THESIS

UNDERSTANDING THE ROLES OF TAL EFFECTORS IN *XANTHOMONAS ORYZAE* INTERACTIONS WITH RICE

Submitted by

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ABSTRACT

UNDERSTANDING THE ROLES OF TAL EFFECTORS IN *XANTHOMONAS ORYZAE* INTERACTIONS WITH RICE

Transcription activator like (TAL) effector proteins are virulence factors that are secreted by *Xanthomonas oryzae.* Some TAL effectors contribute to virulence by activating transcription of plant host susceptibility genes thereby modulating the plant's physiology and creating a more pathogen favorable environment. Some TAL effectors activate transcription of disease resistance genes. Because most *X. oryzae* strains encode many (between 8 and 26) genes for TAL effectors, it is difficult to evaluate the function of individual TAL in the plant-pathogen interaction. In this study, we introduced the use of a TAL deficient strain of *X. oryzae* that allows study of individual TAL effectors. We demonstrated that the TAL deficient strain could deliver TAL to rice, and that subtle differences in TAL virulence functions could be measured in interactions with rice when delivered by this strain.

Plants have evolved resistance genes that detect or recognize TAL effectors and activate resistance responses. Using the TAL deficient *X. oryzae* delivery system, we isolated a TAL that activates resistance in plants which are homozygous for the recessive resistance gene *xa5*. The TAL, Avrxa5P$_{86}$, is predicted to target many transcription factors. Avrxa5P$_{86}$ not only interacted with *xa5* to confer resistance, but also exhibited a novel resistance interaction with Azucena and Nipponbare, both homozygous for the *Xa5* allele. Our discovery of Avrxa5P$_{86}$ is of presents a novel interaction that raises new evolutionary questions about TAL effectors and/or resistance genes in rice.

TABLE OF CONTENTS

CHAPTER ONE

INTRODUCTION: A RECOVERY PLAN FOR *XANTHOMONAS ORYZAE* CAUSING BACTERIAL BLIGHT AND BACTERIAL LEAF STREAK OF RICE*

Executive Summary

Bacterial blight (BB) and bacterial leaf streak (BLS) are the two most important bacterial diseases of rice worldwide. The diseases are caused by two pathovars of *Xanthomonas oryzae*: *X. oryzae* pv. *oryzae* (*Xoo*) that causes BB, and *X. oryzae* pv. *oryzicola* (*Xoc*) that causes BLS. *Xoo* and *Xoc* are not found in the USA. A third group of *X. oryzae*, referred to herein as *Xo*-USA, is found in LA and TX in the USA, causes very weak disease symptoms that resemble BB, is genetically distinct from *Xoo* and *Xoc*, and currently has no pathovar designation.

Xoo and *Xoc* are widely distributed and endemic in many countries in Asia, Africa and Australia, but they have not been found in North America. There are sporadic and/or single reports of *Xoo* in several other rice producing countries, but these have not been systematically verified.

Alternate hosts for both pathogens include weed species commonly found in rice production systems, including *Leersia* spp. and wild rice species (*Oryza* spp.). In only a few cases have Koch's postulates been performed to demonstrate that other weed species are indeed symptomatic or asymptomatic hosts for *Xoo* and *Xoc*.

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Xo-USA, *Xoo* and *Xoc* can be reliably distinguished from one another using PCR-based approaches, and improved protocols for detecting the pathogens in seed- and plant-tissue are being evaluated internationally.

Control of BB and BLS are typically through genetic resistance. BB is most effectively controlled through the use of qualitative resistance governed by single resistance genes. Changes in the race structure of *Xoo* populations can render *R* genes ineffective, so there are continual efforts to identify new sources of resistance, including sources of quantitative resistance. To date, the sources of qualitative resistance for BLS are very limited, and no race structure for *Xoc* has been reported. Thus, most sources of resistance for BLS are quantitative.

Rice is an important commodity for USA agriculture, valued at approximately $2.63 billion in 2011. To protect this important commodity, quarantine efforts are in place to prevent entry of BB and BLS into the USA. As BB and BLS have not occurred in the USA, there have been no concerted efforts to incorporate resistance to these diseases into widely used USA germplasm. Over 30 single gene resistance sources are available for controlling BB, and a few sources of QTL-based resistance for both BLS and BB. Judicious use of resistance, however, requires understanding of the local pathogen populations to be controlled. Thus, if *Xoo* or *Xoc* were detected in the USA, a first critical effort would be to identify effective sources of resistance. To date, this can only be accomplished by analysis of virulence spectrum through plant inoculation.

The best protection of the USA rice industry from BB and/or BLS will be achieved by exclusion through effective statutory quarantines, early detection, and eradication by host

destruction. Identification and development of resistant germplasm and improvement of detection and race monitoring tools are key components of this recovery plan.

Recommended Actions:

1. Develop improved field-level detection tools, seed-detection protocols, and certification approaches. These must reliably distinguish *Xoo* and *Xoc* from each other, and from other *Xo* and *Xanthomonas* species.

2. Improve tools for rapid and accurate characterization of the race structure of the *Xoo* pathogen population. Understanding the effector repertoire is important to knowing what *R* gene combination will be effective in controlling disease [65, 31].

3. Educate and train extension personnel, growers and crop advisors in the symptomatology and detection of BB and BLS in field conditions.

4. Assess key germplasm used in the USA in countries where BB and BLS are indigenous, to screen for resistance. An important caveat is that the resistance sources detected and integrated may not prove effective against the specific race introduced.

5. Improve genetic resistance by incorporating widely effective *R* genes, and identifying and incorporating sources of broad-spectrum resistance (effective against both *Xoo* and *Xoc*, and effective against all races of *Xoo*). This could be through novel transgenic approaches or through the introgression of novel QTL-based resistance.

6. Adopt uniform detection/diagnosis protocols among quarantine agencies worldwide.

7. Develop the physical resources to test, conserve, store, and maintain strains or DNA of *Xo* pathogens in the USA.

INTRODUCTION

Xanthomonas oryzae is currently classified by two pathovars based on symptoms on the same host (rice, *Oryza sativa*). *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) causes bacterial blight (BB, formerly called bacterial leaf blight, BLB) and *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*) causes bacterial leaf streak (BLS). Several excellent reviews of the two diseases and the pathogens that cause them are available [40,49,42,53]. Of the two diseases, BB is currently the more economically important [41]. Under disease-conducive conditions and in rice hosts with ineffective resistance, BB can cause yield losses up to 70% [55,42], although more typical reports range from 20-50% [53]. Collectively, because of its economic impact and its role as a well-established model system, *Xoo* has been ranked in the top-10 list of bacterial plant pathogens [39].

Relative to BB, BLS disease caused by *Xoc* is less widespread, occurring in tropical and subtropical regions of Asia, Africa and Australia [16], and is less severe, with losses usually ranging between 10-20%. However, in recent years, the disease has been observed with increasing frequency and wider distribution in Asia and Africa, likely due to the planting of susceptible varieties, including new high-yielding hybrids, and possibly, as a result of a changing environment [76,63,16,75].

BB and BLS have not been found in rice in the USA. Rice is an important commodity for USA agriculture. In 2011, rice production in the USA was valued at approximately $2.63 billion, half of which was exported (USDA National Ag Statistics Service, 2012). Rice production in the USA occurs on more than 2 million acres in Arkansas, California, Louisiana, Mississippi, Missouri, and Texas. Thus, with the value of rice, the potential for introduction of BB or BLS into the USA is of great concern.

In the 1980s, there was concern that BB had entered the USA. Outbreaks of a disease with weak symptoms that were similar to BB occurred in Texas and Louisiana [25]. Yellow pigmented, Gram negative bacteria isolated from infested leaves were shown to be *Xanthomonas*, and these bacteria caused weak BB-like lesions on a few susceptible rice varieties [25]. Yield losses were less than 1 %. Although the organism was diagnosed as *X. campestris* pv. *oryzae* (note that *Xoo* was previously named *X. campestris* pv. *oryzae*), the authors emphasized that the organism and the disease it caused were clearly distinct from BB caused by Asian strains of *Xoo* [25]. The USA strains were much less virulent than the Asian *Xoo* or *Xoc,* and the symptoms caused by the USA strains were similar among each other but different from those caused by Asian *Xoo*. The genomic fingerprint of the USA strains, as detected by restriction enzyme digestion of genomic DNA, or Restriction Fragment Length Polymorphism analysis using IS elements or an avirulence effector gene as probes, is clearly distinct from the ones reported for Asian and African *Xoo* and *Xoc* strains [25,33,16]. Later, using draft genome sequences in a comparative analysis, the bacterial pathogen from the USA rice was confirmed to be distinct from *Xoo* and *Xoc,* but declared to be within the species *Xanthomonas oryzae* (more below)[64].

Relationships of *X. oryzae*

Pathovars *Xoo* and *Xoc* are highly related, with over 85% DNA homology, and they are distinguished by only a few phenotypic features [68]. Presently, complete genome sequences for three strains of *Xoo* - Japanese strain MAFF311018 [51], Korean strain KACC10331 [34], and Philippine strain PXO99A [60] - and one strain of *Xoc,* Philippine strain BLS256 [9], are available. Draft genome sequences of two *Xo*-USA strains are published [64]. Phylogenetic analyses using these sequences defined three major genetic lineages among the species *X. oryzae*: Asian strains

of *Xoo,* African strains of *Xoo*, and *Xoc* (from Asia and Africa) [20,16]. The *Xo*-USA are now grouped into a fourth genetic lineage of *X. oryzae* [64] but these strains are not yet designated a pathovar [64].

Pathogenic specialization

Xoo, but not *Xoc,* is characterized by a high degree of physiological race-cultivar specificity; races are classified by inoculation to a standard differential set of rice cultivars that contain single BB resistance genes (*Xa* genes) in the same rice genetic background [40,52]. For example, the differential set of near isogenic lines designated as IRBB contain a single *Xa* gene designated by the gene's number, e.g., IRBB10 contains the BB *R* gene *Xa10* [52]. Race designation is built from the complement of effector (avirulence) genes in the pathogen and the *R* genes in the host differential. This is consistent with the fact that as *Xoo* acquire or lose effector gene function, which happens frequently in the field, sources of resistance may no longer be effective [48,47,66,45,65,54,72].

Distribution

Xoo is widely distributed throughout rice growing countries in Africa (Benin, Burkina Faso, Cameroon, Egypt, Gabon, Gambia, Mali, Niger, Nigeria, Senegal, and Togo), Asia (Bangladesh, Cambodia, China, India, Indonesia, Iran, Japan, Korea, Laos, Malaysia, Myanmar, Nepal, Pakistan, Philippines, Sri Lanka, Taiwan, Thailand, and Vietnam), and Oceania (Australia) (CABI, 2011; EPPO, 2007). Although a few old reports describe *Xoo* in Mexico and parts of Central and South America, consistent and validated reports from those areas, particularly within the past 30 years, are lacking, suggesting the disease is not endemic in those areas [38,80,53]. Rice

with symptoms similar to BB were first reported in the United States (Texas and Louisiana) in the late 1980s [25]. However, molecular and genomic methods confirmed that these symptoms were caused by an undesignated pathovar of *Xo*, and not by *Xoo* or *Xoc* [30,57,64]. Currently, the *Xo*-USA strains are known to be present only in the United States.

The distribution range of *Xoc* includes Africa (Burkina Faso, Madagascar, Mali, Nigeria, and Senegal), Asia (Bangladesh, Cambodia, China, India, Indonesia, Laos, Malaysia, Myanmar, Nepal, Pakistan, Philippines, Thailand, and Vietnam), and Oceania (Australia) (CABI, 2012; EPPO, 2007).

Alternate hosts

The primary host of the *Xanthomonas oryzae* pathovars is rice (*Oryza sativa*). In Asia, perennial weeds are considered a possible source of inoculum [53]. These minor hosts can be monocots such as wild rice (*Oryza* spp.) and wild grasses of the *Poaceae* family, Bermuda grass (*Cynodon dactylon*), sedges (*Cyperaceae*), small-flowered nutsedge (*Cyperus difformis*), purple nutsedge (*Cyperus rotundus*), barnyard grass (*Echinochloa crus-galli*), southern cut grass (*Leersia hexandra*), rice cutgrass (*Leersia oryzoides*), Chinese sprangletop (*Leptochloa chinensis*), red sprangletop (*Leptochloa filiformis*), Guinea grass (*Panicum maximum*), ricegrass paspalum (*Paspalum scrobiculatum*), buffelgrass (*Pennisetum ciliare*), grasses (*Poaceae*), tall panicum (*Urochloa mutica*), annual wildrice (*Zizania aquatica*), northern wild rice (*Zizania palustris*), and zoysiagrass (*Zoysia japonica*) [53]. The *Xo*-USA pathovar has been isolated from *Leersia* spp., a weed that can serve as a host in the southern United States (Louisiana and Texas) [17].

Wild hosts of *Xoc* that have been reported include southern cut grass, grasses (*Poaceae*), annual wildrice, red sprangletop, ricegrass paspalum, northern wild rice, and zoysiagrass, although their significance in the life cycle of the pathogen is not known [53]; (Wonni, Detemmerman et al. in preparation).

SIGNS AND SYMPTOMS

The disease cycle of bacterial blight is shown in Figure 1.1. Despite the similarities of *Xoo* and *Xoc,* the two pathogens enter and reproduce in very different rice tissues. *Xoo* is a vascular pathogen [61,62]. It can enter the vessels directly through wounds generated during transplanting or by the wind-driven rains during typhoons. Alternatively, *Xoo* can gain access to vessels by moving with guttation fluids through natural openings called hydathodes, water pores located on the edges of rice leaves [19,43,62]. Once *Xoo* has entered the epithem, the chamber beneath the water pore, the bacteria multiply, move through the vascular pass and into the xylem vessels where they multiply and spread throughout the vascular system [62]. *Xoo* accumulates in high numbers in advance of lesions [6,70]. Younger plants are very susceptible to *Xoo*, particularly because of injuries caused during transplanting or by typhoons [41].

BB usually develops in the field at the tillering stage of rice plants. The first symptom of the disease is a water-soaked spot near the margins of fully expanded leaves [42]. BB lesions have a wavy margin and expand through the vascular tissue of the plant. The lesions rapidly enlarge in length and width along the veins, merging into wavy, elongated lesions. Older lesions appear as bleached white to straw colored necrotic areas that may cover most of the leaf (Figure 1.2A, C). The symptoms may be difficult to distinguish from physiological problems such as saline toxicity

and drought sensitivity.

If infected at the seedling stage, a 'kresek symptom' may occur from 1 or 2 weeks after transplanting; in this case, the diseased leaves become greyish-green in color and fold then roll up along the midrib; the plants die within 2 to 3 weeks. Plants that survive kresek appear stunted in height and are overall yellowish-green in color [41,42]. A pale yellow leaf symptom may also occur under highly favorable conditions, usually occurring on the youngest leaf of infected tiller; however, no *Xoo* can be isolated from these leaves. This could be attributed to accumulation of the bacteria at the bottom of the stem or it could be due to toxin produced by *Xoo*, although no toxins have been verified to be produced by the pathogen [44,45]. Movement between leaves and plants occurs as bacterial exudates are blown by strong winds or splashing rains, or as leaves rub against one another. The *Xo* pathovars may also be present in asymptomatic tissue, passively multiplying for a quorum to initiate the synthesis of virulence factors and start a pathogenic function [70,23,85,6].

In case of severe BB infection, yellow bacterial exudates are visible in the guttation fluid, which oozes from the leaves' natural openings in the morning. These bacterial exudates form dried up clumps of bacteria. Exudates may also become a secondary source of inoculum as they are moistened by high local humidity or precipitation. A yellowish stream emerging from the lower end of the lesion of a cut, infected leaf when placed in a tube with water is indicative of *Xoo* presence [53].

In contrast to *Xoo, Xoc* is an intercellular pathogen that enters plants either through wounds or by invading the open stomata [41,77,46] (Figure 1.3). Once inside the plants, *Xoc* multiplies between the mesophyll parenchyma cells, spreading up and down the leaf between the vascular bundles. *Xoc* can invade the host plant xylem tissue, but only at later stages of infection, when multiplication is limited [41]. BLS lesions may begin anywhere on the leaf between the veins as water soaked symptom and extend generally lengthwise throughout the leaf. Older lesions may extend over veins. The BLS lesion margin is characterized by fine water-soaked streaks.

The progression of BB or BLS lesions is determined by the susceptibility or resistance of the cultivar of rice. In areas where both *Xoo* and *Xoc* occur, BB and BLS symptoms may be present on the same leaf, which can complicate diagnosis [41].

Currently, it is not known if *Xo*-USA is a xylem-limited or an intercellular pathogen, although based on phenotype, it is predicted to be a xylem-limited pathogen [70]. Symptoms of susceptible rice infected with an *Xo*-USA strain begin as water-soaked lesions, typically associated with adult leaf margins [25]. Lesions turn chlorotic yellow, then necrotic (tan to white). Early in disease, the lesions are wavy, but mature lesions are vein delimited, and bounded by a necrotic red-brown stripe [25].

SPREAD AND RISK MAP

Leaves infected with both *Xoc* and *Xoo* exhibit exudates from lesions. Leaves with lesions and/or exudates may fall into the irrigation water of the flooded field, enabling pathogen spread. Irrigation water from one field can move the pathogen into another field, although free bacteria

(outside of the leaf) do not survive long in the irrigation waters. Strong winds associated with rainstorms or typhoon also spread the bacteria to healthy leaves of host plants near-by the infected plant and may also wound the plant to allow an infection of the pathogen. Previously infected rice stubble may also serve as a source of inoculum [53].

Xoo is reported to overwinter on alternate hosts [53,40]. *Xoo* has been reported to survive in leaves in the soil from 1 to 3 months depending on humidity and acidic properties of the soil [53]. Infected leaf straw may also serve as inoculum for *Xoo* [53]. Humans walking through a field may also move exudates from an infected leaf to healthy leaf tissue.

While both *Xoo* and *Xoc* can be associated with the rice seed coat, only *Xoc* has been confirmed to be both seedborne and seed-transmitted [44,78]. The evidence that *Xoo* is seed-transmitted is controversial, and the epidemiological significance of *Xoo* for seedborne transmission has not been determined [44,59,13,67]. Pathogen-related symptoms of *Xo* are not observed on plants grown from infected seed; this may be due to a decline in bacterial populations during soaking of the seed in water prior to sowing [13,28]. Although reported, dissemination following insect infestations or by birds has not been confirmed.

Risk maps for *Xoo* and *Xoc* in the USA that were developed by NAPPFAST are shown in Appendix.

DETECTION AND IDENTIFICATION

Differentiating among the *X. oryzae* pathovars is impossible by colony appearance, as they all form bright yellow, mucoid colonies (Figure 1.4). Furthermore, there are non-pathogenic xanthomonads on rice leaves and seeds that are similar in appearance, and easily mistaken for *X. oryzae* [67]. The most definitive method for differentiation of the *Xo* pathovars is by microbiological and molecular tests [67].

The simplest method to distinguish which pathovar of *X. oryzae* is present in infected leaves is by studying the symptoms (Figure 1.2). However, if symptoms are observed on a late stage of infection, identifying the causal pathovar is difficult.

Protocols from the European and Mediterranean Plant Protection Organization are currently recommended to isolate the bacterium from tissue or suspected seed (EPPO, 2007). However, a recently published set of protocols using rigorously tested methods for isolation and diagnosis, particularly from seed, should be considered for adoption [67].

After the bacterium is isolated, pathogenicity tests can be performed to distinguish the pathovars *Xoo* vs. *Xoc* vs. *Xo*-USA, which cause BLS, BB, or BB-like symptoms, respectively. To assess BLS causing *Xoc*, leaf infiltration (Figure 1.5) or mist inoculation are performed [56,79]. Leaf infiltration is used as a qualitative means to study pathogenicity of *Xoo* or the *Xo*-USA strains [56], while the leaf clip inoculation method provides a quantitative measure of pathogenicity [27] (Figure 1.5). It is important to use a susceptible cultivar of rice when evaluating pathogenicity. For

example, many studies use Nipponbare, IR24, Kitaake, and Azucena as susceptible hosts for *Xoo*, while Kitaake is frequently used for *Xoc*.

Several diagnostic tools are currently available for identification of *Xoo* and *Xoc*, and for *Xo*-USA. In the late 1980s, a set of monoclonal antibodies was developed and widely used for diagnosis and distinction of *Xoo* and *Xoc* [7,4,3,5,15].

More recently, emphasis turned to DNA-based approaches to distinguish *Xoo* and *Xoc.* Early approaches involved amplification of the 16S rDNA followed by digestion with restriction enzymes [29,73]. However, as 16S rDNA sequences exhibit 98.6% similarity within the genus *Xanthomonas* [22], this approach cannot accurately distinguish these two *Xo* pathovars. The approach is only useful if supported by other sequence information such as the 16S-23S rRNA internal transcribed spacers. Primers based on the 16S-23S rDNA spacer region were designed for *Xoo*, but their design and testing were based on *Xoo* isolates from only one country, and did not include *Xoc* isolates [1]. Hence, the reliability of these primers for accurate identification of geographically diverse collections is unknown. Repetitive DNA sequences, usually insertion sequence (IS) elements [32,58], can differentiate *Xo* pathovars from each other and from other *Xanthomonas* species by polymorphic hybridization [32] or PCR-amplification patterns [59,2,69]. However, the high degree of diversity of *Xanthomonas* isolates within and between countries, partially driven by movement of these mobile elements, complicates the analysis of patterns for diagnosis.

Early PCR-based assays developed for single gene targets, e.g., a membrane fusion protein [26], a putative siderophore receptor, and a *hrpF* gene common to *X. campestris* species [8,84], while potentially reliable, were not validated on a diverse and wide array of strains.

More recently, a multiplex PCR with pathovar-specific primers, designed by *Xoo*/*Xoc* genome comparison, was developed [30] and is widely used in detection and diagnosis of *Xoo* and *Xoc* [74] (Wonni, Detemmerman et al. in preparation). Additional primers were developed to distinguish the *Xo*-USA from Asian and African *Xoo* and *Xoc* [64]. Since the primers are based on comparative genome analyses, these primers are highly specific to the *X. oryzae* pathovars.
Currently used methods of detection and diagnosis for *Xoo* do not differentiate the races of the pathogen. Determining race structure for *Xoo* is important because it informs the specific resistance genes to be deployed to control the disease (see below). Determination of race is still best achieved by inoculation of rice differential hosts that contain single BB resistance genes (*Xa* genes).

Near-isogenic lines (NILs) in the *indica* rice IR24 background (also known as IRBB lines for International Rice-Bacterial Blight) were developed at IRRI (International Rice Research Institute) and are commonly used to identify *Xoo* races [52]. Each NIL carries one specific resistance gene (*Xa* gene), which was incorporated into the recurrent backcross parent IR24 by conventional breeding techniques and/or using Marker-Assisted Selection (MAS). Similar NIL sets are available in *japonica* and *indica-japonica* genetic backgrounds [24,35,50]. New *Xoo* races are continuously being reported in countries where BB is endemic and are usually identified because they overcome deployed *R* genes. The development of a universal set of rice NILs and

utilization of a set of *Xoo* reference strains for race typing on a global scale are needed to characterize and compare existing or emerging *Xoo* races.

Many seed-testing methods have been developed for diagnosis of *X. oryzae* pathovars, including growing-on tests [44], host inoculation with seed washings [78], semi-selective media [15] and serological assays [7]. These methods are time-consuming and often lack the needed sensitivity or specificity for routine seed testing. For example, the growing-on test, which involves detecting symptoms of infection from seeds sown on sterile soil, sand or water agar and allowing them to germinate, while uncomplicated, is relatively insensitive. Direct plating of seed extracts on semi-selective medium is usually not sensitive enough for detecting low pathogen levels because both bacteria grow slowly and are easily overgrown by the saprophytic flora (6). Nucleic acid-based methods that use PCR offer greater sensitivity and a shorter response time than conventional assays [30].

One method that has been recently adopted for plant pathogen diagnostics is loop-mediated isothermal amplification (LAMP). LAMP is isothermal and can be performed in a heating block or water bath thereby removing the need for specialized equipment, and allowing for implementation in the field. Adaptation of LAMP to *Xo*-USA*, Xoo,* and *Xoc* is in progress to facilitate rapid, accurate and sensitive detection and diagnosis as well as field surveys (Lang et al., *unpublished results*).

RESPONSE

While this plan is focused primarily on recovery, response to a new disease detection involves a continuum of activities from response to recovery. The response is under USDA, APHIS, Plant Protection and Quarantine's authority delegated from the Secretary under the Plant Protection Act of 2000.9

The ultimate authority for confirming a diagnosis of the disease rests with the Plant Protection and Quarantine (PPQ) division of APHIS: http://www.aphis.usda.gov/ppq. As such, this agency must use the most efficient and effective means to diagnose and differentiate *Xo* pathovars. At least two independent diagnostic methods are recommended to confirm the presence of *Xoc* or *Xoo.*

After a detection of *Xoo* or *Xoc* is confirmed by a USDA, APHIS, PPQ recognized authority, APHIS, in cooperation with the State Department of Agriculture, is responsible for the response. The response is immediate in the form of advance assessment teams of experts and survey personnel sent to the site of initial detection to place holds, conduct investigations, and initiate delimiting surveys. Actions that may be taken include regulatory measures to quarantine infected or potentially infected production areas, stop the movement of infected or potentially infected articles in commerce, and control measures which may include host removal and destruction, and/or insuring adherence to required sanitary practices. APHIS imposes quarantines and regulatory requirements to control and prevent the interstate movement of quarantine-significant diseases or regulated articles, and works in conjunction with states to impose these actions parallel to state regulatory actions which restrict intrastate movement.

The presence of the *Xo*-USA strains, which are indigenous to Louisiana and Texas, and which produce symptoms resembling BB argues that considerable care must be made to avoid raising unnecessary concern. The *Xo*-USA has not been a threat to rice grain yield. Once a sample displaying BLS or BB symptoms is confirmed as positive for *Xoo* or *Xoc* by an APHIS recognized authority, an advanced technical team may be sent to the site as the first step in a response. A larger team would then be deployed, consisting of state and federal regulatory personnel operating under a unified command within the Incident Command System. Survey teams will conduct delimiting surveys in the area using trace back and trace forward information and with various appropriate stratified delimiting sampling schemes for surveys in the area of detection. It is important as part of the response to control the movement of diseased host tissues of infested counties since this may serve as an avenue to infect other rice production areas.

After the results of the delimiting survey are known, if the disease is considered generally distributed through commercial rice in an area, options for control are very limited. If the disease is isolated to a small area, eradication may be effective if all infected rice plants and grain are destroyed.

While rice germplasm with bacterial blight and bacterial leaf streak resistance is available, most US germplasm is susceptible to both diseases. The USDA World Collection has approximately 18,000 entries of which some are known sources of resistance. However, no recent screening has been done on the collection. Resistance sources are available in the International Rice Research Institute's T.T. Chang Genetic Resources Center which houses the world rice collection of over 120,000 accessions. Plant quarantine restrictions and the strict quality standards

of US rice will delay the incorporation of resistance into the US germplasm. No breeding program is actively incorporating resistance to these diseases into the US lines.

There are no active surveys to detect BB or BLS in the US. Current monitoring programs are limited to training cooperative extension service personnel on the existence of these diseases, proper sample collection, and identification techniques.

