Understanding the virulence mechanisms of bacterial phytopathogens: from *Pseudomonas to Xanthomonas*

by

Li Wang

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Program of Study Committee:
Adam J. Bogdanove, Major Professor
Gwyn A. Beattie
Madan K. Bhattacharyya
Steve A. Whitham
Dan Voytas

Iowa State University
Ames, Iowa
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Dedicated to my husband, Hui, my son, David and my parents Zhengling and Fengmei, who made my life joyful and meaningful.
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CHAPTER 1.

Molecular basis of interaction of bacterial phytopathogens and plants – a review of literature related to the research presented in this thesis

Thesis Organization

The purpose of this research is to try to understand the molecular mechanisms of plant-bacterium interactions, furthermore, to support development of new strategies for the plant disease control. The research presented in this dissertation focuses on two different plant-pathogen systems. The first one is Pseudomonas syringae pv. glycinea and soybean (Glycine max.) system; another is Xanthomonas oryzae and rice (Oryza sativa) system. This dissertation comprises a general review related to this research (this chapter), three manuscripts (chapter 2-4), as well as a general summary and two appendices. In this general review chapter, I will review the relevant literature regarding the bacterial pathogens used in this research, their host plants, and the current opinions about the interactions between bacterial pathogens and their host. Chapter 2 addresses functions in soybean of two P. syringae effectors avrB and avrPto using soybean mosaic virus (SMV) adapted vector. This manuscript has been published in Molecular Plant-Microbe Interactions (MPMI). Chapter 3 is a manuscript submitted to Applied and Environmental Microbiology, which describes the construction of a virulent defective mutant library of X. oryzae pv. oryzicola (Xoc) using the TN5 transposon and a quantitative screening method. Chapter 4 presents the functional characterization of the O-chain of lipopolysaccharides-deficient mutant TN38C10 of Xoc, which will be submitted to Journal of Bacteriology. Summary provides general discussion of the results and conclusions drawn from them. Appendices A and B describe the strain-
dependent effects on the function of specific avirulent gene in X. oryzae pv. oryzae strains and the construction of hrpG defective mutant of X. oryzae pv. oryzae PXO99A strain, respectively.

1.1 Bacterial Virulence Mechanisms and Plant Resistance

Plants are a good source of nutrition and water for bacteria. Plants suffer from bacterial diseases caused by many bacterial pathogens from both the Proteobacteria and Actinobacteria. Most intensive studies of bacterial diseases focus on members of Proteobacteria such as Agrobacterium, Erwinia, Pseudomonas, Xanthomonas, Ralstonia and Xylella because they cause more severe economic damage (1). Bacterial diseases impact our food from flavor to yield loss. For example, at least 350 different plant diseases were caused by Xanthomonas spp. X. oryzae pv. oryzae cause rice bacterial blight, which may cause about 30% yield loss in tropical areas like India and South China. Bacteria enter plant through natural opening such as stomata, hydrothodes or wounding and cause disease in apoplast or xylem. The disease symptoms include wilts, spots, blights, cankers and galls. Bacterial pathogens use several different kinds of virulence factors to fulfill their successful infection. Next, we will discuss these factors detailed.

Bacterial factors involved in pathogenesis and virulence

Virulence factors are the most important characteristics of the bacterial pathogens, which determine disease severity. The major virulence factors include type III secretion system (T3SS), polysaccharides, extracellular enzymes, toxins and plant hormones. Most of
known bacterial pathogens possess several different kinds of virulence factors. *Pseudomonas* and *Xanthomonas* species usually do not use the plant hormones as virulence factors. In the following part, the discussion will focuses on all of mentioned virulence factors except plant hormones, with a focus on *Xanthomonas* spp.

**Type III secretion system (T3SS)**

Many Gram-negative bacterial pathogens use the T3SS to export bacterial effectors into host, and cause disease. The T3SS is conserved between pathogens of plant and animal (26). The T3SS encoded by hypersensitive response and pathogenicity (*hrp*) gene cluster which is required for both hypersensitive response and pathogenicity. The physical structure of the T3SS, called hrp pilus, is similar to the flagellar biosynthetic complex in phytopathogens (37). This hrp pilus structure was observed under electron microscope in *Xanthomonas campestris* pv. vesicatoria 85E* and *P. syringae* pv. tomato (61, 117). The hrp pilus of *Xanthomonas campestris* pv. vesicatoria 85E* is up to 4µm in length and can potentially cross the plant cell wall (117). The *hrp* gene cluster is usually comprised of at least 20 genes (15). In *Xanthomonas campestris* pv. vesicatoria, the *hrp* cluster includes 22 genes arrayed in 6 operons named from *hrpA* to *hrpF* (118). The expression of *hrp* gene is induced in plant and in certain minimal media like XOM2, XVM2 but not in rich media like GYE, NBY (92, 110). In *Xanthomonas*, the expression of these *hrp* genes is tightly controlled by the regulators HrpG and HrpX, which are encoded by genes located outside of *hrp* gene cluster (92). The HrpX protein, an AraC-type transcriptional activator, regulates the expression of genes in *hrp* cluster and other effector genes (119). Most of the HrpX-regulated genes have a PIP box as the trans-regulatory element in their promoter regions,
which is supposedly involved in the regulation by HrpX. The consensus sequence of the PIP box in *Xanthomonas* is TTCGC-N$_{15}$-TTCGC (15). The expression of *hrpX* is regulated by HrpG, which belongs to the OmpR family of two-component regulatory systems (120). cAFLP analysis has indicated that genes regulated by HrpG encode proteins covering transcriptional regulators, degradative enzymes, and type III effectors (75). So far, the regulation of expression of *hrpG* is still an enigma.

The main function of the T3SS is to deliver T3SS effectors into plant cells. The delivery of some effectors needs the help of molecular chaperons. In *Xanthomonas*, the successful secretion of XopJ and XopF1 requires HpaC (*hrp*-associated) (17). HpaB, another Hpa protein specifically promotes the translocation of AvrBs3, AvrBs1, AvrBsT, XopC, XopA (16).

**Bacterial Polysaccharides**

Bacterial polysaccharides include lipopolysaccharides (LPS), lipooligosaccharide (LOS), and extracellular polysaccharide (EPS) (82). LPS presents in most Gram-negative bacteria and comprises three core components: lipid A, core oligosaccharide and O-antigen. Lipid A anchors the LPS on the outer membrane and is composed of sugars and fatty acid side chain. The core polysaccharide is made up of sugars and sugar derivatives. The O-antigen which extends to the cell surface consists of repeating oligosaccharide units. LPS without O-antigen is called LOS (97). EPS composed of the repeat sugar units can form a capsule outside of outer membrane or be released to the environment as slime (82).

EPS are secreted by both Gram-negative and Gram-positive bacterium. All *Xanthomonas* species secrete numerous EPS outside the cell membrane. Mutants of
*Xanthomonas* which lose the ability to produce EPS usually lose their virulence function (25). The EPS synthesis genes usually cluster in genome as *gum* operon. In Xoo, this operon consists of 13 genes called *gumBCDEFGHIJKLN* (58). A mutant of *gumG* of Xoo made by transposon insertion loses the synthesis of EPS as well as its virulence on rice (31).

LPS can contribute to the virulence of bacteria, even it does not have the same importance as the EPS. LPS was well studies in human pathogen as one endotoxin of host (86). However, LPS functions in phytopathogenic bacteria are still not clear. Plants recognize certain LPS as microbe-associated molecular patterns (MAMPs), and thus trigger innate defense responses (65, 97). Pretreatment with LPS can “prime” pepper plants, enhancing the defense response to a subsequently inoculated non-host strain of *Xanthomonas campestris* (72). In Arabidopsis suspension cell cultures, LPS activates a subset of defense genes that are coupled to *AtNOS1*-dependent nitric oxide synthesis (NOS). In plants, disruption of *AtNOS1* compromises resistance to *Pseudomonas syringae* (124). Lipid A is sufficient to induce NOS. In Arabidopsis, both the core and lipid A of *X. campestris* lipooligosaccharide trigger independently to the induction of defense genes *PR1* and *PR2* (97). This observation provides evidence for recognition of core polysaccharides and lipid A by distinct receptors. In cultured rice cells LPS from plant and non-plant pathogens can upregulate defense genes and genes involved in the generation of reactive oxygen. Unexpectedly, and differently from the case in Arabidopsis, LPS also induce genes involved in programmed cell death in rice cell suspension culture, typical of gene-for-gene interactions and common to non-host defense responses (30).

In contrast to O-antigens in animal pathogens whose functions are well studied, functions of O-antigens in phytopathogens are still unclear. In the *Rhizobium*-legume
symbiosis, structural changes in LPS O-chain during nodulation suggest an adaptive role of O-antigens (60). When synthetic O-antigens repeat units are used to pretreat Arabidopsis Col-0, they suppress the hypersensitive responses triggered by interaction of AvrRpm1 and Rpm1 and induce PR-1 gene expression (8). All these observations indicate that elucidating the functions of O-antigens of LPS is important to better understand the virulence mechanisms of phytopathogens.

**Extracellular enzymes**

The plant cell wall is the first barrier of protection against pathogen attack. In order to infect host plant effectively, some pathogens secrete extracellular enzymes to degrade plant cell wall. Cell walls in higher plants contain three major classes of polysaccharides: cellulose, hemicellulose, pectins and proteins (99). Each of them is easily degraded by extracellular enzymes secreted by some plant pathogens. Pectolytic enzymes, cellulase and proteases are the major groups of extracellular enzymes. Extracellular enzymes are important virulence factors of bacterial pathogens. Some bacterial pathogens such as Erwinia carotovora use cell wall-degrading enzymes as the main virulence factors. About 30-40 genes encoding cell wall-degrading enzymes exist in the genomes of Xanthomonas campestris pv. campestris and X. axonopodis pv. citri (28). More genes encoding cell wall-degrading enzymes have been found in the Xoo genome (58). Xylanase is the enzyme that degrades xylan, a component of xylem vessels. Xoo strains with a mutant xynB, the gene for xylanase secretion, have an abolished ability to accumulate xylanase in planta and attenuated virulence in rice (81).
Toxins

Almost all toxins produced by phytopathogenic bacteria are secondary metabolites, and often involved in virulence and symptom development. Toxins usually are thought to be secreted outside of pathogen cell *in vitro* or *in planta* (34). Toxins can affect many aspects of physiology and biochemistry of host plant and are capable of causing disease symptoms such as wilting, water-soaking, necrosis and chlorosis. Most of the studies on toxins focus on species of *Pseudomonas*. For example, coronatine, the toxin secreted by *Pseudomonas syringae* species, acts as the mimic molecule of plant signals jasmonate (JA) and methyljasmonate (MeJA) after it is cleaved inside the plant cell(54). Recently it was reported that coronatine is able to prevent the ABA-induced stomata closure during the pathogen attack in order to facilitate the entry of bacteria into plant (70). *X. albilineans* is the agent responsible for a serious disease of sugar cane called leaf scald disease. It produces a toxin called albicidin which is extremely efficient in causing symptoms of the disease on the leaves. Albicidin is a major pathogenicity factor in *X. albilineans*. Although bacterial toxins contribute to successful infections, they differ from T3SS effectors. They do not exhibit host specificity, nor directly contribute to the bacterial growth (3).

Plant defense responses

During the co-evolution of bacterial pathogens and their plant host, the plant develops a series of defense responses against the attack of pathogens. From non-host resistance to gene-for gene race specific resistance, defense responses in plants are layered and continuing responses and signal transduction pathways are overlapped (27). The first barrier of plants is natural passive defenses including the surface wax, cell wall and some pre-existing toxins.
and enzymes (108). Plants also develop the ability to express the induced defense responses through recognizing certain microbial molecules. This kind of molecules usually have repeated conserved features like LPS and flagellin (1) and are referred as pathogen-associated molecular patterns (PAMP) or microbe-associated molecular patterns (MAMP) (65). However, gene-specific resistance has been best characterized in last sixty years.

**MAMP-induced plant innate immunity**

In some way, plant mimics the animal immunity system, which is capable of acquiring resistance by recognizing the non-self chemical components. MAMP-induced innate immunity takes place at the early stages of bacterial attack (70). MAMPs are usually recognized directly by receptors called plasma-membrane-localized pattern recognition receptors (PRRs) which contain a leucine-rich repeat (LRR) domain outside of the plasma membrane and a cytoplasmic kinase domain (1).

The best studied MAMP is flagellin. Flg22, a 22-amino acid peptide of flagellin, was reported to interact with FLS2 in Arabidopsis (21, 39). This recognition triggers a series of defense responses such as the motigen-activated protein kinase (MAPK) cascades and the inhibition of multiplication of the pathogen (4, 131).

LPS is another well known MAMP. It was reported that purified LPS of *Xanthomonas campestris* pv. campestris induces the oxidative burst in tobacco cells (14). The purified LOS of *Xanthomonas campestris* pv. campestris strain 8004 induces PR-1 and PR-2 gene expression in Arabidopsis (97). Recently, it was reported that purified LPS of *Pseudomonas syringae* pv. tomato DC3000 induces the stomata closure which block the entrance of bacteria (70).
**Gene-specific resistance**

Plant disease resistance often depends on specific recognition of the pathogen and a rapid induction of general defense responses. Usually such a recognition event is a gene-for-gene interaction, depending on a resistant gene (*R* gene) in the plant and its corresponding *avr* gene in the pathogen. More than 40 plant disease *R* genes have been cloned in different plant species (67). These *R* genes confer resistance to diverse pathogens and can be classified based on their protein structures into several groups with several exceptions (67).

The recognition of an Avr protein by an R protein triggers a series of defense responses that enable the plant to protect itself and defeat the pathogen in order to survive. Hypersensitive responses (HR), a kind of localized programmed cell death, is proposed to play an important role in disease resistance because of the rapid deprivation of nutrients and collapse of pathogen infected tissues (101). Induction of reactive oxygen species (ROS) is often the first response detected following pathogen attack. The typical ROS include superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and the hydroxyl radical (OH). In incompatible interaction, the induction of ROS occurs in two oxidative bursts, several hours apart (57). These local responses often trigger systemic acquired resistance (SAR) through the whole plant, which provides non-specific, broad-spectrum protection against pathogens. Accompanied with SAR are secondary metabolic changes such as reinforcement of cell wall including lignification and papillae formation, rapid and strong expression of pathogenesis-related (PR) proteins and the accumulation of phytoalexins (103). PR proteins, which have been found in many plant species to date, are classified into seventeen families based on the sequence similarities, serologic or immunologic relationships, and enzymatic properties.
The rapid and strong expression of PR proteins have been found in many incompatible interactions. This suggests the general role of these proteins in defense responses.

1.2 Overview of Literature Related to *Pseudomonas syringae* and Soybean Interaction

**Soybean bacterial blight and *Pseudomonas syringae***

Soybean is an important crop and its yield potential is limited by microbial pathogens. Soybean bacterial blight, caused by *Pseudomonas syringae* pv. glycinea (Psg), occurs every year in rainy areas. Disease symptoms usually appear on the upper, newly developed leaves first as small, water-soaked, yellow-to-brown spots and then developing a yellow halo around the lesion (56). Main control methods of soybean bacterial blight include the usage of resistant cultivars and crop rotation.

*Pseudomonas syringae* pv. glycinea belongs to the Gram-negative phytopathogenic bacterium group. Its effective infection depends on the type III secretion system (T3SS). T3SS pilus delivers numerous effector proteins into the plant cell. Some of these effector proteins are identified as the avirulence (*avr*) effectors first due to their ability to trigger defense responses in the host plant carrying its corresponding resistance (*R*) gene. Then, they are confirmed as the virulence (*vir*) effectors because of their ability to enhance the disease symptoms and bacterial multiplication (20, 121).

**Transient expression of bacterial effector genes in plant***

Transient expression of individual gene in plant was originally developed to understand gene function. The function of several *avr* genes have been identified using the
transient expression approaches (2, 106). Identification of the function of \textit{avr} genes not only helps us understand mechanisms of the plant resistance further benefit potentially finding new means to the disease control. However, transient expression techniques are limited for many crop species.

Three main methods for transient expression in plants have been established. The \textit{Agrobacterium}-mediated transformation is the most widely used method. Introduction of plant-specific promoter-driven gene on vector into plant cells by particle bombardment is the second method, which overcomes the host limitation of \textit{Agrobacterium}-mediated expression (89). A new tool for transient expression is inoculation of plant with recombinant viruses or infectious derivatives carrying transgenes (91). When compare to other approaches, virus-mediated methods uniquely allow the systemic expression in whole plants, and often achieve expression to higher levels and of greater duration (80).

Soybean is a major crop species for which transient expression methods are limited. \textit{Agrobacterium}-mediated transient expression in live plants has not been reported. \textit{Clover yellow vein virus} (ClyVV) was developed as an expression vector for clover and was reported to function in soybean (69), but has not been used widely. Bean pod mottle virus (BPMV) was also developed as a vector and confers soybean resistance to 0.1% amino glufosinate through carrying transgenic \textit{bar} gene (125). In my research, I used the modified soybean mosaic virus (SMV) as a transient expression vector to express \textit{avrPto} or \textit{avrB} gene individually in soybean and investigate further the function of these two \textit{avr} genes in soybean.
**avrPto and avrB**

*avrPto*, an avirulence effector eliciting resistance in Pto-expressing tomato, was first isolated from *P. syringae* pv. tomato race 0 (85). Inoculation of a virulent strain of *P. syringae* pv. tomato race 1 carrying *avrPto* into tomato carrying *Pto* induces resistance associated with hypersensitive reaction (HR). *avrPto* encodes a 164 amino acid hydrophilic protein without significant similarity with other known proteins (85). *avrPto* functions as a virulence effector in susceptible tomato, enhancing the multiplication of bacteria. It has also been shown to suppress cell-wall based defense responses in Arabidopsis (45, 93). It was previously reported that transfer of *avrPto* into virulent strain of *P. syringae* pv. glycinea renders the transferred strain avirulent on most tested soybean cultivars including Harosoy (55, 63, 85).

