***Bacillus cereus* AR156 Activates Defense Responses to *Pseudomonas syringae* pv. *tomato* in *Arabidopsis thaliana* Similarly to flg22**

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Bacillus cereus AR156 (AR156) is a plant growth-promoting rhizobacterium capable of inducing systemic resistance to *Pseudomonas syringae* pv. *tomato* in *Arabidopsis thaliana*. Here, we show that, when applied to *Arabidopsis* leaves, AR156 acted similarly to flg22, a typical pathogen-associated molecular pattern (PAMP), in initiating PAMP-triggered immunity (PTI). AR156-elicited PTI responses included phosphorylation of MPK3 and MPK6, induction of the expression of defense-related genes *PR1*, *FRK1*, *WRKY22*, and *WRKY29*, production of reactive oxygen species, and callose deposition. Pretreatment with AR156 still significantly reduced *P. syringae* pv. *tomato* multiplication and disease severity in NahG transgenic plants and mutants *sid2-2*, *jar1*, *etr1*, *ein2*, *npr1*, and *fls2*. This suggests that AR156-induced PTI responses require neither salicylic acid, jasmonic acid, and ethylene signaling nor flagella receptor kinase FLS2, the receptor of flg22. On the other hand, AR156 and flg22 acted in concert to differentially regulate a number of AGO1-bound microRNAs that function to mediate PTI. A full-genome transcriptional profiling analysis indicated that AR156 and flg22 activated similar transcriptional programs, coregulating the expression of 117 genes; their concerted regulation of 16 genes was confirmed by real-time quantitative polymerase chain reaction analysis. These results suggest that AR156 activates basal defense responses to *P. syringae* pv. *tomato* in *Arabidopsis*, similarly to flg22.

variety of defense responses including activation of mitogen-activated protein kinases (MAPKs), oxidative burst, callose deposition, and defense-related gene expression. On the other hand, to successfully infect plants, pathogens suppress PTI by delivering specific virulence proteins generically termed effectors into the plant cell (Altenbach and Robatzek 2007; Thomma et al. 2011). In response to effectors, some plants have evolved resistance proteins to recognize specific pathogen effectors, thereby eliciting effector-triggered immunity (ETI), usually accompanied by a hypersensitive response at the infection site (Chisholm et al. 2006; Jones and Dangl 2006) to restrict biotrophic pathogen growth.

The typical PAMP flg22 induces PTI in wild-type plants but not in plants carrying mutations in the gene encoding flagellin sensing 2 (FLS2), a leucine-rich repeat (LRR)-type receptor kinase required for flagellin binding and signaling (Gómez-Gómez et al. 2001). FLS2 forms a complex with flg22 and brassinosteroid-associated kinase 1 (BAK1), therefore activating downstream defense-signaling pathways (Sun et al. 2013), which leads to MAPK activation, a transient oxidative burst (reactive oxygen species [ROS]) (Felix et al. 1999), callose (β -1,3-glucan) deposition at the cell walls (Brown et al. 1998; Kim et al. 2005), and the expression of defense-related genes (Gravino et al. 2015; Zipfel et al. 2004).

To survive pathogen attack, plants possess distinct defense signaling pathways, which can interact antagonistically, synergistically, or in parallel, depending on the type of pathogen encountered, resulting in optimization of the specificity of plant defense responses. One of the best-studied examples of defense signaling pathway crosstalk is the antagonistic interaction between the salicylic acid (SA) and the jasmonic acid-ethylene (JA/ET) response pathways (Kazan and Manners 2008; Koornneef and Pieterse 2008; Pieterse et al. 2012). Flg22-induced resistance appears to be independent of SA, JA, and ET signaling pathways in the analysis of the pathways in parallel, so knocking out a single pathway alone does not abolish the induction of resistance. A recent study showed that flg22-triggered oxidative burst and callose deposition are regulated positively by the SA pathway and negatively by the JA pathway (Yi et al. 2014). JA signaling can be activated by coronatine (COR), a structural mimic of JA-isoleucine produced by *Pseudomonas syringae* pv. *tomato*, which is antagonistic to activated SA signaling, thus suppressing flg22-triggered oxidative burst; hence, COR-mediated suppression of flg22-triggered oxidative burst involves the mechanism of JA-SA antagonism (Yi et al. 2014).

Small RNAs (sRNAs) are key regulators of plant defense responses against bacterial infection (Niu et al. 2015). microRNAs

To defend themselves against pathogens, plants have evolved multiple layers of defense mechanisms to protect themselves from pathogen infection (Jones and Takemoto 2004). The first layer is comprised of the plant surface-localized receptors termed pattern-recognition receptors (PRRs) that perceive pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs), consisting of lipopolysaccharide (Dow et al. 2000), glycoprotein, peptide, and polysaccharide (Kaku et al. 2006; Zipfel and Felix 2005). Well-studied PAMPs include flg22, a peptide comprising 22 amino acids of the highly conserved elicitor-active epitope of flagellin (Felix et al. 1999). Recognition of PAMPs by PRRs initiates PAMP-triggered immunity (PTI), which involves a

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(miRNAs), a class of noncoding sRNAs of 21 to 24 nucleic acids, regulate defense-related gene expression through miRNA-mediated gene silencing pathways. miR393 is the first sRNA found to participate in PTI, which can be induced by flg22 and positively regulates PTI by suppressing auxin signaling through downregulation of the gene expression of the F-box auxin receptors TIR1, AFB2, and AFB3 (Navarro et al. 2006). In addition, miR160a, miR398b, and miR773 are involved in the regulation of callose deposition, thus acting in PTI signaling (Li et al. 2010). miR393b* facilitates exocytosis of an antimicrobial pathogenesis-related (PR) protein by targeting a Golgi-localized SNARE gene, thereby functioning in ETI signaling (Zhang et al. 2011). Moreover, some miRNAs have been shown to guide the regulation of disease resistance genes (Li et al. 2012; Niu et al. 2016b; Zhai et al. 2011). On the other hand, argonaute (AGO) proteins are the core molecules of the effector complex in sRNA-directed gene silencing, which is a widespread mechanism of gene regulation. For example, AGO1 is primarily associated with miRNAs and plays an important role in flg22-induced resistance to bacteria (Li et al. 2010), and AGO2 is strongly induced in *Arabidopsis* upon infection with avirulent *P. syringae* pv. *tomato* (*avrRpt2*) strains and is indispensable to antibacterial immune responses (Zhang et al. 2011).

