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3 **Coronatine contributes *Pseudomonas cannabina* pv. *alisalensis* virulence by**
 4 **overcoming both stomatal and apoplastic defenses in dicot and monocot plants**

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23 Abstract

24 *P. cannabina* pv. *alisalensis* (*Pcal*) is a causative agent of bacterial blight of
 25 crucifer including cabbage, radish, and broccoli. Importantly, *Pcal* can infect not only a
 26 wide range of Brassicaceae, but also green manure crops such as oat. However, *Pcal*
 27 virulence mechanisms have not been investigated and are not fully understood. We
 28 focused on coronatine (COR) function, which is one of the well-known *P. syringae* pv.
 29 *tomato* DC3000 virulence factors, in *Pcal* infection processes on both dicot and
 30 monocot plants. Cabbage and oat plants dip-inoculated with a *Pcal* KB211 COR mutant
 31 (Δ *cmaA*) exhibited reduced virulence compared to *Pcal* WT. Moreover, Δ *cmaA* failed
 32 to reopen stomata on both cabbage and oat, suggesting that COR facilitates *Pcal* entry
 33 through stomata into both plants. Furthermore, cabbage and oat plants
 34 syringe-infiltrated with Δ *cmaA* also showed reduced virulence, suggesting that COR is
 35 involved in overcoming not only stomatal-based defense, but also apoplastic defense.
 36 Indeed, defense related genes, including *PR1* and *PR2*, were highly expressed in plants
 37 inoculated with Δ *cmaA* compared to *Pcal* WT, indicating that COR suppresses
 38 defense-related genes of both cabbage and oat. Additionally, SA accumulation increases
 39 after Δ *cmaA* inoculation compared to *Pcal* WT. Taken together, COR contributes to
 40 cause disease by suppressing stomatal-based defense and apoplastic defense in both
 41 dicot and monocot plants. This is the first study to investigate COR functions in the
 42 interaction of *Pcal* and different host plants (dicot and monocot plants) using
 43 genetically and biochemically defined COR deletion mutants.

45 Author summary

46 Disease outbreaks caused by new *Pseudomonas syringae* isolates are problems
 47 worldwide. *P. cannabina* pv. *alisalensis* (*Pcal*) causes bacterial blight on a wide range
 48 of cruciferous plants and bacterial brown spot on oat plants. Although *P. syringae*
 49 deploys a variety of virulence factors, *Pcal* virulence factors have not been investigated.
 50 We focused on coronatine (COR) function, which is one of the well-known *P. syringae*
 51 virulence factors. COR is a non-host-specific phytotoxin and contributes to *P. syringae*
 52 growth and lesion formation or expansion in several host plants. COR function has been

mainly studied in the model pathogen *P. syringae* pv. *tomato* DC3000 and model the plant *Arabidopsis thaliana*. Thus, COR roles in *Pcal* infection especially on monocot plants have not been well studied. Therefore, we investigated COR role in *Pcal* interaction with both dicot and monocot plants. Here, we revealed that COR functions as a multifunctional suppressor to manage *Pcal* virulence on both plants.

Introduction

Pseudomonas syringae is divided into approximately 60 pathovars which cause a variety of symptoms including blight, cankers, leaf spots, and galls on different plants [1]. Successful plant infection by *P. syringae* includes multiple processes which 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 [1, 2]. In the disease cycle, *P. syringae* deploys various virulence factors for successful infection. *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000) studies have revealed a large virulence factor repertoire, including the type III secretion system (TTSS) and the phytotoxin coronatine (COR) [2-5].

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 [3, 6-10]. COR is a hybrid molecule consisting of two components: coronafacic acid (CFA) and coronamic acid (CMA) [10-12]. The CFA and CMA moieties are linked through an amide bond. COR functions as a structural and functional analogue of jasmonic acid (JA) and related signal compounds, such as methyl jasmonic acid (MeJA) and JA-isoleucine [13-15]. 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 (MAMPs), abscisic acid (ABA), or darkness [16-20]. The plant hormones salicylic acid (SA) and JA play major roles in defense response activation to pathogens [21-23]. SA and JA have an antagonistic relationship [22, 24], and because of the SA-JA crosstalk, COR has multiple roles during infection, including facilitating bacterial entry through stomata,

83 promoting bacterial multiplication and persistence *in planta*, and disease symptom
84 induction [16, 17, 25-29].

85 COR biosynthesis has been well investigated in *P. syringae* pv. *glycinea* PG4180
86 and *Pst* DC3000 [4, 30-32]. In these strains, *corR* encodes a response regulator which
87 positively regulates CFA and CMA biosynthesis [32, 33]. In addition to *corR*, *hrpL*
88 encodes an alternate RNA polymerase sigma factor (σ^L) required for TTSS expression,
89 which also regulates COR biosynthesis [32, 34]. In *Pst* DC3000, *hrpL* regulates the
90 COR biosynthesis cluster via *corR* [32]. These results indicate a close regulatory
91 relationship between COR production and TTSS expression [2, 35].

