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ORIGINAL ARTICLE

Carbonic anhydrases CA1 and CA4 function in atmospheric CO_2-modulated disease resistance

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Abstract

Main conclusion **Carbonic anhydrases CA1 and CA4 attenuate plant immunity and can contribute to altered disease resistance levels in response to changing atmospheric CO_2 conditions.**

Abstract β-Carbonic anhydrases (CAs) play an important role in CO_2 metabolism and plant development, but have also been implicated in plant immunity. Here we show that the bacterial pathogen *Pseudomonas syringae* and application of the microbe-associated molecular pattern (MAMP) flg22 repress *CA1* and *CA4* gene expression in *Arabidopsis thaliana*. Using the CA double-mutant *ca1ca4,* we provide evidence that CA1 and CA4 play an attenuating role in pathogen- and flg22-triggered immune responses. In line with this, *ca1ca4* plants exhibited enhanced resistance against *P. syringae*, which was accompanied by an increased expression of the defense-related genes *FRK1* and *ICS1*. Under low atmospheric CO_2 conditions (150 ppm), when CA activity is typically low, the levels of *CA1* transcription and resistance to *P. syringae* in wild-type Col-0 were similar to those observed in *ca1ca4*. However, under ambient (400 ppm) and elevated (800 ppm) atmospheric CO_2 conditions, *CA1* transcription was enhanced and resistance to *P. syringae* reduced. Together, these results suggest that CA1 and CA4 attenuate plant immunity and that differential *CA* gene expression in response to changing atmospheric CO_2 conditions contribute to altered disease resistance levels.

Keywords Arabidopsis · CO_2 metabolism · Defense signaling · Plant immunity · *Pseudomonas syringae*

Abbreviations

CA	Carbonic anhydrase
ET	Ethylene
JA	Jasmonic acid
MAMP	Microbe-associated molecular pattern
Pst	*Pseudomonas syringae* pv. *tomato* DC3000
Psm	*Pseudomonas syringae* pv. *maculicola* 4326
PTI	Pattern-triggered immunity
SA	Salicylic acid

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Introduction

Plants have evolved a complex immune system to regulate survival from attack by pathogenic microbes and herbivorous insects. Upon perception of microbe-associated molecular patterns (MAMPs) by pattern recognition receptors (PRRs), defense responses are activated, including stomatal closure, production of reactive oxygen species, MAP kinase activation, hormonal signaling, and massive transcriptional reprogramming, which leads to the production of defensive compounds that limit pathogen ingress (Tsuda and Katagiri 2010; Zipfel and Robatzek 2010; Couto and Zipfel 2016). These induced signal outputs collectively lead to pattern-triggered immunity (PTI), which forms the first layer of plant defense to invading microbes. Evidence is accumulating that changing climate conditions can have profound effects on plant resistance pathways (Noctor and Mhamdi 2017; Kazan 2018; Velasquez et al. 2018). Atmospheric CO_2 is an important parameter of climate change. Changes in atmospheric CO_2 levels can affect disease development in diverse plant-pathogen interactions (Chakraborty et al. 2000; Garrett et al. 2006; Zavala et al. 2013; Mhamdi and Noctor 2016; Zhou et al. 2017; Williams et al. 2018b). Elevated CO_2 caused reduced multiplication of *potato virus Y* in tobacco plants (Matros et al. 2006) and decreased downy mildew severity in soybean plants (Eastburn et al. 2010). In contrast, the susceptibility of wheat plants to *Fusarium pseudograminearum* was increased by elevated CO_2 (Melloy et al. 2014). In tomato, elevated CO_2 levels rendered the plants more resistant to *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*), while the level of resistance against *Botrytis cinerea* decreased (Zhang et al. 2015). Furthermore, exposure of the model plant species *Arabidopsis thaliana* (hereafter Arabidopsis) to pre-industrial, current and future levels of atmospheric CO_2 uncovered marked effects on plant immunity against diverse (hemi) biotrophic and necrotrophic pathogens (Mhamdi and Noctor 2016; Zhou et al. 2017, 2019; Willams et al. 2018b). Changes in atmospheric CO_2 levels not only affect plant-pathogen interactions, but also impact the interaction of plants with mutualistic mycorrhizal fungi and plant growth-promoting rhizobacteria (Werner et al. 2018; Williams et al. 2018a). Hence, to produce climate resilient crops in the future, it is important to understand how changes in atmospheric CO_2 levels impact plant–microbe interactions.

Carbonic anhydrases (CAs) are metalloenzymes that were initially purified from red blood cells and mainly function as catalysts in the interconversion of CO_2 and bicarbonate (Meldrum and Roughton 1933). There are at least five distinct CA families (α, β, γ, δ, and ε CAs), three of which (α, β, and γ CAs) are ubiquitously distributed among animal, plant, and bacterial species. The widespread distribution and abundance of these CA families underline their evolutionary importance throughout the kingdom of life. βCAs represent the most prominent group of CAs in plants. They are involved in a wide range of biological processes, including CO_2 homeostasis, stomatal aperture, respiration, photosynthesis, pH regulation, and anther cell differentiation (Henry 1996; Smith and Ferry 2000; Hu et al. 2010; Engineer et al. 2014; Huang et al. 2017). Arabidopsis contains six *βCA* genes (*AtβCA1-6*), which are mostly expressed in aboveground tissues (Wang et al. 2014). Several studies reported on the implication of CAs in plant defense. For instance, the expression of a plastidic *CA* gene was found to be repressed in potato leaves upon challenge with the potato late blight pathogen *Phytophthora infestans* (Restrepo et al. 2005). Similarly, expression of the *CA* gene *TC52686* in grapevine was suppressed during infection with the grapevine downy mildew pathogen *Plasmopara viticola* (Polesani et al. 2008). In contrast, five CA proteins were shown to be more abundant in a proteomic analysis of non-heading Chinese cabbage infected with the downy mildew *Hyaloperonospora parasitica* (Sun et al. 2014). These reported alterations in CA transcript or protein levels in pathogen-infected plants point to a potential role of CAs in plant defense. Indeed, a positive role in plant defense was demonstrated for a chloroplast-localized CA of tobacco, also known as SALICYLIC ACID (SA)-BINDING PROTEIN 3 (SABP3), as silencing of this *CA* gene in *Nicotiana benthamiana* leaves suppressed the hypersensitive response mediated by the *Pto:avrPto* resistance gene:effector gene pair (Slaymaker et al. 2002). Moreover, *CA*-silenced *N. benthamiana* was more susceptible to *P. infestans* than the wild type, supporting the notion that CAs contribute to disease resistance (Restrepo et al. 2005).

Despite the accumulating evidence for a role of CAs in plant immunity (Wang et al. 2009), little is known about how their regulation or action affects plant-pathogen interactions. CAs are mainly known as responders and actors in atmospheric CO_2-mediated signaling. For example, under elevated CO_2 conditions, both transcript abundance and enzymatic activity of CAs have been shown to decrease in several plant species (Porter and Grodzinski 1984; Webber et al. 1994; Majeau and Coleman 1996). Moreover, stomatal closure under high CO_2 conditions and stomatal opening under low CO_2 conditions is hampered in the Arabidopsis CA double-mutant *ca1ca4* (Hu et al. 2010). Stomata are entry points of many leaf pathogens. Since activation of PTI triggers the closure of stomata to prevent pathogen entry (Melotto et al. 2008), changes in CA-mediated stomatal aperture may impact disease resistance. Recently, Medina-Puche et al. (2017) reported that several Arabidopsis CAs interact with the transcriptional coregulator NONEXPRESSOR

OF PATHOGENESIS-RELATED GENES 1 (NPR1) and NONRECOGNITION OF BTH-4 (NRB4), thereby modulating the perception of the plant defense hormone salicylic acid (SA) in plants. SA is produced during the onset of PTI and plays an important regulatory role in plant immunity (Klessig et al. 2018). Hence, under changing atmospheric CO_2 conditions, CA-mediated changes in SA responses may have an effect on the level of disease resistance. Previous observations that SA-dependent defenses in Arabidopsis are modulated under changing atmospheric CO_2 conditions (Mhamdi and Noctor 2016; Williams et al. 2018b) support this hypothesis.

