Plant growth-promoting rhizobacterium Bacillus cereus AR156 - induced systemic resistance against multiple pathogens by priming of phytoalexin synthesis and secretion

Chunhao Jiang[1], Zi-Jie Li[1], Shu-Ya Tang[1], Jin-Yao Ren[1], Wen-Pan Dong[1], Pei-Ling Xu[1], Wei Yang[2], Ying Zheng[1], Yi-Yang Yu[1], Jianhua Guo[1], Yu-Ming Luo[2], and Dongdong Niu[1]

[1]Key Laboratory of Integrated Management of Crop Disease and Pests Ministry of Education/Key Laboratory of Integrated Pest Management on Crops in East China Ministry of Agriculture/Key Laboratory of Plant Immunity Nanjing Agricultural University 210095 Nanjing China
[2]Jiangsu Collaborative Innovation Center of Regional Modern Agriculture and Environmental Protection/Jiangsu Key Laboratory for Eco-Agricultural Biotechnology around Hongze Lake Huaiyin Normal University 223300 Huai'an China

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Abstract

Phytoalexin plays an important role in plant immunity. However, the mechanism of how phytoalexin is induced by beneficial microorganisms against broad-spectrum pathogens remains elusive. This study showed that *B. cereus* AR156 could trigger ISR against broad-spectrum disease. RNA-seq and camalexin content assays showed that AR156-triggered ISR can induce the accumulation of phytoalexin such as camalexin synthesis and secretion-related genes. Moreover, it was found that AR156-triggered ISR elevates camalexin accumulation by increasing the expression of camalexin synthesis genes upon pathogen infection. Further studies revealed that *WRKY33* was required for the induction of camalexin accumulation by AR156 during the pathogen infection. Compared to the control inoculated with *Phytophthora capsici* and *Botrytis cinerea* only, the biomass of *P. capsici* and *B. cinerea* in AR156 pretreated *wrky33* mutant plants were quite similar. AR156-induced ISR resistance to *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) was significantly attenuated in the *wrky33* mutant. Furthermore, the study reveals that AR156 could up-regulate the expression level of *PEN3* and *PDR12*, which act as camalexin transporter. In addition, we found that PEN3 and PDR12 served as positive regulators involved in AR156-triggered ISR against pathogens. Specifically, PEN3 and PDR12 participated in AR156-triggered ISR against fungi and oomycetes, while PEN3 was involved in AR156-triggered ISR against *Pst* DC3000. In summary, *B. cereus* AR156 triggered induced systemic resistance against *B. cinerea*, *Pst* DC3000 and *P. capsici* by priming of phytoalexin synthesis and secretion. Our study first proposed that the WRKY33 as a core factor is involved in regulating AR156-induced accumulation and secretion of phytoalexin, and we deeply elucidated the mechanism of AR156-induced phytotoxin accumulation resistance to broad-spectrum pathogens.

1 INTRODUCTION

In the natural environment, terrestrial plants are under relentless challenge by a great many biological organisms including viruses, bacteria, fungi, and oomycetes (Paulo Jose Pl Teixeira, 2019). Throughout a long evolutionary period, plants have acquired a suite of defense mechanisms including the ability to distinguish beneficial microbes from pathogens and to respond defensively when attacked by pathogens. Higher plants perceive microbial or host-derived immunogenic molecular patterns (MAMPs or DAMPs) and more variable pathogen effector proteins delivered to plant cells by PRRs (pattern recognition receptors) on the cell surface

and intracellular immune receptors NLRs (nucleotide-binding, leucine-rich repeat receptors) (Ausubel, 2005; Bacete et al., 2018; Kourelis and Van der Hoorn, 2018; Van de Weyer et al., 2019). Triggering of plant surface PRRs and intracellular NLRs activates downstream signaling events, such as rapid phosphorylation of receptor-like cytoplasmic kinases (RLCKs), calcium inward flow, production of reactive oxygen species, activation of calcium-dependent kinases (CPKs), mitogen-activated protein kinase (MAPK) cascade reaction heterotrimeric G proteins, and production of phytohormones, activation of defense genes, phytoalexin induction. These signaling events contribute to plant resistance to pathogens (Bürger, 2019; Meng and Zhang, 2013; Qi et al., 2017; Seybold et al., 2014; Tang et al., 2017).

At the same time, plants are equipped with a range of induced defense mechanisms to withstand complex and harsh environments (Pieterse et al., 2014). The more established studies on induced resistance include systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Conrath et al., 2002). In general, SAR is directly elicited by plant exposure to toxic, non-toxic and non-pathogenic microorganisms (Conrath et al., 2002), whereas ISR is triggered by some non-pathogenic rhizosphere microorganisms, such as plant growth-promoting bacteria (PGPR) (Van der Ent et al., 2008). ISR and SAR act through different signaling pathways. It was found that ISR induced by *P. fluorescens* WCS417r was blocked in the *Arabidopsis* JA signaling pathway mutant jar1, the ET signaling pathway mutant etr1, and the PR gene nonexpressing mutant *npr1* (Pieterse et al., 1998), suggesting that *P. fluorescens* WCS417r-mediated ISR is dependent on the JA/ET signaling pathway and the *NPR1* gene. The *P. fluorescens* WCS417r has been shown to trigger ISR in a variety of plants. It was shown that *P. fluorescens* WCS417r enhanced resistance to *Fusarium oxysporum* in the above-ground parts of the plant and produced more phytoalexin at the site of pathogen infection after root treated by *P. fluorescens* WCS417r (Van Wees et al., 1999). The results showed that PGPR strain *P. fluorescens* S97 triggered leaf ISR after root colonization in Leguminosae. The *P. fluorescens* WCS417r has been described to elicit ISR against a range of pathogens, including *Xanthomonas campestris* pv. *campestris*and *Pst* DC3000 (Van der Ent et al., 2008). It was revealed that oxalic acid secreted by*Bacillus* sp. resisted *B. cinerea* by activating the JA and ET signaling pathways in tomato (Yu et al., 2022). The plant growth-promoting rhizobacteria *Bacillus amyloliquefaciens* HK34 can trigger ISR against *P. cactorum* in *Panax ginseng* (Lee et al., 2015). There was a study showing that lncRNAs played an important role in biocontrol bacteria *P. putida*Sneb821 to induce tomato resistance against *Meloidogyne incognita*infection (Yang et al., 2020). In addition to *Pseudomonas* , various strains of *Bacillus* , fungi, and viruses can also stimulate ISR, reducing the incidence and severity of crop diseases significantly. A wealth of studies has investigated the molecular mechanism of rhizosphere-triggered ISR. *P. fluorescens*WCS417r-ISR in *Arabidopsis* was shown to have no activation of PR genes in systemic leaf tissue (Pieterse et al., 1996) and be independent of SA (Pieterse et al., 2000). However, in opposition to the ISR triggered by *P. fluorescens* WCS417r, it was found that plant growth-promoting bacteria *B. cereus* AR156 triggered the ISR in*Arabidopsis thaliana* by activating both SA and JA/ET signaling pathways in an NPR1-dependent manner, thereby enhancing plant resistance to *Pst* DC3000 (Niu et al., 2011). Further studies showed that WRKY11 and WRKY70 play essential roles in regulating the signaling pathway of *B. cereus* AR156-triggered ISR through activation of JA and SA signaling pathways, respectively (Jiang et al., 2015). In addition, recent studies have shown that *B. cereus* AR156 triggers ISR against *Pst* DC3000 by suppressing miR472 and activating CNLs-mediated basal immunity in *Arabidopsis* (Jiang et al., 2020).

