

## Priming corn resistance to Goss's wilt using a weakly aggressive strain of *Clavibacter nebraskensis*

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### HIGHLIGHTS

- Goss's wilt caused by *Clavibacter nebraskensis* is a serious yield-limiting factor in corn.
- Priming susceptible plants with small concentrations of a weak strain of *C. nebraskensis* significantly inhibited further infection by a highly aggressive strain.
- Induced plant defense mechanisms occurred along with the primed state of corn plants.

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### ABSTRACT

Goss's wilt, caused by the gram-positive bacterium *Clavibacter nebraskensis*, has become one of the most widely spread corn diseases in the USA and Canada. Considered as a relatively new disease, with limited knowledge about the more recently renamed pathogen causing it, it is today a serious yield-limiting factor in corn crops in North America. The diversity of *C. nebraskensis* strains translates into the differential plant responses it causes, ranging from hypersensitive-like response to partial resistance, or otherwise susceptibility expressed as dark, water-soaked spots and shiny patches of dried bacterial ooze on the lesions. Weakly aggressive strains of *C. nebraskensis* typically appear as the season winds down and the crop is about to be harvested, whereas highly aggressive strains can be found at any point during the growing season. Previous research showed that plant's ethylene and reactive oxygen species (ROS) are essential in limiting the spread of Goss's wilt symptoms near infection sites by triggering programmed cell death (PCD). Preliminary observations that certain strains induce strong defense responses with very little to no symptoms fed the idea to use them for disease resistance priming. The induction of biosynthetic or responsive genes of ethylene, ROS, and salicylic acid (SA) both at the local and distal tissues is in line with the limited disease symptoms development in response to priming with the weakly aggressive strain before further inoculation with a highly aggressive one. The results further expand our understanding of differential host responses to strains with different levels of aggressiveness. The priming process led to an inhibition of the elongated lesions in the susceptible corn plants in comparison with the unprimed plants. The expression patterns of a lectin receptor-like kinase, *Salt Intolerance 2* (*ZmSIT2*), and blue copper binding protein (*ZmBCP*) suggests them as key mediators of ethylene and ROS homeostasis in corn tissues upon infection. This adds to the pool of knowledge on corn defenses against bacterial pathogens and the mechanism of its bioprime, which could serve as an environment-friendly alternative method to control Goss's wilt.

### 1. Introduction

*Clavibacter nebraskensis* infects corn, causing Goss's wilt disease. Infested plant parts, such as roots, stems, leaf blades, sheaths, and kernels, that fall on the soil surface can serve as inoculum sources for

*C. nebraskensis*. For northern United States and Ontario maize farmers, Goss's wilt was among the three most devastating diseases from 2012 to 2015, causing a total projected yield loss of more than 1,270,000 tons with an estimated loss of \$76.51 USD per acre (Ilkley, 2018; Mueller et al., 2016). More recent surveys in the United States and Canada have

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also placed this disease among the top ten most damaging corn diseases (Mueller et al., 2020). Individual field losses might reach as much as 60%, depending on factors such as disease severity, hybrid susceptibility, disease emergence stage, and environmental conditions (Malwick et al., 2014). Heavy rain, hail, and sand blasting during rainstorms are common causes of plant damage that facilitates subsequent infection by bacteria resting on the soil or on plant residues (Malloua et al., 2016; SMIDT and VIDAYER, 1986; Vidaver and Mandel, 1974). Pathogens also can spread by wind, which can transfer infected residues to neighboring fields (Jackson et al., 2007). In addition to wounds, hydathodes and stomata provide additional entry points for these bacteria to infect uninjured plants (Mullens and Jamann, 2021).

The pathogenicity of *Clavibacter* species depends on enzymes and exopolysaccharides, among others (Stevens et al., 2021). In plants with different xylem sap compositions, i.e. maize vs. tomato, distinct secretomes have been identified, showing that aggressive *C. nebraskensis* strains generate a higher concentration of virulence factors in the xylem sap of maize. These included cellulases,  $\beta$ -glucosidases,  $\beta$ -galactosidases, chitinases,  $\beta$ -1,4-xylanases, and proteases, which were otherwise either absent or at lower levels in the xylem sap of the non-host tomato plants, suggesting that the expression of these virulence factors is due to a host-dependent interaction (Soliman et al., 2021b). Strains of *C. nebraskensis* vary in their virulence on corn, with more severe symptoms and crop damage caused by highly aggressive strains. The highly aggressive *C. nebraskensis* strain Cn14-5-1 caused much more severe symptoms in a susceptible corn line, compared to the less aggressive strain DOAB232 (Shumilak, 2021; Soliman et al., 2021b). The variation in aggressiveness between these two strains was explored further by studying their secreted proteomes (Soliman et al., 2021b), showing that the secretome of Cn14-5-1 contains more unique and differentially-abundant proteins and virulence factors than that of DOAB232 and corroborating the disparity in their phenotypic aggressiveness.

Plant defense mechanisms can be induced either directly, i.e., immediate response to an invading pathogen, or indirectly (primed), i.e., defense activation prior to an actual infection event (Conrath et al., 2006; Frost et al., 2008). Priming makes plants acquire a greater ability to activate induced defense responses, which can result into resistance to a wide variety of pathogens (Conrath, 2009; Durrant and Dong, 2004). Priming plants to better withstand biotic and abiotic stress occurs via distinct pathways involving a wide range of metabolic processes, i.e., avirulent *Pseudomonas syringae* increasing the expression of PR genes in *Arabidopsis* plants, thereby priming them for systemic acquired resistance (SAR) (Van Wees et al., 1999). In addition to SAR, priming can also establish induced resistance at the site of infection, forming localized acquired resistance (LAR) (Hammerschmidt, 2009). Direct defense mechanisms are often rapid and effective at preventing pathogen infection, but they can also be costly in terms of resource consumption (Heil, 2002). In contrast, primed defenses, which involve defense response activation in advance of an actual infection, can be much less costly, although they are not always as rapid or effective as direct defense. The advantages of priming far outweigh its drawbacks, as primed plants are resilient to stress without affecting their commercially- and ecologically- important traits (Conrath et al., 2006). Consequently, priming can be one of the best ways to balance the benefits of disease prevention with the costs of defense activation (Conrath et al., 2006; Martinez-Medina et al., 2016).

Transcriptomic analysis of an entire genome in a specific tissue, during a particular stage of its growth or under stress, provide valuable insights into the intricate regulatory networks that contribute to plants' ability to handle stress (Wang et al., 2020). For example, transcriptomic analysis shows that polygalacturonase inhibitor 1 (PGIP), Barwin proteins, PR proteins, and secondary metabolites are key elements of the primed state activation of the maize-*Colletotrichum graminicola* pathosystem. *C. graminicola* triggers SAR in inflorescences through a signaling process dependent on salicylic acid (SA), which was represented by the increased expression of the PR1 gene locally in leaves and roots upon *C.*

*graminicola* exposure, as well as systemically in female inflorescences, suggesting the possibility of a similar transgenerational SAR signaling pathway occurring in maize, similar to that observed in *Arabidopsis* (Miranda et al., 2017).

