Host target genes of the Xanthomonas oryzae pv. oryzae type III effectors for bacterial blight in rice

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Host target genes of the Xanthomonas oryzae pv. oryzae type III effectors for bacterial blight in rice

by

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A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY

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NOMENCLATURE

PRR  Pattern Recognition Receptors
TAL  Transcription Activator-like
PTI  PAMP-triggered Immunity
PAMP Pathogen-associated Molecular Patterns
ETS  Effector-triggered Susceptibility
NB-LRR Nucleotide-binding Leucine-rich Repeat
HR  Hypersensitive Response
SAR Systemic Acquired Resistance
ETI  Effector-triggered Immunity
RLCK Receptor-like Cytoplasmic Kinases
MAPK Mitogen-activated Protein Kinase
UPS Ubiquitin-proteasome System
RING Really Interesting New Gene
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ABSTRACT

*Xanthomonas oryzae* pv. *oryzae* is the causal agent of the bacterial blight in rice, which invades the host rice plant through injecting a myriad of effector proteins through a structure called type III secretion system, thereafter, these effectors are also called type III effectors. There are two groups of type III effectors: TAL (Transcription Activator-Like) effectors that specifically bind to the promoter region of particular host genes and regulate host gene transcription and Non-TAL effectors that bind to host gene products and manipulate the host immune system.

In this thesis, I present the work of three projects. First, I, and my colleagues demonstrated two TAL effectors, PthXo3 and AvrXa7 mediate susceptibility in rice by specifically binding to the promoter EBE (Effector-Binding Element) and activating transcription of the host gene Os-11N3 (Chapter 2). Second, I elucidated that another major virulence effector PthXo2 specifically targets EBE in the promoter of another host gene OsSWEET13 and induces susceptibility to bacterial blight only in *indica* rice but not in *japonica* rice, functional analysis confirmed that OsSWEET13 is a sucrose transporter that mediates the sucrose efflux from cytoplasm to apoplasm (Chapter 3). Finally, I investigated the XopZ-mediated virulence function in rice and found that it stabilizes a putative host E3 ubiquitin ligase PBP in the nucleus (Chapter 4). The revelation of modes of action of these bacterial effectors in rice leads to a better mechanistic understanding of host-pathogen interactions and the plant immune system, as well as effective strategies to defeat plant pathogens.
CHAPTER 1. GENERAL INTRODUCTION

Introduction

Plant pathogens and their hosts have been involved in an evolutionary arms race, in which the plant pathogens always try to invade the host plants and nourish themselves, while plants always co-evolve new strategies to defeat invaded pathogens and survive in nature. There are three sub-groups of plant pathogens: Biotrophic plant pathogens colonize living plant organs or tissues and obtain nutrients from living host cells; Necrotrophic plant pathogens extract nutrients from the dead tissues or cells; Hemi-biotrophic plant pathogens live in living tissues for some time and then colonize in dead tissues (Bailey et al., 1992). Simultaneously, plants have evolved two different strategies to against microbial pathogens (Chisholm et al., 2006; Jones and Dangl, 2006). The first strategy is called PAMP-triggered immunity (PTI), in which the pattern recognition receptors (PRRs) at the plasma membrane recognize the pathogen-associated molecular patterns (PAMPs) including bacterial flagellin, peptidoglycan (PGN) and fungal chitin (Jones and Dangl, 2006; Bohm et al., 2014). Recognition of PAMPs elicits a series of immune responses known as plant innate immunity (PTI), or plant basal defense. PTI is usually overcome by specific pathogens which secrete and directly deliver a large number of effector proteins into the host cells via an apparatus called type III secretion system (T3SS), a process also called effector-triggered susceptibility (ETS). To defeat ETS, plants have evolved a myriad of resistance (R) proteins, such as nucleotide-binding leucine-rich
repeat (NB-LRR proteins), which are capable of recognizing pathogen effectors directly or indirectly and activating robust immune responses against specific plant pathogens. These defense response includes local hypersensitive response (HR) and systemic acquired resistance (SAR) mediated by SA accumulation. Thus, the second type of immunity is also called effector-triggered immunity (ETI) (Chisholm et al., 2006; Coll et al., 2011; Feng and Zhou, 2012). Currently, the ‘zigzag’ model provides a conceptual framework to view the plant immune system, and the co-evolution of plant-pathogen interactions (Figure 1; Jones and Dangl, 2006).

HR and SAR are two important immune responses in plants. HR is characterized by rapid cell death at the local infection sites, leading to restriction of growth and spread of plant pathogens. HR is triggered by the recognition of plant pathogens by the plant R proteins, followed by ion flux, oxidative burst, cellular membrane damage, deposition of lignin and callus, cell wall reinforcement and inhibiting spread of the pathogens (Pennell and Lamb, 1997; Pontier et al., 1998; Heath et al., 2000). SAR is a type of broad-spectrum resistance to pathogens followed by signal perception in plants. Salicylic acid (SA), an important messenger of SAR signaling, is generated in the primary infection sites and travels from cell to cell via plasmodesmata or phloem in the plants (Kiefer et al., 2003). Research indicated that accumulation of Pip (piecolic acid) might serve as an early SAR signal upstream of SA after pathogen infection (Navarova et al., 2012). A series of mobile SAR inducers (methyl SA (MeSA), dicarboxylic acid, azelaic acid (AzA), abietane diterpenoid, dihydroabetinal (DA), and glycerol-3-phosphate (G3P) etc.) accumulate to mediate SAR. SA accumulates in the infected tissues then bind to its receptor NPR3 (Nonexpressor of PR gene-3) and NPR4 (Nonexpressor of PR gene-3) and promotes the degradation of NPR1 (Nonexpressor of PR genes-1) oligomer complex mediated by Cullin 3 ubiquitin E3 ligase. The active NPR1 monomers then are
translocated into the nucleus and function as a cofactor for TGA transcription factors that lead to the activation of immediate-early defense-related genes and latter defense-related genes including \textit{PR}-1 (Lebel et al., 1998; Fu et al., 2012). SAR is conserved across different plant species and is effective in against a broad range of plant pathogens (Durrant and Dong, 2004; Kachroo and Robin, 2013).

In nature, plants detect the pathogen invasion through recognizing their particular pathogen-associated molecular patterns (PAMPs) and triggering the plant basal defense. Increasing members of PAMPs have been identified in recent years, including lipopolysaccharide, flagellin, and elongation factor-Tu (EF-Tu) from bacterium; glucans and glycoproteins from oomycetes; chitin from fungal cell wall, etc. (Danna et al., 2011; Zhang et al., 2010; Segonzac et al., 2011). PAMPs are recognized by pattern recognition receptors (PRRs), which lead to the activation of specific defense signals and triggering defense signal transduction from PRR to downstream responsive genes. Great progress has been made in recent years in the signal-transduction pathways underlying PTI. The cell-surface-localized PRRs are classified as RLCKs (receptor-like kinases) (Fritz-Laylin et al., 2005, Zhang et al., 2010), which contain an extracellular domain, a transmembrane domain (TM), and an intercellular kinase domain. The best-understood PRRs are FLS2 (flagellin sensing 2), EFR (EF-Tu Receptor) and CERK1 (LysM receptor kinase) that recognize bacterial flagella peptide flg22 (N-terminal 22-amino-acid Peptide), bacterial elongation factor EF-Tu peptide elf18 (Acetylated N-terminal 18-amino-acid peptide), and fungal cell wall components chitin, respectively (Chinchilla et al., 2007; Zipfel et al., 2006; Zhang et al., 2010; Lu et al., 2010; Segonzac et al., 2011). Receptor-like cytoplasmic kinase BIK1 was identified as a key component integrating signals from FLS, EFR, and CERK1 (Zhang et al., 2010; Lu et al., 2010). Recent studies show that the BIK1 constitutively interacts
with the kinase domain of FLS2 and EFR by forming a preformed immune receptor complex, (Zhang et al., 2010; Lu et al., 2010), which recruits another receptor-like kinase BAK1 during perceiving PAMPs flg22 and elf18. Studies show that BIK1 is phosphorylated by BAK1 at Ser236 and Thr237 in the activation loop, leading to activation of downstream signaling, as loss-of-function of BAK1 significantly reduces flg22- and elf18-triggered MAPK activation (Chinchilla et al., 2007; Heese et al., 2007; Roux et al., 2011). Additionally, another study shows that OsCERK1 initiates immune response to both bacterial PGN and fungal chitin in Arabidopsis (Gimenez-Ibanez et al., 2009), suggesting an overlapping receptor system in rice that perceives PGN and chitin (Liu et al., 2012).

Plant pathogens subvert PTI through secreting and injecting a myriad of type III effector proteins into the plant cells. Considering the pivotal role of PTI in plant defense, key components of plant immune signaling pathways are frequently attacked by various type III effectors. For example, AvrPto and AvrPtoB subvert PTI by directly targeting PAMP receptors FLS2, EFR, and CERK1 in Arabidopsis and tomato (Xiang et al., 2008; Göhre et al., 2008; Zeng et al., 2009). Other reports also suggest that AvrPto and AvrPtoB act at the early stage of immune signaling pathway and function redundantly with other effectors (Kvitko et al., 2009; Cunnac et al., 2011).

It is well-acknowledged that many type III effectors are capable of recognizing specific RLCKs) and suppressing PTI. P. syringae effector AvrPphB, for example, a cysteine protease, was shown to target and cleave BIK1 and several BIK1 family members (PBL1, PBL2), leading to PTI inhibition (Zhang et al., 2010). Another P. syringae effector AvrB interacts with RIPK, a RIN4-interacting RLCK, and triggers AvrB-specified ETI (Liu et al., 2011). In addition, the Xanthomonas campestris pv. vesicatoria (Xcv) effector XopN was identified to interact with the
tomato receptor like kinase TARK1/TFT1 complex, suggesting TARK1 may play a role in PTI signaling pathway (Kim et al., 2009; Taylor et al., 2012). In addition, AvrAC from *X. campestris pv campestris* (Xcc) was also reported to suppress plant immunity by targeting and adding uridine 5'-monophosphate to the conserved phosphorylation sites in BIK1 and RIPK, leading to the inhibition of downstream immune signaling (Feng et al., 2012).

Plant MAPK cascades are composed of a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MKK), and a MAP kinase (MPK) (Ichimura et al., 2002), play pivotal roles in plant immunity signal transduction from upstream receptor to the downstream MAPK substrates. It is shown that MAPK cascades are involved in multiple defense responses, such as the plant hormone signaling, reactive oxygen species (ROS) generation, defense gene activation, phytoalexin biosynthesis, cell wall strengthening and HR. Plant MAPKs are comprised of several groups. Group A MAPKs, including *Arabidopsis* MPK3 and MPK6 and their orthologs in other plant species, are involved in plant responses to stress as well as growth and development (Asai et al., 2002; Pitzschke et al., 2009; Rodriguez et al., 2010; Tena et al., 2011). Group B MAPKs, containing *Arabidopsis* MPK4 and MPK11, are involved in pathogen defense and abiotic stress response (Gao et al., 2008; Rodriguez et al., 2010; Tena et al., 2011; Bethke et al., 2012). Both flg22 and elf18 activate MAPK in *Arabidopsis* upon perception by PRRs. Perception of flg22 activates two MAPK cascades in *Arabidopsis*: the first cascade of MEKK1, MKK4/MKK5, and MPK3/MPK6 is involved in initiating defense signaling (Asai et al., 2002), and the second cascade of MEKK1, MKK1/MKK2, and MPK4 negatively regulates plant immunity (Petersen et al., 2000; Ichimura et al., 2006; Nakagami et al., 2006; Qiu et al., 2008; Suarez-Rodriguez et al., 2007).
Plant MAPK cascades have been implicated in plant defense protein activation and plant hormone signaling. For example, Arabidopsis MPK3/MPK6 or tobacco SIPK/Ntf4/WIPK subsets were also shown to induce many defense-related (PR) genes by modulating hormone biosynthesis or direct phosphorylation of downstream transcription factors (Ishihama et al., 2011; Kim et al., 2004). Report indicated that MPK3 phosphorylates VIP1, a bZIP transcription factor, and re-localizes it from the cytoplasm to the nucleus, leading to activation of PRI gene expression (Djamei et al., 2007). Another MAPK, MPK6, was shown to phosphorylate an ethylene response factor ERF104 and activate defense genes PDF1.2a and PDF1.2b (Bethke et al., 2009). In addition, a recent study also indicates that MPK3/MPK6 phosphorylates and stabilizes another substrate protein ERF6 and mediates the activation of multiple defense-related genes (PDF1.1, PDF1.2a, PDF1.2B, CbiB, and HEL) (Meng et al., 2013). Plant MAPK cascades also play a pivotal role in hormone signaling. Arabidopsis MPK3/MPK6 and tobacco SIPK/WIPK, for example, were indicated to be dispensable for ethylene biosynthesis (Han et al., 2010; Li et al., 2012) and SA-mediated disease resistance (Beckers et al., 2009) upon infection by pathogens. MPK4 cascade, in contrast, was determined to negatively regulate SA signaling based on reverse genetics (Petersen et al., 2000; Kong et al., 2012). JA biosynthesis and signaling pathways are also regulated by MAPKs, one report showed that wounding- and herbivore- induced JA production requires WIPK and SIPK expression (Seo et al., 2007; Wu et al., 2007). Another report shows that MPK6/MKK3 mediates the suppression of JIN1/MYC2 expression and root growth by JA, while MPK4 positively regulate JA-inducible responses (Petersen et al., 2000). MAPK cascades are also involved in hypersensitive response (HR) in plant cells, direct evidence is constitutive activation of SIPK, Ntf4 and WIPK by plant pathogens.
will promote ROX generation and HR signaling in plants (Zhang et al., 2000; Liu et al., 2007; Ren et al., 2006; Yang et al., 2001).

MAPKs can be activated upon recognition of pathogen effectors by R proteins in plants, a process called effector-triggered MAPK activation (Chisholm et al., 2006, Dodds et al., 2010). One striking example is the fungal Avr9 effector activates SIPK and WIPK upon its recognition by the cognate Cf-9 resistance protein in tobacco (Romeis et al., 1999). Another example is the recognition of bacterial effectors AvrPto or AvrPtoB by the intracellular Ser/Thr protein kinase Pto in tomato, leading to activation of R protein Prf and initiation of ETI responses (Oh et al., 2011). In addition, some R proteins also recognize pathogen invasion by detecting the MAP kinase cascade, SUMM2 (NB-LRR protein), for example, has been recently shown to be activated upon disruption of MEKK1-MKK1/MKK2-MPK4 cascade caused by pathogen effector HopAI1 (Zhang et al., 2012).

Nevertheless, MAPK cascades are host targets for many effectors that suppress immune signaling pathways. The study on *P. syringae* effectors HopAI1 suggested it is related to MPK3 and MPK6 and inactivates their kinase activities, as well as suppresses MAPK-mediated PTI (Zhang et al., 2007). Another report on *P. syringae* effector HopF2 demonstrated its role in ADP-ribosylating MKK5 and blocking its kinase activity (Wang et al., 2010). Recent study also demonstrated that another *P. syringae* effector, AvrB, in contrast, specifically induces MPK4 phosphorylation and modulates JA responses (Cui et al., 2010). Additionally, Xoo effector XopYxo19 (Xoo1488) was recently reported to strongly suppress host PTI in rice by targeting OsRLCK185, which functions as a rice receptor-like cytoplasmic kinase that interacts with OsCERK1. Study shows that XopYxo19 (Xoo1488) suppresses OsCERK1-mediated
phosphorylation of OsRLCK185 and inhibits immune response including MAP kinase activation (Yamaguchi et al., 2013a; Yamaguchi et al., 2013b).

Some other type III effectors employ post-translational modification of host proteins to manipulate host defense system. For example, HopU1 ADP-ribosylates GRP7 on Arg^{47} and Arg^{49}, which abolishes the RNA-binding activity mediated by Arg^{49} (Fu et al., 2007). Similarly, HopF2 ADP-ribosylates MKK5 at Arg313 and inhibits its kinase activity, a recent study also suggests that HopF2 also ADP-ribosylates RIN4 and blocks RPS2-specified ETI (Wilton et al., 2010). Xcv effector XopX has been shown to be a virulence factor that targets basic innate immune system and contributes to the virulence of Xcv on host pepper and tomato plants (Metz et al., 2005). Recently, XopX has also been shown to promote ethylene production and plant cell death (PCD) in tomato and in *Nicotiana benthamiana* (Stork et al., 2015).

Effector-triggered susceptibility (ETS) was overcome by a group of resistance (R) proteins that are co-evolved in plants. R proteins, especially NB-LRR type R proteins, recognize effectors from diverse plant pathogens and trigger defense response. Plant NB-LRR type R proteins include two groups based on their N-terminal domains: TIR (Toll and interleukin-1-like receptor)-NB-LRR type R protein and CC (coiled-coil domain)-NB-LRR type R protein (Meyers et al., 2003; Marone et al., 2013). The N-terminal TIR and CC domain are responsible for protein-protein interactions with adaptor proteins. NB domain contains several conserved motifs (P-loop, kinase-2 and Gly-Leu-Pro-Leu motifs) and are required for ATP and GTP binding and hydrolysis (Tameling et al., 2002). The C-terminal LRR motif shows different functions, including modulating activation and acting as platform for upstream activation (Tameling et al., 2002; Belkhadir et al., 2004). These distinct domains are responsible for specific recognition of pathogen invasion and initiation of defense responses.
Plant immune receptors monitor pathogen effector targets through diverse subcellular localizations, such as plasma membrane, nucleus, cytoplasm, endoplasmic reticulum, and chloroplast. Interestingly, some immune receptors even show nuclear-cytoplasmic localizations, including RPS4, RRS1, Rx, N and MLA10. For example, RPS4 is predominately localized to the endomembranes before activation, but translocated to the nucleus to activate EDS1 (Enhanced Disease Susceptibility-1) -mediated immune response (Wirthmueller et al., 2007; Heidrich et al., 2011). It has been shown that RPS4 mediates the recognition of EDS1-AvrRps4 complex (Bhattacharjee et al., 2011; Heidrich et al., 2011), and the recognition of nuclear-localized AvrRps4 leads to suppression of bacterial growth, but recognition in cytoplasm mediates HR (Heidrich et al., 2011). Another receptor RRS, correlates with RPS4 in defense response against fungal and bacterial pathogens (Narusaka et al., 2009). CC-NB-LRR type protein MLA10 has been shown to trigger HR when localized in cytoplasm, whereas to initiate defense response against plant pathogens when associated with WRKY transcription factors in the nucleus (Bai et al., 2012). Similarly, potato Rx protein has also been shown to mediate HR and transcriptional reprogramming through nuclear-cytoplasmic partitioning, as Rx1-emdiated resistance response is activated in cytoplasm that is determined by LRR domain and attenuated it in nucleus that is mediated by CC domain (Slootweg et al., 2010).

The ETI responses are usually triggered upon the recognition of cognate pathogen effectors by plant R proteins directly or indirectly. One of the most striking examples of direct recognition is type Ⅲ effector PopP2, which physically interacts with R protein RRS1-R, conferring resistance to bacterial wilt (Deslandes et al., 2003). Another example is AvrL567 protein from flax rust fungus, which is recognized by Flax R proteins L5, L6, and L7, and the high variable amino acid sequence differences directly affect the R protein and Avr protein
interaction (Dodds et al., 2006). In addition, *Arabidopsis thaliana* RPP1 (RECOGNITION OF PERONOSPORA PARASITICA1) resistance protein was indicated to directly recognize its cognate effector ATR1 (ARABIDOPSIS THALIANA RECOGNIZED1) through its leucine-rich repeat (LRR) domain and trigger immune response (Krasileva et al., 2010).

On the other hand, most R proteins recognize the invading effector proteins by detecting their modification on host target proteins (indirect interaction), as described by the guard hypothesis (Van der Biezen and Jones, 1998). The most striking example of indirect interaction is RIN4 (RPM1 interacting protein 4), which is associated with two NB-LRR proteins RPM1 and RPS2 (Mackey et al., 2002), and has been shown to be targeted by several effector proteins. RIN4 undergoes different modifications when interacting with different effectors. The *P. syringae* effectors AvrB and AvrRpm1, for example, promote phosphorylation of RIN4 and activate RPM1-mediated immune response (Chung et al., 2011; Liu et al., 2011). AvrRpt2, another *P. syringae* effector has been demonstrated to activate RPS2-mediated immunity by cleavage of RIN4 through its cysteine protease activity (Axtell and Staskawicz, 2003; Mackey et al., 2003). Interestingly, RIPK1 (RPM1-induced protein kinase 1) has also been found to partially promote RIN4 phosphorylation (Liu et al., 2011).

The ETI response partially overlaps with PTI in their signaling pathways, such as activation of downstream MAPK cascade and activation of WRKY transcription factors, which lead to transcriptional activation of a myriad of PR genes and biosynthesis of hormone: SA, JA, ET, as well as cell wall strengthening, production of various antimicrobial compounds, and lignifications etc. The production of SA in the infected tissues leads to the localized HR (Pennell and Lamb, 1997) and SAR (Durrant and Dong, 2004).
As the important players in suppressing the plant immune response, type III effectors are pivotal to mediating bacterial pathogenesis and triggering plant immune response. Therefore, elucidation of molecular mechanisms underlying type III effector-mediated virulence function is of great importance to demonstrate host-pathogen co-evolution and plant immunity against pathogens, as well to identify new plant immune components and immunity pathways.

**Dissertation Organization**

This dissertation is organized in 5 chapters as described below:

**Chapter 1** presents a literature review of present advancement of plant-pathogen interaction and plant immune system.

**Chapter 2** presents a study of *Rice xa13 Recessive Resistance to Bacterial Blight Is Defeated by Induction of the Disease Susceptibility Gene Os-11N3* that has been published in *The Plant Cell* in 2010 by Ginny Antony*, Junhui Zhou*, Sheng Huang, Ting Li, Bo Liu, Frank White and Bing Yang. In this collaborative work, my major contribution is analysis of the interaction between the promoter region of *Os11-N3* and type III effectors AvrXa7 and PthXo3, determining the gene induction of *Os11-N3* by AvrXa7 and PthXo3 in wild type and mutant rice plants, performing the virulence assays in rice plants, as well as examining the promoter-specific GUS activity induction with the *Os-8N3* and *Os-11N3* promoters.

**Chapter 3** presents a study of *Gene Targeting by the TAL Effector PthXo2 Reveals Cryptic Resistance Gene for Bacterial Blight of Rice* that has been published in *The Plant Cell*...
Journal in 2015 by Junhui Zhou, Zhao Peng, Juying Long, Davide Sosso, Bo Liu, Joon-Seob Eom, Sheng Huang, Sanzhen Liu, Casiana Vera Cruz, Wolf B. Frommer, Frank F. White and Bing Yang. I tested the virulence function of PthXo2 that is associated with host gene OsSWEET13 in indica rice species, assayed the sucrose transport activity in N. benthamiana leaves, examined the nucleotide polymorphism in EBE region of japonica and indica rice, examined the disease phenotype that are induced by modified version of pthXo2, as well as isolated the new PthXo2 homologs (PthXo2-1) from PXO163 and PXO339 that shows the similar function to PthXo2 in rice.


Chapter 4 presents a study of Xanthomonas Effector XopZ Targets a Host E3 Ligase and Subverts Immunity in Rice that is in preparation for submission by Junhui Zhou, Huanbin Zhou, Bo Liu, Shinian Char and Bing Yang. I contributed most of the studies except the Co-IP assay of XopZ-PBP interactions, the PXO99△VN mutant and PXO99△VNZ mutant construction. These works are contributed by Dr. Huanbin Zhou.


Chapter 5 presents the general conclusions of this dissertation and gives a short discussion on plant-pathogen interactions.
References


CHAPTER 2 RICE XA13 RECESSIVE RESISTANCE TO BACTERIAL BLIGHT IS DEFEATED BY INDUCTION OF THE DISEASE SUSCEPTIBILITY GENE OS-11N3

A paper published in *The Plant Cell*

Ginny Antony*, Junhui Zhou*, Sheng Huang, Ting Li, Bo Liu, Frank White and Bing Yang

* These authors contribute equally to this work

Abstract

The rice (*Oryza sativa*) gene *xa13* is a recessive resistance allele of Os-8N3, a member of the *NODULIN3 (N3)* gene family, located on rice chromosome 8. Os-8N3 is a susceptibility (*S*) gene for *Xanthomonas oryzae* pv. *oryzae*, the causal agent of bacterial blight, and the recessive allele is defeated by strains of the pathogen producing any one of the type III effectors AvrXa7, PthXo2, or PthXo3, which are all members of the transcription activator-like (TAL) effector family. Both AvrXa7 and PthXo3 induce the expression of a second member of the N3 gene family, here named Os-11N3. Insertional mutagenesis or RNA-mediated silencing of Os-11N3 resulted in plants with loss of susceptibility specifically to strains of *X. oryzae* pv *oryzae* dependent on AvrXa7 or PthXo3 for virulence. We further show that AvrXa7 drives expression of Os-11N3 and that AvrXa7 interacts and binds specifically to an effector binding element within the Os-11N3 promoter, lending support to the predictive models for TAL effector binding specificity. The result indicates that variations in the TAL effector repetitive domains are driven by selection to overcome both dominant and recessive forms of resistance to bacterial blight in rice. The finding that Os-8N3 and Os-11N3 encode closely related proteins also provides evidence that N3 proteins have a specific function in facilitating bacterial blight disease.
Introduction

Plants have evolved mechanisms that protect against pathogen effector-mediated susceptibility of which the resistance ($R$) genes are an important component (Chisholm et al., 2006; Ellis et al., 2009). $R$ gene products have been proposed to guard important defense signaling complexes that are targeted by virulence effectors by sensing perturbations upon the interaction of the complex with a pathogen virulence effector or, alternatively, by acting as target decoys, intercepting effectors upon their entry into the host (Hogenhout et al., 2009). In either event, perception triggers rapid defense responses that are typically associated with localized cell death, commonly known as a hypersensitive reaction. Bacterial pathogens can evade or defeat effector-triggered resistance by a variety of genetic changes, which include alterations in effector structure, resulting in loss of $R$ gene–mediated resistance; outright loss or inactivation of cognate effector genes and loss of effector recognition; and acquisition of new effector genes that mediate suppression of $R$ gene–mediated resistance. Recent evidence in rice (Oryza sativa) and wheat (Triticum aestivum) indicate that host resistance to disease also involves genetic variability in dominant traits that are targeted by virulence effectors, which we refer to here as susceptibility ($S$) genes and are commonly revealed as recessive resistance genes (Liu et al., 2009; White and Yang, 2009). In contrast with the numerous examples of dominant $R$ gene–mediated resistance, few genetic variations in effector-triggered susceptibility have been characterized (Deslandes et al., 2002; Piffanelli et al., 2004; Iyer-Pascuzzi and McCouch, 2007; White and Yang, 2009).

