CHARACTERIZATION OF PATHOGEN EFFECTORS AND HOST ENDOGENOUS PEPTIDE ELICITORS IN THE RICE-MAGNAPORTHE ORYZAE INTERACTION

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by
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ABSTRACT

The arms race is going on forever between plants and pathogens. Along their everlasting co-evolutionary path, plants have developed multilayered surveillance systems and defense lines, whereas pathogens have acquired vast capabilities of evading perception and breaking defense lines. In the interaction of *Magnaporthe oryzae* and rice, *M. oryzae* delivers a phalanx of effectors that could enter rice cells and subvert rice defenses, and rice cells deploy an array of surveillance receptors that could sense pathogen attack and initiate defense responses against *M. oryzae*. This study aims to shed some new light on the rice-*M. oryzae* interaction from both the pathogen and the host perspectives.

A family of one-finger zinc finger proteins was identified as pathogen effectors from the secretome of *M. oryzae* isolate 70-15. Rice leaf sheath live-cell imaging showed that zinc finger effectors were first accumulated in the biotrophic interfacial complex (BIC) and then translocated into rice cells during *M. oryzae* infection. The underlying mechanism for translocation was likely associated with two host-targeting motifs L/IPXP and L/IXAR, as demonstrated by the analysis of the localizations of BAX- and GFP-tagged fusion proteins. Yeast two-hybrid screening of a blast-induced rice cDNA library revealed that the rice nucleus-localized protein HIRA was one of the interactors of this family of zinc finger effectors. The interaction between zinc finger effectors and HIRA was in the nucleus of the rice cell, as shown by bimolecular fluorescence complementation (BIFC). In addition, this interaction was also confirmed by a GST pull-down assay. Zinc finger effectors are in nature DNA-binding proteins, as unveiled by systematic evolution of ligands by exponential enrichment (SELEX). Moreover, zinc finger effectors inhibited BAX-induced
programmed cell death (PCD) when transiently expressed in *Nicotiana benthamiana*. And rice transgenic lines overexpressing zinc finger effectors exhibited lowered defense gene expression and increased susceptibility to *M. oryzae*. A plausible model has been proposed to elucidate how zinc-finger effectors suppress rice immunity by recruiting HIRA for chromatin remodeling.

Rice, like Arabidopsis, harbors a family of defense-related genes encoding plant elicitor peptides (Peps). On one hand, *M. oryzae* induced the expression of the OsPep1-encoding precursor gene *OsPROPEP1* via the ethylene (ET) and jasmonate (JA) pathways. On the other hand, synthetic OsPep1 elicited defense response in rice and thereby enhanced resistance against *M. oryzae*. Furthermore, rice transgenic lines overexpressing *OsPROPEP1* showed constant defense gene expression and increased resistance to *M. oryzae*. All these findings indicate that Pep signaling as an amplifier of plant defense is an integral part of rice innate immunity against *M. oryzae*. 
TABLE OF CONTENTS

List of Abbreviations ...........................................................................................................ix
List of figures .......................................................................................................................xi
List of tables .........................................................................................................................xiv
Acknowledgements .............................................................................................................xv

Chapter 1  Literature review ...............................................................................................1
  1.1  Plant pathogen effector delivery systems .................................................................1
    1.1.1  Bacterial effector secretion/translocation systems .............................................2
    1.1.2  Oomycete and fungal effector delivery venues ...............................................5
  1.2  The subcellular locations of plant pathogen effectors .............................................8
  1.3  The host targets of plant pathogen effectors ............................................................9
    1.3.1  PRRs and Rs .........................................................................................................10
    1.3.2  The MAP3K-MAP2K-MAPK cascade ...............................................................11
    1.3.3  The crosstalk between phytohormones ............................................................11
    1.3.4  The chromatin and transcription machinery ....................................................13

Chapter 2  A family of *M. oryzae* zinc-finger effectors are secreted and
            translocated inside plant cells .................................................................................16
  2.1  Introduction ...............................................................................................................16
  2.2  Results .......................................................................................................................21
    Identification and cloning of a family of putative *M. oryzae*
    one-finger zinc finger proteins ..............................................................................21
    Zinc finger effectors accumulate in BIC and translocate into rice
    cells during fungal infection ..............................................................................23
    Motifs L/IPXP and/or L/IXAR may play a role in translocation
of effectors from fungus to plant cells.................................27

Chapter 3

Zinc finger effectors are nuclear HIRA recruiters and DNA binding factors.................................38

3.1 Introduction.................................................................38

3.2 Results.................................................................45

Zinc finger effectors interact with HIRA and other nuclear proteins.........................................................46

The interactions between zinc finger effectors and HIRA occur in the nuclei of rice cells..........................50

Zinc finger effectors are DNA binding factors.................................52

3.3 Discussion.............................................................56

3.4 Materials and methods.................................................60

Yeast two hybrid screen.....................................................60

GST pull-down assay.........................................................60

Bimolecular fluorescence complementation assay.................................................................60

Systematic evolution of ligands by exponential enrichment (SELEX) assay.................................................62

Chapter 4

Zinc-finger effectors suppress PTI when expressed heterologously in plants.................................63

4.1 Introduction.................................................................63

4.2 Results.............................................................................68

Zinc finger effectors inhibit BAX induced PCD in
Zinc finger effectors inhibit BAX induced PCD in rice protoplasts.

The induction of defense genes is compromised in MGG_07834 or MGG_09035 transgenic lines.

MGG_07834 or MGG_09035 transgenic lines are more susceptible to M. oryzae infection.

4.3 Discussion

4.4 Materials and methods

Rice protoplast preparation, transfection and FDA staining
Rice transformation
RNA extraction and cDNA synthesis
Quantitative RT-PCR for defense-related genes
Rice blast inoculation and disease severity assay

Chapter 5 Role of the rice endogenous peptide elicitor OsPep1 in defense signaling and disease resistance

5.1 Introduction

OsPep1 belongs to a 7-member family of potential endogenous peptide elicitors in rice.

OsPROPEP1 is induced by M. oryzae and JA and ET.

OsPep1 induces the expression of its precursor gene and rice defense genes and enhances disease resistance against M. oryzae.

OsPROPEP1-overexpression lines exhibit growth inhibition and seed-set reduction.

An array of defense genes are constitutively expressed in OsPROPEP1-overexpressing lines.
OsPROPEP1-OX lines enhance disease resistance against

*M. oryzae*………………………………………………………………………….100

5.3 Discussion………………………………………………………………………..105

5.4 Materials and methods………………………………………………………108

*OsPROPEP1* construct and rice transformation………………...108

Plant materials and fungal isolates………………………………………108

Peptide synthesis and chemical treatments.........................109

Assessment of Agronomic traits and disease severity……109

Quantitative real-time PCR analysis of relative gene expression
Levels………………………………………………………………………………110

References…………………………………………………………………………….112
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AA</td>
<td>Amino Acid</td>
</tr>
<tr>
<td>ABA</td>
<td>Abscisic Acid</td>
</tr>
<tr>
<td>AS1</td>
<td>Asymmetric Leaves 1</td>
</tr>
<tr>
<td>AS2</td>
<td>Asymmetric Leaves 2</td>
</tr>
<tr>
<td>A. tumefaciens</td>
<td>Agrobacterium tumefaciens</td>
</tr>
<tr>
<td>AVR</td>
<td>Avirulence</td>
</tr>
<tr>
<td>BAX</td>
<td>BCL-2 Associated X Protein</td>
</tr>
<tr>
<td>BIC</td>
<td>Biotrophic Interfacial Complex</td>
</tr>
<tr>
<td>BiFC</td>
<td>Bimolecular Fluorescence Complementation</td>
</tr>
<tr>
<td>CEBiP</td>
<td>Chitin Elicitor Binding Protein</td>
</tr>
<tr>
<td>CERK1</td>
<td>Chitin Elicitor Receptor Kinase</td>
</tr>
<tr>
<td>CHIP</td>
<td>Chromatin Immunoprecipitation</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage Associated Molecular Pattern</td>
</tr>
<tr>
<td>ET</td>
<td>Ethylene</td>
</tr>
<tr>
<td>ETI</td>
<td>Effector Triggered Immunity</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S Transferase</td>
</tr>
<tr>
<td>HIRA</td>
<td>Histone Regulatory Protein A</td>
</tr>
<tr>
<td>HDA</td>
<td>Histone Deacetylase</td>
</tr>
<tr>
<td>HMT</td>
<td>Histone Methyltransferase</td>
</tr>
<tr>
<td>HR</td>
<td>Hypersensitive Response</td>
</tr>
<tr>
<td>HTM</td>
<td>Host Targeting Motif</td>
</tr>
<tr>
<td>IH</td>
<td>Invasive Hyphae</td>
</tr>
<tr>
<td>JA</td>
<td>Jasmonate</td>
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</table>
L/IPXP     Leucine/Isoleucine-Proline-any amino acid-Proline
L/IXAR     Leucine/Isoleucine-any amino acid-Alanine-Arginine
MAMP       Microbe Associated Molecular Pattern
M. oryzae  Magnaporthe oryzae
N. benthamiana  Nicotiana benthamiana
Os         Oryza sativa
OX         Over-expression
PAMP       Pathogen Associated Molecular Pattern
Pep        Plant Elicitor Peptide
PEPR       Plant Elicitor Peptide Receptor
PPR        Pentatricopeptide Repeat
PR         Pathogenesis Related Protein
PRR        Pattern Recognition Receptor
PTI        Pattern Triggered Immunity
qRT-PCR    Quantitative Reverse Transcription-Polymerase Chain Reaction
SA         Salicylic acid
SELEX      Systematic Evolution of Ligands by Exponential Enrichment
STOP1      Sensitive to Proton Rhizotoxicity 1
Ta2A       Thosea asigna 2A
T3SS       Type III Secretion System
T4SS       Type IV Secretion System
T6SS       Type VI Secretion System
Xoo        Xanthomonas oryzae pv. oryzae
Y2H        Yeast Two Hybrid
ZFE        Zinc Finger Effector
LIST OF FIGURES

Figure 1-1 Bacterial T3SS, T4SS and T6SS translocate effectors into host cells……2
Figure 1-2 Oomycete and fungal effector-exporting specialized structures…………….5
Figure1-3 Subcellular locations and virulence targets in host cells of oomycete and fungal effectors………………………………………………………………………9
Figure 2-1 M. oryzae hemibiotrophic life cycle…………………………………………17
Figure 2-2 A family of M. oryzae zinc-finger effectors………………………………………23
Figure 2-3 Zinc finger effectors accumulate in BIC ………………………………………….25
Figure 2-4. Zinc-finger effectors translocate inside rice cells…………………………….26
Figure 2-5 Protein-lipid overlay assay……………………………………………………………30
Figure 2-6 Localizations of MGG_07834 : EGFP and MGG_10020 : EGFP when expressed in rice protoplasts………………………………………………………30
Figure 2-7 The role of L/IPXP in BAX fusion protein translocation…………………..31
Figure 2-8 The role of L/IPXP in EGFP fusion protein translocation………………….32
Figure 2-9 The role of L/IPXP and L/IXAR in MGG_07834 or its mutant EGFP fusion protein translocation………………………………………………………33
Figure 3.1 Major transcription factors of defense hormone pathways in Arabidopsis…………………………………………………………………………………………39
Figure 3.2 Pathogen effectors and their targets in the nucleus………………………….44
Figure 3-3 The repressive chromatin state of the promoter of KNOX genes established by AS1/AS2/HIRA silencing complex………………………………………45
Figure 3-4 Y2H screen for MGG_07834 interactors……………………………………..48
Figure 3-5 Predicted motifs in HIRA, OsSTOP1 and OsPPR1……………………………49
Figure 3-6 In vitro GST pull-down assay demonstrated zinc-finger effectors interact
with HIRA in vitro……………………………………………………………………………50
Figure 3-7 Interactions between zinc-finger effectors and HIRA were localized to rice
cell nuclei………………………………………………………………………………………51
Figure 3-8 Coimmunoprecipitation assay for the interaction between effectors and
HIRA in rice protoplasts…………………………………………………………………………52
Figure 3-9 3-D structures of zinc finger motifs of MGG_07834, MGG_10020, AVR-
Pii and AS2……………………………………………………………………………………54
Figure 3-10 One-finger zinc finger proteins can specify a sequence of DNA………55
Figure 3-11 MGG_07834 binds to double stranded DNA probes after five rounds of
SELEX reactions…………………………………………………………………………56
Figure 3-12 A model for establishing a chromatin repressive state at the promoters of
certain defense-related genes by ZFE/HIRA silencing complex………………….59
Figure 4-1. Zinc-finger effectors suppress BAX-induced PCD when transiently
expressed in N. benthamiana via Agrobacterium infiltration……………………………70
Figure 4-2. Zinc-finger effectors suppress BAX-induced PCD in rice protoplasts….71
Figure 4-3 Suppression of induction of defense related genes in MGG_07834 and
MGG_09035 transgenic lines after M. oryzae inoculation………………………………76
Figure 4-4 Lesion sizes and Lesion numbers of MGG_07834 and MGG_09035
transgenic lines post M. oryzae inoculation…………………………………………….77
Figure 4-5 Disease severity of MGG_07834 and MGG_09035 post spray and punch
inoculation inoculation inoculation of M. oryzae strains, respectively………………………….78
Figure 4-6 Model of M. oryzae ZFEs suppress chitin (GlcNAc)n-CEBiP/OsCERK1
mediated rice immunity……………………………………………………………………80
Figure 5-1 OsproPep1 and its mature peptide OsPep1…………………………………..88
Figure 5-2 Induction of OsPROPEP1 by various inducers……………………………..90
Figure 5-3 OsPep1 (1µM) induces expression of its precursor gene and defense genes……………………………………………………………………………………………………94

Figure 5-4 OsPep (1µM) induces resistance against M. oryzae………………………………95

Figure 5-5 Comparison phenotypes of wild-type and OsPROPEP1-OX plants………98

Figure 5-6 OsPROPEP-OX lines constitutively express defense genes and enhance disease resistance against M. oryzae…………………………………………………………………………105
LIST OF TABLES

Table 2-1 Effectors cloned as AVR proteins in *M. oryzae*.................................18
Table 2-2 Effector accumulation and movement pattern........................................20
Table 3-1 Chromatin modulins in plant defense...................................................41
Table 3-2 Summary of rice proteins that interact with MGG_07834.........................48
Table 4-1 Rice M/PAMPs and PRRs in PTI...........................................................66
Table 5-1 *OsPROPEPs* and the putative mature peptides.....................................87
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Chapter 1

Literature review

Plant invading organisms, living in a pathogenic relationship with the host plants, often produce an array of proteins that can enter or are injected into host cells. These proteins, predominately living up to the name “effectors”, can turn host biological processes to the advantage of the invaders. In some cases, upon inside plant cells, effectors are recognized by the host surveillance system and trigger defense responses. In this context, effectors are given the name “avirulence” proteins (AVRs) that can directly or indirectly interact with their cognate resistance proteins (Rs), central components of the plant surveillance system. The plant defense response, initiated by AVR-R interaction, is designated as effector-trigger immunity (ETI). Prior to that is another layer of disease resistance called pathogen associated molecular pattern (PAMP) triggered immunity (PTI). PTI and ETI are coming in two flavors with the former being weaker and slower and the latter being much stronger and faster against pathogen infections (Jones and Dangl, 2006). Successful pathogens deploy a whole host of effectors that promote infection by targeting signaling components of PTI and/or ETI pathways from the receptors in the cytosol or at the cell surface all the way through to the chromatin and gene transcription machineries in the nucleus.

1.1 Plant pathogen effector delivery systems

Plant pathogens have adopted a variety of mechanisms to deliver their effectors during the process of co-evolution with their hosts. Effectors from viruses are expressed in plant cells following the whole organism’s cellular entry. Bacteria by and large use type III and IV secretion systems to deliver their effectors into plant cells.
Oomycetes and fungi churn out effectors with N-terminal host targeting motif (HTM), which may guide effectors into plant cells possibly via HTM-mediated cellular uptake. Effectors from nematodes are injected into plant cells through the small orifice of the piercing stylet.

1.1.1 Bacterial effector secretion/translocation systems

Figure 1-1 Bacterial T3SS, T4SS and T6SS translocate effectors into host cells (adapted from Govers et al., 2008, Citovsky, et al., 2007 and Kapitein et al., 2012).

A. Bacterial T3SS; B. *Agrobacterium tumefaciens* T4SS; C. Bacterial T6SS.

Type III secretion system (T3SS), also called injectisome, is a nanomachine specialized in protein secretion and/or translocation in Gram negative bacteria. It operates not only across bacterial genera but also across host kingdoms, dictating pathogenicity and virulence in a wide range of animal and plant pathogens such as *Salmonella, Yersinia, Shigella, Pseudomonas, Erwinia, Ralstonia, Xanthomonas* and
Rhizobia. T3SS, having a common evolutionary origin with the flagellum, consists of up to 25 proteins, of which 9 are conserved across all the seven families found in plant and animal bacteria (Cornelis, 2006). Plant bacterial pathogen, defective in T3SS, often forfeits its ability to induce hypersensitive response (HR) in incompatible plants and pathogenesis in compatible plants. As a result, T3SS is well known as hypersensitive reaction and pathogenicity (Hrp) pilus in plant pathogens. The Hrp pilus, projecting from the bacterial envelope all the way up to the plant cell wall, is home to a hollow channel between the bacterial cytoplasm and the host cell plasma membrane. A protein, secreted via this channel and dubbed translocon, then forms a pore through the host plasma membrane that enables finishing up the last step of assembling the effector delivery machine (Figure 1-1 A).

In comparison with other secretion systems, type IV secretion system (T4SS) is unique in its ability to deliver nucleic acids in addition to proteins (Figure 1-1 B). While Type IV functional similarities are present in such a wide range of bacteria as Helicobacter pylori, Pseudomonas aeruginosa, Bordetella pertussis, Escherichia coli, Legionella pneumophila and Mesorhizobium loti (Christie et al, 2005; Hubber et al, 2007), T4SS in Agrobacterium tumefaciens has made its way to the stardom in basic plant research and modern plant biotechnology. Agrobacterium T4SS, also called T-pilus, is made up of VirB2-VirB11 and VirD4. And VirB1 assists the assembly of T-pilus by remodeling the peptidoglycan layer. Upon recognition of plant signals by the bacterial VirA/VirG sensory system, T-pilus is assembled through which T-DNA in cis with a covalently attached VirD2 and in trans with several other proteins such as VirE2, VirE3, VirF and VirD5, is exported into the plant cell (Citovsky et al., 2007).
Once inside the plant cell, the VirD2-T-strand binds to many molecules of VirE2 to form a mature T-complex. By hijacking many host cell fundamental processes such as nuclear importing, ubiquitin-26S proteasome system (UPS)-mediated proteolysis and DNA transcription and repair machineries, VirD2, VirE2, VirF, VirE3 and VirD5 facilitate T-DNA integration and expression in the host genome (Magori et al., 2012).

Type VI secretion system (T6SS), recently discovered in human bacterial pathogens *Pseudomonas aeruginosa* and *Vibrio cholerae* (Mougous et al., 2006; Pukatzki et al., 2006) is widespread among Gram negative bacteria including plant pathogens and symbionts such as *A. tumafaciens*, *pseudomonas syringae*, *Xanthomonas oryzae*, and *Rhizobacterium leguminosarum* (Records, 2011). T6SS is an inverted phage-like structure, through which effectors are injected into host cells to promote virulence or interbacterial outcompetition (Figure 1-1C).

