Modulation of plant immunity by atmospheric CO_2

Yeling Zhou

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INVITATION

You are cordially invited to

attend the public defence of

my PhD thesis.

Modulation of plant immunity by atmospheric CO_2

Tuesday, May 10th, 2016,

at 2:30 pm in Utrecht University,

Academy Building,

Domplein 29, Utrecht

There will be a reception afterwards

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Paranymphs:

Yujuan Du and Erqin Li

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Modulation of plant immunity by atmospheric CO_2

Modulatie van het afweersysteem van planten door atmosferische CO_2

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen
op dinsdag 10 mei des middags te 2.30 uur

door

Yeling Zhou

geboren op 17 oktober 1986 te Hunan, China

Promotor: Prof. dr. ir. C.M.J. Pieterse

Copromotoren: Dr. S.C.M. van Wees
Dr. P.A.H.M. Bakker

迷眼乱花，脂粉年华。

暖玉顽石，混沌蒙世。

All the hustling and bustling, seemingly unrelated and useless, is what makes you in the world.

to my husband, my parents,
my sister and brother.

CONTENTS

CHAPTER 1

General introduction:

Modulation of plant immunity by atmospheric CO_2

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FOOD PRODUCTION AND THE INCREASING ATMOSPHERIC CO_2 LEVEL

Over the last decade, the world population has increased one billion and it has been projected to continue to grow, reaching 11.2 billion by 2100. To feed this growing world population, action is urgently needed to secure food production. Agricultural production of crops is suffering from diseases caused by plant pathogenic microorganisms. It has been estimated that more than 10% of global food production is lost due to plant diseases (De Wolf and Isard, 2007). In addition, crop production is influenced by global climatic changes, including increasing temperature, declining precipitation, and rising atmospheric CO_2 level (Change, 2014). These climate changes are predicted to reduce the yields of many primary crops (Brown and Funk, 2008), but they can also directly affect pathogens and the diseases caused by them (Newton et al., 2011).

The increase in atmospheric CO_2 is predicted to continue, and reach around 560 ppm by the year of 2050 (Hofmann et al., 2009). Whereas increased temperatures and declining precipitation may reduce crop yield, elevated atmospheric CO_2 is more likely to increase crop yield. Experimental evidence is accumulating that increased atmospheric CO_2 stimulates plant photosynthetic activities and enhances plant production (Ainsworth and Long, 2005; Ainsworth and Rogers, 2007). However, the stimulatory effects of elevated atmospheric CO_2 can vary significantly among plant species and experimental conditions (Nowak et al., 2004; Ainsworth and Long, 2005). In this Chapter current knowledge on effects of atmospheric CO_2 levels on plant growth and plant-microbe interactions is reviewed, with an emphasis on pathogenic interactions.

GENERAL PLANT RESPONSES TO INCRESED ATMOSPHERIC CO_2 LEVELS

Effects of atmospheric CO_2 levels on aboveground plant parts

Exposure of plants to elevated CO_2 leads to changes in leaf chemistry and physiology. Relatively consistent responses include the stimulation of photosynthesis, accumulation of sugar and starch content, and the decrease in stomatal conductance (Zavala et al., 2013). Other changes in leaf epidermal characteristics induced by elevated CO_2 include reduced stomatal density, guard cell length and trichome numbers (Teng et al., 2006; Lake and Wade, 2009). The majority of plants showing a positive relationship with CO_2 enrichment with respect to canopy photosynthesis rate also exhibit an increased ratio of carbon:nitrogen (C:N) in their leaves, resulting in decreased N availability (Ainsworth and Rogers, 2007). The reduced N availability is likely to limit the stimulatory effects of high CO_2 on plant growth (Luo et al., 2004; Reich et al., 2006). Indeed, many C_3 plant species respond to enriched atmospheric CO_2 with an initial stimulation of photosynthesis, which subsequently declines to levels similar to that of plants grown under ambient conditions, a phenomenon known as photosynthetic acclimation (Baker and Allen, 1994).

Increased atmospheric CO_2 can also induce changes in the secondary chemistry of plant leaves (Zavala et al., 2013). For instance, many defense-related chemical compounds, such as phenolics and antioxidant flavonoids tend to increase under elevated CO_2 and this response varies with plant species (Penuelas et al., 1997; Ghasemzadeh et al., 2010; Zavala et al., 2013). In addition, increased atmospheric CO_2 significantly increases multiple hormone levels in *Arabidopsis thaliana* (Arabidopsis), such as indol-3-acetic acid (IAA), gibberellic acid (GA), zeatin riboside (ZR), and indole-3-propionic acid (Li et al.), while the opposite was observed for the abscisic acid (ABA) content (Teng et al., 2006). The plant hormones salicylic acid (SA) and jasmonic acid (JA) are two hormones that play a pivotal role in disease resistance (Pieterse et al., 2012). The SA-mediated signaling pathway is generally enhanced by high CO_2, whereas the JA-mediated signaling pathway is reduced under elevated CO_2 (Casteel, 2010; Ghasemzadeh et al., 2010; Sun et al., 2011b; Zavala et al., 2013). In addition, components associated with the redox status within the cell can be affected by atmospheric CO_2 conditions. High CO_2-exposed oak and pine trees or the crop plant barley had significantly reduced activities of superoxide dismutase (SOD) and catalase and other antioxidative metabolites (Polle et al., 1996; Azevedo et al., 1998; Schwanz and Polle, 2001).

The influence of atmospheric CO_2 on photosynthesis is expected to change levels of organic carbon in the aboveground part of plants. Since a large part of carbon is lost by the plant in the soil, it seems likely that alterations in atmospheric CO_2 levels will also influence processes in and around the plant root.

Effects of atmospheric CO_2 levels on belowground plant parts

The enhanced photosynthesis and aboveground biomass production in plants grown under increased atmospheric CO_2 is likely to induce higher carbon allocation to the belowground plant tissues, resulting in enhanced root growth and increased root to shoot ratios (Pritchard, 2011). In line with this, increased fine root longevity, and changes in root architecture as well as the composition of root exudates were observed in CO_2-enriched atmosphere (Pritchard, 2011).

Direct effects of increased atmospheric CO_2 levels on soil borne microbes are unlikely in view of the high CO_2 concentrations in the soil, between 2,000 and 38,000 ppm in the pore part of active soil (Drigo et al., 2008; Pritchard, 2011). Nonetheless, the enhanced root growth and increased amounts of root exudates under elevated atmospheric CO_2 enrich soil carbon resources, ultimately changing population densities, structure and activities of root-associated microbial communities (Pendall et al., 2004; Carney et al., 2007; Phillips, 2007). Emerging studies have demonstrated that the composition and density of soil microbial communities is altered under elevated atmospheric CO_2 conditions (Drigo et al., 2009; Deng et al., 2012; He et al., 2012). In a semiarid shrub land ecosystem, soil fungal diversity, in terms of both richness and evenness, was increased in response to elevated atmospheric CO_2, and this was tightly linked to plant root production (Lipson et al., 2014). In addition, the

higher C:N ratio of rhizodeposition under elevated atmospheric CO_2 is likely to favor soil fungi over bacteria, which have a higher requirement for N than fungi (Drigo et al., 2009). Thus, a general shift from bacterial- to fungal-dominated soil food webs is expected in future CO_2-enriched scenarios. This shift could further result in significant changes in the structure and diversity of the microbial communities in the rhizosphere (Drigo et al., 2010; Drigo et al., 2013).

Thus, growth stimulation of aboveground plant tissues by elevated atmospheric CO_2 drives significant changes in the rhizosphere microbiome. These changes in the aboveground and belowground part of plants grown under elevated atmospheric CO_2 conditions are also expected to affect disease resistance in the future atmosphere. Effects of atmospheric CO_2 levels on plant disease resistance and the underlying potential mechanisms are reviewed below.

EFFECTS OF ATMOSPHERIC CO_2 LEVELS ON PLANT IMMUNITY

For the successful development of an infectious disease on a susceptible host, environmental conditions are extremely important (De Wolf and Isard, 2007). Changes in climate conditions, such as humidity, temperature, and atmospheric CO_2 level, can influence the severity of the disease. Moreover, environmental conditions not only affect the plant or the pathogen, but also their interaction, resulting in as yet unpredictable effects on disease incidence and severity (De Wolf and Isard, 2007). The direct impact of atmospheric CO_2 on plant pathogens is generally marginal (Wells, 1974; Drigo et al., 2008; Zhang et al., 2015). The *in vitro* growth of the bacterial plant pathogen *Pseudomonas syringae* and the fungal plant pathogen *Botrytis cinerea* was not affected by elevated CO_2 (Zhang et al., 2015). However, several studies have demonstrated that increased CO_2 can affect plant disease resistance, with the effects varying greatly in different plant pathosystems. For example, in oilseed rape (*Brassica juncea*) exposed to elevated CO_2 (550 ppm), a lower incidence and severity of Alternaria blight, caused by *Alternaria brassicae*, and downy mildew, caused by *Hyaloperonospora brassicae*, was observed (Mathur et al., 2013). In contrast, white rust, caused by *Albugo candida*, was increased at 550 ppm CO_2 (Mathur et al., 2013). Moreover, it was noticed that changes in atmospheric CO_2 levels affect disease resistance against foliar and soil borne pathogens in a very different manner.

For many foliar pathogens, plant leaf epidermal characteristics (wax layers, stomata, and trichomes) are important for their ability to infect successfully (Lake and Wade, 2009). Changes in atmospheric CO_2 induce stomatal closure and increase wax layers, which are likely to alter the performance of foliar pathogens under increased atmospheric CO_2 levels. Indeed, enriched atmospheric CO_2 significantly reduced disease incidence of the fungal pathogen *Phyllosticta minima* on *Acer rubrum*, which correlated with the reduced stomatal conductance in high CO_2-grown red maple leaves (Mcelrone et al., 2005). Downy mildew severity was decreased at high CO_2 levels likely due to the changes in the canopy density and leaf age of soybean plants (Eastburn et al., 2010). Also, high CO_2 significantly reduced primary penetration rates

of *Erysiphe graminis* in barley, which was in accordance with the increased production of papillae and accumulation of silicon at the sites of appressorial penetration (Hibberd et al., 1996). In a recent study, elevated CO_2 induced stomatal closure in tomato leaves, which partly contributed to the reduced entry of *P. syringae* pv. *tomato* DC3000 (*Pst*) in tomato plants (Li et al., 2014). Moreover, elevated atmospheric CO_2 levels could affect leaf morphological changes not only at pre-infection stage but also at post-infection stages. These changes might also contribute to differential disease severity under high CO_2. For instance, the aggressiveness of *E. cichoracearum* increased under elevated CO_2, correlated with increased stomata and trichome densities on newly developed leaves after infection (Lake and Wade, 2009).

In addition to induced leaf morphological changes, CO_2 enrichment in the atmosphere could also change leaf physiology and chemistry, resulting in altered disease resistance. Exposure of a red maple to elevated CO_2 increased the C:N ratio, and total phenolics and tannins, leading to reduced disease severity (Mcelrone et al., 2005). In tobacco elevated CO_2 induced accumulation of phenylpropanoids, resulting in reduced multiplication of *potato virus Y* (PVY) (Lorenzo et al., 2004). It is known that SA-mediated defense signaling is effective against biotrophic pathogens whereas the JA/ethylene (ET)-mediated defense signaling is mainly responsible for disease resistance against necrotrophic pathogens and insect herbivores (Glazebrook, 2005). Therefore, an increase in SA levels and up-regulated SA-related transcripts in plants grown under elevated CO_2 conditions is likely to cause reduced disease susceptibility to biotrophic pathogens, while the decreased JA-related defenses are likely to increase severity of necrotrophs and performance of herbivores (Zavala et al., 2008; Sun et al., 2011b; Huang et al., 2012; Zhang et al., 2015). Other defense-related hormones, such as ABA (Arteca et al., 1980; Leymarie et al., 1999; Li et al., 2011b) and ET (Guo et al., 2014), were also shown to be modulated by atmospheric CO_2 levels. Interplay between the different plant hormones in complex cellular signaling networks determine the defense responses that are activated upon encounter with a pathogenic microbe or herbivorous insect (Pieterse et a., 2012). Thus, the CO_2-induced modulations of hormonal signaling pathways contribute to CO_2-regulated plant disease resistance.

Soil borne pathogens, including fungi, oomycetes and bacteria are a major cause of diseases that inflict enormous agricultural losses (Lewis and Papavizas, 1991). They cause disease symptoms occurring in all parts of the plants, including root blackening, root or shoot rot, wilting of leaves, stunted growth or damping-off of seedlings (Haas and Défago, 2005). Many soil borne pathogens have a broad host range and their survival is tightly associated with root exudates and other soil microbes in the rhizosphere (Chakraborty et al., 2012). Whereas previous studies have shown that elevated CO_2 can affect soil borne diseases, effects also vary in different plant pathosystems. For example, tomato plants grown under elevated atmospheric CO_2 showed enhanced tolerance to *Phytophthora parasitica* (Jwa and Walling, 2001). In contrast, higher disease incidence and severity, and increased population densities of *Fusarium pseudograminearum* were observed in wheat plants

grown under elevated CO_2 (Melloy et al., 2014). Moreover, in maize, elevated CO_2 increased susceptibility to *F. verticillioides* (Vaughan et al., 2014). However, in soybean, the incidence of sudden death syndrome caused by *F. virguliforme* was not significantly affected by elevated CO_2 (Eastburn et al., 2010). Also, for the interaction between lettuce and *F. oxysporum* f.sp. *lactucae* it was observed that elevated atmospheric CO_2 had no significant impact (Ferrocino et al., 2013). It seems that there are no consistent effects of increased atmospheric CO_2 on diseases caused by soil borne plant pathogens.

Potential players in plant defense against pathogens under changing atmospheric CO_2

Stomata

Allowing gas (CO_2 and water vapor) exchange between plant tissues and the atmosphere, stomata are essential for the functioning of plants. Stomata are responsive to a spectrum of environmental cues, such as drought, CO_2 concentration, humidity and light (Hetherington and Woodward, 2003; Araújo et al., 2011). The effect of atmospheric CO_2 on stomata has been studied extensively. Generally, low atmospheric CO_2 levels lead to higher stomata aperture whereas high CO_2 concentrations commonly associate with more closed stomata. Previous studies have identified several components involved in CO_2-triggered signal transduction in guard cells. For example, HIGH LEAF TEMPERATURE 1 (HT1), a protein kinase expressed mainly in guard cells, was shown to be important in CO_2-controlled stomatal conductance (Hashimoto et al., 2006). In addition, three CO_2-binding carbonic anhydrase (CA) proteins CA1, CA4, and CA6 were found to act as early regulators in the CO_2-mediated signaling pathway in guard cells (Hu et al., 2010).

Stomata also serve as an important point of entry for pathogens, especially for bacteria that lack the ability to directly penetrate the plant epidermis (Melotto et al., 2006; Melotto et al., 2008). The predicted rising CO_2 concentrations in the atmosphere, resulting in reduced stomatal conductance and opening, could thus promote disease resistance against foliar pathogens that enter through the stomata. Indeed, red maple showed reduced stomatal opening under elevated CO_2 and thus an enhanced resistance to *P. minima* that infects the plant by directing germ tubes to the stomata (Mcelrone et al., 2005). Also it was recently shown that elevated atmospheric CO_2 enhanced resistance of tomato to *Pst*, in line with decreased stomatal opening (Li et al., 2014).

Furthermore, it was found that stomatal defense was activated after the recognition of pathogen/microbe-associated molecular patterns (PAMPs/MAMPs), such as flg22 (a peptide derived from bacterial flagellin) and lipopolysaccharide (LPS) (Melotto et al., 2006). By production of the virulence factor coronatine (COR) the *P. syringae* pathogen suppresses the stomatal closure response and effectively induces stomatal reopening (Melotto et al., 2006). Interestingly, many signaling components that are involved in PAMPs/MAMPs-induced stomatal closure, including the

phytohormones ABA, SA and JA, the guard cell-specific OPEN-STOMATA 1 (OST1) kinase, and the second messengers reactive oxygen species (ROS) and nitric oxide (NO), have also been implicated in CO_2-mediated signaling (Melotto et al., 2008; Neill et al., 2008; Zeng et al., 2010; Montillet et al., 2013). These overlapping signaling components triggered by both pathogens and atmospheric CO_2 in guard cells indicate that stomata act as a key checkpoint of plant defense during the future climate change.

Hormonal signaling pathways

Plant defense responses are controlled by hormone signaling pathways that can interact with one another (Anderson et al., 2004; Robert-Seilaniantz et al., 2011). Phytohormones that are implicated in the plant immunity signaling network include SA, JA, ET, ABA, auxin, cytokinin, brassinosteroids, and GA (Pieterse et al., 2009). Moreover, these hormonal signaling pathways are important in plant responses to abiotic stress and combinations of biotic and abiotic stresses (Fujita et al., 2006; Suzuki et al., 2014). Therefore, the potential roles of hormonal signaling pathways in atmospheric CO_2-regulated plant defense responses should be considered.

SA. The plant hormone SA has been well characterized as a critical defense signal (Delaney et al., 1994; Durner et al., 1997; Pieterse and van Loon, 1999; Vlot et al., 2009; An and Mou, 2014). In general, SA-mediated defense signaling is triggered by biotrophic pathogens (Glazebrook, 2005). Signaling downstream of SA is mostly regulated by NONEXPRESSOR OF PR GENES1 (NPR1), which is monomerized upon SA-induced cellular redox changes, subsequently translocated to the nucleus and then further activates a large number of defense-related genes (Dong, 2004).

