Coronatine Contributes to *Pseudomonas cannabina* pv. *alisalensis* Virulence by Overcoming Both Stomatal and Apoplastic Defenses in Dicot and Monocot Plants

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Pseudomonas cannabina pv. *alisalensis* is a causative agent of bacterial blight of crucifers including cabbage, radish, and broccoli. Importantly, *P. cannabina* pv. *alisalensis* can infect not only a wide range of Brassicaceae spp. but, also, green manure crops such as oat. However, *P. cannabina* pv. *alisalensis* virulence mechanisms have not been investigated and are not fully understood. We focused on coronatine (COR) function, which is one of the well-known *P. syringae* pv. *tomato* DC3000 virulence factors, in *P. cannabina* pv. *alisalensis* infection processes on both dicot and monocot plants. Cabbage and oat plants dip-inoculated with a *P. cannabina* pv. *alisalensis* KB211 COR mutant (Δ *cmaA*) exhibited reduced virulence compared with *P. cannabina* pv. *alisalensis* wild type (WT). Moreover, Δ *cmaA* failed to reopen stomata on both cabbage and oat, suggesting that COR facilitates *P. cannabina* pv. *alisalensis* entry through stomata into both plants. Furthermore, cabbage and oat plants syringe-infiltrated with Δ *cmaA* also showed reduced virulence, suggesting that COR is involved in overcoming not only stomatal-based defense but also apoplastic defense. Indeed, defense-related genes, including *PR1* and *PR2*, were highly expressed in plants inoculated with Δ *cmaA* compared with WT, indicating that COR suppresses defense-related genes of both cabbage and oat. Additionally, salicylic acid accumulation increases after Δ *cmaA* inoculation compared with WT. Taken together, COR contributes to causing disease by suppressing stomatal-based defense and apoplastic defense in both dicot and monocot plants. Here, we investigated COR functions in the interaction of *P. cannabina* pv. *alisalensis*

and different host plants (dicot and monocot plants), using genetically and biochemically defined COR deletion mutants.

Keywords: cabbage, coronatine, oat, *Pseudomonas cannabina* pv. *alisalensis*, SA-mediated defense, stomatal-based defense

Pseudomonas syringae is divided into approximately 60 pathovars that cause a variety of symptoms, including blight, cankers, leaf spots, and galls on different plants (Xin et al. 2018). Successful plant infection by *P. syringae* includes multiple processes that involve epiphytic colonization, entry into the host plant through natural opening sites such as stomata and wounds, establishment and multiplication in the apoplast, and disease symptom production (Xin and He 2013; Xin et al. 2018). In the disease cycle, *P. syringae* deploys various virulence factors for successful infection. *P. syringae* pv. *tomato* DC3000 studies have revealed a large virulence factor repertoire, including the type III secretion system (T3SS) and the phytotoxin coronatine (COR) (Bender and Scholz-Schroeder 2004; Buell et al. 2003; Collmer et al. 2002; Xin and He 2013).

COR is a non-host specific phytotoxin produced by multiple *P. syringae* pathovars. COR contributes to *P. syringae* growth and lesion formation or expansion in several host plants, including ryegrass, soybean, tomato, and several crucifers (Bender et al. 1987; Budde and Ullrich 2000; Elizabeth and Bender 2007; Mittal and Davis 1995; Sato et al. 1983). COR is a hybrid molecule consisting of two components, coronafacic acid (CFA) and coronamic acid (CMA) (Bender et al. 1999; Ichihara et al. 1977; Parry et al. 1994). The CFA and CMA moieties are linked through an amide bond. COR functions as a structural and functional analog of jasmonic acid (JA) and related signal compounds, such as methyl jasmonic acid and JA-isoleucine (Feys et al. 1994; Laurie-Berry et al. 2006; Weiler et al. 1994). COR inhibits stomatal closure through guard cell-specific inhibition of NADPH oxidase-dependent reactive oxygen species (ROS) production triggered by bacterial microbe-associated molecular patterns, abscisic acid, or darkness (Egoshi et al. 2016; Melotto et al. 2006; Mino et al. 1987; Panchal et al. 2016; Toun et al. 2016). The plant hormones salicylic acid (SA) and JA play major roles in defense response activation to pathogens (Dong 1998; Glazebrook 2005; Thomma et al. 2001). SA and JA have an antagonistic relationship (Kunkel and Brooks 2002) and, because of the SA-JA crosstalk, COR has multiple roles during infection, including facilitating bacterial entry through stomata, promoting bacterial multiplication and persistence in planta, and disease symptom induction (Brooks et al. 2005; Cui et al. 2005;

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Ishiga et al. 2012; Melotto et al. 2006; Mino et al. 1987; Uppalapati et al. 2007; Zheng et al. 2012).

COR biosynthesis has been well-investigated in *P. syringae* pv. *glycinia* PG4180 and *P. syringae* pv. *tomato* DC3000 (Bender et al. 1993; Brooks et al. 2004; Buell et al. 2003; Sreedharan et al. 2006). In these strains, *corR* encodes a response regulator that positively regulates CFA and CMA biosynthesis (Peñaloza-Vázquez and Bender 1998; Sreedharan et al. 2006). In addition to *corR*, *hrpL* encodes an alternate RNA polymerase sigma factor (σ^L) required for T3SS expression, which also regulates COR biosynthesis (Sreedharan et al. 2006; Xiao et al. 1994). In DC3000, *hrpL* regulates the COR biosynthesis cluster via *corR* (Sreedharan et al. 2006). These results indicate a close regulatory relationship between COR production and T3SS expression (Peñaloza-Vázquez et al. 2000; Xin and He 2013).

