA fusion assay of AvrXa10 secretion from *X. oryzae* pv. oryzicola into rice cells

The calmodulin-dependent adenylate cyclase (CyaA) domain of the cyclolysin toxin from *Bordetela pertussis* binds calmodulin in eukaryotic cells and catalyzes conversion of ATP to cyclic AMP (cAMP), which can be measured using a modified ELISA protocol (Engvall 1980). Since CyaA itself is not secreted and bacteria do not produce calmodulin, fusion of CyaA to a pathogen protein of interest and subsequent measurement of cAMP accumulation in infected host tissues can be used as an assay for protein translocation assay from bacterial cells to eukaryotic cells (Casper-Lindley et al. 2002; Ladant and Ullmann 1999; Sory and Cornelis 1994).

AvrXa10 has a type III-dependent translocation signal at the N-terminus that is conserved in the AvrBs3/PthA family (Buttner and Bonas 2002). To determine if AvrXa10 is secreted into plant cells from *X. oryzae* pv. oryzicola harboring pHMavrXa10, CyaA encoding sequence was fused to *avrXa10* in pHMavrXa10 to generate pKEB44. Then, pKEB44 was introduced by electroporation into strain BLS256 of *X. oryzae* pv. oryzicola, and as controls, strain BLS256*hrcC*⁻ of *X. oryzae* pv. oryzicola and strains PXO99^{A} and PXO99ME7 (a *hrpC* mutant) of *X. oryzae* pv. oryzae, and these strains were inoculated to susceptible rice cultivar IR24 plants. CyaA activity of inoculated tissue was measured at 4 and 8 hours after inoculation.

In pHMavrXa10, *avrXa10* is downstream of and oriented with the *lacZ* promoter. In pKEB44, the 1232 bp *cyaA* sequence, including a *Sal*I site and a linker sequence (5'-GTCGAC TTGCGGGCGTTGGT-3') is inserted at a *Sal*I restriction site 2945 bp

downstream of the *avrXa10* start site. For the CyaA assay, rice leaves were infiltrated with bacterial suspensions at $O.D._{600} = 0.5$ with needleless syringes. 4 h and 8 h after inoculation, three replicate samples of five centimeter lengths of rice leaves containing four inoculated spots each were collected, frozen in liquid nitrogen and ground with a plastic pestle to a powder. The ground leaf tissues were resuspended in 250 µl of 0.1 M HCl and 50 µl of the supernatants were assayed with a correlate-EIA cAMP immunoassay kit (Assay Designs, Ann Arbor, MI) according to the manufacturer's directions with three technical repeats for each sample. The amount of total proteins in each sample for normalization was determined by the BCA Protein Assay kit (Pierce, Rockford, IL).

Both BLS256(pKEB44) and $PXO99^A$(pKEB44) showed high cAMP amounts at 8 hrs after inoculation but not at 4 hrs after inoculation (Fig. A.2). None of the *hrp* mutants showed significant amounts of cAMP and amounts did not increase between 4 h and 8 h. The results indicate that *X. oryzae* pv. oryzicola can secrete AvrXa10 expressed from pHMavrXa10. The cAMP amount for $PXO99^A$(pKEB44) was higher than that for BLS256(pKEB44). Failure of *X. oryzae* pv. oryzicola harboring pHMavrXa10 to elicit HR on rice IRBB10 plants may be due to a failure to secrete threshold levels of the protein. The experiment should be repeated to confirm the results. Also, it will be important to compare the amount of AvrXa10 secreted from cells harboring pHMavrXa10 with that from cells harboring *avrXa10* in the higher copy plasmid pDD62.

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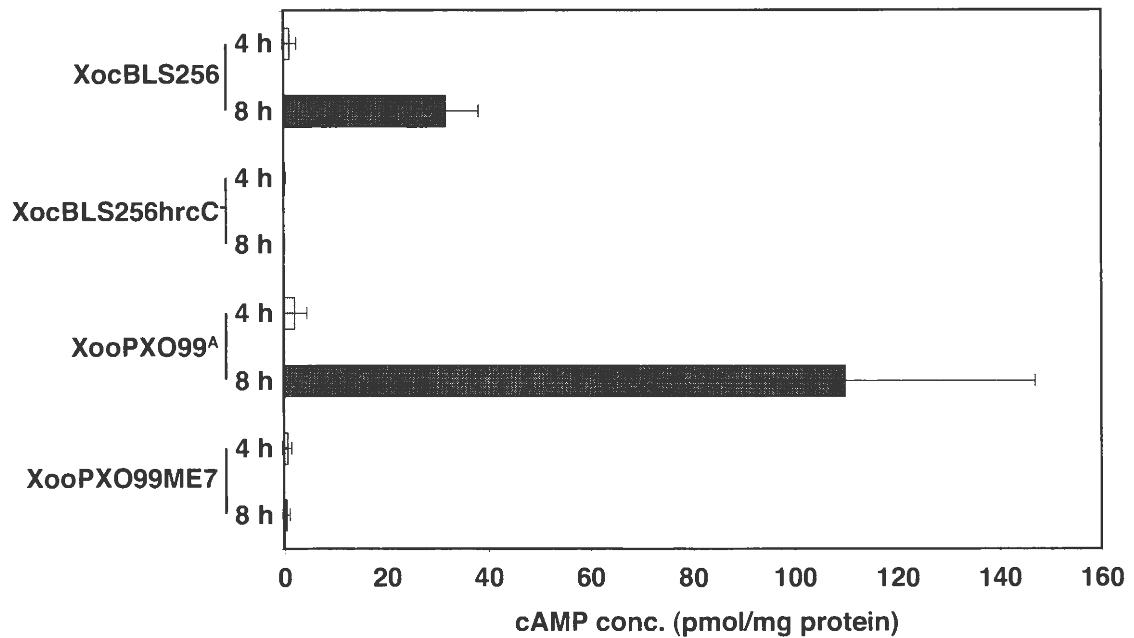

Figure A.2. CyaA fusion assay of AvrXa10 secretion. All strains contain pKEB44. Two week old IR24 plants were infiltrated with needleless syringes with bacterial suspensions of *X. oryzae* pv. oryzicola (Xoo) BLS256, BLS256*hrcC-*, *X. oryzae* pv. oryzae (Xoo) PXO99^{A}, or, PXO99ME7 at $O.D._{600} = 0.5$.

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