The recessive $R$ gene $xa13$ occurs as a series of natural alleles of the $S$ gene Os-8N3, whose expression is induced by strains of Xanthomonas oryzae pv oryzae carrying the gene $pthXo1$, which encodes the transcription activator-like (TAL) effector PthXo1 (Chu et al., 2006;
Yang et al., 2006; Yuan et al., 2009). The \textit{xa13} alleles are unresponsive to PthXo1, and plants with \textit{xa13} are resistant to strains of the pathogen that rely solely on PthXo1 as the essential effector for virulence (Yang et al., 2006). PthXo1 is secreted via the bacterial type III secretion system and is a member of the TAL effector family, which consists of a large number of closely related nuclear-localized DNA binding proteins (White et al., 2009). TAL effectors mediate host gene expression and function as transcription factors within the host cells (Kay et al., 2007). Individual TAL effectors induced expression of specific host genes, and differences in host gene specificity are determined by the repetitive central region of each effector, which consists of direct repeats of 34– to 35–amino acid residues. The repetitive regions have been proposed to determine the sequence specificity within the promoters of the affected genes (Boch et al., 2009). PthXo1 has 23.5 repeats and is encoded by one of 19 TAL effector genes in the genome of \textit{X. oryzae pv oryzae} strain PXO99\textsuperscript{A} (Yang and White, 2004; Salzberg et al., 2008). PthXo1 is the only effector of PXO99\textsuperscript{A} that is capable of Os-8N3 induction, and mutants of \textit{pthXo1} in PXO99\textsuperscript{A} are severely reduced in virulence on all otherwise susceptible rice cultivars (Yang and White, 2004).

\textit{xa13}-mediated resistance is race-specific resistance, meaning that \textit{xa13}-mediated resistance has been defeated by some strains of \textit{X. oryzae pv oryzae} (Lee et al., 2003; Chu et al., 2006). How \textit{xa13} is defeated is unknown. In this regard, PthXo1 is one of four known TAL effectors from different strains of \textit{X. oryzae pv oryzae} that have major contributions to virulence, which we refer to as major TAL effectors (Yang and White, 2004). The major TAL effectors also include AvrXa7, PthXo2, and PthXo3, and each contain unique repetitive regions. AvrXa7 further differs among the four as the cognate effector for the dominant \textit{R} gene \textit{Xa7}. Furthermore,
the three alternate major TAL effectors were identified in races of the pathogen that are compatible on rice lines containing \textit{xa13}, and we previously demonstrated that introduction of the gene \textit{avrXa7} into PXO99\textsuperscript{A} was sufficient to overcome \textit{xa13}-mediated resistance (Yang et al., 2006). Here, we analyzed the ability of additional major TAL effectors to circumvent \textit{xa13}-mediated resistance and attempted to identify induced host genes that circumvent the need for Os-8\textit{N3} function in susceptibility to bacterial blight disease of rice.

Results

Alternate Major TAL Effectors \textit{AvrXa7, PthXo2, and PthXo3 Do Not Induce Os-8N3}

To test whether individual major TAL genes other than \textit{pthXo1} determined compatibility of \textit{X. oryzae pv oryzae} in plants with \textit{xa13}, derivatives of PXO99\textsuperscript{A}ME2, a \textit{pthXo1} mutant derivative of PXO99\textsuperscript{A} (hereafter, ME2), containing the vector pHM1 alone or the vector with one of the major TAL effector genes \textit{avrXa7}, \textit{pthXo2}, or \textit{pthXo3} were tested for virulence on IRBB13, a rice line that is derived from the recurrent susceptible parental line IR24 and homozygous for \textit{xa13}. The allele of \textit{xa13} in rice line IRBB13 has a 253-bp insertion/38-bp deletion within the promoter region of Os-8\textit{N3} in comparison to IR24 (Chu et al., 2006; Yang et al., 2006). (Strains and plasmids are provided in Supplemental Table 1 online.) ME2 itself fails to form lesions on either IRBB13 or IR24 due to the lack of at least one major TAL effector gene for virulence (Figure 1A, treatment 1). Reintroduction of \textit{pthXo1} to ME2 restored virulence on IR24 (Figure 1A, treatment 2, white column) but not on IRBB13 due to the inability of PthXo1 to induce Os-8\textit{N3} in this line (Figure 1A, treatment 2, black column; Yang et. al., 2006). Addition of \textit{avrXa7}, \textit{pthXo2}, or \textit{pthXo3} to ME2 restored virulence on both IR24 and IRBB13 (Figure 1A, treatments 3 to 5, respectively). The strains were then tested for the ability to induce
Os-8N3 in either IRBB13 or IR24 as measured by quantitative RT-PCR (qRT-PCR) and RNA gel blot hybridization (Figure 2B). Os-8N3 expression was 168-fold greater in IR 24 after inoculation with ME2(pthXo1) compared with ME2 (Figure 1B, treatment 2, white column), and no increase in Os-8N3 expression was detected in IRBB13 with ME2(pthXo1) (Figure 1B, treatment 2, black column) or any combination of rice lines with strains with the alternate TAL effectors (Figure 1B, treatments 3 to 5).

AvrXa7 and PthXo3 Induce Os-11N3, Another Member of the N3 Gene Family

Os-8N3 is one of 17 N3 genes in rice (Yang et al., 2006), and the ability of the alternate TAL effectors to promote the expression of other members of the N3 gene family in infected rice leaves was examined. cDNA was prepared from leaf mRNA after individual inoculations of cultivar Nipponbare with strains ME2, ME2(avrXa7), ME2(pthXo2), and ME2(pthXo3) and subjected to qRT-PCR using gene-specific primers derived from the 3′-untranslated region (UTR) sequences of N3 gene family members, starting with the members most similar to Os-8N3 (Yang et al., 2006). The gene Os11g31190 (hereafter, Os-11N3) was induced both in an AvrXa7- and PthXo3-dependent manner (Figure 2A, treatments 6 and 7). Control cDNA samples were also prepared from uninoculated leaves, mock-inoculated (water) leaves, and leaves inoculated with bacteria deficient in type III secretion (ME7), ME2(pthXo1), and ME2(pthXo2). All failed to induce Os-11N3 (Figure 2A, treatments 1 to 5). ME2(pthXo3) induced Os-11N3 an average of 71-fold over uninoculated plants (Figure 2A, treatment 6), while ME2(avrXa7) induced Os-11N3 52-fold over ME2 (Figure 2A, treatment 7).

Os-11N3 is represented in databases by a 1494-base full-length cDNA (National Center for Biotechnology Information accession number AK101913), has four introns, and a predicted
BLAST analysis was performed with the predicted protein product of Os11N3 (Os-11N3), and 18 of the most similar proteins from monocotyledonous species and three sequences of the most similar proteins from representative dicotyledonous species were subjected to phylogenetic analysis (Figure 3). While closely related to Os-8N3, Os-11N3 is a member of a distinct clade of N3 proteins (clade II) that are separated from the Os-8N3 clade (clade I) prior to the divergence of dicots and monocots as some members from Arabidopsis thaliana (At5g23660), pepper (Capsicum annuum; CaUPA16), and soybean (Glycine max; GmABT17358) are more similar to Os-11N3 (Figure 3). Os-11N3 is more closely related to another clade represented by the rice N3 gene Os12g0476200. The separation of Os-11N3 from Os12g0476200 occurred prior to the divergence of rice, sorghum (Sorghum bicolor), and maize (Zea mays; Figure 3). The separation of Os-S11N3 from the other members within rice indicates a possible specialization of this protein in plant development or environmental responses.

**Loss or Suppression of Os-11N3 Expression Results in Loss of TAL Effector-Specific Susceptibility in Rice**

The T-DNA insertion event PFG_3D-03008 was previously reported to have occurred within the first intron of Os-11N3 in rice cultivar Hwayoung (Jeong et al., 2006). The line containing the insertion was genotyped using primers that were derived from sequence on either side of the insertion within the wild-type locus (Figure 4A, black and red arrows) and a third derived from within the T-DNA element (Figure 4A, blue arrow). One combination of primers amplified a 440-bp product from just within the right T-DNA border to the right of the insertion site (Figure 4A, PCR1), and the second set amplified a 563-bp product across the wild-type locus.
(Figure 4A, PCR2). A heterozygous plant was self-crossed, and the progeny were genotyped for the presence and absence of the T-DNA. All three PCR patterns, indicative of homozygous T-DNA insertion, homozygous wild type, and heterozygous, were observed (Figure 4B, examples in lanes 1, 5, and 6, respectively). All plants homozygous for the insertion, as indicated by the single PCR product specific for the T-DNA/Os-11N3 boundary (Figure 2B, lanes 1 to 4 and 8), were resistant to ME2(\textit{avrXa7}) and ME2(\textit{pthXo3}) (Figure 4B, phenotype R). Heterozygous plants (Figure 4B, lanes 6, 7, and 9), homozygous wild-type plants (Figure 4B, lanes 5 and 10), and the parent preinsertion line (Figure 4B, lane 11) were susceptible to infection by ME2(\textit{avrXa7}) (Figure 4B, phenotype S). Average lesion length measurements were obtained from six homozygous insertion plants and six heterozygous progeny with the PF_3D-00308 event after inoculation with either ME2(\textit{pthXo1}), ME2(\textit{avrXa7}), or ME2(\textit{pthXo3}) (Figure 4C). Heterozygous and homozygous plants were susceptible to infection by ME2(\textit{pthXo1}) (Figure 4C, treatment 1), while only heterozygous plants were susceptible to ME2(\textit{avrXa7}) and ME2(\textit{pthXo3}) (Figure 4C, treatments 2 and 3, respectively). Homozygous plants have normal-appearing lesion phenotypes with ME2(\textit{pthXo1}) (Figure 4D, leaf 1) and almost no lesion phenotypes with ME2(\textit{avrXa7}) or ME2(\textit{pthXo3}) (Figure 4D, leaves 2 and 3, respectively). Although normal in appearance, plants homozygous for T-DNA insertion are small seeded (Figure 4D, top right panel) and delayed in growth, requiring ~30 more days to reach the size of 14-d-old heterozygous (normal) plants (Figure 4D, bottom right panel). The average weight of 25 seeds from heterozygous plants was $571.48 \pm 26.39$ mg and that of homozygous plants $313.02 \pm 11.62$ mg.

The requirement for Os-11N3 in AvrXa7- and PthXo3-mediated virulence was also assessed by RNA-mediated gene silencing (RNAi). Transgenic rice plants were generated that
expressed a unique 341-base portion of the 3′-UTR of Os-11N3 as a small double-stranded RNA to initiate silencing of the full transcript. Two plants were selected that showed high expression of the double-stranded RNA construct alone based on qRT-PCR of the 341-bp 3′-UTR fragment (Figure 5A, columns 1 and 2, black) in comparison to the plant with only vector sequences (Figure 5A, column V, black). Both plants failed to show induction of Os-11N3 upon inoculation with ME2(avrXa7) based on amplification of the 5′-UTR region (Figure 5A, columns 1 and 2, white). Induced Os-11N3 expression was observed in the infected control plants (Figure 5A, column V, white). Quantitative measurements based on lesion lengths following inoculation of progeny plants indicated that the control plants were equally susceptible to ME2(avrXa7) (Figure 5B, column V, black) and ME2(pthXo1) (Figure 5B, column V, white). The progeny from the RNAi lines 1 and 2, however, had short lesion lengths upon infection by ME2(avrXa7), indicative of loss of the susceptibility phenotype (Figure 5B, columns 1 and 2, black), while remaining fully susceptible to ME2(pthXo1) (Figure 5B, columns 1 and 2, white). RNAi plants were scored visually as resistant to infection by ME2(avrXa7) and ME2(pthXo3), while control plants were susceptible (Figure 5C, showing phenotype of line 1 only). The RNAi lines remained susceptible to ME2(pthXo1) (Figure 5C).

**Os-11N3 Promoter Contains Candidate Effector Binding Elements for AvrXa7 and PthXo3**

As members of the TAL effector family, PthXo1, PthXo3, and AvrXa7 are predicted to bind effector binding elements (EBEs) in gene promoters and drive expression of their respective S genes. The consensus EBEs for PthXo1, PthXo3, and AvrXa7, as previously noted, was predicted from the order of the 12th and 13th repeat residues of the protein (Figure 6A; Boch et al., 2009). The promoter regions of Os-11N3 and Os-8N3 are distinct in comparison to each other.
The candidate site in the Os-8N3 promoter region lies upstream of the TATA box (Figure 6B). The element overlaps the 243-bp insertion/deletion within the Os-8N3 promoter that occurs in the IRBB13 allele of xa13 (Figure 6B). The predicted promoter region for Os-11N3 is based on the first base of the full-length cDNA. A consensus TATA box lies 25 bases upstream of the predicted transcription start site (Figure 6C). The start sites of transcription of Os-11N3 during infection with ME2(avrXa7) or ME2 without avrXa7 were analyzed by 5′ rapid amplification of cDNA ends (5′-RACE-PCR). Despite low expression of the locus in normal plant leaves, six 5′-RACE cDNA were obtained from ME2-treated tissue, and three of six began at the A located 31 residues from the TATA box and the same base as predicted from the full-length cDNA AK101913 (Figure 6C). The remaining three cDNAs have unique start sites (see Supplemental Figure 2 online). None of 13 5′-RACE cDNAs obtained from ME2(avrXa7)-treated tissue started at A31. Seven of 13 cDNAs, and the largest class, started at the G located 65 residues downstream from the TATA box (Figure 6C), indicating that AvrXa7 may alter the normal transcription start site of Os-11N3. The remaining six cDNAs fell into five different classes of start sites (see Supplemental Figure 2 online). (An alignment of all cDNAs is presented in Supplemental Figure 2 online.) A consensus EBE for AvrXa7 lies in the Os-11N3 promoter within the EBE for PthXo3, starting at the second A base and ending at the second to last base of the EBE for PthXo3, and both encompass the TATA box (Figure 6C).

**PthXo1 and AvrXa7 Drive Promoter-Specific Expression of Os-8N3 and Os-11N3**

*Agrobacterium tumefaciens*–mediated transient expression was used to determine if PthXo1, AvrXa7, and PthXo3 can drive *S* gene promoter-specific expression of a reporter gene in *Nicotiana benthamiana* leaves and whether the consensus EBEs discriminate between the two
effectors. Promoter proximal sequences of Os-8N3 and Os-11N3 were fused at the start codons to the coding sequence of the *uidA* gene (β-glucuronidase [GUS]). Four promoter constructs were prepared for Os-11N3, including the wild type, mutant, and two hybrid constructs where the EBE for PthXo1 in the Os-8N3 promoter was replaced with the wild type or mutant EBE for AvrXa7 and PthXo3 (Figure 7A). Three additional Os-8N3 promoter constructs were prepared, including the wild-type sequence, a mutant version, and the version found in the *xa13* allele in IRBB13 (Figure 7A). Expression was monitored by histological staining for GUS activity (Figure 7B), and average GUS activity was measured using the fluorescence substrate 4-methylumbelliferyl-β-d-glucuronide (MUG) from excised leaf tissue (Figure 7C).

AvrXa7 induced strong GUS activity with the wild type Os-11N3 promoter fragment (Figure 7B, left site 1; Figure 7C, promoter 1, gray column). PthXo3 also induced activity at a lower level based on enzyme activity assays (Figure 7C, promoter 1, white column). Replacement of CCC with GGT within the overlapping EBE region for AvrXa7 and PthXo3 resulted in the loss of GUS activity for both AvrXa7 (Figure 7B, left site 2; Figure 7C, promoter 2, gray) and PthXo3 (Figure 7C, promoter 2, white). The hybrid Os-11N3/Os-8N3 promoter fragment, containing the overlapping EBE for AvrXa7 and PthXo3 from Os-11N3 in place of the EBE for PthXo1 (Figure 7A, promoter 3), resulted in AvrXa7- and PthXo3-dependent expression of GUS (Figure 7B, left site 3; Figure 7C, promoter 3, gray and white, respectively) and loss of PthXo1-dependent expression (Figure 7B, right site 3; Figure 7C, promoter 3, black). Inclusion of the mutant Os-11N3 EBE in the hybrid (Figure 7A, promoter 4) resulted in the loss of both AvrXa7- and PthXo3-mediated expression of GUS activity (Figures 7B and 7C, promoter 4, black). GUS activity was observed with the wild-type (Os-8N3pWT) Os-8N3 promoter when coinoculated with 35S-pthXo1 (Figure 7B, right site 5; Figure 7C, promoter 5, black). A mutant
version of the Os-8N3 EBE (Figure 7A, promoter 6) or the promoter fragment from IRBB13 (Figure 7A, promoter 7) was unable to support GUS expression (Figure 7C, promoters 6 and 7, respectively, black).

**AvrXa7 Binds to the Promoter of Os-11N3**

Previously, AvrBs3 was shown to preferentially bind the EBEs derived from AvrBs3 upregulated (UPA) genes and that binding is likely to occur within the plant cell. To determine if AvrXa7 preferentially binds the consensus EBE, DNA binding assays based on electrophoretic mobility shift (EMS) measurements were performed in combination with double-stranded oligonucleotides encompassing the predicted binding sites. AvrXa7 protein was produced in *Escherichia coli* and subjected to gel electrophoresis in the presence of $^{32}$P-labeled double-stranded oligonucleotides derived from predicted binding sites of the wild-type candidate EBE for AvrXa7 from the Os-11N3 promoter (Os-11N3oWT), a mutant version (Os-11N3oM2), and the candidate EBE for PthXo1 from Os-8N3 (Os-8N3oWT) (Figure 8A). AvrXa7 preferentially show greater retardation of labeled Os-11N3oWT in comparison to Os-8N3oWT (Figure 8B). Furthermore, the binding of the Os-11N3oWT could be competed with unlabeled Os-11N3oWT, but binding was not competitive with excess of the variant oligonucleotide Os-11N3oM2 (Figure 8C).

Chromatin immunoprecipitation (ChIP) assays were performed with AvrXa7 to determine if AvrXa7 is associated in vivo with the respective promoter region of Os-11N3. Double FLAG-tagged genes for AvrXa7 (AvrXa7-2F) and PthXo1 (PthXo1-2F) were constructed and introduced into ME2, and rice leaves were inoculated with the respective strains. Prior to the ChIP analysis, FLAG-tagged versions of both *avrXa7* and *pthXo1* were found
positive for expression by immunoblot analysis, the ability to induce the respective $S$ genes, and TAL effector-specific virulence (Figures 9A to 9C). Rice chromatin complexes were retrieved by immunoprecipitation using anti-FLAG antibody and subjected to qPCR analyses using two respective sets of primers: one set for the promoter region (Figure 9D, Os-11N3p), including the predicted DNA binding elements; and a set for downstream untranslated sequences of Os-$11N3$ (Os-$11N3$-3'). A primer set was included for the promoter region and predicted PthXo1 binding site from Os-$8N3$ as a control (Figure 9D, Os-$8N3$p). Enrichment of Os-$11N3$p was greatest in leaf samples prepared with AvrXa7-2F and FLAG antibody (Figures 9D, Os-$11N3$p). The promoter sequences of Os-$11N3$ showed an ~7-fold increase over the same DNA in anti-IgG complexes. Little or no amplification was observed in the same samples for Os-$11N3$-3' (Figure 9D) or the promoter region of Os-$8N3$ (Figure 9D, Os-$8N3$p). A similarly tagged version of PthXo1 (PthXo1-2F) was also constructed, introduced into ME2, and used to prepare immunoprecipitated complexes with FLAG antibodies. Complexes with PthXo1-2F were not enriched for Os-$11N3$p, indicating specificity of the AvrXa7-2F/Os-$11N3$p interaction (Figure 9D, Os-$11N3$p/PthXo1-2F).

**Discussion**

We demonstrated that strains of *X. oryzae* pv *oryzae* can defeat the recessive resistance of *xa13* by the deployment of any one of the alternate major type III TAL effectors PthXo2, PthXo3, or AvrXa7. Furthermore, the ability of PthXo3 and AvrXa7 to defeat *xa13* is shown to be specifically due to the induction of the alternate *S* gene Os-$11N3$, a member of the *N3* gene family. Similar to previous results with Os-$8N3$, interference with Os-$11N3$ expression during infection, either due to T-DNA insertion or RNA-mediated silencing, provided resistance against
strains of the pathogen that rely solely on AvrXa7 or PthXo3 as the major TAL effectors for virulence. The circumvention of \( xa13 \)-mediated resistance by AvrXa7 and PthXo3 involved the wholesale change in gene targets, in this case, the switch from Os-8N3 to Os-11N3. Nevertheless, the actual basis of the switch, at least as demonstrated for AvrXa7, is the change in DNA sequence recognition as mediated by the repetitive regions of the two effectors. Although DNA binding was not measured specifically, we hypothesize that PthXo3 interacts specifically with the predicted PthXo3 binding site in the Os-11N3 promoter. Compatibility, in the case of all three alternate major TAL effectors, did not entail the induction of Os-8N3. However, existence of PthXo3 illustrates a class of TAL effectors that arise due to recognition of variant sequences within the same promoter. PthXo3 is hypothesized to have arisen as an adaptation to evade \( Xa7 \)-mediated resistance (Yang et al., 2005). The relatively small differences in the EBEs for PthXo3 and AvrXa7 permit induction of Os-11N3, while avoiding elicitation of \( Xa7 \), which, based on previously demonstrated requirements of the TAL effector transcription activation properties, is hypothesized to require induction similar to \( Xa27 \) and \( Bs3 \) (Yang et al., 2000; Gu et al., 2005; Römer et al., 2007). Evidence for this hypothesis awaits further analysis of the binding specificities of AvrXa7 and PthXo3 as well as the characterization of \( Xa7 \).

The use of Os-11N3 by \( X. \) oryzae \( pv \) oryzae also illustrates the dilemma faced by host plants. Simple inactivation of Os-11N3 or Os-8N3 is not an option for achieving resistance, since complete loss, in the case of Os-11N3, resulted in pleiomorphic and severe consequences for the plant, presumably due to the normal function in plant development. Homozygous plants for the Os-11N3 insertion were stunted in several aspects of their development. The most conspicuous phenotype is delayed growth. No T-DNA mutants were available for the Os-8N3 locus. However,
silencing of Os-8N3 resulted in plants with poor fertility. We are unaware of rice germplasm with recessive mutations for Os-11N3 similar to xa13 alleles of Os-8N3. Nonetheless, the finding that base substitutions within the AvrXa7 EBE disrupt effector function in the transient assays provides evidence that it may be possible to incorporate recessive mutations into the Os-11N3 promoter using a variety of approaches. Recessive resistance might have advantages if it provided protection against both AvrXa7- and PthXo3-mediated virulence and did not interfere significantly with normal Os-11N3 expression. The fact that growth aberrancies were not observed in Os-11N3–silenced plants, possibly due to leaky expression of Os-11N3 in comparison to the T-DNA insertion line, also indicates that some change in Os-11N3 expression levels are probably not severely detrimental to plant growth and development.

As variants of the prototype TAL effector AvrBs3, the TAL effectors AvrXa7, PthXo1, PthXo2, and PthXo3 are predicted to bind specifically to host DNA elements that are defined by the sequence of the central repeats (Boch et al., 2009). AvrXa7, PthXo1, PthXo2, and PthXo3 each have unique repetitive regions, and, based on the prediction of the alignments of the AvrBs3 repeats and the cognate consensus target DNA element, each has a unique predicted binding site within the respective promoter regions (Boch et al., 2009; Moscou and Bogdanove, 2009). The results with AvrXa7 and PthXo1 corroborate the EBE model, and multiple approaches corroborate the link between AvrXa7, in particular, the EBE in the Os-11N3 promoter, and the induction of Os-11N3. ChIP analysis of AvrXa7-associated complexes yielded enrichment for the Os-11N3 promoter in comparison to sequences distal to the 3′-UTR region of Os-11N3, the promoter region of Os-8N3, or complexes retrieved by nonspecific IgG or PthXo1-2F. Additionally, AvrXa7 protein showed preferential retardation of a short oligoduplex
representing the EBE in the EMS analysis. Furthermore, the effector-specific induction of the reporter gene was observed in the heterologous *N. benthamiana* expression system, and mutations within the EBE for either PthXo1 or AvrXa7 abolished reporter gene induction, and replacement of the PthXo1-specific element with that for AvrXa7 resulted in AvrXa7-dependent gene induction. Although not examined in detail here, the results also indicated that the *xa13* allele of IRBB13 indeed had an effect on Os-8N3 expression in the *N. benthamiana* assay, supporting the hypothesis that the promoter polymorphisms within the predicted EBE for PthXo1 are the cause of the resistance phenotype in *xa13* plants. The predicted EBEs of Os-8N3 and Os-11N3 promoter regions were also recently and independently demonstrated to direct reporter gene expression in *N. benthamiana* (Römer et al., 2010). Detailed analyses of the Os-11N3 and Os-8N3 promoter sequences in relation to TAL effector-mediated expression should add more insight to TAL effector binding properties.

A limited number of recessive resistance genes have been characterized in various plant species, and fewer have demonstrated involvement in effector-mediated susceptibility. One previous case that involves type III effector-dependent recessive resistance is the *Arabidopsis RRS1-R* gene, which requires the Pop2 type III effector from *Ralstonia solanacearum* (Deslandes et al., 2002). However, the recessive nature of *RRS1-R* has yet to be explained, and the function of Pop2, either for virulence or avirulence, has not been deduced (Deslandes et al., 2003). It also should be noted that a susceptibility function for *RRS1-S*, the susceptible and dominant allele of *RRS1-R*, has not been identified. Recent progress in the analysis of effector-mediated virulence in two fungal diseases of wheat has also revealed evidence for gene-for-gene susceptibility. Both the tan spot pathogen *Pyrenophora tritici-repentis* and the stagonospora nodorum blotch
pathogen *Stagonospora nodorum* are dependent on a family of effector (toxin) genes whose virulence effect is dependent on a set of corresponding host susceptibility genes (Chu et al., 2010). The recessive resistance to specific fungal races is due to the lack of the cognate susceptibility gene. Two additional recessive naturally occurring resistance genes have been cloned. *mlo* provides resistance against all races of powdery mildew of barley, while the *xa5* gene from rice provides resistance against a broad range of strains of *X. oryzae pv oryzae* (Iyer and McCouch, 2004; Piffanelli et al., 2004; Jiang et al., 2006). Neither gene has been demonstrated to interfere with effector-mediated susceptibility. *xa5* encodes a single amino acid residue variant of the transcription factor TFIIAγ5 and, as part of the preinitiation complex of eukaryotic transcription, has potential to function in altering TAL effector function; evidence has been presented that *xa5* can interfere with TAL effector-mediated resistance (Gu et al., 2009).