P. cannabina pv. *alisalensis* is a causative agent of bacterial blight of crucifer including cabbage, radish, and broccoli (Bull et al. 2010; Cintas et al. 2002; Sarris et al. 2013; Takahashi et al. 2013; Takikawa and Takahashi 2014) and was formerly classified into *Pseudomonas syringae* pv. *maculicola*. However, *P. cannabina* pv. *alisalensis* is distinct in some bacteriological characteristics and genetic traits and its ability to infect monocot plants such as oat (*Avena sativa*) and timothy (*Phleum pratense*) (Bull et al. 2010; Ishiyama et al. 2013; Cintas et al. 2002). *P. cannabina* pv. *alisalensis* also produces COR (Ishiyama et al. 2013). Furthermore, acibenzolar-S-methyl (ASM), a well-known plant defense activator, protects cabbage and Japanese radish from *P. cannabina* pv. *alisalensis* KB211 by activating stomatal-based defense (Ishiga et al. 2020, 2021; Sakata et al. 2020). These results imply that overcoming stomatal-based defense is important for successful *P. cannabina* pv. *alisalensis* infection. However, studies of COR function in *P. cannabina* pv. *alisalensis* KB211 have not been conducted. Moreover, not all mutants that showed reduced virulence on cabbage, exhibited reduced virulence on oat (Sakata et al. 2019). This result indicated that *P. cannabina* pv. *alisalensis* optimizes its virulence factors on different host plants (Sakata et al. 2019). Elizabeth and Bender (2007) examined the role of COR in DC3000 virulence on different hosts including collard and turnip. COR functioned as a virulence factor in both plants, although COR was essential for DC3000 to maintain high populations in turnip but not in collard (Elizabeth and Bender 2007), indicating that COR may function differentially in different host plants. We investigated the role of COR in *P. cannabina* pv. *alisalensis* KB211 infection processes in different host plants, including cabbage and oat. Here, we demonstrated that COR contributes to disease by suppressing stomatal-based and apoplastic defenses on both cabbage and oat plants.

RESULTS

P. cannabina pv. *alisalensis* KB211 colonizes around stomata in both cabbage and oat.

First, we observed disease symptoms and measured bacterial populations in dip-inoculated cabbage and oat plants. Cabbage inoculated with *P. cannabina* pv. *alisalensis* KB211 exhibited water-soaked lesions at early timepoints and showed chlorotic and necrotic lesions at 5 days postinoculation (dpi) (Supplementary Fig. S1A). Bacterial populations in cabbage increased gradually (Supplementary Fig. S1C). Unlike cabbage, oat showed brown leaf spots at 5 dpi (Supplementary Fig. S1B) and bacterial populations suddenly increased between 3 and 4 dpi (Supplementary Fig. S1D). These results indicate that cabbage and oat differ considerably with respect to symptom development and bacterial multiplication during *P. cannabina* pv. *alisalensis* KB211 infection.

We also observed the leaf surface at 3 dpi by confocal microscope and observed multiple stomata on the cabbage leaf surface (Fig. 1A). A *P. cannabina* pv. *alisalensis* KB211 bacterial colony was detected in the substomatal chamber in cabbage (Fig. 1A).

Conversely, oat has fewer and larger stomata compared with cabbage, however, bacterial colonies were also observed around the stomata (Fig. 1B). These results suggest that *P. cannabina* pv. *alisalensis* KB211 enters mainly through stomata. Thus, overcoming stomatal-based defense might be critical in the *P. cannabina* pv. *alisalensis* KB211 infection process on cabbage and oat.

COR biosynthesis-related genes are greatly expressed during *P. cannabina* pv. *alisalensis* KB211 infection.

COR facilitates bacterial entry through stomata (Melotto et al. 2006). Therefore, we hypothesized that COR is important for *P. cannabina* pv. *alisalensis* KB211 infection processes. We investigated the expression profiles of COR biosynthesis-related genes, including *cmaA*, *cfl*, *corR*, and *hrpL*, which were greatly expressed during *P. cannabina* pv. *alisalensis* KB211 infection on both cabbage and oat compared with culture conditions (Fig. 1C, D, E, and F). All genes were greatly expressed at 4 hpi (Fig. 1C, D, E, and F), at which timepoint bacteria are supposed to overcome stomatal-based defense. Moreover, these genes also showed great expression at 48 hpi (Fig. 1C, D, E, and F). Therefore, we hypothesized that COR is involved in overcoming both early stages and later infection processes.

COR contributes to disease on cabbage and oat.