USDA PATHOGEN PERMITS AND REGULATIONS

USDA/APHIS/PPQ permit and registration requirements for plant diseases and laboratories fall under two authorities, the Plant Protection Act (7 CFR Part 330) and the Agricultural Bioterrorism Protection Act of 2002 (7 CFR Part 331). Laboratories receiving suspect infected plant material or cultures are required to have PPQ permits. Laboratories possessing, using, or transferring select agents such as *Xanthomonas oryzae*, the causal agents of BLS and BB and the weak BB-like disease found in the USA, are required to be registered. Diagnostic laboratories that identify select agents are exempt from this requirement as long as they complete an APHIS/CDC Form 4 and destroy the culture within 7 days.

The Plant Protection Act permit requirements apply to all plant pests and infected material, including diagnostic samples, regardless of their quarantine status, which when shipped interstate require the receiving laboratory to have a permit. For further guidance on permitting of plant pest material, consult the PPQ permit website at: http://www.aphis.usda.gov/ppq/permits/ or contact PPQ Permit Services at (301) 734-8758.

The Agricultural Bioterrorism Protection Act of 2002 (7 CFR Part 331) specifies requirements for possession, use, and transfer of organisms listed as select agents such as the *Xanthomonas oryzae* pathovars. Once an unregistered diagnostic laboratory identifies a select agent, they must immediately notify the APHIS Select Agent Program, complete an APHIS/CDC Form 4 within 7 days, and either destroy or transfer the agent to a registered entity within 7 days. In compliance with this Act, if a diagnostic laboratory holds back part of a screened sample or culture for voucher purposes, and that sample, when forwarded to the USDA Beltsville Laboratory, comes back as positive for a select agent, the diagnostic laboratory is required to notify the APHIS Select Agent Program immediately. This must take place within seven (7) days of results notification and a PPQ Officer must be given the opportunity to witness the destruction of the sample or culture within that time period. Clarification of this and other information related to adherence to the select agent regulations is available on the following APHIS website: http://www.aphis.usda.gov/programs/ag_selectagent/index.html, or call (301) 734-5960.

ECONOMIC IMPACT AND COMPENSATION

Crop insurance covers production losses due to BB and BLS if the losses are unavoidable and result from naturally occurring events during the insurance period. Producers must follow good farming practices, and should work with agricultural experts and document all actions to control and manage the diseases.

The Risk Management Agency (RMA) defines what constitute good farming practices. To determine if producers followed good farming practices, agricultural experts answer, at least, the following questions: Will the control measure:

1) allow the insured crop to make normal progress toward maturity?
2) produce at least the yield used to determine the production guarantee?
3) not reduce or adversely affect the yield?

The answers to these questions must be "Yes." If the answer to any of the above questions is "No," RMA may not consider the control measure as a good farming practice. RMA does not consider the cost or economics of the control measure in determining good farming practices. RMA recommends that producers document their actions and the data they used in making their decisions, including data from:

- Local weather stations;
- Farm Service Agency (FSA) reports;
- Published articles in newspapers, newsletters, magazines, and Web information from:
    - Land grant universities;
    - Extension Service;
    - Crop consultants; or
    - Other agricultural experts
- Journals and logs that list the date of control measures, application method(s), product(s) (include labels), and conditions, etc.

RMA does not prevent producers from mitigating their losses and taking care of their crop as they see fit. Insurance covers losses due to unavoidable circumstances during the insurance period, assuming the producer followed good farming practices. Production losses due to bacterial leaf streak are covered by the insurance.

MITIGATION AND DISEASE MANAGEMENT

Any disease mitigation strategy that is employed should be coordinated with federal, state and local regulatory officials.

Chemical control measures are available [10,12], but their use and effectiveness are limited by cost and high variability in response or susceptibility among strains [14,12,18,81]. Recommended cultural controls include field sanitation, drainage, plant spacing, and fertilizer management [36].

The rice germplasm system in the United States is one of the most secure in the world. No foreign-produced rice is allowed for direct planting in USA fields. Small amounts may be allowed for research purposes only after going through strict quarantine procedures that may include inspection of the seed, hot water treatment, de-hulling, surface sterilization, and growing out of contaminated-free seedlings in a quarantine greenhouse. Rough rice (with hulls), brown rice, and white rice are allowed in the United States for consumption. Most of the germplasm in the United States is not evaluated for BB and BLS because these diseases are not known to occur in the USA.

The most reliable means of controlling BB is through the use of resistant germplasm. In rice producing areas where the disease occurs, several sources of single gene resistance that can control BB are available. The currently available *R* genes (Table 1.1) were recently reviewed [71]. Deployment of appropriate genes requires an understanding of the race structure of the invading *Xoo* population; without such knowledge, it would be impossible to predict which *R* genes would be effective. Breeding programs in the US have not focused on introduction of resistance to BB

into germplasm because the risk of disease is not considered high. However, introduction of available genes into USA rice varieties through breeding can produce resistant varieties in 4-8 years. Efficient genetic transformation techniques are available for rice, but very few BB *R* genes have been cloned, and their effectiveness against the *Xoo* population (races) needs to be evaluated. New genome editing technologies using engineered nucleases are allowing for novel approaches to developing disease resistance that is not considered transgenic [37].

Currently, no single rice resistance gene source is available that can control BLS; the only resistance sources from rice are multi-genic or quantitative resistance [11,21,63]. An *R* gene, *Rxo1,* which is effective against Asian and some African *Xoc* populations, was identified from maize [82,83,16]. This gene functions in rice, and, if introduced into local varieties by biotechnology approaches, can be useful in controlling BLS, given that the pathogen population carries the corresponding *avrRxo1* effector [82].

RESEARCH, EDUCATION, AND EXTENSION PRIORITIES

The following lines of research are needed to enhance detection and management of BB and BLS. They would improve our ability to block the entrance, detect the presence, and help manage the impact of BB and/or BLS. The research priorities are listed according to their relative importance.

1. Develop improved field-level detection tools, seed-detection protocols, and certification approaches. These must reliably distinguish *Xoo* and *Xoc* from each other, and from other *X. oryzae* and other *Xanthomonas* species.

2. Improve tools for rapid and accurate characterization of the race structure of the *Xoo* pathogen population. Understanding the repertoire of avirulence effectors is important to knowing what *R* gene combination will be effective in controlling disease [65,31].
3. Educate and train extension personnel, growers and crop advisors in the symptomatology and detection of BB and BLS in field conditions.
4. Assess key germplasm used in the USA in countries where BB and BLS are indigenous, to screen for resistance. An important caveat is that the resistance sources detected and integrated may not prove effective against the specific race introduced.
5. Improve genetic resistance by incorporating widely effective R genes, and identifying and incorporating sources of broad-spectrum resistance (effective against both *Xoo* and *Xoc*, and effective against all races of *Xoo*). This could be through novel transgenic approaches or through the introgression of novel QTL-based resistance.
6. Adopt uniform detection/diagnosis protocols among quarantine agencies worldwide.
7. Develop the physical resources to test, conserve, store, and maintain strains or DNA of *X. oryzae* strains in the USA.
8. Determine the feasibility of bacteriophage for biocontrol. Phage can be easily selected, propagated, and mutated to overcome any developed resistance.

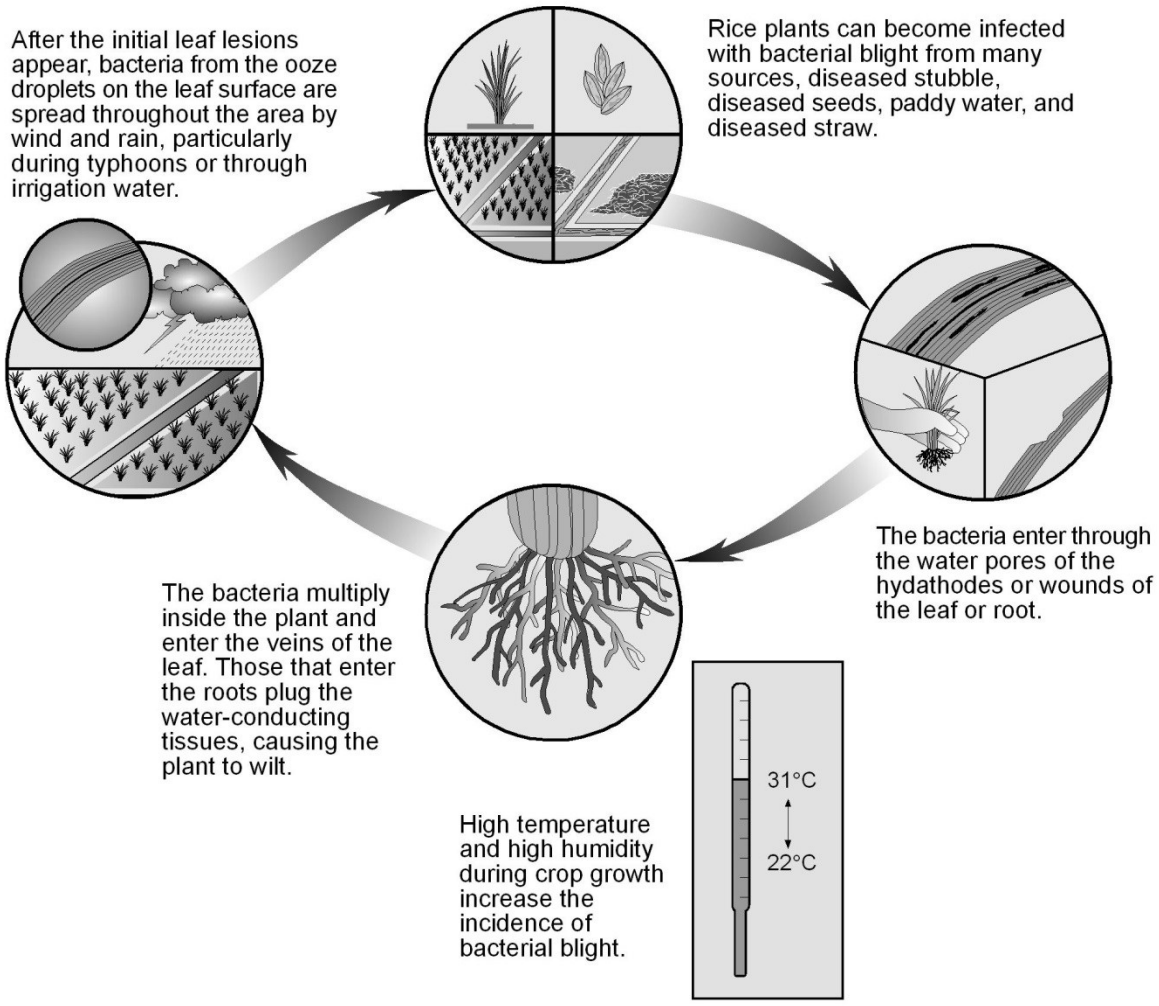

Figure 1.1. Disease cycle of bacterial blight of rice caused by *Xanthomonas oryzae* pv. *oryzae.* Reprinted from [67] with permission from APS Press.

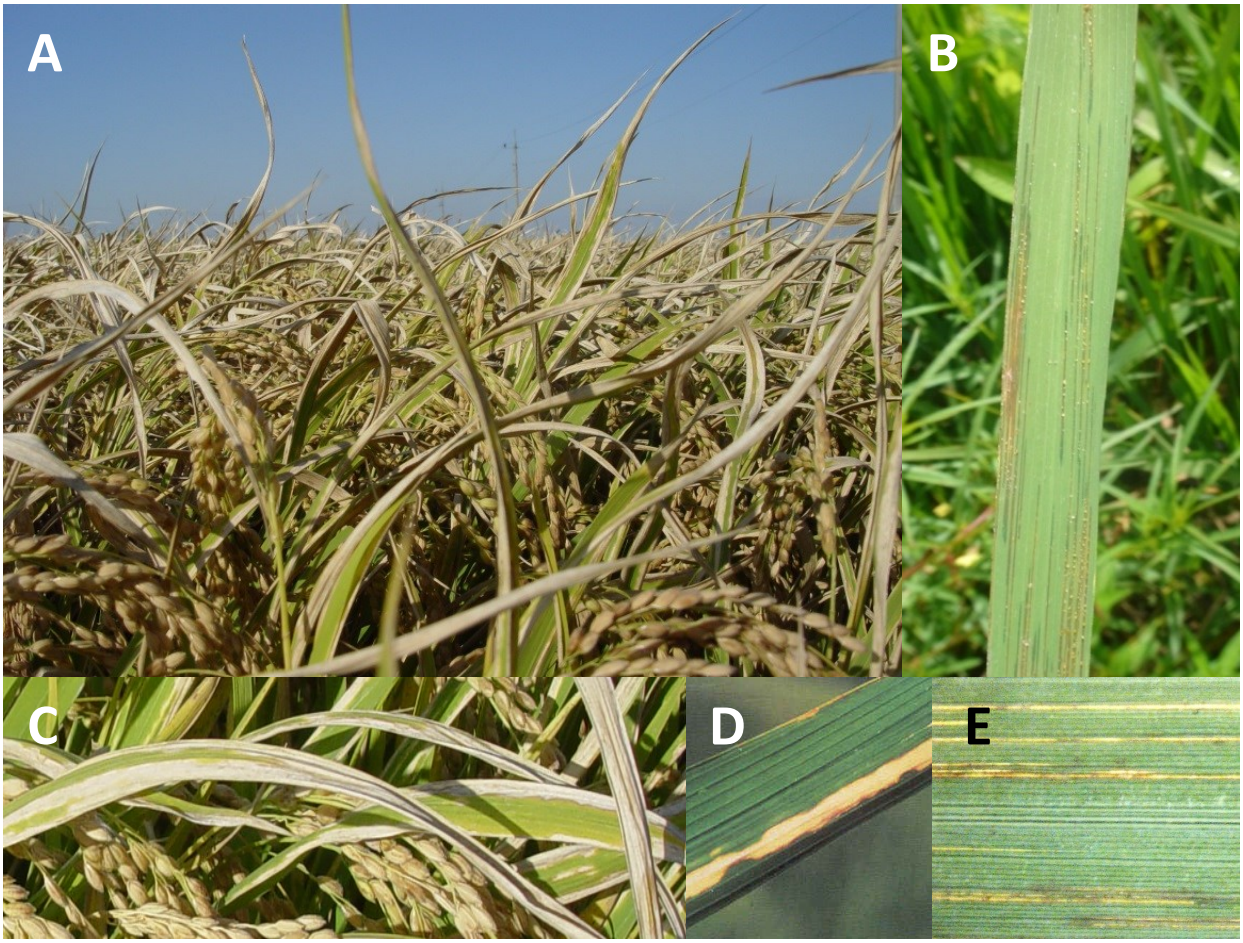

Figure 1.2. Field symptoms of BB and BLS. A. BB epidemic in Korea (photo by J. E. Leach). B. BLS symptoms; note beads of yellow exudate (photo by V. Verdier). C and D are enlarged leaf symptoms of BB (Photos by J. E. Leach). E shows enlarged symptoms of BLS (Photo by V. Verdier).

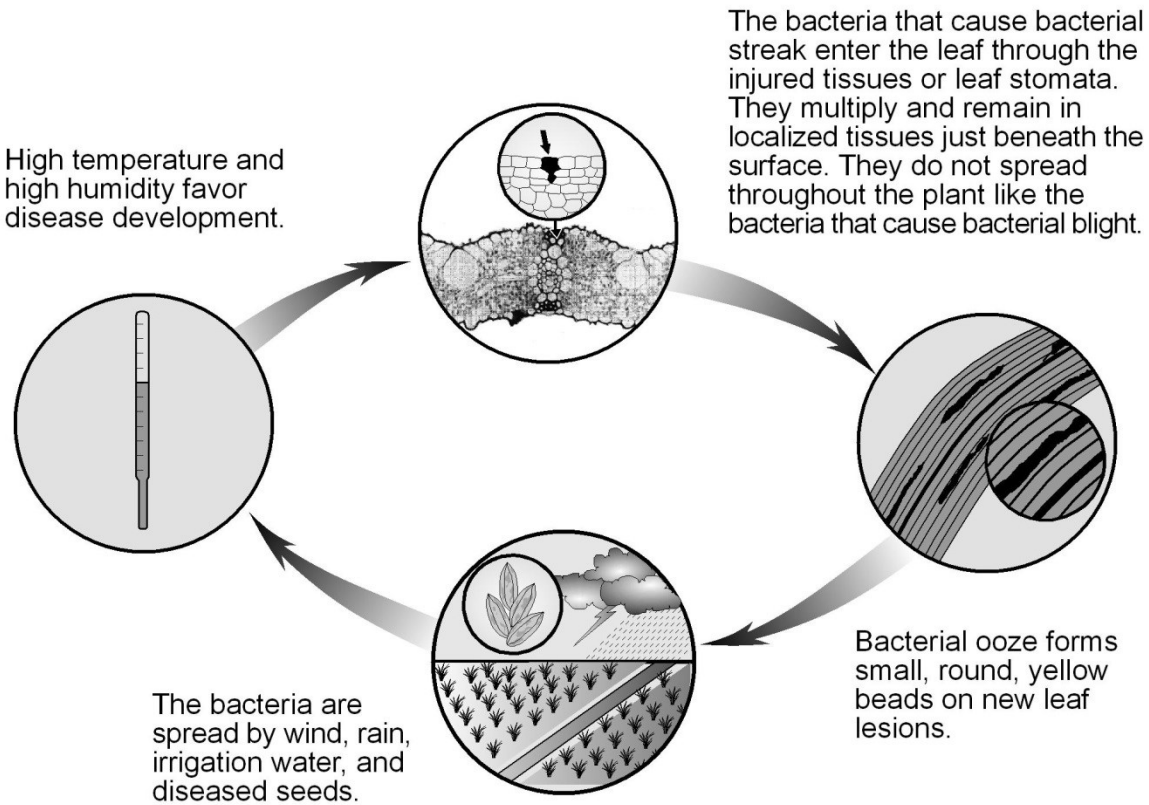

Figure 1.3. Disease cycle of bacterial leaf streak of rice caused by *Xanthomonas oryzae* pv. *oryzicola* [67].

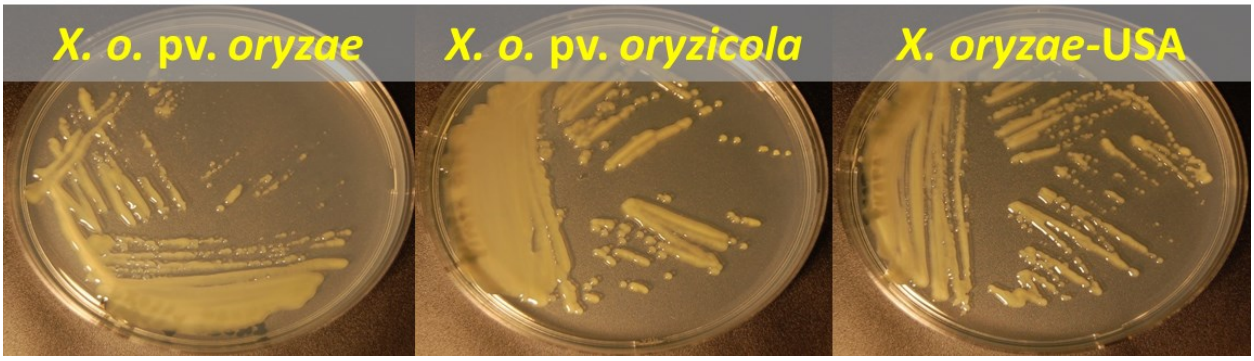

Figure 1.4. Colony morphology of 72-hr old *Xanthomonas oryzae* pathovars on peptone sucrose agar (photos by R. Corral).

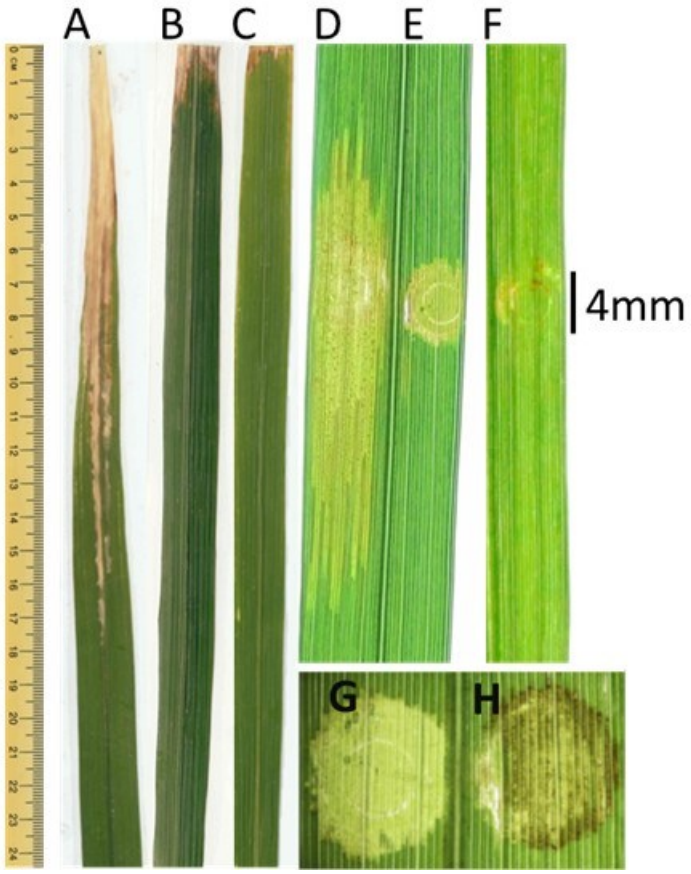

Figure 1.5. Inoculated rice leaf symptoms of BB, BB-like, BLS, and nonpathogenic *Xanthomonas*. Leaves inoculated by scissor clip inoculation [27] A, *Xoo;* B, *Xo*-USA*;* C, nonpathogenic *Xanthomonas* or leaf infiltration D, *Xoc*; E, *Xo*-USA; F, nonpathogenic *Xanthomonas*; G, susceptible reaction to *Xo*; and H, hypersensitive reaction resistance response to *Xo* (Photos by R. Corral).

Table 1.1. *Xa R* genes currently available for BB resistance and their characteristics.

R gene	Subpopulation	Accession name
Xa2, Xa4, Xa11, Xa16, Xa18, Xa25(a) and (b), Xa26, xa28, xa34(t)*	*indica*	Tetep, TKM6, IR8, IR944, IR24, Tetep, HX-3, Minghui63, LotaSail, BG1222
Xa3, Xa14, Xa17, Xa18, Xa31(t)	*japonica*	Wase Aikoku, TN1, Asominori, Toyonishiki, Zhachanglong
Xa1,Xa12	temperate *japonica*	Kogyoku
xa5, Xa7*, xa24*	aus	DZ192, DV85, DV86-DV85-Aus295
Xa21, Xa23, Xa27, Xa29, Xa30(t), Xa32(t), Xa35*	wild species	*O. longistaminata, rufipogon, minuta, officinalis, nivara, australiensis, minuta*
Xa6/Xa3, xa8, xa9, Xa10, xa13, Xa15, Xa22(t), xa33(t), Xa36(t)*	-	Zenith, PI231129, Khao Lay Nhay, CAS209, BJ1, XM41, Zhachanglong, Ba7, C4059
xa19, xa20	mutant	XM5, XM6

- : No data, ND: not determined

**: Xa gene released in Asia*

Note: Xa30(t) from *O. nivara* is now designated as *Xa38*; *Xa9* is a dominant gene and is allelic to *Xa3* and *Xa6;* BJ1 carrying *xa13* is an *aus* cultivar; *Xa15* from XM41 and also a mutant line.

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CHAPTER TWO.

TAL EFFECTORS TARGETING *OsSWEET* GENES ENHANCE VIRULENCE ON DIVERSE RICE VARIETIES WHEN EXPRESSED INDIVIDUALLY IN A TAL EFFECTOR-DEFICIENT STRAIN OF *XANTHOMONAS ORYZAE*[*]

SUMMARY

- Genomes of the rice xylem and mesophyll pathogens *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and pv. *oryzicola* (*Xoc*) encode numerous secreted transcription factors called TAL effectors. In a few studied rice varieties, some of these contribute to virulence by activating corresponding host susceptibility genes. Some activate disease resistance genes. The roles of *X. oryzae* TAL effectors in diverse rice backgrounds, however, are poorly understood.
- *Xoo* TAL effectors that promote infection by activating *SWEET* sucrose transporter genes were expressed in TAL effector-deficient *X. oryzae* strain X11-5A, and assessed in 21 rice varieties. Some were also tested in *Xoc* on variety Nipponbare. Several *Xoc* TAL effectors were tested in X11-5A on four rice varieties.
- *Xoo* TAL effectors enhanced X11-5A virulence on most varieties, but to varying extents depending on the effector and variety. *SWEET* genes were activated in all tested varieties,

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Verdier V, Triplett LR, Hummel AW, Corral R, Cernadas RA, Schmidt CL, Bogdanove AJ, Leach JE. 2012. TAL effectors expressed individually targeting *OsSWEET* genes enhance virulence on diverse rice varieties when in a TAL effector-deficient strain of *Xanthomonas oryzae.* New Phytologist 196:1197-1207. My contributions to the manuscript were the planning, design, execution, and analysis of the pathogenicity experiments, and the isolation of RNA for the qRT-PCR experiments. I also helped with manuscript and figure preparation.

but increased virulence did not correlate with activation level. *SWEET* activators also enhanced *Xoc* virulence on Nipponbare. *Xoc* TAL effectors did not alter X11-5A virulence.

- *SWEET*-targeting TAL effectors contribute broadly and non-tissue specifically to virulence in rice, and their function is affected by host differences besides target sequences. Further, the utility of X11-5A for characterizing individual TAL effectors in rice was established.

Keywords: TAL effectors, AvrXa7, PthXo1, TalC, *Xanthomonas oryzae*, *OsSWEET*, OryzaSNP

INTRODUCTION

Pathogenic *Xanthomonas oryzae* cause two important bacterial diseases of rice. *X. oryzae* pv. *oryzae* (*Xoo*) enters through leaf hydathodes to invade xylem vessels and cause bacterial leaf blight, while *X. oryzae* pv. *oryzicola* (*Xoc*) enters through stomata to colonize the leaf mesophyll and cause bacterial leaf streak. *X. oryzae* is diverse, with distinct phylogenetic clades comprising *Xoo* from Asia, *Xoo* from Africa, and *Xoc* from Asia and Africa (Triplett *et al.*, 2011; Hajri *et al.*, 2012). Strains of weakly pathogenic *X. oryzae*, with no pathovar designation, were also isolated in the United States from rice showing mild symptoms of bacterial blight (Jones *et al.*, 1989). These strains were recently placed in a clade distinct from all other subgroups, and it was hypothesized that they descended from a common ancestor of *Xoc* and *Xoo* (Triplett *et al.*, 2011).

Asian and African *Xoo* and *Xoc* genomes contain multiple members of a gene family encoding transcription activator-like (TAL) effectors. TAL effectors enter host cells via the bacterial type III secretion system, and turn on specific host genes by binding to effector-specific sites in the host genome. Some TAL effectors contribute to virulence by activating host genes that

enhance susceptibility, and some, not mutually exclusively, function as avirulence factors by activating resistance genes (Boch & Bonas, 2010; Bogdanove *et al.*, 2010). TAL effector target specificity is determined by a central region of the effector that is composed of conserved, 33-34 amino acid repeats. Two variable amino acids at positions 12 and 13 in each repeat, called the repeat-variable diresidue (RVD), together specify a nucleotide in the target so that the number and composition of RVDs defines the length and nucleotide sequence of the target (Boch *et al.*, 2009; Moscou & Bogdanove, 2009).