In our previous study (Niu et al. 2011), pretreatment of wild-type *Arabidopsis* Col-0 roots with *Bacillus cereus* AR156 induced systemic resistance (ISR) in *Arabidopsis thaliana* by simultaneously activating salicylate- and jasmonate/ET-dependent signaling pathways. Recently, we found that leaf infiltration of AR156 induced systemic acquired resistance (SAR) in systemic leaves of *Arabidopsis* through a NPR1- and SA-dependent signaling pathway but independently of JA and ET signaling pathways (Niu et al. 2016a). We also showed that pretreatment with AR156 primed ISR to *P. syringae* pv. *tomato* infection by suppressing miR825 and miR825* and activating the defense-related target genes (Niu et al. 2016b). Interestingly, pretreatment with AR156 drastically reduced the abundance of miR398b and miR773 (Niu et al. 2016b), as did flg22 (Li et al. 2010). This phenomenon inspired us to investigate whether AR156 shares the same function of flg22 in triggering PTI, given that miR398b and miR773 are negative regulators of PTI against bacterial pathogens (Li et al. 2010). Our results indicated that AR156 functioned similarly to flg22 in inducing basal defense responses in *Arabidopsis*, which involve PTI response and miRNA-mediated gene expression. Furthermore, a transcriptional profiling analysis revealed that 117 genes were differentially regulated by both AR156 and flg22, indicating that they elicited highly correlated transcriptional responses.

RESULTS

AR156 induces plant resistance to *P. syringae* pv. *tomato*.

We previously demonstrated that AR156, applied as leaf infiltration, triggered SAR by priming systemic leaves to activate multiple PTI components (Niu et al. 2016a). In this study, we performed infection assays to investigate whether it shares the function of flg22 to confer local resistance to *P. syringae* pv. *tomato* in the pretreated leaves. Leaves of wild-type *Arabidopsis* Col-0 plants were syringe-infiltrated with either AR156 or flg22 1 day before challenge-inoculation with *P. syringae* pv. *tomato*. Compared with the nontreated control, both AR156 and flg22 treatments reduced the disease symptoms on leaves at 2 days post-treatment (dpi) (Fig. 1A). In agreement with the reduction in disease symptoms observed on the leaf surface, *P. syringae* pv. *tomato* population density also significantly declined by about 100-fold in leaves pretreated with AR156 or flg22 at 2 dpi relative to that in control leaves (Fig. 1B), signifying that AR156 conferred effective local resistance against *P. syringae* pv. *tomato* infection in the pretreated leaves, as did flg22. On the other hand, the lower *P. syringae* pv. *tomato* densities observed in AR156 treatment may be due, in part, to niche-occupation by AR156.

AR156 elicits PTI defense responses.

To investigate the mechanisms underlying AR156-induced antibacterial resistance, we determined the effect of AR156 on several PTI components in wild-type *Arabidopsis* Col-0 leaves. Callose deposition and H₂O₂ accumulation were noticeable at 24 h post-treatment (hpt) in leaves infiltrated with AR156 or flg22 but were absent in control leaves (Fig. 2A). Consistently, in the *PR1::GUS* transgenic *Arabidopsis* plants with *PR1* promoter fused to a *GUS* (β -glucuronidase) reporter, *PR1* protein was expressed in the leaves infiltrated with AR156 or flg22, as manifested by the precipitate indicating GUS activity, but was absent in control leaves (Fig. 2A).

We also examined whether AR156 can trigger MAPK activation, an early event in PTI signaling. As a result, phosphorylated MPK3 and MPK6 were first detected in the leaves at 15 minutes post-treatment (mpt) with AR156 applied as a foliar spray, while treatment with flg22 induced their phosphorylation as early as 5 mpt, remaining robust until 30 mpt (Fig. 2B). This indicates that AR156 treatment triggers MAPK activation less strongly than flg22 treatment.

We also determined the expression patterns of the PAMP-response genes *FRK1* (encoding flg22-induced receptor-like kinase 1), *WRKY22*, and *WRKY29* (Asai et al. 2002) in wild-type

Fig. 1. *Bacillus cereus* AR156 conferred resistance to *Pseudomonas syringae* pv. *tomato* (Pst) in *Arabidopsis*. **A**, Images of *Arabidopsis* Col-0 leaves with disease symptoms at 2 days postinoculation (dpi) with *P. syringae* pv. *tomato* (5×10^5 CFU/ml). Leaves were syringe-infiltrated with AR156 at 5×10^7 CFU/ml, 1 μ M flg22, or water at 24 h before *P. syringae* pv. *tomato* infection. **B**, Bacterial population density in leaves was measured at 2 dpi. Student's *t* test was carried out to determine the significance of the differences between treatment AR156 or flg22 and the control. Asterisks (**) indicate significant differences at a *P* value <0.01. The data shown are means $\pm$ standard deviation (*n* = 12) from one representative experiment among three independent repetitions.

Col-0 plants treated with AR156 or flg22, using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Both treatments substantially enhanced the accumulation of *FRK1* transcript at 30 mpt, and the transcript levels of *WRKY22* and *WRKY29* increased in seedlings at 10 mpt (Fig. 2C). Collectively, the above results revealed striking parallels between AR156 and flg22 in their function to induce PTI responses and defense-related gene expression.

AR156-induced resistance to *P. syringae* pv. *tomato* requires AGO1 and DCL but neither FLS2 nor SA-, JA-, and ET-pathways.

