The Role of Ubiquitination in the Interaction between Rice and *Magnaporthe oryzae*

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Abstract

Rice is one of the most important food crops and feeds about 50% of the world population. Using rice as a model plant, we studied the structure and function of ubiquitination-related proteins in rice innate immunity. The first part of the dissertation is on the genome-wide analysis of the U-box proteins in rice, and the second part involves in the molecular analysis of the interaction between the effector protein AvrPiz-t and its two interacting E3 ligase proteins. To have a comprehensive assessment of the rice U-box proteins, we explored the genome sequences of two rice subspecies using a battery of whole genome analysis algorithms. From the analysis, we identified a total of seventy seven putative U-box genes in the rice genome. To determine whether the U-box genes are involved in host innate immunity, we conducted cell death assays using the rice protoplast system and semi-quantitative RT-PCR (SqRT-PCR) analysis with RNA from the Magnaporthe oryzae-infected rice leaves. Among the seven randomly selected rice U-box genes, silencing of OsPUB57 caused cell death in rice protoplasts, suggesting it might be a negative regulator in the cell death signaling. In addition, SqRT-PCR results showed that OsPUB57 is strongly induced in the resistant plants carrying the Pi9-resistant gene after inoculation with the avirulent M. oryzae isolate PO6-6. These results suggest that OsPUB57 is a good candidate for further functional analysis in the rice defense pathway to M. oryzae.
The molecular mechanism underlying how effectors from bacterial and oomycete plant pathogens interfere with the host defense system has been well studied. However, the function and host targets of the effectors from plant fungi are still poorly understood. We have recently cloned the broad-spectrum resistance gene \textit{Piz-t} in rice and its cognate \textit{Avr} gene \textit{AvrPiz-t} from \textit{M. oryzae}. During early stages of infection, the AvrPiz-t protein preferentially accumulates in a special structure, named biotrophic interfacial complex (BIC), and is translocated into rice cells at late stages of infection. Ectopic expression of \textit{AvrPiz-t} in transgenic rice suppresses flg22- or chitin-induced reactive oxygen species (ROS), indicating its virulence function in the suppression of pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI). In the yeast two-hybrid screens, we identified 12 AvrPiz-t interacting proteins (APIPs). Among them, APIP2, 6, and 10 are predicted to be E3 ligases. We have demonstrated that APIP2, 6 and 10 ubiquitinate AvrPiz-t while their E3 ligase activity is suppressed by AvrPiz-t \textit{in vitro}. Transient expression assays showed that AvrPiz-t is degraded in rice protoplasts and the degradation is inhibited by the 26S proteasome inhibitor MG132. Agroinfiltration experiments revealed that co-expression of AvrPiz-t with APIP6 or APIP10 in \textit{Nicotiana benthamiana} leads to the degradation of AvrPiz-t. Furthermore, AvrPiz-t also promotes the degradation of the APIP6 and APIP10 proteins in \textit{N. benthamiana}. In the APIP6 and \textit{APIP10} RNAi transgenic rice, the flg22- or chitin-induced ROS generation is significantly reduced compared to the non-transgenic control plants, suggesting that APIP6 and APIP10 are positive regulators of PTI. Consistent with the ROS data, APIP6 and APIP10 RNAi plants showed enhanced susceptibility to virulent isolates. Strikingly,
silencing of \textit{APIP10} but not \textit{APIP6} in the \textit{Piz-t} background is negatively correlated with the accumulation of the \textit{Piz-t} protein, suggesting that \textit{APIP10} is a negative regulator of \textit{Piz-t}. Taken together, our results suggest that AvrPiz-t is secreted from \textit{M. oryzae} into rice cells and suppresses host innate immunity through the manipulation of the two host E3 ligases \textit{APIP6} and \textit{APIP10}, which are the positive regulators of PTI in rice. In rice cells containing the \textit{Piz-t} protein, AvrPiz-t suppresses the E3 ligase activity or promotes the degradation of \textit{APIP10}, which leads to the accumulation of \textit{Piz-t} and triggers a battery of rapid defense responses to prevent rice blast infection. Our results have provided novel insights into the molecular basis of the interactions between fungal effectors and host resistance proteins in plants.
Dedication

Dedicated to my family
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I would like to thank my adviser, Dr. Guo-Liang Wang for all his supports, patient guidance and intellectual encouragement through the entire Ph. D program. I would also like to express my thanks to the members of Student Advisory Committee, Dr. Jyan-chyun Jang, Dr. Terrence Graham and Dr. Thomas Mitchell for the stimulation to think critically and for many suggestions on my research. My special thanks go to Dr. John Lindbo who was a member of Student Advisory Committee till he left. Also I would like thank to Dr. David Mackey for letting me use the equipment in his lab and for giving me countless creative ideas all the time, even in the hallway.

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Chapter 1: The role of ubiquitination in plant innate immunity

Eukaryotic cells employ a variety of post-translational modifications of proteins such as phosphorylation, acetylation, glycosylation and ubiquitination to regulate turnover and activity of proteins for growth and development (Moon et al, 2004; Vierstra, 2003). Ubiquitination is a post-translational modification of proteins, in which a 76 amino acids of the highly conserved small protein called ubiquitin is attached to the lysine residues of target proteins through the isopeptide bond between the C-terminus of ubiquitin and ε-amino group of a lysine residue (Smalle & Vierstra, 2004). Ubiquitination is a unique process in that most of ubiquitinated proteins are subject to degradation by the ubiquitin/26S proteasome system.

The ubiquitination process usually requires participation of three enzymes, i.e., ubiquitin activating enzymes (E1), ubiquitin-conjugating enzymes (E2 or UBC) and ubiquitin ligases (E3). E1 enzymes activate ubiquitin by the hydrolysis of ATP energy, generating high-energy thiol intermediates, while E2 enzymes carry an activated ubiquitin to E3 enzymes, which interact with and ubiquitinate the substrates. In successive reactions, other ubiquitin moieties get conjugated to the lysine residues of ubiquitin previously bound to the target proteins, resulting in the formation of polyubiquitinated substrates (Hatakeyama & Nakayama, 2003; Smalle & Vierstra, 2004). Ubiquitin contains seven lysine residues (K6, K11, K27, K29, K33, K48 and K63) and
each can serve as a ubiquitin acceptor to form the polyubiquitin chain. Whereas K48-linked polyubiquitination is generally recognized by the ubiquitin/26S proteasome as the signal for the protein degradation, monoubiquitination or polyubiquitination of other lysine residues of ubiquitin is usually associated with other functional roles. For example, monoubiquitination has been shown to be required for translocation of cargo proteins into the secretory pathway (Hicke, 2001), while K11-, K29- and K63-linked polyubiquitination is associated with cellular functions as diverse as endocytosis, activation of the NF-κB signaling pathway, DNA damage repair, and ribosomal protein synthesis (Pickart & Fushman, 2004). Therefore, regulatory functions of the ubiquitin signaling pathway extend beyond simple protein degradation.

**Diversity of plant E3 ligases**

The ubiquitination process has a hierarchical structure in terms of the number of E1, E2 and E3 enzymes in a given genome (Glickman & Ciechanover, 2002). For example, *Arabidopsis* genome encodes two E1s, 41 E2s and over 1200 E3 components (Kraft et al, 2005; Vierstra, 2003; Zeng et al, 2008). Each E3 ligase confers substrate specificity and recognizes a subset of substrates/targets. These E3 ligase enzymes can be classified into four families based on the mechanism of actions and the presence of a specific subunit or domain: RING/U-box, HECT, SCF and APC (Hatakeyama & Nakayama, 2003; Vierstra, 2003). The RING (Really Interesting New Genes) finger proteins contain a well-conserved C3HC4 or C3H2C motif chelating Zn$^{2+}$ to form a tertiary structure to mediate the interaction with E2 enzymes (Jackson et al, 2000). The U-box proteins contain a 75-amino acid domain forming a tertiary structure similar to the
RING finger domain (Patterson, 2002). Although the U-box domain lacks of the characteristic Zn$^{2+}$-chelating residues of the RING finger domain, it contains hydrogen-bonds and salt bridges to stabilize it (Aravind & Koonin, 2000; Ohi et al, 2003). The HECT (homologous to E6-AP COOH terminus) domain consists of about 350 amino acids and is characterized by a conserved catalytic cysteine residue in the C-terminal required for E3 ligase activity (Hatakeyama & Nakayama, 2003; Huibregtse et al, 1995). HECT E3 ligases are unique among other E3 ligases in that they can form an E3-ubiquitin thioester intermediate prior to the transfer of ubiquitin to the target proteins. The RING finger/U-Box and HECT classes represent a single subunit E3 ligase, while SCF and APC form multi-subunit E3 complexes. Interestingly, all multi-subunit E3 ligases contain a RING finger protein as part of their complex. The SCF (SKP1-CDC53-F-box) complexes are composed of four proteins that together form a functional E3 ligase: SKP1 (or ASK in plants), CDC3 (also referred as Cullin), F-box, and RBX1 (Deshaiies, 1999). The RBX1 subunit is a RING finger protein and SKP1 acts as an adapter and binds to the F-box, which selects the target protein, and Cullin serves as a scaffold to bring together E2s and RBX1 to the SKP1-F-box unit bound to substrates (Deshaiies, 1999). The APC (anaphase promoting complexes) are E3 ligases composed of roughly 11 subunits in which APC2 and APC11 are homologous to Cullin and a RING finger protein, respectively (Tang et al, 2001). While only a few HECT and APC-type E3 ligases have been found, hundreds of SCF and RING finger/U-box-type E3 ligases have been identified in plants (Downes et al, 2003; Joazeiro & Weissman, 2000; Stone et al, 2005). For example, *Arabidopsis* genome encodes seven HECT, 18 APC, 61 U-box,
about 500 RING finger-type E3 ligases and over 700 F-box proteins for SCF type E3 ligases (Mazzucotelli et al, 2006).

The importance of ubiquitination in plants is reflected by the abundance of ubiquitination machinery proteins in a given plant genome. For example, the analysis of Arabidopsis genome revealed that nearly 6% of the Arabidopsis proteome is devoted to proteins involved in ubiquitination. This is almost nine and two times more than that of yeast and Drosophila, respectively (Vierstra, 2003). Considering the abundance of ubiquitination-related proteins in a plant genome, it is not surprising that ubiquitination is implicated in a variety of processes such as cell cycle, circadian rhythm control, hormone signaling pathway, senescence, responses to biotic and abiotic stresses. Since the focus of this dissertation is on the roles of rice E3 enzymes in the plant innate immunity against the rice blast fungus Magnaporthe oryzae, recent advances in the last decade on how the ubiquitination process contributes to disease resistance in plants and how pathogens manipulate this process to suppress the host defense will be reviewed in the sections below.

**Functions of ubiquitination in the disease resistance in plants**

Since only a few E1 enzymes exist in a given plant genome and their main function is to activate ubiquitin, the involvement of E1s in the defense response against pathogens was not predicted. However, the induction of two tobacco E1 genes, NtE1A and NtE1B, at the transcription level was observed after the inoculation with TMV (tobacco mosaic virus) or ToMV (tomato mosaic virus), suggesting that E1 enzymes may
function in the plant defense signaling against specific pathogens. More direct evidence came from the analysis of the Arabidopsis mos5 (modifier of snc1) mutant. The mos5 mutant carries a deletion of 15 bp in UBA1, one of two Arabidopsis E1 genes and shows susceptibility to Pseudomonas syringae carrying AvrRpt2, not AvrB or AvrRps4, suggesting a functional ubiquitin machinery is required for the activation of downstream signaling of certain R proteins (Goritschnig et al, 2007). In case of E2s, the transcript of OsUBC5b, a rice E2 gene, is induced by the treatment of a biotic elicitor, N-acetylchitoheptaose, in suspension-cultured rice cells, suggesting its possible function in the basal defense (Takai et al, 2002). Apart from above few examples, there is limited amount of evidence for the role of E1s or E2s in the plant defense signaling pathway.

On the contrary, there is emerging evidence from various studies demonstrating that E3 ligases are closely involved in defense responses against various plant pathogens. Arabidopsis coi1 (coronatine insensitive 1) mutant is defective in the JA-signaling, which plays an important role in resistance to herbivore and necrotrophic fungal pathogens such as Alternaria brassicicola and Botrytis cinerea. The COII gene encodes a protein containing N-terminal similar to F-boxes and a leucine-rich-repeat (LRR) close to the C-terminus (Xie et al., 1998). The COII protein associates with AtCUL1, AtRbx1 and one of Arabidopsis Skp1-like proteins to form the SCFCOII E3 ligase complexes (Xu et al., 2002). The COII protein interacts with JAZ proteins, transcriptional repressors of jasmonic acid signaling. Genetic analysis with a coi1 mutant suggests that the degradation of JAZ proteins is processed through the ubiquitin/26S proteasome system involving SCFCOII E3 ligase complexes (Thines et al, 2007). Therefore, it has been proposed that COII forms the SCFCOII E3 ligase complexes, which interacts with and
ubiquitinates JAZ proteins to promote their degradation through ubiquitin/26S proteasome system. As a result, the jasmonic acid signaling pathway is activated for defense responses (Thines et al, 2007).

The TIR1 protein in Arabidopsis is another example of an F-box protein that is implicated in the plant defense by the auxin signaling pathway. The TIR1 F-box protein contains a LRR domain and interacts with AtCUL1, AtASK1 or AtASK2, and AtRBX1 to form the SCF^{TIR1} complexes (Gray et al, 1999; Ruegger et al, 1998). AUX/IAA transcriptional repressors are the substrates of SCF^{TIR1} complexes confirmed by both genetic and biochemical analysis (Dharmasiri et al, 2005). Interestingly, when Arabidopsis plants are challenged with a bacterial pathogen Pseudomonas syringae or flg22, a PAMP (pathogen-associated molecular patterns) elicitor, the level of microRNA miR393 transcript targeting TIR1 transcripts is elevated, which is followed by the reduction of the TIR1 transcript, resulting in stabilization of the AXR3/IAA17 transcriptional repressor, decreasing of the auxin-activated transcripts, and reducing the growth of the pathogen (Navarro et al, 2006). Another interesting example is the SON1 protein, which also contains the F-box motif (Kim & Delaney, 2002). The son1 (suppressor of nim1-1) mutant in Arabidopsis was identified by the suppressor screening of nim1-1 (non-inducible immunity1), which shows enhanced susceptibility to Peronospora parastica and P. syringae pv. tomato strain DC3000. Since the son1 mutant shows the SAR-independent, constitutive resistance against the two obligate pathogens mentioned above, it has been suggested that the SON1 protein functions as a negative
In addition to the F-box proteins, the RING finger and the U-box proteins also have the functions in disease resistance in plants. A cDNA-AFLP study identified three *Avr9/Cf-9 Rapidly Elicited (ACRE)* genes essential for Cf-9– and Cf-4–dependent hypersensitive response (HR) production in *Nicotiana benthamiana*. Interestingly, two of them encode E3 ligase components such as an F-box protein (*ACRE189*) and a U-box domain containing protein (*ACRE276*), respectively. Significantly, silencing of *ACRE276* compromised Cf-9/Avr9- and Cf-4/Avr4-induced hypersensitivity response (HR) in *N. benthamiana*. (van den Burg et al, 2008; Yang et al, 2006), suggesting that the ACRE276 protein is required for the cell death. Importantly, the expression of *AtPUB17*, the closest homolog of *ACRE276* in *Arabidopsis*, was able to restore the HR in tobacco plants silenced for *ACRE276*, while the expression of the U-box domain mutated *ACRE276* lacking ubiquitination activity failed to, demonstrating that the E3 ligase activity is required for the HR induction (Yang et al, 2006). Moreover, the knock-out of *AtPUB17* compromised RPM1/AvrB- and RPS4/AvrRPS4-mediated resistance against *P. syringae pv tomato*, indicating that it functions in the R protein-mediated resistance in plants.

More intensive investigation on *ACRE* genes associated with the ubiquitination process led to the identification of *NtCMPG1* (also referred as *ACRE74*), which encodes a U-box E3 ligase with a high homology to the parsley CMPG1 protein. Overexpression and silencing of *NtCMPG1* enhanced and reduced the HR triggered by CF9/Avr9 system, respectively (Gonzalez-Lamothe et al, 2006), suggesting that it is required for the
CF9/Avr9-triggered HR. Interestingly, overexpression and silencing of the tobacco
NtCMPG1 gene also led to enhanced Pto/AvrPto-triggered HR and reduced inf1-
dependent HR, respectively, suggesting the involvement of CMPG1 in multiple defense
pathways triggered by a variety of elicitors (Gonzalez-Lamothe et al, 2006).

In rice, the Spl11 gene, identified in the genetic screening of lesion mimic mutants
(Yin et al, 2000), encodes an E3 ligase protein with a U-box domain for E3 ligase activity
as well as ARM repeat domains for the interaction with other proteins (Zeng et al., 2004).
The spl11 mutant shows spontaneous cell death as well as enhanced resistance to M.
oryzae and Xanthomonas oryzae pv. oryzae in a race-nonspecific pattern in rice. Recently
it was reported that the AtPUB13 protein, the Arabidopsis ortholog of SPL11, functions
as a negative regulator of defense by promoting the degradation of FLS2 (Flagellin-
Sensing 2) via the ubiquitin/26S proteasome system (Lu et al, 2011). Consistently,
pub13 mutant shows an elevated amount of reactive oxygen species (ROS) after the
flagellin treatment as well as enhanced resistance to the bacterial pathogens P. syringae
pv. tomato DC3000 and P. maculicola ES4326. The Xa21 gene is the first disease
resistance gene cloned from rice. The Xb3 protein, one of the Xa21-interacting proteins,
is a RING finger E3 ligase required for full function of Xa21 (Wang et al, 2006).
However, it is largely unknown about its possible downstream substrates and whether it
ubiquitinates the Xa21 protein for either activation or degradation.

One more direct evidence that the ubiquitin/26S proteasome system is closely
involved in plant innate immunity came from the degradation of the resistance protein
after pathogen inoculation. The barley stem rust resistance protein RPG1 confers
resistance to the rust fungus Puccinia graminis f. sp. tritici (Pgt). Interestingly, the RPG1
protein disappears upon infection only with avirulent rust pathotypes, but not with
virulent ones, and this degradation is inhibited by MG132 (Nirmala et al, 2007), an
inhibitor of the ubiquitin/26S proteasome system. In addition, two non-functional RPG1
mutants, K1 and K2 do not disappear even with the challenge of avirulent rust pathotypes,
supporting the fact that degradation of RPG1 is required for the R gene-mediated defense.
Although polyubiquitination and degradation of the RPG1 protein are correlated with
disease resistance, a specific E3 ligase targeting RPG1 is still unknown.

**Exploitation of host ubiquitination machinery by plant pathogens to suppress innate
immunity in plants**

Plants are sessile and cannot escape from a spectrum of hostile parasites around them. Therefore, they have developed multi-layers of defenses to invading pathogens such as bacteria, fungi and viruses. Upon pathogen infection, the recognition of PAMPs such as flagellin, chitin and lipopolysaccharides by the pattern recognition receptors (PRRs) triggers the first layer of defense response referred as the PAMP-triggered immunity (PTI), which includes the accumulation of ROS and the deposition of phenolic compounds (Jones & Dangl, 2006). However, pathogens deliver proteins called effectors to target PRRs or key components in PTI signaling pathway to suppress the PTI-mediated defense. Some of the effectors referred as avirulence (Avr) proteins are recognized either directly or indirectly by the corresponding resistance (R) gene products, resulting in the second layer of defense called effector-triggered immunity (ETI). ETI is a more robust and effective defense but less stable compared to the resistance mediated by PTI because
effector proteins are relatively prone to mutation that leads to avoid the perception by the host R proteins (Jones & Dangl, 2006).

As mentioned above, ubiquitination plays an essential role in the plant PTI and ETI against pathogens. It is not surprising that plant pathogens try to manipulate the host ubiquitination machinery to suppress innate immunity by delivering effector proteins into host cells. These effector proteins can be classified into five categories according to their interference mechanisms (Angot et al, 2007; Rytkonen & Holden, 2007). The first class utilizes the host ubiquitin/26S proteasome system to degrade the signaling components required for the host defense. One of the examples is HopM1, an effector protein from *P. syringae* that suppresses host defense by promoting the degradation of its interacting proteins such as AtMIN2, AtMIN7, and AtMIN10 through the ubiquitin/26S proteasome system. Among them, AtMIN7 is an ADP ribosylation factor guanine nucleotide exchange factor (ARF GEF), which is a key component of the vesicle trafficking system in eukaryotic cells. In addition, the *Atmin7* knockout mutant shows defects in cell wall-mediated defense. Taken together, these results suggest that the HopM1 protein inhibits the host vesicle trafficking system that is associated with cell wall-mediated defense by promoting the degradation of AtMIN7 through the host ubiquitin/26S proteasome system (Nomura et al, 2006). A recent study showed that the HopM1 mediated-degradation of AtMIN7 protein is blocked by ETI, which is triggered by three different effectors, AvrRpt2, AvrPphB and HopA1, indicating a new mechanism for the ETI to counter the bacterial suppression of the PTI (Nomura et al, 2011).
The second type of effectors mimics host E3 ligases to modulate host defense. AvrPtoB is one of such effector proteins from *P. syringae pv. tomato* and its deduced protein shows no substantial homology to any known proteins. However, a crystal structure study revealed that the C-terminus of the AvrPtoB protein shows a similar structure to plant U-box E3 ligases and it has *in vitro* E3 ligase activity (Abramovitch et al, 2006; Janjusevic et al, 2006). Recently, the kinase protein Fen, a Pto family member, was identified as a target of AvrPtoB. Upon infection, the AvrPtoB protein ubiquitinates Fen and promotes its degradation in a ubiquitin/26S proteasome-dependent manner to suppress Rsb (Resistance suppressed by AvrPtoB C-terminus)-mediated immunity triggered by the N-terminal region of AvrPtoB. It is speculated that the C-terminal region of AvrPtoB was acquired in the evolutionary process to evade the trigger of immune response by the Fen protein (Rosebrock et al, 2007). Another study found that AvrPtoB ubiquitinates CERK1 and FLS2 to suppress PTI as well (Gimenez-Ibanez et al, 2009; Gohre et al, 2008). In the absence of AvrPtoB, CERK1 and FLS2 trigger PTI by the recognition of chitin and flagellin, respectively. Upon delivery into host cells, AvrPtoB suppresses PTI by promoting ubiquitination and degradation of CERK1 and FLS2 via the vacuolar and the ubiquitin/26S proteasome degradation pathways, respectively (Gimenez-Ibanez et al, 2009; Gohre et al, 2008).

The third class of effector proteins directly targets E3 ligases that function in defense signaling pathway. As mentioned above, CMPG1 is required for efficient activation of defense responses as well as cell death mediated by INF1- and CF9/Avr9-dependent HR (Gonzalez-Lamothe et al, 2006). Avr3a protein was delivered from *Phytophthora infestans* into host cell to suppress INF1-mediated-cell death (ICD) through...
the stabilization of CMPG1 (Bos et al, 2010). CMPG1 protein exists in low abundance in the absence of Avr3a due to its constant degradation through the ubiquitin/26S proteasome system. At the biotrophic stage of infection, Avr3a manipulates E3 ligase activity of CMPG1 to suppress INF1-mediated cell death. However, at the necrotrophic stage, *P. infestans* expresses less Avr3a and more INF1 to facilitate the cell death mediated by CMPG1 for its successful infection (Bos et al, 2010). A recent study showed that CMPG1 is also required for cell death by a variety of PAMPs such as cellulose-binding elicitor lectin (CBEL) and the R/Avr proteins such as Cf-9/Avr9, Cf-4/Avr4, and Pto/AvrPto and Avr3a can suppress all these types of cell death by stabilizing CMPG1 (Gilroy et al, 2011).