Common agricultural practices that can help with Goss's wilt management include weed management, tillage, crop rotation, and using tolerant plant varieties (Jackson et al., 2007). The use of chemicals has been helpful to some extent with fungal diseases, making it more challenging for alternative biocontrol agents (molecules, microorganisms) to surge in the market due to costs for formulation and application. In absence of registered chemicals to control Goss's wilt and given the limited success of cultural practices, biocontrol alternatives should be explored besides the development of resistant varieties. Exploiting the plant's own defense arsenals using response induction strategies is worth exploring. Priming studies can identify new genes that breeders can use to develop new resistant varieties and might allow growers to use susceptible corn lines that already have the desirable traits. As well, priming facilitates the rapid and efficient activation of defense responses in plants, providing a broad-spectrum resistance against secondary stresses without significantly impacting growth or yield. Consequently, priming can contribute to new strategies for disease control in the future.

The objectives of this study were (i) to test the hypothesis that a weakly aggressive strain of *C. nebraskensis* (DOAB232) could help prime corn defenses and lessen the disease severity brought upon by further infection by more aggressive *C. nebraskensis* strains such as Cn14-15-1 and (ii) to investigate the defense mechanisms involved in such priming. The tested defense mechanisms were selected based on our previous work that showed genes, peptides, or other molecules with potential role in corn defense against Goss's wilt (Owusu et al. 2019; Soliman et al. 2021b).

## 2. Materials and methods

### 2.1. Plant growth conditions

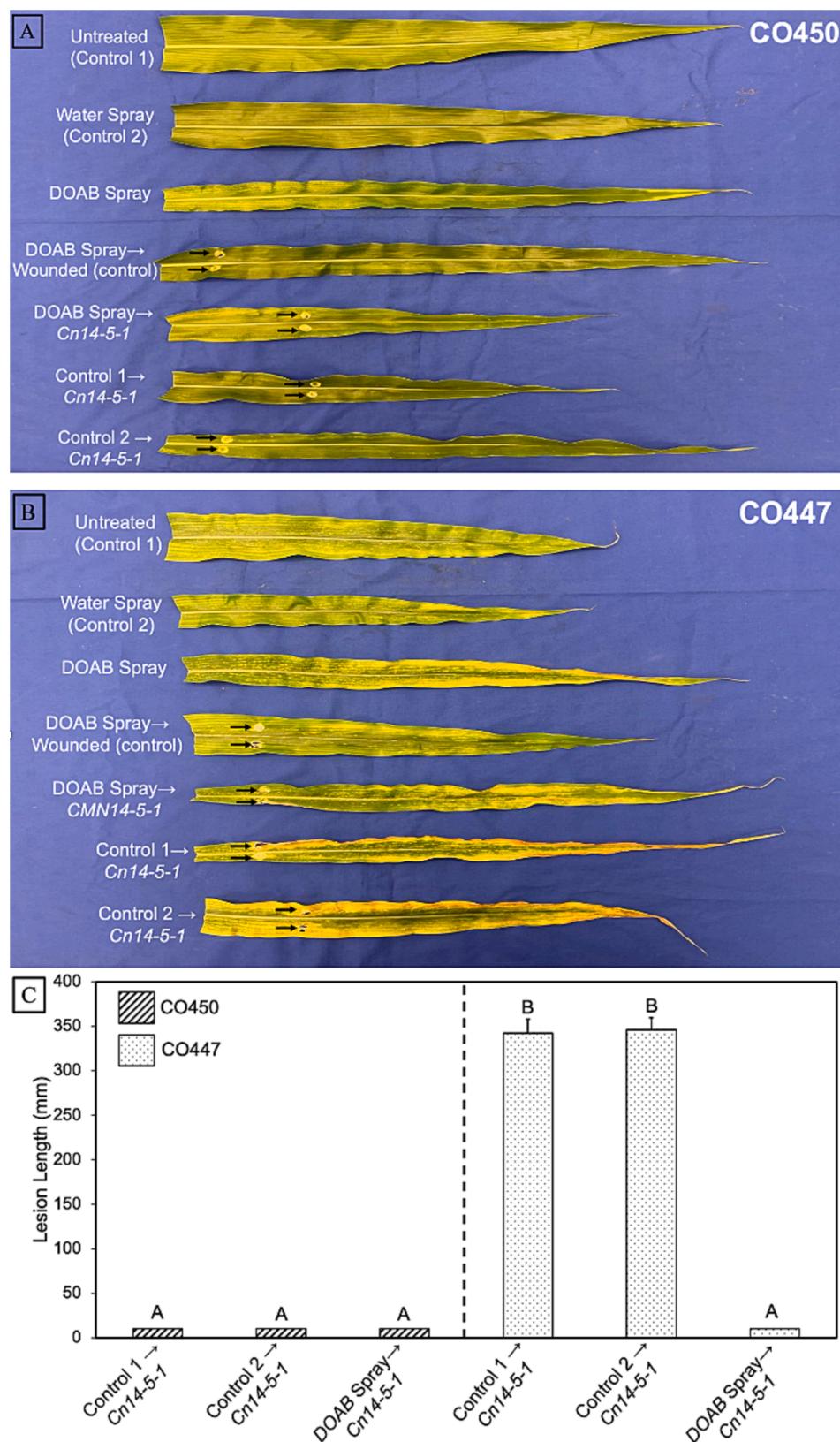
The two inbred corn (*Zea mays L.*) lines used in this study, CO447 and CO450, as susceptible and resistant lines, respectively, were provided by Dr. Aida Kebede (Agriculture and Agri-Food Canada, Ottawa, Ontario) (Jindal et al., 2019). The plants were grown in a growth chamber (at 22 °C during the day and 18 °C at night, as well as a 16-hour light/8-hour dark cycle).