Given the importance of CA1 and CA4 in Arabidopsis' responsiveness to changing CO_2 levels (Hu et al. 2010), we chose to investigate the role of these two CAs in plant immunity and determined their effect on CO_2-modulated defense using the model plant-pathogen system Arabidopsis *P. syringae*. We provide evidence that suppression of *CA1* and *CA4* gene expression is involved in the plant defense response to *P. syringae* infection and that CA1 and CA4 act as negative regulators of plant immunity, likely through antagonizing SA-mediated signaling. We also found that differential expression of *CA1* under different atmospheric CO_2 conditions is correlated with an altered level of disease resistance against *P. syringae* and that CA1 and CA4 are required for the effects of CO_2 on disease resistance against *P. syringae*.

Materials and methods

Cultivation of plants and bacterial strains

For experiments with soil-grown plants, seeds of *Arabidopsis thaliana* accession Col-0 (Arabidopsis Biological Resource Center (ABRC) stock number CS1092) and mutant *ca1ca4* (Hu et al. 2010; kindly provided by Julian Schroeder, UCSD, San Diego, CA, USA) were sown on autoclaved river sand. Two weeks later, seedlings were transferred to 60-ml pots containing a sand/potting soil mixture that was autoclaved twice for 20 min. Plants were grown in a climate chamber with a 10-h day at 20 °C and 14-h night at 18 °C cycle (350 µmol m^{-2} s^{-1}) with 70% relative humidity. For experiments with different atmospheric CO_2 treatments, 2-week-old seedlings in 60-ml pots either stayed in the growth room (ambient; 450 ppm) or were transferred to similar growth rooms with exactly the same conditions, except for CO_2 levels, which were high (800 ppm), or low (150 ppm; Zhou et al. 2017). Plants were grown for the remainder of the experiment under different CO_2 conditions. The technical specifications of the CO_2-controlled growth chambers used in this study were described in detail by Temme et al. (2015).

For experiments with in vitro-grown plants, seeds of Arabidopsis accession Col-0 and mutants *aba2-1* (Koornneef et al. 1982; ABRC stock number CS156), *coi1-1* (Feys et al. 1994; kindly provided by Jane Glazebrook, University of Minnesota, St. Paul, MN, USA), *npr1-1* (Cao et al. 1994; ABRC stock number N3726), *ein2-1* (Guzman and Ecker 1990; ABRC stock number N3071), and *fls2* (Shan et al. 2008; ABRC stock number SALK_141277) were surface sterilized in gas of a mixture of household chlorine (Glorix original, Unilever, Vlaardingen, the Netherlands) and HCl (37%; 97:3) for 3–4 h. Sterile seeds were subsequently sown on agar plates or in liquid. The agar plates contained Murashige and Skoog (MS) medium (Duchefa Biochemie, Haarlem, the Netherlands), pH 5.9, supplemented with 5 mM MES buffer, 10 g l^{-1} sucrose and 0.85% (w/v) plant agar (Duchefa Biochemie). When plants were 2-weeks old, they were transferred to liquid medium to be treated with flg22 (see "MAMP treatment"). For experimental conditions in which seeds were sown immediately in liquid MS, see "MAMP treatment".

Pseudomonas syringae pv. *tomato* DC3000 (*Pst*) and its corresponding effector-deficient mutant *Pst hrpA*$^-$ (de Torres et al. 2003; Truman et al. 2006), and *Pseudomonas syringae* pv. *maculicola* ES4326 (*Psm*) and its corresponding coronatine-deficient mutant *Psm cor*$^-$ (Dong et al. 1991) were grown on King's B medium (King et al. 1954) agar plates supplemented with 50 µg ml^{-1} rifampicine at 28 °C.

Pseudomonas syringae inoculation and bioassay

Pseudomonas syringae inoculation and the disease resistance assay were performed as described (Van Wees et al. 2013). For dip inoculation, the bacterial inoculum was diluted to a final concentration of 5×10^7 cfu ml^{-1} of 10 mM $MgSO_4$ containing 0.015% (v/v) Silwet L-77 (Van Meeuwen Chemicals, Weesp, the Netherlands). For pressure infiltration, the bacterial suspension was adjusted to a concentration of 4×10^7 cfu ml^{-1} unless specified otherwise. Bacterial growth *in planta* was determined as described (Zhou et al. 2019). Eight biological replicates were included for each time point.

Pseudomonas syringae disease symptoms were scored in the following classes according to their severity: class 1, 0–10% chlorotic or water-soaked area per leaf; class 2, 10–50% chlorotic or water-soaked area per leaf; class 3, > 50% chlorotic or water-soaked area per leaf. Six fully grown and morphologically similar leaves per plant were chosen for scoring and 12 plants were scored per treatment. The average *P. syringae* disease index per plant was calculated using the formula:

$$\sum_{c=1}^{3}(c \times \text{the number of leaves in class } c)/6$$

c, the value of the class (1, 2, or 3). The resulting continuous data were tested for normal distribution by the Shapiro–Wilk test and if they passed, they were subsequently subjected to parametric tests for statistical analysis as indicated in the legends.

Botrytis cinerea bioassay

Botrytis cinerea strain B05.10 (Van Kan et al. 1997) was used for the inoculation of 4-week-old plants. Spore inoculation and disease resistance assay were performed as described previously (Van Wees et al. 2013; Zhou et al. 2019). Disease symptoms were scored at 3 days after inoculation. The average disease index was calculated similarly as described above for the *P. syringae* disease index.

MAMP treatment

For gene expression analysis of plants treated with the MAMPs flg22 or nlp20, 2-week-old in vitro-grown seedlings were transferred from agar plates to 24-well plates containing 1.5 ml of liquid MS with 5 mM MES per well and kept overnight at room temperature. Subsequently, a solution of 0.5 ml of MS + MES supplemented with flg22 (Sigma), or nlp20 (kind gift of Thorsten Nürnberger, Universität Tübingen, Germany (Böhm et al. 2014)) was added to obtain the final concentration (as indicated in the figure legends). The rosette leaves or the whole seedlings were harvested for RNA extraction at indicated time points.

For the growth inhibition assay, seeds of Col-0 and *ca1ca4* were surface sterilized and sown in 96-well plates with 200 ml liquid MS + MES per well, supplemented or not with flg22 (1, 10, or 100 nM) from a 100 mM stock solution of flg22. The dry weight was measured when the seedlings were 2-weeks old.

ROS measurement

For the ROS assay, plants were grown at 20 °C in an 8-h light/16-h dark cycle in growth chambers. Leaf discs from 5-week-old plants were floated on water overnight. The water was replaced with 100 µl of a solution containing 20 µM luminol (Sigma), 1 µg horseradish peroxidase (Fluka, Buchs, Switzerland) and 100 nM flg22. ROS production was measured as previously described (Mersmann et al. 2010; Roux et al. 2011). Twelve leaf discs from 5-week-old plants were used for each condition. Luminescence of each sample was measured over 60 min continuously using a high-resolution photon counting system (HRPCS218, Photek, East Sussex, UK) coupled to an aspherical wide lens (Sigma).