*avrB*, an avirulence gene from *P. syringae* pv. glycinea, confers cultivar-specific resistance to bacterial blight on soybean with its corresponding *R* gene *Rpg1-b* (6, 107). *avrB* is also recognized by the Arabidopsis *R* gene RPM1 (10). Both interaction of AvrB and its corresponding *R* gene products elicit the localized programmed plant cell death and enhanced expression of pathogenesis-related (PR) genes (44). As most of avirulent genes, *avrB* is a dual-functional effector. *avrB* increases bacterium growth eight fold in soybean plants that do not harbor *Rpg1-b* gene (5) or produce a cytotoxic yellowing response in susceptible Arabidopsis plants (73).
1.3 Overview of Literature Related to *Xanthomonas oryzae* and Rice Interaction

**Bacterial blight of rice and *Xanthomonas oryzae* pv. *oryzae**

Bacterial blight (BB) of rice caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo) is one of the most important bacterial diseases of rice. BB originates in Asia and has been reported to spread to worldwide rice culture area such as Austria, Africa and Latin America (74). BB has become a major disease of rice in tropical Asia in the last several decades because of the extensive use of nitrogen-responsive modern cultivars. In tropics, the damage caused by the bacteria is more severe than in the temperate regions because of frequent rainstorms and high humidity. A loss of 50% yield on very susceptible cultivars and 10% on moderately susceptible cultivars was reported in South China. Compare to susceptible cultivars, the yield loss was insignificant in resistant cultivars (76, 78). BB is a vascular disease. Xoo enters the rice through hydathodes, multiplies in the epitheme and then accumulates in the xylem vessels. It may also spread into non-vascular adjoining tissues in the late stage of disease. The typical symptoms of BB caused by the accumulation of Xoo are leaf blight and wilting of seedling due to the block of the xylem vessels (74). A main source of natural infection in fields is infected seed. In BB disease management, several control methods such as physical or chemical treatment of seeds and biological control with antagonistic bacteria have been used. However, the efficiency ranges from 10% to 60% (36, 114). The most efficient control approach for BB is rice breeding and pyramiding one or more *R* genes in new resistant cultivars.

Xoo use several general virulence factors such as T3SS, EPS, and extracellular enzymes to increase the disease symptoms. T3SS is the most important pathogenicity and
virulence factor of Xoo. Xoo use T3SS to deliver effector proteins into the plant cell. To date, all of the identified effector proteins belong to AvrBs3/PthA family. Members of AvrBs3/PthA family effectors share a highly conserved protein structure. They can activate the expression of certain specific genes after they are delivered into the plant cell (74). EPS is another important virulence factor. A mutant of *gumG* of Xoo made by transposon insertion loses the synthesis of EPS and possesses the reduced virulence (31). Xoo also use extracellular enzymes as the virulence factors. Xylanase is the enzyme that degrades xylan, a component of xylem vessels. Xoo strains with a mutant *xynB*, the gene for xylanase secretion, have an abolished ability to accumulate xylanase in planta and attenuated virulence in rice (81).

**Bacterial leaf streak and *Xanthomonas oryzae* pv. oryzicola**

Bacterial leaf streak (BLS) of rice caused by *Xanthomonas oryzae* pv. oryzicola (Xoc) has been found in humid tropical areas like Southern China, India and Africa but not in temperate regions such as Japan, Korea and Northern China (71, 77). BLS causes less severe economical loss compare to BB. As opposed to BB, BLS is a non-vascular disease. Xoc enters rice through the stomata and multiplies in the leaf arechyma tissues of leaves. The symptoms of BLS include small, water-soaked lesions along leaves at the beginning of the infection and grey, transparent streaks along leaves at later stages. Unlike BB, few studies have been conducted on the disease control of BLS. Right now, the major control approach is breeding resistant varieties based on the quantitative trait loci (QTL) (96).
Studies on virulence mechanisms of Xoc just started. It was reported that Xoc has the ability to inhibit the avirulence function of Avr proteins from Xoo (66). This observation suggests that Xoc may have more complicated virulence mechanisms.

**Host resistance to Xoo and Xoc**

In rice, about 29 R genes that confer resistance to Xoo have been identified (74). Based on the gene-for-gene hypothesis, Xoo should contain the corresponding avr genes that interact with rice R genes. From the 29 identified R genes, six of them, Xa1, xa5, xa13, Xa21, Xa26 and Xa27, have been cloned (23, 24, 40, 49, 100, 105, 123). Whereas, in Xoo, only three avr genes have been cloned. avrXa7 and avrXa10, the first cloned Xoo avr genes, were cloned from the Philippine race 2 strain PXO86 and avrXa27 was cloned from Philippine race 6 strain PXO99A (40, 46). These three avr genes encode proteins belonging to AvrBs3/PthA family which are highly conserved in Xanthomonas species. In addition to their avirulence function, they contribute to virulence of Xoo in distinct quantitative ways. PXO86 mutants that carry a disrupted avrXa7 gene have a dramatic decrease in virulence on the susceptible rice cultivar IR24. Unlike avrXa7, avrXa10 is not the major virulence effector in PXO86 (7). The product of recently cloned avrXa27 is able to induce the expression of Xa27, its corresponding R gene in rice, through binding to the specific promoter region directly or indirectly (40).

Members of AvrBs3/PthA family effectors share a highly conserved protein structure. The N-terminus functions as the secretion signal directing the translocation from bacterial cell into plant cell. The 34 amino acid near-perfect repeats in the central part of the effector vary in number and participate in the determination of Avr specificity. The repeat region of
avrXa7 is involved in both the race specificity and avirulence activity (122). The C-terminus contains two motifs: the nuclear localization signal domain (NLSs) and the acidic transcription activator domain (AAD). In most cases, the Avr activity of AvrBs3-like proteins relies on the NLS and AAD domains. Mutant strains carrying a defective NLSs or AAD in the AvrXa7 or AvrXa10 effectors also possess an abolished Avr activity (129, 130). Members of the AvrBs3/PthA effector family can also activate the transcription of some specific host genes and therefore are also called transcription activator-like (TAL) effectors. Two newly characterized TAL effectors pthXo6 and pthXo7 in Xoo induce the expression of the rice transcription factors OsTFX1 and OsTFIIA71, respectively (104). In Xoc genome, there are 28 gene encoding TAL effectors, although to date, none of them has been characterized.

From the six cloned R genes to BB of rice, Xa21 and Xa26 are receptor-like kinases with a serine/threonine kinase domain, a transmembrane domain and an extracellular leucine-rich repeat domain (100, 105). Xa1 belongs to typical cytoplasmic NBS-LRR R gene group (nucleotide binding site and leucine repeat region). The sequence of Xa27 is unique and has no similarity with any known plant proteins (40). xa5 and xa13 are recessive genes and do not belong to any of the traditional R genes. xa5 encodes the γ subunit of general transcription factor IIA (49). xa13 is homolog of MtN13 of Medicago truncatula which is required for normal pollen development (23, 24).

To date, no R gene to BLS has been identified in rice. However, the recently cloned Rxol gene from maize confers non-host resistance to Xoc in maize and in transgenic rice cultivar Kitaake carrying Rxol. avrRxol, the first cloned avr gene from Xoc, triggers a strong HR on transgenic Kitaake rice plant with Rxol when expressed in Xoo (126, 127)
1.4 Research Presented in This Dissertation

In my first project, I used the modified soybean mosaic virus (SMV) as a transient expression vector to express \textit{avrPto} or \textit{avrB} gene individually and investigate the function of these two \textit{avr} genes in soybean. In this project, I concluded that SMV is a very efficient tool for the expression of foreign genes transiently in soybean. This is a significant technical advance in soybean research. I also concluded that early defense responses such as HR induced by the AvrB and Rpg1-b interaction, can effectively block the movement of virus, therefore, also prevent the infection of virus. Finally, I concluded that AvrPto does not have avirulent function in all tested soybean cultivars, which challenges previous reported result (85). These results provided us with new knowledge about the functions of non-host effectors.

In my second project, 10,000 Xoc Tn5 transposon mutants were screened to identify candidate virulence factors. From the screened mutants, 21 mutants showed an apparent reduced virulence. These identified virulence factors include genes involved in pathogenesis by other plant pathogenic bacteria, virulence factors of animal pathogens, and genes not previously associated with virulence. From these 21 virulence-impaired mutants, one carrying a mutation in the \textit{WxocB} gene, which is localized in the \textit{lps} gene cluster, possessed a dramatically reduced virulence. My third project focused on the functional assay of the \textit{wxocB} gene, which demonstrated its involvement in the synthesis of the O-antigen of the LPS molecule. The mutation in this gene affected the structure of LPS as well as the production of EPS. Finally, I demonstrated that the T3SS is defective in this \textit{wxocB} mutant strain, which may be due to the poor attachment efficiency of the hrp pilus, its degradation
or the down regulatory expression of hrp genes. These results assign a new insight role to LPS in bacterium cell protection, and also reveal new relationship between LPS and the T3SS.

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CHAPTER 2.

_Pseudomonas syringae_ effector _avrB_ confers soybean cultivar-specific avirulence on _Soybean mosaic virus_ adapted for transgene expression but effector _avrPto_ does not

A paper published in *Molecular Plant-Microbe Interactions*\(^1\)

Li Wang\(^2,3\), Alan Eggenberger\(^3\), John Hill\(^3\), and Adam J. Bogdanove\(^3,4\)

ABSTRACT

_Soybean mosaic virus_ (SMV) was adapted for transgene expression in soybean and used to examine the function of avirulence genes _avrB_ and _avrPto_ of *Pseudomonas syringae* pathovars glycinea and _tomato_, respectively. A cloning site was introduced between the _PI_ and _HC-Pro_ genes in 35S-driven infectious cDNAs of strains SMV-N and SMV-G7. Insertion of the _uidA_ gene or the green fluorescent protein gene into either modified cDNA and bombardment into primary leaves resulted in systemic expression that reflected the pattern of viral movement into uninoculated leaves. Insertion of _avrB_ blocked symptom development and detectable viral movement in cultivar Harosoy, which carries the _Rpg1-b_ resistance gene corresponding to _avrB_, but not in cultivars Keburi or Hurrelbrink, which lack _Rpg1-b_. In Keburi and Hurrelbrink, symptoms caused by SMV carrying _avrB_ appeared more quickly and were more severe than those caused by the virus without _avrB_. Insertion of _avrPto_ enhanced symptoms in Harosoy, Hurrelbrink, and Keburi. This result was unexpected because _avrPto_ was reported to confer avirulence on _P. syringae_ pv. glycinea inoculated to

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\(^2\) Generated 50% of the data presented, prepared 70% figures and tables and wrote 50% of the draft.

\(^3\) Department of Plant Pathology, Iowa State University, Ames, IA, 50011

\(^4\) Author for Correspondence
Harosoy. We inoculated Harosoy with *P. syringae* pv. glycinea expressing *avrPto*, but observed no hypersensitive reaction, *avrPto*-dependent induction of pathogenesis-related protein 1a, or limitation of bacterial population growth. In Hurrelbrink, *avrPto* enhanced bacterial multiplication and exacerbated symptoms. Our results establish SMV as an expression vector for soybean. They demonstrate that resistance triggered by *avrB* is effective against SMV, and that *avrB* and *avrPto* have general virulence effects in soybean. The results also led to a reevaluation of the reported avirulence activity of *avrPto* in this plant.

**INTRODUCTION**

To establish conditions favorable for colonization of plants, gram-negative phytopathogenic bacteria depend on the type III secretion system, encoded by *hrp* genes, to deliver suites of effector proteins into host cells. Collectively, effectors are required for pathogenesis, but their individual functions are only beginning to be understood. Many effectors were first identified as avirulence proteins by virtue of their ability to trigger plant defense in host varieties expressing corresponding resistance proteins (59). Except for a handful of these, virulence functions have been difficult to discern genetically due to their quantitative and probably redundant nature (10).

Transient expression of bacterial effector genes in plants has been a fruitful approach to understanding their functions (e.g., 1, 23, 50). In fact, transient expression experiments first demonstrated that effectors function within plant cells, establishing the role of type III secretion in plant pathogenesis (reviewed in 8). Pathogens of different plants rely on distinct inventories of effectors (31). Dissecting the roles that different effectors play in diseases of different plants will advance our understanding of plant molecular processes and potentially
reveal targets for new approaches to disease control. However, transient expression techniques are limited for many crop plant species.

Methods for transient expression in plants include transformation mediated by Agrobacterium infiltrated into plant tissues (25), introduction of DNA into plant cells by particle bombardment (45), introduction of expression constructs into plant protoplasts by electroporation (18), and inoculation of plants with recombinant viruses or infectious derivatives carrying transgenes (47). Transient expression takes less time and is generally less costly than stable transformation. Also in contrast to stable transformation, which relies on regeneration of whole plants from individual, transformed cells, transient expression can accommodate gene expression that results in developmental defects or cell death. Compared to other approaches, virus-mediated methods uniquely allow for systemic expression in whole plants, and often achieve expression to higher levels and of greater duration (39).

Soybean is a major crop species for which transient expression methods are limited. Agrobacterium-mediated transient expression has not proven to be robust. Particle bombardment has been used for transient expression in soybean (38, 58), but it is costly and results in expression only in a small number of cells. Clover yellow vein virus (ClyVV) was developed as an expression vector for clover and reported to function in soybean (36), but has not seen widespread use.

We adapted Soybean mosaic virus (SMV) for transgene expression in soybean and used it to examine the function of avirulence genes avrB and avrPto of Pseudomonas syringae pv. glycinea and tomato, respectively. A potyvirus, SMV consists of a single positive-sense RNA in a filamentous virion. The RNA is translated to yield a polyprotein that is self-processed by the proteinase components P1, HC-Pro, and Nla to yield eight mature
proteins, two of which undergo partial further processing (55). Potyviruses are transmitted by aphids, but aphid transmissibility is readily lost upon serial mechanical passage (e.g., 43). 

*avrB* governs race-cultivar-specific resistance to bacterial blight of soybean in conjunction with its corresponding resistance (*R*) gene *Rpg-1b* (3, 53). *avrB* is also recognized by the *Arabidopsis* gene *RPM1* (5). Typical of most gene-for-gene interactions, the interaction of *avrB* and *Rpg1b* (or *avrB* and *RPM1*) elicits the defense-associated localized plant cell death known as the hypersensitive reaction (HR) and enhanced expression of PR (pathogenesis-related) proteins (21). In soybean cultivars lacking *Rpg1*-b, *avrB* contributes to pathogen virulence (2). *avrPto* triggers resistance to bacterial speck of tomato mediated by the corresponding *Pto* gene (42). As a transgene in potato virus X (PVX), *avrPto* prevented systemic infection by the virus in tomato plants carrying *Pto* (54). AvrPto was reported to elicit resistance in several soybean cultivars when expressed in *P. syringae* pv. glycinea (32, 42). In addition to its avirulence function, AvrPto was observed to enhance the virulence of *P. syringae* pv. tomato in tomato plants lacking the *Pto* gene (48), and more recently shown to suppress cell wall-based defense responses in *Arabidopsis* (22).

**RESULTS**

**Biologically active cDNA clones of SMV-N and SMV-G7**

SMV strain SMV-N causes mosaic, rugosity, and stunting on susceptible cultivars, whereas SMV-G7 causes mild mosaic or no symptoms (12). SMV-N therefore was
considered a good candidate for use as a vector to examine the effects of transgenes on the
viral-host interaction, and SMV-G7 potentially useful as a general expression vector.

Plasmids containing 35S promoter-driven cDNAs of non-aphid-transmissible isolates of
SMV strains SMV-N and SMV-G7 (Table 1) were constructed and designated as pSMV-N
and pSMV-G7 (see Fig. 1 and Materials and Methods). Introduction of the plasmids by
particle bombardment into primary leaves of intact soybean plants of cv. Williams 82
resulted in systemic viral infection within two weeks, equivalent to that following inoculation
with virions.

SMV-mediated systemic expression of β-glucuronidase and green fluorescent protein in
soybean

pSMV-N and pSMV-G7 were modified to permit insertion of a transgene between the
P1 and HC-Pro coding regions and to allow subsequent processing of the product from the
SMV polypeptide (see Materials and Methods). The resulting constructs, pSMV-Nv and
pSMV-G7v, were confirmed to be infectious. Insertion of the \textit{uidA} gene into pSMV-G7v
(Fig. 2a) or pSMV-Nv (not shown) and introduction of the resulting plasmids into primary
leaves of Williams 82 plants by particle bombardment resulted in systemic expression of β-
glucuronidase (GUS) activity (Fig. 2) that reflected the general pattern of viral replication
and movement (9). Expression of GUS was evident along the veins in young expanding
leaves, spreading into the interveinal tissue in newly expanded leaves, and finally, in fully
expanded leaves, confluent throughout interveinal tissue and reduced along the veins.
Insertion of the \textit{GFP} gene resulted in similar, systemic expression of green fluorescent
protein. Fluorescence in systemic leaves was greater for pSMV-Nv::\textit{GFP} than pSMV-
G7v::GFP (Fig. 2b), consistent with the greater replication of SMV-N in Williams 82 relative to SMV-G7 (A.E. and J.H., unpublished observation). As determined by immunocapture RT-PCR (see Materials and Methods), the GFP gene, 0.7 kb, was stable in both pSMV-Nv (Fig. 2c) and pSMV-G7v (not shown), with no detectable deletion of the gene in the eighth trifoliate approximately six weeks following bombardment. Subpopulations of virus having undergone spontaneous deletion of the larger, 1.8 kb uidA gene were apparent in second and later trifoliate leaves of plants bombarded with either pSMV-Nv::uidA (Fig. 2c) or pSMV-G7v::uidA (not shown), but GUS activity was substantially maintained through the eighth trifoliate leaf (Fig. 2).

**Functions in soybean of avrB and avrPto in pSMV-Nv**

avrB and avrPto were amplified by PCR and cloned into pSMV-Nv using primers designed such that processing would preserve the native N-termini (see Fig. 1 and Materials and Methods), because activities of AvrB and AvrPto depend on an N-terminal myristylation motif (37, 49). Introduction of pSMV-Nv::avrB into primary leaves of soybean cv. Hurrelbrink (Fig. 3) or cv. Keburi (not shown), which are susceptible to *P. syringae* pv. glycinea strains containing avrB (Fig. 4 and data not shown), resulted in systemic symptoms. In contrast to the mild mosaic that became apparent approximately two weeks following introduction of pSMV-Nv (not shown) or pSMV-Nv::GFP (Fig. 3), symptoms following introduction of pSMV-Nv::avrB first became apparent at ten days and developed into relatively severe veinal chlorosis and interveinal necrosis (Fig. 3). The presence of virus containing avrB in upper, non-inoculated leaves was confirmed by immunocapture RT-PCR (Fig. 3). Introduction of pSMV-Nv::avrB into cv. Harosoy, which carries Rpg1-b, did not
result in systemic symptoms of any kind, and virus was undetectable in upper, non-
inoculated leaves. This result contrasts with pSMV-Nv::GFP in cv. Harosoy, which caused
mild systemic mosaic accompanied by detectable amounts of virus in upper leaves. \textit{avrPto} in
pSMV-Nv, pSMV-Nv::\textit{avrPto}, enhanced virus-associated symptoms in cvs. Hurrelbrink (Fig.
3) and Keburi (not shown) which were reported to be susceptible to \textit{P. syringae} pv. glycinea
expressing this gene, but also in cv. Harosoy (Fig. 3), which was reported to be resistant (32).
Black necrosis of veins and adjacent tissue, and rugosity were apparent in cv. Hurrelbrink,
and brown and spotty necrosis developed along veins and within interveinal tissues in cv.
Harosoy (Fig. 3). Immunocapture RT-PCR indicated presence of the virus containing \textit{avrPto}
in upper, non-inoculated leaves of both Hurrelbrink and Harosoy (Fig. 3). Thus, \textit{avrB} but not
\textit{avrPto} confers corresponding soybean cultivar-specific avirulence on SMV, and both \textit{avrB}
and \textit{avrPto} in pSMV-Nv enhance virus-associated symptoms in susceptible plants, albeit
differently.