The fourth class of effector proteins directly changes the ubiquitination status of signaling components in the host immune system to block the defense response. This class of effectors has been reported only in mammalian system so far. In the mammalian innate immune system, the recognition of microbial-associated molecular patterns (MAMPs) by the membrane-anchored Toll-like receptors (TLRs) triggers host defenses involving mitogen-activated protein kinase (MAPK) and the nuclear factor κB (NF-κB) (Chen, 2005; Dong et al, 2002). YopJ protein from *Yersinia pseudotuberculosis* and its homolog YopP protein from *Y. enterocolitica* function as deubiquitinating enzymes and cleave ubiquitin from polyubiquitinated key components in the MAPK and NF-κB pathways, preventing the proinflammatory host immune response (Haase et al, 2005; Thiefes et al, 2006; Zhou et al, 2005).

The last class of effector proteins utilizes the host ubiquitin/26S proteasome system for their delivery into host cells or timely degradation inside host cells. Although
they have not been identified from plant pathogens, they have been well-studied in mammalian systems. SopE and SptP, two effector proteins from *Salmonella enterica* serova Typhimurium, are required for its pathogenicity and alter the structural organization of host actin cytoskeleton by showing opposite activities (Kubori & Galan, 2003; Patel et al, 2005). SopE functions as a GTP-GDP exchange factor (GEF) activating the host Rac1 and Cdc42 proteins for cytoskeleton reorganization to allow bacterial internalization. On the other hand, SptP functions as a GTPase activating protein (GAP) deactivating host Rac1 and Cdc42 proteins for the recovery of the host actin cytoskeleton to normal formation at the late stage of infection. Even though the SopE and SptP proteins are delivered into host cells in same amount, their degradations are under quite different temporal regulations within host cells. The SopE protein required for early infection undergoes the degradation right after the translocation into host cells while the SptP protein required for later infection stage shows milder rate of degradation (Kubori & Galan, 2003; Patel et al, 2005). Also it has been reported that ubiquitination of SopA, another effector protein from *Salmonella*, by the host E3 ligase HsRMA1 is required for its delivery into the cytosol of epithelial cells (Zhang et al, 2005; Zhang et al, 2006). There are more reports that pathogen effector proteins are ubiquitinated by host E3 ligases such as YopE from *Yersinia sp.* and ExoU from *Pseudomonas aeruginosa* (Ruckdeschel et al, 2006; Stirling et al, 2006). However, it is not clear whether ubiquitination of these effectors by host E3 ligases is favorable for pathogens or host.

Rice, feeding nearly 50% of the world’s population, continues to be the lifeline of rice growing countries. At the same time, rice is gradually becoming an important stable food for people in sub-Saharan Africa (see IRRI strategic plan 2007-2015,
http://www.irri.org). However, rice diseases continue to be a major constraint in rice production. The rice blast disease is caused by the fungus *M. oryzae*. It is one of the most devastating diseases and occurs in most rice growing areas worldwide (Talbot, 2003). Up to now, eighteen rice blast *R* genes and six *M. oryzae* *Avr* genes have been cloned and characterized (Hayashi et al, 2010; Liu et al, 2010; Takahashi et al, 2010; Yuan et al, 2011; Zhai et al, 2011). Despite of significant progress on the isolation of *R* and *Avr* genes in rice and rice blast, respectively, the molecular mechanism underlying the recognition between *R* and *Avr* proteins has not yet been fully understood. Moreover, both the downstream signaling upon recognition and the function of the *Avr* proteins in the absence of their cognate *R* protein are largely unknown in rice. Pi-ta/*Avr-Pi-ta* is the only *R*/*Avr* pair that bind directly to each other in a far-western blot assay (Jia et al., 2000). Genomic and evolutionary dynamics of both *AvrPi-ta* and *Pi-ta* has also been investigated recently (Huang et al., 2008; Khang et al., 2008). However, how *AvrPi-ta* and *Pi-ta* interact in rice cells and how the interaction triggers subsequent defense responses have not been reported. Cloning of both *Piz-t* and *AvrPiz-t* provides the second pair of *R*/*Avr* in the rice blast system to investigate their recognition and defense activation mechanisms. Furthermore, the *Piz-t* locus comprises of multiple *R* alleles with extreme sequence similarity to each other (Qu et al., 2006; Zhou et al., 2006). For example, *Piz-t* has only 8 amino acid differences from its allele Pi2 (Zhou et al., 2006). Therefore, the knowledge gained from this dissertation will not only unravel the interaction mode between *Piz-t* and *AvrPiz-t* but also shed new light on understanding the mechanism of different resistance specificity controlled by different *R* alleles.
As discussed above, ubiquitination is emerging as a key regulatory process in both plant host immunity and pathogen invasion. Although considerable progress has been made towards the understanding of how ubiquitination is intimately involved in plant host immunity to bacteria and oomycetes, how fungal plant pathogens manipulate host ubiquitination machinery for pathogenesis is not completely understood. The overall goals of my dissertation project are to identify the function of the E3 ligase genes in defense responses to the fungal pathogen and understand how fungal avirulence effectors interfere with host immunity. In Chapter 2, we hypothesized that rice genome contains E3 ligases functioning in host innate defense. The specific objectives of the research presented in Chapter 2 were: (i) to identify all U-box protein genes in rice using a genome-wide analysis, (ii) to classify them into different groups based on their domain organization, (iii) to determine E3 ligase activity of selected U-box proteins using in vitro assay, and (iv) to assess their function in defense responses using a rice protoplasts system for cell death assay. In Chapter 3, we hypothesized that the interference activity and/or ubiquitination of AvrPiz-t is required for its avirulence and/or suppression of host defense responses in rice. The specific objectives of the research presented in Chapter 3 were: (i) to identify host targets of AvrPiz-t by yeast-two hybrid (Y2H) and to confirm the interaction in rice cells using bimolecular fluorescence complementation (BiFC) method, (ii) to identify the mechanism responsible for the interference activity and to map ubiquitination target sites using biochemical approaches, and (iii) to investigate the function of AvrPiz-t’s ubiquitination in its avirulence and virulence functions using lysine-less AvrPiz-t protein. In Chapter 4, we hypothesized that AvrPiz-t is translocated into rice and it interacts with its host target proteins to suppress basal defense responses.
The specific objectives of the research presented in Chapter 4 were: (i) to visualize the translocation of AvrPiz-t into rice cells using live-cell imaging approach, (ii) to assess AvrPiz-t’s virulence function in rice cells by its ectopic expression in rice cells, (iii) to investigate the function of its interacting proteins, two host E3 ligases APIP6 and APIP10, in host innate immunity by knocking down their transcripts using the RNAi approach, and (iv) to understand how the interaction is recognized by its cognate R protein Piz-t to trigger a robust host defense response or ETI.

References


Introduction

Ubiquitination is a major type of post-translational modification of proteins that occur in eukaryotic cells (Ciechanover, 2005). The ubiquitination process involves in-concert catalytic activities of three types of enzymes, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-ligase (E3). The ubiquitin molecule, a highly conserved 76-amino acid protein, is initially activated by E1 in an ATP-dependent manner, and the activated ubiquitin is then transferred to an E2. With the help of an E3, the ubiquitin molecule is covalently attached to the target protein through the ε-amino group of a lysine residue (Ciechanover & Schwartz, 1998). The attachment of the first ubiquitin moiety to the substrate protein is usually followed by the addition of more ubiquitin molecules to form a polyubiquitin chain. A polyubiquitin chain with at least four sequentially attached ubiquitins linked through the lysine 48 (K48) residue of the ubiquitin molecules will be sufficient to allow the ubiquitinated target protein to be recognized and degraded by the 26S proteasome (Thrower et al, 2000). Importantly, polyubiquitination that links through other lysine residues of the ubiquitin than K48 as well as mono-ubiquitination regulate non-proteolytic processes in the cell (Bray et al, 2005; Huang et al, 2006; Li et al, 2003; Terrell et al, 1998).
Of the three enzymes that are implicated in the ubiquitination process, E3 ubiquitin ligase plays a central role in determining the high specificity of the system by selecting appropriate candidate proteins (Ciechanover, 1998). Not surprisingly, given the myriad of different substrates, E3s are the most diverse components in the ubiquitination pathway. For example, over 1,300 distinct E3 components are encoded by the *Arabidopsis* genome (Smalle & Vierstra, 2004). During the transfer of activated ubiquitin molecule from E2 to the substrate, the ubiquitin molecule may or may not be covalently attached to the E3 ligase before it is attached to the substrate. Accordingly, E3 ligases can be largely classified into two classes, the HECT type and the family of RING/U-box types ligases, based on the occurrence of such covalent linkage. The HECT-type ligases are the only known E3 ubiquitin ligases that form an E3-ubiquitin thioester intermediate before the final attachment of ubiquitin to the substrate (Huibregtse et al, 1995; Scheffner et al, 1993). They contain an approximately 350-amino-acid in-length HECT (homologous to E6-AP COOH terminus) domain that was initially identified in the viral E6-associated protein (E6-AP). A conserved active cysteine residue that is required for the ligase function is presented at the C-termini of these ligases (Scheffner et al, 1993). Depending on their subunit composition and mechanism of actions, members of the RING/U-box family can be further classified into three sub-classes, the CRL (Cullin-RING-Ligase) subgroup, including SCF type (Skp1-Cullin (CDC53)-F-box protein complex) (Bai et al, 1996; Skowyra et al, 1997) and other cullin-based types (such as ElonginC-CUL2-SOCS box type, CUL3-BTB type etc) (Pintard et al, 2003; Stebbins et al, 1999); the RING (really interesting new gene) (Freemont et al, 1991)/U-box (Koegl et al, 1999) subgroup; and the APC (anaphase promoting complex) subgroup (Irniger et al, 1999).
1995; King et al, 1995; Sudakin et al, 1995). The family of RING/U-box types E3 ligases can be either single-subunit proteins or multi-subunits complexes. The RING type E3 ligases belong to the class of single-polypeptide (subunit) E3s that contain a RING motif. The RING motif is maintained by the arrangement of eight cysteine and histidine residues in a cross-brace manner that chelate two zinc ions, which is distinct from other zinc finger domains (Borden, 2000). It is proposed that the RING type E3 ligases serve as scaffolds bringing together the activated ubiquitin-E2 intermediate and the substrate protein to promote the transfer of the ubiquitin molecule to the substrate protein (Borden, 2000; Lorick et al, 1999).

The U-box is a more recently identified, E3 ligase activity-related protein domain that was first shown in yeast to be involved in polyubiquitin chain assembly (Koegl et al, 1999). The U-box contains ~70 amino acids and possesses a tertiary structure resembling that of the RING domain (Aravind & Koonin, 2000; Ohi et al, 2003). The major difference between U-box and RING domains is that the U-box lacks the characteristic Zinc-chelating cysteine and histidine residues. Consequently, U-box E3s use intramolecular interactions other than zinc chelation to maintain the RING finger motif (Ohi et al, 2003). U-box proteins are present in yeast, plants, and animals (Azevedo et al, 2001; Koegl et al, 1999; Meacham et al, 2001; Stone et al, 2003). Nevertheless, only a few U-box proteins have been functionally studied to date. UFD2 (ubiquitin fusion degradation protein-2) is responsible for the assembly of polyubiquitin chains in yeast (Koegl et al, 1999). The Prp19p (precursor RNA processing 19p) is a subunit of the spliceosome and is involved in pre-mRNA splicing (Ohi & Gould, 2002). The human homolog of Prp19p, SNEV (senescence evasion factor), has been proven as a
multifaceted protein playing a role in cellular senescence, cellular life span, pre-mRNA splicing, DNA double-strand break repair and the transport of ubiquitinated substrate to the proteasome (Loscher et al, 2005; Mahajan & Mitchell, 2003; Voglauer et al, 2006). The CHIP (C-terminal of Hsc70-interacting protein) protein acts as chaperone or co-chaperone in the cell to ensure protein homeostasis and has been implicated in stress responses and several neurodegenerative diseases (Ballinger et al, 1999; Dickey et al, 2007; Imai et al, 2002; Jiang et al, 2001; Miller et al, 2005; Rosser et al, 2007; Sahara et al, 2005). The plant proteins CMPG1 and ACRE276 are implicated in defense against pathogen attack (Gonzalez-Lamothe et al, 2006; Kirsch et al, 2001; Yang et al, 2006). SPL11 is related to cell death and other cellular processes in rice, such as flowering time control (Vega-Sanchez et al, 2008; Zeng et al, 2004). PHOR1 (photoperiod-responsive 1) from potato is a general component of the gibberellins signaling pathway (Amador et al, 2001). The protein ARC1 (arm repeat containing 1) was shown interacting with the S-locus kinase and is involved in self-incompatibility of Brassica (Gu et al, 1998; Stone et al, 2003).

The completion of sequencing the rice genome allows us to study rice U-box proteins at the genome scale (Goff et al, 2002; Yu et al, 2002). By using a battery of extensive whole genome analysis algorithms, we identify 77 genes encoding U-box domain-containing proteins from the rice genome in this study. Expressed sequence tags (ESTs) or full-length cDNAs are found for most of the identified U-box proteins. These proteins are classified into eight major groups based on their domain organization. Phylogenetic analysis establishes the homologies and paralogies of rice and *Arabidopsis* U-box proteins, which will help functionally characterize plant U-box protein genes in
the future. *In vitro* ubiquitination assay indicate that five out of seven randomly picked rice U-box proteins display E3 ligase activity in an E2-dependent manner. Interestingly, silencing one of the U-box genes causes cell death in rice protoplast, suggesting that it is involved in rice cell death control.

**Results**

*Identification of U-box-containing proteins from the rice genome*

To have a comprehensive assessment of the rice U-box proteins, the genome sequences of two rice subspecies were explored in our survey (Goff et al, 2002; Yu et al, 2002). To have the latest genome information, the rice genome pseudomolecules release 5 sequences from The Institute of Genome Research (TIGR, now J Craig Venter Institute) were used in the identification of U-box genes in the japonica subspecies. The U-box proteins from the two annotated genomes were identified with high confidence using the same battery of algorithms. The two sets of identified U-box proteins were then compared and combined. It has been reported that on average there is approximately 1% polymorphism in amino acid sequence of the same protein encoded by the japonica and indica genomes (Feng et al, 2002; Han & Xue, 2003). However, proteins from the same loci of the *japonica* and *indica* genomes may have lower protein sequence identity due to the outcome of different annotation procedures. Therefore we considered U-box proteins from the two genomes that show at least 95% protein sequence identity are likely encoded by the same loci and the corresponding genomic DNA sequences of these proteins were then identified and compared manually. The putative U-box proteins identified in the *indica* genome were eliminated from the combined list after their
counterparts are confirmed to exist in the annotated *japonica* genome. Such analyses indicated that all U-box proteins identified from the *indica* genome have their corresponding counterparts in the japonica genome. Seventy seven rice U-box proteins were identified using this approach (Table 1). Using the same algorithms, we identified 61 U-box proteins from the *Arabidopsis* genome. Among them, 60 are common to those identified by other groups (http://www.arabidopsis.org/info/genefamily/pub.html), corroborating the efficacy of the algorithms we used in the survey (Table 2).

*Domain organization of the rice U-box proteins*

The amino acid sequences of the rice U-box proteins were used to search against the PFAM database (Bateman et al, 2004) and the National Center for Biotechnology Information (NCBI) protein database to identify other domains and motifs. Besides the U-box, there are other many various protein domains/motifs present in these proteins (Table 3). The rice U-box proteins are grouped into eight classes based on the presence of common motifs/domains other than the U-box. The numbering of these classes is mandated so that the previously described classes in *Arabidopsis* are incorporated (Azevedo et al, 2001). It is noteworthy that, in addition to U-box and the domain used for classifying, proteins in the same class could also possess other domains. For example, the U-box/ARM type protein SPL11 was shown containing a coiled-coil motif in the central region of the protein (Zeng et al, 2004). In general, E3 ubiquitin ligases have a protein-protein interaction domain for association with their substrates for ubiquitination (Patterson, 2002).
Similar to *Arabidopsis*, class I consists of one rice gene, *OsUFD2*. *OsUFD2* is highly homologous to *AtUFD2* (Azevedo et al, 2001), with 62% identity at the amino acid level. Previous studies indicated that yeast UFD2 interacted with CDC48, a member of AAA-type ATPase family that is implicated in multiple cellular processes (Koegl et al, 1999). The region of yeast UFD2 that is proposed to interact with CDC48 and its human orthologs p97 is conserved in *OsUFD2*.

The largest class of rice U-box proteins contain the armadillo (ARM)/HEAT repeats. The ARM repeat is an approximately 40 amino acids long tandemly repeated sequence motif first identified in the *Drosophila melanogaster* segment polarity gene armadillo (Riggleman et al, 1989). It was shown to be involved in protein-protein interactions (Huber & Weis, 2001). HEAT repeats derive their name from four diverse eukaryotic proteins in which they were first identified: huntingtin, elongation factor 3, PR65/A subunit of protein phosphatase A, and the TOR (target of rapamycin) (Andrade & Bork, 1995). ARM and HEAT repeats are grouped into the same class in this study due to their structural similarity (Andrade et al, 2001). It is noteworthy that the ARM/HEAT repeats found in these proteins are quite divergent, similar to what was described previously (Mudgil et al, 2004).

The second largest class of U-box proteins in rice and *Arabidopsis* show no significant domain or motif hits other than U-box in the PFAM or NCBI databases. Nevertheless, the sequence alignments do detect a conserved domain containing ~ 100 amino acid residues that is located close to the C terminus of these proteins (Figure 1). In addition to the high percentage of leucine residues described before (Azevedo et al, 2001), a high percentage of homology and several highly conserved residues were
detected. We named this putative domain GKL-box after the conserved Glycine (G), Lysine (K)/Arginine (R) residues and its leucine-rich feature. Sequence alignments of the *Arabidopsis* proteins from the same class gave a highly similar pattern. NtCMPG1, the *Nicotiana benthamiana* homolog to the *Arabidopsis* U-box proteins AtPUB20 and AtPUB21 from this class, was shown to be essential for plant defense and disease resistance (Gonzalez-Lamothe et al, 2006). It remains unknown, however, if members of the corresponding class from have similar functions.

The third largest class of rice U-box-containing proteins has a kinase domain at the N-terminal region of the proteins. Given the involvement of the phosphorylation modification in most, if not all, cellular processes, we speculate that members of this class of rice U-box proteins could play a broad-range of roles in the cell.

In addition to the three classes mentioned above, there are another two classes that have more than one protein from each genome. These two classes contain a tetratrico peptide repeat (TPR) motif and a WD40 repeat domain, respectively. Both TPR and WD40 domains have been shown to be involved in protein-protein interactions (Das et al, 1998; Li & Roberts, 2001). The rice and *Arabidopsis* U-box proteins that contain the WD40 repeats are homologous to the animal and human Prp19p protein, which has been shown to be involved in pre-mRNA splicing and other biological processes (Loscher et al, 2005; Mahajan & Mitchell, 2003; Ohi & Gould, 2002; Voglauer et al, 2006).

**Difference of the rice and Arabidopsis U-box proteins in domain contents**

The domain contents of *Arabidopsis* U-box proteins were also investigated using the same method as described above (Table 2). One significant difference between rice
and *Arabidopsis* U-box proteins is that there is only one TPR-U-box type protein in *Arabidopsis* while six are identified in the rice genome. The TPR is a structural motif present in a wide range of proteins (Lamb et al, 1995). It mediates protein-protein interactions and the assembly of multi-protein complexes (D'Andrea & Regan, 2003). The only *Arabidopsis* TPR-U-box protein is homologous to the mammalian carboxyl terminus of Hsc70-interacting protein (CHIP)(AtCHIP) and it is involved in abiotic stress responses and in the control of chloroplast protein turnover (Luo et al, 2006; Shen et al, 2007a; Shen et al, 2007b; Yan et al, 2003). In humans and animals, CHIP interacts with the protein chaperones such as Hsp70 and Hsp90 and acts as chaperone or co-chaperone in the cell to ensure protein homeostasis. CHIP has been implicated in stress responses and several neurodegenerative diseases (Ballinger et al, 1999; Dickey et al, 2007; Imai et al, 2002; Jiang et al, 2001; Miller et al, 2005; Rosser et al, 2007; Sahara et al, 2005).

Nevertheless, no study regarding the biological functions of the rice TPR-U-box type protein has been reported. Interestingly, we identified an expressed protein (OsPUB70) which contains both TPR and kinase domains in addition to the U-box domain. It will be intriguing to find out whether this protein is a newly evolved protein that acquired either the TPR or kinase domain relatively recently.

Another difference of rice and *Arabidopsis* U-box domain contents is that two MIF4G type U-box proteins exist in *Arabidopsis* but the same type protein is absent in rice. The MIF4G domain is named after middle domain of eukaryotic initiation factor 4G (eIF4G) and it is implicated in RNA metabolic processes (Ponting, 2000). The absence of such protein in the rice genome suggested that the arising of the corresponding genes in *Arabidopsis* occurred after speciation of the two species.
Relationship between rice U-box/ARM proteins

The U-box/ARM type proteins constitute the largest family of U-box-containing proteins in both rice and Arabidopsis genomes. Moreover, most plant U-box-containing proteins that have been functionally characterized belong to this class (Amador et al., 2001; Gu et al, 1998; Stone et al, 2003; Yang et al, 2006; Zeng et al, 2004). This prompted us to further study the relationship of members in this class. Phylogenetic analysis using full-length protein sequences indicated that rice U-box/ARM proteins largely fall into four clusters in the phylogenetic tree. Consistently, members that are evolutionally closer in the phylogenetic map (Figure 2A) also show higher similarity in their number and arrangement of ARM repeats (Figure 2B). Unlike clusters I, II, and III in which the ARM repeats in most members occur successively, most of the ARM repeats of cluster IV members are distributed discretely. The phylogeny of rice U-box/ARM proteins are slightly different when only the U-box sequences of each protein are used to construct the phylogenetic tree (data not shown), suggesting that the U-box domain might have evolved differently to other parts of those proteins. Similar to what have been observed in Arabidopsis (Mudgil et al, 2004), the domain organizations (Figure 2B) indicated that the ARM repeats of the rice U-box/ARM proteins are divergent in both number and arrangement.

Relationship between rice and Arabidopsis U-box/ARM proteins

The identification of corresponding set of U-box containing proteins from both rice and Arabidopsis genomes allows us to evaluate the evolutionary relationships within
the U-box gene families of both species. Therefore, we performed a combined phylogenetic analysis of both rice and Arabidopsis U-box/ARM proteins to obtain a joint tree. As shown in Figure 3, most rice U-box/ARM proteins have their corresponding putative homologs in Arabidopsis. Moreover, most rice U-box/ARM proteins show closer phylogenetic distance to their putative rice homologs than to their corresponding putative Arabidopsis homologs. Nevertheless, the rice proteins OsPUB4, OsPUB12, OsPUB29, and OsPUB30 show closer phylogenetic relationship to the Arabidopsis proteins than to their rice paralogs, suggesting that these rice proteins and their corresponding Arabidopsis homologs have evolved from a common ancestor before the speciation of the two species.

In the analysis, rice SPL11 (OsPUB11) displays the closest phylogenetic distance to AtPUB13 (encoded by locus At3g46510). Importantly, a T-DNA insertion mutant of AtPUB13 shows similar phenotype (L. Zeng & GL Wang, unpublished data) to what we observed in rice the spl11 mutant: cell death/enhanced defense and altered flowering time. This suggests that the function of SPL11 is conserved in monocot and dicot plants.