Gene expression by qRT-PCR

Total RNA isolation and qRT-PCR were performed as described previously (Oñate-Sánchez and Vicente-Carbajosa 2008; Zhou et al. 2017), using the constitutively expressed reference gene *At1g13320* (Czechowski et al. 2005), encoding protein phosphatase PP2AA3, and the $2^{-\Delta\Delta C_T}$ method (Schmittgen and Livak 2008) to calculate relative changes in gene expression. Three biological replicates were taken for each data point. Primers used for qRT-PCR are listed in Supplemental Table S1.

Stomatal aperture measurement

To measure stomatal aperture, a modified protocol of dental resin impressions was used (Geisler et al. 2000; Zhou et al. 2017). Stomata were photographed under an Olympus microscope. Analysis D Olympus Software was used to examine the stomata on the pictures taken. Stomatal aperture was assessed by measuring the width and length of the stomata. At least 20–30 observations per leaf were recorded on at least six leaves per treatment.

Results

Repression of *CA1* and *CA4* expression upon infection by *P. syringae* independently of type-III effectors and coronatine

Transcriptional repression of *CA* genes in response to attack by diverse pathogens has been reported for various plant species. Genevestigator analysis (Zimmermann et al. 2004) of the six β-group members of the Arabidopsis *CA* genes shows that *CA1*, *CA2*, *CA4*, and *CA5* display a predominantly reduced expression pattern in response to infection by diverse plant pathogens, while *CA3* and *CA6* show a more variable profile (Supplemental Table S2). For this study, we chose to investigate the role of *CA1* and *CA4* in Arabidopsis immunity to *P. syringae* under ambient and altered CO_2 conditions, because of their previously reported role in CO_2-mediated responses (Hu et al. 2010). We first monitored the expression of *CA1* and *CA4* upon *P. syringae* infection of Arabidopsis cultivated under ambient CO_2 conditions. In mock-infiltrated Col-0 leaves, *CA1* and *CA4* showed a similar basal expression pattern over time, in which the highest level was reached at 24 h after mock treatment (11:00 am) (Fig. 1a, b). This corroborates with a previously reported finding on diurnal rhythm of *CA* gene expression in *Chlamydononas reinhardtii* (Fujiwara et al. 1996), which may

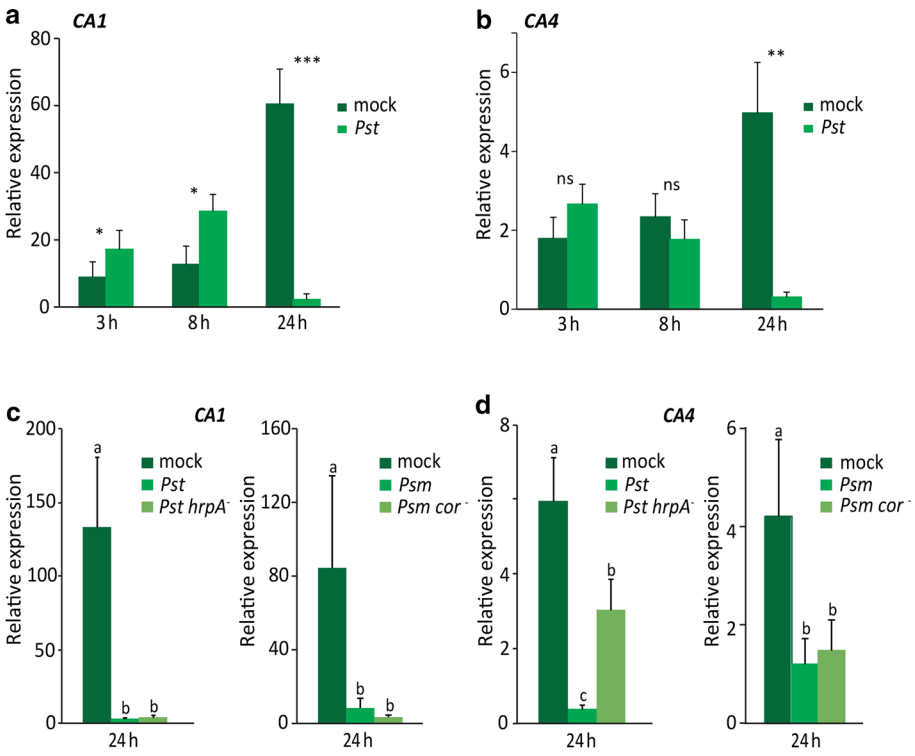

Fig. 1 *Pseudomonas syringae* represses the expression of *CA1* and *CA4* independently of hrpA-dependent effectors and coronatine. **a** *CA1* and **b** *CA4* expression levels relative to the reference gene *At1g13320* in leaves of 4-week-old Col-0 plants at 3, 8, and 24 h after pressure infiltration with mock (10 mM $MgSO_4$) or *Pst* (4×10^6 cfu ml^{-1}). Asterisks indicate statistically significant differences between mock and *Pst* treatment at specific time points (Student's *t* test; $*P<0.05$; $**P<0.001$; $***P<0.0001$; *ns* not significant). **c** *CA1* and **d** *CA4* expression levels relative to the reference gene *At1g13320* in leaves of 4-week-old Col-0 at 24 h after pressure infiltration with mock (10 mM $MgSO_4$), *Psm* or *Psm cor*$^-$ (1×10^7 cfu ml^{-1}), or *Pst* or *Pst hrpA*$^-$ (1×10^8 cfu ml^{-1}). Different letters indicate statistically significant differences between treatments (one-way ANOVA; Fisher's LSD test; $P<0.05$). Error bars represent SD, $n=3$ plants

be associated with diurnal variations in cellular CO_2 levels. At 24 h after pressure infiltration of Arabidopsis leaves with *Pst*, the expression levels of *CA1* and *CA4* were significantly suppressed in comparison to the mock treatment (Fig. 1a, b). Together with the Genevestigator results (Supplemental Table S2), these results suggest that repression of *CA* gene expression is a common plant response to pathogen infection.

Pseudomonas syringae produces a suite of effector molecules, including the phytotoxin coronatine, which act to suppress plant defenses and promote infection (Mittal and Davis 1995; Brooks et al. 2005; Dou and Zhou 2012). To determine whether these virulence factors have a role in the suppression of *CA* gene expression, we tested the effect of infection by a *Pst hrpA*$^-$ mutant, which is defective in the type-III secretion system that translocates effectors into the plant host cell, and a *Psm cor*$^-$ mutant, which is defective in coronatine production. We compared the *CA1* and *CA4* expression levels after infiltration of the leaves with the mutant strains versus their respective wild-type *P. syringae* strains *Pst* and *Psm*. Confirming the findings displayed in Fig. 1a, b, *CA1* and *CA4* were significantly repressed 24 h after infection with wild-type *Pst* and *Psm* (Fig. 1c, d). Infection by the mutant strains *Pst hrpA*$^-$ and *Psm cor*$^-$ repressed *CA1* to the same extent as the respective wild-type *P. syringae* strains (Fig. 1c). Also, *CA4* expression was significantly suppressed by the *P. syringae* mutants, although the effect of *Pst hrpA*$^-$ was less pronounced than that of wild-type *Pst* (Fig. 1d). Together, this suggests that repression of *CA1* and *CA4* in Arabidopsis by infection with *P. syringae* is largely independent of effectors and coronatine.