To assess the importance of COR in *P. cannabina* pv. *alisalensis* KB211 virulence, we constructed a COR-defective *cmaA* mutant ($\Delta cmaA$) and confirmed, by high-pressure liquid chromatography (HPLC) assay, it does not produce COR (Supplementary Fig. S2). To investigate whether COR plays an important role in causing disease on cabbage and oat, we dip-inoculated cabbage and oat with *P. cannabina* pv. *alisalensis* KB211 wild-type (WT) and $\Delta cmaA$. Cabbage leaves inoculated with $\Delta cmaA$ exhibited no necrosis and developed less chlorosis (Fig. 2A). We also investigated whether COR contributes to bacterial multiplication in cabbage. $\Delta cmaA$ populations were significantly reduced compared with WT at 3 and 5 dpi (Fig. 2B). $\Delta cmaA$ also exhibited reduced disease symptoms and reduced bacterial multiplication in oat, as compared with WT at 3 and 5 dpi (Fig. 2C and D). Moreover, to further investigate whether WT-induced disease-associated necrotic cell death is associated with accelerated ROS, 3,3'-diaminobenzidine (DAB) staining was carried out to detect H₂O₂. ROS accumulation was detected in dip-inoculated cabbage and oat in response to WT but not to $\Delta cmaA$ (Supplementary Fig. S3A, D, G, and J). ROS accumulation was detected in cabbage mesophyll cell chloroplasts inoculated with WT (Supplementary Fig. S3B and C) but was not detected in those inoculated with $\Delta cmaA$ (Supplementary Fig. S3E and F). Furthermore, ROS accumulation was observed in oat mesophyll cells inoculated with WT (Supplementary Fig. S3H and I) but not with $\Delta cmaA$ (Supplementary Fig. S3K and L). These results indicate that COR facilitates ROS accumulation and causes visible disease symptoms in both cabbage and oat. Taken together, COR contributes to bacterial multiplication and disease symptoms on both cabbage and oat.

COR reopens cabbage and oat stomata.

Plants are able to respond to bacterial pathogens by actively closing the stomatal pore, so-called stomatal-based defense (Arnaud and Hwang 2015; Gudesblat et al. 2009; McDonald and Cahill 1999; Melotto et al. 2006; Roy et al. 2013; Sawinski et al. 2013; Zhang et al. 2010). COR is responsible for suppressing stomatal-based defense and reopens stomata (Melotto et al. 2006; Mino et al. 1987). Therefore, to investigate whether COR facilitates stomatal reopening in cabbage and oat, we observed the stomatal aperture of both plants dip-inoculated with WT and $\Delta cmaA$ at 1 and 4 hpi. Stomatal reopening was observed in both cabbage and oat inoculated with WT, whereas stomatal reopening was not observed in cabbage and oat inoculated with $\Delta cmaA$ (Fig. 3A

Fig. 1. Visualization of *Pseudomonas cannabina* pv. *alisalensis* KB211 and expression profiles of coronatine-related genes. **A**, Bacterial multiplication in cabbage and **B**, oat leaves. Cabbage and oat were dip-inoculated with bacterial suspension (5×10^7 CFU/ml) of tdTomato-labeled KB211 inoculum containing 0.025% SilwetL-77. Inoculated leaves were observed 3 days postinoculation, using a Leica TCS SP8 confocal microscope equipped with a white light laser (Leica, Wetzlar, Germany), revealing bacterial colonization of the substomatal chamber. Scale bar shows 100 μ m. **C**, Expression profiles of *cmaA*, **D**, *cfl*, **E**, *corR*, and **F**, *hrpL* genes in liquid Luria-Bertani broth (culture) or in dip-inoculated cabbage and oat plants (1×10^8 CFU/ml) at 4, 24, and 48 h. Total RNA was extracted for use in real-time quantitative reverse transcription PCR with gene-specific primer sets. Expression was normalized using *oprE* and *recA*. Vertical bars indicate the standard error for three biological replicates. Different letters indicate a significant difference among treatments based on a Tukey's honestly significant difference test ($P < 0.05$).

and B). However, even when we treated plants with stomatal opening buffer, the oat stomatal reopening rate was quite low (Supplementary Fig. S4), which results in the extremely small oat stomatal aperture width. Although the stomatal response to pathogens might be different, these results suggest that COR has an important role in overcoming stomatal-based defense in early *P. cannabina* pv. *alisalensis* KB211 virulence in cabbage and oat.

COR contributes to apoplastic multiplication and disease on cabbage and oat.

To determine whether COR is only important for overcoming stomatal-based defense, the effect on disease development was investigated using syringe-infiltration to bypass stomatal-based defense. Cabbage leaves inoculated with both WT and $\Delta cmaA$ showed necrosis at 5×10^5 CFU/ml (Supplementary Fig. S5A). However, at 5×10^3 CFU/ml, cabbage inoculated with $\Delta cmaA$

Fig. 2. Disease phenotypes and population dynamics of *Pseudomonas cannabina* pv. *alisalensis* KB211 wild type (WT) and $\Delta cmaA$ in cabbage and oat after dip-inoculation. **A**, Disease symptoms and **B**, bacterial populations in cabbage dip-inoculated with WT and $\Delta cmaA$. **C**, Disease symptoms and **D**, bacterial populations in oat dip-inoculated with WT and $\Delta cmaA$. Cabbage and oat were dip-inoculated with 5×10^7 CFU/ml of WT and $\Delta cmaA$ containing 0.025% SilwetL-77. The leaves were photographed at 5 days postinoculation (dpi). Vertical bars indicate the standard error for three independent experiments. Different letters indicate a significant difference among treatments based on a Tukey's honestly significant difference test ($P < 0.05$). ns = no significant difference between samples. Scale bar shows 1 cm.

showed no symptoms, although cabbage inoculated with WT showed chlorosis (Supplementary Fig. S5A). Conversely, oat leaves inoculated with WT showed orange to brown spots at 5×10^5 and at 5×10^4 CFU/ml (Supplementary Fig. S5B). Oat inoculated with $\Delta cmaA$ showed no symptoms at all bacterial inoculum levels (Supplementary Fig. S5B). Therefore, we next examined bacterial multiplication in cabbage and oat inoculated with 5×10^3 and 5×10^5 CFU/ml, respectively. In cabbage, the $\Delta cmaA$ bacterial populations were significantly reduced at 1 and 2 dpi (Fig. 4B). Interestingly, although $\Delta cmaA$ showed no symptoms, the bacteria multiplied almost to the same level as WT at 3 dpi (Fig. 4A and B). In contrast, $\Delta cmaA$ showed no symptoms and did not grow until 5 dpi in oat (Fig. 4C and D). These results indicate COR plays an important role in overcoming apoplastic defenses and may function differentially in cabbage and oat.

