SnRK1.1-mediated resistance of *Arabidopsis thaliana* to clubroot disease is inhibited by the novel *Plasmodiophora brassicae* effector PBZF1

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Abstract
Plants have evolved a series of strategies to combat pathogen infection. Plant SnRK1 is probably involved in shifting carbon and energy use from growth-associated processes to survival and defence upon pathogen attack, enhancing the resistance to many plant pathogens. The present study demonstrated that SnRK1.1 enhanced the resistance of *Arabidopsis thaliana* to clubroot disease caused by the plant-pathogenic protozoan *Plasmodiophora brassicae*. Through a yeast two-hybrid assay, glutathione S-transferase pull-down assay, and bimolecular fluorescence complementation assay, a *P. brassicae* RxLR effector, PBZF1, was shown to interact with SnRK1.1. Further expression level analysis of SnRK1.1-regulated genes showed that PBZF1 inhibited the biological function of SnRK1.1 as indicated by the disequilibration of the expression level of SnRK1.1-regulated genes in heterogeneous PBZF1-expressing *A. thaliana*. Moreover, heterogeneous expression of PBZF1 in *A. thaliana* promoted plant susceptibility to clubroot disease. In addition, PBZF1 was found to be *P. brassicae*-specific and conserved. This gene was significantly highly expressed in resting spores. Taken together, our results provide new insights into how the plant-pathogenic protist *P. brassicae* employs an effector to overcome plant resistance, and they offer new insights into the genetic improvement of plant resistance against clubroot disease.

**KEYWORDS**
*Arabidopsis thaliana*, effector, PBZF1, *Plasmodiophora brassicae*, SnRK1.1

1 | INTRODUCTION

Plants have evolved a series of strategies to combat pathogen infection, such as physical barriers, constitutive chemical defences, and inducible defences (Bednarek et al., 2009; Chen et al., 1993; Gaffney et al., 1993; Hematy et al., 2009; Sugio et al., 2011; Vorwerk et al., 2004; Wittstock & Gershenzon, 2002). SNF1-related kinase 1 (SnRK1) and target of rapamycin (TOR) kinase have critical functions in the regulation of cell growth and in response to environmental stresses. These two kinases act in opposite ways as described by the
“yin–yang” model (Rodriguez et al., 2019). SnRK1 is a central integrator of transcription networks in plant stress and energy signalling (Baena-Gonzalez et al., 2007), which is activated by lack of energy or nutrients and inhibits cell growth (Hulsmans et al., 2016). Many key enzymes, such as sucrose phosphate synthase (SPS), nitrate reductase (NR), trehalose phosphate synthase (TPS), fructose-2,6-biphosphatase (F2KP), and suppressor of gamma response 1 (SOG1), are involved in this process (Hamasaki et al., 2019; Kulma et al., 2004; Martinez-Barajas & Coello, 2020; Sugden et al., 1999). In response to biotic stresses, SnRK1 is probably involved in shifting carbon and energy use from growth-associated processes to survival and defence upon pathogen attack (Hulsmans et al., 2016). To date, SnRK1 has been reported to be involved in the resistance to many plant pathogens. SnRK1 from wheat contributes to resistance against Fusarium graminearum (Jiang et al., 2020). SnRK1-overexpressing barley plants exhibit enhanced resistance to Blumeria graminis f. sp. hordei (Han et al., 2020). Constitutive expression of a SnRK1.2 transgene results in enhanced resistance to geminivirus infection in Nicotiana benthamiana (Hao et al., 2003). Overexpression of SnRK1 in rice confers broad-spectrum disease resistance to Xanthomonas oryzae pv. oryzae (Xoo), Magnaporthe oryzae, Cochliobolus miyabeanus, and Rhizoctonia solani (Filipe et al., 2018).

Plant pathogens secrete proteins to overcome plant defences, and these proteins are known as effector proteins (Sugio et al., 2011). To date, many effectors have been identified from pathogenic fungi, oomycetes, bacteria, nematodes, and parasitic plants. These effectors interfere with the function of host targets to facilitate infection (Chen, Ullah, et al., 2020, Clark et al., 2018; Guo et al., 2019; Huang et al., 2020; Jiang et al., 2020; Li et al., 2013, 2019; Lin et al., 2016; Ma et al., 2015; Qiao et al., 2013; Song et al., 2015; Su et al., 2020; Wang et al., 2010; Yang et al., 2018, 2020; Yin et al., 2019). With the identification of these effectors, the infection mechanisms of the corresponding plant pathogens are gradually being revealed. However, little is known about the function and molecular mechanisms of effectors from plant-pathogenic protozoa.