### 2.2. Preparation of bacterial inoculum

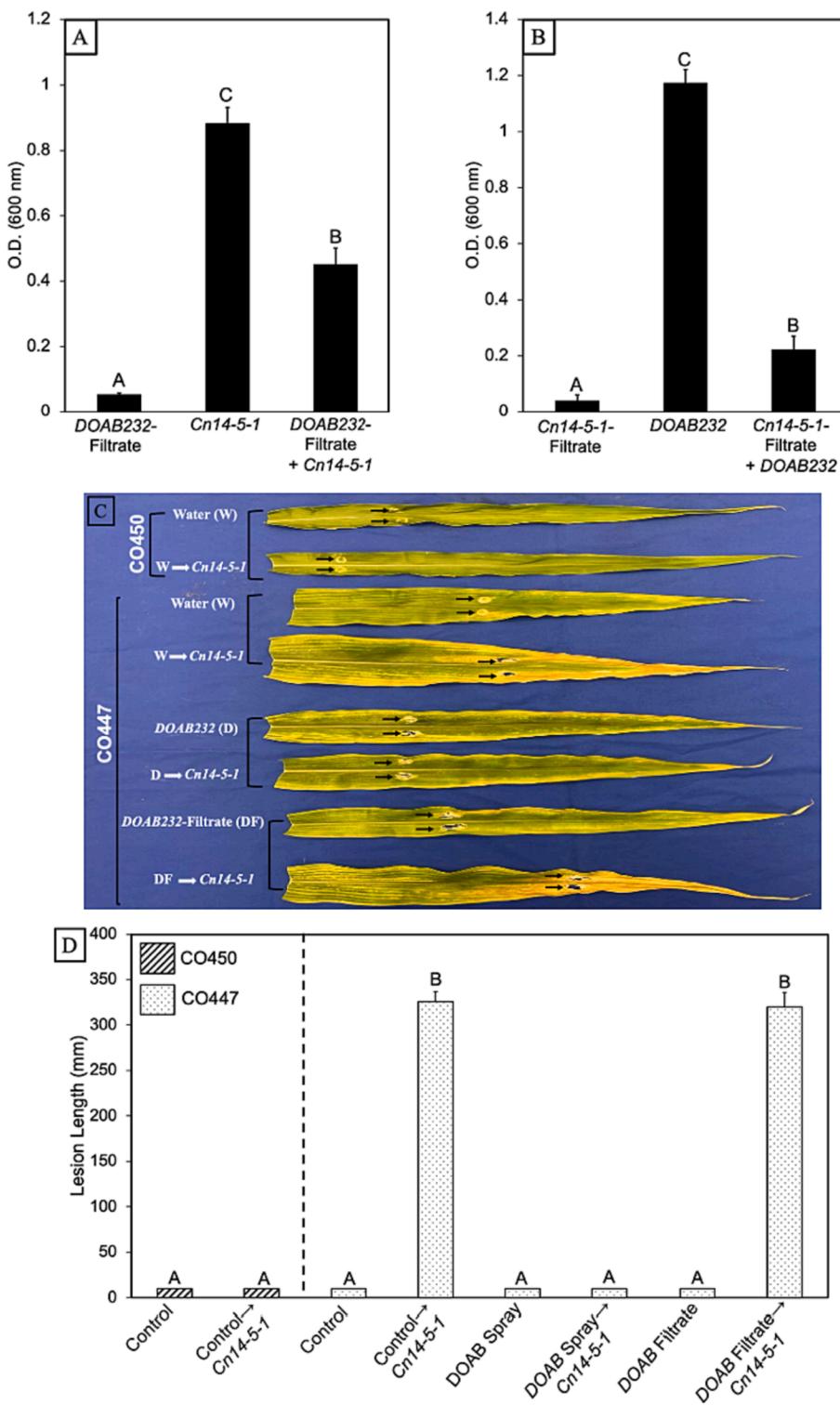
*C. nebraskensis* strains were grown for 2 days at 25 °C in a nutrient broth yeast medium (NBY) containing 8 g/L nutrient broth (Becton, Dickinson and company, cat. no. DF0003178), 2 g/L yeast extract (Becton, Dickinson and company, cat. no. B11929), 15 g/L agar (Becton, Dickinson and company, cat. no. DF0812179), 2 g/L K<sub>2</sub>HPO<sub>4</sub> (Fisher, cat. no. P288-500), 0.5 g/L KH<sub>2</sub>PO<sub>4</sub> (Fisher, cat. no. P-284, CAS-7778-77-0), 5 g/L glucose (Sigma, cat. no. G8270), and 0.246 g/L MgSO<sub>4</sub>·7H<sub>2</sub>O (Fisher, cat. no. M-67, CAS-7487-88-9) (Agarkova et al., 2011; Gross and Vidaver, 1979). Bacterial inoculum was made by suspending the bacterial pellet in the phosphate buffer solution (PBS; 1.74 g/L dipotassium phosphate and 1.36 g/L monopotassium phosphate, pH 6.7). The concentrations of DOAB232 and Cn14-5-1 were determined and adjusted to 1 × 10<sup>3</sup> and 1 × 10<sup>7</sup> cfu/mL for inoculation, respectively.

### 2.3. Bacterial filtrates

Bacterial cultures were diluted to an OD<sub>600</sub> of 1.00, centrifuged at 3000 rpm for 10 min, and the supernatants were filtered via 25 mm syringe filters (Fisher, cat. no. 09-719C) to obtain pure filtrates that were used as substrates or in plant pretreatment.



**Fig. 1.** Priming disease resistance to *C. nebraskensis* (Cn14-5-1) in the susceptible CO447 corn line. (A and B) Phenotype of the tested corn plants. (C) Lesion size was measured on CO450 and CO447 leaves at day 14 after inoculation with Cn14-5-1. Lesions from nine leaves of three different plants were used to calculate the mean and standard error. Letters on bars indicate statistically significant differences; one-way ANOVA; post hoc least significant difference;  $P < 0.05$ .

