A fungal effector and a rice NLR protein have antagonistic effects on a Bowman–Birk trypsin inhibitor

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Summary
Bowman–Birk trypsin inhibitors (BBIs) play important roles in animal and plant immunity, but how these protease inhibitors are involved in the immune system remains unclear. Here, we show that the rice (Oryza sativa) BBI protein APIP4 is a common target of a fungal effector and an NLR receptor for innate immunity. APIP4 exhibited trypsin inhibitor activity in vitro and in vivo. Knockout of APIP4 in rice enhanced susceptibility, and overexpression of APIP4 increased resistance to the fungal pathogen Magnaporthe oryzae. The M. oryzae effector AvrPiz-t interacted with APIP4 and suppressed APIP4 trypsin inhibitor activity. By contrast, the rice NLR protein Piz-t interacted with APIP4, enhancing APIP4 transcript and protein levels, and protease inhibitor activity. Our findings reveal a novel host defence mechanism in which a host protease inhibitor targeted by a fungal pathogen is protected by an NLR receptor.

Introduction
Phytopathogens have evolved multiple strategies to attack plants, including the secretion of effector proteins into host cells. During their coevolution with pathogens, plants have developed receptors and other proteins that recognize pathogen factors to trigger immune responses (Jones and Dangl, 2006; Jones et al., 2016). Pathogens and plants both employ proteases and protease inhibitors to modulate invasion and defence, respectively (Hou et al., 2018). Some pathogen effectors inhibit host proteases, thus subverting plant immunity (Hou et al., 2018; Misas-Villamil and Hoorn, 2008). For instance, the potato (Solanum tuberosum) late blight pathogen Phytophthora infestans secretes two divergent Kazal-like protease inhibitors, EP1P and EP110, which target the tomato (Lycopersicon esculentum) subtilase P69B (Tian et al., 2005; Tian et al., 2004). Similarly, the cystatin-like protease inhibitors EPIC1 and EPIC2B from P. infestans target the tomato defence proteases C14 and Rcr3 (Kaschani et al., 2010; Song et al., 2009). In addition, the Ustilago maydis effector protein Pt12 inhibits a set of maize (Zea mays) cytotoxic proteases to undermine host resistance (Mueller et al., 2013). These studies indicate that the suppression of host proteases by pathogen inhibitors is a common strategy to subvert host immunity. By contrast, only a few studies have demonstrated that pathogen proteases target host protease inhibitors during infection. For example, the P. sojae apoplastic effector PsXEG1 targets a soya bean (Glycine max) apoplastic glucanase inhibitor protein, GmGIp1, to impair infection (Ma et al., 2014). To protect PsXEG1 from inhibition by GmGIp1 in the apoplast, P. sojae secretes PsXLP1, a paralog of PsXEG1 (Ma et al., 2016). However, it is unknown whether the cytoplasmic effectors from pathogens target host protease inhibitors to subvert immunity.

Although proteases and protease inhibitors are involved in pathogen-associated molecular pattern-triggered immunity or basal resistance against pathogens (Chen et al., 2014; Hoorn, 2008; Quilis et al., 2014), only a few studies have reported their functions in resistance (R) protein-mediated immunity. A well-studied example comes from research on the interaction between the tomato R protein Cf-2 and its cognate effector Avr2 from the fungal pathogen Cladosporium fulvum (Rooney et al., 2005). Avr2 physically interacts with the protease Rcr3 and inhibits its activity. Absence of Rcr3 activity in rcr3 mutants does not trigger the resistance response mediated by Cf-2 (Rooney et al., 2005), suggesting that the R protein Cf-2 guards the virulence target Rcr3 of the effector Avr2. Another example is the Arabidopsis cysteine protease RD19, which re-localizes to the nucleus after interacting with the Ralstonia solanacearum effector PopP2. PopP2 and RD19 form a complex in the nucleus to activate the RRS1-mediated resistance response (Bernoux et al., 2008). However, it has not yet been reported that a protease inhibitor is the target of an avirulence effector and is modulated by the corresponding R protein in plants.

The Bowman–Birk trypsin inhibitors (BBIs) are widely known in soya bean and belong to the serpin superfamily of protease inhibitors, which possess trypsin and chymotrypsin activity (Birk, 1985). A growing body of evidence suggests that BBIs act as anticancer and chemopreventive agents by suppressing cancer cell proliferation (Fereidunian et al., 2014). Studies in animal models have proven that dietary BBIs from several legume species, including soya bean, pea (Vicia faba), lentil (Lens culinaris) and chickpea (Cicer arietinum), can prevent or suppress carcinogenic and inflammatory processes within the gastrointestinal tract (Clemente and Arques Mdel, 2014). Moreover, BBIs from plants such as wheat (Triticum aestivum) and maize can strongly inhibit the growth of plant pathogenic fungi (Chen et al., 1999; Chilosi et al., 2000). These results suggest that the BBIs play an important role in animal and plant immunity.
Rice blast, caused by the fungal pathogen *Magnaporthe oryzae*, is a devastating disease of rice, a crop that feeds half the world’s population (Talbot, 2003; Wang and Valent, 2017). *Piz-t* encodes a nucleotide-binding and leucine-rich repeat (NLR) R protein that confers broad-spectrum disease resistance and is widely used in rice breeding (Wu et al., 2017; Zhou et al., 2006). The effector AvrPiz-t from *M. oryzae* is the corresponding avirulence effector of *Piz-t* (Li et al., 2009), but these two proteins do not interact directly (Park et al., 2012). In this study, we report that the *M. oryzae* effector AvrPiz-t targets and inhibits the activity of the rice BBI APIP4 (AvrPiz-t Interacting Protein 4) to facilitate infection. Interestingly, the rice NLR protein *Piz-t* interacts with APIP4 and enhances its accumulation and trypsin inhibitor activity. Our study thus revealed a novel mechanism in which a fungal pathogen effector suppresses a host protease inhibitor to attack the plant immune system, and in which an NLR protein weakens this attack by acting on the same protease inhibitor.