The mechanisms by which bacterial effectors are recognized and delivered by these systems are poorly understood. However, some insights are emerging from the study of T3SS effectors. In brief, effector N-terminal peptide, effector RNA and its cognate chaperone are independently or coordinately involved in effector recognition and delivery by T3SS. In *Pseudomonas syringae*, an unusual bias in N-terminal amino acid composition has been identified and exploited successfully to identify new effectors, emphasizing the N-terminal signal as a general prerequisite for T3SS recognition and delivery (Vinatzer et al., 2005; Schechter et al., 2012). In some cases, the N-terminal signal can be either encoded in the underlying mRNA or the untranslated leader RNA (Anderson et al., 1999; Niemann et al., 2013). And in some
other cases, the binding of a cognate chaperone is necessary for an effector to be delivered by T3SS, but this does not seem to be a universal rule (Guo et al., 2005).

1.1.2 Oomycete and fungal effector delivery venues

Figure 1-2 Oomycete and fungal effector-exporting specialized structures (adapted from Rafiqi, et al., 2012). (a). Haustorium; (b). Invasive hypha; (c). Arbuscule.

Many biotrophic or hemibiotrophic oomycetes and fungi develop a bulbous feeding structure, i.e. haustorium or invasive hypha inside the plant cell by invaginating the host plasma membrane. These specialized structures are bustling venues where effectors are exported and nutrients are ingested.

Haustorium (Figure 1-2 A) is a globular structure formed from a terminal side branch of a hypha and surrounded by an extra-haustorial membrane (EHM), which is continuous with the plasma membrane of the plant cell. In between the pathogen cell
wall and host plasma membrane rests the biotrophic interface (BI), a matrix of secreted effectors from the pathogen and nutrients from the host cell. Although originated from plant plasma membrane, EHM differs in composition from the remaining plant plasma membrane, suggesting a selection driven by the plant pathogen (Lu et al., 2012). And this selection for EHM composition may contribute to effector uptake by the host cell (Bozkurt et al., 2012).

Invasive hypha (Figure 1-2 B) is a bulbous structure and, like haustorium, is also enveloped by host plasma membrane-derived extra invasive hyphal membrane (EIHM). Effectors are often observed to accumulate and latter translocate at the biotrophic interface. In *Magnaporthe oryzae*, first formed on the tip of the first host-invading hyphal cell and latter left beside the newly developed bulbous invasive hypha is a 1-2 μm-diameter dome-shaped biotrophic interfacial complex (BIC), which is full of effectors waiting for translocation (Kankanala et al., 2007; Mosquera et al., 2009; Khang, C. H. et al., 2010). Similarly, interfacial bodies, structures of ~ 500 nm in diameter are also observed at the biotrophic interface as depositories for *Colletotrichum higginsianum* effectors (Kleemann et al., 2012).

Arbuscule (Figure 1-2 C) is a highly branched structure in vascular plant root cells and developed from a hypha of a mutualistic arbuscular mycorrhizal fungus (AMF) during symbiosis. Like haustorium and invasive hypha, arbuscule is also enclosed by plant plasma membrane-derived periarbuscular membrane (PAM). In addition to serving as a nutrient exchange venue, arbuscule is also expected to secret fungal effectors into plant cells (Kloppholz et al., 2011; Plett et al., 2011).
Fungal and oomycete effectors are secreted via the classical endoplasmic reticulum (ER) pathway, i.e. from ER to Golgi apparatus to vesicles to biotrophic interface where they re-enter plant cell cytosol. The mechanisms by which effectors enter plant cells are intensively pursued and yet not known. Based on the study of oomycete RXLR effectors, Kale et al. (2010) proposed a model in which oomycete RXLR motif or its highly degenerate fungal homologs, binds to phosphatidylinositol-3-phosphate (PI3P) at the plant cell plasma membrane outer surface and internalizes into plant cell cytosol via endocytosis. This model, however, is not widely accepted and often challenged by the oomycete and fungal pathogen communities. In flax rust fungus *Melampsora lini*, it is not required for the host targeting motifs of AvrM and AvrL567 to bind phospholipids to guide effectors’ entry into plant cells in the absence of the pathogen (Gan et al., 2010). In oomycete *Phytophthora infestans*, phospholipid binding of Avr3a is not through its N-terminal RXLR but through its C-terminus, and this binding is not for effector entry but for its stability and function inside plant cells (Yaeno et al., 2011). It appears that there exist multiple mechanisms of effector entry and the search for plant-side receptors continues for this class of effectors. Recently, in oomycete fish pathogen *Saprolegnia parasitica*, it was discovered that effector SpHtp1 enters fish cells via binding of tyrosine-O-sulphate on the host cell surface (Wawra et al., 2012). This is another twist en route to reveal the mystic and elusive effector entry mechanisms.

1.2 The subcellular locations of plant pathogen effectors

Once inside the plant cell, effectors can reside in almost all the subcellular compartments: cytosol, nucleus, chloroplast and mitochondrion (Figure 1-3). In some
cases the evidence for cytoplasmic localization is directly supported by experiments showing physical interactions between effectors and R proteins or other proteins in the plant cytoplasm. Examples include *M. oryzae* AvrPita. AvrPita was demonstrated to directly interact with its cognate R protein, Pita in rice cell cytoplasm by transient gene expression and immunoprecipitation (Jia et al., 2000). In other cases, effectors have been identified histochemically by antibody staining or via a fluorescent tag. These include the bacterial effectors AvrPtoB (de Vries et al., 2006) and HopU (Fu et al., 2007); and the oomycete effectors Avr1b (Dou, 2008) and Avr3a (Armstrong, et al., 2005). In *Xanthomonas* spp., a large family of effectors, called transcription activator-like effectors (TALEs), was identified to function in the host nucleus (Kay et al., 2007; Yang et al., 2006). This family of effectors contains a central repeat region for DNA binding, a C-terminal nuclear localization signal and an acidic activation domain, showing their nuclear localization as plant transcription activators. Four putative effectors, Nuk6, Nuk7, Nuk10 and Nuk12, from the oomycete *P. infestans* were also predicted to be nucleus localized (Kanneganti et al., 2007). SP7 and MiSSP7, two effectors from arbuscular fungi, were found to be translocated into the plant root cell nucleus (Kloppholz et al., 2011; Plett et al., 2011). The *P. syringae* effector HopI1, when expressed in the plant cell, is targeted to the chloroplast where it remodels the chloroplast and suppresses defenses (Jelenska et al., 2007).
1.3 The host targets of plant pathogen effectors

The all-over-the-map subcellular locations of plant pathogen effectors suggest that they perform a variety of roles in promoting infection. In the face of a pathogen, plant usually mounts two layers of defense responses: i.e. PTI and ETI. Although different in timing and amplitude, the signaling pathways underlying PTI and ETI to a large extent are the same, involving recognition of effectors by receptors, activation of mitogen-activated protein kinases (MAPKs), crosstalk of stress hormones and
expression of defense genes. All these fundamental cellular processes are natural targets of plant pathogen effectors (Figure 1-3).

1.3.1 PRRs and Rs

Pattern recognition receptors (PRRs) are usually receptor-like kinases (RLKs) or receptor-like proteins (RLPs) in the plant plasma membrane, which recognize PAMPs and initiate PTI. Rs are largely nucleotide binding-leucine rich repeat proteins (NB-LRRs) that recognize corresponding Avr proteins and elicit ETI. Therefore, both PRRs and Rs are the convenient targets for pathogen effectors. Indeed, in Arabidopsis, tomato and other higher plants, FLS2 (flagellin sensitive 2) is the PRR for flg22 (a conserved 22 amino-acid N-terminal stretch of flagellin) and the target of P. syringae effectors AvrPto and AvrPtoB. While AvrPto disrupts PTI by inhibiting the kinase activity of FLS2 or interfering with the formation of FLS2-BAK1 complexes (Xiang et al., 2008; Shan et al., 2008), AvrPtoB blocks PTI by eliminating FLS2 via its C-terminal E3 ligase activity (Gohre et al., 2008). Recently, AvrPtoB has also been demonstrated to inhibit chitin triggered PTI by marking LysM chitin receptor CERK1 for degradation (Gimenez-Ibanez, et al., 2009). Although its major function is to target PRRs for degradation, AvrPtoB can overcome ETI by targeting a protein kinase Fen, an ancient homolog of R protein Pto and a part of a unique ETI pathway (Rosebrock et al., 2007). Tomato leaf mold fungus Cladosporum fulvum secretes a lysin motif (LysM) domain-containing effector Ecp6 to compete with PRR receptor for binding chitin oligosaccharides to avoid eliciting PTI (De Jonge et al., 2010).

Similarly, in M. oryzae, LysM effector Slp1 plays the same role by sequestering chitin
oligosaccharides from rice PRR receptors CEBiP and OsCERK1, short-circuiting PTI (Mentlak et al., 2012).

1.3.2 The MAP3K-MAP2K-MAPK cascade

The perception of PAMPs or Avr proteins by PRRs or Rs is usually followed by the activation of MAP3K-MAP2K-MAPK signaling transduction cascades. Therefore, terminating or intercepting MAPK signaling is a natural strategy for pathogen effectors to promote pathogenicity. HopAI1, present in many *P. syringae* strains, is a phosphothreonine lyase that irreversibly dephosphorylates MAPK3 and MAPK6 to terminate PRR signaling (Zhang et al., 2007). HopPtoD2, another effector from *P. syringae*, has an in vitro protein tyrosine phosphatase activity and is likely to promote pathogenicity through inactivation of MAPK signaling (Espinosa et al., 2003). Also in *Pseudomonas syringae*, HopF2 uses ADP ribosyl-transferase activity to inhibit MKK5 and other MAPKKs, eluding PTI (Wang et al., 2010).

1.3.3 The crosstalk between phytohormones

The crosstalk between plant hormones auxins, salicylic acid (SA), jasmonates (JA), ethylene (ET), abscisic acid (ABA) and gibberellin (GA) delicately fine-tunes plant defense responses in PTI and ETI. In *Arabidopsis*, the basic crosstalk between SA and JA/ET is beginning to emerge: SA-dependent resistance is summoned against biotrophic and hemibiotrophic pathogens, while JA/ET-dependent resistance is mounted against necrotrophic pathogens and herbivorous insects. The crosstalk reflects coherent trad-offs between hormone signaling pathways. Pathogen effectors
can exploit these trade-offs to their benefits. The *P. syringae* effector HopI1 resides in the chloroplast, where it remodels the thylakoid structure and suppresses SA accumulation. Reduced levels of SA cannot effectively activate SA-dependent pathway against hemibiotrophic *P. syringae* (Jelenska et al., 2007). Again, *P. syringae* avrPtoB transcriptionally suppresses *MIR393*, which shuts off auxin responsive pathway. By going through this delicate, multi-step process, avrPtoB ultimately activates auxin responsive pathway to inhibit SA-dependent pathway (Navarro et al., 2006; 2008). In addition to deploying effectors, pathogens also directly interfere with the crosstalk between hormone signaling pathways by producing hormones or hormone mimics. *A. tumefaciens*, the causal agent of crown gall, can genetically transform its host plant by transferring a segment of the tumor-inducing (Ti) plasmid into the host’s genome. The transferred T-DNA carries genes that code for the production of auxins and cytokinins, resulting in uncontrolled host cell proliferation from which the pathogen can benefit. Necrotrophic fungus *Gibberella fujikuroi*, the eponym of GA, was demonstrated to produce significant amount of GA, possibly to disable JA-dependent necrotroph resistance through GA-mediated destabilization of DELLA proteins that play a role in controlling the crosstalk between SA-and JA-dependent defenses (Navarro et al., 2008). *P. syringae* produces the phytotoxin coronatine, a JA mimic and suppresses SA-dependent defenses, thereby promoting susceptibility of the plant to this pathogen (Brooks et al., 2005). Coronatine also plays a key role in re-opening stomata to facilitate pathogen penetration after ABA-mediated stomatal closure by antagonizing the SA/ABA signaling pathways (Melotto et al., 2006). *Heterodera glycine*, the causal agent of soybean cyst root-knot, injects into the plant cell an effector HG-SYV46, which functions as the plant peptide hormone *CLAVATA3/ESR (CLE)* to alter plant
morphology in favor of the pathogen. Effector Cmu1 from maize smut fungus *Ustilago maydis* is a functional chorismate mutase, which diverts chorismate metabolism away from SA production and thereby evade plant defense (Djamei et al., 2011).

1.3.4 The chromatin and transcription machinery

Both PTI and ETI require extensive transcriptional reprogramming by recruiting chromatin modulins and transcription machineries. The nucleus, at the heart of the plant cell, is a major target for pathogen effectors. In *Xanthomonas* and *Ralstonia*, there is a large family of transcription activator-like effectors (TALEs). They bear the same characteristic structural features: the N-terminal secretion signal for delivery by the bacterial T3SS; a central repeats of 34 or 35 amino acids for DNA binding, and the C-terminal region with a nuclear localization signal and an acidic activation domain. Several members of this TALE family have been demonstrated to function as transcription factors or by recruiting basal transcription machinery to promote disease. AvrBs3, the founding member of TALEs from *X. campestris pv. vescicatoria* (Xcv) activates the expression of more than 11 genes, causing enlargement of mesophyll cells in the leaf interior to facilitate pathogen dispersal (Kay, et al., 2007). PthXo1, a major virulence effector and a TALE from *X. oryzae pv. oryzae* (Xoo), induces the expression of a susceptibility gene *Os8N3* (also called *xa13, OsSWEET11*) by binding to its promoter (Yang et al., 2006). *Os8N3*, renamed *OsSWEET11*, encodes a plasma membrane-bound sugar transporter that exports into the apoplast sucrose which the pathogen exploits (Chen et al., 2010; 2012). Avrxa5, another TALE from Xoo, acts as a transcription factor and recruits the $\gamma$ subunit of the general transcription factor
TFIIA for gene transcription to promote pathogenicity (Jiang et al, 2006). XopD, an effector from Xcv, also has a modular structure: an N-terminal secretion signal for delivery by the bacterial T3SS, followed by a helix-loop-helix domain for DNA binding and two ERF (ethylene response factor)-associated amphiphilic repression (EAR) motifs and a C-terminal small ubiquitin-like modifier (SUMO) protease. XopD is translocated into the nucleus and regulates gene expression by interacting with transcription factors. By binding the transcription factor MYB30 and desumoylating the transcription factor SIERF4, XopD suppresses defense responses in Arabidopsis and tomato, respectively (Canonne et al., 2011; Kim et al., 2013). SP7, an effector from arbuscular mycorrhizal fungus Glomus intraradices interacts with the transcription factor ERF19 in the nucleus to evade plant defense and promote symbiosis (Kloppholz et al., 2011). Pathogen effectors also regulate host gene expression to their own benefits by acting as chromatin modulins. Shigella flexneri OspF has a phosphor-Thr lyase (eliminylase) activity that targets nuclear MAPKs. In addition, it alters chromatin structure at specific genes by inducing histone dephosphorylation and deacetylation (Arbibe et al., 2007). It is tempting to propose that HopAI1, a Pseudomonas syringae effector similar to OspF (Li et al., 2007), also can affect histone post-translational modifications and limit chromatin access for specific gene transcription. In Agrobacterium, 6b protein, encoded by T-DNA, is an effector targeted to the plant cell nucleus where it binds to histone H3 and other nuclear proteins and probably functions as a histone chaperone to regulate gene expression (Terakura et al., 2007).

In this study, we try to characterize a family of zinc finger effectors in Magnaporthe oryzae secretome and answer the following questions. Are zinc finger effectors translocated into rice cells during fungal infection? What is the mechanism by which
zinc finger effectors enter rice cells? What are the targets of zinc finger effectors inside rice cells? How to understand the role of zinc finger effectors in disease promotion along the line of suppression of PTI by pathogen effectors?
Chapter 2

A family of *Magnaporthe oryzae* zinc finger effectors are secreted and translocated inside plant cells

2.1 Introduction

*M. oryzae* is a hemibiotrophic ascomycete fungus, the causal agent of rice blast disease. *M. oryzae* can infect about 50 grasses including some agro-economically important crops such as rice, wheat, barley and millet (Couch et al., 2005). Each year 10~30% of rice yield worldwide is usually lost to this fungal disease (Zeigler et al., 1994). In the field, *M. oryzae* infection cycle is as follows: A conidium from a conidiophore in the lesion spot falls onto a nearby plant site, where it attaches firmly and germinates within a few hours. By 12 hours, at the tip of the germination tube, appressorium forms and within 24 hours appressorium forces a penetration peg into the underlying cell. From 24 hours to 36 hours, a thin primary filamentous hypha first appears and subsequently new bulbous invasive hyphae (IH) quickly branch out to fill the whole cell. From 36 hours on, the IH begin to invade the neighboring cells. Later at the infection site plant cells die and the fungus enters the necrotrophic phase. About 5~7 days disease lesions appear, from which, conidia are born and the cycle start anew (Figure 2-1). Great efforts have been focused on the initial infection steps such as how appressorium forms and how penetration peg forces into plant cells and some aspects of these initial steps are well understood (Wilson et al., 2009). However, little is known about how invasive hyphae in the biotrophic phase slyly propagate in living rice cells under the nose of the plant surveillance systems such as PTI and how
transition occurs to a pathogen from a biotrophic life style to a necrotrophic one. These are fundamental questions and effectors are the keys to the mysteries.

Figure 2-1 *M. oryzae* hemibiotrophic life cycle

(adapted from Dean et al., 2011)

*M. oryzae* strain 70-15 (a derivative of the field strain Guy11) has been sequenced and the genome sequence is available to the public (Dean et al., 2005). Of the predicted proteome, 739 (7% of 11109), 1360 (12% of 11109), 1546 (12% of 12481), or 2470 (22% of 11069) proteins are independently predicted to comprise the fungus’ secretome based on different signal peptide prediction and false positive filter softwares (Dean et al., 2005; Yoshida et al., 2009; Soanes et al., 2008; Choi et al., 2010). *M. oryzae* secretome is a rich source for effector identification.
<table>
<thead>
<tr>
<th>Name</th>
<th>Size (aa)</th>
<th>Molecular feature</th>
<th>Location</th>
<th>Avrulence function</th>
<th>Virulence function</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE1</td>
<td>4035</td>
<td>Secondary metabolite enzyme</td>
<td>Fungal cytoplasm</td>
<td>Pi33 (ETI)</td>
<td>None</td>
</tr>
<tr>
<td>PWL2</td>
<td>145</td>
<td>None</td>
<td>Host cytoplasm</td>
<td>NA (ETI)</td>
<td>NA</td>
</tr>
<tr>
<td>AVR-CO39</td>
<td>89</td>
<td>None</td>
<td>Host cytoplasm</td>
<td>PiCO39 (ETI)</td>
<td>NA</td>
</tr>
<tr>
<td>AVR-Pita1</td>
<td>224</td>
<td>Zinc-metalloprotease</td>
<td>Host cytoplasm</td>
<td>Pita (ETI)</td>
<td>NA</td>
</tr>
<tr>
<td>AVR-Piz-t</td>
<td>108</td>
<td>LXAR</td>
<td>Host cytoplasm</td>
<td>Piz-t (ETI)</td>
<td>Destabilize E3 ubiquitin ligase and inhibit PTI</td>
</tr>
<tr>
<td>AVR-Pii</td>
<td>70</td>
<td>LXAR; L/IPXP; Incomplete C_2H_2</td>
<td>Host cytoplasm</td>
<td>Pii (ETI)</td>
<td>NA</td>
</tr>
<tr>
<td>AVR-Pia</td>
<td>85</td>
<td>None</td>
<td>Host cytoplasm</td>
<td>Pia (ETI)</td>
<td>NA</td>
</tr>
<tr>
<td>AVR-Pik/km/kp</td>
<td>113</td>
<td>None</td>
<td>Host cytoplasm</td>
<td>Pikm (ETI)</td>
<td>NA</td>
</tr>
</tbody>
</table>

Due to the large pool of effectors and gene redundancy in *M. oryzae*, direct gene knock-out mutations usually give few clues to effectors’ functions. To date, only a few effectors were cloned by exploiting their functions as AVR proteins in the rice-*M. oryzae* pathosystem (Table 2-1). ACE1 triggers hypersensitive response (i.e. ETI) in rice cultivars containing the resistance gene *Pi33*. It encodes a hybrid polyketide synthase-nonribosomal peptide synthase, a putative secondary metabolite biosynthetic enzyme (Bohnert et al., 2004; Collemare J., et al., 2008). PWL2, a cysteine rich 145aa protein, belongs to a small family with different number of members in different *M. oryzae* strains (Kang et al., 1995). During fungal biotrophic growth, it is expressed and secreted into biotrophic interfacial complex (BIC) and later enters cytoplasm and moves from the first infected cell to the neighboring cells in advance of invasive hyphae (Khang et al., 2010). *AVR-CO39* gene is cloned from weeping lovegrass-infecting *M. oryzae* strain and encodes an 89 aa protein that can be recognized in rice
cultivars containing *PiCO39* resistance gene (Farman et al., 1998). AVR-CO39 can be translocated into rice cell cytosol in the absence of *M. oryzae* (Ribot et al., 2012). AVR-Pita1 encodes a 224 aa protein with similarity to zinc metalloprotease (Orbach et al., 2000). Besides its signal peptide, it contains a propeptide that must be cleaved for its activity to produce a mature 176 aa protein. AVR-Pita176 was demonstrated to interact directly with its cognate resistance protein Pita in vivo (Jia et al., 2000). AVR-Pita is accumulated in BIC and translocated into plant cytoplasm during fungal invasive growth (Khang et al., 2010).