Besides its importance in plant resistance to biotic assailants, SA is also involved in plant responses to various environmental cues (Borsani et al., 2001; Hayat et al., 2010). The effect of increased atmospheric CO_2 on SA signaling varies among plant species and studies. Elevated atmospheric CO_2 increased levels of SA in ginger (Ghasemzadeh et al., 2010). Also in tomato, SA accumulation, as well as SA-mediated signaling, was enhanced by elevated CO_2 (Sun et al., 2011b; Huang et al., 2012). In contrast, both SA levels and SA-mediated defense gene expression remained unchanged in tobacco plants grown at elevated CO_2 levels (Matros et al., 2006). The altered SA levels in plants grown under elevated atmospheric CO_2 was associated with increased resistance of tomato plants to *Tomato yellow leaf curl virus* (TYLCV) (Huang et al., 2012) and *Pst* and tobacco mosaic virus (TMV), which was accompanied with up-regulation of the SA-mediated defense marker genes *PR1* and *NPR1* (Zhang et al., 2015). These results together highlight the significance of SA signaling in plant defense at altered atmospheric CO_2 levels.

JA. Jasmonic acid, derived from the fatty acid linolenic acid, has also been recognized as one of key hormones in regulating plant responses to biotic and abiotic stresses (Glazebrook, 2005; Wasternack and Hause, 2013). Upon synthesis, JA can

be conjugated to isoleucine, resulting in the active JA derivative, JA-Ile. There are two major branches in the JA-regulated signaling pathway during defense activation: the MYC branch and the ERF branch. The MYC branch is dependent on MYC-type transcriptional factors and includes the activation of JA-responsive marker gene *VEGETATIVE STORAGE PROTEIN2* (*VSP2*), which is responsible for the wound-response and defense against insect herbivores (Lorenzo et al., 2004; Pieterse et al., 2009). The ERF branch is regulated by the APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) family of transcriptional factors and contains the JA-responsive marker gene *PLANT DEFENSIN1.2* (*PDF1.2*), and its activation results in enhanced resistance to necrotrophic pathogens (Lorenzo et al., 2003; Anderson et al., 2004; Pré et al., 2008).

JA levels and JA-regulated transcripts were found to be altered by elevated atmospheric CO_2, with variation among plant species (Zavala et al., 2008; Vaughan et al., 2014). This may contribute to the observed variable performance of insect herbivores and pathogens under elevated CO_2. Exposure of tomato plants to elevated CO_2 suppresses the JA pathway, resulting in an increased susceptibility to *B. cinerea* (Zhang et al., 2015). Similarly, elevated CO_2 dampened lipoxygenase (*LOX*) gene expression and the JA production in maize, which correlated with increased susceptibility to *F. verticillioides* (Vaughan et al., 2014).

***ABA*.** The plant hormone ABA mainly functions by regulating developmental processes, such as seed germination, senescence and dormancy (Wasilewska et al., 2008). ABA has also been characterized as an important regulator of plant defense (Mauch-Mani and Mauch, 2005; Ton et al., 2009; Cao et al., 2011). Mutants of tomato plants that are deficient in ABA biosynthesis displayed enhanced resistance to the biotrophic pathogen *Oidium neolycopersici* and the necrotrophic pathogen *B. cinerea* (Achuo et al., 2006). In Arabidopsis, ABA signaling was reported to be essential for the susceptibility to *Pst* by antagonizing SA defense signaling (De Torres-Zabala et al., 2007). However, ABA also contributes positively to resistance to *P. syringae* by mediating the stomatal closure defense response as was shown using Arabidopsis mutants defective in ABA signaling (Melotto et al., 2006).

The accumulation of ABA has been recognized as a typical response that is induced under abiotic stresses (Verslues and Zhu, 2005). Drought stress caused a two-fold increase in endogenous ABA in tomato plants (Achuo et al., 2006). It has been reported that elevated CO_2 alters the ABA signaling pathway differently in different plant species. In Arabidopsis, increased CO_2 reduced the ABA content but significantly enhanced the expression level of several ABA-responsive genes (Teng et al., 2006). Furthermore, ABA signaling interacts with CO_2 signaling in the guard cell (Leymarie et al., 1998; Israelsson et al., 2006; Kim and Maik, 2010; Hubbard et al., 2012; Merilo et al., 2013; Merilo et al., 2015), which might contribute to an altered disease resistance under increased atmospheric CO_2.

***ET*.** Like the above mentioned hormones, the gaseous hormone ET also functions as

an important regulator of developmental processes and responses to both biotic and abiotic stresses (Alonso et al., 1999; Berrocal-Lobo et al., 2002; Alonso and Stepanova, 2004; Broekgaarden et al., 2015). Four rice genes encoding ET-responsive transcription factors participate in mediating disease resistance and responses to salt, cold, drought stress and wounding (Cao et al., 2006). Overexpression of ETHYLENE RESPONSE FACTOR1 (ERF1) in Arabidopsis increased resistance to necrotrophic pathogens such as *B. cinerea* and *Plectosphaerella cucumerina* (Berrocal-Lobo et al., 2002). Two Arabidopsis transcription factors, ETHYLENE INSENSITIVE3 (EIN3) and ETHYLENE INSENSITIVE3-LIKE1 (EIL1), negatively regulate PAMP defense responses and resistance to *Pst* (Chen et al., 2009).

ET signaling has also been reported to be affected by atmospheric CO_2 levels. For example, soybean plants exposed to elevated atmospheric CO_2 showed strong down-regulation of the aminocyclopropane-1-carboxylic acid (Leakey et al.) synthase (*ACS*) gene that encodes for the enzyme that catalyzes the production of ACC, the precursor for ET biosynthesis (Casteel et al., 2008). It was recently reported that elevated CO_2 down-regulated expression of *ACC* and *ERF* genes in *Medicago truncatula*, which was accompanied by an increased abundance of pea aphid (Guo et al., 2014). Therefore, the ET signaling might also be involved in atmospheric CO_2-altered disease resistance.

Hormone crosstalk. The most well-studied interaction between hormonal signaling pathways involved in plant defense is the antagonism between the SA and the JA pathway, which can optimize downstream immune responses (Beckers and Spoel, 2006; Pieterse et al., 2012). In addition, the ABA, JA, and ET signaling pathways have been demonstrated to interact with the SA/JA signaling network during plant defense responses (Kunkel and Brooks, 2002; De Torres-Zabala et al., 2007; Yasuda et al., 2008; Cao et al., 2011). Exogenous ABA treatment repressed JA/ET-responsive genes, and the JA/ET-responsive genes were up-regulated in ABA deficient mutants, indicating an antagonism relationship between ABA and JA/ET signaling pathway (Anderson et al., 2004). In addition, ABA acts antagonistically with SA-dependent signaling, mediating downstream gene expression and disease resistance (Audenaert et al., 2002; De Torres-Zabala et al., 2009).

The crosstalk between different hormone signaling pathways might play a role in disease resistance under increased atmospheric CO_2 levels. Indeed, it was recently reported that elevated CO_2 levels modulated the crosstalk between the SA and JA pathways in tomato plants, resulting in increased and decreased resistance to *Pst* and *B. cinerea*, respectively (Zhang et al., 2015). Thus, the role of crosstalk between hormonal signaling pathways should also be considered when investigating plant defense responses under altered atmospheric CO_2 conditions.

Redox signaling

ROS are small oxygen-containing chemicals, such as superoxide, hydrogen peroxide (H_2O_2), and hydroxyl radicals, that are formed as byproducts of oxygen metabolism and function in plant stress signaling (Baxter et al., 2014). In plant cells, oxidants (mainly ROS) and antioxidants operate in a complex signaling network that includes multiple signaling pathways to fine tune the redox status at the cellular level, ultimately regulating plant growth and defense responses (Foyer and Noctor, 2013). The involvement of ROS in plant defense has been well studied. For example, the activation of both PAMP-triggered immunity (PTI) and effector-triggered immunity (Pérez-López et al.) involves ROS production, which also occurs in plant defense against nematodes and insects (Jones and Dangl, 2006; Torres et al., 2006; Torres, 2010). Besides, ROS and its associated redox signals interact with SA-mediated defense signaling, NO, calcium metabolism and ET signaling (Klessig et al., 2000; Desikan et al., 2005; Torres et al., 2006), thus functioning at multiple levels in plant immunity.

A major source of ROS derives from photosynthesis through processes associated with energy transfer and electron transport and also through the photorespiratory pathway. Elevated atmospheric CO_2 is expected to affect both photosynthetic rate and photorespiration. Thus, exposure of plants to altered atmospheric CO_2 levels is likely to interfere with the cellular redox status (Munné-Bosch et al., 2013). Indeed, both Arabidopsis and soybean plants grown under elevated CO_2 showed protein carbonylation, a marker of oxidative stress (Qiu et al., 2008). Considering the importance of ROS signaling in plant defense, the altered redox status in plants grown under increased CO_2 is anticipated to have profound effects on disease resistance.

CAs

Carbonic anhydrases in plants mostly belong to the beta-CAs (βCAs) group, and like other groups, they mainly function in catalyzing the reversible reaction from carbon dioxide to bicarbonate. There is emerging evidence that CAs are modulated during interactions between plants and pathogens. For instance, *CA* genes were down-regulated in potato and grape plants that were challenged with *P. infestans* and *Plasmopara viticola*, respectively (Restrepo et al., 2005; Polesani et al., 2008). In contrast, five CA proteins were reported to be increased in Chinese cabbage upon infection with the downy mildew *H. parasitica* (Sun et al., 2014). These results suggest that CAs may be involved in plant defense. Indeed, *Nicotiana benthamiana* plants silenced for a CA exhibited an enhanced susceptibility to *P. infestans*, supporting the hypothesis that CA contributes to disease resistance (Restrepo et al., 2005).

Interestingly, CAs also respond to atmospheric CO_2 levels, with different responses in different plant species. Both transcript levels and the enzymatic activity of CAs decrease in C_3 plants exposed to high CO_2 conditions (Porter and Grodzinski, 1984; Bowes, 1991; Webber et al., 1994; Majeau and Coleman, 1996). In contrast,

the steady-state level of *CA*s mRNAs was increased in Arabidopsis plants grown at elevated CO_2 (Raines et al., 1992). It should be mentioned that different experimental conditions such as duration of CO_2 exposure, can result in different CA responses (Majeau and Coleman, 1996; Wang et al., 2014). As CO_2-binding proteins, βCAs have been demonstrated to be important regulators in CO_2-mediated signaling in guard cells, determining stomatal aperture (Hu et al., 2010). Considering the importance of CAs in CO_2 signaling, a role of CAs in plant defense under future increased atmospheric CO_2 conditions is to be expected.

Other components

Other components that may be involved in plant defense under altered atmospheric CO_2 levels include NO and mitogen activated protein kinases (MAPKs) (De Vos et al., 2005; Neill et al., 2008; Pitzschke et al., 2009; Baxter et al., 2014). Recently, it was found that NO plays a role in elevated CO_2-induced stomatal closure and that silencing of key genes involved in NO generation resulted in significant increases in *Pst* infection under both ambient and high CO_2 conditions (Li et al., 2014). Thus, to investigate the molecular mechanisms underlying atmospheric CO_2-regulated disease resistance, it is important to consider a complicated defense signaling network, which involves multiple signaling pathways that may interact with each other.

ATMOSPHERIC CO_2 IN THE PAST: PLANT ADAPTATIONS TO LOW CO_2 LEVELS

Before the industrial revolution, over 100 years ago, the CO_2 level in the atmosphere was below 300 ppm, and for the past million years before that, atmospheric CO_2 was even lower (Sage and Coleman, 2001). The atmospheric CO_2 concentration has strong direct effects on photosynthesis and plant productivity. Under optimal growing conditions, photosynthetic capacity and yield production drops by 50% when atmospheric CO_2 is reduced from 360 to 180 ppm (Sage, 1995). It can be argued that many plants might still be adapted to lower atmospheric CO_2 levels given their evolutionary history in a low CO_2 atmosphere (Sage and Coleman, 2001). On the other hand, they may have adapted to increasing levels of CO_2, but possibly this has come with a trade-off regarding their responsiveness to other environmental conditions. This adaptation to low CO_2 may affect plant responses to the ever rising atmospheric CO_2 levels. Considering this, it is important to include low atmospheric CO_2 conditions when investigating effects of increasing atmospheric CO_2.

OUTLINE OF THE THESIS

Atmospheric CO_2 concentration has been steadily increasing since the industrial revolution, and it will continue to rise. It is predicted that by 2050 atmospheric CO_2 levels will double the pre-industrial levels (Eastburn et al., 2011). As an essential component in plant photosynthesis, the altered atmospheric CO_2 level is expected to change a variety of plant responses, including plant-pathogen interactions. Indeed

altered atmospheric CO_2 levels can impact disease severity but the effects are ambiguous. Moreover, the molecular mechanisms underlying effects of atmospheric CO_2 on plant defense responses are largely unknown. While most studies to date were conducted under elevated CO_2 conditions, the effect of atmospheric CO_2 levels below ambient received little attention. Such experimental conditions may reveal adaptations of plants to low or increasing CO_2 levels and whether such adaptations have consequences for plant defense responses under the future elevated atmospheric CO_2 levels. Using three atmospheric CO_2 concentrations (800 ppm, 450 ppm, 150 ppm), the experiments described in this thesis aim to elucidate how alterations in atmospheric CO_2 levels affect plant defense against both foliar and soil borne pathogens, and to explore which signaling mechanisms are involved.

In Chapter 2, effects of atmospheric CO_2 on resistance of Arabidopsis to *Pst* and the role of ABA signaling therein were investigated. The resistance of Arabidopsis to *Pst* was decreased under high CO_2 and enhanced under low CO_2 conditions. By analyzing stomatal responses and the function of COR, ABA signaling appeared to play an essential part in atmospheric CO_2-altered resistance to *Pst*. Potential adverse influences of future atmospheric CO_2 on plant disease resistance are discussed.

Beta carbonic anhydrases (CAs) are abundant in plant leaves and play important roles in early CO_2 signaling (Hu et al., 2010). In Chapters 3 and 4, we investigated the possible role of CAs in atmospheric CO_2-altered Arabidopsis resistance. First the involvement of CAs in plant immune responses was studied under ambient conditions. In Chapter 3, we showed that down-regulation of *CA1* and *CA4* is a general response during PTI. Using the *ca1ca4* double mutant, it was demonstrated that CA1 and CA4 play a negative role in Arabidopsis defense against *Pst* likely through antagonizing SA signaling.

In Chapter 4, the importance of SA and JA signaling pathways in atmospheric CO_2-mediated defense responses was investigated. This study showed that expression of the JA-responsive gene *PDF1.2* and resistance against *B. cinerea* were enhanced under high atmospheric CO_2 conditions, while resistance to this pathogen was decreased at low CO_2 levels. Enhanced SA signaling and NPR1-mediated resistance to *Pst* were enhanced under the low CO_2 condition, while SA-associated defenses were reduced by high CO_2. This was associated with enhanced versus reduced *Pst*-induced down-regulation of *CA1* and *CA4* by low CO_2 and high CO_2, respectively. Moreover, resistance of the *ca1ca4* mutant to *Pst* was high under all three CO_2 conditions, indicating the involvement of CAs in atmospheric CO_2-altered SA-dependent defenses in Arabidopsis. CAs did not control *B. cinerea* resistance.

In Chapter 5, effects of atmospheric CO_2 on soil borne diseases were investigated. Alterations in atmospheric CO_2 levels had no significant impact on disease severity and growth reduction in Arabidopsis infected with either *Rhizoctonia solani* or *Fusarium oxysporum* f.sp. *raphani*. Previous findings revealed the importance of ET signaling in Arabidopsis defense against *F. oxysporum* (Berrocal-Lobo and Molina, 2004; Pantelides et al., 2013). This was confirmed by the current experiments with Arabidopsis mutants defective in ET signaling. However, systemic colonization of

Arabidopsis by *F. oxysporum* was not affected by the different atmospheric CO_2 levels in hormone signaling mutants. Together, the findings in this Chapter validate our hypothesis that future increases in atmospheric CO_2 are likely to have little influence on this soil borne disease.

In Chapter 6, results presented in this thesis are discussed with respect to the current state of knowledge on plant defense responses under altered atmospheric CO_2 conditions.

CHAPTER 2

Atmospheric CO_2 alters resistance of Arabidopsis to *Pseudomonas syringae* by changing abscisic acid signaling and stomatal responsiveness to coronatine

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ABSTRACT

Atmospheric CO_2 influences plants performance, whereby high CO_2 levels generally lead to enhanced plant growth and reduced stomatal aperture, while oppositely, low CO_2 level results in decreased growth and increased stomatal aperture. Relatively little is known about the effect of CO_2 on disease resistance. Therefore, we set out to study how three different CO_2 levels (high (800 ppm), ambient (450 ppm) and low (150 ppm) alter the resistance of *Arabidopsis thaliana* (Arabidopsis) to the foliar bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*), which naturally gains access to the plant through stomatal openings. Under all three tested atmospheric CO_2 conditions, infection with *Pst* resulted in stomatal closure at 1 h after dip inoculation. Interestingly, subsequent stomatal reopening at 4 h after inoculation, which is caused by the *Pst*-produced phytotoxin coronatine (COR), occurred only under ambient and high CO_2 conditions, but not under low CO_2 conditions. Accordingly, infection was significantly reduced in low CO_2-grown plants, whereby proliferation of the wild-type *Pst* strain was restricted to a similar level as that of the COR-deficient mutant *Pst cor⁻*. In contrast, high CO_2-grown plants exhibited enhanced susceptibility to *Pst*. Under all three CO_2 conditions tested, the ABA mutants *aba2-1* and *abi1-1* were resistant to *Pst*, to the same extent as wild-type plants grown at low CO_2. Moreover, like the wild-type plants grown at low CO_2, the ABA mutants were affected in COR-mediated stomatal reopening. ABA levels in low CO_2-grown plants were significantly reduced both in control and *Pst*-infected plants. Our results emphasize the importance of ABA homeostasis in defense regulation to *Pst* and suggest that reduced ABA signaling under low CO_2 conditions contributes to the enhanced resistance to *Pst* by deregulation of COR-mediated reopening of the stomata. The observed negative correlation between the level of atmospheric CO_2 and disease resistance implies that the global rise in CO_2 levels may impact crop production by weakening plant defense.