Phytoalexin is a small-molecule secondary metabolite synthesized*de novo* after the plant senses the invasion of pathogens. Camalexin (3-thiazol-2'-yl-indole) is a sulfur-containing indole alkaloid unique to cruciferous plants and the predominant phytoalexin in the model plant *A. thaliana* (Ahuja et al., 2012). It was first isolated from the leaves of *Camelina sativa* after infection by*Alternaria brassica* (Browne et al., 1991). After the discovery of phytoalexins from potatoes infected with blast molds, a large number of phytoalexins have been identified from various plants, such as camalexin, capsidiol, scopoletin, resveratrol and pisatin (Ahuja et al., 2012; Holland and O'Keefe, 2010; Pedras et al., 2011; Yang et al., 2009). The biosynthesis of camalexin originated from Tryptophan (Piasecka et al., 2015). Tryptophan is converted to indole-3-acetaldoxime (IAOx) by two cytochrome P450 enzymes, CYP79B2 and CYP79B3 (Glawischnig, 2006; Glawischnig et al., 2004). When plants are infected by pathogenic bacteria, two other cytochrome P450 enzymes, CYP71A12 and

CYP71A13, are produced, which transform IAOx to indole-3-acetonitrile (IAN) (Mller et al., 2015; Nafisi et al., 2007). IAN is further activated and derivatized. Finally, GS-IAN is transformed into camalexin under the machining of γ-glutamylpeptidase and cytochrome P450 enzyme CYP71B15 (PAD3) (Böttcher et al., 2009; Mucha et al., 2019; Parisy et al., 2007; Schuhegger et al., 2006).

Camalexin production has been reported to be induced by many pathogens, such as *P. syringae* , *Alternaria brassicicola* , *B. cinerea* , *P. brassicas* , and *Sclerotinia sclerotiorum* . Camalexin is also an important immune response against the invasion of these pathogens (Jun Tsuji, 1992; Schlaeppi et al., 2010; Stotz et al., 2011; Thomma et al., 1999). The synthetic gene of camalexin is expressed at low levels in the absence of biotic or abiotic stresses and is highly induced once subjected to pathogen invasion (Nafisi et al., 2007; Schuhegger et al., 2006). WRKY33, known for being one of the pivotal transcription factors in the camalexin synthesis pathway, can be induced to be up-regulated by a variety of fungi, oomycetes, and bacteria (Mao et al., 2011; Qiu et al., 2008). WRKY33 can bind to the *CYP71A13*and *PAD3* promoter regions to regulate its expression (Birkenbihl et al., 2017; Mao et al., 2011; Qiu et al., 2008). Mitogen-activated protein kinases (MAPKs) and calcium-dependent protein kinases (CDPKs), activated during plant perception of pathogens, play a crucial role in the induction of camalexin synthesis. Acting upstream of WRKY33, MPK3/6 and CPK5/6 enhance WRKY33 activity by phosphorylating the N-terminal Ser residue and Thr-229 of WRKY33 protein, respectively, with the former increasing its transactivation activity and the latter enhancing its DNA binding activity (Mao et al., 2011; Zhou et al., 2020). The jasmonate and ethylene signaling pathways are also involved in the induction of camalexin synthesis upon pathogen infection. The transcription factor ETHYLENE RESPONSE FACTOR1 (ERF1) integrates the jasmonic acid and ethylene signaling pathways and induces camalexin by upregulating the expression of *CYP71A13* and *PAD3* . ERF1 and WRKY33 interact in the nucleus and they mediate the synthesis of camalexin by targeting the promoters of *CYP71A13* and *PAD3* , interdependently and cooperatively (Zhou et al., 2022).

However, the mechanism of how phytoalexin is induced by beneficial microorganisms against broad-spectrum pathogens remains elusive. In the present study, we found that AR156 could induce ISR against broad-spectrum pathogens, such as *P. capsici* , *B. cinerea, Pst* DC3000. RNA sequencing found that AR156-triggered ISR could induce the accumulation of phytoalexin such as camalexin synthesis and secretion-related genes. Deeply research demonstrated that AR156-induced ISR is impaired in *wrky33* , probably because the induction of camalexin accumulation by AR156 is dependent on *WRKY33*.Meanwhile, AR156 induced up-regulated expression of PEN3 and PDR12. Nevertheless, PEN3 and PDR12 were jointly involved in AR156-triggered ISR against fungi and oomycetes, while PEN3 was involved in AR156-triggered ISR against *Pst* DC3000. Our study firstly raises that WRKY33 is involved in regulating the accumulation and secretion of phytoalexin induced by AR156 as a core factor. Besides, our study systematically elucidates the mechanism of PGPR-triggered ISR resistance to broad-spectrum pathogens and offers a theoretical basis for developing and applying new biopesticides.

2 MATERIALS AND METHODS

2.1 Plants, bacterial strains, fungi, oomycete, and growth conditions

All *Arabidopsis* lines used in this study were as follows: Col-0 (*Arabidopsis* wild type); *wrky33* (Zheng et al., 2006);*pen3-3* , *pdr12-2* , the *PEN3* and *PDR12* gene double mutant line *pen3-3/pdr12-2* (He et al., 2019).*Arabidopsis* seeds were surface sterilized (soaked in 70% ethanol solution for 10 min and rinsed 5-8 times in sterile water) and then incubated at 4 dark conditions. After 4 d of vernalization, seeds were transferred to a half-strength Murashige and Skoog (1/2 MS) plate using a pipette and incubated for about a week. Seedlings were then transferred into black pots containing sterilized vermiculite: nutrient-rich soil (in a 2:1 volume ratio) for cultivation at an ambient temperature of 22 under a 16-h light/8-h dark photoperiod and 70% relative humidity. The PGPR strain *B. cereus* AR156 was grown on Luria-Bertani (LB) agar plates for 24 h at 28. Single colonies were picked and inoculated into liquid LB medium and incubated at 28 and 200 r for 24 h. Subsequently, the bacterial solution was collected by centrifugation at 4500 rpm and resuspended with 0.85% NaCl to a concentration of about 5 x 10^7 CFU mL^{-1}. *Pst* DC3000 was grown in KB agar medium containing 50 mg*L^{-1} rifampicin and 50 mg*L^{-1} kanamycin for 2 d. Single colonies were

picked and inoculated into KB liquid containing 50 mg*L^{-1} rifampicin and 50 mg*L^{-1} Kanamycin grown overnight at 28.*Pst* DC3000 organisms were collected by centrifugation and resuspended with 10 mM $MgCl_2$ (containing 0.02% (v/v) Silwet L-77) and adjusted to 5 x 10^7 CFU mL^{-1} for use. The gray mold fungus was grown on potato dextrose agar (PDA) medium at 25 for about 10 d. Conidia were then collected and resuspended in 10 mM $MgCl_2$, filtered through three layers of gauze to remove mycelium, and the number of conidia was counted using a hemocytometer plate and adjusted to 1 x 10^6 conidia mL^{-1} (Aziz et al., 2003). *P. capsici* LT263 was grown in dark culture on V8 juice agar medium at 25 for 3 d. 2-mm disks of 4-day growth medium were cut along the edges with a scalpel and incubated in V8 liquid medium for 2 ; the culture solution was discarded, the mycelium was washed three times with sterilized tap water, and incubated in sterile tap water for 12 h until mature sporangia were induced. Zoospores were obtained by incubating at 4 for 20 min followed by 2 h incubation at 25.