92 *P. cannabina* pv. *alisalensis* (*Pcal*) is a causative agent of bacterial blight of
93 crucifer including cabbage, radish, and broccoli [36-40]. *Pcal* can infect not only a wide
94 range of Brassicaceae but also green manure crops such as oat (*Avena strigosa*) and
95 causes brown spot on oat plants [41]. *Pcal* also produces COR [41]. Furthermore,
96 acibenzolar-S-methyl (ASM), a well-known plant defense activator, protects cabbage
97 and Japanese radish from *Pcal* KB211 by activating stomatal-based defense [42-44].
98 These results imply that overcoming stomatal-based defense is important for successful
99 *Pcal* infection. However, studies of COR function in *Pcal* KB211 have not been
100 conducted. Moreover, some cabbage virulence factors were also needed for oat
101 infection, but others were not [45]. Elizabeth and Bender [8] examined the role of COR
102 in *Pst* DC3000 virulence on different hosts including collard and turnip. COR
103 functioned as a virulence factor in both plants, but COR was essential for *Pst* DC3000
104 to maintain high populations in turnip, but not in collard [8], indicating that COR may
105 function differentially in different host plants. We investigated the role of COR in *Pcal*
106 KB211 infection processes in different host plants, including cabbage and oat. Here, we
107 demonstrated that COR contributes to disease by suppressing stomatal-based and
108 apoplastic defenses on both cabbage and oat plants.

109

110 **Results**

111 *P. cannabina* pv. *alisalensis* KB211 causes disease on both cabbage and oat

112 Importantly, *Pcal* KB211 can cause disease on both cabbage and oat [41]. Firstly,
113 we observed disease symptoms and measured bacterial populations in dip-inoculated
114 plants. Cabbage inoculated with *Pcal* KB211 exhibited water-soaked lesions at early
115 time points and showed chlorotic and necrotic lesions at 5 days post-inoculation (dpi)
116 (Fig 1A). Bacterial populations in cabbage increased gradually (Fig 1B). Unlike
117 cabbage, oat showed brown leaf spots at 5 dpi (Fig 1C), and bacterial populations
118 suddenly increased between 3 dpi and 4 dpi (Fig 1D). These results indicate that
119 cabbage and oat differ considerably with respect to symptom development and bacterial
120 multiplication during *Pcal* KB211 infection.

121 We also observed the leaf surface at 3 dpi by confocal microscope, and observed
122 multiple stomata on the cabbage leaf surface (Fig 1E). A *Pcal* KB211 bacterial colony
123 was detected in the substomatal chamber in cabbage (Fig 1F). Conversely, oat has fewer
124 and larger stomata compared to cabbage (Fig 1G), however bacterial colonies was also
125 observed around the stomata (Fig 1H). These results suggest that *Pcal* KB211 enters
126 mainly through stomata. Thus, overcoming stomatal-based defense might be critical
127 steps in the *Pcal* KB211 infection process on cabbage and oat.

128

129 **COR biosynthesis-related genes are greatly expressed during *Pcal* KB211 infection**

130 COR facilitates bacterial entry through stomata [16]. Therefore, we hypothesized
131 that COR is important for *Pcal* KB211 infection processes. We investigated the
132 expression profiles of COR biosynthesis-related genes, including *cmaA*, *cfl*, *corR*, and
133 *hrpL*, which were greatly expressed during *Pcal* KB211 infection on both cabbage and
134 oat compared to culture conditions (Figs 2A, 2B, 2C, and 2D). All genes were greatly
135 expressed at 4 hpi, at which time point bacteria are supposed to overcome
136 stomatal-based defense (Figs 2A, 2B, 2C, and 2D). Moreover, these genes also showed
137 great expression at 48 hpi (Figs 2A, 2B, 2C, and 2D). Therefore, we hypothesized that
138 COR is involved in overcoming both early stages and later infection processes.

139

140 **COR contributes to disease on cabbage and oat**

141 To assess the importance of COR in *Pcal* KB211 virulence, we constructed a
 142 COR defective *cmaA* mutant ($\Delta cmaA$), and confirmed it does not produce COR by
 143 HPLC assay (S1 Fig). To investigate whether COR plays an important role in causing
 144 disease on cabbage and oat, we dip-inoculated cabbage and oat with *Pcal* KB211
 145 wild-type (*Pcal* WT) and $\Delta cmaA$. Cabbage leaves inoculated with $\Delta cmaA$ exhibited no
 146 necrosis and developed less chlorosis (Fig 3A). We also investigated whether COR
 147 contributes to bacterial multiplication in cabbage. $\Delta cmaA$ populations were significantly
 148 reduced compared to *Pcal* WT at 3 dpi and 5 dpi (Fig 3B). $\Delta cmaA$ also exhibited
 149 reduced disease symptoms and reduced bacterial multiplication in oat compared to *Pcal*
 150 WT at 3 dpi and 5 dpi (Figs 3C and 3D). Moreover, to further investigate whether *Pcal*
 151 WT-induced disease-associated necrotic cell death is associated with accelerated
 152 reactive oxygen species (ROS), 3,3'-diaminobenzidine (DAB) staining was carried out to
 153 detect H₂O₂. ROS accumulation was detected in dip-inoculated cabbage and oat in
 154 response to *Pcal* WT, but not $\Delta cmaA$ (Figs 4A, 4D, 4G, and 4J). ROS accumulation was
 155 detected in cabbage mesophyll cell chloroplasts inoculated with *Pcal* WT (Figs 4B and
 156 4C), but not detected in those inoculated with $\Delta cmaA$ (Figs 4E and 4F). Furthermore,
 157 ROS accumulation was observed in oat mesophyll cells inoculated with *Pcal* WT (Figs
 158 4H and 4I), but not with $\Delta cmaA$ (Figs 4K and 4L). These results indicate that COR
 159 facilitates ROS accumulation and causes visible disease symptoms in both cabbage and
 160 oat. Taken together, COR contributes to bacterial multiplication and disease symptoms
 161 on both cabbage and oat.