INTRODUCTION

The atmospheric CO_2 level has been rising at an accelerating rate since the Industrial Revolution. According to the Coupled Climate-Carbon Cycle Model Intercomparison Project (C^4MIP), atmospheric CO_2 is predicted to rise to levels varying between 730 and 1020 ppm at the end of 21st century. During recent years, various Free-Air CO_2 Enrichment (FACE) studies were conducted to assess the long term impact of elevated CO_2 levels on plant performance. These studies showed that elevated CO_2 levels typically resulted in promoted plant growth, a decreased transpiration rate, and higher water use efficiency (Coleman et al., 1993; Dermody et al., 2006; Reich et al., 2006; Jain et al., 2007; Leakey et al., 2009; Wang et al., 2012; Schmid et al., 2015). In contrast, lower levels of CO_2 are often associated with a reduction in photosynthesis (Sage and Coleman, 2001). Despite these general effects of atmospheric CO_2 on plants, there have been diverging observations depending on plant species and species ecotypes (Murray, 1995; Li et al., 2006). For example, the levels of major metabolites such as fructose, galactose, and glucose decreased significantly in *Arabidopsis thaliana* (Arabidopsis) ecotype Cvi-0 grown under elevated CO_2, in contrast to two other ecotypes, Col-0 and Ws-0 (Li et al., 2006). Another main effect of atmospheric CO_2 on plant performance is the regulation of stomata. Stomata are small pores that control the exchange of gases, such as water vapor and CO_2, between the atmosphere and plant leaves, thereby being of fundamental importance for plant photosynthesis. Elevated atmospheric CO_2 levels generally lead to a lower stomata density and reduced stomatal aperture, whereas reduced atmospheric CO_2 levels lead to a higher stomata density and increased stomatal aperture (Israelsson et al., 2006).

The impact of the atmospheric CO_2 concentration on plant disease resistance is highly variable (Chakraborty et al., 2000; Garrett et al., 2006; Kobayashi et al., 2006; Yáñez-López et al., 2014). In general, high CO_2 levels increase canopy size and leaf humidity, resulting in a microclimate that is favorable for the development of many pathogenic microbes (Manning and Tiedemann, 1995). Nevertheless, at elevated CO_2 the infection rate of the anthracnose *Colletotrichum gloeosporioides* on the pasture *Stylosanthes scabra* was significantly reduced (Chakraborty and Datta, 2003). Intriguingly, in a FACE study assessing the effects of elevated CO_2 on soybean diseases, it was observed that high CO_2 increased the susceptibility to brown spot *Septoria glycines*, whereas the susceptibility to downy mildew (*Peronospora manshurica*) was reduced (Eastburn et al., 2010). Moreover, high CO_2 induced susceptibility of Arabidopsis to powdery mildew (*Erysiphe cichoracearum*) was dependent on the Arabidopsis ecotype (Lake and Wade, 2009). These results indicate that the effect of changes in atmospheric CO_2 levels on disease resistance is depending on plant genotype, pathogen species and environmental conditions.

Formation and aperture of stomata are, as previously mentioned, regulated by atmospheric CO_2. Stomata serve as important passages for many foliar plant pathogenic bacteria and fungi to enter plant leaves (Melotto et al., 2008; Grimmer et

al., 2012). It was found that stomata actively close 1 to 2 h after infection with the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*), thereby restricting the entry of this pathogen and reducing its colonization of the host tissue (Melotto et al., 2006). Therefore, the changes in stomata that are caused by atmospheric CO_2 are likely to affect plant resistance to pathogens that use stomata to enter the plant. Indeed, red maple leaves showed enhanced resistance to the fungus *Phyllosticta minima* under elevated CO_2 levels, which was associated with reduced stomatal aperture (Mcelrone et al., 2005). In the tomato-*Pst* interaction, it was shown that elevated CO_2 induced resistance against *Pst*, possibly by reducing the stomata-mediated entry of *Pst* (Li et al., 2014). However, recently a role for stomata-independent hormone-mediated defense was also suggested to play a role in this enhanced resistance (Zhang et al., 2015). In contrast, negative effects of elevated CO_2 on stomatal defense have been suggested as well. In a study using Arabidopsis and the fungal pathogen *E. cichoracearum*, it was found that elevated CO_2 enhanced disease severity, which was accompanied by increased stomatal density and guard cell length in leaves that developed post-infection (Lake and Wade, 2009).

A wide range of hormones play pivotal roles in plant regulatory networks that control responses to biotic and abiotic stress conditions (Fujita et al., 2006). Salicylic acid (SA) and jasmonic acid (JA) are considered the two key players in plant immune signaling. Other hormones such as abscisic acid (ABA), ethylene, auxins, gibberellins and cytokinins have also been implicated in the plant immune system through modulation of the SA-JA backbone of the defense signaling network (Vlot et al., 2009; Robert-Seilaniantz et al., 2011; Pieterse et al., 2012). ABA can function negatively in the post-invasive defense phase through its antagonism of SA- and JA-controlled pathogen defenses (Ton et al., 2009; Pieterse et al., 2012). For example, tomato and Arabidopsis mutants that are defective in ABA signaling are less susceptible to hemi-biotrophic bacteria like *Pst* and necrotrophic fungi like *Botrytis cinerea* (Audenaert et al., 2002; Thaler and Bostock, 2004; De Torres-Zabala et al., 2007; Liu et al., 2015). ABA can also function positively in plant immunity by co-regulating the pre-invasive defense phase through controlling papillae formation at the site of infection and controlling stomatal behavior (Melotto et al., 2008; Ton et al., 2009; Pieterse et al., 2012). For example, Melotto et al. (2006) found that the ABA-deficient mutant *aba3-1* was defective in stomatal closure following infection with *Pst*, suggesting that ABA signaling is required for *Pst*-induced stomatal defense.

Elevated CO_2 has been shown to influence hormone levels and signaling, whereby generally SA signaling is enhanced and JA signaling is reduced (DeLucia et al., 2012). For example, in tomato plants that are exposed to high CO_2, SA levels were enhanced and were further induced by infection with tobacco mosaic virus (TMV) or *Pst*, whereas effects on JA content were minimal (Zhang et al., 2015). However, JA-mediated defenses were decreased under high CO_2 conditions, which can likely be attributed to the enhanced antagonism by elevated SA levels (Zhang et al., 2015). Moreover, a reduction in the ABA content of Arabidopsis leaves at elevated CO_2 was reported (Teng et al., 2006). Nevertheless, in a study using three Arabidopsis

accessions it was found that the transcript abundance of ABA-responsive genes was increased at high CO_2 levels (Li et al., 2006). Convergence of ABA and CO_2 signaling was shown to occur at the level of GROWTH CONTROL BY ABA2 (GCA2) in Arabidopsis guard cells, since the ABA-insensitive mutant *gca2* was strongly impaired in high CO_2-induced stomatal closure (Israelsson et al., 2006).

Despite growing efforts on studying plant disease resistance under high atmospheric CO_2, the exact signaling mechanisms underlying the effects of different CO_2 levels on plant defense remain elusive. Moreover, up to now studies on the effects of low CO_2 on plant immune responses are scarce. Using Arabidopsis-*Pst* as a model, we set out to investigate whether and how atmospheric CO_2 affects the disease resistance to this bacterial pathogen that gains access to the plant through stomatal openings. We observed that high CO_2-grown Arabidopsis plants exhibited enhanced susceptibility to *Pst*, whereas plants grown under low CO_2 conditions were more resistant. The role of ABA signaling in the atmospheric CO_2-regulated disease resistance was further investigated by using ABA mutants. We observed attenuation of coronatine (COR)-triggered stomatal reopening, and inhibition of ABA signaling at low atmospheric CO_2, suggesting that at the current global rise of atmospheric CO_2, plants may suffer from enhanced disease susceptibility due to the ABA-regulated suppression of plant innate immunity.

RESULTS

Effect of high and low atmospheric CO_2 levels on Arabidopsis growth and stomatal behavior

Numerous studies have been conducted to assess the effect of high CO_2 on plant performance, including plant growth, stomatal behavior and disease resistance. However, only limited information is available on the effects of low CO_2 on the plant. Here, we studied the effects of three different CO_2 levels (high (800 ppm), ambient (450 ppm) and low (150 ppm) on Arabidopsis plants in the absence and presence of pathogens. Plants were cultivated under ambient CO_2 until they were 2 weeks old, after which they were transferred to other CO_2 conditions. We noticed that plants that had grown under low CO_2 conditions for three weeks had much smaller rosette sizes compared to plants grown under high and ambient CO_2 conditions (Fig. 1a). The dry weight of the rosettes was significantly decreased by the low atmospheric CO_2 level (Fig. 1b). In contrast, there were no effects on rosette growth by high CO_2, which is rather unexpected since most previous studies have reported an increase in biomass (Bowes, 1991; Leakey et al., 2009). However, our experimental conditions may not have been optimal for stimulated growth by elevated CO_2 and moreover, the Col-0 accession that we used may respond differently to high CO_2 than other plants (Li et al., 2006; Leakey et al., 2009).

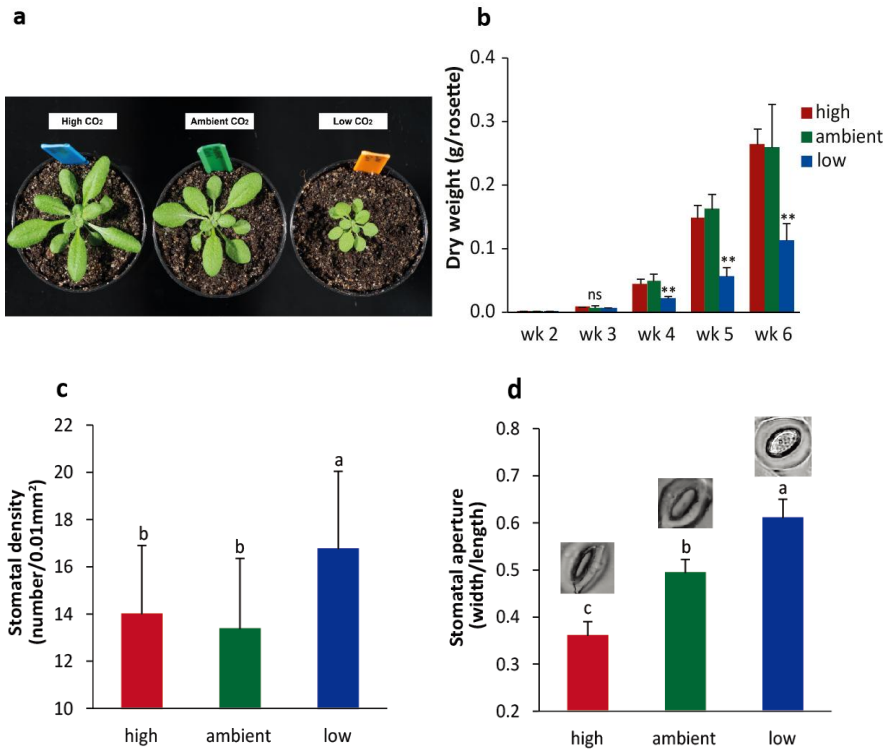

Figure 1: Effect of different atmospheric CO_2 levels on growth and stomatal behavior of Arabidopsis.
(a) Pictures of 4-week-old Arabidopsis plants grown under high (800 ppm), ambient (450 ppm) or low CO_2 (150 ppm) conditions. **(b)** Dry weight of Arabidopsis rosettes at different developmental stages (from week 2 to week 6) under three different CO_2 conditions. Asterisks indicate statistically significant differences between the CO_2 treatments at the specific time points (ANOVA, Duncan's multiple range test, $^{**}P<0.01$; ns, no significant difference). Error bars represent SD, $n=10$ plants. **(c)** Stomatal density and **(d)** stomatal aperture in 4-week-old Arabidopsis plants grown under three different CO_2 conditions. Depicted are the averages of stomatal density and aperture (±SD) of six leaves. In (d) examplar pictures of stomatal aperture typical for the CO_2 conditions are depicted. Different letters indicate statistically significant differences between the CO_2 treatments (ANOVA, Duncan's multiple range test, $P<0.05$).

Stomatal density and aperture were investigated under the three different CO_2 conditions as well. At elevated atmospheric CO_2, stomatal density was not influenced but a significant decrease in stomatal aperture was found (Fig 1c, 1d and S1). At low atmospheric CO_2 an increase in both stomatal density and stomatal aperture was detected. These results are in line with previous studies that found that the inverse relationship between atmospheric CO_2 and stomatal behavior was more evident under sub-ambient CO_2 conditions than under elevated CO_2 conditions (Royer, 2001). This phenomenon is referred to as CO_2 'ceiling' phenomenon. The major effects on plant growth and stomatal behavior observed at especially the low CO_2 level prompted us to introduce a pathogen into the system in order to study the effects of CO_2 on plant immunity.

Low atmospheric CO_2 inhibits COR-triggered stomatal reopening

To explore whether the differential stomatal behavior at the three tested atmospheric CO_2 levels affects defense responses, we examined the stomatal responsiveness of Arabidopsis plants to infection by the bacterial leaf pathogen *Pst* under the different atmospheric CO_2 conditions. Previously, it has been shown that at ambient CO_2 the stomata close at 1 to 2 h after dip inoculation with *Pst*, and reopen again at 3 to 4 h due to the action of coronatine (COR), a virulence factor produced by *Pst*, which regulates stomatal reopening and thereby promotes bacterial infection (Melotto et al., 2006). Our results are in line with this, since we found that under ambient conditions, the stomata closed at 1 h after dip inoculation with both wild-type *Pst* and the COR-deficient mutant *Pst cor*$^-$ (Fig. 2). Moreover, at 4 h after inoculation, the stomata were reopened by the wild-type strain *Pst* but not by the *Pst cor*$^-$ mutant (Fig. 2). High CO_2-grown plants contain stomata that are generally more closed (Fig. 1d and Fig. 2), but upon attack by *Pst* the stomatal closure and reopening were similar to that of ambient CO_2-grown plants. At low CO_2 levels, stomata are opened more widely (Fig. 1d and Fig. 2), but still they responded with closure at 1 h after inoculation with *Pst* wild-type or *Pst cor*$^-$ mutant, which is comparable to the ambient and high CO_2 conditions. However, in contrast, at 4 h after inoculation, the stomata of both *Pst*- and *Pst cor*$^-$-challenged leaves remained closed under the low CO_2 condition. These data show that plants grown under either high or low CO_2 conditions initially respond to *Pst* infection by closing their stomata, despite their original differences in stomatal aperture. However, the subsequent COR-mediated stomatal reopening of the stomata occurs only at high and ambient CO_2 levels, whereas it is blocked at low CO_2 levels.

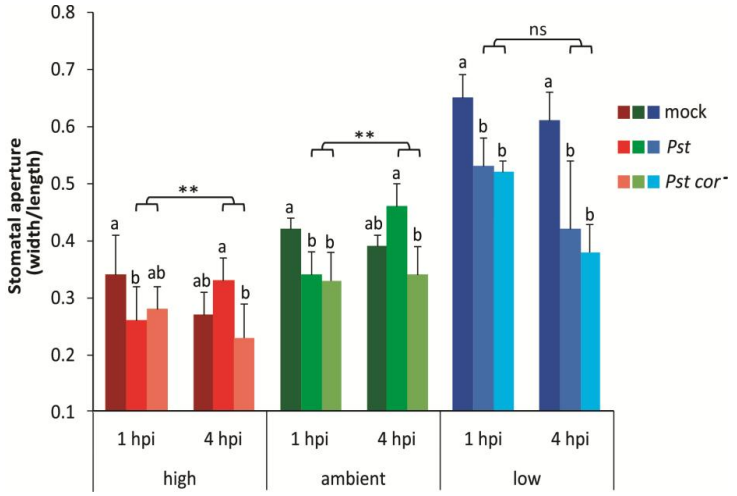

Figure 2: Effect of different atmospheric CO_2 levels on stomatal aperture upon infection by *Pst* or *Pst cor*$^-$. Arabidopsis leaves of 4-week-old plants grown under three different CO_2 conditions were dip inoculated with a mock solution, *Pst* or *Pst cor*$^-$. Stomatal aperture was determined 1 h and 4 h after dip inoculation (hpi, h post inoculation). Depicted are the averages of stomatal aperture (±SD) of six leaves. Different letters indicate statistically significant differences between the treatments at specific time points within the same atmospheric CO_2 level. Indications above the brackets specify the interaction (bacterium genotype × time) between the two *Pst* genotype treatments (wild-type and mutant) and the time (1 h and 4 h) under the same atmospheric CO_2 condition (two-way ANOVA, Fisher's LSD test, **, P <0.01; ns, not significant).