2.2 Biocontrol bacterial treatment and pathogen infection assays Four-week *Arabidopsis* was pretreated with *B. cereus* AR156 as previously described (Jiang et al., 2015). For bacterial treatments, 10 mL of 5 x 10^7CFU*mL^{-1} cell suspension of AR156 was irrigated on the soil around the roots of *Arabidopsis* in each pot, with an equal volume of sterile 0.85% NaCl as a control. The pathogens were inoculated after 5 d of pretreatment with *B. cereus* AR156. The leaves were challenged with a 10-il droplet containing approximately 500*P. capsici* zoospores, with 10 μL droplets of *B. cinerea* at 1 × 10^6 conidia mL^{-1} or by spraying*Pst* DC3000 at 1 × 10^8CFU·mL^{-1} to evaluate disease symptoms. The biomass of*P. capsici* and *B. cinerea* was determined as previously reported and further improved slightly. Briefly, leaf discs (1 cm in diameter) around spore droplets were collected from different leaves of each treatment using a punch. Genomic DNA was extracted by the CTAB method and pathogen biomass was quantified by real-time PCR. To calculate colonization of *Pst* DC3000 on *Arabidopsis*leaves, samples were collected with a puncher at 0 and 3 days (dpi) after inoculation with *Pst* DC3000. *Arabidopsis* leaf discs were surface sterilized in 70% ethanol for 30 seconds and washed five times with sterile distilled water. The samples were then ground and gradient diluted with 0.9 ml of 10 mM $MgCl_2$. The samples were then ground and gradient diluted with 0.9 ml of 10 mM $MgCl_2$. Subsequently, the dilutions of 100 μL were applied uniformly to a KB agar medium containing 50 mg·L^{-1} rifampicin and 50 mg·L^{-1}kanamycin and incubated at 28. Colonies were counted after 48 h and the density of *Pst* DC3000 in the leaves was calculated.

2.3 Construction of RNA-Seq library and RNA sequencing

Four-week-old *A. thaliana* were treated with AR156 by root irrigation and the same volume of 0.85% NaCl was used as control. Arabidopsis leaves from different treatments were gathered after 3 d, with three replicates of each treatment. The total RNA extraction method was referenced to the RNA simple Total RNA Kit (TIANGEN, Cat. No. DP419) instructions. RNA-seq experiments were performed at BGI Genomics (Shenzhen, China). The RNA-seq results were uploaded to the NCBI database (BioProject accession number: PRJNA879188).

2.4 RNA extraction and RT-qPCR About 100 mg of treated*Arabidopsis* leaves were harvested in a 2 mL centrifuge tube, ground to a fine powder with liquid nitrogen, and mixed with 1 mL of TRIZOL followed by 200 μL of chloroform. Total RNA samples were reverse transcribed using the HiScript Q Select RT Super Mix kit (Vazyme, Cat. No. R323). Real-time quantitative PCR was performed using the ChamQ SYBR Color qPCR Master Mix kit (Vazyme, Cat. No. Q711) and the ABI 7500 system. Primers used for RT-qPCR are shown in Supplementary Table S1.**2.5 Trypan blue staining** To evaluate *Arabidopsis* leaf lesion infected with *P. capsic* i. The trypan blue stock solution (a mixture of 10 g phenol, 10 mL glycerol, 10 mL lactic acid, 10 mL deionized water, and 0.02 g trypan blue) was mixed with 96% ethanol at a dilution ratio of 1:2 (v/v). *Arabidopsis* leaves were collected to the staining solution, boiled in a water bath for 2 min, and stained overnight in the dark. The leaves were decolorized with chloral hydrate solution, and then the lesion area was measured while the results were photographed.**2.6 Camalexin analysis** The detection of camalexin is referred to the previous description. At a certain time point, 0.1 g of *Arabidopsis* leaves inoculated with pathogens were taken. The leaves were ground to powder in liquid nitrogen. Add 1 mL extraction buffer to the sample and shake tubes at 4 (30 min, 100 rpm). Add another 2 mL dimethyl sulfoxide

to the sample, followed by a shaking tube for the same time and conditions. The samples were centrifuged at 4 and 5000 g for 10 min. The lower phase was pipetted and the sample was dried in a nitrogen evaporator. The samples were dissolved with 100 μL methanol and filtered through a 0.45 μm filter membrane. The content of camalexin was determined by UFLC-MS/MS system.

2.7 Statistical analyses

All data were analyzed with IBM SPSS (Version 23). The Student's*t*- test (two-tailed) was performed to analyze the difference between Mock and AR156. One-way ANOVA followed by Tukey's post hoc test was performed to the difference between three or more independent groups. The data are presented as mean ± SD.

3 RESULTS 3.1*B. cereus* AR156 pretreatment improves broad-spectrum disease resistance in *Arabidopsis* Plants are exposed to a variety of pathogens during growth and development. To survive, plants have evolved a series of defense mechanisms to resist these pathogens, such as recruiting beneficial microorganisms to the rhizosphere. It is revealed that the plant growth-promoting rhizobacterium *B. cereus* AR156 enhances plant resistance to *Pst* DC3000 by triggering the ISR in*Arabidopsis* . In order to demonstrate that *B. cereus* AR156 can trigger ISR to improve broad-spectrum disease resistance in*Arabidopsis* , *A. thaliana* plants, wild-type Col-0 were inoculated with *B.cinerea* , *Pst* DC3000 and *P. capsici* 5 d after pretreated with *B. cereus* AR156 or 0.85% NaCl. Compared to the control, *B. cereus* AR156 treatment significantly reduced the lesion area caused by *P. capsici* (Figure 1 A and B), the lesion area caused by *B. cinerea*(Figure 1 C and D), and bacterial growth of *Pst* DC3000 (Figure 1 E and F) in *Arabidopsis* leaves. *B. cereus* AR156 pretreatment reduced *Arabidopsis* sensitivity to *P. capsici* , *Pst* DC3000, and *B. cinerea* . Taken together, these results confirmed that *B. cereus* AR156 could trigger ISR against broad-spectrum disease.**3.2 *B. cereus* AR156 induces Arabidopsis phytoalexin biosynthesis signaling pathway** To explore the mechanisms of AR156-triggered ISR response to broad-spectrum diseases, we performed RNA-sequencing experiments and also analyzed the transcriptome changes initiated by AR156 pretreatment. We analyzed the induction of phytoalexin-related genes in *Arabidopsis* after AR156 pretreatment as it plays a vital role in plant response to pathogens. Compared to Mock, phytoalexin-related genes, including camalexin synthesis and transport-related genes, accumulated in the leaves pretreated with AR156 (Figure 2 A). The results of RT-qPCR showed that, several P450 enzymes associated with camalexin syntheses, such as*CYP79B2* , *CYP79B3* , *CYP71A13* and *PAD3* (Figure 2 B˜F), accumulated significantly at different points after AR156 treatment compared to Mock. Meanwhile, the transcripts of*WRKY33* , *PEN3* , and *PDR12* were up-regulated after AR156 treatment (Figure 2 G and H). These results were consistent with the RNA-sequencing results. In addition, we investigated the effect of AR156 on camalexin synthesis in *Arabidopsis* , the results demonstrated that the AR156-triggered ISR could induce the accumulation of camalexin (Figure 2 I). Taken together, AR156-triggered ISR can induce the accumulation of phytoalexin such as camalexin synthesis and secretion-related genes.**3.3 *B. cereus* AR156 prime camalexin accumulation after pathogens infection** To further investigate the role of *B. cereus* AR156 triggered ISR in pathogen-induced camalexin biosynthesis, we examined the camalexin induction in wild-type plants pretreated with *B. cereus* AR156 after infection by *P. capsici* , *Pst* DC3000, and *B. cinerea* . As shown in Figure 3 A, compared with the Mock, *B. cereus* AR156-triggered ISR increased the content of *Arabidopsis*camalexin at different time points of *P. capsici* infection. There was still significant induction of camalexin accumulation by AR156-ISR 96 h after pathogen infection compared to the control. Consistently, the induction of camalexin biosynthetic genes*CYP71A13* and *PAD3* by *P. capsici* infection was also significantly increased under pretreated with *B. cereus* AR156 (Figure 3 B and C). As shown in Figure 3 D, compared with the control, AR156-triggered ISR increased the content of *Arabidopsis*camalexin at different time points of *B. cinerea* infection, with significant differences after 24 h of *B. cinerea* infection. Notably, AR156 still enhanced camalexin synthesis in *Arabidopsis*96 h after *B. cinerea* challenge. Consistent with this, the induction of camalexin biosynthetic genes *CYP71A13* and*PAD3* by *B. cinerea* infection were also significantly increased under pretreated with *B. cereus* AR156 (Figure 3 E, and F). As shown in Figure 3 G, compared with the Mock, AR156-triggered ISR increased the content of *Arabidopsis* camalexin at different time points of *Pst* DC3000 infection, with