Suppression of *CA* gene expression is a MAMP-induced response

Next, we tested whether the suppression of *CA* gene expression by *P. syringae* might be a MAMP-induced response. To this end, we examined the expression pattern of *CA1* and *CA4* in response to treatment with flg22, the 22-amino acid immunogenic epitope of the bacterial MAMP flagellin. As shown in Fig. 2a, the expression of the flg22-induced marker gene *FRK1* was significantly enhanced in Col-0 plants from

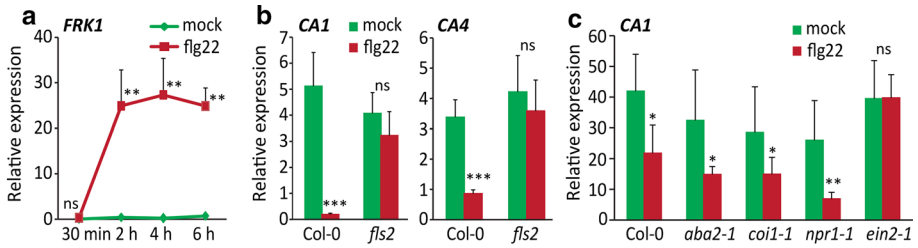

Fig. 2 Expression of defense-related marker gene *FRK1* and *CA1* and *CA4* in response to flg22 treatment. **a** Expression of *FRK1* relative to the reference gene *At1g13320* in response to treatment with flg22 (500 nM) or water (mock) in 2-week-old Col-0 seedlings at specified time points after treatment (30 min and 2, 4, and 6 h). Asterisks indicate statistically significant differences between mock and flg22 treatment at specific time points (Student's *t* test; $*P<0.05$; $**P<0.01$; *ns* not significant). **b** *CA1* and *CA4* expression levels relative to the reference gene *At1g13320* in 2-week-old seedlings of Col-0 and *fls2,* 24 h after flg22 (125 nM) or mock treatment. **c** *CA1* expression levels relative to the reference gene *At1g13320* in 2-week-old seedlings of Col-0, *aba2-1*, *coi1-1*, *npr1-1*, and *ein2-1* at 8 h after flg22 (500 nM) or mock treatment. Asterisks indicate statistically significant differences between mock and flg22 treatment within each genotype (two-way ANOVA; Fisher's LSD test; $***P<0.001$; $**P<0.01$; $*P<0.05$; *ns* not significant). Error bars represent SD, $n=3$ plants

2 h after flg22 treatment onwards, indicating that the flg22 treatment had been effective. The expression of *CA1* and *CA4* was examined at 24 h after flg22 application in both wild-type Col-0 and the flg22 receptor mutant *fls2* (Gómez-Gómez and Boller 2000; Shan et al. 2008). At 24 h after flg22 application, both *CA1* and *CA4* were significantly suppressed in Col-0 plants, whereas this repression by flg22 was compromised in the *fls2* mutant (Fig. 2b). This indicates that the suppression of *CA* genes occurs downstream of the recognition of the MAMP flg22. Besides flg22, analysis of available Genevestigator microarray data also show repression of *CA1* and/or *CA4* by other defense elicitors, such as EF-Tu (elf18), necrosis-inducing *Phytophthora* protein 1 (NPP1), lipopolysaccharide (LPS), oligosaccharides (OGs), *Serratia plymuthiaca* HRO-C48 volatiles, and peptide 2 (Pep2; Supplementary Table S3). Also, the *Hyaloperonospora arabidopsidis* MAMPs necrosis and ethylene-inducing peptide (Nep1)-like proteins (HaNLPs) significantly repress *CA1* and *CA4* gene expression in Arabidopsis (Oome et al. 2014). Collectively, these results suggest that suppression of *CA1* and *CA4* gene expression is a general MAMP-induced response in Arabidopsis.

Plant hormones such as SA, ethylene (ET), jasmonic acid (JA), and abscisic acid (ABA) have all been implicated in the regulation of PTI (Tsuda and Katagiri 2010; Cao et al. 2011). To investigate whether these hormones play a role in MAMP-induced suppression of *CA* genes, we determined the expression of *CA1* in response to flg22 treatment in mutants impaired in synthesis of ABA (*aba2-1*), or responsiveness to JA (*coi1-1*), SA (*npr1-1*), or ET (*ein2-1*). We observed that the suppression of *CA1* by flg22 occurred to the same extent in the mutants *aba2-1*, *coi1-1*, and *npr1-1* as in wild-type Col-0 (Fig. 2c). In contrast, the *ein2-1* mutant did not display suppression of *CA1* gene expression in response to flg22 treatment; however, it has been demonstrated that ET signaling is required for the steady-state expression of the Arabidopsis flg22 receptor gene *FLS2* (Boutrot et al. 2010; Mersmann et al. 2010). The lack of flg22-mediated suppression of *CA1* in *ein2* may, therefore, be explained by a diminished recognition of flg22 rather than diminished signaling downstream of recognition by FLS2. The results with the ABA-, JA-, and SA-related mutants suggest that ABA-, JA-, and SA-dependent signaling are not likely to be important for in flg22-mediated suppression of *CA1* gene expression.

Enhanced MAMP responsiveness in mutant *ca1ca4*

After MAMP perception, multiple responses are activated (e.g. oxidative burst, stomatal closure, and SA accumulation), which are often accompanied by a substantial transcriptional reprogramming (Yu et al. 2017). To gain insight in the function of CAs in plant immunity, we examined several flg22-induced responses in the *ca1ca4* double mutant, which carries T-DNA insertions in the *CA1* and *CA4* genes (Hu et al. 2010). Figure 3a shows that flg22 induced *FRK1* to a significantly higher level in *ca1ca4* than in Col-0. Likewise, significantly augmented transcript levels of the SA biosynthesis gene *ICS1* were induced in the *ca1ca4* mutant after flg22 application (Fig. 3b). Transcript levels of the flg22 receptor gene *FLS2* remained unaltered in *ca1ca4* compared to Col-0 (Fig. 3c), indicating that the effects of CA1 and CA4 on defense-related gene expression are not due to differences in *FLS2* expression. Similar to flg22, the oomycete MAMP nlp20, which is the active 20-amino acid immunogenic epitope of HaNLPs (Böhm et al. 2014), also induced enhanced transcript levels of the SA-responsive genes *FRK1* (Fig. 3a) and *ICS1* in *ca1ca4* (Fig. 3b), suggesting that CA1 and CA4 broadly affect MAMP-induced transcription of the defense-related marker genes.