Atmospheric CO_2 alters resistance to *Pst* in a COR-dependent manner

The resistance of Arabidopsis plants to *Pst* infection under the different atmospheric CO_2 conditions was tested by determining the growth of *Pst* in plants cultivated at different CO_2 levels. Initially, at 4 h after dip inoculation, plants grown at high CO_2 levels contained significantly less *Pst* than plants grown at low CO_2 (Fig. 3a), which coincided with the lower stomatal density and aperture in leaves of high CO_2-grown plants (Fig. 1c, 1d and Fig. 2). However, at 4 d after inoculation, the *Pst* bacterial titer in high CO_2-grown plants was significantly higher compared to that in ambient and low CO_2-grown plants (Fig. 3a and 3c). In low CO_2-grown plants multiplication of *Pst* was inhibited, albeit not always statically significantly (Fig. 3a and 3c). Moreover, there were fewer chlorotic disease symptoms on plants grown at low CO_2 compared to plants grown at ambient and high CO_2 (Fig. 3b).

The role of COR in successful infection by *Pst* through facilitation of stomatal reopening, suppression of SA-mediated defense signaling and disease symptom development has been well established (Mittal and Davis, 1995; Brooks et al., 2005). When plants were dip inoculated, the COR mutant strain *Pst cor*$^-$ grew to a significantly lower level compared with the wild-type strain of *Pst*, as was reported previously (Fig. 3c; (Melotto et al., 2006)). Moreover, although the bacterial titer of wild-type *Pst* was significantly higher in high CO_2-grown plants and lower in low CO_2-grown plants, growth of the *Pst cor*$^-$ mutant strain was severely limited under all three CO_2 conditions, resulting in equivalently low bacterial titers, which were comparable to those of wild-type *Pst* in low CO_2-grown plants (Fig. 3c). The interaction of atmospheric CO_2 with the *in planta* growth difference between *Pst* and *Pst cor*$^-$ suggests that atmospheric CO_2 regulates the plant's sensitivity to COR, leading to enhanced responsiveness at high CO_2 and impaired responsiveness at low CO_2. This differential responsiveness to COR could play a role in the observed differences in resistance levels to *Pst* under the different CO_2 conditions.

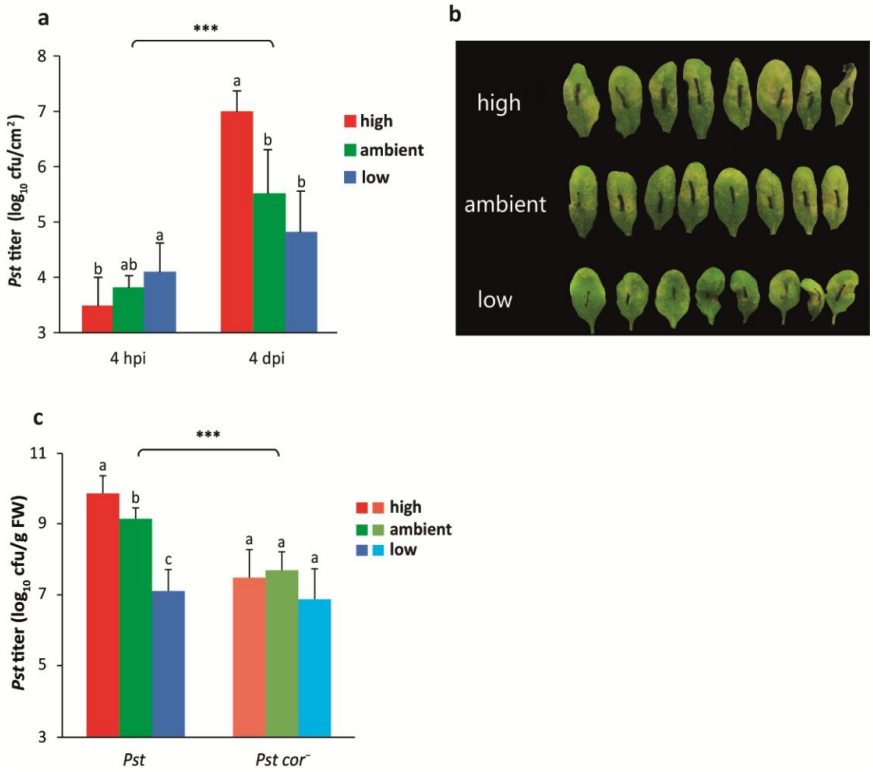

Figure 3: Effect of different atmospheric CO_2 levels on resistance of Arabidopsis plants to *Pst*.
(a) Growth of *Pst in planta* at 4 h and 4 d after dip inoculation of plants grown under three different CO_2 conditions. Depicted are the averages of log_{10}-transformed bacterial titer (±SD; per leaf area) from eight biological replicates. Different letters indicate statistically significant differences between the CO_2 treatments at the indicated time point. Indications above the brackets specify the interaction (CO_2 condition × time) between the three CO_2 conditions and the time (4 hpi and 4 dpi) (two-way ANOVA, Fisher's LSD test, ***, $P<0.001$). **(b)** Pictures of the disease symptoms of plants grown under three different CO_2 conditions at 4 d after dip inoculation with *Pst*. **(c)** Different atmospheric CO_2 grown plants were dip inoculated with *Pst* or *Pst cor*$^-$. Bacterial growth was measured 4 days later. Indicated are the averages of log_{10}-transformed bacterial titer (±SD; per g of leaf fresh weight) from eight biological replicates. Different letters indicate statistically significant differences between the CO_2 treatments within the same bacterial treatment. Indications above the brackets specify the interaction (CO_2 condition × bacterium genotype) between the three CO_2 conditions and the two *Pst* genotype treatments (wild type and mutant) (two-way ANOVA, Fisher's LSD test, ***, $P<0.001$).

A novel role for ABA signaling in COR-mediated stomatal reopening

To gain more insight into how the differential responsiveness to COR, which was apparent under different atmospheric CO_2 levels, could alter plant immunity, we studied the role of ABA in *Pst*-triggered stomatal closure and subsequent reopening. ABA is known to influence disease resistance against *Pst*, by regulating stomatal closure, responsiveness to type III effectors (T3Es) and antagonism of the SA signaling pathway (Brooks et al., 2005; Melotto et al., 2006; Adie et al., 2007; De Torres-Zabala et al., 2007; Zheng et al., 2012). It has been previously shown that the stomata of ABA insensitive mutants *abi1-1* can be more open than that of wild-type plants (Merlot et al., 2002). We found that the stomatal aperture of the ABA mutants *aba2-1* and *abi1-1* under ambient CO_2 conditions was in some experiments

significantly greater than in Col-0 plants (Fig. 4, Fig. 5a and Fig. S2). Other papers also report varying results regarding stomatal aperture of ABA mutants in control conditions (Merlot et al., 2002; Melotto et al., 2006). When challenged with *Pst*, both wild-type and the *aba2-1* and *abi1-1* mutant plants responded with stomatal closure at 1 h after *Pst* dip inoculation (Fig. 4 and Fig. S2a). Moreover, while stomata reopened in wild-type plants at 4 h after inoculation in a COR-dependent manner, stomata did not reopen in the *aba2-1* and *abi1-1* mutants when treated with *Pst* or *Pst cor*$^-$ (Fig. 4 and Fig. S2a). A recent report (Montillet et al., 2013) showed that the ABA-defective mutants *aba2-1* and *ost1-2* closed their stomata upon treatments with *Pst* and flg22, the active epitope of bacterial flagellin which supports our results for the existence of an ABA-independent pathway leading to stomatal closure. Furthermore, our data reveal a novel role of ABA signaling in COR-mediated stomata reopening.

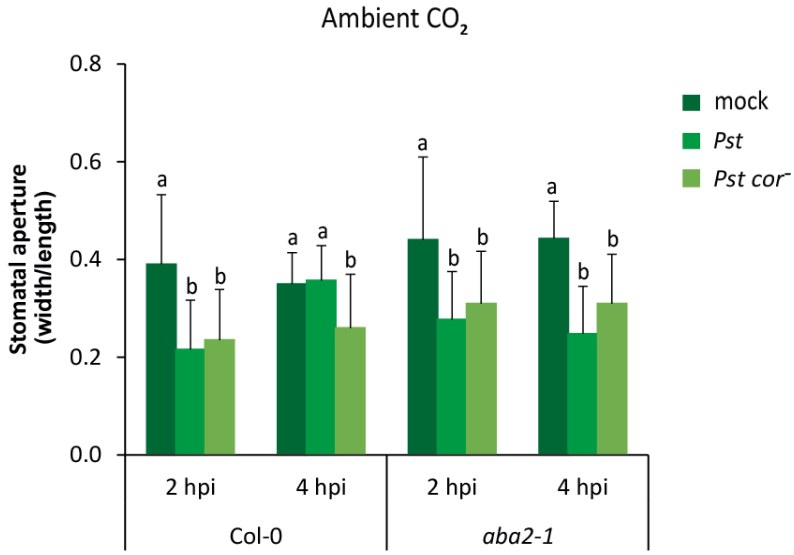

Figure 4: Effect of ABA signaling on stomatal aperture in response to *Pst* and *Pst cor*$^-$ under ambient CO_2 condition.
Stomatal aperture was measured at 2 h and 4 h after dip inoculation with *Pst* or *Pst cor*$^-$ in wild-type Col-0 and the ABA deficient mutant *aba2-1*. Indicated are the averages of the stomatal aperture (±SD) of six leaves. Different letters indicate statistically significant differences between treatments within one plant genotype at the indicated time point (two-way ANOVA, Fisher's LSD test). The interaction (bacterium genotype × time) between the two *Pst* genotype treatments (wild-type and mutant) and the time (1 h and 4 h) in the same plant genotype was 0.26 for wild-type Col-0 and 0.95 for *aba2-1*.

ABA-dependency of atmospheric CO_2 controlled stomatal aperture and disease resistance against *Pst*

Based on our finding that under ambient CO_2 conditions ABA mutants show a stomatal response pattern to *Pst* infection that is similar to that of wild-type Arabidopsis plants grown under low CO_2 conditions, we hypothesized that there could be a role for ABA signaling in atmospheric CO_2-altered disease resistance to *Pst*. To test this, we first measured stomatal aperture of the mutant *aba2-1* when cultivated at

different levels of atmospheric CO_2 without *Pst* infection. We observed that the stomata were relatively open in the *aba2-1* mutant under all three different CO_2 conditions, to a level that was comparable to wild-type Col-0 plants grown at low CO_2 (Fig. 5a). This interaction between ABA signaling and CO_2 levels indicates that ABA signaling controls the differential stomatal aperture under different atmospheric CO_2 conditions.

Subsequently, we tested whether atmospheric CO_2 can alter disease resistance to *Pst* in ABA mutants. Under ambient and high CO_2 conditions both *aba2-1* and *abi1-1* exhibited reduced growth of *Pst* compared with wild-type plants (Fig. 5b and S3), supporting a negative role for ABA signaling in the defense response against *Pst*. More importantly, under all three CO_2 conditions *Pst* growth in both ABA mutants was as low as in the wild-type plants grown at low CO_2 (Fig. 5b and S3). Together, these results suggest that ABA signaling plays an essential role in atmospheric CO_2-regulated plant defense responses against *Pst*.

Previously, ABA has been reported to accumulate upon *Pst* infection (De Torres-Zabala et al., 2007). Moreover, enrichment in atmospheric CO_2 can also change ABA levels or ABA signaling, although variable effects in Arabidopsis have been described (Li et al., 2006; Teng et al., 2006). We assayed the ABA content in leaves infected with *Pst* under different atmospheric CO_2 conditions. Interestingly, in the absence of *Pst*, the ABA levels in low CO_2-grown plants were significantly reduced compared with ambient and high CO_2-grown plants (the ABA content was 4-fold lower; Fig. 5c). In *Pst*-challenged leaves the ABA concentrations rose significantly compared with mock-treated leaves under all three CO_2 conditions. However, the ABA levels in the low CO_2-grown plants were still lower than those in the ambient and high CO_2-grown plants upon infection by *Pst*. These results confirm that ABA signaling contributes to the suppression of defense to *Pst* in Arabidopsis and furthermore, suggest that the reduced ABA levels in low CO_2-grown plants may be responsible for the observed enhanced resistance.

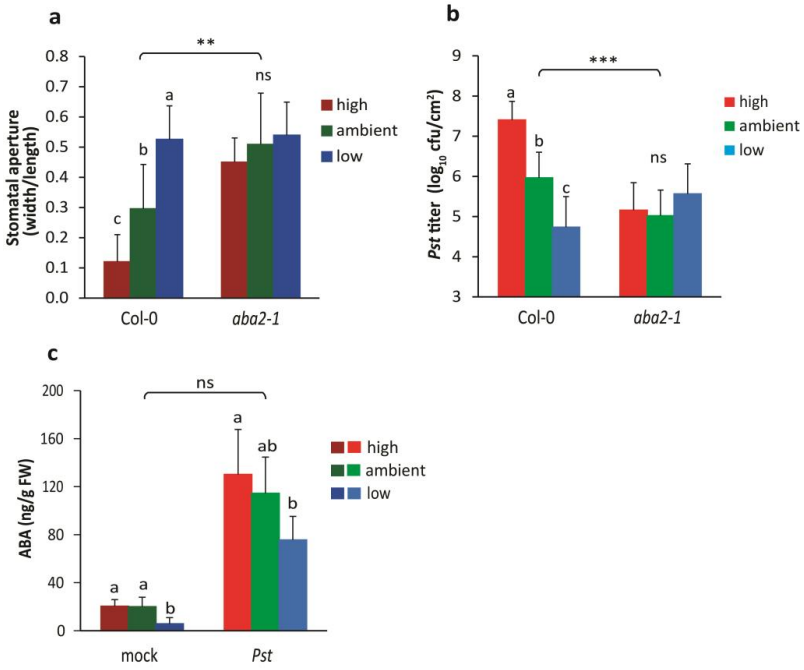

Figure 5: The effect of ABA signaling on atmospheric CO_2-altered stomatal aperture and Arabidopsis resistance to *Pst*.

(a) Stomatal aperture of Arabidopsis wild-type Col-0 and the ABA deficient mutant *aba2-1* grown under different atmospheric CO_2 conditions. Depicted are the averages of the stomatal aperture (±SD) of six leaves. Different letters indicate statistically significant differences between the CO_2 treatments within one genotype (ns, not significant). Indications above the brackets specify the interaction (CO_2 condition × Arabidopsis genotype) between the three CO_2 conditions and the two Arabidopsis genotype (wild type Col-0 and mutant *aba2-1*) (two-way ANOVA, Fisher's LSD test, **, $P<0.01$). **(b)** *Pst* growth in wild-type Col-0 and the mutant *aba2-1* measured at 4 d after dip inoculation. Indicated are the averages of the log_{10}-transformed bacterial titer (±SD; per leaf area) from eight biological replicates. Different letters indicate a statistically significant difference between the CO_2 treatments within one genotype. Indications above the brackets specify the interaction (CO_2 condition × Arabidopsis genotype) between the three CO_2 conditions and the two Arabidopsis genotype (wild type Col-0 and mutant *aba2-1*) (two-way ANOVA, Fisher's LSD test, ***, $P<0.001$). **(c)** Levels of ABA in leaves of wild-type Col-0 plants grown under three different atmospheric CO_2 conditions. Leaves of four-week-old plants were pressure infiltrated with *Pst* (1×10^8 cfu/ml) or mock (10 mM $MgSO_4$) solution and after 24 h assayed for ABA levels. Different letters indicate a statistically significant difference in ABA levels between the CO_2 conditions within the same treatment Indications above the brackets specify the interaction (CO_2 condition × bacterium treatment) between the three CO_2 conditions and the treatments (*Pst* and mock) (two-way ANOVA, Fisher's LSD test, ns, not significant). Error bars represent SD, $n=3$ plants.

DISCUSSION

As one of the best known characteristics of the current global climate change, the continually rising atmospheric CO_2 concentration has received extensive attention during the past decade. Studies range from the effects of atmospheric CO_2 on global vegetative production to its influence on plant-pathogen systems (Coleman et al., 1993; Chakraborty et al., 2000; Dermody et al., 2006; De Boer et al., 2011; DeLucia et al., 2012). The effects of atmospheric CO_2 on plant disease development are highly variable and dependent on the genotypes of plant and pathogen as well as on environmental conditions. Atmospheric CO_2 affects stomatal aperture and hormone

homeostasis and these factors are also known key players in plant defense. Here, we investigated the interplay between different CO_2 conditions and defense mechanisms of Arabidopsis to infection by *Pst*. By including a low CO_2 condition in our study, besides ambient and high CO_2 conditions, we also fill in a gap of knowledge on the effects of low CO_2 on plant performance and plant disease resistance, for which studies have been scarce until now (Tissue and Lewis, 2012). Using the Arabidopsis-*Pst* model system, we reveal that at low atmospheric CO_2, resistance of Arabidopsis to *Pst* is enhanced whereas at high atmospheric CO_2 plants are more susceptible. Furthermore, our date suggest that ABA signaling plays an important role in this differential effect, at least partly through interference with COR-mediated stomatal reopening.

