MOLECULAR BASIS OF *avrXa7* MEDIATED VIRULENCE IN BACTERIAL BLIGHT OF RICE

by

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B.S., Kerala Agricultural University, 2001
M.S., Indian Agricultural Research Institute, 2004

AN ABSTRACT OF A DISSERTATION

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Abstract

Plants have evolved sophisticated mechanisms to protect against microbial invaders of which resistance genes (R) are an important component. R genes mediate specific recognition of pathogens possessing cognate avirulence (avr) gene products, which leads to the induction of plant defense responses and the arrest of pathogen ingress. In contrast to numerous examples of R gene–avr interactions, the susceptible interaction is less well examined. Recent studies on rice and wheat indicate that host resistance to pathogens also involves genetic variability in dominant traits for susceptibility. *Xanthomonas oryzae* pv.*oryzae* (Xoo) causes bacterial blight disease in rice, a serious threat in major rice growing regions of Asia. The pathogenicity of Xoo depends on the translocation of a cocktail of effector proteins into rice cells by a type III secretion system. The family of transcription activator like (TAL) effectors is the most intriguing due to their eukaryotic features and their function as major virulence determinants. The specificity of the effectors is determined by the nearly identical repeat units at the center of each protein. The major virulence determinant of the strain PXO99^{A} is PthXo1, and this effector hijacks the transcription of the host susceptibility (S) gene *Os8N3*, an allele of recessive resistance gene *xa13*. The strains that overcome *xa13*-mediated resistance harbor alternate major TAL effectors including PthXo2, PthXo3 and AvrXa7. Alternate effectors do not induce *Os8N3.* This study identified the alternate S gene *Os11N3*, which is dependent on the effectors AvrXa7 and PthXo3. The effectors bind to specific elements in the proximal promoter regions of the respective S genes and act as transcriptional activators. Our results suggest that Rice–Xoo interaction also involve gene-for-gene susceptibility to bacterial blight in addition to gene-for-gene resistance.

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Plants have evolved sophisticated mechanisms to protect against microbial invaders of which resistance genes (R) are an important component. R genes mediate specific recognition of pathogens possessing cognate avirulence (avr) gene products, which leads to the induction of plant defense responses and the arrest of pathogen ingress. In contrast to numerous examples of R gene–avr interactions, the susceptible interaction is less well examined. Recent studies on rice and wheat indicate that host resistance to pathogens also involves genetic variability in dominant traits for susceptibility. *Xanthomonas oryzae* pv.*oryzae* (Xoo) causes bacterial blight disease in rice, a serious threat in major rice growing regions of Asia. The pathogenicity of Xoo depends on the translocation of a cocktail of effector proteins into rice cells by a type III secretion system. The family of transcription activator like (TAL) effectors is the most intriguing due to their eukaryotic features and their function as major virulence determinants. The specificity of the effectors is determined by the nearly identical repeat units at the center of each protein. The major virulence determinant of the strain PXO99^{A} is PthXo1, and this effector hijacks the transcription of the host susceptibility (S) gene *Os8N3*, an allele of recessive resistance gene *xa13*. The strains that overcome *xa13*-mediated resistance harbor alternate major TAL effectors including PthXo2, PthXo3 and AvrXa7. Alternate effectors do not induce *Os8N3.* This study identified the alternate S gene *Os11N3*, which is dependent on the effectors AvrXa7 and PthXo3. The effectors bind to specific elements in the proximal promoter regions of the respective S genes and act as transcriptional activators. Our results suggest that Rice–Xoo interaction also involve gene-for-gene susceptibility to bacterial blight in addition to gene-for-gene resistance.

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Chapter One

Host and pathogen determinants of rice bacterial blight

Abstract

Rice is an important food crop for half the world's population and has been in cultivation for over 10,000 years. During the long period of monoculture rice has evolved intricate relationships with associated pathogens and pests, bacterial blight (BB) being one of the most important among them. Of the 29 different resistance genes (R genes) identified to confer BB resistance, six have been isolated and cloned. On the pathogen side, a few of the avirulence genes and a large number of candidate pathogenicity genes have been isolated. The host targets and the function of most of these bacterial effectors are unknown. The complete genome sequences of two different rice subspecies japonica and indica and three different races of BB pathogen are available. The rice–Xoo interaction, therefore, represents an excellent model for the study of molecular aspects of bacterial pathogenesis and host counter measures including innate immunity and R gene-mediated immunity. The large number of candidate bacterial effectors from newly available genomes presents an enormous opportunity for more studies and insights into the complex interaction between rice and Xoo.

Introduction

Rice is the most widely consumed food crop and is thesecond largest cereal crop after maize. The cultivation of rice is constrained by biotic and abiotic factors. Bacterial blight (BB) of rice, caused by Gram-negative proteobacterium *Xanthomonas oryzae* pv. *oryzae* (Xoo) is a serious threat in major rice growing regions of Asia, particularly in South and Southeast Asia and Japan and has also been reported to a lesser extent in Africa, Northern Australia, Central and North America. In Asia, the disease became more prevalent after the introduction of high yielding, dwarf, nitrogen-responsive cultivars during the period of the green revolution. As one of the most important diseases of rice, BB can lead to severe crop losses of up to 50% depending on the stage of the crop, cultivar susceptibility, and the environmental conditions (Mew, 1987; Gnanamanickum *et al*., 1999; Gonzalez *et al*., 2007). The BB pathogen invades the host through natural openings in leaves, including hydathodes or wounds, and colonizes the xylem vessels. The symptoms first appear as water-soaked lesions on the edges of leaf blades due to the release of water from infected cells, which later become chlorotic and wilted as the disease progresses. BB is most destructive when occurring in the seedling stage and can lead to total crop failure. Recognizing its potential to impact rice production and exports, Xoo is classified as a select agent by the United States Animal and Plant Health Service (APHIS) and as a quarantine pest by the European and Mediterranean Plant Protection organization (www.eppo.org).

Xoo is a γ-proteobacterium belonging to the genus *Xanthomonas,* which includes 27 different species of plant pathogens. Each species comprises many pathovars, infecting over 390 different host plants (Parkinson *et al*., 2007; Hayward, 1993; Salzberg, *et al*., 2008). The pathovars are, in turn, often further classified into races, which are defined by a set of host cultivars that are resistant to the particular strain. Over 30 different races of Xoo have been reported based on cultivar specificity, and a high

degree of genetic diversity exists among the different strains (Nino-Liu *et al.,* 2006). This genetic diversity is hypothesized to reflect the long evolutionary and adaptive history of the pathogen and host. Genome sequences are great aides in identifying genes involved in host adaptation and for insight into the diversity in Xoo pathotypes. To date, complete genome sequences of three different strains, representing three races, of Xoo have been published, and many more are being sequenced. The three completed strains are the Japanese strain MAFF311018 (also referred to in the literature as T7174; Ochiai *et al*., 2005), Korean strain KACC10331 (also called KXO85; Lee *et al*., 2005) and the Phillippine strain PXO99^{A}, which is a 5-azacytidine-resistant derivative of PXO99 (Salzberg *et al.,* 2008). The genomes vary from 4.94 Mb to 5.24 Mb in size with an average G+C content of ~ 63.7 %. Sequence analyses of published genomes indicate duplications, insertions, deletions, genome rearrangements generated by numerous IS elements, rapidly evolving Clustered Regularly Interspaced Short Palidromic Repeats (CRISPR) elements and homologous recombination between large number of repeat dominated Transcription Activator Like (TAL) effectors as important evolutionary forces behind the amount of genetic diversity and genome plasticity of Xoo strains (Salzberg *et al*., 2008).

Virulence factors of Xoo

In the past 15 years considerable progress has been made in the understanding of virulence and avirulence determinants of Xoo. The factors known to contribute to virulence of Xoo include genes for the synthesis of adhesins, extracellular polysaccharide (EPS) production and regulation, regulation of pathogenicity factors (rpf) gene cluster, type I, II and III secretion systems and their associated secreted proteins. Xoo also encodes adhesin like proteins Xad A and B, which are homologous to adhesins of the mammalian pathogen *Yersinia enterocolitica* and required for virulence. *xad A* mutants are less virulent compared to wild type when inoculated at

low bacterial numbers (Ray *et al*., 2002). Both *xad A* and *xad B* mutants are less virulent on surface inoculation but are completely virulent upon wound inoculation, indicating that these genes may be important for surface attachment and entry into the host (Das *et al*., 2009). When cultured on glucose media, Xoo, like other xanthomonads, produces large amounts of EPS, known as xanthan gum. EPS protects bacteria from desiccation and also aids in wind and water dispersal (Ou, 1972; Swings *et al*., 1990). In advanced stages of infection, EPS fills the xylem tissue of the rice plant, clogging the vessel resulting in wilting. EPS synthesis is directed by genes at multiple chromosomal loci, one of the loci is called the *gum* cluster (Tseng *et al.*, 1999). The Xoo *gum* cluster in strain KACC10331 is composed of 14 open reading frames (ORFs) arranged in a tandem array. The *gum* cluster constitutes an operon expressed from a promoter located upstream of *gumB* but internal promoters can also be found upstream of *gum G*, *gum H* and *gum M*, respectively (Lee *et al*., 2005; 2008). EPS may play a role in the attachment of bacteria to the host surface during initial stages of infection. Two different Xoo mutants, one with a transposon insertion in *gum G* and another with a spontaneous mutation due to the insertion of endogenous IS element in *gum M,* were incapable of EPS production and less virulent. Both EPS production and virulence were restored in the *gum G* mutant by complementation using a genomic clone (Dharmapuri and Sonti, 1999; Rajeswari and Sonti, 2000).

The *rpf* gene cluster regulates cell-to-cell signaling, which involves the synthesis and detection of small diffusible signal molecules. Cell-to-cell communication is believed to allow bacteria to monitor their population density; a process called quorum sensing, and also allows the population to synchronize its behavior to environmental stimuli (Waters and Bassler, 2005). In *Xanthomonas campestris* pv. *campestris* (Xcc) the *rpf* gene cluster (*rpf* A-I) is required for full virulence. Two of the genes in this cluster *rpf B* and *F* are involved in the synthesis of a diffusible signal factor (DSF). Knock-out studies on *rpf F* indicate the role of DSF in the regulation of levels of extracellular enzymes and

EPS (Wang *et al*., 2004). The *rpf* gene cluster in *Xcc* is also important in the dispersal of biofilms (Dow *et al*., 2003). In Xoo, *rpf* F encodes the DSF and is required for full virulence. Unlike Xcc, *rpf F* of Xoo is not involved in the regulation of extracellular enzymes and EPS (Chaterjee and Sonti, 2002). Further studies are required to characterize the remaining genes in this cluster and their effect on pathogenicity.

Type I secretion systems of Gram negative bacteria are secretion systems that transport proteins directly to the extracellular environment from the bacterial cytoplasm through inner and outer bacterial membranes. Three highly conserved components of type I secretion systems are an ABC transporter, which forms a channel across the inner membrane, a membrane fusion protein (MFP) and an outer membrane protein called To1C (reviewed in Holland *et al*., 2005). PXO99^{A} contains a type I secretion system that is involved in triggering resistance in rice cultivars that carry the *Xa21* resistance gene. Transposon induced mutations in PXO99^{A} and subsequent screening for mutants that lost *Xa21*-mediated avirulence activity identified eight genes *raxA*, *raxB*, *raxC* , *rax ST, rax Q* , *rax P, rax H* and *rax R (*da Silva *et al*., 2004*)*. The expression of *rax ST* is regulated by a two component regulatory system encoded by *rax H* and *rax R (*Burdmann *et al*., 2004*)*. Of these, *raxA, raxB* and *rax C* genes encode the MFP, ABC transporter and outer membrane protein, components of the type I secretion system, respectively (da Silva *et al*., 2004). *rax ST* encodes a sulphotransferase enzyme and *rax Q* and *rax P* are involved in the production of the sulfuryl donor phosphoadenosine phosphor sulfate (PAPS). Recently, the elicitor of *Xa21* immunity (called Ax21) was characterized as a 194 amino acid sulfated protein, which is secreted into the extracellular environment (Lee *et al*., 2009). Rice plants perceive Ax21 through *Xa21* product, which is a receptor linked kinase (RLK; Song *et al*., 1995). The N terminal 17 amino acid peptide of Ax21 is sulfated at a tyrosine residue and is sufficient to trigger *Xa21*-mediated resistance. The active region of Ax21 is highly conserved in Xoo

strains and other plant pathogenic members of *Xanthomonas* species (da Silva *et al.*, 2009).

The bacterial type II secretion system mediates a two step process. The proteins that are secreted through this system carry a secretion signal at their N termini and are transported into the periplasmic space through the inner membrane by either the General Secretion Pathway (GSP) or the Twin Arginine Pathway (TWP) (Voulhoux *et al.*, 2001; Jha *et al.*, 2005). Transport across the outer membrane is facilitated by the proteins of main terminal branch (MTB) of general secretion pathway (GSP). The Xoo genome encodes a single type II secretion system in contrast to two different type II systems in *Xanthomonas axonopodis* pv. *citri* (Xac) and Xcc (da Silva *et al., 2002*). Mutations in the gene cluster *xps,* which is required for a functional type II system, result in strains defective in virulence (Ray *et al.*, 2000). Type II secreted proteins are mainly toxins and enzymes that target different components of the host cell, and some of these enzymes, including xylanase, cellulase, cysteine protease, cellobiosidase and lipases, have been characterized as contributors to Xoo virulence (reviewed in Subramoni *et al.,* 2006). Although gene knock-out studies on genes of type II secreted proteins did not result in significant reduction in virulence, mutations in more than one type II secreted proteins resulted in severe effect on the pathogenicity of Xoo (Rajeswari *et al.*, 2005). Experimental evidences indicate that rice plants perceive some type II secreted proteins and respond by hyper sensitive reactions (HR), and these responses are suppressed by type III secreted effectors (Jha *et al.,* 2005).

The best characterized virulence determinants of Xoo are components of the Type III secretion system (T3SS). T3SS and the associated effectors (T3 effectors) are major players in the pathogenicity of plant and animal bacterial pathogens (reviewed in Cornelis, 2006). T3SS is a molecular syringe made of a complex of proteins that extends from the inner membrane of the bacterium to the host cell cytoplasm. In plant pathogenic bacteria, the T3SS injection apparatus is also called the Hrp pilus. The genes

that encode the T3SS apparatus are generally organized in gene clusters. In plant pathogenic bacteria, the genes that are essential for a functional T3SS are characterized by their requirement for both the induction of a HR on nonhost, specific resistant host cultivars, and pathogenicity on host plant genotypes, hence the Hrp acronym for hypersensitive reaction and pathogenicity. T3SS genes that are highly conserved in plant and animal pathogens are called hypersensitive response conserved (*hrc)* genes (reviewed in Gurlebeck *et al*., 2006). The *hrp* genes are, for the most part, only expressed in minimal media or in contact with host. Expression of *hrp* genes in *Xanthomonas* are regulated by an Ara C type transcriptional activator known as Hrp X. HrpX recognizes a palindromic consensus sequence called plant inducible promoter (PIP) box located upstream of Hrp X-regulated genes (Wengelik *et al*., 1996). Hrp X regulates the expression of *hrp B* to *F* and is in turn regulated by Hrp G, which also regulates the expression of another T3SS structural gene *hrpA*. HrpG is related to members of the two component regulatory system, more specifically, members of the OmpR family. A recent study identified a novel gene, *trh* (transcriptional regulator for *hrp),* as a regulator of *hrpG* (Tsuge *et al*., 2006). Studies on *Xcv* indicate that HrpF functions as a translocon and is proposed to create pores in the plant cell membrane (Rossier *et al*., 2000). Xoo mutants of *hrpF* are severely reduced in pathogenicity and elicit much weaker resitance reactions (Sugio, *et al*., 2005). The signals present in the effectors that mediate their recognition by T3SS are located at the N terminus of the protein (Mudgett *et al*., 2000).

A recent review classified all known and candidate T3SS effectors from strains of *Xanthomonas spp* into 39 groups based on sequence and structural differences and similarities (White *et al*., 2009). Of these, 23 different groups are represented in the Xoo genome MAFF311018, although the roles played by most of the T3 effectors in virulence and their associated biochemical function remains to be determined (White *et al*., 2009). A class of T3 effectors called Transcription Activator Like (TAL) effectors was first identified due to their relatedness to the T3 effector AvrBs3 from *Xanthomonas campestris*

pv. *vesicatoria* (Bonas *et al*., 1989). Some of these TAL effectors have avirulence activity and three different TAL effectors *avrXa7, avrXa10* and *avrXa27* from Xoo have been cloned (Hopkins *et al*., 1992; Gu *et al*., 2005). More TAL effectors and their role in virulence were identified from Xoo strains by marker-exchange mutagenesis and complementation (Yang and White, 2004). TAL effectors are highly conserved between and within the plant pathogenic xanthomonads. All TAL effectors have nuclear localization signals (NLS) and an activation domain rich in acidic amino acids (AAD) at their C terminus. Mutational studies on *avrBs3*, *avrXa7* and *avrXa10* have demonstrated the requirement of a least one functional NLS and an intact AAD for their biological activity (Yang *et al*., 2000; Szurek *et al*., 2001). TAL effectors are critical effectors in variety of diseases caused by xanthomonads including bacterial blight of rice. The effectors differ in the number of 102bp repeats, which have variable 12th and the 13th codons on each repeat of the central repetitive region. The N and C termini are highly conserved and can be interchanged between the effectors in most cases.

The biological activity of each effector is determined by its central repetitive region (Herbers *et al*., 1992; Yang and White, 2004). Deletions or rearrangements within the repetitive region can result in the loss of the original function and can sometimes lead to the acquirement of new functions (Herbers *et al*., 1992; Yang *et al*., 2005). Evidence indicates that TAL effectors are translocated to the host cell nucleus where the proteins interact with host transcriptional machinery and alter specific host gene expression. Recently, AvrBs3, the type member of TAL effector family, was demonstrated to act as transcriptional factor by directly interacting with specific DNA elements upstream of the target host genes (Kay *et al*., 2007). A deletion derivative of AvrBs3 recognizes a different element that overlaps with the original element in 11 bases, which indicates that repetitive region determines the DNA binding activity of TAL effectors. Xoo strains harbor multiple TAL effectors, and the sequenced genomes of Xoo strains PXO99^{A}, KACC10331, MAFF311018 encode 19, 15 and 17 TAL effectors,

respectively (Salsberg *et al.*, 2008). The retention of a large number of TAL effectors by these strains and the diversity of the repeat regions are likely to reflect their importance in pathogen survival and host adaptation. In fact, experimental studies indicate that TAL effectors PthXo1, PthXo2, PthXo3 and AvrXa7 have dramatic effects on the virulence phenotype of strains PXO99^{A}, JXO1^{A}, PXO61 and KXO85, respectively (Yang and White, 2004) and are referred to here as major TAL effectors (White and Yang, 2009). The major TAL effectors are required for full virulence and are interchangeable between strains (Yang and White, 2004).

Despite the dependence of some Xoo strains on a single TAL effector for virulence, each strain contains large numbers of TAL effector genes. Multiple copies of TAL effectors in each strain may act as reservoirs for virulence factors for new host genotypes or for the generation of new effectors by recombination in case of host recognition or avoidance. The potential for rearrangements has been demonstrated experimentally. AvrXa7 derivatives with novel virulence specificity were generated *in vitro* and had either deletions (PthXo4) or rearrangements by recombination (PthXo5) of the central repetitive region (Yang *et al.*, 2005). At the same time, some TAL effector genes were found to have minor effects on virulence (Bai *et al.*, 2000; Sugio *et al.*, 2007). Other TAL effectors like PthA of *Xcc*, Avrb6 of *Xanthomonas campestris* pv. *malvacearum* and AvrXa7 of Xoo play dual roles in the virulence and avirulence activities of the respective pathogens (Yang *et al.*, 1994; Yang *et al.*, 2000). AvrXa7 triggers HR in plants harboring cognate R gene *Xa7* and is also critical for the virulence of some strains of the pathogen on non-Xa7 host genotypes (Yang *et al.,* 2005).