Flg22 treatment causes strong growth inhibition in Arabidopsis seedlings (Gómez-Gómez et al. 1999). To assay for flg22-mediated growth inhibition, Col-0 and *ca1ca4*

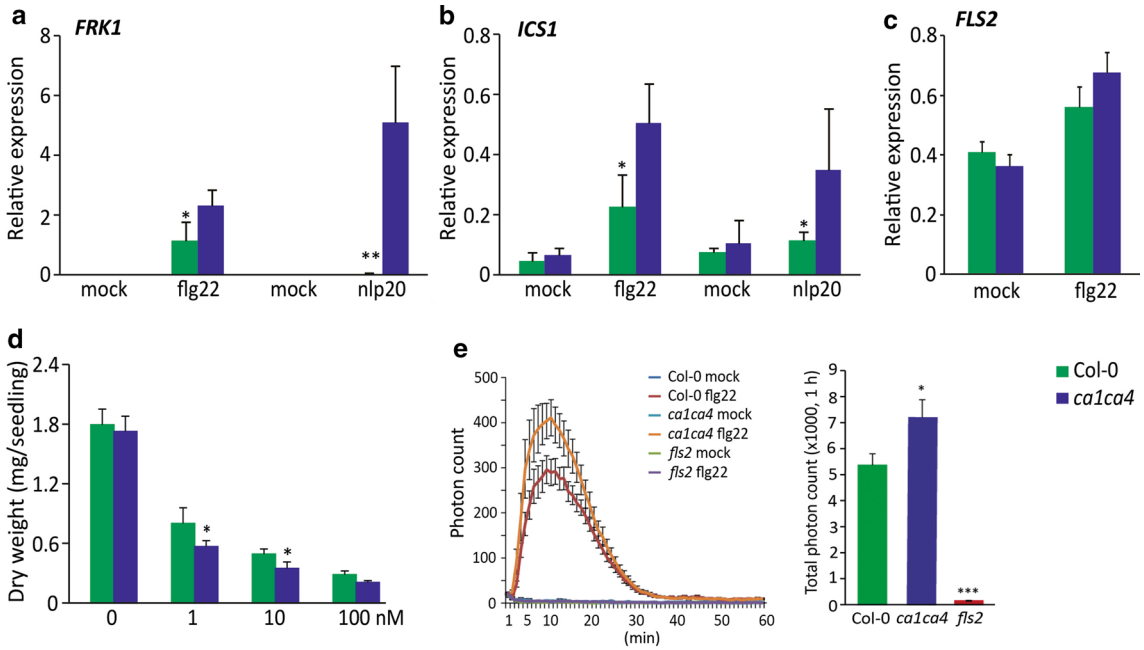

Fig. 3 Augmented defense responses in the *ca1ca4* mutant upon flg22 and nlp20 treatment. **a** *FRK1*, **b** *ICS1*, and **c** *FLS2* expression levels relative to the reference gene *At1g13320* in 2-week-old seedlings of wild-type Col-0 and mutant *ca1ca4* plants at 24 h after flg22 (500 nM), nlp20 (100 nM) or mock treatment. Asterisks indicate statistically significant differences between Col-0 and *ca1ca4* within the same treatment (Student's *t* test; $*P<0.05$; $**P<0.01$). Error bars represent SD, $n=3$ seedlings. **d** Dry weight of 2-week-old seedlings of Col-0 and *ca1ca4* cultivated in the presence of 0, 1, 10, or 100 nM flg22. Depicted are the averages of dry weight per seedling. Asterisks indicate statistically significant differences between Col-0 and *ca1ca4* within the same treatment (Student's *t* test; $*P<0.05$). Error bars represent SD, $n=8$ seedlings. **e** ROS burst induced by flg22 (100 nM) or mock treatment in leaf discs of Col-0, *ca1ca4*, and *fls2*. Depicted in the left panel are photon counts in each genotype after mock or flg22 treatment at indicated time points after flg22 treatment. The right panel depicts cumulative ROS production (photon counts) within 1 h after flg22 treatment. Asterisks indicate statistically significant differences between mutants and wild-type Col-0 (one-way ANOVA, Fisher's LSD test; $*P<0.05$; $***P<0.001$). Error bars represent SE, $n=4/12$ (mock/flg22) leaf discs

seedlings were grown for 2 weeks in the presence of flg22 after which their dry weight was determined. Col-0 displayed more than 50% growth reduction after treatment of 1 nM flg22 (Fig. 3d). Interestingly, growth of *ca1ca4* was reduced to a significantly greater extent than Col-0 after treatment with 1 nM and 10 nM flg22. Another feature of the flg22-induced defense response is the generation of reactive oxygen species (ROS; Nühse et al. 2007). The flg22-triggered ROS burst was significantly enhanced in the *ca1ca4* mutant compared to that in Col-0 (Fig. 3e). Together, these results show that *ca1ca4* plants display an augmented response to flg22 treatment, resulting in enhanced defense-related gene expression and a greater MAMP-mediated inhibition of seedling growth. From this, we conclude that CA1 and CA4 play a role in repressing MAMP-mediated defense responses.

CA1 and CA4 reduce resistance to *Pst*

To further investigate the function of CAs in plant disease resistance, we tested the responsiveness of Col-0 and *ca1ca4* to infection with *Pst*. Pathogen-induced stomatal closure to inhibit pathogen entry has been established as an important defense response in plant resistance against *P. syringae* pathogens (Melotto et al. 2006). Therefore, we first determined whether CA1 and CA4, which are highly abundant in guard cells and control stomatal aperture (Hu et al. 2010), play a role in *Pst*-induced stomatal closure and opening. We tested stomatal responsiveness in Col-0 and *ca1ca4* after treatment with *Pst* by dip inoculation, upon which the bacteria enter the leaf interior through stomatal openings. Consistent with previous results (Melotto et al. 2006), Col-0 plants reacted by closing their stomata between 1 and 2.5 h after *Pst* inoculation, and subsequent reopening at 4 h (Fig. 4a, b). As demonstrated previously (Hu et al. 2010), we observed that the stomatal aperture of *ca1ca4* is significantly higher than that of Col-0 (Fig. 4a). Moreover, *Pst*-induced stomatal closure was delayed in *ca1ca4* and became only apparent at 7 h after inoculation (Fig. 4a, b). The delayed stomatal closure triggered by *Pst* infection in *ca1ca4* supports the notion that *CA1* and *CA4* are involved in *Pst*-induced stomatal movements.

Next, we performed disease resistance assays with Col-0 and *ca1ca4* in which growth of *Pst* and disease symptoms