AVR-Piz-t is a 108 aa protein with no similarity to any known proteins and recognized by the cognate R protein Piz-t to fulfill its avirulence function (Li et al., 2009). AVR-Piz-t was recently also assigned a virulence function of suppressing chitin elicited PTI by interacting with and destabilizing the E3 ubiquitin ligase APIP6 (Park et al., 2012). AVR-Pii, AVR-Pia and AVR-Pik/km/kp were cloned by re-sequencing of a Japanese rice blast isolate Ina 168 and the ensuing association genetic analysis (Yoshida et al., 2009). Of note, AVR-Pia was also independently isolated using map-based cloning (Miki et al., 2009). AVR-Pii is 70 aa protein with a LXAR motif and an incomplete monodactyl C2H2 zinc-finger. AVR-Pia and AVR-Pik/km/kp are typical fungal effectors (a.k.a novel proteins) with no similarity to any known proteins. All three proteins are translocated into rice cells during infection.
An alternative way to isolate putative *M. oryzae* effectors was created in Dr. Barbara Valent’s lab at Kansas State University (Mosquera et al., 2009). By using hand-trimmed sheath tissue containing first invaded cells as RNA materials, Mosquera and colleagues obtained RNA with ~20% RNA from invasive hyphae. *M. oryzae* whole genome microarray hybridization showed that 59 genes encoding putative secreted proteins are upregulated by at least 10-fold, and of the 59 genes one is a known effector gene *PWL2*. They named the induced and secreted proteins during fungal biotrophic growth BAS proteins (biotrophy-associated, secreted proteins). They further characterized four BAS proteins, BAS1-4, and some known effectors such as AVR-Pita1 and PWL2 by carefully observing their accumulation at the biotrophic interface and cell-to-cell movement from first invaded cell to neighboring cells. The accumulation and movement patterns of these effectors fall into four classes (Table 2-2). First, effectors like AVR-Pita1 accumulate in BIC and translocate into plant cytoplasm. Second, effectors like BAS2 and BAS3 accumulate in BIC and later in invasive hyphal host cell wall crossing points (likely plasmodestama). Third, effectors like PWL2 and BAS1 accumulate in BIC and later move to neighboring cells ahead of

### Table 2-2 Effector accumulation and movement pattern

<table>
<thead>
<tr>
<th>Name</th>
<th>BIC</th>
<th>Location</th>
<th>movement</th>
</tr>
</thead>
<tbody>
<tr>
<td>AVR-Pita1</td>
<td>Yes</td>
<td>cytoplasm</td>
<td>NA</td>
</tr>
<tr>
<td>PWL2</td>
<td>Yes</td>
<td>cytoplasm</td>
<td>Move ahead of IH</td>
</tr>
<tr>
<td>BAS1</td>
<td>Yes</td>
<td>cytoplasm</td>
<td>Move ahead of IH</td>
</tr>
<tr>
<td>BAS2</td>
<td>Yes</td>
<td>plasmodesmata</td>
<td>NA</td>
</tr>
<tr>
<td>BAS3</td>
<td>Yes</td>
<td>Plasmodesmata</td>
<td>NA</td>
</tr>
<tr>
<td>BAS4</td>
<td>No</td>
<td>Lining IH</td>
<td>NA</td>
</tr>
</tbody>
</table>
invasive hyphae. And forth, effectors like BAS4 do not accumulate in BIC but outline the growing invasive hyphae. It is tempting to ascribe some virulence functions to these different classes of effectors. Effectors that outline invasive hyphae are fungal cell wall protectors, say, like C. fulvum Avr4 which bind fungal cell wall component chitin to prevent degradation from host chitinases. Effectors accumulate at plasmodesmata are fungal movement proteins that like virus movement proteins modify plamodesmata to allow hyphal crossing. Effectors that move ahead of invasive hyphae are heralds that prime neighboring cells for imminent fungal invasion. All this is hypothetic and the fact will be revealed with the finding of the virulence targets of these effectors.

In this study, a family of one finger zinc finger proteins was identified as putative effectors. Zinc finger effectors accumulate first in BIC and then translocate into rice cells. Once inside rice cells, zinc finger effectors can move to neighboring cells. And the translocation of zinc finger effectors may depend on two putative host targeting motifs L/IPXP and L/IXAR.

2.2 Results

**Identification and cloning of a family of putative M. oryzae one-finger zinc finger proteins**

In the predicted proteome of M. oryzae strain 70-15, there is a family of putative effector proteins with seven paralogs: MGG_07834, MGG_09035, MGG_08230, MGG_10556, MGG_12038, MGG_13665 and MGG_10020 (Figure 2-2 A). They are modular proteins with an N-terminal signal peptide (SP), an immediate region of two
conserved motifs L/IPXP and L/IXAR and a C-terminal one-finger C$_2$H$_2$ motif. It should be noted that MGG_15926 with similar motifs except for two C$_2$H$_2$ fingers is not included in this family. AVR-Pii, an ortholog from a Japanese strain Ina168, was demonstrated to perform avirulence function inside rice cells, suggesting that this protein is translocated into rice cells during fungal infection (Yoshida et al., 2009). This host targeting property might be extended to its orthologs in M. oryzae isolate 70-15. Another indication that this family of zinc finger proteins might be functional effectors is that $MGG_{07834}$ and $MGG_{09035}$ were upregulated 74 and 35 folds, respectively, in invasive hyphae of the first invaded rice cells in comparison to axenic mycelium grown in vitro (Mosquera et al., 2009). We cloned this family of putative zinc finger effector genes for further study. Sequence alignment and phylogenetic analysis (Figure 2-2 B) revealed that MGG_10238, the closest member to AVR-Pii, also has an incomplete zinc finger motif, suggesting zinc-finger motif may play a role in deciding effector’s virulence and avirulence functions.

A

![Sequence Alignment and Phylogenetic Analysis](image-url)
Figure 2-2 A family of *M. oryzae* zinc-finger effectors. A. Alignment of protein sequences of 8 members of zinc-finger effectors. B. Phylogenetic tree of zinc-finger effectors.

**Zinc finger effectors accumulate in BIC and translocate into rice cells during fungal infection**

The biotrophic interfacial complex (BIC) is initially developed at the tip of the first host-invading hyphal cell and later left on the side of the newly developed bulbous invasive hypha. BIC is the depository for most, if not all, of fungal effectors that
function inside plant cells. Under the constitutive promoter $P_{27}$ of *M. oryzae* ribosomal protein 27, MGG_07834 : EGFP and MGG_10020 : EGFP were observed to accumulate in BIC 27 hours and 30 hours post inoculation, respectively (Figure 2-3).

To further study the translocation and movement of effectors inside rice cells during fungal infection, we made new constructs $P_{PWL2} : MGG_07834 : mCherry : NLS : Ter : P_{BAS4} : BAS4 : EGFP$ and $P_{PWL2} : MGG_10020 : mCherry : NLS : Ter : P_{BAS4} : BAS4 : EGFP$. We exploited the promoter of a known effector gene $PWL2$ instead of their native promoters to drive the expression of fusion proteins MGG_07834 : mCherry : NLS and MGG_10020 : mCherry : NLS. Three tandem repeats of the nuclear localization signal (NLS) of the simian virus large T-antigen were added to enrich the fusion proteins in the nucleus in order to observe more clearly their translocation and movement inside rice cells. BAS4 : EGFP, an effector fusion protein outlining invasive hyphae, was used to distinguish fungal growth inside rice cells.

MGG_07834 : mCherry : NLS and MGG_10020 : mCherry : NLS were translocated into the nuclei of rice cells when observed 28 hours and 33 hours post inoculation, respectively (Figure 2-4). Furthermore, traces of MGG_07834 : mCherry : NLS and NGG_10020 : mCherry : NLS began to appear in the nuclei of neighboring cells of the first invaded cell in advance of the invasive hyphae (Figure 2-4), suggesting this family of zinc finger effectors may play a role in priming the rice cells for imminent fungal invasion.
Figure 2-3 Zinc finger effectors accumulate in BIC. A. MGG_07834 : EGFP accumulates in BIC in rice sheath epidermal cell un-plasmolyzed (first row) or plasmolyzed (second row). B. MGG_10020 : EGFP accumulates in BIC in rice sheath epidermal cell un-plasmolyzed (first row) or plasmolyzed (second row). Arrows point to BIC.
Figure 2-4. Zinc-finger effectors translocate inside rice cells. A. MGG_07834: mCherry : NLS translocates into the nuclei of rice sheath epidermal cells unplasmolyzed (first row) or plasmolyzed (second row). B. MGG_10020: mCherry : NLS translocates into the nuclei of rice sheath epidermal cells unplasmolyzed (first row) or plasmolyzed (second row). Arrows point to the nuclei of the uninvaded neighboring cell.
Motifs L/IPXP and/or L/IXAR may play a role in translocation of effectors from fungus to plant cells

Finding host-targeting motifs is one of the major goals in pathogen effector biology study. In the proteomes of oomycete pathogens including *Phytophthora*, *Hyaloperonospora*, *Pythium* and *Albugo*, hard on the heel of the signal peptide, comes a certain conserved motif such as RXLR, LXLFLAK, or CHXC. These motifs are involved in translocation across the host cell plasma membrane and proteins containing one of these motifs are effectors functioning inside plant cells (Rehmany et al., 2005; Haas et al., 2009; Kemen et al., 2011). In fungal pathogens, to date, host-targeting motifs were only identified in four effectors: ToxA from *Pyrenophora tritici-repentis*, MiSSP7 from *Laccaria bicolor* and AvrL567 and AvrM from *Melampsora lini* (Manning et al., 2008; Plett et al., 2011, Rafiqi et al., 2010). Unlike oomycete RXLR, LXLFLAK and CHXC motifs, the motifs identified in these four fungal effectors are not widespread in the pathogen’s proteome. Recently, in barley powdery mildew *Blumeria graminis f. sp. hordei*, the tripeptide Y/F/WXC four-amino acid away from the signal peptide cleavage site were found in many secreted proteins, but experiments demonstrating its involvement in host translocation is still lacking (Godfrey et al., 2010).

We set out to find some possible host targeting motifs in this family of zinc finger effectors. There are two conserved motifs following the signal peptide but preceding the zinc finger domain: L/IPXP and L/IXAR. We searched the Broad Institute fungal database and found L/IPXP exist in another 52 proteins in *M. oryzae*’s secretome and at the same position as in zinc finger effectors, i.e. immediately after the signal peptide cleavage site (Figure 2-1 A). In addition, L/IPXP is found in the secretomes of
a wide range of fungal pathogens such as *Melampsora larici*, *Botryotinia fuckeliana* and *Puccinia graminis* f. *sp. tritici*. The second motif L/IXAR is also widely present in dozens of *M. oryzae* secreted proteins including a known effector AVR-Piz-t. The relatively ubiquitous presence of L/IPXP and/or L/IXAR suggests a conserved function possibly in secretion and/or translocation.

We demonstrated that this family of proteins can bind to various phospholipids with different affinity by using protein-lipid overlay assay (Figure 2-5). GST-MGG_07834 can bind strongly with Phosphatidylinositol-4-phosphate (PI4P), PI3P, PI5P and phosphatidylinositol-3,5-bisphosphate (PI(3,5)P), while GST-MGG_09035 can bind to all –phosphates, -bisphosphates, -triphosphate and phosphatidylylserine (PS). Phospholipids are major components of cell membranes and involved in protein cross-membrane trafficking. From this result, we can infer that zinc finger effectors may interact with plant cell membrane components to enter plant cells.

When expressed in rice protoplasts, MGG_07834 : EGFP and MGG_10020 : EGFP accumulated largely in the nucleus, although we cannot rule out the effect of EGFP because EGFP is all over the map including the nucleus and cytoplasm (Figure 2-6). But these results clearly revealed two interconnected events: first, zinc finger effectors are secreted in rice cells via the ER secretary pathway, and second, zinc finger effectors can re-enter rice cells via host targeting motifs. In other words, zinc finger effectors, like most oomycete and fungal effectors including one known *M. oryzae* effector AVR-CO39 (Ribot et al., 2012), may enter plant cells in the absence of the pathogen.
Knowing zinc finger effectors possibly translocate into plant cells in the absence of the pathogen, we started to test the two most conserved motifs L/IPXP and LXAR for potential host-targeting activity. We chose BAX as a macroscopic indicator for fusion proteins being in or out of plant cells based on the assumption that BAX induces cell death inside but not outside plant cells. Using pMDC32 as a backbone vector, five constructs, \textit{MGG\_07834SP : BAX, MGG\_07834SP-LPAPVN : BAX, MGG\_07834SP-AAAAVN : BAX, MGG\_07834SP-LPAP-IQARSA : BAX} and \textit{BAX} were made. They were introduced by agroinfiltration and expressed in \textit{Nicotiana benthamiana}. In comparison with BAX, \textit{MGG\_07834SP : BAX} and \textit{MGG\_07834SP-AAAAVN : BAX} no longer induced cell death, whereas \textit{MGG\_07834SP-LPAPVN : BAX} and \textit{MGG\_07834SP-LPAP-IQARSA : BAX} did (Figure 2-7). The plausible explanation is that due to a lack of or a mutation of the LPAP motif, \textit{MGG\_07834SP : BAX} and \textit{MGG\_07834SP-AAAAVN : BAX} were not capable of re-entering into plant cells when secreted via the plant ER secretary pathway. By observing the localizations of EGFP fusion proteins, we tested L/IPXP for host-targeting function again. Constructs \textit{EGFP, MGG\_10020SP : EGFP, MGG\_10020SP : IPTP : EGFP, and MGG\_10020SP : AAAA : EGFP} were recombined into vector pMDC32 and introduced biolistically into onion epidermal cells. Again, different localization patterns were observed between IPTP and its alanine substitution mutant AAAA EGFP fusion protein (Figure 2-8). Finally, we tested both L/IPXP and L/IXAR motifs for host-targeting activity using wild-type full-length effector or mutant EGFP fusion proteins. Six constructs \textit{EGFP} (control), \textit{MGG\_07834 SP : LPAP : IQAR : ZF : EGFP} (Wild type), \textit{MGG\_07834 SP : AAAA : IQAR : ZF : EGFP} (LPAP single mutant), \textit{MGG\_07834 SP : LPAP : AAAA : ZF : EGFP} (IQAR single mutant), \textit{MGG\_07834 : AAAA : AAAA : ZF : EGFP} (LPAP and IQAR double mutant) and \textit{ZF :
EGFP (deletion of SP, LPAP and IQAR) were recombined into pMDC32 and transiently expressed in *N. benthamiana*. In comparison to wild type that is localized largely in the nucleus and deletion mutant that is in the cytoplasm, single and double mutants appeared at a different location, probably outside of the cells (Figure 2-9). This indicates that both L/IPXP and L/IXAR motifs may play a role in effector translocation into plant cells.

![Figure 2-5 Protein-lipid overlay assay. From left, Spot positions of phospholipids on PIP strips; GST control; GST-MGG_07834 and GST-MGG_09035.](image1.png)

![Figure 2-6 Localizations of MGG_07834 : EGFP and MGG_10020 : EGFP when expressed in rice protoplasts](image2.png)
Figure 2-7  The role of L/IPXP in BAX fusion protein translocation

1. MGG_07834SP : BAX
2. MGG_07834SP-LPAPVN : BAX
3. MGG_07834SP-AAAAVN : BAX
4. MGG_07834SP-LPAP-IQARSA : BAX
5. BAX
Figure 2-8 The role of L/IPXP in EGFP fusion protein translocation.

A. EGFP

B. MGG_10020 SP : EGFP

C. MGG_10020 SP : IPTP : EGFP

D. MGG_10020 SP : AAAA : EGFP
Figure 2-9 The role of L/IPXP and L/IXAR in MGG_07834 or its mutant EGFP fusion protein translocation

A. GFP.


C. MGG_07834 SP : AAAA : IQAR : ZF : EGFP.

D. MGG_07834 SP : LPAP : AAAA : ZF : EGFP.

E. MGG_07834 : AAAA : AAAA : ZF : EGFP.

F. ZF : EGFP.
2.3 Discussion

We have identified a family of zinc finger effectors from *M. oryzae* strain 70-15. Live-cell imaging showed that they first accumulate in BIC, then are translocated into rice cells, and probably move from the first invaded cell to neighboring cells prior to invasive hyphal invasion. To figure out if there are conserved motifs that can be recognized for secretion and/or translocation, we have tested two relatively widespread conserved motifs in this family of zinc finger effectors for host-targeting activity. Our results showed that L/IPXP and L/IXAR may play a role in host translocation. Based on these results, we propose a model for the secretion, translocation and movement of this family of zinc finger effectors during *M. oryzae* infection: First, zinc finger effectors are expressed and secreted via the ER secretory pathway out of the hyphal cell into BIC. Second, certain receptors in the plant plasma membrane-derived BIC membrane recognized host targeting motifs L/IPXP and L/IXAR and mediate translocation of effectors into the plant cell. And finally, inside the plant cell, zinc finger effectors localize to a subcellular compartment or continue the journey to neighboring cells where they reprogram the host cells to the benefits of fungal biotrophic growth.

Although we demonstrated that this family of zinc finger effectors can bind phospholipids, we cannot conclude that phospholipids are the receptors mediating the translocation of this family of zinc finger effectors. Phospholipid-mediated endocytosis was proposed as a mechanism by which pathogen effectors enter host cells (Kale et al., 2010), but has since been challenged. Yaeno et al. (2011) demonstrated that phospholipid binding is required not for effector entry but for
effector’s function inside host cells. Another study showed that effector SpHtp1 enters host cells by binding tyrosine-O-sulphate but not phospholipids on the host cell surface (Wawra et al., 2012). An early study reported that L/V/IPXP motif in peroxidases is a binding site for aromatic amino acids (Veitch et al., 1994). It will be interesting to investigate if L/IPXP in fungal effectors can also bind some aromatic amino acids or their derivatives like tyrosine-O-sulphate.