PthXo1 is the major TAL effector in many strains, including the common laboratory strain PXO99^{A}, and induces the expression of host gene *Os8N3*, a member of Nodulin 3 (N3) gene family and encode a predicted membrane protein (Yang *et al.*, 2006). Mutations in *pthXo1* result in incompatible interactions on all hosts of PXO99^{A}. PXO99^{A} is also incompatible in rice lines with recessive resistance gene *xa13*, which is

an allele of *Os8N3* and is unresponsive to PthXo1. Silencing of *Os8N3* also results in plants resistant to strains that have PthXo1 as their major virulence effector (Yang *et al.,* 2006). Various lines carrying *xa13* had insertions, deletions or substitutions within the *Os8N3* promoter, indicating that these mutations may abolish the ability of PthXo1 to induce *Os8N3* expression (Chu *et al.,* 2006). Both *xa13* and *Os8N3* (*Xa13)* are expressed to high levels in panicles and anthers, and silencing of either allele resulted in plants with low fertility. Although important for pollen development, how the induction of *Os8N3* expression leads to host susceptibility is unknown. The strains with other major effectors AvrXa7, PthXo2 and PthXo3 are virulent on *xa13* rice lines and do not depend on *Os8N3* induction for pathogenicity (Yang *et al.,* 2006). *Xa13,* thus, confers race-specific resistance, and *Os8N3* and *PthXo1* is the first pair of gene-for-gene susceptibility reported (Yang *et al.*, 2006). However, the mechanism of *Os8N3* induction by PthXo1 remains to be clarified. The rice genome encodes atleast 17 paralogs of *Os8N3* (Yang *et al.*, 2006).

In addition to PthXo1, two other TAL effectors of $PXO99^{A}$ also contribute to virulence by inducing the expression of two different host genes (Sugio *et al.*, 2007). PthXo6 and PthXo7 elevate the transcription of host genes *OsTFX1* and *OsTFIIAγ1* respectively. *OsTFX1* is a member of bZIP family of transcription factors, which are involved in the regulation of many developmental and physiological processes. Among five different Xoo strains tested, all induced *OsTFX1*, which appears then to be a common phenomenon of bacterial blight. Expression of *OsTFIIAγ1*, which encodes a subunit of general transcription factor IIA, was induced only by $PXO99^{A}$ and seems to be strain specific. Loss of PthXo7, however, did not have a measurable effect on the virulence of the $PXO99^{A}$. The introduction of PthXo7 into strain PXO86 had a small effect on the ability of the strain to cause disease lesions on otherwise resistant *xa5* plants. Alterations in the NLS motifs of both TAL effectors resulted in loss of the respective target host gene induction indicating the requirement of nuclear localization

for their activity. Ectopic expression of *OsTFX1* enhanced the susceptibility of the host plants and abrogated the need for PthXo6 for full virulence of PXO99^{A}. Another TAL effector PthXo8 was recently identified with a moderate influence on the pathogenicity of PXo99^{A}, and this effector induces the expression of gene involved in miRNARNA biosynthesis (White and Yang, 2009). These studies indicate that Xoo targets different host genes to alter the host physiology and different TAL effectors have qualitatively different effects on host susceptibility (Sugio *et al.*, 2007). Virulence targets of major TAL effectors PthXo2, AvrXa7 and PthXo3 are not known.

Rice BB resistance genes

Plants have evolved to defend TAL effector-mediated susceptibility by adapting novel defense responses. To date 29 resistance (R) genes *Xa1* to *Xa29* have been identified from different rice cultivars that confer resistance to bacterial blight (reviewed in Nino – Liu *et al.*, 2006). Nine are reported to be recessive, and six have been cloned (reviewed in Iyer and McCouch, 2007). Some recessive R genes are effective against a limited number of strains whereas others impart broad spectrum resistance. The cloned R genes encode five different classes of proteins indicating that rice has adapted to Xoo in a variety of ways (Table 1-1*). Xa21* was the first bacterial blight R gene cloned, and the encoded receptor linked kinase (RLK) is a member of a large family of genes involved in pathogen associated molecular pattern (PAMP) triggered immunity (Song *et al.*, 1995). This dominant R gene was transferred to rice cultivars from wild species *Oryzae longistaminata* (Khush *et al.*, 1989) and confers broad spectrum resistance, effective only in adult plants. *Xa21* protein has an extracellular leucine rich repeat domain, a transmembrane domain and an intracellular kinase domain (Song *et al.,* 1995). Similar kinases involved in innate immunity signaling have been identified in *Arabidospsis* (FLS2 and EFR) and are named pattern recognition receptors (PRR) based

on their ability to recognize molecular PAMP signatures (Gomes– Gomez, L. and Boller, 2000; Zipfel *et al.*, 2006). As noted above, the extracellular LRR domain of Xa21 recognizes the sulfated 17 amino acid N terminal peptide derivative of Ax21 (Lee *et al.*, 2009).

*Xa26 (*also called *Xa3*) is another RLK and, like Xa21, confers broad spectrum resistance (Sun *et al.*, 2004). The effectiveness of *Xa26* mediated resistance is influenced by the genetic background and the developmental stage of the plant. *Xa26* was more effective in japonica background and in the adult plant stage than in the seedling. Evidence suggests that the resistance conferred by *Xa26* is dosage dependent. The expression level of *Xa26* was much higher in japonica cultivars, and the *Xa26* expression increased from seedling stage to the adult (Cao *et al.*, 2007). The enlarged resistance spectrum of *Xa26* and its similarity to *Xa21* suggest that it also represents a component of PTI pathway of rice (White and Yang, 2009). The corresponding PAMP or the activator of *Xa26* triggered immunity has not been identified.

Xa1 a member of the large nucleotide-binding site – leucine rich repeat (NBS – LRR) class of plant disease resistance genes, is induced upon pathogen challenge, and confers race-specific resistance to race1 strain of Xoo in Japan (Yoshimura *et al.*, 1998). Although rice genome encodes ~ 500 NBS –LRR members, *Xa1* is the only NBS-LRR gene identified to confer resistance against bacterial blight. The cognate effector in Xoo that triggers *Xa1* mediated resistance has not been characterized.

Xa27 encodes a novel R gene that is induced upon bacterial inoculation and specifically induced by the TAL effector AvrXa27 (Gu *et al.*, 2005). Similar to *xa13*, the susceptible and the resistant alleles of *Xa27* have identical open reading frames but difference exists in their expression level. Only the resistant allele is induced by strains with *avrXa27,* and polymorphisms exist in the promoter of induced allele. AvrXa27 is widespread in a number of strains from Korea, Japan, China and Philippines, and

therefore, *Xa27*-mediated resistance is broad spectrum. Ectopic expression of *Xa27* under *PR1* promoter (not induced specifically by AvrXa27) resulted in plants resistant to otherwise compatible strains (Gu *et al.*, 2005). Also transgenic plants expressing *Xa27* had thickened secondary cell walls in the vascular elements.

The recessive R gene *xa5* was originally identified in the Aus–Boro group of rice cultivars from Bangladesh and confers broad spectrum resistance (Garris *et al.*, 2003). The dominant allele of *xa5* is *OsTFIIAγ5*, which is located on the telomeric region of chromosome 5 and encodes the small (γ) subunit of the general transcription factor IIA. Both *OsTFIIAγ5* and *xa5* are constitutively expressed in rice plants but differ in the amino acid at position 39. The resistant cultivars have glutamic acid instead of valine at this position, and this substitution from a hydrophobic to hydrophilic amino acid resides in the solvent exposed surface suggesting its significance in protein –protein interaction (Iyer and McCouch, 2005; Jiang *et al.*, 2006). Rice, unlike most other organisms, has two genes encoding similar forms of TFIIAγ subunit. The second gene is located on chromosome 1 and hence named Os*TFIIAγ1*. The encoded proteins are 84% identical to each other and differ at the C-termini. OsTFIIAγ1 has multiple residues of glutamic acid at the C-terminus compared to OsTFIIAγ5 of rice and other grass species including wheat, barley, maize and sorghum. Interestingly, Xoo strain PXO99^{A}, and more specifically, the TAL effector PthXo7 induces Os*TFIIAγ1* expression (Sugio *et al.*, 2007). *OsTFIIAγ5* is the predominant form of TFIIAγ in rice plants, and *xa5* may be a host adaptation to evade bacterial invasion by preserving the general transcription function of TFIIAγ (Sugio *et al.*, 2007).

Conclusions

Studies on rice–Xoo interactions have provided valuable insights into host adaptation and pathogenicity mechanisms. Although several of the type III effectors of Xoo have been characterized and the host targets of a few of them identified, a

relatively large number of candidate effectors remain to be studied and their functions examined (White *et al.*, 2009; Salzberg *et al.*, 2008). A closely related organism *Xanthomonas oryzae* pv. *oryzicola* (Xoc), which causes bacterial leaf streak on rice, also has a large number of TAL effector genes. The leaf streak pathogen has been shown to deliver Xoo TAL effector AvrXa10 (Makino *et al.*, 2006). A comparative analysis of the transcriptome changes during Xoo-rice and Xoc -rice interactions may reveal common factors involved in host adaptation to these related pathogens. Alternatively, Xoo and *Xoc* may use distinct mechanisms in their interactions with the host. The exploitation of *Os8N3* as a race specific susceptibility gene is unique to the bacterial blight disease (Yang *et al.*, 2006). The mechanism by which *Os8N3* expression promotes susceptibility is not known, and investigation into this will shed light on the biochemical processes and physiological changes involved in BB susceptibility. A few models have been predicted to explain by which *Os8N3* mediates susceptibility. The enhanced expression of *Os8N3*, a membrane protein, may lead to changes in membrane dynamics resulting in (a) leakage of nutrients into extracellular spaces (BB pathogen habitats) and consequently greater bacterial growth, or (b) weaken cells leading to the collapse of the tissue and further spread of bacteria beyond the initial infection zone (White, and Yang 2009). Another possibility is that *Os8N3* expression may lead to the suppression of host defense mechanisms. Also *Os8N3* may not be the only factor involved in susceptibility and may have additional downstream components. Further studies are needed to determine if *Os8N3* is a direct target of PthXo1, the biochemical mechanism of gene activation, and other host or pathogen components that are involved in this interaction (Yang *et al.*, 2006; Jiang *et al.*, 2006). The other major TAL effectors AvrXa7, PthXo2 and PthXo3 are capable of replacing PthXo1 with full complementation of virulence and can overcome *xa13* mediated resistance (Yang *et al.,* 2006; Yang and White, 2004). Host genes that are targeted by these TAL effectors are not known.

Research in the field of molecular plant–microbe interactions were largely focused on identifying resistance genes, understanding the nature of resistance and the use of this information for the generation of broad spectrum and durable resistance. The study into the nature plant disease susceptibility is limited in comparison, and only a few genes conferring disease susceptibility have been identified (Yang *et al.*, 2006; Lorang *et al.*, 2007; Liu *et al.*, 2009). Recently, a dominant locus that conditions susceptibility to Victoria blight was reported in *Arabidopsis thaliana* (Lorang *et al.*, 2007). The pathogen, *Cochliobolus victoriae* produces a toxin victorin and requires the presence of *LOV1*, an NBS-LRR gene in the host for full virulence. However, in this case the pathogen is necrotrophic and exploits an R gene to trigger HR and benefits from resulting cell death. A recessive resistance *mlo* gene confers broad spectrum resistance to barley powdery mildew. One hypothesis is that the dominant locus, *Mlo*, conditions susceptibility by acting as a negative regulator of defense response (Kim *et al.*, 2002). Like *Os8N3*, *Mlo* is a transmembrane protein and may have similar functions.

Studies on pepper-*Xanthomonas campestris* pv. *vesicatoria* (*Xcv)* interactions indicate AvrBs3, a type member of TAL effector family induce the expression of host genes (Kay *et al.*, 2007). AvrBs3 triggers HR on pepper plants with cognate R gene *Bs3* (Bonas *et al.*, 1989). Gel shift assays and chromatin immunoprecipitation (ChIP) assay demonstrate that AvrBs3 binds to specific elements in the promoter of *upa 20* and *BS3*. *Upa20* is involved in the symptom formation and ectopic expression of *upa20* leads to hypertrophy of host mesophyll cells. *upa* 20 is a basic helix loop helix transcription factor and, therefore, may regulate the expression of other host genes. A similar element was also found upstream of *BS3* resistance gene, suggesting a host adaptation that mimics the binding element of TAL effector to activate resistance response (Kay *et al.*, 2007; Romer *et al.*, 2007). Recent studies propose that the variable residues (12th and 13th amino acids) in the central repetitive region of TAL effectors recognize single nucleotide in the corresponding target DNA elements (Boch *et al.*, 2009). Additional three

dimensional structural studies are needed to clarify how this specificity is achieved when TAL effector protein function as dimers (Gurlebeck *et al.*, 2005).

TAL effectors act as key factors in the pathogenicity of Xoo on rice. Functional analysis of the repertoire of TAL effectors and the identification of their host targets may be key to developing broad-spectrum and durable resistance to BB. AvrXa7, a major TAL effector with virulence and avirulence functions of the strain PXO86 has been cloned and characterized (Hopkins *et al.*, 1992; Yang *et al.*, 2000) but the host target of this effector has not been identified. **This study was conceived with the following objectives:**

1. Identification of virulence target(s) of the major TAL effector AvrXa7

2. Characterization of the interaction between host target and AvrXa7

3. Determination of the differences in the effector function of PthXo1 and AvrXa7 in the presence of *xa5*

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Table 1-1. Cloned R genes for resistance to Xoo[1]

R gene	Gene family	Comments	Cognate effector	Reference
Xa21	Receptor like Kinase (RLK)	Dominant, broad spectrum resistance to Philippine races.	Ax21, secreted sulfated peptide.	Song *et al*, 1995 Lee *et al*, 2009
Xa1	NBS-LRR	Dominant, resistance to Japanese race1	Unknown	Yoshimura *et al*,1998
xa5	Subunit of transcription factor IIA	Recessive, broad spectrum resistance to Philippine races.	Unknown	Iyer and McCouch, 2004 Jiang *et al*, 2006
Xa27	Novel transmembrane protein	Semi – dominant, Resistance to Philippine races 2&5 Dependent on *Xa5*	AvrXa27-TAL effector	Gu *et al*, 2005 Gu *et al*, 2009
Xa26/Xa3	Receptor like kinase	Dominant, broad spectrum resistance to chinese and phillippine races.	Unknown	Sun *et al*, 2004
xa13	Nodulin 3	Recessive non inducible allele of susceptibility gene	PthXo1 – TAL effector	Yang *et al*, 2006 Chu *et al*, 2006

1 Modified from White and Yang, 2009

Chapter Two

Gene-for-gene interaction between host susceptibility genes and bacterial type III TAL effectors

Abstract

The recessive resistance gene *xa13* confers race-specific resistance against *Xanthomonas oryzae* pv. *oryzae* (Xoo) strains with major TAL effector PthXo1. The strains that depend on alternate TAL effectors are able to defeat *xa13*-mediated resistance. Here, we report that alternate TAL effectors PthXo3 and AvrXa7 target a susceptibility (S) gene other than *Os8N3*. The newly identified S gene is specifically induced by AvrXa7 and PthXo3 and, like *Os8N3*, belongs to nodulin 3 (N3) family. Gene silencing and insertional mutation of *Os11N3* resulted in race-specific resistance, and strains with PthXo1 were able to overcome this resistance. The phenotype of *Os11N3* mutants suggest that this gene is involved in plant development. ChIP assays indicate the direct association of effectors with the S gene proximal promoter regions and transient expression assays corroborate effector driven expression of host target genes. The effector-mediated activation of host gene is dependent on the sequence specificity of the target DNA element. The results suggest a gene-for-gene model for susceptibility in addition to gene-for-gene resistance in rice-*Xanthomonas* interactions.

Introduction

Bacterial blight is caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo) and depends on major transcription activator like (TAL) effectors, which are secreted through type III secretion system (Yang and White, 2004). TAL effector proteins resemble eukaryotic transcription factors; have nuclear localization signals and transcription acidic activation domains (van den ackerveken *et al.*, 1996; Zhu *et al.*, 1998). The TAL effectors differ from each other in the number and nature of repeats in the central repetitive region. The 12th and 13th amino acids of each repeat are the most variable residues within the repeats. The specificity of effector function is associated with the central repetitive region, and rearrangements in the repeat region can affect the virulence and avirulence function of the effector protein (Yang and White, 2004). Several of these TAL effectors were identified based on their ability to trigger host resistance responses in the presence of cognate resistance genes.

Six different resistance genes for bacterial blight have been cloned to date and have provided insight into the diverse resistance mechanisms in the host. Most of the studies on bacterial blight have focused on identifying the resistance genes. However, little is known about the host genes conferring disease susceptibility. Recent studies on major cereal crops, including rice, wheat and oats, indicate that host resistance can also occur due to loss of function of dominant susceptibility genes. In oats, susceptibility to *Cochliobolus victoriae* is conferred by the interaction of the pathogen toxin victorin and an NBS-LRR protein LOV1 (Lorang *et al.*, 2007). In wheat, the necrotrophic fungus *Stagonospora nodorum* produces a toxin SnTox3, which triggers susceptibility in wheat lines carrying *Snn3* gene (Liu *et al.,* 2009). In these two cases, the pathogens are necrotrophic in nature, and they use host selective toxins to induce a hypersensitive reaction or necrotic reaction that result in a compatible interaction. Unlike these, in rice a dominant susceptibility gene *Os8N3* has been reported that is induced by bacterial

blight effector PthXo1 (Yang *et al.*, 2006). Here, the resistance in rice to strains with PthXo1 as major TAL effector is due to lack of inducibility of S gene *Os8N3*. *Os8N3* encodes a membrane protein related to a N3 protein of *Medicago truncatula* (Yang *et al.*, 2006). Sixteen other paralogs of *Os8N3* are present in the rice genome. The recessive resistance gene *xa13* is a natural allele of *Os8N3,* and different versions of *xa13* occur in nature. All *xa13* alleles have polymorphisms in the promoter region (-69 to -86), suggesting that this region may be important for pathogen induced expression. *Os8N3* is expressed in parenchyma cells surrounding the vascular tissue and at higher levels in panicles and anthers (Chu *et al.*, 2006). *Os8N3*-silenced plants are resistant to Xoo strains that have PthXo1 as the major effector (PXO99^{A}) and have low pollen viability, resulting in low fertility (Yang *et al.,* 2006; Chu *et al.,* 2006). The introduction of AvrXa7 into PXO99^{A} defeated the effect of *xa13* and enabled compatible interactions on both IRBB13 and *Os8N3* silenced plants (Yang *et al.*, 2006).

