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DISEASE CONTROL

Acibenzolar-*S*-methyl activates stomatal-based defense against *Pseudomonas cannabina* pv. *alisalensis* in cabbage

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Abstract

Pseudomonas cannabina pv. *alisalensis* (*Pcal*), which causes bacterial blight of brassicaceous plants, is an economically important pathogen worldwide. Copper fungicide and antibiotics are major strategies to manage the disease caused by *Pcal*; however, a *Pcal* strain resistant to these chemicals has already been found, and severe outbreaks of bacterial blight have been reported on cabbage in Japan. Therefore, there is an urgent need to develop new *Pcal* management strategies. Plant defense activators could be useful not only to protect plants against invading pathogens, but also to reduce the amount of copper fungicides and antibiotics applied. However, the mechanisms by which plant defense activators contribute to controlling diseases remains unclear. In this work, we focused on cabbage and acibenzolar-*S*-methyl (ASM), a well-known plant defense activator. Expression profiles revealed that ASM induced expression of systemic acquired resistance (SAR) marker genes including *PR1*, *PR2*, and *PR5* in cabbage plants. We also demonstrated that a soil drench with ASM 2 h before transplanting clearly reduced bacterial blight symptoms and reduced *Pcal* bacterial populations in cabbage. ASM application was also able to prime cabbage for *Pcal* resistance by activating stomatal-based defense. Our findings highlight that ASM protects plants from bacterial pathogens by activating stomatal-based defense.

Keywords Acibenzolar-*S*-methyl · *Pseudomonas cannabina* pv. *alisalensis* · Cabbage · Stomatal-based defense · Plant defense activator

Introduction

In the natural environment, plants are constantly surrounded by numerous microorganisms, including potential pathogens. Although plants have not acquired immune systems like those of animals, they have developed monitoring systems that recognize potential pathogens and activate a wide range of immune responses for self-protection (Hacquard et al. 2017; Jones and Dangl 2006). The first layer of plant immune response against invading pathogens is pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI). Plants recognize conserved molecules such as flagellin and elongation factor Tu (EF-Tu) of an invading bacterial pathogen using plant pattern-recognition receptors (PRRs), such as FLS2 and EFR, respectively (Zipfel 2004, 2008; Zipfel and Felix 2005; Zipfel et al. 2006). Following the recognition of an invading pathogen with PRR, plants then activate immune responses. One of the earliest immune responses in PTI is stomatal-based defense to restrict bacterial pathogen entry through stomata (Melotto et al. 2006, 2008; Underwood et al. 2007). However, bacterial pathogens have successfully acquired multiple virulence factors such as phytotoxins and type III secretion system (TTSS) effectors to overcome stomatal-based defense (Ishiga et al. 2018; Lozano-Durán et al. 2014; Melotto et al. 2017).

Recent outbreaks of plant diseases caused by bacterial pathogens have been reported worldwide. Bacterial blight of plants in the Brassicaceae family, caused by *Pseudomonas cannabina* pv. *alisalensis* (*Pcal*), is becoming an economically important disease (Sarris et al. 2013; Takahashi et al. 2013a; Takikawa and Takahashi 2014). Chemical treatments such as copper fungicides and antibiotics

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are major strategies to manage *Pcal* diseases (Horinouchi 2010), but a *Pcal* strain resistant to these chemicals has already been found (Takahashi et al. 2013b). Therefore, there is an urgent need to develop new *Pcal* management strategies because severe bacterial blight disease outbreaks have been reported on cabbage in Japan (Takahashi et al. 2013b; Takikawa and Takahashi 2014). Among potential new control strategies against bacterial pathogen diseases, plant defense activators are rising stars. Known to activate the natural immune responses of plants, such as systemic acquired resistance (SAR) against invading pathogens without direct activity against pathogens (Bektas and Eulgem 2015; Zhou and Wang 2018), plant defense activators could be useful not only to protect plants against invading pathogens, but also to reduce the use of copper fungicides and antibiotics against *Pcal*.