Plasmodiophora brassicae is an economically important, soilborne, obligate plant-pathogenic protozoan that causes clubroot disease in Cruciferae crops worldwide (Chai et al., 2014; Dixon, 2009). Similar to other obligate plant pathogens, a large number of proteins from P. brassicae have been found to match the characteristics of secreted proteins, encoding N-terminal signal peptides with no extra transmembrane regions (Rolle et al., 2016; Schwelm et al., 2015). The secretory activity of several putative secreted proteins from P. brassicae has been previously confirmed (Chen et al., 2019; Perez-Lopez et al., 2020). It is well known that secreted proteins are candidate effectors. However, to date, only one effector, P. brassicae benzoic acid/salicylic acid methyltransferase (PbBSMT), has been reported in P. brassicae. PbBSMT contains a salicylic acid methyltransferase, benzoic acid methyltransferase and theobromine methyltransferase-type methyltransferase domain and catalyses the conversion of salicylic acid (SA) to methyl salicylate (me-SA) to alter host susceptibility to P. brassicae (Bulman et al., 2019; Ludwig-Muller et al., 2015). There are many effector candidates without known functional domains encoded by P. brassicae (Perez-Lopez et al., 2018). The roles and molecular mechanisms of these effector candidates during P. brassicae infection should be further studied.

The present study demonstrates that SnRK1.1 enhances the resistance of Arabidopsis thaliana to clubroot disease. We found that the novel effector PBZF1 interacts with SnRK1.1 and inhibits the biological function of SnRK1.1. These results provide novel knowledge about how a plant-pathogenic protozoan, P. brassicae, overcomes plant defence, and they provide new clues for the genetic improvement of brassica crop resistance against clubroot disease.

## RESULTS

### 2.1 | SnRK1.1 plays a positive role in A. thaliana resistance to clubroot disease

SnRK1 is a central integrator of transcription networks in plant stress and energy signalling. Recent studies have shown that SnRK1 promotes plant resistance against many plant pathogens, such as geminivirus, B. graminis f. sp. hordei, F. graminearum, and several rice pathogens (Filipe et al., 2018; Han et al., 2020; Hao et al., 2003; Jiang et al., 2020). Because SnRK1.1 is the main SnRK1 kinase in A. thaliana (Jossier et al., 2009), to determine whether SnRK1 is involved in resistance to clubroot disease, two SnRK1.1-RNA interference (RNAi) lines and two SnRK1.1-overexpression (OE) lines were inoculated with P. brassicae resting spores, and the occurrence of clubroot disease was investigated at 28–30 days postinoculation (dpi). In wildtype A. thaliana (Ler), the percentage of medium to severe galls (disease classes 3 and 4) was 72.6%, and the percentage of small galls (disease classes 1 and 2) was 27.4%. The percentage of medium to severe galls was higher in the two SnRK1.1-RNAi lines but lower in the two SnRK1.1-OE lines than in the wild type with a value of 93.4% in SnRK1.1-RNAi line 1, 96.5% in SnRK1.1-RNAi line 7, 49.3% in SnRK1.1-OE line 1, and 50% in SnRK1.1-OE line 2. Moreover, the percentage of small galls decreased to 6.6% in SnRK1.1-RNAi line 1 and 3.5% SnRK1.1-RNAi line 7. The percentage of small galls increased to 50.7% in SnRK1.1-OE line 1 and 50% SnRK1.1-OE line 2. Therefore, the disease index increased in both SnRK1.1-RNAi lines but lower in the two SnRK1.1-OE lines. Because SnRK1.1 enhances the resistance of A. thaliana to clubroot disease and that overexpression of SnRK1.1 promotes the resistance of A. thaliana to clubroot disease.

### 2.2 | The P. brassicae secreted protein PBZF1 physically interacts with A. thaliana SnRK1.1

Because SnRK1 plays a positive role in plant resistance, it often serves as a target of plant pathogens (Hao et al., 2003; Jiang et al., 2020; Szczesny et al., 2010). Previous studies have shown that some plant pathogens encode proteins that target or degrade host SnRK1. In our study, SnRK1.1 promoted the resistance of A. thaliana to clubroot
disease, and it may be the target of some *P. brassicae* proteins. In a yeast two-hybrid (Y2H) screening assay using PBCN_001987 (without signal peptide) as bait, a fragment from SnRK1.1 was obtained. Further verification of this interaction was performed with PBCN_001987 (without a signal peptide) and full-length SnRK1.1. Yeast cotransformed with PBCN_001987 and SnRK1.1 recombinant plasmids grew on synthetic dropout medium (SD−Leu−Trp−His−Ade plates, and these clones showed blue colonies on X-α-Gal-containing plates. Moreover, the clones of the negative control did not grow on SD−Leu−Trp−His−Ade plates (Figure 2a). These results indicated that PBCN_001987 interacts with SnRK1.1 in yeast. Our previous work confirmed PBCN_001987 as a secretory protein (Chen et al., 2019). Conserved domain analysis indicated a zinc finger Myeloid-Nervy-DEAF1 domain at the N-terminus of PBCN_001987, and we named this domain PBZF1.