Plant and stomata performance under low and high CO_2 conditions

Previously, FACE studies showed that plant growth was significantly promoted by elevated atmospheric CO_2 concentrations (Leakey et al., 2009; Eastburn et al., 2010). In our study we show that the low CO_2 condition significantly reduced growth of Arabidopsis (Fig. 1a and 1b). Moreover, stomata at low CO_2 were more open than those at ambient CO_2 (Fig. 1d). We also found that Arabidopsis grown at high CO_2 displayed a reduced opening of their stomata, as has been reported previously (Araújo et al., 2011). However, in contrast to other studies, we found no increase in rosette dry weight under high CO_2 conditions. It has been demonstrated that different Arabidopsis ecotypes respond to elevated CO_2 differently and all the three Arabidopsis accessions including Col-0 developed at the same rate under ambient and elevated CO_2 conditions (Li et al., 2006). Besides, this could be related to the previously reported time-dependent nature of high CO_2-stimulated growth in which initial enhanced growth is followed by no effect or even inhibition (Poorter, 1993). Furthermore, in our experimental setup plants were cultivated in 60-ml pots and enhanced growth induction by high CO_2 may have been bound by space or nutrients constraints. We observed that there was also no effect of high CO_2 on the density of the stomata, whereas we detected a significant increase at low CO_2 (Fig. 1c). Our divergent observation at high CO_2 for stomatal density may be explained by the same reasons as for the lack of stimulated growth. Additionally, our findings are in line with the previously described CO_2 'ceiling' phenomenon, which refers to the effect that stomatal density reaches a maximum at atmospheric CO_2 concentrations around 400 ppm and that stomata tend to respond more strongly to subambient than to elevated CO_2 concentrations (Kürschner et al., 1997; Royer, 2001).

The differential stomatal aperture responses employed under the three different atmospheric CO_2 conditions suggest that stomatal behavior is finely coordinated in Arabidopsis plants, thereby optimizing plant water use and growth (Haworth et al., 2013). Moreover, the fact that the low and high CO_2 levels tested in our study affect plant and/or stomata performance indicates that our study system is responsive to CO_2 levels and thus suitable for assaying immune responsiveness under the different CO_2 conditions.

***Pst*-induced stomatal closure can be independent of ABA**

For many foliar pathogens, the stomata are important passages into plant leaves. Besides CO_2, ABA signaling is an important determinant of stomatal aperture. This is confirmed by a greater stomatal opening in ABA biosynthesis mutants compared to wild-type plants, which is evident under all three tested CO_2 conditions (Fig. 5a). The stomatal aperture of *aba2-1* was and *abi1-1* was under all three CO_2 conditions the same as that of wild-type plants grown under the low CO_2 condition (Fig. 5a and S2). This indicates the dominant role of ABA signaling in stomatal closure in un-infected plants grown under ambient and high CO_2 conditions. This confirms previous reports on the interrelationship of ABA with CO_2-regulated signaling in guard cells (Leymarie et al., 1999; Nishimura et al., 2010; Xue et al., 2011; McLachlan et al., 2014).

Activation of stomatal closure has been demonstrated to be an essential defense response in various plant species (Melotto et al., 2006; Lee et al., 2013; Li et al., 2013; Du et al., 2014). The ABA-deficient *aba3-1* mutant was previously shown to be compromised in its ability to close its stomata in response to *Pst* infection, suggesting that ABA biosynthesis is required for *Pst*-induced stomatal closure (Melotto et al., 2006). However, in our experiments at ambient CO_2, both wild-type plants and the ABA mutants *aba2-1* and *abi1-1* responded to *Pst* infection with closure of their stomata (Fig. 4 and S2a), indicating that *Pst*-induced stomatal closure occurred at least partly independent of ABA. This is in line with a recent finding (Montillet et al., 2013) that demonstrated that an ABA-independent oxylipin pathway is responsible for PAMP- and *Pst*-induced stomatal closure. Moreover, a generic screen of Arabidopsis mutants that are impaired in closing their stomata upon *Pst* infection generated, some mutants that still exhibited ABA-induced stomatal closure (Zeng et al., 2011). Taken together, these results support the notion that besides ABA signaling, additional mechanisms that are independent of ABA are crucial for the stomatal closure response upon *Pst* infection.

Furthermore, we found that the ABA insensitive mutants *abi1-1* grown under high and low CO_2 conditions displayed the same *Pst*-induced stomatal closure as when grown under the ambient CO_2 condition (Fig. S2b and S2c). This corroborates our finding that other mechanisms than ABA signaling are essential for *Pst*-induced stomatal closure and that atmospheric CO_2 does not influence those mechanisms.

COR-induced stomatal reopening is blocked at low CO_2 and depends on ABA

The phytotoxin COR that is produced by *Pst* induces stomatal opening. We found that, comparable to ambient CO_2 conditions, initial *Pst*-induced stomatal closure was also followed by COR-triggered stomatal reopening under high CO_2 conditions (Fig. 2). Interestingly, while stomata in low CO_2-grown plants still responded to *Pst* with closing at 1 hpi, they did not reopen at 4 hpi, resulting in a stomatal aperture very similar to that of *Pst cor*$^-$-infected plants (Fig. 2). Thus, sensitivity to COR is compromised under low CO_2 conditions.

Previous reports demonstrated that under ambient CO_2 conditions, COR and ABA signaling often influence each other's activity either negatively or positively. For instance, ABA-induced stomatal closure is inhibited by COR (Melotto et al., 2006; Zheng et al., 2012), while both COR and ABA repress SA-regulated defense signaling (Brooks et al., 2005) (De Torres-Zabala et al., 2007). Also, ABA and COR both activate three NAC transcription factors genes that suppress *Pst*-induced SA biosynthesis and stomatal closure (Zheng et al., 2012). Here we show that the two ABA mutants *aba2-1* and *abi1-1* closed their stomata upon inoculation with *Pst* and were unable to reopen them in response to COR production by *Pst* at 4 hpi (Fig. 4 and S2). This unexpectedly pointed to an important novel role for ABA signaling in COR-mediated stomatal reopening. Possibly, reduced expression of the three NAC transcription factors gene *ANAC019*, *ANAC055* and *ANAC072* was reduced in *aba2-1* and *abi1-1*, because these genes are induced by COR in an ABA-dependent way (Zheng et al., 2012). This reduction may contribute to the ABA-dependency of COR-mediated stomatal reopening.

Similarly to the ambient condition, also under high and low CO_2 conditions the *abi1-1* mutants exhibited impaired COR-mediated stomatal reopening (Fig. S2b and S2c), confirming the importance for ABA signaling in COR-mediated stomatal reopening, which is apparent under all tested atmospheric CO_2 conditions.

Low CO_2 and defective ABA signaling enhance resistance to *Pst*, while high CO_2 reduces resistance

In accordance with the blocked stomatal reopening, low CO_2-grown plants exhibited significantly lower amounts of *Pst* at 4 dpi compared with ambient CO_2-grown plants (Fig. 3 and 5a). The growth of *Pst* under low CO_2 conditions was arrested to the same level as that of *Pst cor*$^-$ under low, ambient or high CO_2 conditions (Fig. 3c). This demonstrates that the impairment of COR-mediated defense suppression that is apparent under low CO_2 conditions severely reduces the virulence of *Pst*. Likewise, the inability of the ABA mutants *aba2-1* and *abi1-1* to respond with stomatal reopening to COR was associated with enhanced resistance to *Pst* (Fig. 5b, S2a and S3). Interestingly, the ABA mutants were under all three CO_2 conditions as resistant to *Pst* as wild-type plants grown at low CO_2 (Fig. 5b) and *Pst* and *Pst cor*$^-$ grew to a similar level in the *aba2-1* mutant (data not shown). Thus, the resistance phenotype of low CO_2-grown wild-type plants resembles that of ABA mutants. Moreover, plant growth was inhibited in Col-0 grown at low CO_2 as well as in the ABA mutants (Fig. 1a and 1b; (Chatfield et al., 2000; LeNoble et al., 2004). Together with our finding that ABA levels in low CO_2-grown plants were reduced in both control and *Pst*-infected plants (Fig. 5c), these results suggest that the enhanced resistance to *Pst* that is evident under low CO_2 conditions is related to a decrease in ABA signaling.

In addition to a role in COR-triggered stomatal reopening that we demonstrated, ABA is also know to suppress SA defense signaling, possibly in part by activation of the three above-mentioned NAC transcription factors (De Torres-Zabala et al., 2007; Zheng et al., 2012). Not only did we show that the ABA mutants *aba2-1* and a*bi1-1*

are more resistant to *Pst* infection, but we also demonstrated that the ABA hypersensitive mutant *abi1-2* is more susceptible to *Pst* (Fig. S4). The increased susceptibility of Col-0 at high CO_2 (Fig. 3 and 5b) may be related to an enhancement of ABA signaling, although we did not detect increased levels of ABA (Fig. 5c). Under high CO_2 conditions, fewer *Pst* bacteria entered the leaf, due to the decreased stomatal aperture, but at 4 dpi higher bacterial titers were measured (Fig. 1c and 3a). The enhanced *Pst* growth may be caused by a favorable endophytic environment for the bacteria in terms of nutrition and water availability in high CO_2-grown plants (Lake and Wade, 2009; Pangga et al., 2011). In contrast to our findings, it was recently reported that in tomato plants reduced numbers of *Pst* were detected at elevated CO_2 levels (Li et al., 2014). Thus, enrichment of the endophytic environment alone unlikely explains the full effect of high CO_2 on enhanced *Pst* growth in Arabidopsis that we demonstrated. A possible explanation for the discrepancy regarding susceptibility to *Pst* at high CO_2 between Arabidopsis and tomato plants is that the effects of high CO_2 on the immune response may depend on the plant genotype, as has been demonstrated previously (Johnson et al., 2014). Furthermore, Li et al. (2014) exposed the tomato plants to high CO_2 only for 2 days prior and 2 days after inoculation, while in our assays the Arabidopsis plants were exposed to the different CO_2 levels for 2 weeks prior and 4 days after inoculation.

Conclusion

Our results show that atmospheric CO_2 influences plant resistance to *Pst*, whereby pre-industrial, low CO_2 levels lead to a decrease, and oppositely forth-coming, high CO_2 levels lead to an increase in susceptibility of Arabidopsis to *Pst*. ABA signaling is demonstrated to be a key regulator of COR-mediated stomatal reopening and susceptibility to *Pst*. Under low CO_2 conditions ABA levels are reduced, which could explain the defect in COR-mediated stomatal reopening and the enhanced resistance to *Pst*. The global rise in atmospheric CO_2 may be causal for the detected increase in ABA levels under ambient and high CO_2 conditions compared to the low CO_2 condition. Hence, if, as our data suggest, susceptibility to *Pst* correlates with increased ABA levels at rising atmospheric CO_2, plant disease resistance in general may be threatened by the current rise in global atmospheric CO_2. Further research on how atmospheric CO_2 alters ABA signaling and possibly other important components of plant immune signaling, is required to better predict the global impact of elevated CO_2 levels on plant health to fine tune plant immune responses.

MATERIALS AND METHODS

Plant materials and cultivation

Seeds of *Arabidopsis thaliana* accessions Col-0 and Landsberg *erecta* (Ler-0), and mutants *aba2-1* [Col-0] (Koornneef et al., 1982), *abi1-2* [Col-0] (Gosti et al., 1999) and *abi1-1* [Ler-0] (Koornneef et al., 1984) were sown in quartz sand under ambient CO_2 conditions (450 ppm). Two weeks later, seedlings were transferred to 60-ml pots containing a sand/potting soil mixture (v/v, 5:12) that was autoclaved twice for 20 min, and were transferred to high (800 ppm), ambient (450 ppm) or low (150 ppm) atmospheric CO_2 conditions. Plants grew at a 10-h day (350 μmol/m^2/s) and 14-h night cycle at 20°C with 70% relative humidity. Plants were watered every other day and receive with half-strength Hoagland solution (Hoagland and Arnon, 1938) once a week. Plants were treated when four weeks old in all experiments. For dry weight measurement, 10 rosettes per time point were put separately in a paper bag and dried for 3 days at 60°C.

Stomata measurement

Stomatal aperture and density were measured by a modified protocol of dental resin impressions (Geisler et al., 2000). Two components of the dental resin Present Light Body (Coltène, Altstatten, Switzerland) were mixed thoroughly (v/v, 1:1) and the abaxial side of the plant leaves was softly pressed onto the dental resin immediately after harvesting. Leaves were removed 10 min later when the mixture had hardened. Transparent nail polish was applied to the dental resin molds to create casts, which were fixed on microscope slides with Anutex modelling wax (Kemdent, Purton, Swindon, Wiltshire, UK) for further observation.

Stomatal aperture and density were examined using an Olympus microscope and analyzed with the Olympus Software (Analysis D) on the pictures taken. Stomata aperture was determined by measuring the width and length of the stomata. At least six leaves were harvested for each treatment and 20-30 observations were made of each leaf.

Cultivation of bacteria and bioassays

Pst and *Pst cor*$^-$ strains (Whalen et al., 1991; Brooks et al., 2005) were grown on KB medium (King et al., 1954) supplemented with 50 μg/ml rifampicine. Bacterial strains were then cultured in liquid KB medium for 24 h at 28˚C. Bacteria were collected by centrifugation for 10 min at 4,000 rpm, and resuspended in 10 mM $MgSO_4$. The suspension was adjusted to OD_{600}=1. For dip inoculation, the bacterial inoculum was diluted to a final concentration of 5×10^7 cfu/ml containing 0.015% (v/v) Silwet L-77 (Van Meeuwen Chemicals, Weesp, Netherlands). For pressure infiltration, a needleless syringe with a bacterial inoculum of 6×10^5 cfu/ml (unless specified otherwise) was used. A 10 mM $MgSO_4$ solution was used as the mock treatment.

Leaf discs from infected plants were harvested and surface sterilized in 70% ethanol for 8 sec and washed with water immediately. Eight biological replicates were

included for each data point. Subsequently, 200 µl of 10 mM $MgSO_4$ was added to the samples, after which they were ground thoroughly. Ten-µl aliquots of different dilutions were plated onto KB agar plates containing 25 µg/ml rifampicine. After 48 h incubation at room temperature, bacterial colonies were counted. Growth data were log_{10} transformed and subsequently subjected to statistical analysis.

ABA measurement

For ABA quantification, 60-250 mg leaf material was harvested at 24 h after treatment and ground thoroughly to powder using liquid nitrogen. ABA was extracted as described (Scala et al., 2013). The samples were suspended in 0.5 ml of 70% methanol by shaking at 6,000 rpm for 40 s. Subsequently, the homogenates were centrifuged at 10,000 rpm for 20 min at 4˚C. The supernatants of two extraction steps were pooled together. Hormone levels were quantified by liquid chromatography-mass spectrometry (LC-MS) analysis on Varian 320 Triple Quad LC/MS/MS. The endogenous ABA levels were quantified with a standard curve made from an external ABA standard.

ACKNOWLEDGEMENTS

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SUPPORTING INFORMATION

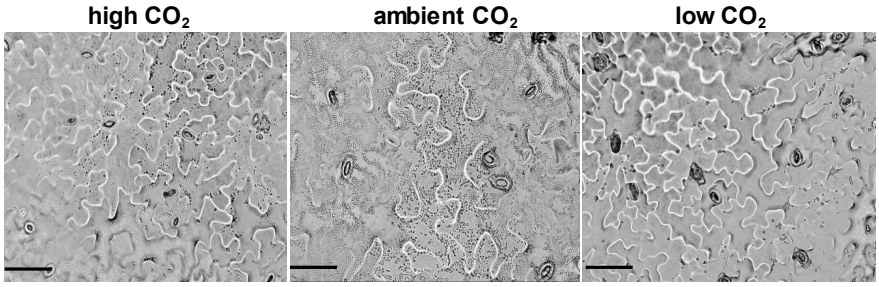

Figure S1: Effect of atmospheric CO_2 levels on stomatal density of Arabidopsis plants.
Shown are microscopic images of imprints from leaves of 4-week-old Arabidopsis plants grown under three different atmospheric CO_2 conditions. Bars depicts 50 µm in all three pictures.

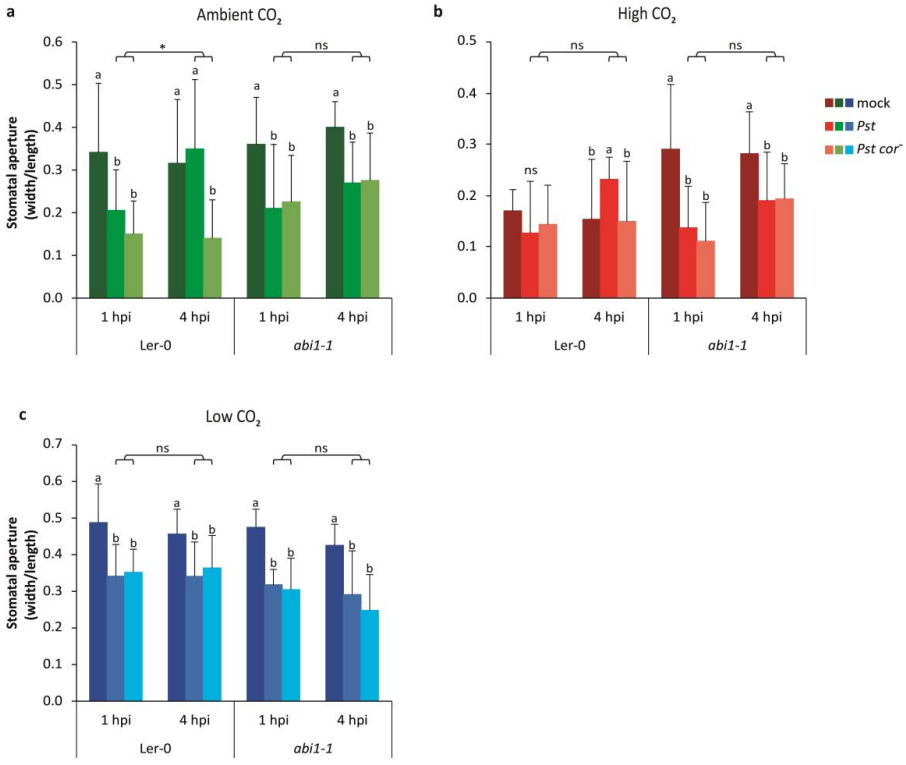

Figure S2: Effect of ABA signaling on stomatal aperture in response to *Pst* and *Pst cor*⁻ under three different CO_2 conditions.
(S2a-c) Stomatal aperture in wild-type Ler-0 and the ABA insensitive mutant *abi1-1* in response to *Pst* and *Pst cor*⁻ under ambient **(S2a)**, high **(S2b)**, and low CO_2 **(S2c)** conditions. Stomatal aperture was measured at 1 h and 4 h after dip inoculation with *Pst*, *Pst cor*⁻ (5×10^7 cfu/ml) or a mock solution in wild-type Ler-0 and the ABA insensitive mutant *abi1-1*. Indicated are the averages of stomatal aperture (±SD) of six leaves. Different letters indicate statistically significant differences between the treatments of one plant genotype at the indicated time point. Indications above the brackets specify the interaction (bacterium genotype × time) between the two *Pst* genotype treatments (wild-type and mutant) and the time (1 h and 4 h) within the same Arabidopsis genotype (two-way ANOVA, Fisher's LSD test, *, $P<0.05$; ns, not significant).