163 COR reopens cabbage and oat stomata

164 Plants are able to respond to bacterial pathogens by actively closing the stomatal
 165 pore, so called stomatal-based defense [16, 46-51]. COR is responsible for suppressing
 166 stomatal-based defense and reopens stomata [16, 17]. Therefore, to investigate whether
 167 *Pcal* WT and $\Delta cmaA$ facilitate to reopen cabbage and oat stomata, we observed the
 168 stomatal aperture of both plants dip-inoculated with *Pcal* WT and $\Delta cmaA$ at 1 hpi and 4
 169 hpi. Stomatal reopening was observed in both cabbage and oat inoculated with *Pcal* WT,
 170 whereas stomatal reopening was not observed in cabbage and oat inoculated with
 171 $\Delta cmaA$ (Figs 5A and 5B). The oat stomatal reopening rate was quite low in this

172 experimental condition (S2 Fig). Although the stomatal response to pathogens might be
173 different, these results suggest that COR has an important role in overcoming
174 stomatal-based defense in *Pcal* KB211 early virulence in cabbage and oat.

175

176 **COR contributes to apoplastic multiplication and disease on cabbage and oat**

177 To determine whether COR is only important for overcoming stomatal-based
178 defense, we also conducted syringe-infiltration. Cabbage leaves inoculated with both
179 *Pcal* WT and $\Delta cmaA$ showed necrosis at 5×10^5 CFU/ml (S3A Fig). However, at $5 \times$
180 10^3 CFU/ml, cabbage inoculated with $\Delta cmaA$ showed no symptoms, although cabbage
181 inoculated with *Pcal* WT showed chlorosis (S3A Fig). Conversely, oat leaves
182 inoculated with *Pcal* WT showed orange to brown spots at 5×10^5 CFU/ml, and at $5 \times$
183 10^4 CFU/ml (S3A Fig). Oat inoculated with $\Delta cmaA$ showed no symptoms at all
184 bacterial inoculum levels (S3B Fig). Therefore, we next examined bacterial
185 multiplication in cabbage and oat inoculated with 5×10^3 CFU/ml and 5×10^5 CFU/ml,
186 respectively. In cabbage, the $\Delta cmaA$ bacterial populations were significantly reduced at
187 1 and 2 dpi (Fig 6B). Interestingly, although $\Delta cmaA$ showed no symptoms, the bacteria
188 multiplied almost to the same level as *Pcal* WT at 3 dpi (Figs 6A and 6B). In contrast,
189 $\Delta cmaA$ showed no symptoms and did not grow until 5 dpi in oat (Figs 6C and 6D).
190 These results indicate COR plays an important role in overcoming apoplastic defenses,
191 and may function differentially in cabbage and oat.

192 We further tested whether exogenous COR application restores disease
193 susceptibility to cabbage and oat. Co-inoculation of $\Delta cmaA$ with COR 500 μ M
194 increased bacterial growth to a similar level as *Pcal* WT in cabbage at 2 dpi (Fig 6B). In
195 oat, this reduced multiplication could be partly recovered by COR application from 4
196 dpi (Fig 6D). Therefore, these results suggest that COR is strongly involved in
197 multiplication in oat, especially in later infection stages, compared to cabbage.

198

199 **COR regulates defense-related gene expression in cabbage and oat**

200 To evaluate the effect of COR on defense-related gene expression in cabbage and
201 oat, we first investigated *PR* gene expression profiles, including *PR1* and *PR2*, in

response to the SA analog plant activator, acibenzolar-S-methyl (ASM). *BoPR1* and *BoPR2* expression were induced by ASM treatment in cabbage (Figs 7A, and 7B), and *AsPR1a*, *AsPR1b*, and *AsPR2* were induced in oat (Figs 7C, 7D, and 7E). Therefore, we investigated the expression profiles of these in response to *Pcal* WT and $\Delta cmaA$. Cabbage *BoPR2* genes showed significantly greater expression levels at 24 h and 48 h in response to $\Delta cmaA$ compared to *Pcal* WT (Fig 7G). *BoPR1* genes showed no significant difference but tended to increase at 48 h when we inoculated with $\Delta cmaA$ compared to *Pcal* WT (Fig 7F). In oat, all *AsPR1a*, *AsPR1b*, and *AsPR2* genes showed significantly greater expression levels at 24 h and 48 h in response to $\Delta cmaA$ compared to *Pcal* WT (Figs 7H, 7I, and 7J). These results indicate that COR suppresses SA-mediated signaling pathways leading to PR protein accumulation.