To determine whether PBZF1 directly interacts with SnRK1.1, maltose-binding protein (MBP)-PBZF1, glutathione S-transferase (GST)-SnRK1.1, MBP-SnRK1.1, and GST-PBZF1 were expressed in *Escherichia coli*, and GST pull-down assays were performed. MBP-PBZF1 was pulled down by GST-SnRK1.1-bound agarose but not GST-bound agarose. MBP-SnRK1.1 was pulled down by GST-PBZF1-bound agarose but not GST-bound agarose (Figure 2b). These results indicated that PBZF1 directly interacts with SnRK1.1 in vitro.

To further validate the interaction of PBZF1 and SnRK1.1 in plants, PBZF1-mCherry and SnRK1.1-GFP were coexpressed in *Nicotiana benthamiana* leaves, and fluorescent signals were detected by confocal microscopy. The results showed that green fluorescent protein (GFP) and mCherry fluorescent signals were colocalized in the cell nucleus and cytoplasm (Figure 2c). A bimolecular fluorescence complementation (BiFC) assay was then performed in *N. benthamiana* leaves. The fluorescent signal was detected only in cells coexpressing the cYFP-PBZF1 (without signal peptide) fusion protein and nYFP-SnRK1.1 fusion protein, while no fluorescent signal was detected in the controls (Figure 2d). Furthermore, to further verify the localization of the interaction between PBZF1 and SnRK1.1, leaves coexpressing cYFP-PBZF1 and nYFP-SnRK1.1 were stained with 4,6-diamidino-2-phenylindole (DAPI). As shown in Figure 2e, the yellow fluorescent protein (YFP) signals were not colocalized with DAPI signals, which indicated that PBZF1 interacted with SnRK1.1 in the plant cytoplasm, but not the nucleus.

### 2.3 Heterogeneous expression of PBZF1 disrupts the expression of genes regulated by SnRK1.1 in *A. thaliana*

Because PBZF1 interacts with *A. thaliana* SnRK1.1, we generated transgenic *A. thaliana* lines expressing PBZF1 (without a signal peptide) to further investigate the effect of PBZF1 on the biological
function of SnRK1.1. The expression of PBZF1 was confirmed by quantitative reverse transcription PCR (RT-qPCR) and western blotting (Figure S4). The expression levels of a series of genes regulated by SnRK1.1 were quantified in the transgenic lines and the wild type. As a central integrator of transcription networks in plant stress and energy signalling, SnRK1.1 regulates global gene expression. The expression of glutamine-dependent asparagine synthetase dark inducible 1 (DIN1), glutamine-dependent asparagine synthetase dark inducible 6 (DIN6), putative auxin-regulated protein (AXP), pollen germination-related protein PGPD14 (PGPD14), and senescence-associated protein 5 (SENS) is induced by SnRK1.1 in A. thaliana. In contrast, the expression of MYB75 transcription factor (MYB75), MYB30 transcription factor (MYB30), cell wall modification expansin 10 (EXP10), 60S ribosomal protein L34 (L34), steroid 22-α-hydroxylase dwarf4 (DWF4), histone deacetylase HD2 family (HD1), Jasmonic acid carboxyl methyltransferase (JMT), and IAA biosynthesis SUPERROOT 1 (SUR1) is suppressed.