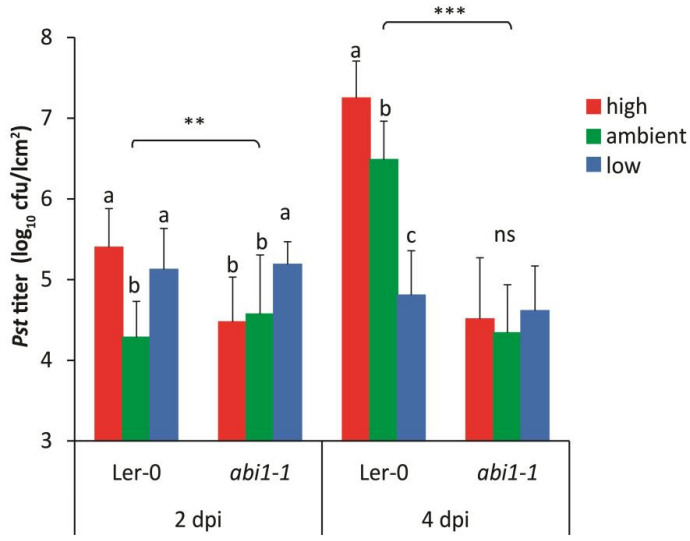

Figure S3: Effect of ABA signaling on atmospheric CO_2-altered resistance to *Pst* in Arabidopsis.
Pst growth in wild-type Ler-0 and the mutant *abi1-1* measured at 2 d and 4 d after dip inoculation. Indicated are the averages of the $\log_{10}$-transformed bacterial titer (±SD; per leaf area) from eight biological replicates. Different letters indicate statistically significant differences between the CO_2 treatments within one line at the indicated time point. Indications above the brackets specify the interaction (CO_2 condition × Arabidopsis genotype) between the three CO_2 conditions and the two Arabidopsis genotype (wild-type Ler-0 and the mutant *abi1-1*) at the same time point (two-way ANOVA, Fisher's LSD test, **, P<0.01; ***, P<0.001).

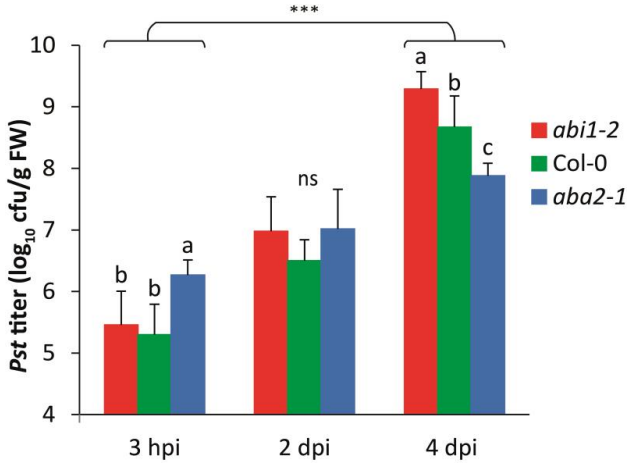

Figure S4: The role of ABA signaling in Arabidopsis resistance to *Pst*.
Pst growth in the ABA hypersensitive mutant *abi1-2*, wild-type Col-0 and the ABA deficient mutant *aba2-1* measured at 3 h, 2 d and 4 d after dip inoculation under the ambient CO_2 condition. Indicated are the averages of the $\log_{10}$-transformed bacterial titer (±SD; per g of leaves) from eight biological replicates. Different letters indicate statistically significant differences between the genotypes at the specific time point. Indications above the brackets specify the interaction (Arabidopsis genotype × time) between the three Arabidopsis genotype (wild-type Col-0 and the mutants *abi1-2*, *aba2-1*) and the time (3 hpi and 4 dpi) (two-way ANOVA, Fisher's LSD test, ***, P<0.001).

CHAPTER 3

Beta-carbonic anhydrases CA1 and CA4 inhibit PAMP-triggered immunity through antagonizing salicylic acid-dependent defense

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ABSTRACT

Changes in atmospheric CO_2 levels have been shown to affect plant immunity. Beta-carbonic anhydrases (CAs) play an important role in CO_2 metabolism and there is some evidence that they are involved in plant immunity. Here we provide evidence that two CAs of *Arabidopsis thaliana* (CA1 and CA4) negatively impact basal plant immunity. Inoculation of Arabidopsis with different strains of the bacterial pathogen *Pseudomonas syringae* or a coronatine minus mutant of this pathogen resulted in the inhibition of *CA1* and *CA4* gene expressions. Similarly, application of the pathogen-associated molecular pattern (PAMP) flg22 resulted in down-regulation of the *CAs*, suggesting that suppression of *CA* gene expression is a PAMP response. The *CA* double mutant *ca1ca4* showed enhanced disease resistance against *P. syringae*. Furthermore, *P. syringae* infection or flg22 treatment of the *ca1ca4* double mutant resulted in enhanced accumulation of SA and enhanced transcriptional activation of the SA-responsive defense-related genes *PR1*, *PR2*, *FRK1*, *ICS1, WRKY22* and *WRKY29*. Together, these results indicate that in wild-type plants, the combined activity of CA1 and CA4 down-regulates PAMP-triggered immunity through antagonizing SA-dependent defense signaling.

INTRODUCTION

Plants have developed a complex immune system to survive from attacks by a wide range of pathogenic microbes. Upon perception of pathogen-associated molecular patterns (PAMPs), defense responses are activated, including stomatal closure, oxidative burst generation, MAP kinase activation, hormonal signaling, and massive transcriptional reprogramming (Tsuda and Katagiri, 2010; Zipfel and Robatzek, 2010). These induced signal outputs collectively lead to a PAMP-triggered immunity (PTI), which forms the first layer of plant defense, and thus is critical to the survival of plants exposed to various potential pathogens.

Carbonic anhydrases (CAs) are metalloenzymes that were initially purified from red blood cells, and mainly function as catalysts in the interconversion of carbon dioxide (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, 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. In plants, it has been shown that CAs are involved in a wide range of biological processes, including CO_2 homeostasis, stomatal aperture, respiration, photosynthesis and pH regulation (Henry, 1996; Smith and Ferry, 2000; Hu et al., 2010).

βCAs represent the most prominent group of CAs in plants. In several cases they have been implicated in plant defense. For instance, a plastidic *CA* gene was found to be severely down-regulated in potato leaves upon challenge with the potato late blight pathogen *Phytophthora infestans* (Restrepo et al., 2005). Similarly, the *CA* gene *TC52686* in grapevine was shown to be suppressed during infection with the grapevine downy mildew pathogen *Plasmopara viticola* (Polesani et al., 2008). In contrast, five CA protein spots 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 alterations of CA protein or *CA* transcript levels in plants upon attack by pathogens suggest a role of CAs in plant defense. Indeed, for a chloroplast-localized CA of tobacco, also known as salicylic acid (SA)-binding protein 3 (SABP3), a positive role in plant defense was suggested as silencing of this *CA* gene in *Nicotiana benthamiana* leaves suppressed the hypersensitive response mediated by the *Pto: avrPto* resistance gene: avirulence gene pair (Slaymaker et al., 2002). Moreover, *CA*-silenced *N. benthamiana* showed enhanced susceptibility to *P. infestans*, which supports the hypothesis that CAs contribute to disease resistance (Restrepo et al., 2005). Although evidence is accumulating that CAs play a role in plant immunity (Wang et al., 2009), little is known about how CAs are regulated and how they affect plant immunity during plant-pathogen interactions.

Here we investigated the role of two *CA* genes of Arabidopsis (*CA1* and *CA4*) in plant immunity using the model plant-pathogen system *Arabidopsis thaliana*-*Pseudomonas syringae*. We provide evidence that down-regulation of *CA1*

and *CA4* is part of the PTI response and that these *CAs* act as negative regulators of PTI, likely through antagonizing SA-mediated signaling.

RESULTS

Pseudomonas syringae represses the expression of *CA1* and *CA4* independently of coronatine

In a previous study we investigated the effect of different levels of atmospheric CO_2 on the level of resistance of Arabidopsis to the bacterial speck disease pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) (Chapter 2). Because CA1 and CA4 play an important role in CO_2 metabolism (Hu et al., 2010) and CAs are implicated in plant immunity (Restrepo et al., 2005), we monitored the expression of the *CA* genes *CA1* and *CA4* upon *Pst* infection. As shown in Fig. 1, in mock-infiltrated leaves *CA1* and *CA4* showed a similar basal expression pattern, in which expression was highest at 24 h after treatment (11:00 am during the day). This corroborates the previous report showing a diurnal rhythm of the *CA* gene expression in *Chlamydononas reinhardtii* (Fujiwara et al., 1996). Upon pressure infiltration with *Pst*, the expression levels of the *CAs* were suppressed in comparison to the mock treatment (Fig. 1a and 1b). This is consistent with previous reports that showed down-regulation of *CA*s in potato plants infected with *P. infestans* (Restrepo et al., 2005) and grapevine plants infected with *P. viticola* (Polesani et al., 2008). These results suggest that repression of *CA* gene expression is a general plant response induced by various pathogens.

Several strains of *P. syringae* produce the phytotoxin, coronatine, which has been demonstrated to suppress plant defenses during *P. syringae* infection (Mittal and Davis, 1995; Brooks et al., 2005). To test whether the virulence factor coronatine plays a role in the suppression of *CA* gene expression we inoculated Arabidopsis with the wild-type strain of *P. syringae* pv. *maculicola* ES4326 (*Psm*) and its mutant *Psm cor*$^-$, which is defective in coronatine production. Upon pressure infiltration of the leaves with these bacterial strains, the expression level of *CA1* and *CA4* was monitored. At 24 h after inoculation, *CA1* and *CA4* were repressed to the same extent by *Psm cor*$^-$ as by *Psm* (Fig. 1c and 1d). This suggests that the virulence factor coronatine does not play a role in the suppression of the *CA* genes.

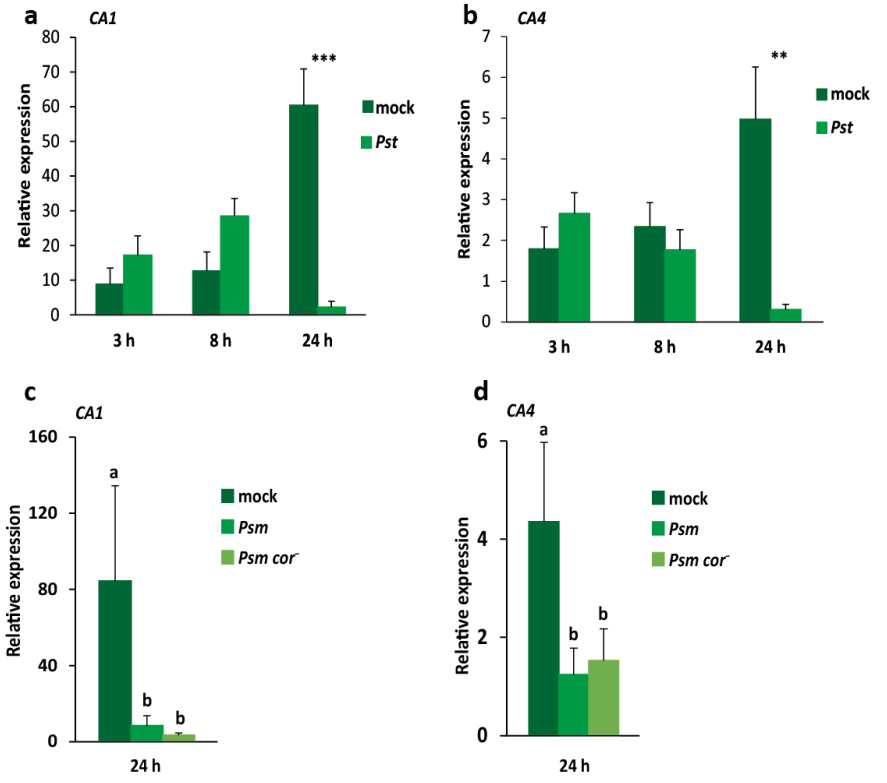

Figure 1: *Pseudomonas syringae* represses the expression of *CA1* and *CA4* independently of the virulence factor coronatine.
(a) and **(b)** RT-qPCR analysis of *CA1* **(a)** and *CA4* **(b)** transcript levels in 4-week-old Arabidopsis leaves at 3, 8, and 24 h after infiltration of the leaves with mock (10 mM $MgSO_4$) or *Pst* (4×10^6 cfu/ml). Indicated are expression levels relative to the reference gene *At1g13320*. Asterisks indicate statistically significant differences between mock and *Pst* treatment at specific time points (Student's *t* test; **, $P<0.001$; ***, $P<0.0001$). **(c)** and **(d)** Relative expression of *CA1* **(c)** and *CA4* **(d)** in leaves of 4-week-old plants 24 h after infiltration with 10 mM $MgSO_4$, *Psm* or *Psm cor*$^-$(1×10^7 cfu/ml). Different letters indicate statistically significant differences between treatments (one-way ANOVA; Fisher's LSD test; **, $P<0.01$; *, $P<0.05$; ns, not significant). Error bars represent SD, $n=3$ plants.

CA1 and *CA4* negatively regulate plant defense responses to *Pst*

To further investigate the function of CAs in plant defense, we tested defense responses in the *ca1ca4* double mutant, carrying homologous T-DNA insertion in *CA1* and *CA4* (Hu et al., 2010). The CA1 and CA4 proteins are localized to the vicinity of the plasma membrane and function redundantly in CO_2-regulated stomatal movements (Hu et al., 2010). Therefore, we reasoned that it is likely that CA1 and CA4 also have redundant functions in plant defense. Pathogen-induced stomatal closure has been established as an important defense response in plant resistance against *P. syringae* (Melotto et al., 2006). We hypothesize that the highly abundant of CA1 and CA4 proteins in guard cells (Hu et al., 2010) also play a role in *Pst*-triggered stomatal closure. We tested stomatal responsiveness in both the double mutant *ca1ca4* and the single mutants *ca1* and *ca4* to dip inoculation with *Pst*, by which the bacteria can enter the plant leaves only through the stomata. Consistent with previous

results (Chapter 2), stomata in wild-type Col-0 plants reacted with closing between 1 h and 2.5 h after *Pst* inoculation, and reopening at 4 h (Fig. 2a). The single *ca1* and *ca4* mutant showed slightly but not significantly different *Pst*-induced stomatal responses compared to wild-type Col-0 (data not shown). As demonstrated by Hu et al. (2010), we also observed that the stomatal aperture of *ca1ca4* is significantly higher than that of Col-0 (data not shown). However, *Pst*-induced stomatal closure was delayed in the double *ca1ca4* mutant and became only apparent at 4 h after inoculation, after which the stomata remained closed until at least 7 h after inoculation (Fig. 2a). The delayed yet sustained stomatal closure triggered by *Pst* infection in the *ca1ca4* mutant supports the notion that *CA1* and *CA4* are involved in *Pst*-induced stomatal regulation.

We also performed disease resistance assays with the *ca1ca4* mutant in which growth of *Pst* and disease symptoms were monitored. We found that the single *ca* mutants showed bacterial numbers that were comparable to wild-type plants (data not shown). However, the *ca1ca4* double mutant exhibited a bacterial titer that was significantly lower than that of Col-0 plants at 4 d after dip inoculation with *Pst* (Fig. 2b). Plants were also inoculated with *Pst* by pressure infiltration, which bypasses stomatal defense. We monitored the disease progress by scoring disease symptoms and presented it as disease index, indicating the leaf area showing chlorosis or watersoaked lesions. Fig. 2c shows that the *ca1ca4* mutant developed significantly fewer symptoms than Col-0 plants at 3 d and 4 d after infiltration. Together, these results suggest that CA1 and CA4 play a negative role in disease progression in response to *Pst* infection, thus negatively impacting resistance to *Pst*.