Bacterial blight disease of rice is proving to be an excellent system for the study of coadaptation of host and bacterial pathogen. On the pathogen side, the evidence indicates that repeat shuffling among the TAL effector genes and the targeting of new DNA elements within the *S* gene promoters has provided the pathogen with a mechanism to circumvent resistance and promote virulence. The TAL effector family of genes is unusual in the large number of variant genes within strains and in different pathovars and species, perhaps owing to their mechanism of action. Strains of *X. oryzae pv oryzae* have high copy numbers of the genes within their genomes, and the presence of multiple genes with their corresponding repetitive regions may facilitate the appearance of TAL effectors through homologous recombination. It is also interesting to note that AvrBs3, AvrXa7, and AvrXa27 bind EBEs that either have a TATA-like sequence within the element, and the predicted EBEs for PthXo3, as shown here, also encompasses the sequence
(Boch et al., 2009; Römer et al., 2009). PthXo1 alone targets a non-TATA element. The effectors have been proposed to potentially compete for the TATA binding protein. Alternatively, the occurrence of the motif in EBEs may reflect the presence of the consensus with many host promoters, reducing the complexity of the target for newly evolved effector genes. Targeting TATA motifs may also reflect pathogen adaptation to host sequences that are more difficult to change.

On the host side, the fact that two major S genes of rice for bacterial blight are all closely related members of the N3 family points to an intrinsic property of the proteins conferring the ability of the bacterium to grow within the host. All strains of *X. oryzae pv oryzae* may require induction of at least one member of the N3 gene family for virulence. Os-11N3 does not appear to be a recent duplication or the result of selection for a pathogen resistant replacement for Os-8N3. Phylogenetic analysis indicates that Os-11N3 is a member of a distinct clade of N3 genes, possibly serving similar function to Os-8N3 in a different developmental context. Alterations in Os-11N3 expression resulted in different developmental phenotypes in comparison to Os-8N3. Previous studies indicated that Os-8N3 is upregulated during pollen development, which is consistent with the observation of reduced fertility in silenced plants (Chu et al., 2006; Yang et al., 2006). Although not analyzed here, published microarray expression data reveal that Os-11N3 is upregulated in root tissues, indicating a primary function in roots (Li et al., 2009). The fact that some apparent full-length 5′-RACE cDNA clones were obtained in this study from leaf tissue may indicate some low level expression in multiple tissue types. The cognate S gene was not identified for PthXo2, and host gene expression studies for PthXo2 are in progress. It is interesting to note that *UPA16*, a bell pepper gene upregulated by AvrBs3, is also a member of
the N3 gene family (Kay et al., 2009). N3 relatives are found throughout the plant kingdom as well as mammals, arthropods, and nematodes (Yang et al., 2006). Recent evidence has revealed two possible functions for N3 proteins in plants and animals. One report indicates that Os-8N3 interacts with copper transport proteins of rice, functioning to redistribute copper and reduce the copper ion concentration within the xylem. The authors propose that normal copper levels in the xylem are inhibitory to bacterial growth, and the reduction in level facilitates bacterial growth within the host (Yuan et al., 2010). Another report indicates that N3 proteins, including Os-8N3 and Os-11N3, can function as low-affinity glucose transporters, allowing ingress or efflux of glucose into or out of cells according to the glucose concentration gradient. In the latter model, the pathogen induces the host to release glucose into the apoplastic and xylem fluids, stimulating pathogen growth and virulence (Chen et al., 2010). Further experimentation will be required to embellish either of these new models, although they are not necessarily mutually exclusive.

Methods

Plant Material, Plasmids, and Bacterial Strains

Rice (Oryza sativa) varieties IR24, IRBB13, Nipponbare, Hwayoung, and Kitake were used in the study. Line PFG_3D-03008 was derived from Hwayoung (Jeong et al., 2006). Seeds of rice variety Nipponbare (accession number PI 514663) were provided by the USDA-Agricultural Research Service National Small Grains Collection. IR24 and IRBB13 seeds were obtained from the International Rice Research Institute (courtesy of Casiana Vera Cruz). Kitake seeds were provided by Pamela Ronald (University of California, 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 h. *Xanthomonas oryzae pv oryzae* strains and plasmids are listed in Supplemental Table 1 online.

**Expression Analyses**

The rice leaves were inoculated with indicated bacterial strains and used for total RNA extraction at indicated time points as described in the text. RNA was extracted using the TRI reagent from Ambion, and RNA concentration and quality were measured using an ND-1000 Nanodrop spectrophotometer (Nanodrop Technologies). 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 probe for Os-8N3 was prepared from cDNA using the primer set of RT-8N3-F and 8N3Probe-R. RT-PCR was performed on RNA extracted from leaves inoculated with bacteria as indicated in the text. Total RNA was extracted 24 h after inoculation, and 1 μg of RNA from each inoculation was treated individually with amplification grade DNase1 (Invitrogen) followed by cDNA synthesis using the iScript Select cDNA synthesis kit (Bio-Rad). Primers 11N3RNAi-F and 11N3RNAi-R were used for Figure 2A, and primers RT-TF2-5F and RT-TF2-5R were used to PCR amplify *TFIIAγ5*. Primer sequences are provided in Supplemental Table 2 online. qPCR and qRT-PCR were performed on DNA or RNA extracted from leaves 24 h after inoculation, respectively. For qRT-PCR, 1 μg of total RNA was subjected to DNase I (Invitrogen) treatment to eliminate the genomic DNA contamination and then to first-strand cDNA synthesis using the iScript cDNA Synthesis kit (Bio-Rad). cDNA derived from 25 ng of total RNA was used for each real-time PCR, which was performed on Stratagene’s Mx4000 multiplex quantitative PCR system using the iQ SYBR green Supermix kit (Bio-Rad).
The gene-specific primer sequences are provided in Supplemental Table 2 online. The average threshold cycle (Ct) was used to determine the fold change of gene expression. TFIIAγ5 expression was used as an internal control. The 2^{ΔΔCt} method was used for relative quantification (Livak and Schmittgen, 2001).

**Phylogenetic Analysis**

Alignment and phylogenetic analyses were conducted using ClustalW (Thompson et al., 1994) and MEGA version 4 for unrooted phylogenetic tree construction using the minimum evolution method (Tamura et al., 2007). The tree is depicted in rooted format using the midpoint between each node. Alignments are provided in Supplemental Figure 1 and Supplemental Data Set 1 online. Bootstrap support value for 1000 reiterations is indicated above each node.

**Genotyping of T-DNA Line PFG_3D-03008**

DNA was extracted from a single leaf of each progeny plant and genotyped with the following primers: Os11g-F (wild-type locus forward primer) and Os11g-R (wild-type locus reverse primer); and 2772 RB-F (pGA2772 right border T-DNA primer). Primer sequences are provided in Supplemental Table 2 online.

**Rice Transformation and Gene Construction**

For construction of Os-11N3 RNAi plants, a 341-bp fragment specific to Os-11N3 was PCR amplified with primers 11N3RNAi-F and 11N3RNAi-R. The product was cloned into pTOPO/D-ENTR vector, sequenced, and recombined into pANDA (Miki and Shimamoto, 2004) through LR recombinase according to the instructions of the manufacturer (Invitrogen). The construct was transformed into *Agrobacterium tumefaciens* strain EHA105. Calli from immature
embryos of rice cultivar Kitaake were initiated and transformed using *Agrobacterium* as described (Hiei et al., 1997).

**Virulence Assays**

The fully expanded rice leaves at the stages indicated in the text were inoculated by leaf tip clipping with scissors that were immersed in bacterial suspensions of optical density of 0.5 at 600 nm (≈5.0 × 10^7 cell forming units per mL) immediately prior to each clipping as described (Kauffman et al., 1973). Symptoms were scored by measuring lesion length. Significance between treatments as assessed on the basis of a P value of <0.05 using the Tukey test after analysis of variance (ANOVA).

**5′-RACE cDNA Analysis**

The 5′-RACE cDNAs were derived from leaf tissue of cultivar Nipponbare 24 h after inoculation with ME2 or ME2(*avrXa7*). RNA was extracted using the TRI reagent (Ambion) and subjected to 5′-RACE RT-PCR analysis using the primer 5′-CTTGCTTGCAAGTAACAAGAG-3′ in place of a poly-dT primer and the SMARTer RACE cDNA amplification kit (Clontech). Individual cDNAs were cloned in pCR2.1 using the TOPO cloning kit (Invitrogen) and sequenced.

**Transient Expression Assays**

Promoter-*GUS* constructs were made by amplifying the promoter regions using specific primers given below, and amplicons obtained were digested with *HindIII* and *XbaI* and cloned into *HindIII* and *XbaI* sites in pBI121 by replacing the 35S promoter (Jefferson et al., 1987). The specific primers (sequences provided in Supplemental Table 2 online) for each promoter
construct are as follows: OS8N3pWT (8pG-F and 8pG-R), OS8N3pM (8pMGF and 8pG-R), OS8N3pBB13 (BB13F and 8pG-R), OS11N3pWT (11pG-F and 11pG-R), OS11N3pM (11pMGF and 11pG-R), OS11N3WT-OS8N3p' (11-8PG-F and 8pG-R), and OS11N3M-OS8N3p' (11M-8pG-F and 8pG-R). All constructs were sequenced before introducing into *Agrobacterium*. For each assay, *Agrobacterium* transformants with various constructs were streaked on Luria-Bertani (LB) agar supplemented with kanamycin (50 μg/mL) and rifampicin (15 μg/mL) antibiotics and grown at 28°C for 2 d. A single colony was inoculated in 5 mL liquid LB media supplemented with kanamycin (50 μg/mL) and rifampicin (15 μg/mL), 1 mL of the overnight culture was subcultured in 50 mL liquid LB supplemented with kanamycin (50 μg/mL) to an OD$_{600}$ of 0.6. The bacterial cells were then collected by centrifugation at 4°C for 10 min at 3000 rpm. The cells from each centrifugation were resuspended in 50 mL *Agrobacterium* inoculation buffer (4.8 gm MES, 5 mL 1 M MgCl$_2$, and 0.147 g acetosyringone in 500 mL water, pH 5.6) and activated at 28°C for 3 h. Coinoculation was done by mixing the cultures in 1:1 ratio prior to inoculation. Bacterial suspension (100 μL) was infiltrated into the leaf at each inoculation site. The inoculation was done on fully opened leaves (three leaves per treatment), and the leaves were harvested 40 h after inoculation and incubated at 37°C in GUS reagent (100 mM phosphate buffer with 0.5% Triton X-100, 10 mM EDTA, 0.5 mM each of X-gluc [5-bromo-4-chloro-3-indolyl-β-d-glucuronic acid cyclohexylammonium salt] and potassium ferri- and ferrocyanide) for 7 h and thereafter cleared using 70% ethanol (Jefferson et al., 1987). Average GUS activity was measured in triplicate from extracts of the inoculated portions of leaves. The tissue (~50 mg) was excised and homogenized in 1.5-mL microcentrifuge tubes with 1 mL of extraction buffer (50 mM NaPO$_4$, pH 7.0, 1 mM Na$_2$EDTA, 10 mM DTT, 0.1% sodium lauryl sarcosine, and 0.1%
Triton X-100. GUS activity was measured using the MUG substrate and expressed as pmol 4-methylumbelliferone/μg protein.

EMS Assays

Six-His-tagged AvrXa7 protein was expressed and purified from *Escherichia coli* BL21 with Ni-NTA agarose (Qiagen). Protein concentration was determined using a Bradford reagent kit (Bio-Rad). Complementary oligonucleotides were annealed and 5′ end labeled with [γ-^{32}P]ATP catalyzed by T4 kinase. Labeled double-stranded DNA was mixed with AvrXa7 in a reaction containing Tris-HCl (15 mM, pH 7.5), KCl (60 mM), DTT (1 mM), glycerol (2.0%), MgCl_2 (2.5 mM), poly(dI.dC) (50 ng/μL), EDTA (0.2 mM), labeled DNA (50 fmol), unlabeled DNA (0 to 2.5 pmol), and AvrXa7 (350 fmol). The binding reactions were kept at room temperature for 30 min before being loaded on an 8% TBE polyacrylamide gel.

ChIP Assays

Versions of AvrXa7 and PthXo1 with two FLAG epitope coding sequences were constructed, and the genes for the effectors were introduced into strain ME2. For immunoblot analysis, *X. oryzae* pv. *oryzae* grown in TSA media was adjusted to the same OD_{600} = 0.5 using the same medium. Bacteria (150 μL) was boiled with the same amount of 2× SDS-PAGE loading buffer, and 20 μL was loaded onto a 10% SDS-PAGE gel. Proteins separated by SDS-PAGE were transferred to a polyvinylidene fluoride membrane and immunoblotted using monoclonal FLAG antibody (Sigma-Aldrich). The secondary antibodies (goat anti-mouse horseradish peroxidase–conjugated; Millipore) bound to the membrane were detected using ECL protein gel blotting reagents (Pierce, Thermo Scientific) and x-ray film (Kodak). Two-week-old rice plants were syringe-inoculated with ME2(*avrXa7*) or ME2(*pthXo1*) with the suspension of OD_{600} = 0.5.
S gene induction was performed as described above using gene-specific primers. Virulence assays were performed as described with the following modifications (Haring et al., 2007): 3.0 g of inoculated leaf material was harvested 20 h after inoculation, fixed using 1.5% formaldehyde. The tissue was sonicated using Virsonic 50 (output control 6) for $8 \times 15$ s. The protease inhibitor cocktail for plant cell and tissue extracts (Sigma-Aldrich; P9599) was used. Immunoprecipitation was done using monoclonal M2 FLAG antibody (12 μg/mL; Sigma-Aldrich). The mouse serum (12 μg/mL) was used as control. PCR on enriched DNA sample was done using the following primers: 11N3-5′ (11N3- 5′F and 11N3-5′R), 11N3-3′ (11N3-3′F and 11N3-3′R), 8N3-5′ (8N3-5′F and 8N3-5′R), and 8N3- 3′ (8N3-3′F and 8N3-3′R). Primer sequences are provided in Supplemental Table 2 online. Quantitative real-time PCR measurements used the average threshold cycle (Ct) to determine the fold change of DNA content. The $2^{ΔΔCt}$ method was used for relative quantification (Livak and Schmittgen, 2001).

Accession Numbers

Sequence data from this article can be found in the National Center for Biotechnology Information database under the following accession numbers: AK070510 (Os-8N3), AK101913 (Os-11N3), and NM_001060961 (Os-TFIIAγ5).

Acknowledgments

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Biotech Center (Pohang University of Science and Technology). The research was supported by funds from the Kansas Agriculture Experiment Station and the United States Department of Agriculture Cooperative State Research, Education, and Extension Service National Research Initiative (Award 2007-01518; F.W.), the National Science Foundation (Award 0820831; F.W. and B.Y.), and the Iowa State University faculty startup fund (B.Y.). This article is publication number 10-237-J of the Kansas Agriculture Experiment Station.
References


Figures and Tables

Figure 1. Major TAL effector genes defeat *xa13* in rice without induction of *Os8N3*. (A) Disease leaf lesions on ten 28 d plants of cultivars IRBB13 (solid bars) and IR24 (white bars) were measured 14 d after inoculation at the leaf tips with bacterial suspensions (0.5 X 10^8 cfu/ml) of the following strain treatments: 1, ME2 (pHM1); 2, ME2 (*pthXo1*); 3, ME2 (*pthXo2*); 4, ME2 (*pthXo3*); 5, ME2 (*avrXa7*). Values with the same small case letters above columns do not differ significantly at the <0.5 level using Tukey statistic and ANOVA analysis. I-bars indicate one standard deviation. (B) qRT-PCR measurements of *Os8N3* expression following strain treatments as for (A). RNA was prepared 24 h after inoculation of leaves, and qRT-PCR was performed using gene specific primers for rice locus *Os8N3*. Northern hybridization analysis of *Os8N3* following bacterial inoculation is shown below the qRT-PCR data for each treatment. Total RNA was prepared from ten leaves of rice cultivars IRBB13 and IR24 and measured spectrophotometrically. Equal amounts from each treatment were loaded in an agarose gel, fractionated and subjected to hybridization analysis using ^32^P-labeled fragment of *Os8N3*.
Figure 2. PthXo3 and AvrXa7 induce Os11N3. (A) qRT-PCR analysis of Os11N3 expression from RNA prepared 24 h after inoculation of leaves of cultivar Nipponbare using gene specific primers for rice locus Os11g31990 (Os11N3). Strains used in each inoculation are indicated below each lane. OsTFIIAγ5 expression as an internal control for the quantity and quality of RNA sample. Strains for each treatment were: Lanes 1, H₂O; 2, ME7; 3, ME2(pHM1); 4, ME2(pthXo1); 5, ME2(pthXo2); 6, ME2(pthXo3); 7, ME2(avnXa7). RNA was extracted twenty-four hours after inoculation. (B) Schematic of cDNA AK101913 corresponding to Os11N3 aligned with genomic sequence. Numbers indicate the bases in the indicated region.
Figure 3. Os11N3 represents a distinct clade of the N3 family. (A) Alignment and phylogenetic analyses were conducted using ClustalW (Thompson et al., 1994) and the Minimal Evolution program in MEGA version 4 for unrooted phylogeny tree construction (Tamura et al., 2007). Bootstrap support for 1000 re-iterations is provided above each line.
Figure 4. A T-DNA insertion in Os11N3 confers AvrXa7- and PthXo3-specific recessive resistance. (A) Position of T-DNA insertion PFG_3D-03008 within the first intron of Os11N3. Schematic is not to scale. PCR product across the right border of insertion is indicated by blue and red arrows (PCR1). PCR product of wild type locus is indicated by the black and red arrows (PCR2). (B) PCR analysis of progeny of rice cultivar Hwayoung with T-DNA insertion PFG_3D-03008. Homozygous mutant progeny are indicated by presence of fragment in upper panel (PCR1) and absence of fragment in lower panel (PCR2). The presence of both fragments is indicative of a heterozygous individual. The absence of PCR1 is indicative of a homozygous wild type locus. The template for sample in lane 11 was prepared from the parent line Hwayoung. Phenotype of the line whose genotype is shown is indicated below lanes. R, resistant to infection by ME2(avnXa7) and ME2(pthXo3); S, susceptible to infection by ME2(avnXa7) and ME2(pthXo3). (C) Average lesion length measurement of six heterozygous (white-fill) and six homozygous plants (black-fill) after inoculation with (1) ME2(pthXo1), (2) ME2(avnXa7), or (3) ME2(pthXo3). (D) Phenotypes of homozygous T-DNA insertion mutant inoculated with the following strains: leaf 1, ME2(pthXo1), leaf 2, ME2(avnXa7); and leaf 3, ME2(pthXo3). Panel at left shows leaves on intact 50 day old plant. Red arrows indicate sites of inoculation. Phenotypic differences in seed sizes are shown in upper right panel: He – heterozygous plant; Ho – homozygous mutant plant. Panel at lower right shows stature comparison of 90-day old heterozygous (He) and homozygous (Ho) mutant individuals.
Figure 5. RNAi knockdown expression of Os11N3 provides AvrXa7- and PthXo3-specific resistance. (A) qrtPCR analysis of two transgenic rice lines (1 and 2) expressing a portion of the Os11N3 3′-UTR as a double-stranded RNA. A control line was also examined containing only the vector T-DNA sequences (V). RNA was prepared from plants generated without the insert (column V, vector alone) and two transgenic lines with the insert (columns 1-2). Black-fill columns indicate analysis of RNA from uninfected plants, and expression of sequences from the over-expressed ds Os11N3 3′-UTR was amplified using 3′-specific primers. White-fill columns indicate analysis of Os11N3 5′-UTR region 24 h following inoculation of the same lines with ME2(avrXa7). (B) Lesion lengths were measured nine days after inoculation of lines V, 1 and 2 with ME2(pthXo1) (white-fill) or ME2(avrXa7) (black fill). Measurements are averages of ten plants. Values with same letter do not differ significantly at the P<0.5 level using the Tukey statistic following ANOVA analysis. (C) Phenotypes of progeny of RNAi line 1 challenged with ME2(pthXo1), ME2(pthXo3) or ME2(avrXa7). Line V, containing only vector sequences, is shown after inoculation with ME2(avrXa7). S, susceptible; R, resistant. Arrow indicates site of inoculation. Plants were photographed nine days after inoculation.
Figure 6. Candidate effector binding elements in the promoters of *Os8N3* and *Os11N3*.  
(A) The predicted EBE of effectors *PthXo1*, *AvrXa7* and *PthXo3* aligned with the corresponding two amino acid variable residues of the respective repeats. Consensus nucleotides are indicated by the single letter code; N, A, C, G, or T; n – unassigned.  
(B) The promoter region of *Os8N3* (-397 to +3) from cultivar *Nipponbare* is shown. Predicted *PthXo1* binding element is underlined, and the site of insertion in IRBB13 is indicated by triangle next to the first nucleotide of the EBE. The start site for normal transcription is indicated in bold large font immediately downstream of TATA box.  
(C) *Os11N3* promoter sequence (-336 to +3) from cultivar *Nipponbare* with *AvrXa7* and *PthXo3* binding elements underlined. The start sites for normal transcription (A) and the alternate transcription in the presence of *AvrXa7* (G) are indicated in bolded large font.
Figure 7. AvrXa7 drives TAL effector-specific host gene induction. (A) Promoter fragments used in the transient expression assay. The consensus effector binding elements are underlined. The non-consensus changes introduced in the mutant versions are in lower case letters. Os8N3pBB13 contains the insertion/deletion of xα13 allele in IRBB13 and sequence is not shown. The first ten (Os11N3) or twelve (Os8N3) bases upstream of the EBE and between the EBE region and the ATG are not shown. Each fragment was fused to the ATG of the uidA coding sequence. (B) GUS assay on N. benthamiana using 35S-avrXa7 (left sites) or 35S-pthXo1 (right sites). Sites are stained with X-gluc. Co-inoculations with 35S-pthXo3 and combinations with promoter fragment Os8N3pBB13 (7) are not shown. The T-DNA vector containing 35S-avrXa7 or 35S-pthXo1 alone was infiltrated at sites indicated with an asterisk (*). (C) Average GUS activity was calculated from triplicate co-inoculations of the promoter fragments in (A) with 35S-pthXo1, 35S-avrXa7, or 35S-pthXo3. The numbers for each treatment with the indicated plant-expressed effector corresponds to the promoter fragments in (A). Average GUS activity from three excised leaf disks with hybrid promoters indicated in (A) and 35S-pthXo1 (black-fill columns), 35S-avrXa7 (gray-fill columns), or 35S-pthXo3 (white-fill). Activity on MUG substrate is expressed as pmol of MU per ug of protein.
Figure 8. AvrXa7 interacts with the Os11N3 promoter. (A) Oligonucleotides used in electromobility shift assays (EMSA). The predicted effector binding elements within the respective promoters are underlined. The nucleotide changes in the mutant version Os11N3oM2 are in small case. (B) EMSA of oligonucleotides [shown in (A)] were incubated with 6His-AvrXa7. + or – indicate the presence or absence of the AvrXa7 or the oligonucleotides included in the reaction for each lane. (C) Competition assay with increasing amounts of unlabeled Os11N3oWT or Os11N3oM2.
Figure 9. AvrXa7 interacts with the Os11N3 promoter in rice. (A) Western blot analysis of expression of avrXa7-2F and pthXo1-2F, containing an internal double FLAG epitope, using the M2 FLAG monoclonal antibody. Analysis of protein from two colonies are shown. Coomassie stained gel is shown at right. M, protein molecular size standards. Numbers at left indicate kilodaltons. Lanes: ME2 (pHM1); 2, ME2(avnXa7-2F-1); 3, ME2(avnXa7-2F-2); 4, ME2(pthXo1-2F-1); 5, ME2(pthXo1-2F-2). (B) FLAG-tagged versions of AvrXa7 and PthXo1 induce the respective S genes when produced in ME2. Induction was measured in $2^{\Delta \Delta Ct}$. Three fourteen day old rice seedlings were inoculated with the indicated strain and total RNA was isolated from three leaves and subjected to qRT-PCR. (C) avrXa7-2F and pthXo1-2F confer virulence on ME2. Four weeks old rice plants were inoculated with the respective strains (indicated below each column) by leaf tip-clipping inoculation. Lesion lengths were measured 12 days post inoculation on 10 inoculated leaves for each treatment. (D) qPCR analysis of AvrXa7-2F (first three columns) and PthXo1-2F (fourth column) immunoprecipitated complexes from leaf infection sites using primers for the indicated DNA fragment. Fold changes in average cycle numbers were compared to average cycle numbers of the same PCR products in complexes immunoprecipitated with IgG control antibodies. The values are the averages of three independent leaf inoculations with the exception of the fourth column, which is the average of two inoculations. Values that do not differ significantly at p<.05 level are indicated by the same lower case letter. Significance was determined using ANOVA and the TUKEY HSD test (F-statistic,13.68,;p= 0.0026)

Supplemental Table 1. Strains and plasmids used in the study.

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Supplemental Table 2. Primers used in this study.

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Supplemental Figure 1. ClustalW alignment of N3 proteins.
Supplemental Figure 2. Alignment of Os11N3 transcription sites after inoculation with ME2 or ME2 (avrXa7). 5'-RACE cDNAs were derived from leaf tissue of cultivar Nipponbare 24 h after inoculation with ME2 or ME2(avrXa7) are aligned above and below, respectively, to the predicted 5'-UTR of Os11N3 (letters in bold). Numbering starts at base 28 downstream of the TATA box.
CHAPTER 3. GENE TARGETING BY THE TAL EFFECTOR PTHXO2 REVEALS CRYPTIC RESISTANCE GENE FOR BACTERIAL BLIGHT OF RICE

A paper published in the Plant Journal

Junhui Zhou, Zhao Peng, Juying Long, Davide Sosso, Bo Liu, Joon-Seob Eom, Sheng Huang, Sanzhen Liu, Casiana Vera Cruz, Wolf B. Frommer, Frank F. White and Bing Yang

Abstract

Bacterial blight of rice is caused by the γ-proteobacterium Xanthomonas oryzae pv. oryzae, which utilizes a group of type III TAL (transcription activator-like) effectors to induce host gene expression and condition host susceptibility. Five SWEET genes are functionally redundant to support bacterial disease, but only two were experimentally proven targets of natural TAL effectors. Here, we report the identification of the sucrose transporter gene OsSWEET13 as the disease susceptibility gene for PthXo2 and the existence of cryptic recessive resistance to PthXo2-dependent X. oryzae pv. oryzae due to promoter variations of OsSWEET13 in japonica rice. PthXo2-containing strains induce OsSWEET13 in indica rice IR24 due to the presence of a novel and unpredicted effector binding site not present in the alleles in japonica rice Nipponbare and Kitaake. The specificity of effector-associated gene induction and disease susceptibility is attributable to a single nucleotide polymorphism (SNP), which is also found in a polymorphic allele of OsSWEET13 known as the recessive resistance gene xa25 from the rice cultivar Minghui 63. The mutation of OsSWEET13 with CRISPR/Cas9 technology further corroborates the requirement of OsSWEET13 expression for the state of PthXo2-dependent disease susceptibility to X. oryzae pv. oryzae. Gene profiling of a collection of 104 strains revealed OsSWEET13 induction by 42 isolates of X. oryzae pv. oryzae. Heterologous expression of OsSWEET13 in Nicotiana benthamiana leaf cells elevates sucrose concentrations in the
apoplasm. The results corroborate a model whereby *X. oryzae* pv. *oryzae* enhances the release of sucrose from host cells in order to exploit the host resources.