To further clarify the molecular mechanisms by which AR156 induces plant resistance to *P. syringae* pv. *tomato*, we carried out infection assays using wild-type Col-0, NahG transgenic plants, mutants with impaired SA-, JA-, or ET-signaling pathway (*jar1*, *etr1*, *ein2*, *sid2-2*, *npr1*), and the transgenic line NahG. Two-way analysis of variance (ANOVA) with Tukey's test was used to compare the levels of AR156- and flg22-induced resistance to *P. syringae* pv. *tomato* in these plants. Pretreatment with AR156 or flg22 significantly reduced disease symptoms (Fig. 3A) and *P. syringae* pv. *tomato* population densities in leaves (Fig. 3B) of wild-type plants as well as all mutants. On the other hand, *P. syringae* pv. *tomato* population densities were significantly

higher in *sid2-2*, *npr1*, and NahG plants than in Col-0 in both AR156 and flg22 treatments; meanwhile, this parameter was also significantly higher in *sid2-2*, *npr1*, and NahG plants than in Col-0 in the control group. The two-way ANOVA revealed that the interaction between treatment (AR156, flg22, or the control) and genotype was not statistically significant and that the overall levels of AR156- and flg22-induced resistance to *P. syringae* pv. *tomato* were not significantly different. Taken together, these results demonstrate that SA-, JA-, and ET-mediated signaling pathways are not essential for AR156-induced basal defense responses to *P. syringae* pv. *tomato*.

Acting as the receptor of flg22, FLS2 is essential for flg22-induced resistance to *P. syringae* pv. *tomato*. To determine whether FLS2 is also required for AR156-induced PTI responses to *P. syringae* pv. *tomato*, we assessed the effect of AR156 pretreatment on *fls2* plants. Consequently, the disease symptoms were significantly attenuated by AR156 at 2 days postinoculation (dpi) but not by flg22 in *fls2* plants in which AR156 pretreatment also significantly reduced bacterial proliferation relative to the control (Fig. 3C and D). This pointed to the fact that AR156-induced resistance does not require a functional FLS2.

Previous studies showed that miRNAs function to regulate plant resistance to bacterial pathogens (Li et al. 2010; Navarro et al. 2006; Niu et al. 2016b; Zhang et al. 2011). As the key

Fig. 2. AR156 activated defense responses and pathogen-associated molecular pattern-triggered immunity components. **A**, AR156- and flg22-induced callose deposition, reactive oxygen species burst, and PR1 expression in *Arabidopsis* Col-0 and transgenic *PR1::GUS* leaves. Plants were infiltrated with 5×10^7 CFU of AR156 per milliliter, 1 μ M flg22, or water. Samples were collected at 24 h post-treatment. **B**, AR156- and flg22-induced activation of mitogen-activated protein kinases in Col-0. Two-week-old seedlings grown on Murashige and Skoog (MS) medium were sprayed with 5×10^8 CFU of AR156 per milliliter or 10 μ M flg22. Samples were collected at 0, 5, 15, and 30 min. **C**, Quantitative reverse transcription-polymerase chain reaction analysis of AR156- and flg22-induced expression of *FRK1*, *WRKY22*, and *WRKY29* in Col-0. Plants grown on MS medium for two weeks were sprayed with 5×10^8 CFU of AR156 per milliliter or 10 μ M flg22. Samples were collected at 0, 30, 60, 120, 240, and 360 min. The data are shown as means $\pm$ standard deviation from three technical replicates. Similar results were obtained in three biological replicates. Student's *t* test was performed to determine the significance of the differences between treatment with AR156 and flg22. Asterisks (*) and (**) indicate significant differences at *P* values <0.05 and <0.01, respectively.

components of miRNAs biogenesis, both AGO1 and Dicer-like (DCL) proteins play an important regulatory role in flg22-induced antibacterial resistance (Li et al. 2010). Recently, we uncovered overlap between AR156- and flg22-mediated miRNAs (Li et al. 2010; Niu et al. 2016b). Thus, in this study, we carried out infection assays using *ago1-27* and *dcl1-9* mutants to clarify the role of AGO1 and DCL in AR156-induced resistance to *P. syringae* pv. *tomato*. At 2 dpi, both treatments AR156 and flg22 caused a significant reduction in disease severity in wild-type Col-0 and Ler plants but not in *ago1-27* and *dcl1-9* mutants. In agreement with the differences in disease severity between wild-type plants and mutants, bacterial population size significantly decreased by approximately 100-fold in wild-type Col-0 and Ler plants but to a significantly lesser extent in mutants *ago1-27* and *dcl1-9* (Fig. 3C and E). This result implied that AGO1 and DCL are required for AR156-induced resistance to *P. syringae* pv. *tomato*.

AR156 and flg22 differentially regulate miRNA expression in a similar manner.

Several AGO1-bound sRNAs, whose expression is modulated by flg22, exert regulatory function in plant defense (Li et al. 2010). Using Northern blotting, the current research determined the effect of AR156 on the expression patterns of eight miRNAs (miR158a, miR160a, miR167, miR169, miR391, miR393, miR396, and miR398b), which are known to be up- or downregulated by flg22 (Li et al. 2010). Compared with the control, AR156 and flg22 stimulated the expression of all these miRNAs except miR398b, whose expression was repressed by the two elicitors (Fig. 4A).

Subsequently, we assessed the impact of AR156 on expression of target genes of these miRNAs. miR393 targets *AFB2* and *AFB3*, genes encoding F-box auxin receptors (Navarro et al. 2006). The *ARF* genes are known to encode auxin response factors (Mallory et al. 2005). miR160a targets *ARF16* and *ARF17* (Mallory et al. 2005), as indicated by their mRNA