\textbf{Lack of observed avirulence activity in soybean of \textit{avrPto} expressed in \textit{Pseudomonas}
syringae pv. glycinea}

Given the \textit{avrB} result and the ability of \textit{avrPto} to block PVX infection in tomato (54),
failure of \textit{avrPto} to prevent SMV-associated symptom development and viral movement in
cv. Harosoy was puzzling in light of the reported resistance of Harosoy to \textit{P. syringae} pv.
glycinea containing cloned \textit{avrPto} (32). The effects of \textit{avrPto} on \textit{P. syringae} pv. glycinea
interaction with soybean were therefore reexamined. Plasmids pPtE2, a 2.6 kb clone
containing \textit{avrPto} downstream of the \textit{lacZ} promoter in the vector pRK404 (Table1), and
pPtE2\textTheta, containing an omega fragment in place of \textit{avrPto} (Table1), were introduced into the
*P. syringae* pv. glycinea race 4 strain PsgR4 (Table 1). PsgR4 is virulent on most soybean cultivars, including Harosoy. Neither of the resulting strains, PsgR4(pPtE2) and PsgR4(pPtE2Ω), elicited the HR in cultivars Acme, Centennial, Faribault, Harosoy, or Norchief, at any of several bacterial titers ranging as high as approximately 5 x 10⁸ cfu/ml (O.D₆₀₀ = 1.0) (data not shown). Population growth over six days of PsgR4(pPtE2) and PsgR4(pPtE2Ω) inoculated at a lower titer to Harosoy plants, as determined with selection for the plasmids, was highly variable within and across four replicate experiments. In two of these, the mean population growth of PsgR4(pPtE2) was 10- to 20-fold less than that of PsgR4(pPtE2Ω), but in the other two roughly the opposite was true, and in no experiment were these differences statistically significant. Plating without selection for the plasmids greatly reduced variability and resulted in higher values for the populations that were not significantly different from one another (see Supplementary Fig. S1). These patterns suggested that the results were influenced by plasmid instability.

The effects of *avrPto* were therefore reexamined using different plasmids: pPtE6, a 0.6 kb subclone of the pPtE2 insert containing the *avrPto* open reading frame, oriented with the lacZ promoter in the vector pDSK519 (Table 1), and pDSK519 without any insert. pPtE6 failed to confer HR-eliciting activity on PsgR4 in Harosoy (Fig. 3), or Acme, Centennial, Faribault, or Norchief (see Supplementary Figure S2). In cultivar Harosoy inoculated with a low titer of bacterial cells, population growth was not statistically different for the transformant with pPtE6, PsgR4(pPtE6), and the transformant with pDSK519, PsgR4(pDSK519) (Fig. 4). Comparison of populations measured with and without selection for these plasmids revealed no indication of plasmid loss *in planta* over six days (see Supplementary Figure S1). On Harosoy leaves inoculated with PsgR4(pPtE6), bacterial
blight symptoms developed that were equivalent to those caused by PsgR4(pDSK519) (Fig. 4) or the parental strain (not shown).

Northern blot analysis of Harosoy leaves sampled over a time course following inoculation with PsgR4(pAVRB), PsgR4(pPtE6), or PsgR4(pDSK519) revealed no effect of avrPto on the pattern of expression of the PR1a gene, in contrast to avrB, which triggered rapid, enhanced accumulation of PR1a transcript (Fig. 5).

Western blotting of proteins in PsgR4(pPtE2) and PsgR4(pPtE6) and immunodetection of AvrPto confirmed that AvrPto was expressed in both strains (see Supplementary Figure S3). Plasmid rescue and testing in P. syringae pv. tomato inoculated to tomato plants with or without Pto confirmed that avrPto in both plasmids was functional (not shown). PsgR4 transformed with the pDSK519 derivative pAVRB containing the avrB gene, PsgR4(pAVRB), elicited a robust HR in cultivars Harosoy (Fig. 4), Faribault, and Norchief (see Supplementary Figure S2). In Harosoy, PsgR4(pAVRB) multiplied approximately 10^4-fold less than PsgR4(pDSK519) over four days, and caused no bacterial blight symptoms (Fig. 4).

In summary, no evidence of avirulence function of avrPto in soybean was observed.

**Virulence activity in soybean of avrPto expressed in Pseudomonas syringae pv. glycinea**

PsgR4(pAVRB), PsgR4(pPtE6), and PsgR4(pDSK519) were inoculated to cv. Hurrelbrink and populations monitored over four days (Fig. 4). PsgR4(pDSK519) grew to approximately 10-fold lower levels after four days in this cultivar relative to Harosoy. A small but significant difference in bacterial population growth was observed between PsgR4(pAVRB) and Psg(pDSK519), as demonstrated previously in other susceptible
cultivars (2): the population levels for PsgR4(pAVRB) in Hurrelbrink were higher and similar similar to those of PsgR4(pDSK519) in Harosoy. AvrPto caused a small but significant (t=3.3845, p=0.0277 on day four) increase in bacterial population growth as well. Also, by two weeks after inoculation, plants inoculated with PsgR4(pAVRB) or PsgR4(pPtE6) had developed more extensive necrosis and more severe chlorosis than plants inoculated with PsgR4(pDSK519) (representative leaflets are shown in Fig. 4). Thus, like avrB, avrPto can contribute to virulence in bacterial blight of soybean.

DISCUSSION

In this study, SMV was adapted for transgene expression in soybean and used to examine the function of avirulence genes avrB and avrPto of Pseudomonas syringae pv. glycinea and tomato, respectively. The results establish SMV as an efficient expression vector for soybean. They demonstrate that avrB triggers R gene mediated resistance that is effective against SMV, and that avrB and avrPto have general virulence effects in soybean. Finally, the results led to a reassessment of previously reported avirulence activity of avrPto in soybean.

Expression of uidA and GFP via SMV reflected the pattern of viral replication and movement in plants, resulting in confluent, homogenous activity in fully expanded trifoliate leaves following transfection by bombardment of primary leaves. In addition to enabling rapid, systemic expression of transgenes, SMV has other important advantages. The availability of non-aphid transmissible strains, such as those used here, mitigates the risk of unintended transmission, as does the fact that SMV is not readily transmitted accidentally by standard practices used in a greenhouse due to its relative instability outside of a host (Plant
Viruses Online: Descriptions and Lists from the VIDE Database, http://image.fs.uidaho.edu/vide/). At the same time, simple methods for (intentional) mechanical transmission (as in 20) would permit use of SMV for transgene expression in large-scale experiments, using sap from a plant transfected by particle bombardment to inoculate others. Strains such as SMV-G7 which cause mild or no symptoms can be useful as general expression vectors, while more virulent strains, such as SMV-N, can be useful for assaying the effect of transgenes on the viral-host interaction. The modifications necessary to adapt SMV as a vector were straightforward and could be used with other strains as needed for studies in different soybean cultivars.

Another advantage of SMV is that since it is a filamentous virus, there is no strict limit on the size of an inserted gene. Nevertheless, as observed of uidA in this study, some transgenes may suffer spontaneous deletion. Spontaneous deletions can be a function not only of insert size but of structural characteristics such as GC content (30). Therefore, stability will be difficult to predict for specific transgenes, but may be manipulable (30).

Gene silencing would not be expected to occur to a significant extent with SMV because it encodes HC-Pro, a strong suppressor of silencing (57).

Finally, incorporation of a NIa proteinase site at the 5’ end of a transgene in SMV permits expression of the product with an unmodified or minimally modified N-terminus. The heptapeptide sequence recognized by NIa is (E or N)xxVxxQ/(A, G, or S), with the cleavage site denoted by the forward slash mark (17, 40). If the N-terminal amino acid of the protein of interest is A, G, or S, which was the case for AvrPto and AvrPtoB, the first six codons for the site can be added and the protein will be released without additional amino
acids at its N-terminus. If not, the complete N1a site can be added, and the protein will have an additional (A, G, or S) residue at its N–terminus.

Insertion of avrB into the adapted SMV genome blocked symptom development and detectable viral movement in cultivar Harosoy, which carries Rpg1-b. The efficacy of avrB and Rpg1-b mediated resistance against SMV, like Pto-dependent resistance of tomato to PVX containing avrPto (54), bolsters the notion that despite specific triggers, R gene mediated responses are general, and effective against a diversity of pathogens. This notion derives also from observations that some R genes have multiple specificities, overexpression of R genes or components of R gene mediated defense signaling confers broad-spectrum disease resistance, and R genes to viral, bacterial, and fungal pathogens share downstream signaling components (for a review, see 34). Even R genes that rely on distinct signaling pathways generate largely identical output (16), though timing and intensity of responses may differ for different R genes (16, 41).

The mechanism whereby SMV-associated symptoms and movement were blocked by avrB and Rpg1-b interaction is not clear. Very rarely, mild mosaic developed on emerging trifoliate leaves three to four weeks following bombardment of primary leaves. By immunocapture RT-PCR, virus was usually undetectable, but occasionally could be detected at a size consistent with it having undergone partial deletion of avrB (data not shown). It is tempting to speculate based on the infrequency of these escapes that resistance is mediated at the level of replication, perhaps by rapid cell death triggered by AvrB. Analysis of the effect of avrB and Rpg1-b mediated resistance compared to responses mediated by other R genes that differ in timing or intensity will shed light on the viral processes that are disrupted.
In susceptible cultivars transfected with SMV containing \textit{avrB}, symptoms appeared more quickly and were more severe than those caused by the virus without \textit{avrB}. \textit{avrB} contributes to multiplication of \textit{P. syringae} pv. glycinea in susceptible soybean plants (2 and this study) and enhances bacterial blight symptoms (this study). Enhanced bacterial blight symptoms could be a consequence of greater bacterial multiplication, but a cytotoxic effect of AvrB has been noted in \textit{Arabidopsis} (37). AvrB also induces phosphorylation of RIN4, a negative regulator of basal defense in \textit{Arabidopsis} (33). RIN4 is not required for the cytotoxicity of AvrB, so AvrB likely targets other plant proteins as well, but targeting, possibly stabilization, of RIN4 by AvrB suggests a role for the effector in foiling plant defense (4). Either a cytotoxic effect of AvrB (resulting directly in necrosis), or a defense-suppressive effect (leading to greater viral activity), or both, might explain the enhancement of SMV-associated symptoms. The more rapid spread of symptoms caused by SMV carrying \textit{avrB} relative to SMV carrying \textit{GFP} suggests that \textit{avrB}-dependent symptoms were not a consequence of AvrB triggering delayed or incompletely effective defense response, \textit{i.e.}, lethal systemic HR (19), rather that \textit{avrB} enhanced spread of the virus itself. AvrB enhancement of bacterial virulence in susceptible plants also weighs in favor of this interpretation. An indirect effect due to structural alteration of the virus by insertion of \textit{avrB} cannot formally be ruled out, however, insertion of \textit{uidA} or \textit{GFP} had no such effect.

Insertion of \textit{avrPto} into SMV caused necrosis in cultivars Harosoy, Hurrelbrink, and Keburi. As for \textit{avrB}, this effect of \textit{avrPto} may be due to necrogenic activity of the effector, or a dampening of defense that permitted greater viral activity. In previous studies, \textit{avrPto} rendered PVX necrogenic (54) and increased the number of lesions caused by \textit{P. syringae} pv. tomato (11, 48) in tomato plants lacking \textit{Pto} or the \textit{Prf} gene required for \textit{Pto}-mediated
resistance. Similarly however, whether these effects occurred because AvrPto is necrogenic, or because it prevents or counteracts plant defense could not be distinguished. Suppression of cell wall-based basal defenses by AvrPto in Arabidopsis (22) and the lack of necrogenesis by avrPto transiently expressed in prf plants (11) support the latter conclusion.

Like avrB, in P. syringae pv. glycinea inoculated to cultivar Hurrelbrink, avrPto also enhanced bacterial multiplication and exacerbated symptoms. Taken together with the enhancement of virus-associated symptoms, the results suggest that both avrB and avrPto play general roles in virulence in soybean (notably, however, avrPto has not been detected in any of several races of P. syringae pv. glycinea, 42). This notion, combined with the virulence and virulence-related effects of these proteins in Arabidopsis and tomato, further suggests that the respective plant targets are broadly conserved across diverse plant taxa.

Enhancement rather than preclusion of SMV-associated symptoms by avrPto in cultivar Harosoy prompted a reexamination of the avirulence function of avrPto in soybean that was reported by Ronald et al. (42) and Lorang et al. (32). In their paper demonstrating that the cloned gene triggers HR on tomato, Ronald et al. reported (but did not show) that avrPto (in pPtE2) transferred to PsgR4 elicited HR also in soybean cultivar Centennial, and in one experiment presented, caused a reduction in bacterial multiplication of approximately 100-fold relative to PsgR4(pPtE2Ω) in this cultivar over four days. Lorang et al., examining the possibility that the four then known avr genes of P. syringae pv. tomato accounted for non-host resistance in soybean, tested whether these genes individually would trigger HR in several soybean cultivars when expressed in PsgR4 (using pPtE2 as the avrPto clone), and reported their results in a table, indicating that avrPto elicited HR in Harosoy and several other cultivars, but not in Hurrelbrink or Keburi. We obtained no evidence of avirulence
function for *avrPto* in soybean using strain PsgR4 and either of two *avrPto*-containing plasmids, including pPtE2: no *avrPto*-mediated HR occurred in Harosoy, Centennial, or any of several other cultivars, and, tested in Harosoy, no induction of *PR1a*, or limitation of bacterial population growth due to *avrPto* was observed. Expression of AvrPto in the PsgR4 transformants used in each of these experiments, and integrity of the gene following transformation, were confirmed by Western blot analysis (Supplemental Fig. S3) and testing of re-isolated plasmid in *P. syringae* pv. *tomato* inoculated to tomato, respectively (not shown).

We found that instability of pPtE2 can result in stochastic, apparent limitation of bacterial populations when measured with selection for the plasmid. This fact may have contributed to the discrepancy between our and the prior results, but can not explain it completely. The SMV result however, agrees with a lack of avirulence activity of *avrPto* in *P. syringae* pv. glycinea.

Though there was no limitation of bacterial growth in Harosoy due to *avrPto*, there was also no augmentation, as there was in Hurrelbrink. The population of PsgR4 without *avrPto* (carrying pDSK519) was approximately ten fold higher in Harosoy than in Hurrelbrink four days after inoculation (Fig. 4), suggesting lower basal defense in Harosoy. If AvrPto contributes to virulence by suppressing defense, already low basal defense in Harosoy might explain the lack of an observable effect of *avrPto* in this cultivar.

Apparent failure of soybean to mount resistance in response to *avrPto* supports interesting speculation regarding the potential functional conservation of *Pto* outside of the Solanaceae. *Pto* encodes a serine-threonine protein kinase, and *Pto* homologs are present in many plant species, including soybean (35). Soybean expresses at least two homologs of Pti1
(52). Pti1 is a Pto-like Pto substrate thought to be involved in signaling downstream of AvrPto recognition (60). Both Pti1 homologs are phosphorylated by Pto, but, in contrast to Pti1, neither were active in vitro (52). Thus, soybean may contain a functionally conserved homolog of Pto that is not effective due to the lack of an active Pti1 homolog. SMV offers an efficient means to test whether expression of Pti1 in soybean might confer (or restore) the ability to resist *P. syringae* pv. glycinea expressing *avrPto*.

Adaptation of SMV as an expression vector for soybean provides a powerful new tool for understanding gene function in this important crop plant. As shown here, it also allows tagging of the virus to better understand its biology in planta, and offers a fruitful approach to determining the roles of effectors of other plant pathogens. SMV will be an effective means to continue exploring the functions of AvrB and AvrPto. Expressing *avrB* and *avrPto* in soybean in isolation from other bacterial proteins, combined with mutagenesis and genetic analyses of both the effectors and the plant, promises further elucidation of effector activity and identification of targets that will lead to a better understanding of soybean resistance and susceptibility to disease.

**MATERIALS AND METHODS**

**Viral and bacterial strains and plasmids used, growth conditions, and transformation**

Viral and bacterial strains and plasmids used are presented in Table 1. *E. coli* was cultured in Luria-Bertani (LB) medium at 37° C, or, for strains containing viral cDNA, in LB supplemented with 20mM glucose at 30° C. *Pseudomonas syringae* was grown in King’s B medium at 28° C. Ampicillin (100 μg/ml), kanamycin (50 μg/ml), rifampicin (750 μg/ml), spectinomycin (25 μg/ml), and tetracycline (10 μg/ml), were added to growth media as
appropriate for selection. Plasmids were introduced into *E. coli* by electroporation and into *Pseudomonas syringae* by electroporation or triparental mating.

**Construction of pSMV-N, pSMV-G7, and derivatives**

For expression of SMV cDNA *in planta* following particle bombardment (see below), an *Eco*RI-*Sph*I fragment containing the 35S promoter and NOS terminator from pAGUS1 (Table 1) was inserted between these sites in pBR322 (Table 1), and oligonucleotide-directed mutagenesis was used to expand the multiple cloning site within this fragment to include *Stu*I, *Bam*HI, *Dra*I, and *Cla*I sites. The resulting plasmid was designated as pBR322-35S. The *Stu*I site in pBR322-35S is proximal to the 35S promoter.