**Introduction**

Many proteobacterial plant pathogens depend on type III secretion systems (T3SS) to deliver a suite of virulence proteins, so-called T3SS effectors, into host cells to facilitate acquisition of nutrients and to dampen host innate immunity (Feng and Zhou, 2012; Block, et al., 2008). At the same time, host plants have evolved genes for recognition of T3SS effectors (Jones and Dangl, 2006). Transcription activator-like (TAL) T3SS effectors of *Xanthomonas* directly target specific host genes for expression, which, in turn, condition disease susceptibility in the host. TAL effector genes are limited to *Xanthomonas* species and *Ralstonia solanacearum*, and the genes comprise the largest group of T3SS effectors of all plant bacteria on the basis of sequence relatedness (White, et al., 2009). TAL effectors can function either as virulence factors or avirulence elicitors, and, when functioning as virulence factors, TAL effectors recognize the DNA sequence specific binding sites, here referred to as effector binding sites or EBEs (Bogdanove and Moscou 2009; Boch et al., 2009). The contribution of TAL effectors to pathogen virulence varies from being dramatic (major), moderate, to not detectable [reviewed in (Boch and Bonas, 2010)].

Bacterial blight of rice, caused by *Xanthomonas oryzae* pv. *oryzae*, is a major disease of rice in South Asia and West Africa. Bacterial virulence is dependent on the bacterial type III effectors and effector interactions with the host target genes or gene products (White and Yang, 2009). An important component of the disease under field and experimental conditions is the induction of at least one member of the clade III of sugar transporters by one of the so-called
major TAL effectors found in all strains of *X. oryzae* pv. *oryzae* (Anthony et al., 2010; Chen et al., 2010; Yu et al., 2010; Streubel et al., 2013). The host genes, as a class, have been termed host disease susceptibility (S) genes, which vary in a gene-for-gene manner, depending on the inciting strains of the pathogen. Initially identified as S genes coding for homologs of the nodulin 3 (N3) family of genes of *Medicago truncatula*, the genes were termed *Os8N3* and *Os11N3* based on the chromosome location of the gene in the rice genome. Simultaneously, alleles of *Os8N3* were identified as recessive resistance genes to specific strains of *X. oryzae* pv. *oryzae* and appropriately named *xa13*, in the case of *Os8N3*, and the compatible allele was called *Xa13*. It should be noted that recessive alleles of *xa13* and, more recently, *xa25* (recessive allele of *Xa25* or *OsSWEET13*, see below) are not null alleles but rather alleles with promoter polymorphisms. Some members of the N3 proteins have been shown to be sugar transporters, hence the most recent nomenclature SWEET, and, specifically, the members of the clade III, where examined, have been shown to preferentially transport sucrose (Chen et al., 2012; Cohen et al., 2014). Here, we refer to the genes using the SWEET nomenclature to reflect the normal physiological function of the gene products.

Experimentally, any one of five SWEET genes of rice has been shown to be competent for conditioning disease susceptibility (Yang, et al., 2006; Antony, et al., 2010; Yu, et al., 2011; Li, et al., 2013; Streubel, et al., 2013). In nature, only two host genes have been identified that correspond to major TAL effectors. *OsSWEET11* is activated by PthXo1 (Yang, et al., 2006), while *OsSWEET14* by multiple TAL effectors, including AvrXa7, PthXo3, TalC and Tal5, individually (Antony, et al., 2010; Yu, et al., 2011; Streubel, et al., 2013). A third SWEET gene, *OsSWEET13* (*Xa25* or *Os12N3*), has been implicated as a target of a major effector due to the
PthXo2 is the product of a major TAL effector gene for virulence in *X. oryzae* pv. *oryzae* strains JXO1 and MAFF 311018 (T7174) (Yang and White, 2004; Ochiai, et al., 2005). The cognate S gene to PthXo2 has not been identified and experimentally validated. An EBE in the promoter of the incompatible allele (xa25) of *OsSWEET13* was predicted for two TAL effectors (YP_200767.1 and YP_451027.1) rather than PthXo2, while PthXo2 was predicted to target three rice genes (LOC_Os01g63820, LOC_Os03g16920 and LOC_Os05g51470) but not *OsSWEET13*, leading to the conclusion that effector binding site predictions for natural TAL effectors and cognate targets, in the absence of functional analyses, are unreliable or speculative (Perez-Quintaro et al., 2013). A second effector binding site for PthXo2 in the promoter of the compatible allele of *OsSWEET13* (or Xa25) in Zhenshan 97 was proposed (Richter et al., 2014). Artificially designed TAL effectors (dTALes) that targeted the promoter sequences in the resistant and susceptible alleles could activate expression in promoter analyses. However, PthXo2 was not tested either for promoter activity on the proposed site or induction and susceptibility of *OsSWEET13* (Richter et al., 2014). Here, we hypothesize that the target of PthXo2 target was the *OsSWEET13* allele in the compatible rice cultivar IR24 based on gene expression data and tested the hypothesis using a variety of approaches including CRISPR-mediated genome editing.
Results

**Virulence Function of PthXo2 is Associated with Expression of the OsSWEET13 Locus on Chromosome 12 of Rice**

*X. oryzae* pv. *oryzae* strain ME2 is a mutant of strain PXO99A and lacks the major TAL effector gene *pthXo1*. Introduction of the major TAL effector gene *pthXo2* restores virulence to ME2 on the rice cultivar IR24. However, ME2(*pthXo2*) is not compatible on the cultivar Nipponbare. Inoculation of the leaf tip of Nipponbare variety results in short lesions, while lesions extend 12-15 cm in the susceptible variety IR24 after 12 days (Figure 1a). We hypothesized that the incompatibility of ME2(*pthXo2*) and Nipponbare was attributable to differential S gene expression in IR24 and Nipponbare. Microarray expression analysis of inoculated leaves showed elevated expression of the gene associated with the probe set Os.5491.1.A1_s_at, which corresponds to the SWEET gene *OsSWEET13*. No expression of *OsSWEET13* was detected either in leaves of cultivar Nipponbare or in either cultivar in the absence of PthXo2 (Table 1). *OsSWEET13*, as represented by the probe set Os.5491.1.A1_s_at, was induced approximately 34 fold by ME2(*pthXo2*) compared to ME2 in IR24 (Table 1). The expression of the internal control gene (*OsTFIIAγ5*, a gene on chromosome 5 that encodes the general transcription factor TFIIAγ5) did not change (Table 1). PthXo2-dependent expression of *OsSWEET13* was also detected in IR24 after inoculation with ME2(*pthXo2*) but not in Kitaake using real time quantitative reverse transcription PCR (qRT-PCR) (Figure 1b).

An F2 mapping population derived from an IR24 and Nipponbare cross was generated and screened with the makers SM1 and SM2 on chromosome 12 and by inoculation with ME2(*pthXo2*) (PCR products by primers SM1-F/SM1-R and SM2-F/SM2-R, Table S3). SM1 is a molecular marker at *OsSWEET13*, while SM2 is derived from sequence 32 kb away from SM1.
None of 456 susceptible progeny harbored the 235 bp fragment of the resistant cultivar Nipponbare at SM1, while one individual possessed the 249 bp allele of Nipponbare at the distal SM2 marker (Figure 1c).

The $\text{OsSWEET13}$ mRNA is represented by the full length 1656 bp cDNA clone J0090073D19 (Accession number AD242853) (Figures 1d, S1a). The predicted protein is 296 amino acids with seven transmembrane domains (Figure 1e). Sequence comparison of a PCR-amplified 5865 bp fragment with $\text{OsSWEET13}$ from IR24 and the corresponding region in Nipponbare revealed 46 single nucleotide polymorphisms (SNPs) and 8 insertion/deletions of >2 bp (indels) in the nontranscribed regions; 21 SNPs and 6 indels in introns; 28 SNPs and 4 indels in 5’- and 3’-UTRs (untranslated regions); and 15 SNPs and 2 indels in the coding regions (Figure S1b).

The amplified 5865 bp fragment spanning $\text{OsSWEET13}$ in IR24, including approximately 2426 bp upstream of the predicted mRNA start site and 1092 bp downstream of the predicted mRNA stop site, was transferred to the fast cycling $\text{japonica}$ rice cultivar Kitaake, which is a close relative of Nipponbare and also resistant to ME2($\text{pthXo2}$). Sixteen primary transgenic plants were obtained and eleven were positive as genotyped with the SM1 marker. Upon inoculation, the transformed plants (T0 lines and T1) were resistant to ME2, while susceptible to ME2($\text{pthXo2}$) (Figure 2a), indicating the allele from IR24 confers disease susceptibility to Kitaake in response to PthXo2. $\text{OsSWEET13}$ expression in two representative lines was dependent on the presence of PthXo2 by qRT-PCR of the transformant mRNA after inoculation with ME2($\text{pthXo2}$). No gene induction was detected upon inoculation with water or ME2 (Figure 2b)
OsSWEET13 Shows Sucrose Transport Activity

Since OsSWEET13 is a member of SWEET clade III subgroup in which some members have been shown to mediate sucrose transport in phloem loading and nectar secretion, we tested whether OsSWEET13 could transport sugars. OsSWEET13 was transiently expressed in Human Embryonic Kidney T293 (HEK293T) cells along with sucrose or glucose Förster Resonance Energy Transfer (FRET) sensors (Chen, et al., 2010; Chen, et al., 2012). Sucrose induced a ratio change (emission intensity of yellow over cyan) in OsSWEET13-expressing HEK293T cells similar to that of positive control AtSWEET12 (Figure 3a). Sensor responses to glucose addition indicated that OsSWEET13 also can function as a weak glucose transporter (Figure 3b).

We tested the ability of OsSWEET13 to mediate sucrose efflux in plant cells using CaMV35S-driven transient expression of OsSWEET13 in Nicotiana benthamiana leaves. The treated leaves were infiltrated with water 36 hours post inoculation, and the leaves were subjected to centrifugation to recover apoplasmic fluids. The sucrose content of the supernatant was 2.3 fold higher in wash fluids from N. benthamiana leaves expressing OsSWEET13 compared to control (Figure 3c).

Multiple Japonica Rice Cultivars Harbor A Cryptic Recessive Resistance Allele of the S Gene OsSWEET13

Sequence analysis revealed a candidate EBE for PthXo2 in the promoter and, more specifically, the TATAA box region of OsSWEET13 of IR24 (Figure 4a). The corresponding promoter sequence in the OsSWEET13 allele from Nipponbare has a single adenosine nucleotide deletion, disrupting the predicted alignment with the RVDs (Figure 4a). We hypothesized that the single nucleotide polymorphism in EBE_{PthXo2} region of IR24 and Nipponbare influences the
expression of \textit{OsSWEET13} in the presence of PthXo2. First, a full length promoter fusion was tested using 2240 bp promoter region that included the 5’-UTR and a 1092 bp terminator region of the \textit{OsSWEET13} allele from IR24 fused with the gene for green fluorescence protein (GFP). GFP fluorescence was observed upon transient co-expression of the reporter construct and 35S-pthXo2 (\textit{pthXo2} under the CaMV 35S promoter) (Figures 4b, c, P1). A 40-bp region containing the predicted EBE\textsubscript{PthXo2} from IR24 was then used and co-expressed with 35S-pthXo2. The GFP fluorescence intensity was as strong as that with the 2.2 kb promoter region (Figures 4b, c, P2). In contrast, the corresponding 39-bp sequence from Nipponbare, lacking the fourth A nucleotide, failed to direct GFP fluorescence (Figures 4b, c, P3). The EBE\textsubscript{PthXo2} regions of \textit{OsSWEET13} alleles were PCR-amplified and sequenced from additional six rice cultivars revealing that all seven rice cultivars of the \textit{japonica} subspecies designation, contained the PthXo2-incompatible promoter (Figure 4d). Disease assays with ME2(\textit{pthXo2}) showed that all the \textit{japonica} rice varieties were resistant to the bacterial infection, while, at the same time, susceptible to ME2 with \textit{avrXa7} (Figure 4d).

\textbf{PthXo2 Binds to the IR24 \textit{OsSWEET13} Promoter}

The physical interaction between PthXo2 and the EBE\textsubscript{PthXo2} sequence was performed using EMSA (electrophoretic mobility shift assay). Purified recombinant PthXo2 was expressed with an N-terminal 6-histidine tag in \textit{Escherichia coli}. A 29-bp double-stranded DNA fragment containing the EBE\textsubscript{PthXo2} (pEBE\textsubscript{PthXo2}) and a fragment with a 5-bp mutation (pSWEET13-m) were labeled and used to test interaction with 6xHis-PthXo2 (PthXo2H6) (Figure 5a). The EMSA results indicate that PthXo2 preferentially binds to pEBE\textsubscript{PthXo2} but not pSWEET13-m (Figure 5b). Using competition binding assay, the intensity of labeled SWEET13 was reduced
with increasing amount of unlabeled pSWEET13 (Figure 5b). In contrast, the unlabeled pSWEET13-m could not compete with the labeled pSWEET13 for binding by PthXo2H6 (Figure 5b).

**Modified Versions of *pthXo2* Enable Switch of *OsSWEET13* Induction and Allelic Disease Resistance/Susceptibility**

The deduced OsSWEET13 protein products from Nipponbare and IR24 differ by eight amino acids (Figure S1). To determine whether *OsSWEET13* in Nipponbare (and Kitaake) can function as a S gene for bacterial blight, a PthXo2 derivative was constructed wherein the fifth repeat of the central repetitive region was deleted, resulting in a RVD configuration that conformed to the EBE region in Nipponbare (and Kitaake) (Figure 6a). Similarly, a designer TAL effector (dTALe-13) was synthesized to target optimally the same 24 nucleotides of the TATAA box region of *OsSWEET13* in Kitaake (and Nipponbare) (Figure 6a). ME2 with the deleted *pthXo2* derivative (*pthXo2D*) gained the ability to induce *OsSWEET13* and a state of disease susceptibility in Nipponbare, and concomitantly lost the same abilities in IR24 (Figures 6b, c). Similarly, the gene for dTALe-13, when introduced into ME2, enabled the induction of *OsSWEET13* and conferred virulence to ME2 in Kitaake but not in IR24 (Figures 6d, e). Thus, changing the effector to match a binding site in Nipponbare and Kitaake resulted in a concomitant gain of *OsSWEET13* expression and disease susceptibility, but loss of the same functions in IR24. Our results are consistent with that in a prior study that *OsSWEET13* is an S gene, when induced by an artificial TAL effector, in Azucena wherein *OsSWEET13* carries an identical sequence to Nipponbare and Kitaake (Streubel et al., 2013).
To further test the requirement of \textit{OsSWEET13} expression for disease susceptibility in the presence of PthXo2, in addition to probing developmental and productive function of \textit{OsSWEET13}, we generated knockout mutants of \textit{OsSWEET13} by targeting its coding region using CRISPR technology. Two independent null mutants, one carrying an 11-bp deletion (\textit{sweet13-1}) and one with a 4-bp deletion (\textit{sweet13-2}), both of which disrupt the coding sequence for OsSWEET13 but have no detectable phenotypic effect on plant morphology under normal growth condition, were tested for disease resistance (Figure 7a). The bacterial strain ME2 expressing dTALe-13, which targets the promoter in Kitaake and other \textit{japonica}-related lines, was used to test the competence of the null mutant lines for disease susceptibility. ME2, which lacks any major TAL effector gene, is weakly virulent, based on lesion length, in Kitaake, \textit{sweet13-1} and \textit{sweet13-2} (Figures 7b, c). Infection with ME2(<\textit{dTALe-13}) resulted long lesions in Kitaake leaves but was incompatible in \textit{sweet13-1} and \textit{sweet13-2}, indicating that an intact coding sequence for OsSWEET13 or gene product in addition to its inducibility is required for TAL effector-mediated susceptibility (Figures 7b, c).

\textbf{Prevalence of \textit{OsSWEET13} Expression in Infections by \textit{X. oryzae} pv. \textit{oryzae} Field Isolates}

The gene \textit{pthXo2} was previously identified only in strains JXO1 and T7174/MAFF 311018 (Yang and White, 2004; Ochiai, et al., 2005). We investigated the distribution of \textit{pthXo2} or functional equivalents (TAL effectors potentially targeting other promoter elements of \textit{OsSWEET13}) within isolates of \textit{X. oryzae} pv. \textit{oryzae} representing diverse geographic areas by profiling \textit{OsSWEET13} induction (Figure S2). A total of 104 strains were used to inoculate IR24 leaves, and mRNA from individual inoculated leaf samples were subjected to qRT-PCR analysis.
for OsSWEET13 expression. Forty-two out of the 104 strains from 11 countries were found to induce OsSWEET13 in IR24 (Table S2). To correlate the OsSWEET13 induction and PthXo2, an attempt was made to identify candidate pthXo2 genes in two strains (PXO339 and PXO163) that were able to cause blight disease in IR24 and not in Kitaake. Whole-genome sequencing of PXO339 and PXO163, using single molecule-real time (PacBio SMRT) sequencing technology, revealed two identical genes that are 99% identical to pthXo2 from T7174/MAFF 311018 and encode TAL effectors nearly identical to PthXo2 in terms of the encoded RVD (Figures 8a, S3). The TAL effector gene capable of conferring susceptibility to IR24 and not Kitaake was retrieved from PXO163 and sequenced (see Materials and Methods). When either the gene or the cosmid clone containing the gene (Cos-70) from PXO163 was introduced into ME2, the strains caused blight symptom and induced OsSWEET13 in IR24 but not in Kitaake (Figures 8b-d). Thus, four strains examined from a collection of X. oryzae pv. oryzae field strains that induce OsSWEET13 all have a pthXo2 related gene and are incompatible on the japonica varieties.

**OsSWEET13 Is Preferentially Expressed in Vascular Tissue**

OsSWEET13 expression under normal growth conditions in rice was assessed by producing transgenic rice with a reporter construct consisting of the Nipponbare OsSWEET13 promoter fused to the coding sequence for β-glucuronidase (uidA or GUS). Twenty-two independent transgenic Kitaake lines were obtained. Representative lines #8 and #12 were analyzed using histochemical and fluorometric assays for β-glucuronidase. When leaves of T1 transgenic plants were inoculated with ME2(dTALe-13), a bacterial strain induce the Nipponbare OsSWEET13 (see details in another section below), the GUS activity was observed to increase compared with the control wherein bacteria lacking the dTAL effector were used, indicating the
functionality of the reporter construct in transgenic plants (Fig. 9A). When root, stem, leaf and floral tissues were stained with 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc), the vascular bundle cells had higher GUS activity in comparison to the surrounding tissues (Fig. 9, B-D).

**Discussion**

We have identified *OsSWEET13* as the targeted S gene for PthXo2 of *X. oryzae* pv. *oryzae* in bacterial blight disease of rice. A number of the findings directly support the conclusion. Susceptibility to a strain with the major TAL effector gene *pthXo2* is tightly linked to polymorphisms at the *OsSWEET13* locus. *OsSWEET13* expression, as demonstrated by microarray and qRT-PCR analyses, was strictly associated both with the presence of *pthXo2* in the inciting strain and host susceptibility to infection. Furthermore, a consensus EBE is present in the *OsSWEET13* promoter in cultivar IR24 and PthXo2 binds specificity to the predicted EBE, supporting the model whereby the promoter is the direct and intended target of PthXo2. Similar to the majority of characterized TAL effectors, EBE$_{PthXo2}$ encompasses the predicted TATAA box of the targeted host gene. Functional analyses of *OsSWEET13* in the host for disease susceptibility also supported the model. Transfer of a DNA fragment that contained the promoter polymorphisms and the *OsSWEET13* coding sequence from IR24 to Kitaake conferred disease susceptibility in a PthXo2-dependent manner. A null mutation in *OsSWEET13* of Kitaake eliminated susceptibility due to a dTALe targeting the TATAA box region of the Kitaake allele. The null mutant plant had no apparent growth or development phenotype of abnormality, indicating SWEETs in rice may be redundant in function or any deleterious effects may not be expressed under well-managed growth conditions. Nonetheless, no null type recessive resistance alleles of SWEET genes are known.
The characterization of the target of PthXo2 has revealed a cryptic recessive gene for resistance in, as yet, all *japonica*-related lines we have examined. A number of polymorphisms are present in both the region corresponding to the EBEs of *OsSWEET13* and the coding sequences of the proteins between IR24 and Kitaake. Similar polymorphisms were noted for the recessive R gene *xa25*, although the results with *xa25* did not pinpoint the critical differences, either promoter or coding sequence polymorphisms between susceptible and resistant lines or the cognate TAL effector (Liu, et al., 2011). In addition, *xa25* was identified in the *indica* cultivar Minghui 63, while the susceptible allele is also in an *indica* background. However, the same sequence region in the susceptible cultivar Zhenshan 97 is different from the sequences of IR24 and Nipponbare. *OsSWEET13* promoter in Zhenshan 97 has only two A residues (2 A’s) compared to four for IR24 (4 A’s) and three for Nipponbare (3 A’s). Thus, either PXO339 has an additional major TAL effector targeting *OsSWEET13* or PthXo2 works on both susceptible alleles. If the latter is true, there are three alleles of *OsSWEET13* EBEs (2, 3 and 4 A’s) for PthXo2, two compatible and one incompatible. Regardless, all the results support a model whereby the difference in susceptibility between IR24 and *japonica*-related lines is due solely to a single base change in the TATAA box region and not due to differences in the SWEET protein product. The change in the *japonica*-related lines throws the predicted alignment of the repetitive domain of PthXo2 out of register with corresponding region in *japonica* lines.

Evidence that EBE variation is indeed the critical difference comes from two observations. First, promoter fusions using the GFP reporter and concomitant expression of PthXo2 function with the IR24 40 bp EBE region and not the same region lacking the fourth A in the TATAA region. Second, targeting the *OsSWEET13* allele of Kitaake by modified PthXo2 derivatives results in host susceptibility, indicating that induction of the endogenous transporter of Kitaake or
Nipponbare is sufficient for host disease susceptibility. The change in PthXo2 that delete a single RVD corresponding to the single nucleotide deletion in Kitaake or Nipponbare switches the effectiveness of PthXo2 to both japonica rice varieties with concomitant loss of effectiveness in IR24. Similarly, a dTALe targeting the TATAA box region of japonica-related allele controls OsSWEET13 expression and disease susceptibility in japonica-related lines but not IR24. The presence of PthXo2 in strains of the pathogen that were isolated in Japan may not be co-incidental as many japonica lines are traditionally deployed in Japan. The presence of genes for both AvrXa7, a major TAL effector targeting OsSWEET14, and PthXo2 in the Japanese type strain T7174 (MAFF 311018) may reflect a virulence shift in the regional pathogen populations at some time in the past due to deployment of japonica-related cultivars. The lack of recognition of the recessive resistance may reflect an ancient selection of japonica lines for resistance.

*OsSWEET13* is one of only three members of clade III sugar transporters in rice that are known to be targeted by field strains of *X. oryzae pv. oryzae*. Sugar transport assays indicate that, like OsSWEET11 and OsSWEET14, sucrose is the preferred substrate of OsSWEET13 in comparison to glucose. Two other SWEETs (OsSWEET11 and OsSWEET12) in the clade III of the family have been demonstrated to be potential *S* genes given as yet unidentified TAL effectors exists in *X. oryzae pv. oryzae* isolates (Li, et al., 2013; Streubel, et al., 2013). A survey of *pthXo2* or functional equivalent genes on the basis of induction of *OsSWEET13* in either IR24 or Kitaake among a collection of 104 pathogen isolates identified 42 of them capable of inducing *OsSWEET13* in IR24 and two additional potential *pthXo2* alleles. One of these was a *pthXo2* homolog from strain PXO339, which was used for the characterization of *xa25*. Our results suggest that targeting *OsSWEET13* is not rare among field isolates. The specificity of each
SWEET exploited by the corresponding TAL effector(s) depends on the EBEs of the S genes and the composition of RVDs of TAL effectors. Disruption of specificity due to the variations of EBEs may confer resistance or the loss of disease susceptibility of host to the TAL effector-dependent strains (Yang, et al., 2006; Chu, et al., 2006).

Ultimately, our goal is to understand SWEET function in disease and development and test our hypothesis that epidemics of bacterial blight and, possibly, other disease with related physiological underpinnings can be hampered by manipulation of SWEET gene expression. OsSWEET13 and members of clade III are sucrose transporters (Chen, et al., 2010; Chen et al., 2012), and the prevailing hypothesis is that some isolates depend on ectopically elevated SWEETs (OsSWEET13 in this case) that primarily leak sucrose into apoplasmic space around the vascular tissue. The efflux of sucrose may provide the carbohydrate nutrient to pathogen or simply deprive the host cell of a source of energy. A surrogate system in *N. benthamiana* leaf cells was tested to determine if, indeed, elevated sucrose concentration in the apoplastic solution result from *OsSWEET13* ectopic expression. Our findings provide evidence supportive of sucrose efflux from plant leaf tissue into the apoplast upon SWEET expression.

Here, we deployed the new CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9) technology to construct a null mutation in *OsSWEET13* [reviewed by (Sander and Joung, 2014)]. Our prior work targeted the EBE sequences in *OsSWEET14* for PthXo3 and AvrXa7 using TALEN (TAL effector nuclease) technology to produce blight resistant rice plants. TALEN-induced disruption of cognition between the RVDs of PthXo3 and AvrXa7 with the EBEs in the *OsSWEET14* promoter resulted unresponsive alleles of *OsSWEET14* to PthXo3 or AvrXa7 and resistance to strains that are
dependent on these major TAL effectors. TALEN-mutagenized rice remains susceptible to pathogen isolates deploying alternative major TAL effectors targeting other SWEET disease susceptibility genes (Li, et al., 2012). Identification of the PthXo2-dependent S gene reveals that, serendipitously, the TALEN generated lines, which have the japonica genetic background, already possess recessive resistance to PthXo2 dependent strains. Further genome editing for multiplexed recessive resistance in the SWEET susceptibility genes may provide broad and durable resistance to bacterial blight as these virulence determinants are conserved among X. oryzae pv. oryzae strains. The effectiveness of this resistance strategy depends on our better understanding the population dynamics of the pathogenic isolates that utilize alternative or combination of the major TAL effectors and could be increased in combination with other R genes.