Fig. 3. AR156-induced resistance does not require salicylic acid (SA)-, jasmonic acid (JA)-, or ethylene (ET)-mediated pathways and FLS2 but involves AGO1 and DCL proteins. **A** and **B**, AR156- and flg22-induced resistance does not require SA, JA, and ET signaling pathways. Leaves of wild-type Col-0, NahG transgenic plants, and mutants (*jar1*, *etr1*, *ein2*, *sid2-2*, *npr1*) were syringe-infiltrated with 5×10^7 CFU/ml AR156, $1 \mu\text{M}$ flg22, or water for 24 h and were then infiltrated with *Pseudomonas syringae* pv. *tomato* (5×10^5 CFU/ml). Images were taken and bacterial population densities in leaves were determined at 2 days postinoculation (dpi) with *P. syringae* pv. *tomato* (5×10^5 CFU/ml). Two-way analysis of variance (ANOVA) with Tukey's test (significance set at $P < 0.05$) was performed (Xin et al. 2016). The bacterial populations in control plants with different genotypes indicated by different lower-case letters (a versus b, c, and d) were significantly different (bc was not significantly different from b or c), whereas differences in this parameter among different genotypes in AR156 and flg22 treatments are indicated by two sets of distinct lower-case letters (a' versus b', c', and d' for AR156 treatment; and a'' versus b'' and c'' for flg22 treatment); ns = not significant. The data presented are means $\pm$ standard deviation (SD) ($n = 12$) from one representative experiment of the four independent repetitions. **C**, **D**, and **E**, Defects in flg22 sensing do not compromise AR156-triggered defense responses. AGO1 and DCL are required for both AR156- and flg22-induced resistance to *P. syringae* pv. *tomato*. FLS2 contributes to the resistance induced by flg22 but not to that by AR156. Leaves of Col-0, Ler, *fls2*, *ago1-27*, and *dcl1-9* were syringe-infiltrated with 5×10^7 CFU of AR156 per milliliter, $1 \mu\text{M}$ flg22, or water for 24 h and were then infiltrated with *P. syringae* pv. *tomato* (5×10^5 CFU/ml). Images were taken and bacterial population densities in leaves were determined at 2 dpi with *P. syringae* pv. *tomato* (5×10^5 CFU/ml). Two-way ANOVA was performed to compare the differences between AR156 and flg22 treatments in plants with different genotypes; ns, not significant. Asterisks (**) indicate significant differences at a P value < 0.01 , respectively. The data presented are means $\pm$ SD ($n = 12$) from one representative experiment of the four independent repetitions.

levels being downregulated in *35S::miR160a* transgenic plants (Li et al. 2010); miR167 targets *ARF6* and *ARF8* (Jones-Rhoades and Bartel 2004; Rhoades et al. 2002). miR398b targets *CDS1* and *CDS2* (Jones-Rhoades and Bartel 2004), as evidenced by their mRNA levels declining in *35S::miR398b* transgenic plants (Li et al. 2010). miR158a targets *AT3G03580*, miR169 targets *AT1G17590* and *AT5G12840*, miR396 targets *GRF3* (Li et al. 2010), and miR391 putatively targets *GDS1*. In addition to the genes targeted by the aforementioned miRNAs, *MET2* is the target gene of miR773 (Fahlgren et al. 2007), whose expression was significantly up-regulated by flg22 treatment, although the abundance of miR773 in plants was below the detection limit of RNA-blot assay (Li et al. 2010). As shown in Figure 4B, both treatments, AR156 and flg22, significantly repressed the expression of all target genes of six miRNAs (miR158a, miR160a, miR167, miR391, miR393, and miR396) relative to the control; conversely, the gene expression of *CDS1*, *CDS2*, and *MET2* was consistently enhanced by both treatments. Expression of *AT1G17590* and *AT5G12840*, the genes targeted by miR169, was not significantly altered by flg22 treatment in the work of other

investigators (Li et al. 2010); the same results were obtained in our study, but both genes showed reduced expression in response to AR156 (Fig. 4B). Taken together, the above results demonstrated that AR156 and flg22 differentially regulate the expression of miRNAs involved in PTI generally, in a similar manner, and that the expression of miRNAs is correlated with the expression of their putative target genes.

AR156 and flg22 activated similar transcriptional programs.

To further compare the function of AR156 and flg22 in triggering PTI responses to *P. syringae* pv. *tomato*, we identified the transcriptional programs regulated by AR156 and flg22 by comparing the genome-wide transcriptional changes in the two treatments. The leaves of 4-week-old wild-type *Arabidopsis* Col-0 plants were syringe-infiltrated with 1 μ M flg22, 5×10^7 CFU of AR156 per milliliter, or water (the control) and were subjected to transcriptome analysis 1 day later. As a result, more than 17,000 genes were demonstrated to be differentially regulated by one or both AR156 and

Fig. 4. Expression of selected microRNAs (miRNAs) and their target genes in response to treatments AR156 and flg22. **A**, Northern blotting analysis of miRNAs. Leaves of four-week-old *Arabidopsis* Col-0 plants were syringe-infiltrated with 5×10^7 CFU of AR156 per milliliter, 1 μ M flg22, or water (the control) and were collected for RNA extraction at 24 h post-treatment (hpt). U6 was used as a loading control. **B**, Quantitative reverse transcription-polymerase chain reaction analysis of mRNA levels of genes targeted by miR158a (*AT3G03580*), miR160a (*ARF16* and *ARF17*), miR167 (*ARF6* and *ARF8*), miR169 (*AT1G17590* and *AT5G12840*), miR391 (*GDS1*), miR393 (*AFB2* and *AFB3*), miR396 (*GRF3*), miR398b (*CDS1* and *CDS2*), and miR773 (*MET2*). The data are shown as means $\pm$ standard deviation from three technical replicates. Similar results were obtained in three biological replicates. Student's *t* test was performed to determine the significance of the differences between treatment with AR156 or flg22 and the control. Asterisks (*) and (**) indicate significant differences at *P* values <0.05 and <0.01, respectively.

flg22 treatment (Supplementary Table S1). Genes with significant changes in expression levels in response to AR156 or flg22 treatment were determined according to a Benjamini-Hochberg false discovery rate (FDR) ≤ 0.05 , with multiple-testing correction, and a differentially expressed gene (DEG) was defined as having at least a twofold change (≤ -1 or ≥ 1 in $\log_2$ ratio value) based on DESeq normalized read counts in response to treatment with AR156 or flg22. Consequently, a total of 1,302 DEGs were selected in AR156 and flg22 treatments (Supplementary Table S2), with 128 and 1,291 DEGs having altered expression levels in treatments with AR156 and flg22, respectively, relative to the control (Fig. 5A). A hierarchical heat-map image was generated to analyze these DEGs in response to different treatments (Fig. 5B). Of the 128 DEGs responsive to AR156, 117 were also responsive to flg22 (Fig. 5A), of which 111 were up-regulated by AR156 and six were down-regulated by it.