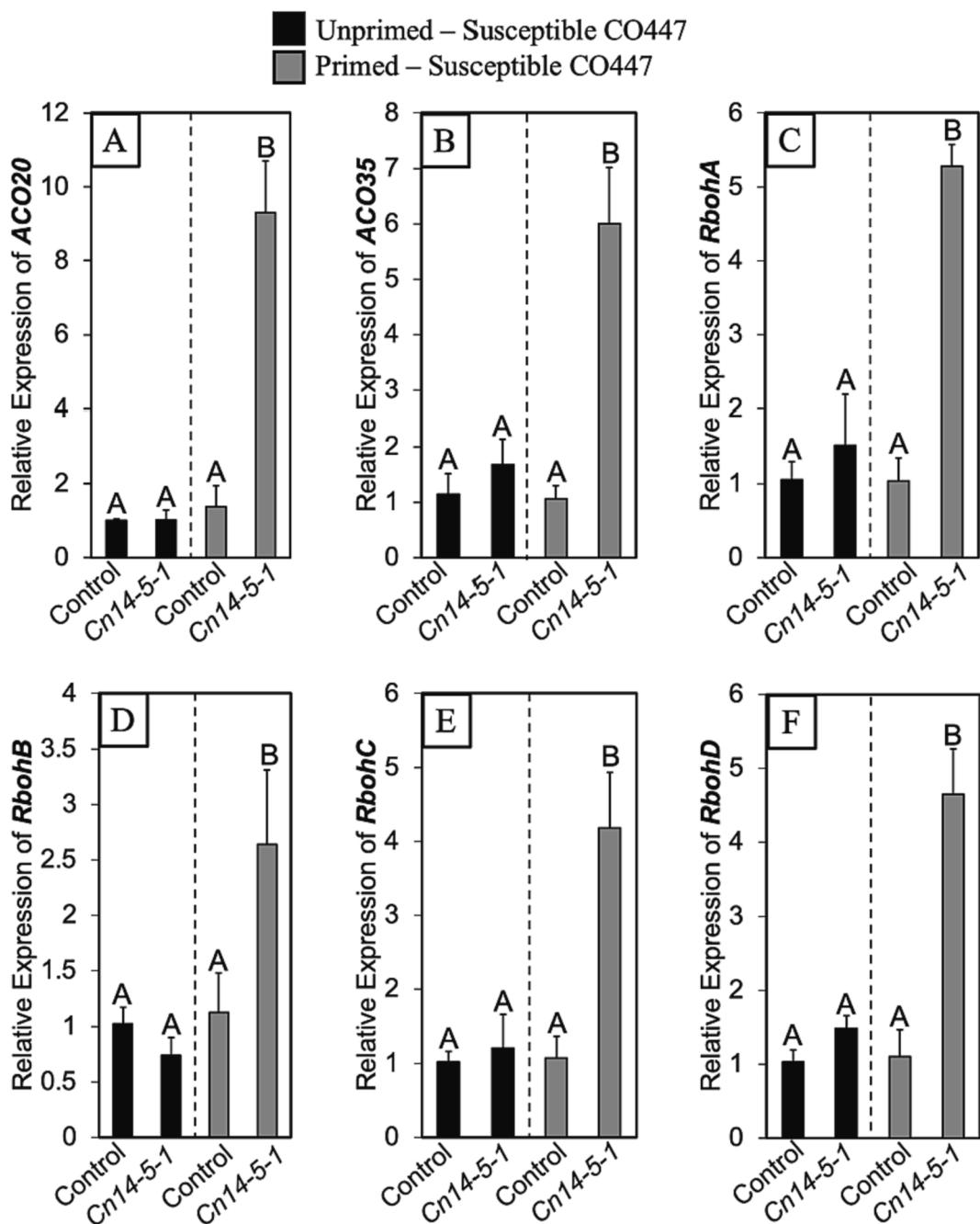


**Fig. 2.** The priming effect of DOAB232 is due to the induced defense response in the susceptible CO447 corn line. Antagonistic activity of *C. nebraskensis* strains against each other (A and B). (C) Phenotype of the tested corn plants. (D) Lesion size was measured on CO450 and CO447 leaves at day 14 after inoculation with Cn14-5-1. Lesions from nine leaves of three different plants were used to calculate the mean and standard error. Letters on bars indicate statistically significant differences; one-way ANOVA; post hoc least significant difference;  $P < 0.05$ .

#### 2.4. Leaf inoculation and measurement of lesion length

Maize plants (3–4-week-old) were sprayed with phosphate buffer solution, DOAB232 inoculum, or DOAB232 filtrate, and 48 h later, plants were mechanically wounded at the V5 leaf stage with a 1-mL disposable syringe plunger. The third, fourth, and fifth leaves were

wounded on both sides next to the midrib of the treated plants (Soliman et al., 2018). Twenty microliters of PBS or Cn14-5-1 inoculum were placed on the wounds. Three biological replicas (plants), each containing three leaves, were used in the experiments. Treated plants were kept overnight in a humidity chamber (100 percent relative humidity) then transferred to a growth chamber for 14 days. At 14 dpi, the length of the



**Fig. 3.** Ethylene and ROS are involved in the early events of corn resistance to *C. nebraskensis*. RT-qPCR transcript levels of the ethylene-synthetic genes, aminocyclopropane-1-carboxylic acid oxidase (ACO), *ZmACO20* (A) and *ZmACO35* (B), and the respiratory burst oxidase homologs (*ZmRboh A–D*) genes, *ZmRbohA* (C), *ZmRbohB* (D), *ZmRbohC* (E), and *ZmRbohD* (F) in response to Cn14-5-1 were measured. Leaves were sampled at 3 h after Cn14-5-1 inoculation. Primed plants were pretreated with DOAB232 prior to Cn14-5-1 inoculation. Reference gene, actin. Three biological replicates were used to calculate the mean and standard error. Letters on bars indicate statistically significant differences; one-way ANOVA; post hoc least significant difference;  $P < 0.05$ .

lesion was measured upward and downward from the site of infection (Soliman et al., 2018).