Methods

Plant Material, Plasmids, Bacterial Strains and Growth Conditions

Rice varieties (Oryza sativa ssp. japonica) Nipponbare, Azucena, Dongjing, Hwayoung, Heijiao, Taipei 309, Kitaake, and IR24 (Oryza sativa ssp. indica) were used in this study. Nicotiana benthamiana was also used in this study. Plasmids, X. oryzae pv. oryzae and E. coli strains are listed in Table S1. All rice plants were grown in growth chamber with the temperature of 28 °C, relative humidity of 85% and a photoperiod of 12 hours. E. coli cells were grown in Luria-Bertani medium with a standard culture technique (Ausubel, et al., 1993). X. oryzae pv. oryzae cells were grown in either nutrient broth (Becton, Dickinson and Company, NJ) or tryptone sucrose medium (tryptone, 10 g/L, sucrose, 10 g/L, glutamic acid, 1 g/L) at 28°C.
Antibiotics used in this study included carbenicillin (100 mg/L), cephalexin (10 mg/L), kanamycin (50 mg/L), tetracycline (12.5 mg/L) and spectinomycin (100 mg/L).

**Gene Expression Analyses**

The rice leaves were inoculated with indicated bacterial strains and used for total RNA extraction 24 hours post inoculation. RNA was extracted using the TRI reagent following the instruction provided by the manufacturer (Ambion/Life Technologies). RNA concentration and quality were measured using an ND-1000 Nanodrop spectrophotometer (Nanodrop Technologies). For microarray hybridization, five micrograms of total RNA of each sample was used for synthesis of cDNA and biotin-labeled cRNA using the One-Cycle Eukaryotic Target Labeling kit according to the manufacturer’s manual (Affymetrix). The processed cRNA was used for hybridization to the GeneChip Rice Genome Array (Affymetrix), which was processed at the Iowa State University DNA core facility. For quantitative reverse transcription PCR (qRT-PCR), one microgram of RNA from each sample was treated with amplification-grade DNase I (Invitrogen) followed by cDNA synthesis using the iScript Select cDNA synthesis kit (Bio-Rad). cDNA derived from 25 ng of total RNA was used for each qRT-PCR reaction with gene-specific primers. The rice gene *TFIIAγ5* was used as an internal control. The real-time qRT-PCR was performed on Stratagene’s Mx4000 multiplex quantitative PCR system using the iQ SYBR Green Supermix kit (Bio-Rad). The average threshold cycle (Ct) was used to determine the fold change of gene expression. The $2^{\Delta\Delta Ct}$ method was used for relative quantification (Livak and Schmittgen, 2001). The primer sequences are provided in Table S3.
**Disease Assay**

Four- to five-week old rice plants with fully expanded leaves were inoculated using leaf tip-clipping method for lesion measurement (Kauffman, et al., 1973) and syringe infiltration for bacterial population counting as described (Yang and White, 2004). Bacterial inoculum with optical density of 0.5 at 600 nm (OD\(_{600}\) = 0.5) was used for inoculation. Bacterial growth in rice leaves was measured by sampling 6 leaves for each treatment 12 days after inoculation by clipping the leaf tip with contaminated scissors. The infected leaves were ground with a mortar and pestle in sterile water, diluted serially, and spread on TSA plates with the appropriate antibiotics. The mean number of colonies in three plates of the proper dilution (10 – 100 colonies) was calculated. One-way analysis of variance (ANOVA) statistical analyses were performed on all measurements. The Tukey honest significant difference test was used for post-ANOVA pair-wise tests for significance, set at 5% (P<0.05).

**FRET Assay on OsSWEET13 Transport Activity Through HEK293T Cells**

Identification of sucrose/glucose transport activity for OsSWEET13 was performed by co-transfecting HEK293T cells with cytosolic FRET sucrose sensor FLIPsuc90μΔ1V plus OsSWEET13 (A) and FLIPglu600μD13V plus OsSWEET13 (B), respectively. Arabidopsis AtSWEET12 and AtSWEET1 were used as positive control for sucrose and glucose uptake, respectively. Each transfection was done in 6-well plates and perfusion experiments were performed as described before (Hou, et al., 2011). HEK293T/ FLIPsuc90mΔ1V cells were perfused with medium, followed by a pulse of 10mM sucrose, while HEK293T/ FLIPglu600μD13V cells were perfused with medium, followed by three pulses of 2.5, 5 and 20 mM glucose.
Electrophoretic Mobility Shift Assay

PthXo2 with an N-terminal 6xHis tag was expressed and purified from *E. coli* BL21 with Ni-NTA agarose (Qiagen) following the manufacturer’s manual. Protein concentration was determined using a Bradford reagent kit (Bio-Rad). Complementary oligonucleotides were labelled with $[^\gamma-^{32}P]ATP$ at the 5’ ends using T4 kinase and annealed. Labeled double-stranded DNA was mixed with 6His-PthXo2 in a reaction containing 15 mM Tris-HCl (pH 7.5), 60 mM KCl, 0.2 mM EDTA, 1 mM DTT, 2.5 mM MgCl$_2$, 2.0% glycerol and 50 ng/µl poly(dI.dC). The concentration of PthXo2, labeled DNA and unlabeled DNA was about 350 fmol, 50 fmol and 0 to 2.5 pmol, respectively.

Construction of Designer TAL Effector and PthXo2 Mutant

A library of four basic repeats derived from *avrXa7* encoding RVDs NI, NG, NN and HD corresponding to nucleotides A, T, G and C, respectively was used to assemble the TAL effector central repeats based on the DNA sequence of target site as described (Li and Yang, 2013). The repetitive fragment was cloned into the repeat-deleted pZWavrXa10, resulting in *dTALe-13* gene consisting of the 5’ and 3’ coding sequences of *avrXa10* and the custom-made repeat domain. The pZWdTALe-13 was further linearized with HindIII and ligated into the HindIII-treated broad host range vector pHM1. For deletion of pthXo2, pZWpthXo2 plasmid DNA was first linearized by restriction enzyme HindIII, and then partially digested with NcoI (New England BioLabs). Fragments in a range of 3.8-4.2 kb were collected through gel electrophoresis, purified, and ligated with T4 ligase (New England BioLabs). The ligated product was transformed into *E. coli*. The desired pZWpthXo2D plasmid with the 5$^{th}$ repeat deletion was
obtained by sequencing the resulting ampicillin resistant colonies. pZWpthXo2D was inserted into pHM1 with HindIII restriction and T4 ligation.

**Construction of OsSWEET13 Reporter Genes**

For promoter analysis, the *OsSWEET13* terminator region was PCR-amplified with primers SWET13P-F3 & SWET13-R3 and genomic DNA of Nipponbare. The amplicon was digested with BamHI and HindIII, and cloned into pCAMBIA1300 (CAMBIA), resulting in pSWT13T. The different fragments of the *OsSWEET13* promoter from Nipponbare were amplified using the specific primers, and amplicons were digested with KpnI and BamHI and cloned into pSWT13T. The primers for each promoter fragment are SWET13P-F1 and SWET13P-R1 for a 2240 bp fragment of IR24, SWET13P<sub>IR24</sub>-F2 and SWET13P-R1 for a 40-bp fragment of IR24, and SWET13P<sub>NIP</sub>-F2 & SWET13P-R1 for the 39 bp fragment of the Nipponbare *OsSWEET13* promoter. The GFP coding region was cloned between the promoter and terminator of *OsSWEET13* at BamHI and XbaI. For ectopic expression of pthXo2, the coding region was cloned downstream of CaMV 35S promoter in vector pBY02 (Antony, et al., 2010). Primers for promoter are provided in Table S3 online.

**Transient Gene Expression in N. benthamiana**

For each assay, *Agrobacterium* transformants with individual constructs were grown in 5 ml Luria-Bertani (LB) broth supplemented with kanamycin (50 µg/ml) and rifampicin (15 µg/ml). One milliliter of overnight culture was subcultured in 50 ml liquid LB supplemented with kanamycin (50 µg/ml) and allowed to grow to an OD<sub>600</sub> of 0.6. The bacterial cells were then collected by centrifugation under room temperature for 10 min at 3000 rpm. The cells from each centrifugation were resuspended in 50 ml *Agrobacterium* inoculation buffer (1/2 Murashige and
Skoog basal salt and 200 µM acetosyringone, pH 5.2) and activated at 28°C for 3 h. Inoculation was performed with single strain or two strains by mixing the cultures in 1:1 ratio prior to inoculation. *N. benthamiana* leaves were infiltrated with bacterial suspension, and the leaves were harvested 48 h after inoculation for subsequent analyses.

**Extraction of Apoplastic Solution from N. benthamiana**

Four-week old *N. benthamiana* plants were grown in growth chamber with a day/night temperature of 25 °C and 18 °C. The *Agrobacterium* cells with p35S:*OsSWEET13* plasmid and p35S:*GFP* (negative control) were separately infiltrated into *N. benthamiana* leaves. Forty-eight hours after infiltration, the inoculated leaves were detached and immersed in water. Repeated pressurization and depressurization with vacuum were applied to infiltrate the HPLC water into the apoplastic space till fully saturated. The leaves were blot dried and weighed, cut into small pieces that were placed in a nylon mesh cell strainer (inside diameter, 2.5 cm). The strainer was placed at the top of the 50 ml polypropylene centrifuge tube. The apoplastic solution was collected through centrifugation at 500 x g for 15 minutes at 4 °C. The concentration of sucrose was determined by using a sucrose assay kit (Sigma-Aldrich).

**Production of Homozygous *ossweet13* Null Mutants**

Embryogenic callus cells derived from the Kitaake immature seed were used for transgenics with CRISPR/Cas9 technology as described (Zhou, et al., 2014). Briefly, gene for the guide RNA targeting a 20-bp region (5’- GCCTGTCCCTGCAGCATCCC -3’) 4 bp downstream of the translation start codon of *OsSWEET13* was constructed and along with Cas9 driven by the maize ubiquitin 1 promoter was transferred into Kitaake callus cells. Primary transgenic plants
with the mutations of 4-bp and 11-bp deletion were self-pollinated and progeny plants were screened for homozygous mutant plants (Zhou, et al., 2014).

TAL Effector Gene Sequencing and Cloning

Genomic DNA of *X. oryzae* pv. *oryzae* strain PXO163 was partially digested with Sau3AI, and DNA fragments larger than 20 kb were separated in 0.8% agarose gel and purified from the gel through electro-elution. The DNA was ligated into BamHI digested and alkaline phosphatase-treated pHM1 and was packaged into MaxPlax™ Lambda Packaging Extracts, and the phages were transduced into X11-blue MRF’ cells, according to the manual of supplier (Epicentre). The library was screened for TAL effector positive clones using a $^{32}$P-labeled repetitive fragment of *pthXo2* as a probe. Individual cosmid clones (n=30) were introduced into ME2 and the transformants were used for disease assay in IR24 and Kitaake. The cosmid that complemented ME2 for virulence in IR24 was digested with BamHI, and the BamHI fragments of TAL effector repeats were subcloned into repeatless pZWavrXa10. The pZW version of TAL effector was subcloned into pHM1 and transferred into ME2 for disease assay in IR24 and Kitaake. The virulent TAL effector clone was sequenced for repetitive region from both ends using the flanking primers. Whole-genome sequencing of PXO339 was performed using the bacterial genomic DNA with standard PacBio-SMRT sequencing method at the Yale Center for Genome Analysis. The genome assembly was carried out using the SMRT Analysis pipeline (Pacific Biosciences).

Histochemical Assay of GUS Activity in Different Organs and Tissues

Histochemical localization of the *OsSWEET13* promoter driven GUS was performed following the method described by Jefferson et al. (Jefferson, et al., 1986) with minor
modifications. The anthers of transgenic rice were stained in the GUS staining buffer (100 mM phosphate buffer (pH 7.0) containing 10mM EDTA, 0.5mM K₃[Fe(CN)₆], 0.5mM K₄[Fe(CN)₆], 0.1% Triton X-100 and 1mM X-Gluc (Sigma)) for 1 hour. The leaves and roots of mature transgenic plants were sectioned into 40-80µm thick pieces using vibratome (Leica VT1000 P) and stained in GUS staining buffer for 3 hours. The stained tissues were destained with 70% ethanol and visualized by using Microscope Axio Imager Z2 ACR.

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References


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Figures and Tables

a

b

![Diagram](image)

Exp. of OsSWEET13 (24hpc)

- Kitaake
- IR24

H₂O ME2 ME2/(pthX02)

0.00 50.00 100.00 150.00 200.00

1.00 1.02 0.58 0.78 1.02

158.31

Chr 12

32kb

OsSWEET13

SM1 (0/456) SM2 (1/456)

191 132 92

949 104 211

2240

pSWT13IR24

- Untranscribed region
- UTR
- Coding region
- Intron

e

>OsSWEET13 GenBank ABA98216

MAGLSLQDLSAPEFLNLSENSFPPYLPFTYAPATTFTFYRITYKISRTGQ5Q5VYWALFSAMLWI

FVLRKREVETVAMGCETITTVNYLAVAMRKAVYFTKTRLLNNGGSGVTLIUTJLEIEKQNVIGLOWSASASSIVYFVQPGILHRVIQSGGWYMRGSLLTJLAVWEI

YGLLIDRKYWAPNLILGTGEM3LVYVYNNATTVAGKGRKGGKLAAARELPWWG

KLAAATFRIIGSRAVHVHHPVSCHAEENAAEAEPEVLWDIPPEPPRPAVAAV
Figure 1. Bacterial blight resistance gene in Nipponbare is linked to OsSWEET13.
(a) Phenotypes of disease reactions in Nipponbare and IR24 upon inoculation with ME2(pthXo2). Arrows indicate the lesion progression. Nip, Nipponbare.
(b) OsSWEET13 induction by PthXo2-expressing X. oryzae pv. oryzae strains in IR24 and Kitaake as measured by qRT-PCR on total RNA extracted from plants 24 hours post inoculation. Numbers above the bars indicate the fold changes in OsSWEET13 expression relative to water treatment.
(c) Linkage of the molecular markers SM1 (Os12g29220/OsSWEET13) and SM2 to cryptic disease resistance gene in Nipponbare. A population of 228 F2 plants (456 gametes) resistant to ME2(pthXo2) was used to determine the recombination event associated with the markers. One recombination occurred with marker SM2 and none with SM1.
(d) Gene structure of OsSWEET13.
(e) Deduced amino acids of OsSWEET13. Regions underlined represent trans-membrane domains.
Figure 2. Transfer of the OsSWEET13 allele from IR24 to Kitaake confers disease susceptibility in a PthXo2-dependent manner.

(a) Disease phenotypes of IR24, Kitaake, and transgenic Kitaake plant containing pSWT13_{IR24} inoculated with ME2(pthXo2). Arrows point to the edges of disease progression. Photographs were taken 14 days post inoculation of 35 day old plants.

(b) Relative gene expression of OsSWEET13 in rice in response to *X. oryzae* pv. *oryzae* strains ME2 and ME2(pthXo2). Values with the same lowercase letters above columns do not differ significantly at the <0.5 level using Tukey statistic and ANOVA analysis. Bars indicate standard deviation (SD).
Figure 3. OsSWEET13 functions as a sugar transport activity.

(a) The sucrose transport activity was assayed in human HEK293T cells which co-expressed the cytosolic FRET sucrose sensor FLIPsuc90μ∆1V and Arabidopsis AtSWEET12 (positive control for sucrose uptake; Chen, et al., 2012) and OsSWEET13, respectively. Individual cells were analyzed by quantitative ratio imaging of eCFP and Venus emission (at 10 second intervals). Cells transfected with sensor only were used as negative controls (“Control”).
Glucose transport activity was assayed by co-expression of the cytosolic FRET glucose sensor FLIPglu600μD13V with AtSWEET1 (positive control for glucose uptake; Chen et al., 2010) and OsSWEET13, respectively, in HEK293T cells. Note that the citrus SWEET1 was tested in parallel; the controls shown here are from the same experiment as shown in Fig. 3b in Hu et al., 2013. Image acquisition and analysis were performed similarly as for sucrose transport activity of OsSWEET13. Accumulation of sucrose (a) or glucose (b) is indicated by a negative FRET ratio change. All experiments were performed at least three times. Error bars are SD.

(c) Transient expression of OsSWEET13 resulted in elevated sucrose concentrations in apoplasms of N. benthamiana leaves. Agrobacterium strain containing the CaMV 35S promoter with OsSWEET13 (35S-OsSWEET13) or without OsSWEET13 (35S-GFP, as negative control) was used in the assay. Sucrose was measured in the apoplastic wash solution of inoculated N. benthamiana leaves. Lowercase letters above columns indicate significant difference at the <0.5 level using Tukey statistic and ANOVA analysis. Bars indicate SD.
Figure 4. EBE of OsSWEET13 from IR24 directs PthXo2-dependent expression in N. benthamiana.

(a) Alignment of PthXo2 RVDs with the predicted OsSWEET13 promoter from IR24 (OsSWEET13IR24) and Nipponbare (OsSWEET13Nip). The dashed line in OsSWEET13Nip denotes the one nucleotide deletion compared to OsSWEET13IR24.

(b) Schematics of the reporter construct containing the GFP gene fused with different promoter regions of OsSWEET13 alleles. Open box represents the promoter region of OsSWEET13 allele from IR24. The red box illustrates the small region upstream of the OsSWEET13 transcription initiation site (+1); the grey box denotes the untranslated region of OsSWEET13, and black box represents the terminator region of OsSWEET13 from IR24. Construct P1 contains a 2240-bp promoter region of the IR24 OsSWEET13 allele; P2 contains only 40 bp of the promoter region of IR24 (nucleotides shown below the bar); P3 contains the 39-bp OsSWEET13 promoter element from Nipponbare.

(c) Transient activation of GFP in reporter constructs with effector constructs of the CaMV 35S promoter directing pthXo2 (35S-pthXo2) or vector control 48 hours post inoculation of Agrobacterium. The images were taken under fluorescence microscope with magnification of 20X.
(d) DNA alignment of the OsSWEET13 promoter elements from eight rice cultivars. The PthXo2-dependent EBE from IR24 is underlined. The hyphen indicates the deletion of 1 bp compared to IR24. “+” and “-” indicate disease susceptibility and resistance, respectively, to X. oryzae pv. oryzae strain expressing TAL effector PthXo2 or AvrXa7.
Figure 5. PthXo2 binds the EBE of OsSWEET13<sub>IR24</sub>.

(a) Oligonucleotide sequences used in EMS assays. The predicted OsSWEET13 EBE for PthXo2 is underlined, and the number above the sequence indicates the positions of nucleotides upstream of the transcription initiation site of OsSWEET13. A mutated version (SWEET13-m) of OsSWEET13 was used as a control.

(b) Competition assay with increasing amounts of unlabeled SWEET13 or SWEET13-m against labeled SWEET13 in the binding reactions with PthXo2.
Figure 6. Designer TAL effector and mutated PthXo2 induce OsSWEET13 and disease susceptibility in *japonica* rice.

(a) Alignment of the RVDs of dTALe-13 and PthXo2D and EBEs in the OsSWEET13 promoters of the IR24 and Nipponbare (SWEET13<sub>IR24</sub> and SWEET13<sub>Nip</sub>).

(b) and (c) *X. oryzae* pv. *oryzae* ME2 containing *pthXo2D* caused blight disease as indicated by lesion length and induced *OsSWEET13* in Nipponbare but not in IR24. Values with the same lowercase letters above columns do not differ significantly at the <0.5 level using Tukey statistic and ANOVA analysis. Bars indicate SD.

(d) and (e), *X. oryzae* pv. *oryzae* ME2 containing *dTALe-13* caused blight disease as indicated by lesion length and induced *OsSWEET13* in Kitaake but not in IR24. Values with the same lowercase letters above columns do not differ significantly at the <0.5 level using Tukey statistic and ANOVA analysis. Bars indicate SD.
Figure 7. Null mutations of *OsSWEET13* confer disease resistance to *X. oryzae* pv. *oryzae*.

(a) DNA sequence alignment of *OsSWEET13* alleles among wild type Kitaake and derived null mutants. Start codon of *OsSWEET13* is in red font.

(b) Plants homozygous for the allele *sweet13-1* or *sweet13-2* were inoculated with *X. oryzae* pv. *oryzae* strains specified, and the lesion length measurement was performed 10 day post inoculation. Values with the same lowercase letters above columns do not differ significantly at the <0.5 level using Tukey statistic and ANOVA analysis. Bars indicate SD.

(c) Disease phenotypes of Kitaake and *OsSWEET13* mutants after inoculation with ME2 and ME2(*dTALe-13*). Photos were taken 12 days post inoculation. Arrows indicate the lesion progression.
Figure 8. PXO339 and PXO163 contain a homolog to PthXo2.

(a) Alignment of EBE nucleotides of the IR24 *OsSWEET13* promoter with RVDs of PthXo2 homologs from JXO1, PXO163, and PXO339.

(b) Disease phenotype induced by *X. oryzae* pv. *oryzae* strains containing TAL effector from PXO163. Leaves of rice cultivars Kitaake and IR24 were inoculated with *X. oryzae* pv. *oryzae* strains ME2 (leaves 1 and 4), ME2(Cos-70) (leaves 2 and 5) and ME2(*pthXo2.1*) (leaves 3 and 6).

(c) Length lesions of disease leaves inoculated with *X. oryzae* pv. *oryzae* strains as specified in (b) and below each graph. The lesions were measured 10 days post inoculation. Values with the same lowercase letters above columns do not differ significantly at the <0.5 level using Tukey statistic and ANOVA analysis. Bars indicate SD.

(d) Gel images of semi-quantitative RT-PCR products of *OsSWEET13* in response to *X. oryzae* pv. *oryzae* inoculation as specified in (b).
Figure 9. *OsSWEET13* promoter directs the GUS reporter gene expression in vascular tissue. a, *OsSWEET13p-GUS* transgene induced by dTALE-13 expressing *X. oryzae* pv. *oryzae* bacterium. The Transgenic plants were inoculated with bacteria as specified below the a axis. b, GUS staining of root. c, GUS staining of cross section of leaf blade. d, GUS staining of anther.
Table 1. Microarray scores of gene expression from rice leaf treated with *X. oryzae* pv. *oryzae*

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a. The score is the average of three samples.
b. Fold change is calculated by dividing the average score of ME2(*pthXo2*) by that of ME2.
Figure S1. Alignment of *OsSWEET13* alleles between IR24 and Nipponbare. The PCR-amplified 5865 bp in IR24 was aligned with the corresponding region from Nipponbare genome (Nip, retrieved from The Rice Annotation Project [http://rapdb.dna.affrc.go.jp/](http://rapdb.dna.affrc.go.jp/)). The Exon sequences in Nipponbare are in bold upper case letters; introns are in lower case letter. The start and stop codons in Nipponbare are in red. The putative TATA box in IR24 is in red at the position of 2202 bp.

(a) sequence information of the Nipponbare *OsSWEET13*

| IR24 | GGATCCGCGGTTTGCCATCGTCACCGCCCTTGTCCCCTCCTGTCGCTGGCACCATCGTCA |
| Nip | GGATCCGCGGTTTGCCATCGTCACCGCCCTTGTCCCCTCCTGTCGCTGGCACCATCGTCA |

(b) Alignment of *OsSWEET13* alleles between IR24 and Nipponbare.

| IR24 | CAGCTTGCCATCGCCGTCGCACCGCCCTTGTCCCCTTCTCGTCATCGGCATCGTCGCCGTTGCCACCGCGGTCATCGTCA |
| Nip | CAGCTTGCCATCGCCGTCGCACCGCCCTTGTCCCCTTCTCGTCATCGGCATCGTCGCCGTTGCCACCGCGGTCATCGTCA |
Nip   CTCGCTTTTACAACCTACACACGAGTATGTGTGCTGCTATATTTAAGAACAATTAAATA 1140
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IR24  CGAGTACCTGTATCAATTTTGGATTTAAAATATATGTATGATCATATATATTTCAAAAT 1196
Nip   CGAGTACCTGTATCAATTTTGGATTTAAAATATATGTATGATCATATATATTTCAAAAT 1200
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IR24  TAAATGATGTATATATATGCGATAGTTGCTGATATATATTTGTGCTGATGCTTATAGGT 1256
Nip   TAAATGATGTATATATATGCGATAGTTGCTGATATATATTTGTGCTGATGCTTATAGGT 1260
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IR24  TGATGGAAAGGCAAGACCGCGCCGGCCAGAGGGAAAAAGTAGAGAGAGAAAAAAGCAG 1316
Nip   TGATGGAAAGGCAAGACCGCGCCGGCCAGAGGGAAAAAGTAGAGAGAGAAAAAAGCAG 1318
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Nip   ATCCACTAGCTTGTGTCTTATACGTGATGAGTCTTTAGTACAGGGGTGTGCTGAGAAG 1378
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IR24  CAGAAATCGACCTGTCTTCCCAACAAACTGTGTATGATAGGTTAGTCGACAGGGATGCT 1436
Nip   CAGAAATCGACCTGTCTTCCCAACAAACTGTGTATGATAGGTTAGTCGACAGGGATGCT 1438
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Nip   GAAACTATAGTATACCTACATGTATCTATCACCCAATAATTGCAAATCTATGCTGACAG 1498
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Nip   CTGACAATGCAAAAGAATATTCTGCTAATACTTGGGAGAGTGAAGATCTCATCTTA 1918
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IR24  GCAAAAGGCAATCTTCAGATCTTCAGACTTTCGCTAATTGCTGTAAGAGAGATCTCATCTTA 2036
Nip   GCAAAAGGCAATCTTCAGATCTTCAGACTTTCGCTAATTGCTGTAAGAGAGATCTCATCTTA 2038
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Nip   TAGTTTTAACACTGTTCATGTACAATGTTTGACCGTTCTTCTTAT TTGAGAAATAATTAT  5791

IR24  -----------------------------                      ---------------
Nip   ATGTATTCAAAC--T  5625

IR24  -----------------------------                      ---------------
Nip   GATATCTATTGCTGCACTTATTTTGGGACGAAGTTAGCATGTAGT ATGTATTCAAAC--T  5685

IR24  -----------------------------                      ---------------
Nip   GATGCTTTCCAGGTTCATTAATTAGGAAAATGACGTAGTTTATGA TTGGTCTAAACGGTT  5971

(c) Predicted protein alignment of OsSWEET13 between IR24 and Kitake
Kit  MAGLSLQHPWAFAFGLLGNLISFTTYLAPIFTFYRIYKSKSTEGFQ SVPYVVALFSAMLWIFYA
IR24 MAGLSLQHPWAFAFGLLGNLISFTTYLAPIFTFYRIYKSKSTEGFQ SVPYVVALFSAMLWIFYA

Kit  LIKSNEALLITINAAGCVIETIYIVMYLAYAPKKAKVFTTKILLLL NVGVFGVILLLTLLLSHG
IR24 LIKSNEALLITINAAGCVIETIYIVMYLAYAPKKAKVFTTKILLLL NVGVFGVILLLTLLLSHG

Kit  EQRVVSLGWVCVAFSVSVFVAPLSIIKRIQVQSRVEYMPFSLSLTLTSLAVVWFLYGLLIKDKY
IR24 EQRVVSLGWVCVAFSVSVFVAPLSIIKRIQVQSRVEYMPFSLSLTLTSLAVVWFLYGLLIKDKY

Kit  VALPNILGFTGFVVQMGVYMNAPVAGEGKEGKGLAAAEELPVVNVGKLAATPDRSTG
IR24 VALPNILGFTGFVVQMGVYMNAPVAGEGKEGKGLAAAEELPVVNVGKLAATPDRSSG

Kit  AVHVHSVPRSCAAEAEEAEPEVLVDIPPPPPRAVEVAAV
IR24 AVHVHSVPRSCAAEAEEAEPEVLVDI--PPPPRAVEVAAV
Figure S2. The global locations of 104 *X. oryzae* pv. *oryzae* strains collected and assessed for *OsSWEET13* induction in IR24
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Table S2. OsSWEET13 induction in IR24 by different X. oryzae pv. oryzae field strains.
Table S3. Primers and sequences used in this study.