2.4 Materials and methods

Genetic constructs

Gene fusions $P_{27} : MGG\_07834/10020$, $P_{PWL2} : MGG\_07834/10020$ and gene mutants $L/IPXP/AAAA$, $L/IXAR/AAAA$ were obtained by overlap extension PCR.

EcoRI- $P_{27} : MGG\_07834/10020$-BamHI were ligated with vector fragment EcoRI- $pBV126$-BamHI to obtain $MGG\_07834/10020 : EGFP$ expression constructs. XbaI- $P_{PWL2} : MGG\_07834/10020$-BamHI, XbaI- $mCherry : Ter : P_{BAS4} : BAS4$-BamHI (PCR from $pBV591$), and EcoRI-$pBV126$-BamHI were ligated together to obtain $MGG\_07834/10020 : mCherry$ expressing constructs. All these 4 constructs were used to transform blast isolate O-137. $MGG\_07834/10020 : EGFP$ fragments were recombined into vector $pUGW11$ by the gateway cloning strategy. These two constructs were used to transfect rice protoplasts. $L/IPXP : BAX$ fusion gene fragments: $G\_07834SP : BAX$, $MGG\_07834SP-LPAPVN : BAX$, $MGG\_07834SP$-AAAAVN : BAX, and $MGG\_07834SP-LPAP-IQARSA : BAX$ were obtained by overlap extension PCR. These $L/IPXP : BAX$ fusion genes were recombinant into gateway vector $pMDC32$ and used for transient expression in $N. benthamiana$. $L/IPXP : EGFP$
frusion genes $MGG_{10020} \, SP:\, EGFP$, $MGG_{10020} \, SP:\, IPTP:\, EGFP$ and $MGG_{10020} \, SP:\, AAAA:\, EGFP$ were also constructed in pMDC32 vector and used to transform onion epidermal cells by particle bombardment. Full-length effector : $EGFP$ gene constructs $MGG_{07834} \, SP:\, LPAP:\, IQAR:\, ZF:\, EGFP$, $MGG_{07834} \, SP:\, AAAA:\, IQAR:\, ZF:\, EGFP$, $MGG_{07834} \, SP:\, AAAA:\, AAAA:\, ZF:\, EGFP$ and $ZF:\, EGFP$ were constructed in pMDC32 and used to transform $N. \, benthamiana$ by agroinfiltration.

Live-cell imaging of zinc finger effectors in rice cells

Fungal spores from O-137 strains containing $MGG_{07834}/10020: \, mCherry: \, NLS: \, BAS4: \, EGFP$ were inoculated into the hollow interior of detached leaf sheath of rice cultivar YT16 at a concentration of $3 \times 10^4$ spores/ml in 0.25% of gelatin. 24–36 hours post inoculation, inner epidermal layers were trimmed off and observed with Zeiss Axiovert 200M confocal microscope. Excitation/emission wave lengths were 488nm/505~550 for EGFP and 543nm/560nm for mCherry.

Protein-lipid overlay assay of zinc finger effector-phospholipid interaction

GST-MGG_{07834} and GST-MGG_{09035} were purified by Glutathione sepharose 4B (GE Healthcare Life Sciences). PIP strips were purchased from Echelon Biosciences Inc (Salt Lake City, UT). Protein-overlay assay was conducted according to Echelon’s procedures. PIP strips were blocked in 3% fatty-acid free BSA in TBS for one hour at room temperature. The blocking solution was discarded and PIP strips were incubated
with 1µg/ml GST-MGG07834/09035 in TBS for 1 hr at RT. The strips were washed with TBST three times for 10 minutes each and then incubated with an anti-GST antibody 1: 8,000 in blocking solution for 1 hr at RT. The strips were washed three times for 10 minutes each and incubated with an anti-mouse HRP antibody diluted 1: 10,000 in blocking solution for 1 hr at RT. After the strips were washed 3 times for 10 minutes each, chemiluminescence was detected using SuperSignal®West Pico Chemiluminescent substrate (Thermo Scientific) and the ChemiDoc XRS system (Bio-Rad).

Agrobacterium-mediated transformation of *N. benthamiana* and biolistic transformation of onion epidermis

*A. tumefaciens* GV3101 carrying gene constructs were grown on YEP media plates (5 g of Yeast extract, 10 g of NaCl, 10 g of bacteriological peptone, 15 g of agar in 1 liter) overnight. Cells were harvested and resuspended in MMA media (5 g of MS salts, 1.95 g of MES, 5 g of glucose in 1 liter, pH 5.6, filter sterilized) containing 200 µM of acetosyringone at an OD$_{600}$ of 0.3. Cell suspensions were placed at RT for at least 3 hours before infiltration. Cells were infiltrated into leaves with a 1 ml disposable syringe without a needle. For biolistic transformation of onion epidermis, gold particles (1 micron) were coated with gene constructs (~5µg) and bombarded into onion epidermal cells via Model PDS-1000/He biolistic particle delivery system (Bio-Rad, Hercules CA).
Chapter 3

Zinc finger effectors are nuclear HIRA recruiters and DNA binding factors

3.1 Introduction

Receptors of pathogen/microbe-associated molecular patterns (PAMPs/MAMPs) are usually at the cell surface and are a class of receptor-like kinases (RLKs) or receptor-like proteins (RLPs), whereas receptors for avirulence (AVR) proteins are usually nucleotide binding leucine-rich repeat (NB-LRR) proteins in the cytoplasm. RLKs/RLPs are called pattern recognition receptors (PRRs) and NB-LRR proteins resistance (R) proteins. Upon recognition of their cognate ligand, PRR initiates pattern triggered immunity (PTI), and R initiates effector triggered immunity (ETI) usually with a hypersensitive response (Jones and Dangl, 2006). Although different in timing and amplitude at the infection site, both types of defenses can trigger systemic acquired resistance (SAR) in the neighboring and distal tissues to prime the cell for secondary infection (Mishina and Zeier, 2007). Signaling for PTI, ETI or SAR leads to extensive transcriptome reprogramming in the nucleus. For example, SAR in Arabidopsis involves upregulation or downregualtion of ~2000 genes, i.e., ~10% of the transcriptome (Fu and Dong, 2013). This feat is accomplished to a large extent by plant transcription (co)factors and chromatin modulins.

Transcription (co)factors regulating plant defenses are major components of plant defense hormone signaling pathways or interlocutors between these pathways. In Arabidopsis, plant defenses are mainly determined by three plant defense hormone
pathways and their crosstalk (Figure 3.1). SA largely regulates defense against (hemi)biotrophic pathogens and functions in SAR. Recently, NPR3 and NPR4, paralogs of NPR1 (nonexpressor of PRI genes), were shown to be SA receptors and act as BTB domain-containing adaptors for cullin3 ubiquitin E3 ligase to regulate the level of transcription cofactor NPR1 in the nucleus. In infected cells, SA enhances NPR3-NPR1 interaction by binding to NPR3 and promotes NPR1 degradation. In neighboring or distal cells, SA disrupts NPR4-NPR1 interaction and release NPR1 for gene activation. Transcription cofactor NPR1, along with transcription factors TGAs activates defense gene expression such as PR1 (Fu et al., 2012). JA alone activates responses to wounding and herbivory, but in the presence of ET, it activates defense against necrotrophs. Transcription factors ERF1 and ORA59 consolidate the JA/ET signaling to one pathway and control disease resistance to necrotrophs (Pre et al., 2008). Components in SA signaling such as transcription (co)factors NPR1, TGAs and WRKY70 can suppress components in the JA/ET signaling pathway, resulting in a negative crosstalk between SA and JA/ET pathways.

Figure 3.1 Major transcription factors of defense hormone pathways in Arabidopsis
Chromatin modulins are the other essential part of the chromatin/transcriptional regulatory machinery and play important roles in plant immunity. Chromatin modulins are a plethora of regulators of chromatin structure such as histone chaperones, histone modifying enzymes and ATP-dependent remodelers. Histone modifications include acetylation, methylation, phosphorylation, ubiquitination, sumoylation, carbonylation and glycosylation (Kouzarides, 2007). In general, histone acetylation is associated with gene activation, whereas deacetylation is linked to gene suppression. Histone methylation or ubiquitination can either activate or suppress transcription (Smolle and Workman, 2013). Epigenomic mapping of histone modifications in Arabidopsis showed that tri-methylations of H3K4 (H3K4me3) and H3K36 (H3K36me3) are correlated with active genes, while H3K27me3 and H3K9me2 are connected to repressed genes (Zhang et al., 2009; Roudier et al., 2011).

Net histone acetylations result from antagonistic histone acetyltransferases and histone deacetylases (HDAs), while histone methylations are catalyzed by histone methyltransferase (HMTs). Chromatin modulins play important roles in plant disease resistance (Table 3.1). In Arabidopsis, HDA6 and HDA19 increase basal level expression of JA/ET responsive genes and enhance resistance to fungal pathogen *Alternaria brassicicola* (Zhou et al., 2005; Wu et al., 2008). Interaction of HDA19 with WRKY38 and WRKY62 leads to an increase of PR gene expression and resistance to *P. syringae* DC3000 (Pst DC3000) (Kim et al., 2008). In rice, HDT701, a H4 deacetylase, negatively regulates histone acetylation levels at the promoters of two PRRs, CEBiP and FLS2, and one R chaperone complex unit SGT1, thereby conferring resistance to *M. oryzae* and *X. oryzae pv. oryzae* (Xoo) (Ding et al., 2012). In Arabidopsis, SDG8, a major HMT for H3K36me3, increase the level of
H3K36me3 at the loci of R genes, such as RPM1 and LAZ5, and is thus required for R initiated ETI (Palma et al., 2010). SDG8 also mediates H3K36me3 enrichment in the loci of M KK3, M KK5 and several PR genes, thereby establishing a permissive state for defense-related gene expression (Berr et al., 2010). H3K4me3 established by an unknown HMT at WRKY6, WRKY29 and WRKY53 serves as a stress memory in SAR (Jaskiewicz et al., 2011). And H3K9 acetylation as a stress memory in SAR even can be passed on to the next generation (Luna et al., 2012).

Table 3-1 Chromatin modulators in plant defense

<table>
<thead>
<tr>
<th>Name</th>
<th>Plant</th>
<th>Consequence</th>
<th>Association</th>
<th>Effect on plant defense</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDA6</td>
<td>Arabidopsis</td>
<td>Histone deacetylation</td>
<td>Transcription repression</td>
<td>Increase basal expression of JA/ET responsive genes; enhance resistance to A. brassicicola</td>
</tr>
<tr>
<td>HDA19</td>
<td>Arabidopsis</td>
<td>Histone deacetylation</td>
<td>Transcription repression</td>
<td>Same as HDA6; Increase PR gene expression and enhance resistance to Pst DC3000</td>
</tr>
<tr>
<td>HDT701</td>
<td>Rice</td>
<td>H4 deacetylation</td>
<td>Transcription repression</td>
<td>Negative regulator of PTI and ETI</td>
</tr>
<tr>
<td>SDG8</td>
<td>Arabidopsis</td>
<td>H3K36me3</td>
<td>Transcription activation</td>
<td>Negative regulator of ETI; Increase JA/ET responsive genes and resistance to A. brassicicola</td>
</tr>
<tr>
<td>HMT?</td>
<td>Arabidopsis</td>
<td>H3K4m3</td>
<td>Transcription activation</td>
<td>Positive regulator of SAR</td>
</tr>
<tr>
<td>HAT?</td>
<td>Arabidopsis</td>
<td>H3K9ac</td>
<td>Transcription activation</td>
<td>Positive regulator of transgenerational SAR</td>
</tr>
</tbody>
</table>

As mentioned above, the nucleus is a major venue for the host cell to interpret cues and rapidly respond to an ever changing environment by transcriptional reprogramming. Many pathogens deliver effectors into the nucleus to manipulate the chromatin/transcriptional machinery in order to suppress host defense and/or siphon off nutrients (Figure 3.2). In addition to transferring T-DNA, A. tumefaciens also delivers several effectors along the way. In the nucleus, the T-complex, a compound of T-DNA, VirD2 and VirE2, is pulled to the chromatin by the interaction of the
VirE2 interacting protein 1 (VIP1) with core histones. Then, T-DNA seems to be integrated into the host genome by the non-homologous end-joining (NHEJ) mechanism (Magori and Citovsky, 2011). T-DNA transformed plant cells undergo neoplastic growth and synthesize opines as a carbon and nitrogen source for the bacteria. In some strains of *Xanthomonas spp.* and *Ralstonia solanacearum*, several or dozens of transcription activator-like effectors (TALEs) are delivered into the nucleus, where they act as eukaryotic transcription factors. TALEs specifically bind to a stretch of DNA sequence in the promoters of their target genes in a simple and decodable manner (Boch et al., 2009; Moscou and Bogdanove, 2009). AvrBs3 from Xcv, acting as a transcription factor, regulates dozens of genes, of which some are expansin genes. The activation of AvrBs3 target genes changes host cell morphology to facilitate bacterial dispersal for the next cycle of infection (Marois et al., 2002; Kay et al., 2007). PthXo1 and AvrXa7/PthXo3/Talc from Xoo, functioning as transcription factors, target *OsSWEET11* and *OsSWEET14*, respectively, two genes encoding sugar transporters for normal sugar flow between host cells. The bacteria co-opt these sugar transporters to pump more sugars into the apoplast where the bacteria thrive (Yang et al., 2006; Chen et al., 2010; 2012). XopD from Xcv interacts with two transcription factors Myb30 and SIERF4, suppressing their roles in plant disease resistance (Canonne et al., 2011; Kim et al., 2013). SP7, an effector from arbuscular mycorrhizal fungus *Glomus intraradices* interacts with the transcription factor ERF19 to evade plant defense and promote symbiosis (Kloppholz et al., 2011). RKN16D10, a 13 aa secretory effector from a root knot nematode *Meloidogyne incognita*, acts as a ligand for two plant scarecrow-like transcription factors, but the biological role of this binding is yet to be known (Huang et al., 2006). OspF, an effector from human pathogen *Shigella flexneri* and a homolog of *pseudomonas syringae* HopAI1,
functions as a phosphothreonine lyase, preventing histone H3 phosphorylation and acetylation (Arbibe et al., 2007). In addition, it also interacts with a chromatin remodeling factor Rb to suppress NF-κB regulated immune responsive genes. NUE, an effector from another human pathogen *Chlamydia trachomatis*, acts as a histone modifier, using its SET domain-containing methyltransferase activity to induce histone methylation (Pennini et al., 2010). LntA, an effector from human pathogen *Listeria monocytogenes*, interacts with a heterochromatin-forming BAH1 silencing complex to regulate interferon-stimulated gene expression (Lebreton et al., 2011). Characterization of LntA unveiled the role of this novel BAH1 silencing complex in interferon signaling pathway. *Agrobacterium* T-DNA encoded 6b protein is localized to the nucleus and interacts with several nuclear proteins such as H3. It was first proposed that 6b might affect gene expression by acting as a chromatin chaperone or a transcription regulator (Terakura et al., 2007). Recently, 6b was demonstrated to have ADP-ribosylating activity and to function as a RNA silencing suppressor by targeting two components in the miRNA pathway for ADP-ribosylation (Wang et al., 2011).
Figure 3.2 Pathogen effectors and their targets in the nucleus. Names of bacterial, fungal/oomycete and nematode effectors are in red, purple and green, respectively.

The transcriptional repression of *KNOX* genes mediated by the AS1/AS2/HIRA silencing complex is a model that smart pathogen effectors are likely to emulate in suppressing plant defense genes (Figure 3-3). *KNOX* genes encode homeodomain proteins that promote stem cell activity, and their expression must be repressed during lateral organogenesis such as in the development of leaves. In Arabidopsis, this process is initiated by the formation of a DNA binding complex of ASYMMETRIC LEAVES1 (AS1) and ASYMMETRIC LEAVES2 (AS2). AS1, the ortholog of maize ROUGH SHEATH2 (RS2), is an unusual myb domain-containing protein and AS2 is a zinc finger and leucine zipper-like domain-containing protein (Timmermans et al., 1999; Byrne et al., 2000; Iwakawa et al., 2002). AS1/AS2 binds to two sites in the
promoter of KNOX gene and recruits HIRA to form an AS1/AS2/HIRA silencing complex (Phelps-Durr et al., 2005; Guo et al., 2008). This silencing complex interacts with histone methyltransferase PRC2 and/or histone deacetylase HDA6 to establish a repressive chromatin state at the promoter loci of KNOX genes (Lodha et al., 2013; Luo et al., 2012). This repressive chromatin state is stably maintained all the way through the many rounds of cell divisions during leaf development.

Figure 3-3 The repressive chromatin state of the promoter of KNOX genes established by AS1/AS2/HIRA silencing complex

In this study, the interaction between zinc finger effectors and rice HIRA is reminiscent of the interaction between AS1/AS2 and HIRA. Furthermore, like AS1/AS2, zinc finger effectors bind to DNA with specificity. Therefore we hypothesize that zinc finger effectors promote disease by recruiting HIRA for defense gene expression.

3.2 Results
Zinc finger effectors interact with HIRA and other nuclear proteins

To find the host virulence targets of this family of zinc finger effectors, we screened the rice yeast two hybrid (Y2H) cDNA library made from transcriptome induced by *M. oryzae*. In total, $2 \times 10^7$ independent clones were screened for their interactions with MGG_07834. Yeast transformants harboring the bait pDB-Trp/MGG_07834 and the prey pAD-GAL4-2.1/cDNA were tested for all three Y2H markers (His$^+$, Ura$^+$, Laz$^+$). In total, four independent clones were obtained as MGG_07834 interactors (Figure 3-4; Table 3-2).

HIRA (histone regulatory protein A) is one of the three interactors of MGG_07834. HIRA is encoded by a single-copy gene in most eukaryotes except yeast. In yeast, two proteins HIR1 and HIR2 function together complementarily as homologues of HIRA. HIRA is known not only to establish and maintain heterochromatin loci but also to control the spatial and temporal expression of some euchromatic genes (Spector et al., 1997; Magaghi et al., 1998; Phelps-Durr et al., 2005). The interaction between MGG_07834 and HIRA suggests a new role that HIRA may play in plant immunity. Rice HIRA (OsHIRA) is a 975 aa nuclear proteins. Like its orthologs in Arabidopsis (AtHIRA) and maize (ZmHIRA), OsHIRA contains 7 canonical or degenerate WD40 repeats and one C-terminal HIRA motif conserved in all eukaryotes. One nuclear localization signal (NLS) is predicted in the near middle of the protein. OsHIRA is closer to Zm HIRA than is AtHIRA in the primary structure (Figure 3-5 A).

The second interactor of MGG_07834 is an ortholog of Arabidopsis STOP1 and named OsSTOP1 (Figure 3-5 B). OsSTOP1 contains four C$_2$H$_2$ zinc finger motifs in
its C-terminus, suggesting it may function as a transcription factor in rice. In Arabidopsis, STOP1 regulates multiple genes associated with detoxifying photon and aluminum toxicities (Sawaki et al., 2009). In rice, ART1, a paralog of OsSTOP1, also plays a role in regulating genes linked to detoxifying aluminum toxicity (Yamaji et al., 2009). Due to its interaction with MGG_07834, it is tempting to investigate whether OsSTOP1 also plays a role in plant immunity.

Another interactor of MGG_07834 is OsPPR. Pentatricopeptide repeat (PPR) containing proteins comprise a large family of proteins in plants. There are 450 and 470 PPR protein-encoding genes in Arabidopsis and rice, respectively. A few studied PRR proteins are implicated in various plant developmental processes via their functions in RNA editing in plant organelles. But some might also have novel functions such as in disease resistance and abiotic stress tolerance (Laluk et al., 2011).