We further tested whether exogenous COR application restores disease susceptibility to cabbage and oat. Coinoculation of $\Delta cmaA$ with 500 μ M COR increased bacterial growth to a similar level as WT in cabbage at 2 dpi (Fig. 4B). In oat, this reduced multiplication could be partly recovered by COR application from 4 dpi (Fig. 4D). Therefore, these results also suggest that COR functions differentially in different host plants.

COR regulates defense-related gene expression in cabbage and oat.

To evaluate the effect of COR on defense-related gene expression in cabbage and oat, we first investigated pathogenesis-related (*PR*) gene expression profiles, including *PR1* and *PR2*, in response to the SA analog plant activator ASM. The expression of *BoPR1* and *BoPR2* were induced by ASM treatment in cabbage (Supplementary Fig. S6A and B), and *AsPR1a*, *AsPR1b*, and *AsPR2* were induced in oat (Supplementary Fig. S6C, D, and E). Therefore, we investigated the expression profiles of these in response to WT and $\Delta cmaA$. Cabbage *BoPR1* genes showed no significant difference but tended to increase at 48 h when we inoculated with $\Delta cmaA$ compared with WT inoculation (Fig. 5A), whereas *BoPR2* genes showed significantly greater expression levels at 24 and 48 h in response to $\Delta cmaA$ compared with the response to WT (Fig. 5B). In oat, all *AsPR1a*, *AsPR1b*, and *AsPR2* genes showed significantly greater expression levels at 24 and 48 h in response to $\Delta cmaA$ compared with WT (Fig. 5C, D, and E). These results indicate that

COR suppresses defense-related gene expression in both cabbage and oat.

COR is involved in suppression of SA biosynthesis.

We demonstrated that SA-induced gene markers, including *PR1* and *PR2*, are greatly expressed in plants after inoculation with $\Delta cmaA$ compared with WT (Fig. 5A, B, C, D, and E). Thus, we next examined SA accumulation levels in cabbage and oat when inoculated with WT and $\Delta cmaA$, using liquid chromatography-tandem mass spectrometry (LC-MS/MS). In cabbage, SA accumulation with mock treatment was less than 20 ng per gram and increased around 20-fold at 24 h after WT inoculation (Fig. 6A). $\Delta cmaA$ inoculation induced greater SA levels than WT inoculation at 24 hpi (Fig. 6A), indicating that COR delays SA accumulation in cabbage. Conversely, SA accumulation triggered by bacterial pathogens caused subtle changes and showed no statistical difference between WT and $\Delta cmaA$ inoculations in oat at 24 hpi (Fig. 6B). However, at 48 h, SA accumulation was also suppressed by COR in oat (Fig. 6B). Therefore, although COR contributes to SA suppression differently, COR can suppress SA accumulation and allow *P. cannabina* pv. *alisalnesis* KB211 to multiply to great levels in both cabbage and oat.

DISCUSSION

Plant pathogens deploy a variety of virulence factors. *P. cannabina* pv. *alisalnesis* KB211 multiplied mainly around stomata and COR biosynthesis-related genes were greatly expressed during early and later infection processes (Fig. 1). Therefore, we hypothesized that COR contributes to KB211 virulence for overcoming both stomatal-based and apoplastic defenses. COR is involved in suppressing stomatal closure (Fig. 3) and SA-mediated defenses (Figs. 5 and 6) in both cabbage and oat. These results shed light on spatiotemporal COR function in different hosts, monocot and dicot.

The $\Delta cmaA$ multiplication defect was observed at 3 dpi, and this defect became greater at 5 dpi in both cabbage and oat (Fig. 2). $\Delta cmaA$ was defective in reopening stomata in both cabbage and oat (Fig. 3), indicating that COR suppresses stomatal-based defense and facilitates *P. cannabina* pv. *alisalnesis* entry through stomata into both cabbage and oat. Melotto et al. (2006) demonstrated that *P. syringae* pv. *tomato* DC3118 *cor* mutant populations were greatly reduced compared with those of *P. syringae* pv. *tomato* DC3000 when dip-inoculated on

Fig. 3. Stomatal aperture width on cabbage and oat in response to *Pseudomonas cannabina* pv. *alisalnesis* KB211 wild type (WT) and $\Delta cmaA$. **A**, Stomatal aperture width on intact cabbage and **B**, oat leaves 1 and 4 h after dip-inoculation with 1×10^8 CFU/ml of WT and $\Delta cmaA$. OB = stomatal opening buffer (Lee et al. 2013). Different letters indicate a significant difference among treatments based on a Tukey's honestly significant difference test ($P < 0.05$).