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CHAPTER 4. XANTHOMONAS EFFECTOR XOPZ TARGETS A PUTATIVE HOST E3 UBIQUITIN LIGASE AND SUBVERTS IMMUNITY IN RICE

A manuscript is in preparation

Junhui Zhou, Huanbin Zhou, Bo Liu, Shinian Chr, Zhiyuan Ji, and Bing Yang

Abstract

Pathogen effector XopZ is one of 18 non-TAL effectors identified from Xanthomonas oryzae pv. oryzae strain PXO99A that contribute virulence to bacterial blight of rice. Here we show that XopZ interacts with a putative host E3 ubiquitin ligase protein PBP (s-ribonuclease) in vitro and in vivo. The N-terminus of XopZ, containing two NLS signals, is sufficient to interact with PBP in the nuclei of plant cells. PBP is a negative regulator of host immune response based on the disease phenotype in PBP-knockout rice plants. Degradation of PBP is not dependent on 26S proteasome but on C1A, a cysteine protease C1A (papain-proteases, family C1, clan CA). Our study shows that C1A directly interacts and strongly degrades PBP through its cysteine protease activity, leading to a homeostatic state of PBP in plant cells. The function of XopZ is to stabilize PBP in the nucleus and inhibit PBP degradation-mediated by C1A. These results suggest that XopZ may function to interfere with the homeostatic state of the negative regulator (PBP) in immune system in rice, and subvert the plant immune response.

Introduction

Plant pathogens and their hosts have been engaged in an evolutionary “tug-of-war”, in which the pathogens always try to invade the host plants and overcome Pathogen-Associated-Molecular-Pattern Triggered Immunity (PAMP-triggered Immunity, or PTI), also called plant
basal defense, mostly through secreting effector proteins into host cells. On other hand, host plants have co-evolved a myriad of specific resistance (R) proteins that directly or indirectly recognize pathogen effectors, leading to so-called effector-triggered immunity (ETI) and restriction of further proliferation of pathogens (Chisholm et al., 2006; Bouwmeester et al., 2011).

In *Xanthomonas* bacteria, about 39 families of type III effectors were classified and were named as *Xanthomonas* outer proteins (Xop) (White and Yang, 2009). However, only a few of them were documented to be involved in regulating host immunity. AvrBs2 is the first Xop effector that is reported to enhance bacterial growth (Kearney and Staskawicz, 1990; Wichmann and Bergelson, 2004). Another *Xanthomonas* effector XopD encodes a cysteine protease that cleaves the small ubiquitin modifier (SUMO) precursors and compromises the expression of defense-related genes in tomato (Hotson et al., 2003; Kim et al. 2008). XopN from Xcv was also shown to interact with a tomato atypical receptor-like kinase (TARK1) and Tomato Fourteen-Three-Three isoforms (TFT1) (Kim et al., 2009). A recent report shows that XopN facilitates the TARK1/TFT1 complex formation in planta and promotes bacterial pathogenesis (Taylor et al., 2012). XopR from Xoo strain MAFF311018 was recently found to suppress basal defense responses in plants (Akimoto-Tomiyama et al., 2012). In addition, Xoo1488 from *Xanthomonas oryzae pv. oryzae* was indicated to inhibit peptidoglycan (PGN)- and chitin-induced immunity by suppressing OsCERK1-mediated phosphorylation of OsRLCK185 and inhibiting chitin-induced MAP kinase activation (Yamaguchi et al., 2013).

Ubiquitination or Proteolysis caused by ubiquitin system and 26S proteasome has been well-acknowledged for regulation of a myriad of biologic processes, such as gene induction, hormone (JA, ET) signaling, Programed cell death (PCD), and plant immunity. Ubiquitination is
achieved through an enzymatic cascade that transfers ubiquitin (Ub) from ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin-ligating enzymes (E3), leading to the production of Poly-Ub chain on the substrates. Usually, Lys(11), Lys(29)- and Lys(48)-linked poly-Ub chains target substrate proteins for degradation by 26S proteasome, which consists of a 20S barrel-shaped proteolytic core and two 19S regulatory “caps” at two ends (Zeng et al., 2006; Vierstra et al., 2009; Dudler et al., 2013).

During the process of protein ubiquitination, E3 ligases confer specificity to the ubiquitin-proteasome system (UPS) by selecting the specific substrate proteins for ubiquitination (Deshaies et al., 2009; Vierstra et al., 2009). There are four major groups of E3s in eukaryotes based on the structures and modes of action: HECT, RING, U-box, and Cullin-RING ligase (CRLs) sub-groups. The RING/U-box type (PUB) E3 ligases, the most abundant E3 ligase group in eukaryotes, are characterized by forming a rigid, globular scaffold for direct transfer of Ub from E2 to the substrates (Deshaies et al., 2009; Trujillo et al., 2010; Hua et al., 2011). Each organism contains a large number of E3-coding genes, it is reported that about 600 different RING domain E3 ligases are potentially expressed in human (Mazzucotelli et al., 2006; Li et al., 2008; Hua et al., 2011).

Accumulating evidence indicate that ubiquitination also plays an important role in pathogen-host interaction and pathogenesis, not only by pathogen effectors but also by host gene products. From the effector aspect, one of the most striking examples is the *Pseudomonas syringae* pv. *tomato* (Pst) effector AvrPtoB, which contains a C-terminal E3 ubiquitin ligase activity domain that can target and ubiquitinate a host kinase Fen and disrupt plant immunity (Abramovitch, et al., 2006; Janjusevic et al., 2006; Rosebrock et al., 2007). Other reports show that AvrPtoB is also involved in ubiquitinating FLS2 and CERK1 in *Arabidopsis* and
suppressing not only ETI but also PTI (Göhre et al., 2008; Gimenez-Ibanez et al., 2009). Recently, another type III effector XopL from *Xanthomonas campestris* pv. *vesicatoria* was shown to be a new class of E3 ubiquitin ligases that mediates the formation of K11-linked polyubiquitin chain via its C-terminal region, leading to suppression of plant immunity (Singer et al., 2013). Some F-box containing T3Es, like XopI from *X. campestris* pv. *vesicatoria* strain 85-10, was also identified during Xcv infection in resistant pepper plants (Schulze et al., 2012; Ustun et al., 2014)

In addition, some virulence effectors may serve as small ubiquitin-like modifier proteases. The effector XopD, for example, from Xcv was reported to encode a Cys protease localized in subnuclear foci that cleaves the small ubiquitin modifier (SUMO) precursors in tomato (Hotson et al., 2003; Kim et al., 2008), leading to the delayed tissue degeneration and reduced chlorophyll loss, as well as decreased hormone (salicylic acid) levels. XopD prevents ethylene-mediated defense response by targeting tomato transcription factor SIERF4 and catalyzing SUMO1 hydrolysis of SIERF4 (Kim et al., 2013). It was also shown that both the conserved ERF-associated amphiphilic motif and protease activity were required for the XopD-dependent leaf necrosis in *N. benthamina* (Hotson et al., 2003; Kim et al., 2008). Intriguingly, XopD from *X. campestris* pv. *campestris* (8004) was shown to target and suppress the proteasome-mediated degradation of DELLA protein RGA (repressor of ga1-3) in the nucleus, leading to repressed GA signaling pathway and enhanced plant tolerance to biotic and abiotic stresses (Tan et al., 2014).

Several plant RING/U-box type (PUB) E3 ligases were reported to serve as positive or negative regulators in the plant immune system (Trujillo et al., 2010). For example, *Avr9/Cf-9 Rapidly Elicited* genes *ACRE74 (CMPG1)* and *ACRE276 (PUB17)* were shown to be
indispensable for HR response caused by the perception of Avr9 peptide by Cf-9- and Cf-4-receptor-like protein in *N. benthamiana* (González-Lamothe et al., 2006; Yang et al., 2006). Recently, Bos et al (2010) showed that CMPG1 physically interacts and is stabilized by the *P. infestans* effector AVR3a, which is essential for pathogen virulence by preventing host cell death during pathogen infection. Prior study also suggests that homodimerization of N-terminal translocation region (RxLR leader) in AVR3a plays an important role in this process (Wawra et al., 2012). Another group of three U-box type E3 ligases, PUB22, PUB23, and PUB24 were reported to negatively regulate PTI and ETI, as the triple mutant *pub22/pub23/pub24* was shown to decrease the downstream regulation of PTI (Trujillo et al., 2008). The rice XA21 binding protein 3 (XB3) is another reported E3 ligase that is recognized by XA21 Ser/Thr kinase. Study showed that XB3 physically interacts with XA21 through its Ankyrin domain and positively regulates the level of XA21 protein (Wang et al., 2006).

Several other type III effectors are also revealed to interact with UPS components. For example, XopJ from Xcv strain 85-10, a member of cysteine protease/ acetyltransferases (Hotson and Mudgett, 2004; Lewis et al., 2011), was recently shown to interact with RPT6 (RP ATPase 6) of the 26S proteasome and inhibit the proteasome activity, leading to decreased SA accumulation (Üstün et al., 2013). YopJ from *Yersinia pestis* shows de-sumoylating and de-ubiquitinating activities to their target proteins (Mukherjee et al., 2006), implying its roles in manipulation of plant UPS. AvrBsT from Xcv 75-3 was identified to acetylate ACIP1 and mediate immune response in *A. thaliana* (Cheong et al., 2014). In addition, PthA 2 and 3 from *X. axonopodis pv. citri* were recently shown to interact with ubiquitin-conjugation enzyme complex Ubc 13-Uev that is involved in ubiquitination of target proteins related to DNA repair (Domingues et al., 2010). A recent report also revealed XopP* xoo* inhibits the ubiquitin ligase
activity of rice U-box E3 ligase OsPUB44, leading to suppression of PGN- and chitin- triggered immunity in rice (Ishikawa et al., 2014).

RING finger E3 ligases act as monomers, dimers or multi-subunit complexes, and homodimerization is the most common way to activate their E3 ligase activity (Deshaies et al., 2009; Metzger et al., 2012). It seems that homodimerization usually occurs on RING finger domain or its surrounding regions, such as of the RING finger E3 ligases TRAF2 (TNF receptor-associated factor 2) (Polekhina et al., 2002), RNF4 (ring finger protein 4) (Mackey et al., 2002; Mace et al., 2008), cIAP (cellular inhibitor of apoptosis) (Liew et al., 2010), and Cullin3 (Choo et al., 2012). Dimerization promotes E3 ubiquitin ligases probably through their abilities to form a scaffold for substrate (SCF) or to increase the substrate affinity (Fbw7/Hdc4) (Tang et al., 2007; Welcker et al., 2007; Deshaies, et al., 2009; Liew et al., 2010; Choo et al., 2012; Johnson et al., 2012).

Auto-ubiquitination is another common feature of E3 ubiquitin ligases (Lorick et al., 1999; Bie et al., 2011), which plays a pivotal role in maintaining the homeostatic state of E3 ligases in organisms, though the mechanism of self-catalyzed ubiquitination remains largely elusive. A group of E3 ligases like E6-AP and RING ligase SIAH1 were shown to employ intermolecular transfer of ubiquitin (Nuber et al., 1998; Hu et al., 1999). Another group of E3 ligases like TRAF requires the homodimerization for its auto-ubiquitination activity (Galan et al., 1999; Lamothe et al., 2007; Yin et al., 2009). Up to date, there are mainly three major hypotheses to address the mechanism of auto-ubiquitination: (1). Substrate-independent auto-ubiquitination such as for Mdm2, in which the E3 ligase self-degradation activity is determined by its abundance and too much of E3 accelerates the auto-ubiquitination (Fang et al., 2000); (2). Substrate-dependent auto-ubiquitination such as for CBL, in which both of the E3 ligase and its
substrates are ubiquitinated (Ryan et al., 2006); and (3). Auto-ubiquitination of E3 ligase can be inhibited by binding with substrates (Petroski et al., 2005).

Plant cysteine-proteases (CysProt), especially CysProt papain-proteases of the C1A group (family C1, clan CA), were shown to be involved in diverse biologic processes such as senescence, abscission, programmed cell death, fruit ripening and systemic defense (Van der Hoorn 2008; Martinez et al., 2012; Dudler, 2013). Therefore, manipulation of host proteasomes is usually a virulence mechanism for plant pathogens. One of the most striking examples is AvrPtoB, by mediating the ubiquitination of Fen via its C-terminal U box domain, it marks Fen for degradation through 26S proteasome (Rosebrock et al., 2007). Other reports revealed two structurally related kinase-binding domains at the N terminus of AvrPtoB, with the first one binding to CERK1 and the second binding to FLS2 and BAK1 (Gohre et al., 2008; Gimenez-Ibanez et al., 2009; Cheng et al., 2011). In addition, P. syringae effectors AvrPphB and AvrRpt2 function as cysteine proteases that recognize and cleave specific amino acid sequences in their respective substrate proteins BIK1 and RIN4 (Shao et al., 2003; Day et al., 2005; Kim et al., 2006). HopM1 and HopZ1 have been shown to induce the degradation of host target proteins MIN7 and GmHID1, respectively, although their pathogenic mechanisms are still elusive (Nomura et al., 2006; Zhou et al., 2011).

*Xanthomonas oryzae* pv. *oryzae* (Xoo) is the causal agent of bacterial blight in rice, also a model pathosystem studying plant-pathogen interactions (Nino-Liu et al., 2006; Zhang and Wang, 2013). Xoo depends on a type III secretion system (T3SS) to deliver a number of effector proteins into host cells and promote disease susceptibility. Previous study of Type III effectors in *Xanthomonas oryzae* pv. *oryzae* had identified 18 non-TAL effectors from Xoo strain PXO99A based on the presence of plant-inducible promoter (PIP) sequence and the sequence
similarity to effectors from other bacteria. Mutagenesis of 18 non TAL T3 effectors in Xoo strain PXO99A revealed XopZ(PXO99A) contributes virulence to Xoo strain PXO99A for bacterial blight of rice (Song and Yang., 2010). There are two identical loci of XopZ$_{PXO99}$ in PXO99A due to a duplication of 212kb in the genome (Song and Yang, 2010), and knockout of both XopZ copies reduces virulence of PXO99A. A recent report (Sinha et al., 2013) indicated that XopZ also functions as a suppressor of LipA-induced innate immune responses since the mutation of XopZ partially compromises virulence while quadruple mutant of XopN/XopQ/XopX/XopZ induces calluses deposition just similarly to Xoo T3S- mutant in rice leaves.

Previous data suggest that XopZ plays a conserved role in Xanthomonas spp., even though, the molecular mechanism of XopZ in contributing the virulence to PXO99A in rice remains elusive. In this study, we demonstrated that the host protein PBP is directly targeted by the XopZ effector. We show that XopZ stabilizes the putative E3 ligase PBP and prevents its degradation-mediated by a cysteine protease in plant cells. The roles of PBP in plant immune response were also examined.

**Results**

*Xanthomonas* Effector XopZ Is a Virulence Factor in Rice

Previous mutagenesis of XopZ indicated that it contributes to the virulence to Xoo strain PXO99A by suppressing PTI in *N.benthamiana* leaf (Song and Yang, 2010). Given the possible functional redundancy of XopZ, XopN and XopV as detected in our virulence assay (unpublished data), and to further evaluate the virulence function of XopZ in rice cells, we constructed a XopZ/XopV/XopN triple mutant PXO99A strains (referred to as PXO99A$\Delta$ZVN) and a XopV/XopN double mutant PXO99A strains (referred to as PXO99A$\Delta$VN). These two
mutant strains and wild type PXO99A strain were inoculated into rice cultivar Kitaake leaves. The leaves lesion caused by strain PXO99AΔZVN is significantly shorter compared to these caused by wild type strain PXO99A and double mutant PXO99AΔVN (Fig 1), suggesting the virulence function of XopZ in PXO99A strain.

**XopZ Is Distributed in Both Cytoplasm and Cell Nucleus in Plant Cells**

To investigate the subcellular localization of XopZ in plant cells, we transiently expressed GFP-XopZ fusion protein in *N. benthamiana* through *Agrobacterium*-mediated gene transfer, but could not detect a fluorescence signal under confocal microscopy. We, thereafter, transiently expressed XopZ in rice protoplasts and performed cell fractionation following the protocols as described previously (Yanagisawa et al., 2003). XopZ was almost equally distributed in cytoplasmic fractionation and nuclear fractionation (Fig 2A), as indicated by anti-Tubulin (cytoplasmic marker) and anti-Histone 3 (nuclear marker) antibodies, respectively. The results validate the prediction of cellular localization of XopZ that contains two predicted Nuclear Localization Signals (NLS) at the N-terminus of XopZ (Fig. 2B) and several predicted Nuclear Export Signals (NES) (Fig. S1A). The existence of NLS and NES in XopZ suggests that XopZ may shuttle between cytoplasm and nucleoplasm in plant cells (Fig S1B). To test this hypothesis, we generated a XopZ mutant with the two putative NLSs changed (KRRK->AAAA, KRR->AAA) (Fig. 2C) and performed the cell fractionation assay in rice protoplasts. As expected, the mutation of NLS signals in XopZ almost abolished its nuclear localization (Fig. 2D).
XopZ Interacts with a Putative Host E3 Ligase PBP

To elucidate the mode of action underlying XopZ-mediated virulence function in rice, we performed the yeast two-hybrid (Y2H) screening to identify the potential host targets of XopZ with a rice cDNA library. In total 120 positive yeast clones were acquired from ~10^7 yeast clones (Table 1). Among them, 44 positive prey clones contain different nucleic acid fragments that match the rice gene Os03g46570. These clones contain different 5’ end truncations of 22 bp, 76 bp, 111 bp, 351 bp and 423 bp, respectively. Os03g46570 is predicted to encode a putative \textbf{P}rotein \textbf{B}inding \textbf{P}rotein (PBP), a member of S-ribonuclease binding proteins (SBP1) family that contains a C terminal RING-HC domain. Database search reveals that PBP matches the *Oryza sativa* SKIP interacting protein 31 (SIP31), one of 30 proteins that interact with OsSKIPa (a rice homolog of human Ski-interacting protein) in Y2H; OsSKIPa has been implicated in regulation of stress tolerance and cell viability (Hou et al., 2009). Phylogenetic analysis indicated that PBP shares the highest amino acid similarity with XM_006651607 from *Oryza brachyantha*, a BOI-related E3 ubiquitin ligase 3-like protein (Fig S2), which usually is involved in suppression of caspase activation and attenuates cell death. We, thereafter, chose PBP for further characterization as the host target of XopZ.

To further confirm the interaction between effector XopZ and PBP, the full-length PBP was cloned from the rice cDNA library and cloned into GAL4 AD containing vector pGAD T7, followed by co-transforming with a construct expressing XopZ (pGBK T7-XopZ) in yeast strain AH109. As shown in Fig 3A, the reporter genes (*His3*, *Ade2* and *LacZ*) were activated in the presence of XopZ and PBP in yeast, but not in yeast containing the empty vector control. To pinpoint the specific region in XopZ that interacts with PBP, a series of XopZ with the N-terminal and C-terminal truncations were co-expressed with PBP in yeast AH109. The results
indicated that the N-terminus of XopZ (XopZ_{1-265aa}) was sufficient to interact with PBP (Fig S3). \textit{In vitro} Glutathione S-transferase (GST) pull down assay was also performed to test this interaction, GST-XopZ, but not GST alone, pulled down a significant amount of MBP-PBP protein (Fig 3B), indicating a direct interaction between PBP and XopZ.

The \textit{in vivo} specific interaction between PBP and XopZ was further examined using the bimolecular fluorescence complementation (BiFC) assay in \textit{N. benthamiana} leaves. The N-terminal YFP fused with PBP (PBP-nYFP) and C-terminal YFP fused with the N-terminal region of XopZ (XopZ_{1-309aa}-cYFP) were co-expressed in \textit{N.benthamiana} leaves using \textit{Agrobacterium}-mediated infiltration. As shown in Fig. 3C, the re-constitution of florescence signal was visualized in the tobacco leaves expressing constructs PBP-nYFP and XopZ_{1-309aa}-cYFP, but not in leaves with empty vector control. The physical interaction between XopZ and PBP was also examined by \textit{in vivo} co-immunoprecipitation (Co-IP) assay. Both anti-FLAG magnetic bead that bind PBP-3*FLAG protein specifically pulled down the XopZ-3*HA protein, as detected by antibody against HA (Fig 3D). Taken together, the pathogen effector XopZ physically interacts with the host proteins PBP in plant cells.

To investigate the domain in PBP that interacts with XopZ, a series of N- and C-terminally deleted PBP gene fragments were generated and co-transferred with XopZ_{1-309aa} in yeast AH109. The results suggest that regions containing 193 aa-225 aa of PBP is required for interacting with XopZ_{1-309aa} (Fig S4A). As many E3 proteins require dimerization for the E3 ubiquitin ligases activities (Welcker et al., 2007; Yin et al., 2009; Liew et al., 2010; Wawra et al., 2012; Johnson et al., 2012), we also examined if PBP, by functioning as a putative E3 ligase, involves in self-association. As shown in Fig S4B, PBP forms homo-dimers or homo-polymers
based on Y2H assay, and the motif containing peptide 137aa-192aa of PBP is responsible for its self-association. The predicted motifs in PBP were shown in Fig S4C.

XopZ-PBP Interaction Primes the Distribution of XopZ

Rice gene *pbp* (*Os03g46570*), localized to the short arm of rice chromosome 3, is predicted to encode a polypeptide of 319 amino acids. To test the subcellular localization of PBP in rice cells, the construct expressing 35S:GFP/PBP was transiently expressed in rice protoplasts isolated from Kitaake and the fluorescence signal was predominately visualized in the cell nucleus in the rice protoplasts 12 hours post transfection (Fig 4A and Fig S5), and a significantly large proportion of GFP-PBP proteins is also visualized in cytoplasm after 18 hours post transfection (Fig 4A.). To corroborate this result, a cell fractionation assay was performed in rice protoplasts expressing PBP proteins activated by 35S promoter (35S: PBP). The enrichment of PBP protein in each fraction was indicated by immunoblotting with anti-PBP primary antibody (GeneScript Inc.) (Fig S6), The PBP signal was predominately detected in the nuclear fraction in rice protoplast 5 hours post transfection. However, the cytoplasmic fractionation also exhibits immunoblot signal 12 hours post transfection (Fig 4B). The similar result was validated in the rice protoplasts expressing PBP protein induced by native PBP promoter (PBP pro: PBP) (Fig 4C), although the immunoblot signal is much weaker compared to that induced by 35S promoter. Thereafter, we are applying 35S promoter in our subsequent PBP transient expression experiments. The localization pattern of PBP was also examined in *N. benthamiana* leaves, by transiently expressing binary vector of 35S:GFP/PBP through *Agrobacterium*-mediated gene transfer, the result shows that fluorescence signal caused by PBP/GFP fusion protein is
predominately visualized in nuclei 40 hours post infiltration, but is obvious in nucleus, cytoplasm and plasma membrane 2.5 days post infiltration (Fig S7).

Sequence analysis of PBP revealed a NLS signal at the N-terminus of PBP (108-111aa) (Fig 4D) ([http://www.sbc.su.se/~maccallr/nucpred/cgi-bin/single.cgi](http://www.sbc.su.se/~maccallr/nucpred/cgi-bin/single.cgi)). To examine if this NLS signal is responsible for the nuclear distribution of PBP, an NLS mutant (RKRK to AAAA) was generated through PCR-mediated site-directed mutagenesis. The subcellular localization of PBPΔNLS was tested by transiently expressing GFP-PBPΔNLS in rice protoplasts and visualizing fluorescence signal through confocal microscopy, as expected, PBP lacking NLS motif only shows cytoplasmic distribution but not nuclear distribution (Fig 4E). This result was also confirmed by cell fractionation assay performed in rice protoplasts, which showed that PBPΔNLS protein is predominately distributed in the cytoplasmic fractionation 12 hours post transfection (Fig 4F), and the interaction between XopZ and PBPΔNLS is confirmed in Y2H assay (Data not shown). Taken together, the NLS signal (108-111aa) determines the nuclear distribution of PBP.

Given the predicted nucleo-cytoplasmic distribution of XopZ as described above (Fig 1), we tested if the XopZ-PBP interaction will affect XopZ cellular localization. For this purpose, we co-transfected XopZ with empty vector pUC19 and two pUC19 constructs expressing 35S:PBP-3*FLAG and 35S:PBPΔNLS-3*FLAG in to rice protoplasts and performed cell fractionation assay in these three batches of protoplasts, respectively. As shown in Fig 4G, the interaction of XopZ-3*HA and PBP-3*FLAG leads to the predominately nuclear distribution of XopZ, nevertheless, the association of XopZ-3*HA and PBPΔNLS-3*FLAG results in the predominately cytoplasmic distribution of XopZ. Considering the distribution of XopZ in rice
protoplasts that only expressing construct 35S:XopZ-3*HA (Fig 2A and Fig 4G), we proposed that the interaction of XopZ and PBP primes the localization of XopZ.