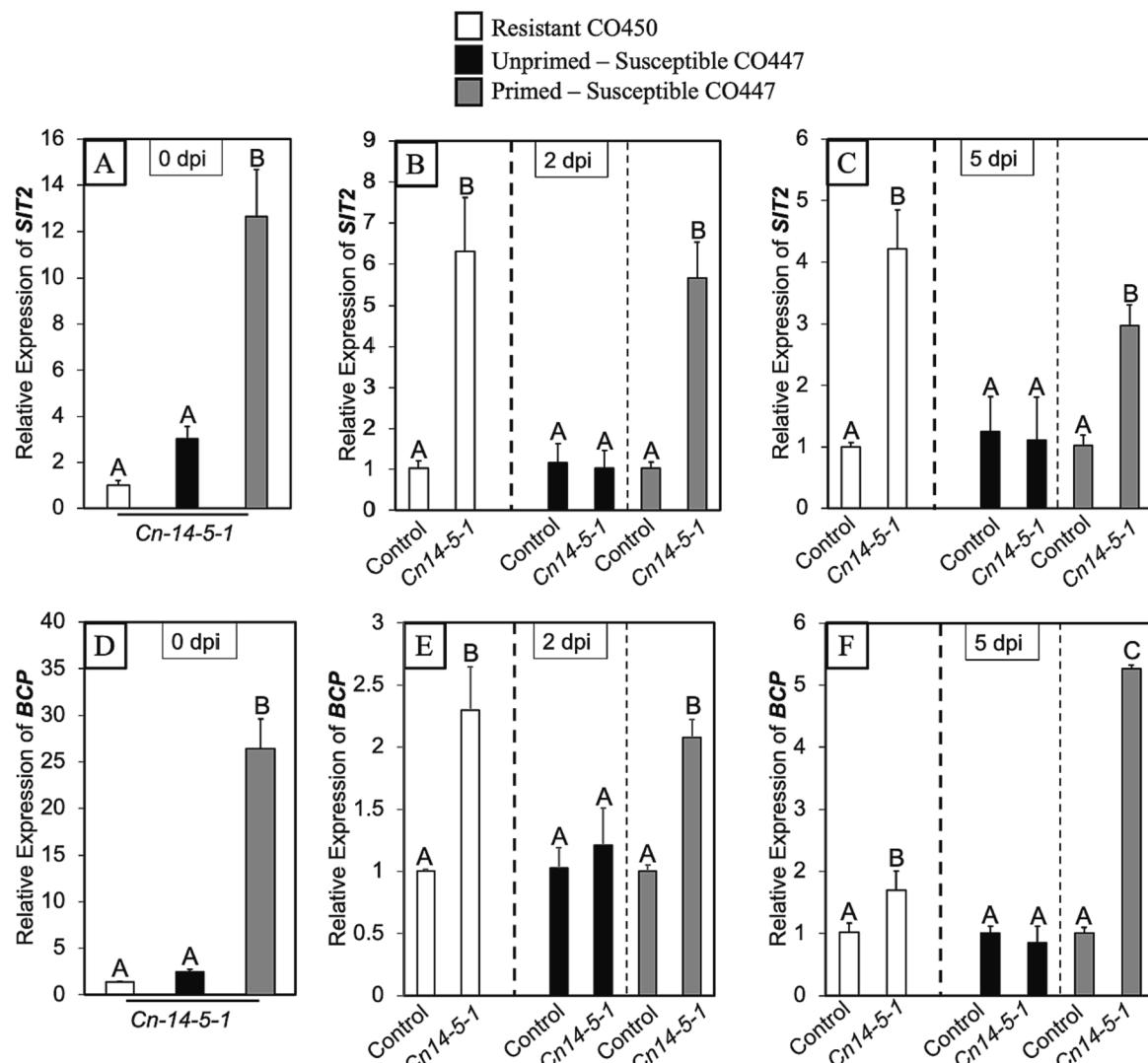
#### 2.5. Antagonistic activity test

Three ml of the supernatant or the filtrate of either DOAB232 or Cn14-5-1 were added to new falcon tubes, and 10  $\mu$ l of the opposite bacterial strain that was adjusted to an OD<sub>600</sub> of 1.00 were added to each tube. Tubes were incubated for 24 h at 25 °C. Then, the bacterial concentration was measured at OD<sub>600</sub>. NBY medium alone; uninoculated supernatants or filtrates served as controls. The experiments were

carried out in three biological replicas.

#### 2.6. Sampling and measurement of transcript levels

Plant tissues were collected at 3 hpi and at 0, 2, and 5 dpi of Cn14-5-1. TRI Reagent (Invitrogen, cat. no. AM9738) was used to extract the total RNA, which was treated with DNase I (ThermoFisher Scientific, cat. no. EN0521), and then reverse-transcribed using the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific, cat. no. K1621) to synthesize cDNA. All the primers used in gene expression studies were listed in supplementary Table S1. The relative gene expression was



**Fig. 4.** Cellular balance of ethylene and reactive oxygen species is dependent on *ZmSIT2* and *ZMBCP*. RT-qPCR transcript levels of the L-type lectin-domain containing receptor kinase (*ZmSIT2*), *ZmSIT2* (A-C) and the blue copper protein gene, *ZMBCP* (D-F) in response to Cn14-5-1 of CO450 and CO447 corn lines were measured at 0, 2, and 5 dpi. Primed CO447 plants were pretreated with DOAB232 prior to Cn14-5-1 inoculation. Reference gene, actin. Three biological replicates were used to calculate the mean and standard error. Letters on bars indicate statistically significant differences; one-way ANOVA; post hoc least significant difference;  $P < 0.05$ .

determined using the  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen, 2001), and actin was used as a reference gene.

## 2.7. Statistical analysis

Statistical analyses were carried out using the IBM SPSS Statistics software (version 22). The one-way ANOVA; post hoc least significant difference ( $\alpha=0.05$ ) was used to compare treatment means. Each experiment was repeated two times.

## 3. Results

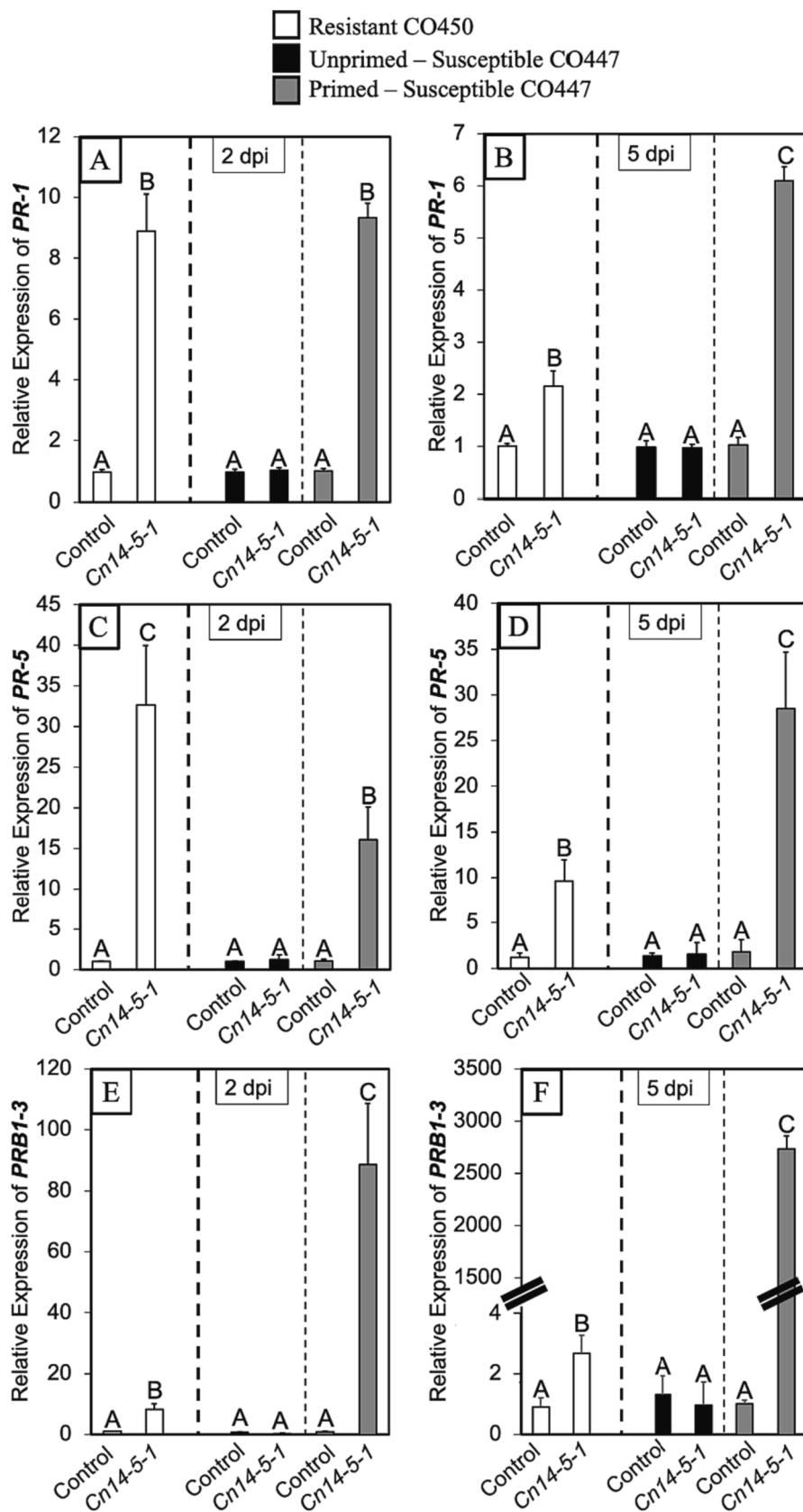
### 3.1. Corn priming with the weakly aggressive strain DOAB232 for potentiated responses to the highly aggressive strain Cn14-5-1