Acibenzolar-*S*-methyl (ASM), a synthetic salicylic acid (SA) functional analog, can function as a plant defense activator (Kunz et al. 1997; Oostendorp et al. 2001). ASM activates plant resistance against a wide variety of pathogens, including viruses, fungi, and bacterial pathogens (Friedrich et al. 1996; Görlach et al. 1996; Kunz et al. 1997; Lawton et al. 1996). ASM does not act directly on plant pathogens in vitro (Friedrich et al. 1996). Although it does not induce SA biosynthesis, ASM triggers *NPR1*-dependent SAR (Lawton et al. 1996).

In the present study, we demonstrate that ASM induces *PR* gene expression in cabbage. We further demonstrate that ASM-treatment suppresses *Pcal* disease development by activating stomatal-based defense. Thus, our results provide new insights into the mechanisms by which plant defense activators contribute to protecting plants against bacterial pathogens.

Materials and methods

Plant materials and chemical treatment

Cabbage (*Brassica oleracea* var. *capitata*) cv. Kinkei 201 plants were used for all experiments. Two-week-old seedlings were grown in 200-hole trays at 24 °C with a light intensity of 200 μE m^{-2} s^{-1} and 16 h light/8 h dark. Acibenzolar-*S*-methyl (ASM, marketed as ACTIGARD®) was supplied courtesy of Syngenta. To evaluate the effect of ASM on plant defense, we treated 2-week-old cabbage seedlings by directly drenching the soil with ASM [as 50% active ingredient (a.i.)] suspended in 500 ml of water (100 mg/l) at the rate of 50 mg a.i./200 plants 2 h before *Pcal* inoculation. Soils were drenched with water or mock-inoculated with water as controls.

Bacterial strains and growth conditions

Pseudomonas cannabina pv. *alisalensis* strain KB211 (*Pcal*), a pathogenic strain, was kindly provided by the Nagano Vegetable and Ornamental Crops Experiment Station, Nagano, Japan. *Pcal* was grown at 28 °C on mannitol-glutamate (MG; Keane et al. 1970) agar. For inoculum, bacteria were suspended in sterile distilled H_2O, and cell densities measured at 600 nm (OD_{600}) using a JASCO V-730 spectrophotometer (JASCO, Tokyo, Japan) just before the inoculation.

Bacterial inoculation

Intact leaves on plants were dipped into a bacterial suspension (OD_{600} of 0.1) in sterile distilled water containing 0.025% Silwet L-77 (OSI Specialties, Danbury, CT, USA). The plants were then incubated in growth chambers at ~100% RH for the first 24 h, then at ~70% RH for the rest of the experiment. At 5 days post-inoculation (dpi), symptoms on the inoculated plants were evaluated.

To quantify bacterial populations in cabbage plants, the internal bacterial population in two harvested leaves was measured at several times. Total mass of each leaf was measured, then the leaves were surface-sterilized with 5% v/v H_2O_2 for 3 min, then washed three times with sterile distilled water. The plants were then homogenized in sterile distilled water, and these diluted samples were plated onto solid MG agar. The bacterial colony forming units (CFU) were normalized as CFU/g based on the total leaf mass. The bacterial population at 0 dpi was estimated using leaves harvested 1 h post-inoculation (hpi) without surface-sterilization. The bacterial populations were evaluated in three independent experiments.

Real-time quantitative RT-PCR

For expression profiles of cabbage defense genes in response to ASM, cabbage plants were treated by drenching the soil with ASM. After 2, 6, 12, 24, 48, 72, and 168 h, total RNA was extracted from leaves and purified. Total RNA was extracted using RNAiso Plus (Takara Bio, Shiga, Japan) according to the manufacturer's protocol and used for real-time quantitative RT-PCR (qRT-PCR) as described (Ishiga and Ichinose 2016). Two micrograms of total RNA was treated with gDNA Eraser (Takara Bio) to eliminate genomic DNA, and the DNase-treated RNA was reverse transcribed using the PrimeScript RT reagent Kit (Takara Bio). The cDNA (1:20) was then used for qRT-PCR using the primers shown below with SYBR Premix Ex Taq II (Takara Bio) on a Thermal Cycler Dice Real Time System