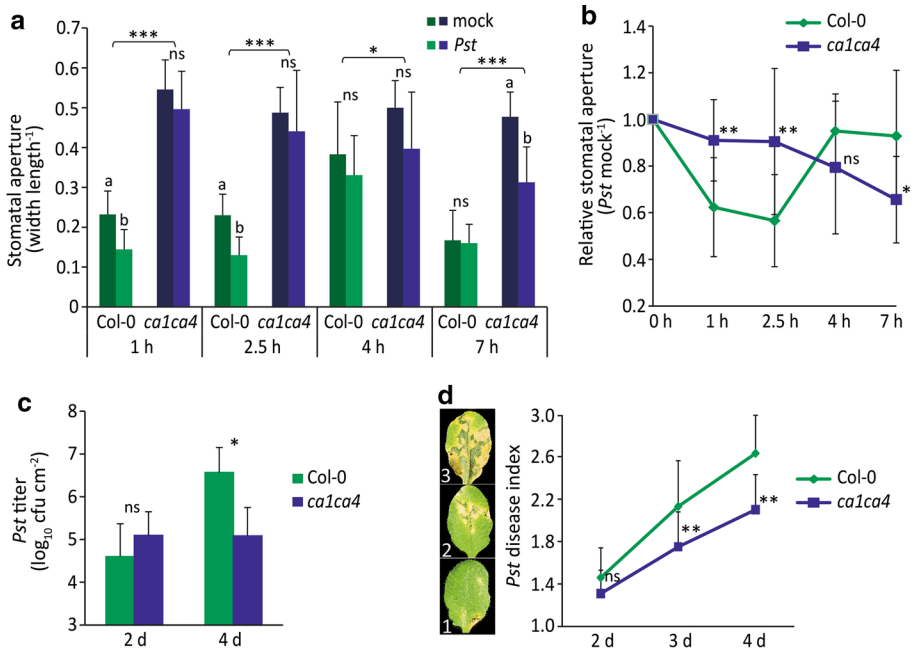

Fig. 4 CA1 and CA4 influence stomatal aperture and resistance to *P. syringae*. **a** Stomatal aperture in leaves of 4-week-old wild-type Col-0 and mutant *ca1ca4* plants at 1, 2.5, 4, and 7 h after dip inoculation with *Pst* (5×10^7 cfu ml^{-1}). Indicated are the averages of stomatal aperture (±SD) of six leaves. Different letters indicate statically significant differences between mock and *Pst* treatment within the same genotype (two-way ANOVA; Fisher's LSD test; $P<0.01$; *ns* not significant). Indications above the brackets specify whether there is an overall statistically significant difference between Col-0 and *ca1ca4* at specific time points (***$P<0.001$; *$P<0.05$). Error bars represent SD, $n=6$ leaves. **b** Stomatal apertures in *Pst*-treated leaves relative to mock (10 mM $MgSO_4$)-treated leaves at 0, 1, 2.5, 4, 7 h after treatment. Asterisks indicate statistically significant differences between Col-0 and *ca1ca4* at specific time points (Student's *t* test; **$P<0.01$; *$P<0.05$; *ns* not significant). Error bars represent SD, $n=6$ leaves. **c** Bacterial growth in 4-week-old Col-0 and *ca1ca4* plants at 2 and 4 days after dip inoculation with *Pst* (5×10^7 cfu ml^{-1}). Indicated are the averages of log_{10}-transformed bacterial titers per leaf area. Asterisks indicate statistically significant differences between Col-0 and *ca1ca4* at specific time points (Student's *t* test; *$P<0.05$; *ns* not significant). Error bars represent SD, $n=8$ plants. **d** Disease symptom severity on 4-week-old Col-0 and *ca1ca4* plants at 2, 3, and 4 days after pressure infiltration with *Pst* (6×10^5 cfu ml^{-1}). Indicated is the average of the disease index calculated from the percentage of leaves in three different disease severity classes. Class 1, 0–10% chlorotic or water-soaked area per leaf; class 2, 10–50% chlorotic or waters-oaked area per leaf; class 3, >50% chlorotic or waters-oaked area per leaf. Asterisks indicate statistically significant differences between Col-0 and *ca1ca4* at specific time points (Student's *t* test; **$P<0.01$; *ns* not significant). Error bars represent SD, $n=12$ plants

were monitored. Double-mutant *ca1ca4* exhibited a bacterial titer that was significantly lower than that of Col-0 plants at 4 d after dip inoculation with *Pst* (Fig. 4c). Plants were also inoculated with *Pst* by pressure infiltration, which bypasses stomatal defense. Figure 4d shows that the *ca1ca4* mutant developed significantly fewer disease symptoms than Col-0 plants at 3 and 4 days after infiltration. Together, these results show that CA1 and CA4 negatively impact disease resistance to *Pst*, with no clear role for stomatal defense, suggesting that post-invasion defenses are antagonized.

CA1 and CA4 antagonize SA-responsive gene expression upon *Pst* infection

In Arabidopsis, SA plays an important role in activating defense against *P. syringae* (Pieterse et al. 2012). To investigate whether CA1 and CA4 interfere with SA-dependent defenses, we infiltrated leaves of Col-0 and *ca1ca4* plants with *Pst* and subsequently monitored expression levels of the SA-responsive genes *PR1, PR2, FRK1*, *ICS1*, *WRKY22*, and *WRKY29*. Figure 5 shows that all tested SA-responsive genes were induced by *Pst* to a significantly higher level in *ca1ca4* than in Col-0 and most of the genes showed a slightly enhanced basal expression level in the *ca1ca4* mutant. Conversely, the JA-responsive marker gene *VSP2* was significantly suppressed in *Pst*-infected *ca1ca4* plants (Fig. 5). These data suggest that CA1 and CA4 may modulate plant immunity by affecting SA- and JA-dependent plant responses.

CA1 and CA4 are involved in atmospheric CO_2-affected disease resistance against *Pst*

Atmospheric CO_2 levels have been shown to influence plant development and defense (Velasquez et al. 2018). In Arabidopsis, plant growth and the level of resistance against *P. syringae* is also impacted by changes in atmospheric CO_2

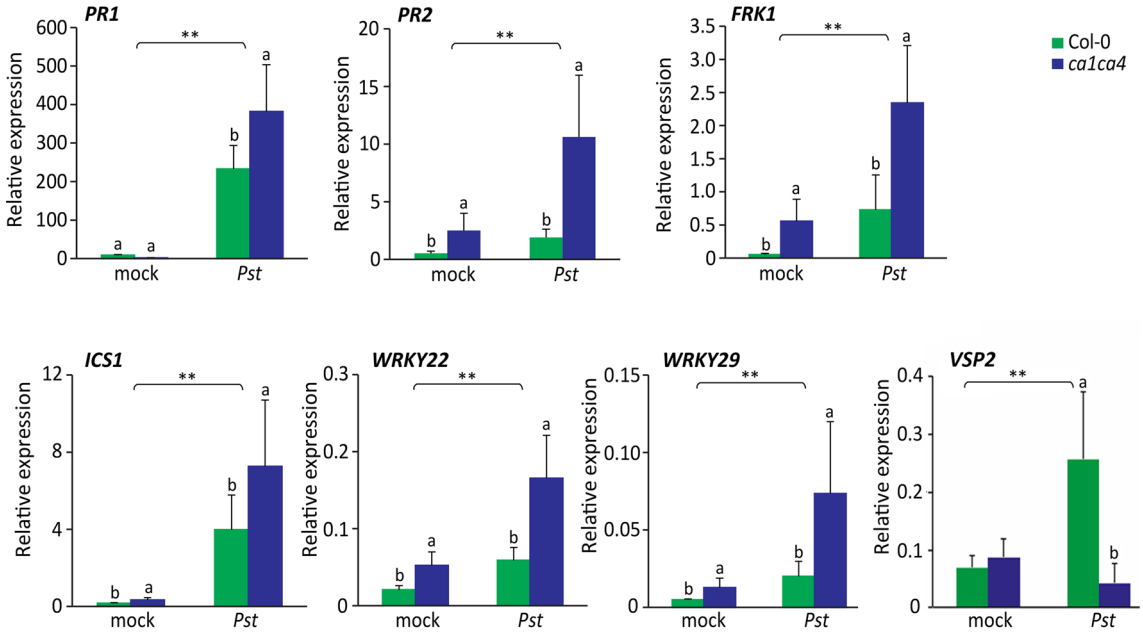

Fig. 5 SA- and JA-responsive gene expression in the mutant *ca1ca4* upon infection by *P. syringae*. *PR1*, *PR2*, *FRK1*, *ICS1*, *WRKY22, WRKY29,* and *VSP2* expression levels relative to the reference gene *At1g13320* in 4-week-old Col-0 and *ca1ca4* plants 24 h after infiltration with *Pst* (4×10^7 cfu ml^{-1}) or 10 mM $MgSO_4$ (mock). Error bars represent SD, $n=3$ plants. Different letters indicate statically significant differences between Col-0 and *ca1ca4* within the same treatment (two-way ANOVA; Fisher's LSD test; $P<0.05$). Indications above the brackets specify whether there is an overall statistically significant difference between mock and *Pst* treatment ($^{**}P<0.01$)

levels (Zhou et al. 2017, 2019). In the present study, we tested the role of CA1 and CA4 in the effect of changes in atmospheric CO_2 levels on Arabidopsis disease resistance against *Pst*. We found that the disease resistance of Arabidopsis Col-0 plants against *Pst* was decreased under high CO_2 (800 ppm) compared with that under ambient CO_2 (450 ppm), whereas it was enhanced under low CO_2 conditions (150 ppm; Fig. 6a), confirming previous findings (Zhou et al. 2017). In the *ca1ca4* double mutant, the level of *Pst* resistance was at all three atmospheric CO_2 levels as high as