**Results**

**AvrPiz-t interacts with APIP4 in vitro and in vivo**

*Oryza sativa* AvrPiz-t is the avirulence effector of *Piz-t* in rice (Li et al., 2009) and is secreted into the cytoplasm of rice cells at the early stages of infection (Park et al., 2012). APIP4 was identified as one of the AvrPiz-t-interacting proteins in a yeast two-hybrid Y2H screen in our previous study (Park et al., 2012). APIP4 contains a signal peptide, transmembrane domain (TM domain) and three BowB domains (Figure 1a). To confirm the interaction between AvrPiz-t and APIP4, a yeast two-hybrid assay was conducted using the AvrPiz-t (without the signal peptide) and APIP4-C (containing the full intracellular domain) fragments. Colony growth on selective media containing 35 mM 3-amino-1,2,4-triazole (3AT) validated the interaction between AvrPiz-t and APIP4-C (Figure 1b). Two unrelated effectors, AvrPi9 and AvrPii (Wu et al., 2015; Yoshida et al., 2009), did not interact with APIP4-C (Figure 1a), demonstrating that AvrPiz-t specifically interacts with APIP4.

Next, we performed a GST pull-down assay with purified maltose-binding protein (MBP)-fused AvrPiz-t (MBP-AvrPiz-t) and GST-fused APIP4-C (GST-APIP4-C), as well as the empty control GST. Immunoblot analysis showed that the GST-fused APIP4-C proteins bound to MBP-AvrPiz-t, whereas GST alone did not (Figure 1c). Finally, we confirmed the interaction between AvrPiz-t and APIP4 in rice protoplasts using a co-immunoprecipitation (Co-IP) assay. Plasmids of GFP:AvrPiz-t and HA:Luc:APIP4 were co-transfected into *N. benthamiana* (NBP) rice protoplasts. Immunoblot analysis specifically detected AvrPiz-t in the APIP4 immunocomplex proteins (Figure 1d, upper panel in the last lane). Overall, these results suggest that AvrPiz-t specifically interacts with APIP4 in vitro and in vivo.

AvrPiz-t was found to be secreted into rice cells during rice infection (Park et al., 2016). To determine whether APIP4 is also localized in the cytoplasm, we transfected APIP4-GFP and mCherry empty plasmids into rice protoplasts. Fluorescence signals of APIP4-GFP were clearly observed mainly in the cytoplasm (Figure S2a), indicating that APIP4 is a cytoplasmic protein. The protein level of APIP4 in rice protoplasts was confirmed by immunoblot analysis (Figure S2b). Furthermore, we found that APIP4 had overlapped signals in the cytoplasm with AvrPiz-t (Figure S2c).

**AvrPiz-t interferes with APIP4 trypsin inhibitor activity in vitro and in vivo**

APIP4 encodes a Bowman–Birk-type trypsin inhibitor (BBI), which is classified as a member of the serine protease inhibitors (Clemente et al., 2011). To determine whether APIP4 is a functional inhibitor, we tested its activity in *Escherichia coli* and in rice protoplasts. Trypsin inhibitory ability was determined by end-point analysis (James et al., 2017). Both the APIP4 and the APIP4 mutant, APIP4K198A (Terada et al., 1978), which bears a K-to-A substitution in the protease-binding site (Figure S3), were fused with the MBP at its N terminus. The trypsin inhibitor assay showed that increasing amounts of the MBP-APIP4 fusion protein led to decreasing trypsin activity, indicating it is a functional trypsin inhibitor, which is similar to the positive control, soya bean BBI (SBBI) (James et al., 2017) (Figure 2a). However, the mutant MBP-APIP4K198A showed much lower trypsin inhibitor activity than that of MBP-APIP4 (Figure 2a), indicating that the protease-binding site in APIP4 is essential for its trypsin inhibitor activity.

To explore the APIP4 biochemical function in planta, we conducted a trypsin inhibitor activity assay for APIP4 and APIP4K198A in rice protoplasts. The APIP4 and APIP4K198A fragments were cloned into the pRTVnHA vector under the control of the maize Ubiquitin promoter (He et al., 2018). Plasmids of APIP4 and APIP4K198A fused with a HA tag were transfected into NBP protoplasts, and a HA-Luc construct was used as the negative control. Compared to HA-Luc, the APIP4 protein in 50 µL of rice protoplasts produced about a 57% reduction in trypsin inhibitor activity (Figure 2b). In contrast, APIP4K198A had about a 28% reduction in activity. The protein levels of APIP4 and APIP4K198A in the rice protoplasts were confirmed by immunoblot analysis (Figure S4a). These results demonstrate that APIP4 functions as a trypsin inhibitor both in vitro and in vivo.