(Takara Bio). Cabbage *UBQ1* gene was used as an internal control. Average CT values, calculated using the 2nd derivative maximum method from triplicate samples, were used to determine the fold expression relative to the controls. Primers used in gene-specific PCR amplification for *PR1* (XM_013770002.1) were 5′-GGTCAACGAGAAGGCTAACTATAA-3′ and 5′-GCTTTGCCACATCCAATTCTC-3′; for *PR2* (XM_013747042.1), 5′-GAAGAGTGGAACTCCGAGAAAG-3′ and 5′-AGGCTGTTGACTAGGAAGAAAC-3′; for *PR5* (XM_013734443.1), 5′-GACGGCTACAACGTCAAGAT-3′ and 5′-CCATGACACGAAGCTCGTTA-3′; and for *UBQ1* (XM_013746806.1), 5′-GTCAAGGCCAAGATCCAAGA-3′ and 5′-GGATGTTGTAGTCAGCCAGAG-3′.

Stomatal assay

A modified method was used to assess stomatal response as described previously (Chitrakar and Melotto 2010). Briefly, cabbage plants were grown for 2 weeks after germination as described previously. *Pcal* was grown at 28 °C for 48 h on MG agar, then suspended in sterile distilled water to an OD_{600} of 0.2. Dip-inoculated cabbage leaves were directly imaged at 1 hpi or 4 hpi using a Leica TCS SP8 confocal microscope equipped with a white light laser (Leica, Wetzlar, Germany). A reflected image of the leaf surface was obtained by illuminating the sample with 561 nm wavelength, and reflected light was detected through a 558–566 nm filter. The aperture width of at least 60 stomata was measured. The average and standard error for the stomatal aperture width were calculated. The stomatal apertures were evaluated in three independent experiments.

Results

ASM induces defense-related gene expression in cabbage

ASM treatments are well known to induce defense-related gene expression including PR proteins. *PR1* is one of the most widely used genes to monitor the induction of SAR in plants (Lawton et al. 1996; Tripathi et al. 2010). To evaluate the effect of ASM on defense gene induction in cabbage, we investigated the expression profiles of *PR1*, *PR2*, and *PR5* in response to ASM. Two-week-old cabbage plants were treated with water as a control (mock), or ASM, and then total RNA was isolated from samples fixed at 2, 6, 12, 24, 48, 72, and 168 h post-treatment (hpt). Soil was drenched with ASM. *PR1* expression was induced in leaves within 2 h after ASM treatment and reached a maximum at 48 hpt (Fig. 1a). Similar to the *PR1* expression profile, *PR2* and *PR5* expression was induced by ASM treatment and peaked at 48 hpt (Figs. 1b, c). These results indicate that ASM activates SA-mediated signaling pathways leading to PR protein accumulation.

ASM suppresses *Pcal* disease development

To assess the effect of ASM on bacterial disease development, cabbage was challenged with *Pcal* 2 h after the soil drench with ASM, and disease symptoms were monitored. Figure 2a shows that control water-treated cabbage plants inoculated with *Pcal*, had typical bacterial blight symptoms at 5 dpi. However, symptoms were less severe on ASM-treated plants (Fig. 2a). In ASM-treated cabbage leaves, *Pcal* populations were significantly lower than in the water-treated control (Fig. 2b), suggesting that decreased bacterial multiplication was responsible for the decrease in severity. These results clearly indicate that ASM has a critical effect on suppressing *Pcal* disease development.