The susceptible CO447 corn lines pre-inoculated with DOAB232 strain showed no symptoms following inoculation with the aggressive Cn14-5-1 strain, contrary to plants inoculated only with latter strain. The resistant CO450 corn lines, whether pre-inoculated with DOAB232 strain or not, showed no symptoms against Cn14-5-1 strain. The size of the lesions observed on unprimed susceptible CO447 corn lines reflected

the level of aggressiveness of the Cn14-5-1 strain, whereas tiny lesions at the inoculation sites were recorded in response to Cn14-5-1 strains in either primed CO447 or resistant CO450 (Fig. 1A-C).

### 3.2. Primed state of CO447 is due to induced plant defense mechanisms prior to Cn14-5-1 inoculation

The enhanced disease resistance of the primed CO447 corn plants against the aggressive strain, Cn14-5-1, prompted us to investigate whether the inability of Cn14-5-1 to cause disease on the primed CO447 came from the antagonistic effect of DOAB232 strains against Cn14-5-1 on the plant surface, or whether DOAB232 induced plant defense mechanisms prior to Cn14-5-1 inoculation. Filtrates from either strain were efficient in inhibiting the bacterial growth of the other one, compared to controls (Fig. 2A and B). As a next step, CO447 plants pre-treated either with the weakly aggressive DOAB232 strain or with its filtrate were tested against Cn14-5-1. In fact, pretreatment with DOAB232 filtrate did not confer disease resistance in CO447 against Cn14-5-1 (Fig. 2C and D).



**Fig. 5.** Salicylic acid regulates the late events of corn resistance to *C. nebraskensis*. RT-qPCR transcript levels of the SA-regulated genes, pathogenesis-related proteins *ZmPR-1* (A and B), *ZmPR-5* (C and D) and *ZmPRB1-3* (E and F) in response to Cn14-5-1 of CO450 and CO447 corn lines were measured. Leaves were sampled at 2, and 5 dpi of Cn14-5-1. Primed CO447 plants were pretreated with DOAB232 48 h prior to Cn14-5-1 inoculation. Reference gene, actin. Three biological replicates were used to calculate the mean and standard error. Letters on bars indicate statistically significant differences; one-way ANOVA; post hoc least significant difference;  $P < 0.05$ .

### 3.3. Plant defense in primed CO447 plants in response to Cn14-5-1

The defense response of the primed CO447 plants to inoculation with the aggressive strain, Cn14-5-1, was analyzed by investigating the expression levels of well-known corn defense-related genes using real-time PCR in primed CO447 plants compared to unprimed CO447 and resistant CO450 plants. The transcript levels of ethylene biosynthetic genes, *ZmACO20* (Fig. 3A) and *ZmACO35* (Fig. 3B), as well as the ROS-related genes, *ZmRbohA* (Fig. 3C), *ZmRbohB* (Fig. 3D), *ZmRbohC* (Fig. 3E) and *ZmRbohD* (Fig. 3F) were highly induced in primed CO447 plants at 3 hpi of Cn14-5-1 with 6.7-, 5.6-, 5-, 2.3-, 3.8- and 4.2-fold enhancement, respectively in comparison with control plants. On the contrary, there was no significant increase in the expression of ethylene and ROS genes in CO450 and unprimed CO447 plants (Fig. 3A–F). All CO450 and primed CO447 plants showed either no increase or a significant downregulation of all tested ethylene and ROS genes at 2 and 5 dpi of Cn14-5-1 (Fig. S1A–F, supplementary material).

Treatment with Cn14-5-1 caused a 12-fold increase in *ZmSIT2* expression at 0 dpi in primed CO447, and no increase or a minor increase (3-fold) was recorded in CO450 and unprimed CO447, respectively, compared with the control uninoculated plants (Fig. 4A). Also, the *ZmSIT2* expression in CO450 and primed CO447 increased by 6.1- and 5.5-fold, respectively, at 2 dpi and by 4.1- and 2.8-fold, respectively, at 5 dpi compared to their relative control plants (Fig. 4B, C). Likewise, Cn14-5-1 caused 26-fold increase in the transcript abundance of the *ZmBCP* gene at 0 dpi in primed CO447 and no significant increase in CO450 or unprimed CO447 compared with their relative control uninoculated plants (Fig. 4D). Also, the *ZmBCP* transcript levels in CO450 and primed CO447 increased by 2.2- and 2-fold, respectively, at 2 dpi and by 1.6- and 5.2-fold, respectively, at 5 dpi in comparison with their relative control plants (Fig. 4E, F). The fold increase of *ZmSIT2* and *ZmBCP* in primed CO447 plants at 2 and 5 dpi was significant but at a much lower extent compared to 0 dpi, whereas unprimed CO447 plants did not show an increase in *ZmSIT2* or *ZmBCP* expression levels at 2 and 5 dpi (Fig. 4A–F). Moreover, only primed CO447 plants showed a 9.4-fold increase of the probable LRR receptor-like serine/threonine-protein kinase, *ZmLPK*, at 0 dpi, whereas no increase in the transcript abundance of *ZmLPK* was recorded in all lines at 2 dpi (Fig. S2A and B, supplementary material). *ZmSIT2*, *ZmBCP*, and *ZmLPK* genes were selected based on a transcriptome analysis that has been done in the susceptible CO447 plants inoculated with the highly aggressive strain Cn14-5-1 at 5 dpi compared to control plants, which showed a huge significant upregulation of *ZmSIT2* at 4.79E + 11-fold and a downregulation of *ZmBCP* and *ZmLPK* at 0.055136- and 8.47E-08-fold, respectively (Table S2, supplementary material).