Several *Xoo* TAL effectors that contribute to virulence have been identified and their relevant targets in rice characterized. PthXo6 from Philippine *Xoo* strain PXO99^{A} contributes moderately to virulence by activating the transcription factor TFX1 (Sugio *et al.*, 2007). PthXo7 from that strain contributes to virulence specifically in a variety that carries a mutation in the gene for the gamma subunit of general transcription factor TFIIA (Iyer & McCouch, 2004). This mutation confers resistance to many *Xoo* strains, hypothetically by reducing the ability of TAL effectors to recruit the host transcriptional machinery (Iyer & McCouch, 2004). PthXo7 induces expression of a TFIIAγ paralog that presumably restores full TAL effector activity (Sugio *et al.*, 2007). *Xoo* TAL effectors with more substantial effects on virulence uniformly activate members of the *OsSWEET* family of sucrose transporter genes (Chen *et al.*, 2010) to promote disease. PthXo1 from strain PXO99^{A} activates *OsSWEET11* (also called *Os8N3*) and PthXo3 from Japanese strain MAFF31101 and AvrXa7 from Philippine strain PXO86 activate *OsSWEET14* (also called *Os11N3*) from overlapping binding sites (Yang *et al.*, 2006; Antony *et al.*, 2010; Romer *et al.*, 2010). The TalC protein from the African *Xoo* strain BAI3 activates *OsSWEET14* from a binding site distinct from those of AvrXa7 and PthXo3 (Yu *et al.*, 2011).

In addition to the TFIIAγ mutation mentioned above (also known as the *xa5* resistance gene), which confers resistance in a general way, plants have evolved highly specific mechanisms to defend against pathogens that deploy TAL effectors. Resistance genes *Xa27* in rice and *Bs3* in pepper are specifically transcriptionally activated by TAL effectors AvrXa27 and AvrBs3, respectively, and block disease progression by triggering a localized host cell death (Gu *et al.*, 2005; Romer *et al.*, 2007). Polymorphisms that provide resistance by destroying a TAL effector binding site in a major susceptibility gene promoter and preventing its activation also occur. For example, a large insertion in the promoter of *OsSWEET11* prevents PthXo1-mediated susceptibility and constitutes the recessive *xa13* gene for bacterial blight resistance (Yang *et al.*, 2006). *Xa7* confers a strong hypersensitive reaction (HR), a programmed cell death response, when inoculated with bacteria harboring the *OsSWEET*-activating TAL effector *avrXa7* (Hopkins *et al.*, 1992), but to date, the resistance gene has not been cloned.

Despite these major advances in our understanding, the targets and roles of most TAL effectors from *X. oryzae* (and other *Xanthomonas* species) are still unknown. Sequenced *X. oryzae* genomes harbor between 8 and 26 individual TAL effector genes (White *et al.*, 2009; Bogdanove *et al.*, 2011). The strain from which most TAL effectors have been characterized, PXO99^{A}, has 11 additional yet uncharacterized TAL effectors. Furthermore, no studies of the virulence functions of TAL effectors from any *Xoc* strain have yet been reported. TAL effector functional studies are complicated by the possibility of functional redundancies and by the quantitative nature of their contributions to virulence (Yang *et al.*, 1996; Bai *et al.*, 2000). Also, as exemplified by PthXo7 discussed above, functions of some TAL effectors may only be apparent in certain host genotypes. Indeed, studies of *X. oryzae* TAL effector function to date have been conducted only a relatively

narrow range of rice varieties, including Azucena, Taipei, Nipponbare, and isogenic lines derived from IR24.

The weakly virulent U.S. *X. oryzae* strains contain no TAL effector genes (Ryba-White & Leach, 1995; Triplett *et al.*, 2011). Because these strains are otherwise highly genetically similar to African and Asian *Xoo* and *Xoc* strains (Triplett *et al.*, 2011), they offer the opportunity to study the virulence (or avirulence) properties of TAL effectors individually. We used one of these, strain X11-5A, to ask whether the major virulence roles of *Xoo* TAL effectors that target *OsSWEET11* and *OsSWEET14* are conserved across a diverse collection of rice varieties, and whether any of these TAL effectors triggers defenses in one or more of these varieties that could reveal novel resistance loci. We also asked whether the virulence function of *SWEET*-targeting *Xoo* TAL effectors is specific to the xylem, or if they might enhance virulence of *Xoc* in the leaf mesophyll as well. Finally, we tested whether selected *Xoc* TAL effectors affect the behavior of X11-5A.

MATERIALS AND METHODS

Strains, plasmids and rice varieties

The bacterial strains and plasmids used for this study are described Table 2.1. *Escherichia coli* cells were grown in Luria-Bertani (LB) medium at 37°C. *X. oryzae* was grown in peptone-sucrose agar (PSA) medium at 28°C (Karganilla *et al.*, 1973). Antibiotics were used at the following concentrations (μg l^{-1}): tetracycline, 2; gentamycin, 20; ampicillin, 50.

Rice varieties used included a set of 20 diverse rice lines (the OryzaSNP set) that represent the genotypic and phenotypic diversity of cultivated rice (McNally *et al.*, 2009). We also included

Kitaake, an early flowering japonica rice from northern Japan that is widely used in rice transformation studies (Manosalva *et al.*, 2009). In addition, rice isogenic lines IRBB7 (with *Xa7*) and IR24 (the recurrent parent background) were used to assess delivery of AvrXa7 by X11-5A. Finally, *UXO-3*, a transgenic Kitaake line containing the *Xa27* resistance gene driven by a custom promoter that is responsive to several TAL effectors from *Xoo* and *Xoc*, including PthXo1 and Tal2g (Hummel *et al.*, 2012), was used to test translocation of those TAL effectors.

Cloning of TAL effector genes

Except for *talC*, the repeat region of each TAL effector gene was first cloned as a *Bam*HI or *Sph*I fragment into pCS466, a derivative of the Gateway entry vector pCR8-GW (Invitrogen) that contains a truncated form of the *Xoc* BLS256 *tal1c* gene, from which the *Sph*I fragment that comprises the repeat region had been removed. *Sph*I fragments were cloned into the single *Sph*I site in the truncated *tal1c* gene. *Bam*HI fragments replaced the *Bam*HI fragment of the truncated gene. The reconstituted genes were then transferred to the broad host-range destination vector pKEB31 (Cermak *et al.*, 2011); Addgene plasmid 31224, www.addgene.org) using Gateway LR Clonase (Invitrogen) according to manufacturer's instructions, for expression in *Xanthomonas* (Table 2.1). In pKEB31, expression is driven by the *lac* promoter, which is constitutive in *Xanthomonas*.

Clones of *avrXa7* and *pthXo1* repeat regions were obtained from F. White, Kansas State University) and B. Yang (Iowa State University). Repeat region clones of *tal1c*, *tal2a*, *tal2g*, and *tal8* were selected from a TAL effector gene repeat region library generated by digesting the genomic DNA of *Xoc* BLS256 with *Bam*HI, gel purifying all fragments ranging from 2.5 to 5 kb,

ligating them to pBlueScript SK(-) (Agilent Technologies) linearized with *Bam*HI, and then screening by PCR and sequencing. For *talC*, a complete gene clone in broad host range vector pSKXI was obtained from B. Szurek (personal communication).

Transformation of *X. oryzae* X11-5A

Plasmids were introduced into *X. oryzae* strain X11-5A by electroporation (Choi & Leach, 1994). For each transformation event, at least six single colonies were selected, purified twice on selective media, and confirmed by PCR. Templates for PCR amplification were derived by resuspending cells from single colonies in sterile water and boiling for 6 min at 95 °C. Each PCR reaction was prepared with 20.6 µl of water, 0.4 µl of each primer at 10 µmol l^{-1}, 0.5 mmol l^{-1} of dNTPs, 2.5 µl Buffer 10x (Thermopol Buffer, New England BioLabs, Ipswich, Massachusetts, USA), and 3.5 µl of denatured cells. PCR amplification was performed in a GeneAmp® PCR System 2700 (Applied Biosystems Carlsbad, California, USA) using the following program: an initial denaturation step of 1 min at 95 °C followed by 32 cycles of 30" at 95 °C, 45" at 62.5 °C, and 1 min at 72 °C, and ending with a final elongation step for 5 min at 72 °C. The primers used for amplification were P270 (5'-GCCAAGTCCTGCCCGCG- 3') and P271 (5'-CCTCCAGGGCGCGTGC -3'), which correspond to the N-terminus of *talC*. The 695-bp PCR products and an *ex*ACTGene 100-bp DNA ladder (ThermoFisher Scientific, Waltham, MA, USA) were separated in a 1% agarose gel and visualized by staining with ethidium bromide. Images were captured using the Gene Genius digital image capture system and the GeneSnap Software (SynGene, Cambridge, England) version 7.12.

To confirm plasmid presence and stability, plasmid DNA was extracted from transformed X11-5A strains after several rounds of culture and after re-isolation from inoculated plant tissue using the PureYield™Plasmid Miniprep System (Promega, Madison, WI, USA). 200 ng of plasmid DNA was transformed into *E. coli* DH5α competent cells using a heat-shock protocol. Plasmids were extracted and digested with *Sph*I, and the products were visualized as described above for PCR products.

Plant inoculations

X. oryzae strains were grown on PSA medium supplemented with antibiotics for 24 h at 30°C, then resuspended in sterile water at 0.2 OD_{600} (approximately 10^8 CFU ml^{-1}). Inoculations were conducted in growth chambers maintained at 28°C 12-h-day and 24°C 12-h-night with 85% relative humidity. For leaf clip assays, the two youngest, fully expanded leaves on each tiller of four-week-old rice plants were inoculated as described (Kauffman *et al.*, 1973). Lesion length (centimeters) was measured 15 days post inoculation (dpi). Each strain was assessed on a set of 21 rice lines, including the OryzaSNP lines (McNally *et al.*, 2009) and Kitaake. For each bacterial treatment, 10 plants per accession were evaluated with at least two fully expanded leaves inoculated per plant. For syringe infiltration assays, strains were introduced with a needleless syringe into the intercellular spaces of rice leaves from 2-wk-old plants at concentrations of 10^8 cfu ml^{-1} as described (Reimers & Leach, 1991), HR was scored at 72 hours after inoculation (hai), and lesions were measured at 10 days. Experiments were repeated three independent times.

Multiplication of *Xoo* X11-5A and X11-5A(*pthXo1*) *was measured in planta* at three time points (0, 8 and 15 days after inoculation, dai). Rice varieties Azucena and Nipponbare were

inoculated by leaf clipping of 4-week-old plants. The top 20 cm of each leaf was cut into four 5-cm sections, designated as A, B, C, and D, starting from the point of inoculation "A". The leaf pieces were ground in 1 ml of sterile water, and bacterial numbers were assessed in serial dilutions that were spread onto PSA agar plates supplemented with antibiotics. The plates were incubated at 28°C until single colonies could be counted. The experiment was repeated three times.

To visually differentiate lesions from resistance responses, the terminal five cm of each inoculated leaf was cleared by suspending overnight in a solution of 95% ethanol and 5% glycerol. A resistance response was visualized as a blackening of the inoculation site and rated as (+). Disease lesions that appear as light brown after clearing were rated as (-) while lesions with a light blackening and light browning were rated as (+/-) (Supporting Information, Fig. S2.1).

Sequence, RT-PCR, and qPCR analysis of target promoters

Published primers (Romer *et al.*, 2010) were used to amplify the sequences upstream of *OsSWEET11* and *OsSWEET14* from genomic DNA extracted from rice varieties Dular, Minghui, Nipponbare, Moroberekan, and FR13A (McNally *et al.*, 2009). 50 µl reactions containing 25 ng genomic DNA, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM each primer, 1x PCR buffer (Life Technologies, Grand Island, NY) and 2 units Taq DNA polymerase were cycled in the following conditions: 1 min. at 94° C followed by 28 cycles of 30 s at 94° C, 30 s at 55° C, and 1 min. at 72° C. PCR products were sequenced at the Colorado State University proteomics and metabolomics facility and deposited in GenBank (accession numbers JQ968614-JQ968623).

For Reverse Transcriptase (RT)-PCR and quantitative Real-Time RT-PCR (qPCR), RNA was extracted from 10 cm of infiltrated leaf tissue collected at 48 hai using the Spectrum™ Plant Total RNA Kit according to the manufacturer's recommendations (Sigma-Aldrich, St. Louis, MO, USA). The RNA was quantified by spectrophotometer (NanoDrop, ND-2000, ThermoFisher Scientific, Waltham, MA, USA).

Two micrograms of total RNA were treated with DNaseI (Invitrogen) and reverse transcribed using SuperScript III (Invitrogen). For the semi quantitative RT-PCR analysis, one microliter (5%) of the RT-PCR reaction was used as a template with Phire Hot Start II DNA polymerase (Thermo Scientific) together with gene-specific oligos (*OsSweet11F*, CAAGCCCCACCAGGTCAAGGT; *OsSweet11R*, TTGTGCACGCCGAGGATCG; *OsSweet14F*, GCCTTCGCCTTTGGTCTCCT; *OsSweet14R*, ATCTGGATGACGCCGAAGGA; *ActinAS1*, CAAAATTCACGTCCGTACATCG; *ActinS1*, AAACTTTGTTACGTCGCGGC) as follows: 30 s at 98°C; 23 cycles of 10 s at 98°C, 5 s at 60°C, and 10 s at 72°C.

PCR was performed using an iCycler Thermo Cycler (Bio-Rad) with 50 ng total RNA as template for cDNA synthesis and PCR amplification using the iScript One-Step RT-PCR kit with SYBR Green (Bio-Rad) according to the manufacturer's protocol. Gene-specific primers for *OsSWEET11* (P941, ATGGCTAACCCGGCGGTCACCCT; P942, GCGTTGATGGTCAGCAGCGGCCT), *OsSWEET14* (P943, GGCGACCGCCGCATCGTGGTT; P944, GCCCAGCACGTTGGGAAGAGCG) , and rice Actin-6 for normalization (EU215044; P787, CCGGTGGATCTTCATGCTTACCTGG; P788, CGACGAGTCTTCTGGCGAAACTGC) were used for amplification. A minimum of three

independent biological replicates, each with three qPCR technical replicates, was tested for each treatment. The $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) was used to quantify expression of *OsSWEET11* or *OsSWEET14* transcript for each treatment relative to abundance in mock-inoculated tissue.

RESULTS

X11-5A expresses and delivers heterologous TAL effector AvrXa7

The sequenced US *X. oryzae* strain X11-5A, which does not contain TAL effectors, was selected for heterologous TAL effector expression. To confirm the ability of this strain to express and deliver TAL effectors, we transformed it with a broad host range, *avrXa7* expression construct, pKEB31-*avrXa7*. The resulting strain, X11-5A(*avrXa7*), was inoculated to rice variety IRBB7, which contains the cognate resistance gene *Xa7*, and to IR24, which does not. X11-5A(*avrXa7*) triggered an *Xa7*-dependent hypersensitive reaction (HR) (Fig. 2.1). Thus, X11-5A functionally expresses and delivers TAL effectors from a plasmid vector.

OsSWEET-targeting TAL effectors increase *X. oryzae* X11-5A virulence to varying extents in different rice varieties

TAL effectors from Asian and African *Xoo* strains known to target members of the *OsSWEET* family (AvrXa7, PthXo1, and TalC) were selected for expression in *X. oryzae* X11-5A. *X. oryzae* X11-5A elicits weak or delayed symptoms on susceptible rice, and we hypothesized that the absence of TAL effectors in the strain may partially account for the low virulence relative to *Xoo* and *Xoc* (Triplett *et al.*, 2011). To determine whether addition of TAL effectors could increase the virulence of X11-5A in diverse host backgrounds, the OryzaSNP set of 20 rice varieties

(McNally *et al.*, 2009) plus Kitaake were clip-inoculated with X11-5A and X11-5A containing *avrXa7, pthXo1* and *talC*. X11-5A alone caused blight symptoms (lesion of ≥1 cm) on nine of the 21 varieties, six of which are in the *O. sativa* japonica group (Fig. 2.2, Supporting Information, Table S2.1). Eight varieties showed increased susceptibility in response to all three TAL effectors, and five of these varieties are phylogenetically clustered in the japonica group (Fig. 2.3). Another seven varieties did not show a TAL effector-dependent increase in susceptibility (Fig. 2.3 and Table S2.1). Varieties Cypress, Aswina, and Pokkali were more susceptible in the presence of PthXo1 but not other TAL effectors. Dular susceptibility was affected by PthXo1 and AvrXa7, while Kitaake susceptibility was affected by PthXo1 and TalC. In all, 14 of 21 varieties showed increased susceptibility in response to at least one TAL effector, including five of the varieties that did not develop lesions (>1 cm) in response to X11-5A alone.

We hypothesized that TAL effectors might be ineffective in some varieties if a strong resistance response were triggered by *X. oryzae* X11-5A. To discern resistance from short lesions, inoculated leaf tips were cleared with ethanol at 15 dpi and rated as dark brown (DB), suggestive of a hypersensitive reaction (HR), or beige, indicative of a susceptible response (Fig. S2.1). We also tested X11-5A(*avrXa7*) by using leaf infiltration assays, and observed an HR at 48 h post inoculation in those lines also exhibiting the DB response (Fig. S2.1). When TAL effectors did not contribute to virulence, the dark brown tips were observed in many cases; of the eight varieties in which TAL effectors made no virulence difference, five had a clearly blackened leaf tip in response to X11-5A (Table S2.1). On the other hand, seven of the eight varieties in which all TAL effectors increased virulence did not exhibit this resistance response. However, TAL effectors were ineffective in several varieties despite the lack of any obvious resistance response. In the varieties

Minghui and Dular, the DB resistance response was observed in the absence, but not in the presence, of one or more TAL effectors (Table S2.1). AvrXa7 reduced lesion length and induced the DB response in the varieties Cypress and Sadu-cho, suggesting that these lines harbor resistance mediated by *Xa7* activity. AvrXa7 also caused a reduction in lesion size in Pokkali plants without inducing leaf browning (Table S2.1). TalC also reduced lesion length slightly in Pokkali. Among those varieties responding to TAL effectors, the relative degree to which each TAL effector affected virulence varied (Table S2.1). Even though the TAL effectors each target one of two host genes in the same *OsSWEET* family, there was variation among the effect of different TAL effectors on the same varieties. For example, on some varieties, PthXo1 caused the largest increase in lesion size among the TAL effectors, while on others, TalC consistently caused a greater increase (Table S2.1). These experiments demonstrate that while OsSWEET-targeting TAL effectors increase the virulence of X11-5A on many rice varieties, the degree of increase caused by each TAL effector varies among varieties.

Increased X11-5A virulence conferred by *Xoo* TAL effectors is associated with increased multiplication and movement.

X11-5A(*pthXo1*) caused a substantial increase in lesion length on some varieties, such as Azucena, compared to the wild-type X11-5A, but no increase in lesion length on other varieties, such as Nipponbare (Fig. 2.2). To determine the effect of PthXo1 on bacterial population growth and movement, colony counts were determined from four 5-cm sections of clipped leaves (sections A, B, C, and D) at 0, 8, and 14 dpi. On Azucena, there was no difference in population growth between X11-5A and X11-5A(*pthXo1*) in the clipped section of leaf (Azucena leaf section A, Fig. 2.4). However, X11-5A(*pthXo1*) numbers were significantly greater than the wild type in leaf

section B on days 8 and 14, and only X11-5A(*pthXo1*) spread to leaf sections C and D (Fig. 2.4). In Nipponbare, there was no significant difference between the population growth of X11-5A and X11-5A(*pthXo1*) in leaf section A, and neither strain was detected beyond this section (Fig. 2.4). Together, these results show that X11-5A bacterial spread and virulence are increased by *pthXo1* in Azucena, but not in Nipponbare rice.

TAL effector binding sites in targeted *OsSWEET* promoter sequences are conserved and the target genes are activated in diverse rice varieties.

The three TAL effectors that affect virulence activate transcription of two members of a family of sucrose transporters in rice. PthXo1 binds to the promoter of *OsSWEET11*, and AvrXa7 and TalC target the *OsSWEET14* promoter. RT-PCR confirmed that both promoters are activated in leaves of Azucena infiltrated with *X. oryzae* X11-5A expressing the appropriate TAL effector (Fig. S2.2). TAL effectors similarly activated transcription in Nipponbare leaves, although there was no TAL effector-mediated increase in lesion development or bacterial multiplication in this variety.

We next tested whether the differential effectiveness of TAL effectors in some other varieties could arise from variations in the promoter TAL effector binding site or from differences in transcriptional activation levels. We sequenced roughly 500 bp upstream of the *OsSWEET14* and *OsSWEET11* coding sequences in five varieties with different patterns of TAL effector-mediated symptoms, including the indica variety Minghui, japonica varieties Nipponbare and Moroberekan, and aus varieties Dular and FR13A. Except for a single mutation in the Moroberekan AvrXa7 binding site, among these varieties, there were no other differences in the

known binding sites for AvrXa7, PthXo1 or TalC (accession numbers JQ968614-JQ968623). The single difference in the Moroberekan AvrXa7 binding site is a G to A substitution at a position corresponding to an "NN" RVD in AvrXa7; this RVD has dual specificity for G and A (Boch *et al.*, 2009), so this substitution can be expected to be inconsequential. Thus, barring differences in epigenetic marks, there should be no difference in promoter binding efficiency among the varieties. Quantitative RT-PCR was performed to determine relative levels of TAL effector-dependent gene activation among four of these varieties (Minghui, Moroberekan, Dular and FR13A). Each TAL effector upregulated its corresponding *SWEET* gene target strongly (> 50 fold vs. mock inoculation) in each variety (Fig. 2.5). However, there was no observed relationship between lesion length increase and relative fold-change in target expression. For example, *OsSWEET14* is upregulated to a similar degree by AvrXa7 and TalC in Dular even though these effectors cause different increases in lesion length, and the effectors cause similar increases in lesion length in Minghui and Moroberekan despite differences in *OsSWEET14* activation levels in that variety. Thus, the differences in TAL effector-mediated virulence levels are likely not due to differences in target activation levels.

OsSWEET-targeting *Xoo* TAL effectors enhance *Xoc* virulence

Although the US *X. oryzae* strains cause weak bacterial blight symptoms on rice, we previously found that US strains are phylogenetically distant from both *Xoo* and *Xoc* strains (Triplett *et al.*, 2011). Therefore, it is not clear whether US strains are more like *Xoo*, and normally invade the vascular system, or like *Xoc*, colonize the spaces between leaf mesophyll cells. This raised the question whether the contribution of the *Xoo* TAL effectors to X11-5A virulence might be tissue specific, selectively facilitating invasion of the xylem. To test this possibility, we assayed

the effect of *avrXa7* and *pthXo1* on the virulence of *Xoc* BLS256, by leaf syringe infiltration typically used for quantitative measures of bacterial leaf streak susceptibility, and separately by leaf clip inoculation, used for bacterial blight. Both *avrXa7* and *pthXo1* significantly enhanced the lengths of lesions caused by *Xoc* BLS256 following syringe inoculation, however, they did not render BLS256 capable of causing bacterial blight symptoms following clip inoculation (Fig. 2.6).

Selected *Xoc* TAL effectors do not affect virulence of X11-5A in four rice varieties

We next asked whether X11-5A behavior could be modified by selected TAL effectors from *Xoc*. Preliminary mutagenic studies implicated *Xoc* effectors Tal2a, Tal2g, and Tal1c from strain BLS256 as potential virulence factors (R.A.C., L. Wang, and A.J.B., *unpublished*). We transformed X11-5A with gene expression constructs corresponding to these effectors and syringe infiltrated the resulting strains into leaves of rice varieties Nipponbare, Lemont, Kitaake and Azucena. Over 11 days, watersoaking localized to the inoculated spot was the only symptom that developed, and no differences were observed between the wild type X11-5A strain and the transformants carrying the *Xoc* TAL effector genes, despite the fact that *Xoc* strain BLS256 developed lesions > 2 cm on all four varieties (Kitaake and Nipponbare results are shown in Fig. 2.7). Furthermore, no effects on X11-5A virulence were observed after clip inoculation, although *Xoo* strain PXO145 induced lesions of greater than 10 cm (Kitaake and Nipponbare results are shown in Fig. 2.7). The *Xoc* TAL effector constructs are identical to the *Xoo* TAL effector constructs except for the repeats, and would therefore be expected to be delivered by X11-5A. Nonetheless, to examine the possibility that the lack of effect of the *Xoc* TAL effectors on X11-5A virulence was due to lack of delivery, we took advantage of transgenic Kitaake rice line *UXO-3*, which mounts an HR in response to several *Xoo* and *Xoc* TAL effectors, including PthXo1 and

Tal2g (Hummel *et al.*, 2012), to test whether X11-5A is indeed able to deliver the Xoc effector Tal2g. X11-5A expressing *tal2g*, *pthXo1*, a central repeat domain deletion control (*tal1c ΔCRR*), or no *tal* gene, as well as *Xoc* BLS256 were inoculated to *UXO-3* plants by syringe infiltration (Fig. 2.7). Only X11-5A(*tal2g*), X11-5A(*pthXo1*), and *Xoc* BLS256 triggered the resistance gene mediated HR. Thus, X11-5A expresses and delivers the *Xoc* effector Tal2g effectively.

DISCUSSION

Xanthomonas TAL effectors induce plant genes that promote disease, or, in some genotypes, genes that provide disease resistance. Understanding their roles in diverse plant genetic backgrounds will help define key plant disease susceptibility factors, and may identify novel sources of resistance. In this study, we used a strain from the US clade of *X. oryzae* as a TAL effector-free platform for characterizing the effects of individual TAL effectors on diverse rice varieties. We found that three *Xoo* TAL effectors targeting the *OsSWEET* family of sucrose transporters conferred increased virulence to the weakly pathogenic US *X. oryzae* strain X11-5A on a majority of rice varieties tested. Importantly, the plant genetic background affected the level of virulence enhancement by these TAL effectors, despite sequence conservation of the targets. Variation did not correlate with quantitative differences in the fold-change of *OsSWEET* expression.

The increase in plant susceptibility to X11-5A that was conferred by the *Xoo* TAL effectors validates our previous hypothesis that the absence of TAL effectors is a major factor limiting the virulence of US *X. oryzae* strains (Triplett *et al.*, 2011), and confirms that the *OsSWEET* family is a critical set of bacterial disease susceptibility genes in rice. Evidence suggests that *SWEET* family

members are functionally equivalent; *SWEET14*-activating TAL effectors can rescue the *SWEET11* activation deficiency of a *pthXo1* mutant of *Xoo* strain PXO99^{A}, and PthXo1 can rescue the *SWEET14* activation deficiency of a *talC* mutant of *Xoo* strain BAI3 (Antony *et al.*, 2010; Yu *et al*., 2011). Our data (Fig. 2.4) show that PthXo1 contributes in the same way to virulence as TalC (Yu *et al*., 2011), allowing invasive colonization of the xylem, further corroborating the functional equivalence of their respective targets.

The TAL effector binding sites in the *OsSWEET* promoters were identical among diverse rice varieties. Despite polymorphisms in other parts of the promoters (and possibly epigenetic differences), the fact that each gene was strongly activated by its corresponding TAL effector(s) in all varieties tested suggests that *X. oryzae* TAL effectors have evolved to target highly conserved, functional promoter elements. The quantitative differences in virulence contribution that we observed across varieties might be explained by differences in genes that modulate *SWEET* activity, in yet unidentified additional targets of the TAL effectors that affect the plant-bacterial interaction, or, except in the case of AvrXa7 and TalC, which target the same gene, in the coding sequences of the *OsSWEET* genes.

Some patterns of differential virulence contribution were associated with rice varietal group or phylogeny, suggestive of a genetic basis (Fig. 2.3, Table S2.1). For example, while most japonica varieties showed increased susceptibility to all three TAL effectors, Minghui is the only indica variety in which susceptibility was enhanced by all three. SNP analysis previously showed that Minghui has several large japonica introgressions not found in other indica varieties (McNally *et al.*, 2009). These results show that characterization of plant responses to TAL effectors might

be useful to identify susceptibility loci in breeding programs. One of the future goals of our group is to use advanced mapping lines to identify rice genetic regions associated with differential susceptibility to TAL effectors.