ASM activates stomatal-based defense against *Pcal*

The stomatal-based defense mechanism in plants is responsible for closing stomata in response to the perception of PAMPs of invading pathogens, especially bacterial pathogens (Melotto et al. 2017). Since we demonstrated that a soil drench with ASM 2 h before inoculation can suppress disease development, we next examined cabbage stomatal-based defense after *Pcal* inoculation with or without ASM. As shown in Fig. 3a, ASM-triggered stomatal closure was not observed on mock-inoculated cabbage leaves at 1 or at 4 hpi. In contrast, stomata on both water- and ASM-treated cabbage leaves inoculated with *Pcal* were closed at 1 hpi, indicating that PTI, including stomatal-based defense, is induced in cabbage leaves against *Pcal*. Interestingly, stomata had reopened on water-treated, *Pcal*-inoculated leaves by 4 hpi, whereas stomata were still closed on *Pcal*-inoculated leaves treated with the ASM soil drench (Fig. 3a). Consistent with this stomatal-based defense in ASM-treated cabbage leaves, *Pcal* bacterial populations at 4 hpi were significantly lower than in the water-treated inoculated control (Fig. 3b). These results indicate that ASM activates stomatal-based defense against *Pcal*.

Discussion

In our functional analysis of ASM, a well-known plant defense activator in the plant immune system, we found that a soil drench with ASM led to the accumulation of *PR1*, *PR2*, and *PR5* mRNAs in cabbage leaves and effectively suppressed *Pcal* lesion formation on those leaves (Figs. 1 and 2). Surprisingly, ASM induced SAR against *Pcal* within 2 h after the soil drench, suggesting that mobile signals generated by ASM triggered SAR in untreated leaves. Several