**PBP Degradation Is not Dependent on 26S Proteasome**

Protein structure analysis suggests that PBP protein is a putative E3 ligase containing a typical RING finger motif at its C-terminus, which is usually indicative of the E3 ubiquitin ligase activity and auto-ubiquitination activity that mediates protein degradation through 26S proteasome. To test the stability of PBP in plant cells, 35S:GFP-PBP-3*FLAG was transiently expressed in *N. benthamiana* leaves through *Agrobacterium*-mediated gene delivery and visualized at different time courses (20 hpi (hours post infiltration), 40 hpi, 60 hpi, 3 dpi (days post infiltration), and 4 dpi) using confocal microscopy. As shown in Fig S8A, fluorescence signals were gradually diminished after 40 hpi co-agroinfiltration with or without P19 (P19 is an RNA silencing suppressor to boost expression of PBP), fluorescence signal in *N. benthamiana* leaves co-agroinfiltrated with construct expressing 35S:GFP-3*FLAG was not decreased in the same time courses, indicating that decreased protein levels of GFP-PBP-3*FLAG protein were not caused by gene silencing. Similar observations were noticed when detecting PBP and GFP protein levels using anti-FLAG antibody in *N. benthamiana* leaves (Fig S8B). To test if instability of PBP was mediated through the 26S proteasome, 26S proteasome inhibitor MG132 was co-infiltrated with *Agrobacterium* strains carrying construct expressing 35S:PBP-3*FLAG*. PBP protein level was not increased after MG132 treatment in *N. benthamiana* leaves, indicating that PBP degradation is not 26S proteasome-dependent (Fig S8C), but through other protein degradation pathways.
PBP Is Negatively Regulated by a Host Cysteine Protease C1A

To investigate the roles of PBP in plant immune system, we performed another round of Y2H assay to identify candidate PBP-interacting proteins in rice, particularly focusing on the protein degradation components. Twelve positive yeast clones were acquired that contain the gene fragments encoding seven groups of different host proteins, including two putative RING type E3 ubiquitin ligases, suggesting PBP might interact with other E3 ligases and form an E3 ligase complex in the rice cells. In addition, 2 yeast clones containing the DNA fragments that encode a C1A type protein, a specific cysteine protease in plant cells that missing the N-terminal 80aa (C1A<sub>80-458aa</sub>) (Table2).

The gene (Os04g0650000) that encodes a 364 aa C1A protein is located in chromosome 4 in <i>japonica</i> rice. The C1A protein contains a cathepsin propeptide inhibitor I29 domain at its N-terminus (40 aa-99 aa) and a peptidase C1A domain [cd02248] following the I29 domain and a GRAN domain [smart00277] at its C-terminus. The interaction between PBP and C1A was further tested by yeast two-hybrid assay. As indicated in Fig 5A, the interaction between the full-length C1A protein and PBP is barely detected, however, C1A missing the N-terminal 80aa (C1A<sub>80-458aa</sub>), in which the I29 inhibitor domain was truncated, strongly interacts with PBP in the Y2H assay. A Far Western blotting was also performed to confirm the physical interaction between PBP and C1A, the results showed that the C1A-containing spots strongly pull down PBP proteins (Fig 5B, spots #3 and #4), but the spot containing MBP protein could not pull down PBP protein (Fig 5B spots #5). We failed to detect PBP-C1A interaction <i>in vivo</i> (Co-IP and BiFC) probably due to the protein instability of PBP and C1A in plant cells.

We proposed that C1A might act downstream of PBP (substrate) or upstream of PBP. To investigate the interaction mechanism between C1A and PBP, the constructs for transiently over-
expressing C1A-3*MYC and PBP were co-transfected into protoplasts of rice and *N. benthamiana* leaves, respectively. As expected, C1A down-regulates PBP dramatically in both rice (host) protoplasts (Fig 6A, lane 1 to 3) and *N. benthamiana* (non-host) protoplasts (Fig S9, lane 1 to 3). However, PBP does not dramatically affect C1A protein level (Fig 6A). Transcriptional levels of PBP in the protoplasts expressing these constructs were similar based on semi-quantitative RT-PCR assay (Fig S10). These results indicate that C1A down-regulates PBP at the post-transcriptional level.

To test if PBP degradation-mediated by C1A is dependent on the cysteine protease activity, we generated the C1A mutant with mutation on the active cysteine residue (C153A) that is responsible for cysteine protease activity in C1A, and co-transfected the constructs expressing C1A<sup>C153A</sup>-3*MYC and PBP in rice protoplasts and tobacco protoplasts, respectively. Both of them show that PBP degradation was almost totally abolished by C1A<sup>C153A</sup> mutant (lane 5 of Fig 6A and Fig S9). In addition, we applied different concentration of cysteine proteinase inhibitors E64 (0uM, 1uM, 5uM, 10uM and 20uM) in the rice protoplasts co-expressing C1A and PBP, as shown in Fig 6B, the inhibition of PBP degradation is positively related to E64 concentration. These data indicate that PBP degradation caused by C1A is dependent on cysteine protease activity of C1A.

To investigate the subcellular localization of C1A protein in rice cells, the construct expressing C1A-GFP fusion protein was transfected into rice protoplasts and visualized by confocal microscopy, as shown in Fig 7, C1A is predominately localized in autophagic vacuole which is indicated by the Dansylcadaverine (MDC) staining. Interestingly, dots of C1A-GFP florescence signal were mobile in vacuole, as shown by different locations of same dots from the view in Fig 7. A cell fractionation assay in rice protoplast confirmed that C1A protein was not
distributed in nuclear fractions (Fig S11). Considering the localization feature of PBP (Fig 4), we are proposing that C1A may function to degrade the excessive PBP proteins in vacuole.

**XopZ Stabilizes PBP in Cell Nucleus and Inhibits PBP Degradation Caused by C1A in Rice**

Since XopZ has no clear effect on PBP stability when we co-expressed XopZ and PBP in the rice protoplasts (Fig S12), we intend to investigate if the expression of XopZ will affect PBP degradation-mediated by C1A. To this purpose, a cassette construct (Fig 8A) expressing both C1A-3*MYC and PBP was generated and co-transfected with the construct expressing XopZ-3*HA in rice protoplasts. As shown in Fig 8B, PBP degradation-mediated by C1A was partially compromised by co-expressing with XopZ-3*HA (Lane 1 to 3). However, the XopZ mutant lacking two NLS signals (XopZ\(\Delta\)NLS) had no obvious effect on this degradation (Lane 4). Given the co-localization of PBP and XopZ during their interaction (Fig 4G) and the cytoplasmic distribution of XopZ\(\Delta\)NLS and C1A (Fig 2D and Fig 7), we proposed that XopZ binds PBP in cell nucleus and stabilizes it, decreases the degradation of excessive PBP mediated by C1A in vacuole.

**PBP Is a Negative Regulator of Host Immunity**

Our efforts to generate the PBP over-expressing and gene-silencing transgenic rice lines failed as we could not detect the changes in transcripts of PBP in these transgenic lines (Fig S13). To determine the roles of PBP in XopZ-mediated virulence function to rice upon PXO99\(^{\Lambda}\) infection, the transgenic rice lines with knock of PBP (KO) (Fig 9A) are generated by using the CRISPR/Cas9 system developed in our lab (Zhou et al., 2014). Two independent homologous PBP knock-out lines (Fig 9B, #1 and #2) show significantly reduced lesion length upon PXO99\(^{\Lambda}\) and PXO99\(^{\Lambda}\)\(\Delta\)VN infection compared to that in wild-type Kitaake, however, the lesion lengths
upon infection by PXO99ΔVNZ (Fig 9B) and PXO86 (Fig S14) are not significantly different among wild type Kitaake plants and two KO-PBP lines. This result suggests that PBP negatively regulates host immunity in rice. The stabilization of negative regulator PBP by effector XopZ results in the enhanced susceptibility in rice.

**Discussion**

*Xanthomonas* type III effector XopZ has been demonstrated to be a virulence factor in host rice plant, probably through suppressing innate immune response induced by products of cell wall degrading enzyme (Song and Yang, 2010; Sinaha et al., 2013). Previous study in our lab had revealed that mutagenesis of XopZ in PXO99A significantly reduces its virulence to rice. However, its role in mediating virulence and its mode of action have yet to be elucidated. Here we show that XopZ physically interacts with a putative host protein PBP in rice. The interaction between XopZ and PBP was further confirmed by *in vitro* GST pull-down assay, assays of *in vivo* BiFc and Co-IP. In plant cells, PBP functions as negative regulator of host immunity based on disease assay on the PBP knock-out plants. We hypothesize that PBP, a putative E3 ligase in the nucleus, might target particular plant immunity component(s) in the nucleus. In addition, PBP is also predicted to be a S-ribonuclease binding protein that is involved in gametophytic self-incompatibility, suggesting that PBP may need to maintain a homeostatic state in plant cells for balancing productive development and plant defense. Based on our results, we provide a putative mode of action for PBP-mediated immune pathway as described in Fig S15. PBP protein is predominately localized in the nucleus in rice cell, which is degraded by C1A through its cysteine protease activity only after PBP is excessively expressed and translocated into the vacuole, leading to a homeostatic state of PBP in plant cell. Further studies on XopZ-PBP
interaction suggest a model in which XopZ binds to PBP in nucleus and inhibits PBP degradation-mediated by C1A in the autophagic vacuole, causing increased PBP protein level in the nucleus and enhanced disease susceptibility in rice (Fig S15).

**PBP Is an Unstable Protein**

By functioning as a putative host E3 ubiquitin ligase, PBP was shown to be very unstable in plants, since when we transiently expressed GFP-PBP fusion proteins in *N. benthamiana* leaves, the GFP fluorescence signals were quickly degraded after 2.5 dpi, and the RNA silencing suppressor P19 cannot suppress this degradation (Fig S8). We proposed that PBP proteins might carry self-ubiquitination activity and are sent to the 26S proteasome for degradation. Intriguingly, when we co-infiltrated the 26S proteasome inhibitor MG132 and the *Agrobacterium* cells expressing PBP in *N. benthamiana* leaves, MG132 do not increase PBP protein levels even by applying 100μM of MG132, suggesting that PBP degradation might be involved in other protein degradation pathways.

In order to investigate the role of PBP in regulating host immunity, we performed a second round of Y2H screening to investigate the PBP-interacting proteins in rice. We proposed that PBP might function as a host E3 ubiquitin ligase and bind to pivotal protein components in plant immune system. Interestingly, we acquired a host cysteine protease C1A from Y2H screening. C1A specifically binds and strongly degrades PBP in rice protoplast cells, while over-expression of PBP does not affect C1A protein levels, suggesting that C1A functions upstream of PBP in plant cells. Considering that PBP protein accumulates in nucleus at the early stage of transient over-expression, and then “diffused” to cytoplasm at the late stage of transient expression (Fig 4A), we postulated that C1A functions as a negative regulator of PBP abundance.
in host cells by degrading the excessive PBP in vacuole, leading to a homeostatic state of PBP in plant cells. This hypothesis was supported by another factor: Theoretically, PBP is predicted to be a putative S-ribonuclease binding protein (SBP1) that plays a role in gametophytic self-incompatibility, suggesting PBP might maintain a steady state in the host cells in order to balance development and defense in plants.

**E3 Ubiquitin Ligase Activity Analysis in PBP**

Based on protein sequence analysis and structure prediction, PBP is a putative E3 ubiquitin ligase with a typical C3HC4 RING motif at its C-terminus, which is characterized by the zinc ligation system and is defined by the “cross-brace” motif C-X2-C-X(9-39)-C-X(1-3)-H-X(2-3)-(N/C/H)-X2-C-X(4-48)C-X2-C. This might explain why we could only detect the fluorescence signals when the GFP was fused at the N-terminal of PBP, but not for that fused at the C-terminus in our transient expression assay performed in *N. benthamiana* leaves and rice protoplasts.

We failed to detect the E3 ubiquitin ligase activity in PBP probably due to the low yield of PBP protein acquired *in vitro* purification system, neither by using the MBP (Maltose-binding protein) expression system nor by using the SUMO expression system. In our future work, we expect to synthesize the PBP protein and perform the E3 ubiquitin ligase assay by following the protocol as described in a previous report (Zhao et al., 2013).

**XopZ-mediated Suppression of PBP Degradation Caused by C1A**

We demonstrated that XopZ functions to stabilize PBP and inhibit PBP degradation-mediated by C1A. However, the chemical activities underlying this remain unknown. It is highly possible that XopZ may inhibit the ubiquitin E3 ligase activity of PBP and compromise its
auto-ubiquitination activity, leading to accumulation of negative regulator of host immunity PBP in nucleus. Previous reports indicated that dimerization usually plays an important role in E3 ubiquitin ligase activity (Welcker et al., 2007; Yin et al., 2009; Liew et al., 2010; Wawra et al., 2012; Johnson et al., 2012). Given the fact that PBP dimerization motif and XopZ-binding motif are closely related (Fig S4C), one possibility is that XopZ binding to PBP may interfere with the homo-dimerization of PBP. Obviously, this hypothesis remains to be examined.

**XopZ is a Virulence Effector Involves in Multiple Defense Pathways**

It is shown that XopZ directly interacts with the negative regulator PBP in the nucleus through its N-terminal domain and stabilizes it, and our another study also indicated that XopZ is involved in known positive regulator XB3-mediated immune response (Wang et al., 2006). Given the specific nucleic-cytoplasmic localization of XopZ, we hypothesize that XopZ suppresses host immunity through different pathways: either by stabilizing negative regulator PBP, or by regulating XB3-mediated immune response, or other pathways. And it is highly possible that these different pathways might over-lap with each other.

In the future, we plan to examine the host substrates of PBP in rice cells, we propose that PBP may function as an E3 ubiquitin ligase and mediate the degradation of particular host immune components in rice cells. Also this work facilitates us to better understand the roles of PBP in plant immune system. Our studies on XopZ function suggest that pathogen effector mediates virulence through interfering with both negative and positive regulators in the plant immune system, which provides new insights on pathogenesis and immune responses in plants.
Methods

Plant Material and Bacterial Strains

Rice (*Oryza sativa*) variety Kitaake and tobacco variety *Nicotiana Benthamiana* were used in this study. Kitaake seed was provided by Dr. Pamela Ronald (UC, Davis). All wild type rice plants and transgenic plants were grown in growth chamber (temperature 28 °C, relative humidity 85%, and photoperiod 12 hours). *Xanthomonas oryzae* pv. *oryzae* strains PXO99<sup>A</sup> was grown in either NB (Difco Laboratories, Detroit) or trypton sucrose (TSA) medium (tryptone, 10 g; sucrose, 10 g; glutamic acid, 1 g; Difco Bacto agar, if solid, 15 g per liter) at 28°C. Antibiotics used in this study for *X. oryzae* pv. *oryzae* were ampicillin (100 µg/ml), cephalexin (10 µg/ml), kanamycin (50 µg/ml), and spectinomycin (100 µg/ml).

Construction of Mutants for PXO99<sup>A</sup> Effector Genes

The PXO99<sup>A</sup> mutant strains were constructed following the protocol as described (Song and Yang, 2010). The PXO99<sup>A</sup> double-mutant strain PXO99<sup>A</sup>ΔNV and triple-mutant strain PXO99<sup>A</sup>ΔNVZ were generated by mutating the effector genes following this order: XopN the first, XopV the second, and XopZ the last. The PXO99<sup>A</sup> mutants were confirmed by multiple restriction enzyme digestion and sequencing.

Site-directed Mutagenesis

To generate different mutated version of PBP and XopZ (PBP<sub>ΔNLS</sub>, PBP<sub>ΔRING</sub>, XopZ<sub>ΔNLS</sub>), gBlock Gene Fragments containing the corresponding nucleotides were synthesized by Integrated DNA Technologies. The active cysteine amino acid in C1A was mutated by PCR based site-directed mutagenesis according to a previous report (Zheng et al.,...
2004). The mutated DNA fragments were then sequenced in the DNA facility at Iowa State University and cloned into corresponding vectors.

**Rice Transformation and Disease Assay of Transgenic Rice Plants**

Rice transformation was performed following the protocol as described by Hiei et al. (1994) with some modifications. 40-50 seeds (without glumes) of rice cultivar Kitaake were sterilized by 2.5% bleach for 20 min and were cultured on a plate containing solid N6 medium (Sucrose 30g/L; CHU[N6] basal mixture 3.98g/L; Myo-inositol 100mg/L; Casamino acids 300mg/L; L proline 2.878g/L; N6 vitamins (1000ˣ) 1ml/L and 2,4-D (0.2mg/ml) 10ml/L; pH 5.7-5.8) for 4 weeks under lights at 28°C. The *Agrobacterium* strain EHA105 expressing particular constructs were collected and resuspended in 30ml of 2N6 liquid medium containing 20 mg/L acetylsyringone (AS). The rice calli were placed onto a sterile wire mesh and soaked with *Agrobacterium* suspension (shake gently for 3-5 min) and cultured with on filter paper on solid 2N6AS medium (Sucrose 30g/L; Glucose 10g/L; CHU[N6] basal mixture 3.98g/L; Myo-inositol 100mg/L; Casamino acids 300mg/L; N6 vitamins (1000ˣ) 1ml/L and 2,4-D (0.2mg/ml) 10ml/L; pH 5.2) for 7 days at 28°C in dark. Calli were then collected and washed for 3-5 times with sterile water, followed by washing with sterile water containing 150mg/L Timentin. The calluses were transferred in N6D medium (containing 35 mg/mL Hygromycin and 150 mg/mL Timentin) and re-transferred every 10-14 days. Normally, resistant calli show small nodular embryos after four weeks of selection. The calluses were transferred to regeneration medium (Sucrose 30g/L; Sorbitol 30g/L; MS-basal salts 4.33g/L; Casamino acids 2.0g/L; B5 vitamins (1000ˣ) 1ml/L; NAA (stock 0.1mg/ml) 200ul/L and Kinetin (stock 0.2mg/ml) 10ml/L; pH 5.7-5.8) containing 35 mg/ml hygromycin B and 150 mg/ml Timentin and are re-transferred to fresh media every 10-14
days for several weeks. The regenerated plantlets were transferred to MS medium containing hygromycin B (35mg/mL) and grow for several days, the plantlets were transferred to soil and grow in growth chamber.

The PBP knock-out rice lines were generated through CRISPR-Cas9 system following the protocol as described (Zhou et al., 2014). For construction of PBP-RNAi transgenic plants, a 516bp DNA fragment of PBP (3’ end) was cloned into pTOPO/D-ENTR vector, sequenced and recombined into pANDA vector (Miki and Shimamoto, 2004) through LR recombinase according to the instruction of the manufacturer (Invitrogen). These constructs were transformed into Agrobacterium strain EHA105.

**SUMO Fusion Protein System for Expressing Recombinant Proteins**

SUMO fusion protein expression system was performed following the previous protocol (Lee et al., 2008) with few changes: *XopZ-3*MYC gene fragment and *PBP* full length DNA fragment were cloned into vector pSUMO and transformed into *E.coli* strain BL21, followed by induction by 0.2 mM IPTG at 16 °C for 16 hours. The BL21 cells were collected by centrifuge and broken down by lysis buffer (50mM Tris-HCl [pH 7.4], 300mM NaCl, 0.2mM EDTA), the supernatant was mixed with 0.5ml of HisPur™ Ni-NTA resin (Thermo Sci) with rotation at 4 °C for 1-1.5 hour, followed by 3 washes with washing buffer (50mM Tris-HCl [pH 7.4], 300 mM NaCl, 0.2 mM EDTA, 40 mM imidazole [pH 8.0]) and incubate with 0.1-1 mg of His6-Ulp403-621-His6 protein for 10h at 4 °C. The purified XopZ/3MYC and PBP were dialyzed against buffer Q (50 mM Tris-HCl pH 8.0, 10% glycerol, 1mM dithiothreitol (DTT)) and stored in -80 °C for further study.
Far Western Blotting (WB) Assay

Far-western blotting (WB) assay was performed following the protocol as described previously (Wu et al, 2007) with few changes: The new nitrocellulose membrane were cut into expected size and loaded with the 1μg prey protein (MBP-CIA, MBP as control, PBP-3FLAG are input protein), and incubated at 4 °C for 30 min until dry. The non-specific areas of the nitrocellulose membrane were blocked by 5% non-fat milk at 4 °C for 3 hours. Membrane was incubated in the solution with 20-50μl of bait protein (PBP-3FLAG, 0.5μg/μl) at 4 °C for 2 hours, followed by wash with MBP washing buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.4) for 3-5 times, with 10min each time. The standard western blot procedure was performed on the nitrocellulose membranes by applying particular primary antibodies.

GST Pull-down Assay

GST pull-down assay was performed as described (Einarson et al., 2005). The purified GST-fusion proteins were incubated with Glutathione-Sepharose 4B beads (Thermo Inc.) in GST washing buffer (50 mM Tris pH7.4, 100 mM NaCl, 1 mM EDTA, 1 mM DTT) for 2 h at 4 °C, followed by wash with GST pull-down buffer (50 mM Tris pH7.4, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5% NP-40) for 4 times and resuspending in 1 x SDS PAGE loading buffer. The samples were boiled and loaded in SDS-PAGE gel. 10% input of the test protein was used in the western blot assay.
Yeast Two-hybrid Screening

The yeast transformation was performed following the protocol as described by Gietz and Woods (2002) with few changes: The yeast strain AH109 was streaked out from the stock and pre-inoculated twice in the YPAD medium. The final yeast cells should reach to $1 \times 10^7$ cells/ml (OD. 1.0), wash the yeast cells twice with ddH2O and mix vigorously with transformation mix (Each transformation mix contains PEG3500 (50%w/v) 24 μl, 1.0 M LiAc 3.6μl, boiled 1.0 M ss-carrier DNA 5μl, plasmid DNA plus water up to 36μl), followed by incubation at 42 °C water bath for 40 min. The transformed yeast cells were plated onto appropriate SC selection medium and grew at 30 °C for 3-4 days.

For screening the XopZ-interacting proteins, full length XopZ DNA sequence was PCR amplified using the primer pair 1041orf-F1 and 1041orf-R and cloned into the GAL4 BD-containing vector pAS2-1. A yeast two-hybrid library was constructed from rice IRBB7 following the protocol (HybriZAP® -2.1 XR Library Construction Kit and HybriZAP®-2.1 XR cDNA Synthesis Kit, Stratagene Inc.). Yeast strain AH109 carrying vector pAS2-1-XopZ was used as competent cells for transformation with the rice cDNA library DNA. In total about $10^7$ yeast clones are plated on 50 plates (150 mm x 50 mm). The positive yeast clones were confirmed by β-galactosidase assays as described in the yeast protocols handbook (PT3024-1, Clontech Inc). The plasmids containing the candidate genes were isolated and sequenced by Sanger sequencing methods at the ISU DNA facility.

We screened PBP-interacting proteins via the same method except using construct pGBK T7-PBP as the bait, the plasmids extracted from candidate yeast clones were transferred into XL-blue and DNA was sequenced.
**In vivo BiFC Assay**

We performed the BiFC assay following the procedure previously reported by Waadt et al. (2008) and made a few changes. PBP full length DNA fragment was cloned into pENTR4-3*HA by BamHI and KpnI, following digestion by BamHI and XbaI (containing PBP-3*HA fragment) and cloned into nYFP-containing vector pXY106. The 5’ fragment of *XopZ* (*XopZ*1-927nt) was cloned into pENTR4-3*HA vector by BamHI and EcoRI, followed with digestion by BamHI and XbaI (containing XopZ-3*HA) and cloned into cYFP-containing vector pXY105. The BiFC assay was performed by co-expressing the constructs expressing both pXY105-*XopZ*1-927nt-*3HA and pXY106-PBP-3*HA through *Agrobacterium*-mediated gene transfer. Leaf disks were visualized 48 h after infection by Leica LAS AF confocal microscopy in confocal microscopy facility at ISU, with excitation wavelength 514nm and emission wavelength 527nm.

**Co-IP Assay**

Proteins were extracted with 0.5 ml of IP buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10% glycerol, 0.1% NP-40, 0.5 mM DTT, 1 mM PMSF and an EDTA-free protease inhibitor cocktail tablet (Roche). After vortex vigorously for 10 second, cell debris was pelleted at 20,000 ×g for 15 min at 4°C. The supernatants were incubated with 40 μl of EZviewTM Red ANTI-FLAG® M2 Affinity Gel (Sigma-Aldrich) for at least 1 h at 4°C with gentle shaking, the gel was spun down at 1200 ×g and the supernatant was discarded, gel was washed for three times with IP buffer. A total of 25μl of 2xSDS loading buffer was added to each sample and boiled for 5 min, immunobloting was carried out with anti-HA or anti-FLAG antibody as described below.
Phylogenetic Analysis

Sequence alignment and phylogenetic analyses were conducted by using ClustalW (Thompson et al., 1994) and EMBL-EBI clustalw2phylogeny for phylogenetic tree construction using the neighbor-joining method. The tree is depicted in rooted format using the midpoint between each node. Bootstrap support value for 1000 re-iterations is indicated above each node.

Transient Gene Expression in N. benthamiana

For each assay, Agrobacterium transformants with specific constructs were grown in 5ml Luria-Bertani (LB) broth supplemented with kanamycin (50μg/ml) and rifampicin (15μg/μl) and acetosyringone (AS) (20ug/mL). One milliliter of overnight culture was subcultured in 50 ml liquid LB supplemented with kanamycin (50μg/μl) and acetosyringone (AS) (20ug/mL) and allowed to grow to an OD$_{600}$ of 0.6. The bacterial cells were then collected by centrifugation under room temperature for 5 min at 3000 rpm. The cells from each centrifugation were resuspended in 50 ml Agrobacterium inoculation buffer (1/2 Murashige and Skoog basal salt and 200 μM acetosyringone, pH 5.2) and activated at 28°C for 3 h. Inoculation was performed with single strain or two strains by mixing the cultures in 1:1 ratio prior to inoculation. N. benthamiana leaves were infiltrated with bacterial suspension, and the leaves were harvested 48 h after inoculation for subsequent analyses.

For assessing stabilization of PBP by MG132, we agro-infiltrated strains expressing 35S:PBP-3*FLAG construct in N.benthamiana leaves, infiltration sites were challenged with different concentration of MG132 (0μM, 10mM, 100μM in 1% DMSO) after 2.5 days infiltration. Leaf discs were harvested 16 hours after MG132 treatment.
Rice Protoplast Isolation and Transfection

The seed of rice cultivar Kitaake was sterilized with liquid bleach (2.5% sodium hypochlorite) for 25 minutes and incubated on 1/2 MS medium with a photoperiod of 12h light at 28 °C for six days. Green tissues from stem and sheath of 50-60 rice seedlings were cut into approximately 0.5 mm strips and immediately immersed into 0.6M mannitol for 15 minutes in the dark. Enzyme solution (1.5% Cellulose RS, 0.75% Macerozyme R-10, 0.6 M mannitol, 10 mM MES at pH 5.7, 10 mM CaCl2 and 0.1% BSA) was added on the green tissues and inoculated for 6-7 hours in the dark with gentle shaking (60-80 rpm). Protoplasts were released by filtering through 40μm nylon meshes and enriched by 21% Sucrose solution, followed by wash with equal volume of W5 solution (154 mM NaCl, 125 mM CaCl2, 5 mM KCl and 2 mM MES at pH 5.7) twice and kept in ice for a half hour. 20 μl purified plasmids DNA (10 μg) were mixed with 200 μl protoplasts (about 2 x 105 cells) in MMG solution (0.4 M mannitol, 15 mM MgCl2 and 4 mM MES at pH 5.7), the protoplast transfection was performed by using 40 % PEG solution (W/V) PEG 4000, 0.2 M mannitol and 0.1 M CaCl2) with mixture, and incubated at room temperature for 15 minutes in the dark, 440μl and 880 μl W5 solution were used to wash the protoplasts twice, followed by centrifugation at low speed (1200rpm) for 2 min. Then, the protoplasts were transferred into multi-well plates and cultured under dark at room temperature for 6-16 hours. The fluorescence in protoplasts was visualized using confocal microscopy (Leica LAS AF), with excitation (ex) and emission (em) wavelengths as these: GFP: ex 488nm, em 508nm; YFP: ex 514nm, em 527nm; DAPI: ex 345nm, em 455nm. MDC: ex 335nm, em 508nm. The protein samples were prepared by adding SDS-PAGE loading buffer and vertexing at high speed.
Gene Expression Analyses

The rice leaves were inoculated with particular Xoo strains and used for total RNA extraction 24 hours post inoculation. RNA was extracted using the TRI reagent following the instruction provided by the manufacturer (Ambion/Life Technologies). RNA concentration and quality were measured using an ND-1000 Nanodrop spectrophotometer (Nanodrop Technologies). For semi-quantitative reverse transcription PCR (semi-RT PCR), one microgram of RNA from each sample was treated with amplification-grade DNase I (Invitrogen) followed by cDNA synthesis using the iScript Select cDNA synthesis kit (Bio-Rad). cDNA derived from 25 ng of total RNA was used for each qRT-PCR reaction with gene-specific primers. The rice gene $TFIIA_5$ was used as an internal control. The primer sequences are provided in Table S1.