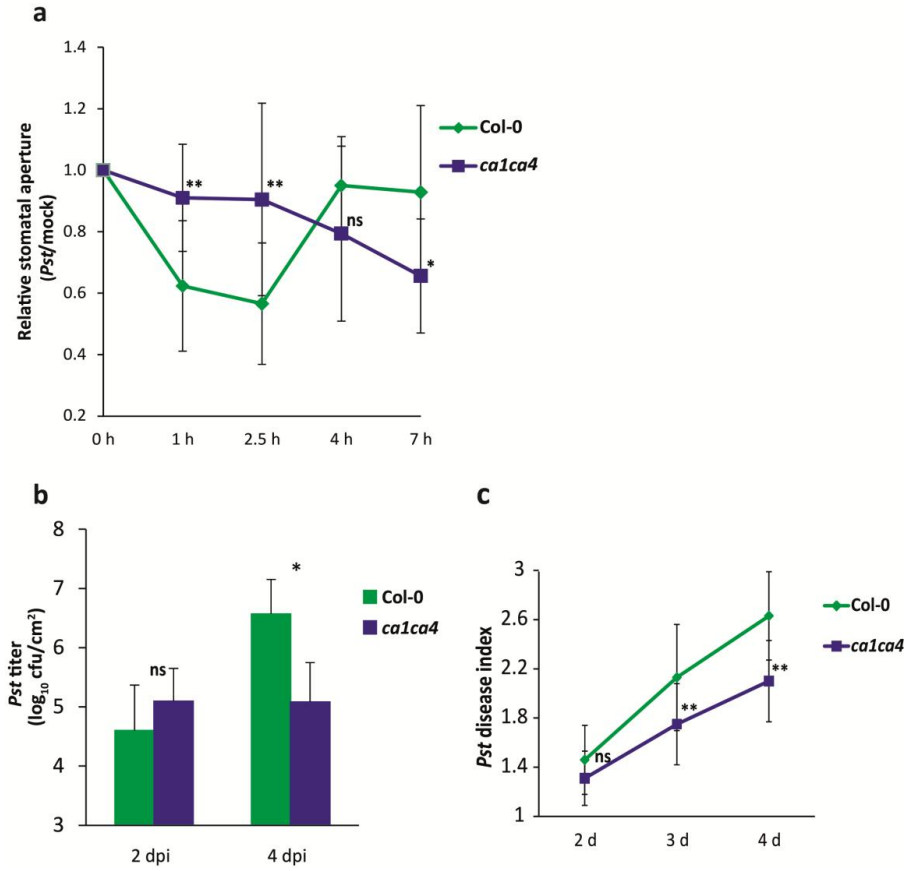

Figure 2: CA1 and CA4 are involved in *Pst*-induced stomatal responses and resistance to *Pst*.
(a) Stomatal closure response in leaves of 4-week-old wild-type Col-0 and mutant *ca1ca4* plants at 1, 2.5, 4, and 7 h after dip inoculation with *Pst*. Depicted are averages of stomatal apertures in *Pst*-treated leaves relative to mock-treated leaves. Error bars represent SD, *n*=6 leaves. **(b)** Four-week-old Col-0 and *ca1ca4* plants were dip inoculated with *Pst* (5×10^7 cfu/ml). Bacterial growth was measured at 2 and 4 d after inoculation. Indicated are the averages of $\log_{10}$-transformed bacterial titers per leaf area. Error bars represent SD, *n*=8 plants. **(c)** Four-week-old Col-0 and *ca1ca4* plants were pressure infiltrated with *Pst* (6×10^5 cfu/ml). Disease symptoms were scored at 2, 3, and 4 d after infiltration. 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 watersoaked area per leaf; class 2, 10-50% chlorotic or watersoaked area per leaf; class 3, >50% chlorotic or watersoaked area per leaf. Error bars represent SD, *n*=12 plants. Student's *t* test was used to compare means of relative stomatal aperture, $\log_{10}$-transformed bacterial numbers, or the disease index between Col-0 and *ca1ca4* at specific time points (**, $P<0.01$; *, $P<0.05$; ns, not significant).

CA1 and *CA4* antagonize *Pst*–induced SA signaling

In Arabidopsis, SA plays an important role in defense against *Pst* (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 SA levels and expression levels of the SA-responsive genes *FRK1*, *ICS1*, *PR1*, *PR2*, *WRKY22* and *WRKY29*. Fig. 3A shows that *Pst*-induced SA accumulation was significantly enhanced in the *ca1ca4* mutant compared to the wild-type Col-0 plants. In addition, all tested SA-responsive genes were induced by *Pst* to a significantly

higher level in *ca1ca4* than in Col-0 (Fig. 3B-3G). Moreover, nearly all genes showed a slightly enhanced basal expression level in the *ca1ca4* mutant. These data suggest that CA1 and CA4 interfere with plant defense through counteracting SA biosynthesis and signaling.

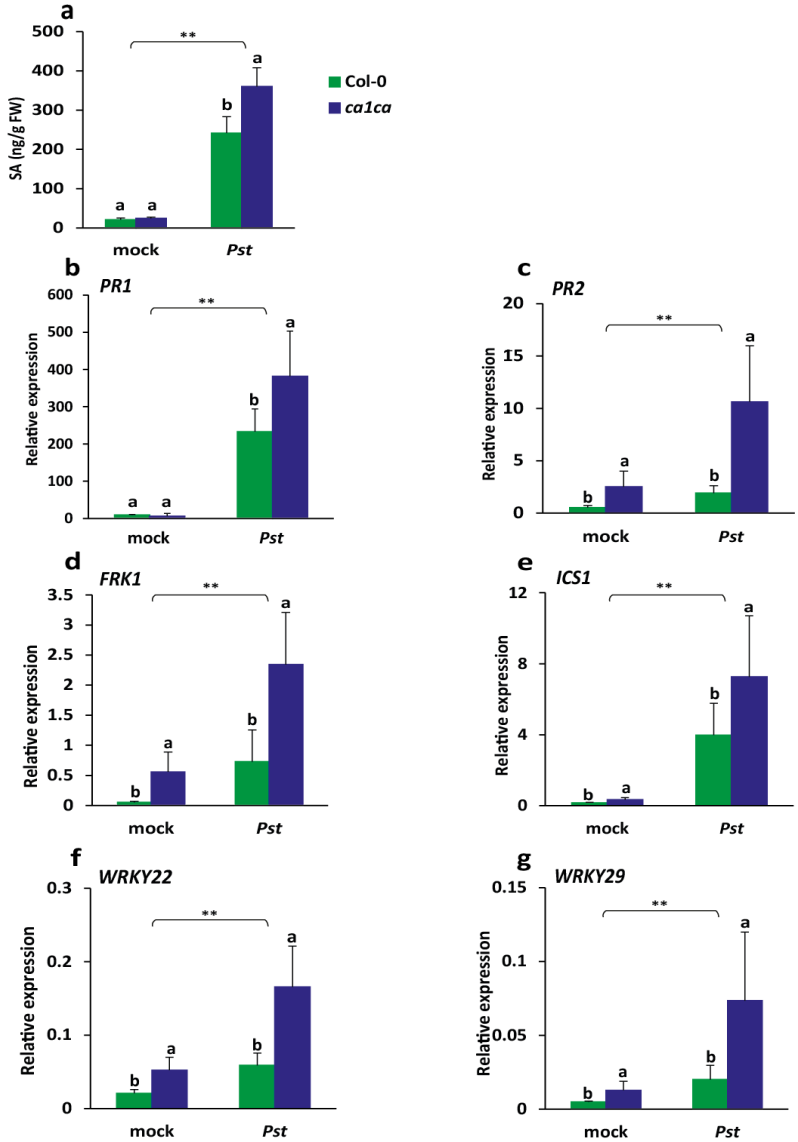

Figure 3: Increased SA accumulation and enhanced SA-responsive gene expression in mutant *ca1ca4* upon infection by *Pst*.

(a) SA levels in leaves of 4-week-old Col-0 and *ca1ca4* plants 24 h after *Pst* infiltration (1X10^{8}cfu/ml). Depicted are the average SA levels in leaves of mock- and *Pst*-treated Col-0 and *ca1ca4* plants. Error bars represent SD, *n*=5 plants. **(b-g)** RT-qPCR analysis of transcript levels of the SA-responsive genes *PR1* **(b)**, *PR2* **(c)**, *FRK1* **(d)**, *ICS1* **(e)**, *WRKY22* **(f)** and *WRKY29* **(g)** in 4-week-old Col-0 and *ca1ca4* plants 24 h after mock treatment or *Pst* infiltration (4X10^{7}cfu/ml). Indicated are expression levels of the tested genes relative to the reference gene *At1g13320*. 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 (**, $P<0.01$).

Suppression of *CAs* is a PAMP-mediated response

The down-regulation of *CA* gene expression by *P. syringae* is unlikely mediated by the virulence factor coronatine (Fig. 1a) and CAs seems suppress SA defense signaling (Fig. 1 and 3). Therefore, we speculated that suppression of the *CA* genes might be a PAMP-mediated response. To investigate this, we examined the expression pattern of *CA1* and *CA4* in response to flg22, which is the functional epitope of the bacterial PAMP flagellin. As shown in Fig. 4a, the expression of the flg22-induced marker gene *FRK1* was significantly enhanced in Col-0 wild-type plants from 2 h after flg22 treatment onwards. Interestingly, *CA1* exhibited an opposite expression pattern compared to *FRK1*, showing a noticeable suppression, which started at 2 h (Fig. 4b). The expression pattern of *CA4* in response to flg22 was different from that of *CA1*, as expression during the first two hours was enhanced, followed by a decline to similar levels as the mock treatment at 4 h (Fig. 4c). In the next experiment, the expression of *CA1* and *CA4* was examined at 24 h after flg22 application in both the wild-type Col-0 and mutant *fls2-1* that, lacks the receptor for flg22 and thus is defective in flg22-induced downstream signaling. Fig. 4d and 4e shows that 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 *fls2-1* mutant. This indicates that the suppression of *CA* genes occurs downstream of the recognition of the PAMP flg22.

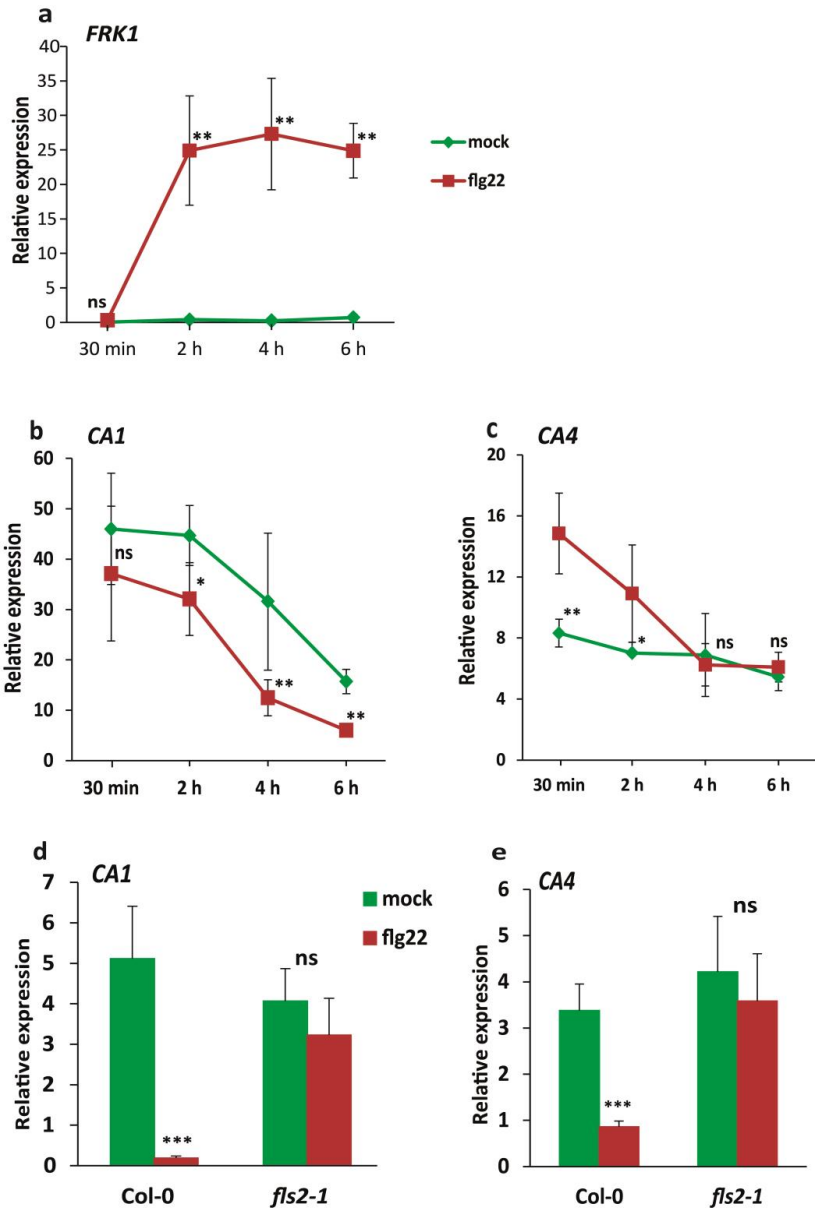

Figure 4: Expression of *CA1*, *CA4* and the flg22 marker gene *FRK1* in response to flg22 treatment.
Kinetics of the expression of *FRK1* **(a)**, *CA1* **(b)**, and *CA4* **(c)** in response to flg22 (500 nM) treatment in 2-week-old Col-0 seedlings at specified time points (30 min and 2, 4 and 6 h). Indicated are the expression levels of the tested genes relative to the reference gene *At1g13320*. Student's *t* test was used to compare means of relative expression between mock and flg22 treatment at specific time points (**, $P<0.01$; *, $P<0.05$; ns, not significant). Error bars represent SD, $n=3$ plants. **(d)** and **(e)** RT-qPCR analysis of *CA1* **(d)** and *CA4* **(e)** gene expression in 2-week-old seedlings of Col-0 and *fls2-1*. Leaf tissue was harvested 24 h after water or flg22 treatment (125 nM). Indicated are the expression levels relative to the reference gene *At1g13320*. Fisher's LSD test was used to compare means of relative expression between mock and flg22 treatment within genotypes (two-way ANOVA; ***, $P<0.01$; ns, not significant). Error bars represent SD, $n=3$ plants.

Flg22-mediated repression of *CA1* requires ethylene signaling

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 PAMP-induced suppression of the *CA* genes, we tested the expression of *CA1* and *CA4* 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 at 8 h after application occurred to the same extent in the mutants *aba2-1*, *coi1-1*, and *npr1-1* as in wild-type Col-0 (Fig. 5). In contrast, *ein2-1* mutant did not display suppression of *CA1* gene expression. We did not detect profound differences in *CA4* expression (data not shown), which is likely due to the relatively early sampling time point (Fig. 4). It has been demonstrated that ET signaling is required for the accumulation of FLS2, the Arabidopsis receptor kinase that recognizes and physically interacts with flg22 and activates subsequent responses (Mersmann et al., 2010), which supports the link between ET and the observed flg22-mediated down-regulation of *CA1*.

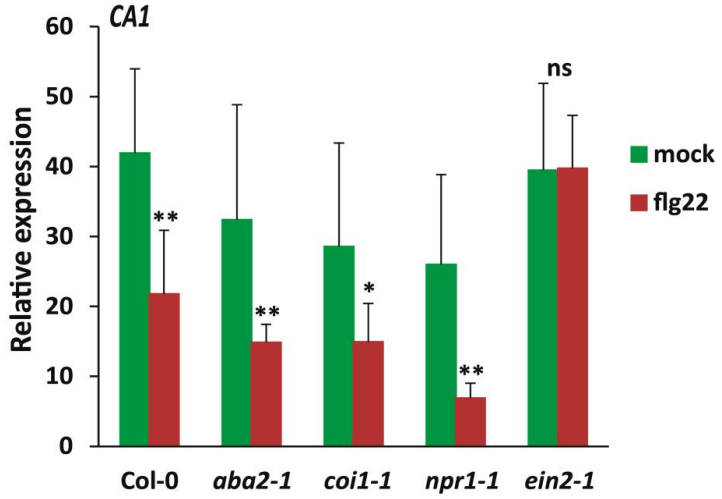

Figure 5: Suppression of *CA1* by flg22 in different hormone signaling mutants.
Relative expression of *CA1* in 2-week-old seedlings of Col-0, *aba2-1*, *coi1-1*, *npr1-1*, and *ein2-1*. Leaf tissue was harvested 8 h after mock or flg22 treatment. Fisher's LSD test was used to compare means of relative expression between mock and flg22 treatment within each genotype (two-way ANOVA; **, $P<0.01$; *, $P<0.05$; ns, not significant). Error bars represent SD, $n=3$ plants.

Enhanced PAMP-induced responses in the *ca1ca4* mutant

After PAMP perception, multiple responses are activated (oxidative burst, stomatal closure and SA accumulation), often accompanied with substantial transcriptional reprogramming (Zipfel and Robatzek, 2010). To better understand the function of CAs in PAMP-triggered immunity, we examined several flg22-induced responses in the *ca1ca4* mutant including the expression levels of defense-related genes (*FRK1* and *ICS1*), reactive oxygen species (ROS) production, and flg22-mediated inhibition of plant growth. Fig. 6a shows that *ca1ca4* displayed a nearly three times level of flg22-induced *FRK1* transcript level than Col-0. Likewise, significantly augmented

transcript levels of the SA biosynthesis gene *ICS1* were induced in the *ca1ca4* mutant after flg22 application (Fig. 6b). These results resemble the expression patterns of these genes upon infection with *Pst* (Fig. 3), thus confirming that suppression of *CAs* by flg22 is part of the PAMP-triggered immunity response leading to induction of SA signaling.