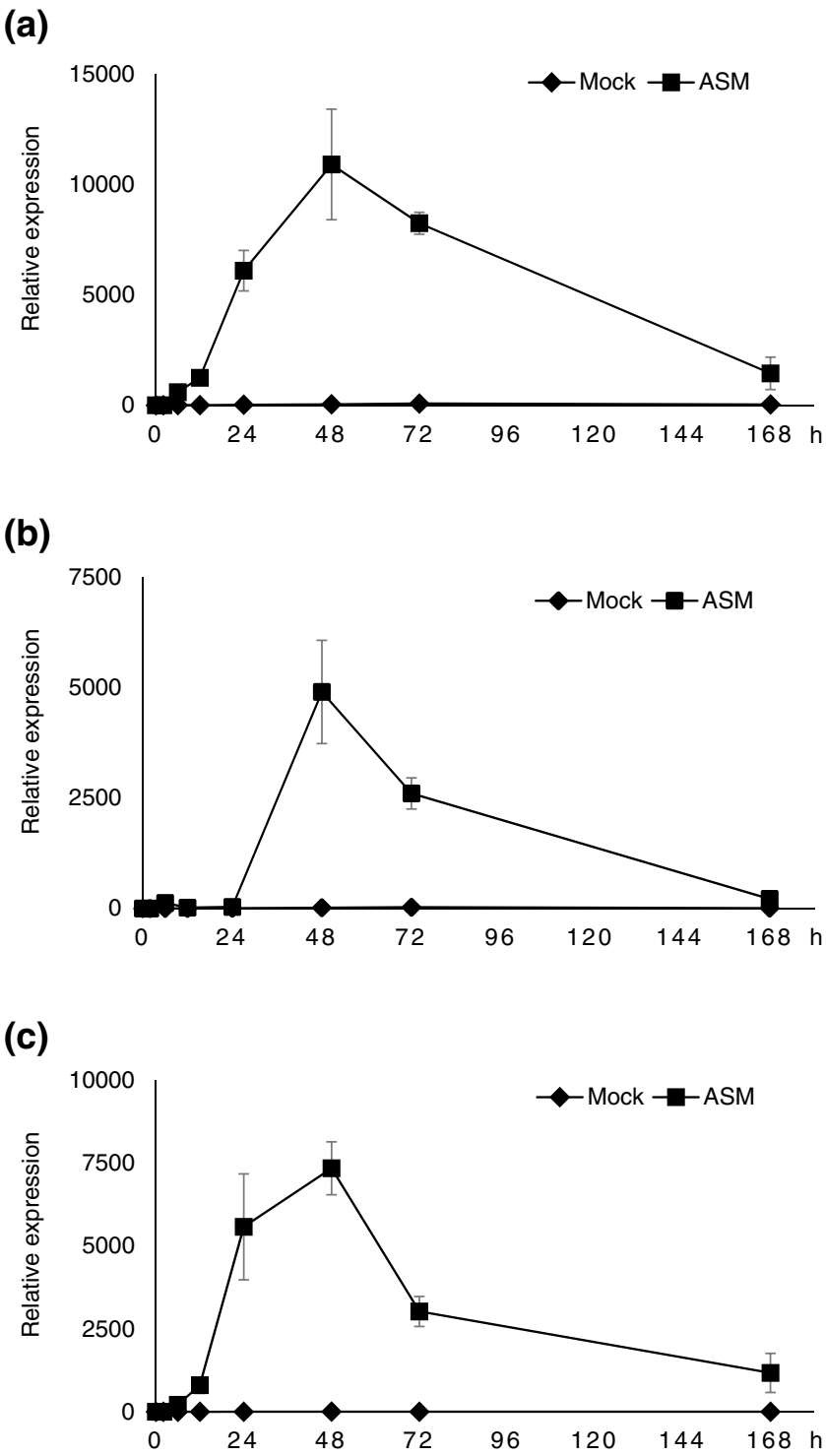

Fig. 1 Gene expression profiles involved in plant defense of 2-week-old cabbage plants in response to ASM. Total RNA was isolated at 2, 6, 12, 24, 48, 72, and 168 h post-treatment (hpt). Expression of **a** *PR1*, **b** *PR2*, and **c** *PR5* was determined using RT-qPCR with gene-specific primer sets. Expression was normalized using *UBQ1*. Vertical bars indicate the standard error for three biological replicates

mobile SAR signals including methyl SA (MeSA), azelaic acid (AzA), dihydroabetinal (DA), and glycerol-3-phosphate (G3P) have been identified (Chanda et al. 2011; Chaturvedi et al. 2012; Jung et al. 2009; Yu et al. 2013). On the other hand, unlike SA, ASM is highly mobile in tobacco plants (Friedrich et al. 1996). Tripathi et al. (2010) reported that ASM is converted to acibenzolar by SA-binding protein 2 (SABP2), and functional SABP2 is required for ASM-mediated SAR in tobacco plants. Homologs for SABP2 have also been identified in *Arabidopsis*, and production of these proteins is induced in response to infection with avirulent *Pseudomonas syringae*, suggesting that cabbage plants also have homologs for SABP2 to catalyze the conversion of ASM to acibenzolar (Vlot et al. 2008). Moreover, ASM might be