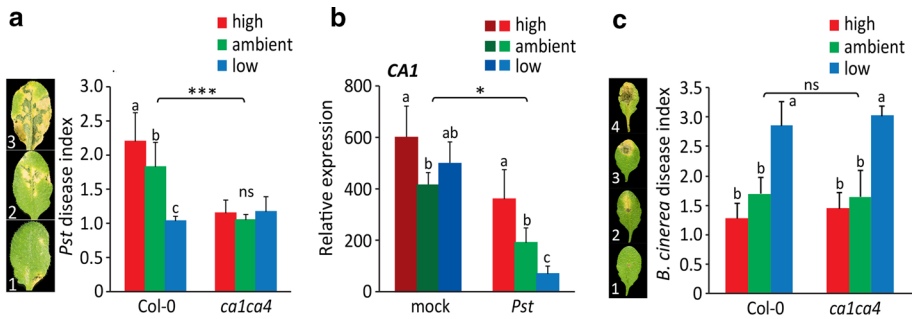

Fig. 6 The role of CAs in atmospheric CO_2-modulated disease resistance to *Pst* and *B. cinerea*. Arabidopsis wild-type Col-0 and mutant *ca1ca4* plants were grown under high (800 ppm), ambient (450 ppm) and low (150 ppm) levels of atmospheric CO_2 until 4-week old and dip inoculated with *Pst* (**a**, **b**) or drop inoculated with *B. cinerea* (**c**). **a** Disease severity in Col-0 and *ca1ca4* at 4 d after dip inoculation with *Pst* (4×10^7 cfu ml^{-1}). Shown is the average *Pst* disease index calculated from the percentage of six leaves per plant belonging to different disease severity classes. Error bars represent SD, $n=12$ plants. **b** *CA1* expression levels relative to the reference gene *At1g13320* in Col-0 grown under high, ambient and low levels of atmospheric CO_2 at 24 h after dip inoculation with *Pst* (1×10^8 cfu ml^{-1}) or 10 mM $MgSO_4$ (mock). Error bars represent SD, $n=3$ plants. **c** Disease severity in Col-0 and *ca1ca4* plants inoculated with *B. cinerea* (1×10^6 spores ml^{-1}). Disease symptoms were scored 4 days after inoculation. Shown is the average of the disease index calculated from the percentage of leaves in four different disease severity classes. Error bars represent SD, $n=12$ plants. Different letters indicate statistically significant differences between CO_2 treatments within the same genotype. Indications above the brackets specify the interaction (Arabidopsis genotype × CO_2 conditions) between Col-0 and *ca1ca4* and the three CO_2 conditions (two-way ANOVA; Fisher's LSD test; $^{*}P<0.05$; $^{***}P<0.001$; *ns* not significant).

that observed in Col-0 under low CO_2 (Fig. 6a), suggesting that in wild-type plants, the CAs play a role in the modulation of atmospheric CO_2-affected disease resistance to *Pst*. This was confirmed by the observation that the *Pst*-mediated suppression of *CA1* gene expression becomes stronger with decreasing CO_2 concentrations in the atmosphere (Fig. 6b).

In Arabidopsis, changes in atmospheric CO_2 levels can also affect disease resistance against necrotrophic fungi (Williams et al. 2018b; Zhou et al. 2019). To test whether CAs play a role in this process, we tested the resistance of Arabidopsis plants to the necrotrophic pathogen *B. cinerea* at three different atmospheric CO_2 levels. We found that high CO_2-grown Col-0 plants developed less disease symptoms compared with plants grown under ambient and low CO_2 conditions (Fig. 6c), confirming that Arabidopsis disease resistance against *B. cinerea* increases as atmospheric CO_2 levels increase (Zhou et al. 2019). Mutant *ca1ca4* also displayed increasing levels of disease severity with increasing CO_2 levels, which was similar to that observed in Col-0 (Fig. 6c), suggesting that CA1 and CA4 do not influence atmospheric CO_2-altered disease resistance to *B. cinerea*.

Discussion

During the last decade, our understanding of the mechanisms involved in plant immune signaling greatly increased (Couto and Zipfel 2016; Cheng et al. 2019; Nobori and Tsuda 2019). Evidence is accumulating that climate change parameters can have profound effects on plant immunity (Noctor and Mhamdi 2017; Kazan 2018; Velasquez et al. 2018). As one of the core characteristics of global climate change, the increasing atmospheric CO_2 level has been shown to affect various plant-pathogen systems (Chakraborty et al. 2000; Garrett et al. 2006; Yáñez-López et al. 2014). In the present study, we revealed that the β-carbonic anhydrases CA1 and CA4 of Arabidopsis modulate plant immune responses and that they are likely involved in CO_2-modulated plant defense against *Pst*.

Upon *P. syringae* infection, expression of the *CA1* and *CA4* genes in Arabidopsis was strongly repressed (Fig. 1). This occurred largely independently of hrpA-dependent effectors and coronatine (Fig. 1c, d). We further demonstrated that repression of *CA1* and *CA4* is triggered by the MAMPs flg22 and nlp20 (Fig. 2). This suggests that repression of *CA1* and *CA4* is part of the Arabidopsis defense response when under attack by *P. syringae*. The inability of the *ein2-1* mutant to repress *CA1* expression (Fig. 2c) most likely results from the strongly reduced expression of *FLS2* in *ein2-1* (Boutrot et al. 2010; Mersmann et al. 2010). However, a role for ET signaling in repression of *CA1* expression downstream of flg22 recognition cannot be ruled out. Nonetheless, the repression of *CA1* by flg22 occurred independently of ABA, JA, or SA signaling (Fig. 2c).

In Arabidopsis, perception of flg22 triggers multiple responses, such as activation of defense-related genes and growth inhibition (Yu et al. 2017). Our results with the double-mutant *ca1ca4* show significantly enhanced expression levels of two defense-related marker genes, *FRK1* and *ICS1*, as well as stronger growth inhibition compared to wild-type plants upon treatment with flg22 (Fig. 3). The *FLS2* expression levels were unaffected by the *ca1ca4* mutation (Fig. 3c), suggesting the enhanced activation of plant immune responses by flg22 is not likely due to an enhanced capacity of flg22 recognition. This is supported by the observation that another MAMP, nlp20, also triggered enhanced expression of the defense-related marker genes *FRK1* and *ICS1* in *ca1ca4* (Fig. 3a, b). Collectively, these data indicate that suppression of *CAs* is part of the basal plant immune response, thereby positively contributing to the activation of defenses against the pathogen encountered.

CAs have been reported to control CO_2 homeostasis and stomatal aperture. Consistent with previous findings (Henry 1996; Smith and Ferry 2000; Hu et al. 2010), our results showed that the *ca1ca4* mutant displayed greater stomatal aperture than wild-type Col-0 plants (Fig. 4a). Moreover, stomatal closure, which is part of the defense response induced upon inoculation with *Pst*, is delayed in *ca1ca4* as it is detected starting at 4 h while in Col-0, closure is already evident at 1 h after inoculation (Fig. 4a, b). This points to a positive role of the CAs in stomatal defenses. However, despite the larger opening of the stomata throughout the first 7 h and the delay in the stomatal closure response, the *ca1ca4* mutant exhibited enhanced resistance to *Pst* compared to wild-type Col-0 in both the *Pst* dipping and infiltration assays (Fig. 4c, d). Although CA1 and CA4 positively regulate stomatal defenses, these results suggest that they negatively influence other post-invasion plant defense responses, likely those mediated by SA signaling, as the *ca1ca4* mutant showed significantly augmented SA-responsive gene expression upon infection with *Pst* (Fig. 5).