To understand the biological function of the 117 genes differentially regulated by both AR156 and flg22, the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology database was used to identify the metabolic pathways involving them. Twenty of these genes were found to act in 15 pathways under three biologically functional categories: environmental information processing, metabolism processing, and organismal

systems processing (Fig. 5C; Supplementary Table S3). Most of these pathways are associated with metabolism processing. Specifically, phenylpropanoid biosynthesis and phenylalanine metabolism pathways play an important role in plant immunity, since they are involved in the biosynthesis of significant secondary metabolites. On the other hand, amino sugar and nucleotide sugar metabolism, galactose metabolism, pentose and glucuronate interconversions, and starch and sucrose metabolism are related to the formation of plant cell walls (Lee et al. 2016).

The selected 1,302 DEGs regulated by one or both AR156 and flg22 were used to determine correlation coefficients between any two of the three treatments (AR156, flg22, and the control). AR156 and flg22 treatments induced highly correlated responses, indicated by the correlation coefficient of 0.76 (Fig. 5D), despite the considerable difference in the number of DEGs between the two treatments (Fig. 5A). Notably, most of the DEGs were differentially regulated to a larger amplitude by treatment with 1 μM flg22 than by treatment with AR156 at 5×10^7 CFU/ml, in the transcriptome sequencing analysis (Fig. 5B). To verify the result of transcriptome sequencing, qRT-PCR was conducted to examine the expression of 16 DEGs coregulated by AR156 and flg22, of which ten were most strongly up-regulated by AR156 and six were down-regulated by it

Fig. 5. Transcript profiles of *Arabidopsis* Col-0 plants treated with AR156 or flg22. Leaves of four-week-old *Arabidopsis* Col-0 plants were syringe-infiltrated with AR156 at 5×10^7 CFU of AR156 per milliliter, 1 μM flg22, or water (the control). Samples were collected at 24 h post-treatment (hpt). **A**, Overlap between AR156- and flg22-mediated differentially expressed genes (DEGs) (false discovery rate of ≤ 0.05 and $\log_2$ fold change $\geq |1|$). **B**, Hierarchical cluster analyses of expression levels of DEGs based on log ratio FPKM (fragments per kilobase of exon per million fragments mapped) data. The color key represents (FPKM+1) normalized $\log_2$ transformed counts. Red and green indicate high- and low-level gene expression, respectively. Each column represents a treatment, and each row represents a gene. **C**, The Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology database pathways involving the 117 DEGs coregulated by AR156 and flg22. **D**, DEGs correlation coefficients (Pearson correlation coefficient) between different treatments.

(Table 1). Figure 6A and B show expression patterns in response to AR156 or flg22 relative to the control in the qRT-PCR analysis, which aligned with transcriptome sequencing data.

DISCUSSION

Bacillus cereus AR156 is a plant growth-promoting rhizobacterium that triggers both ISR and SAR in *Arabidopsis* against bacterial infection (Niu et al. 2011, 2016a). Our earlier study showed that pretreatment with AR156 suppressed the expression of miR398b and miR773 in wild-type *Arabidopsis* plants (Niu et al. 2016b), as did flg22. These two miRNAs negatively modulate PTI against bacterial pathogens (Li et al. 2010). In this study, pretreatment of *Arabidopsis* Col-0 plants with AR156 or flg22 effectively inhibited *P. syringae* pv.

tomato multiplication and also ameliorated the disease symptoms (Fig. 1), suggesting that AR156 behaves similarly to flg22 in activating immune responses, leading to reduced pathogen growth. Flg22 has been demonstrated to trigger PTI responses, such as activation of MAPK signaling, induction of hydrogen peroxide accumulation, callose deposition, and activation of defense-related gene expression (Asai et al. 2002; Gómez-Gómez et al. 1999), which all contribute to restriction of pathogen growth in plants (Nürnberger et al. 2004). In this study, we detected evident hydrogen peroxide accumulation, callose deposition, and PR1 expression in *Arabidopsis* Col-0 leaves infiltrated with AR156. Moreover, AR156, applied as foliar spray, led to phosphorylation of MPK3 and MPK6 and expression of early defense genes *FRK1*, *WRKY22*, and *WRKY29*, as did flg22 (Fig. 2).

Table 1. Characteristics of 16 differentially expressed genes (DEGs) examined in the quantitative reverse transcription-polymerase chain reaction analysis

DEG ID	Primary sequence	Log ₂ fold change		Function of DEGs
		AR156	flg22	
AT3G55150	ATEXO70H1	5.41351	6.65342	A member of the EXO70 gene family, putative exocyst subunits, conserved in land plants; <i>Arabidopsis thaliana</i> contains 23 putative EXO70 genes, which can be classified into eight clusters on the phylogenetic tree
AT1G44160	...	5.05182	7.06413	Encodes a HSP40/DnaJ peptide-binding protein
AT5G43290	ATWRKY49	4.96243	5.9502	Encodes a WRKY transcription factor in Group II-c
AT1G18860	ATWRKY61	4.86998	5.64852	Encodes a WRKY transcription factor in Group II-b
AT5G39580	...	4.60275	6.15912	Encodes a peroxidase superfamily protein functioning in peroxidase activity and heme binding, involved in defense response to fungus
AT1G31885	NIP3;1	4.49333	7.20497	Encodes a NOD26-like intrinsic protein 3;1 (NIP3;1) functioning in transporter activity, involved in transport and transmembrane transport
AT1G68150	ATWRKY9	4.49332	6.29881	Encodes a WRKY transcription factor in Group II-b; its mRNA is cell-to-cell mobile
AT1G69810	ATWRKY36	4.41993	5.34704	Encodes a WRKY transcription factor in Group II-b
AT4G09770	...	4.23681	6.08637	Encodes a tumor necrosis factor receptor-associated factor (TRAF)-like family protein
AT2G39530	CASPLAD1	4.22446	6.80413	Unknown
AT3G09260	LEB	-3.8872	-7.08336	Encodes β -glucosidase, the major constituent of endoplasmic reticulum (ER) bodies and one of the most abundant proteins in <i>Arabidopsis</i> seedlings, which exists in soluble (inactive) and insoluble (active) form, most probably formed in a polymerization process
AT1G52040	MBP1	-3.1418	-4.93361	Encodes a myrosinase-binding protein expressed in flowers
AT2G10940	...	-2.89134	-7.11807	Encodes a bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein, functioning in lipid binding and involved in lipid transport
AT3G28220	...	-2.65761	-3.13113	Encodes a TRAF-like family protein, involved in response to salt stress and located in vacuole and chloroplast envelope
AT5G15780	...	-2.55822	-5.61531	Encodes a Pollen Ole e 1 allergen and extensin family protein
AT1G52400	BGLU18	-2.54111	-4.34809	Encodes a member of glycosyl hydrolase family 1, located in inducible ER bodies which are formed after wounding, required for inducible ER body formation; its mRNA is cell-to-cell mobile