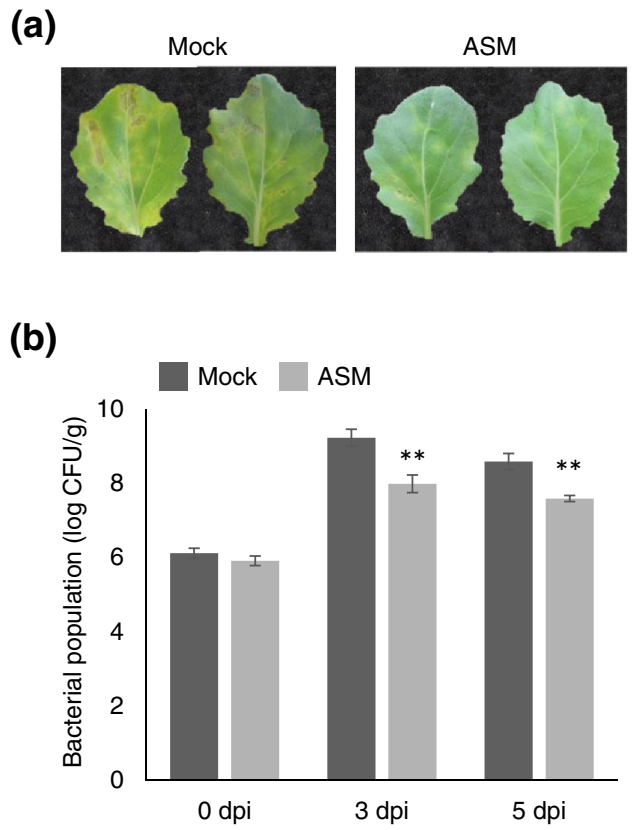

Fig. 2 Disease symptoms and bacterial population dynamics in cabbage plants dip-inoculated with a *Pcal* (5×10^7 CFU/ml) suspension after ASM treatment. Two-week-old cabbage plants were treated with ASM as a soil drench. Two hours after ASM treatment, cabbage plants were dip-inoculated, and bacterial populations were estimated by dilution plating on selective medium as described in the methods. **a** Necrotic lesions surrounded by chlorotic haloes at 5 days post-inoculation (dpi). **b** Bacterial populations in leaves at 0, 3, and 5 dpi. Vertical bars indicate the standard error for three independent experiments. Asterisks indicate a significant difference from the water treatment in a *t* test (** $P<0.01$)

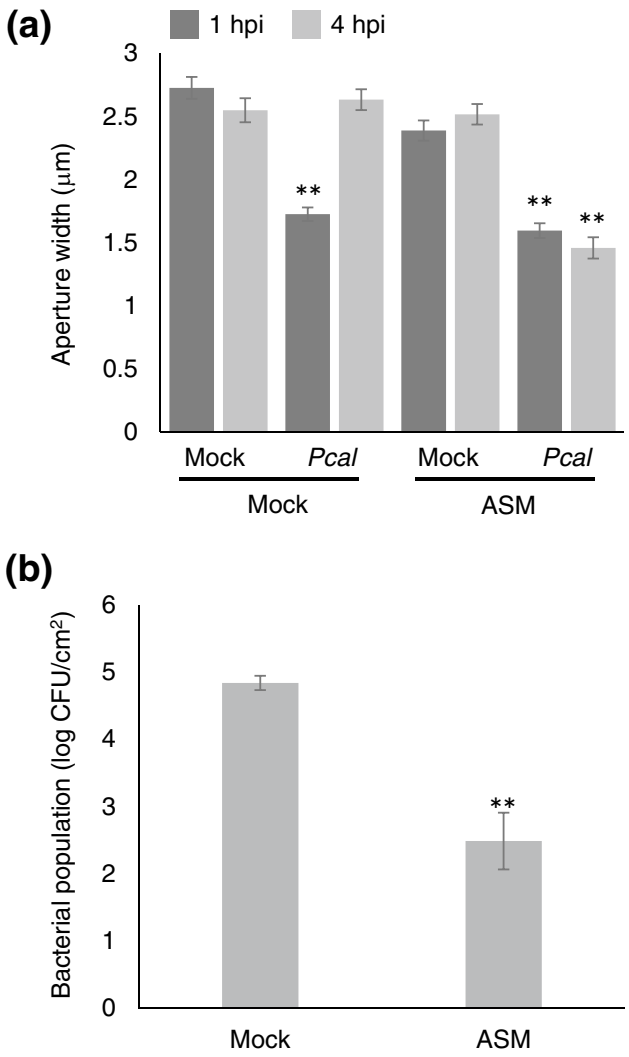

Fig. 3 Stomatal aperture width (μm) and bacterial population dynamics in cabbage plants dip-inoculated with a *Pcal* suspension (1×10^8 CFU/ml) after ASM treatment. **a** Aperture width on intact cabbage leaves 1 h and 4 h after *Pcal* dip-inoculation. Two-week-old cabbage plants were treated with a soil drench of ASM. Two hours after ASM treatment, cabbage leaves were dip-inoculated, then imaged using a Leica TCS SP8 confocal microscope equipped with a white light laser. In all bar graphs, vertical bars indicate the standard error for three biological replicates. Asterisks indicate a significant difference from the water treatment in a *t* test (**$P<0.01$). **b** Bacterial populations in leaves were estimated by dilution plating on selective media at 4 h post inoculation (hpi). Vertical bars indicate the standard error for three independent experiments. Asterisks indicate a significant difference from the water treatment in a *t* test (** $P<0.01$)

absorbed by roots and translocated to aboveground parts via transpiration and act directly to induce SAR. Together, these results suggest that ASM or acibenzolar is a highly mobile signal that triggers SAR in cabbage plants.