AvrXa7 is a major TAL effector present in PXO86, a race 2 strain of Xoo from Philippine (Hopkins *et al.*, 1992; Bai *et al.*, 2000; Yang *et al.*, 2000). AvrXa7 is unique among all known TAL effectors from Xoo due to dual virulence and avirulence activities. In host plants with cognate R gene *Xa7*, AvrXa7 elicits defense responses characterized by hypersensitive reaction (HR) and an incompatible host interaction (Ogawa *et al.*, 1997). In rice plants without a functional *Xa7* gene, *avrXa7* is required for full virulence, and a loss of *avrXa7* can lead to a dramatic reduction in the virulence of the strain (Bai *et al.*, 2000; Yang *et al.*, 2000). A few type III effectors in related organisms have known to have dual activity, including AvrBs2 from *Xanthomonas campestris* pv. *vescatoria*, *PthA* from *X. axonopodis* pv. *citri* and *Avrb6* from *X. campestris* pv. *malvacearum* (Gassmann *et al.*, 2000; Yang *et al.*, 1994 and 1996). The virulence and avirulence specifities of *avrXa7* are associated with the central repeat domain, which consists of 25.5 repeats (Yang *et al.*, 2000). Experimental evidence indicates that functional nuclear localization signals and acidic activation domain are required for both the virulence and

avirulence activities of *avrXa7*. The activation domain of herpes simplex viral protein VP16 can replace the endogenous activation domain of AvrXa7 without affecting avirulence function. The nuclear localization signal motifs of simian virus (SV) 40 T antigen restored the loss of function mutations in the endogenous nuclear localization signals of *avrXa7* for both virulence and avirulence activities (Yang *et al.*, 2000). Previous studies have determined that the C-terminus region of AvrXa7 plays an important role in avirulence specificity. AvrXa7 retained the virulence activity but lost the avirulence function when the C-terminus was replaced with the corresponding region of AvrBs3 (Yang *et al.*, 2005). Internal reorganization within the central repetitive domain of *avrXa7* by deletion or recombination can result in new effectors with novel specificity. Four different effectors were derived from *avrXa7 in vitro,* and all of them have alterations in the central repetitive region. PthXo4, which was derived from *avrXa7* following the deletion of 8th -19th repeats of the central repetitive domain, lost avirulence specificity but acquired new virulence specificity (Yang *et al.*, 2005). PthXo5 is another derivative of AvrXa7 with deletions and rearrangements in the repeat region that has lost *Xa7* dependent elicitor activity but acquired new virulence function. AvrXa7- Δ38 retained the *Xa7* dependent activity but lost the virulence activity, and the fourth variant, *avrXa7*-sacB50, lost both virulence and *Xa7* dependent avirulence function but acquired avirulence activity on cultivar IR24 (Yang *et al.*, 2005; White and Yang, 2009).

The targets of TAL effectors PthXo1, AvrXa27, PthXo7, PthXo6 and AvrBs3 have been identified (Yang *et al.*, 2006; Chu *et al.,* 2006; Gu *et al.*, 2005; Sugio *et al.*, 2007; Kay *et al.*, 2007; Romer *et al.,* 2007). Among these AvrBs3 was experimentally shown to bind to specific elements in the promoter of it targets *Bs3* and *upa20* (Kay *et al.*, 2007; Romer *et al.*, 2007). Most of the targets of TAL effectors identified so far are either transcription factors or components of eukaryotic transcriptional machinery. AvrBs3 targets *upa20*, which is a basic helix-loop -helix transcription factor and a regulator of cell hypertrophy (Kay *et al.*, 2007). The Xoo effector PthXo6 induces the expression of *OsTFX1*, a bZIP

transcription factor, and PthXo7 targets *OsTFIIAγ1*, a subunit of general transcription factor IIA (Sugio *et al.*, 2007). TFIIA is one of the basal transcription factors required for RNA polymerase II dependent transcription events. TFIIA interacts directly with TFIID and enhances binding to the TATA box (Buratowski *et al.*, 1989). TFIIA also functions to exclude repressors and, in some cases, function as co-activator by interacting with other transcription activators (reviewed in Hoiby *et al.*, 2007). TFIIA is composed of three subunits α, β and γ. The γ subunit in rice is encoded by two genes, one on chromosome 1 and the other on chromosome 5. The allele on chromosome 5, *OsTFIIAγ5,* is the predominant form in rice and is constitutively expressed (Iyer and McCouch, 2004; Jiang *et al.*, 2006; Sugio *et al.*, 2007). Interestingly, a single amino acid substitution variant (V39E) of *OsTFIIAγ5* confer race-specific resistance to bacterial blight (Iyer and McCouch, 2004; Jiang *et al.*, 2006).

Strains without *avrXa7* depend on other major TAL effectors for virulence activity. PthXo1, PthXo2 and PthXo3 are major virulence determinants of PXO99^{A}, JXO1^{A} and PXO61 respectively (Yang *et al.,* 2004). PthXo1, PthXo2 and PthXo3 are known to have only virulence function, and AvrXa7 is the only major TAL effector with R gene specific activity. Although the strains of Xoo contain several TAL effector genes, they are highly dependent on a single major TAL effector. Major virulence determinants are therefore attractive targets for functional analysis as the intervention of its function can lead to substantial cost to the fitness of the pathogen upon loss or alteration of these effectors (Yang *et al.*, 2004). The structural features and the requirements of AvrXa7 suggest that, like other TAL effectors from Xoo and *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*), AvrXa7 may also function by manipulating the host transcriptional machinery and by reprogramming the transcription of specific genes (Kay *et al.*, 2007). DNA binding activity of AvrXa7 was demonstrated by gel shift assay. However, the target gene or the specific DNA element has not been identified (Yang *et al.*, 2000). This

study reports the identification of the host target of AvrXa7 and also characterizes its interaction with the target gene.

Materials and Methods

Plant material

Seeds of rice variety Nipponbare (accession no. PI 514663) were provided by the U.S. Department of Agriculture-Agricultural Research Service National Small Grains Collection. IR24 and IRBB13 seeds were obtained from the International Rice Research Institute (courtesy of Nollie vera Cruz). Kitake seeds were provided by Pamela Ronald (UC, Davis). Seeds of the T-DNA insertion line PFG_3D-03008 and its parental strain Hwayoung were provided by the POSTECH Biotech Center in Pohang University of Science and Technology. All rice plants were grown in growth chambers with temperature of 28°C, relative humidity of 85%, and photoperiod of 12 hr on Baccto® premium potting soil. *Nicotiana benthamiana* plants were grown on the same medium as mentioned above at temperature cycles of day 24°C /night 19°C at relative humidity of 70%. After inoculation with *Agrobacterium,* temperature was raised to 27°C to allow proper infection.

Plasmids and bacterial strains

The *Xanthomonas oryzae* pv. *oryzae* (Xoo) strains and plasmids used in this study are listed in Table.2-1. *Escherichia coli strain* DH5α was cultivated in Luria broth (LB) at 37°C (Bertani , 1951), and Xoo strains in TSA medium (tryptone 10 g/L, sucrose 10 g/L and glutamic acid 1 g/L) at 28°C. *Agrobacterium* strain LBA 4404 was grown on LB medium supplemented with rifampicin 15ug/ml. Transformed *Agrobacterium* were selected using Kanamycin 50 ug/ml and Rifampicin 15 ug/ml. The mutant Xoo strains were grown on TSA supplemented with Kanamycin 100 ug/ml and the one with pHM1

plasmids were cultured on TSA supplemented with Spectinomycin 100 ug/ml and Kanamycin 100 ug/ml (Yang and White, 2004).

Generation of *Os11N3*-silenced plants

Calli from immature embryos of rice cultivar Kitake were initiated and transformed by using *Agrobacterium trumefaciens* as described in Hiei *et al*, 1997. For construction of Os11N3RNAi plants, a 341-bp fragment specific to *Os11N3* was PCR amplified with primers 11N3RNAi-F (5'–GAGAAGAAGGTAGCTGCATGAGTG-3') and 11N3RNAi-R (5'–CAACCTGTAAGGGTTCCTTCCATGA-3'). The product was cloned into pTOPO/D-ENTR vector, sequenced and recombined into pANDA (Miki and Shimamoto, 2004) through LR recombinase according to the manufacturer's instruction (Invitrogen). The RNAi vector was electroporated into *Agrobacterium trumefaciens* strain EHA105.

Northern hybridization and semi-quantitative RT-PCR analysis

The rice leaves were inoculated with indicated bacterial strains and used for total RNA extraction at indicated time points as described in the text. The RNA extract buffer was TRI Reagent from Ambion. Fifteen micrograms of total RNA for each sample were separated in 1% agarose gel and blotted on Hybond N+ membrane (Amersham Pharmacia). The blot hybridization was performed with specific probes as indicated in the text at 65°C with appropriate buffer. The probes of *Os11N3* were prepared from cDNA by using primer sets of 11N3RNAi-F (5' GAGAAGAAGGTAGCTGCATG AGTG-3') and 11N3RNAi-R (5'–CAACCTGTAAGGGTTCCTTCCATGA-3') for 3' probe and 11N3-5-F (5'–ATCAAGCCTTCAAGCAAAGC-3') and 11N3-5-R (5'CTAGGAGA CCAAAGGCGAAG-3') for 5' probe, respectively. Semi-quantitative RT-PCR (qRT-PCR) was performed on RNA extracted from leaves inoculated with bacteria as indicated in the text. Primers 11N3RNAi-F and 11N3RNAi-R were used for detecting *Os11N3*

transcript and RT-TF2-5F (5′-GGGTTT GCCTGGTATTTGTTAG-3′) and RT-TF2-5R (5′-GTTGCTGCTGTGATATACTCTG-3′) was used to amplify *OsTFIIAγ5*.

Genotyping of T-DNA line PFG_3D-03008

Seeds from PFG_3D-03008 were germinated and selfed. DNA was extracted from a single leaf of each progeny plant and genotyped with the following primers: Os11g-F 5′GTCTCCTAGGTGTGTTG CCTTTG-3′ Os11g-R (wild type locus forward primer); 5′-GTGGGTGACGTCATG TTACACT-3′ (wild type locus reverse primer); and 2772 RB-F 5′-CGTAACATAAGGGACTGACCTACC-3′ (pGA2772 right border T-DNA primer). The progeny of plants heterozygous for the insertion were analyzed for the presence of T-DNA at *Os11N3* locus and found that the insertion segregated in 3:1 ratio.

Virulence assay

The fully expanded rice leaves at the stages indicated in the tests were inoculated by leaf tip-clipping with scissors that were immersed in bacterial suspensions of optical density of 0.5 at 600 nm (≈$5.0x10^7$ cell forming units per ml). The optical density of bacterial suspensions were measured using Genesys20 (Thermo scientific) spectrophotometer immediately prior to each clipping as described (Ogawa and Yamamoto, 1987). Symptoms were scored by measuring lesion length.

Generation of promoter–GUS constructs

Various promoter–GUS constructs for the transient expression assay were made using standard recombinant DNA techniques (Sambrook *et al.,* 1989). The promoter regions were amplified the using specific primers given Table 2-1 and amplicons obtained were digested with HindIII and XbaI (Invitrogen) and cloned into HindIII and XbaI site in pB121 (kindly provided by Dr. Xiaoyan Tang, KSU) replacing the 35S promoter in the same. Each construct was sequenced to confirm the identity prior to

transforming into *Agrobacterium* strain LBA4404. The 35S- effector T -DNA vectors were provided by Dr. Bing Yang, Iowa State University.

Transient expression assay

For each assay *Agrobacterium* transformants with various constructs were streaked on LB agar supplemented with kanamycin (50 µg/mL) and rifampicin (15 µg/mL) antibiotics and grown at 28°C for 2 days. A single colony was inoculated in 5mL liquid LB media supplemented with kanamycin (50 µg/mL) and rifampicin (15 µg/mL), 1mL of the overnight culture was sub cultured in 50mL liquid LB supplemented with kanamycin (50 µg/mL) to an OD_{600} of 0.6. The bacterial cells were then collected by centifugation at 4°C for 10 minutes at 3000 rpm. The cells from each centifugation were resuspended in 50ml *Agrobacterium* inoculation buffer (4.8gm MES, 5mL 1M MgCl2 and 0.147g acetosyringone in 500ml water, pH 5.6) and activated at 28°C for 3 hrs. Co – inoculation was done by mixing the cultures in 1:1 ratio prior to inoculation. 100µl of bacterial suspension was infiltrated into the leaf at each inoculation site. The inoculation was done on fully opened leaves(3 leaves per treatment) and leaves harvested 40hrs post inoculation and incubated at 37°C in GUS reagent (100mM phosphate buffer with 0.5% triton X-100,10mM EDTA,0.5mM each of X-gluc, potassium ferri and ferro cyanide) for 7 hrs and there after cleared using 70% ethanol.

Chromatin immunoprecipitation (ChIP) assay

avrXa7 was tagged with double FLAG sequence in tandem using primers FL-F and FL-RL (Table 2-3) at EcoR1 and Sal1 sites and introduced into Xoo strain ME2 (Horton *et al*, 1989). This strain was tested for the expression of FLAG tagged AvrXa7 (*avrXa7*-2F) by western blotting and its ability to induce *Os11N3* on IR24 plants and HR on IRBB7. Previously AvrXa7 with single FLAG epitope in the same C terminus region was found to be biologically active (Zhu *et al*., 1998). Similar double FLAG version of *pthXo1* was made by replacing the SphI fragment of pZW*avrXa7*-2F with the SphI fragment of

pthXo1. The biological activity of resulting *pthXo1*-2F was tested on its ability to induce *Os8N3* on IR24 plants and its incompatibility on IRBB13. Two week old rice plants were syringe inoculated with ME2 (*avrXa7*-2F) or ME2 (*pthXo1*-2F) suspension with OD_{600} 0.5. ChIP assay was performed as described in Haring, M. *et al.*, 2007 with the following modifications. 2.5 gms of inoculated leaf material was harvested 18 hpi, fixed using 1.5% formaldehyde. The tissue was sonicated using Virsonic 50 (output control 6) for 8x15 sec. Ix protease inhibitor cocktail for plant cell and tissue extracts (sigma) was used as protease inhibitor. Immunoprecipitation was done using monoclonal FLAG antibody (Sigma, 12µg/ml). 12µg/ml mouse serum was added in the control IP tube. PCR on enriched DNA sample was done using the primers listed in Table 2-3.

Results

AvrXa7 and PthXo3 dependent differential host gene expression

Previous studies in our lab have shown that the introduction of AvrXa7 into $PXO99^A$ (strain originally incompatible on *xa13* plants) enabled it to overcome *xa13* mediated resistance. Also the newly derived strain, $PXO99^A$ (*avrXa7*) failed to induce S gene *Os8N3* (Yang *et al.*, 2006). The strains with major effectors PthXo2 and PthXo3 are known to be virulent on *xa13* plants. This prompted us to investigate the possibility that these major TAL effectors may target alternate host gene(s) to induce a state of susceptibility. Rice genome encodes 17 members of Nodulin 3(N3) family. Rice cultivar Nipponbare was inoculated with a *pthXo1* mutant ME2 and the strains obtained by introducing each of the major TAL effectors individually into ME2 (Table 2-1). RT-PCR analysis was done on RNA extracted 24hrs post inoculation using 3'UTR specific primers for each of the 17 Nodulin 3(N3) genes. One of the N3 members *Os11g31190* showed elevated expression when inoculated with ME2 (*pthXo3*) and ME2 (*avrXa7*) (Fig.2-1). Inoculation with water, type III secretion mutant ME7 ME2 (*pthXo2*) and ME2(*pthXo1*)did not induce *Os11g31190*. The constitutively expressed rice gene

OsTFIIAγ5 was used as internal control. 15 other N3 genes showed no differential expression in the presence of *pthXo1*, *pthXo2*, *pthXo3* and *avrXa7*(data shown).

_Os11N3_ encodes a putative membrane localized Nodulin 3 (N3) protein.

Os11g31190 is located on chromosome 11 of rice genome and hence named *Os11N3. Os11N3* is represented by the 1,494 base full length cDNA (NCBI accession AK101913) and has four introns (Fig.3a). It is predicted to encode a putative N3 family protein of 303 amino acids with seven transmembrane domains. *Os11N3* is closely related to one of the two MTN3 genes located on chromosome 12, Os12g29220 (Os12aN3) and their encoded proteinsare 72% identical (Fig.2-2). *Os11N3* is also 47% similar to the known S gene *Os8N3*. Additional N3 genes with very high similarity to *Os11N3* are also found in sorghum and maize.

_Os11N3_ silenced plants are resistant to strains with AvrXa7.

The elevated expression of *Os11N3* by PthXo3 and AvrXa7 and it being an N3 family member like *Os8N3*, indicated its role in susceptibility. To test if *Os11N3* expression is a requirement for effector mediated susceptibility, *Os11N3* silenced plants were generated by RNAi approach. A hairpin construct was made in pANDA vector using a unique 341 bp region from the 3'UTR of *Os11N3* (Fig 3a). Thirteen independent lines were obtained that expressed the double stranded RNA and of these data from 3 representative lines are shown in Fig. 3b. The lines were assessed for the expression of the transgene using 3' specific probe (Fig.2-3a) and all transgenic lines showed an increased expression of *Os11N3* 3' region by northern analysis where as the control plant generated using empty pANDA vector showed no enhanced expression. The endogenous *Os11N3* transcript was barely detected in the control (Fig.2-3b). The transgenic lines and the control line were inoculated with ME2 (*avrXa7*) and Northern

analysis was done on RNA extracted 24hrs post inoculation using 5′ UTR specific probe (Fig.2-3a). AvrXa7 failed to induce *Os11N3* in all transgenic lines tested where as the control line showed an enhanced expression of *Os11N3* following inoculation. Transgenic lines were subjected to virulence assay using strains ME2 (*pthXo1*) and ME2 (*avrXa7*). The susceptibility of the plants was measured on the basis of lesion length observed 20 days post inoculation (Fig.2-3c). Plants expressing iRNA corresponding to *Os11N3* were resistant to ME2 (*avrXa7*) but remained susceptible to ME2 (*pthXo1*) (Fig.2-3c column 1 and 2). The control line was susceptible to both strains (Fig.2-3c, column CK). *Os11N3* silenced plants were also resistant to ME2 (*pthXo3*) (data not shown). The transgenic plants were normal in appearance and were affected only in their response to the pathogen. The results suggest that the virulence function of TAL effectors PthXo3 and AvrXa7 is dependent on its ability to induce *Os11N3*.

T-DNA insertion mutant of *Os11N3* has TAL effector dependent resistance.

T-DNA insertion event PF_3D-03008 resulted in the insertion of pGA2772 T-DNA in the first intron of *Os11N3* gene in cultivar Hwayoung (Jeon *et al.*, 2000) (Fig.2-4a). The plants obtained from this line were genotyped using two sets of primers. A set of primers flanked the insertion site and were specific to the *Os11N3* gene (Fig.2-4, blue arrows). The third primer corresponds to the right border of T-DNA (Fig.2-4, red arrow) and in combination with the gene specific reverse primer amplified 440bp product spanning the insertion site. The plants that were heterozygous had both amplicons (Fig.2-4b, Lanes 6, 7 and 9) were as the plants that were homozygous for the insertion amplified only the 440bp product (Fig.2-4b, Lanes 1, 2, 3, 4 and 8). The plants 5 and 10 had no insertion and had only the gene specific band similar to the wild type. The plants were challenged with Xoo strain ME2 (*pthXo1*), ME2 (avrXa7) and ME2 (*pthXo3*). The plants homozygous for the T-DNA insertion remained lesion free 9 days after inoculation with ME2 (*avrXa7*) (Fig.2-5Ab, Bb) and ME2 (*pthXo3*) (Fig.2-5Ac, Bc). The same plants developed lesions in response to challenge with ME2 (*pthXo1*) (Fig.2-5Aa,

Ba). The progeny of the heterozygous plants with insertion in one allele or no insertion the wild type plants were susceptible to all three strains (data not shown). The virulence of different strains on *Os11N3* mutants were also determined by measuring the lesion lengths. The lesion lengths were measured 9 days post inoculation. The plants homozygous for the insertion showed no lesions in response to strains with AvrXa7 or PthXo3 (Fig.2-6). The average lesion length of the homozygous plants in response to ME2 (*pthXo1*) was 12.8 cms (Fig.2-6, blue bar). All three strains were highly virulent on the heterozygous plants, each of them causing long lesions (Fig.2-6, blue, green and orange bars). The results indicate that the mutation in *Os11N3* provides race specific resistance and the mutation is effective against strains with AvrXa7 and PthXo3. Also the induction of *Os11N3* seems to be the key to AvrXa7 and PthXo3 mediated susceptibility. Strains with other major effectors like PthXo1 are compatible on *Os11N3* mutant plants and the evidence suggests that N3 members and major TAL effectors interact in a gene for gene manner.