*In silico analysis of the rice and Arabidopsis U-box protein genes expression*

Expression of the identified U-box protein genes was investigated by searching the rice cDNA (http://cdna01.dna.affrc.go.jp/cDNA/), EST (http://combio.dfci.harvard.edu/tgi/ and http://www.ncbi.nlm.nih.gov/), and massively parallel signature sequencing (MPSS) databases (http://mpss.udel.edu/rice/). Evidence for expression was found for the majority of the rice U-box protein genes. Corresponding ESTs or full-length cDNAs or both could be identified for 61 out of the 77 rice U-box
genes, amongst which 55 have full-length cDNAs (Table 1). Based on the origin of the ESTs, the rice U-box protein genes were expressed in a variety of tissues, such as root, leaf, flower, calli etc., suggesting that they are likely involved in a broad-range of biological functions.

To have a comprehensive idea on the expression pattern of the rice U-box genes, we used the same method as described (Nobuta et al, 2007) to analyze their MPSS tags in 61 MPSS libraries that were prepared with different rice tissues or rice leaves challenged by various abiotic or biotic stresses (http://mpss.udel.edu/rice/mpss_index.php). Of the seventy seven rice U-box genes, sixty-four of them showed the significant expression in one or more libraries with only four of the rice U-box genes having corresponding MPSS tags presented in the libraries (data not shown). Some U-box genes were only expressed at a low level under specific conditions. For example, OsPUB58 has low transcript only in salt-challenged young roots and OsPUB65 is only expressed in calli and leaves challenged by the incompatible fungal pathogen. This may explain why ESTs were not identified for these genes.

By utilizing the collection of microarray data available at Genevestigator database (Zimmermann et al, 2004), we also investigated the expression of 52 Arabidopsis U-box genes on the Affymetrix Genechips under various experimental conditions (data not shown). Interestingly, many of the Arabidopsis U-box genes have over two-fold change in their expression when the plant is challenged by various abiotic (such as chitin treatment or salt stress) or biotic stresses (such as Pseudomonas syringae infection).

Role of rice U-box gene in cell-death signaling
To date, a few plant U-box proteins implicated in defense against pathogen attack have been identified (Gonzalez-Lamothe et al., 2006; Kirsch et al., 2001; Yang et al., 2006; Zeng et al., 2004). To assess the expression of rice U-box genes during pathogen infection, we chose nine rice U-box genes that showed differential expression in our microarray hybridizations (C.H. Park and G.L. Wang, unpublished) and examined their expression in rice leaf tissue challenged by the rice fungal pathogen *Magnaporthe oryzae*. As shown in Figure 4, seven of them showed differential expression patterns after inoculation with the fungal isolate PO6-6. Specifically, *OsPUB4*, *12*, and *23* had a similar expression in both resistant and susceptible plants. *OsPUB5*, *64*, and *73* showed stronger expression in the susceptible plants. Nevertheless, *OsPUB57* showed a stronger expression only in the resistant plants carrying the *Pi9*-resistant gene (Qu et al., 2006). These results suggest some of the genes might play an important role in the signaling or regulation of rice defense against *M. oryzae*.

To further confirm their function in defense responses, a rice protoplast system for cell death assay was used (Chen et al., 2006). Rice protoplasts were transiently co-transfected with a U-box gene-silencing construct and a beta-glucuronidase (GUS) gene expression construct. The strength of GUS activity was monitored as an indicator of viability of the protoplasts. Among the eight genes tested, *OsPUB57* cause cell death when it is knocked down, which suggests that it might act as a negative regulator in rice cell death signaling (Figure 5).

*E3 ubiquitin ligase activities of rice U-box proteins*
The U-box-containing proteins are predicted to possess E3 ubiquitin ligase activity. To determine if the rice U-box proteins do encode functional E3 ligases, *in vitro* ubiquitination assays were performed. Five rice U-box proteins, OsPUB4, OsPUB12, OsPUB57, OsPUB73 and OsPUB77 were selected. These proteins were expressed with GST-tags in *Escherichia coli* and tested for E3 ligase activity using an *in vitro* ubiquitination assay as described (Zeng et al, 2004). The polyubiquitination bands presented in the reaction, as shown by ladder of higher molecular weight bands was used as an indicator of E3 ligase activity (Hatakeyama et al, 2001).

As shown in Figure 6, the five rice U-box proteins were found to possess E3 ligase activity. Importantly, like the observation with mammalian U-box proteins (Hatakeyama et al, 2001), the ligase activity of the rice U-box proteins also depends on the E2 enzyme used for the assay. The preference for a particular E2 enzyme did not show any correlation with the presence of a particular domain in the rice U-box proteins. In the control experiment, omission of E1, E2, or ubiquitin, no ubiquitination was observed (data not shown). Therefore, the ubiquitination observed was due to the E2-dependent E3 ligase activity of the rice U-box proteins.

**Discussion**

The distribution of U-box proteins among species of different kingdoms is uneven. There are a larger number of genes encoding such proteins in the plant genomes. Three U-box genes were identified among the 6300 annotated genes in the yeast genome. Similarly, less than twenty U-box proteins were predicted in the human genome (Patterson, 2002). Only two putative U-box proteins were identified in the rice fungal
pathogen *M. oryzae* genome (L. Zeng, unpublished). In contrast, sixty one U-box proteins were identified in *Arabidopsis* when a series of analyses were performed (Azevedo et al, 2001) (http://www.arabidopsis.org/info/genefamily/pub.html). The U-box domain is structurally analogous to the RING domain. The large number of RING-type proteins constitutes a super-family in plants (Stone et al, 2005). The high similarity between U-box and other sub-family members of RING-type proteins makes it a daunting challenge to correctly identify U-box sequences from the RING-type proteins pool. In this study, seventy seven U-box-containing proteins were identified from the annotated rice genome using a series of algorithms in six steps. Of the seventy seven U-box protein genes, either cDNA or EST were detected for sixty of them and only four of them do not have matching MPSS tags, corroborating the effectiveness of the algorithms used in the survey of this study. Sixty-one U-box proteins were identified from *Arabidopsis* when data from this study and other classes (http://www.arabidopsis.org/info/genefamily/pub.html) were combined. The identification of two nearly complete sets of the U-box-containing proteins from the genomes of the model monocot and dicot plants provided the basis for studying the phylogeny of U-box proteins in plants.

Sequence alignment indicated that there exists a class of U-box proteins with highly conserved sequences at their C termini in both rice and Arabidopsis genomes (Figure 1). We named this region as GKL domain based the presence of highly conserved Glycine, Lysine/Arginine residues and leucine rich feature. To date, however, no biological function has been associated to such domain. It is generally considered that the U-box domain serves as the E2 recruitment domain, while another distinct domain in U-box proteins, such as ARM repeats or TPR domain, serve to recognize and recruit the
substrate. However, no other known domain/motif was significantly identified in these proteins. Therefore, we propose that of the GKL domain might be involved in protein-protein interaction. Recently, one tobacco homolog of U-box/GKL type protein, NtCMPG1, was implicated in plant immunity (Gonzalez-Lamothe et al, 2006). Identification of a substrate for NtCMPG1 and testing if mutation in GKL domain will compromise the interaction of NtCMPG1 to the substrate will help verify our hypothesis.

The ARM repeat is an approximately 40 amino acid long tandemly repeated sequence motif first identified in the *Drosophila melanogaster* segment polarity gene armadillo involved in signal transduction (Riggleman et al, 1989). Structural characteristics of the ARM motif suggest its involvement in protein-protein interactions, which has been demonstrated in several cases (Huber et al, 1997). In a few cases, HEAT repeats were detected in proximity to the ARM repeats. Phylogenetic analysis indicated most rice U-box proteins are evolutionally much closer to their homologs from the rice genome than those from the Arabidopsis genome (Figure 3). The establishment of such relationship between the rice and *Arabidopsis* U-box proteins could help in characterizing these genes. For example, the identification of the putative *Arabidopsis* homolog of rice Spl11 gene (*AtSpl11*) based on the combined phylogenetic tree led us to the identification of a loss-of-function mutant of *AtSpl11* that displayed similar cell death and altered flowering phenotype to the rice *spl11* mutant (L. Zeng & G.L, Wang, unpublished). Given the advantage of *Arabidopsis* over rice in terms of plant growth and genetic resources, we expect that an in-depth characterization of the *Atsp11* mutant will help elucidate the connection of the immunity and the reproductive development in rice. Similarly, it would
be interesting to investigate if the putative rice homolog of AtPUB17 (ACRE276), OsPUB4, has a similar function in rice cell death and defense (Yang et al, 2006).

Why plants require more U-box proteins than animals is unclear at present. One speculation, given the lack of animal-like immune system and the static life of plants, is that the U-box proteins may play an important role in plants’ responding to various environmental stresses (Patterson, 2002). Domain organization analysis indicated that the U-box family could be divided into at least nine classes. A variety of other motifs/domains, such as protein-protein interaction domains, protein phosphorylation-related domain, and transcription initiation-related domain were found to be associated with the U-box domain in the rice and Arabidopsis U-box proteins (Table 3). U-box proteins with a similar domain organization from other organisms have been demonstrated to function in the different cellular processes (Amador et al, 2001; Kirsch et al, 2001; Ohi & Gould, 2002; Stone et al, 2003). These data suggested that the biological functions of the U-box proteins might be diverse rather than be limited to stress response signaling. Of the rice and Arabidopsis U-box proteins identified hereby, only a few of them have been functionally characterized. In addition, the existence of multiple members in each class of the U-box proteins raised the possibility of functional redundancy among the members. Such function redundancy could make the study of biological roles for plant U-box genes a daunting challenge. Among the eight rice U-box genes analyzed for their role in cell death signaling pathway, only one was found causing cell death when it is silenced in rice cells (Figure 5). The failure of the other seven genes to display cell death phenotype could be due to such functional redundancy as well. Therefore, a systemic approach, using knockout mutants or transgenic RNAi lines, to
modify the expression of most or all members in the same class might be essential to functionally characterize these genes in large-scale.

U-box-containing proteins are predicted to possess E3 ubiquitin ligase activity. In *Arabidopsis*, several U-box proteins were revealed to possess E3 ligase activity (Mudgil et al., 2004; Yan et al., 2003; Yang et al., 2006). Of the seventy seven rice U-box proteins, only SPL11 has been demonstrated to possess E3 activity in previous studies. In this study, we tested five randomly picked rice U-box proteins for their E3 ligase activity *in vitro*. Such ubiquitination assay indicated that all five proteins have E3 ligase activity and they demonstrate different preferences for E2 enzymes in these assays (Figure 6). Unlike the E3s in the HECT family, RING finger and U-box E3s facilitate the transfer of ubiquitin molecule by precise spatial orientation of the E2 and the substrate (Wu et al., 2003). The U-box or RING-finger domain serves as the E2 recruitment domain, while another distinct domain, such as ARM repeats or TPR domain, serve to recognize and recruit the substrate. The crystal structure of the U-box protein Prep19 indicated that, for those U-box E3s that oligomerize to function, the U-box and the substrate-recruiting domain are arranged in such architecture that brings the E2 recruitment domain into close proximity with a substrate recognition domain. Therefore, such E2 preference may reflect the minor difference in the U-box domains of these proteins. Nevertheless, such preference could make it difficult to test the *in vitro* E3 ligase activity of some U-box proteins due to the availability of the best E2s they required for the assay.

The diverse motifs/domains presented in the rice U-box proteins support the notion that the rice U-box family may target a myriad of substrates. Biochemical assays of the ligase activity of the U-box proteins, identification of their substrates, and
elucidation of the mechanisms under which they work in the cell will be the next study in order to fully understand these genes.

Materials and methods

Identification of U-box containing proteins in the rice genome.

The U-box proteins in the genomes of rice *japonica* and *indica* subspecies were identified using a battery of algorithms. The complete results are available as supplementary materials at the following address: http://supfam.org/ubox/ubox.html. Taxonomically, we place the U-box family as a member of the RING domain superfamily. U-box family shows some distant sequence homology to other member families under the RING domain superfamily. To identify them correctly, the U-box proteins must be distinguished from other members in the same superfamily. A crucial difference between the U-box proteins and other superfamily members is the presence of fewer cysteine/histidine residues. The following seven procedures were used in the identification process: (1) WU-BLAST of known U-box proteins against the genome, (2) searching PFAM models of non-U-box families within the RING domain superfamily (Bateman et al, 2004), (3) scoring a SAM hidden Markov models (HMM) built from the PFAM seed alignment of U-box domains (Sonnhammer et al, 1998), (4) a SAM T99 'family' level model was built for the U-box proteins (Karplus & Hu, 2001), (5) scoring an HMM built from the PFAM 'full' alignment, (6) a SAM T99 'superfamily' level model was built and scored. The final results of the above seven sequential analyses were further examined with respect to the number of aligned cysteine residues and multiple sequence alignments annotated with the structural key residues highlighting the
differences between U-box family members and other RING domain superfamily members. Additional evidence such as structural information was consulted in some specific cases.

PHYLOGENETIC ANALYSIS.

The rice and Arabidopsis U-box proteins were used as queries to search against the PFAM or GenBank database and were classified into the different classes based on their domain contents. Rice and Arabidopsis proteins from the ARM class were then aligned using the clustal_X program (Thompson et al, 1997). The aligned sequence data was then inputted into the MEGA4 program (Kumar et al, 2001; Tamura et al, 2007) to build the phylogenetic tree.

PREPARATION OF MPSS LIBRARIES AND DATA ANALYSIS

The growth of rice plants, collection of rice tissues, preparation of MPSS libraries, and data mining were done described (Nobuta et al, 2007).

CELL DEATH ANALYSIS OF THE CANDIDATE GENES IN RICE PROTOPLASTS

RNAi constructs containing the candidate gene fragments and the construct containing the beta-glucuronidase (GUS) gene under the control of maize ubiquitin promoter were transiently expressed in rice protoplasts as described (Chen et al, 2006).

IN VITRO UBQUITINATION ASSAY
The *in vitro* ubiquitination assays were performed as described (Zeng et al, 2004) with some modifications. In brief, the full length *OsPUB* proteins were expressed as N-terminal GST-tagged in BL21 (DE3) cells (Stratagene) and affinity-purified with glutathione matrix (Sigma, USA). The *in vitro* ubiquitination reactions were performed by adding 1μg of protein of interest, 40ng of wheat E1, 100ng of Arabidopsis E2, 10μg of ubiquitin and 1.5μL of 20X reaction buffer (1 M Tris HCl pH 7.5, 40 mM ATP, 100 mM MgCl2, 40 mM DTT, 600 mM creatine phosphate and 1mg/ml creatine phosphokinase). The reaction was incubated at 30 ºC for 1.5 hr in 30μl volume before being stopped with SDS sample loading buffer and heated to 100 ºC for 5min. samples of the reactions were then separated by 10% SDS–PAGE. Ubiquitinated substrates were detected by western blotting with detected by ubiquitin antibody (Biomol, USA) followed by detection by chemiluminescence with the ECL kit (Pierce, USA).

References


**Table 1. Rice U-box gene names, their corresponding groups, rice genome locus, GenBank GI, GenBank Protein ID, cDNA tags, EST, and MPSS tags.**

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Table 2. Arabidopsis U-box gene names, their correspondence to sub-class, the Arabidopsis genome locus, GenBank protein accession, and accessions for their cDNAs and cognate ESTs. Analysis was conducted by Lirong Zheng.
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Table 3. Domain organizations of rice and *Arabidopsis* U-box proteins

*Analysis was conducted by Lirong Zheng*
Figure 1. Identification of a putative conserved domain located close to the C terminus of the rice U-box leucine-rich type U-box proteins. The 150 amino acids from the C-terminal end of each protein were used for alignment by the program Clustal_X (Larkin et al, 2007; Thompson et al, 1997). The high percentage of leucine residues presented in the ~100 amino acid residues located at the C-terminal half of these proteins and highly conserved Glycine and Lysine/Arginine residues are outline in the consensus. We named this putative domain GKL-box domain after the conserved G and K/R residues and its leucine rich feature. The GKL-box is denoted by the empty black box. The highly conserved Glycine, Lysine/Arginine and Leucine residues are marked as either blue or red in the alignment and bold black in the consensus line. Analysis was conducted by Lirong Zheng.
Figure 2. Phylogenies of the U-box sequences and domain organizations of the rice U-box/ARM proteins. The phylogenies were generated in MEGA 4 (Tamura et al, 2007) with neighboring joining with 400 bootstrap replicates and were rooted at midpoint based on full-length protein sequence analysis. The bootstrap values are shown as percentages.

A, Phylogeny was generated using the U-box protein sequences from the rice U-box/ARM proteins. The three clusters are indicated with light yellow, green, and purple respectively. B, Schematic representation of domain organizations in the rice U-box/ARM proteins, ordered according to A. The ARM repeats were identified by searching the Pfam database (Bateman et al, 2004) with cutoff E-value set at 1.0. The red box indicates the U-box domain, and the individual ARM repeat of the ARM domain is indicated by blue box. The scale bar represents length of protein in amino acids. The relative position of U-box and armadillo (ARM) repeat on the bold midnight blue line indicates their position in primary structure of the protein. **Analysis was conducted by Lirong Zheng**
Figure 3. Phylogenetic relationship of rice and Arabidopsis U-box/ARM proteins. The amino acid sequences of the U-box/ARM proteins from the two genomes were used for analysis. The same method used for generating Figure 2A was used to construct the phylogeny tree. The four rice proteins that have closer phylogenetic relationship to their putative Arabidopsis homologs than their rice ones are marked with shaded box. Rice SPL11 protein and its closest Arabidopsis homolog AtPUB13 are marked with black arrows. Analysis was conducted by Lirong Zheng
Figure 4. RT–PCR analysis of the rice U-Box protein genes. Three-week-old seedlings of susceptible variety TP309 and resistant transgenic line harbouring the resistance gene Pi9 (Qu et al, 2006) were inoculated with M. oryzae isolate PO6-6. RNA samples from infected plant leaves at indicated time points were used for RT–PCR analysis using gene-specific primers designed to amplify the corresponding the U-box protein genes. PCR reaction for each gene was performed using 25 cycles of amplification, and the PCR products were separated on 1.4% agarose gel. The amplification of the ubiquitin gene was used as control for an equal amount of first-strand cDNA being used for each PCR reaction. This experiment was repeated for twice, with similar results.
Figure 5. Analysis of the role of the rice U-box genes in the cell death signaling using rice protoplast system. The candidate rice U-box gene was transiently knocked-down in rice protoplast using RANi method. The beta-glucuronidase (GUS) gene under the control of maize ubiquitin promoter was co-transiently expressed in rice protoplasts. The strength of GUS activity was monitored as an indicator of viability of the protoplasts. Protoplast treated with an *M. oryzae* elicitor known to cause rice cell death was used as the positive control. Cells maintain high GUS activity denote low rate of cell death.
Figure 6. The rice U-box proteins possess E3 ligase activity. Affinity-purified recombinant rice U-box proteins were tested for E3 ligase activity in an in vitro ubiquitination assay. The wheat E1 enzyme, E2 enzymes from Arabidopsis (AtUBC) and ubiquitin were used in the reactions. The E2 enzyme used in each reaction is indicated above each lane, and the rice protein tested is listed below each panel.
Chapter 3: Understanding the mechanisms underlying ubiquitination of the *Magnaporthe oryzae* effector protein AvrPiz-t and its suppression of host E3 ligase activity.

**Introduction**

Plants have evolved multiple layers of preformed and induced defenses to protect themselves from pathogen attack. One of the most effective defenses is activated through so-called resistance genes (*R*), which primarily trigger the hypersensitive response (HR) at the infection site to inhibit the proliferation of pathogens (Chisholm et al, 2006; Jones & Dangl, 2006). The well-known “gene-for-gene” theory of disease resistance predicted that successful disease resistance is mounted only if both the *R* gene and its cognate avirulence (*Avr*) gene coexist during the interaction (Flor, 1971). In the last two decades, enormous efforts have been devoted to the mapping and the cloning of *R* genes in plants, and over 70 *R* genes in many plants have been cloned and characterized so far (Sanseverino et al, 2010). Based on their protein structures, cloned *R* genes can be divided into several classes, and most of them encode proteins with conserved nucleotide-binding sites (NBS) and leucine-rich repeats (LRR) (Liu et al, 2007).

While much has been learned about *R* genes, little is known about the *Avr* genes, of which about 35 have been identified (Block et al, 2008; Grant et al, 2006; Kamoun, 2007; Tyler, 2009). As the number of cloned *Avr* genes is increasing, studies on their functions in host plants are receiving more attention. The *Avr* proteins, also referred to as
effectors, often have dual functions. In plants with the cognate R protein, they elicit an HR response by associating either directly with the R protein or indirectly through interacting with another protein, which binds to or forms a complex with the R protein. In the absence of the appropriate R protein, these Avr proteins can suppress host basal defenses mainly via three main strategies (Gohre & Robatzek, 2008). First, they may alter host protein turnover either by direct cleavage of their host targets or by modulating the host ubiquitination pathway. Second, they may interfere with RNA metabolism by transcriptional activation or ADP-ribosylation of RNA-binding proteins. Finally, they may inhibit kinases involved in plant signal transduction networks that regulate the activation of defense responses. Although these results have advanced our understanding on the functions of pathogen effectors, these three questions remain to be answered: 1) What is the function of the host targets of effector proteins during plant and microbe interactions? 2) What fitness advantages do effector proteins confer to the pathogen? 3) How do effector proteins suppress host defense responses?

Rice blast disease, caused by the fungal pathogen Magnaporthe oryzae, became a model pathosystem for understanding the molecular basis of plant-fungal interactions due to the availability of both host and pathogen genome sequences, a wealth of genetic resources available, and the feasibility of molecular manipulation of both species (Caracuel-Rios & Talbot, 2007). Up to now, eighteen rice blast R genes and six M. oryzae Avr genes have been cloned and characterized (Hayashi et al, 2010; Liu et al, 2010; Takahashi et al, 2010; Yuan et al, 2011; Zhai et al, 2011). With the exception of Pi-d2, which encodes a B-lectin kinase protein, all the rice R genes encode NBS-LRR proteins. With regard to the pathogen side, the molecular function of the identified M. oryzae Avr
genes varies extensively. The $ACE1$ avirulence gene recognized by the $Pi33$ resistance gene encodes a putative hybrid between polyketide synthase, and a nonribosomal peptide synthetase, and is a member of an infection specific gene cluster involved in microbial secondary metabolism (Bohnert et al, 2004; Collemare et al, 2008). $ACE1$ is expressed exclusively in appressoria, suggesting its role in the penetration process (Fudal et al, 2007). The deduced amino acid sequence of $AvrPi-ta$ exhibits high similarity to fungal neutral metalloproteases (Orbach et al, 2000). However, the other four avirulence genes do not exhibit any known functions nor have any identifiable functional domains. Despite significant progress on the isolation of the $R$ and $Avr$ genes in rice and rice blast, respectively, the molecular mechanism underlying the recognition between $R$ and $Avr$ proteins has not yet been well understood. Moreover, both downstream signaling upon the recognition and the function of $Avr$ proteins in the absence of cognate $R$ proteins are largely unknown in the rice blast pathosystem. $Pi-ta/AvrPi-ta$ has been the only $R/Avr$ pair that was found to bind directly to each other in a far-western blot assay (Jia et al, 2000). Genomic and evolutionary dynamics of both $AvrPi-ta$ and $Pi-ta$ has also been investigated recently (Huang et al, 2008; Khang et al, 2008). However, how $AvrPi-ta$ and $Pi-ta$ interact in rice cells and how the interaction triggers subsequent defense responses have not been reported. $AvrPiz-t$, the cognate $Avr$ gene for the blast $R$ gene $Piz-t$, has been cloned recently (Li et al., 2009). $AvrPiz-t$ encodes a predicted 108-amino acid polypeptide with the 18-aa secretion signal at its N-terminus. $AvrPiz-t$ is a single-copy gene in $M. oryzae$ genome and its deduced amino acid sequence shows no sequence homology to any known protein in fungi or other organisms (Li et al, 2009). When $AvrPiz-t$ was expressed in the leaves of $Nicotiana benthamiana$, it significantly
suppressed programmed cell death (PCD) that was triggered by the mouse BAX protein in *Agrobacterium tumefaciens*-mediated transient assays, suggesting that *AvrPiz-t* may contribute to virulence fitness in the fungus by suppression of defense signaling in plants (Li et al, 2009). Cloning of both *Piz-t* and *AvrPiz-t* provides the second pair of R/Avr genes in the rice blast pathosystem to investigate the mechanisms of their recognition and subsequent activation of defense responses.