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 to *Pcal* WT (Figs 7F, 7G, 7H, 7I, and 7J). Thus, we next examined SA accumulation levels in cabbage and oat when inoculated with *Pcal* WT and $\Delta cmaA$ by using LC-MS/MS. In cabbage, SA accumulation with mock treatment was less than 20 ng/g, and increased around twenty-fold at 24 h after WT inoculation (Fig 8A). $\Delta cmaA$ inoculation induced greater SA levels than *Pcal* WT inoculation at 24 hpi (Fig 8A), indicating that COR suppresses SA biosynthesis in cabbage within 24 h after inoculation. Conversely, SA accumulation triggered by bacterial pathogens caused subtle changes, and showed no statistical difference between *Pcal* WT and $\Delta cmaA$ inoculations in oat (Fig 8B). However, at 48 h, SA accumulation also tended to be suppressed by COR in oat (Fig 8B). Therefore, although COR contributes to SA suppression differently, COR can suppress SA accumulation and allow *Pcal* KB211 to multiply to great levels in both cabbage and oat.

Discussion

Plant pathogens deploy a variety of virulence factors. *Pcal* KB211 multiplied mainly around stomata (Figs 1), and COR biosynthesis-related genes were greatly

expressed during early and later infection processes (Figs 2). Therefore, we hypothesized that COR contributes to *Pcal* KB211 virulence for overcoming both stomatal-based and apoplastic defenses. COR is involved in suppressing stomatal closure (Figs 5) and SA-mediated defenses (Figs 7 and 8) in both cabbage and oat. These results shed light on spatiotemporal COR function in different hosts, monocot and dicot.

The Δ *cor* multiplication defect was observed at 3 dpi, and this defect became greater at 5 dpi in both cabbage and oat (Figs 3). Δ *cor* was defective in reopening stomata in both cabbage and oat (Figs 5), indicating that COR suppresses stomatal-based defense and facilitates *Pcal* entry through stomata into both cabbage and oat. Melotto et al. [16] demonstrated that *Pst* DC3118 *cor* mutant populations were greatly reduced compared with those of *Pst* DC3000 when dip-inoculated on *A. thaliana*. This multiplication defect was observed at early infection stages (3 dpi), suggesting that the COR-mediated suppression of stomatal defense is critical for *Pst* DC3000 host plant infection [16]. COR is required for *Pst* DC3000 growth and persistence within tomato and *A. thaliana* [27, 31]. These results highly support our results that COR is important for disease symptom development and growth in both cabbage and oat. Elizabeth and Bender [8] investigated the role of COR in the interaction of *Pst* DC3000 and different brassicas, collard and turnip. They demonstrated that COR contribution to bacterial multiplication in collard was more subtle than in turnip [8]. 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 (S2 Fig). Roles of stomata in plant disease resistance are not fully understood, especially in monocot plants. Zhang et al. [52] 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 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.

Δ *cor* populations were significantly less than those of *Pcal* WT in both syringe-inoculated cabbage and oat (Figs 6). 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 the *Pst* AK7E2 (*cmaA* mutant) and *Pst* DB4G3 (*cfa6* mutant) at 4 dpi, but not at 2 dpi [31], indicating that COR may be important for apoplastic bacterial persistence. However, Melotto et al. [16] observed no multiplication defect at 3 dpi when the *cor* mutant *Pst* DC3118 was infiltrated directly into the apoplast compared to *Pst* DC3000. Additionally, Mittal and Davis [7] also showed multiplication impairment of *Pst* 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 highly motivated us to investigate the bacterial multiplication at different time points and different inoculum concentrations. $\Delta cmaA$ -inoculated cabbage leaves exhibited similar disease symptoms to *Pcal* WT-inoculated leaves at 5×10^5 CFU/ml (S3A Fig). Conversely, oat infiltrated with $\Delta cmaA$ did not show any symptoms even at the greatest concentration we tested, 5×10^5 CFU/ml (S3B Fig). Helmann et al. [53] demonstrated that disease symptoms were observed only when local internal population sizes in excess of about 10^5 cells/cm² were present, suggesting that bacterial concentrations above a specific “threshold” were required for visible lesion formation. Thus, the 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 we inoculated cabbage and oat with $\Delta cmaA$ compared to *Pcal* WT (Figs 7), indicating that COR suppresses defense-related gene expression. Also, we showed that SA accumulation was greater in cabbage and oat inoculated with $\Delta cmaA$ compared to *Pcal* WT at 24 h (Fig 8A). SA accumulation in oat showed subtle changes against the bacterial pathogen, and no statistical change between the *Pcal* WT and $\Delta cmaA$ inoculations (Fig 8B). However, concerning bacterial multiplication, the $\Delta cmaA$ population was 10-fold less than those of *Pcal* 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*

296 *planta* in dicot plants, such as *A. thaliana* and tomato [27, 29, 54, 55]. However, COR
297 function in SA-mediated defense in monocot plants is less studied. Moreover, in
298 monocot plants, the role of SA biosynthesis in disease resistance is still poorly
299 understood [56]. Consistent with our results on oat, rice plants accumulated increased
300 basal SA levels (8-37 mg/g FW) that did not change significantly upon pathogen attack
301 [57]. In contrast, in tobacco and *A. thaliana*, basal SA levels were less (less than
302 100ng/g FW), but increased by two orders of magnitude following pathogen infection
303 [58]. However, several reports indicated that SA-JA antagonism is conserved in
304 monocot plants such as rice and wheat. In wounded rice, JA levels elevated whereas SA
305 levels decreased, indicating negative crosstalk between the JA and SA pathways during
306 early wounding response in rice [59]. Furthermore, Ding et al. [60] also demonstrated
307 that as in dicot plants, SA and JA acted antagonistically in pathogen-inoculated wheat
308 plants. Also, Iwai et al. [61] demonstrated that SA accumulation in rice was induced
309 after probenazole (PBZ) treatment (one of the synthetic SA analogs) in adult leaves, but
310 not in young leaves. Therefore, SA-mediated defense might be quite different
311 depending on leaf age and plant species. To understand the mechanism by which COR
312 suppresses SA accumulation, further investigation of SA-JA interactions in monocots
313 will be necessary.