***Os11N3* is important for normal plant development**

During the genotyping of PF_3D-03008 plants we noticed that all plants were heterozygous for the insertion but one which died after germination. In the next generation the selfed progeny were germinated on MS media and transplanted at three leaf stage and we were able to recover homozygous plants. The homozygous plants were weak and developed slowly compared to the heterozygous plants (Fig.2-7B). They also took an additional 30 days to complete their life cycle and the matured plants were shorter than the plants heterozygous for the insertion (Fig.2-7C). Homozygous plants had small spikelets and the seeds were smaller and deformed (Fig.2-7A). The seeds from the homozygous plants had poor germination rate (32%). The average weight of 25 seeds from heterozygous plants is 571.48 ± 26.39 mg and that of homozygous plants is 313.02 ± 11.62 mg.

TAL effectors interact with host target gene promoter *in planta*

Previous studies in our lab have demonstrated the requirement of NLS and activation domain (AD) of AvrXa7 for its virulence and avirulence function (Yang *et al.*, 2000). Also related effector AvrBs3 was demonstrated to bind to the promoter of its target genes (Kay *et al.*, 2007; Romer *et al.,* 2007). To determine whether TAL effectors PthXo1 and AvrXa7 associate with their target gene promoter chromatin immunoprecipitation (ChIP) assay was performed. Rice chromatin complexes were isolated from leaf tissue individually inoculated with ME2 (*pthXo1*-2F) and ME2 (*avrXa7*-2F) 18 hrs post inoculation. Effector associated complex was immuno-precipitated using monoclonal anti-FLAG antibody and the enriched DNA was analyzed using promoter and 3'UTR specific primers (Fig.2-8A and B). Fragments containing the promoter region of *Os11N3* were significantly enriched in both the α-FLAG precipitated replicates compared to the control from ME2 (avrXa7-2F) inoculated sample. (Fig.2-8A). The *Os11N3* 3'UTR specific primers and *Os8N3* promoter specific primers showed no amplification in IgG as well as α-FLAG precipitated complexes. Strong amplification of the promoter region of *Os8N3* was observed in only in α-FLAG retrieved complex from ME2 (*PthXo1*-2F) inoculated tissue (Fig.2-8B). No differential amplification was observed with *Os8N3* 3'UTR specific primers or with *Os11N3* promoter specific primers. This study demonstrates that the TAL effector binding is specific and the effectors associates with the target gene promoter *in vivo*.

Promoter target sequences of AvrXa7 and PthXoI

A recent study demonstrated that variable two amino acid residues in the central repetitive domain of TAL effectors recognize and bind to specific nucleotides in the target gene promoter (Boch *et al*, 2009). A stretch of nucleotides determined by the variable residues of each repeat of effector protein defines the target element. The 5' region of *Os11N3* was scanned for the presence of AvrXa7 binding element (hereafter

A7BE) and a stretch of 26 nucleotides with one to one correspondence with di variable amino acid residues were found at -278 to -252 positions relative to the predicted start codon. A stretch of 29 nucleotides matching the di variable residues of 29 repeats of PthXo3 is found at position – 280 to -251 of *Os11N3* gene (Fig.2-9). The predicted PthXo3 binding element (here after P3BE) overlaps with A7BE and both binding elements overlap the TATA box of the predicted ORF.

TAL effectors drive promoter specific expression of target gene

The binding elements of PthXo1 and AvrXa7 were testedusing transient expression assay. The promoter regions of *Os8N3* and *Os11N3* were fused individually to the coding sequence of reporter gene *uidA* and the ability of the respective effector to drive promoter specific expression was analyzed. A 282 bp fragment of *Os8N3* promoter from -24 to -306 positions relative to the start codon which includes the predicted PthXo1 binding element (P1BE) -227 to -250 was used (Fig.2-10A, denoted 8N3). A mutant version was also made with three consecutive non consensus bases inserted into position -249 to -247 (Fig.2-10A, denoted $8N3^{m}$). The 8N3 construct when co- inoculated with *pthXo1* under the control of CaMV 35S promoter produced strong GUS activity. The construct $8N3^{m}$ showed no activity or rarely weak activity (Fig.2- 10B). Similarly a 306 bp fragment from position -24 to -330 of *Os11N3* promoter which included the A7BE -252 to -279 (Fig.2-11A, denoted 11N3) and a mutated version with non-consensus nucleotide substitution at -267 to -269 (Fig.2-11A, denoted $11N3^{m}$) was used. 11N3 construct in combination with 35S-*avrXa7* showed strong GUS expression where as the mutant version showed no activity (Fig.2-11B).

A7BE mediated recognition is independent of the promoter context.

To see if the A7BE retained its function when moved to a different location and to determine if the neighboring nucleotide sequence influenced the binding of AvrXa7, a chimeric promoter construct 11/8 was generated in which the P1BE from 8N3 promoter

was replaced with A7BE (Fig.2-12A). Similarly a mutant version $11^m/8$ was also made with three nucleotide changes as mentioned above within the binding element. A7BE element in the new construct is located -227 to -256, different from its position in the wild type 11N3, relative to the start site. The chimeric constructs were then infiltrated into *Nicotiana benthamiana* plants in combination with either effector (*avrXa7* or *pthXo1*) expressed from 35S promoter. Transient expression assay demonstrated that 35S-*avrXa7* activated the expression of the reporter gene in combination with 11N3 or 11/8 where as it failed to activate both the mutant versions $11N3^m$ and $11^m/8$ (Fig.2-12 B). The data also demonstrates the specificity of effector binding as no GUS activity was observed in the combinations 35S-*avrXa7*: 8N3, 35S-*pthXo1*: 11N3 or 35S-*pthXo1*:11/8. The results also confirm that the adjacent nucleotide sequence, the context of the promoter or its relative distance from the start site had no influence on the effector mediated recognition and activation of target host genes.

Discussion

Our study identified a N3 gene paralog located on chromosome 11 (*Os11N3*) to be upregulated in an effector-specific manner. AvrXa7 and PthXo3, two major TAL effectors of Xoo strains PXO86 and PXO61, respectively, elevated the expression of *Os11N3*. AvrXa7 is previously shown to overcome *xa13*-mediated resistance without the induction of host susceptibility gene *Os8N3* (Yang *et al.*, 2006). We hypothesized that *Os11N3* is an alternate susceptibility gene targeted by *avrXa7* and *pthXo3*. Consistent with this hypothesis, *Os11N3* silencing led to effector-specific resistance. The strains with a dependence on either *avrXa7* or *pthXo3* for virulence were unable to induce *Os11N3* and were incompatible on *Os11N3*-silenced transgenic lines. This evidence was further confirmed by the evaluation of *Os11N3* T-DNA insertion mutants.

The results from ChIP assays support a direct association of effectors PthXo1 and AvrXa7 with the promoter regions of their respective target genes *Os8N3* and *Os11N3*.

We have also demonstrated through heterologous transient expression system in *Nicotiana benthamina* that induction of the reporter gene was both promoter-specific and effector-specific. The TAL effectors PthXo1, PthXo3 and AvrXa7 have unique repeats in their central repetitive region. The variable amino acids in repetitive regions of each effector are predicted to define specific nucleotide sequences in their respective target gene promoter similar to prototype TAL effector AvrBs3 (Boch *et al.*, 2009). Mutations within the predicted binding element of PthXo1, PIBE and AvrXa7, A7BE abolished effector-specific reporter gene expression. Also the replacement of P1BE with A7BE in the *Os8N3* promoter led to AvrXa7 dependent reporter gene expression. Therefore, host plant susceptibility is the outcome of the recognition of specific DNA element and activation of S gene by the cognate effector.

TAL effectors are critical determinants of Xoo, though the bacterium harbors multiple TAL effectors, few of these have shown to have dramatic effects on the virulence (Yang and White, 2004). Rice has adapted to bacterial blight disease by evolving dominant R genes, which can recognize and interfere with effector- -mediated virulence. AvrXa7 is recognized by *Xa7* (Sidhu *et al.*, 1978). PthXo3 has a similar virulence function as AvrXa7 but does not trigger *Xa7*-mediated resistance. The existence of two effectors that target the same host gene reflects the adaptation of the pathogen to maintain the virulence function while avoiding recognition by the host. Apart from acquiring dominant R genes, rice has also evolved to adapt to bacterial disease by genetic changes in susceptibility genes. One excellent example is the recessive resistance gene *xa13,* which is a non-inducible allele of susceptibility gene *Os8N3. Os8N3 (Xa13)* is required for normal pollen development and, therefore, critical for reproduction (Chu *et al.*, 2006). The occurrence of several variants of *xa13* in rice cultivars indicates several separate events of host adaptation to evade bacterial manipulation of an essential gene (Chu *et al.*, 2006). This study identified a second host susceptibility gene with an essential function in plant development. Natural alleles of

Os11N3 that provide recessive resistance are not known. At the same time, genetic variations in the EBE that result in the evasion of effector-mediated susceptibility may also interfere with normal gene regulation. Similar to *Os8N3, Os11N3* inactivation can result in abnormal plant development as is the case with T-DNA insertion mutant. *Os11N3*-silenced plants are normal in appearance, possibly due to incomplete silencing of expression. As demonstrated with *Os11N3* knock out plants and T-DNA mutants, the resistance is race-specific, recessive and is defeated by strains with PthXo1. The resistance is due to the lack of induction of the susceptibility gene. This is different from the host susceptibility mechanisms reported recently in wheat and oat, where necrotrophic pathogen effectors interact with host susceptibility gene products to trigger HR like reaction (Lorang *et al.*, 2007; Liu *et al.*, 2009). A third S gene *Os12aN3* closely related to the host target of *avrXa7*, *Os11N3* was identified as the cognate susceptibility gene of major TAL effector *pthXo*2 (Yang and White, unpublished). It is intriguing that the virulence targets of all four major TAL effectors PthXo1, PthXo2, AvrXa7 and PthXo3 are closely related members of N3 family. Furthermore, in a screen of forty Xoo strains from different locations around the world, all of them induced either one or more of the three susceptibility genes indicating a common theme in effector mediated virulence (Yang and White, unpublished). Taken together, the results indicate that the S genes of the N3 family encode proteins with some conserved properties that enable bacterial growth and proliferation inside the host.

The EBEs of the susceptibility genes of major TAL effectors can be used to design complex R gene promoters that can recognize multiple TAL effectors and provide broad spectrum resistance as recently demonstrated for the Bs3 and *Xa27* promoters (Romer *et al.*, 2009). However, repeat shuffling and recombination within the repetitive region can result in effectors with new specificity enabling the pathogen to avoid recognition and to target alternate binding sites in the S gene promoter. The potential for rearrangements was demonstrated in the case of *avrXa7,* where repeat rearrangements

resulted in effectors with new specificity (Yang *et al.,* 2005). Our results indicate that host susceptibility or resistance is determined by the TAL effector repertoire of the bacterium, and Xoo depends on the N3 genes for virulence. Further studies on susceptibility genes and the proteins they encode will provide insights into the molecular basis of host susceptibility.

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Table 2-1. Strains and Plasmids used

Strain or Plasmid	Comments	Reference or Source
***Xanthomonas oryzae* pv. *oryzae* strains**		
PXO99^{A}	5-Azacytidine resistant, race 6	Hopkins *et al*., 1992
PXO99^{A}ME7	hrpC mutant, Kn^{r}	Yang *et al*., 2000
ME2	*pthXo1* mutant of PXO99^{A}	Yang and White, 2004
ME2 (*pthXo1*)	ME2 with pZWpthXo1 in pHM1	Yang and White, 2004
ME2 (*avrXa7*)	ME2 with pZWavrXa7 in pHM1	Yang and White, 2004
ME2 (*pthXo3*)	ME2 with pZWpthXo3 in pHM1	Yang and White, 2004
Plasmids		
pBluescript II KS(+)	Phagemid, pUC derivative, Cb^{r}	Stratagene
pENTR/D-TOPO	Phagemid, Cb^{r}, Kn^{r}	Invitrogen
p35S-pthXo1	*pthXo1* under CaMV 35S promoter in pCAMBIA1300	This study

p35S-avrXa7	*avrXa7* under CaMV 35S promoter in pCAMBIA1300	This study
pBI121-8N3	*Os8N3* promoter fusion with GUS reporter gene in pBI121	This study
pBI121-11N3	*Os11N3* promoter fusion with GUS reporter gene in pBI121	This study
pANDA	Gateway RNAi vector, Kn^r	Miki and Shimamoto, 2004
pCAMBIA1300	Binary T-DNA vector for rice transformation	Cambia
pBI121	T-DNA vector	Jefferson *et al.*, 1987

Table. 2-2. Primers used for promoter constructs

Construct	Primer name	Sequence (5′ to 3′)
8N3	8pG-F	TACAAGCTTATATCAGAGTGAAAAAGAAATATCAAGC
	8pG-R	ATCTCTAGAACTAACTCTAAGGTGTTAATCAGTGAGAAGG
8N3^{m}	8pMG-F	TACAAGCTTATATCAGAGTGAAAAAGAAATATCAAGC ACAAGAAAAAAAAAGCAAAGGTTAGATATGGTGCTCCCC
	8pG-R	
11N3	11pG-F	TACAAGCTTCAGCTGGTCATGTGTGCCTTTTCATTCC
	11pG-R	ATCTCTAGACTAGCTCGGAATATGAGAGAGAGAGAG AGAGT
11N3^{m}	11pMG-F	TACAAGCTTCAGCTGGTCATGTGTGCCTTTTCATTCCC TTCTTCCTTCCTAGCACTATATAAACCGGTTCCAACC
	11pG-R	
11/8	11-8pG- F	TACAAGCTTATATAAACCCCCTCCAACCAGGTGCT AACCAAAGTGGAG
	8pG-R	
11^{m}/8	11M-8pG-F	TACAAGCTTATATAAACCGGTTCCAACCAGGTGCT AACCAAAAGTGGAG
	8pG-R	
FLAG	FL-F	GTCACCGATTCGAAGCCCCGCTAC
	FL-RL	GGCTGGGTCGACCCTTCTTGTCGTCGTCGTCCTT GTAATCCTTCTTGTCGTCGTCGTCCTGTAATC

Table 2-3. Primers used for PCR analysis on ChIP DNA

Amplicon	Primer name	Sequence (5′ to 3′)
Os11N3-5′	Os11N3-5′-F	GTCAGCAGCTGGTCATGTGT
	Os11N35′-R	GTTTGGTGGGAGGAGATCA
Os11N3-3′	Os11N3-3′-F	AGGGACAAAACAAGACAGGTCA
	Os11N3-3′-R	GTGCACCACACCAGTTCTCT
Os8N3- 5′	Os8N3-5′-F	CAGAGTGAAAAAGAAATATCAAGC
	Os8N3-5′-R	GGTGTTAATCAGTGAGAAGG
Os8N3- 3′	Os8N3-3′-F	GGAGAGTGCTGTATGGCGA
	Os8N3-3′-R	GTCTGCCCTGTTCAAACACA

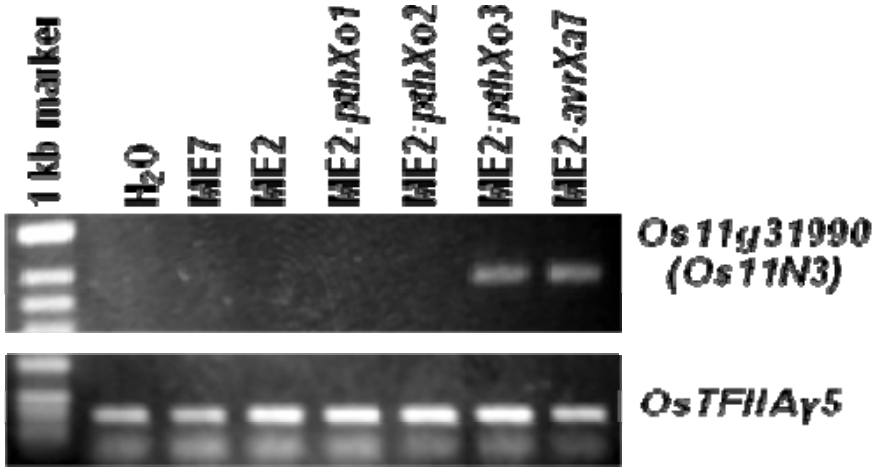


Fig.2-1. TAL effector AvrXa7 and PthXo3 induce the expression of *Os11N3*

Semi quantitative RT-PCR analysis on rice plants (Nippon bare) inoculated with strains ME2, ME2 (*pthXo1*), ME2 (*pthXo2*), ME2 (*pthXo3*) and ME2 (*avrXa7*). Mock inoculation with water and inoculation with type III secretion mutant ME7 were used as control. Total RNA was extracted from each treatment 24hrs post inoculation. *OsTFIIAγ5* served as control for the amount of cDNA used.