To identify the host targets of *AvrPiz-t*, we conducted yeast-two hybrid (Y2H) screens of a rice cDNA library using *AvrPiz-t* as a bait and found twelve putative interacting proteins named *AvrPiz-t* interacting proteins (APIPs). Among them, APIP2, 6 and 10 are predicted to be E3 ligases and are likely involved in the ubiquitination-mediated protein degradation pathway. Surprisingly, *AvrPiz-t* suppresses the ligase activity of the three E3 ligases, while the three APIP E3 ligases ubiquitinate *AvrPiz-t* in vitro. In this chapter, we aimed to identify the mechanism how *AvrPiz-t* interferes with host E3 ligase activity and to map the ubiquitination target sites in the *AvrPiz-t* protein by the APIP6 protein in vitro.

**Results**

*AvrPiz-t interacts with three E3 ligases in yeast and rice protoplasts*

To search for the host targets of *AvrPiz-t*, we performed Y2H screens of a rice cDNA library using *AvrPiz-t* as a bait and identified twelve APIPs in the screens (Table 4). Interestingly, four APIPs (APIP2, 6, 8, and 10) are likely involved in the ubiquitination-mediated protein degradation pathway because APIP2, 6, and 10 are putative C3H4-type RING E3 ligases, and APIP8 is a homolog of the yeast UFD1.
protein, which is responsible for the retrotranslocation of the ubiquitinated proteins with their partners (Ye et al., 2003).

To further confirm the interaction between AvrPiz-t and each APIP in rice cells, we conducted the bimolecular fluorescence complementation (BiFC) experiment by cloning the AvrPiz-t full-length cDNA fragment lacking the signal peptide (NS-AvrPiz-t) and full-length of APIP2, APIP6 or APIP10 cDNA into the NYFP and CYFP vectors, respectively. The AvrPiz-t-NYFP with APIP-CYFP and AvrPiz-t-CYFP with APIP-NYFP vector pairs were transfected into rice protoplasts, respectively. About 20 hrs after the transfection, a very faint GFP signal in the transfected rice protoplasts was detected in all combinations, suggesting the interaction between AvrPiz-t and APIP proteins (data not shown).

AvrPiz-t and all three E3 ligases are localized in the whole rice cell

To determine the subcellular localization of AvrPiz-t and E3 APIPs in rice cells, the full-length cDNA fragments of NS-AvrPiz-t, APIP2, APIP6 and APIP10 were cloned into the pX-DG vector, respectively (Chen et al., 2009). Each construct was transfected into rice protoplasts and evaluated for green fluorescence 20 hrs after transfection. With all the constructs tested, a weak GFP fluorescent signal was evidently observed throughout the cells with enhanced florescence in the nucleus (Figure 7).

All three E3 ligases show E3 ligase enzyme activity with selected E2 proteins

Bioinformatic analysis indicated that APIP2, 6, and 10 all contain a RING finger domain responsible for E3 ligase activity. To assay their E3 ligase activity in vitro, we
expressed the full-length cDNA fragments of \textit{APIP} 2, 6 and 10 in \textit{Escherichia coli} as a fusion protein with the GST tag. Each fusion protein was purified by the affinity chromatography and mixed with the wheat E1, one of the seven E2s, and ubiquitin. Since the ubiquitination process has a hierarchical structure in terms of the number of E1, E2 and E3 enzymes in a given genome (Glickman & Ciechanover, 2002), an E3 ligase can show various levels of ligase activity with different E2s. As expected, the GST:APIP 2, 6 and 10 proteins only showed ligase activity in the presence of selected E2s, not with all E2s. For example, APIP2 showed E3 ligase activity with most of the E2s except for OsUBC16 while APIP6 and 10 showed activity only with AtUBC8 and 9 (Figure 8), indicating that these APIPs possess E3 ligase activity and have preference for E2s.

\textit{AvrPiz-t interferes with E3 ligase activity and is ubiquitinated by E3 ligases APIP2, 6 and 10.}

To assess whether AvrPiz-t has any biochemical function in the ubiquitination process, we included the purified MBP:AvrPiz-t protein in the \textit{in vitro} E3 ligase assay reactions of APIP2, APIP6 and APIP10, respectively. Surprisingly, the western blot analysis with anti-ubiquitin antibody showed that the E3 activity of all three APIPs was significantly reduced when the MBP:AvrPiz-t fusion protein was included in the reactions (Figure 9A), suggesting that AvrPiz-t interferes with E3 ligase activity. Furthermore, high molecular weights of multiple bands above the MBP:AvrPiz-t protein were detected by western blot analysis with anti-MBP antibody in the presence of the MBP:AvrPiz-t recombinant protein while no signal was detected in the absence of the MBP:AvrPiz-t protein in all three E3 ligase activity reactions (Figure 9B). The difference
in size between any given two bands is about the size of ubiquitin (8.5 KDa), indicating that the MBP-AvrPiz-t is ubiquitinated by the APIP E3 ligases in vitro and is a substrate of these E3 ligases. Since APIP6 shows the strongest E3 ligase activity among the three E3 ligases, it was chosen for further in vitro biochemical assay with the AvrPiz-t protein. To confirm the specificity of the interference and ubiquitination, two E3 ligases involved in the host basal defense, RING4 (Li et al, 2011) and AtPUB13 (Lu et al, 2011), were used for the E3 ligase assay reaction and the APIP6 and MBP proteins were used as positive and negative controls, respectively. The assays showed that AvrPiz-t interferes with the E3 ligase activity of three APIPs (Figure 10A) and at the same time AvrPiz-t is ubiquitinated by the three E3 ligases (Figure 10B).

AvrPiz-t is neither a protease enzyme nor a deubiquitinase (DUB) in vitro

Based on the fact that AvrPiz-t suppresses the activity of E3 ligases in vitro, we hypothesized that the AvrPiz-t protein may act as a protease enzyme or a deubiquitinase (DUB) on APIP E3 ligases. To determine whether AvrPiz-t functions as a protease for the E3 ligase enzymes, we reprobed the western blot membrane showing the interference activity of AvrPiz-t with the anti-GST antibody to detect the E3 ligase protein. The assay did not show any significant difference in the amount of E3 ligase protein between the absence and presence of the AvrPiz-t protein (Figure 10C). Then we assayed whether AvrPiz-t contains deubiquitinating activity. There are two main DUBs counteracting ubiquitination in living cells (Evans et al, 2003; Love et al, 2007). The ubiquitin C-terminal hydrolase DUBs (Type I) are small (20-30 KDa) and cleave α-amino linked (linear) polyubiquitin chains while the ubiquitin-specific processing proteases (UBP)

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(Type II) are relatively large (60-300 kDa), which are able to hydrolyze branched polyubiquitin chains. To determine whether AvrPiz-t has isopeptidase (Type I DUB) or peptidase activity (Type II DUB), we used GST-Rubi3 for Type I DUB and K48-linked polyubiquitin as a substrate for type II DUB activity assay. Isopeptidase T and ubiquitin aldehyde were used as a positive control and an inhibitor, respectively, in the type II DUB assay. The western blot analysis indicates that AvrPiz-t did not degrade either GST-Rubi3 (Figure 11A and B) or K48-linked polyubiquitin (Figure 11C), suggesting that AvrPiz-t does not function as a DUB enzyme to interfere APIPs’ E3 activity in vitro.

Ubiquitination of AvrPiz-t by APIP6 involves in non-K29, K48 and K63 lysine residues in ubiquitin

Among the seven lysine residues in ubiquitin, most of K48- and some of K29- and K63-polyubiquitination of the substrate are known to be involved in the degradation of target proteins through the ubiquitin/26S proteasome system (UPS) while the ubiquitination involving other lysine residues in ubiquitin has non-proteolytic functions such as protein trafficking, DNA damage tolerance and translation (Hicke, 2001; Pickart & Fushman, 2004). To investigate whether K29, K48 and K63 in ubiquitin are also involved in the ubiquitination of AvrPiz-t by APIP6, mutated ubiquitins in these three lysine residues, either individually (K29R, K48R or K63R) or all three mutated to arginine (K29R-K48R-K63R), were used for the ubiquitination assays by APIP6. For the reduction of the background in western blots, the AvrPiz-t fragment was fused with the HA tag and then the entire fragment was cloned into pGex-6p-1 for protein expression in E. coli. In addition, the APIP6 cDNA fragment fused with the MBP tag sequence was
cloned into pMAL-c2x. Even with the K29R-K48R-K63R mutated ubiquitins in which all three were substituted with arginine, APIP6 showed E3 ligase activity and AvrPiz-t was still ubiquitinated by APIP6 (Figure 12), suggesting the polyubiquitination of AvrPiz-t by APIP6 is carried out by other lysine residues of ubiquitin rather than K29, K48 and K63.

_Lysine-less AvrPiz-t (LL AvrPiz-t) is still ubiquitinated by APIP6_

The C-terminal glycine (Gly76) of ubiquitin forms isopeptide bonds with a lysine residue(s) of target proteins. Since lysine is the only amino acid having ε-NH₂ group that forms isopeptide bonds with ubiquitin, it is highly possible that a lysine-less target will not be ubiquitinated by an E3 ligase (Breitschopf et al, 1998). AvrPiz-t contains totally six lysine residues, which are named K1 to K6 according to their orders in the AvrPiz-t protein. To determine which lysine residue(s) of AvrPiz-t is responsible for the ubiquitination by APIP6, we mutated each one individually or all six into arginine. Unexpectedly, the AvrPiz-t protein without any lysine was still ubiquitinated by APIP6 _in vitro_ (Figure 13). To test the possibility that the GST or MBP tag in AvrPiz-t is ubiquitinated, we used 6X His:LL AvrPiz-t:HA as a substrate in the ubiquitination assay by APIP6 and found out that the mutated form was still ubiquitinated by APIP6 (data not shown).

_AvrPiz-t contains at least two possible ubiquitination target sites_

To determine how many residues of the AvrPiz-t protein can be ubiquitinated, we used LL ubiquitin in the ubiquitination assay of AvrPiz-t. LL ubiquitin does not have any
\(\varepsilon\)-NH\(_2\) groups and only forms monoubiquitination in multiple sites of the substrate as illustrated in Figure 14. Therefore, an E3 ligase assay was conducted with AvrPiz-t and LL ubiquitin. The western blot analysis with the anti-HA antibody revealed a strong and a weak band above the AvrPiz-t fusion protein (Figure 15), indicating that AvrPiz-t has at least two ubiquitination sites. Also two bands above LL AvrPiz-t were observed, further confirming lysine residues are not the ubiquitin acceptor of the AvrPiz-t protein by APIP6 in vitro.

The 30 amino acids of AvrPiz-t’s N-terminus are required for the ubiquitination by APIP6

It has been reported that cysteine, tyrosine, threonine and serine residues as well as lysine can be ubiquitinated through esterification of either hydroxyl or thio group by E3 ligases (Grou et al, 2008; Wang et al, 2007). Since AvrPiz-t contains seventeen amino acids of the five types of residues as shown in Figure 16A, we generated a series of 10-aa deletion fragments both from both N- and C-termini of the LL AvrPiz-t protein as illustrated in Figure 16B. All of the nine deletion mutants along with wild type and LL AvrPiz-t were assayed for the ubiquitination by APIP6. While all of the C-terminal deletion mutant proteins were ubiquitination by APIP6, AvrPiz-t lacking N-terminal 30, 40 or 50 amino acids was not ubiquitinated by APIP6 and a mutant lacking the N-terminal 20 amino acids showed a weak ubiquitination (Figure 17).

To pinpoint the ubiquitination target sites, all the three possible target sites in N-terminal 30 amino acids of the AvrPiz-t protein were mutated into alanine and the resulting mutants were named AvrPiz-t Y1A-T1A-Y2A in which the first threonine, the first and the second tyrosine in the AvrPiz-t protein were mutated into alanine. In
addition, all the ten amino acids between 11th and 20th or those between 21st and 30th were mutated into alanine and named ND2 Ala or ND3 Ala, respectively, as shown in Figure 18A. Surprisingly, all the mutated AvrPiz-t proteins assayed with LL ubiquitin were still ubiquitinated by APIP6 (Figure 18B), suggesting that all those three amino acids are not the ubiquitination target sites and the 30 amino acids in the N-terminus of AvrPiz-t might only provide the platform for the interaction between AvrPiz-t and APIP6. Since all of the four C-terminal mutants of AvrPiz-t are ubiquitinated and the 30 amino acids in the N-terminus does not contain any ubiquitination target site, only two possible candidate sites in AvrPiz-t were the second cysteine (C2) and the second serine (S2). Therefore, we mutated these two candidate residues into alanine (C2A-S2A), and then assayed their ubiquitination by APIP6. In addition, we mutated all four cysteine residues in AvrPiz-t into alanine, resulting in cysteine-less (CL) AvrPiz-t. Surprisingly, all these mutant proteins were still ubiquitinated by APIP6 (Figure 18C).

*AvrPiz-t is degraded in vivo through the ubiquitin/26S proteasome system in rice protoplasts.*

Since the ubiquitination of AvrPiz-t by APIP6 involves in non-K29, K48 and K63 lysine residues in ubiquitin, we speculated that the ubiquitination might be required for the activation or transportation of the AvrPiz-t protein in rice cells rather than its degradation. To test this possibility, *AvrPiz-t:HA* and *LL AvrPiz-t:HA* were cloned into transient expression vectors for rice protoplast as well as for agroinfiltration into *N. benthamiana* leaves. Even with several times of attempts with either MG132 (inhibitor of the 26S proteasome system) or BAF (inhibitor of vacuolar degradation) treatment, neither
of AvrPiz-t:HA nor LL AvrPiz-t:HA was detected by western blot with the anti-HA antibody while the expression of the control GFP-HA was easily detectable in both rice protoplasts (Figure 19) and *N. benthamiana* leaves (data not shown).

To test the possibility that AvrPiz-t cleaves its C-terminus, we cloned the wild type as well as the AvrPiz-t mutants into the expression vectors designed for fusing with a variety of tags such GFP, HA and Myc either to its N-, C-terminus or both. In addition, we made an anti-AvrPiz-t antibody (BioSyn) with two epitopes to detect, even if it undergoes the cleavage of tag in both N- and C-termini. The western blot showed that GFP:LL AvrPiz-t:HA was detected by western blot either by anti-HA or anti-AvrPiz-t antibody as the expected size (Figure 20), suggesting that there is no cleavage in the N- or C terminal of AvrPiz-t and the lysine residues in AvrPiz-t may serve as ubiquitin acceptors, which might be required for the proteasome-mediated degradation in rice protoplasts.

Since we were able to detect GFP:LL AvrPiz-t:HA protein in rice protoplasts, we investigated whether we can detect GFP:AvrPiz-t:HA in rice protoplasts with the treatment of either MG132 or BAF. A faint band of the GFP:AvrPiz-t:HA protein was detected by western blot with anti-HA or anti-GFP-antibody when treated with MG132, not BAF (Figure 21), indicating that the degradation of the AvrPiz-t protein in rice protoplasts is through the UPS.

*Lysine residues of AvrPiz-t are required for its avirulence function.*

Since the GFP:LL AvrPiz-t:HA protein is more stable than wild type protein in rice protoplasts, we reasoned that lysine residues might be important for its virulence or
avirulence function. To test this speculation, we made the mutant *LL AvrPiz-t* and wild type *AvrPiz-t* constructs under the control of its native promoter and transformed them into the *M. oryzae* isolate RB22 lacking the *AvrPiz-t* gene. Four *LL AvrPiz-t* transformants were chosen by PCR screening and each of them was inoculated on Toride (Piz-t⁺) and Nipponbare (Piz-t⁻) plants with the wild type *AvrPiz-t* transformants and RB22 as negative and positive controls for disease symptom on the Toride plants, respectively. Interestingly, all four *LL AvrPiz-t* transformants showed susceptible type symptoms on Toride while the wild type transformants showed resistant phenotypes as expected (Figure 22), suggesting *LL AvrPiz-t* completely loses its avirulence function in the Piz-t⁺ background. All of the tested transformants showed good infection symptoms on Nipponbare plants (Piz-t⁻), ensuring the pathogenicity of these transformants (Figure 22).

**Discussion**

Compared to the effector proteins from plant pathogenic bacteria, much less is known about host targets of effectors from eukaryotic plant pathogens such as oomycetes and fungi. In this chapter, we investigated the relationship between the blast effector *AvrPiz-t* and its E3 ligase targets *in vitro*. We found that the *AvrPiz-t* protein interacts with and suppresses the activity of three host E3 ligases (Figure 9 and 10). In mammalian system, the YopJ protein from *Yersinia pseudotuberculosis* deubiquitinates ubiquitin molecules from polyubiquitinated key component proteins in host immune system, blocking the defense response (Haase et al, 2005; Thiefes et al, 2006; Zhou et al, 2005). Therefore, we hypothesized that the *AvrPiz-t* protein might have a similar biochemical
function as a deubiquitination enzyme. However, no deubiquitinating activity of the AvrPiz-t protein either for K48-linked polyubiquitins or for linear polyubiquitins, Rubi3, was observed the in vitro experiments (Figure 11), suggesting that AvrPiz-t might not be a deubiquitination enzyme. However, we also found that the polyubiquitination bands formed by APIP6 involves non-K29, K48 and K63 lysine residues in ubiquitin (Figure 12), indicating that AvrPiz-t may be able to deubiquitinate polyubiquitin formed through non-K48 residues. Since only K-48 and K-63 linked polyubiquitin are commercially available, we are not able to assay AvrPiz-t’s deubiquitination ability on other polyubiquitins. The application of the polyubiquitins formed by APIP6, after the immunoprecipitation (IP) with the anti-ubiquitin antobody, as a substrate in a deubiquitination assay might help to understand the biochemical mechanism for its interference function.

Another interesting finding is that the AvrPiz-t protein is ubiquitinated by host E3 ligases (Figure 9 and 10). Avr3a from Phytophthora infestans interacts with and stabilizes NtCMPG1, a host E3 ligase, in vivo (Bos et al, 2010). However, it is unknown if NtCMPG1 ubiquitinates Avr3a either in vitro or in vivo. Therefore, AvrPiz-t is the first report that an effector protein from a plant pathogen is ubiquitinated by host E3 ligases and affects their activity at the same time. Since AvrPiz-t is ubiquitinated by host E3 ligases and interferes with their activity, it is highly possible that the ubiquitination of their original substrates is also affected. Thus identification of the E3 ligase’s substrates followed by the functional analysis of these genes in disease resistance will shed light into the AvrPiz-t-mediated suppression mechanism of the host UPS.

To confirm the interaction between the APIP proteins and the AvrPiz-t protein, we
employed the BiFC approach in rice protoplasts. As a preliminary experiment, we also
determined their subcellular localization to assess whether there will be any change in the
subcellular localization after the interaction between AvrPiz-t and APIPs. For example,
SPL11 localizes in whole cells but it moves to the nucleus after the interaction with
SPIN1 protein (Vega-Sanchez et al, 2008). However, we only observed a weak GFP
fluorescent signal throughout the cell in rice protoplasts transfected with the AvrPiz-t or
APIPs GFP fusion constructs (Figure 7) and in the BiFC experiments. The weak GFP
signal of the three APIP GFP fusion proteins in rice protoplasts might be caused by self-
ubiquitination and degradation that were observed in E3 ligases (Jackson et al, 2000;
Vaux & Silke, 2005). The weak GFP signal from the AvrPiz-t:GFP transfected rice
protoplasts most likely due to degradation of AvrPiz-t in rice protoplasts.

Although the ubiquitination of AvrPiz-t by APIP6 is non-canonical in vitro (Figure 12), we were not able to detect the AvrPiz-t protein in planta when it was
transiently expressed either in rice protoplasts or N. benthamiana leaves even with the
presence of MG132 or BAF (Figure 19). Therefore, we hypothesized that the affinity tags
might be removed from the AvrPiz-t fusion protein when expressed in rice protoplasts or
N. benthamiana plants. It was reported that Cladosporium fulvum effectors such as Avr2,
Avr4E and ECP2 undergo cleavage, removing tags, when they are expressed as
recombinant fusion proteins in the tomato leaf apoplast, irrespective of the N- or C-
terminal fusion (van Esse et al, 2006). Among all the AvrPiz-t protein fused with a
variety of tags in either C-, N-terminus or both, only GFP:LL AvrPiz-t:HA was detected
by western blot with either anti-AvrPiz-t or anti-HA antibody (Figure 20). Since we did
not detect any band of GFP size in either GFP:AvrPiz-t:HA or GFP:LL AvrPiz-t:HA-
transfected rice protoplasts by western blot with the anti-GFP antibody (Figure 21A Bottom), we don’t think that there is any cleavage for either N- or C-terminal of the AvrPiz-t protein. However, we cannot explain why we could not detect AvrPiz-t:HA with MG132 either in rice protoplasts or *N. benthamiana* plants. One possible explanation is that the turnover of AvrPiz-t is so rapid that the residual activity of the UPS inhibited by MG132 might be enough for the degradation of the ubiquitinated AvrPiz-t protein. In case of GFP:AvrPiz-t:HA, host E3 ligases targeting AvrPiz-t such as APIP6 and APIP10 might be less accessible to ubiquitin acceptor residues of AvrPiz-t due to the dimerization or the size of GFP, which makes the AvrPiz-t protein more stable with either the MG132 treatment or the substitution of lysine for arginine of the protein.

Substitution of lysine residues in the substrate protein with arginine and E3 ligase assay with LL ubiquitin is a traditional method to map the ubiquitination target lysine (Ciechanover & Ben-Saadon, 2004; Patel et al, 2009). Unexpectedly, we found that even GST:LL AvrPiz-t:HA was still ubiquitinated by APIP6 *in vitro* (Figure 13). 6X HIS:LL AvrPiz-t:HA without any extra lysine residue in the tag was also ubiquitinated by APIP6 (data not shown), ruling out the possibility that ubiquitination involves target lysine amino acid residues in the GST tag region. There are only a handful of reports that lysine-less proteins also are ubiquitinated through esterification of either hydroxyl or thio group in tyrosine, threonine, serine or cysteine (Grou et al, 2008; Wang et al, 2007). With a series of AvPiz-t deletion mutants, we narrowed down the ubiquitination target residues in amino acids from 11th to 30th of AvrPiz-t. However, mutant proteins with a point mutation of three possible ubiquitination sites as well as two substitution mutants, alanine substitution for all amino acids either from 11th to 20th or 21st to 30th, were also
ubiquitination by APIP6 (Figure 18B). Therefore, we speculated that amino acids from
11th to 30th of AvrPiz-t might provide the platform for the interaction between AvrPiz-t
and APIP6 protein. Two epitopes predicted for the production of the anti-AvrPiz-t
antibody covers amino acids between 11th and 29th, supporting this possibility. Internal
deletion mutant of 11th to 30th in the N-terminal of AvrPiz-t might be a good starting
material for the Y2H to assess the interaction with the APIP proteins. Once we found the
deletion mutant that doesn’t interact with the APIP proteins, point mutation can be
applied to narrow down the interaction site. There could be two possible ways to explain
why we could not pinpoint the ubiquitination target sites. First, the N-terminus of AvrPiz-
t contains a free NH2-group in methionine that can be ubiquitinated via so called N-
terminal ubiquitination when all internal lysine residues were substituted into arginine or
other amino acids (Breitschopf et al, 1998; Fajerman et al, 2004). N-terminal
ubiquitination is a novel pathway involving a new proteolytic pathway described in yeast
as ubiquitination fusion degradation (UFD) pathway (Ciechanover & Ben-Saadon, 2004;
Fajerman et al, 2004). This possibility is supported by the fact that APIP8 encodes UFD1
like protein. However, AvrPiz-t protein contains at least two possible ubiquitination
target sites (Figure 15) and N-terminal ubiquitination can explains only one target site
even if it occurs. Second, mutation of the original target sites might render other
previously non-acceptor amino acids to be ubiquitinated. Therefore, another alternative
way to find the acceptor amino acid residues will be protein sequencing of the in vivo
ubiquitinated AvrPiz-t protein using mass spectrometry. However, the amount of in vivo
ubiquitinated AvrPiz-t proteins after MG132 treatments in rice protoplasts was very low
and not enough to be detected even by the western blot analysis. Therefore, we are
generating rice transformants expressing \textit{GFP:AvrPiz-t:HA} to get enough amount of the AvrPiz-t protein after the MG312 treatment for protein sequencing to identify ubiquitination target residues. Alternatively, \textit{in vitro} ubiquitinated wild type AvrPiz-t can be chosen as a starting material for protein sequencing. After GST pull-down or IP, the ubiquitinated AvrPiz-t by APIP6 can be analyzed by mass spectrometry after trypsin digestion. However, \textit{in vivo} expression of the mutant \textit{AvrPiz-t} with mutations in the ubiquitin-target sites should be conducted to confirm the result from the \textit{in vitro} assay.