**FIGURE 2** PBZF1 interacts with SnRK1.1 from Arabidopsis thaliana. (a) PBZF1 interacts with SnRK1.1 in yeast. Yeast (strain AH109) cotransformed with pGBK7T (BD) or the BD-PBZF1 bait vector and pGADT7 (AD) or the AD-SnRK1.1 prey vector was grown on SD−Trp−Leu, SD−Trp−Leu−His, SD−Trp−Leu−His−Adex + X−α−Gal for interaction confirmation. Only yeast cotransformed with PBZF1 and AD-SnRK1.1 grew on the SD−Trp−Leu−His and SD−Trp−Leu−His−Adex medium, yielding X−α−Gal activity. BD + AD, BD + AD-SnRK1.1, and AD + BD-PBZF1 were used as negative controls. BD-SWP1 + AD-TC14 was used as positive control. Three independent experiments showed consistent results. (b) PBZF1 directly interacts with SnRK1.1 in vitro. GST-PBZF1 or GST-SnRK1.1 protein purified by glutathione agarose was incubated with Escherichia coli lysates containing MBP-SnRK1.1 or MBP-PBZF1 for 4 hr at 4 °C. Pulled-down proteins were detected by western blot to determine the presence of glutathione S-transferase (GST)-tagged proteins and maltose-binding protein (MBP)-tagged proteins. The following controls were used for specific interactions: GST incubated with E. coli lysates containing MBP, MBP-PBZF1, and MBP-SnRK1.1; GST-PBZF1 incubated with E. coli lysates containing MBP; and GST-SnRK1.1 incubated with E. coli lysates containing MBP. (c) PBZF1 colocalizes with SnRK1.1 in planta. Leaves of Nicotiana benthamiana were infiltrated with Agrobacterium GV3101 strains containing pBIN-mCherry-PBZF1 and pCAMBIA1302-SnRK1.1. The infiltrated leaves were observed via confocal microscopy (Nikon, C2) at 48 hr postinoculation (hpi). Three independent experiments showed similar results. Bars = 50 μm. (d) PBZF1 interacts with SnRK1.1 in planta. Leaves of N. benthamiana were infiltrated with Agrobacterium GV3101 strains containing pBIN-mCherry-PBZF1 and pCAMBIA1302-SnRK1.1. The infiltrated leaves were observed via confocal microscopy (Nikon, A1) at 48 hpi. Three independent experiments showed similar results. Bars = 50 μm. (e) PBZF1 interacts with SnRK1.1 in cytosol. Leaves of N. benthamiana were infiltrated with Agrobacterium GV3101 strains containing nYFP-SnRK1.1 + cYFP-PBZF1. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). The infiltrated leaves were observed via confocal microscopy (Nikon, C2) at 48 hpi. Three independent experiments showed similar results. Bars = 50 μm.
by SnRK1.1 (Baena-González et al., 2007). Therefore, the expression profiles of these genes were quantified in PBZF1-heterogeneous expression (HE) lines by RT-qPCR. Compared with the levels in the wild type, the expression levels of SEN5, PGPD14, AXP, DIN6, and DIN1, which were induced by SnRK1.1, decreased to different degrees in both PBZF1-HE lines. The expression levels of these genes decreased from 22.3% to 87.6% in PBZF1-HE line 1 and 44.8% to 87.1% in PBZF1-HE line 2. Consistent with the above results, the expression levels of MYB75, MYB30, EXP10, L34, DWF4, HDT1, JMT, and SUR1, which were suppressed significantly by SnRK1.1, increased in the two PBZF1-HE lines. The expression levels of these genes increased from 27% by 7.5 times in PBZF1-HE 1 and from 26% by 1.8 times in PBZF1-HE line 2 (Figure 3). These results showed that heterogeneous expression of PBZF1 disrupts the expression of genes regulated by SnRK1.1 in Arabidopsis thaliana, indicating that PBZF1 inhibits the biological function of SnRK1.1.

2.4 | Heterogeneous expression of PBZF1 enhances the virulence of P. brassicae

Because PBZF1 inhibits the biological function of SnRK1.1, which plays a positive role in the resistance of A. thaliana to clubroot disease, we speculated that PBZF1 could promote the virulence of P. brassicae. To test this hypothesis, two PBZF1-HE lines and the wild type were inoculated with P. brassicae, and the occurrence of clubroot disease was investigated at 28–30 dpi. In the wild type, the percentage of medium to severe galls (disease classes 3 and 4) was 69.6%, and the percentage of small galls (disease classes 1 and 2) was 30.4%. In the PBZF1 heterogeneous expression lines, the percentage of medium to severe galls was higher, with a value of 91% in PBZF1-HE line 1 and 91.4% in PBZF1-HE line 2. The percentage of small galls decreased to 9% in PBZF1-HE line 1 and 8.6% in PBZF1-HE line 2. Consequently, the disease index in both PBZF1-HE lines was higher than that of the wild type (Figures 4 and S5). These results illustrate that PBZF1 enhances the virulence of P. brassicae and promotes plant susceptibility to clubroot disease in A. thaliana. In addition, these findings suggested that PBZF1 is a pathogenicity factor.

2.5 | PBZF1 is a novel, conserved protein in P. brassicae

The above results indicate that PBZF1 is an effector that contributes to the pathogenicity of P. brassicae. Some effector proteins, called core effectors, are highly conserved within species and even among different species. These core effectors generally play important roles in pathogenicity (Chen, Li, et al., 2020; Nie et al., 2019). To investigate whether PBZF1 is a core effector, we searched for proteins similar to PBZF1 in the nonredundant protein sequence database of GenBank using the BlastP program. Only two proteins (PBRA_008189 and PBRA_008198) from P. brassicae were similar to PBZF1 with E-values less than 10^{-10}. Proteins similar (E-value less than 10^{-10}) to PBZF1 were not found in other pathogens. From the perspective of amino acid sequence similarity, PBZF1 is a P. brassicae-specific effector. The sequences of PBZF1 proteins from

![Figure 3](image-url)

**Figure 3** Heterogeneous expression of PBZF1 disrupts the expression of genes regulated by SnRK1.1 in Arabidopsis thaliana. Four-week-old A. thaliana leaves were harvested for quantifying the expression of genes regulated by SnRK1.1. The expression levels were normalized to the transcript level of the A. thaliana actin gene, and the relative transcript levels of genes regulated by SnRK1.1 were calculated using the comparative threshold (2^{-ΔΔCt}) method. The transcript level of genes regulated by SnRK1.1 in wildtype A. thaliana (Col-0) was standardized to 1. Values represent the mean ± SE of three independent biological replicates, and each biological replicate contained three technique replicates. Student’s t test, *p < .05, **p < .01
different *P. brassicae* isolates were analysed. Among the 69 isolates analysed, the PBZF1 gene from 43 isolates shared identical nucleotide sequences, and PBZF1 from 66 isolates shared identical amino acid sequences. Only four polymorphism sites in the nucleotide sequence were found in the PBZF1 gene from three Canadian isolates and eight Chinese isolates (Figure 5a). Among PBZF1 proteins from these 11 isolates, two polymorphism sites in the amino acid sequence were found in two Canadian isolates and a Chinese isolate (Figure 5b). These results showed that the PBZF1 proteins share high sequence identity, indicating that PBZF1 is a conserved core protein in *P. brassicae*.

