Susceptibility factor RTP1 negatively regulates Phytophthora parasitica resistance via modulating UPR regulators bZIP60 and bZIP28

Xiaoyu Qiang,1,2 Xingshao Liu,1,2 Xiaoxue Wang,1,2 Qing Zheng,2,3 Lijuan Kang,1,2 Xianxian Gao,1,2 Yushu Wei,1,2 Wenjie Wu,1,2 Hong Zhao,2,3 and Weixing Shan1,2,*

1 College of Agronomy, Northwest A&F University, Yangling, Shaanxi 712100, China
2 State Key Laboratory of Crop Stress Biology for Arid Areas, Northwest A&F University, Yangling, Shaanxi 712100, China
3 College of Plant Protection, Northwest A&F University, Yangling, Shaanxi 712100, China

*Author for communication: wxshan@nwafu.edu.cn
†Senior author.
X.Q, X.L., and X.W. contributed equally to this work.
X.Q. and W.S. conceived and designed the research. X.Q., X.L., X.W., Q.Z., L.K., X.G., Y.W., and H.Z. performed the experiments. X.Q., X.L., X.W., X.G., Y.W., and W.W. analyzed data. X.Q. and W.S. wrote the article with help of all authors.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (https://academic.oup.com/plphys/pages/general-instructions) is: Weixing Shan (wxshan@nwafu.edu.cn).

Abstract

The unfolded protein response (UPR) is a conserved stress adaptive signaling pathway in eukaryotic organisms activated by the accumulation of misfolded proteins in the endoplasmic reticulum (ER). UPR can be elicited in the course of plant defense, playing important roles in plant–microbe interactions. The major signaling pathways of plant UPR rely on the transcriptional activity of activated forms of ER membrane-associated stress sensors bZIP60 and bZIP28, which are transcription factors that modulate expression of UPR genes. In this study, we report the plant susceptibility factor Resistance to Phytophthora parasitica 1 (RTP1) is involved in ER stress sensing and rtp1-mediated resistance against P. parasitica is synergistically regulated with UPR, as demonstrated by the simultaneous strong induction of UPR and ER stress-associated immune genes in Arabidopsis thaliana rtp1 mutant plants during the infection by P. parasitica. We further demonstrate RTP1 contributes to stabilization of the ER membrane-associated bZIP60 and bZIP28 through manipulating the bifunctional protein kinase/ribonuclease IRE1-mediated bZIP60 splicing activity and interacting with bZIP28. Consequently, we find rtp1bzip60 and rtp1bzip28 mutant plants exhibit compromised resistance accompanied with attenuated induction of ER stress-responsive immune genes and reduction of callose deposition in response to P. parasitica infection. Taken together, we demonstrate RTP1 may exert negative modulating roles in the activation of key UPR regulators bZIP60 and bZIP28, which are required for rtp1-mediated plant resistance to P. parasitica. This facilitates our understanding of the important roles of stress adaptive UPR and ER stress in plant immunity.

Introduction

To circumvent invasion by a plethora of pathogens, plants have evolved a two-layered innate immune system. Perception of pathogen-associated molecular patterns (PAMPs) constitutes the first layer of plant innate immunity and is referred to as PAMP-triggered immunity (PTI). The
second layer is effector-triggered immunity that is often accompanied by hypersensitive response (HR; Jones and Dangl, 2006; Dodds and Rathjen, 2010). These innate immune systems often rely on basic cellular processes to defend pathogenic invasion, such as the endoplasmic reticulum (ER) quality control (ER-QC) system (Li et al., 2009) and hormone signaling (Tsuda et al., 2009). Thus, insights into the key regulators of these cellular processes during plant–pathogen interactions reveal orchestrating factors in plant immunity and defense mechanisms.

In eukaryotic cells, secreted and transmembrane proteins are translocated into the ER, where they are properly folded and modified through the sophisticated ER-QC system before being transported to their functional destination (Liu and Howell, 2010). Under abiotic or biotic stress, unfolded or misfolded proteins accumulate in the ER lumen, leading to the occurrence of ER stress. To release ER stress and restore ER homeostasis, ER membrane-associated stress sensors, such as the transcription factors (TFs) bZIP28 and bZIP60 subsequently activate the unfolded protein response (UPR; Liu et al., 2007; Iwata et al., 2008; Howell, 2013). The UPR comprises induction of ER chaperones and foldases (e.g. binding proteins, BiP), attenuation of protein translation and potentiation of protein secretion as well as degradation (Liu and Howell, 2010, 2016; Jäger et al., 2012; Korner et al., 2015). In plants, there are at least two signaling pathways of UPR that are mediated either by inositol requiring enzyme 1 (IRE1)/BiP or by bZIP28 (Korner et al., 2015). IRE1 is a bifunctional protein kinase (PK)/ribonuclease (Deng et al., 2013). The activation of the IRE1-mediated unconventional splicing of mRNA is the most conserved UPR pathway in eukaryotes (Chen and Brandizzi, 2013; Ruberti et al., 2015). In plants, the sensor domain of IRE1 binds to the ER-luminal BiP and the full-length bZIP60 is anchored to the ER membrane under normal conditions. In response to ER stress, BiP dissociates from IRE1 to assist proper folding of the accumulated unfolded proteins (Iwata and Koizumi, 2005; Howell, 2013). The released IRE1 is dimerized to align its cytosolic kinase domain in such a way that they trans-autophosphorylate each other to activate further splicing of bZIP60 mRNA (Nagashima et al., 2011; Iwata and Koizumi, 2012; Mishiba et al., 2013). The spliced bZIP60 mRNA derived protein is transported into nucleus, which functions as TF to activate UPR genes (Deng et al., 2011; Humbert et al., 2012). In the other pathway of UPR, bZIP28 TF acts as ER stress sensor and is crucial to activate UPR (Iwata and Koizumi, 2012). Under normal conditions, the bZIP28 and cochaperone protein are anchored to the ER membrane by interacting with BiP (Williams et al., 2010; Li et al., 2017). In response to ER stress, BiP dissociates from bZIP28 and the released bZIP28 traffics to the Golgi and is proteolytically cleaved by site-1 (S1P) and site-2 proteases (S2P), which further translocates into the nucleus, where it functions to activate UPR genes (Liu and Howell, 2010; Srivastava et al., 2012; Iwata et al., 2017).

Increasing evidence demonstrates that proper ER function and UPR regulation play crucial roles in plant immunity. For instance, disturbance of ER-QC results in improper processing of the pattern-recognition receptor EFR and impairs plant immunity (Nekrasov et al., 2009; Saijo et al., 2009). Furthermore, the IRE1/bZIP60-mediated UPR pathway is of importance for inducing systemic-acquired resistance (SAR) against bacterial pathogens and abiotic stress tolerance (Moreno et al., 2012). Intriguingly, in rice (Oryza sativa L.), the underlying SAR-mediated priming effect depends on WRKY33, a gene that is known to be involved in salicylic acid (SA)-mediated defense in A. thaliana (Wakasa et al., 2014). Moreover, OsWRKY46 was reported to play a key role in SA-induced plant immunity and is induced by OsIRE1-mediated ER stress, indicating functional integration of plant immunity with ER stress signaling (Hayashi et al., 2012). SA is an important plant hormone, which regulates plant immunity, particularly upon infection by biotrophic pathogens (Glazebrook, 2005). As a master regulator of SA-dependent responses to pathogens, NPR1 was recently reported to interact with UPR TFs bZIP28 and bZIP60 in the nucleus and negatively regulates the activation of UPR independently from SA (Lai et al., 2018). Notably, the ER-localized FKBP15-2 protein is a direct target of P. capsici effector PcAvr3a12 and is a positive immune factor in regulating ER stress-mediated plant immunity (Fan et al., 2018).

During a compatible plant–pathogen interaction, there are key regulators which facilitate pathogen invasion into host plant, namely, susceptibility factors (Pavan et al., 2010; van Schie and Takken, 2014). They could either manipulate the plant target proteins which are recognized by pathogens or negatively regulate plant immune response to facilitate compatible plant–pathogen interaction. Because the loss-of-function or mutation of susceptibility genes enhances plant durable resistance and confers broad-spectrum resistance to host plant, they are potentially applied in molecular breeding for crop disease resistance. For instance, the natural variation in the promoter of Bsr-d1, which is a crucial regulator of rice blast disease resistance, improves plant resistance without obvious loss of rice yield and quality (Li et al., 2017). However, the molecular mechanisms of how ER-associated or ER-regulated processes participate in plant immunity mediated by susceptibility factors remain less understood.