The transcript abundances of *PR-1*, *PR-5*, and *PRB1-3* in primed CO447 plants were promoted by 9.2-, 15.2-, and 87-fold, respectively, at 2 dpi (Fig. 5A, C, and E) and by 5.9-, 15.8-, and 2698-fold, respectively, at 5 dpi compared to control plants (Fig. 5B, D, and F). Furthermore, the *PR-1*, *PR-5*, and *PRB1-3* transcript levels increased by 9.1-, 32.5-, and 8.1-fold, respectively, at 2 dpi (Fig. 5A, C, and E) and by 2.1-, 8.1-, and 2.9-fold, respectively, at 5 dpi in CO450 plants compared to control plants (Fig. 5B, D, and F). On the other hand, *PR-1*, *PR-5*, and *PRB1-3* expression levels were not induced in Cn14-5-1-stressed unprimed CO447 plants at all tested time points (Fig. 5A–F).

## 4. Discussion

Goss's bacterial wilt and blight disease exhibits two distinct stages: the systemic wilt phase and the leaf blight phase (Clafin, 1999). The leaf blight phase is more prevalent and primarily impacts the upper plant canopy. The loss of yield caused by the leaf blight phase is generally not as significant as the loss caused by the systemic wilt phase (Langemeier et al., 2017). On the other hand, the systemic wilt phase typically emerges after early-season injuries to plants and can be lethal to young plants, resulting in decreased stand density (Pataky, 1989).

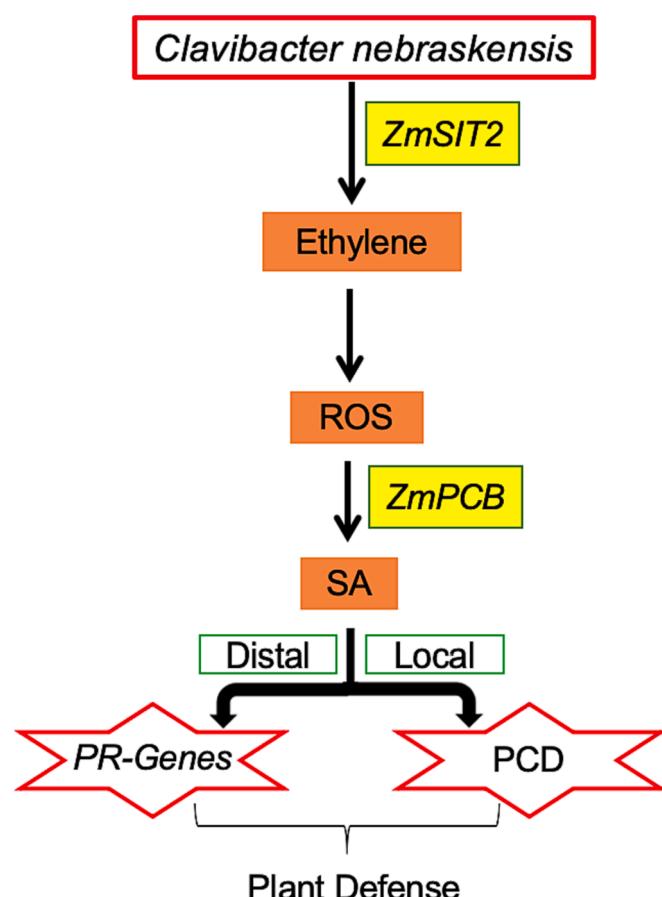
Consequently, we try to prime the defense signaling of plants to limit the spread of leaf blight and wilt phases. We investigated the primed-induced responses of susceptible corn plants treated with a small concentration of the weakly aggressive strain, DOAB232, to inoculation with the highly aggressive strain Cn14-5-1 of *C. nebraskensis*. Induced defense responses occurred in the primed susceptible CO447 corn plants upon inoculation by Cn14-5-1 (Fig. 1). Bacterial filtrates from either DOAB232 or Cn14-5-1 strains were efficient in inhibiting the bacterial growth of the other one compared to controls. This could be attributed to the ability of both strains to synthesize virulence factors, including  $\beta$ -glucosidases,  $\beta$ -galactosidases, chitinases,  $\beta$ -1,4-xylanases, and proteases. However, it is worth noting that the less aggressive strain exhibits a comparatively diminished expression of these factors (Soliman et al., 2021b). Pre-treatment with the filtrate of DOAB232 did not confer disease resistance in CO447 against Cn14-5-1, suggesting that any antagonistic effect of DOAB232 against Cn14-5-1 on the plant surface had no role in potentiating the defense responses of CO447 plants (Fig. 2).

To identify molecular markers of the priming state, we investigated the effect of the DOAB232 stress on the plant transcriptional response of selected defense-related genes to *C. nebraskensis*. SA and PCD are very important in controlling how maize reacts to Goss's wilt. The action of SA and ROS, which is represented by hydrogen peroxide ( $H_2O_2$ ), along with other important components, is likely to trigger the development of disease resistance against Goss's wilt (Shumilak et al., 2023). Also, it has been reported that tolerance against *C. nebraskensis* can be triggered through the substantial repression of the maize phytoglobin *ZmPgb1.1* by inducing a hypersensitive response (HR), itself mediated by the activation of ethylene and ROS genes (Owusu et al., 2019). In the context of Goss's wilt, the expression of RbohD was found to be elevated in maize at the site of infection by *C. nebraskensis*, indicating a potential involvement in the corn defense mechanisms against this causal agent (Owusu et al., 2019; Shumilak et al., 2023). As a result, we assayed the transcript levels of the ethylene biosynthetic genes, *ZmACO20* and *ZmACO35*, and the ROS biosynthetic genes, *ZmRbohA*, *ZmRbohB*, *ZmRbohC*, and *ZmRbohD*, which were induced in primed CO447 plants at 3 hpi of Cn14-5-1, compared to mock-treated and unprimed CO447 and CO450 plants (Fig. 3). The repressed or unenhanced expression levels of all tested ethylene and ROS genes at 2 and 5 dpi of Cn14-5-1 infection in CO450, unprimed CO447, and primed CO447 plants (Fig. S1A–F, supplementary material) suggest that the activation of ethylene and ROS are among the early events that are required to induce the defense mechanisms of corn in response to Goss's wilt.