SMV-N and SMV-G7 were purified from infected soybean leaves as described previously (56), and viral RNA isolated using an RNeasy RNA purification kit (Qiagen, Valencia, CA). cDNA to each strain was generated using AMV reverse transcriptase (Seikagaku, Falmouth, MA) or Superscript II reverse transcriptase (GIBCO-BRL, Gaithersburg, MD) according to the manufacturer's instructions, followed by PCR using virus-specific primers (see Supplemental Material for further details). PCR was carried out using an LA PCR kit (Panvera, Madison, WI). Complete nucleotide sequences are available for SMV-G7 (Genbank accession no. AY216010, 19) and SMV-N (GenBank accession no. NC_002634, our unpublished results). cDNAs were cloned into pBR322-35S such that transcription from the 35S promoter would be initiated at the first nucleotide. The resultant plasmids were designated as pSMV-N and pSMV-G7. Next, to allow for transgene insertion and subsequent release of the transgene product from the SMV polypeptide, an *Avr*II site followed by sequence encoding an NiA proteinase cleavage site were added immediately
downstream of the P1 proteinase cleavage site following the \textit{P1} cistron in these plasmids, yielding pSMV-Nv and pSMV-G7v.

\textit{uidA} and \textit{GFP} were amplified from pRAJ275 and psmGFP (Table 1), respectively, using primers designed to introduce an \textit{AvrII} site at each end, and the resulting products were each inserted into the \textit{AvrII} site in pSMV-Nv and pSMV-G7v. \textit{avrB} and \textit{avrPto} were amplified from pAVRB and pPtE6 (Table 1) and inserted in pSMV-Nv similarly except that an NIa proteinase recognition site (17) was added that includes and directs cleavage immediately preceding the glycine at position 2 in the AvrB and AvrPto proteins (see Fig. 1). Cloning details, including the sequences of all primers used, are included in Supplementary Material.

**Introduction of SMV constructs into plants**

SMV constructs were introduced into the primary leaves of 10-12 day old seedlings by particle bombardment as described (46) using a Biolistic PDS-1000/He system (Bio-Rad Laboratories, Hercules, CA), 1.0 or 1.6 \( \mu \)m gold particles, and 1100 psi rupture disks at a distance of 6 cm. Following bombardment, plants were maintained in a greenhouse.

**Detection of SMV and visualization of GUS activity and green fluorescent protein**

Accumulation of SMV in bombarded and systemic leaves was confirmed using ELISA (14). GUS activity was visualized as described previously (26). GFP was visualized by illumination with a high-intensity longwave UV lamp (Ultraviolet Products, Upland, CA) and photography through a Kodak \#58 Wratten Gel Filter.
Immunocapture RT-PCR

Immunocapture RT-PCR was performed using anti-SMV monoclonal antibody S4 as described previously (20) except that reactions were carried out directly in standard PCR tubes. The primers 5’-GAG CGA TCA TCT TTA ACC AC-3’ and 5’-GTG CAT TCA TGA TTC TCC AC-3’, which correspond to sequences flanking the transgene cloning site in pSMV-Nv and pSMV-G7vS, were used for amplification.

Bacterial HR and virulence assays

Plants were grown in a growth chamber under a cycle of 16 h light at 25°C and 8 h dark at 22°C, HR assay was performed as described previously (6), with bacterial suspensions in 10 mM MgCl₂ at approximately 1 x 10⁸ cfu/ml (O.D._₆₀₀ = 0.2) unless noted otherwise. For virulence assays, three-week-old seedlings were vacuum infiltrated with bacterial suspensions in 10 mM MgCl₂ plus 0.04% Silwet L-77 at approximately 1 x 10⁵ cfu/ml, and following inoculation, plants were returned to the growth chamber with humidity added to 80%. Bacterial populations were measured by dilution plating of homogenated leaf disks excised using a 1 cm cork borer. For each time point per inoculum, the first trifoliate leaves of three inoculated plants were sampled individually and the mean and standard deviation calculated. Experiments were repeated five times.

Western Blot Assay

Bacteria were grown to O.D._₆₀₀ = 0.5, and cells in 1 ml were resuspended in 50 µl 1 X Laemmli buffer and heated to 100°C for 3 min to obtain total cellular proteins. Polyacrylamide (15%) gel electrophoresis and western blotting of samples were performed
using standard protocols. Rabbit anti-avrPto antiserum diluted 1:20,000 (kindly provided by G. Martin, Boyce Thompson Institute) followed by goat anti-rabbit 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 AvrPto.

**Northern blot assay**

Ten day old seedlings of cv. Harosoy were inoculated as described for the virulence assay, above, with bacterial suspensions of approximately $5 \times 10^7$ cfu/ml. RNA was extracted from primary leaves using a modified hot trizol protocol (24) and subjected to standard northern blot analysis using approximately 20 µg total RNA per lane and NYTRAN Supercharge nylon membranes (Schleicher & Schuell Bioscience Inc. NH) with Rapid-Hyb buffer (Amersham, Piscataway, NJ) according to manufacturer instructions. The PR1-a probe was generated by RT-PCR amplification and cloning of a 200 bp fragment of the gene (GenBank accession AF136636) from RNA of cv. Williams using the primers 5’-CGTGAATGCACACAATGCAG-3’ and 5’-GCCACTTAGTTCACCAGTGC-3’. The probe was labeled using the Prime-A-Gene labeling system (Promega, Madison, WI). A probe for actin message (kindly provided by T. Baum, Iowa State University) was labeled in the same way and used as a control. Signal was detected using a Molecular Dynamics (Sunnyvale, CA) phosphorimaging system.
ACKNOWLEDGEMENTS

The authors thank S. Whitham for critical reading of the manuscript and Jaime Holdridge for assistance with photography. This work was supported by a grant from the Iowa State University Plant Sciences Institute to A.J.B. and by a grant from the Iowa Soybean Promotion Board to J.H.

LITERATURE CITED


**e-Xtra ITEMS**

**Figure S1**, Variability in measured population growth of *P. syringae* pv. glycinea strain PsgR4 containing pPtE2 or pPtE2Ω as opposed to pPtE6 or pDSK519 and evidence of instability for pPtE2 and pPtE2Ω in leaves of soybean cv. Harosoy; **Figure S2**, *P. syringae* pv. glycinea harboring *avrPto* fails to elicit HR in soybean cultivars Acme, Centennial, Faribault, or Norchief; **Figure S3**, pPtE2 and pPtE6 direct equivalent expression of AvrPto in *P. syringae* pv. glycinea; **Materials and methods supplement**: Details of construction of pSMV-N, pSMV-G7, and derivatives.
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<sup>a</sup> Ap<sup>f</sup>, Ampicillin resistance; Km, kanamycin; Rf, rifampicin; Tc, tetracycline; Sp, spectinomycin, Sm, streptomycin
FIGURE LEGENDS

**Figure 1.** SMV expression constructs used in this study. (a) Schematic representation of the modified SMV genome. Open boxes represent the indicated cistrons. The shaded box represents sequence modified for transgene insertion. Arrows indicate P1 and NIa proteinase cleavage sites within this sequence. (b) Nucleotide and deduced amino acid sequences of the transgene cloning site. The AvrII site is underlined, and the proteinase cleavage sites are represented by a forward slash mark. (c) Deduced terminal amino acid sequences following release from the polyprotein by the P1 and NIa proteinases for the products of each of the transgenes cloned into the AvrII site in this study. Terminal sequences are separated by ellipses, with the N-terminal sequence on the left. Non-native residues are indicated by italics. The NIa proteinase site added to AvrB and AvrPto is shown in bold. Cleavage at this site results in N-termini for both proteins that correspond to their native sequence beginning at a glycine residue at position two.

**Figure 2.** SMV-mediated expression of *uidA* and *GFP* in soybean cv. Williams 82. (a) Leaflets of the indicated trifoliate leaves, ranging from expanding to fully mature (left to right), stained for GUS activity and cleared six weeks following bombardment of primary leaves with pSMV-G7v::*uidA*. (b) Leaflets of the eighth trifoliate leaf, photographed under UV light six weeks following bombardment of primary leaves with pSMV-G7v::*GFP* or pSMV-G7v (left panel) and pSMV-Nv::*GFP* or pSMV-Nv. (c) Products of immunocapture RT-PCR amplification of the transgene site for leaflets of the second, fifth, and eight trifoliate leaves of plants six weeks following bombardment of primary leaves with pSMV-Nv::*GFP* (lanes 1, 2, and 3) or pSMV-Nv::*uidA* (lanes 4, 5, and 6). The relative amounts of
PCR products do not necessarily reflect the relative amounts of viral RNA, as smaller templates may be preferentially amplified.

**Figure 3.** Effects of *avrB* and *avrPto* as transgenes in pSMV-Nv. Shown are (a) the appearance of leaflets and (b) the products of immunocapture RT-PCR amplification of the transgene site for the third trifoliate leaf of Harosoy (Ha) or Hurrelbrink (Hu) plants two weeks following bombardment of primary leaves with pSMV-Nv carrying the indicated transgenes. The leftmost lane in (b) contains the pooled products of PCR amplification using the indicated plasmids as templates individually.

**Figure 4.** Avirulence and virulence activity of *avrB* and *avrPto* in *Pseudomonas syringae* pv. glycinea in soybean. (a) HR assay of strain PsgR4 transformants with the indicated plasmids in primary leaves of Harosoy and Hurrelbrink plants. The minus sign indicates no plasmid. Bacterial suspensions of approximately $10^8$ cfu/ml were introduced into leaves by using a needleless syringe, and leaves photographed after 48 h. (b) Bacterial population growth in Harosoy and Hurrelbrink leaves over 4 days post inoculation (DPI) of whole plants by vacuum infiltration with PsgR4 transformants carrying the indicated plasmids, at a concentration of approximately $10^5$ cfu/ml. Values are means of three samples per time point. Vertical bars represent standard deviation. (c) Bacterial blight symptoms on leaflets of Harosoy and Hurrelbrink plants 2 weeks following inoculation as in (b). Results were essentially identical in five replicate experiments.
**Figure 5.** Northern blot analysis of *PR1-a* expression in leaves of soybean cv. Harosoy in response to *avrB* and *avrPto* expressed in *P. syringae* pv. glycinea. Ten-day-old plants were inoculated by vacuum infiltration with PsgR4 transformants carrying the indicated plasmids at a concentration of $5 \times 10^7$ cfu/ml. RNA was isolated from whole primary leaves sampled at the times shown following inoculation, and hybridized to probes for PR1-a and actin transcript.

**LEGENDS FOR e-Xtra ITEMS 1-3**

**Figure S1.** Variability in measured population growth of *P. syringae* pv. glycinea strain PsgR4 transformants containing pPtE2 or pPtE2Ω as opposed to pPtE6 or pDSK519 and evidence of instability for pPtE2 and pPtE2Ω in leaves of soybean cv. Harosoy. Inoculation and bacterial growth measurements were carried out as described for Fig. 4, except that samples were plated both with and without selection for plasmid. Values shown are means of four replicate experiments. Vertical bars represent standard deviation. Rf, rifampicin; Tc, tetracycline; Km, kanamycin.

**Figure S2.** *P. syringae* pv. glycinea harboring *avrPto* fails to elicit HR in soybean cultivars Acme, Centennial, Faribault, or Norchief. PsgR4 transformants with the indicated plasmids were introduced at a concentration of $10^8$ cfu/ml into primary leaves by using a needleless syringe, and leaves photographed after 48 h. The minus sign indicates no plasmid.
**Figure S3.** pPtE2 and pPtE6 direct equivalent levels of expression of AvrPto in *P. syringae* pv. glycinea. Shown are the results of immunolabeling AvrPto on a western blot of total cellular proteins from cultures of PsgR4 transformants carrying pPtE2, pPtE6, or pDSK519.

*e-Xtra ITEM 4, MATERIALS AND METHODS SUPPLEMENT*

**Construction of pSMV-N, pSMV-G7, and derivatives**

Complete nucleotide sequences are available for SMV-G7 (Genbank accession no. AY216010, 19) and SMV-N (GenBank accession no. NC_002634, our unpublished results). PCR was carried out using an LA PCR kit (Panvera, Madison, WI). Other nucleic acid manipulations were performed using standard methods (44) or as described below.

For expression of SMV cDNA *in planta* following particle bombardment (see below), an EcoRI-SphI fragment containing the 35S promoter and NOS terminator from pAGUS1 (Table 1) was inserted between these same sites in pBR322 (Table 1), and oligonucleotide-directed mutagenesis was used to expand the multiple cloning site within this fragment to include *Stu*I, *Bam*HI, *Dra*I, and *Cla*I sites. The resulting plasmid was designated as pBR322-35S. The *Stu*I site in pBR322-35S is proximal to the 35S promoter.

Purified SMV-N and SMV-G7 were obtained as described previously (56) and viral RNA isolated using an RNeasy RNA purification kit (Qiagen, Valencia, CA). cDNA to each strain was generated using AMV reverse transcriptase (Seikagaku, Falmouth, MA) or Superscript II reverse transcriptase (GIBCO-BRL, Gaithersburg, MD) according to the manufacturer's instructions. To construct pSMV-N, first strand cDNA to SMV-N was made using the primer 3dt 5’-GTTTCTAGAGCTCAGCGGCCGCATCGA(T)20-3’. Then, 6 kb corresponding to the 3’ end of the virus was amplified from the cDNA by PCR using the
forward primer CI5 (5’-GCGGATCCAGTGTTGATGAGATTACAGA-3’) and the reverse primer 3dt-core (5’-GTTTCTAGAGCTCAGCGGCCGCATCGATT-3’, which contains a ClaI site (underlined). The product was trimmed at the ClaI site and an internal DraIII site and inserted into pBR322-35S. The overlapping 5’ 5.5 kb of SMV-N was then amplified from the cDNA using the forward primer 5’-AAAAATTAAACTACTCATTAAGAC and the reverse primer 5’-GTCAGGTCTGATGTC-3’, trimmed at the same internal DraIII site, and joined to the 3’ end in pBR322-35S using the Stul and DraIII sites. For pSMV-G7, first, partial cDNA to SMV-G7 was generated by reverse transcription using the internal reverse primer 5’-GCGGATCCATCTGTAATTGGACTGCATTCA-3’, and the 5’ end of the cDNA amplified by PCR using this primer and the forward primer 5’-GGTAGGCTTAAATTTAAACTCTCCTATAAGAC-3’, which contains a Dra I site (underlined). Then, this product was trimmed with DraI and BamHI and inserted between the Stul and BamHI sites of pBR322-35S. Next, cDNA was generated using primer 3dT, and from this cDNA, the 3’ end of SMV-G7 was amplified by using 3dT-core and primer CI5. Finally, the resulting fragment was joined to the 5’ fragment in pBR322-35S using ClaI and SalI.

To construct pSMV-Nv and pSMV-G7v, an AvrII site and a sequence encoding a NIa proteinase cleavage site were introduced between the PL and HC-Pro cistrons in pSMV-N and pSMV-G7 by 1) amplifying this region using a forward primer containing these sequences, 5’-

ATGGAGGATATTCAGCCTACTCCCTCCTTAGGAGAATCAGTTGCTCTTACAAATCCCAAAATCCTGAGGCTCAG-3’ (the AvrII site is underlined; the sequence encoding a NIa proteinase cleavage site is in italics) and the reverse primer 5’
GCGAATTTCGCGCCATTCTGCTTCAA-3’, 2) carrying out PCR again using this product as a reverse primer along with a forward primer, 5’-GTGCGGCGACGATAGTCA-3’, corresponding to a vector sequence upstream of the 35S promoter to generate a complete modified insert plus the 35S promoter for both pSMV-N and pSMV-G7, and 3) trimming these final PCR products with *Not*I and *Bgl*II and exchanging them for the corresponding portions of pSMV-N and pSMV-G7, respectively, using these sites.

To insert *uidA* into pSMV-Nv and pSMV-G7v, *Avr*II sites were added to each end of the reporter gene by PCR amplification from pRAJ275 (27) using the primers 5’-TGCAGCCTAGGCGATGCTGTCCGTCTGTAGAAAC-3’ and 5’-GTTGCCTAGGATTGTCTGCCCTCGGTAGAAAC-3’ (*Avr*II sites are underlined in these primers and in the primers following in this paragraph) and the product ligated into the *Avr*II site of each vector, yielding pSMV-Nv::*uidA* and pSMV-G7v::*uidA*. The same method was used to insert *GFP* to generate pSMV-Nv::*GFP* and pSMV-G7v::*GFP* except that *GFP* was amplified from psmGFP (13) using the primers 5’-CCAACCTAGGAAATGTAAAGAGAAGAACT-3’ and 5’-CCTTCCTAGGACCATTTGTAGCTCCATCC-3’. To generate pSMV-Nv::*avrB*, the avirulence gene was amplified from pAVRB (Table 1) using the primer 5’-CGTACCTAGGAGAGAGCGTTAGGCTTCGCTCTCGTCAAAAGGCACCA-3’ which creates a Nla proteinase recognition site (17) that includes and directs cleavage immediately preceding the glycine (codon italicized) at position 2 in the AvrB protein (see Fig. 1), and the primer 5’-CGTTTCCTAGGAAAAGGCAATCAGATCTAGCAGA-3’, and the product trimmed and inserted into pSMV-Nv using *Avr*II. SMV-Nv::*avrPto* was made similarly, with *avrPto* amplified from pPtE6 (Table 1) using the primers 5’-
CGTACCTAGGAGAGCGTTAGCCTTCAGGGAGAATATATGTCGGCGGATCCA
GGATGGGCC-3’ (codon for glycine at position 2 of AvrPto is italicized) and 5’-CGTTCCTAGGAGATTGCCAGTTACGGGTACGGGCT-3’. 
Figure 1. SMV expression constructs used in this study.
Figure 2. SMV-mediated expression of *uidA* and *GFP* in soybean cv. Williams 82.
Figure 3. Effects of \textit{avrB} and \textit{avrPto} as transgenes in pSMV-Nv.
Figure 4. Avirulence and virulence activity of *avrB* and *avrPto* in *P. syringae* pv. glycinea in soybean.
Figure 5. Northern blot analysis of PR1-a expression in leaves of soybean cv. Harosoy in response to *avrB* and *avrPto* expressed in *P. syringae* pv. glycinea.
Figure S1. Variability in measured population growth of *P. syringae* pv. glycinea strain PsgR4 transformants containing (A) pPtE2 or pPtE2W as opposed to (B) pPtE6 or pDSK519 and evidence of instability for pPtE2 and pPtE2W in leaves of soybean cv. Harosoy.
Figure S2. *P. syringae* pv. glycinea harboring *avrPtO* fails to elicit HR in soybean cultivars Acme, Centennial, Faribault, or Norchief.
**Figure S3.** pPtE2 and pPtE6 direct equivalent levels of expression of AvrPto in *P. syringae* pv. glycinea.
CHAPTER 3.