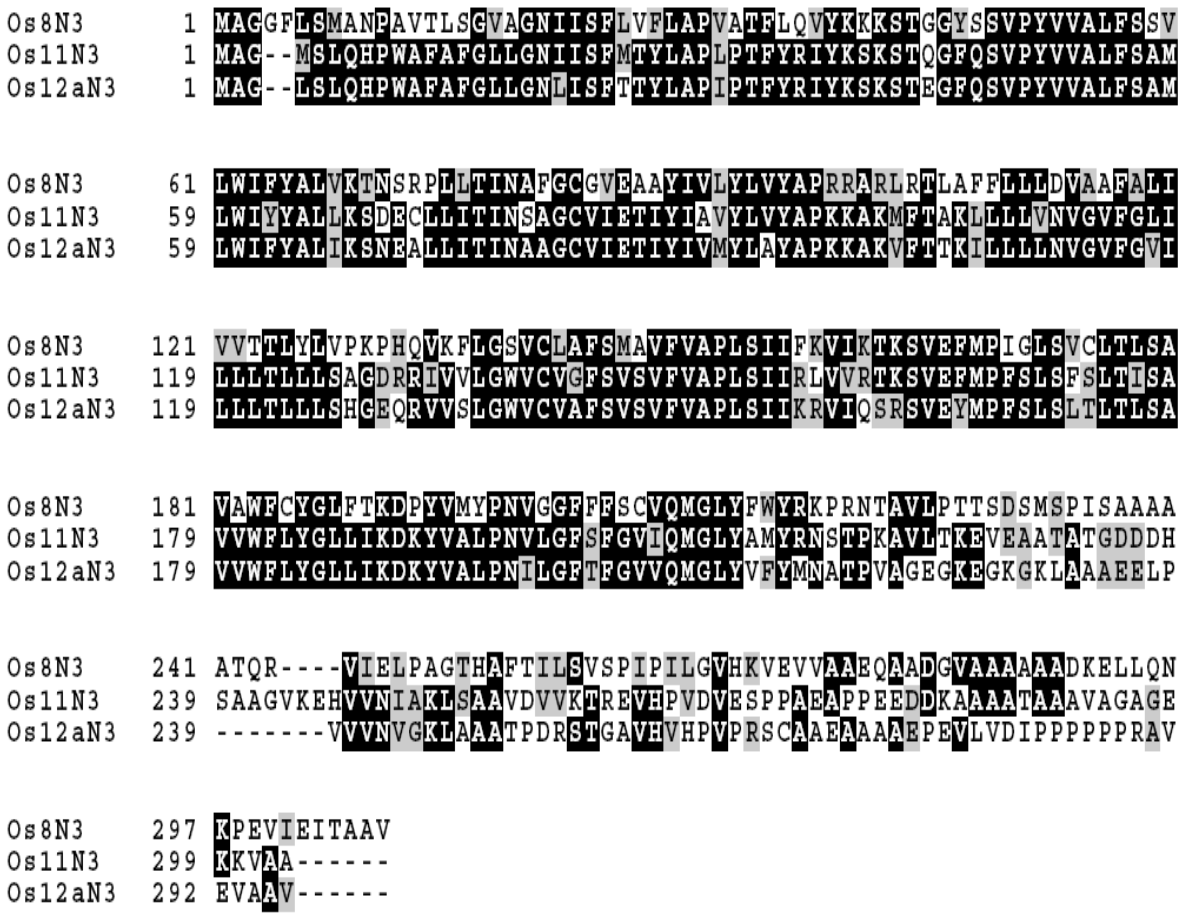
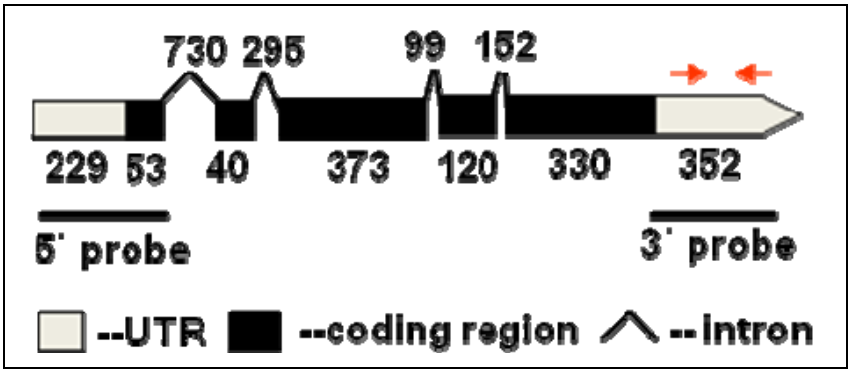


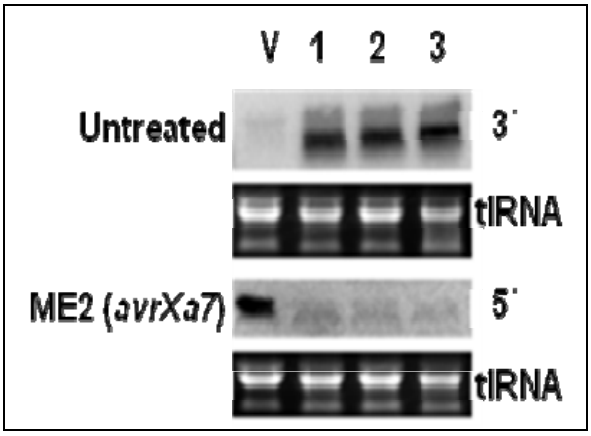
Fig. 2-2. Os11N3 is closely related to two other rice MTN3 members.

Protein sequence of Os11N3 (*Os11g31190*), Os8N3 (Os08g42350) and Os12aN3 **(**Os12g29220) are aligned using CLUSTALW and displayed using BOXSHADE (http://www.ch.embnet.org /software/BOX_form.html).

A



B **C**



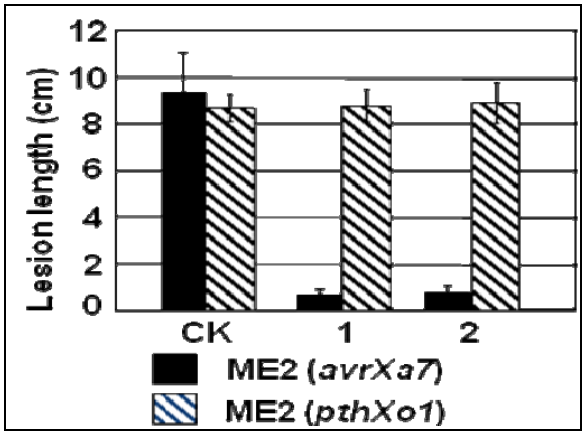


Fig. 2-3. RNAi mediated silencing of *Os11N3* results in AvrXa7 specific resistance.

(A). Schematic of *Os11N3* transcript. The numbers above indicate the length of introns and numbers below the length of exons. The black lines indicate the region used as 5′ and 3′ probes. **(B)** Northern analysis using Os11N3 5′ and 3′ specific probes on transgenic lines. Total RNA extracted from plants generated using empty pANDA vector (V) and three different lines 1,2 and 3 expressing double stranded 3′UTR of *Os11N3* were extracted with and without inoculation with ME2(*avrXa7*). **(C)**. Virulence assay on the transgenic lines using strains with AvrXa7 or PthXo1.

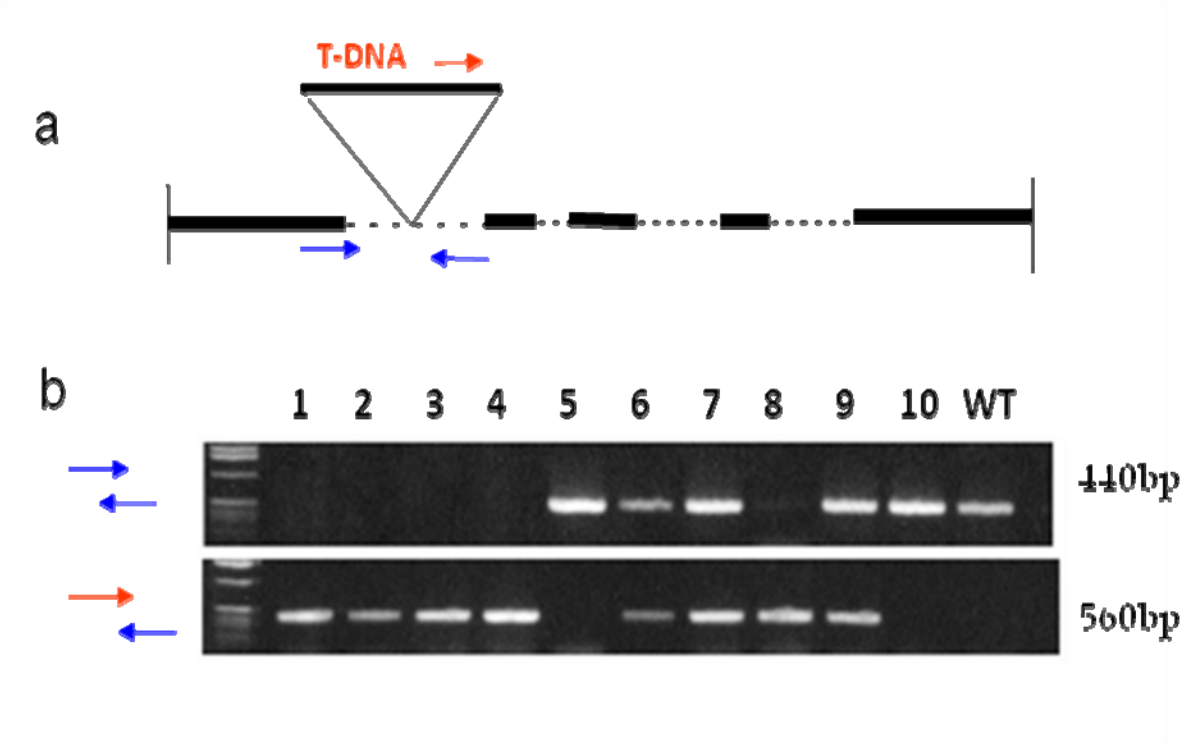


Fig.2-4. Genotyping of *Os11N3* T-DNA insertion mutants (PF_3D-03008)

(a). Pictorial representation of T-DNA insertion in *Os11N3* gene with the position of oligonucleotides used for genotyping. Red arrow indicates T-DNA specific primer and blue arrows represent Os11N3 specific primers flanking the insertion site. **(b).** PCR analysis using T-DNA and gene specific primers. The wild type plant Hwayoung denoted WT served as control. Plants 1, 2, 3, 4 and 8 are homozygous for the insertion; plants 6, 7 and 9 are heterozygous while plants 5 and 10 have no insertion.

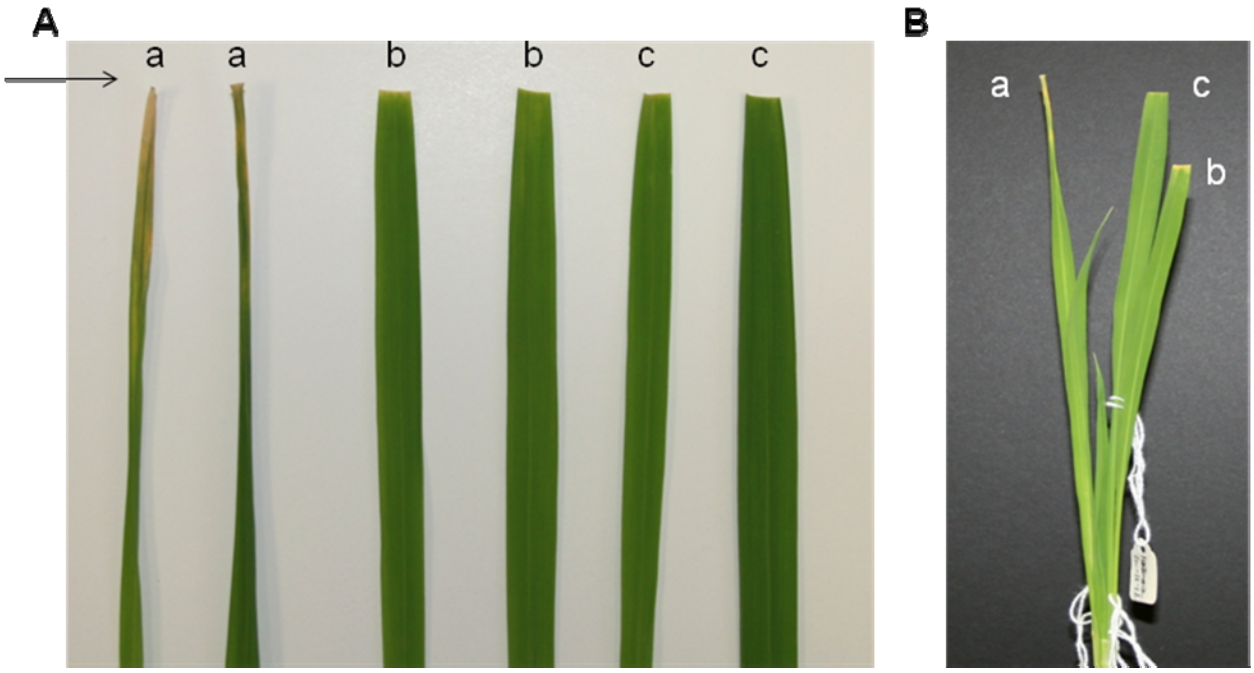


Fig.2- 5. *Os11N3* homozygous mutants are resistant to strains with AvrXa7 or PthXo3.

(A). Inoculated leaves aligned at the site of inoculation. The black arrow indicates the site of inoculation. Inoculations were done using bacterial strains: **a.** ME2 (*PthXo1*), **b**. ME2 (avrXa7) and **c.** ME2 (PthXo3). **(B)**. A single homozygous *Os11N3* mutant plant inoculated with different strains. 50 days old plants were clip inoculated and the photographs were taken 9 days post inoculation.

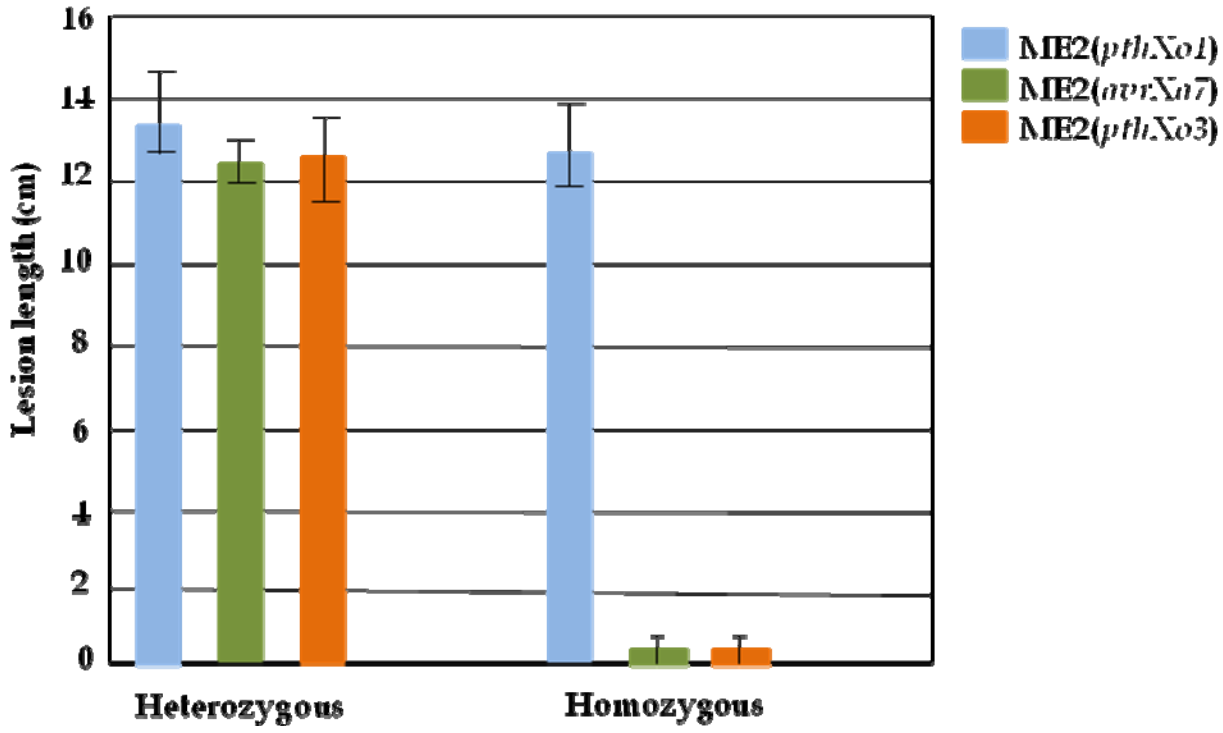


Fig.2-6. Strains with AvrXa7 and PthXo3 are incompatible on *Os11N3* null mutants.

T-DNA insertion mutants (PF_3D-03008) were genotyped prior to inoculation. Clip inoculation was done on 50 day old plants and lesion length measured 9 days after inoculation. Six heterozygous and six homozygous plants were used for each inoculation assay.

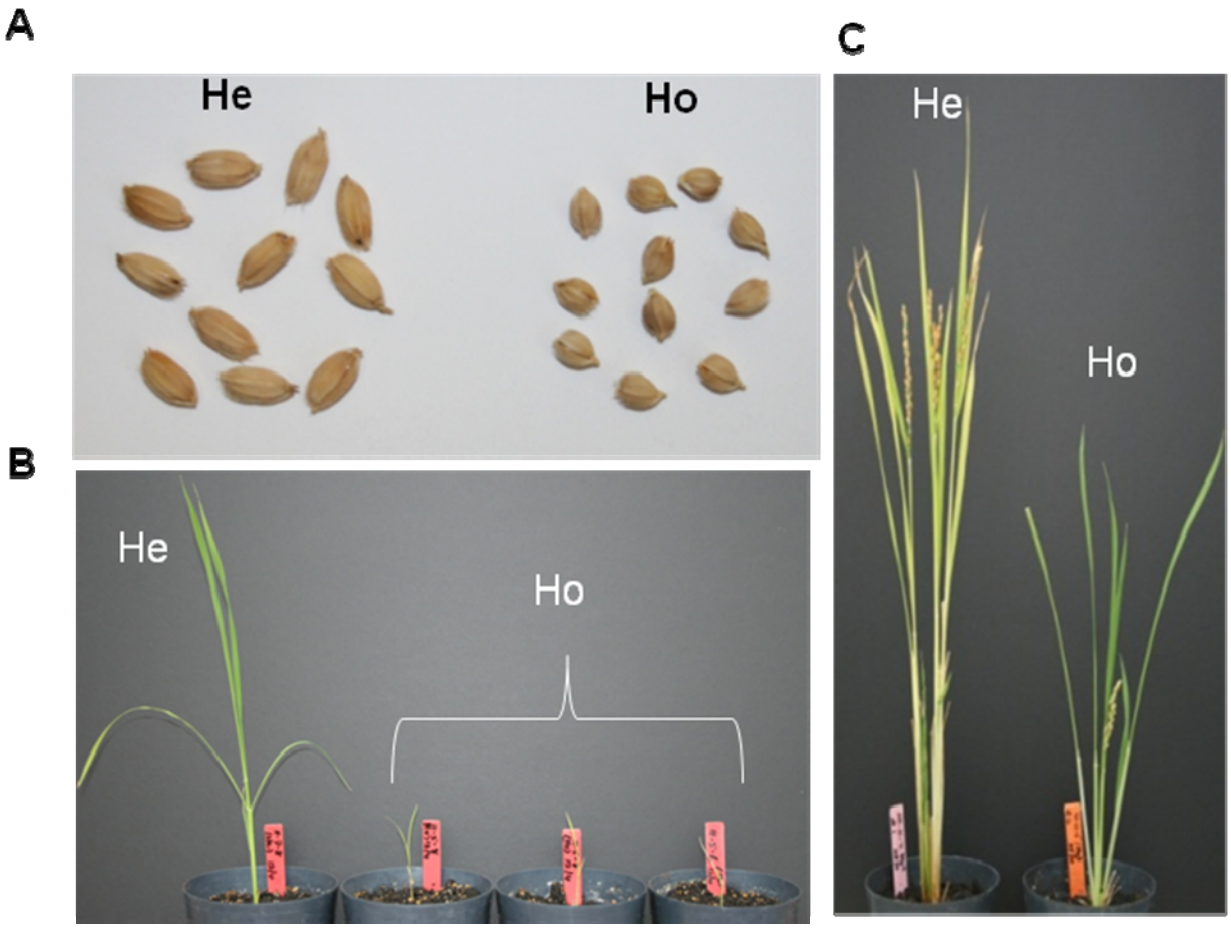


Fig.2- 7. *Os11N3* is important for normal plant development and growth.

(A). Seed morphology of heterozygous and homozygous insertion mutants. **(B)** Heterozygous and homozygous plants 24 days after sowing. **(C)**. Plants at 95 days after sowing. Os11N3 homozygous mutant plants showed remarkable difference in the phenotype like leaf texture, color and plant height. They developed slowly compared to plants that were heterozygous for the insertion.