significant differences 24 h after *Pst* DC3000 infection. We also found that *B. cereus* AR156 induces maximal synthesis of camalexin at 72 h of *Pst* DC3000 infection. Consistent with this, the induction of camalexin biosynthetic genes *CYP71A13* and *PAD3* upon *Pst* DC3000 infection was also significantly increased under pretreated with *B. cereus* AR156 (Figure 3 H and I). These results suggested that AR156-induced ISR increases the expression of camalexin synthesis genes and the accumulation of camalexin.

3.4 *B. cereus* AR156 triggers the accumulation of camalexin dependent on WRKY33 upon pathogen infection

There are reported that the transcription factor WRKY33 act as a key positive regulator of pathogen-induced camalexin biosynthesis. Therefore, we investigated whether WRKY33 was required for enhancing camalexin accumulation by AR156-induced ISR under *P. capsici* ,*Pst* DC3000, and *B. cinerea* infection. As we expected, the expression level of *WRKY33* increased at 6 h and 12 h after *P. capsici* , *Pst* DC3000, and *B. cinerea* infection with or without AR156 pretreated, while the expression level of *WRKY33* was significantly increased under the AR156 pretreatment, compared with the Mock (Figure 4 A, D, and G). These results suggested that AR156-induced ISR increased the expression of *WRKY33* . To further explore whether AR156 regulates camalexin synthesis via *wrky33* , we examined the accumulation levels of camalexin in wild-type *Arabidopsis* and *wrky33* mutants inoculated with pathogenic bacteria 5 d after pretreatment with AR156 or 0.85% NaCl. AR156 enhanced camalexin accumulation in Col-0, while the induction of camalexin by *P. capsici* , *Pst* DC3000, and *B. cinerea* was blocked. Meanwhile, the induction of camalexin accumulation in *wrky33* by AR156 was also impaired under pathogen inoculation (Figure 4 B, E, and H). The compromised camalexin induction in *wrky33* mutant with or without AR156 pretreatment was correlated with the significantly attenuated activation of camalexin biosynthetic genes *CYP71A13* and *PAD3* after *P. capsici* , *Pst* DC3000, and *B. cinerea* infection (Figure 4 C, D, G, H, K, and L). Therefore, these data suggest that *WRKY33* plays a crucial role in AR156-induced ISR-regulated camalexin biosynthesis.

3.5 *B. cereus* AR156-triggered ISR against different pathogens is impaired in the *wrky33* mutant

The above results suggested that *WRKY33* acts as a positive regulator of camalexin synthesis by AR156-triggered ISR, and it remains unclear whether *WRKY33* functions in the process of AR156-triggered ISR against *P. capsici* , *Pst* DC3000, and *B. cinerea* . To further investigate the function of *WRKY33* in the AR156-triggered ISR defense against pathogens, *A. thaliana* Col-0 and *wrky33* were inoculated with *B. cinerea* ,*Pst* DC3000, and *P. capsici* 5 d after pretreated with *B. cereus* AR156 or 0.85% NaCl. The *P. capsici* biomass in the leaves of AR156-treated Col-0 plants were significantly lower than that in the mock plants inoculated only with *P. capsici* ; by contrast, the *P. capsici* biomass in the leaves of AR156-treated *wrky33* mutant plants was similar to that in the mock plants only inoculated with *P. capsici* (Figure 5 A and B), indicating that AR156-triggered ISR against *P. capsici* was abolished in *wrky33* mutant plants. The *B. cinerea* biomass in the leaves of AR156-treated Col-0 plants were significantly lower than that in the control plants inoculated only with *B. cinerea* . However, the *B. cinerea* biomass in the leaves of AR156-treated *wrky33* mutant plants was similar to that in the control plants only inoculated with *B. cinerea* (Figure 5 C and D), indicating that AR156-triggered ISR against *B. cinerea* was abolished in *wrky33* mutant plants. The pathogen density in the leaves of AR156-treated Col-0 plants was significantly lower than that in the control plants inoculated only with *Pst* DC3000. At the same time, the AR156-triggered ISR against *Pst* DC3000 was attenuated in *wrky33* mutant plants (Figure 5 E and F). Taken together, *WRKY33* functions as a positive regulator in the AR156-triggered ISR process against *P. capsici* , *Pst* DC3000, and *B. cinerea* .

3.6 PEN3 and PDR12 serve as positive regulators involved in AR156-triggered ISR against *P. capsici*, *Pst DC3000*, and *B. cinerea*

By analyzing the RNA-seq results, we found that the AR156-triggered ISR induced the accumulation of Pleiotropic Drug Resistance Transporters PEN3 and PDR12 (Figure 2 A, H and I), which were reported to function in immunity in *Arabidopsis* through the transport of camalexin and other Trp metabolites (He et al., 2019). Moreover, WRKY33 could bind to the promoters of *PEN3* and *PDR12* . Therefore, we speculate that AR156-triggered ISR resists *P. capsici* , *Pst* DC3000, and *B. cinerea* by upregulating *PEN3* and

PDR12 . To reveal the mechanism, *pen3* , *pdr12-2* single mutant, and*pen3-3/pdr12-2* double mutant were used. *A. thaliana*plants, wild-type Col-0, *pen3* , *pdr12-2* single mutant, and*pen3-3/pdr12-2* double mutant were inoculated with *B. cinerea* , *Pst* DC3000 and *P. capsici* 5 d after being pretreated with *B. cereus* AR156 or 0.85% NaCl. The *P. capsici* biomass in the leaves of *pen3-3* mutant plants treated with AR156 was slightly reduced compared to the control inoculated with*P. capsici* only. The *P. capsici* biomass in the leaves of*pdr12-2* mutant plants treated with AR156 was similar to the control inoculated with *P. capsici* only. The *P. capsici*biomass in leaves of *pen3-3/pdr12-2* double mutant plants treated with AR156 had shown no significant difference compared to the control inoculated with *P. capsici* only (Figure 6 A). Similarly, we found that The *B. cinerea* biomass in the leaves of *pen3-3* mutant plants treated with AR156 was slightly reduced compared to the control inoculated with *B. cinerea* only. The *B. cinerea* biomass in the leaves of *pdr12-2* mutant plants treated with AR156 was similar to the control inoculated with *B. cinerea* only. The*B. cinerea* biomass in leaves of *pen3-3/pdr12-2* double mutant plants treated with AR156 had shown no significant difference compared to the control inoculated with *B. cinerea* only. These results suggest that *PEN3* and *PDR12* function as positive regulators of AR156-triggered ISR resistance to *P. capsici* and*B. cinerea.* However, the density of AR156-treated *pen3-3*was similar compared to the control inoculated with *Pst* DC3000 only, similar in *pdr12-2* and *pen3-3/pdr12-2* mutant leaves. The results suggested that it is PEN3 but not PDR12 acting as a positive regulator of AR156 triggering ISR resistance to *Pst* DC300. In summary, PEN3 and PDR12 serve as positive regulators involved in AR156-triggered ISR against pathogens. Specifically, PEN3 and PDR12 were jointly involved in AR156-triggered ISR against fungi and oomycetes, while PEN3 was involved in AR156-triggered ISR against *Pst* DC3000 (Zheng et al., 2006).