We also demonstrated that bacterial entry through stomata was more than 100 times greater in water-treated leaves compared with leaves after the ASM soil drench (Fig. 3b), indicating that stomatal-based defense has a crucial role in PTI against invading bacterial pathogens. It is important to note that when virulent *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000) was used to infiltrate the apoplastic space in ASM-treated *Arabidopsis* leaves, thus bypassing the stomatal-based defense, symptom severity and bacterial populations were both reduced (Lawton et al. 1996), indicating that ASM application contributes not only to stomatal-based defense, but also to other plant immune systems. Therefore, the responses to ASM in various crop plants needs to be further characterized to understand the mechanisms by which plant defense activators contribute to disease control.

Our stomatal response assay demonstrated that stomatal closure was induced in cabbage leaves after the ASM soil drench in response to *Pcal* infection at 4 hpi (Fig. 3a). Interestingly, the stomata did not close after the ASM treatment

unless *Pcal* was present (Fig. 3a). Stomatal-based defense is characterized by stomatal closure after perception of PAMPs of invading pathogens, especially bacterial pathogens (Melotto et al. 2017). We also showed stomatal closure of cabbage leaves in response to *Pcal* as a PTI at 1 hpi (Fig. 3a). Successful bacterial pathogens, however, have acquired virulence factors including phytotoxins and type III secretion system (TTSS) effectors, to overcome stomatal-based defense (Lozano-Durán et al. 2014; Melotto et al. 2017). Genome analysis of multiple *Pcal* strains revealed that *Pcal* also has these virulence factors (Sarris et al. 2013). Consistent with previous studies on *P. syringae*, stomata had reopened by 4 hpi on water-treated, inoculated cabbage leaves, but not on ASM-treated inoculated leaves (Fig. 3a), indicating that *Pcal* can overcome stomatal-based defense. Prodhan et al. (2017) showed the involvement of endogenous SA in PAMP-induced apoplastic ROS production in *Arabidopsis thaliana*, which resulted in stomatal closure. Furthermore, SA-activated SHAM-sensitive peroxidases generate apoplastic ROS, leading to stomatal closure in *A. thaliana* (Prodhan et al. 2018; Toum et al. 2016), suggesting that ASM or ASM-related SAR signals can trigger SHAM-sensitive peroxidases associated with ROS production in cabbage. Further characterization of ASM or ASM-related SAR signals in stomatal-based defense is needed to understand the plant immune system, especially PTI.

PR1, *PR2*, and *PR5* transcripts began to accumulate in cabbage within 2–6 h after the ASM soil drench (Fig. 1), in agreement with the expression of *PR1*, *PR2*, and *PR5* in *A. thaliana* after ASM application and during SAR (Lawton et al. 1996; Uknes et al. 1992). ASM also induced *PR1* and *PR5* expression in maize leaves (Morris et al. 1998). These results suggest that *PR1*, *PR2*, and *PR5* can serve as SAR marker genes in cabbage.

In this study, we first demonstrated that an ASM soil drench protects cabbage plants against *Pcal*, a causal agent of bacterial blight disease, by enhancing their inherent disease resistance mechanisms, such as stomatal-based defense. Because numerous foliar bacterial pathogens target stomata as an entry site, we believe that ASM and/or ASM-related SAR signals will provide an additional disease management tool to prevent crop disease losses against bacterial pathogens.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human participants or animals This article does not contain any studies with human participants or animals performed by any of the authors.

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