In addition to variation in susceptibility, this study identified several cultivars on which some TAL effectors either reduce virulence, trigger a resistance response, or have no effect; all of these are phenotypes that could be associated with resistance loci. The introgression of disease resistance (*R*) genes is currently the most economically feasible way to control bacterial diseases of plants and strain X11-5A can serve as a useful platform for screening for novel sources of major gene resistance to individual TAL effectors. As advances in high throughput sequencing come to enable characterization of TAL effector gene inventories across a pathogen population, regionally conserved TAL effectors that would make the most logical candidates for such resistance screens might emerge (Bart *et al.*, 2012). Though we did not identify any cases here, this approach might also identify sources of resistance such as *xa13*, in which the susceptibility gene promoter sequence for an important TAL effector is disrupted. While screening large germplasm collections for sources of resistance or susceptibility to individual virulence factors is an important step in selecting plant resistance genes for deployment, screening for the identification of individual resistance genes to *Xoo* strains has usually been conducted on near-isogenic rice lines in IR24 (indica) and Toyonoshiki (temperate japonica) cultivar backgrounds. X11-5A will be useful for characterizing interactions of multiple TAL effectors with diverse rice varieties to rapidly identify accessions for resistance gene discovery and deployment.

In addition to enhancing susceptibility to xylem invasion and bacterial blight development caused by X11-5A, each of two tested *OsSWEET*-targeting *Xoo* TAL effectors, *avrXa7* (targeting *OsSWEET14*) and *pthXo1* (targeting *OsSWEET11*), enhanced virulence of the mesophyll pathogen *Xoc*, resulting in increased length of bacterial leaf streak lesions. However, the effectors did not enable *Xoc* to cause bacterial blight. These observations indicate that the capacity of SWEET proteins to contribute to susceptibility is not tissue-specific and, at the same time, is not sufficient to allow the mesophyll pathogen *Xoc* to colonize the xylem. A role for SWEET proteins in phloem loading, as sugar transporters to export photosynthate from phloem parenchyma cells for uptake by companion cells, was recently demonstrated in *Arabidopsis* (Chen *et al.*, 2012). Their activation in xylem parenchyma cells by *Xoo* TAL effectors was proposed to contribute to bacterial blight susceptibility by pumping sugar into the xylem for use by the bacterium (Chen *et al.*, 2010). A similar mechanism could account for the contribution of those TAL effectors to bacterial leaf streak susceptibility, flooding the mesophyll apoplast with a ready carbon source for the pathogen. Curiously though, no native *OsSWEET*-targeting TAL effectors have been identified in *Xoc*.

Our study also addressed whether selected *Xoc* TAL effectors could have a virulence role in X11-5A. These TAL effectors made no difference in susceptibility to X11-5A in either clip or syringe inoculations, on four tested rice varieties. Although the *Xoc* TAL effectors were not previously confirmed virulence factors like AvrXa7, PthXo1, and TalC, the effectiveness of *Xoo* but not *Xoc* TAL effectors in X11-5A suggests that this strain may be predisposed to act as a *Xoo*-like vascular pathogen. Because X11-5A branched off from the ancestor of both *Xoo* and *Xoc* (Triplett *et al.*, 2011), we hypothesize that *Xoo*-like vascular pathogenicity is the ancestral state of the *X. oryzae* group, and that the *Xoc* group lost some factor that enables xylem invasion or gained

one that precluded it to evolve towards efficient colonization of the leaf mesophyll. Furthermore, the absence of any TAL effector fragments or pseudogenes in the X11-5A genome suggests that TAL effectors were not present in the genome of the common ancestor. A single gene transfer event incorporating a TAL effector into the *X. oryzae* ancestral genome could have conferred a major selective advantage, increased by gene duplication and diversification. Whether the TAL effectors that emerged in *Xoo* and *Xoc*, and their respective plant targets, drove the divergence in plant tissue specificity between *Xoo* and *Xoc*, or were shaped by it, remains an open question.

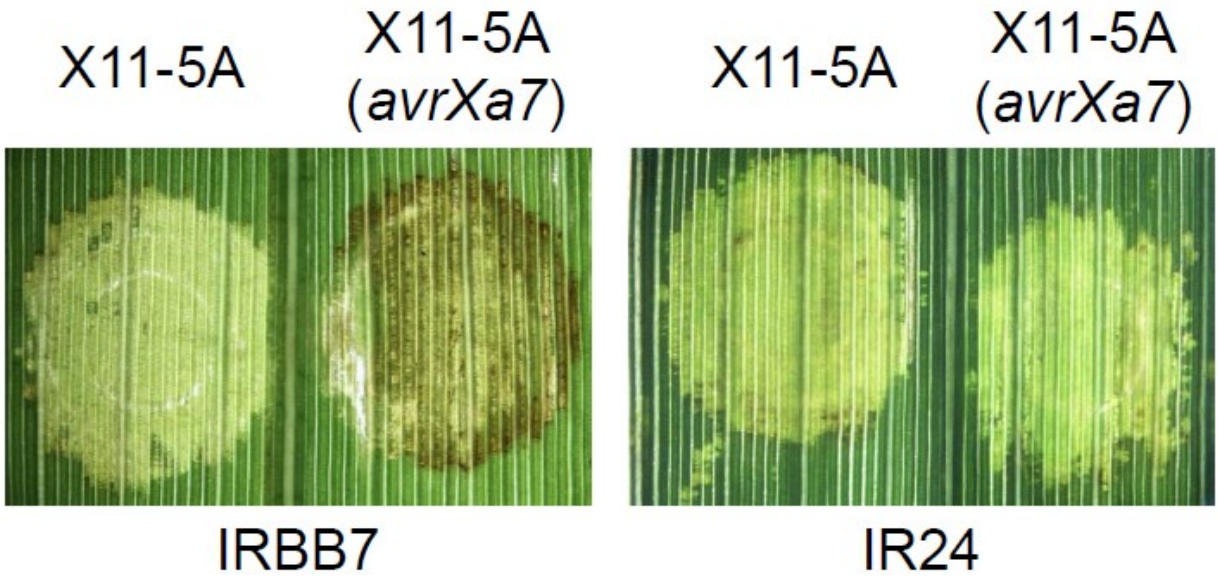

Figure 2.1. Heterologous TAL effector expression and delivery by TAL effector-deficient *X. oryzae* strain X11-5A. Reactions in leaves of rice varieties IRBB7 (left) carrying the *Xa7* resistance gene and IR24 (right) 72 h after infiltration with X11-5A or X11-5A(*avrXa7*) were photographed on a light box. *Xa7* mediates a hypersensitive reaction (HR), visible as browning, in response to AvrXa7 delivered into the plant cell.

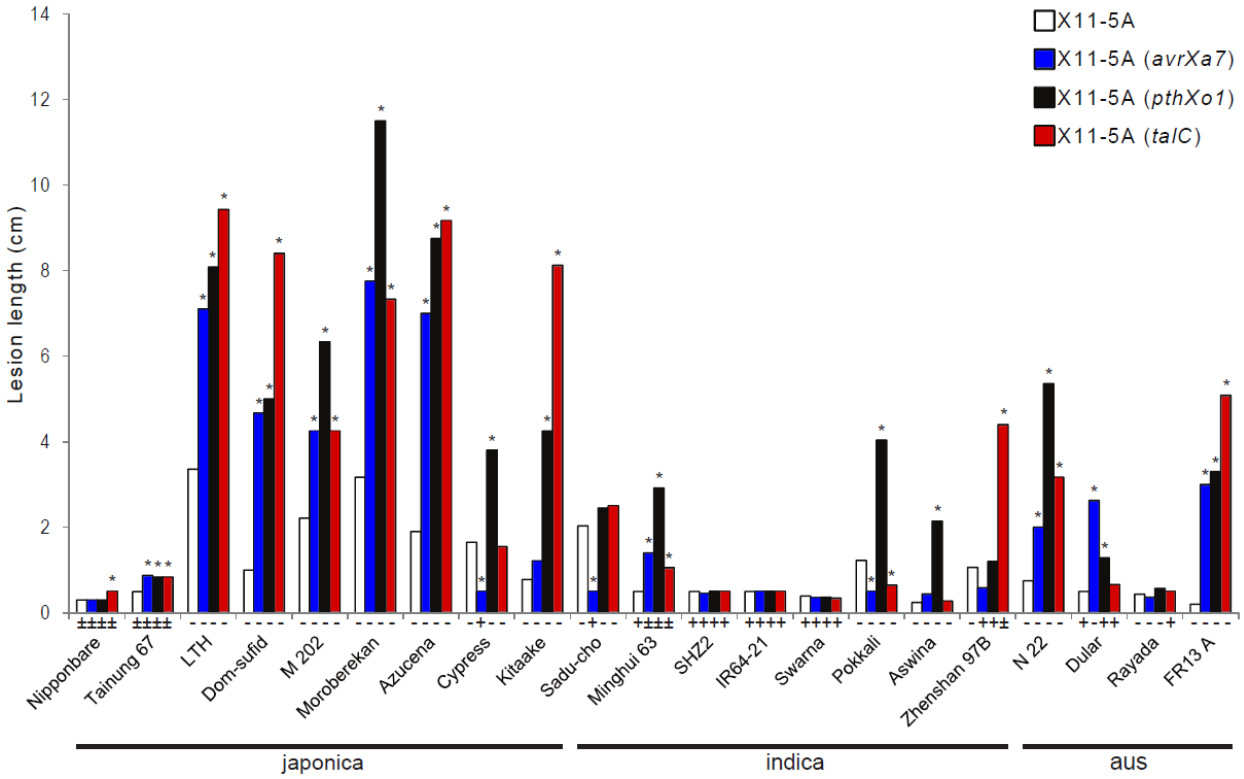

Figure 2.2. Lengths of lesions caused by *X. oryzae* X11-5A and derivatives carrying *avrXa7*, *pthXo1*, or *talC* on 21 diverse varieties of rice, grouped by type (japonica, indica or aus). Lesions were measured 15 days after clip inoculation. The (+) below the horizontal axis indicates a dark brown (DB) phenotype after leaf clearing and the (–) indicates no dark brown color (see Fig. S2.1 for detail). An asterisk denotes significant difference between the wild type and the TAL effector-expressing derivative (P<0.05). Values represent averages of at least 10 different inoculated leaves. The experiment was repeated three times with similar results.

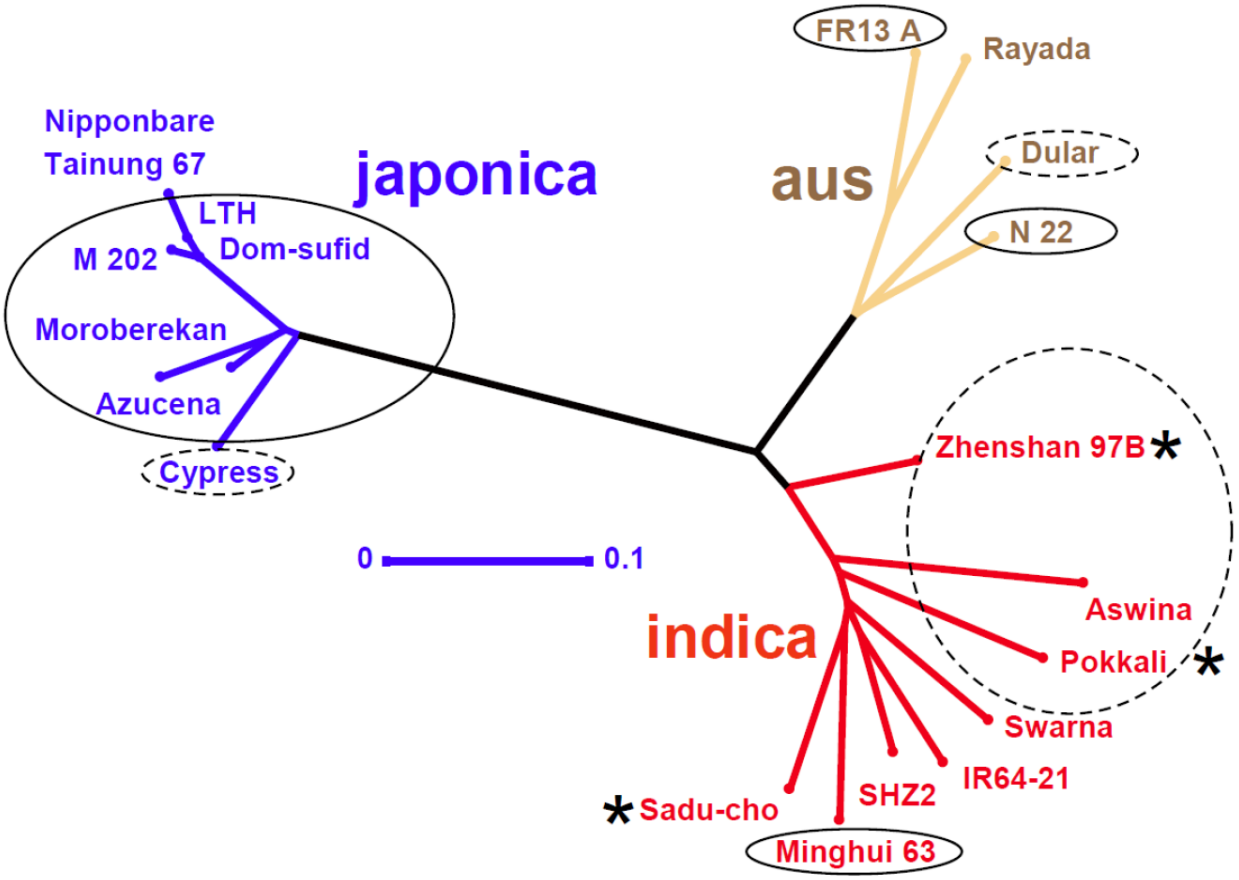

Figure 2.3. Phylogenetic relationships of the 20 OryzaSNP varieties (adapted from (McNally *et al.*, 2009) and the changes in their responses to *X. oryzae* X11-5A due to *avrXa7*, *pthXo1*, or *talC*. Varieties are circled according to the nature of the changes in their response due to the TAL effector. A solid line indicates enhanced susceptibility in response to each TAL effector; a dotted line indicates enhanced susceptibility in response to one or two of the TAL effectors; no line indicates no enhanced susceptibility. Asterisks (*) indicate decreased lesion length in response to one or more TAL effectors. See Supporting Information Tables S2.1 and S2.2 for additional details.

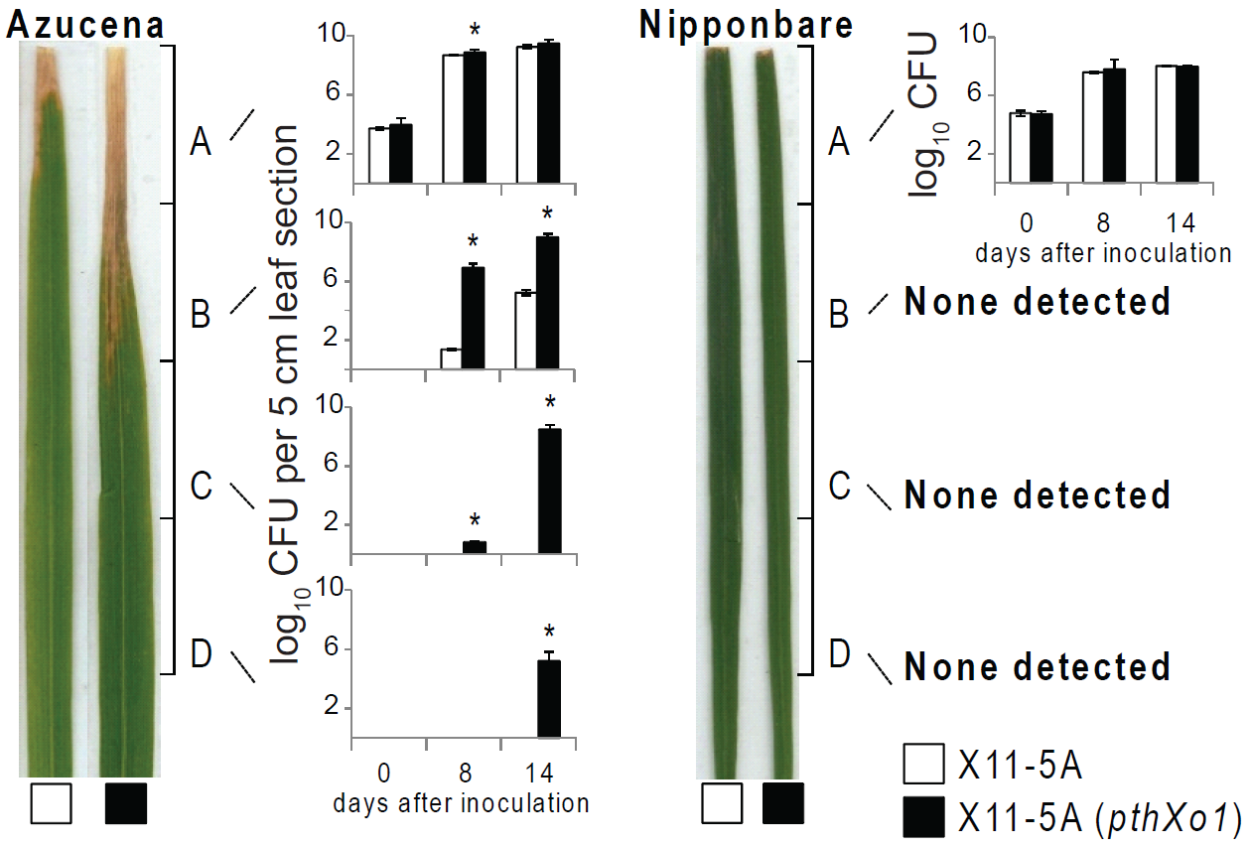

Figure 2.4. Population size and distribution of *X. oryzae* X11-5A and X11-5A(*pthXo1*) in leaves of Azucena (left) and Nipponbare (right) rice plants following clip inoculation. Four-week-old plants were inoculated and bacterial populations in five-centimeter leaf segments were measured at 0, 8, and 14 day after inoculation. Asterisks indicate significant differences between the two strains (P<0.05). Error bars represent standard deviation. Values represent averages of at least three inoculations. The experiment was repeated three times with similar results.

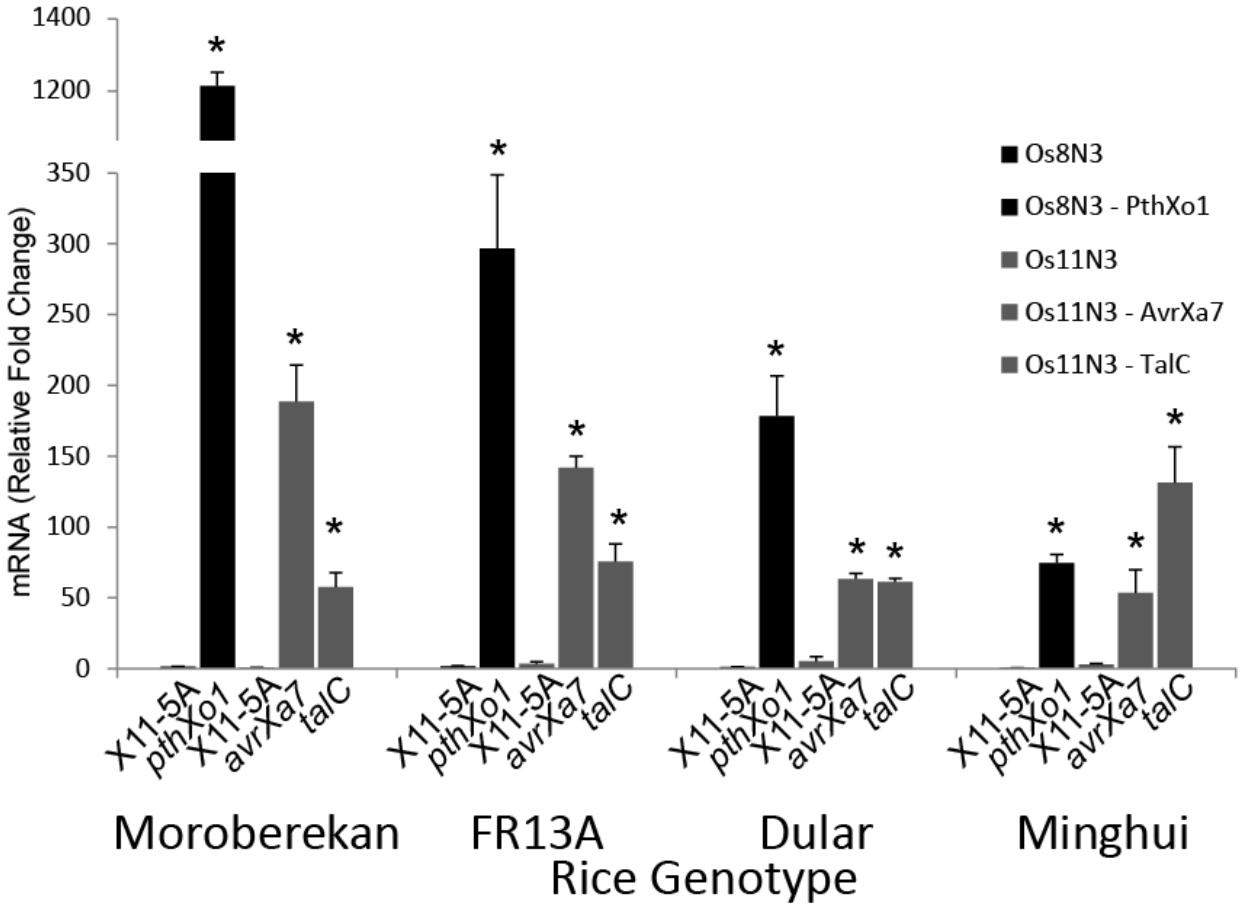

Figure 2.5. Transcription activation of (a) *OsSWEET11* and (b) *OsSWEET14* in four rice varieties in response to *X. oryzae* X11-5A containing no TAL effector gene (none), *avrXa7, pthXo1*, or *talC*. Bars represent fold-change in mRNA abundance in leaves of each variety at 48 h after inoculation relative to mock inoculated plants, measured by qPCR. Each bar is the average of three technical replicates of at least three biological replicates. Error bars denote standard deviation. Asterisks indicate values significantly greater than 1.0 as determined by two-tailed, heteroscedastic *t* tests. Relative fold-change was calculated by the $2^{-\Delta\Delta Ct}$ method.

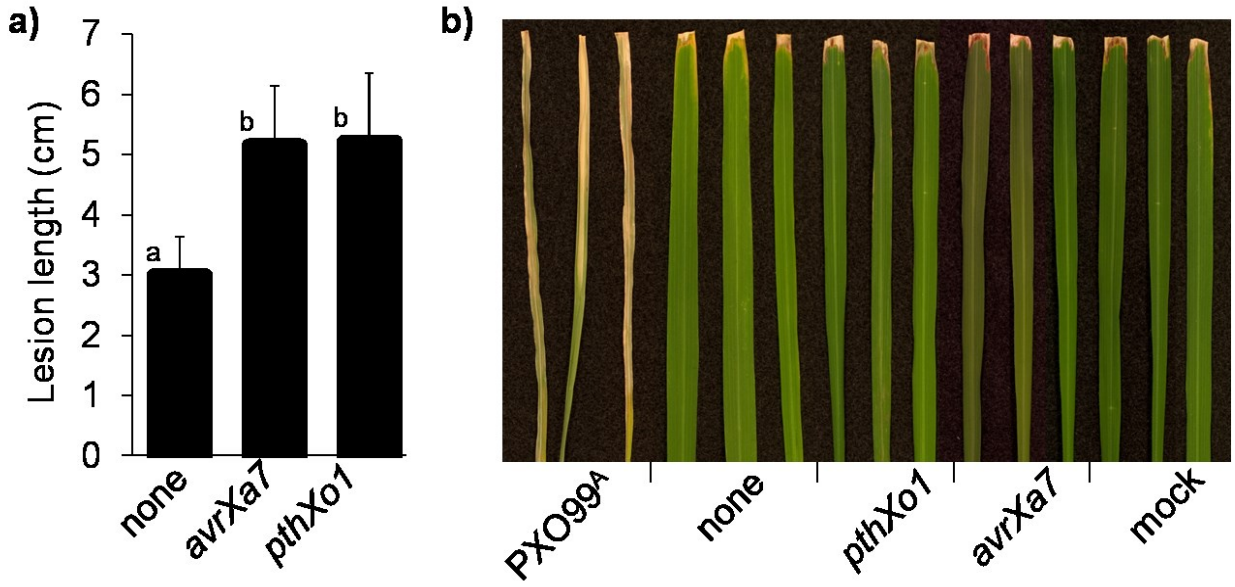

Figure 2.6. Effects of *X. oryzae* pv. *oryzae* (*Xoo*) TAL effectors on *X. oryzae* pv. *oryzicola* (*Xoc*) virulence and pathogenicity. (a) Lengths of lesions caused by syringe infiltration of *Xoc* BLS256 carrying no Xoo TAL effector gene (none), *avrXa7*, or *pthXo1* on rice variety Nipponbare. Lesions were measured 10 days after inoculation. Error bars denote standard deviation. Values represent means of at least eight inoculations. The experiment was repeated twice with similar results. Different lower case letters indicate that the means are significantly different ($P<0.01$) by two-tailed, heteroscedastic *t* test. (b) Failure of *Xoo* TAL effectors to render *Xoc* capable of inciting bacterial blight. Leaves were photographed 14 days following leaf clip inoculation with *Xoo* PXO99^{A}, *Xoc* BLS256 with no *Xoo* TAL effector gene, *pthXo1*, or *avrXa7*, and a buffer only control (mock). The experiment was repeated three times with similar results.