4 DISCUSSION

Plant roots are colonized by a large number of diverse microorganisms in the soil. Plant growth-promoting bacteria (PGPR) can survive in the plant rhizosphere, attach to the plant surface, and develop a symbiotic relationship with the plant, promoting plant growth and improving plant resistance to biotic and abiotic stress (De Zelicourt et al., 2013). ISR triggered by PGPRs is a vital mechanism for enhancing plant resistance to stress. The mechanism of ISR tended to vary in different PGPRs and different pathogens. For example, ISR signaling triggered by *P. fluorescens* WCS417r in *A.* thaliana requires JA/ET but not SA (Ton et al., 2001; Zamioudis and Pieterse, 2012). In contrast, some PGPRs induce ISR signaling via SA instead of JA/ET (Audenaert et al., 2002; Barriuso et al., 2008). it was found that plant growth–promoting rhizobacterium *B. cereus* AR156 triggered the ISR in *A. thaliana* by activating both SA and JA/ET signaling pathways in an NPR1-dependent manner, thereby enhancing plant resistance to *Pst*DC3000 (Niu et al., 2011).

In this study, we showed that AR156 could induce ISR against different lifestyle pathogens, including fungi and bacteria, which was in accordance with the results of previous studies (Jiang et al., 2015; Jiang et al., 2020; Nie et al., 2019; Niu et al., 2011). Simultaneously, we discovered that AR156 triggers ISR against *P. capsici* (Figure 1). However, the mechanism of AR156-induced ISR resistance to different types of pathogens remains unclear.

4.1 Phytoalexin is implicated in AR156-triggered ISR against broad-spectrum pathogens

Plant secondary metabolites were earlier considered to be the end-product of metabolic or products of detoxification, and research in recent decades has demonstrated their role in plant regulation of their growth and development and response to various biotic and abiotic environmental stresses in the regulation of plant growth and development and response to various biotic and abiotic environmental stresses. A large proportion of them is involved in the defense response of plants against pathogenic microorganisms, including phytoalexin (Dixon, 2001). Although it is reported that beneficial bacteria *P. fluorescens*PTA-CT2 and *B. subtilis* PTA-271 can induce resistance to *B. cinerea* and *Pst* DC3000 (Nguyen et al., 2022), together with induction of camalexin accumulation and *CYP71A12* expression, many issues remain to be addressed. For example, could *B. cereus* AR156 contribute to broad-spectrum pathogens resistance by regulating phytoalexin-related pathways? How is phytoalexin induced by beneficial bacteria to accumulate and secrete? To deal with the above concerns, we analyzed the *Arabidopsis* RNA-seq results and found that a large number of genes related to the synthesis and regulation of phytoalexin (particularly the camalexin) were up-regulated in expression

after AR156 treatment compared to the control. Meanwhile, we confirmed the transcriptome results by qPCR assays (Figure 2 B-H). Known as the dominant phytoalexin in *A. thaliana* , camalexin has been implicated in resistance to a wide range of pathogens including bacteria, fungi, and oomycetes (Glawischnig, 2007; Jun Tsuji, 1992; Schlaeppi et al., 2010; Stotz et al., 2011; Thomma et al., 1999). In addition, we found the content of camalexin modest increase in*Arabidopsis* leaves after AR156 treatment compared to the control (Figure 2 I). It was reported that root-colonizing *P. fluorescens* strain SS101 (Pf.SS101) relies on camalexin to defend against *Pst* DC3000 and the insect pest*Spodoptera exigua* (Van de Mortel et al., 2012). We hypothesize that AR156 triggers the accumulation of camalexin to defend against different pathogens upon invasion by different pathogens. As was expected, when inoculated with different pathogens, camalexin accumulated markedly in *Arabidopsis.* At the same time, we found that the content of camalexin in *Arabidopsis* Col-0 leaves treated with AR156 was significantly increased compared to the control inoculated with *P. capsici* , *B. cinerea,* or *Pst*DC3000 only. In this study, we found that enhanced camalexin accumulation was attributed to the activation of *CYP71A13* and*PAD3* .

4.2 WRKY33 functions as a positive regulator in the AR156-triggered ISR against broad-spectrum pathogens

Plant transcription factors play an essential role in plant growth and development, morphogenesis, and stress resistance responses. It has been widely reported that WRKY transcription factors are involved in plant immunity. SlWRKY8 promotes resistance to *Pst* DC3000 and enhances tolerance to drought (Gao et al., 2020). Overexpression of*AtWRKY48* confers resistance to *Pst* DC3000 in*Arabidopsis* (Xing et al., 2008). AtWRKY70 and AtWRKY11 were essential for AR156 elicited ISR response to *Pst* DC3000 (Jiang et al., 2015). It was shown that the *WRKY33* transcription factor is a dominant regulator of SAR triggered by local *Pst* DC3000 invasion, while the SAR triggered by local invasion of *Pst* DC3000 was depleted in the *wrky33* mutant (Wang et al., 2018). In the current study, we identified WRKY33 transcription factor upregulation by AR156 treatment at different time points. Moreover, *WRKY33* was required for AR156-mediated accumulation of camalexin during pathogenic infection. Concurrently, AR156-induced CYP71A13 and PAD3 upregulation was significantly attenuated in the *wrky33* mutant, which fully illustrated that *WRKY33* was critical for AR156-mediated synthesis of camalexin upon pathogens invasion (Figure 4). AR156 was found with a diminished function in inducing resistance to different pathogens. As the result is shown in Figure 5 compared to the control inoculated with*P. capsici* and *B. cinerea* only, the biomass of*P. capsici* and *B. cinerea* in AR156 pretreated *wrky33* mutant plants were quite similar. AR156-induced ISR resistance to *Pst* DC3000 was significantly attenuated in the *wrky33* mutant. In parallel, we found that Col-0 and *wrky33* mutants displayed similar susceptibility to pathogens when inoculated only with *P. capsici*and *Pst* DC3000, which was in line with the results of previous studies (Zheng et al., 2006).