Large scale mutational analysis reveals novel candidate virulence factors in the rice pathogen *Xanthomonas oryzae* pv. *oryzicola*

A paper submitted to *Applied and Environmental Microbiology*

Li Wang¹,², Seiko Makino²,³, Ashim Subedee², and Adam Bogdanove²,⁴

ABSTRACT

Bacterial leaf streak, caused by *Xanthomonas oryzae* pv. *oryzicola*, is an important disease of rice. Transposon-mediated mutational analysis of the pathogen using a quantitative assay revealed candidate virulence factors including genes involved in pathogenesis in other phytopathogenic bacteria, virulence factors of animal pathogens, and genes not previously associated with virulence.

INTRODUCTION

Bacterial leaf streak is an important disease of rice (*Oryza sativa*) for which control measures are limited (24). In particular, no simply inherited gene for resistance to the disease has been reported. The disease is caused by *Xanthomonas oryzae* pv. *oryzicola*, a member of the gamma subdivision of the proteobacteria. The pathogen enters through leaf stomata or

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¹ Played a major role in conception of the work, generated 80% of the data presented, prepared all figures and tables, wrote the first draft and participated in revisions resulting in the final draft.
² Department of Plant Pathology, Iowa State University, Ames, IA, 50010
³ Current Address: Cardiovascular Research Center, 4F Massachusetts General Hospital 149, 13th St., Charlestown, MA 02129
⁴ Author of correspondence
wounds and colonizes the parenchyma apoplast, causing lengthening, interveinal lesions that appear water soaked initially then develop into translucent, yellow to white streaks. Leaf streak is prevalent in Asia and parts of Africa, where it can decrease yields by as much as 30%. In the US, the pathogen is quarantined and designated as a select agent under the Agricultural Bioterrorism Act of 2002. To lay the groundwork for disease prevention and control strategies based on interference with bacterial virulence, transposon-mediated mutational analysis of *X. oryzae* pv. oryzicola was carried out to identify candidate virulence factors.

**RESULTS**

**Identification and characterization of reduced-virulence mutants**

Strain BLS303 of *X. oryzae* pv. oryzicola (C. Vera-Cruz, International Rice Research Institute) was mutagenized using the EZ::TN <R6K\γori/KAN-2> Tn5 insertion kit (Epicentre). BLS303 cells were transformed by electroporation as described previously (32). Insertion mutants were selected on glucose yeast extract (GYE) agar(20) containing 25 µg/ml kanamycin, then cultured overnight in liquid GYE with kanamycin. Cells were washed twice and resuspended in sterile water to an OD$_{600}$ of 0.5 and spot-infiltrated in duplicate into leaves of 4-week-old rice plants of the Indica variety IRBB10 using a needleless syringe. Symptoms were observed after 4 days. A total of 10,000 mutants were screened. For 153 mutants, symptoms appeared reduced relative to wild type, or were absent. These were characterized further using a more stringent quantitative assay. In this assay, leaves of 8-week-old rice plants were inoculated with a mutant on one side of the midrib and the wild type directly opposite on the other. For each mutant, five replicate, paired inoculations on
each of two leaves were made. After 10 days, lesion lengths were measured for each paired inoculation and a paired, 2 tails student’s t-test performed across all replicates. By this test, 21 mutants were confirmed as virulence-impaired (\( p<0.1 \)) (Figure 1).

**Rescue and sequence analysis of disrupted genes**

Each of the 21 mutants contained just one insertion, as determined by Southern blot hybridization of EcoRI-digested genomic DNA with the 1kb Xhol/BamHI fragment of the transposon. To rescue the DNA containing the transposon, which carries the pir protein–dependent origin of replication R6K and the \( nptII \) gene and lacks EcoRI sites, EcoRI-digested DNA was treated with T4 ligase and electroporated into \( E. coli \) S17 \( \lambda \) pir. Transformants were selected on LB agar containing kanamycin (25 \( \mu g/ml \)). Nucleotide sequences flanking the transposon were determined using transposon-specific primers. Insertions were mapped and oriented by aligning the sequences to the whole genome sequence of \( X. oryzae \) pv. oryzicola available through the Comprehensive Microbial Resource (http://tigrblast.tigr.org/cmr-blast/). Insertions mapped to genes encoding components of the type III secretion system (T3SS), a lipopolysaccharide (LPS) synthesis enzyme, a two-component system response regulator, type IV pilus assembly proteins, enzymes involved in carbohydrate metabolism, and enzymes for fatty acid and aromatic amino acid synthesis (Table 1).

**Type III secretion genes**

Six insertions mapped to the \( hrp/hrc \) gene cluster, which encodes proteins involved in regulation and assembly of the T3SS, a macromolecular, syringe-like complex that delivers
effector proteins into host cells. In many plant pathogens the T3SS is essential for eliciting the host hypersensitive reaction and for pathogenicity (6, 15). In mutant strains 41C9 and 6H4, the insertions occurred in \textit{hrcU} and \textit{hrcV}, respectively, which encode inner membrane components of the complex. The insertions in mutant strains 42C3, 22H2, 6H7, and 26B10 reside in \textit{hrcC}, whose product is a key outer membrane component. As expected, and shown previously for \textit{hrcC} in BLS303, (20), each of the six mutants were non-pathogenic (not shown).

**Lipopolysaccharide synthesis gene**

LPS is a component of the bacterial cell surface thought to protect against environmental stresses and antimicrobial compounds by restricting membrane permeability. It comprises three covalently linked components: an outer membrane–bound moiety called lipid A, a core oligosaccharide, and an outermost polysaccharide known as the O-chain (25). The core and lipid A without the O-chain constitute lipo-oligosaccharide (LOS). In mutant 38C10, the transposon disrupts gene \textit{wxocB}, which encodes a predicted member of the rhamnose-glucose polysaccharide assembly protein F (RgpF) family (34). Members of the RgpF family are involved in assembly of the O-chain, but immediately downstream of \textit{wxocB} resides \textit{wxocE}, predicted to be involved in synthesis of the core. So the insertion, if polar, might block assembly of even LOS. LPS has been implicated previously in plant pathogenesis owing to the isolation of reduced virulence mutants that exhibited LPS deficiencies. Plants recognize certain LPS as microbe-associated molecular patterns (MAMPs), triggering innate defense responses (19, 28). In the \textit{Rhizobium}-legume symbiosis, structural changes in the O-chain take place during nodulation, suggesting an adaptive role
Changes in LPS structure can affect the efficiency of the T3SS and the expression and function of some other virulence factors (4, 33). Rice leaves inoculated with 38C10 developed lesions dramatically reduced in length relative to the wild type. Though structural consequences of the mutation in this strain and the reason for its reduced virulence remain to be elucidated, this observation implies that LPS plays a central role in the virulence of *X. oryzae* pv. oryzicola.

**Two-component regulatory gene rpfG**

In mutant 25C4, the transposon inserted into *rpfG*, which encodes the regulatory component of the RpfG/RpfC two-component system shown to positively regulate the synthesis of virulence factors and dispersal of biofilms in *X. campestris* pv. campestris in response to cell-to-cell signaling mediated by a diffusible signal factor, DSF (27, 31). The mutant caused lesions in rice leaves about half the length of wild type.

**Type IV pilus assembly and twitching motility genes**

Twitching motility in bacteria is movement independent of flagella that occurs by extension, tethering, and retraction of type IV pili (18). Twitching motility plays an important role in host colonization by several animal pathogens (22). Reports for plant-associated bacteria are fewer, and to our knowledge, limited to bacteria that colonize the vascular system (7, 16, 23). Six of the mutant strains reported here carry insertions in type IV pilus assembly and twitching motility genes. In mutants 5G9 and 17B9, the transposons disrupt the coding and promoter regions, respectively, of *pilY1*. Disruption of *pilY1* in *Xylella fastidiosa* caused a reduction in but not complete loss of type IV pili and twitching motility (18).
Mutants 14H9 and 25A6 carry insertions in \textit{pilQ} and \textit{pilM}, respectively, which reside at either end of an operon conserved across several species and required for pilus assembly, twitching motility, and phage sensitivity (12, 21, 26). The transposons in mutants 17D5 and 25D2 inserted in \textit{pilZ} and \textit{pilT}, respectively. PilZ is a predicted receptor for the secondary messenger bis-(3’-5’)-cyclic dimeric guanosine monophosphate (c-di-GMP), which regulates processes such as biofilm formation, twitching motility, photosynthesis, and virulence (1, 8, 13). \textit{pilT} encodes a putative hexameric ATPase required for type IV pilus retraction (2). With the exception of 25A6, which showed a relatively severe (greater than 50\%) reduction in virulence, the impairment in virulence in each of these mutants was slight to moderate. Although the contribution of individual genes to virulence may generally be small, to our knowledge this is the first time that type IV pili (and twitching motility) have been implicated in non-vascular plant pathogenesis. The mechanistic basis for the role of type IV pili in bacterial leaf streak, whether attachment, motility, biofilm formation, or some combination, remains to be determined.

\textbf{Carbohydrate synthesis genes}

In 5 mutants, transposons inserted in genes encoding enzymes for sugar metabolism. In mutant 24A2, the disrupted gene encodes fructose-bisphosphate aldolase class-I. In mutant 22G10, the transposon resides in \textit{pgk}, which encodes phosphoglycerate kinase. Mutants 1A8 and 20E6 both carry an insertion in the \textit{gapA} gene for type 1 glyceraldehyde-3-phosphate dehydrogenase. Mutants 24A2, 22G10, 1A8, and 20E6 each resulted in moderately to dramatically reduced lesion lengths in inoculated rice leaves. Interestingly, the affected genes co-localize in the genome in a cluster involved in the glycolytic and glycconeogenesis
pathway (10). Products of gap, pgk, and another gene in the cluster, pyk, were identified as candidate virulence factors in Yersinia pestis by proteomic analysis of the low calcium response (11). In Streptococcus pneumoniae the gapA gene product is both cytoplasmic and cell-wall associated and contributes to virulence through a role in plasminogen binding, recruiting proteolytic activity to the bacterial cell surface important for invasiveness (5). gapA also plays a role in adhesion and invasiveness in Paracoccidioides brasiliensis, a fungal pathogen of humans, possibly by binding to host surface matrix components (3). A role for the gapA product in plant pathogenesis has not been reported previously. Whether it recruits protease activity to the X. oryzae pv. oryzicola cell surface, binds to rice cell surfaces, or contributes in some other way remains to be determined. Although functional analysis remains, isolation of these four mutants suggests that some housekeeping and metabolic proteins play more complex roles than previously thought. In the fifth mutant of this class, 24D10, the disrupted gene is ppsA, which encodes phosphoenolpyruvate synthase A, a key enzyme in gluconeogenesis. ppsA was reported as important for virulence in X. campestris pv. campestris (30). In rice leaves inoculated with 24D10, a slight reduction in lesion length relative to wild type was observed.

Other genes
Two genes identified among the reduced-virulence mutants encode enzymes for basic metabolism. In mutant 29D3, the transposon inserted in a gene encoding 3-oxoacyl-[acyl-carrier-protein] synthase III, an enzyme involved in fatty acid and phospholipid biosynthesis. A homolog of this gene regulates virulence factors in the tobacco pathogen P. syringae pv. tabaci, potentially through an effect on the synthesis of acyl homoserine lactones (AHL) that
are involved in quorum sensing (29). AHL-mediated quorum sensing has not been reported in any xanthomonads, however. In mutant 38A5, the disrupted gene encodes the P-protein involved in the prephenate pathway for aromatic amino acid biosynthesis. A number of aromatic amino acid auxotrophic mutants of other plant and animal pathogens also have attenuated virulence (9, 14). The moderate reduction in virulence in mutants 29D3 and 38A5 likely reflects general defects in cellular physiology.

CONCLUSION

Transposon-mediated mutagenesis has been used to identify virulence factors in many plant pathogenic bacteria. With our screen for virulence-impaired transposon insertion mutants of _X. oryzae_ pv. oryzicola, we identified several factors associated with virulence in other plant pathogens, including the T3SS, the _rpfG/rfpC_ two-component regulatory system, LPS, and type IV pili. Importantly, ours is the first reported indication of a virulence function for type IV pili in a non-vascular plant pathogen. Also, we identified factors not previously associated with plant pathogenesis, but important or implicated in the virulence of animal pathogens, namely, selected enzymes for sugar metabolism, of which some appear also to play roles in binding host substrates at the cell surface. Finally, we isolated mutants affected in fatty acid and aromatic amino acid synthesis, processes that likely contribute to virulence through their roles in basic metabolism.

In all, despite the large scale of the screen, only 21 mutants were confirmed as virulence-impaired. Ten thousand mutants represent roughly a 2-fold coverage for a targeted insertion rate of 1 per 1,000 bp (the genome is just under 5 x 10^6 bp), so the screen likely was not saturating. Also, leaf-to-leaf variability and the early time point used for scoring in the
initial screen might have precluded capture of subtle virulence deficiencies. These factors were accounted for in the more stringent quantitative assay used for further characterization of mutants. Another possibility is that strains with mutations that cause a marked effect on colony size or morphology (e.g., loss of EPS) may have been overlooked. Finally, functional redundancy of some virulence genes may have precluded their isolation.

The genes identified will require confirmation by genetic complementation, since the reduced virulence phenotypes may be due to polar effects of insertions on downstream genes, or to spontaneous, ectopic mutations. However, we have observed the transposon to be a non-polar mutagen when in the forward orientation (Figure 2; LW and AJB, unpublished), ostensibly due to the outreading promoter of the terminator-less \textit{nptII} gene that it contains. Also, for several genes or gene classes, we isolated multiple independent insertions, strongly supporting the conclusion that the insertions are the cause of the virulence deficiencies, and that genes to which they localize are the relevant virulence factors.

\textit{X. oryzae pv. oryzicola} is a pathogen of emerging importance that constrains production of the world’s most important food crop. Because rice is also an important biological model, bacterial leaf streak can serve as a representative system for understanding non-vascular pathogenesis of other plants. The identification of candidate virulence factors reported here is an important first step toward elucidating molecules and mechanisms important in disease that may be targeted for the development of novel means of control and prevention. It should be noted that the inoculation technique used to identify these factors bypasses survival on the leaf surface and entry into the apoplast. Screening of the mutant library, using alternative methods, for mutants affected in these processes is likely to reveal yet additional virulence factor candidates.
ACKNOWLEDGEMENTS

The authors are grateful to J. Helgerson, Z. Sayre, E. Flemmig, J. Paulson, and J. Lorence for assistance with mutant screening, to K. Vogel for technical assistance, and to D. Meyer and D. Niño-Liu for critical reading of the manuscript. This work was supported by award 0227357 from the Plant Genome Research Program of the National Science Foundation.

REFERENCES


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TABLE 1. *X. oryzae* pv. oryzicola transposon insertion mutants affected in virulence.
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\(^1\) Virulence rated as lesion length relative to wild type (%). -, 0%; +, 10%–30%; ++, 31%–60%; ++++, 61%–95%.

\(^2\) Relative to the first nucleotide in open reading frame.

\(^3\) Orientation of *nptII* promoter in transposon relative to orientation of disrupted gene. F, forward; R, reverse.
FIGURE LEGENDS

**Figure 1.** Lengths of lesions in rice leaves caused by reduced-virulence mutants of *X. oryzae* pv. oryzicola as percent of wild type inoculated side-by-side. Error bars represent the standard deviation of 10 replicate inoculations. *p*-values resulting from a paired, 2 tails student’s *t*-test for each mutant are shown at the top. Mutants that caused no lesions are omitted.

**Figure 2.** Genomic locations and orientations of transposon insertions in reduced-virulence mutants of *X. oryzae* pv. oryzicola (A-L). Each mutant carries only one insertion. Disrupted genes are represented by empty block arrows. Transposon insertions are represented by inverted triangles. Orientation of the *nptII* promoter in each insertion is shown by an arrow above the triangle. Mutant designations corresponding to each insertion are given at the top. Numbers to the left and right are genome coordinates in base pairs. Gene symbols or products are noted below disrupted genes and selected flanking genes.
Figure 1. Lengths of lesions in rice leaves caused by reduced-virulence mutants of *X. oryzae* pv. oryzicola as percent of wild type inoculated side-by-side.
Figure 2. Locations and orientations of transposon insertions in reduced-virulence mutants of *X. oryzae* pv. oryzicola. Each mutant carries only one insertion.
CHAPTER 4.

The lipopolysaccharide O-chain of Xanthomonas oryzae pv. oryzicola is required for type III secretion–mediated protein delivery and virulence

A paper to be submitted to Journal of Bacteriology

Li Wang and Adam J. Bogdanove

ABSTRACT

Xanthomonas oryzae pv. oryzicola causes bacterial leaf streak of rice (Oryza sativa). A previously reported, reduced virulence mutant, TN38C10, with a disruption in the \textit{wxocB} gene predicted to play a role in lipopolysaccharide (LPS) synthesis was characterized in this study. TN38C10 caused much shorter lesions than the wild type, but multiplied to a similar extent in inoculated rice leaves. The composition of LPS in TN38C10 and the wild type was characterized, and \textit{wxocB} was shown to play a role in assembly of the O-chain. The \textit{wxocB} mutant was partially impaired in extracellular polysaccharide (EPS) production, but characterization of a distinct, EPS-deficient mutant indicated that that impairment could not solely account for the virulence deficiency of TN38C10. TN38C10 was more sensitive to osmotic stress but less sensitive to oxidative stress than the wild type, and equally or not as sensitive as the wild type to several phytoalexins. No differences in elicitation of defense-related gene expression in rice were observed between TN38C10 and the wild type. Type III secretion system (T3SS)–mediated protein delivery into rice cells, however, was reduced nearly 4-fold in the \textit{wxocB} mutant relative to the wild type. These findings indicate that the O-chain of LPS is required for wild-type virulence, and that its requirement likely involves
structural and/or regulatory interactions with other virulence components, notably EPS and the T3SS.

INTRODUCTION

Bacterial polysaccharides include lipopolysaccharides (LPS) and extracellular polysaccharides (EPS) (48). Lipopolysaccharides are important components of the outer membrane of Gram-negative bacteria. They cover about 75% of the bacterial surface area (35). The usual LPS structure is composed of hydrophobic lipid A, which anchors LPS to the outer membrane and a covalently attached hydrophilic polysaccharide portion. The amphiphilic LPS provide a permeability barrier to large, negatively charged or hydrophobic molecules. Lipid A plays a role in many human and animal diseases as toxin (49). The polysaccharide portion usually includes two parts: core oligosaccharide attached to lipidA and the outermost O-polysaccharide (O-chain). The O-chain is composed of repeat oligosaccharide units which may contain three to five residues. The O-chain is the main hydrophilic component of LPS. The sugar residues in the O-chain are the most variable part of LPS. They may be different between species, strains, and even within strains (35). O-chains of most Gram-negative bacteria are antigenic. EPS, composed of the repeat sugar units, can form a capsule outside of outer membrane or be released to the environment as slime (48). Both LPS and EPS can contribute to the virulence of bacteria (13, 22).