Oomycetes, particularly Phytophthora species, cause serious crop diseases, such as potato late blight, and threaten the sustainable crop production worldwide (Fry, 2008). Because of the prominent variation of pathogen virulent genes and loss-of-function of genotype-specific plant resistant genes during evolution, understanding and utilization of novel factors that confer broad-spectrum and durable resistance have attracted great attention in the field of plant–oomycete interaction researches. Phytophthora parasitica is a typical hemibiotrophic oomycete pathogen, which has a wide range of hosts and seriously threatens agricultural
production (Meng et al., 2014). With the established compatible interaction system between A. thaliana and P. parasitica (Wang et al., 2011), the susceptibility factor resistance to Phytophthora parasitica 1 (RTP1) was identified to render A. thaliana more resistant to infection by pathogens including P. parasitica (Pan et al., 2016). Further studies have demonstrated that RTP1 encodes an ER membrane-localized protein and affects the transcription of SA-responsive gene PR1 during the early infection stage (Pan et al., 2016). However, the mechanism of how RTP1 negatively regulates plant resistance remains largely unknown.

In this study, we address the question whether rtp1-mediated plant resistance is synergistically regulated with ER stress signaling. We show that the loss of functional RTP1 exhibits altered tolerance to ER stress induced by tunicamycin (TM), a well-established ER stress inducer. Simultaneously, it displays stronger induction of UPR and ER stress-associated immunity in response to early infection by P. parasitica. Our analyses further demonstrate that RTP1 contributes to stabilization of the ER membrane-bound stress sensors bZIP60 and bZIP28 through manipulating the bifunctional protein kinase/ribonuclease IRE1-mediated bZIP60 splicing activity and interacting with bZIP28. On the basis of these results and the evidence of compromised resistance in rtp1bzip60 and rtp1bzip28 mutants shown in this work, we propose that RTP1 functions as a negative regulator of the activation of bZIP60 and bZIP28 UPR TFs, this work, we propose that RTP1 functions as a negative regulator of the activation of bZIP60 and bZIP28 UPR TFs, which are required for rtp1-mediated plant resistance to P. parasitica. Together, this work identifies the susceptibility factor RTP1 as a critical modulator of the plant UPR and facilitates to understand convergence of signaling decoding in UPR and plant immunity.

**Results**

**RTP1 is involved in general ER stress and UPR regulation**

According to a previous study, RTP1 encodes an ER-localized protein that mediates plant susceptibility to P. parasitica (Pan et al., 2016). This finding prompted us to question whether RTP1 is involved in ER stress and the subsequent UPR signaling pathway, which mediates the immune function of RTP1 against P. parasitica. To test this, 5-d-old seedlings of A. thaliana wild-type (WT) Col-0 and rtp1 mutant were treated with TM to induce ER stress by inhibiting N-linked glycosylation. Seedlings treated with dimethyl sulfoxide (DMSO) were used as controls. At 5 d post treatment, the fresh weight of the seedlings was measured. The results showed ~40% of reduction in fresh weight for the TM-treated WT Col-0 seedlings compared with that of the DMSO-treated seedlings. In contrast, TM treatment resulted in around 55% of biomass reduction in the rtp1 mutants compared with control DMSO treatment (Figure 1A), suggesting that rtp1 mutants seem hypersensitive to TM-induced ER stress.

To further examine whether RTP1 is involved in ER stress, seedlings of WT Col-0 and rtp1 mutant were initially grown on medium supplemented with TM, followed by 10 d of growth without TM. Thereafter, the recovery rate of seedlings was calculated as described (Moreno et al., 2012). Around 80% of WT Col-0 seedlings were rescued from TM treatment. In comparison, rtp1 mutants exhibited distinct chlorosis and noticeable growth retardation, showing merely 20% of seedlings recovered. Notably, this reduced recovery rate was complemented in RTP1-OE transgenic lines, since in these lines ~85% of seedlings rescued from TM treatment (Figure 1B). Arabidopsis thaliana γvpe had been documented to be involved in ER stress-mediated cell death and γvpe mutants were shown insensitive to TM (Qiang et al., 2012). Expectedly, γvpe mutants were less affected by TM, exhibiting ~70% recovery rate (Figure 1B). In parallel, we examined the recovery rate of seedlings upon pretreatment with another ER stress inducer, dithiothreitol (DTT), a reducing agent that blocks disulfide bridge formation. Consistently, rtp1 mutants exhibited much less recovery rate in comparison to WT Col-0, while the recovered seedlings in RTP1-OE transgenic lines were increased to the comparable level of WT Col-0 (Figure 1C). These results imply that RTP1 is involved in the TM- and DTT-induced ER stress responses.

To further examine whether RTP1 regulates ER stress sensing and the subsequent UPR signaling, 2-week-old WT Col-0 and rtp1 mutant seedlings were treated with TM and the transcript levels of ER stress sensor genes bZIP28 and bZIP60, as well as UPR marker genes BiP3 and DnaJ (ER stress-induced genes, participating in ER-localized protein folding; Iwata et al., 2008) were analyzed by reverse transcription quantitative PCR (RT-qPCR). The results showed that expressions of bZIP60, BiP3, and DnaJ were clearly induced in WT Col-0 upon treatment of TM. In comparison, levels of their expressions were significantly increased in TM-treated rtp1 mutants, particularly at 12-h post treatment (Figure 1D). Although the expression of bZIP28 was slightly induced by TM treatment, its transcript level was elevated in rtp1 mutants compared with WT Col-0 (Figure 1D). Collectively, these results indicate that RTP1 functions in general ER stress and UPR regulation.

**RTP1 negatively modulates activation of UPR and ER stress-responsive plant immunity during infection by P. parasitica**

To further investigate whether RTP1 functions in UPR signaling in A. thaliana upon infection by P. parasitica, 2-week-old WT Col-0 and rtp1 mutant seedlings were inoculated with P. parasitica zoospores and the transcript levels of ER stress sensor genes bZIP28, bZIP60, as well as UPR marker genes BiP3 and DnaJ in WT Col-0 and rtp1 mutants during early colonization of P. parasitica were analyzed by RT-qPCR. The results showed expression of bZIP28, bZIP60, BiP3, and DnaJ

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**References**


Wang, Z., Chen, J., Sun, Y., Li, Y., & Pan, Z. (2011). vpe mutants are less affected by TM compared with WT Col-0 (Figure 1C). These results imply that RTP1 is involved in the TM- and DTT-induced ER stress responses.


**Figure 1** RTP1 is involved in sensing TM- or DTT-induced ER stress and UPR regulation. A. The *Arabidopsis thaliana* WT Col-0 and rtp1 mutants show reduced biomass upon treatment by ER stress inducer TM. Plant biomass was determined at 5 d post treatment. Relative fresh weight was plotted by calculating the biomass of treatment/control seedlings. Data presented show means of three independent experiments ± SE. For each experiment, 20 plants were analyzed per treatment. Asterisks indicate significance at *P < 0.05* analyzed by Student’s t test. The phenotype of Col-0 and rtp1 mutant seedlings at 5 d post treatment by TM was recorded. B and C, *Arabidopsis thaliana* seedlings of WT Col-0, rtp1 mutants,
was induced in WT Col-0 during the early colonization (Figure 2A). In comparison, we found generally increased expression of these genes in *P. parasitica*-colonized *rtp1* mutants. For instance, expression levels of *BiP3* and *DnaJ* were significantly elevated in *rtp1* mutants at 3 and 12 hpi, respectively, compared with those in WT Col-0 (Figure 2A). To complement this, we further examined the level of BiP protein accumulation in both WT Col-0 and *rtp1* mutants colonized by *P. parasitica*. Similarly, the BiP protein accumulation was increased in *rtp1* mutants by 73% and 76% at 3 and 12 hpi, respectively, compared with that in WT Col-0 (Figure 2B). Furthermore, we found that the expression levels of both *bZIP28* and *bZIP60* were significantly increased in *rtp1* mutants at 24 hpi, compared with WT Col-0 (Figure 2A). These results indicate that plant UPR signaling is activated upon early infection by *P. parasitica*, while *RTP1* participates in modulating UPR activation in response to infection.