314 Suppression of SA-mediated defense by COR results in reduced apoplastic
315 defense in cabbage and oat (Figs 6, 7, and 8). However, COR may contribute to
316 suppress not only SA-mediated defense, but also other apoplastic defenses, such as
317 plant secondary metabolites synthesis. Geng et al. [62] showed that COR inhibited the
318 SA-independent pathway, contributing to callose deposition by reducing accumulation
319 of an indole glucosinolate. Consistent with this study, when *A. thaliana* was challenged
320 by *Pst* DC3000, COR affected gene expression involved in the synthesis of Trp-derived
321 glucosinolates [63]. Brassica species, including cabbage, produce elevated levels of
322 secondary metabolites all proposed to originate from indole glucosinolate [64].
323 Therefore, there is a possibility that COR regulates secondary metabolites in cabbage.
324 These plant-derived secondary metabolites are known to have an important role in plant
325 defense through their antimicrobial activities. Indeed, brassinin, one of the indole-sulfur
326 phytoalexins produced in cabbage, can cause 50% growth inhibition of *Lectosphaeria*
327 *maculans* [65]. Moreover, Wang et al. [66] reported that an *A. thaliana* secondary

metabolite, sulforaphane, directly targets TTSS 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 [67-71]. Avenantheramide levels in vegetation were enhanced by BTH treatment, which is a SA analog same as ASM [68]. This study implied the possibility that COR might suppress avenanthramide biosynthesis, because of the SA-JA antagonistic relationship. Therefore, there is a possibility that COR contributes to overcome 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 *Pcal* 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 *Pcal* 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 which 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 S1 Table. *Pseudomonas cannabina* pv. *alisalensis* strain KB211 (*Pcal* KB211) was used as the pathogenic strain to inoculate cabbage and oat. *Pcal* KB211 wild-type (WT) and the Δ *cmaA* mutant were grown on King's B (KB; [72]) medium at 28°C, and *E. coli* strains were grown on Luria-Bertani (LB; [73]) medium at 37°C. Antibiotics used for selection of *Pcal* and *E. coli* strains included (in µl/ml): rifampicin, 50; kanamycin, 25; ampicillin, 100; chloramphenicol, 10. Before *Pcal* 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, Japan).

358

359 **Generation of tdTomato-labeled *Pcal* KB211 and microscopic observation**

360 A pDSK-*tdTomato* plasmid was generated by replacing the *GFPuv* gene with
361 *tdTomato* (S1 Table) in pDSK-*GFPuv* [74] for tdTomato fluorescent protein expression.
362 The pDSK-*tdTomato* vector was introduced into *Pcal* KB211 by electroporation.
363 Dip-inoculated cabbage and oat leaves were directly imaged using a Leica TCS SP8
364 confocal microscope equipped with a white light laser (Leica, Wetzlar, Germany). A
365 reflected image of the leaf surface was obtained by illuminating the sample with 480 nm
366 wavelength, and reflected light was detected through 475-486 nm laser lines. tdTomato
367 red fluorescence was detected by 554-611 nm laser lines.

368

369 **Plant materials**

370 Cabbage (*Brassica oleracea* var. *capitate*) cv. Kinkei 201 and oat (*Avena*
371 *strigosa*) cv. Hayoat plants were used for *Pcal* virulence assays. Cabbage and oat were
372 grown from seed at 23-25°C with a light intensity of 200 $\mu\text{Em}^{-2}\text{s}^{-1}$ and a 16 h light/8 h
373 dark photoperiod. Cabbage and oat plants were used for dip-inoculation assays around
374 two weeks after germination and for syringe-inoculation assays around three weeks
375 after germination.

376

377 **Generation of $\Delta cmaA$ mutant**

378 The mutants were generated as described previously [75]. The genetic regions
379 containing *cmaA* and surrounding regions were amplified using PCR primer sets (S2
380 Table) that were designed based on the *Pcal* ES4326 registered sequence with Prime
381 Star HS DNA polymerase (TaKaRa, Otsu, Japan), then dA was added to the 3' end of
382 the PCR product with 10 x A-attachment mix (TOYOBO, Osaka, Japan). The resultant
383 DNA was inserted into the pGEM-T Easy vector (Promega, Madison, WI, USA). The
384 recombinant plasmid DNA was then used to obtain pGEM-*cmaA* as templates, and
385 inverse PCR was carried out using a primer set (S1 Table) to delete an open reading
386 frame for *cmaA*. Then, the PCR product and template DNA were digested with BamHI
387 and DpnI. The resultant DNA was self-ligated with T4 DNA ligase

(Ligation-Convenience kit, Nippon Gene, Tokyo, Japan). The *cmaA*-deleted DNA constructs were introduced into the EcoRI site of the mobilizable cloning vector pK18*mobsacB* [76]. 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 [77]. Transconjugants were selected on KB agar containing 30 µg/ml of kanamycin (Km) and 30 µg/ml of rifampicin (Rif). We generated mutants by incubating the transconjugants on a KB agar plate containing 25 µg/ml of rifampicin and 10% sucrose. The specific deletions in the Δ *cmaA* mutants were confirmed by PCR using primers (S1 Table).