To test whether AvrPiz-t affects APIP4 trypsin inhibitor activity in vitro and in vivo, we purified MBP-APIP4, His-fused AvrPiz-t, and AvrPiz-t (C70A) (Wang et al., 2016), which bears a C-to-A substitution and leads to a weak interaction with APIP4 in yeast (Figure S1a). PCR results confirmed the presence of AvrPiz-t, AvrPiz-t (C70A) and APIP4 in yeast cells (Figure S1b). A trypsin inhibitor activity assay showed that the difference in the activity of APIP4 in the absence and presence of AvrPiz-t was substantial (Figure 2c). When AvrPiz-t was added to the reaction, the trypsin inhibitor activity of APIP4 decreased dramatically, whereas adding AvrPiz-t (C70A) did not affect the activity substantially. This suggests that AvrPiz-t inhibits the activity of APIP4, while the mutant AvrPiz-t (C70A) has lost this inhibitory ability.

Subsequently, we co-transfected plasmids of HA-APIP4 with Myc-AvrPiz-t, Myc-AvrPiz-t (C70A) and Myc-Luc to determine whether AvrPiz-t affects APIP4 activity in rice protoplasts. Compared to the control Myc-Luc, APIP4 trypsin inhibitor activity was significantly reduced in cells co-expressing AvrPiz-t, but was only reduced to about 50% when co-expressed with AvrPiz-t (C70A) (Figure 2d). The protein levels of Myc-AvrPiz-t, Myc-AvrPiz-t (C70A) and HA-APIP4 in the reactions were confirmed using immunoblots (Figure S4b). Overall, these results demonstrate that AvrPiz-t suppresses APIP4 trypsin inhibitor activity in vitro and in vivo.

**APIP4 positively regulates resistance to *M. oryzae***

To investigate the role of APIP4 in the immunity of rice to *M. oryzae*, we generated transgenic rice plants that contained...
insertion and deletion mutations generated by CRISPR/Cas9, or that overexpressed the APIP4 gene, using Agrobacterium-mediated transformation (Qu et al., 2006). To knock out APIP4, we designed a guide RNA to target a 20-nt sequence in the APIP4 gene for Cas9 cleavage (Figure S5) and cloned it into the pBYY02-OsCas9-ccdB vector (Zhou et al., 2014). After confirming the mutation types of the transgenic lines with PCR and sequencing analyses, we selected two apip4 T1 knockout lines (apip4-1 and 5) for disease phenotype evaluations. apip4-1 carried a 7-bp deletion in one strand and a 29-base deletion in the second strand, and apip-5 carried a 1-bp insertion in one strand and a 2-bp deletion in the second strand (Figure S5). These knockout lines grew normally without any obvious growth penalty. Then, these two APIP4 knockout lines were punch-inoculated with the virulent strain RB22 using NPB as the control. The mutant plants showed elevated susceptibility to RB22, with increased lesion area and fungal biomass, compared with NPB (Figure 3a–c).

To overexpress APIP4, we cloned the full-length gene into the pCXUN vector under the control of the maize Ubiquitin promoter (Chen et al., 2009). We selected two of the T2 homozygous APIP4 overexpression lines (APIP4-OX 7-6 and 16-2) and confirmed their elevated APIP4 RNA levels by qRT-PCR, and their protein expression levels by immunoblotting (Figure S6a,b). Similarly, these overexpression lines also grew normally without any obvious phenotype changes. Next, when the APIP4-OX plants were inoculated with the M. oryzae virulent isolate RB22, smaller lesions over a smaller area and a decrease in fungal biomass were observed in the overexpression plants compared with the control NPB plants (Figure 3d–f).

The expression levels of defence marker genes such as OsPR1a and OsPAD4 were consistently up-regulated in the APIP4-OX lines 7-6 and 16-2 compared with NPB plants (Figure 3g, h). Thus, the results from the knockout and overexpression lines suggest that APIP4 functions as a positive regulator in the resistance to M. oryzae.

**APIP4 trypsin inhibitor activity is modulated in the AvrPiz-t transgenic rice plants**

To further determine the APIP4 biochemical function in rice, we detected APIP4 trypsin inhibitor activity in the protoplasts of the two APIP4-OX lines (7-6 and 16-2) generated above using NPB as the control. The assay revealed that the APIP4 proteins in the two overexpression lines exhibited a higher ability to inhibit trypsin with increasing amounts of the protoplasts isolated from the two overexpression lines (Figure 3i). To confirm the above results that AvrPiz-t suppresses APIP4 trypsin inhibitor activity, we attempted to detect APIP4 activity in the AvrPiz-t transgenic plants generated in our previous study (Park et al., 2012). The HA-APIP4 plasmids were transfected into protoplasts isolated from NPB and the AvrPiz-t transgenic plants. The assay showed that APIP4 activity in the AvrPiz-t transgenic rice protoplasts was significantly reduced compared with that in the NPB protoplasts when equal amounts of rice protoplasts were tested (Figure 4a), confirming that AvrPiz-t genuinely inhibits APIP4 activity. The protein levels of APIP4 were the same in the NPB and AvrPiz-t rice protoplasts (Figure S7a). To exclude the possibility that AvrPiz-t affects APIP4 stability, we investigated the APIP4 transcript and protein levels in the AvrPiz-t transgenic plants and found that AvrPiz-t did not affect either APIP4 transcription or protein stability (Figure 4b,c).