In animal cells, although the O-chain is non-toxic, it has been reported to contribute to virulence. The O-chain may prevent interaction of host antibodies with antigens on the membrane surface, and therefore, prevent bacterial cell lysis or protect from phagocytic engulfment (35). Recent studies on Salmonella enterica serovar typhimurium show that the
length of the O-chain side chain influences uptake by macrophages, and the optimal length to provide protection from the complement is 4-15 repeat units (42). Bacteria also mimic host components through modification of the O-chain to avoid the host immune defenses.

Research on *Helicobacter pylori* shows that *H. pylori* can express Lewis x, Lewis y, and H type I blood group structures (3). Recently, glucosylation of O-chain was found in *Shigella* to be required for the ability of type III pili to extend beyond it to deliver effectors into the host cell. This observation gave rise to the interpretation of these two features as coordinately wielded sword (type III pilus) and shield (LPS) (59).

As one of the main components of the outer membrane, LPS have diverse functions in the interaction of plant-associated bacteria with their hosts. LPS form a semi-permeable barrier which may protect phytobacteria from adverse environmental conditions (22). Plants recognize certain LPS as microbe-associated molecular patterns (MAMPs), which trigger innate defense responses (37, 52). In *Arabidopsis*, both the core and lipid A of *Xanthomonas campestris* LPS trigger independently the induction of defense genes *PR1* and *PR2* (52). This observation provides evidence for recognition of core polysaccharides and lipid A by distinct receptors.

In contrast to O-chains in animal pathogens whose functions are well studied, functions of O-chains in phytopathogens are still unclear. In the *Rhizobium*-legume symbiosis, structural changes in LPS O-chain occur during nodulation, suggesting an adaptive role (35). When synthetic O-chain repeat units are used to pretreat *Arabidopsis Col-0*, they suppress the hypersensitive response normally triggered by a gene-for-gene interaction with an avirulent pathogen, and induce *PR-1* gene expression (5). All these
observations indicate that elucidating the functions of O-chains of LPS is important to better understand the virulence mechanisms of phytopathogens.

EPS are secreted by both Gram-negative and Gram-positive bacteria (13). Mutants of bacterial phytopathogens, especially those that colonize the plant vascular system, that lose the ability to produce EPS usually suffer a reduction in virulence (21, 31, 32). One characteristic of *Xanthomonas* species is the secretion of copious EPS, called xanthan. Genes involved in EPS synthesis in *Xanthomonas* include a large cluster known as *gum* genes. The product of *gumD* catalyzes the transfer of glucosyl-1-phosphate from UDP-glucose to polyprenol phosphate, which is the first step in the biosynthesis of lipid intermediates involved in the synthesis of xanthan (32).

The type III secretion system (T3SS) is conserved among pathogens of plants and animals (14). It is encoded by the *hrp* gene cluster in plant-associated bacteria, which is required for both hypersensitive response and pathogenicity. The T3SS is responsible for delivering bacterial effectors from the bacterial cytosol directly into the interior of host cells. The efficiency of T3SS may directly affect the virulence of bacteria.

The phytopathogenic bacterium *Xanthomonas oryzae* pv. oryzicola is the causal agent of bacterial leaf streak on rice. The pathogen enters through leaf stomata or wounds and colonizes the parenchyma apoplast, causing lengthening, interveinal lesions that appear translucent and water soaked initially then develop into necrotic streaks that may be white, or yellowish due to the pigmented bacteria within them. *X. oryzae* pv. oryzicola can cause severe yield losses in tropical area of Asia (40). Some quantitative trait loci for resistance to bacterial leaf streak have been identified (50), but due to the lack of rice cultivars with major resistance genes to bacterial leaf streak, disease management methods are limited.
Understanding the virulence mechanisms of *X. oryzae* pv. oryzicola is important to improving disease management. We reported previously (Wang et al., submitted; Chapter 3 of this dissertation) the isolation of a transposon insertion mutant of *X. oryzae* pv. oryzicola, TN38C10, with a disruption of the *wxocB* gene, which resides in an LPS synthesis locus. The predicted product of *wxocB* is a glucosyltransferase, a member of the rhamnose-glucose polysaccharide assembly protein (RgpF) family (62). TN38C10 is dramatically impaired in its ability to cause lesion in rice, relative to the wild type. Here we present characterization of the *wxocB* gene and its role in virulence that reveals a functional relationship between LPS and other virulence components, namely EPS and the T3SS.

**RESULTS**

**The *wxocB* gene is required for full virulence**

To confirm and further characterize the contribution of *wxocB* to the virulence of *X. oryzae* pv. oryzicola, the gene was cloned in the broad host range expression vector pUFRO47, resulting in pWxocB. In this plasmid, expression of *wxocB* is driven by the *lac* promoter, which is constitutive in *Xanthomonas* spp (54). Wild-type strain BLS303, *wxocB* mutant strain TN38C10, and TN38C10 transformed with pWxocB were inoculated into leaves of rice variety IR24 (Figure 1). After 10 days, lesions caused by TN38C10 were only 25% as long as those caused by the wild type. pWxocB restored lesion lengths to wild type. Bacterial populations of the *wxocB* mutant measured at 0, 4, and 8 days, however, were not significantly different from those of BLS303, increasing almost 1,000 fold over this time period.
**wxocB functions in synthesis of the O-chain of lipopolysaccharide**

*wxocB* resides in a locus predicted to function in lipopolysaccharide biosynthesis (44, 58). It encodes a member of the rhamnose-glucose polysaccharide assembly protein (RgpF) family. Members of this family in other bacteria have been shown to function in assembly of the LPS O-chain (43, 51). The effect of the *wxocB* mutation on LPS composition was therefore examined. LPS from wild type BLS303, *wxocB* mutant TN38C10, and complemented strain TN38C10 (pWxocB) were purified and visualized by tricine SDS-PAGE followed by silver staining (Figure 2). The resulting profiles revealed that TN38C10 is missing the diffuse, high apparent molecular weight species that typically represent complete and partially degraded LPS (58), but retains the low apparent molecular weight bands that represent fragments of lipooligosaccharide (LOS) containing the lipid A and core oligosaccharide (58). The wild-type profile was restored in the complemented strain. In a separate study, to confirm that the altered profile for TN38C10 is due to loss of the O-chain, the preparations were analyzed by GLS-MS (Gas-liquid chromatograph-mass spectrometry) and NMR. The O-chain of the wild-type strain was found to consist of a rhamnose backbone with a xylose side chain attached every third rhamnose. The core oligosaccharide structure was also determined. Results of this analysis showed that the *wxocB* mutant indeed lacks the O-chain, but retains the core oligosaccharide and lipid A (E. Vinogradov, LW, and AJB, unpublished). The *wxocB* gene therefore functions specifically in synthesis of the O-chain, ostensibly in assembly of the rhamnose backbone subunits. Disruption of *wxocB* causes a reduction in extracellular polysaccharide.
Colony morphology of TN38C10 on glucose yeast extract (GYE) agar differs from the wild type.

By 4 days after plating, TN38C10 colonies appear smaller and less mucoid than wild-type colonies. By 8 days, however, colonies appear similar to wild-type. This observation suggested that the \textit{wxocB} mutation causes a reduction in, but not complete loss of, extracellular polysaccharide (EPS)(48). This prediction was tested by quantification of EPS in supernatants of \textit{wxocB} mutant strain TN38C10 and wild-type cultures grown in liquid GYE for 2 days. The \textit{wxocB} mutant culture supernatant yielded roughly half the EPS of that of the wild type. Transformation of the mutant strain with pWxocB restored EPS production in liquid culture, and colony morphology on GYE plates, to near wild-type, confirming that the \textit{wxocB} mutation causes the reduction in EPS (Figure 3).

Absence of extracellular polysaccharide causes a moderate reduction in virulence

EPS is important for virulence in many plant pathogenic bacteria, particularly xylem-colonizers (19, 32), (12), (6). But for other, non-vascular pathogens, EPS has been shown not to play a significant role (23). To determine whether the virulence deficiency of TN3810C might be attributable to the reduced capacity of this strain to produce EPS, a distinct BLS303 mutant with a transposon insertion in \textit{gumD}, which encodes a key enzyme for EPS synthesis (32), was isolated (see Materials and Methods) and assayed for virulence. This strain, BLS303\textit{gumD}$^-$, was confirmed as non-mucoid (lacking EPS) on GYE agar (not shown). In rice leaves, it showed reduced virulence relative to the wild type, yet was significantly more virulent than TN38C10 (Figure 4), indicating that the virulence defect in TN38C10 could not be accounted for solely by its reduction in EPS.
Sensitivity of the \textit{wxocB} mutant to oxidative stress, osmotic stress, and phytoalexins

To determine whether increased sensitivity to stresses and antimicrobial compounds that might be encountered in the plant could be involved in the reduced virulence of the \textit{wxocB} mutant, TN38C10 was compared to BLS303 for resistance to oxidative stress, osmotic stress, and several phytoalexins. First, survival of cells in suspension following a 15 min. treatment with $H_2O_2$ was assessed (Figure 5A). At each of three different concentrations of $H_2O_2$, TN38C10 survival was markedly greater than that of BLS303. The mutant strain carrying pWxocB showed wild-type sensitivity to $H_2O_2$. Thus, the \textit{wxocB} mutation renders the bacterium more resistant to $H_2O_2$, implying that the reduced virulence is not related to oxidative stress. Next, plating efficiency on a medium containing high concentrations of NaCl was determined relative to a medium without added salt (Figure 5B). TN38C10 showed increased sensitivity relative to BLS303 that was complemented by pWxocB, indicating a possible role for osmotic stress in the reduced virulence of the \textit{wxocB} mutant. Finally, resistance to compounds representing different classes of phytoalexin was tested on supplemented solid media (Table 3). Of 6 phytoalexins assayed, three were found to have significant inhibitory effects on either the wild type or TN38C10: plumbagin (a naturally occurring naphthoquinone isolated from roots of \textit{Plumbago scandens})(16), berberine (an alkaloid found in such herbs as berberis and goldensal) (47) and resveratrol (a compound largely found in the skin of red grapes) (9). No differences in minimal inhibitory concentrations of plumbagin and resveratrol to TN38C10 versus BLS303 were found, but TN38C10 was significantly more resistant to berberine than BLS303. Wild-type sensitivity was restored by pWxocB (data not shown). Differences in sensitivity to phytoalexins, therefore, are unlikely to explain the virulence reduction caused by disruption of \textit{wxocB}. 


Host defense-related gene expression in response to the wxocB mutant

To determine whether the reduced virulence of TN38C10 is due to greater elicitation of rice defense responses by this strain than by the wild type, transcript levels of three defense-associated genes – pathogenesis-related gene 1 (PR1), EL2 (an early chitin oligosaccharide elicitor-responsive gene) (41), and POX22.3, a peroxidase gene (10) – were analyzed by semi-quantitative RT-PCR at several time points over 4 days in rice leaves inoculated with BLS303, TN38C10, or a mock inoculum (Figure 6). For all three genes, the wild type and the wxocB mutant elicited increases in transcript levels equally, suggesting that differences in plant defense responses do not account for the reduced virulence of TN38C10.

Delivery of an AvrXa10:CyaA fusion protein into rice cells is reduced in the wxocB mutant

Structural and regulatory dependencies, respectively, of the T3SS on LPS have been reported in Shigella flexneri (59), and Pseudomonas aeruginosa (4) and Yersinia enterocolitica (7). In light of this, the efficiency of T3SS-mediated protein delivery into rice cells was examined for the wxocB mutant. TN38C10 was transformed with pKEB45 (Table 1), carrying a translational fusion of the type III effector gene avrXa10 with the cyaA gene. The product of cyaA serves as a reporter for protein delivery into the eukaryotic cell by virtue of its calmodulin-dependent adenylate cyclase activity (53). cAMP levels in rice leaves 8 hr following inoculation with TN38C10(pKEB45), BLS303(pKEB45), a T3SS-deficient derivative of BLS303 carrying the plasmid (38), and TN38C10 carrying pHM1, the backbone vector for pKEB45, were measured using an immunoassay (Figure 7). Populations of
TN38C10(pKEB45) and BLS303(pKEB45), measured in separately inoculated leaves, were not significantly different at this time point (not shown). The results revealed a nearly 4-fold reduction in delivery of the \textit{avrXa10:cyA} fusion product into rice cells in the \textit{wxocB} mutant relative to the wild type. cAMP levels for TN38C10 were, nonetheless, above background (indicated by the T3SS mutant and empty vector controls). Thus, mutation of \textit{wxocB} causes a marked reduction but not complete loss of T3SS-mediated protein delivery into rice cells.

**DISCUSSION**

In this study, we investigated functions of the \textit{wxocB} gene, which is located in an LPS synthesis locus in \textit{X. oryzae} pv. oryzicola. We found that disruption of \textit{wxocB} dramatically attenuates virulence but does not affect population levels of the bacterium in planta measured at 4 and 8 days following inoculation. The gene encodes a predicted glucosyl transferase and member of the rhamnose-glucose polysaccharide assembly protein (RgpF) family (62). We determined that the O-chain of \textit{X. oryzae} pv. oryzicola LPS consists of a rhamnose backbone with xylose side chains, and that \textit{wxocB} indeed plays a role in its synthesis. Disruption of \textit{wxocB} also caused a reduction in EPS, but this defect did not fully explain the virulence deficiency of the \textit{wxocB} mutant, because a distinct mutant completely devoid of EPS was still more virulent than the \textit{wxocB} mutant. Loss of the O-chain in the \textit{wxocB} mutant was not associated with changes in resistance to oxidative stress or phytoalexins that could explain the reduced virulence. The mutant was more sensitive to high osmolarity than the wild-type, but it is unclear whether the bacterium is likely to encounter such conditions in the rice apoplast. Also, no differences were observed in the ability of the mutant versus the wild type to elicit expression of several defense-related genes in rice. We
found, however, that loss of the O-chain was associated with a decrease in T3SS-mediated protein delivery into rice cells. T3SS-mediated delivery of proteins (effectors) into the host cell is essential for the pathogenicity of *X. oryzae* pv. oryzicola (38). Thus, a change in the capacity for effector delivery could affect virulence in a quantitative fashion. We conclude that the O-chain of LPS is required for wild-type virulence, and that its requirement is manifested at least in part through structural and/or regulatory interactions that affect EPS and the T3SS.

**Virulence defect of the *wxocB* mutant**

The *wxocB* mutant causes shorter lesions than wild type. Despite this, *wxocB* mutant bacteria *in planta* multiply at the same level as the wild type strain. This result hinted that *wxocB* gene may not affect the basic metabolism of the bacterium. The *wxocB* gene may contribute to virulence via reducing the ability to move in the plant or the ability to cause plant cell death to form lesions. The inoculation titer used here was relatively high. A difference in multiplication might be detected in lower-titer inoculations. Also, the *wxocB* mutation may cause a lag in multiplication that was not detected by our measurements at 4 and 8 days after inoculation. An examination of earlier time points following inoculation is warranted.

Our data suggest impaired T3SS delivery in the *wxocB* mutant, the T3SS is thought to contribute to both symptomagenesis and multiplication of bacteria (26). Yet, T3SS delivery of effectors is reduced, not abolished in the mutant strain. Perhaps the threshold for delivery required for multiplication is lower than that required for watersoaking and lesion development. It is also possible that effector delivery is selectively affected, There is some
evidence that uncouples multiplication of bacteria and lesion development in planta. A mutant of X. oryzae pv. oryzicola disrupted in a gene encoding a transcription-activator-like effector caused reduced water-soaking in rice but multiplied at the same level as wild type (Niño-Liu and Bogdanove, unpublished). The watersoaked phenotype results from loss of cell membrane integrity and the accumulation of fluids in the intracellular spaces of plant tissue (25). Specific plant genes may be involved in this process such as genes involved in ion transporter synthesis and regulation. X. oryzae pv. oryzicola upregulates the expression of two genes encoded Ca+-transporting ATPases during infection (Niño-Liu and Bogdanove, unpublished). The observation that different T3SS effectors may specifically regulate the expression of different target genes in rice (63) suggests that the expression of genes required for watersoaking may be upregulated by specific T3SS effector protein whose delivery or expression has been in the wxocB mutant. Hierarchical ordering of effector delivery by E.coli supports this possibility (17).

**Reduction of EPS in the wxocB mutant strain**

Loss of the O-chain in the wxocB mutant is associated with a reduction in EPS. In fact, this reduction in EPS could contribute to the other phenotypes observed, though as we have demonstrated, it cannot account solely for the reduction in virulence exhibited by the wxocB mutant. The reason for the reduction in EPS is not clear. In X. oryzae pv. oryzae, several EPS defective mutants display modified LPS structures (20). Mutation of wzt, a conserved gene involved in LPS translocation in Xanthomonas species, affects the profile of LPS as well as the production of EPS (P. Patil and R. Sonti, unpublished). In X. oryzae pv. oryzicola, defects in production of EPS were observed in strains carrying mutations in two
other LPS synthesis genes, wzt and metB (LW and AJB, unpublished). One reason single mutations might affect both LPS and EPS is that the synthesis of LPS and EPS share some common substrates and intermediates. Enzymes used in one pathway may be feedback-inhibited by the accumulation of intermediates caused by the disfunction of an enzyme in the other pathway. Another reason may be that some common pathways are involved in the synthesis of both LPS and EPS such as the UDP-glucose synthesis pathway, or the polysaccharide export pathway (45). The disfunction of one enzyme in a shared pathway will affect the production of both LPS and EPS. These two facts may not explain well why wxocB affects both LPS and EPS production because xanthan does not contain rhamnose (32). There may be a more complicated feedback regulatory mechanism which links the production of LPS and EPS such that a defect in LPS triggers down-regulation of EPS production (and vice-versa).