Arabidopsis thaliana. This multiplication defect was observed at early infection stages (3 dpi), suggesting that the COR-mediated suppression of stomatal defense is critical for DC3000 host plant infection (Melotto et al. 2006). COR is required for DC3000 growth and persistence within tomato and *A. thaliana* (Brooks et al. 2004; Uppalapati et al. 2007). These results highly support our results that COR is important for disease symptom development and growth in both cabbage and oat. Elizabeth and Bender (2007) investigated the role of COR in the interaction of DC3000 and different brassicas, collard and turnip. They demonstrated that COR contribution to bacterial multiplication in collard was more subtle than in turnip (Elizabeth and Bender 2007). However, the symptoms on collard appeared earlier than on turnip and bacterial growth in collard reached greater levels earlier than in turnip. They speculated that COR might prolong stomatal aperture opening in turnip but not in collard. The stomatal opening rate is quite

decreased in oat in our experimental conditions (Supplementary Fig. S4). Roles of stomata in plant disease resistance are not fully understood, especially in monocot plants. Zhang et al. (2019) demonstrated that open stomata in rice lead to leaf blight bacterial resistance against *Xanthomonas oryzae* pv. *oryzae* and this resistance was conferred through modulation of host water status. It is possible that the stomatal-based defense response differs between cabbage and oat. Further investigations on stomatal response will be needed to understand stomatal-based defense, especially in monocot plants.

$\Delta cmaA$ populations were significantly less than those of WT in both syringe-inoculated cabbage and oat (Fig. 4). These results indicate COR functions not only in suppressing stomatal-based defense but also in overcoming apoplastic defense. This multiplication defect was also observed in *A. thaliana* plants vacuum-infiltrated with *P. syringae* pv. *tomato* strains AK7E2 (*cmaA* mutant) and DB4G3 (*cfa6* mutant) at 4 dpi but not at

Fig. 4. Disease phenotypes and population dynamics of *Pseudomonas cannabina* pv. *alisalensis* KB211 wild type (WT) and $\Delta cmaA$ in cabbage and oat after syringe-inoculation. **A**, Disease symptoms and **B**, bacterial populations at 1, 2, and 3 days postinoculation (dpi) in cabbage syringe-inoculated with WT and $\Delta cmaA$. Cabbage were syringe-inoculated with 5×10^3 CFU/ml of WT and $\Delta cmaA$. **C**, Disease symptoms and **D**, bacterial populations at 1, 2, and 3 dpi in oat syringe-inoculated with WT and $\Delta cmaA$. Oat were syringe-inoculated with 5×10^5 CFU/ml of WT and $\Delta cmaA$. Additionally, coronatine (COR) (500 μ M) was coinoculated with $\Delta cmaA$. The leaves were photographed at 5 dpi. Vertical bars indicate the standard error for three independent experiments. Different letters indicate a significant difference among treatments based on a Tukey's honestly significant difference test ($P < 0.05$). Scale bar shows 2 cm.

2 dpi (Brooks et al. 2004), indicating that COR may be important for apoplastic bacterial persistence. However, Melotto et al. (2006) observed no multiplication defect at 3 dpi when the *P. syringae* pv. *tomato* *cor* mutant DC3118 was infiltrated directly into the apoplast, as compared with infiltration of DC3000. Additionally, Mittal and Davis (1995) showed multiplication impairment of *P. syringae* pv. *tomato* DC3661, a Tn5 *Cor*⁻ derivative mutant, when dip-inoculated, but multiplication impairment was not observed in *A. thaliana* and tomato inoculated by infiltration. In these studies, the inoculum concentration was 1×10^6 CFU/ml. These results motivated us to investigate bacterial multiplication at different timepoints and different inoculum concentrations. $\Delta cmaA$ -inoculated cabbage

leaves exhibited similar disease symptoms to WT-inoculated leaves at 5×10^5 CFU/ml (Supplementary Fig. S5A). Conversely, oat infiltrated with $\Delta cmaA$ did not show any symptoms even at the greatest concentration we tested, 5×10^5 CFU/ml (Supplementary Fig. S5B). In field conditions, the concept of an “infection threshold” has been used to predict disease incidence and severity (Lindemann et al. 1984). Thus, the infection threshold might be different between cabbage and oat. Therefore, our results support the importance of selecting the proper inoculum levels depending on the host plant and bacterial pathogen.

We demonstrated that defense-related genes, including *PR1* and *PR2*, were greatly expressed when cabbage and oat were inoculated with $\Delta cmaA$ compared with WT (Fig. 5), indicating

Fig. 5. Gene expression profiles involved in cabbage and oat plant defense after syringe-inoculation with water (mock), *Pseudomonas cannabina* pv. *alisalnesis* KB211 wild type (WT), and $\Delta cmaA$. **A**, Expression profiles of *BoPR1*, **B**, *BoPR2*, **C**, *AsPR1a*, **D**, *AsPR1b*, and **E**, *AsPR2* were determined 24 and 48 h after inoculation with 5×10^5 CFU/ml of WT and $\Delta cmaA$ or mock water-inoculated control, using real-time quantitative reverse transcription-PCR with gene-specific primer sets. Expression was normalized using *BoUBQ1* and *PsAct* in cabbage and oat, respectively. Vertical bars indicate the standard error for three biological replicates. Different letters indicate a significant difference among treatments based on a Tukey's honestly significant difference test ($P < 0.05$).