Two reports prompted us to focus our attention on HIRA being an important regulator of plant immunity. Yang et al. (2008) found that βC1, a pathogenicity factor from tomato yellow leaf curl China virus, associated with AS1 to suppress JA responsive genes by competing with AS2 for AS1 binding. And AS1 was demonstrated to be a negative regulator of plant immunity to necrotrophic fungus Botrytis cinerea by suppressing JA responsive genes (Nurmberg et al., 2007). These studies suggested that a similar silencing complex to AS1/AS2/HIRA may play a role in plant immunity by selectively suppressing a set of defense genes. We conducted in vitro GST pull-down assay with GST-MGG_07834 and His-HIRA. His-HIRA can be co-precipitated with GST-MGG_07834 by Glutathione sepharose 4B beads (Figure 3-6). HIRA in the fusion protein has no N-terminal WD40 domain and it is encoded by the original
partial ORF we identified in Y2H screening. The C-terminal region of HIRA is sufficient for it to interact with MGG_07834.

Figure 3-4 Y2H screen for MGG_07834 interactors. Clones 1. STOP1. 2. PPR. 3. STOP1. 4. HIRA. A. SC-Trp-Leu. B. SC-Trp-Leu-His+3AT (25mM). C. SC-Trp-Leu-Ura. D. SC-Trp-Leu+5-FOA (0.2%). E. β-Gal filter lift assay.

Table 3-2 Summary of rice proteins that interact with MGG_07834

<table>
<thead>
<tr>
<th>Interacting Proteins</th>
<th>MGG_07834-D(25mM3AT)</th>
<th>MGG_07834-D(0.2% 5-FOA)</th>
<th>Number of Colonies</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIRA</td>
<td>+ +</td>
<td>- -</td>
<td>1</td>
<td>AK065913</td>
</tr>
<tr>
<td>STOP1</td>
<td>+ +</td>
<td>- -</td>
<td>2</td>
<td>AK072417</td>
</tr>
<tr>
<td>TPR</td>
<td>+ +</td>
<td>- -</td>
<td>1</td>
<td>AK065769</td>
</tr>
</tbody>
</table>

++ demonstrates strong growth. - - indicates no or weak growth.
Figure 3-5 Predicted motifs in HIRA, OsSTOP1 and OsPPR1. Marks in brown indicate nuclear localization signal (NLS). Marks in pink indicate low-complexity sequence. Mark in green shows a coiled-coil motif. BLAST, degenerate WD40.
Figure 3-6 In vitro GST pull-down assay demonstrated zinc-finger effectors interact with HIRA in vitro.

The interactions between zinc finger effectors and HIRA occur in the nuclei of rice cells

HIRA and the other two MGG_07834 interactors OsSTOP1 and OsPPR are all predicted to have at least one nuclear localization signal, suggesting that this family of effectors interact with their virulence targets in the nucleus. And the zinc finger effector MGG_10020 was observed to be localized into the nucleus during fungal infection with live-cell imaging microscopy (Barbara Valent, personal communication). We set out to test the possible nuclear localization of the interaction between this family of zinc finger effectors and HIRA using bimolecular fluorescence complementation (BIFC). Three zinc finger effectors MGG_07834, MGG_10020 and AVR-Pii were N-terminally fused to cEYFP, and HIRA was both N-terminally and C-terminally fused to nEYFP. BIFC constructs for effector and HIRA were appropriately combined and transfected into rice protoplasts. As expected, the interaction between MGG_07834 and HIRA is almost exclusively in the nucleus (Figure 3-7 A). The interaction between MGG_10020 is to a large extent in the nucleus (Figure 3-7 B). The interaction between AVR-Pii and HIRA is in the cytoplasm of rice cells (Figure 3-7 C). Compared with MGG_07834 and MGG_10020 that are expected to be virulent effectors, AVR-Pii is avirulence protein (AVR) that can be recognized by its cognate resistance (R) protein Pii in rice cells. The difference in their functions is likely to be used to explain the different patterns of their
interaction with HIRA, vice versa. The molecular mechanism underlying this difference will be proposed in the following section. Coimmunoprecipitation (CoIP) was attempted unsuccessfully to demonstrate the interaction between MGG_07834 and HIRA in rice protoplasts (Figure 3-8). It seems that the fusion protein HIRA-HA was not appropriately expressed in rice protoplasts.

Figure 3-7 Interactions between zinc-finger effectors and HIRA were localized to rice cell nuclei. A, MGG07834-cEYFP+HIRA-nEYFP. B, MGG10020-cEYFP+nEYFP-HIRA. C, AvrPii-cEYFP+HIRA-nEYFP. Each row from left to right: GFP, bright and composite. Scale bar, 20 µM.
Figure 3-8 Coimmunoprecipitation assay for the interaction between effectors and HIRA in rice protoplasts. 10% input was left as control for western blot. Left panel, blotted membrane was detected with α-HA. Right panel, blotted membrane was detected with α-FLAG.

**Zinc finger effectors are DNA binding factors**

One-finger zinc finger proteins are usually known as protein interactors, and not as DNA binding factors. But our study showed that in addition to interacting with proteins, one-finger zinc finger proteins such as this family of zinc finger effectors can be DNA binding factors.

AS2 in the AS1/AS2/HIRA silencing complex is a one-finger zinc finger protein and its zinc finger motif shares similarities with zinc finger effectors MGG_07834, MGG_10020 (Figure 3-9 A and B). AS2 contributes a major part to the DNA binding
activity of the AS1/AS2 complex, because AS1 has changed the conserved amino acids in the third helix of the R3 MYB motif that are essential for MYB-DNA interaction. Furthermore, mutant as2 is genetically epistatic to as1, suggesting AS1 function depends on AS2 (Phelps-Durr et al., 2005; Guo et al., 2008). From the above analysis, it is plausible to propose that fungal zinc finger effectors are mimics of AS2 or AS1/AS2 complex in DNA-binding.

There is a precedent that a one-finger zinc finger protein specifies a stretch of DNA sequence. GAGA transcription factors function either as transcription activators or repressors by inducing changes of chromatin composition and conformation at promoters (Adkins et al., 2006). A typical GAGA protein has an 82 aa segment containing a C2H2 motif and two stretches of basic amino acids and this segment is sufficient for it to bind to consensus sequence GAGAG (Pedone et al., 1996). Specifically, amino acids in the zinc finger motif make contacts with GAG in the DNA major groove, while amino acids in basic region 1 (BR1) interact with A in the minor groove and those in BR2 make contact with G in the minor groove (Figure 3-10 B). Through these interactions, the C2H2 zinc finger and the basic stretches of amino acids make high-affinity specific DNA-binding. Zinc finger effectors MGG_07834, MGG_10020, MGG_08230, MGG_13665 and MGG_10238 have flanking stretches of basic amino acids (Figure 3-10 A), suggesting that these zinc finger effectors can specifically bind to certain DNA sequences with their C2H2 motif and nearby basic stretches of amino acids.

We conducted systematic evolution of ligands by exponential enrichment (SELEX) to try to find some consensus DNA sequences for this family of zinc finger effectors.
Random DNA oligomers of 16 nucleotides were synthesized and used as SELEX probes. GST-MGG_07834 was used to bind the probes and pull down specific probes. GST-MGG_07834-SELEX probe complexes were washed and SELEX probes were eluted. Eluted SELEX probes were PCR amplified and added to the next round of binding reaction. After 5 rounds of SELEX reactions, GST-MGG_07834, like transcription factor EIL1 (positive control), pulled down DNA, while GST alone (negative control) could not precipitate DNA (Figure 3-11). This result showed that zinc finger effectors are DNA binding factors.

Figure 3-9 3-D structures of zinc finger motifs of MGG_07834, MGG_10020, AVR-Pii and AS2. A. Sequence arrangement of zinc finger motifs of effectors and AS2. Shaded letters indicate conserved amino acids. B. 3-D structures of zinc finger motifs of effectors and AS2 modeled on homologous zinc finger proteins with known structures.
Figure 3-10 One-finger zinc finger proteins can specify a sequence of DNA. A.
Sequence alignment of zinc finger effectors MGG_07834, MGG_10020, MGG_08230, MGG_13665, MGG_10238 and GAGA-DB. Basic stretches of amino acids are indicated in red, zinc finger motif in blue. B. 3-D structure of complex of GAGA-DB and DNA sequence GAGAG. BR, basic region. (Adapted from Omichinski et al., 1997).
3.3 Discussion

From yeast two hybrid screening, we found that HIRA is an interactor of zinc finger effectors. HIRA is well known to induce a suppressive chromatin state in promoters of genes such as the KNOX genes. This prompted us to ask these questions: Whether does HIRA play a role in suppression of plant defense genes? If so, how is it recruited to the promoters of certain defense genes? The latter question led us to the discovery of the molecular/biochemical function of zinc finger effectors, i.e., DNA binding factors with specificities. Most fungal effectors are usually small novel proteins with no similarities to any known proteins. And fungal gene knock-out mutants usually have no changes in disease symptoms due to redundancy of genes or functions. As a result, it is not easy to characterize their molecular/biochemical functions, much less the mechanisms by which they interact with their virulence targets in host cells. To date, only a few virulence targets and/or functions have been ascribed to fungal
effectors. Cmu1, an effector from maize smut fungus *U. maydis*, acts as a chorismate mutase to divert the shikimate pathway towards production of aromatic amino acids and away from production of SA (Djamei et al., 2011). SA is a key plant defense hormone in maize. In rice- *M. oryzae* pathosystem, an E3 ubiquitin ligase APIP6 was found to be a virulence target of AVR-Piz-t, but the mechanism by which AVR-Piz-t destabilizes APIP6 to suppress PTI is not known (Park et al., 2012). Our finding of HIRA as a virulence target of zinc finger effectors will provide new insights into how fungal effectors defeat plant immunity.

The function of AS1/AS2/HIRA silencing complex is well established in suppression of *KNOX* genes. AS1 and AS2 interact to form an AS1/AS2 complex and this heterodimer complex specifically binds to two sites in the promoters of *KNOX* genes (Guo et al., 2008). Although neither AS1 nor AS2 alone can bind DNA in vitro, the zinc finger protein AS2 likely contributes more to the activity and specificity of the DNA binding complex AS1/AS2. In this study, zinc finger effector MGG_07834 has been demonstrated to bind DNA in vitro, suggesting that zinc finger effectors fulfil the function of the AS1/AS2 complex in DNA binding. It needs to be pointed out that differences in DNA binding likely exist in this family of zinc finger effectors based on the amino acid sequence of zinc-finger motif and the flanking basic regions. MGG_07834, MGG_10020, MGG_08230, and MGG_13665 contain an intact C2H2 zinc finger motif and basic regions that precede, follow, or flank this motif (Figure 3-10 A). These four zinc finger effectors are presumably active DNA-binding factors. In contrast, AVR-Pii contains an incomplete zinc finger motif and cannot form a typical 
\( \alpha \)-helix in the modeled structure that is critical for zinc finger motif to make contacts with bases in the DNA double helix (Figure 3-9). In addition, AVR-Pii lacks basic
stretches of amino acids that also take part in determining DNA binding specificity as in GAGA transcription factors and TALEs (Pedone et al., 1996; Nga-Sze Mak et al., 2013). In this study, the BiFC assay demonstrated that the localization of the interaction between AVR-Pii and HIRA is in the cytoplasm, while MGG_07834 and MGG_10020 interplay with HIRA in the nucleus. The prediction that AVR-Pii has no DNA binding activity is a possible explanation for this observation.

Well known as two conserved factors functioning in leaf development in plants, AS1 and AS2 were also demonstrated to be involved in suppression of JA responsive defense genes (Nurmberg et al., 2007; Yang et al., 2008). However, the picture of AS1 or AS2 in suppression of plant defense genes is incomplete. Our findings that fungal zinc finger effectors were AS2 or AS1/AS2 mimics, and that HIRA was recruited by these DNA binding factors make this picture clearer, richer and more colorful. To explain the mechanism by which zinc finger effectors suppress plant defense genes, we propose the following model. Zinc finger effectors bind specifically to the promoters of certain defense-related genes and recruit HIRA to the promoters to form a zinc finger effector (ZFE)/HIRA silencing complex. This ZFE/HIRA silencing complex then interacts with histone modifiers such as histone methyltransferase PRC2 and histone deacetylase HDA6 to establish a repressive chromatin state at the promoters. As a result, the promoters are closed to the transcriptional machinery and defense genes are silenced (Figure 3-12).
Figure 3-12 A model for establishing a chromatin repressive state at the promoters of certain defense-related genes by ZFE/HIRA silencing complex

It is pressing to map the binding sites of zinc finger effectors across the rice genome. Chromatin immunoprecipitation-sequencing (CHIP-seq) will help us obtain this goal. Recently, CHIP-exo, a new technique that combines standard CHIP and DNase footprinting, was used to map the binding sites of transcription factors at one base resolution (Rhee and Pugh, 2011). One-finger zinc finger proteins are likely to bind shorter sequences, say, 3~8 bp as demonstrated by the GAGA transcription factors. CHIP-exo will help us narrow and pin down the binding sites of this family of fungal zinc finger effectors in the rice genome. Other must-do things are to find the interactors of the ZFE/HIRA silencing complex by Y2H screening and map the histone methylation and acetylation status at promoters of some key players in plant immunity. All these efforts will lead to a deeper understanding of how fungal effectors act to avoid, suppress and defeat plant defenses.
3.4 Materials and methods

Yeast two hybrid screen

*MGG_07834* was amplified using forward and reverse primers with SalI and NotI sites, respectively. PCR fragment of *MGG_07834* was cut with SalI and NotI and inserted into the bait vector pDB-Trp such that the GAL4 DNA binding domain and MGG_07834 were fused in frame. The cDNA library contains cDNAs that were synthesized from *M. oryzae* induced mRNAs and cloned into HybriZAP-2.1 vector. The cDNA library was converted to pAD-GAL4-2.1 phagemid library by mass excision using ExAssist helper phage. Approximately $2 \times 10^7$ pAD-GAL4-2.1-cDNA phagemids were used to transform yeast strain MaV203 carrying pDB-Trp/*MGG_07834* according to the protocol described by Gietz and Schiestl (2007). Transformants were grown on SC-Trp-Leu-His+25mM 3-AT plates for 3-8 days. His prototrophic clones were selected for further tests. His$^+$ colonies were transferred to SC-Trp-Leu-Ura plates to screen for Uracil prototrophy. His$^+$ Ura$^+$ colonies were spotted on SC-Trp-Leu-5-FOA (0.2%W/V) media for counter-selection of Ura$^+$. β-galactosidase filter lift assay was performed to test for activity of lacZ gene. In total, all three marker genes were tested for MGG_07834 interactors. Plasmids were extracted from His$^+$ Ura$^+$ lacZ$^+$ clones by using the glass bead method.

GST pull-down assay

*MGG_07834* was cloned into pGEX-4T-2 (GE healthcare), making an N-terminal fusion with GST. HIRA was cloned into pDEST17 (Invitrogen), creating an N-terminal fusion with 6×His. 10 µg of GST or GST-07834 was incubated with 50 µl of
glutathione sepharose 4B beads (GE healthcare) for 1 h at 4°C in 500 µl of 20 mM Tris, pH7.5, 500 mM NaCl and 0.5% NP40. After washing once, the beads were incubated with 20 µl of bacterial extract His-HIRA at 4°C for 2 h in 500 µl of the same buffer. Beads were washed three times in 200 µl of 10 mM Tris, pH7.5, 150 mM NaCl and 0.1% NP40. Precipitated proteins were separated by SDS-PAGE and blotted onto PVDF membrane. Anti-His antibody (H1029, Sigma, St. Louis, MO) was used to detect the pull-downed 6×His-HIRA.

Bimolecular fluorescence complementation assay

*MGG_07834, MGG_10020* and *AVR-Pii* were cloned into Gateway BiFC vector pUGW2/cEYFP such that *MGG_07834, MGG_10020* and *AVR-Pii* were N-terminally fused to cEYFP. HIRA was cloned into Gateway BiFC vectors pUGW0/nEYFP and pUGW2/nEYFP for N-terminal and C-terminal fusion to nEYFP, respectively. Rice protoplasts were prepared from the stems of 10-day-old seedlings. Approximately 1X10^5 protoplasts were transfected with 5µg of *MGG_07834-cEYFP* and 5 µg of *HIRA-nEYFP*. Two combinations: 5 µg of *MGG_10020-cEYFP* and 5µg of nEYFP-HIRA, 5 µg of *AVR-Pii-cEYFP* and 5µg of *HIRA-nEYFP*, were also transfected into the same amount of protoplasts. Protoplast transformation was conducted as described in standard protocol for PEG/Ca^{2+} method. Fluorescence was detected by using a Zeiss Cell Observer SD Spinning Disk Confocal microscope equipped with a YoKogawa CSU-X1 spinning disk head and a Photoetrics QuantEM 512SC EMCCD camera. Excitation/emission wave lengths for EGFP were 488nm/525-550nm, respectively. The objective was Apochromat 100X 1.4 NA oil immersion objective.
Systematic evolution of ligands by exponential enrichment (SELEX) assay

Random sequence Oligo DNAs of 72 nucleotides 5’-GGA TTT GCT GGT GCA GTA CAG TGG ATC C-(N)16-GGA TCC TTA GGA GCT TGA AAT CGA GCA G-3’ were synthesized and purified by HPLC (Integrated DNA Technologies). Random sequence Oligo DNAs were made double stranded by Taq DNA polymerase and reverse primer. Double-stranded DNAs were separated by native PAGE and recovered from the gel. 10 ng of dsDNA and 5 ng of GST-MGG_07834 (GST and GST-EIL1 as negative and positive controls, respectively) were incubated on ice for 30 m in 50 µl of protein-DNA binding buffer: 10 mM of Tris, pH7.5, 50 mM of NaCl, 1 mM of EDTA, 5% of glycerol, 10 mM of DTT, 10mg/ml of BSA, 1µg/µl of poly (dI/dC). Equilibrated glutathione sepharose 4B beads (GE healthcare) were used to pull down GST-MGG_07834 and its binding probes. After washing, beads were collected and suspended in 20 µl water. 5µl were used for PCR at the following program: 5 m at 94°C; 20 cycles (round 1 of SELEX) or 15 cycles (other rounds) of 1 m denaturation at 94°C; 1 m at 50°C; 30 s at 72°C; and a final extension of 20 m at 72°C, followed by 4°C incubation. After 5 rounds of SELEX reactions, GST-MGG_07834 (GST and GST-EIL1) binding DNAs were separated by PAGE and recovered from the gel for sequencing.
Chapter 4

Zinc-finger effectors suppress PTI when expressed heterologously in plants

4.1 Introduction

Pattern triggered immunity (PTI) is the first line of plant active defense response. Potential pathogens fail to cause disease on a certain plant are usually due to PTI and these pathogens are called nonadapted pathogens to the plant. Adapted pathogen usually delivers a plethora of effectors into its host to defeat PTI. PTI is activated when conserved microbe/pathogen associated molecular patterns (M/PAMPs) are recognized by pattern recognition receptors (PRRs) at the host cell surface. The past decade has witnessed the identification of many M/PAMPs and PRRs. In rice, several pairs of M/PAMP and PRR provide innate immunity to rice bacterial and fungal pathogens (Table 4-1).