Very recently, the crystal structure of effector proteins such as ATR1 from \textit{Hyaloperonospora arabidopsisidis} and AVR3a11 and PexRD2 from \textit{Phytophthora} was determined (Boutemy et al, 2011; Chou et al, 2011). Especially, the determination of ATR1 crystal structure made a substantial progress to understand the basis of its recognition by its cognate R protein RPP1 and the mechanism how pathogen escapes host recognition through surface polymorphism. Similarly, the determination of the crystal structure of APIP6 and AvrPiz-t might help us to identify the motifs for the ubiquitination in AvrPiz-t and reveal the mechanism of its suppression activity.

It was reported that a class of effectors from mammalian bacterial pathogens utilizes the host ubiquitination machinery for their delivery into host cell and/or timely degradation inside of host cell (Angot et al, 2007; Rytkonen & Holden, 2007). For example, ubiquitination of SopA, an effector protein from \textit{Salmonella}, by the host E3 ligase HsRMA1, is required for its delivery into the cytosol of epithelial cells (Zhang et al, 2005; Zhang et al, 2006). Also the SopE and SptP effector proteins from \textit{Salmonella enterica} serova Typhimurium, are ubiquitinated by host E3 ligases and undergo different rates of degradation in host cells, allowing the structural organization of host actin.
cytoskeleton at different infection stage for its successful infection (Kubori & Galan, 2003; Patel et al, 2005). Therefore, we reasoned that the ubiquitination of the AvrPiz-t protein by host E3 ligases might be required for its avirulence or virulence function. Indeed, *M. oryzae* transformants with *LL AvrPiz-t* under native promoter completely lost their avirulence on Piz-\(t^+\) background rice Toride (Figure 22), indicating that lysine residues are required for its avirulence function, even though it is supposedly more stable in rice cells. However, we do not have any direct evidence to show that ubiquitination of AvrPiz-t followed by degradation is required for its avirulence function because any of the six lysine residues of AvrPiz-t might be required for its translocation into rice cells. To answer this question, we can use the following two approaches. The first one is to express *LL AvrPiz-t* in the Piz-\(t^+\) background with the XVE inducible promoter. If lysine is required for AvrPiz-t/Piz-t triggered-HR, induction of *LL AvrPiz-t* in the Toride background will not show any cell death after the treatment of the inducer estradiol. The other approach is that the detection of the translocation of *LL AvrPiz-t:mCherry:NLS* in rice sheath using a live-cell imaging approach (Khang et al, 2010). If any of the lysine residues are required for the translocation of AvrPiz-t into rice cells, the red florescence signal will not be observed in the nucleus of the transformed rice cells.

**Materials and methods**

*Plant material*

Rice (*Oryza sativa*) seeds were sterilized by the treatment in 75% ethanol for 1 minute followed by the immersion in 2% of sodium hypochlorite for 40 minutes. After
washing with sterile water, seeds were germinated on 1/2 MS medium for 1 week and then transferred to soil. Rice plants were maintained in the growth chamber at 26 °C, 80% relative humidity under 13 hours photoperiod, or in the greenhouse under similar growing conditions.

Yeast two-hybrid screening

The ProQuest Two-Hybrid system (Invitrogen) was used for screening of the AvrPiz-t interacting proteins. The full-length of \textit{NS-AvrPiz-t} was cloned in-frame into the bait vector pDBleu and was transformed into yeast strain Mav203. Yeast cells carrying pDBleu-\textit{NS-AvrPiz-t} was transformed again with a rice cDNA library that was built with the prey vector pPC86 using mRNA isolated from the seedlings of rice line 75-1-127 (Vega-Sanchez, et al., 2008). Candidates selected onto SD/-Leu-Trp+3AT medium were subjected to β-galactosidase assays for the confirmation following the manufacturer’s protocol (Invitrogen).

Protein purification and E3 ubiquitin ligase activity assays

The full-length cDNAs of each \textit{APIP} gene and \textit{NS-AvrPiz-t} were expressed as MBP as well as GST tagged proteins in \textit{E. coli} Rosetta2 (DE3) strain and were affinity-purified with maltose (NEB) and glutathione matrix (Sigma), respectively. \textit{In vitro} ubiquitination reactions were performed by adding 1μg each of the MBP:APIP proteins, 1μg of GST:AvrPiz-t:HA, 40 ng of yeast E1 (Biomol), 100 ng of \textit{Arabidopsis} E2 (AtUBC10), 1 μg of ubiquitin and 1.5μL of 20X reaction buffer (1 M Tris HCl, pH 7.5, 40
mM ATP, 100 mM MgCl₂, 40 mM DTT, 600 mM creatine phosphpate and 1 mg ml⁻¹ creatine phosphokinase). The reaction was incubated at 30°C for 1.5 hours in 30 μL reaction volume before being stopped with the SDS sample loading buffer and heated to 100°C for 5 min. Samples of the reactions were then separated in 10 or 15% SDS–PAGE gel. Polyubiquitin bands were detected by western blotting with anti-ubiquitin antibody (Biomol, USA) followed by chemiluminescence with ECL kit (Promega, USA).

Deubiquitination assay.

Type I *in vitro* deubiquitination reactions were performed by adding 1μg of MBP:AvrPiz-t, 1μg of GST:Rubi3 (Os04g53620.1) and 1.5μL of 20 X reaction buffer (1 M Tris HCl, 20 mM DTT and 200 μg ml⁻¹ ovalbumin, pH7.5). GST:Rubi3 with GST-HA protein was used as a negative control.

For type II *in vitro* deubiquitination reactions, the purified AvrPiz-t and commercial isopeptidase T (positive control) proteins in reaction buffer (50mM Tris, 1mM DTT, 10μg/mL ovalbumin, pH7.5) were pre-incubated 37°C for 30 minutes in the presence or absence of 2mM of ubiquitin-aldehyde as an inhibitor. And then, an excess of K48-linked polyubiquitin is added as a substrate.

Both reactions were incubated at 37°C for 1.5 hours in 30 μL reaction volumes before being stopped with SDS sample loading buffer and heated to 100°C for 5 min. GST:Rubi3 and K48-linked polyubiquitin were revealed by western blot with anti-GST and anti-ubiquitin antibody, respectively.

*BiFC in rice protoplasts*
BiFC assay was conducted to confirm the \textit{in vivo} interaction between the three APIPs and AvrPiz-t in rice protoplasts isolated. To avoid the orientation problems in the YFP fusion protein, the \textit{NS-AvrPiz-t} was cloned into both NYFP and CYFP vectors. Co-transfection of \textit{AvrPiz-t-NYFP} with \textit{APIP-CYFP} or \textit{AvrPiz-t-CYFP} with \textit{APIP-NYFP} was performed in rice protoplasts. Fluorescence of transfected cells was observed with a Nikon epifluorescence microscope at 20 hours after transfection. Co-transfection of empty vector with each construct was used as negative controls.

\textit{Reverse transcription-PCR analyses}

Total RNA was extracted using Trizol reagent (Invitrogen) following the manufacturer’s protocol. 1.5 \(\mu\)g of RNA treated with DNase1 (Invitrogen) was used for first strand cDNA synthesis with the reverse transcription system (Promega) according to the manufacturer’s instruction. The \textit{Tap} tag gene was used as an internal control for agroinfiltration samples in RT-PCR.

\textit{Expression and detection of AvrPiz-t in rice protoplasts}

Rice protoplasts were isolated from two weeks old etiolated rice seedling tissues as previously described (Chen et al, 2006). For protoplasts transfection, 10\(\mu\)g of each plasmid DNA was mixed with 200\(\mu\)L of isolated rice protoplasts followed by the addition of 40\% PEG solution. After the incubation for 20 min at room temperature, 1.0mL of W5 medium with a final concentration of 100 \(\mu\)M MG132 or 300 nM Bafilomycin A1 (BAF) was added to the transfected rice protoplasts to inhibit the protein degradation by 26S proteasome and vacuole, respectively. At 16 hours after incubation, rice protoplasts were
spun down with 300g for 5 min and resuspended in 60μL of 3X SDS loading buffer. 20μL of each sample was loaded for western blot analysis with anti-HA antibody.

Plasmids

To express AvrPiz-t in rice protoplasts, PCR-amplified AvrPiz-t with a primer pair AvrPiz-t-HAF1/ AvrPiz-t-HAR was cloned into pXUN-CHA vector (Chen et al, 2009). To mutate all the six lysine residues in AvrPiz to arginine, three truncated fragments of AvrPiz-t were amplified with primer pairs AvrPiz-t-HAF1/ AvrPiz-t-K123RR, AvrPiz-t-K345RF/ AvrPiz-t-K6RR and AvrPlz-t-Link6F/ AvrPiz-t-HAR, respectively. The resulting three fragments were fused to each other by the double-joint PCR (Yu et al, 2004) with primer pairs AvrPiz-t-HAF1/ AvrPiz-t-HAR, and cloned into pXUN-CHA vector. The resulting plasmid, designated pXUN-LL AvrPiz-t:CHA, was used for rice protoplasts assay.

Either AvrPiz-t:CHA or LL AvrPiz-t:CHA was amplified with a primer pair AvrPiz-t-HAF1/ AvrPiz-t-HAR2. After the enzyme digestion with BamHI and XhoI, DNA fragment was inserted into the BamHI/SalI site of pGex-6p-1 vector for the protein expression in E. coli or BglII/SalI site of pGD vector for protein expression in N. benthamiana (Goodin et al, 2002). Also either AvrPiz-t-CHA or LL AvrPiz-t-CHA was amplified with a primer pair PiGFP-F/ AvrPiz-tR and then cloned into the pX-DG vector for the detection of its subcellular localization in rice protoplasts (Chen et al, 2009).

For BiFC, AvrPiz-t amplified with the primer pair Pi-GFP-F/ Pi-NYFP-R was cloned into pA7-NYFP and pA7-CYFP vector. Also APIP6 and APIP10 PCR amplicons were cloned into pA7-NYFP and pA7-CYFP.
For the cloning of **APIP6** into the pMAL-c2x vector to express protein in *E. coli*, **APIP6** was amplified with a primer pair APIP6-ProF/API6-ProR. The amplicon digested with *EcoRI* and *SalI* was inserted into *EcoRI/SalI* site of pMAL-c2x vector.

To generate **APIP6 H58Y** mutation, the RING finger domain was mutated with the primer pair AP6mutF/AP6mutR and digested with *PstI* and *HindIII*. This digested fragment was cloned into the PstI/HindIII site of pMAL-c2x APIP6. The primers used in this chapter are listed in Table 5.

**References**


Caracuel-Rios Z, Talbot NJ (2007) Cellular differentiation and host invasion by the rice


Rytkonen A, Holden DW (2007) Bacterial interference of ubiquitination and
deubiquitination. *Cell Host Microbe* **1**: 13-22


<table>
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<tr>
<td>APIP3</td>
<td>Hypothetical protein</td>
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Table 4. The list of APIPs identified by Y2H. Four genes involved in the ubiquitin/26S proteasome system are highlighted in bold. **Y2H screening was conducted by Bo Zhou**
Figure 7. Subcellular localization of AvrPiz-t and three APIPs in rice protoplasts. The full-length ORF of each APIP gene and NS-AvrPiz-t were cloned to vector containing GFP reporter gene and transfected to rice protoplasts. 24 hours after transfection, GFP signal was observed under the epifluorescence microscope. Scale bar, 20 μm.
Figure 8. E3 ligase assay of APIP2 (A), APIP6 (B) and APIP10 (C) with a variety of the E2 enzymes or negative control (-). GST:APIP2, APIP6 and APIP10 fusion protein were assayed for E3 ligase activity in the presence of Arabidopsis E1 (At5g06460), a variety of the E2s either from rice or Arabidopsis and ubiquitin. The absence of the E2 enzyme was used as a negative control. Western blot was conducted with anti-ubiquitin antibody to detect the polyubiquitin bands.
Figure 9. Suppression of E3 ligase activity by AvrPiz-t and ubiquitination of AvrPiz-t by three APIP proteins. A. Western blot with anti-ubiquitin antibody to detect polyubiquitin bands. B. Western blot with anti-MBP antibody to detect the MBP:AvrPiz-t protein. E3 ligase assay was conducted with E2 enzyme, AtUBC8. A2: APIP2, A6: APIP6 and A10: APIP10. In case of APIP6, two independent reactions were performed and showed similar results.
Figure 10. Ubiquitination of AvrPiz-t by basal defense-related plant E3 ligases and suppression of their E3 ligase activity by MBP:AvrPiz-t. A, Western blot with anti-ubiquitin antibody to detect the suppression of E3 ligase activity by the AvrPiz-t protein. B, Western blot with anti-MBP antibody to detect ubiquitination of MBP:AvrPiz-t. MBP protein was used as a negative control. C, Western blot with anti-GST antibody to monitor each E3 ligase protein. R4: GST:OsRing4, AP: GST:APIP6 and SP: GST:AtSPL11.
Figure 11. *In vitro* deubiquitination assay. MBP-AvrPiz-t protein was assayed to investigate the function of AvrPiz-t as a deubiquitinating enzyme *in vitro*. **A and B**, Type I DUB enzyme assay. OsRubi3 was used as a substrate. For A and B, anti-ubiquitin and anti-HA antibody were used to detect polyubiquitin and AvrPiz-t, respectively. **C**, Type II DUB enzyme assay. 100nM of Isopeptidase T (Biomol, USA) and MBP itself were used as a positive control and a negative control, respectively. 1µg of K48-linked polyubiquitin and 1µM of ubiquitin aldehyde was used as a substrate and an inhibitor, respectively. HA: GST:HA, AvrPiz-t:HA: GST:AvrPiz-t:HA and M, MBP.
Figure 12. Non-canonical ubiquitination of AvrPiz-t by APIP6. A, Western blot with anti-ubiquitin antibody to detect polyubiquitin bands. B, Western blot with anti-HA antibody to detect the ubiquitination of AvrPiz-t:HA. To determine which lysine residue of ubiquitin is involved in polyubiquitination of AvrPiz-t, K29R-, K48R-, K63R-mutated or a triple K29R-K48R-K63R-mutated ubiquitin was used in ubiquitination assay instead of wild type ubiquitin. HA: GST:HA and AvrPiz-t:HA: GST:AvrPiz-t:HA
Figure 13. Ubiquitination of LL AvrPiz-t by APIP6. Six lysine residues of the AvrPiz-t protein were named K1 to K6 according to their orders and were mutated singly or with the combinations. Even LL AvrPiz-t was ubiquitinated by APIP6. Ubiquitination of AvrPiz-t was detected by western blot with anti-HA antibody.
Figure 14. Schemes illustrating how to determine the number of ubiquitination acceptor residues of AvrPiz-t protein using LL ubiquitin. A, ubiquitination of wild type or a lysine mutated AvrPiz-t with wild type ubiquitin B, ubiquitination of wild type or lysine mutated AvrPiz-t with LL ubiquitin. LL ubiquitin forms only monouquitination of a substrate in the multiple sites. Ubiquitination of AvrPiz-t will be revealed by western blot with AvrPiz-t specific antibody. Light blue triangle and red triangle indicate wild type and LL ubiquitin, respectively.
**Figure 15.** Ubiquitination of the lysine-mutated AvrPiz-t proteins by APIP6 with LL ubiquitin to determine the number of ubiquitination target sites. Two arrows indicate mono-ubiquitinated AvrPiz-t by APIP6 with LL ubiquitin. GST:HA was used as a negative control for the ubiquitination by APIP6. Western blot was conducted with anti-HA antibody.
Figure 16. Schemes describing the possible ubiquitination target sites of AvrPiz-t protein and the layout of the AvrPiz-t protein deletion analysis. A, Seventeen possible ubiquitination sites of AvrPiz-t. AvrPiz-t contains four cysteine, seven serine, three tyrosine and three threonine residues as possible ubiquitination sites. Each candidate residue was named according to their amino acid one letter code and order in the AvrPiz-t protein. B, Serial deletion of the AvrPiz-t protein. Serial deletion of ten amino acids in the AvrPzi-t protein generates five and four deletion mutants from N-and C-terminus of AvrPiz-t, respectively. Deletion mutants were generated using the LL AvrPiz-t protein to prevent the ubiquitination of lysine, if possible.
Figure 17. Ubiquitination of the AvrPiz-t deletion mutants by APIP6. ND2 is ubiquitinated weakly and ND3, ND4 and ND5 completely lost their ability being ubiquitinated by APIP6 E3 ligase. Every AvrPiz-t mutated proteins assayed here was fused with the GST tag for the purification. Western blot was performed with anti-HA antibody.
Figure 18. Point mutation of AvrPiz-t and ubiquitination assay of mutated AvrPiz-t proteins. A, A scheme illustrating point mutation of AvrPiz-t. B and C, Ubiquitination assay of point-mutated AvrPiz-t proteins. GST:HA protein and GST:AvrPta:HA, an unrelated effector protein from M. oryzae, were used negative controls for B and C, respectively. LL and WT ubiquitin were used for B and C, respectively. Arrow indicates GST:AvrPiz-t protein. Ubiquitination of AvrPiz-t was revealed by western blot with anti-HA antibody. All assayed proteins were fused with GST for purification. Y1T1Y2 Ala: AvrPiz-t Y1A-T1A-Y2A in which the first threonine, the first and second tyrosine in the AvrPiz-t protein were mutated into alanine, C2S2 A: AvrPiz-t C2A-S2A where the second cysteine and the second serine of the AvrPiz-t protein were mutated into alanine, CL: cysteine-less AvrPiz-t.
Figure 19. Expression of GFP:HA, AvrPiz-t:HA and LL AvrPiz-t:HA in rice protoplasts. GFP:HA, AvrPiz-t:HA or LL AvrPiz-t:HA plasmid DNA was transfected to rice protoplasts. At 20 hours after transfection, rice protoplasts were collected by centrifugation and resuspended in 1X SDS loading buffer for the western blot analysis with anti-HA antibody. Neither AvrPiz-t:HA or LL AvrPiz-t:HA protein was detected even with MG132 and BAF that are inhibitor of protein degradation through the 26S proteasome and vacuole, respectively.
Figure 20. Expression of a variety of the AvrPiz-t fusion proteins in rice protoplasts. *AvrPiz-t* was cloned into different vectors to fuse a variety of tag at either N-, C-terminus or both ends. AvrPiz-t was detected with anti-AvrPiz-t and anti-HA antibody only when LL AvrPiz-t was fused with GFP and HA at its N- and C-terminus, respectively. GST-AvrPiz-t:HA expressed and isolated from *E. coli* was used as a control for anti-AvrPiz-t antibody.
Figure 21. Inhibition of AvrPiz-t degradation in rice protoplasts by MG132. GFP:HA, GFP:AvrPiz-t:HA and GFP:LL AvrPiz-t:HA were expressed in rice protoplasts and detected by western blot with anti-HA or anti-GFP antibody.
Figure 22. Inoculation of RB22, WT and LL AvrPiz-t transformants on NPB (Piz-t') and Toride (Piz-t'). LL AvrPiz-t completely lost its avirulence function on Piz-t' background rice.

Rice blast transformation and following screening by PCR were conducted by Pattavipha Songkunmarn (Nun). Constructs and inoculation were done by the author.
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<th>Primers</th>
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<td>Gene15-Y2H-F</td>
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Table 5. The list of primers used in Chapter 3.
Chapter 4: The fungal effector AvrPiz-t suppresses host innate immunity by targeting the RING-type E3 ligase APIP6 and APIP10 in rice

Plant pathogens secrete numerous effector proteins into host cells during the infection process. These effector proteins manipulate both pathogen-associated molecular patterns (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) through distinct and overlapping modes of actions, which include the inhibition of receptor activation, the interference with MAPK signaling, the exploitation of the host ubiquitin proteasome system (UPS), and the modulation of the defense transcriptome (Gohre & Robatzek, 2008). Among them, the interference with the host UPS by bacterial effectors has been well-documented in the mammalian system (Rytkonen & Holden, 2007). Recently, several lines of evidence showed that bacterial effectors manipulate the host plant UPS as well (Birch et al, 2009). For example, F-box-containing GALA effector proteins from Ralstonia solanacearum interact with Arabidopsis SKP1-like proteins and function as a virulence factor (Angot et al, 2006). The Pseudomonas syringae effector HopM1 does not contain any known conserved domains associated with the UPS, but it mediates the proteasome-dependent degradation of the Arabidopsis protein AtMin7, which is required for cell wall-mediated defense (Nomura et al, 2006). A recent study showed that blocking the degradation of trans-Golgi network/early endosome-associated AtMIN7 by P. syringae is an essential part of the ETI mechanism to counter bacterial
suppression of PTI (Nomura et al, 2011). The function of the *P. syringae* effector AvrPtoB has been well elucidated in the last few years. AvrPtoB suppresses cell death triggered by Pto and it contains a U-box-like domain in its C-terminal region for its E3 ligase activity. The E3 ligase activity of AvrPtoB is required for the degradation of the tomato protein kinase Fen, which transduces defense signals together with the NBS-LRR R protein Prf (Rosebrock et al, 2007). Research showed that Pto is resistant to AvrPtoB-mediated degradation because it inactivates E3 ligase function of AvrPtoB with a higher kinase activity compared to that of the Fen kinase (Ntoukakis et al, 2009), demonstrating a novel host immunity strategy via the enzymatic inactivation of a bacterial effector.

Compared to what is known about effectors of plant pathogenic bacteria, much less is known about the impact of effector proteins secreted from eukaryotic plant pathogens such as oomycetes and fungi on the host UPS. Bos et al. (2010) reported that AVR3a, an effector from oomycetes *Phytophthora infestans*, interacts with and stabilizes host U-box E3 ligase CMPG1, which is required for the efficient activation of defense response as well as INF1- and CF9/Avr9- dependent cell death. The CMPG1 protein is degraded through the 26S proteasome pathway, which is prevented by the modifications of CMPG1 E3 ligase activity by AVR3a upon infection (Bos et al, 2010). These results provide molecular evidence that AVR3a stabilizes host E3 ligase CMPG1 in order to prevent host cell death during the biotrophic phase of infection. However, the impact of the AVR3a/CMPG1 interaction on the function of the cognate resistance protein R3a in potato is still unknown.