### 2.6 PBZF1 is an RxLR effector that is highly expressed in resting spores

Effectors rich in cysteine or containing the RxLR motif are widely studied in plant-pathogenic oomycetes and fungi. These motifs enable the delivery of effector proteins into plant cells (Kale et al., 2010). Although *P. brassicae* is an intracellular parasite, in theory, when proteins are secreted from *P. brassicae* during infection they enter plant cells directly. Although the RxLR motif and cysteine-rich sequence are redundant for *P. brassicae* secretory proteins, we found an RxLR motif in the PBZF1 protein located at amino acids 35–38 in the N-terminus (Figure S6), suggesting that even at the stage before *P. brassicae* invades plant cells after secretion from *P. brassicae*, PBZF1 can also enter plant cells. The resting spore stage is the stage before *P. brassicae* invades plant cells. To test whether PBZF1 is expressed in resting spores and further clarify the expression pattern of PBZF1, the expression levels of PBZF1 in the resting spores and plant roots during *P. brassicae* infection were quantified by RT-qPCR. It is generally recognized that *P. brassicae* resting spores require 5 days to complete primary infection at 25 °C (Sharma et al., 2011). Therefore, we detected the expression levels at 3 and 5 dpi for the primary infection stage. The results showed that the transcripts of PBZF1 were detected in all samples. The highest expression level of PBZF1 was found in resting spores. The expression levels of PBZF1 in the primary infection stage were the lowest with levels of 0.175% (3 dpi) and 0.184% (5 dpi) compared to resting spores. The expression levels of PBZF1 during the secondary infection stage were 1.49% (15 dpi) and 2.26% (30 dpi) of those in resting spores (Figure 6). These results showed that PBZF1 is highly expressed in resting spores.

### 3 DISCUSSION

Plant SnRK1 is a homologue of SNF1 from the yeast *Saccharomyces cerevisiae* and AMP-activated protein kinase α subunit (AMPKα) from mammals (Halford & Hardie, 1998). SnRK1 has been reported to be involved in the resistance to many plant-pathogenic fungi, bacteria, and viruses. In this study, we also found that SnRK1.1 promoted the resistance of *A. thaliana* to clubroot disease, which is caused by the protozoan *P. brassicae*. Thus, SnRK1-mediated resistance is a broad-spectrum disease resistance in plants. However, the SnRK1-mediated resistance mechanism is still unclear. As an energy sensor, SnRK1 globally regulates energy signalling and carbon metabolism, which are typically associated with defence responses (Baena-Gonzalez et al., 2007; Halford & Hardie, 1998; Hulsmans et al., 2016). During
this process, SnRK1 regulates global gene expression and several key enzymes, such as SPS, NR, TPS, F2KP, SOG1, and autophagy-related gene 1 (ATG1) (Hamasaki et al., 2019; Kulma et al., 2004; Martinez-Barajas & Coello, 2020; Sugden et al., 1999), as well as further affects secondary metabolism, growth, development, and autophagy (Baena-Gonzalez et al., 2007; Chen et al., 2017; Hulsmans 2017; Kulma et al., 2004).

**FIGURE 5** Sequence diversity of PBZF1 in different Plasmodiophora brassicae isolates. The sequences of PBZF1 from Chinese *P. brassicae* isolates were obtained by PCR and subsequent sequencing. The sequences of PBZF1 from Canadian, USA, and European *P. brassicae* isolates were obtained from assembly data of the *P. brassicae* genome sequencing project or genome resequencing project in GenBank. The sequence analysis was performed by DNAMAN v. 6.0. (a) Nucleotide sequence of PBZF1 from different *P. brassicae* isolates. (b) Protein sequence of PBZF1 from different *P. brassicae* isolates.
et al., 2016). These regulated genes and enzymes provide clues for the resistance mechanism of SnRK1. SnRK1 directly inhibits SPS activity (Sugden et al., 1999), which is positively associated with the content of sugar (Hall et al., 1995). Galls caused by *P. brassicae* are sites of intense sugar consumption and a strong physiological sink for carbohydrates (Keen & Williams, 1969). Plant sugar efflux machinery is the nutrient supply of *P. brassicae* (Walerowski et al., 2018). SnRK1 negatively regulates the expression of *MYB75* and *SUR1* (Baena-Gonzalez et al., 2007). *MYB75* regulates the biosynthesis of anthocyanin and phenylpropanoids, and it further affects cell wall composition and auxin transport (Bhargava et al., 2010; Buer & Muday, 2004). *SUR1* is involved in auxin biosynthesis, which confers auxin overproduction (Mikkelsen et al., 2004). Moreover, auxin biosynthesis and transport are associated with the formation of galls in clubroot disease (Devos et al., 2006; Ludwig- Muller, 2009; Ludwig- Muller & Schuller, 2008; Xu et al., 2016). In addition, some researchers have shown that SnRK1 phosphorylates and destabilizes WRKY3 to enhance wheat resistance to powdery mildew (Han et al., 2020). SnRK1 from rice positively regulates salicylic acid (SA) and jasmonic acid (JA) pathways after inoculation with the blast fungus *Pyricularia oryzae* (Filipe et al., 2010). These results suggest that the resistance mediated by SnRK1 is complex and diverse.