*Xanthomonas oryzae pv. oryzae* (Xoo) Ax21 is type I secreted sulfated 194aa quorum sensing protein that is conserved in all *Xanthomonads* and related genera. A sulphated 17 aa peptide synthesized based on Ax21 N-terminal sequence is an active epitope for rice XA21 recognition (Lee et al., 2009). To function properly, XA21 interacts with several proteins such as XB3, XB15, XB24 and XB10 (Seo et al., 2011). XB3 is an E3 ubiquitin ligase and phosphorylated by XA21. The biological relevance of this interaction is not known (Wang et al., 2006). XB15 is a PP2C phosphatase and dephosphorylates XA21, and is thus a negative regulator of the XA21-mediated immunity (Park et al., 2008). XB24, a protein with intrinsic ATPase activity, is likely
to keep XA21 in an inactive state by promoting autophosphorylation of XA21 (Chen et al., 2010b). XB10 is the transcription factor WRKY62. Upon activation, the intracellular domain of XA21 is cleaved and translocated into the nucleus where it directly interacts with WRKY62 for gene regulation (Park and Ronald, 2012).

Chitin, a conserved component of the fungal cell wall, is a homopolymer of N-acetylglucosamine (GlcNAc)_n. Although it was well documented that chitin is a potent elicitor of plant defense response, it was only recently that its receptor, the chitin elicitor binding protein (CEBiP) was first identified in rice (Kaku et al., 2006). CEBiP is a lysin motif (LysM) containing receptor-like protein (LysM-RLK), consisting of a signal peptide, three consecutive LysMs, and a C-terminal region without any noticeable signature, followed by a glycosylphosphatidylinositol (GPI) anchor motif. Due to its lack of cytoplasmic signaling domain, CEBiP forms a heterodimer with another LysM containing receptor-like kinase (LysM-RLK) to transduce the signal (Shimizu et al., 2010). This LysM-RLK is called rice chitin elicitor receptor-like kinase (OsCERK1), an ortholog of Arabidopsis CERK1. In Arabidopsis, CERK1 binds chitin and forms homodimer to transduce signal, as opposed to OsCERK1 which has low affinity to chitin and must form heterodimer with CEBiP for signaling (Liu et al., 2012). Adding some complexity to the rice chitin signaling is the finding that two other LysM-RLPs, LYP4 and LYP6, also play a role in chitin signaling (Liu et al., 2012). In addition to binding chitin, LYP4 and LYP6 also bind peptidoglycan (PGN). PGN, a cell wall component of Gram positive and negative bacteria, consists of heteroglycan chains with alternating GlcNAc and N-acetylmuramic acid (MurNAc) units. Heteroglycan chains are crosslinked via a stem peptide connected to two MurNAc moieties. PGN and its degraded soluble derivatives...
muropeptides are potent plant defense elicitors. In Arabidopsis, two LysM proteins LYM1 and LYM3 are identified as PGN receptors and need CERK1 for PGN signaling (Willmann et al., 2011). In rice, LYP4 and LYP6 can be PGN receptors, but whether they need OsCERK1 for signaling is not known.

Flagellin is the major component of the bacterial flagellum and a potent elicitor of plant defense. Flagellin or its active epitope flag22 at the conserved N-terminus serves as a ligand of Arabidopsis flagellin sensitive 2 (FLS2). Arabidopsis FLS2 is a leucine-rich repeat receptor-like kinase (LRR-RLK) and has orthologs in tomato, *N. benthamiana* and rice. Flagellin from two rice bacterial incompatible pathogens *P. avenae* and *Acidovorax avenae* can induce immune response in rice similar to that mediated by flg22-FLS2 in Arabidopsis, suggesting OsFLS2 in rice is also a functional sensor for bacterial flagellin (Che et al., 2000; Tanaka et al., 2003).

In rice, two R proteins mediate wide-spectrum resistance against pathogens and may also serve as PRRs, although their cognate M/PAMPs are not known. Xa26 from *Xoo* is an LRR-RLK and required for broad-spectrum resistance against *Xoo*. Pi-d2 is a bulb-type mannose specific binding lectin (B-lectin) receptor-like kinase and mediates wide-spectrum resistance to *M. oryzae* (Chen et al., 2006).
<table>
<thead>
<tr>
<th>Pathogen</th>
<th>M/PAMP/epitope</th>
<th>Virulence function</th>
<th>PRR</th>
<th>Overall structure</th>
<th>Ligand binding domain</th>
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<tr>
<td>Xoo</td>
<td>Ax21/AxY&lt;sup&gt;”22&lt;/sup&gt;</td>
<td>Quorum sensing</td>
<td>XA21</td>
<td>LRR-RLK</td>
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<td>Fungi</td>
<td>Chitin/(GlcNAc)&lt;sub&gt;n&lt;/sub&gt;; Cell wall integrity</td>
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<td>CEBiP</td>
<td>LysM-RLP</td>
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<td>Bacteria; Fungi</td>
<td>PGN/(GlcNAc-MurNAc)&lt;sub&gt;n&lt;/sub&gt;; Chitin/(GlcNAc)&lt;sub&gt;n&lt;/sub&gt;</td>
<td>Cell wall integrity</td>
<td>LYP4/LYP6</td>
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<tr>
<td>Fungi; Bacteria</td>
<td>PGN/(GlcNAc-MurNAc)&lt;sub&gt;n&lt;/sub&gt;; Chitin/(GlcNAc)&lt;sub&gt;n&lt;/sub&gt;</td>
<td>Cell wall integrity</td>
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<td>Bacteria</td>
<td>Flagellin/flg22</td>
<td>mobility</td>
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Upon binding to M/PAMP, PRR is activated and PTI signaling is initiated. These are followed by the production of reactive oxygen species (ROS), inflow of Ca<sup>2+</sup>, and activation of MAPK cascades. In rice, there are 17 MAPKs (Reyna and Yang, 2006), of which OsMAPK5, OsMAPK6 and OsMAPK12 were demonstrated to be involved in plant immunity. OsMAPK5 is induced by several biotic and abiotic stresses, and acts as a positive regulator of drought tolerance and a negative regulator of plant defense (Xiong and Yang, 2003). OsMAPK6 is chitin-induced and involved in the de novo synthesis of diterpenoid phytoalexin against <i>M. oryzae</i> (Kishi-Kaboshi et al., 2010). OsMAPK12 (a.k.a., BWMK1) is induced by both <i>M. oryzae</i> and Xoo and implicated in plant innate immunity to these two pathogens (He et al., 1999; Seo et al., 2011).

Two major types of transcription factors WRKYs and TGAs are implicated in rice immunity. More than 100 WRKYs are among the rice proteome, several of them such
as WRKYS 62, 28, 71, 76 and WRKYS 53, 89 were investigated for their involvement in plant defense. WRKYS62 is a negative regulator of XA21-mediated basal immunity and physically interacts with the cleaved intracellular domain of XA21 in the nucleus (Park and Ronald, 2012). In contrast, overexpression of all four WRKYS 62, 28, 71, 76 leads to enhanced resistance against Xoo, suggesting their roles in regulating plant immunity negatively or positively (Peng et al., 2010). WRKYS53 is induced by chitin and overexpression of WRKYS53 increases resistance against *M. oryzae* (Chujo et al., 2007). Overexpression of WRKYS89 also results in enhanced resistance to *M. oryzae* (Wang et al., 2007). In Arabidopsis, it is well established that NPR1 and TGAs regulated gene expression that is associated with SAR. In rice, TGAs also play a role in rice immunity. OsTGAP1 is chitin inducible and regulates the expression of genes related to diterpenoid phytoalexin biosynthesis (Okada et al., 2009). And rTGA2.1 plays a negative role in regulating disease resistance against Xoo (Fitzgerald et al., 2005).

Phytohormone signaling often crosstalks with and fine-tunes plant immunity against pathogens. brassinosteroids (BRs) regulate plant growth and development and also have a role in plant disease resistance. Rice plants treated with brassinolide are more resistant to *M. oryzae* and Xoo (Nakashita et al., 2003). Several PRRs such as FLS2, EFR, EIX1/2, VE1, likely as well as LYM1/ LYM3/CERK1 all require BAK1 (BRI1-associated kinase; a. k. a., SERK3) for PTI signaling. BAK1 was originally identified in the BR pathway as an essential component for BR signaling. The involvement of BAK1 in BR signaling and PTI suggests a likely inherent crosstalk between BR and plant defenses. In rice, ET and ABA often act antagonistically to regulate disease resistance and abiotic stress. Usually, ET enhances disease resistance, whereas ABA
weakens it. JA and SA generally have positive roles in regulating resistance against pathogens in rice (Mei et al., 2006; Jiang et al., 2010).

To exert PTI effect, plants mount an extensive reprogramming of their physiology, including production of ROS, deposition of callose, synthesis of phytoalexin and expression of PR genes. There are 17 family of PR genes (PR1-PR17), encoding hydrolytic enzymes (such as glucanase and chitinase) and defensins that hydrolyze pathogen cell wall and disrupt pathogen membrane, respectively. Phytoalexins are secondary metabolites that are de novo synthesized in response to pathogen infection. Callose deposition often occurs in the cell walls at the infection site to restrict pathogen further growth. ROS is produced rapidly also at the infection site to directly kill pathogen and/or functions as a signal for defense response.

In this study, we demonstrated that rice plants over-expressing zinc finger effectors showed more severe disease symptoms and that the induction of defense-related genes was moderately inhibited. Because the induction of defense-related genes is characteristic of PTI, we hypothesized that zinc finger effectors suppress PTI to promote disease.

4.2 Results

**Zinc finger effectors inhibit BAX induced PCD in N. benthamiana**

Due to the functional redundancy between zinc finger effectors, *M. oryzae* mutants with a single knock-out effector gene are likely to show no detectable changes in their virulence. We thus investigated the roles of zinc finger effectors in virulence by
heterologously expressing them in plants. BAX is a proapoptotic factor of the Bcl-2 family and promotes programmed cell death (PCD) in animals. Although the key regulators of animal apoptosis (a typical PCD), such as Bcl-2 family proteins and caspases, have not been found in plant proteomes, some aspects of the PCD molecular machinery are shared by plants and animals. BAX can cause cell death in plants very similar to the hypersensitive response, a mechanism by which plants sacrifice cells at the infection site to restrict pathogen growth and spread. To test whether zinc finger effectors can inhibit BAX-induced PCD in *N. benthamiana*, we adopted two strategies. First, we cloned effector gene and murine *BAX* gene in the viral vector pGR106, respectively. *Agrobacterium* GV3101 carrying the effector gene was infiltrated into a spot of *N. benthamiana* and 24 hours later *Agrobacterium* GV3101 harboring the *BAX* gene was infiltrated into the same spot. A period of 24 hours is a hiatus for zinc finger effectors to be expressed first in the primarily transformed cells and then in the neighboring cells. All zinc finger effectors MGG_07834, MGG_09035, MGG_08230, MGG_10238, MGG_13665, MGG_10020 and MGG_10556 inhibited BAX-induced PCD, while the EGFP control did not (Figure 4-1 A). Second, in order to express the effector and *BAX* genes in the same cell and at the same time, we inserted a ribosomal stuttering signal from *Thosea asigna* virus between the effector gene and *BAX* gene. The effector gene: Ta2A: *BAX* fusions were cloned into pMDC32 vector and introduced into *N. benthamiana* by agroinfiltration. MGG_07834, MGG_10020 completely inhibited BAX-induced PCD, while MGG_09035, MGG_08230, MGG_10238, MGG_13665, MGG_10556 and AVR-Pii did not (Figure 4-1 B). This time, we added AVR-Pii and it did not inhibit BAX-induced PCD. Compared with the results from the first strategy, MGG_09035, MGG_08230, MGG_10238, MGG_13665 and MGG_10556 did not inhibit BAX-
induced cell death. And this may be due to the second strategy being more sensitive and strict.

Figure 4-1. Zinc-finger effectors suppress BAX-induced PCD when transiently expressed in *N. benthamiana* via *Agrobacterium* infiltration.

Zinc finger effectors inhibit BAX induced PCD in rice protoplasts

To further demonstrate that zinc finger effectors are genuine BAX-induced PCD inhibitors, we cloned the effector gene:Ta2A:BAX fusions into pUGW11 vector. We chose MGG_07834 and MGG_10020 for this experiment, because they consistently showed BAX-induced PCD inhibition in N. benthamiana in previous experiments.

Rice protoplasts transfected with pUGW11/MGG_07834 : Ta2A : BAX, pUGW11/MGG_10020 : Ta2A : BAX and pUGW11/GST : Ta2A : BAX (control) were observed for fluorescence after fluorescein diacetate (FDA) staining. FDA accumulates inside the plasmalemma of viable protoplasts can be detected by fluorescence microscopy. Compared with the control, BAX-induced cell death was largely inhibited by MGG_07834 and MGG_10020 (Figure 4-2). These results demonstrated that zinc finger effectors have virulent functions inside N. benthamiana and rice cells.

The induction of defense genes is compromised in MGG_07834 or MGG_09035 transgenic lines

We cloned MGG_07834 and MGG_09035 into pMDC32 (Curtis and Grossniklaus, 2003) and made transgenic rice lines stably expressing MGG_07834 and MGG_09035. We obtained 24 and 20 lines for MGG_07834 and MGG_09035, respectively. For each gene, three lines were chosen for the following experiments. MGG_07834 and MGG_09035 transgenic lines and their genetic background rice cultivar Kitaake (Wide type, WT) were inoculated with M. oryzae virulent isolate 70-15 and leaf tissues were harvested 0, 24 and 48 hours post inoculation for RT-PCR. Five rice defense-related genes, OsPAL, OsCPS4, OsBBI2-3, OsPR-5 and OsJIPR10, were tested for their relative expression levels in wild type and transgenic lines. In plants, phenylalanine ammonia lyase (PAL) is the first and committed step in the phenylpropanoid pathway and involved in the biosynthesis of polyphenol compounds such as flavonoids, phenylpropanoids and lignin, which are antimicrobial and effective against various pathogens. Pathogens and PAMPs such as chitin, PGN and flagellin can induce PAL gene expression. When induced by M. oryzae, OsPAL levels in MGG_07834 and MGG_09035 transgenic lines were lower by about 2 folds than in WT rice plants (Figure 4-3 A). Upon pathogen infection, diterpene phytoalexins such as momilactones, oryzalexins and phytocassanes are de novo synthesized in cells at the infection site. OsCPS4 is the first enzyme in the phytoalexin biosynthesis pathway
leading to production of momilactone A, B and oryzalexin S from a common precursor geranylgeranyl diphosphate. Induction of OsCPS4 gene in MGG_07834 and MGG_09035 transgenic lines were compromised and the expression levels were lower by ~3-6 folds in these transgenic lines than in WT (Figure 4-3 B). OsBBI2-3 is a Bowman–Birk serine protease inhibitor and induced by wounding, pathogens and herbivores in rice. Upon M. oryzae infection, induction of OsBBI2-3 was decreased by about 2 folds in MGG_07834 and MGG_09035 transgenic lines than in WT plants (Figure 4-3 C). PR5 and PR10 are two families of pathogenesis-related genes and encode thaumatin and ribonuclease-like structure proteins, respectively. In rice, OsPR5 and OsJIPR10 are induced by pathogens, herbivores and phytohormones such as ET and JA. After inoculation of M. oryzae, the induction of OsPR5 and OsJIPR10 were slightly compromised. The relative expression of OsPR5 and OsJIPR10 are lower by ~0.5 fold in MGG_07834 and MGG_09035 transgenic lines than in WT plants (Figure 4-3 D, E). In summary, the trend is evident that the induction of rice defense related genes by M. oryzae was moderately compromised in zinc finger effector transgenic lines.
A

OsPAL

B

OsCPS4
Figure 4-3 Suppression of induction of defense related genes in MGG_07834 and MGG_09035 transgenic lines after M. oryzae inoculation. A. OsPAL. B. OsCPS4. C. OsBBI2-3. D.OsPR5. E. OsJIPR10. Values are the means of three replications and error bars represent the se (n=3).

*MGG_07834 or MGG_09035 transgenic lines are more susceptible to M. oryzae infection*

We examined disease severity of MGG_07834 and MGG_09035 transgenic lines caused by M. oryzae isolates 70-15 and Guy11-GFP. Six days post inoculation of M. oryzae 70-15, disease severity indices such as lesion size and lesion number were measured. In comparison with those of WT plants, lesion size and lesion number of MGG_07834 and MGG_09035 transgenic lines were significantly increased (n=6, P<0.01) (Figure 4-4 A, B). The macroscopic disease severity of WT, MGG_07834 and MGG_09035 transgenic lines is shown in Figure 4-5 A. We also inoculated WT and transgenic lines by the punch method that is more suitable to measure the subtle difference caused by basal level disease resistance (Ono et al., 2001). Compared with
WT, \textit{MGG\_07834} and \textit{MGG\_09035} transgenic lines are more susceptible to Guy11-GFP, as shown by the inoculation spot size and GFP intensity (Figure 4-5 B).

Figure 4-4 Lesion sizes and Lesion numbers of \textit{MGG\_07834} and \textit{MGG\_09035} transgenic lines post \textit{M. oryzae} inoculation. A. Lesion sizes. B. Lesion numbers. Values are the means
of three replications and error bars represent the se (n=6, **P<0.01).

Figure 4-5 Disease severity of MGG_07834 and MGG_09035 post spray and punch inoculation of M. oryzae strains, respectively. A. Spray inoculation. B. Punch inoculation.

4.3 Discussion

This study demonstrated that zinc finger effectors suppress rice innate immunity by silencing a wide variety of defense-related genes. The products of these defense genes vary from enzymes of phenylpropanoid synthesis to enzymes of phytoalexin production, from serine protease inhibitors to thaumatin to ribonucleases. All these low molecular weight products, either proteins or secondary metabolites, contribute to the basal defense against pathogens. Is the expression of these defense genes controlled by several master transcription factors or by disparate regulators? Mapping the binding sites across the rice genome for this family of zinc finger effectors is the key to solving this question. Primary SELEX results for MGG_07834 are promising and CHIP-exo mapping of MGG_07834 and MGG_10020 binding sites throughout the rice genome is underway.
Pathogen effector biology study often serendipitously reveals novel components or mechanisms of plant signaling pathways that otherwise defy exploring. In this study, HIRA is identified as an interactor of *M. oryzae* zinc finger effectors, not only expanding the functions of HIRA in gene silencing, but also revealing chromatin status changing as an important mechanism of defense gene regulation in plant innate immunity. The next challenge is to find out all essential players in the ZFE/HIRA silencing complex such as the predicted histone modifiers and transcription repressors. Yeast two hybrid screens and in planta coimmunoprecipitation followed by mass spectrometry will be the chosen practices to find these ZEF/HIRA interactors.

Based on the data obtained in this study, we propose a simple model for how *M. oryzae* zinc finger effectors suppress rice defense, in particular, the chitin (GlcNAc)_n-CEBiP mediated PTI (Figure 4-6). Upon *M. oryzae* infection, rice cells are alerted to the recognition of the fungal chitin fragments by CEBiP and deploy the CEBiP/OsCERK1 initiated PTI against the pathogen. To defeat PTI, zinc finger effectors from *M. oryzae* accumulate in the BIC and later translocate into rice cells. Inside rice cells, zinc finger effectors are localized in the nuclei where they bind specifically to promoters of their target genes such as defense-related genes in the PTI pathway and recruit HIRA to form ZEF/HIRA silencing complex. The ZFE/HIRA silencing complex creates a repressive chromatin state at the promoters of these genes possibly by directing the activities of histone modifiers such as HAD6 and PRC2. Eventually defense-related genes are silenced and PTI suppressed. Rice sheath live-imaging revealed that zinc finger effectors, like *M. oryzae* BAS1, PWL2 and *U. maydis* Cmu1, can move from infected cells to neighboring cells ahead of invasive hyphae. This phenomenon can be explained by this model: zinc finger effectors enter neighboring cells to create a chromatin repressive state at the
promoters of defense-related genes, thereby priming the neighboring cells for an ensuing fungal invasion.