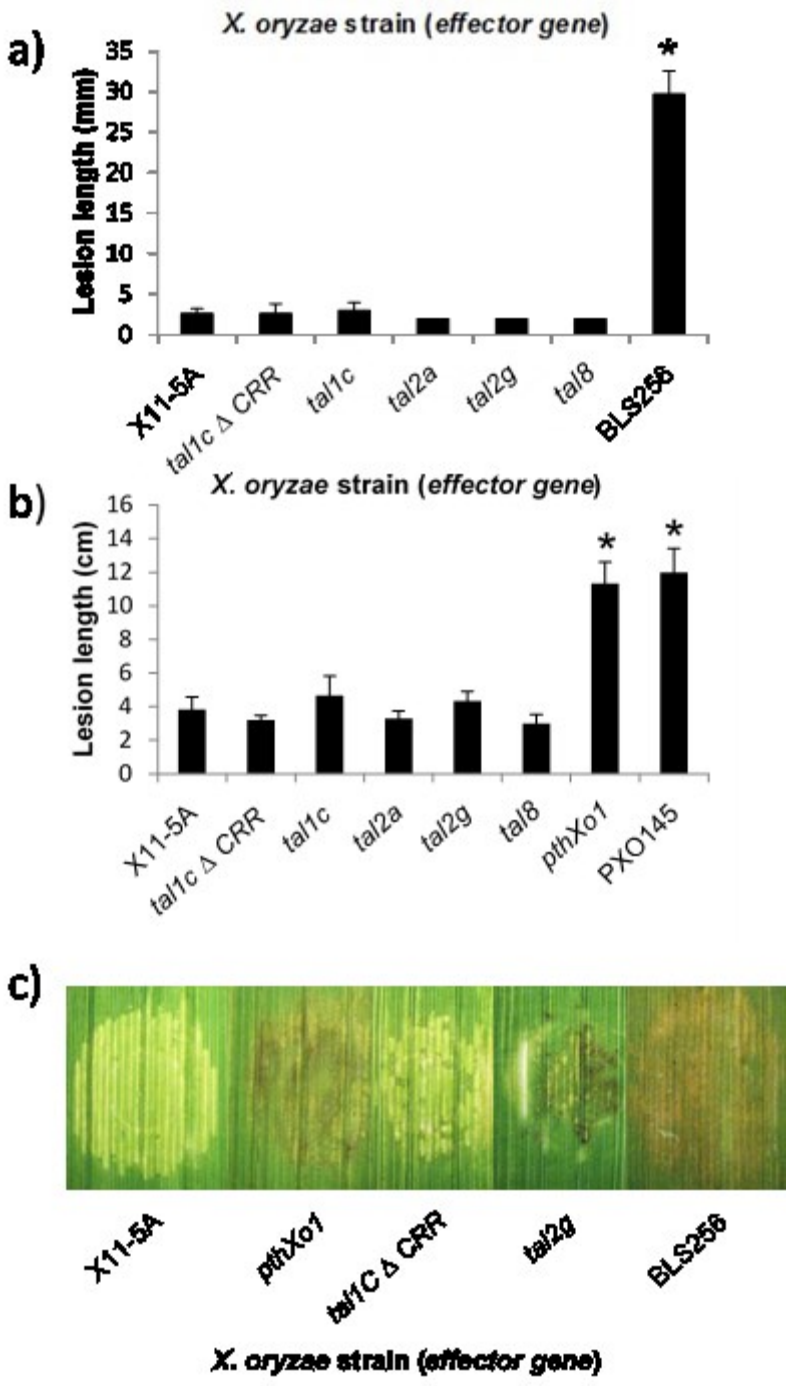

Figure 2.7. ***X. oryzae*** **pv.** ***oryzicola*** **(*Xoc*) TAL effectors (Tal1c, Tal2a, Tal2g, and Tal8) delivered by X11-5A do not affect lesion development after inoculation by a) infiltration of rice variety Nipponbare and b) leaf-clipping of rice variety Azucena.** a) Mean length of spread

beyond the circular infiltration site at 11 days after inoculation, and b) mean lesion length at 15 days after inoculation; error bars are standard deviation. c) Both *X. oryzae* pv. *oryzae* (*Xoo*) and *Xoc* TAL effectors delivered by X11-5A activate resistance gene *Xa27* after infiltration of rice transgenic line *UXO-3*, confirming translocation of the effectors. *UXO-3* is a transgenic line containing the *Xa27* resistance gene with the effector binding sites (EBE) for PthXo1 and Tal2g engineered into its' promoter. Leaves were inoculated with *X. oryzae* strain X11-5A with or without *Xoc* or *Xoo* TAL effectors, or with *Xoc* strain BLS256 or *Xoo* strain PXO145. *UXO-3* leaves infiltrated with X11-5A delivering TAL effectors PthXo1 and Tal2g and *Xoc* BLS256 exhibited an HR (photographed at 72 h after inoculation). For all experiments, three biological replications with more than 10 observations per replication were performed with consistent results. Asterisk indicates significant difference at $P<0.05$.

Supporting Information:

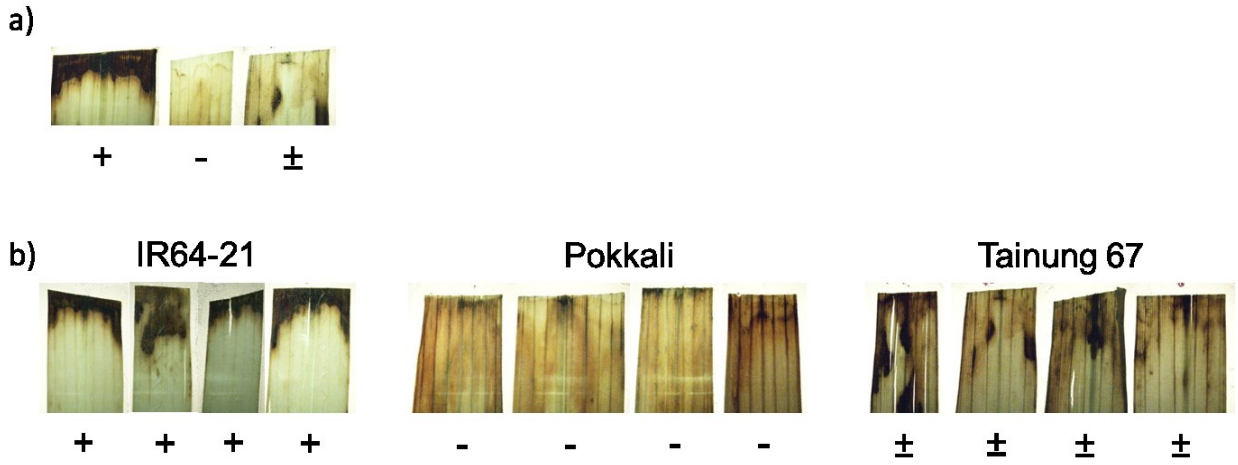

Figure S2.1. (a) Rating system for dark brown (DB) phenotype of cleared leaves. Three independent raters compared lesions with the photographs of a clear DB response and a clear lesion and rated whether the lesions at the tips of ethanol-cleared leaves predominantly matched color in the (+) images or the (–) images. (b) Representative images of inoculated, cleared leaves from three varieties of rice and how they were rated. All raters gave the same scores for each treatment except for the cultivar Tainung 67, which was given an intermediate value of +/- for each treatment. Three leaves were rated for each of four treatments on 21 lines. For each variety, leaves were inoculated with X11-5A, X11-5A(*pthXo1*), X11-5A(*talC*), and X11-5A(*avrXa7*).

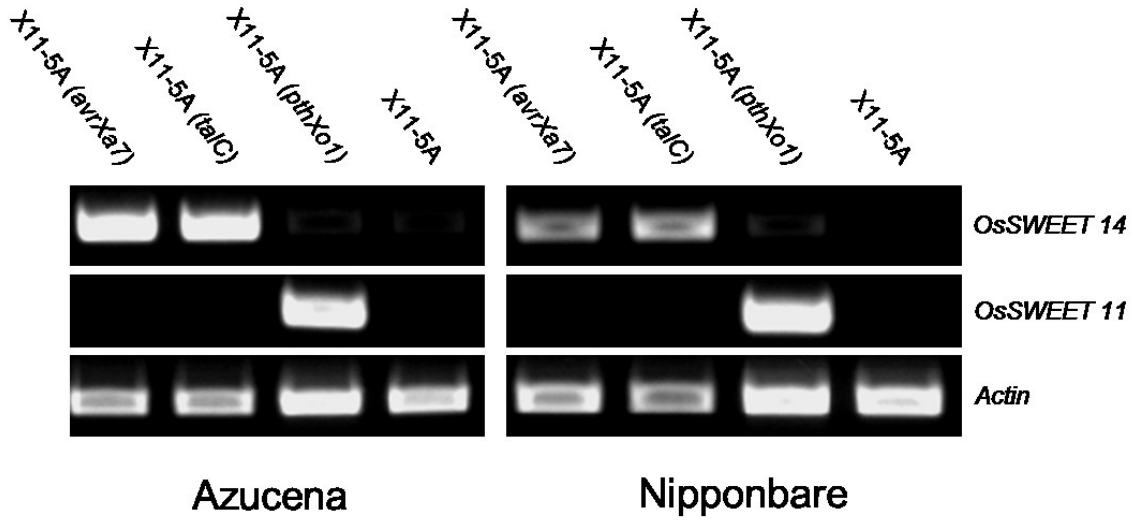

Figure S2.2. Reverse Transcriptase (RT)-PCR of *OsSWEET11* and *OsSWEET14* induction in mRNA isolated from Azucena and Nipponbare tissue inoculated with X11-5A and X11-5A expressing *avrXa7*, *talC*, or *pthXo1*. Tissue was collected 48 hai.

Table S2.1. Host response[a] to *X. oryzae* X11-5A with and without plasmid-borne copies of the *avrXa7, pthXo1,* or *talC* genes on 21 rice varieties.

			TAL effector gene							
			None		*avrXa7*		*pthXo1*		*talC*	
IRIS GID	**Subspecies**	**Variety**	**LL±SD**	**DB**	**LL±SD**	**DB**	**LL±SD**	**DB**	**LL±SD**	**DB**
2254728	japonica	Nipponbare	0.3±0.1	+	0.3±0.1	+	0.3±0.0	+	0.5±0.1*	+
2021623		Tainung 67	0.5±0.0	±	0.9±0.3*	±	0.8±0.3*	±	0.8±0.3*	±
2254732		Li-Jiang-Xin-Tuan-Hei-Gu (LTH)	3.4±0.9	-	7.1±1.2*	-	8.1±2.0*	-	9.4±1.6*	-
2254723		Dom-Sufid	1.0±0.0	-	4.7±0.6*	-	5.0±0.1*	-	8.4±1.7*	-
2254738		M 202	2.2±0.5	-	4.3±0.4*	-	6.3±1.1*	-	4.3±1.1*	-
2254722		Moroberekan	3.2±0.9	-	7.8±0.5*	-	11.5±1.4*	-	7.3±1.1*	-
2254730		Azucena	1.9±0.6	-	7.0±1.3*	-	8.8±0.5*	-	9.2±0.8*	-
2254737		Cypress	1.7±0.4	-	0.5±0.0*	+	3.8±1.7*	-	1.6±0.4	-
2874091		Kitaake	0.8±0.4	-	1.2±0.3	-	4.3±0.8*	-	8.1±1.1*	-
2254719	indica	Sadu-cho	2.0±0.4	-	0.5±0.0*	+	2.5±0.5	-	2.5±1.3	-
2030504		Minghui 63	0.5±0.0	+	1.4±0.4*	±	2.9±0.7*	±	1.1±0.2*	±
2254731		Shan-Huang Zhan 2 (SHZ2)	0.5±0.0	+	0.5±0.1	+	0.5±0.0	+	0.5±0.0	+
2254729		IR64-21	0.5±0.0	+	0.5±0.0	+	0.5±0.0	+	0.5±0.0	+
2254736		Swarna	0.4±0.1	+	0.4±0.1	+	0.4±0.1	+	0.3±0.2	+
2254727		Pokkali	1.2±0.5	-	0.5±0.0*	-	4.0±0.7*	-	0.7±0.5*	-
2254724		Aswina	0.2±0.1	-	0.4±0.1	-	2.1±0.5*	-	0.3±0.1	-
2030525		Zhenshan 97B	1.1±0.4	-	0.6±0.2	+	1.2±0.4	+	4.4±0.9*	±
2254720	aus	N 22	0.8±0.3	-	2.0±0.7*	-	5.4±2.0*	-	3.2±0.7*	-
2254725		Dular	0.5±0.0	+	2.6±0.9*	-	1.3±0.8*	+	0.7±0.2	+

2254726	Rayada	0.4±0.1	-	0.4±0.1	-	0.6±0.2	-	0.5±0.0	+
2254721	FR13 A	0.2±0.0	-	3.0±0.4*	-	3.3±1.1*	-	5.1±1.4*	-

[a] Lesion lengths (LL, mean of 10 replicates in cm) 15 days following clip inoculation; SD, standard deviation; *, greater than corresponding value with no TAL effector gene by student's *t* test, $p < 0.05$. The presence (+) or absence (-) of a dark brown (DB) resistance response was assessed by leaf clearing of leaf samples taken at 15 days after leaf clipping. A weak resistance response is indicated by "±."

Table S2.2. **Summary of changes in responses of 21 rice varieties to X11-5A due to heterologous expression of *avrXa7*, *pthXo1*, or *talC*.**

+++[a]	++0	0++	0+0	000	00+	-+0	-00	-+-
Azucena	Dular	Kitaake	Aswina	Swarma	Zheshan	Cypress	Sadu-cho	Pokkali
Moroberekan				IR64-21				
Dom-Sufid				SHZ-2				
Tainung 67				Rayada				
M 202				Nipponbare				
LTH								
Minghui 63								
FR13 A								
N22								

[a] Each column header shows the response changes due to AvrXa7, PthXo1, and TalC in order, relative to X11-5A with no TAL effector.

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CHAPTER THREE.

TAL EFFECTOR *AVRxa5P*$_{86}$ CONFERS AVIRULENCE FUNCTION TO *XANTHOMONAS ORYZAE* PV. *ORYZAE*

INTRODUCTION

Xanthomonas oryzae pv. *oryzae* (*Xoo*), causal agent of bacterial blight (BB), is the most destructive bacterial pathogen of *Oryza sativa* (rice). BB can cause up to a 75% yield loss depending on the environmental conditions and the developmental stage of the rice plant (Mew et al., 1993; Mizukami and Wakimoto, 1969; Reddy et al., 1979). All *X. oryzae* pathovars are classified as select agents in the United States of America and are species of quarantine importance (Plant Protection Act, 7 CFR Part 330); Agricultural Bioterrorism Protection Act of 2002 (7 CFR Part 331). Although *X. oryzae* strains were found in the USA, they are distinct from *Xoo*, are weakly virulent, and have not been reported to contribute to a significant yield loss (Jones et al., 1989). The USA strains of *Xo* have not been designated a pathovar (Triplett et al., 2011).

Bacterial pathogens use virulence factors to facilitate infection of their host plant. Virulence factors of *Xoo* include TAL (transcription activator-like) effectors and the bacterial type III secretion system (TTSS). The TTSS is a syringe-like mechanism used by *Xoo* to deliver virulence factors into the host cells. TAL effectors are specialized proteins that are injected into the host plant by the TTSS and activate the transcription of specific host genes. The structure of TAL effectors is highly conserved. The N-terminus of each TAL effector contains a nuclear localization signal (NLS) and an acid transcriptional activation domain (AD). The C-terminus contains a translocation signal that mediates transfer across the TTSS secretory apparatus. The

central domain of the TAL effector is formed by a central repeat region (CRR) of 102 base pair (bp) nucleotide repeats. These 102 bp repeats encode for 1.5 to 33.5 repeats in the TAL effector sequence (Boch et al., 2009). The 102 bp repeated motif encodes for 34 amino acids. Amino acids 12 and 13 within each repeat are known as the repeat variable diresidue (RVD) and these are the repeats that dictate the binding to specific nucleotides of the host plant target gene (Boch and Bonas, 2010; Boch et al., 2009; Moscou and Bogdanove, 2009). The RVDs of a TAL can be used to predict the target (binding site) of the TAL (Doyle et al., 2012). The main differences among various TAL effectors are the number of repeats and the amino acids that encode for the RVDs (Boch and Bonas, 2010; Boch et al., 2009).

Depending on the genetic background of the host plant, TAL effectors may induce the expression of both susceptibility genes to support bacterial virulence or resistance genes to confer a resistance response (Boch and Bonas, 2010). TAL effectors such as AvrXa7 from *Xoo* are predicted to support bacterial virulence by activating sucrose transporter genes. Sucrose transporter genes are involved in the efflux of glucose from the plant host cell to the intercellular spaces in which the pathogenic bacterial reside (Chen et al., 2010). TAL effectors may also target the transcriptional machinery of the host plant cell to decrease or impede resistance. For example, the *Xoo* effectors PthXo6 and PthXo7 target a bZIP transcription factor (*OSTFX1*) and the subunit of transcription factor IIA (*OsTFIIAγ1*), respectively (Sugio et al., 2007). Interference or activation of transcriptional machinery by bZIP can benefit pathogens, because these transcription factors may regulate plant defense, development, secondary metabolism, hormone signal transduction, and abiotic stress tolerance (Corrêa et al., 2008; Jakoby et al., 2002).

Plants have evolved resistance genes against virulence factors such as TAL effectors. Pathogenic *Xoo* strain PXO86 is avirulent to rice containing resistance genes *xa5*, *Xa7*, *Xa8*, and *Xa10*. Thus, PXO86 is predicted to harbor the corresponding effectors Avrxa5, AvrXa7, AvrXa8 and AvrXa10; of these, AvrXa7 and AvrXa10 are known to be TAL-type effectors (Hopkins et al., 1992). These effectors are translocated to the host plant cell by the TTSS and the TAL effectors are then delivered into the nucleus. Most *Xoo* effectors are predicted to have a virulence function, although the relative contributions of each effector vary (Bai et al., 2000; Vera Cruz et al., 2000). If the plant has resistance (R) genes corresponding to the effectors, a defense response is activated, frequently culminating in a hypersensitive response (HR). How or where plant R proteins recognize and interfere with the virulence functions of *Xoo* effectors through activation of resistance are still not known. The cloning and characterization of different effector and R genes are important steps in helping to resolve this mystery.

Rice resistance gene *xa5*, located on chromosome 5, is particularly interesting because it is recessive resistance gene. The gene encodes the gamma subunit of the general transcription factor *OsTFIIA* gene (*OsTFIIAγ5*) (Iyer and McCouch, 2004; Iyer-Pascuzzi et al., 2008). Interactions of Avrxa5 and xa5 in rice lead to an HR response (Hopkins et al., 1992). Interestingly, when the TAL effector from *Xoo* PthXo7 is present in a strain with *avrxa5*, an increase in virulence is observed in plants with *xa5* (Sugio et al., 2007).

Previously, Hopkins et al. (1992) cloned a fragment of genomic DNA from *Xoo* PXO86 that conferred *avrxa5* and *avrXa10* activities to *Xoo*. The clone, pXO6-33, was shown to contain two TAL effector genes by using DNA blot hybridization with the TAL effector *avrXa10* as a

probe (Jan E. Leach, *unpublished*). When *Xoo* strain PXO99^{A} (virulent to rice containing *xa5*) containing cosmid pXO6-33 was inoculated on rice line IRBB5 (containing resistance gene *xa5*), the resulting phenotype was an HR, suggesting that pXO6-33 harbors *avrxa5* (Bai et al., 2000; Hopkins et al., 1992). However, the identity of the *avrxa5* gene was not confirmed by subcloning from pXO6-33.

The goal of this study was to identify and characterize the *Xoo* strain PXO86 *avrxa5* gene by subcloning the gene and studying its function in rice with and without the R gene *xa5*. Based on prior work (Bai et al., 2000; Hopkins et al., 1992), we predicted that *avrxa5* was encoded by a TAL effector. Furthermore, during the course of the study, a gene with Avrxa5 function was cloned from *Xoo* strain JXOIII, and that *avrxa5* gene was shown to encode a TAL effector (Zou et al., 2010). Thus, in this study, we used knowledge of the conserved structure of TAL effectors to subclone and characterize the TAL effector gene *avrxa5* from pXO6-33. To study the effect of the isolated TAL effector gene, we expressed *avrxa5* in *Xo* strain X11-5A (Jones et al., 1989), which contains the TTSS but no TAL effector genes (Triplett et al., 2011; Verdier et al., 2012).

MATERIALS AND METHODS

Strains and plasmids

Bacterial strains and plasmids used in this study are listed on Table 3.1. *Xo* strains were grown on PSA media (peptone, 10 g l^{-1}; sucrose, 10 g l^{-1}; glutamic acid, 1 g l^{-1}; pH 7.0) at 28°C. Where appropriate, antibiotics were added to the media to a final concentration of 50 μg l^{-1} of spectinomycin and streptomycin each or 2 μg l^{-1} of tetracycline.

Cloning and sequencing of TAL effector *avrxa5* from cosmid pXO6-33

pXO6-33 was digested by *BamH*I (New England BioLabs, Ipswich, MA, USA) for 2 h. The digestion product was separated on a 0.8% agarose gel stained with ethidium bromide for visualization. Images were captured using Gene Genius digital image capture system and GENESNAP Software (SynGene, Cambridge, UK) version 7.12. The digestion product separated into seven bands; 1.5, 2.1, 2.6, 3.1, 4.5, and 7.0 kbp. The bands were extracted individually using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) using the manufacturer's protocol. The extracted bands were cloned into pBluescript II KS+ (Stratagene Inc. La Jolla, CA, USA), producing pBS1.5b, pBS2.1b, pBS2.6b, pBS3.1b, pBS4.5b, and pBS7.0b plasmids. Pair ends of the insert were sequenced using universal primers M13 forward and reverse.. Sequences were analyzed by using the NCBI BLAST tool (http://blast.ncbi.nlm.nih.gov). Inserts with TAL gene sequence homology were selected. Pair end sequencing of pBS7.0b had no homology with known TAL gene sequence; however, we proceeded to subclone because pair end sequencing revealed 25 percent of insert sequence.

Selected plasmids with TAL gene sequence homology (pBS2.6b, pBS3.1b, and pBS4.5b) or unknown sequence (pBS7.0b), were subcloned following digestion with *Sph*I (New England BioLabs, Ipswich, MA, USA). The conserved regions flanking the CRR of TALs frequently contain *Sph*I endonuclease cut sites (Cermak et al., 2011; Li et al., 2010). The *Sph*I digestion produced no bands from pBS2.6b, two bands (2.1 and 1.0 kbp) from pBS3.1b, two bands (2.5 and 1.0 kbp) from pBS4.5b, and two bands (2.6 and 0.7 kbp) from pBS7.0b. pBS3.1 was no longer subcloned because it contained known TAL *avrXa10* (Hopkins et al., 1992).

pCS466 is a derivative of Gateway entry vector pCR8-GW (Invitrogen, Carlsbad, CA, USA) which contains a truncated form of the *Xoc* BLS256 *tal1c* gene, in which the *Sph*I fragment that comprises the CRR had been removed. *Sph*I fragments from pBS4.5b and pBS7.0b were individually cloned into the single *Sph*I site of the truncated *tal1c* gene, giving rise to pCS2.5s and pCS1.0s from pBS4.5b; and pCS2.6s and pCS0.7s from pBS7.0b. Primer p235 (Forward: 5'-GGAGGCCTTGCTCACGGATGC-3') (courtesy of Raul Andres Cernadas), designed based on the 5' conserved region of TAL shortly before the CRR, and the M13 universal reverse primer were used to sequence pCS2.5s, pCS1.0s, pCS2.6s, and pCS0.7s. Because it contained sequence common to TAL, pCS2.5s was selected for further subcloning. The 2.5 kbp CRR insert within the truncated *tal1c* gene was then transferred to the broad host-range destination vector pKEB31 for expression in *Xanthomonas* (Cermak et al., 2011; Addgene plasmid 31224; www.addgene.org) using Gateway LR Clonase (Invitrogen) following the manufacturer's instructions, producing pK2.5s. Based on information from sequencing with the p235 forward and M13 reverse primers, additional primers were designed by using jPCR (Kalendar et al., 2011) to further sequence into the CRR of pK2.5s (Supplemental Figure 3.1).

Transformation of *Xo* strains

Plasmids were introduced into the *Xoo* strain PXO99A and *Xo* strain X11-5A by electroporation (Choi and Leach, 1994). Plasmid stability and presence in the *Xo* strains was confirmed as previously described (Verdier et al., 2012).

Plant material and plant inoculations

O. sativa subsp. *indica* cultivar IRBB5, which contains the *xa5* resistance gene, and is near-isogenic to the recurrent backcross parent IR24 (Ogawa et al., 1988), was provided by C.M. Vera Cruz from International Rice Research Institute (IRRI). *O. sativa* subsp. *japonica* cultivars Azucena (Philippines) and Nipponbare (Japan), are dominant for the *Xa5* allele (Jiang et al., 2006, Kim et al., 2009), and were used to determine if Avrxa5 increased virulence of strains X11-5A and PXO99A, respectively. Seeds were germinated for 5 days, and the seedlings were then planted in a 3 inch square pots in soil composed of 4 parts peat moss, 1 part fine grain sand, and 4 parts Pro-Mix with Mycorrhizae (Premier Tech Horticulture, Québec, Canada). Plants were grown at 24-28°C with 85% relative humidity with a 12 h day and 12 h night photoperiod.

Xo strains were grown on PSA media with appropriate antibiotics for 24 h at 28°C, then re-suspended in sterile distilled water at an optical density (OD_{600}) of 0.2 (*c*. 10^8 CFU ml^{-1}) to use for infiltration or leaf clipping. For infiltration assays, four-week-old plants were inoculated with a needleless syringe as described previously (Reimers and Leach, 1991). Infiltrated samples were collected at 7 days after inoculation (dai). For leaf clip assays, all fully expanded leaves on each tiller of six-week-old rice plants were inoculated as described previously (Kauffman et al., 1973). Lesion lengths (centimeters) were measured at 15 or 19 dai. Wild type (untransformed) strains PXO99A and X115-A were used as negative controls. Strains supplemented with pXO6-33 and *Xoo* strain PXO339 known to have Avrxa5 activity were used as positive controls.

To assess *in planta* multiplication of *Xo* X11-5A and X11-5A(pK2.5s) on rice lines IR24 and IRBB5, four-week-old plants were inoculated using a needleless syringe. A 5 cm section was

marked in the middle of the three tallest fully expanded leaves. The 5 cm section was then infiltrated with a needleless syringe three times on each side of the central vein with spacing of 2 mm between each infiltration, for a total of six infiltration inoculations per 5 cm section (Supplemental Figure 3.3). Samples were collected 30 min after the infiltration (0 dai) and then at 3 and 7 dai. The 5 cm section was ground in 1 ml of sterile water, and bacterial numbers were assessed as described by (Verdier et al., 2012). The experiment was repeated twice.

To assess *in planta* multiplication of *Xo* X11-5A(pthB), X11-5A and X11-5A(pK2.5s) on Azucena, six-week-old plants were inoculated by leaf clipping. A 10 cm sample was collected 30 minutes after the infiltration (0 dai) and then at 7 dai. The 10 cm section was ground in 1 ml of sterile water, and bacterial numbers were assessed as described by (Verdier et al., 2012).

TAL effector binding site prediction

TALVEZ (version 3.1) (Pérez-Quientero et al., 2013) was used to predict the targets (binding sites) of TAL effectors Avrxa5P$_{86}$, PthXo7, Avrxa5, TalC, PthXo6, and PthXo1 using the *O. sativa* subspecies *japonica* cultivar Nipponbare annotated genome (version 7.0) (Ouyang et al., 2007). From the top ranking 500 predicted targets, which included genes other than transcription factors, only those in the MYB and bZIP transcription factor prediction sites were used from each TAL for analysis.

RESULTS

avrxa5 encodes a TAL effector that is recognized by *xa5.*

To clone *avrxa5* and study the Avrxa5- *xa5* interaction, we used the TAL effector deficient *Xo* strain X11-5A (Verdier et al., 2012). *avrxa5* activity was associated with the 4.5 kb *Bam*HI fragment contained in pXO6-33, however, subcloning of the complete *Bam*HI fragment into entry vector was not successful, as the fragment always inserted in the wrong orientation. Thus, to facilitate cloning of the *avrxa5* gene, we introduced the central repeat region (CRR) from the 4.5 kb *Bam*HI TAL homolog, removed as a *Sph*I fragment from pBS4.5b, into the entry vector pCS466 to recombine with pKEB31, creating plasmid pK2.5s. Wild type X11-5A causes a susceptible response (water soaking) on IRBB5 (*xa5*) plants while X11-5A(pK2.5s), *Xoo* strain PXO339 (*avrxa5*), and X11-5A(pXO6-33) conferred an HR on IRBB5 that was clearly visible between 5-7 days after inoculation (Figure 3.1). Of the subclones tested, only pK2.5s conferred a *xa5*-gene specific HR to X11-5A, indicating the 4.5 kb *Bam*HI fragment in pXO6-33 harbors the *avrxa5* gene, and that *avrxa5* encodes a TAL effector.