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 Specialities, Danbury, CT, USA). The seedlings were then incubated in growth chambers at 85-95% RH for the first 24 h, then at 80-85% RH for the rest of the experimental period. Disease symptom pictures on cabbage and oat seedlings were taken at 5 days post-inoculation. For syringe-inoculation, bacteria were suspended at a final concentration ranging from 5×10^2 to 5×10^5 CFU/ml, and infiltrated with a 1-ml blunt syringe into leaves. The plants were then incubated at 70-80% RH for the rest of the experimental period. Leaves were removed and photographed at 5 days post inoculation.

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% H₂O₂ for 3 minutes. 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

419 pieces were cut from syringe-infiltrated leaf zones and the area (cm²) measured. Leaf
420 extracts were homogenized in sterile distilled water, and diluted samples were plated
421 onto solid KB agar medium. Two or three days after dilution sample plating, the
422 bacterial colony forming units (CFUs) were counted and normalized as CFU per gram
423 or CFU per cm², using the total leaf weight or leaf square meters. The bacterial
424 populations at 0 dpi were estimated using leaves harvested 1 hpi without
425 surface-sterilization. The bacterial populations were evaluated in at least three
426 independent experiments.

427

428 **Real-time quantitative RT-PCR**

429 For expression profiles of cabbage defense genes in response to ASM, cabbage
430 and oat were treated by drenching the soil with ASM. After 24 h, total RNA was
431 extracted from leaves and purified using RNAiso Plus (TaKaRa) according to the
432 manufacture's protocol. For expression profiles of *Pcal* KB211 genes in culture,
433 bacteria were grown in LB broth for 24 h, adjusted to an OD₆₀₀ of 0.1, and grown again
434 in LB broth for 3 hours. Then, bacteria were transferred to fresh mannitol-glutamate
435 broth (MG; [78]) and grown for 15 min. Bacterial RNA was extracted using ReliaPrep
436 RNA Cell Miniprep System Kit (Promega, WI, USA) according to the manufacture's
437 protocol. To analyze *Pcal* KB211 gene expression profiles during infection, we
438 dip-inoculated cabbage and oat with *Pcal* KB211 at 1 x 10⁸ CFU/ml for 4, 24, and 48 h,
439 then the total RNAs including plant and bacterial RNAs were extracted from infected
440 leaves and purified using RNAiso Plus (TaKaRa) according to the manufacture's
441 protocol. The real-time quantitative RT-PCR (RT-qPCR) were done as described
442 previously [79]. Two micrograms of total RNA were treated with gDNA Remover
443 (TOYOBO, Osaka, Japan) to eliminate genomic DNA, and the DNase-treated RNA was
444 reverse transcribed using the ReverTra Ace qPCR RT Master Mix (TOYOBO). The
445 cDNA (1:10) was then used for RT-qPCR using the primers shown in Supplementary
446 Table S1 with THUNDERBIRD SYBR qPCR Mix (TOYOBO) on a Thermal Cycler
447 Dice Real Time System (TaKaRa). *Pcal* KB211 *oprE* and *recA* were used to normalize
448 gene expression. Cabbage *UBIQUITIN EXTENSION PROTEIN 1* (*BoUBQ1*) and Oat

449 *Actin* (*PsAct*) were used as internal controls to normalize gene expression in cabbage
450 and oat, respectively.

451

452 **Hydrogen peroxidase detection**

453 Hydrogen peroxidase generation was detected by 3,3'-diaminobenzidine (DAB)
454 staining as described previously [28, 80]. After inoculation with *Pcal* WT and $\Delta cmaA$,
455 the leaves were placed in DAB-HCL (pH 3.8) at 1 mg/ml. After incubation for 6 h at
456 room temperature, chlorophyll was removed with 95% ethanol.

457

458 **Stomatal assay**

459 A modified method was used to assess stomatal response as described previously
460 [81]. Briefly, cabbage and oat were grown for around 2 weeks after germination as
461 described previously. *Pcal* WT and $\Delta cmaA$ were grown at 28°C for 24 h on KB agar,
462 then suspended in distilled water to an OD₆₀₀ of 0.2 (1 x 10⁸ CFU/ml). Dip-inoculated
463 cabbage and oat leaves were directly imaged at 1 hpi and 4 hpi using a Nikon optical
464 microscope (Eclipse 80i). The aperture width of at least 300 and 600 stomata was
465 measured on cabbage and oat, respectively. The average and standard error for the
466 stomatal aperture width were calculated. The stomatal apertures were evaluated in at
467 least three independent experiments.