Given that SnRK1 mediates broad-spectrum disease resistance, plant pathogens have evolved proteins to overcome the resistance mediated by SnRK1. AL2 from tomato golden mosaic virus and L2 from beet curly top virus interact with SnRK1.2 from *A. thaliana* (Hao et al., 2003). The Osp24 effector from *F. graminearum* interacts with wheat TaSnRK1α (Jiang et al., 2020). AvrBsT from *Xanthomonas campestris* interacts with SnRK1 from pepper (Szczesny et al., 2010). In this research, we reported a *P. brassicae* protein, PBZF1, that interacts with SnRK1.1. The sequences of these proteins share low identity (lower than 10%), and the identity between the AL2 and L2 viral proteins is 18.75%. Although potential 3D conservation of these proteins cannot be ruled out, PBZF1 was identified as a novel protein only from the perspective of amino acid sequence similarity. These findings suggested that different strategies for interfering with SnRK1 biological function have evolved in viruses, bacteria, fungi, and protozoa.

The present study demonstrated that PBZF1 interacts with SnRK1.1 and promotes the virulence of *P. brassicae*. Because our previous work indicated that PBZF1 is a secretory protein, PBZF1 was identified as an effector. Some effectors are highly expressed in the early stage of infection. Previous transcriptomic data have shown that PBZF1 is expressed in the primary infection stage, which is the critical stage for the recognition of *P. brassicae* by hosts and the initiation of resistance or susceptibility responses in plants (McDonald et al., 2014). Further RT-qPCR analyses showed that PBZF1 is mainly expressed in resting spores, which is the stage before infection. Considering the presence of the RxLR motif in PBZF1, PBZF1 could be secreted from resting spores and then enter plant cells, suggesting that the interaction between *P. brassicae* and the host occurs earlier than indicated in previous research. Moreover, this finding suggested that *P. brassicae* may be prepared to infect hosts at the resting spore stage, during which this organism probably does not come in direct contact with its hosts. A similar phenomenon has been reported in another protist, *Plasmodium falciparum.*
Plasmodium sporozoites are transmitted from infected mosquitoes to mammals, but the genes of P. falciparum essential for infecting human cells are highly expressed in sporozoites from mosquitoes (Lindner et al., 2019). These results suggest that the expression of effector genes before pathogen infection also plays key roles during infection.

Although a P. brassicae effector was identified in this paper, few effectors have been reported in P. brassicae (Perez-Lopez et al., 2018). With the completion of genome sequencing and subsequent research, many secretory proteins that were considered as candidate effectors have been identified from P. brassicae (Chen et al., 2019; Perez-Lopez et al., 2020). However, it is difficult to identify the effectors that function during the infection process and subsequent disease progression. One limitation for research progress on effectors from P. brassicae, a soilborne obligate pathogen, is the difficulty in generating transgenic strains to overexpress or silence candidate genes. Another limitation is the lack of a convenient and effective method to evaluate the pathogenicity of different P. brassicae strains. In this paper, we applied heterogeneous expression to overexpress P. brassicae genes and then inoculated P. brassicae spores at a relatively low concentration (4 × 10^5 spores per plant). This experiment accurately showed the differences in disease severity between wild-type and transgenic A. thaliana, allowing a simple evaluation of the function of candidate genes in clubroot disease. In this study, PBZF1 was identified as a pathogenicity factor. These findings may provide references for functional studies of other genes in P. brassicae.

In conclusion, we first reported the positive function of SnRK1.1 in resistance against clubroot disease and then found a P. brassicae-specific and conserved effector, PBZF1, that enhances P. brassicae virulence by interacting with A. thaliana SnRK1.1 and interfering with its biological function. These results may lay a foundation for revealing the interaction between P. brassicae and plants, and they provide important information for breeding clubroot disease-resistant plants.