This prompted us to elucidate whether *RTP1* plays a regulatory role in synergistic signaling between UPR and plant immunity. As *rtp1* plants show broad spectrum resistance against biotrophic pathogens accompanied with faster accumulation of PR1 transcripts, a key SA signaling marker gene (Pan et al., 2016), we further examined transcript levels of several immune-related genes in SA defense signaling and PTI pathways, including WRKY33 (UPR-mediated SAR priming gene; Wakasa et al., 2014), WRKY46 (SA signaling marker gene; Hu et al., 2012), CBP60g (SA signaling marker gene; Truman and Glazebrook, 2012), MPK11 (SA signaling marker gene and PTI marker gene, encoding a MAP kinase activated during PTI; Bethke et al., 2012) and CYP71A12 (PTI marker gene; Millet et al., 2010). The results showed that transcripts of all these immune genes were upregulated in WT Col-0 plants upon infection by *P. parasitica*. In comparison, stronger induction of expression of these genes was detected in *P. parasitica*-infected *rtp1* mutants, especially during the early biotrophic infection stage (Figure 2C).

Notably, based on our analyses on differentially expressed genes according to the RNA-seq data from WT Col-0 with treatment of TM and DMSO (control), the strongly induced immune genes (e.g. WRKY33, WRKY46, CBP60g, MPK11, and CYP71A12) in response to *P. parasitica* were identified to be simultaneously induced by TM (Supplemental Table S1). We assume that these genes are ER stress-responsive genes that may function in plant defense response against *P. parasitica*. Collectively, these data imply that *RTP1* may play a negative regulating role in ER stress-mediated plant immunity, especially during the early biotrophic colonization stage.

**RTP1 negatively regulates bZIP60 splicing activity and stabilizes ER membrane-associated bZIP60 TF**

To further investigate how *RTP1* regulates plant UPR signaling pathways for functional integration with plant immunity, we firstly investigated whether *RTP1* might affect cytoplasmic splicing of the mRNA encoding the bZIP60 TF, which is a hallmark event in IRE1/bZIP60 mediated UPR signaling pathway. Two-week-old WT Col-0 and *rtp1* mutant seedlings were treated with TM and the bZIP60 splicing activity in TM-treated Col-0 and *rtp1* mutant plants were analyzed by quantitative transcript measurement that can distinguish between the forms of *unspliced* bZIP60 (ubZIP60) and *spliced* bZIP60 (sbZIP60), ER stress-activated form of bZIP60; Moreno et al., 2012). The results showed that bZIP60 splicing activity was significantly increased in TM-treated *rtp1* mutants, with ~20% and 40% more than that in WT Col-0 at 5-h and 12-h post treatment, respectively (Figure 3A), suggesting that *RTP1* plays a role in regulating bZIP60 processing upon TM-induced ER stress.

To further elucidate to what extent *RTP1* affects bZIP60 processing upon early infection by *P. parasitica*, we examined bZIP60 splicing activity in WT Col-0 and *rtp1* mutant plants colonized by *P. parasitica*, with seedlings of 2-week-old WT Col-0 and *rtp1* mutants inoculated with *P. parasitica* zoospores at 3, 6, 12, and 24 hpi. Similarly, the quantitative transcript measurement showed an increased bZIP60 splicing activity in *P. parasitica*-colonized *rtp1* mutants, with almost two-fold as that in WT Col-0 at 24 hpi (Figure 3B). These results imply that *RTP1* seems to negatively regulate bZIP60 processing upon infection by *P. parasitica*.

To examine whether the protein encoded by *RTP1* contributes to stabilization of the ER membrane-associated bZIP60 TF, the p35S::myc-bZIP60 construct or p35S::myc-GFP construct as a control was cotransformed with p35S::RTP1-FLAG or empty vector (EV) into *Nicotiana benthamiana* leaves by agroinfiltration. At 2- and 3-d post infiltration, the leaf protein was extracted and equal amount of proteins for each sample was used to evaluate the impact of *RTP1* on bZIP60 protein stability. The immunoblotting (IB) analysis showed that the accumulation of bZIP60 was significantly

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**Figure 1 (Continued)**

RTP1-OE lines, and *vpe* mutants were grown on 1/2 MS medium containing 0.3 μg/mL TM (B) and 2mM DTT (C) for 3 d. Percentage of recovery was plotted by calculating alive/dead seedlings recovered 10 d post TM treatment. Data presented show means of three independent experiments ± se. For each experiment, 25 plants were analyzed per line. Asterisks indicate significance at *P* < 0.05 analyzed by Student’s t test. D. Expressions of BiP3, DnaJ, bZIP60, and bZIP28 were evaluated by RT-qPCR. Two-week-old A. thaliana WT Col-0 and *rtp1* mutant roots were treated with TM (5 μg/mL) or DMSO (control). Total RNA was extracted from treated root samples at 5 and 12 h post treatment. Data shown represent fold changes of genes and display the ratio of candidate gene expression to plant housekeeping gene *AtUBIQUITIN9* using the ΔΔCt method in TM-treated plants relative to DMSO-treated plants. Three independent experiments showed similar results. Error bars indicate se from three biological replicates. Asterisks indicate significance at *P* < 0.05 analyzed by Student’s t test.
Figure 2 The rtp1 mutants show stronger induction of UPR and ER stress-responsive immunity during the early infection by P. parasitica. A, Expressions of BiP3, DnaJ, bZIP60, and bZIP28 were evaluated by RT-qPCR. Two-week-old A. thaliana WT Col-0 and rtp1 mutant roots were dip-inoculated by P. parasitica zoospores or mock treated. Total RNA was extracted from inoculated roots at 3, 6, 12, and 24 hpi. Data shown represent fold changes of genes and display the ratio of candidate gene expression to plant housekeeping gene AtUBIQUITIN9 using the ΔΔCt method in colonized plants relative to mock-treated plants. Fold changes >1 indicate induction of genes. Data presented show means of three independent experiments.
increased when coexpressed with RTP1 compared with that of EV control, with an increase of almost 2- and 2.81-fold protein accumulation at 2- and 3-d post coinfiltration, respectively (Figure 3, C and D), whereas the accumulation of GFP protein was not affected in the presence of RTP1 (Supplemental Figure S1). This suggests that RTP1 stabilizes the ER membrane-associated bZIP60 TF. Consistent with this finding, when we coexpressed either mCherry-bZIP60 with RTP1-GFP or GFP-bZIP60 with mCherry-labeled ER marker in N. benthamiana leaves, the confocal microscopy showed overlapping of mCherry-bZIP60 with RTP1-GFP (Supplemental Figure S2, A and B) as well as GFP-bZIP60 (Supplemental Figure S2, C and D). Asterisks indicate significance at *P < 0.05 and **P < 0.01 analyzed by Student’s t test.

Figure 3 RTP1 negatively regulates bZIP60 splicing activity and stabilizes ER membrane-localized bZIP60 transcription factor. A and B, The bZIP60 splicing activity was evaluated by RT-qPCR. Ten-day-old A. thaliana Col-0 and rtp1 mutant roots were treated by 5 μg/mL TM (A) or dip-inoculated by P. parasitica zoospores (B) and harvested at indicated time points. Total RNA was extracted and AtUBIQUITIN9 was used as the plant reference gene. Ratios of fold induction of spliced and unspliced bZIP60 were plotted, while setting ratio of Col-0 as 100%. Data presented show means of three independent experiments ± se. Asterisks indicate significance at *P < 0.05 and **P < 0.01 analyzed by Student’s t test. C, Protein stability of bZIP60, coexpressed with RTP1 or empty vector, was analyzed by IB. Total proteins were extracted from infiltrated leaves at 2- and 3-d post infiltration. The accumulation of 7×myc-bZIP60 and RTP1-3×FLAG was detected by IB using anti-myc- and anti-FLAG- antibodies, respectively. The protein size of RTP1 was marked by arrowhead. D, Relative myc-bZIP60 protein band intensities (protein accumulation at 2-d post infiltration, when coexpressed 7×myc-bZIP60 and EV, was set to 1) were determined by Image J. Ponceau staining of the membrane was used to show equal loading. Data presented show means of three independent experiments ± se. Asterisks indicate significance at *P < 0.05 analyzed by Student’s t test.