Figure 4-6 Model of *M. oryzae* ZFEs suppress chitin (GlcNAc)_n-CEBiP/OsCERK1 mediated rice immunity.

4.4 Materials and methods

Rice protoplast preparation, transfection and FDA staining

Fusion fragments of *GFP:Ta2A:BAX, MGG_07834:Ta2A:BAX* and *MGG_10020:Ta2A:BAX* were made by overlap extension PCR and cloned into vector pUGW11. Rice protoplasts were prepared and transfected as described in the Materials and methods of Chapter 3. Twenty four hours after transfection, protoplasts were stained
with a solution of 0.01% (w/v) fluorescein diacetate (FDA) in acetone. Fluorescence was observed on an epifluorescence stereomicroscope (Leica) with UV filter. Excitation and emission wave lengths were 360/40 nm and 420 nm, respectively.

Rice transformation

*MGG_07834* and *MGG_09035* were recombined into the gateway vector pMDC32 under the control of the CaMV 35S promoter. Constructs were introduced into *A. tumafesciens* strain EHA 105 by electroporation. Vigorously growing rice calli induced from embryos of the Japanese cultivar Kitaake were used for *Agrobacterium*-mediated gene transformation as described by Xiong and Yang (2003). T₀ lines were self-pollinated and the resulting T₁ seeds were germinated in 50 µg/ml of hygromycin solution. Hygromycin resistant seeds were planted in Metro Mix 360 (Sun Gro) soil for T₂ seeds. A fraction of T₂ seeds were soaked in 50 µg/ml of hygromycin solution for germination. The rest of T₂ seeds with 100% germination rate in hygromycin solution were kept as lines with homozygous genotype for *MGG_07834* or *MGG_09035*.

RNA extraction and cDNA synthesis

Approximately 100 mg of rice leaf tissues were harvested at 0, 24 and 48 hours post inoculation of *M. oryzae* strain 70-15 and ground in liquid nitrogen to powders. RNA was extracted using 1 ml of TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Traces of DNA were removed by treating RNA samples with DNaseI (New England Biolabs). cDNA was synthesized using High Capacity Reverse Transcription Kit (Applied Biosystems) according to the manufacturer’s protocol.
Quantitative RT-PCR for defense-related genes

Quantitative RT-PCR was conducted using Step One Plus Real-Time PCR system (Applied Biosystems). PCRs were conducted following the protocol of DyNAmo SYBR green PCR Kit (New England Biolabs). Programs for qRT-PCR were as follows: 15 min at 94ºC for denaturation; 40 cycles of 10 sec at 94ºC for denaturation and 1 min at 60ºC for amplification and extension. Gene expression levels were calculated using the ∆∆Ct method and normalized relative to rice ubiquitin gene.

Rice blast inoculation and disease severity assay

*M. oryzae* isolates 70-15 and Guy11 were used in this study. Fungi were grown on oatmeal agar under fluorescent light at room temperature. After 7~8 days, conidia were harvested and adjusted to a concentration of 5×10⁵ spores/ml in 0.1% (v/v) of Tween 20 and 0.25% of (w/v) gelatin. 2-week-old plants were used for spray inoculation or punch inoculation. The spray or punch inoculated plants were placed at a container with water to keep moisture for 24 hours at room temperature and then moved to a Conviron growth chamber set at 12:12 photoperiod with temperature of 28ºC/24ºC (day/night). Six to seven days later, disease indices of the spray inoculated plants were scored by measuring and counting the sizes and numbers of lesions on 6 leaves, respectively. Leaves from punch inoculated plants were photographed using fluorescence stereomicroscope (Leica) with a GFP filter. The excitation and emission wave lengths of GFP were 425/60nm and 480nm, respectively.
Chapter 5

Role of the rice endogenous peptide elicitor OsPep1 in defense signaling and disease resistance

5.1 Introduction

Plant endogenous elicitors, also known as damage-associated molecular patterns (DAMPs), are usually molecules released from broken components of plant cell walls or intracellular precursor proteins processed upon pathogen attack and/or wounding. Like pathogen-associated molecular patterns (PAMPs), DAMPs also elicit pattern triggered immunity (PTI) against pathogens or insect herbivores.

Oligogalacturonides (OGs) are released from broken pectin of plant cell walls by bacterial or fungal secreted polygalacturonases and can induce defense response in various plants (D’Ovidio et al., 2004). Exogenous application of OGs protects grapevine leaves against the necrotrophic fungal pathogen Botrytis cinerea (Aziz et al., 2004). In Arabidopsis, the pattern recognition receptor (PRR) for OGs is identified as WAK1 (Brutus et al., 2010). WAK1 is a wall-associated epidermal growth factor (EGF)-domain containing receptor-like kinase and its ectodomain specifically binds to the elicitor active form of “egg-box” type of OGs to transduce the signal (Cabrera et al., 2008). Arabidopsis plants overexpressing WAK1 exhibited increased resistance against necrotrophic pathogens B. cinerea and Pectobacterium carotovorum (De Lorenzo et al., 2011).

In addition to carbohydrate DAMPs, proteinaceous DAMPs are also discovered as strong
plant endogenous elicitors. Systemin, an 18-amino acid (aa) peptide, is processed from the C-terminal region of its precursor prosystemin and can induce defense response against herbivorous insects in many solanaceous plants (Pearce et al., 1991). Systemin is the first peptide signal reported in plants. The receptor for systemin in tomato was isolated as SR160, the tomato ortholog of Arabidopsis brassinosteroid receptor BRI1 (Scheer et al., 2002). However, BRI1/SR160 being the common receptor for both brassinosteroids and systemin is debatable, as demonstrated by the finding that tomato BRI1/SR160 cu-3/curl-3 mutants, while being insensitive to brassinosteroid, were still fully responsive to systemin (Holton et al., 2007).

AtPeps, a 6-member family of 23-aa plant elicitor peptides (Peps) in Arabidopsis, were found to induce expression of pathogenesis-related (PR) gene PRI, defensin gene PDF1.2 and their precursor genes AtPROPEPs (Huffaker et al., 2006; Huffaker and Ryan, 2007). Arabidopsis plants overexpressing PROPEP1 showed enhanced resistance to the necrotrophic oomycete root pathogen Pythium irregulare (Huffaker et al., 2006) and wild-type plants pretreated with AtPep1 became more resistant to the hemibiotrophic bacterial pathogen Pseudomonas syringae pv. tomato DC3000 (Yamaguchi et al., 2010).

Orthologous genes of AtPROPEP were found in many plant species including canola, soybean, potato, maize and rice (Huffaker et al., 2006), suggesting that Pep signaling may be widely involved in plant defense against various biotic stresses. Indeed, ZmPROPEP1, the Arabidopsis AtPROPEP1 gene ortholog in maize, was induced by JA, ZmPep1 and a fungal pathogen Cochliobolis heterostrophus and pretreatment with ZmPep1 of maize leaves and stems enhanced resistance to necrotrophic anthracnose stalk rot pathogen C. heterostrophus and hemibiotrophic southern leaf blight pathogen Colletotrichum graminicola, respectively (Huffaker et al., 2011). Furthermore, ZmPep3, another Pep from
the 5-member family of maize Peps, was shown to induce herbivore-specific defense responses against insect *Spodoptera exigua* and its precursor gene *ZmPROPEP3* was induced by the oral secretions of *S. exigua* (Huffaker et al., 2013). These new findings expand the spectrum of resistance elicited by Peps from against pathogens to insects. Like systemin, lacking a typical N-terminal signal peptide, Peps are likely to be released into the apoplast from injured cells upon pathogen/insect attack and/or wounding. The apoplastic Peps then function as DAMPs, binding receptors at the surface of neighboring cells to elicit defense against pathogens or insects. Two pattern recognition receptors (PRRs) for AtPeps were identified as PEPR1 and PEPR2 with overlapping functionalities (Yamaguchi et al., 2006; Yamaguchi et al., 2010; Krol et al., 2010).

Like the perception of PAMPs by PRRs, upon interaction of AtPeps and PEPR1/2, plant cells rapidly activate defense responses by undergoing a cascade of cellular and molecular events such as Ca\(^{2+}\) influx, production of reactive oxygen species (ROS), MAPK activation, ethylene (ET) production and defense gene expression. Aside from the induction of defense genes, AtPeps as well as many PAMPs such as flg22 also activate the expression of the precursor genes *AtPROPEPs*, generating more AtPeps to act as DAMPs. There is a positive feedback loop inherent in plant innate immunity. A current view is that the AtPep/PEPR system plays the role of an amplifier of the PAMP/PRR initiated plant immunity by facilitating the rapid buildup and maintenance of plant defense responses in the face of invading pathogens (Huffaker and Ryan, 2007).

In rice, it is well known that several PAMP perception systems function as surveillance systems over bacterial and fungal pathogens. PAMPs such as bacterial flagellin, quorum sensing protein Ax21 and fungal chitin are all potent elicitors of rice defense responses. In
rice, PRR for flagellin was identified as OsFLS2, an ortholog of the Arabidopsis FLS2 and heterologous expression of OsFLS2 in Arabidopsis fls2 mutants restores flg22 responsiveness (Takai et al., 2008). Interestingly, the rice Ax21/XA21 system also seems to exist in Arabidopsis where Ax21 is recognized by the same flagellin receptor FLS2 (Danna et al., 2011). Arabidopsis and rice also share chitin elicitor receptor-like kinase (CERK1) in chitin signaling. In Arabidopsis, upon perception of chitin, CERK1 forms homodimers to transduce chitin signaling, whereas in rice chitin elicitor binding protein CEBiP and OsCERK1 interact to signal intracellularly (Liu et al., 2012; Shimizu et al., 2010). In addition, OsWAK1, a distant ortholog of AtWAK1, plays an important role in mediating disease resistance against rice blast pathogen Magnaporthe oryzae (Li et al., 2009), although evidence for OGs as DAMPs of OsWAK1 is yet to be obtained. Therefore many P/DAMP/PRR systems are well conserved across divergent plant taxa and it is plausible to propose Peps may also function in rice and other plants. In this study, we make the first step to demonstrate that rice endogenous elicitor OsPep1 is an active DAMP and plays a role in defense signaling and disease resistance.

5.2 Results

OsPep1 belongs to a 7-member family of potential endogenous peptide elicitors in rice

Based on the 23-aa AtPep1 sequence, we blasted the Rice Genome Annotation Project Database and identified 7 predicted proteins with a C-terminal amino acid sequence close to that of AtPep1(Table 5-1). The genes encoding these 7 proteins are designated OsPROPEP1-7, and the putative mature peptides from the C-termini of these proteins OsPep1-7. OsPROPEP1 resides alone on chromosome 4, while the other 6 OsPROPEPs
are located in tandem within a 33 kb region on chromosome 8. Furthermore, we checked Genevestigator and RED microarray databases for expression of these genes. Except for *OsPROPEP4*, which we cannot find any information on its expression, the others are activated by 2~3 by different inducers such as pathogen, insect and phytohormones (Table 5-1). Of the 7 *OsPROPEPs*, *OsPROPEP1* is induced by *M. oryzae* and ethylene (Table 5-1), and more importantly, *OsPROPEP1* has all the conserved motifs typical of precursors of established bioactive peptide elicitors AtPep1 and ZmPep1 (Figure 5-1 A; Huffaker et al., 2006; Huffaker et al., 2011). Like AtPep1 and ZmPep1, *OsPep1* is 23-aa peptide processed from the C-terminus of its precursor OsproPep1 likely through a biogenesis pathway similar to systemin. *OsPep1* is closely related to ZmPep1 (Figure 5-1 B), suggesting that it probably also functions in rice. We chose *OsPep1* and its precursor encoding gene *OsPROPEP1* for further study.

**Table 5-1 OsPROPEPs and the putative mature peptides**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene locus</th>
<th>Annotated length of precursor protein (AA)</th>
<th>Predicted processed C-terminal 23-aa elicitor peptide</th>
<th>Inducers of expression*</th>
</tr>
</thead>
<tbody>
<tr>
<td>OsPropep1</td>
<td>Os04g54590</td>
<td>159</td>
<td>ARLRPKPPGNNREGSGGNGGH</td>
<td><em>M. oryzae</em>, ET</td>
</tr>
<tr>
<td>OsPropep2</td>
<td>Os08g07600</td>
<td>93</td>
<td>DDSKFTPQGAPAEGSGGNGAIH</td>
<td><em>M. oryzae</em>, Chitin; JA</td>
</tr>
<tr>
<td>OsPropep3</td>
<td>Os08g07630</td>
<td>168</td>
<td>ADSAPQGAPAEGGGNGAVH</td>
<td><em>M. oryzae</em></td>
</tr>
<tr>
<td>OsPropep4</td>
<td>Os08g07640</td>
<td>137</td>
<td>ADSAPQGAPAEGGGNGVDH</td>
<td>N/A</td>
</tr>
<tr>
<td>OsPropep5</td>
<td>Os08g07660</td>
<td>182</td>
<td>ADSAPQGAPAEGGGNGGAVH</td>
<td>Chitin</td>
</tr>
<tr>
<td>OsPropep6</td>
<td>Os08g07670</td>
<td>172</td>
<td>SKKPFEPPGGPREGGGNGGVD</td>
<td><em>N. lugens</em></td>
</tr>
<tr>
<td>OsPropep7</td>
<td>Os08g07690</td>
<td>111</td>
<td>RANPRSERPVLREGGGKGGAHH</td>
<td><em>M. oryzae</em></td>
</tr>
</tbody>
</table>

*Gene induction information is from Genevestigator and RED microarray databases. N/A, not available.*
Figure 5-1 OsproPep1 and its mature peptide OsPep1. A. The amino acid sequence of OsproPep1 and OsPep1. Conserved motifs are KEKE motif (in blue), amphipathic helix motif (in purple) and elicitor active OsPep1 (in red). B. The relationship between OsPep1 and other Peps. The dendrogram was generated based on a multiple sequence alignment of 7 OsPeps, 5 ZmPeps and 7 AtPeps.

**OsPROPEP1** is induced by *M. oryzae* and JA and ET

*OsPROPEP1* have two transcripts as a result of alternative splicing. The splicing occurs
immediately upstream of the C-terminal region encoding OsPep1, producing a precursor with 5 fewer amino acids. The seemingly frequent occurrence of alternative splicing in PROPER gene transcription may have a role in tightly regulating the biogenesis of Peps from their precursors (Huffaker et al., 2011). Time-course analysis of the expression of OsPROPEP1 after M. oryzae 70-15 inoculation showed that the level of OsPROPEP1 in rice leaves increased 1 day post inoculation (DPI) and peaked 2 DPI and then gradually fell. In comparison with mock (water) inoculation, M. oryzae induced just over 2.5-fold change in OsPROPEP1 expression but with statistical significance (P<0.05; Figure 5-2 A). The expression of AtPROPEP1 changed little upon treatment with various pathogens or PAMPs, whereas ZmPROPEP1 and ZmPROPEP3 were highly induced by pathogen and insect oral secretions, respectively (Huffaker et al., 2006; 2011; 2013). The observation of the difference in induction of PROPEPs indicates that Peps as DAMPs play different roles in eliciting defenses against pathogens or insects.

We further tested the responsiveness of OsPROPEP1 to JA and ET. 100 µM of methyl jasmonate (MeJA) or 100 µM of ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC) was sprayed onto 2-week-old rice seedlings. Rice leaves were harvested at the indicated time points and used to prepare RNA for detection of OsPROPEP1 transcripts by qRT-PCR. In comparison with water, both JA and ACC induced the expression of OsPROPEP1 to a moderately high level at 1 hour post treatment. At its peak, OsPROPEP1 transcripts increased 2.5- and 3.5-fold with statistical significance by JA and ACC, respectively (Figure 5-2 B). In rice, defense hormones SA, JA and ET play different roles in disease resistance. With a high basal level in rice, SA changes little under various abiotic and biotic stresses and is regarded as a minor regulator of rice defense (Silverman et al., 1995; Yang et al., 2004). In contrast, ET and JA play prominent roles in rice defense
against pathogens (Singh et al., 2004; Helliwell et al., 2013; Mei et al., 2006).

*OsPROPEP1* is induced by JA and ET (Figure 5-2 B) but not by SA (data not shown), suggesting that ET and JA play major roles in OsPep1 signaling.

![Graph A: Induction of OsPROPEP1 by various inducers. A. Induction of OsPROPEP1 by *M. oryzae*. B. Induction of OsPROPEP1 by JA (100 µM) and ACC (100 µM). Values represent means ± se from three replications. Asterisks indicate statistically significant)](image)

Figure 5-2 Induction of *OsPROPEP1* by various inducers. A. Induction of *OsPROPEP1* by *M. oryzae*. B. Induction of *OsPROPEP1* by JA (100 µM) and ACC (100 µM). Values represent means ± se from three replications. Asterisks indicate statistically significant
differences compared with water treatment (* and ** indicate P<0.05 and 0.01, respectively; Student’s t test).

**OsPep1 induces the expression of its precursor gene and rice defense genes and enhances disease resistance against *M. oryzae***

In addition to *OsPROPEPs*, two putative OsPep receptors OsPEPR1 and OsPEPR2 are also encoded in the rice genome at loci *Os08g34640* and *Os10g06760*, respectively, lending another support to the hypothesis that the Pep/PEPR system also functions in rice. To test whether OsPep1 is perceived in rice and whether OsPep1 is biologically active in eliciting rice defense response, the 23-aa OsPep1 was synthesized and used to feed rice seedlings at a concentration of 1 µM. Rice seedlings at the indicated time points were harvested and RNA were extracted and used to detect the expression of *OsPROPEP1*, *OsPR5* and *OsPAL*. At 6 h, the expression of *OsPROPEP1* was 4.5 fold higher in the OsPep1-supplied plants compared with water-supplied control. *OsPROPEP1* transcripts continued to accumulate and reached a peak at 12 h where they were 6.5-fold richer in OsPep1-supplied plants in comparison with water-supplied plants. And at 24 h, *OsPROPEP1* expression was still 5-fold higher in OsPep1-treated plants relative to control plants (Figure 5-3 A). As a result, consistent with the early findings with AtPep1 and ZmPep1 (Huffaker et al., 2006; 2011), OsPep1 signals via a positive feedback loop. *OsPR5* and *OsPAL* are two well-established pathogenesis-related (PR) genes that are induced by ET, JA and *M. oryzae*. We tested the effect of OsPep1 on the expression of these two genes. The transcripts of *OsPR5* accumulated to a peak level 8-fold higher in the OsPep1-supplied plants compared with water-supplied plants at 6 h. And at 12 h, the expression of *OsPR5* was still higher by 5-fold (Figure 5-3 B). The expression of *OsPAL* is 4-fold higher
at 12 h in OsPep1-supplied plants compared with water-supplied plants (Figure 5-3 C). In
rice, upon pathogen attack, ET and JA often cooperate to regulate the expression of a
plethora of defense genes including OsPR5 and OsPAL. As such, it is unclear whether
OsPep1 induces the expression of its precursor gene and specific defense genes through a
distinct JA or ET pathway or both pathways.