**Piz-t increases APIP4 trypsin inhibitor activity, transcript levels, and protein accumulation**

Because AvrPiz-t is indirectly recognized by the cognate resistance protein Piz-t in rice to induce immunity (Li et al., 2009), we
hypothesized that APIP4 may be associated with Piz-t-mediated resistance. To test this hypothesis, we first tested whether Piz-t can regulate APIP4 activity. We transfected plasmids of Myc-APIP4 into rice protoplasts isolated from Piz-t-HA and NPB plants and measured APIP4 trypsin inhibitor activity. The assay revealed that APIP4 activity increased more quickly in the Piz-t-HA rice protoplasts than in the NPB protoplasts when equal amounts of rice protoplasts were tested (Figure 4d). Next, we determined whether Piz-t can potentiate APIP4 transcript and APIP4 protein levels. RNA and protein were isolated from two-week-old NPB and Piz-t-HA plants. RT-PCR and immunoblot analyses showed that both the transcript and protein levels of APIP4 were constitutively higher in the Piz-t-HA plants than in the NPB plants (Figure 4e, f). To further investigate whether APIP4 is induced during M. oryzae infection, we spray-inoculated two-week-old NPB and Piz-t-HA plants with the M. oryzae virulent isolate RB22 and avirulent isolate RB22-AvrPiz-t, which was a transformant with the AvrPiz-t gene in the RB22 background. Immunoblot analysis using the proteins isolated from RB22-AvrPiz-t-infected plants indicated that more APIP4 accumulated in Piz-t-HA plants compared with NPB plants. APIP4 rapidly accumulated 24 h after inoculation in Piz-t-HA plants but only after 48–96 h in NPB plants (Figure 5a, b). This indicated that APIP4 accumulates earlier and at higher levels in the Piz-t-HA background when infected with an AvrPiz-t-containing strain than when non-infected. Meanwhile, immunoblot analysis using the protein isolated from the RB22-infected Piz-t-HA plants indicated that less APIP4 was accumulated at 24 h after inoculation compared with the avirulent isolate RB22-AvrPiz-t (Figure S8a and Figure 5b). Taken together, these results suggest that Piz-t enhances APIP4 trypsin inhibitor activity, transcript and protein accumulation and APIP4 may contribute to Piz-t-mediated resistance. To further determine whether APIP4 protein accumulation in NPB and Piz-t-HA plants after inoculation with RB22-AvrPiz-t depends on the transcriptional reprogramming, we performed qRT-PCR with the same samples used in Figure 5a,b and found that APIP4 transcription levels were only induced at the early stage, implying that APIP4 protein accumulation does not depend on the transcriptional reprogramming (Figure S8b).

**APIP4 interacts with Piz-t, and knocking out APIP4 affects Piz-t immunity to a virulent M. oryzae strain**

To further determine the relationship between APIP4 and Piz-t, we performed a Co-IP assay using the protein isolated from NPB and Piz-t-HA transgenic plants with the anti-HA and anti-APIP4 antibodies. The immunoblot analyses showed that the expected APIP4 band was detected using the endogenous APIP4 antibody in the Piz-t-HA immunoprecipitated proteins but not in NPB plants (Figure 5c). Furthermore, we used split-luciferase image (LCI) analysis to determine which Piz-t domains interact with APIP4 in Nicotiana benthamiana (Chen et al., 2008). The truncated Piz-t fragments, NT, NBS, and LRR, were fused to the C terminus of the LUC gene in the pCAMBIA-CLuc vector for the generation of CLuc-NT, NBS and LRR constructs. The APIP4 gene was fused to the N terminus of LUC in the pCAMBIA-NLuc vector (APIP4-NLuc). AvrPiz-t fused to the N terminus of LUC in the pCAMBIA-NLuc vector (AvrPiz-t-NLuc) was used as the negative control. The LCI analysis showed that co-expression of APIP4-NLuc with CLuc-NT or CLuc-NBS produced strong LUC activity but not with CLuc-LRR (Figure 5d), indicating that both the NT and NBS fragments of Piz-t interact with APIP4. Strong fluorescence was also detected with a low-light imaging system in N. benthamiana leaves in which the NT and NBS fragments of Piz-t and APIP4 were co-infiltrated, but not the LRR domain (Figure S9a). These results demonstrate that...
APIP4 interacts with the NT and NBS domains of Piz-t in vitro and in vivo.

To examine whether Piz-t-mediated rice blast resistance depends on APIP4, we generated APIP4 knockout lines in the Piz-t-HA background (apip4/Piz-t-HA) using the CRISPR/Cas9 method. We selected two knockout lines (apip4/Piz-t-HA 6 and 21) for phenotype evaluations. The mutations in the two transgenic lines were confirmed by PCR and sequencing analyses. We found that apip4/Piz-t-HA 6 carries a 2-bp deletion in one strand and a 1-bp insertion in the second strand and that apip4/Piz-t-HA 21 is a homozygous line carrying a 1-bp insertion (Figure S9b), both of which truncated the APIP4 open reading frame. When the apip4/Piz-t-HA plants were challenged with the avirulent isolate RO1-1, we found that the transgenic plants had the similar resistance level as the Piz-t-HA plants (Figure S9c). However, when we inoculated the apip4/Piz-t-HA plants with the virulent isolate RB22, the plants showed enhanced susceptibility to M. oryzae with a greater lesion area and more fungal biomass compared with the Piz-t-HA plants (Figure 5e-g). These results imply that knocking out APIP4 does not affect Piz-t-mediated resistance against avirulent isolates, but could decrease the resistance against virulent isolates.