AvrPiz-t, an effector protein from rice blast fungus *Magnaporthe oryzae*, is recognized by the cognate resistance protein Piz-t. *AvrPiz-t* encodes a predicted 108-
amino acid (AA) polypeptide with a secretion signal at the N-terminus. The deduced protein sequence shows no sequence homology to any known proteins. AvrPiz-t suppresses BAX-induced cell death in *Nicotiana benthamiana*, suggesting it may have virulence function as an effector protein in rice cells (Li et al., 2009). To understand the mechanism underlining the function of the AvrPiz-t protein in rice cells, we performed a yeast two-hybrid screen (Y2H) and identified twelve APIPs (AvrPiz-t interacting proteins) protein from a rice cDNA library (Vega-Sanchez, et al., 2008) by using AvrPiz-t as the bait. Interestingly, homology searches against the GenBank databases (http://www.ncbi.nlm.nih.gov/) have revealed that four out of 12 APIPs are predicted to be the key components of the UPS, suggesting that AvrPiz-t may target the host UPS when entering into rice cells. Therefore, in this chapter, we aimed to address the following questions about the function of AvrPiz-t and the E3 ligase APIPs: 1) Is AvrPiz-t translocated into host cells to interact with host proteins such as Piz-t and E3 APIPs? 2) What is the function of AvrPiz-t and APIPs in host defense? 3) What is the relationship among AvrPiz-t, Piz-t and the E3 ligase APIP proteins? Using multiple approaches, we demonstrated that AvrPiz-t is translocated into rice cells where it triggers HR in the presence of Piz-t, whereas it suppresses PTI such as PAMP triggered-reactive oxygen species (ROS) generation and the expression of the defense-related genes in the absence of Piz-t. The analysis of the APIP6 and 10 transgenic RNAi lines revealed that both APIP6 and 10 are positive regulators of PTI and APIP10 is a negative regulator of Piz-t-mediated ETI. We also found the evidence that AvrPiz-t manipulates PTI by promoting the degradation of both APIP6 and 10 *in vivo*. Taken together, we have discovered a previously unknown relationship among a fungal effector, host E3 ligases and an R
protein in plants.

Results

AvrPiz-t is a cytoplasmic effector that functions inside host plant cells

Functional recognition of a pathogen Avr protein by the plant intracellular NBS-LRR R protein leading to hypersensitive response (HR) and defense response suggests that the Avr proteins are translocated into the plant cells. To assess the functional interaction of AvrPiz-t with Piz-t inside rice cells, we generated stable transgenic rice plants expressing the AvrPiz-t gene under the control of an inducible XVE system in the Piz-t background (Figure 23A) (Zuo et al, 2000). The secretion of signal peptide containing proteins are highly conserved in eukaryotes. Therefore, AvrPiz-t lacking eighteen amino acids of the N-terminal signal peptide coding region (designated as NS-AvrPiz-t) was cloned in XVE vector to prevent AvrPiz-t from the secretion into apoplast of transgenic plants. The XVE-NS-AvrPiz-t transgenic plants in the Piz-t background showed normal growth in our growth chambers. Semi-quantitative RT-PCR (sqRT-PCR) revealed that the NS-AvrPiz-t transcripts were increased in the detached transgenic rice leaves after the treatment with estradiol, but not with the solvent DMSO (Figure 23B). Following the induction of NS-AvrPiz-t, the detached NS-AvrPiz-t-leaves exhibited chlorotic cell death (Figure 23C). Conversely, the NS-AvrPiz-t transgenic plants in the Piz-t Nipponbare (NPB) background did not show a cell death phenotype after the treatment with estradiol (Figure 23C), suggesting that the cell death is due to the presence of both AvrPiz-t and Piz-t in rice plants.

To directly assess the translocation of AvrPiz-t into the rice cytoplasm, we used a
live-cell imaging approach (Khang et al, 2010; Valent & Khang, 2010) and observed the localization pattern of the AvrPiz-t protein during the infection of rice leaves by *M. oryzae*. A fusion construct of AvrPiz-t:mCherry:NLS under control of the native AvrPiz-t promoter (P_{AVR-Piz-t}:AvrPiz-tCDS:mCherry:NLS) was created and used for fungal transformation. *M. oryzae* transformants co-expressing AvrPiz-t:mCherry:NLS and a putative interfacial matrix protein BAS4:EGFP (Khang et al., 2010) were analyzed for the localization of the fusion fluorescent proteins in the inoculated rice sheath epidermal cells. Totally 10 independent fungal transformants expressing both the AvrPiz-t:mCherry:NLS and the BAS4:EGFP fusion proteins were used for the observation of fluorescence. Fluorescence analysis showed that BAS4:EGFP uniformly outlined the invasive hyphae at successful infection sites (Figure 24), while AvrPiz-t:mCherry:NLS exhibited clear fluorescence in nuclei of invaded rice cell as well as biotrophic interfacial complex (BIC) (Figure 24, arrow). There was no detectable red fluorescence inside infectious hyphae (IH), indicating successful secretion of the fluorescently labeled AvrPiz-t to BIC (Figure 24, arrowhead) and translocation of AvrPiz-t into the rice cells. These results suggest that host-translocated AvrPiz-t protein functions inside the rice cells.

*AvrPiz-t suppresses PAMP-triggered immunity in rice*

A rapid accumulation of ROS is one of the first reactions triggered by the recognition of PAMPs such as flagellin and chitin by pattern recognition receptors (PRRs) (Nicaise et al, 2009). Several effector proteins interrupt the generation of ROS as a virulence function to perturb host cell immunity (Sohn et al, 2007) and, therefore, we
examined whether ectopically expressed *AvrPiz-t* suppresses PTI activity based on the assay of PAMP-induced ROS response (Schwacke & Hager, 1992). Like other plants such as *Arabidopsis thaliana* (Felix et al, 1999; Gomez-Gomez et al, 1999; Kunze et al, 2004; Miya et al, 2007), rice tissues develop a rapid and transient ROS burst when they are exposed to flg22 or chitin (Figure 25A and 25B). Compared to those in the non-transgenic NPB control plants, both the flg22- and chitin-induced H$_2$O$_2$ accumulation levels in transgenic *NS-AvrPiz-t* plants were significantly reduced (Figure 25A and 25B). In addition, quantitative RT-PCR (qRT-PCR) revealed that ectopic expression of *AvrPiz-t* suppressed the transcription level of two early defense genes, *KS4* and *PAL*, triggered by flg22 and chitin (Figure 25C). *KS4* encodes one of the two diterpene cyclase enzymes involved in momilactone biosynthesis (Hasegawa et al, 2010; Shimura et al, 2007). While the level of *KS4* transcription was rapidly induced up to 30 to 75 fold in wild type plants 1 hr post treatment with either with flg22 or chitin, ectopic expression of *AvrPiz-t* suppressed the transcription of *KS4* to the same level similar to that of the water control treated plants. Phenylalanine ammonia lyase (PAL) is an enzyme that catalyzes the deamination of L-phenylalanine to trans-cinnamic acid and is involved in the biosynthesis of certain classes of low molecular weight antimicrobial compounds called phytoalexins (Dixon & Paiva, 1995; Zhu et al, 1995). In rice, the transcription of *PAL* has been shown to be induced by flagellin from bacteria as well as by chitin elicitor treatment (Chen et al, 2010; Shimizu et al, 2010; Tanaka et al, 2003). While the transcription level of *PAL* was up-regulated 3-4 fold by chitin treatment in the wild type plants, ectopic expression of *AvrPiz-t* reduced the level of *PAL* transcripts to the same level similar to that of the water control plants 1 hr post treatment with chitin. Taken together, these
results indicated that AvrPiz-t suppresses host immunity signaling triggered by PAMPs in rice cells.

We next examined whether ectopic expression of *AvrPiz-t* interferes with rice blast disease resistance. Rice leaf infection assays using the punch inoculation method (Takahashi et al, 1999) showed that *NS-AvrPiz-t* transgenic plants exhibited bigger size of infection lesions when compared to the non-transgenic NPB control (Figure 26A). To further confirm the observation, we performed a spore counting assay as well as estimation of fungal mass by DNA-based qPCR. Three samples of 1cm² area were taken from each infected rice leaf for spore counting. The result showed that the spores produced in the *NS-AvrPiz-t* plants were significantly more than those produced in the non-transgenic plants (Figure 26B, left). Moreover, the relative fungal mass measured by DNA-based qPCR of the *M. oryzae POT2* gene versus the rice *ubiquitin* gene ratio was more in the *NS-AvrPiz-t* plants than in non-transgenic plants (Figure 26B, Right). Taken together, these results suggest that AvrPiz-t contributes to the virulence of *M. oryzae* by the suppression of host PTI during the compatible interaction.

*APIP6 and APIP10 are RING finger E3 ligases*

As mentioned in Chapter 3, we performed Y2H screens to identify potential host targets of AvrPiz-t. Among 12 APIPs (*AvrPiz-t* interacting proteins), two proteins, APIP6 and APIP10, showed the strongest interaction with AvrPiz-t in yeast (data not shown) and were chosen for the further studies. The *APIP6* and *APIP10* identified from the Y2H library consist of a 1,320-bp and a 969-bp of open reading frame encoding 439 and 322-amino acid protein, respectively. A BLAST search using the deduced amino acid
sequence revealed that both APIP6 and APIP10 belong to a large family of conserved and ubiquitous RING finger proteins present in eukaryotes ranging from plants to mammals. However, the BLAST searches against the rice genome database revealed that both APIP6 and APIP10 are single copy in rice genome. While APIP6 has only one predicted domain, a C3H4 RING finger (Figure 27A), APIP10 has multiple predicted conserved domains such as a BRAP2 (BRCA1-Associated Protein 2) and a C3HC4 type RING finger, and a ZnF UBP (Zinc-Finger Ubiquitin Binding Protein (Figure 28A). As shown in Chapter 3, both APIP6 and APIP10 have in vitro E3 ligase activity. To confirm the preliminary result, we tested a series of negative controls missing E1, E2 or ubiquitin in the E3 ligase activity assay. The MBP:APIP6 or MBP:APIP10 fusion protein purified from E. coli was incubated with wheat (Triticum aestivum) E1, Arabidopsis E2 AtUBC9, ubiquitin (Ub), and ATP for the E3 ligase activity assay. Only in the presence of all the components, the signal of polyubiquitin products with high molecular weight was detected either from the APIP6 (Figure 27C) or APIP10 (Figure 28B) assay, confirming both proteins have E3 ligase activity.

To determine whether the E3 ligase activity is dependent on its RING finger domain, an amino acid in the APIP6’s RING finger domain was mutated as shown in Figure 27B. Even with only one mutation of histidine at position 58 to tyrosine (H58Y), the APIP6 protein completely lost its E3 ligase activity (Figure 27C), suggesting an intact RING finger domain is responsible for the E3 ligase activity of APIP6.

Ubiquitination and interference activity is specific to AvrPiz-t protein.

As shown in chapter 3, the AvrPiz-t protein is ubiquitinated by APIP6 and
APIP10 and interferes with their E3 ligase activity in vitro at the same time. To assess the specificity of AvrPiz-t for those two activities, the GST:AvrPi-ta:HA protein of an unrelated M. oryzae effector, AvrPi-ta, was included as a negative control. The signal of high molecular mass bands of ubiquitinated products was detected only in the reaction with GST:AvrPiz-t:HA (Figure 29, lane 7 and Figure 30, lane 8) when hybridized with the anti-HA antibody, while no ubiquitination signal was observed in the reaction with the fusion protein GST:AvrPi-ta:HA (Figure 29, lane 9 and Figure 30, lane 9), indicating the specificity of APIP6 and APIP10’s ubiquitination ability. Further, when the hybridization with anti-ubiquitin antibody for the detection of polyubiquitin bands, the ubiquitination signal became much weaker in the reaction with AvrPiz-t (Figure 29, lane 7 and Figure 30, lane 8), compared with the signal in the reaction with AvrPi-ta (Figure 29, lane 9 and Figure 30, lane 9), suggesting that AvrPiz-t’s suppression of the E3 ligases APIP6 and APIP10 is specific.

APIP6 is degraded in N. benthamiana plants and the degradation requires E3 ligase activity.

Due to the limitations of the rice protoplasts system approach, such as difficulty to normalize samples, we selected N. benthamiana leaves for the expression analysis of the APIP6 protein. First, Agrobacterium carrying FLAG:APIP6 were infiltrated into N. benthamiana leaves and the tissues were harvested 1 to 3 days after infiltration (DAI) to monitor whether the APIP6 protein can be expressed in N. benthamiana leaves or not. To inhibit any possible degradation of APIP6 through the ubiquitin/26S proteasome system, we infiltrated 50μM of MG132 at 2 DAI and the tissues were harvested at 3 DAI. The
TAP tag protein was expressed as an internal control and detected by western blot with peroxidase anti-peroxidase (PAP). Unexpectedly, the FLAG:APIP6 protein was not detected by western blot with anti-FLAG antibody while the expression of the FLAG:APIP6 H58Y mutant protein, whose E3 ligase activity is abolished, was easily detected (Figure 31). It is possible that the APIP6 protein is ubiquitinated and degraded, which is not efficiently inhibited by MG132, an inhibitor of the UPS. sqRT-PCR confirmed the gene expression of the APIP6 and APIP6 H58Y in the infiltrated tissues (Figure 31).

**Co-immunoprecipitation (Co-IP) of APIP6 and APIP10 with the AvrPiz-t protein**

To further validate the interaction results in yeast (Figure 32A and C), we performed in vivo experiments for the interaction between AvrPiz-t and APIP6 or APIP10. Since the transiently expressed AvrPiz-t:HA was not detectable both in rice or in *N. benthamiana*, the GFP:AvrPiz-t:HA protein was co-expressed with FLAG:APIP6 H58Y or Myc:APIP10 in the *N. benthamiana* leaves. We used FLAG:APIP6 H58Y, which harbors a mutation in the RING finger domain, instead of the wild type APIP6 because FLAG:APIP6 protein was not detectable in the infiltrated tissues (Figure 31).

When the GFP-AvrPiz-t-HA fusion protein was immunoprecipitated (IP) from the plant extract using the anti-HA IgG beads, the FLAG:APIP6 H58Y and the Myc:APIP10 proteins were also detected in the immunocomplex of GFP:AvrPiz-t:HA (Figure 32B and D). As a control, no visible background signal was detected in the samples only expressing GST:AvrPiz-t:HA, FLAG:APIP6 H58Y (Figure 32B) or Myc:APIP10 (Figure 32D) after IP. These results indicate that AvrPiz-t interacts with APIP6 or APIP10 in vivo.
It is noteworthy that the interaction between APIP6 and AvrPiz-t in vivo does not require the E3 ligase activity of APIP6.

**APIP6 and APIP10 promote the degradation of AvrPiz-t in N. benthamiana.**

As shown in Chapter 3 (Figure 21), the transient expression analysis in rice protoplasts demonstrated that the AvrPiz-t protein is degraded through the UPS. We also found that AvrPiz-t is ubiquitinated by APIP6 and APIP10 in vitro, suggesting that AvrPiz-t may be APIP6 and APIP10’s target in planta as well. To confirm this hypothesis, we co-expressed GFP:AvrPiz-t:HA either with FLAG:APIP6 or Myc-APIP10 in N. benthamiana leaves. The western blot analysis revealed that the protein level of GFP:AvrPiz-t:HA was significantly lower in the tissue where APIP6 or APIP10 was co-expressed compared to the control where APIP6 H58Y or Myc:GFP was co-expressed, respectively (Figure 33). This result suggests that APIP6 and APIP10 promote the degradation of AvrPiz-t in plant cells.

**Lysine-less (LL) AvrPiz-t is also a substrate of APIP6 in N. benthamiana leaves.**

Since LL AvrPiz-t is ubiquitinated by APIP6 in vitro as shown in Chapter 3 (Figure 13), it was speculated that LL AvrPiz-t might be a substrate of APIP6 in vivo. Therefore, we co-expressed GFP:LL AvrPiz-t:HA with either FLAG:APIP6 or FLAG:APIP6 H58Y using GFP:AvrPiz-t:HA as a positive control for the degradation of the AvrPiz-t protein. Even though GFP:LL AvrPiz-t:HA was more stable than GFP:AvrPiz-t:HA in rice protoplasts, much less amount of GFP:LL AvrPiz-t:HA was detected when co-expressed with APIP6, as compared with the co-expression with APIP6
H58Y (Figure 34), suggesting GFP:LL AvrPiz-t:HA is also a substrate of APIP6 in *N. benthamiana*.

**AvrPiz-t promotes the degradation of APIP6 and APIP10 in N. benthamiana leaves.**

Since Avr3a stabilizes a host E3 ligase CMPG1 by modifications or inactivation (Bos et al, 2010), we reasoned that AvrPiz-t might affect the accumulation of APIP6 and APIP10 in plant cells. Especially, we expected that AvrPiz-t might stabilize the FLAG:APIP6 protein as Avr3a does CMPG1 because we could not observe the accumulation of the FLAG:APIP6 protein in *N. benthamiana* leaves when it was transiently expressed (Figure 35A). However, the FLAG:APIP6 protein was not detected by the co-expression with GFP:AvrPiz-t:HA protein. To the contrary, the accumulation of FLAG:APIP6 H58Y protein was significantly decreased by its co-expression with GFP:AvrPiz-t:HA compared to the controls such as co-expression of APIP6 H58Y either with empty vector or GFP:HA (Figure 35A). A similar trend was also detected with the APIP10 protein when it was co-expressed with GFP:AvrPiz-t:HA (Figure 35B). This implies the AvrPiz-t protein suppresses the accumulation of the APIP6 H58Y and the APIP10 protein *in planta*.

**Knocking-down of APIP10 shows spontaneous cell death phenotype in the Piz-t background**

We generated transgenic RNAi lines of *APIP6* and *APIP10* in the *Piz-t*+ Toride (TRD) background to assess their function in the *R*-gene mediated resistance. The designed RNAi construct targeting the 3’ UTR of each gene was used to generate stable
transgenic rice plants through *Agrobacterium*-mediated transformation. More than 20 independent lines were obtained for each construct. Among them, three independently transformed T2 lines of each RNAi construct were used for the molecular and phenotypic analysis. Interestingly, the *APIP10* RNAi lines showed an obvious lesion mimic phenotype, especially at late developmental stages (Figure 36), whereas the *APIP6* RNAi plants did not show any abnormal growth phenotypes. To determine whether this spontaneous cell death phenotype in the *APIP10* RNAi lines is related to Piz-t, the *APIP10* RNAi construct was also transformed into the *Piz-t*-NPB background. The *APIP10* RNAi lines in *Piz-t*-NPB background showed much less spontaneous cell death compared with those in the *Piz-t* TRD background. To further confirm the relationship between APIP10 and Piz-t, we transformed the *Piz-t:HA* construct in NPB background (NPB-Piz-t:HA) and identified the transgenic lines by the inoculation with a virulent isolate RB22. Then we transformed the *APIP10* RNAi construct into the calli of NPB-Piz-t:HA and obtained over 20 independent lines. As we observed in TRD background, the *APIP10* RNAi lines in NPB-Piz-t:HA background showed more severe cell death and dwarf phenotype compared to those in the NPB background (Figure 37A). To assess whether the severe cell death is related to the Piz-t protein level, we conducted western blot for Piz-t and sqRT-PCR for *APIP10* with three lines that had different levels of cell death: one line with normal growth and two lines with cell death and/or dwarf phenotype. In both backgrounds, lines showing normal growth without or less cell death showed more *APIP10* transcripts compared to ones showing cell death and/or dwarfism (Figure 37B), indicating that cell death was caused by the silencing of *APIP10*. Even though line #72 in NPB-Piz-t:HA background shows higher level of *APIP10* transcription compared
to line #31 or #86 in the NPB background (Figure 37B), more severe growth retardation
was observed (Figure 37A), suggesting that the Piz-t protein affects severity of phenotype
by knock-down of APIP10. In addition, the lines with less APIP10 transcripts in NPB-
Piz-t:HA background showed a higher level of the Piz-t protein Figure 37B), suggesting
that APIP10 is a negative regulator of the Piz-t-mediated cell death.

Knocking-down of APIP6 and APIP10 compromises basal defense in rice

Because AvrPiz-t interferes with APIP6 and APIP10’s E3 ligase activity in vitro
and promotes the degradation of APIP6 and APIP10 in vivo, we reasoned that both
APIP6 and APIP10 may be the targets of AvrPiz-t-mediated suppression of host defense.
Therefore, we monitored the ROS generation in the leaf disks of the 4 week old APIP6
and APIP10 RNAi lines after the treatments of flg22 and chitin using a luminol chemi-
luminescence assay (Schwacke & Hager, 1992). The analysis showed that knocking-
down of APIP6 as well as APIP10 resulted in the suppression of the rapid oxidative burst
in rice tissues induced by flg22 (Figure 38A, top and 39A, top) but not by chitin (Figure
38A, bottom and 39A, bottom).

qRT-PCR also revealed that the knock-down lines of APIP6 and APIP10 showed
significant but distinct transcriptional suppression profiles of PTI-related defense genes 1
hr after treatment with chitin or flg22 (Figure 38C and Figure39C). For example, the
knock-down lines of APIP10 showed the suppression of the KS4 transcripts 1 hr post
incubation of flg22 (Figure 39C, left) whereas there was no significant difference in the
APIP6 RNAi plants and the control plants (Figure 38C, left). The expression of the PAL
gene in the knock-down lines of APIP10 was significantly suppressed 1 hr post
incubation upon the treatment with both flg22 and chitin (Figure 39C, right), whereas the suppression of \textit{PAL}'s expression in the \textit{APIP6} RNAi plants proceeded, only upon treatment with chitin (Figure 38C, right).

Next we investigated the resistance level of the \textit{APIP6} and \textit{APIP10} RNAi plants to rice blast. No obvious difference in the resistance phenotype was observed in the \textit{APIP6} or \textit{APIP10} lines compared to the wild type NPB plants after the spray inoculation with a avirulence isolate RO1-1 (data not shown). However, more severe disease symptoms were observed in the sheath of \textit{APIP10} RNAi plants with a virulent isolate RB22 (Figure 40).

Rice blast inoculation methods involve the humidity treatment for 24 hrs post inoculation (Berruyer et al, 2006; Takahashi et al, 1999). Unfortunately, high humidity accelerated the cell death of the \textit{APIP10} RNAi plants in the \textit{Piz-t}+ TRD background (Figure 40B, compare left and right) and the treated plants eventually died. Therefore, we were not able to test the resistance level of the \textit{APIP10} knock-down plants in the \textit{Piz-t}+ TRD background. It was more feasible to test the resistance phenotype of \textit{APIP10} RNAi plants in the NPB background because they developed less severe cell death (Figure 39, compare left and right panels). We also performed punch inoculations of \textit{APIP6}-RNAi and \textit{APIP10}-RNAi lines with a virulent isolate RB22 and measured the relative fungal mass and sporulation in the disease lesions. The punch inoculation showed that the \textit{APIP6} or \textit{APIP10} RNAi plants produced relatively bigger lesions compared to the non-transgenic control plants (Figure 41A and 41C). Spore counting in the infected rice leaf area also showed that more spores were produced in the \textit{APIP6} RNAi and \textit{APIP10} RNAi plants compared to those in the non-transgenic plants (Figure 41, B and D, left panel). In
addition, the relative fungal mass measured by the DNA-based qPCR of *M. oryzae POT2* versus rice *ubiquitin* gene ratio was more in the *APIP6* or *APIP10* RNAi plants than in the non-transgenic plants (Figure 42, B and D, right). Taken together, these results suggest that knocking-down of the *APIP6* and *APIP10* expression in rice compromises the basal defense to the virulent isolate RB22.