Figure 2 (Continued)
with mCherry-labeled ER marker (Supplemental Figure S2, C and D), indicating the colocalization of bZIP60 and RTP1 in the ER.

RTP1 modulates the induction of A. thaliana IRE1 upon P. parasitica infection and manipulates the phosphorylation of IRE1 protein

The activation of bZIP60 requires unconventional splicing of bZIP60 mRNA executed by ER membrane-associated protein IRE1. IRE1 contains a PK domain and a ribonuclease domain, which is an important UPR regulator in plants (Nagashima et al., 2011). Therefore, we further examined whether RTP1 is involved in regulating the induction pattern of IRE1 under TM-induced ER stress and upon P. parasitica infection. The RT-qPCR results showed that the expression of IRE1a was not obviously induced in either WT Col-0 or rtp1 mutants upon treatment by TM at 5 h and 12 h. In comparison, the expression of IRE1b was significantly induced in TM-treated rtp1 mutants at 12 h (Figure 4, A and B). Interestingly, the transcript levels of both IRE1a and IRE1b exhibited slight elevation in P. parasitica-colonized WT Col-0 at 3 hpi, followed by a gradual reduction during the infection. Nevertheless, the induction peaks of IRE1a and IRE1b did not occur until 12 hpi in P. parasitica-colonized rtp1 mutants. Meanwhile, higher levels of IRE1a and IRE1b transcripts were notable in rtp1 mutants at 12 and 24 hpi, respectively, compared with those in WT Col-0 (Figure 4, C and D). In accordance with this, quantification of P. parasitica biomass by qPCR showed significantly much less pathogen colonization at 12 and 24 hpi in rtp1 mutants compared with Col-0 (Figure 4E). These results imply that RTP1 is involved in modulating the expression pattern of IRE1 in A. thaliana during the early stage of colonization by P. parasitica.

The isoforms of IRE1a and IRE1b in A. thaliana are classified as a single-pass transmembrane protein in the ER membrane and their activation seems to be induced by its trans-autophosphorylation (Koizumi et al., 2001; Zhang et al., 2016). To elucidate whether the ER membrane-localized protein encoded by RTP1 could affect the phosphorylation activity of IRE1, we cotransformed the constructs of p35S::IRE1a-FLAG with p35S::RTP1-HA or p35S::EV as well as p35S::FLAG-IRE1b with p35S::RTP1-HA or p35S::EV, using Agrobacterium-mediated transient transformation in leaves of N. benthamiana. At 3-d post coinfiltration, phosphorylated and unphosphorylated forms of IRE1 were analyzed as described (Kinoshita et al., 2006). The IB results showed that when coexpressed with EV, both IRE1a and IRE1b exhibited clear phosphorylated forms of the protein, with a size of ~130 kDa, indicative of the IRE1 phosphorylation (Figure 4F; Supplemental Figure S3). Interestingly, analysis of relative intensities of phosphorylated IRE1 proteins showed that the phosphorylated form of IRE1b was obviously reduced when coexpressed with RTP1, being around 50% less than that coexpressed with EV, though the phosphorylated form of IRE1a appeared less affected by RTP1 (Figure 4F; Supplemental Figure S3).

As both phosphorylated and unphosphorylated forms of IRE1 were attenuated when coexpressed with RTP1 (Figure 4F; Supplemental Figure S3), we further examined whether RTP1 affects the stability of IRE1 proteins through Agrobacterium-mediated transient cotransformation in N. benthamiana leaves. The IB results exhibited that the accumulation of both IRE1a and IRE1b was significantly reduced when coexpressed with RTP1 compared with that coexpressed with EV at 3-d post coinfiltration (Supplemental Figure S4). Taken together, these in vivo assays imply that RTP1 is involved in manipulating the general phosphorylation of IRE1b and stability of IRE1 proteins, though the interactions between RTP1 and IRE1a or IRE1b were not detected (Figure S5).

RTP1 interacts with and stabilizes ER membrane-associated bZIP28

To further investigate how RTP1 manipulates the induction of plant UPR pathway mediated by the ER-membrane localized stress sensor bZIP28, we analyzed whether RTP1 interacts with bZIP28 protein using the firefly luciferase complementation imaging assay. The constructs of RTP1-NLuc and CLuc-bZIP28, RTP1-NLuc and CLuc, NLuc and CLuc-bZIP28, NLuc and CLuc were cotransformed in leaves of N. benthamiana, respectively, and the relative luciferase activities were measured and recorded at 3-d post cotransformation (Chen et al., 2008). Our results showed that coexpression of RTP1-NLuc and CLuc, NLuc and CLuc-bZIP28, NLuc and CLuc did not show luciferase complementation signals, whereas coexpression of RTP1-NLuc and CLuc-bZIP28 resulted in strong luciferase complementation signal (Figure 5A), implying that the protein encoded by the full-length bZIP28 interacts with RTP1.

To further confirm this interaction, we carried out coimmunoprecipitation (Co-IP) assays. The p35S::myc-bZIP28 construct was cotransformed with either p35S::RTP1-FLAG or p35S::FLAG-GFP in N. benthamiana leaves through agroinfiltration. Total proteins were extracted from infiltrated leaves and were immunoprecipitated with FLAG-Trap agarose beads. The IB results showed that myc-bZIP28 was coimmunoprecipitated in RTP1-FLAG-expressed samples, but not in the FLAG-GFP samples, though it was expressed in all leaves (Figure 5B). These results were further confirmed by the Co-IP of RTP1-FLAG in myc-bZIP28-expressed samples, but not in the myc-GFP samples, using anti-myc magnetic beads, when we cotransformed p35S::RTP1-FLAG construct with either p35S::myc-bZIP28 or p35S::myc-GFP in N. benthamiana leaves (Supplemental Figure S6). Taken together, these results indicate that RTP1 interacts with ER stress-sensing TF bZIP28 in planta.

To further elucidate whether RTP1 targeting stabilizes bZIP28, the myc-bZIP28 fusion construct was cotransformed with RTP1-FLAG or EV into N. benthamiana leaves by
agroinfiltration. At 2- and 3-d post infiltration, the leaf proteins were extracted and equal amount of proteins for each sample was used to evaluate the impact of RTP1 on bZIP28 protein stability. The IB analysis showed that the accumulation of bZIP28 was significantly increased when coexpressed with RTP1 compared to that coexpressed with EV, with an increase of 73% and two-fold protein accumulation at 2- and 3-d post coinfiltration, respectively (Figure 5).