Fig. 6. Total salicylic acid production in **A**, cabbage and **B**, oat after syringe-inoculation with *Pseudomonas cannabina* pv. *alisalnesis* KB211 wild type (WT) and $\Delta cmaA$ (5×10^5 CFU/ml) or with water as a control. Vertical bars indicate the standard error for three biological replicates. Different letters indicate a significant difference among treatments based on a Tukey's honestly significant difference test ($P < 0.05$).

that COR suppresses defense-related gene expression. Also, we showed that SA accumulation was greater in cabbage and oat inoculated with $\Delta cmaA$ compared with WT at 24 h (Fig. 6). SA accumulation in oat showed subtle changes against the bacterial pathogen, and no statistical change between the WT and $\Delta cmaA$ inoculations (Fig. 6B). However, concerning bacterial multiplication, the $\Delta cmaA$ population was 10-fold less than those of WT. Therefore, taken together, our results showed that COR suppressed SA-mediated defense in both cabbage and oat. Several studies have demonstrated that COR functions to suppress SA-mediated defenses via COI1 activation, thus allowing bacteria to grow to greater densities in planta in dicot plants, such as *A. thaliana* and tomato (Kloek et al. 2001; Uppalapati et al. 2005, 2007; Zheng et al. 2012). However, COR function in SA-mediated defense in monocot plants is less studied. Moreover, in monocot plants, the role of SA biosynthesis in disease resistance is still poorly understood (De Vleeschauwer et al. 2014). Consistent with our results on oat, rice plants accumulated increased basal SA levels (8 to 37 mg per gram of FW) that did not change significantly upon pathogen attack (Silverman et al. 1995). In contrast, in tobacco and *A. thaliana*, basal SA levels were lower (less than 100 ng per gram of FW) but increased by two orders of magnitude following pathogen infection (Malamy and Klessig 1992). However, several reports indicated that SA-JA antagonism is conserved in monocot plants such as rice and wheat. In wounded rice, JA levels elevated whereas SA levels decreased, indicating negative crosstalk between the JA and SA pathways during the early wounding response in rice (Lee et al. 2001). Furthermore, Ding et al. (2016) also demonstrated that, as in dicot plants, SA and JA acted antagonistically in pathogen-inoculated wheat plants. Also, Iwai et al. (2007) demonstrated that SA accumulation in rice was induced after probenazole treatment (one of the synthetic SA analogs) in adult leaves but not in young leaves. Therefore, SA-mediated defense might be quite different depending on leaf age and plant species. To understand the mechanism by which COR suppresses SA accumulation, further investigation of SA-JA interactions in monocots will be necessary.

Suppression of SA-mediated defense by COR results in reduced apoplastic defense in cabbage and oat (Figs. 4, 5, and 6). However, COR may contribute to suppression not only of SA-mediated defense but also other apoplastic defenses, such as synthesis of plant secondary metabolites. Geng et al. (2012) showed that COR inhibited the SA-independent pathway, contributing to callose deposition, by reducing accumulation of an indole glucosinolate. Consistent with this study, when *A. thaliana* was challenged by DC3000, COR affected gene expression involved in the synthesis of Trp-derived glucosinolates (Thilmony et al. 2006). Brassica species, including cabbage, produce elevated levels of secondary metabolites all proposed to originate from indole glucosinolate (Klein and Sattely 2017). Therefore, there is a possibility that COR regulates secondary metabolites in cabbage. These plant-derived secondary metabolites are known to have an important role in plant defense through their antimicrobial activities. Indeed, brassinin, one of the indole-sulfur phytoalexins produced in cabbage, can cause 50% growth inhibition of *Lectosphaeria maculans* (Pedras et al. 2017). Moreover, Wang et al. (2020) reported that an *A. thaliana* secondary metabolite, sulforaphane, directly targets T3SS expression to inhibit bacterial virulence. These studies suggest that plant secondary metabolites could have a major role in apoplastic defense. In oat, a group of phenolic antioxidants termed avenanthramides and saponins have been well-characterized as phytoalexins (Collins 1989; Faizal and Geelen 2013; Matsukawa et al. 2002; Osbourn et al. 2011; Wise 2011). Avenanthramide levels in vegetation were enhanced by benzothiadiazole treatment, which is a SA analog, as is ASM (Wise 2011). This study

implied the possibility that COR might suppress avenanthramide biosynthesis, because of the SA-JA antagonistic relationship. Therefore, it is possible that COR contributes to overcoming the apoplastic defense, not only by suppressing SA-mediated defense, but also by modulating plant secondary metabolites. Further exploration will be necessary to better understand the potential function of COR in apoplastic defense.

In summary, COR contributes to *P. cannabina* pv. *alisalnesis* KB211 virulence on dicot and monocot plants in both early and later stages of the infection process by suppressing stomatal-based and apoplastic defenses. Moreover, we suggest that COR might contribute differentially to KB211 virulence between monocot and dicot plants. This differential might be strongly involved in apoplastic defenses in each host plant. We need to investigate the possibilities that lead to these differential results to understand the potential function of COR and host optimization.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

The bacterial strains and plasmids used in this study are described in Supplementary Table S1. *Pseudomonas cannabina* pv. *alisalnesis* KB211 was used as the pathogenic strain to inoculate cabbage and oat. WT and the $\Delta cmaA$ mutant were grown on King's B (King et al. 1954) medium at 28°C, and *Escherichia coli* strains were grown on Luria-Bertani (LB) (Sambrook et al. 1989) medium at 37°C. Antibiotics used for selection of *P. cannabina* pv. *alisalnesis* and *E. coli* strains included (in microliters per milliliter): rifampicin, 50; kanamycin, 25; ampicillin, 100; chloramphenicol, 10. Before *P. cannabina* pv. *alisalnesis* plant inoculation, bacteria were suspended in sterile distilled H₂O, and the bacterial cell densities at 600 nm (OD₆₀₀) were measured using a Biowave CO8000 cell density meter (Funakoshi, Tokyo).