468

469 **SA quantification by RP-LC-ESI-MS/MS**

470 *Pcal* WT, $\Delta cmaA$ bacterial suspension (5 x 10⁵ CFU/ml), or water (mock) were
471 infiltrated into three-week old cabbage and oat third leaf. Eight leaf discs (3.5
472 mm-diameter) from two cabbage leaves and about 3.5 cm² from two oat leaves were
473 collected 0 (untreated), 24 hpi, and 48 hpi, the weight was measured, and samples were
474 frozen in liquid nitrogen and stored at -80°C. Samples were extracted with 300 µl of
475 80 % methanol.

476 SA was measured by using the multiple-reaction monitoring (MRM) mode on the
477 LC-ESI-MS/MS (LCMS-8045; SHIMADZU, Kyoto, Japan) under the following
478 conditions: capillary voltage, 4.5 kV; desolvation line, 250°C; heat block, 400°C;

nebulizer nitrogen gas 3 L/min; drying gas, 10 L/min. Ion source polarity was set in the negative ion mode. The separation was performed with the LC system equipped with a 150 x 2.1 mm ACQUITY UPLC BEH Shield RP18 Column (Waters, Ireland) with a particle and pore size of 1.7 μm and 130Å, 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 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 the column temperature at 40°C were maintained throughout the analysis. The MRM-transition m/z 137→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 msec, 15 V, 17 eV, 15 V, respectively.

COR quantification by HPLC

Pcal WT and ΔcmaA bacteria were cultured in HS medium optimized for coronatine production (HSC; [82]) for 5 days. Culture supernatant was obtained by centrifugation (12,000 x g for 5 min). The culture supernatant was analyzed by HPLC with a Shimadzu LC20A system equipped with a Symmetry C8 column (4.6 x 250 mm; Waters Corporation, MA, USA) under the following conditions: column temperature, 40°C; isocratic elution; mobile-phase composition, 0.05% trifluoroacetic acid (TFA)/acetonitrile (4:6, v/v); flow rate, 1 ml/min; and detection, 208 nm. COR in the culture supernatant was identified, as compared with authentic COR (Sigma) as the standard.

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509

510 **Figure legends**

511 **Figure 1. Disease phenotypes and population dynamics of *Pseudomonas cannabina***
512 ***pv. alisalnesis* KB211 in cabbage and oat. (A) and (C)** Symptoms on cabbage (A) and
513 oat (C) leaves dip-inoculated with *Pcal* KB211. Cabbage and oat were dip-inoculated
514 with 5×10^7 CFU/ml of inoculum. The leaves were photographed at 5 dpi. Scale bar
515 shows 1 cm. **(B) and (D)** *Pcal* KB211 total populations on cabbage (B) and oat (C)
516 leaves when dip-inoculated with 5×10^7 CFU/ml. Vertical bars indicate the standard
517 error for three independent experiments. **(E)-(H)** Bacterial cell location on cabbage (F)
518 and oat (H) leaves. (E) and (G) shows the leaf surface without bacteria cells on cabbage
519 and oat, respectively. Cabbage and oat were dip-inoculated with bacterial suspension (5
520 $\times 10^8$ CFU/ml) of tdTomato-labeled *Pcal* KB211 inoculum containing 0.025%
521 SilwetL-77. Inoculated leaves were observed 3 dpi by using a Leica TCS SP8 confocal
522 microscope equipped with a white light laser (Leica, Wetzlar, Germany), showing
523 bacterial colonization of the substomatal chamber. Scale bar shows 100 μ m.

524

525 **Figure 2. Expression profiles of *Pcal* KB211 COR biosynthesis related genes**
526 **during infection on cabbage and oat plants. (A)-(D)** Expression profiles of *cmaA* (A),
527 *cfl* (B), *corR* (C), and *hrpL* (D) genes in liquid LB broth (culture) or in dip-inoculated
528 cabbage and oat plants (5×10^5 CFU/ml) at 4, 24, and 48 h. Total RNA was extracted
529 for use in real-time quantitative reverse transcription-polymerase chain reaction
530 (RT-qPCR) with gene-specific primer sets. Expression was normalized using *oprE* and
531 *recA*. Vertical bars indicate the standard error for three biological replicates. Asterisks
532 indicate a significant difference from culture cells in a *t* test (* $p < 0.05$, ** $p < 0.01$).

533

534 **Figure 3. COR contributes to cause disease on cabbage and oat. (A) and (B)**
535 Disease symptoms (A) and bacterial populations (B) in cabbage dip-inoculated with
536 *Pcal* WT and $\Delta cmaA$. **(C) and (D)** Disease symptoms (C) and bacterial populations (D)

537 in oat dip-inoculated with *Pcal* WT and $\Delta cmaA$. Cabbage and oat were dip-inoculated
538 with 5×10^7 CFU/ml of *Pcal* KB211 containing 0.025% SilwetL-77. The leaves were
539 photographed at 5 dpi. Vertical bars indicate the standard error for three independent
540 experiments. Asterisks indicate a significant difference between *Pcal* WT and $\Delta cmaA$ in
541 a *t* test (* $p < 0.05$, ** $p < 0.01$). Scale bar shows 1 cm.

542

543 **Figure 4. COR contributes to induce ROS accumulation. (A)-(L)** Reactive oxygen
544 species (ROS) accumulation visualized by staining hydrogen peroxidase using
545 3,3'-diaminobenzidine (DAB). DAB staining was carried out to detect H_2O_2 in cabbage
546 after dip-inoculation with 5×10^7 CFU/ml of *Pcal* WT (A)-(C) and $\Delta cmaA$ (D)-(F), and
547 in oat dip-inoculated with 5×10^7 CFU/ml of *Pcal* WT (G)-(I) and $\Delta cmaA$ (J)-(L).
548 Black and white bars show 1 cm and 500 μm , respectively.