Figure 4 RTP1 modulates the induction of A. thaliana IRE1 upon P. parasitica infection and manipulates the phosphorylation of IRE1. A–D, Expressions of IRE1a and IRE1b was evaluated by RT-qPCR. Ten-day-old A. thaliana Col-0 and rtp1 mutant roots were treated by 5 μg/mL TM (A and B) or dip-inoculated by P. parasitica zoospores (C and D). Total RNA was extracted from treated or inoculated roots at indicated time points. Data shown represent fold changes of genes and display the ratio of candidate gene expression to plant housekeeping gene AtUBIQUITIN9 using the ΔΔCt method in TM-treated or colonized plants relative to DMSO- or mock-treated plants. Data presented show means of three independent experiments ± se. Asterisks indicate significance at *P < 0.05 analyzed by Student’s t test. E, Quantification of P. parasitica biomass in A. thaliana Col-0 and rtp1 mutant roots at 3, 6, 12, and 24 hpi was determined by RT-qPCR. Primers specific for the P. parasitica UBC and the AtUBIQUITIN9 were used. For each experiment, approximately 200 plants were analyzed per line. Bars represent PpUBC levels relative to AtUBC9 levels with se of three biological replicates. Asterisks denote significance in the colonization of rtp1 mutant compared with Col-0 analyzed by Student’s t test ( *P < 0.05; **P < 0.01). F, The phosphorylation levels of IRE1a-3×FLAG and 3×FLAG-IRE1b, coexpressed with RTP1-HA or empty vector, was analyzed by IB. Total proteins were extracted from infiltrated leaves at 3-d post agroinfiltration and then separated by phosphate affinity SDS–PAGE in a 7.5% (w/v) polyacrylamide gel containing 15M Phos-tag. The phosphorylated or unphosphorylated IRE1a-3×FLAG and 3×FLAG-IRE1b were detected by IB using anti-flag antibody. Two independent experiments were performed showing similar results. The band intensities of P and UP were determined by the gray values using Image J. The adjusted band intensities of P in the tested samples (i.e. IRE1a-FLAG or FLAG-IRE1b + RTP1-HA) were calculated as described in M&M. Protein loading is indicated by Ponceau staining. Numbers on top of the immunoblot indicate adjusted band intensities of P in the tested samples normalized to band intensities of P in the control samples (i.e. IRE1a-FLAG or FLAG-IRE1b + EV). UP, unphosphorylated IRE1; P, phosphorylated IRE1.
Figure 5 RTP1 interacts with and stabilizes bZIP28. Proteins were expressed in *N. benthamiana* leaves through infiltration with an *A. tumefaciens* cell suspension with an OD<sub>600</sub> value of 0.3. A, The interaction between RTP1 and bZIP28 in living cells was detected by firefly luciferase complementation imaging assay at 72-h post infiltration. The N terminus of LUC was fused to the C terminal of RTP1, and the C terminus of LUC was fused to the N terminus of bZIP28. Coexpression of RTP1-nLUC and cLUC-bZIP28 resulted in specific fluorescence as detected by a low-light cooled charge-coupled device camera. Three independent biological experiments were performed and showed similar results. B, CoIP assays showing that RTP1 interacts with bZIP28 in planta. Total native protein extracts (input) from agroinfiltrated leaves expressing the indicated proteins were precipitated with anti-FLAG M2 affinity gel (IP: FLAG), separated on SDS-PAGE gels, and blotted with specific antibodies. In immunoprecipitation fractions, 7×myc-bZIP28 was detected in a complex with RTP1-3×FLAG, but not with FLAG-GFP. Protein size markers are indicated in kDa, and protein loading is indicated by Ponceau staining. Three independent biological experiments were performed and showed similar results. C, Protein stability of bZIP28, coexpressed with RTP1 or empty vector, was analyzed by IB. Total proteins were extracted from infiltrated leaves at 2- and 3-d post infiltration. The accumulation of 7×myc-bZIP28 and RTP1-3×FLAG was detected by IB using anti-myc- and anti-FLAG antibodies, respectively. The protein size of RTP1 was marked by arrowhead. D, Relative bZIP28 protein band intensities (protein accumulation at 2-d post
C and D), indicating that targeting of ER membrane-associated bZIP28 by RTP1 enhances its stability.

**Both bZIP60 and bZIP28 are required for rtp1-mediated plant resistance against *P. parasitica***

To investigate the potential function of the key UPR regulators bZIP60 and bZIP28 in rtp1-mediated plant resistance, we generated rtp1bzip60 and rtp1bzip28 double mutants by crossing rtp1 with bzip60 as well as bzip28 mutants. The detached leaves of 6-week-old mutants of bzip60, bzip28, rtp1bzip60, and rtp1bzip28 were compared with WT Col-0 and rtp1 mutant following inoculation with *P. parasitica* zoospores. At 48 hpi, leaves of both bzip60 and bzip28 mutants displayed severer water-soaked lesions compared with that exhibited in WT Col-0, whereas rtp1 mutant showed less visible lesions (Figure 6A). Both disease index statistics and qPCR assay for pathogen biomass (Figure 6, B and C) consistently indicated that both bzip60 and bzip28 mutants were more susceptible than WT Col-0 to *P. parasitica* infection, suggesting their potential functions in plant resistance. In parallel, both rtp1bzip60 and rtp1bzip28 double mutants were significantly more susceptible to *P. parasitica* than the rtp1 mutant, though they exhibited less susceptibility than either bzip60 or bzip28 mutant (Figure 6, A and B). Quantification of *P. parasitica* biomass confirmed that the resistance of rtp1 mutant clearly compromised in both rtp1bzip60 and rtp1bzip28 double mutants, in which the level of *P. parasitica* colonization was dramatically higher than in rtp1 mutant but less than in mutants of bzip60 and bzip28 (Figure 6C). These results indicate that UPR regulators bZIP60 and bZIP28 play a role in rtp1-mediated plant resistance against *P. parasitica* and they may function downstream of RTP1.

**Both bZIP60 and bZIP28 play a role in the activation of ER stress-responsive immunity in rtp1 plants***

The expression of several ER stress-responsive immune genes (e.g. WRKY33, WRKY46, CBP60g, MPK11, and CYP71A12; Supplemental Table S1) showed stronger induction in rtp1 mutants than in WT Col-0 upon early infection by *P. parasitica* (Figure 2C), implying their potential shared function as novel UPR regulators and key components in RTP1-mediated immune signaling. Moreover, the significant induction of expression of these ER stress-responsive immune genes occurred simultaneously with increased expression of bZIP28 and bZIP60 in *P. parasitica*-infected rtp1 mutants (Figure 2A). This prompted us to examine the activation of ER stress-responsive immunity in rtp1 plants by bZIP60 and bZIP28. We performed RT-qPCR assay to examine transcript levels of these ER stress-responsive immune genes in WT Col-0, rtp1, rtp1bzip60, and rtp1bzip28 upon early infection by *P. parasitica*. In comparison to the stronger induction of expression of ER stress-responsive immune genes WRKY33, WRKY46, CBP60g, MPK11, and CYP71A12 in *P. parasitica*-colonized rtp1 mutant, transcript levels of these genes were notably reduced in double mutants rtp1bzip60 and rtp1bzip28 during the early stage of colonization by *P. parasitica* (Figure 7A), suggesting the regulatory roles of bZIP60 and bZIP28 in the activation of ER stress-responsive immune genes in rtp1 during the infection.

As callose deposition and reactive oxygen species (ROS) production are characteristic of PTI mediated by the leucine-rich repeat receptor kinases flagellin-sensing 2 (FLS2; Luna et al., 2011), we assessed callose deposition and oxidative burst in leaves of WT Col-0 and rtp1 mutants triggered by flg22, the cognate ligand of the FLS2 receptor. Using aniline-blue staining and fluorescence microscopy, more callose deposition was found in the leaves of rtp1 mutant than that in WT Col-0 plants by 24 h of treatment (Figure 7, C and E). Simultaneously, luminol-based assay confirmed a stronger transient oxidative burst in rtp1 mutants than in WT Col-0 (Figure 7B). Further analyses on mutants of bzip60, bzip28, rtp1bzip60, and rtp1bzip28 upon flg22 treatment showed that oxidative burst was obviously abolished in bzip60 and bzip28 mutants compared with that in WT Col-0. Moreover, the strong oxidative burst occurred in rtp1 mutant was attenuated in rtp1bzip60 and rtp1bzip28 mutants (Figure 7B). These results suggest that RTP1 is involved in PAMP-triggered immune signaling, in which bZIP60 and bZIP28 might play a regulatory role. Consistently, visible callose deposition was monitored in WT Col-0 leaves upon early infection by *P. parasitica*, while rtp1 mutants showed ~60% more callose deposition. Notably, both mutants rtp1bzip28 and rtp1bzip60 exhibited significantly less callose deposition than rtp1 mutant (Figure 7, D and F), indicating its compromised defense response to pathogen infection. Collectively, these results point to the notion that both bZIP60 and bZIP28 play a role in rtp1-mediated plant resistance through their probable shared function to facilitate synergistic signaling of UPR and ER stress-mediated plant immunity.

**Discussion***

Host cells use an intricate signaling system to respond to invasions by pathogenic microorganisms. Although several signaling components of disease resistance against biotrophic pathogens have been identified, our understanding of molecular components and host processes that contribute to plant disease susceptibility remains elusive. The susceptibility factor RTP1 encodes an ER membrane-localized protein and negatively regulates plant resistance to biotrophic pathogens including *P. parasitica* (Pan et al., 2016). In this study, we further demonstrate a role of *A. thaliana* RTP1 in the UPR. Specifically, we establish the role of RTP1 in infiltration, when coexpressed 7×myc-bZIP28 and EV, was set to 1) were determined by Image J. Poncet al. staining of the membrane was used to show equal loading. Data presented show means of three independent experiments ± se. Asterisks indicate significance at *P*<0.05 analyzed by Student’s t test.****
responding to TM- and DTT-induced ER stress, as demonstrated by altered tolerance to ER stress (Figure 1, A–C) and enhanced induction of UPR marker genes in rtp1 mutants (Figure 1D). Consistently with these observations, induction of expression of UPR marker genes in P. parasitica-colonized Col-0 plants (Figure 2A) indicates UPR activation upon infection. In comparison, the increased expression of these UPR genes in rtp1 mutant during infection (Figure 2A) suggests the role of RTP1 as a negative regulator for UPR activation in A. thaliana upon colonization of P. parasitica.