Figure 3 APIP4 positively regulates rice immunity against M. oryzae. (a) Punch inoculation of the apip4 plants. Leaves of eight-week-old rice plants were inoculated with the virulent isolate RB22. The leaves were photographed at 14 dpi. (b, c) Relative lesion area (left) and relative fungal biomass (right) were measured at 14 dpi. Data shown are means of three replicates. Error bars indicate the SEM (***P < 0.01; n = 3). (d) Punch inoculation of APIP4-OX and NPB plants. Leaves of eight-week-old rice plants were inoculated with the virulent isolate RB22. The leaves were photographed 14 d post-inoculation (dpi). (e, f) Relative lesion area (left) and relative fungal biomass (right) were measured at 14 dpi. Data shown are means of three replicates. Error bars indicate the standard error of the mean (SEM) (***P < 0.01; n = 3). (g, h) Induction of defence-related genes OsPR1a (g) and OsPAD4 (h) in the APIP4-OX transgenic and NPB plants by qRT-PCR. Significance was determined at *P < 0.05 and **P < 0.01 (n = 3) with a t-test. (i) Trypsin inhibitor activity of APIP4 in the APIP4-OX transgenic plants. In vivo trypsin inhibitor activity was detected by incubation of an increasing volume of the protoplasts isolated from APIP4-OX transgenic plants with trypsin at 37°C for 20 min. NPB was used as the negative control.
Discussion

The *M. oryzae* cytoplasmic effector directly binds and interferes host protease inhibitor activity

Plant pathogens secrete both protease inhibitors and proteases into host cells for immune suppression. Many protease inhibitors act as effectors to target the host proteases and thus subvert plant immunity (Shabab et al., 2008). How host protease inhibitors inhibit pathogen proteases for defence is not fully understood. A recent study showed that the *P. sojae* apoplastic effectors PsXEG1 and PsXLP1 target the soya bean apoplastic protease inhibitor GmGIP1 (Ma et al., 2016), suggesting a ‘bodyguard’ strategy in which the paralogous decoy PsXLP1 protects the virulence factor PsXEG1 in the extracellular space (Paulus et al., 2017).

In this study, our results revealed that the cytoplasmic effector AvrPiz-t targets a host trypsin inhibitor, APIP4. We demonstrated that APIP4 is a functional BBI, which exerts the trypsin inhibitor activity in *vitro* and in *vivo*. Furthermore, AvrPiz-t can specifically target and inhibit APIP4 trypsin inhibitor activity in *vitro* and in *vivo*. Notably, the APIP4 overexpression plants show enhanced trypsin inhibitor activity, while the APIP4 trypsin inhibitor activity is significantly reduced in the AvrPiz-t transgenic rice protoplasts. Although the protease from *M. oryzae* that targets APIP4, how AvrPiz-t affects APIP4 trypsin inhibitor activity and whether the trypsin inhibitor activity of APIP4 is indeed involved in rice immunity are still unclear, our study suggests that *M. oryzae* effectors can target host protease inhibitors in the intracellular space.

The rice BBI APIP4 confers enhanced resistance against the destructive rice pathogen *M. oryzae*

Soya bean BBIs confer antitumour, anti-inflammatory and antiviral activities in mammals (Kennedy, 1998; Safavi and Rostami, 2012). For example, a soya bean BBI can suppress MMP-2 and MMP-9 enzyme activity to inhibit the growth of the two adenocarcinoma cells AGS and HT29 (Fereidunian et al., 2014). And the soya bean BBI can block HIV entry into macrophages (Ma et al., 2018). BBI homologs were also identified as antimicrobial proteins in wheat, maize and rice (Chen et al., 1999; Chilosi et al., 2000; Qu et al., 2003). However, the molecular mechanisms of BBI-mediated immunity are largely unknown. In this study, we demonstrated that the rice BBI APIP4 plays a positive role in rice immunity. After *M. oryzae* infection, the level of APIP4 protein greatly increased. Overexpression of APIP4 in transgenic rice consistently enhanced rice blast resistance, while knocking out APIP4 increased susceptibility to this pathogen. The positive role of APIP4 in resistance can be explained by the up-regulation of defence-related marker genes in the APIP4 overexpression plants. However, how APIP4 interacts with other proteins to trigger defence responses remains unknown.

The function of the rice BBI APIP4 is reinforced by the NLR protein Piz-t

In this study, we found that APIP4 interacts with rice blast NLR Piz-t in *vivo* and Piz-t increases APIP4 trypsin inhibitor activity when they are co-expressed. In addition, both the APIP4 and Piz-t proteins are significantly up-regulated in the rice blast infected rice protoplasts. The rice BBI APIP4 confers enhanced resistance against the destructive rice pathogen *M. oryzae*.
transcript level and APIP4 protein accumulation are induced in the Piz-t-expressing plants. This is similar to the relationship between the transcription factor WRKY45 and the rice blast NLR protein Pb1 (Inoue et al., 2013). WRKY45 interacts with Pb1, and transient expression of Pb1 in rice protoplasts stabilized WRKY45 accumulation and enhanced its transactivation activity. Genetic analysis demonstrated that Pb1-mediated blast resistance is dependent on WRKY45 (Inoue et al., 2013). However, different from the relationship between Pb1 and WRKY45, knocking out APIP4 in the Piz-t-HA background does not affect Piz-t resistance to an avirulent strain, while it enhances susceptibility to a virulent strain.