4.3 AR156 induces phytoalexin secretion via ABC transporter protein to resist broad-spectrum pathogens

The ATP-binding cassette (ABC) transporter can transport a large number of substrates in *Arabidopsis* , such as secondary metabolites (Verrier et al., 2008). The PDR subfamily is classified as an ABC transporter subfamily. PEN3 and PDR12 are members of the PDR subfamily (Crouzet et al., 2006; Stein et al.). PEN3 and PDR12 are redundant in function and responsible for transporting camalexin from intracellular to extracellular regions upon *B. cinerea* infection (He et al., 2019). In the current experiment, we found that AR156 induced up-regulated expression of *PEN3* and *PDR12* (Figure 2 A, H, and I). Consistent with previous studies, inoculation with *Pst*DC3000 only, *pen3-3* showed enhanced sensitivity to *Pst*DC3000 compared to Col-0 (Xin et al., 2013). In line with previous studies, we found that *pen3-3/pdr12-2* double mutant showed remarkably less resistance to *B. cinerea* compared to Col-0,*pen3-3* , and *pdr12-2* when inoculated with *B. cinerea*only (Figure 6 B) (He et al., 2019). PEN3 has been reported to mediate the translocation of numerous substances while participating in the defense of pathogens (Kang et al., 2011; Strader and Bartel, 2009), similarly to PDR12 (Campbell et al., 2003; Kang et al., 2010; Lee et al., 2005). We found that PEN3 is required for AR156 induction of ISR against the three pathogens, while PDR12 acts in AR156 elicitation against fungi and oomycetes, but not*Pst* DC3000 (Figure 6). This may result from the different functions of

PEN3 and PDR12 in *Arabidopsis* responding to different pathogens infections. Further studies are necessary to investigate whether other secondary metabolites are involved in the AR156-induced ISR and how AR156 regulates the transport of different secondary metabolites by PEN3 and PDR12 in response to pathogens with different lifestyles.

CONCLUSION

In conclusion, we demonstrated that the WRK33 transcription factor can act as a core regulator of AR156-induced ISR resistance against different lifestyles pathogens (Figure 7). In *Arabidopsis* , AR156 colonizes in *Arabidopsis* roots, inducing ISR signaling, and the signals are transferred to the leaves. *WRKY33* , a positive regulator of AR156-induced systemic resistance, was upregulated by AR156 induced. AR156 regulated the expression of *CYP71A13* and*PAD3* through WRKY33 thereby increasing the intracellular accumulation of camalexin upon infection with different pathogens. On the other hand, AR156 relied on PEN3 and PDR12 to transfer camalexin accumulated outside the cell to protect against different pathogens. Our study firstly raises that *WRKY33* is involved in regulating the accumulation and secretion of phytoalexin induced by AR156 as a core factor. In addition, our study systematically elucidates the mechanism of PGPR-triggered ISR resistance to broad-spectrum pathogens and offers a theoretical basis for developing and applying new biopesticides.

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AUTHOR CONTRIBUTIONS

Zi-Jie Li and Chun-Hao Jiang: designed the experiments; Zi-Jie Li, Shu-Ya Tang, and Jin-Yao Ren performed the experiments; Zi-Jie Li analyzed the data and wrote the manuscript. Wen-Pan Dong, Pei-Ling Xu, Wei Yan, Ying Zhen, and Yi-Yang Yu provide suggestions on figures. Jian-Hua Guo, Yu-Ming Luo, Dong-Dong Niu and Chun-Hao Jiang revise the manuscript.

CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

Data and materials are available on request from the corresponding author.

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SUPPORTING INFORMATION

Table S1 Lists of all bacteria strains used in this study

Table S2 Lists of primers used in this study

Table S3 The RPKM of differential expressed genes (DEGs) related to phytoalexin synthesis and secretion in different samples

FIGURE LEGENDS

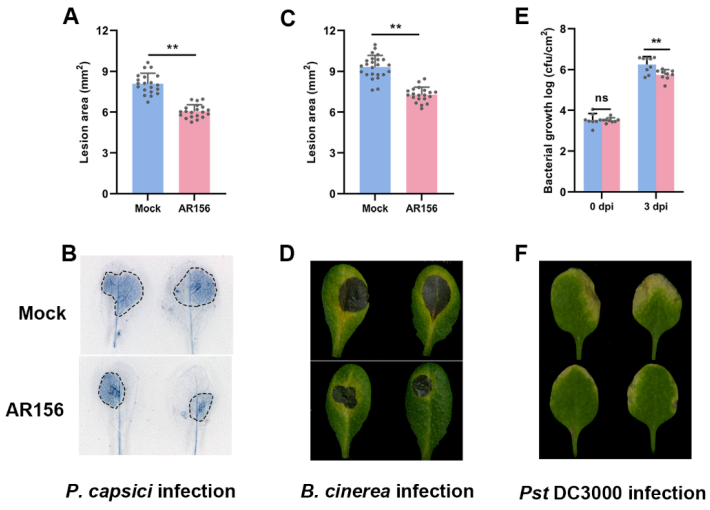

FIGURE 1 *Bacillus cereus* AR156 induces systemic resistance against *P. capsici*, *B. cinerea*, and *Pst*DC3000.

Induction of systemic resistance of *Arabidopsis* ecotype Col-0 wild type (WT) to *P. capsici* , *B. cinerea* , and *Pst*DC3000 by *B. cereus* AR156. Plants were pretreated with 5×10^7 CFU/mL of AR156 and 0.85% NaCl for 5 d. Subsequently, the plants were inoculated with *P. capsici* ,*B. cinerea* , and *Pst* DC3000. (A) The lesion area was determined 2 d after inoculation with *P. capsici* . (B) Phenotypic effects of AR156 ISR on *P. capsici* in *Arabidopsis* . (C) The lesion area was determined 2 d after inoculation with *B. cinerea* . (d) Phenotypic effects of AR156 ISR on *B. cinerea* in*Arabidopsis* . (e) The concentration of *Pst* DC3000 in the

leaves was counted after 3 d. (f) Phenotypic effects of AR156-ISR on*Pst* DC3000 in *Arabidopsis* . Statistical analyses were performed between Mock and AR156 (**, P < 0.01; Student's*t* -test).

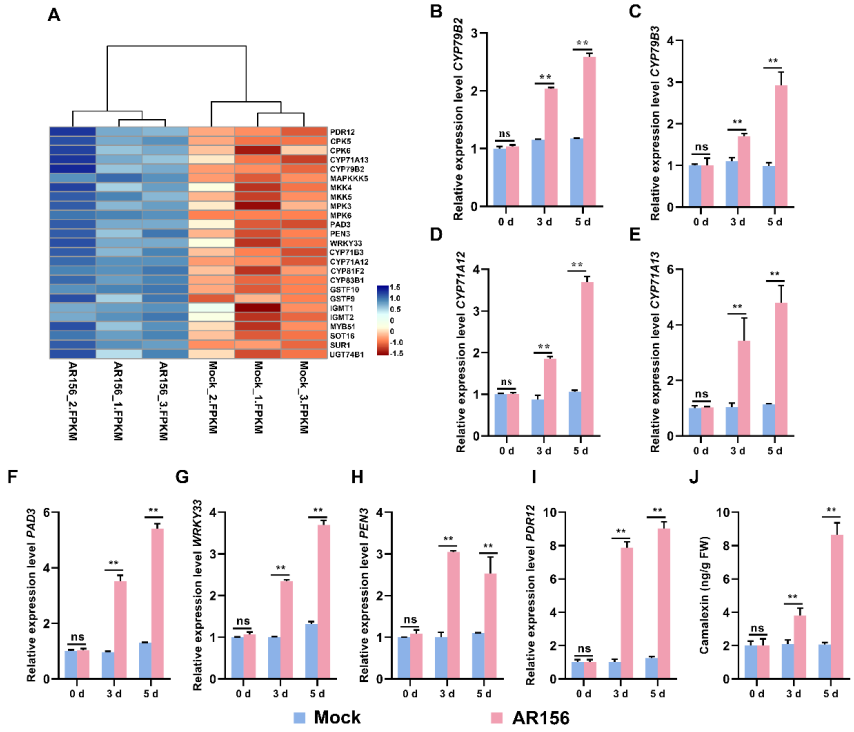

FIGURE 2 AR156-triggered ISR induced the accumulation of phytoalexin synthesis and secretion-related genes.