There is increasing evidence that UPR signaling contributes to plant immunity in different ways, for instance, through the regulation of antimicrobial protein secretion, the processing of pattern-recognition receptors for PTI, the priming of SAR and ER stress-mediated cell death (Wang et al., 2005; Li et al., 2009; Moreno et al., 2012; Qiang et al., 2012). In addition to the stronger induction of UPR in P. parasitica-infected rtp1 mutants (Figure 2, A and B), our study further suggests that RTP1 participates in negative modulation of SA-mediated defense signaling, as demonstrated by the higher expression levels of SA-mediated immune genes in rtp1 mutants than in WT Col-0 plants during the infection (Figure 2C). These results support the notion that RTP1 negatively regulates plant resistance to biotrophic but not necrotrophic pathogens (Pan et al., 2016), as SA signaling is thought to be essential to resist infection from biotrophic pathogens (Dodds and Rathjen, 2010). Furthermore, plant hormone signaling pathways including SA signaling contribute positively to PTI and PTI strongly depends on synergistic interactions between these signaling pathways (Tsuda et al., 2009). Indeed, our results showed that induction of PAMP-activated genes (CYP71A12 and MPK11; Millet et al., 2010; Bethke et al., 2012) as well as PAMP-elicited callose deposition and oxidative burst were dramatically increased in rtp1 mutant plants compared to Col-0 upon early stage of infection (Figures 2, C and 7, B, C, and E), suggesting the potential function of RTP1 in PTI. These data further support the finding that RTP1-mediated plant resistance is broad spectrum (Pan et al., 2016), as PTI is conserved in different plant species and acts as a basal defense (Lacombe et al., 2010). Moreover, the expression levels of the ER stress-mediated plant immune gene EFR, ER stress-mediated cell death gene VPE, and secreted immunity-related protein gene PR1 were increased in rtp1 mutant during the early stage of infection compared with that in Col-0 (Supplemental Figure S7; Pan et al., 2016). Collectively, these results indicate that RTP1 negatively regulates plant resistance, most likely by participating in UPR signaling pathways.

As a master regulator of SA-mediated immune defense, NPR1 functions to antagonize the UPR regulators bZIP28 and bZIP60, independent of its role in SA defense (Lai et al., 2018). This suggests the possibility of cross-signaling between UPR and SA-mediated defense through the critical modulator NPR1. Intriguingly, our study further reveals a scenario

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**Figure 6** Both bZIP60 and bZIP28 are required for rtp1-mediated plant resistance against P. parasitica. A, Detached leaves of bzip28 and bzip60 mutants showed enhanced susceptibility and rtp1 mutant exhibited resistance against infection by P. parasitica, as compared to WT Col-0 plants. Detached leaves of rtp1bzip60 and rtp1bzip28 double mutants showed compromised resistance compared to rtp1 mutant against P. parasitica. Representative images were taken at 48 hpi. B, Disease severity index (DSI) from level 1 to level 4 was recorded at 48 hpi. Level 1: disease symptom is less than 1/3 of whole leaf area; level 2: disease symptom is between 1/3 and 1/2 of whole leaf area; level 3: disease symptom is between 1/2 and 2/3 of whole leaf area; and level 4: disease symptom is more than 2/3 of whole leaf area. Asterisks indicate significant differences between genotypes (Wilcoxon rank-sum test: *P < 0.05, **P < 0.01). C, Quantification of P. parasitica biomass in infected A. thaliana leaves at 48 hpi was determined by RT-qPCR. Primers specific for the P. parasitica UBC and the AtUBIQUITIN9 were used. The biomasses of P. parasitica in all mutants were normalized with that in WT Col-0 (set to 1). For each experiment, approximately 200 plants were analyzed per line. Bars represent SEM of three biological replicates. Asterisks denote significance analyzed by student’s t test (*P < 0.05, **P < 0.01).
Figure 7 Both bZIP60 and bZIP28 play a role in the activation of ER stress-responsive immunity in rtp1 plants. A, Expressions of WRKY33, WRKY46, MPK11, CBP60g, and CYP71A12 in WT Col-0, rtp1, rtp1bzip60, and rtp1bzip28 mutants were determined by RT-qPCR. Two-week-old A. thaliana plant roots were dip-inoculated by P. parasitica zoospores and harvested at indicated time points. AtUBIQUITIN9 was used as the plant reference gene. Data shown represent fold changes of genes and display the ratio of candidate gene expression to plant housekeeping gene AtUBIQUITIN9 using the ΔΔCt method in colonized plants relative to mock-treated plants. Fold changes >1 indicate induction of genes. Data
that the immune function of RTP1 is highly associated with its role in modulating the ER membrane-bound UPR regulators bZIP28 and bZIP60. Through interacting with and stabilizing the ER membrane-tethered bZIP28 (Figure 5; Supplemental Figure S6), RTP1 possibly contributes to retain the bZIP28 in the ER. Additional subcellular localization assays also support their partial colocalization in the ER membrane (Supplemental Figure S8). We speculate that the activation of bZIP28 TF is probably manipulated by RTP1, as evident by the elevated induction of UPR in rtp1 mutant in response to either TM treatment (Figure 1D) or pathogen infection (Figure 2, A and B).

In A. thaliana, bZIP60 is thought to transmit the ER stress signal in the UPR pathway through ER-localized IRE1, an RNA splicing enzyme (Iwata and Koizumi, 2012). AS RTP1 loss-of-function enhances bZIP60 splicing activity induced either by TM treatment (Figure 3A) or upon infection by P. parasitica (Figure 3B), we speculate that RTP1 might be involved in manipulating bZIP60 mRNA processing. Further finding that the expression pattern of IRE1 is associated with significantly enhanced bZIP60 splicing activity and reduced colonization of P. parasitica in rtp1 mutants during the infection (Figures 3, B and 4, C–E) implies bZIP60 as a target of IRE1 nuclease activity in response to P. parasitica infection. Plant IRE1 is assumed to be activated by trans-auto-phosphorylation due to the presence of conserved cytosolic kinase and RNase domains among the eukaryotes (Koizumi et al., 2001; Zhang et al., 2016). Our findings that the general phosphorylation and stability of IRE1 protein are manipulated by RTP1 (Figure 4F; Supplemental Figures S3 and S4) provide evidence that RTP1 might negatively modulate the activation of RNA splicing enzyme IRE1. We speculate that the impact of RTP1 on differential phosphorylation between IRE1a and IRE1b (Figure 4F; Supplemental Figure S3) might be a consequence of their dissimilar PK activation loops (Koizumi et al., 2001). Further in vitro studies are of importance to understand the effect of RTP1 on the autophosphorylation of IRE1. Notably, though the direct interaction between RTP1 and IRE1 is not detected (Supplemental Figure S5), we find that RTP1 interacts with A. thaliana Bax inhibitor-1 (BI-1; Supplemental Figure S9). In mammalian cells, IRE1α is demonstrated to interact with BI-1 to regulate its endoribonuclease activity (Lisbona et al., 2009). It remains to be examined whether BI-1 mediates interaction between RTP1 and IRE1. Consequently, it is logical to find that the ER membrane-associated bZIP60 is stabilized by RTP1 protein (Figure 3, C and D). Further finding that induction of UPR is potentiated in rtp1 mutant in response to either TM treatment (Figure 1D) or pathogen infection (Figure 2, A and B) suggests the activation of bZIP60 TF is manipulated by RTP1.