Our previous studies showed that APIPS positively regulates Piz-t protein accumulation and APIP10 negatively regulates Piz-t accumulation (Park et al., 2016; Wang et al., 2016). Thus, it is possible that other Piz-t-associated proteins, such as APIPS and APIP10, or unknown proteins, have redundant functions in the regulation of Piz-t-mediated resistance when APIP4 is knocked out. APIP4 in the Piz-t-HA background does not affect Piz-t resistance to an avirulent strain, while it enhances susceptibility to a virulent strain.

Our working model for the relationship among the AvrPiz-t, APIP4 and Piz-t proteins during M. oryzae infection

Based on our results, we propose a working model to illustrate the relationship among AvrPiz-t, APIP4 and Piz-t (Figure 6). Upon M. oryzae infection, M. oryzae secretes the effector AvrPiz-t into the cytoplasm, where it interacts with APIP4, inhibiting APIP4 activity to impair plant immunity (Figure 6a). In the Piz-t-expressing plants, Piz-t interacts with APIP4, and enhances its transcript and protein levels, and inhibitor activity, possibly reducing the attenuation by AvrPiz-t (Figure 6b). Our work demonstrates a novel mechanism in which a protease inhibitor from a host is targeted by the effector from a pathogen, and also maybe act as a downstream component of the host NLR protein. Overall, our study reveals a novel host–microbe relationship in which a BBI inhibitor can be modulated by a pathogen cytoplasmic effector, but an NLR receptor can reinforce its inhibitor activity for plant immunity.
**Experimental procedures**

**Plant materials and growth conditions**

Rice (Oryza sativa L.) lines, including NPB and the Piz-t transgenic line in NPB (NPB-Piz-t). Rice transgenic materials for phenotypic analysis, including CRISPR or overexpression plants, are from T2 generation. Rice plants are grown in pots and kept in a growth chamber at 26°C and 70% relative humidity with the cycle of 12-h light/12-h dark.

**Punch inoculation of rice leaves**

Spore concentration in the suspension was adjusted to 5 x 10^5 conidia/mL before punch inoculation. The rice leaves were lightly punched with a mouse ear punch, and a 10 µL volume of spore suspension was dropped onto the punched sites of leaves. The spore suspension was secured by sealing with Scotch tape on both sides. The relative fungal biomass was measured with DNA-based quantitative PCR (qPCR) using the threshold cycle value (CT) of the M. oryzae MoPot2 gene against the CT value of the rice ubiquitin gene (Park et al., 2012). All inoculation experiments were repeated three times independently.

**Rice protoplast isolation and transfection**

Ten-day-old etiolated rice seedlings grown on 1/2 MS medium in the dark were used for protoplast isolation (He et al., 2016). The sheath and stem of seedlings were cut into 0.5-mm strips and soaked in cell wall digestion buffer (1.5% cellulase RS, 0.75% Piz-t. (a) Upon infection, the effector AvrPiz-t is secreted into M. oryzae-containing plants, Piz-t directly binds APIP4, -containing plants, Piz-t directly binds APIP4, which may lead to the enhancement of APIP4 protein level (red solid arrow) and trypsin inhibitor activity to attenuate AvrPiz-t interference (red dashed arrow towards AvrPiz-t) and trigger downstream immune responses.

**Trypsin inhibition activity measurement in vitro and in vivo**

Trypsin inhibition activity was determined as previously described with slight modifications (James et al., 2017). SBBI from soya bean was used as a positive control (Sigma-Aldrich). In the reaction, increasing amounts of proteins or increasing volumes of the protoplast lysate were added to 50 µL of 20 µg/mL bovine pancreatic trypsin (Sigma-Aldrich) dissolved in 50 mM Tris-Cl and 20 mM CaCl₂, pH 8.0, and incubated for 15 min at 37°C. Trypsin activity was determined by adding 125 µL of 1 mM N-a-benzoyl-DL-arginine-p-nitroanilide (BAPNA) substrate (Sigma-Aldrich) dissolved in 50 mM Tris-HCl, 20 mM CaCl₂, pH 8.0, and 1% (w/v) dimethyl sulfoxide, and incubated at 37°C for 20 min. The reaction was stopped with the addition of 25 µL of 30% (v/v) acetic acid. Total reaction volume was 0.25 mL. The absorbance was measured at 410 nm.

**In vitro pull-down assay**

The GST fusion proteins, GST: APIP4-C, and the MBP fusion proteins, MBP:AvrPiz-t and MBP:APIP4, were expressed in E. coli. After cell lysis, GST and GST-APIP4-C were mixed with an equal amount of the MBP fusion proteins, and then, the mixture was added to 1ml binding buffer (50 mM Tris at pH 8.0, 120 mM NaCl, 1 mM DTT, 0.5 % NP-40, 1 mM PMSF). The mixture was incubated at room temperature for 1 h with gentle shaking, and then, 20 µL glutathione-Sepharose beads (GE Healthcare) were added, followed by incubation at room temperature for 1 h (Shi et al., 2018). The beads were then washed five times with washing buffer (50 mM Tris at pH8.0, 500 mM NaCl, 1 mM DTT, 0.5% NP-40, 1 mM PMSF). Immunoblots were used for detection of fusion proteins with the anti-MBP and anti-GST antibodies.