549

550 **Figure 5. COR reopens stomata on both cabbage and oat. (A) and (B)** Stomatal
551 aperture width on intact cabbage (A) and oat (B) leaves 1 h and 4 h after dip-inoculation
552 with 1×10^8 CFU/ml of *Pcal* WT and $\Delta cmaA$. OB indicates opening buffer control.
553 Different letters indicate a significant difference among treatments based on a Tukey's
554 HSD test ($p < 0.05$).

555

556 **Figure 6. COR contributes to cause disease on cabbage and oat. (A) and (B)**
557 Disease symptoms (A) and bacterial populations at 1, 2, and 3 dpi (B) in cabbage
558 syringe-inoculated with *Pcal* WT and $\Delta cmaA$. Cabbage were syringe-inoculated with 5
559 $\times 10^3$ CFU/ml of *Pcal* WT and $\Delta cmaA$. (C) and (D) Disease symptoms (C) and
560 bacterial populations at 1, 2, and 3 dpi (D) in oat syringe-inoculated with *Pcal* WT and
561 $\Delta cmaA$. Oat were syringe-inoculated with 5×10^5 CFU/ml of *Pcal* WT and $\Delta cmaA$.
562 Additionally, COR (500 μM) was co-inoculated with $\Delta cmaA$. The leaves were
563 photographed at 5 dpi. Vertical bars indicate the standard error for three independent
564 experiments. Different letters indicate a significant difference among treatments based
565 on a Tukey's HSD test ($p < 0.05$). Scale bar shows 2 cm.

566

567 **Figure 7. COR suppresses defense-related gene expression in cabbage and oat. (A)-**
568 **(E)** Gene expression profiles involved in cabbage and oat plant defense after ASM

treatment. Cabbage and oat were treated with water as a control and ASM (100 ppm). Expression levels of *BoPRI* (A), *BoPR2* (B), *AsPRIa* (C), *AsPRIb* (D), and *AsPR2* (E) were determined using real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR) 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. Asterisks indicate a significant difference from the water control in a *t*-test (* $p < 0.05$; ** $p < 0.01$). ns stands for no significant difference between samples. (F)-(J) Gene expression profiles involved in cabbage and oat plant defense after syringe-inoculation with water (mock), *Pcal* WT, and $\Delta cmaA$. Expression profiles of *BoPRI* (F), *BoPR2* (G), *AsPRIa* (H), *AsPRIb* (I), and *AsPR2* (J) were determined 24 h and 48 h after inoculation with 5×10^5 CFU/ml of *Pcal* WT and $\Delta cmaA$, or mock water-inoculated control using RT-qPCR 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. Asterisks indicate a significant difference between *Pcal* WT and $\Delta cmaA$ in a *t*-test (* $p < 0.05$; ** $p < 0.01$).

Figure 8. COR suppresses SA accumulation in cabbage and oat. (A)-(B) Total SA production in cabbage (A) and oat (B) after syringe-inoculation with 5×10^5 CFU/ml of *Pcal* WT and $\Delta cmaA$, or treated with water as a control. Vertical bars indicate the standard error for three biological replicates. Asterisks indicate a significant difference between plants inoculated with *Pcal* WT and $\Delta cmaA$ in a *t*-test (* $p < 0.05$). ns indicate no significant difference between samples.

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848 Supporting information

849 **Supplementary Figure 1. HPLC-based quantification of COR production by *Pcal***
850 **WT and Δ *cmaA*.** *Pcal* WT and Δ *cmaA* bacteria were cultured in HS medium optimized
851 for coronatine production (HSC; [81]) medium for 5 days. Culture supernatant was
852 obtained by centrifugation, and analyzed by HPLC with a Shimadzu LC20A system
853 equipped with a Symmetry C8 column under the following conditions: column
854 temperature, 40°C; isocratic elution; mobile-phase composition, 0.05% trifluoroacetic
855 acid (TFA)/ acetonitrile (4:6, v/v); flow rate, 1 ml/min; and detection, 208 nm. COR
856 was identified in the culture supernatant, as compared with authentic COR as the
857 standard. Red, green, and black chromatograms show authentic COR, *Pcal* WT, and
858 *Pcal* Δ *cmaA*, respectively. Arrow indicates COR retention time.

859

860 **Supplementary Figure 2. COR reopens stomata on oat.** Stomatal opening rate of oat
861 1 h and 4 h after inoculation with *Pcal* WT and $\Delta cmaA$. In all bar graphs, vertical bars
862 indicate the standard error for three technical replicates.

863

864 **Supplementary Figure 3. COR contributes to cause disease on cabbage and oat.**
865 **(A) and (B)** Disease symptoms on cabbage (A) and oat (B) 5 days after
866 syringe-inoculation with *Pcal* WT and $\Delta cmaA$. Plants were inoculated with 5×10^2 , $5 \times$
867 10^3 , 5×10^4 , and 5×10^5 CFU/ml of *Pcal* WT and $\Delta cmaA$. Scale bar shows 2 cm.

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