Though complementation of the gumD mutation was not attempted, characterization of the gumD mutant indicates a likely, but apparently minor role for EPS in the virulence of X. oryzae pv. oryzicola. Whether the LPS profile of the gumD mutant strain is changed remains to be determined. In endosymbiotic cyanobacteria, the EPS layer during symbiosis was thinner than that of cultured bacteria and three proteins involved in the synthesis of EPS were downregulated (24). So there is precedence for alteration in EPS production during interactions with a host. In our study, the amount of EPS in planta was not assessed, and we cannot exclude the possibility that the gumD mutant produces some EPS in planta. However, to date, there is no information to suggest that GumD function can be substituted for in planta via plant-induction of a cryptic, analogous gene.
Proposed functions of the O-chain of LPS in virulence in animal pathogens include protection of the pathogen from toxic compounds, as a cell surface permeability barrier to large, negatively charged or hydrophobic molecules (35). Induction of reactive oxygen species (ROS) is often the first plant response detected following pathogen attack. The typical ROS include superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and the hydroxyl radical (OH) (34). Plants also produce antimicrobial phytoalexins constitutively or in response to attack (28). LPS mutants of _X. campestris_ pv. campestris were reported to be more sensitive to antimicrobial compounds in vitro (22). We first tested whether the _wxocB_ mutant is more sensitive than wild type to the ROS H$_2$O$_2$ or to phytoalexins. No rice phytoalexins are commercially available, but several other phytoalexins isolated from other plant species were tested. Our results showed that the lack of O-chain in mutant TN38C10 was associated with greater resistance to oxidative stress caused by H$_2$O$_2$ and the action of berberine, a hydrophilic phytoalexin. But no difference in sensitivity to other, hydrophobic phytoalexins between wild type and _wxocB_ mutant strain was observed. These observations imply that the reduced virulence of the _wxocB_ mutant does not relate to alterations in resistance to oxidative stress or antimicrobials. A possible reason for the enhanced resistance to H$_2$O$_2$ or berberine may be increased hydrophobicity of the cell surface due to absence of the O-chain.

We also examined resistance to osmotic stress. In _Arabidopsis_, the hypersensitive reaction that occurs during the incompatible interaction between a resistant plant and an avirulent pathogen induces severe water stress in the apoplast, which appears causally related to successful plant defense (60). Though our assay was of a compatible interaction, we
reasoned that osmotic stress conditions might arise that could account for the reduced virulence of the \textit{wxocB} mutant if it was less resistant to osmotic stress than the wild type. The \textit{wxocB} mutant was indeed less resistant to osmotic stress than the wild type. This sensitivity may relate either to the lack of the O-chain or to the reduction in EPS. Since both O-chain and EPS are extremely hydrophilic molecules, they may provide protection from osmotic stress by capturing water molecules. Thus, reduced resistance to osmotic stress might contribute to the reduced virulence of the \textit{wxocB} mutant, but it is unknown whether osmotic stress is significant high during the \textit{X. oryzae} pv. oryzicola interaction with rice.

\textbf{LPS as microbe associated molecular patterns (MAMPs)}

Pathogen- or microbe-associated molecular patterns (PAMPs/MAMPs) are conserved structures such as flagellin and LPS that are recognized directly by host-encoded receptors (36), (46, 61), leading to the activation of host defenses (37). In animals, functions of bacterial LPS have been well studied. At low concentration, LPS triggers a variety of host responses to counter bacterial invasion. At high concentration, LPS may induce shock, fever, and severe inflammatory reactions, through the release of endogenous mediators from host cells (39). In some cases, LPS may mimic host components to evade detection (35). Plants recognize certain LPS as MAMPs, resulting in activation of defense responses (37, 52). In \textit{Arabidopsis}, both the core and lipid A of \textit{X. campestris} lipooligosaccharide trigger independently the induction of defense genes \textit{PR1} and \textit{PR2} (52). We reasoned that loss of the O-chain in \textit{X. oryzae} pv. oryzicola might expose components of the remaining LOS that elicit enhanced rice defense responses and thus account for the reduction in virulence of the \textit{wxocB} mutant. Our results showed however, that both the wild type and the \textit{wxocB} mutant
upregulate the expression of certain pathogen-related (PR) genes to a similar extent over 4 days after inoculation, indicating that the *wxocB* mutant likely does not induce enhanced plant defense that could account for its reduced virulence. The observation that type III secretion is reduced in the *wxocB* mutant (see below), but induction of defense genes is unaffected supports the notion that this induction is MAMP-mediated, and the likelihood that MAMPs in addition to LPS are involved.

**O-chain of LPS and the T3SS**

Our most intriguing finding is that loss of the *X. oryzae* pv. oryzicola O-chain is associated with reduced T3SS-mediated protein delivery into rice cells. To our knowledge, this is the first report of such a connection in a plant-pathogen interaction, and the first *in vivo* study pointing to the relationship. Based on in vitro characterization, glucosylation of the O-chain in *Shigella* was postulated to promote bacterial invasion by shortening the LPS molecule and exposing the tip of the T3SS pilus, allowing efficient type III secretion (59). Absence of LPS O-chain was reported to increase T3SS secretion in *P. aeruginosa in vitro* via upregulation of T3SS gene expression (4). Our observation seems to relate to mechanisms distinct from these. We hypothesize that the O-chain protects the type III pilus from apoplastic proteases, or provides a hydrophilic microenvironment that facilitates its assembly. Alternatively, absence of the O-chain could cause negative feedback regulation of *hrp* and other virulence-associated gene expression. The latter observation is consistent with the observed reduction in EPS production, and there is ample precedence for interdependent regulation of virulence factors in both animal and plant pathogens (4), (7), (57), (29, 33, 55), (1, 11). Experiments to test these hypotheses, including a CyaA assay of the mutant strain
coinfiltrated with protease inhibitors, and assessment of hrp gene expression in the mutant in planta, are planned. A third possibility is that attachment of X. oryzae pv. oryzicola to the rice cell is needed for efficient type III delivery, and that the O-chain plays a role in that attachment. Histological observation and assessment of whether type III secretion into the extracellular space (as opposed to delivery into plant cells) is affected in vitro will be necessary.

In summary, we demonstrate that wxocB is involved in O-chain synthesis, and by extension, that the O-chain is necessary for full virulence, EPS production, and normal function of the T3SS. Though the mechanistic basis for these requirements is not yet clear, these findings reveal new roles for the O-chain in plant-bacterial interactions, and contribute to our understanding of the virulence mechanisms of phytopathogenic bacteria.

**MATERIAL AND METHODS**

**Strains, plasmids and growth conditions**

Bacterial strains and plasmids used in this study are presented in Table 1. *E. coli* was cultured in Luria-Bertani (LB) medium at 37°C. *X. oryzae* pv. oryzicola strains were cultured in GYE medium. Kanamycin (50 µg/ml), spectinomycin (25 µg/ml), gentamycin (25 µg/ml), were added to growth media as appropriate for selection. Plasmids were introduced into *E. coli* and *X. oryzae* pv. oryzicola by electroporation.

**Plant material and bacterial virulence assays**

Indica rice variety IR24 plants were grown in a growth chamber under a cycle of 12 h light at 28°C and 12 h dark at 25°C. Plants were inoculated at 6 weeks with bacterial
suspensions in sterile distilled water at approximately $1 \times 10^8$ cfu/ml (O.D.$_{600} = 0.4$) unless noted otherwise using a needleless syringe (38). Following inoculation, plants were returned to the growth chamber. Lesion lengths were measured 10 days after inoculation. For the bacterial population assay, for each sample, a 3 cm long leaf section centered on the inoculation site was collected and ground in 0.5 ml sterile distilled water. Bacterial populations were measured by dilution plating of these homogenates on GYE agar. For each time point per inoculum, three independent inoculations were sampled individually and then mean and standard deviation calculated. Experiments were repeated three times.

**wxocB gene cloning and complementation**

A 1.9 kb fragment containing the *wxocB* gene was amplified from BLS303 genomic DNA using gene specific primers listed in Table 2, and ligated into pCR2.1 TOPO TA (Invitrogen), yielding pLW4. Fidelity of amplification was confirmed by complete sequencing of the cloned fragment. The plasmid was then digested with *Hind*III and *Eco*RI, and the 1.9 kb fragment containing *wxocB* gel purified and ligated into pUFR047, resulting in pWxocB. pWxocB was introduced into TN38C10 by electroporation as described (56).

**LPS purification and analysis**

*X. oryzae* pv. oryzicola BLS303 and TN38C10 cultured overnight in NB medium were diluted to OD=0.04 and then diluted 1000 folds, finally plated at 100 ul per plate on 200 large (175mm ) NB-agar plates and incubated for 5 days at 28°C. Cells were harvested and suspended in 1 ml sterile distilled water, centrifuged for 1 hour at 13000g, washed twice with 0.9% NaCl and once with distilled H$_2$O, and then resuspended in 100 ml of distilled H$_2$O.
LPS were extracted using the hot phenol-water method as described previously, with modification (8). In detail, bacterial suspensions were heated to 80°C using a water bath. 100ml of phenol heated to the same temperature was added, and suspensions kept at 80°C for 90 min. During the heating, suspensions were mixed vigorously every 10 min. Afterwards, resulting solutions were cooled for 30 min. on ice. Mixtures were dialyzed in tubing (MWCO 3,500 D) against running tap water four days, then concentrated by immersing the dialysis tubes in PEG 8000 flakes for several hours. After concentration, solutions were centrifuged 30 min at 10,000g. Then, 150 ul DNase I (3 mg/ml) and 50 ul RNase A (10 mg/ml) were added. Solutions were then incubated at 37°C for four hr, 150 ul Protease K (2.5 mg/ml) added, and incubation at 37°C continued overnight. Solutions were dialyzed for two days against running tap water. LPS were obtained by lyophilization. 5 ml ddH₂O was added into the LPS lyophilized powder, and the concentrated solutions further purified by centrifugation over night at 80,000g. Pellets were resuspended in Tricine sample buffer (Bio-rad) and analyzed by TSDS-PAGE using a 16.5% separating gel (Bio-rad) followed by silver staining (2).

**Isolation of BLS303gumD⁻**

A library of transposon insertion mutants of BLS303 (Wang et al., submitted; Chapter 3 of this dissertation) was plated on GYE agar with kanamycin. Small, non-mucoid colonies were picked. Insertions were mapped as described (Chapter 3). An isolate with a single forward-oriented insertion 584bp from the start codon of the 1455bp gumD gene was selected for analysis.
Quantification of EPS

EPS was quantified essentially as described by He et al. (27). Briefly, 10 ml bacterial cultures were grown in GYE media for two days at 28°C, reaching an approximate OD$_{600}$ of 2.4. Supernatants were collected by centrifugation at 10,000g for 20 min. EPS was precipitated by adding 2 vol. of ethanol and incubation at $-20$°C for 30 min. The precipitated EPS was harvested by centrifugation, lyophilized, and weighed.

Assays for sensitivity to H$_2$O$_2$, osmotic stress, and phytoalexins

For testing sensitivity to H$_2$O$_2$, cells cultured overnight were washed twice with sterile distilled deionized water and diluted in H$_2$O to OD$_{600}$ = 0.2 (approximately $5 \times 10^7$ cfu/ml). Cells were treated by addition of H$_2$O$_2$ to final concentrations of 20 mM, 60 mM, or 100 mM for 15 min. Viable cells were quantified by dilution plating on PSA (peptone-sucrose agar), with incubation for 3 days at 28°C. Survival rates were calculated as percent of untreated wild type and mutant respectively. For testing sensitivity to osmotic stress, cultured cells overnight in PS medium were diluted and plated on PSA with 100 mM or 500 mM NaCl and incubated at 28°C until colonies appeared (3 to 7 days). Colonies were counted, and the survival rate was calculated relative to the colony number on PSA without NaCl. To test the sensitivity to phytoalexins, a macrodilution assay on plates was used to Six different phytoalexins representing different structural classes were tested. Plumbagin, berberine and resveratrol were found to have inhibitory effects at the concentrations tested and were used for comparative analysis of wild type and mutant. Strains were cultured overnight in GYE, diluted to an OD$_{600}$ of 0.04, then dilute 100 fold, finally 10ul (approximately $10^3$ cells) were plated on GYE agar with plumbagin, berberine and resveratrol at different concentrations,
and incubated at 28 °C for 7 days, the MIC was defined as the lowest concentration necessary to prevent colony formation and was determined empirically in repeated trials by narrowing the range of concentrations used.

**RT-PCR analysis of pathogenesis-related gene expression**

Six-week-old IR24 rice plants were inoculated as described for the virulence assay, above, with bacterial suspensions of approximately $10^8$ cfu/ml. Inoculated rice leaves were collected at 2, 8, 16, 24, and 96 hours post inoculation. RNA was extracted from the inoculated leaves using a modified hot trizol protocol (30) and treated with RNase free DNase I (Invitrogen). RT was carried out using 1 ug RNA in a 20 ul reaction using the superscript reverse transcriptase III (Invitrogen) based on the manufacturer instructions. 2 ul RT product corresponding to 100 ng total RNA and Platinum Taq (Invitrogen) were used for PCR. The primer sets used are listed in Table 2.

**CyaA reporter assay for type III effector delivery into rice cells**

Rice leaves were infiltrated with bacterial suspensions at OD$_{600} = 0.5$ by needleless syringe. 8 hours after inoculation, three replicate samples containing eight inoculated spots each were collected using a number 2 punch, frozen in liquid nitrogen, and ground with motor and pestle to powder. The ground leaf tissues were suspended in 250ul of 0.1M HCl and were centrifuged briefly. The supernatants were diluted 100-fold in 0.1M HCl and assayed with the correlate-EIA cAMP immunoassay kit (Assay Designs, Ann Arbor, MI, USA) based on the manufacturer’s directions. The amount of total protein in each sample for normalization was determined by using the BCA protein assay kit (Pierce).
ACKNOWLEDGMENTS

The authors are grateful to L. Hackman for assistance with mutant screening and technical assistance, to Dr. P. Patil for assistance with the LPS silver staining and to D. Niño-Liu for critical reading of the manuscript. This work was supported by award 0227357 from the Plant Genome Research Program of the National Science Foundation.

REFERENCES


Table 1. Bacterial strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
</table>
| *Xanthomonas oryzae* pv. oryzicola  
BLS303 | Philippines isolate, Ap\(^f\) | J. Leach |
| BLS303*hrcC\(^{-}\) | BLS303 with insertion of pTOPO *hrcC*174-781 in *hrcC*, T3SS-deficient, Km\(^f\) | (38) |
| TN38C10 (BLS303 *wxocB*-\(^{−}\)) | BLS303 containing a Tn5 insertion in the *wxocB* gene, Km\(^f\) | Wang et al. submitted (Chapter 3 of this dissertation) |
| BLS303*gumD*- | BLS303 containing a Tn5 insertion in the *gumD* gene, Km\(^f\) | This study |
| *Escherichia coli*  
DH5\(^{α\,}\) | F\(^{−}/endAl hsdR17(rK-mK+) supE44 thi-1 recA1 gyrA (Nalr) relA1 D(laclZYA-argF)U169 deoR (F80dlacD(lacZ)M15) | Invitrogen, Carlsbad, CA |
| Plasmids | | |
| pCR2.1 | TOPO TA cloning vector, Km\(^f\) | Invitrogen, Carlsbad, CA |
| pLW4 | pCR2.1 carrying the cloned *wxocB* gene from BLS303, Km\(^f\) | This study |
| pUFR047 | Broad host range expression vector, Gm\(^f\) | (15) |
| pWxocB | pUFR047 carrying the cloned *wxocB* gene, downstream of and oriented with lacZ promoter of pUFR047, Gm\(^f\). Original designation, pUFRLW4 | This study |
| pHM1 | Derivative of pRI40 (Innes et al. 1988) containing multiple cloning site of pUC19, broad host range, low copy, Sp\(^f\) | R. Innes |
| pKEB45 | pHM1 carrying *avrXa10* with a 1,232-bp fragment encoding residues 2 to 406 of Cya inserted in-frame in the *SalI* site at bp 2,945 of *avrXa10*, Sp\(^f\) | (38) |

\(^{a}\) Ap, Ampicilin; Km, kanamycin; Rf, rifampicin; Sp, spectinomycin; Gm, gentamycin.
<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers used</th>
<th>Reference</th>
</tr>
</thead>
</table>
| *wxocB* | 5'-aag ctt tcc ggc att tgc tga gtt c-3'  
5'-aag aat tcc gct cat acc act get gat gat c-3' | This study |
| actin | 5'-act gcc tgt gaa tgg tac gtc ac-3'  
5'-aat gcc ttc tca ctt tgg tag g-3' | This study |
| POX22.3 | 5'-acg aca taa aac gcc cac ac-3'  
5'-tag tgt cta atg cca tgg ctg-3' | (63) |
| *PR1* | 5'-tgc ca acct tca cct cca aa-3'  
5'-aat acg get gac gat gac a-3' | This study |
| *EL2* | 5'-gat ctt acg tga agg tgt gc-3' | (18) |
Table 3. Sensitivity of *X. oryzae* pv.oryzicola BLS303 (WT) and TN38C10 (*wxocB*-) to antimicrobial compounds.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Minimum inhibitory concentration (µg/ml)</th>
<th>WT</th>
<th><em>wxocB</em>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Berberine</td>
<td></td>
<td>25</td>
<td>40</td>
</tr>
<tr>
<td>Plumbagin</td>
<td></td>
<td>0.95</td>
<td>0.95</td>
</tr>
<tr>
<td>Resveratrol</td>
<td></td>
<td>46</td>
<td>46</td>
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</table>
FIGURE LEGENDS

Figure 1. Characterization and complementation of the virulence defect of the \textit{wxocB} mutant. Leaves of six-week-old rice plants were inoculated by needless syringe with $10^8$ cfu/ml suspensions of \textit{Xanthomonas oryzae} pv. oryzicola strain BLS303 (WT), TN38C10 (\textit{wxocB}-), or TN38C10 with pWxocB, a plasmid carrying \textit{wxocB}, [\textit{wxocB-(pWxocB)}]. A. Symptoms 10 days following inoculation. Leaves were photographed on a light box, resulting in a translucent yellowish appearance of watersoaked areas (lesions). B. Average lesion lengths of 10 replicate inoculations per strain. C. Bacterial populations in inoculated leaves 0, 4, and 8 days after inoculation (DAI). Values represent averages of 10 replicate inoculations per strain. Error bars in B and C represent standard deviation. These experiments were carried out twice with similar results.