**Discussion**

While the delivery mechanism of cytoplasm effector proteins from oomycetes into plant host cells is relatively well-studied, less is known about the effectors from plant fungal pathogens. A subset of cytoplasmic effector proteins in plant pathogenic oomycetes contains the RXLR motif at their N-terminal, which is required for the translocation of effectors into host plant cells (Whisson et al, 2007). Similarly, it has been reported that a set of cytoplasmic effectors from *M. oryzae* contain the LxAR motif, which may function in translocation of effector proteins (Yoshida et al, 2009). Predicted amino acid sequence of AvrPiz-t also contains the LxAR motif, whose mutation led to the loss of avirulence (Bo Zhou, personal communication), suggesting the importance of this motif in the avirulence function of AvrPiz-t. In order to obtain more direct evidence that AvrPiz-t is delivered into host plant cells, we employed a live-cell imaging approach developed by Khang et al. (2010). Since the AvrPiz-t protein is subject to degradation in rice protoplasts and AvrPiz-t fused with GFP gave only a faint signal of fluorescence when it was over-expressed with the ubiquitin promoter in rice protoplasts, we expected that a very weak or even no signal will be observed if a construct with *AvrPiz-t* under its native promoter is used for the fungal transformation and the inoculation. Therefore, we
made a construct carrying AvrPiz-t:mCherry:NLS in which the *AvrPiz-t* fragment is fused with DNA sequences of *mCherry* containing the nuclear localization signal (NLS) under *AvrPiz-t*’s native promoter. If AvrPiz-t is secreted into rice cells, the AvrPiz-t:mCherry:NLS fusion protein will be concentrated in the rice nucleus because of the NLS peptide. After the sheath inoculation, AvrPiz-t-associated red fluorescence was observed in the nuclei of invaded host cells (Figure 24), indicating that AvrPiz-t:mCherry:NLS has been indeed translocated into host cells. Unlike PWL2 which was observed even in the uninfected neighboring cells, red fluorescence from the AvrPiz-t:mCherry:NLS fusion protein was not observed in the nuclei of adjoining cells without IH, indicating AvrPiz-t might not move ahead into the uninfected neighboring cells. It might be because AvrPiz-t is degraded by the ubiquitin/26S proteasome system in the nucleus. Since we found that LL AvrPiz-t is more stable than the wild type in rice cells, it will be interesting to monitor whether the fusion protein LL AvrPiz-t:mCherry:NLS can move ahead into the rice cells without IH. Other experiments can be conducted is to test whether the LXAR motif is required for the translocation of AvrPiz-t into rice cells and whether the non-functional polymorphic AvrPiz-t proteins have lost their translocation ability.

Avirulence protein often has dual functions. In the presence of its cognate R protein, it triggers the rapid defense responses often associated with an HR. On the other hand, avirulence protein may suppress PTI as a virulence function in the absence of its cognate R protein (Gohre & Robatzek, 2008). We previously found that AvrPiz-t suppresses BAX-mediated program cell death in *N. benthamiana* (*Li et al*, 2009). In this project, we expressed the *AvrPiz-t* gene without the signal peptide sequence into the *Piz-
and Piz-t' backgrounds. Cell death phenotype was observed in the Piz-t' background when the expression of AvrPiz-t is induced. Although no cell death was observed in the AvrPiz-t transgenic plants in the Piz-t' background, suppression of ROS generation and early defense responses triggered by the two PAMP elicitors flg22 and chitin was observed in the transgenic plants (Figure 25). In addition, the AvrPiz-t transgenic plants confer enhanced susceptibility to a virulent isolate of M. oryzae. These results clearly demonstrated that AvrPiz-t has virulence function. Further research needs to be carried out to answer how AvrPiz-t triggers HR when Piz-t is present and how it suppresses PTI when Piz-t is absent.

We found that the GFP:AvrPiz-t:HA protein level is low when it was co-expressed with Flag:APIP6 or Myc-APIP10, indicating that the AvrPiz-t protein is degraded and might be the substrate of APIP6 and APIP10 in planta (Figure 33). Intriguingly, the degradation of AvrPiz-t by APIP6 or APIP10 in N. benthamiana was not significantly inhibited by MG132, suggesting that the degradation is independent of the UPS unlike the degradation of AvrPiz-t in rice protoplasts. In addition, while GFP:AvrPiz-t:HA was not detected by western blot in rice protoplasts after transfection, this fusion protein had the same level in N. benthamiana with the GFP:LL AvrPiz-t:HA protein (Figure 33). This discrepancy might be due to the differences of the UPS between rice and N. benthamiana plants. Similar phenomena were observed in the cleavage of affinity tags from the C. fulvum effector proteins. Removal of the affinity tags from effector proteins was observed only when they were expressed in Solanaceous species but not in A. thaliana (van Esse et al, 2006). We speculate that N. benthamiana have APIP6 and APIP10 homologous E3 ligases but they cannot interact and ubiquitinate the
GFP:AvrPiz-t:HA protein efficiently. To address whether APIP6 and APIP10 are the E3 ligases targeting AvrPiz-t for degradation in rice cells, we will generate GFP:LL AvrPiz-t:HA transgenic plants. Rice protoplasts will be isolated from these plants for transient expression of the  

\textit{FLAG:APIP6} and  \textit{FLAG:APIP10} constructs with or without the treatment of MG132. This experiment will let us know whether AvrPiz-t is the true substrate of APIP6 and APIP10.

When \textit{FLAG:APIP6} and \textit{FLAG:APIP6 H58Y} were expressed in \textit{N. benthamiana}, we detected the accumulation of the \textit{FLAG:APIP6 H58Y} protein, whose E3 ligase activity is completely lost \textit{in vitro}, but not the \textit{FLAG:APIP6} protein, suggesting that APIP6 may self-ubiquitinates and is degraded, which were also observed in other plant E3 ligases (Jackson et al, 2000; Vaux & Silke, 2005). However, we believe that the degradation of \textit{FLAG:APIP6} is not complete because it promotes the degradation of the AvrPiz-t protein when they were co-expressed in \textit{N. benthamiana}. Similarly, the CMPG1 protein exists in a low amount in \textit{N. benthamiana} and the 4X myc:CMGP1 protein was detectable by the western blot analysis (Bos et al, 2010). In addition, the detection of 3.5X myc:APIP10 by western blot was successful while that of \textit{FLAG:APIP10} was not (data not shown) Therefore, the generation of an APIP6 fusion protein with the tandem repeats of affinity tags such as 5X Myc and 5X FLAG may facilitate the detection of the wild type APIP6 protein in \textit{N. benthamiana}.

While the ectopic expression of \textit{AvrPiz-t} in rice plants suppressed the ROS induction induced by both flg22 and chitin (Figure 25), knock-down plants of \textit{APIP6} and \textit{APIP10} only showed significant suppression with the treatment of flg22, not chitin (Figure 38 and Figure 39). Furthermore, The \textit{APIP6} and \textit{APIP10} RNAi plants have
distinct expression profiles of selected defense genes between each other and also with that of the AvrPiz-t transgenic plants when treated with flg22 or chitin (Figure 38C and Figure 39C). The difference between the APIP6 RNAi and APIP10 plants may be explained by the fact that that APIP10-RNAi lines are in the NPB background while the APIP6 RNAi lines are in the TRD background. For the no change of ROS induction to chitin in APIP6 or APIP10 RNAi plants, it is possible that AvrPiz-t targets other APIPs for the suppression of the chitin-mediated defense response. For example, totally seven classes of XA21 binding proteins (XB) were identified by Y2H with a truncated kinase domain of XA21 (XA21K690) as a bait. However, silencing of XB3 or over-expression of XB15 alone comprises resistance mediated by XA21 partially (Park et al, 2008; Wang et al, 2006). Therefore, further studies on the function of other APIPs may identify a target of AvrPiz-t for the suppression of chitin-mediated defense pathway. Similarly, deep transcriptome analysis of AvrPiz-t transgenic plants along with the APIP6 and APIP10 RNAi plants after the treatment with PAMPs will reveal more insights into the AvrPiz-t-mediated molecular mechanism of defense suppression in rice.

Although knock-down lines of APIP6 didn’t show any cell death phenotype in Piz-t+ background, it is still possible that APIP6 is involved in the Piz-t-mediated defense. Recently, in silico analysis revealed that an alternative splicing form of APIP6 (APIP6 AS2) missing the N-terminal RING finger domain is expressed only in the lesion mimic mutant spl11 in rice. Furthermore, the attempt to generate dominant negative mutant rice plants by over-expressing APIP6 H58Y in Piz-t+ TRD background was not successful due to cell death in the calli (data not shown), raising the possibility that APIP6 AS2 might be involved in the Piz-t-mediated cell death. Over-expression lines of
APIP6 AS2 in Piz-t NPB and Piz-t+ TRD backgrounds will answer whether APIP6 AS2 is involved in the Piz-t-mediated HR and defense.

In Chapter 3, it has been shown that lysine residues in AvrPiz-t are required for its avirulence function. However, we don’t have any clue whether lysine is required for the virulence function of AvrPiz-t as well. In order to answer this question, two different approaches can be employed. First, LL AvrPiz-t as well as WT AvrPiz-t can be transformed into Piz-t- NPB background. If the ectopic expression of LL AvrPiz-t lost the ability to suppress PTI, it means lysine is required for virulence function. Instead, it might show strong suppression of PTI if lysine is not required for the virulence function because the AvrPiz-t is more stable in the rice plants. The other approach is to co-express GFP:LL AvrPiz-t:HA with FLAG:APIP6 H58Y in N. benthamiana leaves to monitor whether the former can destabilize the APIP6 H58Y protein.

To date, a total of 18 rice R genes, two rice blast quantitative trait loci (QTLs), and six Avr genes in M. oryzae have been cloned (Hayashi et al, 2010; Liu et al, 2010; Takahashi et al, 2010; Yuan et al, 2011; Zhai et al, 2011). However, little is known about the mechanism how the R protein recognize the avirulence protein directly or indirectly in rice blast pathosystem. In this project, we identified the E3 ligase APIP10 is a negative regulator of the AvrPiz-t-mediated resistance. The next question is whether APIP10 directly interacts with the Piz-t protein. If not, what the other protein is between them that is important for the AvrPi-z/Piz-t recognition? This question might be answered if we found that interacting proteins of APIP10 or Piz-t by Y2H or affinity purification. For example, when Pto was used as a bait in the Y2H screening of a cDNA library, several Pto-interacting (Pti) have been identified that are important components for the Pto
resistance (Munkvold & Martin, 2009). The generation of APIP10 over-expression lines with tandem repeat tags such as 5X Myc in NPB-Piz-t:HA lines will help us identify such proteins in the complexes of the Piz-t protein. Using the Co-IP approach, we already identified a band of proteins from *M. oryzae*-infected NPB-Piz-t:HA plant tissues (data not shown). Identification and functional analysis of the gene(s) may lead to the identification of missing protein(s) between APIP10 and Piz-t.

**Materials and methods**

*Fungal material*

For the generation of *M. oryzae* mutant expressing fluorescence fusion proteins, protoplasts of a field isolate O-137 (Valent et al, 1991) were used for transformation via the *Agrobacterium*-mediated procedure as described previously (Khang et al, 2006).

*Plasmids*

In general, plasmid construction was carried out by following standard molecular manipulation procedures (Sambrook & Russell, 2001)

For P\textsubscript{AVR-Piz-t}:AvrPiz-tCDS:mCherry:NLS construct, PCR amplified AvrPiz-t promoter and its entire 108-amino acids coding sequence with a primer pair AvrPiz-t-FPro-MfeI/ AvrPiz-t-R-BamHI was digested with *MfeI* and *BamHI* and cloned into *EcoRI/SalI* site of pBV436 vector which contains P\textsubscript{BAS4}:BAS4CDS:EGFP along with the sequence coding mCherry:NLS released from pBV591 by enzyme digestion with
For the expression of 3.5 X Myc-APIP10 in *N. benthamiana*, *APIP10* amplified with a primer pair AP10-5mycF/AP10-5mycR was cloned into pCAMBIA2300-Ubi-Myc-OCS vector, resulting in 5.5 X Myc:*APIP10*. And *Myc-APIP10* with multiple Myc sequences was amplified with a primer pair 5mycF/ APIP10-5mycR and inserted into *SacI/BamHI* site of pGD after digestion with *SacI* and *BamHI*. By sequencing, 3.5 X Myc:*APIP10* was chosen for further experiment.

For *Myc-GFP* construct, *GFP* amplified from pGD vector with a primer pair NtGFPF/tGFPR was cloned into pXUN-myc. After digestion with *BamHI*, Myc-GFP was inserted into *BamHI* site of pGD vector. For pGD-Flag APIP6 and pGD-Flag APIP6 H58Y constructs, either *APIP6* or *APIP6 H58Y* amplified with a primer pair APIP6-ProF/AP6-ProR was cloned into pXUN-Flag vector. And *BamHI* and *SalI* fragment of this construct was inserted into *BglII/SalI* site of pGD vector.

For *Tap tag* construct, *Tap tag* fragment amplified from the pUbi.nc1300.ntapintron vector with a primer pair Taptag-F/ Taptag-R was cloned into pGD for the expression in *N. benthamiana* as an internal control.

For the expression of GFP::AvrPiz-t::HA or GFP::LL AvrPiz-t::HA in *N. benthamiana*, either *AvrPiz-t-CHA* or *LL AvrPiz-t-CHA* was amplified with a primer pair AvrPiz-t-HAF1/ AvrPiz-t-HAR2. These fragments were digested with *BamHI* and *XhoI* and then inserted into *BglII/SalI* site of pGDG.

The primers used in this Chapter for cloning are described in Table 6.
Transformants of *M. oryzae* isolate O-137 co-expressing AvrPiz-t:mCherry:NLS, and BAS4:EGFP (Khang et al., 2010) were used to inoculate rice leaf sheaths following procedure as described previously (Khang et al., 2010). Leaf sheaths were incubated with fungal spores (3 $\times$ 10^4 spores ml^-1 in 0.25% gelatin) in the hollow interior of detached leaf sheaths of rice cultivar YT16, and the inner epidermal layer was excised immediately prior to microscopy. Confocal microscopy observation was performed on a Zeiss Axiovert 200 M microscope equipped with a Zeiss LSM 510 META system. A C-APOCHROMAT 40 ×/1.2 NA water immersion objective was used. Excitation/emission wavelengths were 488 nm/505 to 550 nm for EGFP, and 543 nm/560 to 615 nm for mCherry.

*Agroinfiltration assay in N. benthamiana*

*Agrobacterium tumefaciens* strain GV3101 carrying different constructs were grown at 28°C in a shaking incubator. After 18 hours of culture, bacterial cells were spun down with 3,200g for 20 minutes and were resuspended in MES buffer (10mM MgCl₂ and 10mM MES, pH 5.6) at a final OD₆₀₀ of 1.5 for testing constructs, 1 for p19 and 0.25 for TAP tag. After a final concentration of 150μM acetosyringone was added, bacteria suspensions were kept at room temperature under dark condition for 3 hours and used to infiltrate *N. benthamiana* plants as previously described (Goodin et al, 2002).

*Rice plant induction treatment, inoculation, and gene expression analysis*

To express *AvrPiz-t* in *Piz-t*⁺ rice, transgenic TRD plants carrying *NS-AvrPiz-t*
controlled by a β-estradiol-inducible system XVE were generated. Leaves of second
generation plants were cut off and cultured onto 1/2 MS medium containing 50 mM β-
estradiol. Chemical treated-leaves were also collected to determine gene expression at 12
hours after induction.

A punch inoculation method (Ono et al., 2001) with a slight modification was
used for infection of rice plants with rice blast fungus. *M. oryzae* isolate RB22 was
cultured on oat meal agar medium for two weeks. 10μl of spore suspension with
concentration of $5 \times 10^5$ spores ml$^{-1}$ was applied on slightly punctured sites of leaves
from 4-6 week-old plants. Lesion size was recorded at 10 days after inoculation. To
measure the sporulation rate, we cut off leaf portion of $3 \times 1$ cm$^2$ of the lesion and
immersed in the microcentrifuge tube containing 100 μl of distilled water with 1%
Tween20. The samples were vigorously vortexed for 2 minutes to dislodge the spores,
and the number of spores ml$^{-1}$ was counted under microscope using a hemacytometer.
Infection ratio was calculated using the method as described previously (Kawano et al.,
2010).

qRT-PCR assay was used to determine gene expression in rice plants or detached
tissues. Detached tissues after appropriate treatment of tissues from rice plants were used
to extract total RNAs using TRIZol reagent (Invitrogen) according to manufacturer’s
instruction. Total RNA samples were treated with DNase I (Invitrogen) to remove DNA
contamination by following manufacturer’s protocol. About 1 μg of DNase I-treated RNA
was subjected to first-strand cDNA synthesis using the Promega Reverse Transcription
System (Promega). qRT-PCR was carried out using iQ5 real-time PCR detection systems
(Bio-Rad). The primers used for qRT-PCR are described in table 6.

The measurement of ROS level

The last second leaf of 4-6 week-old rice plants were used for measurement of reactive oxygen species (ROS). Small circle of leaf discs (approximately 4 mm) were cut from the rice leaves with a cork borer and pre-incubated overnight in sterile distilled water. ROS generation after the treatment of elicitors to the leaf disks was monitored using luminol chemi-luminescence assay (Schwacke & Hager, 1992). Three pre-incubated leaf disks were immersed in the microcentrifuge tube containing 100 μl luminol (Bio-Rad), 1μl horseradish peroxidase (Jackson ImmunoResearch) and elicitor (100 nM flg22, 8 nM hexa-N-acetylchitohexaose or water control). Luminescence was measured continuously with10 sec of interval for 21 min using Glomax 20/20 luminometer (Promega). Three replications were performed for each treatment.

In vivo Co-IP assays

For in vivo Co-IP assay, Agrobacterium strain GV3101 carrying expression vectors of Myc:APIP10, FLAG:APIP6 H58Y or GFP:AvrPiz-t:HA were used to express proteins in N. benthamiana leaves via agroinfiltration. Two days after infiltration, N. benthamiana leaf tissues were harvested, and total proteins were extracted with a native buffer including 50 mM Tris-MES pH8.0, 0.5M sucrose, 1mM MgCl2, 10mM EDTA 5mM DTT and plant protease inhibitor. 10 μl of anti-HA agarose suspension (Sigma) was added to the protein samples and the mixtures were kept at 4 °C with head-to-tail shaking.
for overnight. The samples were washed three times using 1 × IP buffer according to the manufacturer’s instruction (Sigma). After adding 50 μl of 1 × SDS loading buffer, the column was heated to 95 °C for 5 min. About 15 μl of each sample was loaded to the protein gel for immunoblot analysis using anti-HA and anti-Myc antibodies.

In Vitro ubiquitination assay

Two E3 ligases fused with MBP and GST:AvrPiz-t:HA were expressed in E. coli strain Rosetta2 (DE3), and were affinity-purified using maltose (NEB) and glutathione-agarose (Sigma), respectively. In vitro ubiquitination assays were performed as described (Zeng et al., 2004) with the following reaction system: 1μg of MBP:APIP6 or MBP-APIP10, 1μg of GST-AvrPiz-t-CHA, 40 ng of yeast E1 (Biomol), 100 ng of Arabidopsis E2 (AtUBC10), 1 μg of ubiquitin and 1.5 μl of 20 × reaction buffer (1 M Tris HCl, pH 7.5, 40 mM ATP, 100 mM MgCl₂, 40 mM DTT, 600 mM creatine phosphate and 1 mg ml⁻¹ creatine phosphokinase). The reactions were incubated at 30°C for 1.5 hours in 30 μl of final volume before being stopped with SDS sample loading buffer and heated to 100°C for 5 min. The samples were subjected to SDS-PAGE followed by the immunoblot analysis using anti-ubiquitin antibody (Biomol, USA).

References


Felix G, Duran JD, Volko S, Boller T (1999) Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant J* **18**: 265-276


Ntoukakis V, Mucyn TS, Gimenez-Ibanez S, Chapman HC, Gutierrez JR, Balmuth AL,


Tanaka N, Che FS, Watanabe N, Fujiwara S, Takayama S, Isogai A (2003) Flagellin from an incompatible strain of Acidovorax avenae mediates H2O2 generation accompanying hypersensitive cell death and expression of PAL, Cht-1, and PBZ1, but
not of Lox in rice. Mol Plant Microbe Interact 16: 422-428


Figure 23. Cell death in rice cells containing the cognate R gene, Piz-t, by transient expression of AvrPiz-t. A. A map of construct used to generate AvrPiz-t-inducible transgenic rice plants. B. qRT-PCR to confirm induced expression of AvrPiz-t in transgenic rice at 24 hours after the treatment. Estradiol was dissolved in DMSO. Thus, DMSO was used as a mock. For the induction of AvrPiz-t, a piece of rice tissue was took from the same leaf, and cut as two parts for DMSO or 100 μM of estradiol treatment. 1 and 2 are two independent samples. C. Induction assay of transgenic AvrPiz-t rice leaves. D: DMSO and E: estradiol treatment. Experiment was conducted by Songbiao Chen.
Figure 24. Translocation of AvrPiz-t into rice cells. Fungal transformants expressing AvrPiz-t:mCherry:NLS and a putative interfacial matrix protein BAS4:EGFP at 30 hours after inoculation (hpi) in YT16 rice is shown as a projection of confocal optical sections taken over a depth of 4 μm. Merge shows bright-field, mCherry (red), and EGFP (green). The arrow indicates BIC, the arrowhead indicates rice nucleus, and yellow indicates overlapping mCherry and EGFP fluorescence signals. Pinhole settings were 1 airy unit for EGFP and 12.5 airy units for mCherry. Bar = 5 μm. mCherry fluorescence occurred in the BIC and in the nucleus of the invaded rice cell with brighter fluorescence in the presumed nucleolus. Construct and analysis were done by the author and ChangHyun Khang, respectively.
Figure 25. Suppression of basal defense responses to flg22 and chitin by ectopic expression of AvrPiz-t in rice

A, B. PAMP-induced reactive oxygen species (ROS) burst in AvrPiz-t NPB and control NPB lines by flg22 (A) and chitin (B). Rice leaf disks were treated with 100 nM flg22, 8nM chitin (hexa-N-Acetyl-Chitohexaose) or water. ROS were detected using a luminol-chemiluminescence assay. Error bars, s.e.m. (n=3).

C. Induction of defense related genes, KS4 (left) and PAL (right), at 1 hour post incubation (hpi) in either water, flg22 or chitin, respectively (grey bars indicate AvrPiz-t NPB and white bars indicate control NPB lines). DNA-based qPCR was performed using gene-specific primers. Data are means and error bars, s.e.m. (n=3). Constructs and following rice transformation were done by Songbiao Chen and other analyses were conducted by Gautam Shirsekar.
Figure 25. Suppression of basal defense responses to flg22 and chitin by ectopic expression of AvrPiz-t in rice
Enhanced susceptibility to the virulent *M. oryzae* isolate RB22 rendered by the ectopic expression of *AvrPiz-t* in rice. **A.** Infection assay, rice leaves of 6 weeks old plants were inoculated with the virulent isolate RB22 (picture taken 10 days post inoculation, dpi). **B.** Sporulation rate (left) and relative fungal growth (right) were measured 10 dpi. Data represent means, error bars are s.e.m. (*n*=8) Constructs and following rice transformation were done by Songbiao Chen and other analyses were done by Gautam Shirsekar.
Figure 27. Structure and E3 ubiquitin ligase activity assay of APIP6 and its RING finger domain mutant, APIP6 H58Y. A, Structure of APIP6. B, Scheme of APIP6 RING finger composition and the mutation of an amino acid in the RING finger domain. C, E3 ligase assay of MBP:APIP6 and its RING finger domain mutant, MBP:APIP6 H58Y, in the presence of Arabidopsis E1 (At5g06460), E2 (AtUBC10, At5g53300) and ubiquitin. MBP (lane 5) was used a negative control. Western blot was performed with anti-ubiquitin antibody to detect polyubiquitin bands.
Figure 28. Structure and E3 ubiquitin ligase activity assay of APIP10. 

A. Protein structure of APIP10. BRAP2, BRCA1-Associated Protein 2, RING finger, a C3HC4 type zinc-finger, and ZnF UBP, Zinc-Finger Ubiquitin Binding Protein. 