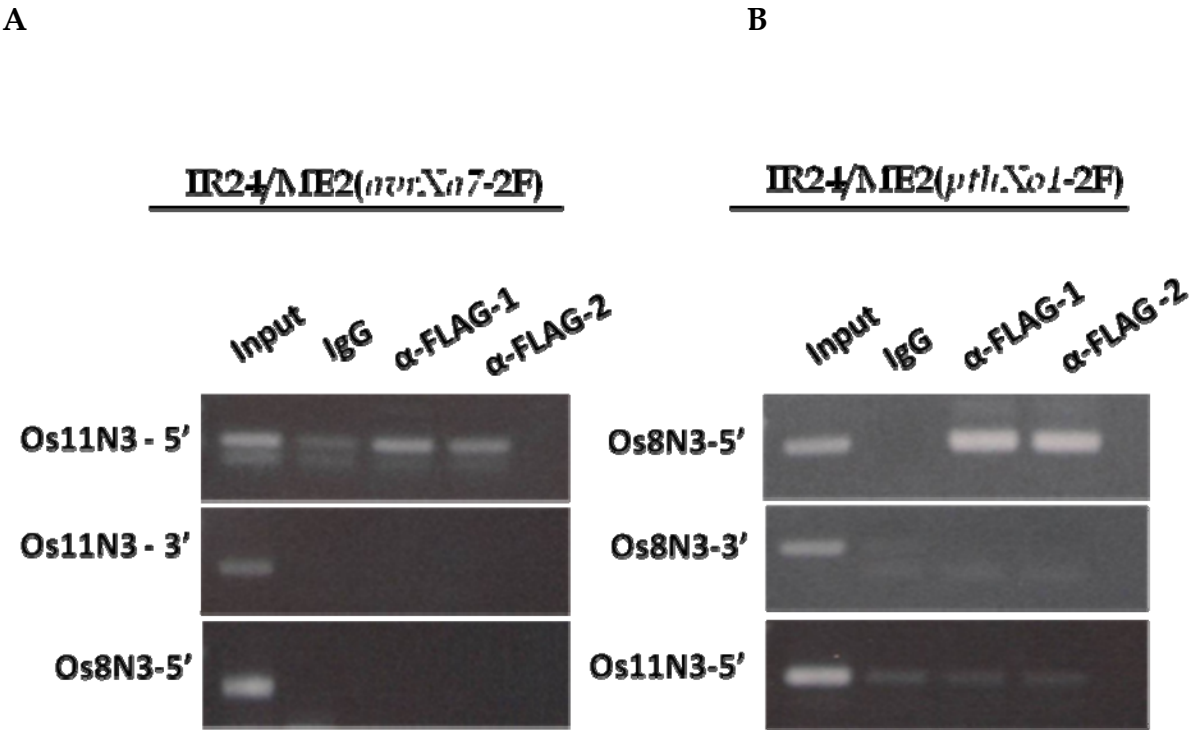


Fig.2- 8. AvrXa7 and PthXo1 bind to their respective S gene promoter *in vivo*

ChIP analyses were performed on cultivar IR24 inoculated with Xoo strains with AvrXa7 or PthXo1 tagged with a double FLAG epitope. Leaves were harvested 18 h post inoculation and immuno-precipitations were done using IgG (Control) and monoclonal FLAG anti body .α-FLAG 1 and 2 represent two replicates of immuno-precipitation. **(A).** Semi quantitative PCR on enriched DNA precipitated from IR24 tissue inoculated with ME2 (*avrXa7*-2F) for 37 cycles and **(B)** with ME2 (*PthXo1*-2F) for 36 cycles. Input to immuno-precipitation chromatin ratio is 1:10.

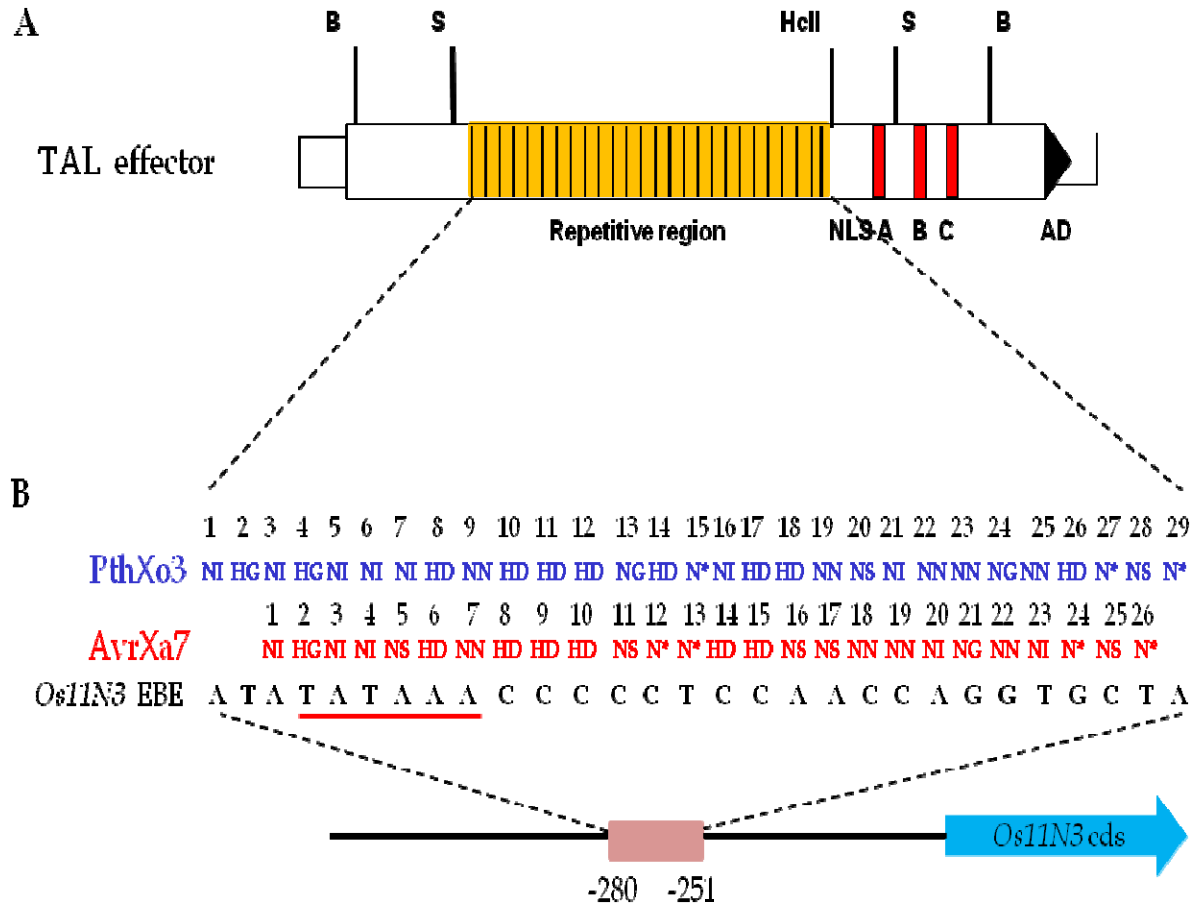
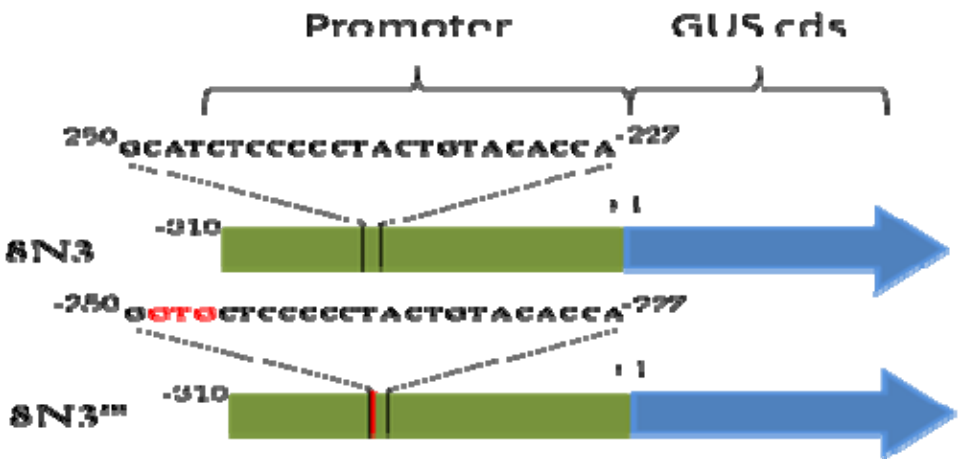


Fig.2-9. Effector binding element (EBE) of *Os11N3* (A). TAL effector with central repetitive domain (orange), nuclear localization signals (red bars) and activation domain (black). **(B).** The variable amino acids of each of the 29 repeats from the repetitive domain of PthXo3 (blue) and the 25.5 repeats of AvrXa7 (red) are aligned with the target sequence/effector binding element (-280 to -251) in the *Os11N3* promoter (pink rectangle). The asterisk in the repeat indicates that the amino acid in that position is missing. TATA box in *Os11N3* promoter is underlined in red. The effector binding elements of AvrXa7 and PthXo1 overlaps with the TATA box.

A



B

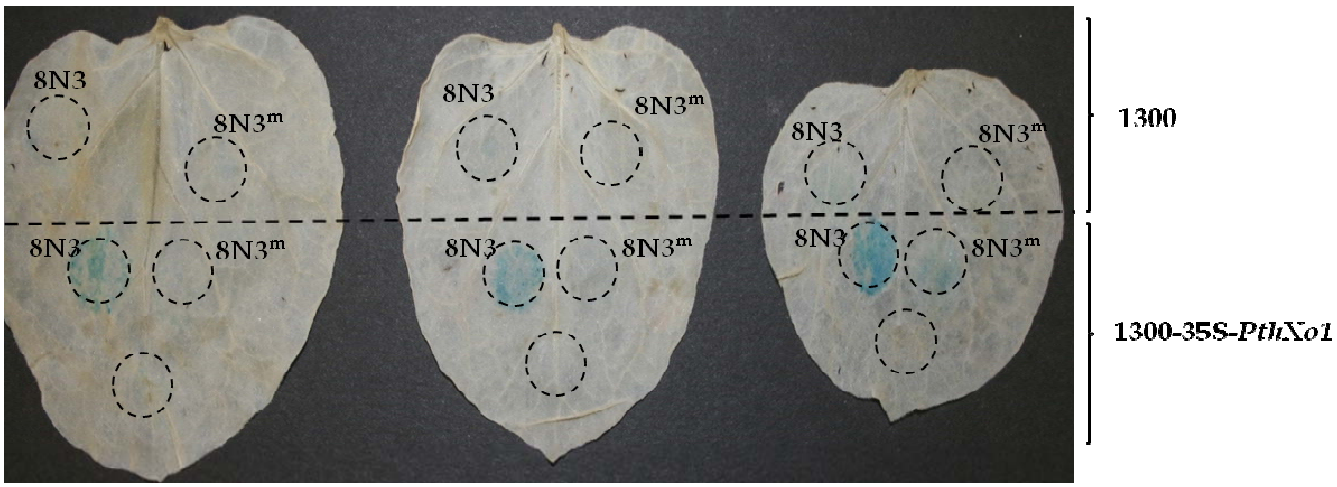
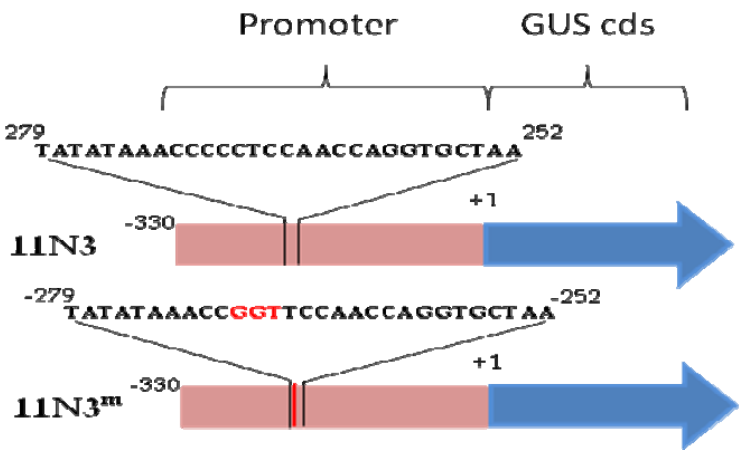


Fig. 2-10. PthXo1 drives Os8N3 promoter specific expression

(A). Promoter – GUS reporter constructs. GUS coding sequence is shown as blue rectangle and the green rectangle represents *Os8N3* promoter. The introduced changes in the predicted binding element are indicated in red. **(B).** *Agrobacterium* mediated transient expression assay using the GUS reporter constructs 8N3 and 8N3^{m}. The reporter constructs were co-delivered with empty T-DNA vector (1300) or 35S driven PthXo1 (1300-35S-*PthXo1*). Three replicates of same experiment are shown.

A



B

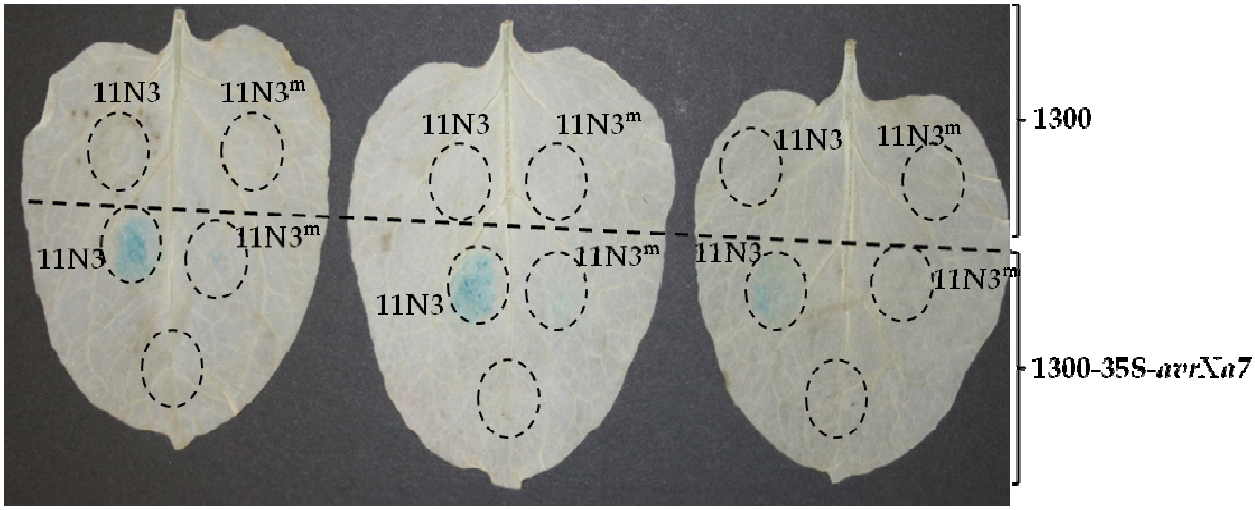
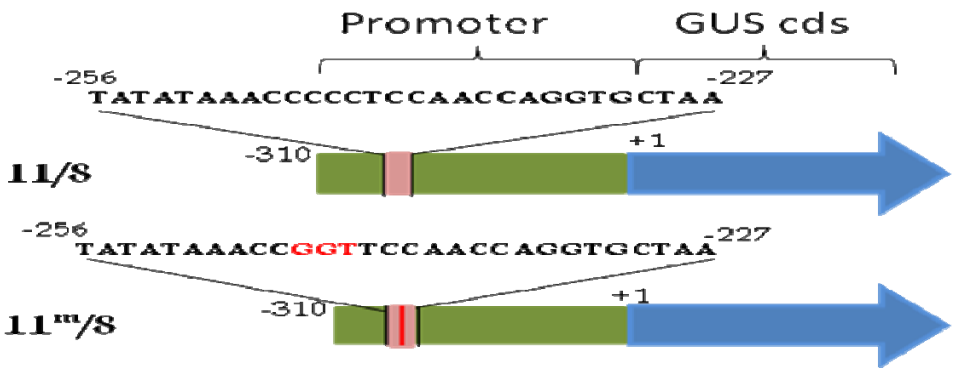


Fig.2- 11. AvrXa7 drives promoter specific expression of the reporter gene

(A). Schematic representation of Promoter – GUS reporter constructs 11N3 and 11N3^{m}. GUS coding sequence is shown as blue rectangle and the pink rectangle represents *Os11N3* promoter. The introduced changes in the predicted binding element are indicated in red. **(B).** *Agrobacterium* mediated transient expression assay using the GUS reporter constructs 11N3 and 11N3^{m}. Th reporter constructs were co delivered with empty T-DNA vector (1300) or 35S driven AvrXa7 (1300-35S-*avrXa7*). Three replicates are shown.

A



B

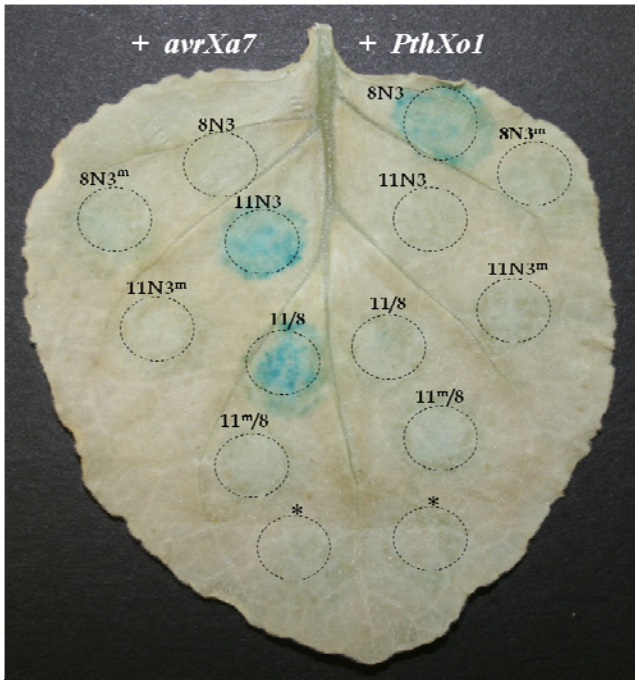


Fig.2- 12. AvrXa7 retain the recognition specificity irrespective of the promoter context. (A). Fusion promoter constructs in which the PthXo1 binding element in the *Os8N3* promoter is replaced with AvrXa7 binding element of *Os11N3* promoter. $11^m/8$ represent the mutant version. **(B).** Transient expression assay on *Nicotiana benthamiana.* The left half of the leaf was co- inoculated with 35S: *avrXa7* and the right half with 35S:*PthXo1.* * denote the sites where the effector alone was injected.

Chapter Three

Differential sensitivity of effector mediated susceptibility in *xa5* plants

Abstract

Xanthomonas oryzae pv. oryzae (Xoo) strains PXO99^{A} and PXO86 have different responses on plants containing the recessive resistance gene *xa5,* which encodes a valine to glutamic acid substitution variant of the γ subunit of general transcription factor TFIIA. Strain PXO99^{A} is virulent or compatible on *xa5*, whereas PXO86 has a weakly virulent or incompatible phenotype on *xa5* plants. Replacement of *pthXo1*, the major TAL effector gene of PXO99^{A}, with *avrXa7*, the major TAL effector of PXO86, converted PXO99^{A} to an incompatible phenotype on *xa5*, indicating that *xa5*-mediated resistance is dependent on the TAL effector content of the pathogen. Comparison of gene expression levels of *Os8N3* and *Os11N3*, which are the targets of the major TAL effectors of PXO99^{A} (PthXo1) and PXO86 (AvrXa7), respectively, in *OsTFIIAγ5* (*Xa5)* and *xa5* backgrounds revealed that resistance is correlated with the level of susceptibility gene expression. The evidence indicates that host pathogen co-evolution and adaptation involves mutational changes to basic components of the host transcription complex in order to modulate pathogen-mediated host gene expression.