Generation of tdTomato-labeled KB211 and microscopic observation.

A pDSK-*tdTomato* plasmid was generated by replacing the *GFPuv* gene with *tdTomato* (Supplementary Table S1) in pDSK-*GFPuv* (Wang et al. 2007) for *tdTomato* fluorescent protein expression. The pDSK-*tdTomato* vector was introduced into KB211 by electroporation. Dip-inoculated cabbage and oat leaves were directly imaged 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 480 nm wavelength, and reflected light was detected through 475 to 486 nm laser lines. *tdTomato* red fluorescence was detected by 554 to 611 nm laser lines.

Plant materials.

Cabbage (*Brassica oleracea* var. *capitata*) cultivar Kinkei 201 and oat (*Avena strigosa*) cultivar Hayoat plants were used for *P. cannabina* pv. *alisalnesis* virulence assays. Cabbage and oat were grown from seed at 23 to 25°C with a light intensity of 200 $\mu\text{Em}^{-2}\text{s}^{-1}$ and a 16-h-light and 8-h-dark photoperiod. Cabbage and oat plants were used for dip-inoculation assays around two weeks after germination and, for syringe-inoculation assays, around three weeks after germination.

Generation of $\Delta cmaA$ mutant.

The mutants were generated as described previously (Ishiga et al. 2018). The genetic regions containing *cmaA* and surrounding regions were amplified using PCR primer sets (Supplementary Table S2) that were designed based on the *P. cannabina* pv. *alisalnesis* ES4326 registered sequence with Prime Star HS DNA polymerase (TaKaRa, Otsu, Japan), then dA was added to the 3' end of the PCR product with 10 $\times$ A-attachment mix

(Toyobo, Osaka, Japan). The resultant DNA was inserted into the pGEM-T Easy vector (Promega, Madison, WI, U.S.A.). The recombinant plasmid DNA was then used to obtain pGEM-*cmaA* as templates, and inverse PCR was carried out, using a primer set (Supplementary Table S2), to delete an open reading frame for *cmaA*. Then, the PCR product and template DNA were digested with *Bam*HI and *Dpn*I. The resultant DNA was self-ligated with T4 DNA ligase (Ligation-Convenience kit, Nippon Gene, Tokyo). The *cmaA*-deleted DNA constructs were introduced into the *Eco*RI site of the mobilizable cloning vector pK18*mobsacB* (Schäfer et al. 1994). The resulting plasmids containing the DNA fragment lacking *cmaA* were then transformed into *E. coli* S17-1. The deletion mutant was obtained by conjugation and homologous recombination according to the method previously reported (Shimizu et al. 2003). Transconjugants were selected on KB agar containing 30 µg of kanamycin per milliliter and 30 µg of rifampicin per milliliter. We generated mutants by incubating the transconjugants on a KB agar plate containing 25 µg of rifampicin per milliliter and 10% sucrose. The specific deletions in the Δ *cmaA* mutants were confirmed by PCR using primers (Supplementary Table S2).

Bacterial inoculation methods.

To assay for disease on cabbage and oat seedlings, dip-inoculation was conducted by soaking seedlings in bacterial suspensions (5×10^7 CFU/ml) containing 0.025% Silwet L-77 (OSI Specialties, Danbury, CT, U.S.A.). The seedlings were then incubated in growth chambers at 85 to 95% relative humidity (RH) for the first 24 h, then at 80 to 85% RH for the rest of the experimental period. Disease symptom pictures on cabbage and oat seedlings were taken at 5 dpi. For syringe-inoculation, bacteria were suspended at a final concentration ranging from 5×10^2 to 5×10^5 CFU/ml and were infiltrated with a 1-ml blunt syringe into leaves. The plants were then incubated at 70 to 80% RH for the rest of the experimental period. Leaves were removed and photographed at 5 dpi.

To assess bacterial growth in cabbage and oat seedlings, the internal bacterial population was measured after dip-inoculation. Inoculated seedlings were collected, and a total of two inoculated leaves were measured chronologically on both plants. The leaves were surface-sterilized with 10% H_2O_2 for 3 min. After washing three times with sterile distilled water, the leaves were homogenized in sterile distilled water, and diluted samples were plated onto solid KB agar medium. For syringe-inoculation, to assess bacterial growth in cabbage seedlings, the internal bacterial population was measured after syringe-inoculation. Leaf discs were harvested using a 3.5 mm diameter cork-borer from syringe-infiltrated leaf zones. To assess bacterial growth in oat, leaf pieces were cut from syringe-infiltrated leaf zones and the area (in square centimeters) was measured. Leaf extracts were homogenized in sterile distilled water, and diluted samples were plated onto solid KB agar medium. Two or three days after dilution sample plating, the bacterial colony forming units (CFUs) were counted and normalized as CFU per gram or CFU per square centimeter, using the total leaf weight or leaf area. The bacterial populations at 0 dpi were estimated using leaves harvested 1 hpi without surface-sterilization. The bacterial populations were evaluated in at least three independent experiments.

Real-time quantitative reverse transcription (RT-qPCR).