It has been shown that flg22-induced resistance to *P. syringae* pv. *tomato* in *Arabidopsis* does not require SA, JA, and ET signaling (Zipfel et al. 2004). In line with this, pretreatment with AR156 still conferred resistance to *P. syringae* pv. *tomato* in NahG transgenic plants and in mutants *jar1*, *etr1*, *ein2*, *sid2-2*, and *npr1*, indicating that SA, JA, and ET pathways are not indispensable to AR156-induced PTI responses. As demonstrated in previous studies, AR156 applied as a root drench primed *Arabidopsis* Col-0 plants for ISR to the biotrophic pathogen *P. syringae* pv. *tomato*, which is dependent on the SA and JA/ET signaling pathways as well as NPR1 (Niu et al. 2011), while JA and ET pathways and NPR1 are involved in ISR against *P. syringae* pv. *tomato* in *Arabidopsis* Col-0 triggered by the rhizobacterium *P. fluorescens* WCS417r applied as a root drench (Pieterse et al. 1998). In addition, another rhizobacterium, *Pseudomonas putida* WCS358r, conferred ISR in *Arabidopsis* but not in radish and carnation (Leeman et al. 1995; van Peer et al. 1991; Van Peer and Schippers 1992; Van Wees et al. 1997). In *Arabidopsis* Col-0 plants, rhizosphere colonization by AR156 elicited an ISR against the biotrophic pathogen *P. syringae* pv. *tomato* and an ISR against the necrotrophic pathogen *Botrytis cinerea*. However, different from the AR156-triggered ISR to *P. syringae* pv. *tomato*, the AR156-elicited ISR to *B. cinerea* does not require the SA signaling pathway (Nie et al. 2017). On the other hand, when infiltrated to leaves, AR156 induced SAR against *P. syringae* pv. *tomato* in *Arabidopsis* Col-0 plants by involving the SA signaling pathway and NPR1 (Niu et al. 2016a). Hence, we infer that the specificity of signaling pathway activation during induction of antibacterial resistance by elicitors depends on, not only the specific elicitor, host plant, and pathogen, but also, the specific method of applying an elicitor. Besides, AR156 still

conferred disease resistance in *fls2* mutants (Fig. 3C and D), suggesting that, unlike flg22, AR156 does not require FLS2, the receptor of flg22, for inducing PTI against *P. syringae* pv. *tomato* (Gómez-Gómez and Boller 2000; Zipfel et al. 2004). In our previous study (Niu et al. 2011), the unique MAMPs of AR156 are assumed to be recognized by several receptors of the plant, leading to generation of distinct signals. In this study, it is possible that AR156 induces the activation of the SA, JA, ET, and FLS2 signaling pathways in parallel and that knocking out a single pathway in the mutant alone does not abolish the induction of resistance. Future research is warranted to identify the receptor for AR156 in *Arabidopsis*.

Host endogenous sRNAs play an important regulatory role in reprogramming gene expression and contribute to plant defense responses (Katiyar-Agarwal and Jin 2010). Perception of flg22 induces the accumulation of miR393, the first identified miRNA that negatively regulates auxin signaling and contributes to the PTI by downregulation of *TIR* and *AFB* transcripts (Navarro et al. 2006). Moreover, several other AGO1-bound miRNAs that are induced or repressed by flg22 have also been identified as mediators of PTI. For example, flg22 induces the accumulation of miR160a, which positively regulates callose deposition by repressing the expression of its target genes *ARF16* and *ARF17*. By contrast, flg22 suppresses accumulation of miR398b and miR773, which negatively regulate callose deposition (Li et al. 2010). Here, we found that AR156 treatment induced accumulation of miR393 and miR160a while downregulating the expression of miR398b and miR773. It was reported that auxin treatment rendered *Arabidopsis* Col-0 plants more susceptible to biotrophic pathogens (Robert-Seilanian et al. 2011). Moreover, overexpression of miR393 led to the redirection of metabolic flow toward glucosinolates (GSs) and away from camalexin

Fig. 6. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis validating transcriptome sequencing results. Leaves of four-week-old *Arabidopsis* Col-0 plants were syringe-infiltrated with AR156 at 5×10^7 CFU/ml, 1 μ M flg22, or water (the control). Samples were collected at 24 h post-treatment (hpt). **A**, The transcript levels of 10 differentially expressed genes (DEGs) most strongly upregulated by AR156 treatment were determined by qRT-PCR. **B**, The transcript levels of six DEGs downregulated by AR156 were determined by qRT-PCR. The data are shown as means $\pm$ standard deviation from three technical replicates. Similar results were obtained in three biological replicates. Student's *t* test was performed to determine the significance of the differences between treatment with AR156 or flg22 and the control. Asterisks (**) indicate significant differences at a *P* value <0.01 .

(CL), which was the direct consequence of the suppression of auxin signaling (Robert-Seilantantz et al. 2011), though both being antimicrobial molecules, GSs are toxic to a wide range of bacteria (Aires et al. 2009a and b) but CL does not contribute to bacterial defense (Nafisi et al. 2007). Taken together, AR156-induced resistance to *P. syringae* pv. *tomato* might involve multiple signal pathways, including the auxin signaling and GS biosynthetic pathways.