Because *M. oryzae* induced the expression of *OsPROPEP1* and because OsPep1-supplied
plants had elevated PR gene expression, we inferred that OsPep1 would enhance disease
resistance against *M. oryzae*. To test this hypothesis, 2-week-old rice seedlings were
sprayed with 1µM OsPep1 and 12 h after OsPep1 treatment, rice seedlings were inoculated
with *M. oryzae* 70-15. Six days after *M. oryzae* inoculation, disease symptoms and severity
were compared between OsPep1- and water-treated plants. For water-sprayed plants, 70%
of leaf tips were wilted, whereas only 10% of leaf tips of OsPep1-sprayed plants were
withered (Figure 5-4 A). More quantitatively, lesion size and lesion number were evaluated
on water- and OsPep1-sprayed plants. Both disease severity indices were lower with
statistical significance for OsPep1-sprayed plants compared with water-sprayed plants
(P<0.01; Figure 5-4 B and C).
Figure 5-3 OsPep1 (1μM) induces expression of its precursor gene and defense genes. A. Time-course induction of *OsPROPEP1*. B. Time-course induction of *OsPR5*. C. Time-course induction of OsPAL. Values represent means ± se from three replications. Asterisks indicate statistically significant differences compared with water treatment (* and ** indicate P<0.05 and 0.01, respectively; Student’s t test).
OsPep1-overexpression lines exhibit growth inhibition and seed-set reduction

One way to ascertain a functioning DAMP/PRR system in plants is to characterize transgenic plants constitutively producing DAMP. Plants overexpressing prosystemin established the role of systemin in plant defense against herbivores (McGurl et al., 1994). Similarly, overexpression of a fungal polygalacturonase in Arabidopsis and tobacco helped to elucidate the role of the OG/WAK1 system in plant defense against pathogens (Ferrari et al., 2008; Capodicasa et al., 2004). Likewise, Arabidopsis plants overexpressing PROPEP1 and PROPEP2 facilitated the revelation of the role of the Pep/PEPR system in plant defense against pathogens (Huffaker et al., 2006). To further establish that an active Pep/PEPR system also exists in rice, we sought to make transgenic plants overexpressing

Figure 5-4 OsPep (1µM) induces resistance against M. oryzae. A. Comparison of effects of water and OsPep1 on M. oryzae infection. B. Lesion size 6 days post inoculation of M. oryzae. C. Lesion number 6 days post inoculation of M. oryzae. Values are the means of three replications and error bars represent the se (n=6, **P<0.01).
OsPROPEP1. The construct CaMV 35S:PROPEP1 was introduced into a fast-growing Japanese rice variety Kitaake by Agrobacterium-mediated transformation. Three independent OsPROPEP1-overexpression (OX) lines, designated OsPROPEP1-OX1, 2 and 3, were chosen for further analysis.

Compared with wild-type (WT) Kitaake, OsPROPEP1-OX lines were dwarf and yellowish (Figure 5-5A). Tracking the growth week by week, we found that the height of OsPROPEP1-OX lines was about half that of the wild-type plants at weeks 7 and 8 when the plants completed the vegetative growth and entered the reproductive stage (Figure 5-5 B). In addition, seed-set rate of OsPROPEP1-OX lines was lowered approximately by half in comparison with that of wild-type Kitaake (Figure 5-5 C). Little difference in plant height or seed-set rate was found between the three OsPROPEP1-OX lines. The dwarf phenotypes were also observed with transgenic tobacco plants overexpressing an OG-generating polygalacturonase and tomato plants overexpressing prosystemin (Capodiscasa et al., 2004; McGurl et al., 1994). Although Arabidopsis plants overexpressing PROPEP1 and PROPEP2 exhibited increased root and aerial growth (Huffaker et al., 2006), seedlings growing in MS medium supplemented with 1µM AtPep1 showed pronounced growth retardation (Krol et al., 2010). Consistent with these early findings, our results revealed a tradeoff in rice between plant development and OsPep/OsPEPR-mediated plant defense.
Figure 5-5 Comparison phenotypes of wild-type and *OsPROPEP1*-OX plants. A. Statures of wild-type and *OsPROPEP1*-OX plants at 10-week-old stage. B. Growth curves of wild-type and *OsPROPEP1*-OX plants. C. Seed-set of wild-type and *OsPROPEP1*-OX plants.

An array of defense genes are constitutively expressed in *OsPROPEP1*-overexpressing lines

Constant presence of DAMPs in plants usually leads to constitutive expression of an array of plant defense genes. Tomato plants overexpressing prosystemin accumulated high levels of serine proteases, peroxidases and many other defense-related proteins (Bergey et al., 1996). Similarly, tobacco plants overexpressing polygalacturonase, which generates OGs by degrading pectin, constitutively expressed *POX* (peroxidase). And Arabidopsis overexpressing the same polygalacturonase had high basal levels of transcripts for *PR-I* (unknown), *PDF1.2* (defensin) and *AtPGIP1* (Polygalacturonase inhibiting protein) (Ferrari et al., 2008). Likewise, Arabidopsis plants overexpressing *PROPEP1* and *PROPEP2* expressed *PR-I* and *PDF1.2* at higher levels than those found in wild-type plants (Huffaker et al., 2007).
Eight rice defense-related genes, *OsPAL, OsCPS4, OsBBI2-3, OsBBI3-3, OsOsPR-5, OsVSP, OsJIPR10* and *PXO22.3*, were tested for their relative expression levels in wild type and *OsPROPEP1* transgenic lines (Figure 5-6 A). In plants, phenylalanine ammonia lyase (PAL) is the first and committed step in the phenylpropanoid pathway and is involved in the biosynthesis of polyphenol compounds such as flavonoids, phenylpropanoids and lignin, which are antimicrobial and effective against various pathogens. Pathogens and PAMPs such as chitin, peptidoglycan and flagellin can induce PAL gene expression. The level of *OsPAL* expression was 2.5~4.5-fold higher in *OsPROPEP1*-OX lines as compared with wild-type plants. Upon pathogen infection, diterpene phytoalexins such as momilactones, oryzalexins and phytocassanes are de novo synthesized in cells at the infection site. *OsCPS4* is the first enzyme in the phytoalexin biosynthesis pathway leading to production of momilactone A, B and oryzalexin S from a common precursor geranylgeranyl diphosphate. *OsCPS4* transcripts were 2.5~3.8-fold more abundant in *OsPROPEP1*-OX lines than in wild-type plants. *OsBBI2-3* and *OsBBI3-3* are two Bowman–Birk serine protease inhibitors and induced by wounding, pathogens and herbivores in rice. The transcripts of *OsBBI2-3* were 3.2~7.8-fold higher and those of *OsBBI3-3* were 1.8~7.5-fold more in *OsPROPEP1*-OX lines compared with wild-type plants. *PR5* and *PR10* are two families of pathogenesis-related genes and encode thaumatin and ribonuclease-like proteins, respectively. VSP2, i.e., vegetative storage protein 2, is an acid phosphatase. In rice, *OsPR5, OsJIPR10* and *OsVSP2* are induced by pathogens, herbivores and phytohormones such as ET and JA. *OsPROPEP1*-OX lines exhibited a 2.8~15-fold greater expression of *OsPR5* than did wild-type plants. Compared with wild-type plants, *OsPROPEP1*-OX lines displayed a 4.2~9.2-fold increase in *OsJIPR10* transcript. Similarly, *OsVSP2* transcripts were 5.5~8.8-fold more abundant in comparison
with wild-type plants. PXO22.3, encoding a peroxidase, is induced by Xanthomonas oryzae pv. oryzae (Xoo) and M. oryzae. OsPROPEP1-OX lines showed a 3.2–6.3-fold increase in PXO22.3 transcript compared with wild-type plants.

*OsPROPEP1-OX lines enhance disease resistance against M. oryzae*

The positive correlation between constitutive expression of defense-related genes and disease resistance prompted us to test *OsPROPEP1-OX* lines for basal resistance to *M. oryzae*. One *M. oryzae* highly virulent isolate IE1K was used to inoculate wild-type plants and *OsPROPEP1-OX* lines. Although *OsPROPEP1-OX* lines showed no R-mediated qualitative resistance, disease symptoms were less severe on *OsPROPEP1-OX* lines as compared with wild-type plants when plant leaves were either spayed or spot-inoculated with IE1K (Figure 5-6 B). Measured by lesion size and lesion number, both disease indices were lower by nearly half with statistical significance on *OsPROPEP1-OX* lines in comparison with wild-type plants (P<0.01; Figure 5-6 C).
Figure 5-6 OsPROPEP-OX lines constitutively express defense genes and enhance disease resistance against *M. oryzae*. A. Fold changes of defense gene expression in 2-week-old *OsPROPEP1*-OX lines compared with wild-type plants. B. Comparison of disease severity of wild-type and *OsPROPEP1*-OX lines 6 days post spray (left) and spot (right) inoculation of *M. oryzae*. C. Lesion sizes 6 days post inoculation of *M. oryzae*. D. Lesion numbers 6 days post inoculation of *M. oryzae*. Values are the means of three replications and error bars represent the se (n=6, **P<0.01).

5.3 Discussion

Pep signaling is activated upon pathogen or insect attack, suggesting that it plays an essential role in plant defense against various biotic agents (Huffaker et al., 2006; 2011; 2013). This seemingly widespread component of plant defense mechanism is demonstrated again by our finding that OsPep1 signaling is also operational in rice basal resistance against *M. oryzae*. A current view holds that Pep signaling works as an amplifier of PTI to help mount and sustain a rapid and prolonged defense at the local infected or infested sites.
(Huffaker and Ryan, 2007; Yamaguchi et al., 2010). In line with this model and based on the experimental data in this study, our explanation for the role of OsPep1 in rice basal resistance against *M. oryzae* could be as follows. Upon *M. oryzae* infection, chitin oligomers or other yet to be discovered fungal PAMPs promote a series of stereotypical PTI events, chief among them being induction of an array of rice defense-related genes encoding PR proteins and signaling molecules. Among the induced signaling molecules are OsPROPEPs and their receptors OsPROPEP1/2. Released into the apoplast from OsPROPEP1, OsPep1, together with its receptors, elicits new waves of PTI in neighboring cells at the infection site. In this way OsPep1 signaling contributes to the basal resistance against *M. oryzae*. Most likely, PTI initiated by chitin and strengthened by OsPep may also play a role in rice basal resistance against another fungal pathogen *Rhizoctonia solani*. Furthermore, because rice also senses PAMPs from bacteria such as flagellin, Ax21 and peptidoglycan, it is plausible to expect a role for OsPep signaling in disease resistance against bacterial pathogens such as Xoo. In addition, Huffaker and colleagues found that OsPep2 induced emission of the same spectrum of volatile organic compounds as did ZmPep3 in maize, indicating a role of OsPep2 signaling in herbivore defense (Huffaker et al., 2013). Besides OsPep2, OsPep6 also most likely shares a role of eliciting herbivore defense, as suggested by the observation that *OsPROPEP6* was induced by *Nilaparvata lugens* (table 5-1).

Pep signaling as an amplifier of PTI is mediated by plant defense hormones, i.e., SA, JA and ET. PAMPs such as flg22 and elf18 often induce the production of SA and ET (Tsuda et al., 2008; Felix et al., 1999), which in turn induce the expression of *PROPEPs* (Huffaker et al., 2006; Huffaker and Ryan, 2007). Recently, the detailed interconnections between ET and Pep pathways were revealed. On one hand, Arabidopsis *ein2* mutants were elf18
insensitive due to the loss of *PROPEP2* induction by ET (Tintor et al., 2013). On the other hand, *pepr1/2* mutants were compromised in ET-mediated defense response and growth inhibition as a result of the defective Pep signaling (Liu et al., 2013). In rice, SA, JA and ET play differential roles in disease resistance. Due to a relatively high basal level of SA and lack of measurable induction upon pathogen infection, SA is usually regarded as a minor contributor to rice disease resistance by serving as an antioxidant (Silverman et al., 1995; Yang et al., 2004). Compared with SA, ET and JA play major roles in regulating rice defenses. ET is inducible by *M. oryzae* and overexpression of OsACS2, a key enzyme of ET biosynthesis, leads to enhanced basal disease resistance against *M. oryzae* and *R. solani* (Iwai et al., 2006; Helliwell et al., 2013). Moreover, ET antagonizes ABA to promote biotic defense over abiotic tolerance (Xiong and Yang, 2003; Bailey et al., 2009).

Similarly, rice plants overexpressing OsAOS2, a key enzyme in JA biosynthesis, exhibited increased PR gene expression and enhanced disease resistance against *M. oryzae* (Mei et al., 2006). In rice, JA also antagonizes gibberellin (GA) to prioritize defense before growth during biotic stress (Yang et al., 2012). Realizing that ET and JA are important players in rice defense, we tested their roles in OsPep1 signaling. The results that *OsPROPEP1* was induced by ET and JA and that *OsPROPEP1*-OX lines constitutively expressed ET-and JA-induced PR genes point to a looming conserved PTI amplification loop of PAMP-ET/JA-Pep in rice defense. Future dissection of OsPep1 signaling by using ET/JA mutants such as *OsEIN2*-RNAi and *OsCOII*-RNAi will provide more insights into the Pep pathway.

In this study, we demonstrated that Pep signaling is conserved and functional in rice defense, promising a prospect of exploiting this integral part of PTI for basal resistance against devastating rice pathogens such as *M. oryzae*, *R. solani* and Xoo. We obtained *OsPROPEP1*-OX lines with constitutive PR gene expression and enhanced *M. oryzae*
resistance. Expectedly, OsPROPEP1-OX lines also showed stunted stature and lowered seed-set rate due to the constant activation of defense. This tradeoff between defense and growth will be overcome by directing OsPROPEP expression with pathogen inducible promoters such as PBZ1 promoter (Mei et al., 2006; Helliwell et al., 2013). To make the most of the Pep signaling in disease resistance, it is also tempting to construct chimeric receptors by swapping ectodomains or intracellular domains between OsPEPR1/2 and other rice PRRs such as OsCERK1 and XA21. Rice transgenic lines harboring these chimeric receptors are likely to provide a new, broadened and strengthened spectrum of basal resistance against pathogens.

5.4 Materials and methods

OsPROPEP1 construct and rice transformation

OsPROPEP1 full-length cDNA clone (AK062886) was obtained from the Rice Genome Resource Center, Tsukuba, Japan and used as template to amplify OsPROPEP1 fragment with primers: 5´-CAC CAT GGA TCG GGT CGA GGA AAA G-3´ and 5´-CTA GTG ATG GTG TCC TCC ATT G-3´. OsPROPEP1 fragment was first incorporated into vector pENTR™/D-TOPO and then combined into the binary Gateway vector pMDC32. Plasmid pMDC32/OsPROPEP1 was introduced into Agrobacterium tumefaciens strain EHA 105 by electroporation. Agrobacterium-mediated rice transformation was conducted using calli derived from rice cultivar Kitaake according to a protocol as described by Xiong and Yang (2003).

Plant materials and fungal isolates
Rice cultivar Kitaake and its OsPROPEP1-OX lines were grown in MetroMix 360 soil (Sun Gro, Bellevue, WA) in greenhouse with alternating 12 h of light (day) at 28 °C and 12 h of dark (night) at 24 °C. *M. oryzae* isolates 70-15 and IE1K were grown on oatmeal agar under constant fluorescent light at room temperature. After 7~8 days, conidia were harvested and adjusted to $5 \times 10^5$ spores/ml in a solution containing 0.1% (v/v) of Tween 20 and 0.25% of (w/v) gelatin. For analysis of the effect of OsPep1 treatment on *M. oryzae* infection, two-week-old Kitaake seedlings were sprayed with 10 µM OsPep1 and inoculated with *M. oryzae* 70-15 24 h post elicitor treatment. For characterization of OsPROPEP1-OX lines, highly virulent *M. oryzae* IE1K was used to inoculate two-week-old seedlings.

Peptide synthesis and chemical treatments

OsPep1 (RAARLRPKPPGNPREGSGGNGGHHH) was synthesized by GenScript (Piscataway, NJ) and dissolved in water at 1 mM as stock solution. Two-week-old rice seedlings grown in soil were sprayed with 100 µM of JA or ACC and rice leaves were harvested at the time points 0, 0.5, 1, 2 and 4 h post hormone treatment. Ten-day-old rice seedlings grown in liquid MS were transferred to fresh liquid MS medium supplied with 1 µM of OsPep1 and leaves were harvested at the time points 0, 0.5, 1, 6, 12 and 24 h post elicitor treatment.

Assessment of Agronomic traits and disease severity

The heights of Kitaake and OsPROPEP1-OX lines were measured every week until
maturity. Height was scored as the length of the plant aerial part and three plants from each line were measured. The seed-set rate was indicated by seeds per panicle and three plants from each line were scored. Two independent biological replicates were conducted in two growing seasons. Disease severity was assayed on the third leaf of each plant 6 days after M. oryzae inoculation. Each leaf was scored for the number of necrotic lesions and the size of each of the three largest lesions. Three independent experiments were carried out to evaluate disease severity.

Quantitative real-time PCR analysis of relative gene expression levels

RNA was isolated from 100~200 mg rice leaves using 1 ml of TRIZol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Traces of DNA were removed by treating RNA samples with DNase I (New England Biolabs, Ipswich, MA). cDNA was synthesized using High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. Quantitative real-time PCR was performed on Step One Plus Real-Time PCR system (Applied Biosystems) with DyNamo SYBR green PCR kit (New England Biolabs). Programs for quantitative real-time PCR were as follows: 15 min at 94ºC for denaturation; 40 cycles of 10 sec at 94ºC for denaturation and 1 min at 60ºC for amplification and extension. Gene specific primers were used to amplify the fragments of OsPROPEP1 (AK062886), OsPR5 (AK241419), OsPAL (AK068993), OsCPS4 (AK100631), OsBBI2-3 (AK064050), OsBBI3-3 (AK243607), OsJIPR10 (AK121376), OsVSP2 (AK100363), PXO22.3 (AK073202) and OsUBQ1 (AK059011). These primers are as follows: OsPROPEP1 forward primer, 5´-GAC ATG AAG ACG GT TAC-3´, and reverse primer, 5´-CTA GTG ATG GTG TCC TCC ATT G-3´; OsPR5 forward primer, 5´-GCA ACA GCA ACT ACC AAG
TC-3′, and reverse primer, 5′-TGT CCC ATG ATA CAT ATA CTA C-3′; OsPAL forward primer, 5′-TGA ATA ACA GTG GAG TGT GGA G-3′, and reverse primer, 5′-AAC CTG CCA CTC GTA CCA AG-3′; OsCPS4 forward primer, 5′- CCC CAC CTC CAC TAC CAA TTC C-3′, and reverse primer, 5′-AAA ACA CGA GGT ACA CCA CAA CAA AC-3′; OsBBI2-3 forward primer, 5′- TGT GTT GTC TCG TGT GAA CGA TGG-3′, and reverse primer, 5′-ACG ATG ACA GCG CAA CAT GGC-3′; OsBBI3-3 forward primer, 5′-CTG CAA GGA CCG CTT CAC CG-3′, and reverse primer, 5′-AGC TGC AAG CTA GGG CGA GT-3′; OsJIPR10 forward primer, 5′- GAC GCT TAC AAC TAA ATC GTC-3′, and reverse primer, 5′-CAA TCA CTG CTT GGA AGC AG-3′; OsVSP2 forward primer, 5′-CCA ACC CTG CCT ACT ACA TCG-3′, and reverse primer, 5′-TAG CTA GGC ATC GTT TGC TTC A-3′; PXO22.3 forward primer, 5′-ATG GCT TCT GCA ACT AAT TCT TC-3′, and reverse primer, 5′-TTA GGA GTT CAC CTT GGA GCA GCT G-3′; and OsUBQ1 forward primer, 5′-AAC CAG CTG AGG CCC AAG AAG-3′, and reverse primer, 5′-ACG ATT GAT TTA ACC AGT CCA TG-3′. Gene expression levels were calculated using the ∆∆Ct method and normalized relative to the level of rice ubiquitin gene transcripts.
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