For expression profiles of cabbage and oat defense genes in response to ASM, cabbage and oat were treated by drenching the soil with ASM (100 ppm) as described previously (Ishiga et al. 2021). After 24 h, total RNA was extracted from leaves and was purified using RNAiso Plus (TaKaRa) according to manufacturer protocols. For expression profiles of KB211 genes in culture, bacteria were grown in LB broth for 24 h, adjusted to an OD_{600} of

0.1, and were grown again in LB broth for 3 h. Then, bacteria were transferred to fresh mannitol-glutamate broth (Keane et al. 1970) and were grown for 15 min. Bacterial RNA was extracted, using ReliaPrep RNA Cell Miniprep system kit (Promega) according to the manufacturer protocols. To analyze KB211 gene expression profiles during infection, we dip-inoculated cabbage and oat with KB211 at 1×10^8 CFU/ml for 4, 24, and 48 h, then, the total RNAs, including plant and bacterial RNAs, were extracted from infected leaves and were purified, using RNAiso Plus (TaKaRa) according to the manufacturer protocols. The real-time RT-qPCR were done as described previously (Ishiga and Ichinose 2016). Two micrograms of total RNA were treated with gDNA Remover (Toyobo) to eliminate genomic DNA, and the DNase-treated RNA was reverse transcribed using the ReverTra Ace qPCR RT master mix (Toyobo). The cDNA (1:10) was then used for RT-qPCR, using the primers shown in Supplementary Table S1 with THUNDERBIRD SYBR qPCR mix (Toyobo) on a Thermal Cycler Dice Real Time System (TaKaRa). KB211 *outer membrane porin F* (*oprF*) and *recombinase A* (*recA*) were used to normalize gene expression. Cabbage *UBIQUITIN EXTENSION PROTEIN 1* (*BoUBQ1*) and oat *Actin* (*PsAct*) were used as internal controls to normalize gene expression in cabbage and oat, respectively.

Hydrogen peroxidase detection.

Hydrogen peroxidase generation was detected by DAB staining as described previously (Ishiga et al. 2009, 2012). After inoculation with WT and Δ *cmaA*, the leaves were placed in DAB-HCl (pH 3.8) at 1 mg/ml. After incubation for 6 h at room temperature, chlorophyll was removed with 95% ethanol.

Stomatal assay.

A modified method was used to assess stomatal response as described previously (Chitrakar and Melotto 2010). Briefly, cabbage and oat were grown for around 2 weeks after germination, as described previously. WT and Δ *cmaA* were grown at 28°C for 24 h on KB agar and were then suspended in distilled water to an OD_{600} of 0.2 (1×10^8 CFU/ml). Cabbage and oat leaves were floated on stomatal opening buffer (10 mM MES-KOH, 20 mM KCl, pH6.3) (Lee et al. 2013). Four hours after treatment, cabbage and oat leaves were dip-inoculated with WT and Δ *cmaA*. Dip-inoculated cabbage and oat leaves were directly imaged at 1 and 4 hpi, using a Nikon optical microscope (Eclipse 80i). The aperture width of at least 300 and 600 stomata was measured on cabbage and oat, respectively. The average and standard error for the stomatal aperture width were calculated. The stomatal apertures were evaluated in at least three independent experiments.

SA quantification by reverse phase LC-electrospray ionization (ESI)-MS/MS.

WT, Δ *cmaA* bacterial suspensions (5×10^5 CFU/ml), or water (mock) were infiltrated into the third leaves of 3-week-old cabbage and oat. Eight leaf discs (3.5 mm diameter) from two cabbage leaves and about 3.5 cm² from two oat leaves were collected 0 (untreated), 24, and 48 hpi, weight was measured, and samples were frozen in liquid nitrogen and were stored at -80°C. Samples were extracted with 300 µl of 80% methanol.

SA was measured, using the multiple-reaction monitoring (MRM) mode on the LC-ESI-MS/MS (LCMS-8045; Shimadzu, Kyoto, Japan), under the following conditions: capillary voltage, 4.5 kV; desolvation line, 250°C; heat block, 400°C; nebulizer nitrogen gas 3 liters min⁻¹; drying gas, 10 liters min⁻¹. Ion source polarity was set in the negative ion mode. The separation was performed with the LC system equipped with a 150 × 2.1 mm ACQUITY UPLC BEH Shield RP18 column (Waters Corp., Milford, MA, U.S.A.) with a particle and pore size of 1.7 µm and

130 Å, respectively. The initial mobile phase was solvent A/solvent B = 95:5 (solvent A, 0.5 mM ammonium formate [pH 3.5]; solvent B, 0.5 mM ammonium formate [pH 3.5] in 90% acetonitrile). The solvent B concentration was increased to 100% for 8 min and was then maintained at that ratio for another 2 min. The column was re-equilibrated for 3 min. The flow rate of 0.4 ml min⁻¹ and column temperature at 40°C were maintained throughout the analysis. The MRM-transition *m/z* 137 to 93 was used as precursor and product ions, respectively. The dwell time, Q1 pre bias, collision energy, and Q3 pre bias were set at 100 ms, 15 V, 17 eV, 15 V, respectively.

COR quantification by HPLC.

WT and *ΔcmaA* bacteria were cultured in HS medium optimized for coronatine production (Palmer and Bender 1993) for 5 days. Culture supernatant was obtained by centrifugation (12,000 × *g* for 5 min). The culture supernatant was analyzed by HPLC with a Shimadzu LC20A system equipped with a Symmetry C8 column (4.6 × 250 mm; Waters Corp.) under the following conditions: column temperature, 40°C; isocratic elution; mobile-phase composition, 0.05% trifluoroacetic acid/acetonitrile (4:6, vol/vol); flow rate, 1 ml min⁻¹; and detection, 208 nm. COR in the culture supernatant was identified, as compared with authentic COR (Sigma) as the standard.

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