To determine if the *avrxa5* function in pK2.5s might be affected by the *Xoo* strain used for delivery, we introduced pK2.5s into *Xoo* strain PXO99A and inoculated 6-week-old IRBB5 plants using the leaf clipping assay (Figure 3.2). When inoculated with PXO99A(pK2.5s), PXO339, or PXO99A(pXO6-33), lesions on IRBB5 were significantly shorter ($P< 0.05$) than lesions on IR24 (Figure 3.2a). When inoculated with the wild type strain PXO99A, lesion lengths were not different on IRBB5 vs. IR24 (Figure 3.2a). Lesion lengths caused by PXO99A(pK2.5s) were significantly shorter on IRBB5 than those caused by the wild type PXO99A ($P< 0.05$) (data not shown). The appearance of lesions on IRBB5 inoculated with PXO99A(pK2.5s), PXO339, and

PXO99A(pXO6-33) was dark brown at 19 days after inoculation; this dark brown response was clearly evident at 7 dai (data not shown). All of the IR24 inoculated leaves had typical bacterial blight symptoms (Figure 3.2b). Thus, *avrxa5* in pK2.5s also confers resistance to *Xoo* strain PXO99A, a strain that contains many endogenous TAL effector genes.

To further validate the phenotypes observed from the X11-5A and PXO99A strains harboring pK2.5s, we assessed *in planta* multiplication of X11-5A and X11-5A(pK2.5s). Rice lines IR24 and IRBB5 were inoculated at 4 weeks after sowing. Bacterial numbers on IR24 plants did not differ among strains at any time point after inoculation ($P< 0.05$) (Figure 3.3a). In contrast, at 3 and 7 days after inoculation of IRBB5, bacterial numbers for strains harboring pK2.5s were lower than the strains without the clone ($P< 0.05$) (Figure 3.3b). Taken together, these results indicate that the CRR region of the TAL effector from the 4.5 kb *Bam*HI fragment in pXO6-33 activates *xa5* to confer resistance in rice line IRBB5; because of this the TAL effector gene in pK2.5s is hereby named *avrxa5P*$_{86}$.

Avrxa5P$_{86}$ is distinct from other TAL effector genes.

In TAL effectors, amino acids 12 and 13 within each repeat are described as the repeat variable diresidue (RVD) (Boch and Bonas, 2010; Boch et al., 2009; Moscou and Bogdanove, 2009), information which can be used to predict the target (binding site) of a given TAL (Doyle et al., 2012). We aligned the RVDs of Avrxa5P$_{86}$ to those of other known TAL sequences including the Avrxa5 recently characterized by (Zou et al., 2010). The unique arrangement of RVDs in Avrxa5P$_{86}$ indicates a new TAL with Avrxa5 activity (Table 3.2). From the RVD alignment,

Avrxa5P$_{86}$ is most similar to PthXo7. The RVDs from PthXo6 (Sugio et al., 2007) and PthXo1 (Yang and White, 2004) were least similar to the Avrxa5P$_{86}$ RVDs (Table 3.2).

Avrxa5P$_{86}$ potentially interacts with transcription factors

TALVEZ, a new prediction software tool, was used to predict targets for the RVDs of Avrxa5P$_{86}$ in the regions 1 kbp upstream of genes in the rice reference Nipponbare genome. Because *xa5* encodes for a subunit of transcription factor IIA, and in interactions with Avrxa5, a series of defense responses are activated, presumably through activation of additional transcription factors, we were interested in identifying other transcription factors that are predicted to be activated by Avrxa5. Among the top 500 predictions, specific targets were predicted in the promoters of several transcription factor families; the most prevalent were members of the MYB and bZIP transcription factor families. Avrxa5P$_{86}$ was predicted to target as many transcription factor genes as other TAL effectors, some of which are known to target transcription factors (PthXo7, Avrxa5, and PthXo6) (Table 3.3). Interestingly, neither the promoters of *R* gene *xa5* or the dominant allele *Xa5*, which encode for the gamma subunit of general transcription factor TFIIA (Iyer & McCouch, 2004), were predicted to interact with Avrxa5.

Avrxa5P$_{86}$ confers a resistance response on *japonica* cultivars with *Xa5* homozygous alleles

Several TAL effectors function as virulence effectors in *Xoo* (Bai et al., 2000; Yang et al., 2000; Yang and White, 2004; Yu et al., 2011). Bai et al. (2000) showed that inactivation of *avrxa5* in strain PXO86 resulted in a reduction of virulence of *Xoo.* To determine if *avrxa5P$_{86}$* present in pK2.5s would enhance virulence, we inoculated *japonica* varieties Azucena and Nipponbare with X11-5A(pK2.5s) and PXO99A(pK2.5s). Azucena (Kim et al., 2009) and Nipponbare (Jiang et al.,

2006) are homozygous for the *Xa5* allele, and are susceptible to both wild type X11-5A (Verdier et al., 2012) and PXO99A (Zou et al., 2010). Surprisingly, when these strains were supplemented with pK2.5s, a resistance response was observed on both Azucena and Nipponbare (Figures 3.4-3.7). The resistance response is unlike the HR resistance that is observed with IRBB5, because there is no darkening of the tissue. However, bacterial numbers are reduced, and lesions lengths are shorter in the novel resistance response. As a control to show that a TAL effector could enhance virulence to *Xo* we inoculated Azucena with X11-5A(pthB); PthB is a TAL effector that is known to enhance virulence (Castiblanco et al., 2013). Indeed, the presence of PthB in X11-5A resulted in higher bacterial numbers in Azucena, confirming that virulence TAL effector could enhance virulence to this cultivar. Taken together, these results indicate that *avrxa5P$_{86}$* is confers a novel, effector-gene specific strong non-HR resistance response in the *japonica* cultivars Azucena and Nipponbare.

DISCUSSION

The homolog of Avrxa5 from *Xoo* strain PXO86 (Avrxa5P$_{86}$) is a TAL effector and confers a strong HR with recessive resistance gene *xa5* when delivered by the TAL deficient *Xo* strain X11-5A. Although the HR induced by Avrxa5P$_{86}$- *xa5* interactions is weaker than other effector-*R* gene interactions such as AvrXa7- *Xa7* and AvrXa10- *Xa10* (Hopkins et al. 1992), the resistance is effector-*R* gene specific, and reduces bacterial multiplication and lesion lengths, particularly for the mildly virulent pathogen X11-5A, in the inoculated leaves.

The phenotype conferred to *Xo* X11-5A by Avrxa5P$_{86}$ is not *Xo* strain-specific because *Xoo* strain PXO99A harboring Avrxa5P$_{86}$ also demonstrated reduced lesion lengths and lower final

bacterial numbers on IRBB5. Initial browning of the leaf tip was observed during the resistant interaction, however, over time, lesions expanded beyond the leaf tip (Figure 3.2). The browning of the cut tips, an HR, is not as strong for interactions of PXO99A(pK2.5s) as that conferred by *Xoo* strain PXO339, which harbors a chromosomal copy *avrxa5*. There may be several reasons for the difference in phenotypes. First, PXO99A contains at least 19 different TAL effectors, including PthXo7, which may interfere with Avrxa5P$_{86}$ function. Previously it was shown that PthXo7 increases virulence in a *Xoo* strain that expresses Avrxa5 when inoculated onto rice line IRBB5 (Sugio et al., 2007). Second, plasmid pK2.5s may not be stably maintained in PXO99A through generations within the plant. Never-the-less, Avrxa5P$_{86}$ does confer the *avrxa5* function on PXO99A, because presence of pK2.5s in PXO99A results in shorter lesions on IRBB5 relative to IR24.

Cosmid pXO6-33 conferred a weaker HR response on rice line IRBB5 than did X11-5A(pK2.5s) (Figure 3.1). This might be because (1) the large cosmid pXO6-33 is present in lower copies per cell than pK2.5s, or (2) pXO6-33 also contains virulence factor AvrXa10 and other unknown virulence factors that could interfere with the interaction of Avrxa5 with *xa5*.

The Avrxa5- *xa5* interaction was reported to be directed by the central repeat region of an *avrxa5* previously identified and characterized from *Xoo* strain JXOIII (Zou et al., 2010). However, the RVDs of Avrxa5P$_{86}$ from *Xoo* strain PXO86 in our study are very different from the Avrxa5 reported by Zou et al. (2010), suggesting that the TAL effector central repeat regions of Avrxa5P$_{86}$ and Avrxa5 may have evolved independently to perform a similar function.

The RVDs of Avrxa5P$_{86}$ are most similar to PthXo7, the very TAL effector that can increase virulence of strain PXO86, which harbors Avrxa5P$_{86}$, on rice with IRBB5 (Sugio et al., 2007). PthXo7 was suggested to suppress host defense functions elicited by PXO86 (Sugio et al., 2007). Alternatively, due to the similarity of RVDs between PthXo7 and Avrxa5P$_{86}$, the two TALs might compete for binding to target sites, with PthXo7 having a higher affinity for genes that lead towards susceptibility pathways than Avrxa5P$_{86}$.

We used TALVEZ TAL prediction target software with TAL effectors Avrxa5P$_{86}$, PthXo7, Avrxa5, TalC, PthXo6, and PthXo1 using the *O. sativa* subspecies *japonica* cultivar Nipponbare annotated genome (version 7.0) (Ouyang et al., 2007; Pérez-Quientero et al., 2013). The promoter of the *Xa5* susceptibility gene in Nipponbare, the dominant allele of *xa5*, is predicted to contain a target of Avrxa5P$_{86}$ and TalC; however, the prediction ranking by TALVEZ was above 4,600 for both TALs and is, therefore, most likely a false positive prediction. Previously, Iyer and McCouch (2004) and Jiang et al. (2006) observed constitutive and similar levels of expression of genes *Xa5* and *xa5* from leaves of homozygous resistant and susceptible varieties of rice when inoculated with strains expressing Avrxa5. Thus, unlike the TAL effector AvrXa27 which binds to a target in the promoter of *R* gene *Xa27* (Römer et al., 2009), Avrxa5P$_{86}$ is not predicted to target and activate the *R* gene *xa5*.

From the top ranking 500 prediction targets, we focused on those in the MYB and bZIP transcription factor prediction sites from each TAL for further analysis. Avrxa5 was predicted to target the most transcriptional machinery of any TAL (Table 3.3). Furthermore, Avrxa5P$_{86}$ was predicted to target more bZIP transcription factors than the other TAL effectors. An Avrxa5P$_{86}$-

xa5 interaction causes an HR, and oxidative stress, which can be regulated by bZIP transcription factors, may be a component of the HR (Corrêa et al., 2008, Tiwari et al., 2002). Perhaps the HR is a result of the combined transcriptional activation of bZIP transcription factors and the interaction of Avrxa5P$_{86}$- *xa5*. Of course, these hypotheses would need to be validated experimentally, as the bZIP transcription factors that are predicted targets of Avrxa5P$_{86}$ may not regulate oxidative stress, or the predictions that they are targets may be erroneous.

At least half of the transcription predicted targets of Avrxa5P$_{86}$, PthXo7, Avrxa5, and PthXo6 were MYB family transcription factors. MYB transcription factors may regulate plant development, secondary metabolism [including lignin activation (Zhao and Dixon, 2011)], hormone signal transduction, disease resistance, abiotic stress tolerance (Katiyar et al., 2012). In its role as a virulence factor, a TAL effector may target these developmental transcription factors to divert or diminish the host cell's transcription or cellular processes (Cannone et al., 2012). Co-opting the transcription factors of the host plant cells could enable the pathogen to divert the plant's resources and enable successful invasion of the infection site.

PthXo6 had the lowest similarity of RVD with Avrxa5P$_{86}$, but it was predicted to target as much transcription machinery as Avrxa5P$_{86}$. Although several TAL effectors, such as PthXo6, PthXo7 target transcription factors (Sugio et al., 2007), and our analysis showed that Avrxa5 was predicted to target transcription factors, not all TAL effectors target transcription factors. TalC and AvrXa7 activate the same sucrose transporter gene (Yu et al., 2011), and PthXo1 also activates a different sucrose transporter gene in the same family (Yang et al., 2004). These three TAL were not predicted to target as many transcription factors. While no known sucrose transporter genes

were found in the top 2000 targets for Avrxa5P$_{86}$, there may be other susceptibility genes besides transcription factors targeted by Avrxa5P$_{86}$. The top 50 potential targets of Avrxa5P$_{86}$ effector are listed in Table 3.4. Experimental validation of potential targets by TAL effectors would help develop hypotheses as to the role of Avrxa5P$_{86}$ in *Xoo*-rice interactions.

Xo X11-5A is weakly virulent to rice, and produces a mild defense response in rice lines with the IR24 background. We previously demonstrated that cultivar Azucena, which has the dominant *Xa5* allele (Kim et al., 2009), is susceptible to X11-5A (Verdier et al., 2012). Furthermore, expressing certain TAL effectors in X11-5A can increase virulence to Azucena. For example, TAL effectors PthXo1 (Verdier et al. 2012) and PthB (Figure 3.5) both increase virulence of X11-5A. We tested if Avrxa5P$_{86}$ has a virulence function in Azucena. Unexpectedly, in Azucena, expression of Avrxa5P$_{86}$ resulted in reduced bacterial numbers and shorter lesions. Similarly, PXO99A, which is highly virulent to Azucena and Nipponbare, both *japonica* cultivars with the dominant *Xa5* allele, exhibited shorter lesions when it carried Avrxa5P$_{86}$. These results suggest that either Azucena and Nipponbare harbor another resistance gene that recognizes Avrxa5P$_{86}$, or that the product of the dominant *Xa5* from those cultivars can recognize and respond to Avrxa5P$_{86}$.

Jiang et al. (2006) and Zou et al. (2010) observed a susceptible response on Nipponbare when inoculated with strains PXO61 and JXOIII, both wild type for the *avrxa5* gene, and the cloned *avrxa5* from JXOIII did not confer a resistant response to PXO99A when inoculated to Nipponbare (Zou et al., 2010). We demonstrated that Avrxa5P$_{86}$ shows sequence differences from Avrxa5 from JXOIII (Zou et al., 2010), particularly in the sequences encoding the critical RVDs

for targeting. Because both *avrxa5* genes confer *R*-gene specific responses in *xa5*–containing varieties of rice, yet Avrxa5P$_{86}$ confers resistance to Nipponbare with the *Xa5* allele, it is possible that Avrxa5P$_{86}$ and Avrxa5 from JXOIII are an example of divergent evolution. The Avrxa5 effector from these strains could represent an evolution to avoid detection from resistance mechanisms in the Nipponbare background; however, it still confers resistance on rice plants homozygous for the *xa5* allele.

Our discovery of *avrxaP$_{86}$*, led to a unique resistance interaction in rice. Studying this interaction will offer new insights into how TAL effectors trigger resistance in rice and may lead towards the characterization of a novel *R* gene in rice. Avrxa5P$_{86}$ and Avrxa5, having the similar function of activating *xa5* with sequence differences, will uncover key aspects of TAL functional genomics. Finding novel TAL effector genes, such as Avrxa5P$_{86}$, will increase our ability to understand how different TAL effectors interact with host DNA and the different specific types of gene targets (Grau et al., 2013). Furthermore, the study of genes targeted by TAL effectors is important to find novel sources or pathways of susceptibility or defense mechanisms (Li et al., 2013). High throughput sequencing of TAL containing strains and function characterization of TAL effectors will provide insight into how TALs have evolved.

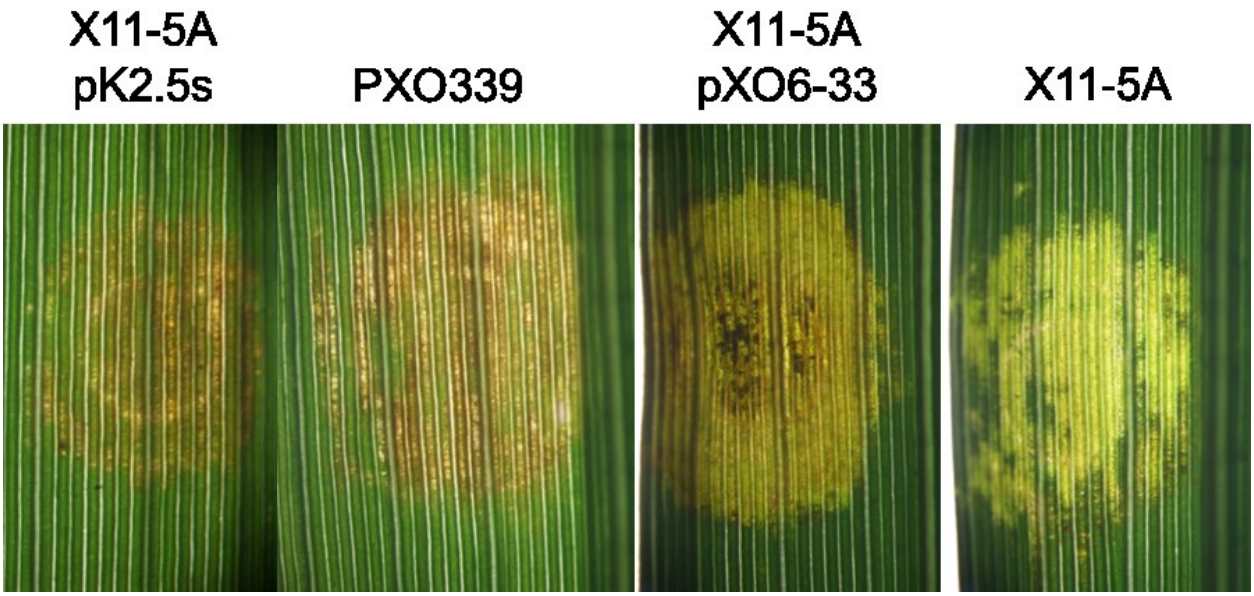

Figure 3.1. Reaction of IRBB5 rice leaves, homozygous for recessive resistance gene *xa5*, to *Xo* strain X11-5A(pK2.5s) (which contains the subcloned *avrxa5P*$_{86}$ gene), *Xoo* strain PXO339, X11-5A(pXO6-33), and wild type X11-5A. Photographs were taken on a light box at 7 days after infiltration. *xa5* confers a hypersensitive response (HR) in interactions with *avrxa5*, visible as a light browning of the leaf tissue at the infiltration site.

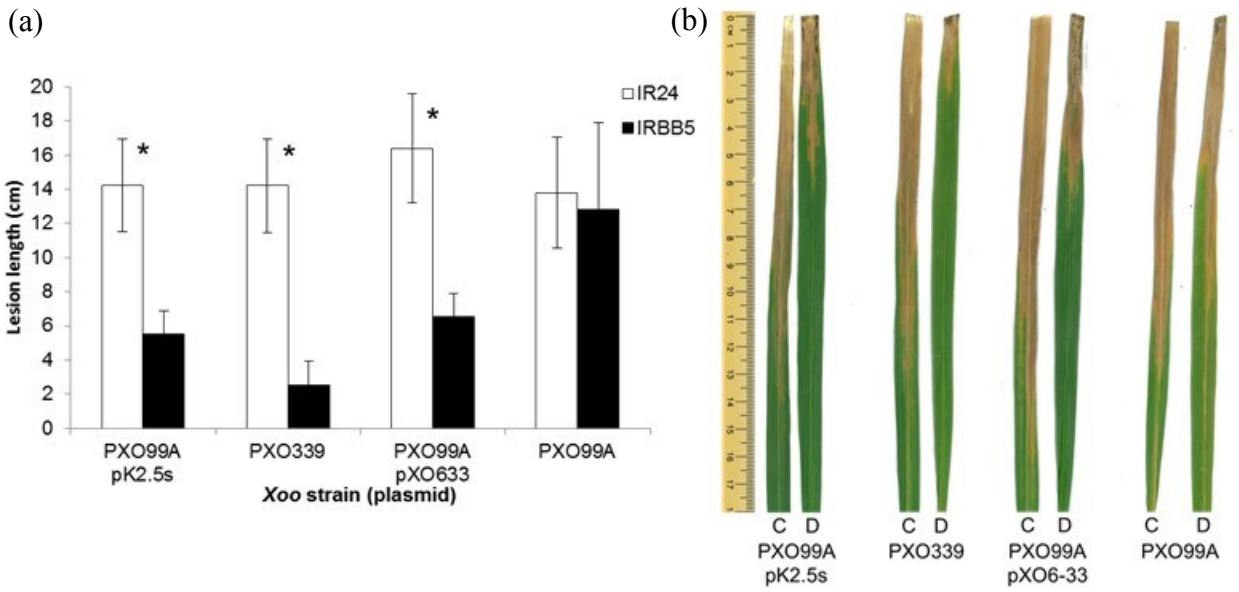

Figure 3.2. Lesion lengths caused by PXO99A(pK2.5s), *Xoo* strain PXO339 (*avrxa5*), PXO99A(pXO6-33), and wild type PXO99A on the near-isogenic rice lines IR24 (without *xa5*) and IRBB5 (*xa5*). (a) Lesions were measured 19 days after inoculation. An asterisk denotes a significant difference between each strain by rice line inoculated ($P < 0.05$). Error bars represent ± standard deviation. **(b)** Leaves are representative of phenotype and lesion length observed. Leaves marked "C" are IR24 and "D" are IRBB5.

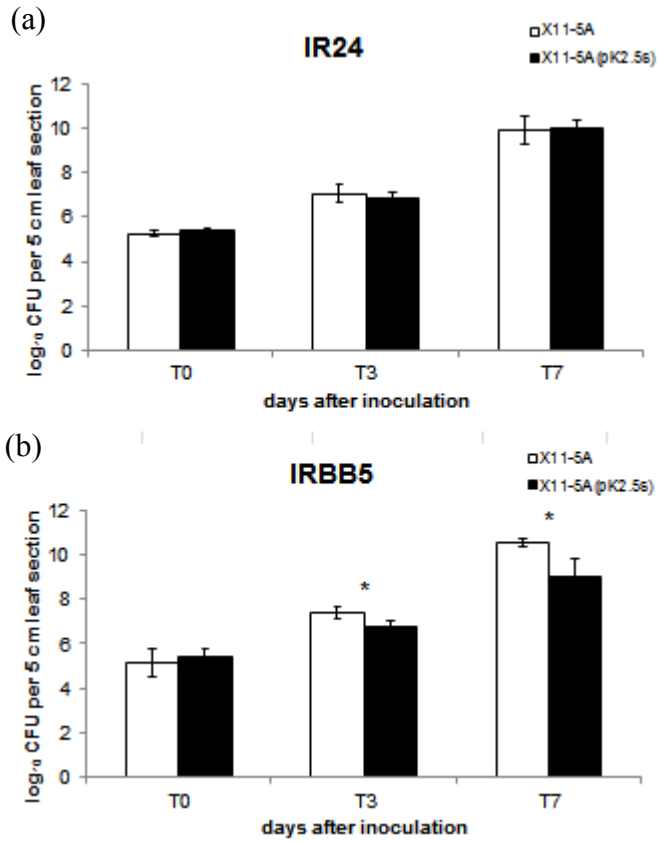

Figure 3.3. Bacterial numbers in rice lines IR24 and IRBB5 inoculated with *Xo* X11-5A and X11-5A(pK2.5s). Four-week-old plants of IR24 **(a)** and IRBBB5 **(b)** were infiltrated at six sites per 5 cm segment per leaf, and bacterial numbers were measured at 0, 3 and 7 days after inoculation. Asterisks indicate a significant difference between the two strains ($P< 0.05$). Error bars represent ± standard deviation. Values are representative of at least three leaves. The experiment was repeated twice.

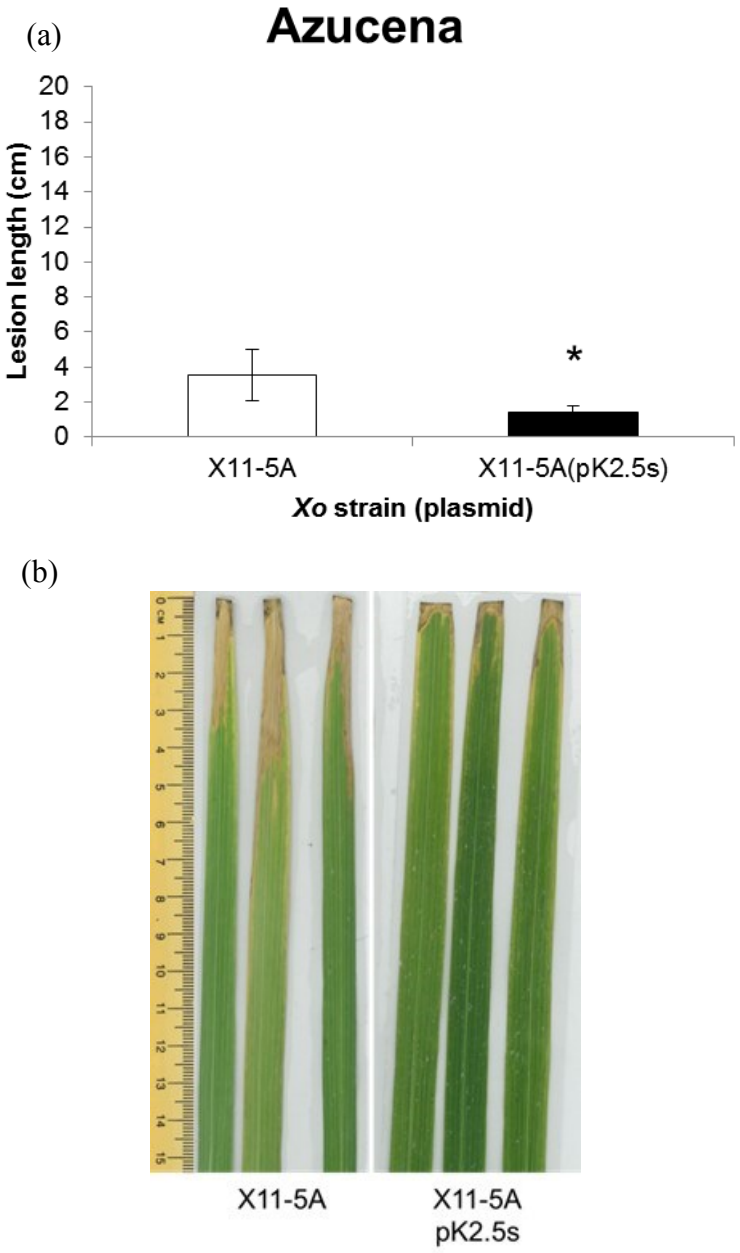

Figure 3.4. Lesion lengths caused by X11-5A(pK2.5s) and wild type X11-5A on Azucena (without *xa5*). (a) Lesions were measured 15 dai. An asterisk denotes a significant difference between each strain inoculated ($P < 0.05$). Error bars represent ± standard deviation. **(b)** Leaves are representative of phenotype and lesion length observed. The experiment was repeated twice.