B. E3 ubiquitin ligase activity of APIP10. The MBP:APIP10 fusion protein was assayed for E3 ligase activity in the presence of Arabidopsis E1 (At5g06460), E2 (AtUBC10, At5g53300) and ubiquitin. MBP itself was used as a negative control (lane5). Western blot was performed with anti-ubiquitin antibody.
Figure 29. Ubiquitination of AvrPiz-t by APIP6 and suppression of APIP6 E3 ligase activity by the AvrPiz-t protein in vitro. In vitro ubiquitination assay of GST:AvrPiz-t:HA by the MBP:APIP6 fusion protein. Ubiquitination of AvrPiz-t by APIP6 was detected by western blot with anti-HA antibody (top). GST:AvrPi-ta:HA of an unrelated *M. oryzae* effector, AvrPi-ta, was used as a negative control for the specificity of AvrPiz-t ubiquitination by APIP6 (top, lane 9). Suppression of APIP6 E3 activity by the presence of AvrPiz-t was determined by western blot with anti-ubiquitin antibody (middle). GST:AvrPi-ta:HA as well as GST:HA was used as negative controls. Western blot with anti-MBP antibody was conducted to determine the amount of MBP:APIP6 or MBP protein loading in each lane (bottom).
Figure 30. Ubiquitination of AvrPiz-t by APIP10 and suppression of APIP10 E3 ligase activity by the AvrPiz-t protein \textit{in vitro}. Ubiquitination of AvrPiz-t by APIP10 was detected by western blot with anti-HA antibody (top). The GST:AvrPi-ta:HA fusion protein of an unrelated \textit{M. oryzae} effector, AvrPi-ta, was used as a negative control for the specificity of AvrPiz-t ubiquitination by APIP10 (top, lane 9). Suppression of APIP10 E3 activity by the presence of AvrPiz-t was determined by western blot with anti-ubiquitin antibody (middle). Western blot with anti-MBP antibody was conducted to determine the amount of MBP-APIP10 or MBP protein loading in each lane (bottom). MBP protein itself was used as a negative control for the ubiquitination of AvrPiz-t.
Figure 31. Expression of FLAG:APIP6 and FLAG:APIP6 H58Y in *N. benthamiana*. *Agrobacterum* carrying FLAG:APIP6 or FLAG:APIP6 H58Y was agroinfiltrated and tissues were harvested at 1 to 3 days after infiltration (DAI) as indicated. 50μM of MG132 was infiltrated at 2DAI and tissues were harvested at 3DAI. The TAP tag protein was expressed as an internal control and was detected by western blot with peroxidase anti-peroxidase (PAP). Transcriptional level of each gene expression was determined by sqRT-PCR.
Figure 32. Interaction between AvrPiz-t and APIP6 or APIP10 in vitro and in vivo. A. The interaction between BD-AvrPiz-t and AD-APIP6 in yeast cells. Cells were plated on SD-Leu-Trp-His media containing 50mM 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of the His3p enzyme to assess the growth of yeast. B. Co-IP analysis of FLAG:APIP6 H58Y and GFP:AvrPiz-t:HA in *N. benthamiana*. Since GFP-AvrPiz-t-HA gets degraded when it is co-expressed with APIP6, GFP:AvrPiz-t:HA and FLAG:APIP6 H58Y were co-expressed in *N. benthamiana* using agroinfiltration. Co-IP experiment was performed with anti-HA antibody and protein was analyzed by western blot using anti-FLAG and anti-HA antibody to detect APIP6 and AvrPiz-t, respectively. C. The interaction between BD-AvrPiz-t and AD-APIP10 in yeast cells. Cells were plated on SD-Leu-Trp-His media containing 50mM 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of the His3p enzyme to assess the growth of yeast. D. Co-IP analysis of Myc:APIP10 and GFP:AvrPiz-t:HA in planta. GFP:AvrPiz-t:HA and Myc:APIP10 proteins were expressed in *N. benthamiana* using agroinfiltration. Co-IP experiment was performed with anti-HA antibody and protein was analyzed by western blot using anti-Myc and anti-HA antibody to detect APIP10 and AvrPiz-t, respectively. Asterisk indicates rat IgG protein detected by the secondary anti-rat antibody from goat.

APIP6 and APIP10 constructs for Y2H were made by the author and AvrPiz-t construct for Y2H was made by Bo Zhou. Y2H between APIP6 and AvrPiz-t was conducted by Pattavipha Songkunmarn (Nun) and Y2H between APIP10 and AvrPiz-t was conducted by Songbiao Chen.
Figure 32. Interaction between AvrPiz-t and APIP6 or APIP10 in vitro and in vivo
Figure 33. Degradation of GFP:AvrPiz-t:HA with the co-expression of FLAG:APIP6 or Myc:APIP10. A. Co-expression of GFP:AvrPiz-t:HA either with FLAG:APIP6 or FLAG:APIP6 H58Y in *N. benthamiana*. FLAG:APIP6, FLAG:APIP6 H58Y, Myc:APIP10 or Myc:GFP was co-expressed with GFP:AvrPiz-t:HA by agroinfiltration and tissues were harvested at 2 DAI. 50µM of MG132 was infiltrated with DMSO as a control at 18hrs before sampling. The TAP tag protein was expressed as an internal control and was detected by western blot with peroxidase anti-peroxidase (PAP). Transcriptional level of each gene expression was determined by sqRT-PCR. H58Y: FLAG:APIP6 H58Y. B. Co-expression of GFP:AvrPiz-t:HA with either Myc:APIP10 or Myc:GFP in *N. benthamiana*. G:Myc:GFP.
Figure 33. Degradation of GFP:AvrPiz-t:HA with the co-expression of FLAG:APIP6 or Myc:APIP10.
Figure 34. Degradation of GFP:LL AvrPiz-t:HA by co-expression of FLAG:APIP6, not APIP6 H58Y. Either GFP:AvrPiz-t:HA or GFP:LL AvrPiz-t:HA was co-expressed with FLAG:APIP6 in N. benthamiana plants by agroinfiltration. Co-expression with FLAG:APIP6 H58Y was used as control. Tissues were collected 2 DAI. GFP:AvrPiz-t:HA and GFP:LL AvrPiz-t:HA was detected by western blot with anti-HA antibody. Transcriptional level of each gene expression was determined by sqRT-PCR.
Figure 35. Degradation of APIP6 and APIP10 with the co-expression of AvrPiz-t in *N. benthamiana*. A. Degradation of APIP6 with co-expression of AvrPiz-t in *N. benthamiana*. B. Degradation of APIP10 with co-expression of AvrPiz-t in *N. benthamiana*. Either Myc:APIP10 or FLAG:APIP6 H58Y was co-expressed with either empty vector, GFP:HA or GFP:AvrPiz-t:HA in *N. benthamiana*. G: GFP and -: empty vector, pGD. TAP tag was used as an internal control and transcriptional level of each gene expression was determined by sqRT-PCR.
Figure 35. Degradation of APIP6 and APIP10 with the co-expression of AvrPiz-t in *N. benthamiana*. 
Figure 36. Lesion mimic phenotype in the late developmental stage by knocking down of APIP10. Transformation and analysis were done by Songbiao Chen.
Figure 37. Comparison of *APIP10* RNAi lines in NPB and NPB-Piz-t:HA backgrounds. A. Cell death and dwarf phenotype of *APIP10* RNAi T1 lines in both NPB and NPB-Piz-t:HA backgrounds. *APIP10* RNAi lines in NPB-Piz-t:HA background showed more severe cell death and dwarf phenotype compared with those in NPB background. B. sqRT-PCR and western blot with *APIP10* RNAi lines from both backgrounds. sqRT-PCR was conducted to assess the knock-down of *APIP10* transcripts and the western blot was conducted with anti-HA to detect the level of the Piz-t proteins. HSP protein was used for the loading control.
Figure 37. Comparison of *APIP10* RNAi lines inNPB and NPB-Piz-t:HA backgrounds.
Figure 38. Compromised basal defense in rice by knocking-down of APIP6 expression. A,B. PAMP-induced reactive oxygen species (ROS) burst in APIP6 RNAi TRD and control TRD lines following flg22 (A) or chitin (B) treatment. Rice leaf disks were treated with 100 nM flg22, 8nM chitin (hexa-N-Acetyl-Chitohexaose) or water. ROS were detected using a luminol-chemiluminescence assay. Error bars, s.e.m. (n=3). C. Induction of defense related genes KS4 and PAL at 1hour post incubation hpi in water, chitin or flg22, respectively (grey bars indicate APIP6 RNAi and white bars indicate control TRD). qRT-PCR was performed using gene-specific primers. Data are means and error bars, s.e.m. (n=3). Constructs and following rice transformation were done by Songbiao Chen and other analyses were conducted by Gautam Shirsekar.
Figure 38. Compromised basal defense in rice by knocking-down of APIP6 expression.
Figure 39. Compromised basal defense in rice by knocking-down of APIP10 expression. A, B. PAMP-induced reactive oxygen species (ROS) burst in APIP10 RNAi NPB and control NPB lines following flg22 (A) or chitin (B) treatment. Rice leaf disks were treated with 100 nM flg22, 8nM chitin (hexa-N-Acetyl-Chitohexaose) and water. ROS were detected using a luminol-chemiluminescence assay. Error bars, s.e.m. (n=3). C. Induction of defense related genes KS4 and PAL at 1 hpi in water, chitin or flg22, respectively. Grey bars indicate APIP10 RNAi and white bars indicate control NPB. qRT-PCR was performed using gene-specific primers. Data are means and error bars, s.e.m. (n=3). Constructs and following rice transformation were done by Songbiao Chen and other analyses were conducted by Gautam Shirsekar.
Figure 39. Compromised basal defense in rice by knocking-down of APIP10 expression.
Figure 40. Spray inoculation of a virulent *M. oryzae* isolate RB22 on three weeks old APIP10 RNAi plants in both TRD and NPB background plants. Disease symptom on leaves and sheaths were observed 5 and 7 days post inoculation, respectively. APIP10i TRD: *APIP10* RNAi-TRD, APIP10i NBP: *APIP10* RNAi-NPB. Transformation and analysis were done by Songbiao Chen.
Figure 41. Enhanced susceptibility to the virulent *M. oryzae* isolate RB22 by the knockdown APIP6 or APIP10 in rice plants. A, C. Infection assay of *APIP6* and *APIP10* RNAi lines, respectively. Rice leaves of 6 weeks old plants were inoculated with a virulent isolate RB22 (picture taken 10 dpi). B, D. Sporulation rate (left) and relative fungal growth (right) on *APIP6* (B) and *APIP10* RNAi lines (D) were measured 10 dpi. Data represent means, error bars are s.e.m. (*n*=8). Grey bars indicate RNAi lines and white bars indicate control plants. **Constructs and following rice transformation were done by Songbiao Chen and other analyses were conducted by Gautam Shirsekar.**
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<th>Sequence</th>
<th>Purpose</th>
</tr>
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<td>Apiz-t-FPro-MfeI</td>
<td><code>5’CCCCAATTGTGATCCGTCGCTCTATCCCGTG3’</code></td>
<td>P\textsubscript{AVR-Piz-t:AvrPiz-tCDS:mCherry:NLS}</td>
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<td>Apiz-t-R-BamHI</td>
<td><code>5’CCCCGGATCTCTTGCGCTGAGCGCTCTG3’</code></td>
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<td>AP10-5mycF</td>
<td><code>5’CTTGAATCCATGGCGACCTCAGCTCCGAG3’</code></td>
<td>Cloning of 3.5 X Myc-APIP10 in pGD</td>
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<td>5mycF</td>
<td><code>5’TGCAGCTCGGTACCGATCGCTACGAAAGCT3’</code></td>
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<td>APIP6-proF</td>
<td><code>5’GCCCAGATCTCGAGAGCTCCGAGGCGG3’</code></td>
<td>APIP6 and APIP6 H58Y in pXUN-Flag</td>
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<td>APIP6-proR</td>
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<tr>
<td>NtGFPF</td>
<td><code>5’GATGGGTAAGGAGAAAAAGCTTTT3’</code></td>
<td>GFP in pCUN-Myc</td>
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<td>tGFPR</td>
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<tr>
<td>TapTag-F</td>
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<td>Cloning of Tap tag in pGD vector</td>
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<tr>
<td>TapTag-R</td>
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<td>AvPiz-HAF1</td>
<td><code>5’CCCCCTGACCTCGAGAGGTACATGCC3’</code></td>
<td>Cloning of AvrPiz-t:HA and LL AvrPiz-t:HA into pGDG vector</td>
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<td>TAP-RTR</td>
<td><code>5’GCTCTGCCATCTCAGGCTCTC3’</code></td>
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<td>sqRT-PCR of APIP6</td>
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<td>sqRT-PCR of APIP10</td>
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<tr>
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<td>HIP-RT-F</td>
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<td>KS4-RT-F</td>
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**Table 6.** List of primers used in Chapter 4.
Chapter 5: Summary and conclusion

Ubiquitination is one of the post-translational modifications and most of the ubiquitinated proteins are subjected to the degradation by the ubiquitin/26S proteasome system (Smalle & Vierstra, 2004). The number of ubiquitination-related proteins in plant genomes is much more than that in yeast and *Drosophila* (Vierstra, 2003), reflecting the importance of the ubiquitination process in plants. It is involved in many different aspects of biological processes in plants such as cell cycle, circadian rhythm control, hormone signaling pathway and senescence (Smalle & Vierstra, 2004). Recently, a growing body of evidence suggests that ubiquitination is also closely implicated in disease resistance in plants and pathogen’s strategies to suppress the innate immunity in host plants (Chapter 1).

In Chapter 2, we described the identification of sixty one and seventy seven putative U-box proteins from *Arabiopsis* and rice genome, respectively, using a battery of whole genome analysis approaches. Based on their domain compositions, the rice and *Arabidopsis* U-box proteins were classified into nine major classes. Most of the rice U-box protein genes are expressed, as supported by *in silico* gene expression analysis of the rice ESTs, full-length cDNAs, and massively parallel signature sequencing (MPSS) tags databases.
We assayed five randomly selected rice U-box proteins for E3 ligase activity assay *in vitro* and found that all of the five proteins have E3 ligase activity with different preferences for E2 enzymes. In semi-quantitative RT-PCR (sqRT-PCR) of seven OsPUBs, OsPUB57 showed a stronger expression only in resistant plants carrying the *Pi9* resistance gene. Furthermore, silencing of the *OsPUB57* gene in rice protoplasts leads to cell death, suggesting that OsPUB57 is a negative regulator of cell death signaling and is a good candidate for further functional analysis.

In Chapter 3, we identified the host targets of AvrPiz-t, an avirulence protein from the rice blast fungus *Magnaporthe oryzae*, by the yeast-two hybrid (Y2H) screens using AvrPiz-t as a bait. The twelve putative interacting proteins were named *AvrPiz*-t interacting proteins (APIPs). Among them, APIP2, 6 and 10 are predicted to be E3 ligases, suggesting that AvrPiz-t may target the ubiquitin proteasome system (UPS) for its virulence in rice cells. To confirm the interaction between AvrPiz-t and APIPs, we conducted the bimolecular fluorescence complementation (BiFC) experiments in rice protoplasts and observed a weak green fluorescence signal in each experiment probably due to the low expression levels of the AvrPiz-t and APIP proteins. In the *in vitro* E3 ligase assays, we found that AvrPiz-t suppresses the ligase activity of the three E3 ligases, while the APIP E3 ligases ubiquitinate AvrPiz-t. With a series of experiments to identify the mechanism of the suppression, we found that the AvrPiz-t protein does not contain either protease activity for E3 ligase or deubiquitination activity for linear and K48-linked polyubiquitin. Interestingly, we also found that lysine residue in the AvrPiz-t protein is not the ubiquitination target site *in vitro*. So far there are only few reports that cysteine, tyrosine, threonine and serine residues as well as lysine can be ubiquitinated by
E3 ligases (Grou et al, 2008; Wang et al, 2007). By using lysine-less ubiquitin (Ciechanover & Ben-Saadon, 2004; Patel et al, 2009), we found that AvrPiz-t contains two ubiquitination sites. In the analysis of the AvrPiz-t mutant proteins with different deletions and point mutations, we identified three deletion mutant proteins that are not ubiquitinated by APIP6. However, we were not able to pinpoint the exact ubiquitination sites, probably due to the limitations of the in vitro assay. Therefore, protein sequencing of the in vivo ubiquitinated AvrPiz-t using mass spectrometry may lead to the identification of the ubiquitination sites in AvrPiz-t.

Even though the lysine residues of AvrPiz-t are not the ubiquitination target sites by APIP6 in vitro, we observed that lysine-less AvrPiz-t mutant is more stable than wild type in rice protoplasts. Furthermore, degradation of AvrPiz-t is inhibited by MG132, an inhibitor of 26S proteasome system. These results suggest that the lysine residues in AvrPiz-t are probably the ubiquitination target sites of host E3 ligases in rice cells and the ubiquitinated AvrPiz-t is degraded through the UPS. Lysine-less AvrPiz-t is more stable than wild type and is most likely to have chances to be recognized by Piz-t in rice cells. Despite this fact, M. oryzae transformants carrying lysine-less AvrPiz-t lost its avirulence on the Piz-t+ Toride rice plants. This suggests that the ubiquitination of AvrPiz-t by host E3 ligases followed by degradation through 26S proteasome system might be required for its functional recognition by the cognate resistance protein Piz-t.

In Chapter 4, we aimed to understand the relationship among AvrPiz-t, Piz-t and two APIP E3 ligases as well as the function of AvrPiz-t and two APIPs in host innate immunity. First, we showed that AvrPiz-t is translocated from the infectious hyphae into rice cells where it supposedly interacts with Piz-t either directly or indirectly, triggering a
hypersensitive reaction (HR). By the ectopic expression of *AvrPiz-t* in transgenic rice, we also showed that *AvrPiz-t* contributes to the virulence for *M. oryzae* by the suppression of pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and reducing the resistance level of rice plants to the virulent isolate RB22. Remarkably, the expression of *AvrPiz-t* in *N. benthamiana* suppresses the accumulation of the APIP6 and APIP10 proteins, which is consistent with the *in vitro* data that *AvrPiz-t* interferes with their E3 ligase activity. Furthermore, APIP6 and APIP10 promote the degradation of *AvrPiz-t* in *N. benthamiana* plants, supporting the fact that *AvrPiz-t* is ubiquitinated *in vitro* by APIP6 and APIP10. With these results, we hypothesized that *AvrPiz-t* suppresses pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) through the manipulation of APIP6 and APIP10. Indeed, the knock-down lines of *APIP6* and *APIP10* showed enhanced susceptibility to the virulent isolate RB22 and suppression of the expression of the PTI-related defense genes, which are the similar phenotypes with the *AvrPi-z-t* transgenic plants. In addition, the knock-down lines of *APIP10* showed more severe spontaneous cell death in the *Piz-t+* background compared to that in the *Piz-t−* background. The western blot and sqRT-PCR analysis with the T1 *APIP10* knock-down plants in both *Piz-t+* and *Piz-t−* backgrounds revealed that the presence of the Piz-t protein affects the severity of the cell death phenotypes. The negative relationship between the transcription level of *APIP10* and the protein level of Piz-t in the *APIP10* knock-down lines in the *Piz-t+* background also supports the APIP10’s negative regulation over the Piz-t protein in rice plants.

Based on the above results, we proposed the following working model to explain the relationship among *AvrPiz-t*, Piz-t, APIP6 and APIP10 as illustrated in Figure 42.
After being secreted and translocated into rice cells, AvrPiz-t interacts and interferes with the E3 ligase activity of APIP6 and APIP10 to suppress the PTI triggered by PAMPs. To fight back, host E3 ligases such as APIP6 and APIP10 ubiquitinate AvrPiz-t, causing degradation of AvrPiz-t in rice cells. In the absence of the AvrPiz-t protein in rice cells, APIP10 regulates the Piz-t protein at a low level directly or indirectly through ubiquitination. However, when AvrPiz-t is delivered into rice cells to promote the degradation of APIP10, the negative regulation of APIP10 on the Piz-t protein fails and a rapid accumulation of the Piz-t protein triggers a strong HR and the activation of defense responses. In the Piz-t- background, the change in APIP10 might be detected by a cryptic resistance protein that controls relatively weak cell death and the activation of defense genes. Therefore, it will be interesting to monitor the accumulation of APIP6, APIP10 and AvrPiz-t in the Piz-t- NPB background rice after inoculating with the AvrPiz-t isogenic M. oryzae transformants. The barley stem rust resistance protein, RPG1, is degraded through the 26S proteasome system in response only to avirulent pathotypes of Puccinia graminis f. sp. tritici (Nirmala et al, 2007). In our case, we expect that the level of the Piz-t protein accumulates upon the infection with an avirulent transformant with AvrPiz-t but not with a virulent one.

Although we have made considerable progress in dissecting the AvrPiz-t-Piz-t interaction pathway, many interesting questions remain to be answered. First, what is the mechanism of AvrPiz-t mediated suppression of the E3 ligase activity of APIP6 and APIP10 and PTI in rice plants? Second, how is AvrPiz-t degraded in rice cells and what is the impact of AvrPiz-t’s ubiquitination by APIP6 and APIP10 on its degradation? To answer the first and second questions, we have generated stable transgenic rice plants
expressing *AvrPiz-t* or *lysine-less AvrPiz-t* in the Piz-t- background. Third, if APIP10 and Piz-t do not interact directly, what are their interacting proteins and their role in blast resistance? To answer this question, we are going to conduct Y2H with APIP10 as a bait and to assess the function of its interacting proteins in rice innate immunity. In addition, we will assess whether Piz-t interacts with APIP10’s interacting proteins in rice plants as well as in yeast. We have already identified a few Piz-t-interacting proteins by immunoprecipitation in rice cells and we will determine whether they interact with APIP10 or not. The final question is what is the function of other APIPs in the AvrPiz-t-Piz-t-interaction pathway? This question will be answered by the generation of the stable over-expression and knock-down lines of these genes in both Piz-t− and Piz-t+ backgrounds. Future experiments designed toward answering these questions will reveal many exciting insights into the plant and fungal interactions.

Rice is a model for molecular biology and genome studies in cereals because of its small genome size, extensive genetic map, available genome sequence, and relative ease of transformation. Furthermore, comparative mapping studies show that most cereal genomes share a similar gene content and long stretches of collinear gene order. For example, shotgun sequencing of Nipponbare genome revealed that homologs of 98% of the known maize, wheat, and barley genes are found in rice. Because of the conservation of gene sequences among cereals, the functional analysis of important rice genes such as APIP6 and APIP10 described in this dissertation may have broad practical implications for the other economically important cereals. The better understanding of the molecular mechanism underlying broad-spectrum resistance mediated by the Piz-t and AvrPiz-t
interaction will help us design new disease control strategies for rice and other economically important cereals.

References


Figure 42. Proposed model for the functions of the APIP6, APIP10 and AvrPiz-t proteins in rice innate immunity. Upon its delivery into rice cells, AvrPiz-t suppresses PTI through the manipulation of host E3 ligases, APIP6 and APIP10, which positively regulate PTI. APIP10 negatively regulates the accumulation of the Piz-t protein, a broad-spectrum resistance gene product. Therefore, the suppression of APIP10 by AvrPiz-t leads to the accumulation of the Piz-t protein, which triggers a strong hypersensitive reaction (HR) and subsequent defense responses. To fight back the suppression by AvrPiz-t protein, APIP10 and APIP6 ubiquitinates the AvrPiz-t protein leading to its degradation. In the absence of Piz-t, the suppression of APIP10 might leads to a weak HR and defense response due to the activation of cryptic R proteins.


Felix G, Duran JD, Volko S, Boller T (1999) Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant J* **18**: 265-276


arabidopsis PLANT U-BOX17 and its functional tobacco homolog ACRE276 are required for cell death and defense. Plant Cell 18: 1084-1098


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