Increasing evidence indicate that IRE1/bZIP60-mediated UPR pathway not only functions in response to ER stress or heat stress (Iwata et al., 2008; Deng et al., 2011), but also may exert a unique role in certain biological processes such as plant–virus interactions and SA-mediated plant defense signaling in response to bacterial pathogens (Tateda et al., 2008; Moreno et al., 2012; Zhang et al., 2015). Our findings that bZIP60 loss-of-function leads to increased susceptibility to P. parasitica indicate that the ER membrane-associated bZIP60 plays a role in the defense response against Phytophthora (Figure 6). This further confirms the role of IRE1/bZIP60 mediated UPR pathway in plant immunity. To the best of our knowledge, although bZIP28-mediated UPR pathway confers overlapping functions with IRE1–bZIP60 UPR pathway in fundamental biology (Liu et al., 2007; Gao et al., 2008), its function in response to biotic stress such as pathogen infection is largely unknown. Notably, we found that bzip28 mutants resembled the susceptible phenotype of bzip60 mutants in response to P. parasitica infection (Figure 6). This implies that bZIP28 UPR pathway may also function in plant immune response to microbial infection. More interestingly, our study further reveals that both bZIP60 and bZIP28 play a role in RTP1-mediated plant immunity, as demonstrated by the attenuated fg22-triggered oxidative burst (Figure 7B), reduced callose deposition upon infection (Figure 7, D and F) and compromised resistance against P. parasitica in mutants rtp1bzip60 and rtp1bzip28 compared with rtp1 (Figure 6).

Notably, our finding that strong induction of expression of ER stress-responsive immune genes in rtp1 mutants during the early infection is significantly attenuated in mutants rtp1bzip60 and rtp1bzip28 (Figure 7A; Supplemental Table S1) further indicates that the activation of these immune-related genes is regulated by bZIP60 and bZIP28 TFs. With analysis on the promoter regions of these genes through plantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/), the CCAAT box, a key motif of ER stress response element-I cis-element required for the interaction with bZIP TFs (Liu and Howell, 2010), is identified in the promoter regions of WRKY33, CBP60g, and CYP71A12 (Supplemental Figure S10). Further studies are needed to understand the interaction of bZIP60 and bZIP28 TFs with the promoter of these ER stress-responsive immune genes presented show means of three independent experiments ± se. Asterisks indicate significance at *P < 0.05 and **P < 0.01 analyzed by Student’s t test. B, ROS burst upon fg22 treatment of leaves of 4-week-old WT Col-0 as well as mutants rtp1, bzip60, bzip28, rtp1bzip60, and rtp1bzip28. At least 12 leaves from 6 plants of each group were measured using a luminol-based chemiluminescence assay. Data presented show means ± se (n = 12). Three independent experiments were repeated with similar results. RLU, relative light units. C–F, Leaves of 4-week-old WT Col-0 and rtp1 plants were treated with 1μM fg22 for 24 h (C and E) and leaves of 4-week-old WT Col-0, rtp1, rtp1bzip60, and rtp1bzip28 plants were incubated by P. parasitica zoospores for 24 h (D and F). Leaves were fixed in ethanol–glacial acetic acid and stained by aniline blue for 2 h. Callose depositions were detected by fluorescence microscopy. Bar = 250 μm (C and D). Callose deposits were quantified as the number of depositions per 4 mm² (E and F). Data presented show means ± se (n = 10). Asterisks denote significant differences analyzed by Student’s t test (*P < 0.05, **P < 0.01). Three independent experiments were repeated with similar results.
that are modulated by the susceptibility factor RTP1. Nevertheless, our results point to the notion that bZIP60 TF can possibly have functional overlapping with bZIP28 TF in rpt1-mediated plant resistance against P. parasitica through regulating the expression of downstream ER stress-responsive immune genes. This may explain why the intermediate susceptible phenotype of rpt1bzip60 resembled that of rpt1bzip28 (Figure 6, A and C), as the absent transcriptional activity of bZIP60 TF might be compensated by the bZIP28 TF in the rpt1bzip60 mutant.

Collectively, we propose a mechanism to explain how plant susceptibility factor RTP1 negatively regulates resistance to biotrophic pathogens: RTP1 is involved in UPR regulation in response to P. parasitica infection, and may exert negative modulating roles in the activation of ER-localized UPR TFs bZIP60 and bZIP28, which are possibly shared to facilitate synergistic signaling between UPR and plant immunity (Figure 8).

Materials and methods

Plant materials, growth conditions, and plant inoculation

The Arabidopsis (Arabidopsis thaliana) RTP1 T-DNA insertion line (SALK_094320) was obtained from ABRC. The mutants of bzip28, bzip60 and vpe (Qiang et al., 2012) were provided by Dr P. Schäfer. The double mutants of rpt1bzip60 and rpt1bzip28 were generated by crossing rpt1 with bzip60 and bzip28. The T-DNA insertion homozygous mutants were confirmed by PCR using primers rpt1-LP, rpt1-RP, bzip28-LP, bzip28-RP, bzip60-LP, bzip60-RP, and LbB1.3 (Supplemental Table S2). For root inoculation or chemical treatment, all A. thaliana seeds were sterilized and grown in squared petri dishes on half-strength Murashige and Skoog (MS). For leaf inoculation or treatment on leaves of A. thaliana and N. benthamiana, the plant growing conditions were as previously described (Pan et al., 2016). The culture and inoculation of P. parasitica strain Pp016, as well as the quantitation of P. parasitica infection by qPCR were conducted as previously described (Wang et al., 2011; Pan et al., 2016).

Plasmid constructs

For the creation of firefly luciferase complementation constructs, the coding regions of RTP1, bZIP28, BI-1, IRE1a, and IRE1b were cloned from A. thaliana WT Col-0 cDNA and inserted into the Kpn1 and Sal1 site of pCAMBIA1300 (Chen et al., 2008). The sequence of RTP1 was fused upstream of N-Luc in the pCAMBIA-NLuc vector, and bZIP28, BI-1, IRE1a, and IRE1b were fused downstream of C-Luc in the pCAMBIA-CLuc vector. To create 7×myc-fusion plasmids, the 7×myc fragment was cloned into pKannibal (Wesley et al., 2001) with Xho1 and EcoR1 sites and Not1 sites were used to release the fragment with the promoter and terminator and then inserted into the binary vector pART27 (Gleave, 1992). The mature bZIP28, bZIP60, IRE1a, and IRE1b coding sequences were inserted into previously modified pART27 at the EcoR1 and Xba1 sites to create 7×myc-bZIP28, 7×myc-bZIP60, 7×myc-IRE1a, and 7×myc-IRE1b. For other plant expression constructs, including RTP1-FLAG and FLAG-GFP, fusion fragments were obtained from overlapping PCR and cloned into the binary vector pART27 replacing the existing sequence.

For the creation of phosphorylation assay constructs, the fusion fragments IRE1a-3×Flag, SP-3×Flag-IRE1b.1, IRE1b.1-3×Flag, and RTP1-HA were obtained through overlapping PCR and inserted into the monocalonic site of pKannibal (IRE1a-3×Flag, XhoI and BamHI; SP-3×Flag-IRE1b.1 and IRE1b.1-3×Flag, Xholand HindIII; RTP1-HA: EcoR1 and XbaI), and thereafter inserted into the binary vector pART27 at the Not1 site.

Plant recovery and growth retardation assays

Seeds were sterilized and treated by TM (0.3 μg/mL) or DTT (2 mM) as previously described (Moreno et al., 2012). After TM or DTT exposure, 30 seeds per line were transferred to 1/2 MS 0.8% (w/v) agar medium supplemented with ampicillin (50 μg/mL), and grown in a horizontal direction. After 10 d growth, survival seedlings were recorded. Percentage of recovery was calculated as described (Moreno et al., 2012). For growth retardation assay, 5-d-old A. thaliana seedlings grown in 1/2 MS medium were transferred into 1/2 MS + 1% sucrose (w/v) liquid medium with TM (75 ng/mL)/DTT (2 mM) or DMSO (control), respectively. These seedlings were placed in 96-well plates. At least 10 plants per line were treated. The seedlings fresh weight was measured at 5 d after treatment. Relative fresh weight was plotted by calculating treatment/control seedlings.
Gene expression analysis
Total RNA was extracted from root material by using TRIzol (Invitrogen) reagent. For RT-qPCR analysis, cDNA was synthesized from 1 μg of total RNA using PrimeScript RT reagent Kit (Takara). Twenty nanograms of cDNA were used as template for the amplification of candidate genes using SYBR premix Kit (Roche) according to the manufacturers’ instructions. The primers we used are listed in Supplemental Table S2. The Ct values of genes were quantified using an iQ7 Real-Time Cycler (Life technologies, USA). Expression fold changes were calculated by the 2−ΔΔCt method (Schmittgen and Livak, 2008).