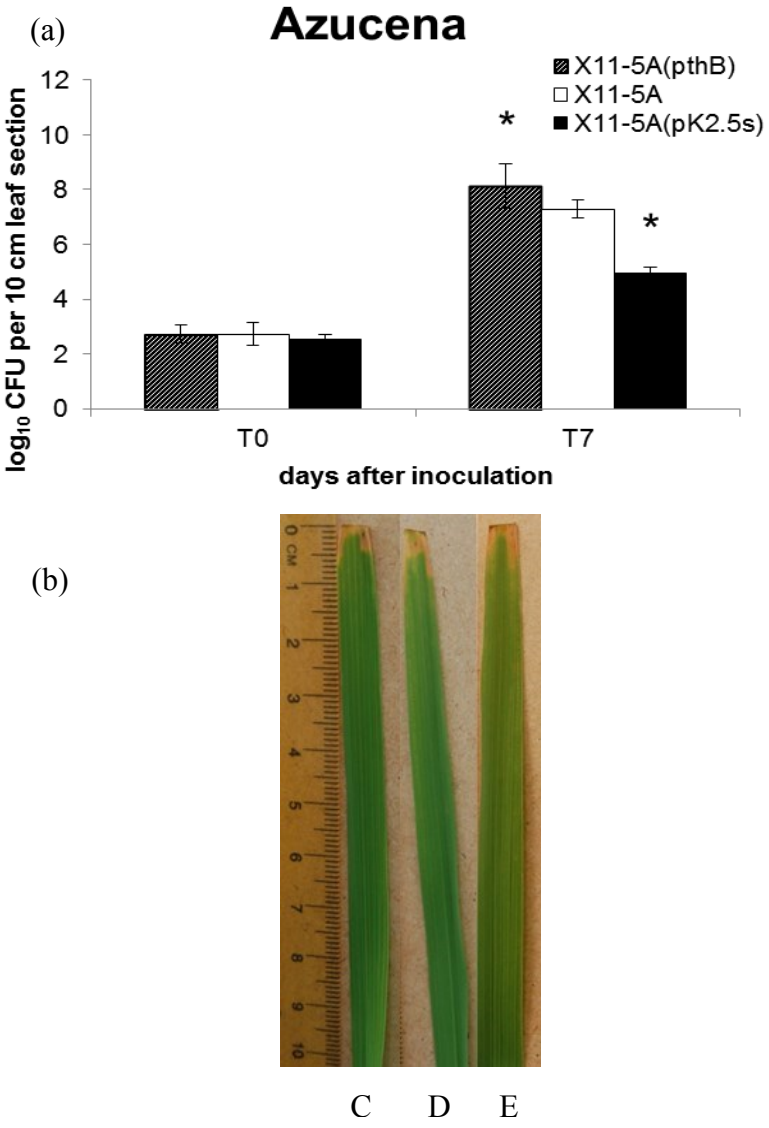

Figure 3.5. Bacterial numbers in Azucena inoculated with *Xo* X11-5A(pthB) (C), X11-5A (D), and X11-5A(pK2.5s) (E). Six-week-old plants of Azucena were inoculated by leaf clipping, 10 cm segment per leaf were taken, and bacterial numbers were measured at 0 and 7 days after inoculation **(a)**. An asterisk denotes significant difference between the wild type and the TAL effector-expressing derivative (P<0.05). Error bars represent ± standard deviation. Values are representative of at least three leaves. **(b)** Leaves are representative of phenotype and lesion length observed.

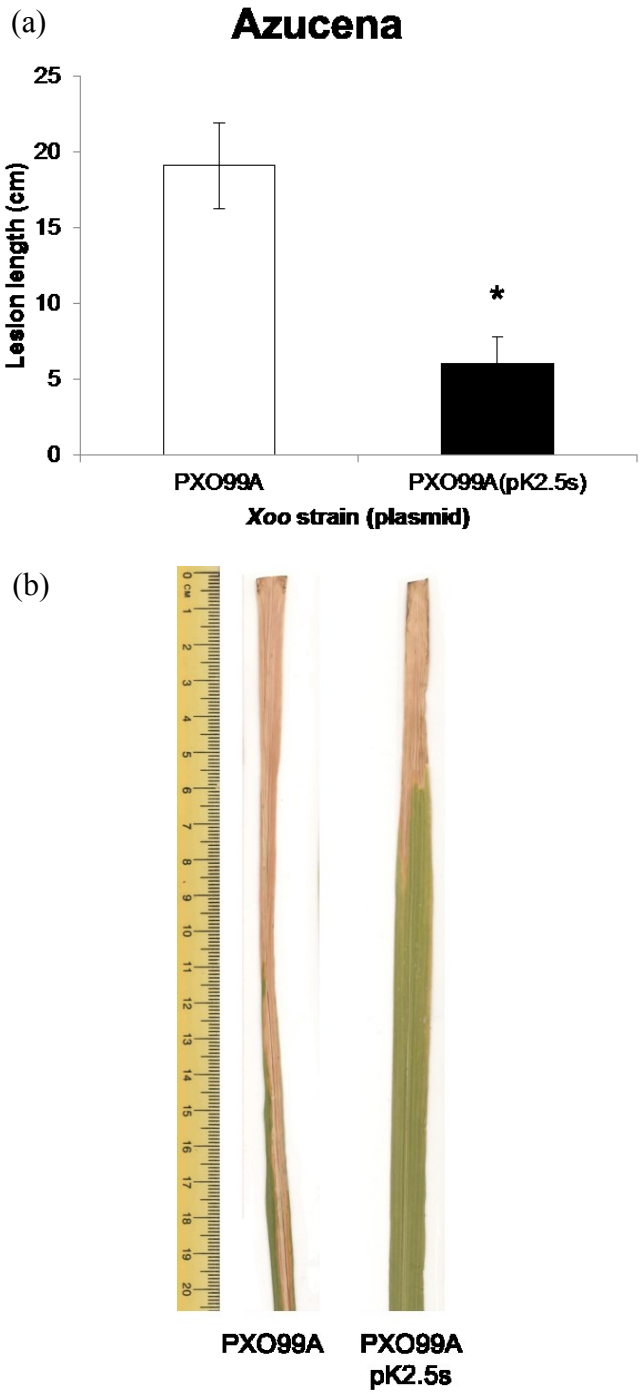

Figure 3.6. Lesion lengths caused by PXO99A(pK2.5s) and wild type PXO99A on Azucena (without *xa5*). (a) Lesions were measured 15 days after inoculation. An asterisk denotes a significant difference between each strain inoculated ($P < 0.05$). Error bars represent ± standard deviation. **(b)** Leaves are representative of phenotype and lesion length observed.

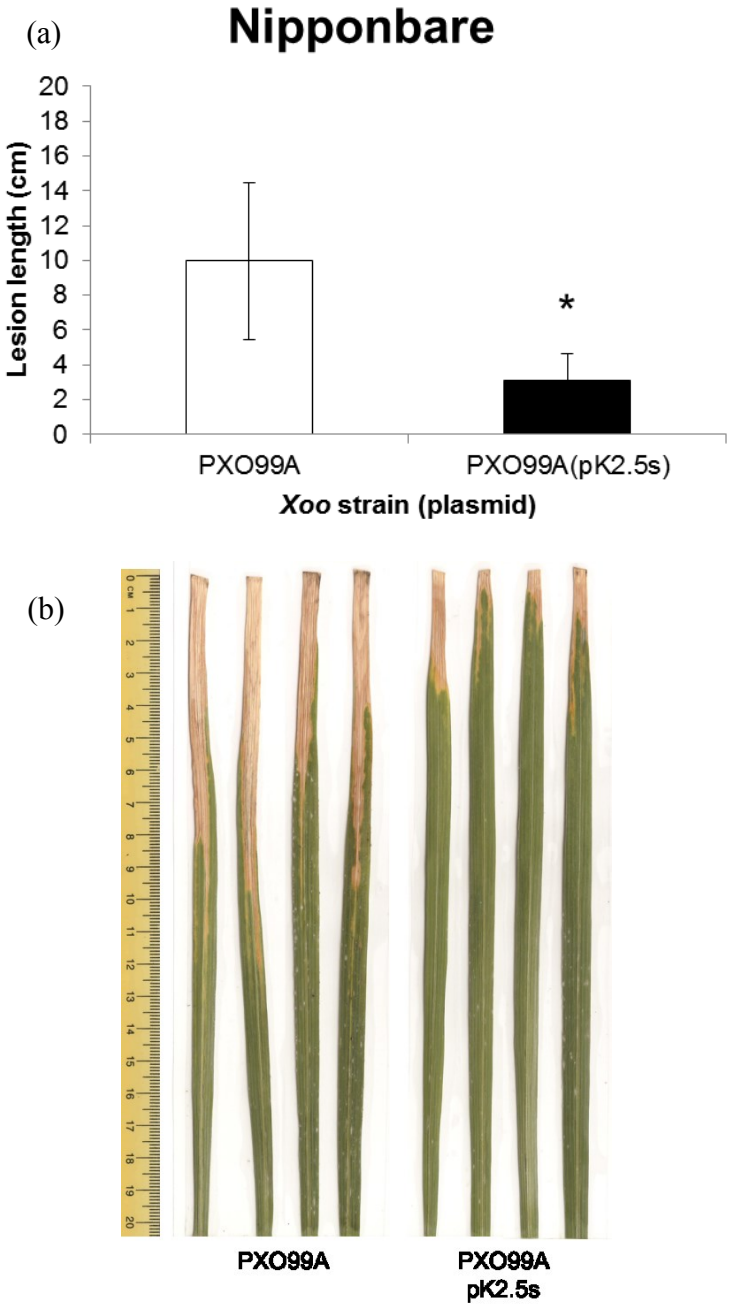

Figure 3.7. Lesion lengths caused by PXO99A(pK2.5s) and wild type PXO99A on Nipponbare (without *xa5*). (a) Lesions were measured 15 days after inoculation. An asterisk denotes a significant difference between each strain inoculated ($P < 0.05$). Error bars represent ± standard deviation. **(b)** Leaves are representative of phenotype and lesion length observed.

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

Strains	Relevant characteristics[a]	Reference or source
E. coli		
DH5α	F⁻ *recA hsdR17* (r_K^-,m_K^+) Φ*80dlacZ* Δ*M1*	Bethesda Research Laboratories, Bethesda, MD, USA
Xanthomonas oryzae		
PXO339	pv. *oryzae* race 9; wild type	International Rice Research Institute
PXO86	pv. *oryzae* race 2; wild type	International Rice Research Institute
PXO99A	pv. *oryzae* race 6; wild type derivative selected for azacytidine resistance	Hopkins et al. (1992)
PXO99A(pK2.5s)	PXO99A derivative containing Avrxa5P$_{86}$	This study
PXO99A(pXO6-33)	PXO99A derivative containing *avrxa5* and *avrXa10*; Sp^r and Sm^r	Hopkins et al. (1992)
X11-5A	no pv. designation; wild type; Ap^r	Jones et al. (1989); Triplett et al. (2011)
X11-5A(pK2.5s)	X11-5A derivative containing Avrxa5P$_{86}$	This study
X11-5A(pthB)	X11-5A derivative containing pthB	This study
X11-5A(pXO6-33)	X11-5A derivative containing *avrxa5* and *avrXa10*; Sp^r and Sm^r	This study
Plasmids		
pBluescript II KS+	Vector that allows for blue/white screening and sequencing into the multi-cloning site using M13 universal primers; Ap^r	Stratagene Inc., La Jolla, CA, USA
pBS4.5b	pBluescript II KS+ clone containing the 4.5 kbp *Bam*HI fragment from pXO6-33	This study
pCS466	Gateway entry vector pCR8-GW (Invitrogen) containing *tal1c* gene of *Xoc* BLS256 without central repeat region-containing *Sph*I fragment; Sp^r	Verdier et al. (2012)
pKEB31	pDD62 derivative containing Gateway destination vector cassette (Invitrogen) between *Xba*I and *Bam*HI sites; Tc^r	Cermak et al. (2011)
pKEB31-*tal ΔCRR*	pKEB31 containing *tal1c* gene of *Xoc* BLS256 without central repeat region-containing *Sph*I fragment; Tc^r	Verdier et al. (2012)
pKEB31-2.5s (pK2.5s)	pKEB-tal1c with central repeat region-containing *Sph*I fragment replaced by that of *avrxa5* gene of *Xoo* PXO86; Tc^r	This study
pthB	pVAB226 containing pthB gene of *Xam*; Tc^r	Restrepo and Verdier (1997)
pXO6-33	pHM1 containing *avrxa5* and *avrXa10* genes of *Xoo* PXO86; Sp^r and Sm^r	Hopkins et al. (1992)

[a]Ap^r, ampicillin resistance; Sp^r, spectinomycin resistance; Tc^r, tetracycline resistance; *tal*, transcription activator-like

Table 3.2. Alignment of the repeated variable diresidues of *Xoo* TAL effector proteins.

	TAL protein RVDs[a]																									
Repeat	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	Source
Avrxa5P$_{86}$	NI	HG	NI	NI	NI	NN	HD	NS	NN	NS	NN	NS	NN	HD	NN	NI	HD	NN	NS	NG							This study
PthXo7	NI	NG	NI	NI	N*	NN	HD	HD	N*	NI	NI	NI	NG	HD	HG	NN	NS	NN	HD	HD	NG	N*					Sugio et al. (2007)
Avrxa5J$_{III}$	HD	HD	HD	NG	N*	NN	HD	HD	HD	N*	NN	HD	HI	ND	HD	NI	HD	HD	NG	NG							Zou et al. (2010)
TalC	NG	NS	HD	NI	NG	NN	NG	HD	NI	NN	N*	NI	NN	HD	NG	NI	NN	N*	HD	NN	NG						Yu et al. (2011)
AvrXa7	NI	HG	NI	NI	NS	HD	NN	HD	HD	HD	NS	N*	N*	HD	HD	NS	NN	NS	NN	NI	NG	NN	NI	N*	NS	N*	Yang et al. (2000)
AvrXa10	NI	HG	NI	HG	NI	NI	NN	HD	NI	HD	NN	HG	NS	NG	HD	N*											Hopkins et al. (1992)
PthXo6	NI	H*	NI	NN	NN	NN	NN	NN	HD	NI	HD	HG	HD	NI	N*	NS	NI	NI	HG	HD	NS	NS	NG				Sugio et al. (2007)
PthXo1	NN	HD	NI	HG	HD	NG	N*	HD	HD	NI	NG	NG	NI	HD	NG	NN	NG	NI	NI	NI	NI	N*	NS	N*			Yang et al. (2004)

[a]Asterisks denote missing amino acid residue. Underlined RVDs are homologous to our Avrxa5.

Table 3.3. TALVEZ top 500 predicted transcription factor family TAL target prediction.

TAL	Total TFs	bZIP	MYB	Source
Avrxa5P$_{86}$	14	3	7	This study
PthXo7	16	0	9	Sugio et al. (2007)
Avrxa5	23	2	13	Zou et al. (2010)
TalC	8	0	3	Yu et al. (2011)
PthXo6	14	1	9	Sugio et al. (2007)
PthXo1	9	1	4	Yang et al. (2004)

Table 3.4. TALVEZ top 50 predicted targets.

TAL effector	Locus ID	Rank	Strand	Annotation
Avrxa5P$_{86}$	LOC_Os12g02260	1	-1	nucleotidyltransferase, putative, expressed
Avrxa5P$_{86}$	LOC_Os06g50370	2	1	zinc finger, C3HC4 type, domain containing protein, expressed
Avrxa5P$_{86}$	LOC_Os04g05030	3	1	serine-rich 25 kDa antigen protein, putative, expressed
Avrxa5P$_{86}$	LOC_Os02g01332	4	-1	ribosomal protein L6, putative, expressed
Avrxa5P$_{86}$	LOC_Os09g11840	5	-1	expressed protein
Avrxa5P$_{86}$	LOC_Os11g40990	6	1	retrotransposon protein, putative, unclassified, expressed
Avrxa5P$_{86}$	LOC_Os10g31630	7	-1	expressed protein
Avrxa5P$_{86}$	LOC_Os03g50560	8	-1	RNA recognition motif containing protein, putative, expressed
Avrxa5P$_{86}$	LOC_Os04g55810	9	-1	expressed protein
Avrxa5P$_{86}$	LOC_Os07g45439	10	1	expressed protein
Avrxa5P$_{86}$	LOC_Os09g03560	11	-1	retrotransposon protein, putative, unclassified, expressed
Avrxa5P$_{86}$	LOC_Os03g08600	12	1	glycosyl transferase, putative, expressed
Avrxa5P$_{86}$	LOC_Os12g37980	13	-1	protein kinase domain containing protein, expressed
Avrxa5P$_{86}$	LOC_Os01g52500	14	1	NADP-dependent malic enzyme, putative, expressed
Avrxa5P$_{86}$	LOC_Os02g37170	15	-1	expressed protein
Avrxa5P$_{86}$	LOC_Os11g24130	16	1	zinc-binding protein, putative, expressed
Avrxa5P$_{86}$	LOC_Os05g23700	17	1	DNA-binding storekeeper protein-related, putative, expressed
Avrxa5P$_{86}$	LOC_Os12g16720	18	-1	cytochrome P450 71A1, putative, expressed
Avrxa5P$_{86}$	LOC_Os01g58540	19	1	expressed protein
Avrxa5P$_{86}$	LOC_Os07g34050	20	1	harpin-induced protein 1 domain containing protein, expressed
Avrxa5P$_{86}$	LOC_Os02g33840	21	1	OsFBX52 - F-box domain containing protein, expressed
Avrxa5P$_{86}$	ChrSy.fgenesh.gene.32	22	-1	expressed protein
Avrxa5P$_{86}$	LOC_Os06g22370	23	1	expressed protein
Avrxa5P$_{86}$	LOC_Os02g22070	24	1	expressed protein
Avrxa5P$_{86}$	LOC_Os02g38970	25	1	expressed protein
Avrxa5P$_{86}$	LOC_Os05g06190	26	1	expressed protein
Avrxa5P$_{86}$	LOC_Os12g04970	27	-1	retrotransposon protein, putative, unclassified, expressed
Avrxa5P$_{86}$	LOC_Os03g47749	28	1	expressed protein
Avrxa5P$_{86}$	LOC_Os04g55590	29	-1	homeobox domain containing protein, expressed
Avrxa5P$_{86}$	ChrSy.fgenesh.gene.45	30	-1	expressed protein
Avrxa5P$_{86}$	LOC_Os12g22520	31	-1	retrotransposon protein, putative, Ty3-gypsy subclass, expressed
Avrxa5P$_{86}$	LOC_Os11g19560	32	-1	transposon protein, putative, CACTA, En/Spm sub-class
Avrxa5P$_{86}$	LOC_Os03g33730	33	-1	retrotransposon protein, putative, Ty3-gypsy subclass, expressed
Avrxa5P$_{86}$	LOC_Os01g62410	34	1	MYB family transcription factor, putative, expressed
Avrxa5P$_{86}$	LOC_Os01g03040	35	-1	expressed protein
Avrxa5P$_{86}$	LOC_Os01g14310	36	-1	expressed protein
Avrxa5P$_{86}$	LOC_Os07g23580	37	1	expressed protein
Avrxa5P$_{86}$	LOC_Os03g29244	38	1	expressed protein
Avrxa5P$_{86}$	LOC_Os11g36410	39	-1	NBS-LRR type disease resistance protein, putative, expressed
Avrxa5P$_{86}$	LOC_Os05g02070	40	1	expressed protein
Avrxa5P$_{86}$	LOC_Os07g45310	41	1	BSD domain-containing protein, putative, expressed
Avrxa5P$_{86}$	LOC_Os10g25330	42	1	expressed protein
Avrxa5P$_{86}$	LOC_Os06g34400	43	1	zinc finger, C3HC4 type domain containing protein, expressed
Avrxa5P$_{86}$	LOC_Os09g23650	44	-1	FAM10 family protein, putative, expressed
Avrxa5P$_{86}$	LOC_Os04g55480	45	-1	BRCA1-associated protein, putative, expressed
Avrxa5P$_{86}$	LOC_Os03g63900	46	1	1-aminocyclopropane-1-carboxylate oxidase 2, putative, expressed
Avrxa5P$_{86}$	LOC_Os04g28260	47	1	Kinesin motor domain domain containing protein, expressed
Avrxa5P$_{86}$	LOC_Os04g38730	48	-1	hypothetical protein
Avrxa5P$_{86}$	LOC_Os03g07330	49	1	pentatricopeptide, putative, expressed
Avrxa5P$_{86}$	LOC_Os10g25420	50	1	GDSL-like lipase/acylhydrolase, putative, expressed

Supplemental Figure 3.1. Primers used to sequence the central repeat region of pK2.5s.

Primer	Sequence (5'- 3')	Primer direction	Source
P235	GGAGGCCTTGCTCACGGATGC	Forward	R. Cernadas, *unpublished*
F4	CGCAATGCACTGACGGGTGC	Forward	This study
R2458	CATGCAAAGACGCCTGATCCGG	Reverse	This study
R2058	TAACTGGGCAACAATGCTCTCC	Reverse	This study

Supplemental Figure 3.2. Putative amino acid sequence of the central repeat region (CRR) and nucleotide sequence of 4.5 kbp *Bam*HI fragment from cosmid pXO6-33. Restriction enzyme cut sites, yellow for *Bam*HI and green for *Sph*I sites, are highlighted. The first repeat of the CRR is underlined and the repeat variable diresidues (RVDs) are highlighted red. Primers used to sequence the CRR are colored blue.

```
         10        20        30        40        50        60
GGATCCCATTCGTTCGCGCACGCCAAGTCCTGCCCGCGAGCTTCTGCCCGGACCCCAACCGGATAGGGTT
         80        90       100       110       120       130
CAGCCGACTGCAGATCGGGGGGGGGCTCCGCCTGCTGGCGGCCCCCTGGATGGCTTGCCCGCTCGGCGGA
        150       160       170       180       190       200
CGATGTCCCGGACCCGGCTGCCATCTCCCCCTGCGCCCTCGCCTGCGTTCTCGGCGGGCAGCTTCAGCGA
        220       230       240       250       260       270
TCTGCTCCGTCAGTTCGATCCGTCGCTTCTTGATACATCGCTTCTTGATTCGATGCCTGCCGTCGGCACG
        290       300       310       320       330       340
CCGCATACAGCGGCTGCCCCAGCAGAGTGCGATGAGGTGCAATCGGGTCTGCGTGCAGCCGATGACCCGC
        360       370       380       390       400       410
CACCCACCGTGCGTGTCGCTGTCACTGCCGCGCGGCCGCCGCGCGCCAAGCCGGCCCCGCGACGGCGTGC
        430       440       450       460       470       480
GGCGCAACCCTCCGACGCTTCGCCGGCCGCGCAGGTGGATCTACGCACGCTCGGCTACAGTCAGCAGCAG
        500       510       520       530       540       550
CAAGAGAAGATCAAACCGAAGGTGCGTTCGACAGTGGCGCAGCACCACGAGGCACTGGTGGGCCATGGGT
        570       580       590       600       610       620
TTACACACGCGCACATCGTTGCGCTCAGCCAACACCCGGCAGCGTTAGGGACCGTTGCTGTCACGTATCA
        640       650       660       670       680       690
GGACATAATCAGGGCGTTGCCAGAGGCGACACACGAAGACATCGTTGGCGTCGGCAAACAGTGGTCCGGC

        710       720       730       740       750       760
           GGAGGCCTTGCTCACGGATGC→p235
GCACGCGCCCTGGAGGCCTTGCTCACGGAGGCGGGGGAGTTGAGAGGTCCGCCGTTACAGTTGGACACAG

        780       790       800       810       820       830
                                                          GCAATGC
GCCAACTTCTCAAGATTGCAAAACGTGGCGGCGTGACCGCAGTGGAGGCAGTGCATGCATGGCGCAATGC

        850       860       870       880       890       900
ACTGACGGGTGC→F4s
ACTGACGGGTGCCCCCCTGAACCTGACCCCGGACCAAGTGGTGGCCATCGCCAGCAATATTGGCGGCAAC
                      L  T  P  D  Q  V  V  A  I  A  S  N  I  G  G  N

        920       930       940       950       960       970
CAGGCGCTGGAGACGGTGCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCATGGCCTGACCCCGGACCAGG
 Q  A  L  E  T  V  Q  R  L  L  P  V  L  C  Q  A  H  G  L  T  P  D  Q

        990      1000      1010      1020      1030      1040
TCGTGGCCATCGCCAGCCATGGCGGCGGCAAGCAGGCGCTGGAGACGGTGCAGCGGCTGTTGCCGGTGCT
V  V  A  I  A  S  H  G  G  G  K  Q  A  L  E  T  V  Q  R  L  L  P  V  L
```

```
      1060      1070      1080      1090      1100      1110
GTGCCAGGACCATGGCCTGACCCCGGACCAGGTGGTGGCCATCGCCAGCAATATTGGCGGCAAGCAGGCG
  C  Q  D  H  G  L  T  P  D  Q  V  V  A  I  A  S  N  I  G  G  K  Q  A


      1130      1140      1150      1160      1170      1180
CTGGAGACGGTGCAACGGCTGTTGCCGGTGCTGTGCCAGGACCATGGCCTGACCCCGGACCAGGTGGTGG
 L  E  T  V  Q  R  L  L  P  V  L  C  Q  D  H  G  L  T  P  D  Q  V  V

      1200      1210      1220      1230      1240      1250
CCATCGCCAGCAATATTGGCGGCAAGCAGGCGCTGGAGACGGTGCAGCGGCTGTTGCCGGTGCTGTGCCA
A  I  A  S  N  I  G  G  K  Q  A  L  E  T  V  Q  R  L  L  P  V  L  C  Q

      1270      1280      1290      1300      1310      1320
GGACCATGGCCTGACCCCGGACCAGGTGGTGGCCATCGCCAGCAATATTGGCGGCAAGCAGGCGCTGGAG
  D  H  G  L  T  P  D  Q  V  V  A  I  A  S  N  I  G  G  K  Q  A  L  E

      1340      1350      1360      1370      1380      1390
ACGGTGCAGCGGCTGTTGCCGGTGCTGTGCCAGGACCATGGCCTGACCCCGGACCAGGTGGTGGCCATCG
 T  V  Q  R  L  L  P  V  L  C  Q  D  H  G  L  T  P  D  Q  V  V  A  I

      1410      1420      1430      1440      1450      1460
CCAACAATAACGGCGGCAAGCAGGCGCTGGAGACGGTGCAACGGCTGTTGCCGGTGCTGTGCCAGGACCA
A  N  N  N  G  G  K  Q  A  L  E  T  V  Q  R  L  L  P  V  L  C  Q  D  H

      1480      1490      1500      1510      1520      1530
TGGCCTGACCCCGGACCAGGTGGTGGCCATCGCCAGCCACGATGGCGGCAAGCAGGCGCTGGAGACGGTG
  G  L  T  P  D  Q  V  V  A  I  A  S  H  D  G  G  K  Q  A  L  E  T  V

      1550      1560      1570      1580      1590      1600
CAGCGGCTGTTGCCGGTGCTGTGCCAGGGCCATGGCCTGACCCCGGACCAGGTGGTGGCCATCGCCAGCA
 Q  R  L  L  P  V  L  C  Q  G  H  G  L  T  P  D  Q  V  V  A  I  A  S

      1620      1630      1640      1650      1660      1670
ATAGTGGCGGCAAGCAGGCGCTGGAGACGGTGCAGCGGCTGTTGCCAGTGCTGTGCCAGGACCATGGCCT
N  S  G  G  K  Q  A  L  E  T  V  Q  R  L  L  P  V  L  C  Q  D  H  G  L

      1690      1700      1710      1720      1730      1740
GACCCCGGACCAGGTGGTGGCCATCGCCAACAATAACGGCGGCAAGCAGGCGCTGGAGACGGTGCAGCGG
  T  P  D  Q  V  V  A  I  A  N  N  N  G  G  K  Q  A  L  E  T  V  Q  R

      1760      1770      1780      1790      1800      1810
CTGTTGCCGGTGCTGTGCCAGGACCATGGCCTGACCCCGGACCAGGTGGTGGCCATCGCCAGCAATAGTG
 L  L  P  V  L  C  Q  D  H  G  L  T  P  D  Q  V  V  A  I  A  S  N  S

      1830      1840      1850      1860      1870      1880
GCGGCAAGCAGGCGCTGGAGACGGTGCAGCGGCTGTTGCCAGTGCTGTGCCAGGCCCATGGCCTGACCCC
G  G  K  Q  A  L  E  T  V  Q  R  L  L  P  V  L  C  Q  A  H  G  L  T  P

      1900      1910      1920      1930      1940      1950
GGACAAGGTGGTGGCCATCGCCAACAATAACGGCGGCAAGCAGGCGCTGGAGACGGTGCAGCGGCTGTTG
  D  K  V  V  A  I  A  N  N  N  G  G  K  Q  A  L  E  T  V  Q  R  L  L

      1970      1980      1990      2000      2010      2020
CCGGTGCTGTGCCAGGACCATGGCCTGACCCCGGACCAGGTGGTGGCCATCGCCAGCAATAGTGGCGGCA
 P  V  L  C  Q  D  H  G  L  T  P  D  Q  V  V  A  I  A  S  N  S  G  G
```

```
       2040      2050      2060      2070      2080      2090
AGCAGGCGCTGGAGACGGTGCAGCGGCTGTTGCCGGTGCTGTGCCAGGACCATGGTCTGACCCCGGACCA
K  Q  A  L  E  T  V  Q  R  L  L  P  V  L  C  Q  D  H  G  L  T  P  D  Q

       2110      2120      2130      2140      2150      2160
GGTGGTGGCCATCGCCAACAATAACGGCGGCAAGCAGGCGCTGGAGACGGTGCAGCGGCTGTTGCCGGTG
  V  V  A  I  A  N  N  N  G  G  K  Q  A  L  E  T  V  Q  R  L  L  P  V

       2180      2190      2200      2210      2220      2230
CTGTGCCAGGACCATGGCCTGACCCCGGACCAGGTGGTGGCCATCGCCAGCCATGATGGCGGCAAGCAGG
 L  C  Q  D  H  G  L  T  P  D  Q  V  V  A  I  A  S  H  D  G  G  K  Q

       2250      2260      2270      2280      2290      2300
CGCTGGAGACGGTGCAGCGGCTGTTGCCGGTGCTGTGCCAGGACCATGGCCTGACCCCGGACCAGGTGGT
A  L  E  T  V  Q  R  L  L  P  V  L  C  Q  D  H  G  L  T  P  D  Q  V  V

       2320      2330      2340      2350      2360      2370
GGCCATCGCCAACAATAACGGCGGCAAGCAGGCGCTGGAGACGGTGCAGCGGCTGTTGCCGGTGCTGTGC
  A  I  A  N  N  N  G  G  K  Q  A  L  E  T  V  Q  R  L  L  P  V  L  C

       2390      2400      2410      2420      2430      2440
CAGAACCATGGCCTGACCCCGGACCAGGTCGTGGCCATCGCCAGCAATATTGGCGGCAAGCAGGCGCTGG
 Q  N  H  G  L  T  P  D  Q  V  V  A  I  A  S  N  I  G  G  K  Q  A  L

       2460      2470      2480      2490      2500      2510
AGACGGTGCAGCGGCTGTTGCCGGTGCTGTGCCAGGACCATGGCCTGACCCCGGACCAGGTGGTGGCCAT
E  T  V  Q  R  L  L  P  V  L  C  Q  D  H  G  L  T  P  D  Q  V  V  A  I

       2530      2540      2550      2560      2570      2580
CGCCAGCCACGATGGCGGCAAGCAGGCGCTGGAGACGGTGCAGCGGCTGTTGCCGGTGCTGTGCCAGGAC
  A  S  H  D  G  G  K  Q  A  L  E  T  V  Q  R  L  L  P  V  L  C  Q  D

       2600      2610      2620      2630      2640      2650
CATGGCCTGACCCCGGCCCAGGTGGTGGCCATCGCCAACAATAACGGCGGCAAGCAGGCGCTGGAGACGG
 H  G  L  T  P  A  Q  V  V  A  I  A  N  N  N  G  G  K  Q  A  L  E  T

       2670      2680      2690      2700      2710      2720
TGCGGCGGCTGTTGCCGGTGCTGTGCCAGGACCATGGCCTGAGCCCGGACCAGGTCGTGGCCATCGCCAG
V  R  R  L  L  P  V  L  C  Q  D  H  G  L  S  P  D  Q  V  V  A  I  A  S

       2740      2750      2760      2770      2780      2790
CAATAGTGGCGGCAAGCAGGCGCTGGAGACGGTGCAGCGGCTGTTGCCGGTGCTGTGCCAGGACCATGGC
  N  S  G  G  K  Q  A  L  E  T  V  Q  R  L  L  P  V  L  C  Q  D  H  G

       2810      2820      2830      2840      2850      2860
CTGACCCCGGCCCAGGTGGTGGCCATCGCCAGCAATGGCGGCGGCAAGCAGGCGCTGGAGAGCATTGTTG
                                                  R2058s←CCTCTCGTAACAAC
 L  T  P  A  Q  V  V  A  I  A  S  N  G  G  G  K  Q  A  L  E

       2880      2890      2900      2910      2920      2930
CCCAGTTATCTCGCCCTGATCCGGCGTTGGCCGCGTTGACCAACGACCACCTCGTCGCCTTGGCCTGCCT
GGGTCAAT

       2950      2960      2970      2980      2990      3000
CGGCGGACGTCCTGCCCTGGATGCAGTGAAAAAGGGATTGCCGCACGCGCCGGAATTGATCAGAAGAATC
       3020      3030      3040      3050      3060      3070
AATCGCCGTATTCCCGAACGCACGTCCCATCGCGTTGCCGACTACGCGCAAGTGGTTCGCGTGCTGGAGT
```