To confirm the similarity between PTI responses to *P. syringae* pv. *tomato* induced by AR156 and flg22, we compared the full-genome transcriptional changes in *Arabidopsis* Col-0 leaves in treatments with AR156 and flg22. Of the 128 DEGs regulated by AR156, a vast majority (117) were also mediated by flg22. Also, the correlation coefficient calculated using DEGs between the two treatments was high (0.76). On the other hand, AR156 treatment differentially regulated a substantially lower number of genes and to a lesser amplitude than flg22 treatment (Figs. 5 and 6A and B). The 10 genes most highly up-regulated (by more than tenfold) by AR156 were identified as components of defense signaling pathways. Among them, *AtEXO70H1* is a member of the *EXO70* gene family. Compared with wild-type *Arabidopsis* Col-0, the mutant *atexo70H1* was more sensitive to *Pseudomonas syringae* pv. *maculicola* infection (Pečenková et al. 2011). *AT5G39580* encodes a peroxidase, a key component of the plant ROS-generating system (Chen and Schopfer 1999). *AT4G09770* encodes a tumor necrosis factor receptor-associated factor-like family protein, which plays important roles in plant development and response to abiotic stress (Bao et al. 2014). A member of the subfamily of nodulin 26-like intrinsic proteins (NIPs) of *Arabidopsis*, i.e., *NIP3;1*, encodes NIP3;1, which was demonstrated to extrude arsenite into the xylem for long-distance distribution by translocation from the root to the shoot in *Arabidopsis* (Xu et al. 2015); arsenite is a metalloid and highly toxic to a wide range of organisms including plants and humans (Abernathy et al. 2003; Tchounwou et al. 2004). Among the 10 DEGs most highly upregulated by AR156, four encode WRKY transcription factors, which exert regulatory function in plant defense response (Eulgem 2005); *Arabidopsis* plants overexpressing *AtWRKY61* showed lower symptom severity and less virus accumulation upon *Turnip crinkle virus* attack compared with *Arabidopsis* Col-0 plants (Gao et al. 2016). Interestingly, the ten DEGs most highly upregulated by AR156 were also up-regulated by flg22, which elicited a greater amplitude of gene expression variation than AR156. Together, the above results indicate that the transcriptional changes triggered by AR156 overlap those elicited by flg22.

In this study, AR156 at 5×10^7 CFU/ml and flg22 at 1 μ M were similarly effective in protecting *Arabidopsis* Col-0 plants from *P. syringae* pv. *tomato* infection, as shown by the *P. syringae* pv. *tomato* population density and disease symptoms in leaves being reduced to a similar degree relative to the nontreated control (Fig. 1). This is inconsistent with the observation that, when compared with AR156 at 5×10^7 CFU/ml, flg22 at 1 μ M triggered stronger PTI responses, including the abundance change of miRNAs involved in plant innate immunity and transcriptional changes. It could easily imagine a dose-dependent effect of AR156 or flg22 treatment on PTI responses that could produce the observed effects. However, if the AR156 dose was increased or the flg22 dose decreased, perhaps the PTI responses would be the same. While the effect of these doses on *P. syringae* pv. *tomato* growth is the same, there could be a threshold effect by which a base level of PTI induction is sufficient to control *P. syringae* pv. *tomato* growth or the lower *P. syringae* pv. *tomato* densities observed may be due in part to niche-occupation by AR156. Perhaps the PTI induced by AR156 coupled with the physical presence of the AR156 bacteria occupying the niche

could combine to give the same effect as the flg22-induced PTI. Therefore, it would be worthwhile to identify the factors causing the discrepancy between the level of disease resistance and the extent of PTI responses triggered by AR156 relative to those elicited by flg22 in the future.

MATERIALS AND METHODS

Plant materials, bacterial strains, and growth conditions.

Arabidopsis plant lines used in this study were wild-type Columbia-0 (Col-0) and Landsberg *erecta* (Ler), transgenic line *PR1::GUS*, NahG (a transgenic line of Col-0 harboring the bacterial *NahG* gene) (Delaney et al. 1994), and mutants *ago1-27*, *fls2*, *dcl1-9* (Ler background), *sid2-2*, *jar1* (Staswick et al. 1992), *etr1* (Bleecker et al. 1988), *ein2* (Alonso et al. 1999), and *npr1* (Bowling et al. 1994). Plants were grown in a controlled growth room at $23 \pm 1^\circ\text{C}$ and with 70% relative humidity in a 12-h light and 12-h dark photoperiod.

Two-week-old *Arabidopsis* Col-0 seedlings grown on Murashige and Skoog medium were used for the MAPK activity assay and qRT-PCR analysis of PAMP-response genes *FRK1*, *WRKY22*, and *WRKY29*. Seedlings were collected at 0, 5, 15, and 30 min for the MAPK activity assay after treatment with AR156 or flg22, while seedlings were collected at 0, 30, 60, 120, 240, and 360 min for the qRT-PCR analysis. Differently from the above experiments, 4-week-old soil-grown plants were consistently used for the other experiments in this study, as indicated below.

Bacillus cereus AR156 was grown in liquid Luria-Bertani medium at 28°C for 24 h. Subsequently, bacterial cells were pelleted by centrifugation and were resuspended in sterile water for use. *Pseudomonas syringae* pv. *tomato* was grown in liquid King's B medium containing, per liter, 50 mg of rifampicin and 50 mg of kanamycin at 28°C overnight. Bacterial cells were pelleted by centrifugation and were resuspended in 10 mM MgCl_2 .

Bacterial infection.

Leaves of 4-week-old *Arabidopsis* plants were syringe-infiltrated with AR156 at 5×10^7 CFU/ml, 1 μ M flg22, or water (the control). One day after pretreatment, the leaves were challenge-inoculated by syringe-infiltration with a *P. syringae* pv. *tomato* suspension at the concentration of 5×10^5 CFU/ml. Bacterial growth assays were performed as previously described, and at least 12 leaf discs were collected for each growth assay.

qRT-PCR.

Leaves of four-week-old *Arabidopsis* Col-0 plants were syringe-infiltrated with AR156 at 5×10^7 CFU/ml, 1 μ M flg22, or water (the control). Leaf tissues were sampled at 24 hpt, from which RNA was extracted using TRIzol reagent (Invitrogen, San Diego, CA, U.S.A.). Total RNA was treated with DNase I (Invitrogen). cDNA was synthesized from 1 μ g of RNA, using the SuperScript III first-strand synthesis system (Invitrogen). qRT-PCR was performed using SYBR Green JumpStart Taq ReadyMix (Sigma, St. Louis). The gene expression level was normalized using *Actin2* as a control. Primers used in real-time PCR are shown in Supplementary Table S4.