Under salt stress, *Salt Intolerance 1 (SIT1)*, a lectin receptor-like kinase, and its homolog *SIT2* (Os04g44900) are activated, causing ethylene and ROS accumulation and MAPKs activation, which lead to oxidative stress and plant death (Li et al., 2014). The high expression levels of blue copper binding protein (BCB; At5g20230) were associated with ROS scavenging in Arabidopsis (Kim et al., 2011). Also, it has been shown that suppressing *GhUMC1*, a blue copper-binding protein, can hinder the accumulation of ROS and considerably weaken the JA and SA signaling pathways, which raises cotton's susceptibility to *Verticillium dahliae* (Zhu et al., 2018). Treatment with Cn14-5-1 caused an increase in the transcript abundance of *ZmSIT2* and *ZmBCP* at 0, 2, and 5 dpi of Cn14-5-1 in primed CO447, compared to unprimed CO447. In the meantime, the resistant CO450 showed high expression levels of both genes at 2 and 5 dpi only, but not at 0 dpi (Fig. 4). The transcriptome profiling of the susceptible CO447 plants inoculated with the highly virulent strain Cn14-5-1 at 5 dpi showed an upregulation and a reduction of the transcript levels of *ZmSIT2* and *ZmBCP* genes, respectively (Table S2, supplementary material). The huge upregulation of *ZmSIT2* at 5 dpi in our transcriptomic analysis is suggested to be either a late response that could not confer resistance to plants or a high resultant accumulation of ethylene and ROS that leads to the abolishment of defense responses because it is reported that free radicals, such as nitric oxide and ROS, mediate SAR in Arabidopsis plants in a concentration-dependent manner where high ROS levels cannot confer SAR (Wang

et al., 2014). Together, these results suggest that *ZmSIT2* and *ZmBCP* are key mediators of ethylene and ROS homeostasis in plant tissues upon infection with *C. nebraskensis*, that ethylene and ROS mediate early signaling defense responses in a dose-dependent manner, and that timing of the triggered plant defenses is important to confer resistance against Goss's wilt in corn.

Priming initiates signal amplification, which results in a more quick and robust activation of defense responses (Conrath et al., 2006). Both local resistance and SAR are frequently associated with PR accumulation (van Loon et al., 2006). Salicylic acid is a crucial mediator of the HR by activating the associated disease resistance mechanisms and is involved in a feedback loop both upstream and downstream of cell death (Alvarez, 2000; Dangl et al., 1996; Rossi et al., 2011). Systemic vascular infections can be caused by *C. nebraskensis*, a biotrophic pathogen that can exist in the xylem vessels of its host plants, if infection starts early at the seedling stage (Eichenlaub and Gartemann, 2011). In general, biotrophic pathogens trigger SA signaling and the production of pathogenesis-related (PR) proteins, while necrotrophic pathogens and chewing insects elicit JA-mediated responses (Thaler et al., 2012). Systemic resistance can prime cells, leading to a more robust elicitation of diverse defense responses, and SA, which plays a crucial role in activating plant defense mechanisms, is produced locally and could elicit SAR in plants. Nevertheless, the quick glycosylation of SA and its phytotoxic properties have hindered the use of SA as a chemical agent for plant protection (Tripathi et al., 2019). Additionally, it has been reported that *PR-1* genes possess the ability to suppress PCD in the first lesion induced by *Pseudomonas syringae* pv. *tabaci*, once the pathogen is successfully controlled through PCD (Lincoln et al., 2018). The induction of *ZmPR-1* expression levels by Cn14-5-1 was significantly higher in the CO450 plants as compared to the CO447 plants (Shumilak et al., 2023). Our data showed that the transcript abundance of pathogenesis-related proteins *PR-1*, *PR-5*, and *PRB1-3* in the primed susceptible CO447 and resistant CO450 plants were highly induced at 2, and 5 dpi of Cn14-5-1 infection in comparison with control plants and unprimed CO447 plants (Fig. 5). These results are consistent with previous studies, which showed that SA induction following *C. nebraskensis* infection triggers plant defense responses in corn (Da Silva et al., 2021). Also, high and quick SA-responsive transcript levels accumulate in the leaves of resistant corn plants to *C. nebraskensis* (Da Silva et al., 2021; De Ilarduya et al., 2003). Similarly, *C. nebraskensis* inoculation resulted in elevated expression levels of the pathogen-responsive genes *PR1* and *PR5*, which serve as molecular markers of the SA pathway (Singh et al., 2019). Rice stripe virus (RSV) and *Phytophthora infestans* infections are reported to induce the expression of *PRB1-3* in *OsCIPK30* [a CBL (calcineurin B-like proteins)-interaction protein kinase protein] and respiratory burst oxidase homolog A (*StRbohA*) in overexpressing rice and potato plants, respectively, in comparison with wild-type (WT) plants (Liu et al., 2017; Soliman et al., 2021a). Our analyses showed a stronger induction of *PRB1-3* than *PR-1* and *PR-5* in primed CO447 plants at 0, 2, and 5 dpi of Cn14-5-1 infection. This is in line with a previous study, which reported that among *PR1*, *PR10*, *PRB1-2*, and *PRB1-3*, RSV inoculation strongly promoted the transcript levels of only *PRB1-3* in WT plants (Liu et al., 2017). The increase in the expression levels of probable LRR receptor-like serine/threonine-protein kinase, *ZmLPK*, at 0 dpi of Cn14-5-1 infection in the primed CO447 plants (Fig. S2, supplementary material) and the significant downregulation of *ZmLPK* at 5 dpi in the unprimed CO447 plants (Table S2, supplementary material) suggest that *PRB1-3* induction is a key player among the PR genes when plant defense responses are associated with protein kinases. Collectively, these data imply that priming and induced resistance are dependent on SA for mediating HR and induction of the late events of the defense mechanisms against Goss's wilt in corn. Our simplified model illustrating chemical signaling during priming and direct defense against Goss's wilt shows that the activation of ethylene and ROS are among the early signaling events of plant defense responses via upregulation of *ZmSIT2*. The excess ethylene and ROS are scavenged by *ZmBCP*. The ethylene and



**Fig. 6.** A proposed model illustrating chemical signaling during corn defense responses to Goss's wilt disease against *C. nebraskensis*. Inoculation of the *C. nebraskensis* pathogen triggers early and late signaling events of plant defense responses. As early events, the L-type lectin-domain containing receptor kinase gene (*ZmSIT2*) is activated, promoting ethylene and ROS accumulation in local tissues, and leading to programmed cell death (PCD) at the infection site. The excess ethylene and ROS are scavenged by the blue copper protein gene, *ZmBCP*. The ethylene and ROS cellular levels are regulated via *ZmSIT2* and *ZmBCP*. As late events, salicylic acid (SA) accumulates in local and distal tissues, which is represented by the upregulation of pathogenesis related proteins (PR). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ROS cellular levels are mediated by both *ZmSIT2* and *ZmBCP*. SA accumulates in local and distal tissues, which is represented by the upregulation of pathogenesis-related proteins (PR). Both SA and ROS trigger PCD at the infection site, and the activation of SA responsive pathways in distal tissues ensures the optimal induction of SAR (Fig. 6). In conclusion, the process of priming corn plants with the less aggressive strain of *C. nebraskensis* promoted the prompt and effective initiation of defensive mechanisms in corn plants, resulting in a comprehensive resistance against the more aggressive strain.

#### CRediT authorship contribution statement

**Mohamed El-Shetehy:** Conceptualization, Methodology, Data curation, Writing – original draft. **Mohammad Sayari:** . **Fouad Daayf:** Conceptualization, Supervision, Resources.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

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