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       3090      3100      3110      3120      3130      3140
TTTTCCAGTGCCACTCCCACCCAGCGTACGCATTTGATGAGGCCATGACGCAGTTCGGGATGAGCAGGAA
       3160      3170      3180      3190      3200      3210
CGGGTTGTTACAGCTCTTTCGCAGAGTGGGCGTCACCGAACTCGAAGCCCGCGGTGGAACGCTCCCCCCA
       3230      3240      3250      3260      3270      3280
GCCTCGCAGCGTTGGGACCGTATCCTCCAGGCATCAGGGATGAAAAGGGCCAAACCGTCCCCTACTTCAG
       3300      3310      3320      3330      3340      3350
CTCAAACACCGGATCAGGCGTCTTTGCATGCATTCGCCGATTCGCTGGAGCGTGACCTTGATGCGCCTAG
 R2458s←GGCCTAGTCCGCAGAAACGTAC

       3370      3380      3390      3400      3410      3420
CCCAATGCACGAGGGAGATCAGACAGGGGCAAGCAGCCGTAAACGGTCCCGATCGGATCGTGCTGTCACC
       3440      3450      3460      3470      3480      3490
GGCCCCTCCGCACAGCAATCTTTCGAGGTGCGCGTTCCCGAACAGCACGATGCGCTGCATTTGCCCCTCA
       3510      3520      3530      3540      3550      3560
GCTGGAGGGTAAAACGCCCGCGTACCAGGATCGGGGGCGGCCTCCATGATCCTGGTACGCCCATCGCTGC
       3580      3590      3600      3610      3620      3630
CGACCTGGCAGCGTCCAGCACCGTGATGTGGGAACAAGATGCGGCCCCCTTCGCAGGGGCAGCGGATGAT
       3650      3660      3670      3680      3690      3700
TTCCCGGCATTCAACGAAGAGGAGCTCGCATGGTTGATGGAGCTATTGCCTCAGTCAGGCTCAGTCGGAG
       3720      3730      3740      3750      3760      3770
GGACGATCTGAGGGGCGGCAGGGATTCGAGCAAGAAACCTTTACTGACAGCAAGTTAGCTCACTTTTGGC
       3790      3800      3810      3820      3830      3840
TGTGTTTTACACGAATCCCTGCCGACCCTCTACTCCAGCGCAGGCGTGAAATGCGGTTATCAGTAGTGGA
       3860      3870      3880      3890      3900      3910
TCGCGCGGGGTTCGCCGACAGAGCGCGTGTGGTCCGGCGTACACGTGGGCCACGAAGTCCACGGCGCGGG
       3930      3940      3950      3960      3970      3980
TGAGGCGTTATCCGTAGGGGCGATGCCCCTACACCCCCTACAATCCCGGAGCATCGTCATTGGGGACCGT
       4000      4010      4020      4030      4040      4050
ATGAGCTACAGACCGCAGAACAACCAAGATGGGCTTTGGTGGGAAATCGCCCTGGGCATCTGCGTCGGCC
       4070      4080      4090      4100      4110      4120
AGCTGATGACCGCAGCGTTCGCAGGTGTGGTGGCCCTGTGCCTGGGCTACTTCACGCTGCGCAGCGTCAG
       4140      4150      4160      4170      4180      4190
CGCAGGACTACCGGCACCACGATTACTGCCGGTCACTCCACAGGAAGCAGACTGAACAATGACCTTTGAC
       4210      4220      4230      4240      4250      4260
ACCTACGAGCGCGTAGACCTGACCGGCCCTTGGGCCGGTTTTGGTTTTCAGGGACATCGATTCTTCACAC
       4280      4290      4300      4310      4320      4330
CAGAAAATTACGACATCGAGCCCTGCGGCATGCGGTACTGGGCGCTGACCTGCGCCATCGCACGGGAGTG
       4350      4360      4370      4380      4390      4400
GTCGCTGCTGATGTCCGAAGAACGCAATACGCGCTCGGCGAACCCGCGAACGCCTACTGCCACCAGGTCT
       4420      4430      4440      4450      4460      4470
CCGGGGTCGCCTTTGTCTCAAGGCGCAGAAATGATCTACCTGCGGGACGTACTCGAGGGTTGGCAGGGAT
       4490      4500      4510      4520      4530      4540
TCGTGTAAAAAACAGCCAAAAGTGGGTTCACTCGCTGTCAGCACAGAAATTTCTCACAACCCTCTGCCGA
       4560      4570      4580      4590      4600      4610
TCCTCCATGCGGGTCCGGGATCGCCTTCATGTCTGCGCCTCACCCTGGTCGTCGAGGGTTGCCAGGATCA
       4630      4640      4650      4660      4670      4680
CCCGAAGTTGTGTACTGCCATGCGGCCTGGGAAGCTATGTAGGGACCACAGACCGCTAGTCTGGAGGCAA
       4700      4710
CCATGTAAAGAGGTATGCCTGATGGATCC
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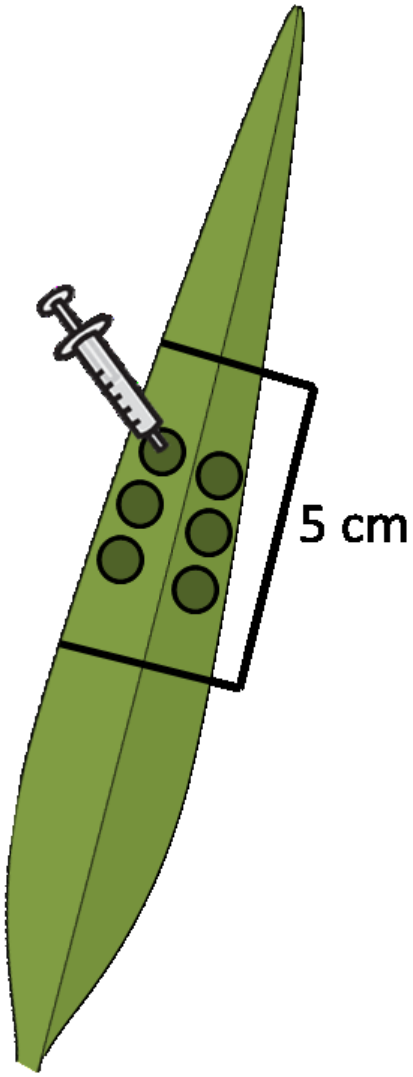

Supplemental Figure 3.3. Leaf infiltration inoculation method for *in planta* multiplication quantification assays of X11-5A and X11-5A(pK2.5s) which contains Avrxa5P$_{86}$.

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CHAPTER FOUR.

CONCLUSIONS AND FUTURE PERSPECTIVES

Xanthomonas oryzae strains may encode for numerous TALs (from 8 to 26). Some TAL effectors may contribute to virulence and others to resistance. This work introduces a new system to study the roles of an individual TAL effector and the characterization of a novel TAL effector which activates resistance in rice plants homozygous for the recessive resistance gene *xa5*.

In this work, we confirmed that TAL effectors were expressed and delivered by TAL deficient *Xanthomonas oryzae*- USA strain X11-5A. The *Xo*- USA strain elicits bacterial blight-like symptoms on infected rice tissue, similar to symptoms caused by *Xanthomonas oryzae* pv. *oryzae*. *Xanthomonas oryzae* pathovars *oryzae* and *oryzicola* infect the host differently, either by colonizing the vascular tissue or intercellular spaces of the host plant, respectively. By introducing *Xoc* TAL effectors into the *Xo*- USA strain and *Xoo* TAL effectors into a *Xoc* strain, we determined that TAL effectors do not change tissue specificity by which the pathogen infects the host.

Xanthomonas oryzae pv. *oryzae* TAL effectors which promote disease by activating *SWEET* sucrose transporter genes, were also shown to activate disease resistance genes depending on the effector and rice variety. *SWEET* gene expression was activated in all tested varieties; however an increase in virulence did not correlate with activation level. *Xanthomonas oryzae* pv. *oryzicola* TAL effectors which were believed to contribute to virulence by preliminary mutagenesis studies (Cernadas et al., *unpublished*), did not affect virulence of X11-5A in four rice

varieties representing the 21 rice varieties. *SWEET*-targeting TAL effectors contribute broadly and non-tissue specifically to virulence in rice, and their function is affected by host differences besides target sequences. X11-5A was established for characterizing individual TAL effectors in rice.

The avirulence protein to recessive resistance gene *xa5* had not been cloned. We predicted that *avrxa5* encoded for a TAL effector based on prior work (Bai et al., 2000; Hopkins et al., 1992). During the course of this study, a gene encoding for a TAL effector that had Avrxa5 function was cloned from *Xoo* strain JXOIII (Zou et al., 2010). Hopkins et al., (1992) cloned genomic DNA from *Xoo* strain PXO86 known to have Avrxa5 function, creating cosmid pXO6-33. Cosmid pXO6-33 had Avrxa5 function and we used our knowledge of the conserved TAL effector structure to subclone *avrxa5P86*. *avrxa5P86* encoded for a TAL with conserved regions of type III translocation signal, central repeat region, three nuclear localization signals, and an activation domain belonging to the *avrBs3* TAL effector family structure (Boch and Bonas, 2010). The RVD sequence from Avrxa5P$_{86}$ encoded for a TAL effector unlike any previously known TAL effectors. Based on the RVD sequence of Avrxa5P$_{86}$, we used TALVEZ TAL target prediction software (version 3.1) (Pérez-Quintero et al., 2013) to predict the type of gene families that were likely to be targeted by Avrxa5P$_{86}$. Our predictions of host gene families targeted by Avrxa5P$_{86}$ were transcription factors belonging to the bZIP and MYB. Modulating the expression of bZIP and MYB transcription factors may be beneficial to the pathogen since these transcription factors regulate hormone signal transduction, plant development, and resistance (Corrêa et al., 2008; Jakoby et al., 2002; Katiyar et al., 2012; Zhao and Dixon, 2011). The promoter region of *Xa5* was not predicted to be a target of Avrxa5 from JXOII or Avrxa5P$_{86}$. This finding, along with previous studies which observed constitutive and similar levels of expression of genes *Xa5* and *xa5* from

leaves of resistant and susceptible rice lines when inoculated with strains expressing Avrxa5 (Iyer and McCouch, 2004; Jiang et al., 2006), suggest that *Xa5* and *xa5* are possibly not a target of Avrxa5. Azucena is homozygous for the susceptible gene *Xa5* (Kim et al., 2009), and mildly resistant to the TAL deficient *X. oryzae* X11-5A strain (Verdier et al., 2012). Nipponbare is also homozygous for the *Xa5* allele (Jiang et al., 2006), and is susceptible to *Xoo* strain PXO99A (Zou et al., 2010). X11-5A and PXO99A complemented with Avrxa5P$_{86}$ conferred resistance onto Azucena and PXO99A complemented with Avrxa5P$_{86}$ conferred resistance onto Nipponbare. *Xoo* strain PXO61 (*avrxa5*) and PXO99A complemented with the previously isolated Avrxa5 TAL effector did not elicit a resistance response when inoculated onto Nipponbare (Jiang et al., 2006; Zou et al., 2010). The unique interaction of Avrxa5P$_{86}$ with Azucena and Nipponbare is suggestive that Avrxa5P$_{86}$ may have diverged from the previously characterized Avrxa5 (Zou et al., 2010).

Altogether, the TAL deficient *X. oryzae*- USA strain was used to study the roles of individual TAL effectors on a diverse panel of rice varieties and to rapidly identify the Avrxa5P$_{86}$ TAL effector which activated resistance in plants that are homozygous for the recessive resistance gene *xa5*. The comparison of phenotype elicited by the wild type strain and the strain complemented with a single type III effector could be used to identify new sources of resistance. I propose an approach to identify the new resistance gene towards the pathogen's type III secreted protein. A resistance and a susceptible rice variety to the strain complemented with the type III secreted protein would be crossed. The segregating population would be again tested with the wild type strain and the strain complemented with a single type III effector. Sequencing the segregating line that gave the resistance response towards the complemented strain and the susceptible parental line would allow for narrowing of a possible resistance gene.

TALVEZ TAL target prediction tool gave some insight to which gene family is being modulated by Avrxa5P$_{86}$. To address what or which host genes are targets modulated by Avrxa5P$_{86}$. I propose that a combination of TALVEZ and promoter-reporter fusion. TALVEZ would be used to list possible TAL targeted gene candidates. Promoters of genes of interest or the top ranking genes from the TALVEZ prediction would be fused to a reporter. The effect of TAL activation would be seen by the reporter.

This study raised the question of TAL effector gene divergent evolution. Longer reads are necessary when sequencing genes with multiple tandem repeats such as TAL genes, to avoid misassembling error due to the high sequence homology between tandem repeats. New sequencing technologies allow for longer reads when sequencing. I proposed that a sequencing project of *Xanthomonas oryzae* species be performed with long read sequencing. TAL effector genes that are identified in this project could provide for TAL effector evolution.

In summary, this work provides a system for understanding the roles of TAL effectors of *Xanthomonas oryzae* interactions with rice. This system was used for the characterization of a novel TAL effector which activates resistance in rice plants homozygous for the recessive resistance gene *xa5*. As research continues, the interactions of *Xanthomonas oryzae* TAL effectors with rice plant mechanisms will continue evolve.

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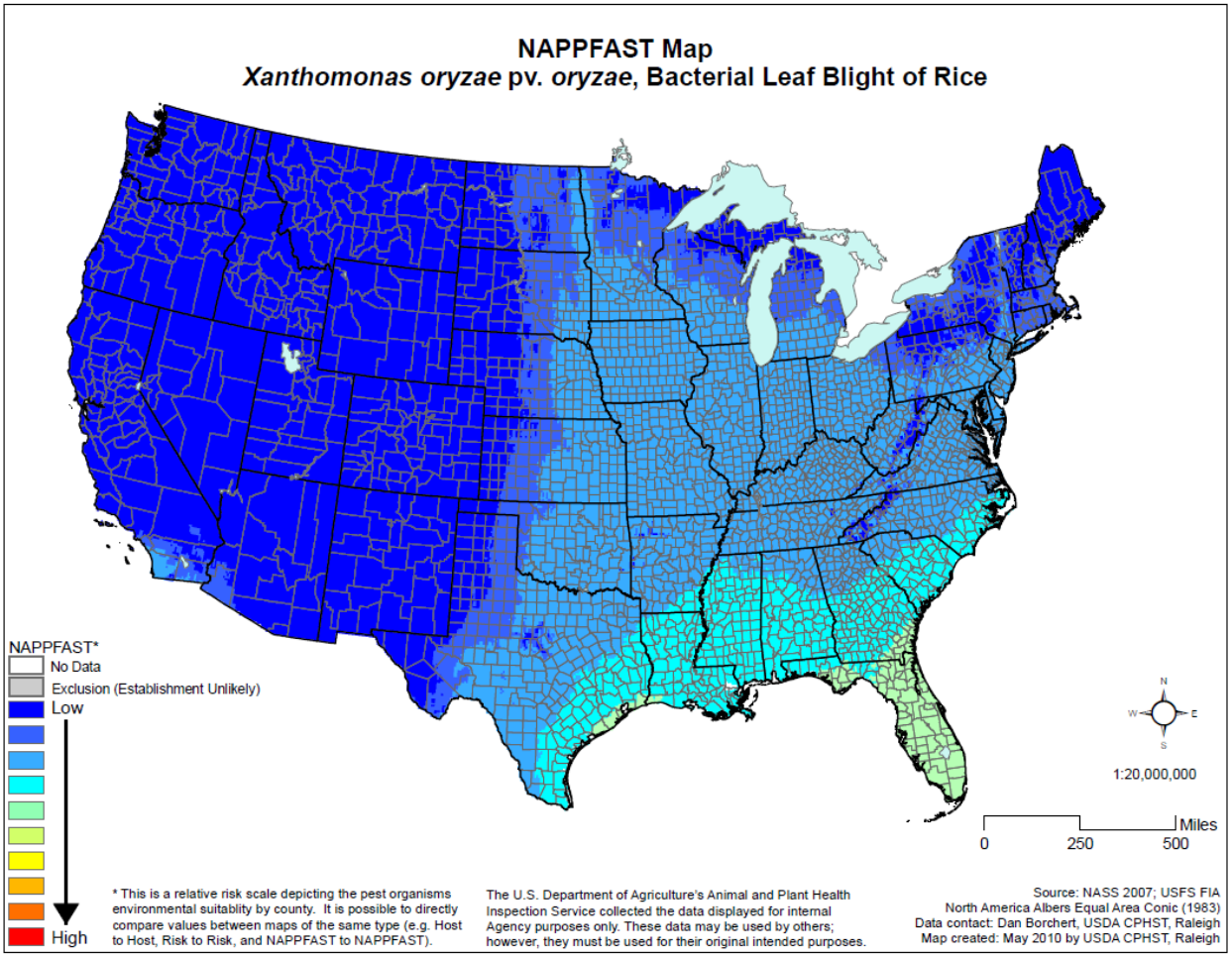
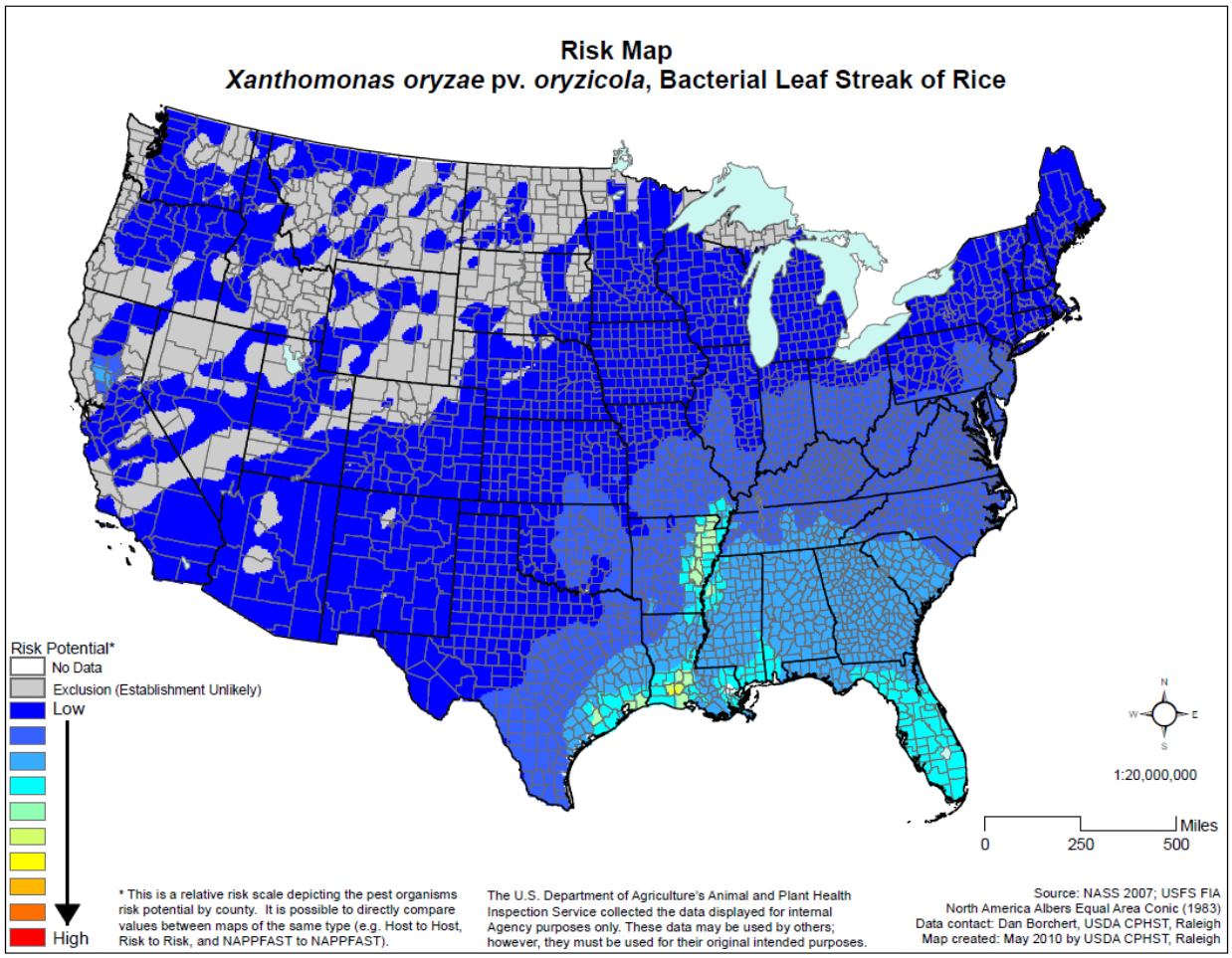
APPENDIX


NAPPFAST Map
Xanthomonas oryzae pv. oryzae, Bacterial Leaf Blight of Rice
NAPPFAST*
No Data
Exclusion (Establishment Unlikely)
Low
High
* This is a relative risk scale depicting the pest organisms environmental suitablity by county. It is possible to directly compare values between maps of the same type (e.g. Host to Host, Risk to Risk, and NAPPFAST to NAPPFAST).
The U.S. Department of Agriculture's Animal and Plant Health Inspection Service collected the data displayed for internal Agency purposes only. These data may be used by others; however, they must be used for their original intended purposes.
1:20,000,000
0
250
500
Miles
Source: NASS 2007; USFS FIA
North America Albers Equal Area Conic (1983)
Data contact: Dan Borchert, USDA CPHST, Raleigh
Map created: May 2010 by USDA CPHST, Raleigh


Risk Map
Xanthomonas oryzae pv. oryzicola, Bacterial Leaf Streak of Rice
Risk Potential*
No Data
Exclusion (Establishment Unlikely)
Low
High
* This is a relative risk scale depicting the pest organisms risk potential by county. It is possible to directly compare values between maps of the same type (e.g. Host to Host, Risk to Risk, and NAPPFAST to NAPPFAST).
The U.S. Department of Agriculture's Animal and Plant Health Inspection Service collected the data displayed for internal Agency purposes only. These data may be used by others; however, they must be used for their original intended purposes.
N
W
E
S
1:20,000,000
0
250
500
Miles
Source: NASS 2007; USFS FIA
North America Albers Equal Area Conic (1983)
Data contact: Dan Borchert, USDA CPHST, Raleigh
Map created: May 2010 by USDA CPHST, Raleigh