Disease Assay

Five week old rice leaves were inoculated using leaf tip-clipping method for lesion measurement (Kauffman, et al., 1973). Bacterial inoculum with optical density of 0.5 at 600 nm ($OD_{600} = 0.5$) was used for inoculation. The lesion length of each leaves were measure 9-12 days post inoculation. One-way analysis of variance (ANOVA) statistical analyses were performed on all measurements. The Tukey honest significant difference test was used for post-ANOVA pair-wise tests for significance, set at 5% ($P<0.05$).

Cell Fractionation Assay in Rice Protoplasts

Nuclear and cytoplasmic fractions in protoplast expressing specific proteins were fractionated following the protocol as described by Yanagisawa et al (2003) with few changes: Protoplasts were lysed with lysis buffer (20 mM Tris-HCl, pH7.0, 250 mM sucrose, 25% glycerol, 20 mM KCl, 2 mM EDTA, 2.5 mM MgCl2, 30 mM β-mercaptoethanol, 1 X protease
inhibitor cocktail, and 0.7% Triton X-100) in room temperature for 15 min and centrifuge at 4 °C
3000g for 6 min. The supernatant (cytoplasmic fraction) was taken out and mixed with 2 X SDS-
PAGE loading buffer. The pellet was further washed twice with suspension buffer (20 mM Tris-
HCl, pH 7.0, 25% glycerol, 2.5 mM MgCl2, and 30 mM β-mercaptoethanol) and mixed with 1 X
SDS-PAGE loading buffer. The nuclear fractionation and the cytoplasmic fractionation were
loaded in SDS-PAGE gel (cytoplasmic fraction: nuclear fraction=4:1) and detected by
immunoblotting with corresponding primary antibodies, with anti-Histone3 and anti-Tubulin as
the marker of nuclear fractionation and cytoplasmic fractionation, respectively.

**MDC Staining for Acidic Bodies in Rice Protoplasts**

Autophagic vacuoles were labeled by auto-fluorescent compound monodansylcadaverine
(MDC) following the protocol (Biederbick et al., 1995), rice protoplast cells were stained with
0.05 mM final concentration of MDC (Sigma) in PBS for 10 min and were washed twice by W5
to remove MDC residue. The protoplast cells were kept on ice in the dark after MDC staining.
Fluorescence of protoplast cells was measured with excitation wavelength of 335nm and an
emission wavelength of 508nm.

**DAPI Staining of Nucleus of Rice Protoplasts**

DAPI (dihydrochloride) nucleic acid staining was performed following the protocol
(Invitrogen Inc): 5mg/ml DAPI stock solution (10 mg DAPI in 2 ml deionized water) was diluted
to 300 nM in PBS and added in the vials containing rice protoplasts. The stained protoplast
samples were visualized under UV light (excitation 358 nm, emission 461 nm) by using confocal
microscopy (Leica LAS AF).
Acknowledgments

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system effectors XopN, XopQ, XopX and XopZ of *Xanthomonas oryzae* pv. *oryzae*. PLOS one 8, e75867.


**Üstün, S., Bartetzko, V., and Börnke, F.** (2013). The *Xanthomonas campestris* type III effector XopJ targets the host cell proteasome to suppress salicylic-acid mediated plant defense. PLOS Pathogens 9, e1003427.


Figures and Tables

Fig 1. Effector XopZ contributes to virulence to rice. Kitaake plants were inoculated with PXO99\textsuperscript{A}, PXO99\textsuperscript{A}\textsuperscript{ΔVN} (double mutant) and PXO99\textsuperscript{A}\textsuperscript{ΔVNZ} (triple mutant). The lesions were measured 8 day post inoculation. The experiment was repeated three times with the similar result.
Fig 2. XopZ protein is distributed in both nucleus and cytoplasm in rice cells.

(A) Cell fractionation assay of XopZ performed in rice protoplasts. Cytoplasmic fractionation and nuclear fractionation were indicated by anti-Tubulin and anti-Histone3 antibodies, respectively.

(B) Two NLS signals at the N-terminal of XopZ were predicted by The NucPred (http://www.sbc.su.se/~maccallr/nucpred/cgi-bin/single.cgi). The underlined amino acids indicate the predicted NLS signals at the N-terminus of XopZ.

(C) The XopZ mutant (XopZ△NLS) with mutations on both NLS signals.

(D) Cell fractionation assay of XopZ△NLS performed in rice protoplasts. Cytoplasmic fractionation and nuclear fractionation were indicated by anti-Tubulin and anti-Histone3 antibodies, respectively.
Fig 3. XopZ interacts with PBP \textit{in vitro} and \textit{in vivo}.

(A) XopZ interacts with PBP in yeast two-hybrid assay.

(B) XopZ interacts with PBP in GST pull-down assay. MBP-PBP proteins were detected by anti-MBP antibody.

(C) XopZ interacts with PBP in BiFC assay. N-terminal fragment of XopZ (1-309 aa) was used in this assay. The fluorescence signals were visualized under confocal microscopy, with excitation wavelengths 514 nm and emission wavelengths 527 nm.

(D) XopZ interacts with PBP in Co-IP assay. IB: anti-FLAG; IP: anti-HA.
D 1 MAVDAHHLRLPPPIQPAEFSMPRPQFCFGAAAGEVVGGAGGAMGLC 50
51 QEQLVQYQRQVFQGGGVRQPAASEEVRQYSQVCAAAADAVNGTFS 100
101 GQEAAPRKRRAEEPVLGAADGAVAAQARQLYDVRVLHLHAAKMA 150
151 ELAEQRGRHARQVATVEAAAARRLRKDEEIER1GRNLWALEERLGMY 200
201 VEAQVWRDLQAESEATANALGEELHVLDARHRGADHGDDAESCRYG 250
251 ENDDLDRAAGDGEASAEERCKCGGAAAVVLLLPCRHLCAACAPCAAAAA 300
301 ACPACGCAKNGSVYNFS 318

E

F 35S:PBP, 3FL 35S:PBB, 3FL 35S:XopC 3HA

G

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Fig 4. XopZ and PBP are co-localized in nuclei of rice protoplasts. 

(A) GFP-PBP localization in rice protoplasts at different time courses after transfection. Scar bar = 10μm.

(B) Cell fractionation assay of rice protoplasts expressing 35S: PBP at 5 hours and 12 hours post transfection. The cytoplasmic fractionation and nuclear fractionation are indicated by anti-Tubulin and anti-Histone 3 antibodies, respectively.

(C) Cell fractionation assay of rice protoplast expressing PBP(pro):PBP at 5 hours and 12 hours post transfection.

(D) A NLS signal was predicted in PBP. (http://www.sbc.su.se/~maccallr/nupred/cgi-bin/single.cgi). Underlined is the XopZ binding site.

(E) Kitaake protoplasts expressing 35S:GFP-PBP△NLS are visualized by confocal microscopy. Scar bar = 10μm.

(F) Cell fractionation assay in Kitaake protoplasts expressing 35S:PBP△NLS.

(G) XopZ-PBP interaction primes XopZ localization in rice protoplasts. The distribution of XopZ are detected by anti-HA antibody. Samples are collected 12 hour post transfection.
Fig 5. PBP interacts with C1A and C1A$_{80-458aa}$ \textit{in vitro}.

(A) Interaction of PBP and C1A in yeast two-hybrid assays. The truncated C1A$_{80-458aa}$ peptide (I29 mutation) shows strong interaction with PBP, but the interaction between full length C1A and PBP are barely detected.

(B) Interaction of PBP and C1A in Far-Western blot assays. 5μg proteins are used in each spot, except the fourth spot (1.67μg).
Fig 6. C1A degrades PBP through its cysteine protease activity.

(A) Co-expression of PBP and C1A shows that C1A strongly down-regulates PBP protein level in rice protoplasts, however, the mutation at the active cysteine (C1A^{C153A}) abolishes its ability to degrade PBP.

(B) Western blot probed with anti-PBP antibody and anti-MYC antibody, following expression of PBP and C1A-MYC. Different concentration of cysteine protease inhibitor E64 (0, 1, 2, 5, 10, 20μM) are applied in each reaction.
Fig 7. C1A is predominately localized in autophagic vacuole in rice protoplasts. The monodansylcadaverine (MDC) staining labels late stage autophagosomes or autophagic vacuoles. MDC (final concentration of 0.05 mM) staining signaling was visualized by 335 nm/508 nm (excitation/emission) filter set. The same spot was shot 4 times at different time points (30s interval).
Fig 8. XopZ stabilizes PBP and inhibits PBP degradation mediated by C1A.

(A) The cassette construct was generated by insertion of 35S:C1A-3*MYC cassette into pUC19-35S:PBP expression vector.

(B) Western blot assay indicates XopZ inhibits PBP degradation-mediated by C1A. Three cassette constructs are used in this assay (PBP-C1A, PBP-C1A\(^{C153A}\), PBP-GFP). Asterisk indicates the non-specific band caused by anti-Myc antibody in rice protoplasts.
Fig 9. PBP is a negative regulator of plant immunity.

(A) DNA sequence of homologous two PBP knock-out (KO) lines that are generated by CRISPR-Cas9 technology. #1 represents -1 bp mutant, #2 represents -10 bp mutant. Underlined AGG represents PAM sequence.

(B) PBP-KO lines show decreased lesion length upon inoculation with PXO99\(^\text{A}\) strain, double mutant PXO99\(^\text{A}\)\(\Delta\)VN strain, compared to that in wild type Kitaake rice line. The lesion lengths were measured 8 days post infection.
Table 1. XopZ-interacting proteins in rice cDNA library.

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Table 2. PBP-interacting proteins in rice cDNA library.

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Supplementary Figures

Fig S1. XopZ distribution in rice cells.
(A) The predicted NES signals in the XopZ full length protein by NetNES 1.1 Server (http://www.cbs.dtu.dk/services/NetNES/).
(B) The predicted shuttle distribution of XopZ. Quadrangle indicates XopZ.

Fig S2. Phylogenetic analysis of PBP (Os03g46570). PBP shares the highest similarity with XM_006651607 from Oryza brachyantha, a putative BOI-related E3 ubiquitin ligase 3-like protein. Protein sequences alignment was performed by ClustalW2. The Phylogenetic tree was generated by EMBL-EBI clustalw2_phylogeny by using neighbor-joining method.
Fig S3. N-terminus of XopZ is sufficient to interact with PBP in Y2H assay. The different truncated fragments of XopZ were cloned into pGBK T7 vector and co-expressed with pGAD T7-PBP in yeast strain AH109.

Fig S4. PBP functional motifs analysis.
(A) PBP$_{193-225\text{aa}}$ is required for interacting with XopZ in Y2H assay.
(B) PBP$_{137-192\text{aa}}$ is required for PBP homo-dimerization.
(C) Putative functional motifs in PBP full length protein.
Fig S5. Rice protoplasts expressing GFP-PBP are visualized by confocal microscopy at 12 hours post transfection. The DAPI (final concentration 300nM) staining indicates the cell nucleus in rice protoplasts. The protoplasts are isolated from young seedling of cultivar kitaake. Scar bar=10μm.

Fig S6. Anti-PBP primary antibody specifically recognizes NLS site in PBP.
Fig S7. PBP localization pattern in *N. benthamiana* leaves. Fluorescence signal caused by GFP-PBP fusion proteins is predominantly visualized in the nuclei 40 hours post infiltration, but is obvious in nucleus, cytoplasm and plasma membrane 2.5 days post infiltration. Blue triangle indicates nucleus. Bar 50μm. Up: magnification, 50; Down: magnification, 257.
Fig S8. PBP degradation is not dependent on 26S proteasome
(A) GFP fluorescence signal is dampened after 2.5 dpi (day post infiltration) when fused with PBP and transiently expressed in the N.benthamiana leaves. RNA silencing suppressor P19 (OD0.3) could not suppress this degradation, suggesting PBP degradation is not caused by gene silencing. Agrobacterium EHA105 strains expressing GFP-PBP and GFP are adjusted to final concentration OD0.3, and OD. 0.1, respectively. (Scale bar, 500 μm).

(B) PBP protein is degraded in a time courses in N.Benthamiana leaves. GFP was used as the control. PBP-3*FLAG and GFP-3*FLAG proteins are detected by antibody against FLAG. 4 leaf discs are usd in each sample.

(C) Western blot probed with anti-FLAG antibodies, following expression of PBP-FLAG. The infiltration sites were challenged with different concentration (0 μM, 10 μM, 100 μM) of MG132 in 1% DMSO at 60 hpi. Leaf discs were harvested 16 h after MG132 infiltration.
Fig S9. PBP and C1A co-expression in *N. Benthamiana* protoplasts.

Fig S10. Transcriptional levels of PBP in the protoplasts expressing PBP, C1A and XopZ and their combination were similar. The mRNA from each sample is extracted 12 hours post transfection and applied for semi-quantitative RT-PCR assay. Rice gene TF II λ5 was used as internal control. PBP primer: PBP-F4 & PBP-R6, 29 cycles. Chr5-TF2-F & Chr5-TF2-R, 25 cycles.
Fig S11. Cell fractionation assay in rice protoplasts expressing C1A. Asterisk indicates non-specific band caused by anti-Myc antibody in rice protoplasts.

Fig S12. Co-expression of XopZ and PBP in rice protoplasts has no clear effect on PBP stability. The protoplast samples are collected 12 hours post transfection.
Fig S13. PBP transcript level is not significantly changed in PBPox plants and RNAi plants. Rice gene TF II λ5 was used as internal control. PBP primer: PBP-F4&PBP-R6, 29 cycles. TF II λ5 primers: Chr5-TF2-F & Chr5-TF2-R, 25 cycles.

Fig S14. PXO86 infection on wild type Kitaake and two PBP knock-out transgenic lines (#1: -1bp mutant, #2: -10bp mutant). The lesions are measured 10 days post infection.
Fig S15. Putative mode of action. PBP is a negative regulator of host immunity, which is predominantly localized in the nucleus. The plant cells maintain the homeostasis of PBP through degradation of excessive PBP protein via a cysteine protease C1A, which is localized in the autophagic vacuole. XopZ interacts with and stabilizes PBP in the nucleus, leading to inhibition of PBP degradation caused by C1A, as well as enhanced disease susceptibility in rice. Green quadrangle represents PBP, blue quadrangle represents XopZ, blue triangle represents C1A.
Chapter 5 GENERAL CONCLUSIONS

Plants and pathogens are engaged in a continual arm race, in which plant-pathogen interactions are highly dynamic in nature. Plant pathogens utilize their effector proteins to overcome the host innate immune system. In contrast, plants have co-evolved particular resistance proteins to recognize the invading effector proteins and trigger immune responses. Thereafter, elucidation of the interaction between plant pathogens and the host genes/proteins plays a pivotal role in understanding the “tug-of-war” between plants and pathogens. In this dissertation, chapter 2 describes the pathogenic mechanism of Xoo TAL effectors PthXo3 and AvrXa7 and the interaction with their cognate host susceptible gene Os11N3. Chapter 3 describes another TAL effector PthXo2-mediated activation of host OsSWEET13 gene in indica but not in japonica due to promoter variation. Chapter 4 describes the elucidation of mechanism of non-TAL effector XopZ-mediated suppression of host defense in rice.

TAL Effectors (PthXo3, AvrXa7 and PthXo2) Activate Host Gene Transcription in rice

TAL effectors PthXo3 and AvrXa7 were demonstrated to induce gene transcription of Os-11N3 (OsSWEET14), a member of MtN3 or SWEET gene family. Loss of function or gene silencing of Os-11N3 results in loss or suppression of PthXo3 and AvrXa7-specific susceptibility in rice. The promoter sequence analysis on Os-11N3 revealed the EBE for AvrXa7 and PthXo3 based on the prediction using the TAL effector DNA recognition code, and the physical interaction between AvrXa7 and Os-11N3 promoter was also confirmed by EMS assay and ChIP assay. These findings indicate that Os-11N3 (OsSWEET14) is a susceptible gene that contributes to bacterial blight in rice, TAL effectors PthXo3 and AvrXa7 promote susceptibility in rice by
targeting the EBE motif in the promoter of \textit{Os11N3} (\textit{OsSWEET14}) and activating its gene transcription.

TAL effectors PthXo2 is another major virulence effector that contributes to bacterial blight in rice. PthXo2 has been found to specifically target the EBE motif in the promoter region of host sucrose transporter gene \textit{OsSWEET13} and transcriptionally activate its expression in \textit{indica}, but not in \textit{japonica}. The specificity of effector-associated gene induction and disease susceptibility are attributable to a single nucleotide polymorphism (SNP) in the EBE of \textit{OsSWEET13} gene, which is also found in a polymorphic allele of \textit{OsSWEET13} known as the recessive resistance gene \textit{xa25}. The elevated OsSWEET13 protein expression promotes sucrose efflux from cytoplasm to apoplasm and facilitates the Xoo growth, leading to the promoted susceptibility to bacterial blight in rice. Modified versions of \textit{pthXo2} enable the switch of \textit{OsSWEET13} induction from \textit{indica} to \textit{japonica}, based on the predictive models for TAL effector binding specificity. \textit{OsSWEET13} null mutants abolished the PthXo2-dependent susceptibility to Xoo, suggesting the specific interaction between PthXo2 and \textit{OsSWEET13}. The prevalence of PthXo2 in 104 Xoo strains has also been examined. Among 104 strains, 42 are able to induce \textit{OsSWEET13} in IR24 based on qRT-PCR assay. The whole-genome sequencing of Xoo strain PXO339 and PXO163 revealed a version of PthXo2-like effector that shares 99% percent amino acid similarity to PthXo2, and the corresponding PthXo2.2 effector was then isolated from strain PXO163 through molecular cloning.

Our studies on TAL effector (PthXo3, AvrXa7 and PthXo2) function have expanded our knowledge on TAL effector-host gene interaction and molecular mechanisms of TAL effector-mediated virulence in rice, our results also confirmed the DNA binding specificity code and the sugar transporter activities of these host SWEET genes in mediating susceptibility in plants. The
host S genes are manipulated by TAL effectors for increasing sugar availability in the apoplast and triggering disease susceptibility. Abolishing host S gene activation will cause a state of “atypical” disease resistance that does not involve in the interaction of R gene-avirulence gene. The studies on PthXo2-mediated virulence function in two rice sub-species *indica* and *japonica* even revealed the cryptic resistance in *japonica* rice lines caused by a nucleotide deletion. Taken together, TAL effectors cause disease susceptibility in rice through specifically activating host S gene transcription.

Recent data support a model that these host S genes encode the SWEET family and facilitate sucrose and glucose efflux from cytoplasm to apoplasm, leading to the increased nutrient availability in the apoplast. However, direct evidence on whether the increased sugar concentration in the apoplast will facilitate Xoo growth in apoplast remains missing. We had tried to test this hypothesis by quantifying Xoo growth in rice plants grown in different sugar concentrations. However, our data are inconsistent due to the poor plant growth caused by high osmotic stress. In the future, we might try to increase sugar concentration in the apoplast through leaf infiltration method. We might also try to transiently express exogenous sugar transporter genes from other organisms and examine the Xoo growth at different sugar concentrations. These works facilitate us to better understand the molecular basis of these S genes in plant susceptibility.

Demonstration of TAL effector-DNA binding specificity code has led to many new techniques for genome editing, gene knock-out and specific gene activation. A chimeric TAL effector nuclease technique was developed and demonstrated to successfully generate a wide variety of gene-specific mutations in various plant genomes. In addition, the gene-specific TAL activators/suppressors have been indicated to positively/negatively regulate host gene
transcription. These TAL effector-based gene targeting reagents have been shown to manipulate gene function efficiently, specifically and of low toxicity.

**Non-TAL Effector XopZ Mediates Virulence to Bacterial Blight in Rice**

XopZ is one of the 18 non-TAL effector identified from Xoo strain PXO99^A that contribute to the bacterial blight in rice. Through yeast two-hybrid screening of rice cDNA library, a putative host E3 ubiquitin ligase PBP has been identified to interact with XopZ. XopZ contains two NLS signals at its N-terminus and the N-terminus of XopZ is sufficient for the specific binding with PBP. Disease assay on PBP knock-out plants indicated that PBP is a negative regulator of host immunity. Intriguingly, degradation of PBP protein is not dependent on 26S proteasome-mediated pathway, but on a host cysteine protease C1A. Our study indicates that C1A specifically binds PBP and strongly degrades it in vacuole through its cysteine protease activity; this process might play a pivotal role in regulating the homeostatic state of PBP in rice cells. The function of XopZ is to stabilize the PBP proteins and inhibit PBP degradation mediated by C1A, leading to the accumulation of negative regulator PBP in the nucleus, as well as enhanced susceptibility to bacterial blight in rice.

Our studies on PBP function suggest that the PBP is different from other E3 ubiquitin ligases because it is not dependent on the 26S proteasome for protein degradation, but is regulated by a cysteine protease C1A that appears to be localized in the autophagic vacuole. This finding highlights a new insight that the localization of host E3 ubiquitin ligase is important for its functions. As suggested in our studies, XopZ stabilizes PBP when they are co-localized in the nucleus. Our additional data also suggests that PBP is predicted to be a S-ribonuclease binding protein that is involved in gametophytic self-incompatibility. PBP also shares high sequence
similarity with the BOI-related E3 ubiquitin-protein ligases that involves in disruption of 
resistance to *Botrytis cinerea*, as well as increased cell death upon pathogen infection. These data 
suggest that PBP might be a pivotal protein in rice that is involved in both flower development 
and plant defense, and its homeostasis in the plant cells is important for plant growth and fitness 
in nature. As PBP acts in the nucleus and mediates its host substrates for degradation, we 
propose that substrates of PBP may include particular positive regulators of host defense and 
many S-ribonuclease proteins. As suggested in our data, excessive PBP proteins are degraded by 
cysteine protease only after PBP are “diffused” and translocated into cytoplasm, suggesting the 
enrichment of PBP protein in plant cells and the E3 ubiquitin ligase are two important factors in 
balancing plant development and plant defense.

The revelation of XopZ-mediated stabilization of PBP paves the way for further studies 
on XopZ-mediated virulence function and PBP-mediated plant immunity pathways. Based on 
our understanding, XopZ enhances disease susceptibility in rice through at least two pathways: 
first of all, XopZ is involved in subverting plant UPS and breaking down the homeostasis of 
negative regulator of plant defense (PBP) in rice cells, even though the molecular basis 
underlying this stabilization is remain unclear. It is most likely that XopZ will affect the E3 
ubiquitin ligase of PBP and suppress the ubiquitin ligase activity and auto-ubiquitin activity of 
PBP, leading to increased PBP protein levels in the nucleus and enhanced disease susceptibility 
of rice plants to bacterial blight. Secondly, our additional data also suggest that XopZ targets 
another host E3 ubiquitin ligase XB3, which is demonstrated to be a positive regulator of host 
defense (Song et al., 2006). The mode of action of XopZ-XB3 interaction remains elusive. 
However, it is obvious that molecular mechanisms underlying XopZ-XB3 interaction and XopZ-
PBP interaction are different due to their opposite roles in plant defense. The demonstration of
XopZ-XB3 interaction is of great importance to examine the host-pathogen interaction. Our findings suggest that effector XopZ-mediated virulence function in rice is involved in both positive regulator(s) and negative regulator(s) of host defense, highlighting a new prospect on host-pathogen interactions.

For our future work, we will focus on determination of the host substrates of PBP through multiple protein-protein interaction techniques, especially focus on these known positive regulator(s) of host defense, we hypothesize that PBP might be an important components in plant that regulates many other plant defense regulators and S-ribonucleases. Our works on PBP functions might provide a possible new insight in the tradeoff between plant growth and plant defense.

In general, *Xanthomonas* strains suppress host basal defense and infect host plants through secreting and injecting a myriad of type III effectors into host plants, which either manipulate host gene expression transcriptionally (TAL effectors) or subvert plant immune-related protein components (Non-TAL effectors). The major virulence TAL effectors (PthXo1, PthXo2, PthXo3, and AvrXa7) usually contribute strong virulence to the bacterial blight by transcriptionally activating the sugar transporter genes and providing more nutrients to pathogen growth in apoplast. Non-TAL effectors suppress host immunity by targeting multiple immune components in plant cells. Revelation of the pathogenic mechanism of these pathogen effectors has allowed the identification of new components in plant immunity, leading to a better understanding of the host-pathogen interactions and host immune system, as well as development of effective strategies to defeat plant pathogens.
APPENDIX
MEDIUM AND REAGENTS FOR YEAST TRANSFORMATION

**SC selection medium:**
4.0 g  Difco Yeast Nitrogen Base (w/o amino acids)
12.0g  Glucose
0.5g   Synthetic complete drop out mix
600ml Distilled water
10.0g  Difco Bacto Agar (Add only for solid medium)

**YPAD medium**
6.0 g  Yeast extract (Difco)
12.0g  Peptone (Difco)
12.0g  Glucose
60mg   Adenine hemisulphate
600ml Distilled water

**Single-stranded carrier DNA (2mg/ml)**
200mg of ssDNA in 100ml TE buffer, boil before use

**1.0M Lithium Acetate stock solution**
1.0M stock in ddH2O

**Poly ethylene glycol (PEG 50\%w/v)**
PEG3500 (MW) is resolved in ddH2O and filter sterile.

**Yeast transformation mix**
PEG3500 (50\%w/v)  24 μl
LiAc 1.0M           3.6μl
ssDNA(2mg/ml)       5μl
Plasmid DNA plus H2O Up to 36μ