(A) Hierarchical clustering analyses of DEGs related to phytoalexin synthesis and secretion-related genes. Color scale denotes FPKM values. (B-H) Validation of RNA-seq data for seven selected genes by RT-qPCR.*AtActin1* was used as an internal control. The expression level of these genes in Col-0 was set to 1. (I) Measurement of camalexin accumulation in *Arabidopsis* ecotype Col-0 wild type treated with 5×10^7 CFU/mL of AR156 and 0.85% NaCl. Data represent mean ± SD (n = 3). Statistical analyses were performed between Mock and AR156 (**, P < 0.01; **, P < 0.01; Student's *t* -test).

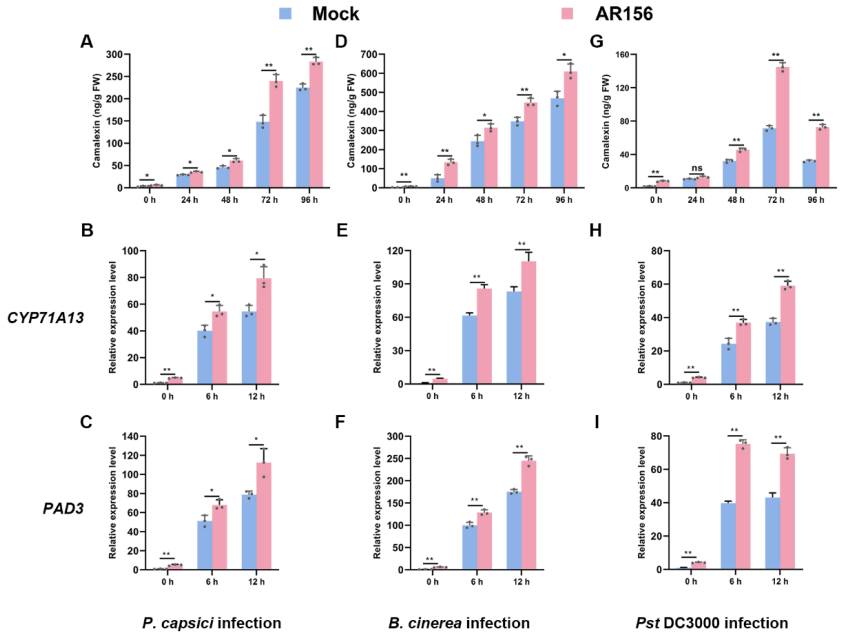

FIGURE 3 AR156 prime *CYP71A13* and *PAD3* expression and camalexin accumulation in the leaves of wild-type Col-0 after *P. capsici*, *B. cinerea*, and *Pst* DC3000 infection.

Induction of systemic resistance of *Arabidopsis* ecotype Col-0 wild type (WT) to *P. capsici* , *B. cinerea,* and *Pst*DC3000 by *B. cereus* AR156. Plants were pretreated with 5×10^7 CFU/mL of AR156 and 0.85% NaCl for 5 d. Subsequently, the plants were inoculated with *P. capsici* ,*B. cinerea* , and *Pst* DC3000. (A) The camalexin production in leaves was quantified at the indicated time points after inoculation with *P. capsici* . (B and C) The transcript levels of*CYP71A13* and *PAD3* were analyzed in leaves pretreated with AR156 or NaCl by RT-qPCR at the indicated time points after inoculation with *P. capsici* . (D) The camalexin production in leaves was quantified at the indicated time points after inoculation with *B. cinerea* . (E and F) The transcript levels of *CYP71A13* and *PAD3* were analyzed by RT-qPCR at the indicated time points after inoculation with *B. cinerea* . (G) The camalexin production in leaves was quantified at the indicated time points after inoculation with *Pst* DC3000. (H and I) The transcript levels of *CYP71A13* and *PAD3* were analyzed by RT-qPCR at the indicated time points after inoculation with*Pst* DC3000. Data represent mean ± SD (n = 3). Statistical analyses were performed between Mock and AR156 (*, $P < 0.05$; **, $P < 0.01$; Student's t -test).

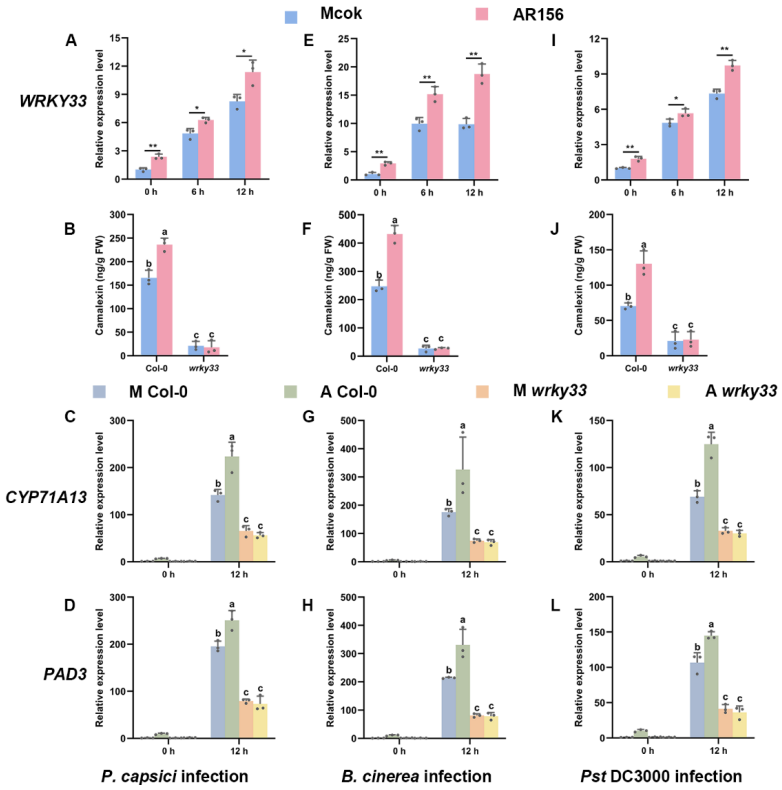

FIGURE 4 *B. cereus* AR156-triggered camalexin accumulation isdependent on WRKY33 upon pathogen infection

Induction of systemic resistance of *Arabidopsis* ecotype Col-0 wild type (WT) and wrky33 mutant to *P. capsici* , *B. cinerea* , and *Pst* DC3000 by *B. cereus* AR156. Plants were pretreated with 5×10^7 CFU/mL of AR156 and 0.85% NaCl for 5 d. Subsequently, the plants were inoculated with *P. capsici* , *B. cinerea* , and *Pst* DC3000. (A, E, and C) The transcript levels of WRKY33 were analyzed in *Arabidopsis* ecotype Col-0 wild-type leaves pretreated with AR156 or NaCl by RT-qPCR at the indicated time points after inoculation with *P. capsici* , *B. cinerea* , and *Pst* DC3000, separately. (B) The camalexin production in *Arabidopsis* ecotype Col-0 and wrky33 leaves was quantified at the indicated time points after inoculation with *P. capsici* . (C and D) The transcript levels of CYP71A13 and PAD3 were analyzed in *Arabidopsis* ecotype Col-0 and wrky33 leaves pretreated with AR156 or NaCl by RT-qPCR at the indicated time points after inoculation with *P. capsici* . (F) The camalexin production in *Arabidopsis* ecotype Col-0 and wrky33 leaves was quantified at the indicated time points after inoculation with *B. cinerea* . (G and H) The transcript levels of *CYP71A13* and *PAD3* were analyzed by RT-qPCR at the indicated time points after inoculation with*B. cinerea* . (J) The camalexin production in *Arabidopsis*ecotype Col-0 and *wrky33* leaves was quantified at the indicated time points after inoculation with *Pst* DC3000. (K and L) The transcript levels of *CYP71A13* and *PAD3* were analyzed by RT-qPCR at the indicated time points after inoculation with *Pst*DC3000. Data represent mean ± SD (n = 3). Statistical analyses were performed between Mock and AR156 (*, $P < 0.05$; **, $P < 0.01$; Student's t -test).