Introduction

Plant interactions with their pathogens have resulted in the evolution of resistance (R) genes that facilitate the initiation of biochemical reactions that result in effective resistance. Extensive research on this mechanism has enabled the isolation of more than 40 resistance genes from different plant species (reviewed in McHale, *et al*, 2006). Most of the cloned R genes confer dominant or semi-dominant race-specific resistance and belong to five major classes of R genes (Martin *et al.*, 2003). Compared to dominant resistance, recessive resistance is less well-studied, and few recessive resistance genes have been characterized (Buschages *et al.*, 1997; Ruffel *et al.*, 2002; Deslandes *et al.*, 2002; Iyer and McCouch, 2004; Jiang *et al.,* 2006; Yang *et al.*, 2006; Chu *et al.,* 2006). In the rice bacterial blight patho-system, over 30 resistance genes have been identified of which nine confer recessive resistance (reviewed in Iyer and McCouch, 2007). The cloned recessive BB resistance genes *xa5* and *xa13* confer race specific resistance, and these genes encode proteins that have essential functions in normal plant development. The dominant allele of *xa13*, which is referred to here as *Os8N3*, and the effector PthXo1 of strain PXO99^{A} interact in a gene-for-gene manner, leading to race-specific susceptibility. *Os8N3* encodes a transmembrane protein and is induced to high levels by PthXo1. The resistance conferred by *xa13* allele is due to the loss of induction mediated by the virulence effector PthXo1 (Yang *et al.*, 2006; Chu *et al.*, 2006). All naturally occurring variants of *xa13* have polymorphism in the promoter region (Chu *et al.*, 2006). Xa13 is there for a dominant susceptibility gene targeted by TAL effector PthXo1 and its induction is critical for virulence of PXO99^{A} (Yang *et al.*, 2006). ChIP assay demonstrate that PthXo1 associates with *Os8N3* promoter, and the introduction of non-consensus nucleotide substitutions within the predicted PthXo1 binding element interfered with its transcriptional activation (Results, Chapter 2). *Xa13* is essential for pollen development and silencing of *Xa13* leads to low pollen viability (Yang *et al.*, 2006; Chu *et al.*, 2006).

xa5 confers broad spectrum resistance to Xoo Philippine races 2, 3 and 5, while race 1 strains, including PXO99^{A}, are compatible on *xa5* plants (Iyer and McCouch, 2004). *Xa5* (*TFIIAγ5*) encodes the small subunit (γ) of general transcription factor *OsTFIIAγ5* and is located on chromosome 5. Rice has two closely related genes that code for the γ subunit of TFIIA, the second one is located on chromosome 1 (*OsTFIIAγ1*). *xa5* is a variant of *OsTFIIAγ5* with a 2 nucleotide substitution that results in a valine to glutamic acid change at position 39 (Iyer and McCouch, 2004; Jiang *et al*., 2006). Both the resistant (*xa5*) and susceptible alleles (*Xa5*) are constitutively expressed in plants, and no differential expression was observed in response to infection by Xoo (Jiang *et al*., 2006). Transformation of the dominant allele into the resistant background (*xa5/xa5*) resulted in BB susceptibility (Jiang *et al*., 2006). TFIIA is highly conserved in plants and animals (Hampsey, 1998). The complex functions to stabilize the binding of another complex (TFIID) to the TATA box of the promoter. TFIIA has two domains, a β barrel that interacts with TATA box binding protein complex and a four helix bundle (Kramer *et al*., 2001). In yeast, the four helix bundles (4HB) domain of TFIIA is important for interaction with transcription co-activators and other transcription associated factors (Ma *et al*., 1996; Kramer *et al*., 2006). Two of these helices are encoded by γ subunit. The mutation in *xa5* that confer resistance to BB lies in the helix 3 (Iyer and McCouch, 2007). Intriguingly, the second *TFIIAγ* gene in rice, *OsTFIIAγ1,* is induced by effector PthXo7 of the compatible strain PXO99^{A} in the inoculated leaf tissue. The introduction of PthXo7 into PXO86, a *xa5* incompatible strain, resulted in slight increase in the virulence of the pathogen (Sugio *et al*., 2007). Therefore, *OsTFIIAγ1* induction may be an adaptation by the pathogen to overcome *xa5* mediated resistance (White and Yang, 2009). We hypothesized that the critical mutation in the resistance allele (*xa5*) may interfere with its ability to interact with transcription activator like (TAL) effectors of the pathogen and thereby preventing effector-mediated induction of susceptibility genes. To further understand the differential response of the two pathogen strains

PXO99^{A} and PXO86 on *xa5* plants, their virulence on near isogenic lines 1R24 (susceptible, *Xa5*) and IRBB5 (resistant, *xa5*/*xa5*) are compared.

Materials and Methods

Plant material and growth conditions

Rice line IR24 and near isogenic line IRBB5 which has the recessive resistant allele *xa5* were grown in baccto® premium potting soil. The plants were raised in growth chamber at 27°C, 80% relative humidity and 12 hr photoperiod. Seeds of both lines were obtained from IRRI, Philippines (courtesy of Nollie vera Cruz).

Virulence assay

Seven- week old rice plants were clip inoculated with bacterial suspensions prepared in sterile distilled water (Ogawa and Yamamoto, 1987). Xoo strains were freshly streaked and grown at 28°C for two days prior to each inoculation assay. Inocula concentrations were adjusted using spectrophotometer (Genesys 20, Thermo scientific) to an optical density of 0.5 at wavelength 600 nm (~ 0.5 x 10^8 colony forming unit/ml). Symptoms were scored by measuring the lesion length 20 days after inoculation.

Microarray analysis

Microarray analysis was done using total RNA isolated from two sets of rice leaves inoculated with strains indicated in Table 3-2. Five micrograms of total RNA extracted 24 hrs post inoculation from each sample was used for cDNA synthesis and biotin labeled cRNA according to manufacturer's protocol using one-cycle eukaryotic target labeling kit (Affymetrix). The hybridization was done at Gene Expression Facility, KSU using gene chip rice genome array (www.affymetrix.com/products/arrays/specific

/rice.affix). The intensities for the probe sets from each array were calculated and normalized using GeneSpring GX software (Agilent Technologies). Mean scores within 2 fold or less of the standard deviation was excluded.

Real time PCR analysis

14 day-old rice plants were inoculated with bacterial suspensions using needless syringes covering the entire top half of fully opened leaves. Total RNA was extracted 24 hrs post inoculation and 1 µg of RNA from each inoculation was treated individually with amplification grade DNase1 (Invitrogen) followed by cDNA synthesis using iscript select cDNA synthesis kit (Bio-Rad). cDNA derived from 0.025 µg of total RNA was used for each reaction. RT-PCR was done using iCycler iQ (Bio-Rad) with iQ SYBR green super mix (Bio-Rad) (Weihong and Saint, 2002). Gene specific primers were designed using Beacon Designer 7.0 software. $2^{-\Delta\Delta ct}$ method was used for calculating the relative values of gene expression. *OsTFIIAγ5* expression was used as an internal control (Iyer and McCouch, 2004). The experiments were repeated with consistent results. The primer sequences used for gene expression analysis are listed in Table 3-1.

Results

Strains with AvrXa7 as the major TAL effector are incompatible on *xa5* plants

The Xoo inoculation phenotype of *xa5* is race-specific. However, the gene confers effective resistance to rice against a broad range of Xoo strains (Iyer and McCouch, 2005; Jiang *et al.*, 2006). To investigate if the resistance gene, *xa5,* had any effect on the effector-mediated susceptibility, the genes for effectors AvrXa7 and PthXo1 were introduced into ME2 and the virulence activity of the strains PXO99^{A}, ME2, ME2 (*pthXo1*) and ME2 *(avrXa7*) was measured on the susceptible cultivar IR24 and the

resistant cultivar IRBB5 (*xa5*/*xa5)*. The strains were individually inoculated and lesion lengths were measured 20 days after inoculation. The *pthXo1* mutant strain ME2 was incompatible on both the cultivars tested whereas the wild type strain PXO99^{A} was compatible on both cultivars but the lesion lengths produced on IRBB5 were shorter than on IR24 (Fig. 3-1, column PXO99^{A}). The strain ME2 (*pthXo1*) in which *pthXo1* was reintroduced was also compatible on both cultivars (Fig. 3-1, column ME2/*pthXo1*).The strain with *avrXa7* hardly produced any lesions on IRBB5 but produced lesions similar to *pthXo1* harboring strain on IR24 (Fig. 3-1, column ME2/*avrXa7*). The data from the virulence assay indicate that the broad spectrum resistance phenotype of *xa5* modifies the effectiveness of some effectors in their ability to induce susceptibility. Here, the virulence function of PthXo1 is reduced but phenotypically pathogenic, whereas the AvrXa7-mediated susceptibility is severely reduced to the point of resistance.

AvrXa7 is unable to induce *Os11N3* in *xa5* background

To further verify this finding and to identify the molecular basis of this differential sensitivity in *xa5* background, we looked at the expression of cognate S genes of each effector in presence of *xa5*. IR24 plants having the *Xa5* allele was used as control. The expressions of *Os11N3*, *Os8N3* and a candidate susceptibility gene 1 (*CSG1*-Os04g19960) were analyzed during challenge with strains containing effectors AvrXa7 and PthXo1 and the strain without any major TAL effector ME2. The relative expressions of the genes were quantified using Real Time PCR analysis and by $2^{-\Delta\Delta ct}$ method. The highest relative expression with respect to inoculation with ME2 was observed for *Os8N3* when the IR24 plants were challenged with ME2 (*pthXo1*) (Fig. 3-2. column: IR24/*pthXo1*, blue bar). *Os8N3* expression showed a reduction in *xa5* background when challenged with the same pathogen but the expression level still remained high, nearly 200 fold compared to the expression of *Os8N3* in IRBB5 plants challenged with ME2 (Fig. 3-2. column: BB5/*pthXo1* , blue bar). The *Os11N3* expression was remarkably reduced in IRBB5 plants compared to IR24 when challenged with ME2

*(avrXa7) (*Fig. 3-1, green bars). *CSG1* was expressed in both cultivars upon inoculation with strain containing *avrXa7* and its expression level was slightly reduced in IRBB5 plants though not to the extent as for Os11N3 (Fig. 3-2, red bars). The data from the real time PCR analysis mirrored the observations for the virulence assay for each effector (Fig. 3-1). This provides the evidence that the incompatibility of *avrXa7* containing strains and the expression level of its cognate S gene *Os11N3* are directly correlated.

Suppression of *Os8N3* expression results in incompatibility on IRBB5

The differential sensitivity of AvrXa7-mediated *Os11N3* expression in background with or without *xa5* indicates that either the AvrXa7-*Os11N3* interaction is particularly sensitive to *xa5* or, alternatively, a threshold of S gene expression, regardless of TAL effector, is required for compatible interactions. We noted that *Os8N3* expression is also reduced significantly in the level of expression in the *xa5* plant due to PthXo1, and the virulence of PXO99^{A} is reduced. However, PXO99^{A} remains pathogenic. We, therefore, hypothesized that those TAL effectors promoting the expression of *Os8N3* to a lesser degree than observed for PthXo1 might be incompatible in the *xa5* background since a general reduction in overall TAL effector effectiveness might prevent *Os8N3* from reaching the hypothesized threshold value. To test this hypothesis, two previously derived TAL effectors, PthXo4 and PthXo5, with novel specificity were analyzed and, fortuitously, found to have switched from induction of *Os11N3* to induction of *Os8N3* (Yang *et al*., 2005, Table 3-2).

PthXo4 is the product of an effector gene derived previously by deletion of the 8th to 19th repeats of *avrXa7* (Fig. 3-3.A). PthXo4 has 14 repeats and has lost *Xa7*-mediated avirulence activity but retained the virulence activity on 1R24 (Yang *et al*., 2005). PthXo5 is the result of deletions in the repeat region of *avrXa7*, followed by recombination with an alternate TAL effector gene of PXO99^{A} (Fig. 3-3.A). PthXo5 also retained virulence activity on IR24 but lost the *Xa7*-dependent elicitor activity (Yang *et*

al., 2005). Microarray hybridization analysis indicates that PthXo4 and PthXo5 induce the expression of *Os8N3* in susceptible interaction. The hybridization values for *Os8N3* levels in the presence of PthXo4 and PthXo5 were much lower than that for PthXo1 (Table 3-2). This result indicated *Os8N3* as a possible cognate S gene for effectors PthXo4 and PthXo5.

We therefore scanned the 5′ region of *Os8N3* gene for binding elements that matched the two-amino acid variable residues in the central repetitive region of both PthXo4 and PthXo5 (Boch *et al*., 2009). Potential effector binding elements with one to one correspondence with the two- amino acid variable residues of PthXo4 and PthXo5 was identified at positions -188 to – 202 and -185 to -204 respectively, relative to the start codon (Fig. 3-3.B). Both effector binding elements overlap with the TATA box and is different from the PthXo1 binding element (P1BE) located at position -227 to -250 (results, Chapter 2; Fig 3-3.B).

The strains with PthXo4 and PthXo5 were assayed for their virulence activity on IRBB5 plants to see if the broad resistance phenotype of *xa5* had any effect on PthXo4- and PthXo5-mediated virulence. The strain ME2 was weakly virulent on both cultivars (Fig. 3-4, column ME2). The strains obtained by the introduction of both effectors individually into ME2, ME2 (*pthXo4*) and ME2 (*pthXo5*) were incompatible on IRBB5 plants (Fig. 3-4, Column: ME2 (*pthXo4*) and ME2 (*pthXo5*), red bars).

Discussion

The response of the two strains PXO86 and PXO99^{A}, incompatible and compatible on plants that are homozygous for *xa5*, respectively, is correlated with TAL effector content, and specifically, the presence of AvrXa7 and PthXo1. The effectors in the same background ME2 produced different virulence phenotype on IRBB5. The

introduction of *pthXo1* into ME2 resulted in compatibility on both cultivars, whereas the introduction of the gene for AvrXa7 resulted in compatibility on IR24 and incompatibility on IRBB5 plants. The disease phenotype was correlated with gene expression studies using real time PCR analysis on the cognate S genes for each TAL effector. The virulence targets of both effectors have been determined to be two different members of the N3 family, and the requirement for elevated expression of the cognate S gene for compatibility on any cultivar, in general, was established (Chapter 2, Yang *et al.*, 2006). The expression patterns of the host targets or susceptibility genes on IR24 and IRBB5 revealed that the effector-mediated virulence and the host target gene expression are directly correlated. A 10 fold reduction in the expression of *Os11N3* was observed in IRBB5 compared to IR24. AvrXa7 fails to induce the expression of its virulence target *Os11N3* in *xa5* background but induces the same in IR24 plants which has the wild type allele *Xa5* where as the Os8N3 expression remained over 400 fold in IR24 and nearly 200 fold on IRBB5 compared to strain ME2 without the major effector (Fig. 3-2).

The reasons for reduced cognate gene expression by AvrXa7, PthXo4 and PthXo5 remain to be determined. The binding element of PthXo1 is located close to the TATA box (24 nucleotides upstream) whereas in case of AvrXa7, TATA box overlaps the binding element (chapter 2, Fig 2-9). The subunit TATA binding protein (TBP) of general transcription factor TFIID binds to the TATA box and TFIIA stabilizes this complex. TFIIAγ5 was shown to interact with transcriptional co–activators and associated factors (Kramer *et al.*, 2006). The close proximity of the effector binding element to the TATA box suggests the possibility of interaction between the general transcription factors that form mRNA pre-initiation complex (PIC) and the TAL effector. It is tempting to speculate that the complex formed at A7BE would be different from that of P1BE due to the difference in the distance between the binding element and the TATA box. Although clear evidence is lacking, the interaction between AvrXa7 and PIC may be determined by *Xa5* and the amino acid variation in *xa5* allele may interfere

with this interaction. This hypothesis is further supported by the results from the virulence assay of PthXo4 and PthXo5 on *xa5* plants. The strains with effector PthXo1 which target *Os8N3* is compatible on *xa5* plants whereas strains with PthXo4 and PthXo5 are not. Similar to AvrXa7, the predicted binding elements of both PthXo4 and PthXo5 overlap the TATA box. Based on the evidence from this study a model is proposed to explain the differential gene expression of *Os11N3* in the presence of *xa5* (Fig. 3-5).

Xa5, the wild type allele for *TFIIAγ5*, either enables a stronger interaction between AvrXa7 and PIC, or better activates gene induction, in general, both of which lead to high levels of S gene transcription and susceptibility. In IRBB5 plants, the substitution of V39E in *xa5* may result in weaker interactions. Alternatively, if there is a general reduction in the effector-mediated expression of S genes in *xa5* background, and AvrXa7-mediated expression of *Os11N3* is below the threshold level of expression required for susceptibility, the same effect would be seen on virulence. The observation that *xa5* also interferes with the level of Os8N3 expression as mediated by PthXo1 supports the model of general reduction in TAL effector-mediated expression. It will be interesting to determine the effect of the *xa5* mutation on the activated expression of other host genes.

PXO99^{A} may also have adapted to *xa5* by targeting the alternate gene for TFIIAγ, *OsTFIIAγ1* (Sugio *et al*., 2007). OsTFIIAγ1 is similar to the susceptible allele product *Xa5* and contain valine or conservative substitutions leucine or isoleucine at the 39th amino acid position (Jiang *et al*., 2006). The induction of *OsTFIIAγ1* by PthXo7 may suppress the ineffectiveness of the *xa5* allele. The presence of two genes that encode TFIIAγ but with divergent roles suggests rice may be adapting to prevent the exploitation of essential components of its transcriptional machinery by pathogen (Sugio *et al*., 2007). The effect of *xa5* on the virulence phenotype of *avrXa7* derivatives, PthXo4 and PthXo5 suggest that broad resistance phenotype of *xa5* may alter the effectiveness of other TAL effectors in inducing their target host genes. This is also supported by a recent study

which suggests that a related TAL effector AvrXa27 which induces the expression of cognate R gene *Xa27*, requires the presence of *Xa5* (*OsTFIIAγ5)* for *Xa27* activation (Gu *et al*., 2009). The activation of resistance response in Xa7 plants requires the nuclear localization signal (NLS) and activation domain (AD) of AvrXa7 (Yang *et al*., 2000). Taken together, it is tempting to speculate that resistance gene *Xa7* may also be transcriptionally activated by AvrXa7 and this induction may likely require the wild type *Xa5* allele. In such a scenario, the pyramiding of *Xa7* and *xa5* for durable resistance would actually compromise the effect of *Xa7* mediated resistance. Further analysis of a physical interaction between the TAL effectors and TFIIAγ protein subunits would provide evidence to corroborate this model.

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Table 3-1. Primers used for gene expression study

Primer name	Sequence (5′ to 3′)
Os11N3–QRT-F	TGCCTCCTCATCACCATC
Os11N3-ORT-R	CAGACCCAACCAAGAACC
Os8N3– QRT-F	GGCTCACGGACAGGATGG
Os8N3-QRT-R	GGCTCACGGACAGGATGG
CSG1-QRT-F	CGCATTGCCATGTGTACG
CSG1-QRT-R	TCGCTTAATTCATACTCCTACC
OsTFIIAγ5-F	GGGTTTGCCTGGTATTTTGTTAG
OsTFIIAγ5-R	GTTGCTGCTGTGATATACTTG

Table 3-2. Microarray analysis of expression levels of selected rice genes with TAL effectors PthXo4 and PthXo5.[a]

Probe Set	Gene	ME2[a]	ME2 (pthXo4)	ME2 (pthXo5)	ME2 (pthXo1)	ME2 (avrXa7)
Os.10401.1.S1_s_at	*Os8N3*	39[b]	7660[c]	15043[c]	30529[b]	129[b]
Os.4974.1.S1_x_at	*Os11N3*	311[b]	91.3[b]	103.8[b]	197[b]	8038[b]
Os.12011.1.S1_at	*OsTFIIAγ5*	6189[b]	5988[c]	5900[c,]	6069[b]	5945

a Data is normalized to Os.12011.1.S.1 expression.

b Data is average of two experiments.

c. Data is single hybridization/chip measurement.