**In vivo Co-IP assay**

For Co-IP experiments using rice protoplasts, total protein was extracted with native extraction buffer [50 mM Tris-MES at pH 8.0, 0.5 M sucrose, 1 mM MgCl₂, 10 mM EDTA, 5 mM DTT and protease inhibitor cocktail (Roche, Mannheim, Germany)]. For anti-HA Co-IP, the extracted proteins were pre-rinsed with 30 µL protein A agarose for 1 h. The pre-rinsed proteins were incubated with 2 µg anti-HA antibody and protein A agarose for 4 h. The agarose was washed five times with PBST buffer. The bound proteins were boiled with SDS loading buffer for 5 min in water and detected by the anti-HA (Roche) and anti-GFP (Roche) antibodies.

**Yeast two-hybrid (Y2H) assay**

The matchmaker GAL4 two-hybrid system (Invitrogen, Carlsbad, CA) was used for Y2H assay. The coding regions without the signal peptide of AvrPiz-t, AvrPiz-t (C70A), AvrPi9 and AvrPii were cloned into the pDBleu vector. The APIP4-C (containing the full
intracellular domain) and OsBBT15-C fragments were cloned into the pPC86 vector. Constructs were co-transformed into the yeast strain MaV203 (Wang et al., 2016). Positive clones were selected on medium without Leu-Tryp-His but with 35mM 3AT.

In vivo split-luciferase assay

APIP4 and AvrPiz-t were fused to the N terminus of LUC in the pCAMBIA-NLuc vector, and Piz-t-NBS/LRR was fused to the C terminus of LUC in the pCAMBIA-CLuc vector (Chen et al., 2008). A. tumefaciens strain EHA105 containing the indicated constructs was infiltrated into 5- to 6-week-old N. benthamiana leaves. Three leaf discs were taken after 2 d, and incubated with 200 μL of 100 μM luciferin in a 96-well plate, and luminescence was measured with the GLOMAX 96 microplate luminometer (Promega, Sunnyvale, CA). Each experiment was performed at least three times.

Rice protein extraction and immunoblotting

Total protein was extracted from the wild-type and transgenic rice seedlings with protein denaturing buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 % NP-40, 4 mM urea and protease inhibitor cocktail) or native buffer (50 mM Tris-MES at pH 8.0, 0.5 M sucrose, 1 mM MgCl2, 10 mM EDTA, 5 mM DTT and protease inhibitor cocktail). Supernatants were collected twice by centrifugation at 15,000 g for 15 min. Total protein was separated in SDS-PAGE and detected by immunoblotting with the antibodies indicated.

Accession number

The accession number of the APIP4 gene reported in this paper is LOC_Os01g03340.

Acknowledgements

This work was supported by grants from the National Key Research and Development Program of China (2016YFD0100600) and the National Natural Science Foundation of China (31822041 and 31972225) to Y.N.

Conflict of interest

The authors declare no conflicts of interest.

Author contributions

C.Z., H.F., X.S., R.W. and F.H. performed the experiments; Y.N., G-L.W. and C.Z. designed the experiments; Y.N., G-L.W. and C.Z. analysed the data; C.Z. wrote the paper. All authors have discussed the results and have read and approved the final manuscript.

References

Bird, Y. (1985) The Bowman-Birk inhibitor. Int. J. Peptide Protein Res. 25, 113–131.

Chen, Z.Y., Brown, R.L., Lax, A.R., Cleveland, T.E. and Russin, I.S. (1999) Inhibition of plant-pathogenic fungi by a corn trypsin inhibitor overexpressed in Escherichia coli. Appl. Environ. Microbiol. 65, 1320–1324.

Chen, H., Zou, Y., Shang, Y., Lin, H., Wang, Y., Cai, R., Tang, X. and et al. (2008) Firefly luciferase complementation imaging assay for protein-protein interactions in plants. Plant Physiol. 146, 368–376.

Chen, S., Songkumarn, P., Liu, J. and Wang, G.L. (2009) A versatile zero background T-vector system for gene cloning and functional genomics. Plant Physiol. 150, 1111–1121.

Chen, P.J., Sentilkhumar, R., Jane, W.N., He, Y., Tian, Z. and Yeh, K.W. (2014) Transplastomic Nicotiana benthamiana plants expressing multiple defence genes encoding protease inhibitors and chitinase display broad-spectrum resistance against insects, pathogens and abiotic stresses. Plant Biotechnol. J. 12, 503–515.

Chilosi, G., Caruso, C., Caporale, C., Leonardi, L., Bertini, L., Buzi, A., Nobile, M. et al. (2000) Antifungal activity of a Bowman Birk type trypsin inhibitor from wheat kernel. J. Phytopathol. 148, 477–481.

Clemente, A. and Arques Midel, C. (2014) Bowman-Birk inhibitors from legumes as colorectal chemopreventive agents. World J. Gastroenterol. 20, 10305–10315.

Clemente, A., Sonnante, G. and Domoney, C. (2011) Bowman-Birk Inhibitors from legumes and human gastrointestinal health: current status and perspectives.Curr. Protein Pept. Sci. 12, 358–373.

Feredian, M., Sadeghalvad, M., Oscoe, M.O. and Mostafaie, A. (2014) Soybean Bowman-Birk protease inhibitor (BBI): identification of the mechanisms of BBI suppressive effect on growth of two adencocarcinoma cell lines: AGS and HT29. Arch. Med. Res. 45, 455–461.

He, F., Chen, S., Ning, Y. and Wang, G.L. (2016) Rice (Oryza sativa) protoplast isolation and its application for transient expression analysis. Curr. Protoc. Plant Biol. 1, 373–383.