4 | EXPERIMENTAL PROCEDURES

4.1 | Plant material and P. brassicae

Seeds of A. thaliana (Col-0 ecotype and Landsberg erecta (Ler) background), N. benthamiana, and the ZS11 oilseed rape line were sown onto a vermiculite and compost mixture (3:1). Plants were grown in a greenhouse at approximately 23 °C with a 16-hr light/8-hr dark cycle. SnRK1.1 RNAi lines (SnRK1.1-RNAi line 1 and SnRK1.1-RNAi line 7, Ler background) and SnRK1.1 overexpression lines (SnRK1.1-OE line 1 and SnRK1.1-OE line 2, Ler background) were kindly donated by Dr Shi Xiao from Sun Yat-sen University, School of Life Science (Chen et al., 2017). The expression level of SnRK1.1 in the SnRK1.1 RNAi lines and SnRK1.1 overexpression lines was quantified by RT-qPCR (Figure S1).

P. brassicae isolates were collected from oilseed rape fields or Chinese cabbage fields located in Hubei Province (Zhijiang city and Enshi Prefecture), Yunnan Province (Heshun town, Tenchong city, and Dali city), Guizhou Province (Jinsha County), and Chongqing Municipality (Fuling District). Clubroots were stored at −80 °C for resting spore extraction. For the A. thaliana inoculation assay and PBZF1 expression pattern analysis, a P. brassicae isolate from Yichang city, Hubei Province was used.

4.2 | Gene cloning and plasmid construction

For the PBZF1 gene clone, DNA extracted from resting spores of a P. brassicae Yichang isolate was used as the PCR template. For the SnRK1.1 gene clone, cDNA isolated from the leaves of 4- to 5-week-old A. thaliana was used as the PCR template. The coding region of PBZF1 without sequences encoding the signal peptide and SnRK1.1 gene were amplified by a high-fidelity DNA polymerase (Tsingke Biotech Co.) using the primers listed in Table S1, and the purified DNA was then cloned into linearized plasmids by the ClonExpress II One Step Cloning kit (Vazyme Biotech Co.). The recombinant plasmids were transformed into E. coli DH5α and sequenced by Sanger sequencing. The correct recombinant plasmids were used for the subsequent research.

4.3 | P. brassicae resting spore isolation, inoculation, and disease investigation in A. thaliana

P. brassicae resting spores were extracted from infected oilseed rape according to the technique provided by Castlebury with several modifications (Castlebury et al., 1994). Approximately 10 clubroots were homogenized in a blender with 100 ml of distilled water and filtered through eight layers of cheesecloth. The filtrate was the resting spore suspension, which was used for the inoculation assay.

For clubroot disease investigation in A. thaliana, the resting spores were adjusted to a concentration of 2 × 10^5 spores/ml. Then, 14-day-old A. thaliana was inoculated with 2 ml of resting spores per plant (inoculating first with 1 ml of spores and then with the remaining spores on the next day). Approximately 28–30 dpi, the disease index was assessed using the following 0–4 scoring system (Siemens et al., 2002): 0 indicates no disease; 1 indicates small galls only on lateral roots; 2 indicates small galls covering the main root and few lateral roots; 3 indicates medium to large galls also on the main root; and 4 indicates severe galls on lateral roots, the main root, or the rosette with complete destruction of the fine roots. The disease index (DI) was calculated according to the following formula: DI = 100 × (N1 + 2N2 + 3N3 + 4N4)/4Nt, where N1 to N4 are the numbers of plants in the indicated class, and Nt is the total number of plants tested. In total, 58–95 plants were analysed in different Arabidopsis lines.

4.4 | Yeast two-hybrid assay

For the Y2H screening assay, the coding region of PBZF1 without sequences encoding the signal peptide was cloned into pGBK7. The
pGBK7-PBZF1 recombinant plasmid was transformed into S. cerevisiae AH109 to generate bait. A transcription self-activation assay was first performed. No transcriptional self-activation function of PBZF1 was found. Then, a cDNA library from A. thaliana was transformed into the bait strain. Five days later, the clones were transferred to SD−Leu−Trp−His plates, SD−Leu−Trp−His−Ade plates, and SD−Leu−Trp−His−Ade + X-α-Gal plates for further confirmation of interaction (Figure S2). The interaction clones were propagated in SD−Leu−Trp−His−Ade medium, and the plasmids were extracted for further sequencing. Then, the full-length coding sequence of the interaction fragments was cloned into pGADT7 and cotransformed into A. thaliana (Col-0) plants by the floral dip method (Clough & Bent, 1998). T0 seeds were screened in Murashige & Skoog (MS) medium containing kanamycin (50 mg/ml). Plants of the T3 generation were used for subsequent studies.