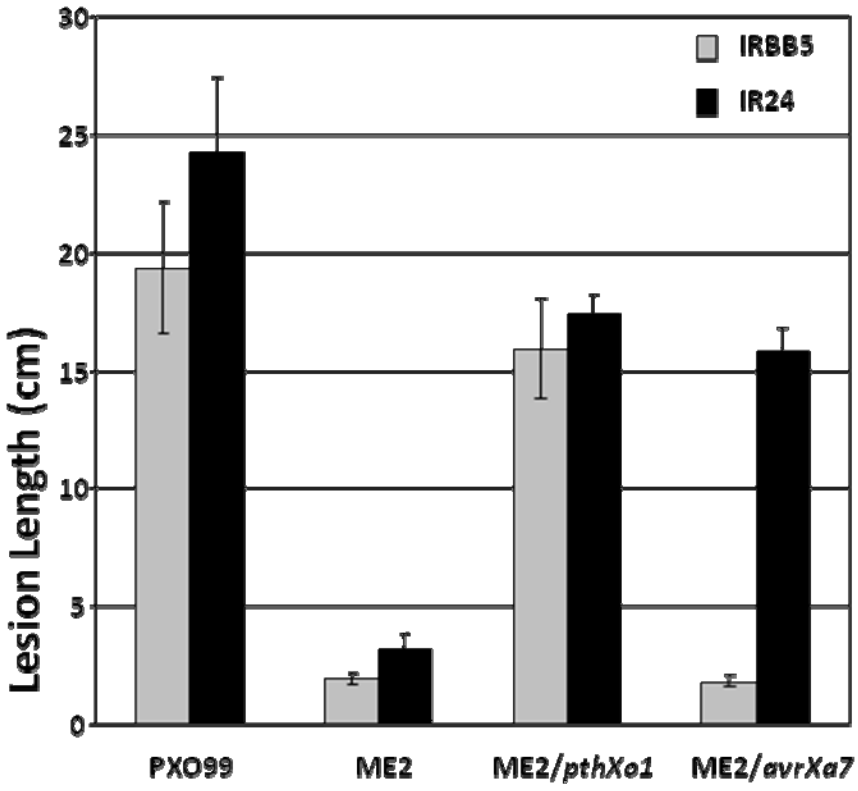


Fig.3-1. AvrXa7 strain is incompatible on IRBB5 plants.

Virulence assay with different strains as indicated was done on seven week old plants by clip inoculation method. The lesion length is the average of seven plants measured 20 days post inoculation. ME2 is a *pthXo1* mutant of PXO99^{A} and ME2 (*pthXo1*) and ME2 (*avrXa7*) are the result of introduction of *pthXo1* and *avrXa7* into ME2.

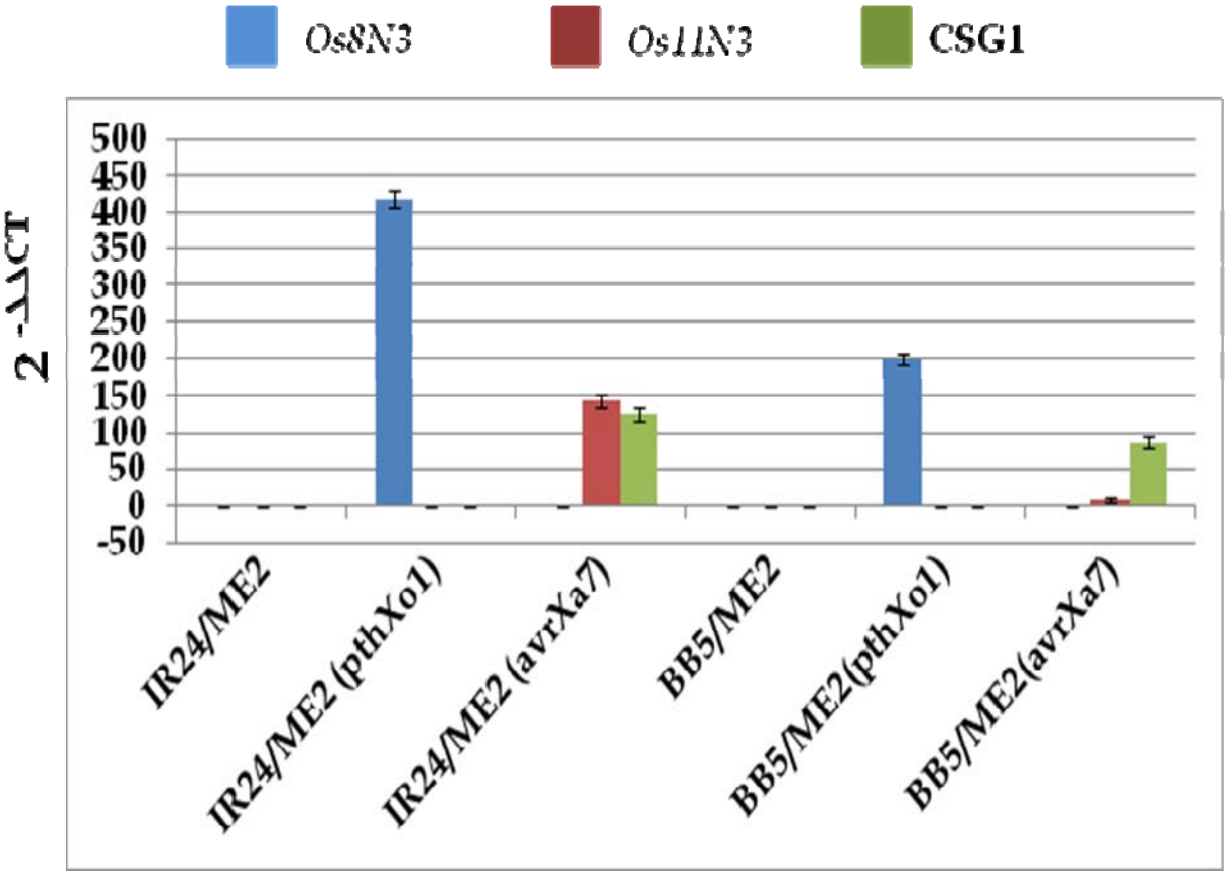
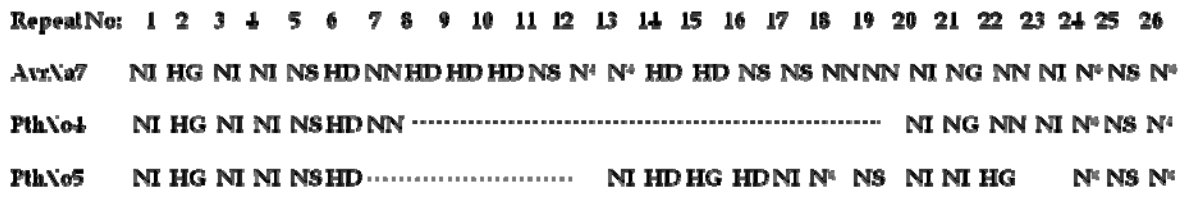


Fig.3-2. The effect of *xa5* (IRBB5 plants) on AvrXa7 mediated expression of *Os11N3*.
Real time PCR analysis using cDNA derived from the samples 24 hrs post inoculation with respective strains as denoted. CSG1 denotes a candidate susceptibility gene (Os04g19960) induced in AvrXa7 specific manner. The relative quantification of gene expression was done using $2^{-\Delta\Delta CT}$ method. The results are the average of two independent experiments.

A



B

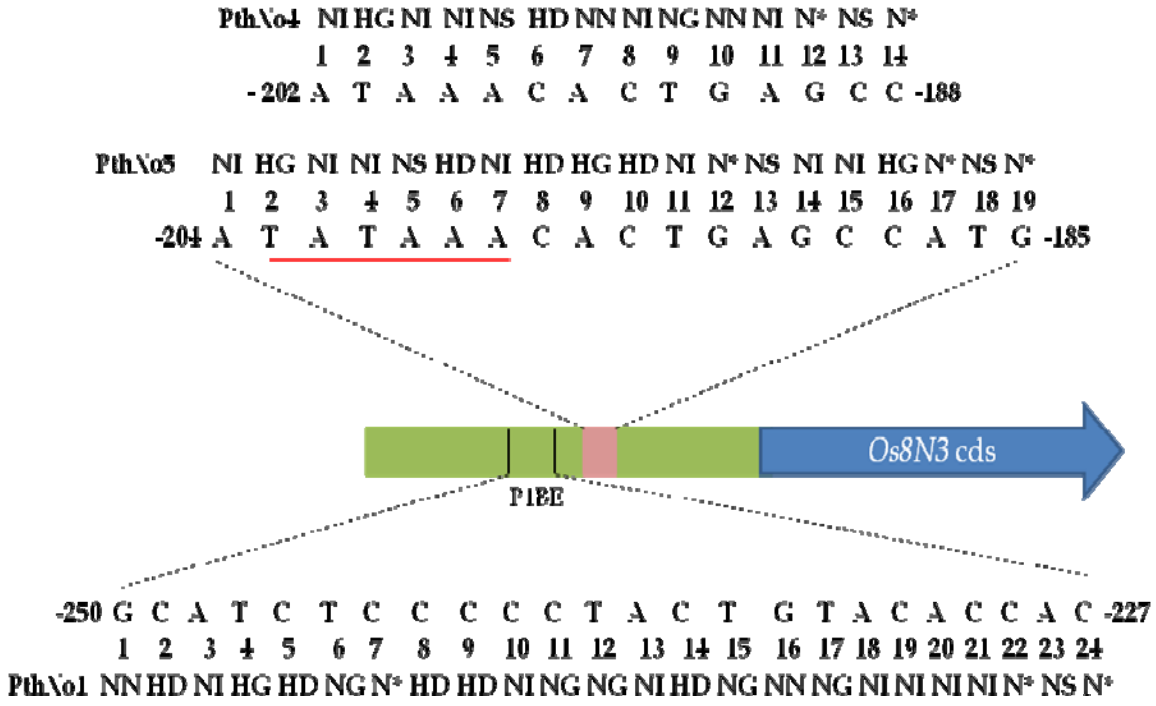


Fig. 3-3. Predicted binding elements of *avrXa7* derivatives PthXo4 and PthXo5

(A). Variable di amino acid residues of the repeat regions of PthXo4 and PthXo5 are aligned with that of AvrXa7. **(B).** The di variable residues of each of the effector PthXo4 and PthXo5 are aligned with putative target DNA elements in the *Os8N3* promoter. The effector binding element of PthXo1 (P1BE) is located close to the TATA box but does not overlap with putative EBE's of PthXo4 and PthXo5.

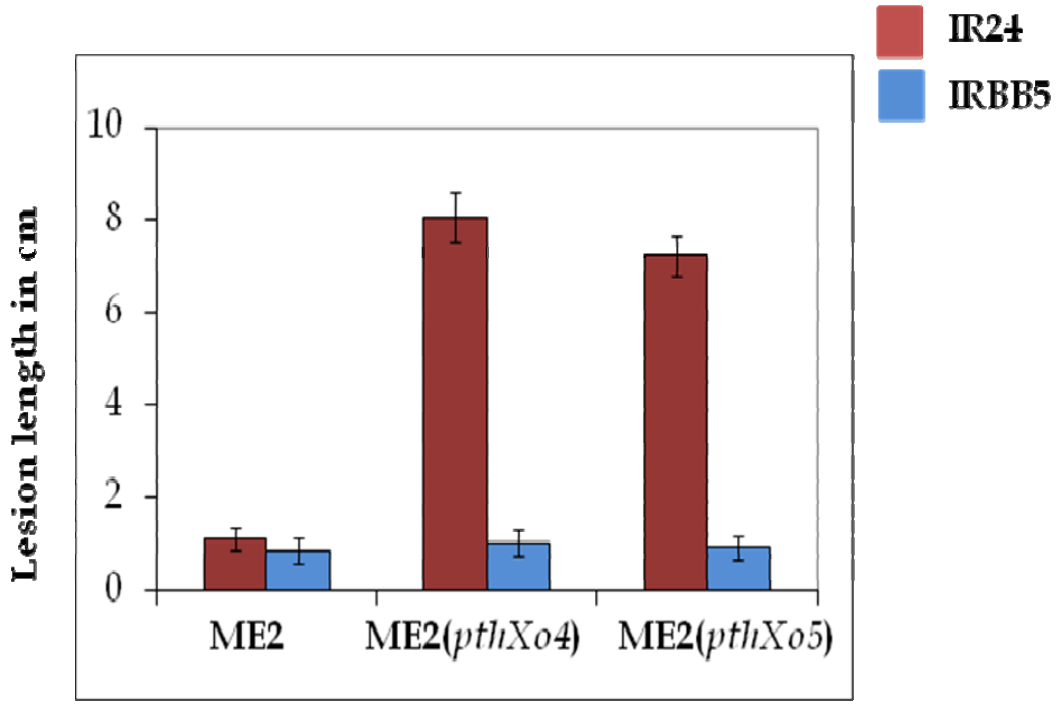


Fig.3-4. Strains with PthXo4 and PthXo5 are incompatible on *xa5* plants.

Four week old plants were inoculated with strains as indicated and lesion lengths measured 12 days post inoculation. Lesion length is the average of seven plants for each assay.

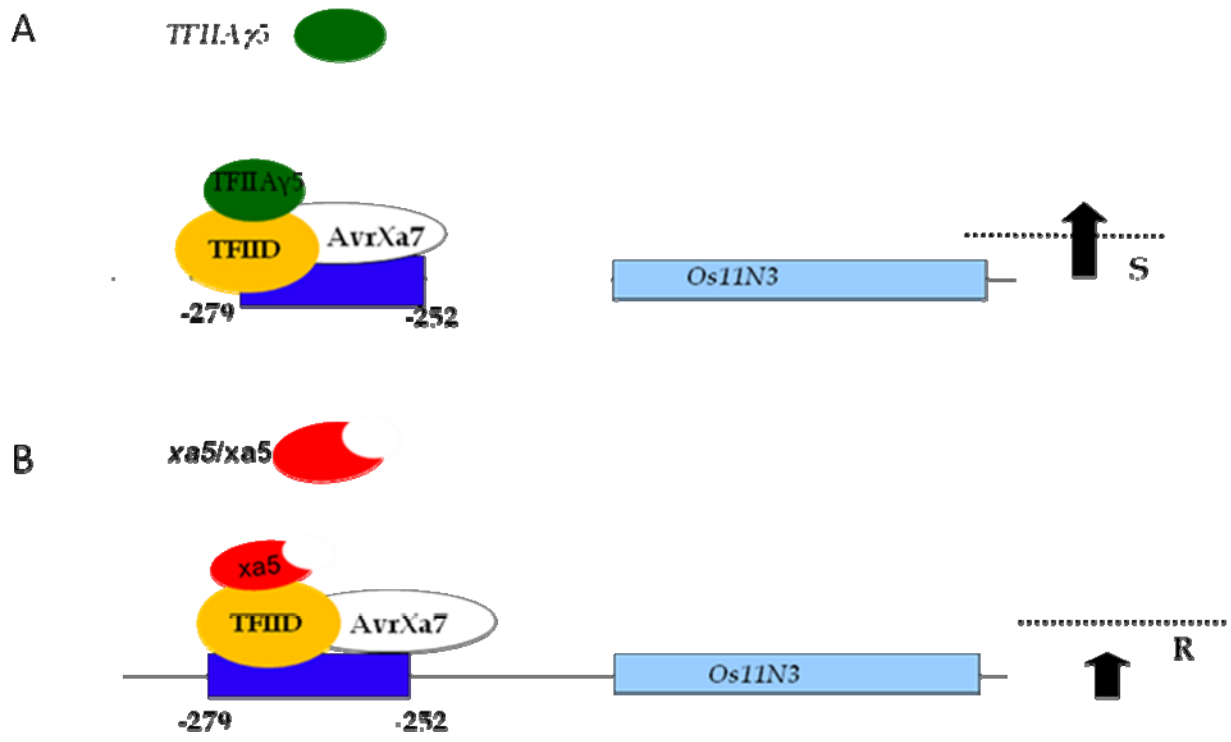


Fig.3-5. Model for AvrXa7 activation of S gene *Os11N3*

(A). TAL effector AvrXa7 binds to the A7BE at position -252 to -279 and interacts with the general transcription factors TFIID and TFIIA that form the pre initiation complex (PIC) at the TATA box. AvrXa7 drives the expression of S gene expression over a threshold level required for inducing the state of susceptibility. **(B).** In the presence of *xa5* allele the interaction between the AvrXa7 and the PIC is weak and results in a weak induction of *Os11N3*. The level of induction does not cross the threshold level required for susceptibility and results in moderate resistance. The dotted line represents the threshold level of gene expression and the black upright arrow indicates the level of host gene expression in each case. The letter R represents resistance and S susceptibility.

Conclusions and Future Prospects

The results presented here demonstrate S gene induction as critical events for successful Xoo colonization of rice, and major TAL effectors AvrXa7, PthXo3 and PthXo1 of Xoo interact with specific N3 genes in a gene-for-gene manner. S gene induction is further corroborated by the identification of a third N3 gene, *Os12aN3* which is induced by effector PthXo2 (Yang and White, unpublished). A promoter variant of *Os12aN3, xa37(t)* confers race specific recessive resistance to bacterial blight in cultivar Nipponbare. Forty Xoo strains from different geographical regions induced either one or more of the three S genes *Os8N3*, *Os11N3* and *Os12aN3* (Yang and White, unpublished). The effectors interact directly with the specific cis elements of S genes and regulate the expression. All three S genes encode membrane proteins, and, although the biochemical functions of these genes are not known, *Os8N3* is essential for pollen development. The phenotype of *Os11N3* T-DNA insertion mutant suggests that this gene may be important for normal plant development. Rice, in adapting to TAL effector function, has adapted to disease by evolving new promoter variants at critical TAL effector targets. These variants of S genes occur in nature as recessive resistance alleles. *xa13* and *xa37(t)* are promoter variants of S genes *Os8N3* and *Os12aN3*. However, the existence of two different effectors PthXo3 and AvrXa7, both of which target the same S gene *Os11N3,* indicate the potential of the effectors to target different binding sites within the same S gene promoter. Though natural recessive resistance at *Os11N3* locus is not known, rice cultivars with recessive resistance gene *xa5* are resistant to strains that depend on AvrXa7 for virulence. *xa5*, which is a V39E variant of γ subunit of transcription factor IIA, reduces the effectiveness of AvrXa7 in inducing the S gene *Os11N3*. The resistance allele *xa5* may be the result of host adaptation to sequester the components of the transcriptional machinery from manipulation by the pathogen.

The fact that the virulence of Xoo strains is dependent on the induced expression of S genes all of which encode N3 protein members is intriguing. Further study into the nature of the proteins encoded by S genes and their cellular function would provide insight into mechanism of susceptibility. This information would also open up new possibilities in engineering durable and broad spectrum resistance.