Detection of ROS burst and callose deposition.

Leaves of 4-week-old *Arabidopsis* Col-0 plants were syringe-infiltrated with 1 μ M flg22, AR156 at 5×10^7 CFU/ml, or water (the control). The leaves were collected at 24 h post-inoculation and were then processed for detection of ROS burst and callose deposition according to previously described procedures (Niu et al. 2011). Briefly, to detect ROS burst, *Arabidopsis* leaves were stained with 1 mg of diaminobenzidine

(DAB) (pH 3.0) per milliliter, in the dark at room temperature for 8 h. Subsequently, leaves were incubated in a solution of ethanol/acetic acid/glycerol (3:1:1, vol/vol/vol) in a 90 to 95°C water bath for 20 min and were then gently rinsed with water before observation. DAB staining visualized hydrogen peroxide as red-brown precipitate under the light microscope. To determine callose deposition, leaves were immersed in 5 ml of a destaining solution (phenol/glycerol/lactic acid/water/ethanol, 1:1:1:1:8, vol/vol/vol/vol/vol) in a bottle and were infiltrated by applying a vacuum for 5 to 10 min. The bottle with samples was incubated in a 60°C water bath for 20 to 30 min to clear chlorophyll. The resultant chlorophyll-free leaves were gently rinsed with water and were then soaked in 3 to 5 ml of a 0.01% (wt/vol) aniline blue staining solution containing 150 mM K₂HPO₄ (pH 9.5) in the dark for 2 to 4 h. After staining, leaves were gently rinsed with water and were then mounted on microscope slides for observation under an epifluorescence microscope equipped with a UV excitation filter.

Histochemical GUS detection.

Histochemical detection of GUS activity was conducted as previously described (Hua et al. 2001), with some modifications. Leaves of 4-week-old transgenic *PRI::GUS* plants of *Arabidopsis* were syringe-infiltrated with AR156 at 5×10^7 CFU/ml, 1 μ M flg22, or water (the control). Samples were collected at 24 hpt and were then incubated in a staining solution (100 mM sodium phosphate, pH 7.0, 10 mM EDTA, 2 mM 5-bromo-4-chloro-3-indoyl glucuronide, 1 mM potassium ferricyanide, and 0.1% triton X-100) at 37°C overnight. After incubation, stained leaf tissues were cleared of chlorophyll in an ethanol series.

MAPK activity assay.

Immunoblot detection of phosphorylated MPK3 and MPK6 was performed as previously described (Niu et al. 2016a). Two-week-old seedlings grown on Murashige and Skoog medium were sprayed with 10 μ M flg22 or AR156 at 5×10^8 CFU/ml. Samples were collected at 0, 5, 10, and 30 min and were analyzed by Western blotting using monoclonal rabbit phospho44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP antibodies (Cell Signaling Technology, Danvers, MA, U.S.A.) (1:2,000 dilution). α -Tubulin was used as a loading control and was detected with a monoclonal mouse anti- α -tubulin (Sigma-Aldrich) (1:4,000 dilution).

Transcriptome sequencing.

Leaves of 4-week-old *Arabidopsis* Col-0 plants were syringe-infiltrated with AR156 at 5×10^7 CFU/ml, 1 μ M flg22, or water (the control). Samples were collected at 24 hpt. Total RNA was extracted from treated *Arabidopsis* Col-0 leaf tissues using TRIzol reagent (Invitrogen), following the manufacturer's instructions. Two biological replicates were used for each sample, and each sample contained tissues from at least ten whole plants.

For RNA-seq, the libraries were constructed and sequenced at Biomarker Technologies (Beijing, China) with an Illumina HiSeqX Ten platform. Paired-end reads from each sample were quality checked using FASTQC and were mapped to the *Arabidopsis* genome. RNA-seq data were normalized by FPKM (fragments per kilobase of exon per million fragments mapped). The Benjamini-Hochberg FDR threshold ≤ 0.05 and a $\log_2$ fold change ≤ -1 or ≥ 1 based on DESeq normalized read counts were used as the criteria to determine differentially expressed genes.

Gene Ontologies were analyzed for term enrichment using the online tool released in the The Arabidopsis Information Resource website and DAVID bioinformatics resources. The

KEGG Orthology database was utilized for pathway mapping. We employed the MapMan package to visualize the metabolic pathways of the DEGs.

RNA gel blot analysis.

Leaves of 4-week-old *Arabidopsis* Col-0 plants were syringe-infiltrated with AR156 at 5×10^7 CFU/ml, 1 μ M flg22, or water (the control). Samples were collected at 24 hpt. Total RNA was isolated from treated leaves and was resolved on a 14% denaturing urea-polyacrylamide gel electrophoresis gel and was then transferred and ultraviolet-crosslinked onto a Hybond N⁺ membrane (GE Healthcare Life Sciences, Piscataway, NJ, U.S.A.). miRNAs were detected using oligonucleotide probes, and miRNA probes were end-labeled with γ -32P-ATP using T4 polynucleotide kinase (New England Biolabs, Beverly, MA, U.S.A.). RNA gel blots were subsequently exposed on a phosphor imager screen.

Statistical analysis.

The specific statistical method used, the sample size, and the results of statistical analyses are described in the relevant figure legends. Statistical analysis was conducted using GraphPad Prism 6.0. Two-way ANOVA analysis with Tukey's test ($P < 0.05$) was performed when treatments comprising different genotypes were compared. Different letters above the columns are used to indicate differences that are statistically significant. The Student's *t* test was performed for comparison of means between two datapoints. Single and double asterisks above the columns indicate differences that are statistically significant ($P < 0.05$) or very significant ($P < 0.01$), respectively.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- The Arabidopsis Information Resource website:
<http://www.arabidopsis.org>
- DAVID bioinformatics resources: <http://david.abcc.ncifcrf.gov>
- FastQC: <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>
- GraphPad Prism 6.0 software: <https://www.graphpad.com>
- The Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology database:
<http://www.genome.jp/kegg/ko.html>
- The MapMan package: <http://MapMan.gabipd.org>