Figure 2. Effect of the \textit{wxocB} mutation on lipopolysaccharide composition. Shown are silver-stained tricine SDS-PAGE profiles of lipopolysaccharide purified from the same strains as in Figure 1. In the WT and \textit{wxocB-(pWxocB)} profiles, the diffuse high apparent molecular weight bands represent intact and partially degraded LPS, and the dark-staining, low apparent molecular weight bands represent major fragments of lipooligosaccharide (LOS), containing lipidA and all or part of the core oligosaccharide. Note in the \textit{wxocB}-profile the absence of the diffuse high apparent molecular weight bands, indicative of loss of the O-chain. An additional low-apparent molecular weight band in this profile likely represents LOS with the initial O-chain subunit attached.
Figure 3. Effect of the \textit{wxocB} mutation on extracellular polysaccharide (EPS) production. Shown are results for the same strains as in Figure 1. A. Colony morphology on glucose yeast extract agar after 4 days and 8 days growth at 28\textdegree{} C. B. Amounts of EPS in supernatants of liquid glucose yeast extract cultures after two days growth at 28\textdegree{} C. Values represent averages of triplicate samples. Error bars represent standard deviation. This experiment was carried out twice with similar results.

Figure 4. Relative virulence of the \textit{wxocB} mutant and a strain devoid of extracellular polysaccharide (EPS). Shown are lengths of lesions caused by \textit{X. oryzae} pv. oryzicola strains TN38C10 (\textit{wxocB}\textsuperscript{-}) and BLS303\textit{gumD::Tn5} (\textit{gumD}\textsuperscript{-}), a strain unable to produce EPS, as a percent of that caused by the wild type inoculated directly opposite on the same leaf. Values represent averages of 10 replicates. Error bars represent standard deviation. This experiment was carried out twice with similar results.

Figure 5. Effects of the \textit{wxocB} mutation on resistance to oxidative and osmotic stress. Survival rate of bacteria strains under different stress conditions. Suspensions of the same strains as in Figure 1 were treated with different concentrations of H\textsubscript{2}O\textsubscript{2} (A) or NaCl (B), as described in Materials and Methods. Survival rates were determined relative to suspensions without treatment, by dilution plating of the suspensions. Values represent averages of triplicate samples. Error bars represent standard deviation. This experiment was carried out twice with similar results.
**Figure 6.** Effect of the *wxocB* mutation on expression patterns of pathogenesis-related genes in rice leaves. Shown are the results of semi-quantitative RT-PCR analysis of expression of *EL2, PR1*, and *POX22.3*, and an actin gene for reference, in rice leaves, at the hours indicated, after inoculation with *X. oryzae* pv. oryzicola strain BLS303 (WT), TN38C10 (*wxocB*-*), or water (Mock).

**Figure 7.** Effect of the *wxocB* mutation on type III protein delivery into rice cells. Rice leaves of susceptible cultivar IR24 were infiltrated by using a needleless syringe with $10^8$ cfu/ml suspensions of *X. oryzae* pv. oryzicola strain BLS303 (WT), TN38C10 (*wxocB*-*), or BLS303*hrcC-* (*hrc*-), a strain deficient in type III secretion, each carrying a plasmid with an *avrXa10::cyaA* translational fusion construct driven by the *lac* promoter (pKEB45), or with the WT strain carrying the empty vector (pHM1). Shown are mean cyclic AMP concentrations for two technical replicates of three replicate leaves for each inoculum. Error bars represent standard deviation. This experiment was carried out twice with similar results.
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**Figure 4.** Relative virulence of the *wxocB* mutant and a strain devoid of extracellular polysaccharide (EPS).
Figure 5. Effects of the wxcB mutation on resistance to oxidative and osmotic stress. Values represent averages of triplicate samples. Error bars represent standard deviation. This experiment was carried out twice with similar results.
Figure 6. Effect of the *wxocB* mutation on expression patterns of pathogenesis-related genes in rice leaves.
Figure 7. Effect of the \textit{wxocB} mutation on type III protein delivery into rice cells.
CHAPTER 5. General conclusions

Bacterial phytopathogens use several different kinds of virulence factors to invade, acquire nutrition, and multiply in plants. Responding to the attack of pathogens, higher plants have developed a series of defense mechanisms to prevent microbial infection, which include lignification of the cell wall, induction of reactive oxygen species, and expression of pathogenesis related genes (3). In incompatible interactions, pathogens are unable to overcome these defense responses, whereas in compatible interactions, pathogens are able to overcome these defenses, and the challenged plant develops disease. My research focused on two important phytopathogenic bacterial species, *Pseudomonas syringae* and *Xanthomonas oryzae*, to investigate mechanisms of virulence and interactions with plant hosts.

GENERAL CONCLUSIONS OF MY STUDY

*Soybean mosaic virus was adapted as new expression vector of soybean*

In the first study, which is described in Chapter 2, in collaboration with others, I transiently expressed the AvrB or AvrPto type III effector proteins of *Pseudomonas syringae* pv. glycinea and pv. tomato, respectively, in soybean by using Soybean Mosaic Virus (SMV) adapted as a vector. Initial step for adapting SMV as an expression vector was adding the cloning site for a foreign gene between the *P1* and *HC-Pro* genes in 35S-driven infectious cDNAs of strains SMV-N and SMV-G7. The next step was to test the expression and stability of this new vector *in planta* using GFP and *uidA* as reporter genes. I found that GFP and *uidA* can be efficiently expressed in plants using the adapted SMV vector, with systemic
spread of the virus and no significant deletion of the transgene. Next, I expressed AvrB and AvrPto using the SMV vector in soybean cultivars Harosoy, which carries the Rpg1-b resistance gene corresponding to avrB, and Keburi or Hurrelbrink, which lack Rpg1-b. From these experiments, I discovered that AvrB blocks symptom development and detectable viral movement in cultivar Harosoy, but enhances and accelerates symptom development in Keburi and Hurrelbrink. These observations emphasized the dual function of avrB in both avirulence and virulence. Unexpectedly, I found that avrPto enhanced viral symptoms in Harosoy, Hurrelbrink, and Keburi. These results were unexpected because avrPto was reported to confer avirulence to P. syringae pv. glycinea when inoculated on Harosoy (2). Finally, I retested the functions of avrPto by inoculating Harosoy with P. syringae pv. glycinea expressing avrPto. Neither hypersensitive reaction nor avrPto-dependent induction of the pathogenesis-related protein 1a (PR 1a) was observed. Restriction of bacterial population growth was not observed either. In fact, when inoculated on Hurrelbrink, avrPto enhanced bacterial multiplication and disease symptoms.

**Novel virulence factor candidates of X. oryzae pv. oryzicola were identified using Tn5 transposon-mediated mutagenesis and quantitative virulence assay**

In Chapter 3, I studied the bacterial pathogen X. oryzae pv. oryzicola, the causal agent of bacterial leaf streak of rice. No major resistance gene for bacterial leaf streak has been reported in rice. To lay the groundwork for control strategies based on interference with bacterial virulence, transposon-mediated mutational analysis of X. oryzae pv. oryzicola was carried out to identify candidate virulence factors. Of 10,000 mutants inoculated on rice
leaves, 21 were confirmed as virulence-impaired using a quantitative assay. Each contained a single transposon insertion in the genome. Among these were several factors associated with virulence in other plant pathogens, including the type III secretion system, the \( \text{rpfG/rfpC} \) two-component regulatory system, LPS, and type IV pili. Importantly, my result is the first reported of a virulence function for type IV pili in a non-vascular plant pathogen. Also, I identified factors not previously associated with plant pathogenesis, but important or implicated in the virulence of animal pathogens, namely, selected enzymes for sugar metabolism, of which some appear also to play roles in binding host substrates at the cell surface. Finally, I isolated mutants affected in fatty acid and aromatic amino acid synthesis, processes that likely contribute to virulence through their roles in basic metabolism. The identification of these virulence factor candidates facilitates our understanding of the virulence mechanisms and pathogenesis of \( X. \text{oryzae pv. oryzicola} \).

**O-chain of lipopolysaccharides of \( X. \text{oryzae pv. oryzicola} \) is required for full virulence and function of other virulence factors**

In chapter 4, I characterized a mutant strain of \( X. \text{oryzae pv. oryzicola} \) that carries a disruption in the \( \text{wxocB} \) gene, which is located in the LPS synthesis locus. This mutant possessed a remarkably reduced virulence while keeping a similar proliferating ability as the wild type strain *in planta*. Structural analysis of the LPS in this strain carried out in collaboration with Dr. Evgeny Vinogradov of the National Research Council, Canada, showed that the O-chain was missing. This mutant also secreted less EPS. Another EPS defective mutant with a disrupted \( \text{gumD} \) gene was included in the study to separate the
functions of LPS from EPS. This EPS defective mutant possessed a moderately reduced virulence but was more virulent than the \textit{wxocB} mutant. These results suggested that the O-chain of LPS contributes to virulence. To understand how the O-chain of LPS contributes to virulence, I compared the survival rate of the \textit{wxocB} mutant to the wild type under different stresses conditions. The results showed that this mutant was more sensitive to osmotic stress but less sensitive to oxidative stress and the phytoalexin berberine. Increased sensitivity to osmotic stress might explain the reduced virulence of the \textit{wxocB} mutant, but it is not knowh whether significant osmotic stress is encountered during a compatible interaction (one that leads to disease) between a plant and a bacterial pathogen. Therefore, I decided to test the efficiency of the type III secretion system in the \textit{wxocB} mutant, perhaps the most important virulence-associated system of most Gram-negative pathogenic bacteria. My results indicated that the efficiency of the T3SS was significantly decreased in the \textit{wxocB} mutant strain.

**OVERALL SIGNIFICANCE**

Bacterial diseases of plants are important. Some of disease may cause the dramatic yield loss. The bacterial blight of rice can cause 70% yiled loss in severe years in south China or India (1). Some of diseases may decrease the market value. When symptoms of citrus canker appear on fruit, the fruit will be used only as the juice orange because of the bad appearance. The understanding the virulence mechanisms will foster the development of new effective methods. To fulfill the efficient disease control to the bacterial disease, creating new tools to study them is important and useful, especially for the plant species which has the limited study tools such as soybean.
Our research established SMV as a new and powerful tool for transient gene expression in soybean, a plant species for which transgenic methods are limited. We successfully characterized the functions of two bacterial effector proteins in soybean using this vector, demonstrating that resistance triggered by \textit{avrB} is effective against SMV, and that \textit{avrB} and \textit{avrPto} have general virulence effects in soybean. These results also led to a re-evaluation of the reported avirulence activity of \textit{avrPto} in this plant.

The identification of candidate virulence factors reported here is an important first step toward elucidating molecules and mechanisms important in disease that may be targeted for the development of novel means of control and prevention. Research on these mutants will facilitate the understanding of virulence mechanisms in \textit{X. oryzae pv. oryzicola}, which is an important pathogen of rice for which disease management methods are limited. Understanding regulatory mechanisms among these virulence factors will shine light on finding new disease management methods. Studies on the \textit{wxocB} mutant strain linked the function of T3SS with the O-antigen of LPS in vivo and reinforced the hypothesis that a complicated regulatory system among virulence factors exists. These results provide a fresh insight into the role of LPS in plant-pathogen interactions by revealing a relationship between LPS and the T3SS. Furthermore, this understanding may also benefit the finding of efficient disease control method.

REFERENCES


APPENDIX A.

Copy number of vector and the genetic background of the host strain influence the expression of specific avirulence genes in *Xanthomonas oryzae* pv. oryzae

*Xanthomonas oryzae* pv. oryzae is the causal agent of rice bacterial blight. In tropical area such as south Asia and Africa, it causes huge yield loss in some years. The main management method for controlling bacterial blight is the use of resistant cultivars. In rice, nearly 30 pathogen race–specific resistance genes have been reported. Based on Flor’s gene-for-gene hypothesis, there may be a corresponding number of pathogen avirulence genes. Right now, only 5 of these R genes and three corresponding avr genes have been characterized. *avrXa10*, one of the earliest cloned avirulence genes from *X.oryzae* pv. oryzae strain PXO86, confers host strain avirulence to rice cultivar IRBB10 carrying the resistance gene *Xa10*. *avrXa10* is one of several *X.oryzae* pv. oryzae avirulence genes in the *avrBs3/pthA* or transcription activator–like (TAL) effector family (2). The cloned *avrXa10* confers upon PXO99A, one virulence race 6 strain, avirulence on Indica rice cultivar IRBB10. pHM1 is a low copy number, broad host range expression vector. pDD62 is a higher copy number broad host vector. In PXO99A, both *avrXa10* cloned in low copy number vector pHM1 and *avrXa10* cloned in high copy number vector pDD62 conferred avirulence to IRBB10 (Figure 1B). When *avrXa10* in pHM1 (pHMavrXa10) was introduced into PXO99A ME10 a mutantstrain disrupted in a distinct TAL effector gene (3), it attenuated virulence toward IRBB10 (B). When it was expressed with the high copy number of plasmid pDD62 (pKEB26), it conferred the IRBB10-specific avirulence function to the strain (Figure 1B). When pHM1vrXa10 was introduced into PXO125, a virulent race 4 strain, it failed to
confer avirulence in IRBB10 (Figure 1A). These observations suggest that the function of \textit{avr} genes of the TAL effector family in \textit{Xanthomonas oryzae pv. oryzae} may be influenced by the genetic makeup of the host strain and the copy number of vectors. TAL effector family members dimerize in the plant cell, and this dimerization is important for function (1). The function of AvrXa10 may depend on or be inhibited by hetero-dimer formation with other TAL effector family members in the plant cytoplast. Overexpression could favor homodimerization.

\textbf{References}


3. \textbf{Sugio, A., B. Yang, T. Zhu, and F. F. White.} 2007. Two type III effector genes of \textit{Xanthomonas oryzae pv. oryzae} control the induction of the host genes \textit{OsTFIIA}\{gamma\}1 and \textit{OsTFXI} during bacterial blight of rice. Proc Natl Acad Sci U S A.
Fig. 1. *avrXa10* confers different phenotypes when introduced into different host strains. **A.** pHMavrXa10 fails to trigger HR on IRBB10 plants when introduced into PXO125 strain. Leaves are shown four days following inoculation with the indicated strains suspensions individually at an OD$_{600}$=0.5. **B.** In PXO99AME10, pHMavrXa10 causes only a weak to null HR on IRBB10 plants; pKEB26 causes HR on IRBB10 plants. Pictures were taken two days following inoculation with the indicated strains suspensions individually at an OD$_{600}$=0.5.
APPENDIX B.

Construction of a $\Delta$hrpG mutant of X. oryzae pv. oryzae PXO99A and complementation with hrpG constructs of X. oryzae pv. oryzae PXO99A and X. oryzae pv. oryzicola BLS256

*Xanthomonas oryzae* pv. oryzae and *Xanthomonas oryzae* pv. oryzicola are important rice pathogens. *X. oryzae* pv. oryzae and *X. oryzae* pv. oryzicola have distinct tissue specificity. *X. oryzae* pv. oryzae enters the plant through hydathodes, colonizes the xylem and causes rice bacterial blight. *X. oryzae* pv. oryzicola enters through stomata, colonizes the parenchyma, and causes rice bacterial leaf streak. In *Xanthomonas*, the expression of hrp genes is tightly controlled by the regulatory genes hrpG and hrpX, which are located outside of the hrp gene cluster (1). The HrpX protein, an AraC-type transcriptional activator, regulates the expression of the genes in the hrp cluster and other effector genes. The expression of hrpX is regulated by HrpG which belongs to the OmpR family of two-component regulatory systems (2). A $\Delta$hrpG mutant of *X. oryzae* pv. oryzae strain PXO99A was constructed through double homologous recombination. This $\Delta$hrpG mutant strain lost its pathogenicity on rice. The hrpG genes from *X. oryzae* pv. oryzae and *X. oryzae* pv. oryzicola were introduced into this $\Delta$hrpG mutant strain, respectively, for complementation. Both of the hrpG genes restored the pathogenicity of the mutant strain.

**Construction of $\Delta$hrpG mutant by double recombination**

The 3,364bp fragment including 1,538 bp upstream sequence of the open reading frame of hrpG, the 792 bp hrpG gene, and 1,034 bp downstream sequence was amplified by
PCR from genomic DNA of *X. oryzae* pv. *oryzae* PXO99A with forward primer 5’-AGGAACGCCCAAATT TGCATTC-3’ and reverse primer 5’-TCGAACTCCAGATTCAGCG-3’. The PCR product was ligated into pCRTOPo2.1 vector (Invitrogen). The internal 1,341 bp AgeI and AatII fragment including the full length *hrpG* ORF was deleted by digestion with these enzymes, fill-in with Klenow, and ligation of the vector-containing fragment. The resulting plasmid was named pLW3. The 1,145 bp XhoI/EcoRV fragment of pCR8/CW-TOPO including a gene encoding spectinomycin resistance and its native promoter was blunted and ligated with the SacII digested and blunted linear pLW3. The resulting plasmid was named pTOPOΔ*hrpG*2 (Fig. 1). Plasmid pTOPOΔ*hrpG*2 was electroporated into *X. oryzae* pv. *oryzae* PXO99A strain and selected on GYE plate with spectinomycin. 100 colonies were picked and transferred onto GYE agar with kanamycin. Six kanamycin-sensitive colonies were picked and infiltrated into rice leaves for the pathogenicity test. All of these six isolates lost the ability to cause disease. One was selected arbitrarily and designated as PXO 99A Δ*hrpG*.

**Complementation with *hrpG* gene from *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola***

pSM1, the pDD62 vector carrying *hrpG* from *X. oryzae* pv. *oryzae* PXO99A strain, and pSM2, pDD62 vector carrying *hrpG* gene from *X. oryzae* pv. *oryzicola* BLS256 strain (S. Makino, M.S. Thesis, Iowa State University) were electroporated into the PXO 99A Δ*hrpG* mutant strain. Each of these two *hrpG* genes restored pathogenicity (Fig. 2).
References


Fig. 1. Map of pTOPOΔhrpG2. Names of genes are indicated on the map. Black arrows indicate ORFs, The white arrow is the replication origin of pUC19. The grey arrows represent promoters of the kanamycin and spectinomycin resistance genes. The grey bar represents the concatenated *X.oryzae* pv. oryzicola (Xoo) genome sequences flanking *hrpG*. The black arrow represents the deleted *hrpG* gene position.
Fig. 2. *hrpG* of vascular and non-vascular *X. oryzae* pathovars functions equivalently in a *X. oryzae* pv. *oryzae* Δ*hrpG* strain. *X. oryzae* pv. *oryzae* strains PXO99AΔ*hrpG*(pSM1) and PXO99A(pSM2) restored development of water-soaking lesions in rice cultivar IR24. Pictures of leaves (on a lightbox) were taken two days after inoculation.
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