For RT-qPCR-based bZIP60 splicing assay, cDNA was used as template. The bZIP60 splicing activity was analyzed as described (Moreno et al., 2012).

Firefly luciferase complementation imaging assay
The constructed vectors were transformed into Agrobacterium strain GV3101 and then infiltrated N. benthamiana leaves. After 3 d, 1 mM beetleluciferin (Promega) was sprayed on leaves and kept dark for 6 min at room temperature to quench the autofluorescence. A low-light cooled charge-coupled device camera (Lumazone Pylon 2048B, Princeton, USA) was used to capture the LUC image. The camera was cooled at −120°C and used to measure the relative LUC activity as described (Chen et al., 2008).

Co-IP and immunoblot assays
Nicotiana benthamiana leaves were harvested at indicated timepoints after agroinfiltration and proteins were extracted with a buffer containing 250 mM sucrose, 50 mM HEPES-KOH, 5% glycerin, 1 mM Na2MoO4 × 2 H2O, 25 mM NaF, and 10 mM EDTA. Co-IP was performed as described (Win et al., 2011). Precipitates by anti-FLAG M2 affinity gel (Sigma-Aldrich) or anti-Myc magnetic beads (Bimake) were washed at least five times with GTEN buffer (10% glycerol, 25 mM Tris [pH 7.5], 1 mM EDTA, 150 mM NaCl) supplemented with 1 mM DTT and 0.15% NP40 (w/v; Sigma). Fusion proteins from crude extracts (input) and precipitated proteins were detected by IB with protein-specific antibodies. For immunodetection of BiP, Arabidopsis root extracts were separated by SDS-PAGE followed by transfer to polyvinylidene difluoride (PVDF) membranes (Roche). The polyclonal antibody anti-BiP2 (Agrisera, Sweden; AS09481) was used at a 1:5,000 dilution and followed by incubation with a second antibody, anti-rabbit Ig-horseradish peroxidase (#AS014, Abclonal). Protein bands were quantified using Image J software (Schneider et al., 2012).

Phosphorylation assay
The constructs of IRE1a-3×FLAG and RTP1-HA, 3×FLAG-IRE1b, and RTP1-HA, IRE1α-3×FLAG and EV, 3×FLAG-IRE1b and EV, were cotransformed using the Agrobacterium-mediated transient transformation in leaves of 6-week-old N. benthamiana. At 3 d post coinfiltration, the leaves were harvested and total proteins were extracted using HEPES buffer containing phosphatase inhibitor cocktail (Sigma) 1:100. The Phos-tag indicator (#300-93523, Wako) and MnCl2 were used to distinguish the phosphorylated and non-phosphorylated proteins (Kaps et al., 2015). After SDS-PAGE, the gel was soaked in a transfer buffer containing 10 mM EDTA and gently incubated for 10 min for three times, and then soaked in a transfer buffer without EDTA and gently shaken for 10 min. The accumulation of phosphorylated and nonphosphorylated proteins was analyzed by IB using protein-specific antibodies. The band intensities of phosphorylated and unphosphorylated IRE1 proteins were determined by the gray values using Image J. In order to compare intensities of IRE1 phosphorylation in different samples in parallel, the band intensities of unphosphorylated IRE1 proteins in control samples (i.e. IRE1a-FLAG or FLAG-IRE1b + EV) were used as the calibrator; whereas the band intensities of phosphorylated IRE1 proteins in the tested samples (i.e. IRE1a-FLAG or FLAG-IRE1b + RTP1-HA) were calculated by an adjusted factor calculated as follows:

\[
\text{Adjusted factor} = \frac{\text{intensity of UP (negative control)}}{\text{intensity of UP (tested sample)}}
\]

\[
\text{Adjusted intensity} = \text{intensity of P (tested sample) \times adjusted factor}
\]

(UP, unphosphorylated IRE1; P, phosphorylated IRE1)

The adjusted band intensities of phosphorylated IRE1 proteins in the tested samples were normalized with the band intensities of phosphorylated IRE1 proteins in control samples, which were set to 1.

Callose deposition assay
The callose assay was performed as described (Luna et al., 2011). Briefly, the 4-week-old A. thaliana plant leaves were infiltrated with 1 μM flg22 for 24 h or infected by P. parasitica zoospores for 24 h. Leaves were cut, fixed, and destained in ethanol:glacial acetic acid (3:1, v:v) with 1× change of the solution until the leaves were transparent. Thereafter, leaves were rehydrated in 70% ethanol for 15 min, and then in 50% ethanol for 15 min. After several washes with water, leaves were incubated for 2 h in 150 mM K2HPO4 (pH 9.5) solution containing 0.01% aniline blue (w/v) in darkness. Callose deposits were detected using Olympus BX63 microscope (excitation, 365 nm; emission, 420 nm) and quantitated from digital photographs with Image J software (Schneider et al., 2012).

Oxidative burst assay
ROS production was measured with a previously reported luminol-based assay (Sang and Macho, 2017). The leaf disks 5 mm in diameter were cut from the 7-week-old A. thaliana plant leaves with sharp puncher and were floated in 200 μL H2O overnight. Water was replaced with reagent containing luminol, peroxidase, and 1 μM flg22. ROS released by leaf discs was detected by luminescence of luminol. Luminescence was measured at 535 nm (excitation 490 nm) in a TECAN Infinite F200 microplate reader (TECAN, Switzerland).
Agroinfiltration and confocal laser scanning microscopy

Agrobacterium tumefaciens strain GV3101 cells transformed with individual vector constructs were grown in Luria–Bertani medium with appropriate antibiotics at 28°C for about 36 h. Agrobacteria were pelleted, resuspended in infiltration buffer (10 mM MES, 10 mM MgCl2, and 200 mM acetosyringone) and adjusted to the required concentration (OD600 0.3) before being infiltrated into the 4- to 6-week-old N. benthamiana leaves.

Nicotiana benthamiana cells expressing fusion proteins were observed 2 or 3 d after infiltration using an Olympus FV3000 confocal microscope (Japan). GFP was detected after excitation with a 488 nm wavelength laser, and emissions were collected at 500–540 nm. The fluorescence of mCherry was excited with a 559 nm wavelength laser to detect specific emissions at 600–680 nm.

Statistical analysis

Results are expressed as means ± standard deviation (SD) or ± standard error (SE) as indicated in the figure legends and represent at least three biological repetitions. Statistical analysis was performed using Student’s t test. P < 0.05 was considered to be significant.

Accession numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL database under the following accession numbers: Arabidopsis: RTP1 (AT1G70260), bZIP60 (AT1G42990), IRE1a (AT2G17520), IRE1b (AT5G24360), bZIP28 (AT3G10800), BIP3 (AT1G09080), DnaJ (AT1G56300), WRKY33 (AT2G38470), WRKYG6 (AT2G46400), CBP60a (AT5G26920), CYP71A12 (AT2G30750) and MPK11 (AT1G01560).

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure S1. Analysis on the protein stability of myc-GFP in the presence of RTP1.

Supplemental Figure S2. RTP1 colocalizes with bZIP60 in the ER membrane.

Supplemental Figure S3. Independent analysis on the phosphorylation of IRE1 protein.

Supplemental Figure S4. RTP1 attenuates the protein stability of IRE1 protein.

Supplemental Figure S5. Analysis on protein interaction between RTP1 and IRE1.

Supplemental Figure S6. Co-immunoprecipitation assay on protein interaction between RTP1 and bZIP28.

Supplemental Figure S7. EFR and γVPE show increased expression in P. parasitica-infected rtp1 mutants.

Supplemental Figure S8. RTP1 colocalizes with bZIP28 in the ER membrane.

Supplemental Figure S9. Analysis on protein interaction between RTP1 and BI-1.

Supplemental Figure S10. Diagrams showing regulatory elements in the promoter regions of immune genes WRKY33, CBP60a, and CYP71A12.

Supplemental Table S1. Immune genes induced more than four-fold by tunicamycin treatment in A. thaliana WT Col-0 plants.

Supplemental Table S2. List of primers used in this study.

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Conflict of interest statement. The authors declare no conflict of interest.

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