He, F., Zhang, F., Sun, F., Ning, Y. and Wang, G.L. (2018) A versatile vector toolkit for functional analysis of rice genes. Rice 11, 27.

van der Hoorn, R.A. (2008) Plant proteases: from phenotypes to molecular mechanisms. Annu. Rev. Plant Biol. 59, 191–223.

Hou, S., Jamieson, P. and He, P. (2018) The cloak, dagger, and shield: proteases in plant-pathogen interactions. Biochem. J. 475, 2491–2509.

Inoue, H., Hayashi, N., Matsuishi, A., Xin provisioning, L., Nakayama, A., Sugano, S., Jiang, C.J. et al. (2013) Blast resistance of CC-NB-LRR protein Pbl1 is mediated by WRKY45 through protein-protein interaction. Proc. Natl. Acad. Sci. USA, 110, 9577–9582.

James, A.M., Jayasena, A.S., Zhang, J., Berkowitiz, O., Secco, D., Knott, G.J., Whelan, J. et al. (2017) Evidence for ancient origins of Bowman-Birk Inhibitors From Selaginella moellendorffii. Plant Cell, 29, 461–473.

Jones, J.D. and Danjil, I.J. (2006) The plant immune system. Nature, 444, 323–329.

Jones, J.D., Vance, R.E. and Danjil, J.L. (2016) Intracellular innate immune surveillance devices in plants and animals. Science 354, 6316.

Kaschani, F., Shabab, M., Bozkurt, T., Shindo, T., Schornack, S., Gu, C., Iyas, M. et al. (2010) An effector-targeted protease contributes to defense against Phytophthora infestans and is under diversifying selection in natural hosts. Plant Physiol. 154, 1794–1804.

Kennedy, A.R. (1998) The Bowman-Birk inhibitor from soybeans as an anticarcinogenic agent. Am. J. Clin. Nutr. 68, 14065–14125.

Li, W., Wang, B., Wu, J., Lu, G., Hu, Y., Zhang, X., Zhang, Z. et al. (2009) The Magnaporthe oryzae avirulence gene AvrPiz-t encodes a predicted secreted protein that triggers the immunity in rice mediated by the blast resistance gene Piz-t. Mol. Plant Microbe Interact. 22, 411–420.

Ma, Z., Zhu, L., Song, T., Wang, Y., Zhang, Q.Xia, Y., Qui, M.et al. (2016) A paralogous decoy protects Phytophthora sojae apoplastic effector PSE1 from a host inhibitor. Science 355, 710–714.

Ma, T.C., Le, G., Zhou, R.H., Wang, X., Liu, J.B., Li, J.L., Zhou, Y. et al. (2018) Soybean-derived Bowman-Birk inhibitor (BBI) blocks HIV entry into macrophages. Virolology 513, 91–97.

Misas-Villamil, I.C. and van der Hoorn, R.A. (2008) Enzyme-inhibitor interactions at the plant-pathogen interface. Curr. Opin. Plant Biol. 11, 380–388.

Mueller, A.N., Ziemann, S., Treitschke, S., Assmann, D. and Doehlemann, G. (2013) Compatibility in the Ustilago maydis-maize interaction requires inhibition of host cysteine proteases by the fungal effector Piz-t. PLoS Pathog. 9, e1003177.
Park, C.H., Chen, S., Shirakara, G., Zhou, B., Khang, C.H., Songkumarn, P., Afzal, A.J. (2012) The Magnaporthe oryzae effector AvrPiz-t targets the RING E3 ubiquitin ligase AP106 as a potential therapy for autoimmune diseases. Plant Physiol. 159, 1433–1443.

Shabab, M., Shindo, T., Gu, C., Kaschani, F., Pansuriya, T., Chintha, R., Harzen, J.D.G. and Wit, P.J.G.M.D. (2005) Cladosporium Avr2 inhibits tomato Rcr3 immunity by modulating a host potassium channel. Mol. Plant Microbe Interact. 18, 1261–1269.

Talbot, N.J. (2003) On the trail of a cereal killer: exploring the biology of plant pathogens target the tomato defense protease Rcr3.

Tosa, Y. et al. (2009) Association genetics reveals three novel avirulence genes from the rice blast fungal pathogen Magnaporthe oryzae. Plant Physiol. 149, 1573–1585.

Wang, G.-L. and Valient, B. (2017) Durable resistance to rice blast. Science 355, 906–907.

Wang, R., Ning, Y., Shi, X., He, F., Zhang, C., Fan, J., Jiang, N. et al. (2016) Immunity to rice blast disease by suppression of effector-triggered necrosis. Curr. Biol. 26, 2399–2411.

Wu, J., Kou, Y., Bao, J., Li, Y., Tang, M., Zhu, X., Ponaya, A. et al. (2015) Comparative genomics identifies the Magnaporthe oryzae avirulence effector AvrP19 that triggers P9-mediated blast resistance in rice. New Phytol. 206, 1463–1475.

Wu, Y., Chen, J., Pan, C., Xiao, N., Yu, L., Li, Y., Zhang, X. et al. (2017) Development and evaluation of near-isogenic lines with different blast resistance alleles at the Piz locus in japonica rice from the lower region of the Yangtze River. China. Plant Dis. 101, 1283–1291.

Tian, M., Benedetti, B. and Kamoun, S. (2005) A Second Kazal-like protease inhibitor from Phytophthora infestans interacts with the apoplastic pathogenness-related protease P69B of tomato. Plant Physiol. 138, 1785–1793.