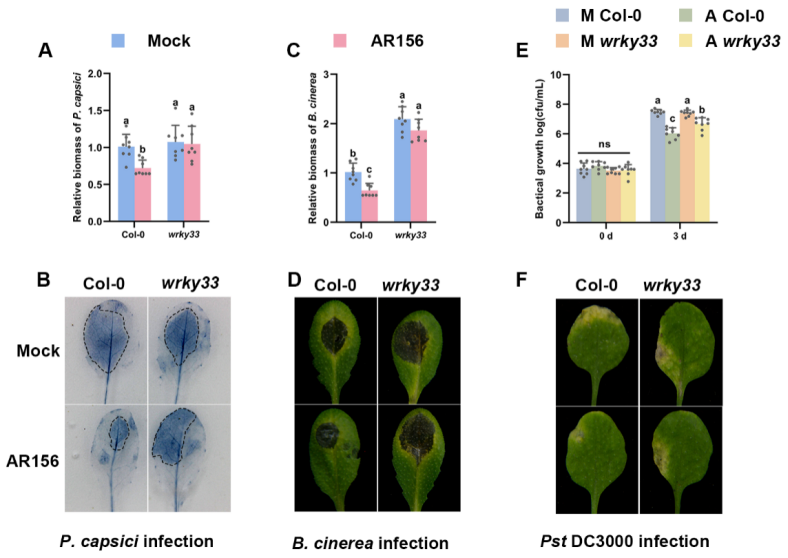

FIGURE 5 AR156-induced ISR resistance to different pathogens is reliant on WRKY33.

Induction of systemic resistance of *Arabidopsis* ecotype Col-0 wild type (WT) and *wrky33* mutant to *P. capsici* , *B. cinerea* , and *Pst* DC3000 by *B. cereus* AR156. Plants were pretreated with 5×10^7 CFU/mL of AR156 and 0.85% NaCl for 5 d. Subsequently, the plants were inoculated with *P. capsici* , *B. cinerea* , and *Pst* DC3000. (A) The degree of*P. capsici* colonization at 48 hpi was determined by RT-qPCR. Primers specific for the *P. capsici* actin gene and the *A. thaliana UBC9* gene were used. Data represent mean ± SD (n = 8). (B) Phenotypic effects of AR156 ISR on *P. capsici* in*Arabidopsis* . (C) The degree of *B. cinerea* colonization at 48 hpi was determined by RT-qPCR. Primers specific for the *B. cinerea* actin gene and the *A. thaliana UBC9* gene were used. Data represent mean ± SD (n = 8). (d) Phenotypic effects of AR156 ISR on *B. cinerea* in *Arabidopsis* . (e) The concentration of*Pst* DC3000 in the leaves was counted after 3 d. (f) Phenotypic effects of AR156-ISR on *Pst* DC3000 in *Arabidopsis* . One-way ANOVA with Tukey's post hoc test was performed ($P < 0.05$). Different letters indicate statistically significant differences.

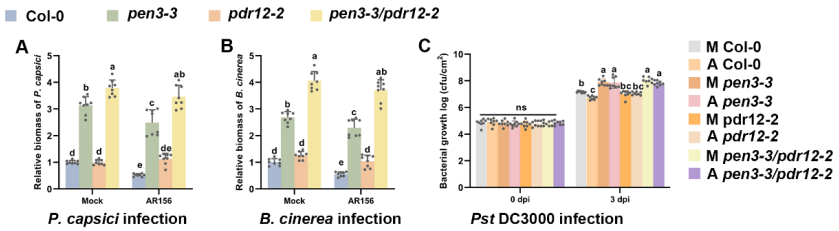

FIGURE 6 AR156-induced ISR resistance to *P. capsici*,*B. cinerea*, and *Pst* DC3000 is dependent on PEN3 and PDR12.

Induction of systemic resistance of *Arabidopsis* ecotype Col-0 wild type (WT), *pen3-3* , *pdr12-2* , and *pen3-3/pdr12-2*mutant to *P. capsici* , *B. cinerea* , and *Pst* DC3000 by*B. cereus* AR156. Plants were pretreated with 5×10^7 CFU/mL of AR156 and 0.85% NaCl for 5 d. Subsequently, the plants were inoculated with *P. capsici* ,*B. cinerea* , and *Pst* DC3000. (A) The degree of *P. capsici* colonization at 48 hpi was determined by RT-qPCR. Primers specific for the *P. capsici* actin gene and the *A. thaliana*UBC9 gene were used. (B)

The degree of *B. cinerea*colonization at 48 hpi was determined by RT-qPCR. Primers specific for the *B. cinerea* actin gene and the *A. thaliana UBC9*gene were used. (C) The concentration of *Pst* DC3000 in the leaves was counted after 3 d. Data represent mean ± SD (n = 8). One-way ANOVA with Tukey's post hoc test was performed ($P < 0.05$). Different letters indicate statistically significant differences.

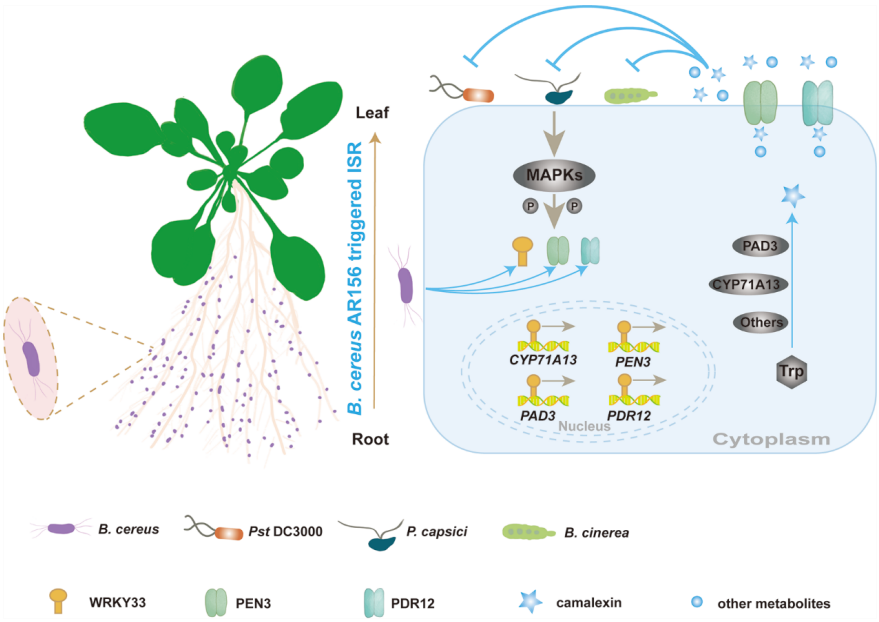

FIGURE 7 A model for *B. cereus* AR156 induces systemic resistance against multiple pathogens by priming of phytoalexin synthesis and secretion.

The root colonization of AR156 induces systemic resistance to different pathogens in *Arabidopsis* . AR156 induces camalexin accumulation by employing the transcription factor WRKY33 to regulate the expression of genes related to camalexin synthesis. On the other hand, AR156 relies on PEN3 and PDR12 to transfer accumulated camalexin outside the cell allowing for protection against multiple pathogens.