4.5 | GST pull-down

Full-length PBZF1 without a sequence encoding a signal peptide and SnRK1.1 were cloned into pGEX-KG and pMAL-c2×. Then, the recombinant vectors were transformed into E. coli BL21 (DE3). The positive bacterial clones were first grown for 2.5 hr at 37 °C, and tagged proteins were then expressed after induction with 1 mM isopropyl-β-D-thiogalactopyranoside overnight at 16 °C. Finally, the bacteria were collected and suspended in phosphate-buffered saline (PBS). After cell disruption by sonication and centrifugation, the supernatant containing GST-tagged proteins was mixed with 100 μl of glutathione agarose and incubated for 2 hr at 4 °C. Then, the agarose was washed six times with 1 ml PBS and incubated with the supernatant containing MBP-tagged proteins for 4 hr at 4 °C. After washing six times with 1 ml PBS, the agarose was boiled in 100 μl of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. The loading proteins were judged by Coomassie brilliant blue staining. Finally, the presence of GST-tagged and MBP-tagged proteins was detected by western blotting using anti-MBP (1:5,000 dilution, AE016; ABlonol Biotechnology Co.), anti-GST (1:5,000 dilution, A02030; Abbkine Scientific Co.), and horseshadish peroxidase-conjugated goat anti-mouse IgG (1:20,000 dilution, A02030; Abbkine Scientific Co.) antibodies.

4.6 | Colocalization and bimolecular fluorescence complementation assay

Colocalization and bimolecular fluorescence complementation (BiFC) assays were performed by transient agroinfiltration into 4- to 5-week-old N. benthamiana leaves. For the colocalization assay, full-length PBZF1 without a sequence encoding a signal peptide was cloned into pBIN61-mCherry, and SnRK1.1 without a stop codon was cloned into pCAMBIA1302. For the BiFC assay, full-length PBZF1 without a sequence encoding a signal peptide was cloned into pBIN61-cYFP, and SnRK1.1 was cloned into pBIN61-nYFP. Then, pBIN61-mCherry-PBZF1, pCAMBIA1302-SnRK1.1, pBIN61-cYFP-PBZF1, and pBIN-nYFP-SnRK1.1 were transformed into Agrobacterium tumefaciens GV3101. For the colocalization assay, A. tumefaciens containing pBIN61-mCherry-PBZF1 and A. tumefaciens containing pCAMBIA1302-SnRK1.1 were coinjected into N. benthamiana leaves at a ratio 1:1. For the BiFC assay, A. tumefaciens containing pBIN-cYFP-PBZF1, A. tumefaciens containing pBIN-nYFP-SnRK1.1, and A. tumefaciens containing pBIN-P19 were coinjected into N. benthamiana leaves at a ratio of 2:2:1. After 48 hr, the fluorescent signal was detected by confocal microscopy (Nikon, A1 and C2). The nuclei were stained with DAPI. To detect YFP and GFP signals, a laser with a 488 nm wavelength was used, and emissions were detected between 490 nm and 560 nm. For mCherry signal detection, a laser with a 561 nm wavelength was used, and emissions were detected between 560 nm and 620 nm. For DAPI signal detection, a laser with a 405 nm wavelength was used, and emissions were detected between 400 nm and 407 nm.

4.7 | Generation of transgenic Arabidopsis plants

The coding region of PBZF1 (without a sequence encoding a signal peptide) and the FLAG tag sequence at the N-terminus were amplified by a high-fidelity DNA polymerase (Tsingke Biotech Co. Ltd), and the fragment was cloned into the pBI121 plant binary vector using the ClonExpress II One Step Cloning Kit (Vazyme Biotech Co. Ltd) to generate pBI121-NFLAG-PBZF1. The recombinant plasmid was transformed into A. tumefaciens GV3101. Finally, A. tumefaciens containing pBI121-NFLAG-PBZF1 was used to transform 5- to 6-week-old A. thaliana (Col-0) plants by the floral dip method (Clough & Bent, 1998). T0 seeds were screened in Murashige & Skoog (MS) medium containing kanamycin (50 mg/ml). Plants of the T3 generation were used for subsequent studies.

4.8 | PBZF1 expression pattern analysis

To investigate the expression pattern of PBZF1, 7-day-old seedlings of the ZS11 oilseed rape line were inoculated with 10⁷ P. brassicae resting spores purified by differential centrifugation and sucrose gradient centrifugation (Castlebury et al., 1994). The clean roots collected at 3, 5, 15, and 30 dpi were used for RNA extraction. Total RNA was extracted by Trizol reagent (Invitrogen), and cDNA was synthesized using the PrimeScript RT reagent kit with gDNA Eraser (Takara). The relative expression level of PBZF1 was defined as the ratio of the expression of PBZF1 to that of the P. brassicae actin gene (accession number AY45218.1) by the 2⁻ΔΔCt method.

4.9 | PBZF1 sequence polymorphism analysis

To investigate the sequence polymorphism of PBZF1, DNA was extracted from 19 P. brassicae isolates from China, and PBZF1 was cloned and sequenced. The resequencing data of 40 P. brassicae isolates from Canada, the USA, and Europe (Sedaghatkish et al., 2019)
were downloaded for analysis of the sequence polymorphisms of PBZF1. Finally, the nucleotide and protein sequences of PBZF1 were aligned by DNAMAN v. 6.0.

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CONFLICT OF INTEREST
The authors declare no competing interests.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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