Previous studies have shown a positive involvement of CAs in plant defense against avirulent *Pst* strains. For instance, in tobacco, silencing of the CA known as SA-BINDING PROTEIN 3 (SABP3), led to suppression of the *Pto:avrPto*-mediated hypersensitive defense response (Slaymaker et al. 2002). Also in Arabidopsis, the orthologue AtSABP3, which is also named CA1 (used in this study), is required for expression of full defense against the avirulent bacterial pathogen *Pst* carrying *avrB* (Wang et al. 2009). Our results showed that *CA1* gene expression was similarly repressed upon infection by wild-type *Pst* and its correspondent effector mutant *Pst hrpA*$^-$ (Fig. 1c). Still, the CA1 protein could be a potential target of type-III effectors of *Pst* as the abundance of CA1 was shown to be reduced to

a lesser extent upon infection by wild-type *Pst* than by its correspondent effector mutant *Pst hrpA*$^-$ (Jones et al. 2006). The dual role of CAs in defense against virulent and avirulent *Pst* strains suggests differential actions of CAs during compatible and incompatible interactions between a host and its pathogens.

One important aspect of prevailing climate changes is the elevation of atmospheric CO_2 and this has boosted research on plant defenses under elevated CO_2 conditions (Restrepo et al. 2005; Polesani et al. 2008; Noctor and Mhamdi 2017; Kazan 2018; Velasquez et al. 2018; Williams et al. 2018b). CAs are important enzymes in CO_2 metabolism and we addressed whether they play a role in atmospheric CO_2—affected plant disease resistance. Previous reports have shown that elevated CO_2 increased the disease resistance of tomato plants against *Pst* (Li et al. 2014; Zhang et al. 2015). In contrast, we found enhanced disease susceptibility to *P. syringae* and increased resistance against *B. cinerea* in Arabidopsis plants grown at increasing CO_2 levels (Fig. 6; Zhou et al. 2017, 2019). This suggests that the response of plants to changes in the level of atmospheric CO_2 is plant species specific.

Arabidopsis defense against *P. syringae* and *B. cinerea* is largely regulated by SA and JA signaling, respectively (Glazebrook 2005). Previously, we showed that increasing atmospheric CO_2 levels lowered the level of resistance against *Pst*, while the level of resistance against *B. cinerea* increased (Zhou et al. 2019). This opposite effect on resistance against the (hemi)biotroph *Pst* and the necrotroph *B. cinerea* is likely due to the antagonistic interaction between the SA and the JA defense pathways (Pieterse et al. 2012). In this study, we confirmed our previous observation that with increasing CO_2 levels, Arabidopsis resistance against *Pst* decreases, while resistance against *B. cinerea* increases (Fig. 6). Moreover, we show that in mutant *ca1ca4,* the effect of the atmospheric CO_2 level on *Pst* resistance is lost, while for *B. cinerea*, the CO_2 effect on resistance remains unaltered in comparison to Col-0 (Fig. 6). These results suggest that CAs predominantly have an impact on SA-mediated resistance.

Williams et al. (2018b) also tested the effect of both elevated and sub-ambient levels of atmospheric CO_2 on disease caused by necrotrophic (*Plectosphaerella cucumerina*) and biotrophic (*H. arabidopsidis*) pathogens. They observed enhanced resistance against the necrotroph under elevated CO_2 conditions, corroborating our findings with *B. cinerea* (Fig. 6c). However, in contrast to our observations, resistance against the biotroph was also enhanced under elevated CO_2 conditions. Similar observations were done by Mhamdi and Noctor (2016) who found enhanced resistance against both *Pst* and *B. cinerea* under conditions of elevated CO_2. However, in the latter, study plants were grown at long day conditions (16 h light/8 h dark versus 10 h light/14 h dark in our study) and at very high CO_2 levels (3000 ppm versus 800 ppm in our study), suggesting that effects of CO_2 on pathogen resistance are conditionally determined by prevailing environmental factors. Interestingly, both Williams et al. (2018b) and Mhamdi and Noctor (2016) provided evidence that the effect of changed atmospheric CO_2 levels on plant immunity is associated with cellular redox status. We found that the mutant *ca1ca4* developed a stronger oxidative burst in response to flg22 treatment than did wild-type plants (Fig. 3e), confirming the notion that CAs may modulate plant immunity via changes in cellular redox processes. Different atmospheric CO_2 levels may also directly affect the growth rate or pathogenicity of the microbial pathogens, but we did not test to what extend this contributed to the disease outcome in our experiments.

In conclusion, our results show that induction of defense responses in *P. syringae*-infected Arabidopsis plants results in the repression of *CA1* and *CA4* gene expression. This leads to the alleviation of CA-mediated suppression of SA-dependent defenses and consequently increased disease resistance against *Pst* (Fig. 7). Changes in atmospheric CO_2 influence CA activity, which as a result impact SA-dependent defenses against *Pst*, possibly via changes in the cellular redox status (Mhamdi and Noctor 2016). Collectively, our results provide new leads for future investigations on plant adaptation to global environmental changes. A more

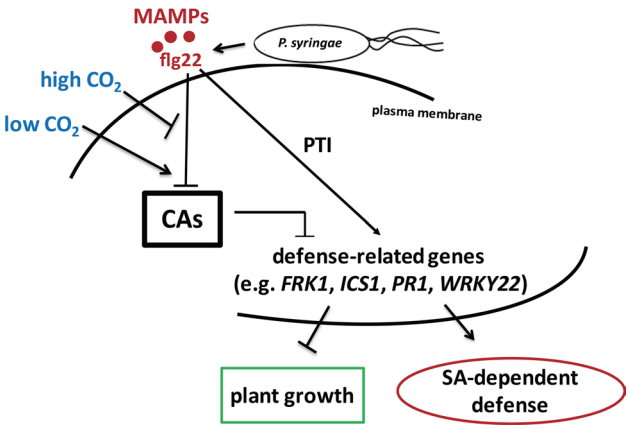

Fig. 7 A model of CO_2-modulated, MAMP-induced suppression of CA1 and CA4 that alleviate attenuation of SA-dependent defenses during the plant immune response to infection by *P. syringae*. Upon attack by *P. syringae*, plants recognize the flg22 epitope of the MAMP flagellin, resulting in repression of *CA1* and *CA4* gene expression. In uninduced plants, CAs have an antagonizing effect on PTI-mediated responses. Recognition of flg22 results in suppression of *CA*s and increased defense-related gene expression, ultimately leading to enhancement of SA-dependent resistance to *P. syringae* and inhibition of plant growth. At a low atmospheric CO_2 level, repression of *CA1* by *Pst* is enhanced, while at a high CO_2 level, *CA1* repression is reduced. This contributes to an increase versus a decrease in resistance levels against *Pst* at low and high CO_2 levels, respectively. Arrows, induction; blocked lines, repression

comprehensive analysis of the exact function of CAs in plant defense, including the βCAs that were not investigated here, will be subject of future study.

Author contribution statement YZ and SCMVW designed the experiments. YZ performed most of experiments and analyzed the data. Other authors assisted in experiments and discussed the results. YZ, IAV-V, CMJP, and SCMVW wrote the manuscript.

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