Flg22 treatment is known to cause strong growth inhibition in Arabidopsis seedlings (Gómez-Gómez et al., 1999). To assay for flg22-mediated growth inhibition, Col-0 and *ca1ca4* seedlings were supplied with flg22. After 2 weeks of growth, the seedlings were weighed. Fig. 6c shows that when treated with 1 nM flg22, Col-0 showed more than 50% growth reduction. Interestingly, growth of *ca1ca4* was reduced to an even greater extent than Col-0 after treatment with 1 nM and 10 nM flg22. Generation of ROS species is another feature of the flg22-induced defense response. Fig. 6d and 6e show that the flg22-triggered ROS burst was significantly enhanced in the *ca1ca4* mutant compared with that in Col-0. Together, these results show that *ca1ca4* plants display an augmented response to flg22 treatment, resulting in enhanced SA-dependent gene expression, augmented ROS production, and a greater inhibition of growth. From this we conclude that in wild-type plants CA1 and CA4 play a role in repressing PAMP-mediated defense responses.

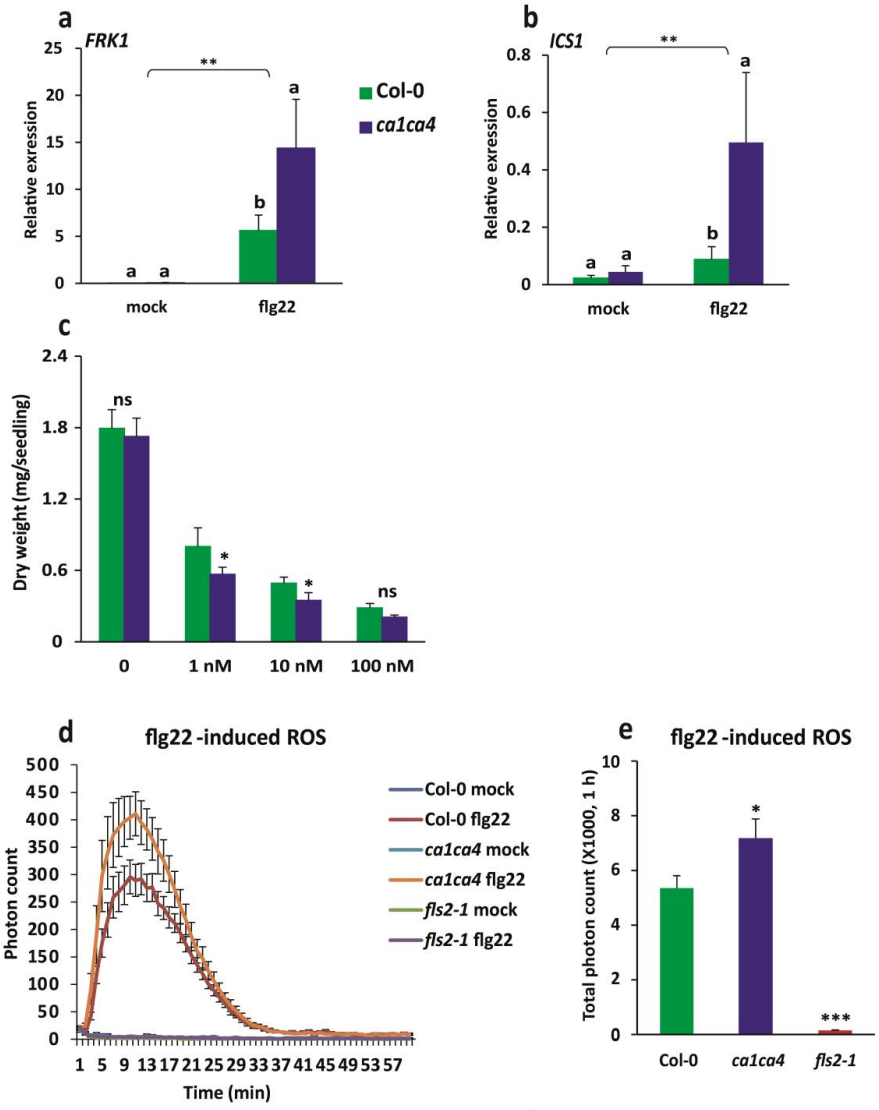

Figure 6: Augmented PTI responses in the *ca1ca4* mutant upon flg22 treatment.
(a-b) RT-qPCR analysis of *FRK1* **(a)** and *ICS1* **(b)** in 2-week-old seedlings of wild-type Col-0 and mutant *ca1ca4* plants. Leaf tissue was harvested 3 h after mock or flg22 treatment (250 nM). Indicated are the expression levels relative to the reference gene *At1g13320*. 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*<0.01). Indications above the brackets specify whether there is an overall statistically significant difference between mock and flg22 treatment (**, *P*<0.01). **(c)** Dry weight of 2-week-old seedlings of Col-0 and *ca1ca4* grown that were treated with 0, 1, 10, or 100 nM flg22. Depicted are the averages of dry weight per seedling. Error bars represent SD, *n*=8 seedlings. Student's *t* test was used to compare means of dry weight between Col-0 and *ca1ca4* with the same concentration (*, *P*<0.05; ns, not significant). **(d)** The ROS production induced by flg22 treatment (100 nM) in 2-week-old seedlings of Col-0, *ca1ca4*, and *fls2-1*. Depicted are photon counts in each genotype after mock or flg22 treatment at specific time points. Error bars represent SE, *n*=4/12 (mock/flg22) seedlings. **(e)** Total ROS production (photon count) in 2-week-old seedlings of Col-0, *ca1ca4*, and *fls2-1* at 1 h after flg22 treatment. Asterisks above the brackets indicate statistically significant differences between mutants and wild-type Col-0 (ANOVA, Fisher's LSD test; *, *P*<0.05; ***, *P*<0.001). Error bars represent SE, *n*=12 seedlings.

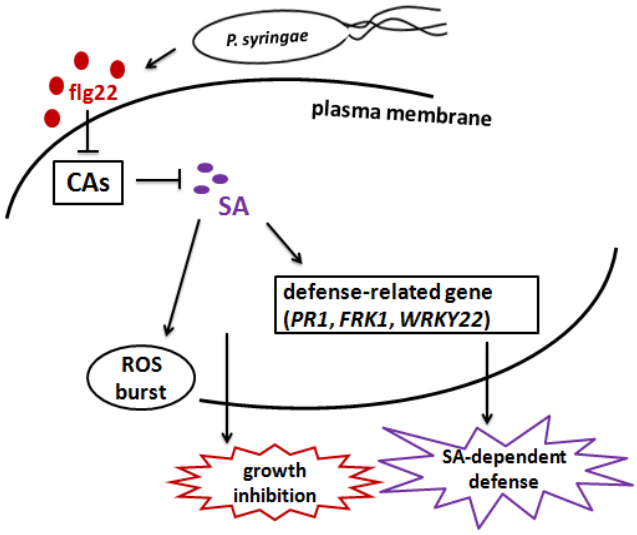

Figure 7: A model of the role of CA1 and CA4 in suppression of SA-dependent defense during PTI.
Upon attack by *P. syringae*, plants recognize the flg22 epitope of the PAMP flagellin, resulting in down-regulation of *CA1* and *CA4*. In healthy plants, CAs have an antagonizing effect on SA signaling, hence, flg22-mediated suppression of the CAs results in enhanced ROS production and increased defense-related gene expression, ultimately leading to enhanced SA-dependent defenses and inhibition of growth. Arrows, induction; blocked lines, repression.

DISCUSSION

PAMP-associated immune responses have been documented extensively during the last decade, from identification and regulation of PAMP receptors (Zipfel et al., 2006; Chinchilla et al., 2007; Heese et al., 2007) to investigations on the downstream signal cascades (Trujillo et al., 2008; Chen et al., 2009; Schwessinger et al., 2011; Kadota et al., 2014). In this study, we revealed that *CA1* and *CA4* of Arabidopsis are players in the modulation of the PTI response.

CAs are important enzymes in CO_2 metabolism and may play a role in the serious effects on plant disease resistance that changes in atmospheric CO_2 levels can have (Restrepo et al., 2005; Polesani et al., 2008). In Arabidopsis, we found that the expression of *CA1* and *CA4* was profoundly repressed upon *P. syringae* infection. The repression of *CA1* and *CA4* was independent of coronatine, indicating that this host immune suppressive virulence factor does not play a role in this phenomenon. We further demonstrated that repression of *CA1* and *CA4* is triggered by flg22 (Fig. 4), a common PAMP in plant innate immunity (Nürnberger et al., 2004; Pel and Pieterse, 2013). Interestingly, a microarray analysis with transgenic Arabidopsis seedlings overexpressing the newly identified PAMP, *HaNLP*, also showed strong down-regulation of both *CA1* and *CA4* (Oome et al., 2014). Moreover, data collected from Genevestigator (Perturbation module) also show suppressed expression patterns of *CAs* in response to multiple biotic stresses (*P. syringae* and *Sclerotinia sclerotiorum*) and elicitors (e.g. flg22, EF-Tu). Collectively, this indicates that

suppression of *CAs* is part of the basal plant immune response that aids in mounting resistance against the pathogen encountered.

Unlike the repression in transcription of *CA* genes in response to pathogens and PAMPs, the CA proteins seem oppositely regulated at the post-transcriptional level. For instance, CA1 protein was more abundant in Arabidopsis plants that are *Plutella xylostella*-resistant than in *P. xylostella*-susceptible plants (El-Shemy et al., 2010). A comprehensive proteomic study revealed that the chloroplast localized CA1 in Arabidopsis decreased in response to PAMPs (Jones et al., 2006). Intriguingly, the abundance of CA1 was reduced to a less extent upon infection by wild-type *Pst* than upon infection with its correspondent effector mutant *Pst hrpA*$^{-}$, indicating CA1 is a potential target of type III effectors (T3Es) (Jones et al., 2006). These findings suggest that CA proteins might be targeted by both PAMPs and T3Es during plant defense responses.

Previous studies have shown the involvement of CAs in plant defense against avirulent *Pst* strains. For instance, silencing of the tobacco CA SA-binding protein 3 (SABP3) led to suppressed *Pto:avrPto*-mediated hypersensitive defense response (Slaymaker et al., 2002). In Arabidopsis, CA1 (also known as AtSABP3) has been demonstrated to be required for the expression of full defense against the avirulent bacterial pathogen *Pst avrB* (Wang et al., 2009). Our study showed that the *ca1ca4* mutant exhibited enhanced resistance to *Pst* compared to wild-type Col-0 (Fig. 2), suggesting a negative role of CA1 and CA4 in plant defense responses to the virulent *Pst* strain. The dual roles of CAs in the defense against avirulent and virulent *Pst* strains suggest that CAs might act differently during compatible and incompatible interactions between host and pathogens.

Besides, our results showed that *ca1ca4* displayed delayed, yet sustained stomatal closure in response to *Pst* inoculation (Fig. 2). During Arabidopsis interactions with *P. syringae*, SA signaling plays a key role in stomatal defense as well as in disease resistance (Zeng et al., 2010). The increase in SA levels was recognized as a major signal output in both PTI and ETI (Tsuda et al., 2008). The *ca1ca4* mutant showed enhanced stomatal defense, enhanced SA accumulation and augmented SA-responsive gene expression upon infection with *Pst* (Fig. 3) or treatment with flg22 (Fig. 6), suggesting an antagonistic relationship between the functions of these CAs and the SA-dependent defense responses.

In addition to reversibly catalyzing CO_2 to bicarbonate, plant CA was also recognized as an antioxidant as it exhibits antioxidant activity (Slaymaker et al., 2002). Thus, it is anticipated that CAs might have a negative effect on cellular ROS levels. Indeed, we observed an augmented ROS burst in the *ca1ca4* mutant compared to those in wild-type plants upon flg22 treatment (Fig. 6d and 6e). The production of ROS, was hypothesized to form a signal amplification loop with SA during the establishment of plant defenses (Bi et al., 1995; Neuenschwander et al., 1995; Slaymaker et al., 2002). Overall, our results on the role of CA1 and CA4 in PTI point to a model (Fig. 7) in which *P. syringae*-infected plants recognize the flg22 epitope of the PAMP flagellin, which then leads to down-regulation of *CA1* and *CA4*

in an ET-dependent manner (Fig. 5). In healthy and *Pst*-infected plants, CAs have an antagonizing effect on SA-dependent defense signaling (Fig. 3 and 6). Thus, the flg22-mediated suppression of the CAs results in increased defense-related gene expression and enhanced ROS production, which ultimately leads to enhanced SA-dependent defenses, and consequently increased disease resistance and inhibition of plant growth.

Together, our study not only supports the suppression of *CA1* and *CA4* as an important strategy employed by Arabidopsis plants under attack by *P. syringae*, but also implicates CAs as players in PTI (Fig. 7). Considering the importance of CAs in plant photosynthesis and stomatal responsiveness to atmospheric CO_2, our findings on the functions of CAs in plant defense may shed new light on the trade-offs between on the one hand plant growth and adaptation to changes in CO_2 levels and on the other hand defense to pathogens.

MATERIALS AND METHODS

Cultivation of plants and bacterial strains

For experiments with soil-grown plants, seeds of *Arabidopsis thaliana* accession Col-0, the single *ca* mutants *ca1* and *ca4*, and the double mutant *ca1ca4* (Hu et al., 2010) were sown in 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 (350 µmol/m^2/s) and 14-h night cycle at 20°C with 70% relative humidity.

For experiments with *in vitro*-grown plants, seeds of Arabidopsis accession Col-0 and mutants *aba2-1* (Koornneef et al., 1982), *coi1-1* (Feys et al., 1994), *npr1-1* (Cao et al., 1994), *ein2-1* (Guzman and Ecker, 1990), *sid2-1* (Nawrath and Métraux, 1999), and *fls2-1* (Shan et al., 2008) were surface sterilized in gas of a mixture of household chlorine (Glorix, original) and HCl (37%) (97:3) for 3-4 h. Sterile seeds were subsequently sown on agar plates or in liquid. The agar plates contain Murashige and Skoog (MS) medium (Duchefa Biochemie, Haarlem, The Netherlands), pH 5.9, supplemented with 5 mM MES buffer, 10 g/l sucrose and 0.85% (w/v) plant agar (Duchefa Biochemie). When plants were 2 weeks old they were transferred to liquid MS to be treated with flg22 (see “Flg22 treatment”). For sowing of the seeds immediately in liquid MS, see “Flg22 treatment”.

Pseudomonas syringae pv. *tomato* (*Pst*) DC3000, *Pseudomonas syringae* pv. *maculicola* (*Psm*) ES4326 and its corresponding coronatine deficient mutant *Psm* ES4326 *cor*$^-$ (Dong et al., 1991) were grown on King’s B medium (King et al., 1954) agar plates supplemented with 50 µg/ml rifampicine at 28°C.

Pseudomonas syringae *infection and bioassay*

To prepare inoculum, bacteria of *Pst*, *Psm*, and *Psm cor*$^-$ strains were streaked from rifampicin selective KB agar plates and subsequently cultured in liquid KB medium in a shaker at 220 rpm at 28°C for 24 h. Bacteria were collected by centrifugation for

10 min at 4000 *g* and resuspended in 10 mM $MgSO_4$. The suspension was adjusted to OD_{600}=1. For dip inoculation, the bacterial inoculum was diluted to a final concentration of 5×10^7 cfu/ml of 10 mM $MgSO_4$ containing 0.015% (v/v) Silwet L-77 (Van Meeuwen Chemicals, Weesp, Netherlands). For pressure infiltration, the bacterial suspension was adjusted to a concentration of 4×10^7 cfu/ml unless specified otherwise. The abaxial side of leaves of 4-week-old Arabidopsis plants was pressure infiltrated with a needleless syringe.

For the bioassay, leaf discs of inoculated plants were harvested, surface sterilized in 70% ethanol for 8 sec, and washed immediately with water. Subsequently, 200 µl of 10 mM $MgSO_4$ was added to the leaf discs after which they were thoroughly ground. Aliquots of 10 µl of different dilutions were plated onto KB plates containing 25 µg/ml rifampicin. After 48 h of incubation at room temperature, bacterial colonies were counted and growth of *Pst* strains was calculated of log-transformed cfu data. Eight biological replicates were included for each time point.

Flg22 treatment

For gene expression analysis of flg22-treated plants, 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. A solution of 0.5 ml of MS+MES supplemented with flg22 (Sigma, the Netherlands) was added to a final concentrations (indicated in the figure legends). The rosette leaves or the whole seedlings were harvested for RNA extraction at indicated time points.

For 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.

For ROS assay, plants were grown at 20-21°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 500 µl of a solution containing 20 µM luminol (Sigma, St. Louis, MO), 1 µg horseradish peroxidase (Fluka, Buchs, Switzerland) and 100 nM flg22 (Sigma, UK). The water solution was included as negative control.

Gene expression by qRT-PCR

Total RNA was isolated as described (Oñate-Sánchez and Vicente-Carbajosa, 2008). SuperScript™ III Reverse Transcriptase was used to convert DNA-free total RNA into cDNA. PCR reactions were performed in optical 384-well plates (Applied Biosystems) with an ABI PRISM® 7900 HT sequence detection system using SYBR® Green to monitor the synthesis of double-stranded DNA. Transcript levels were calculated relative to the reference gene *At1g13320* (Czechowski et al., 2005) using the $2^{-\Delta\Delta CT}$ method described previously (Schmittgen and Livak, 2008). Three biological replicates were taken for each data point. Primers used for qRT-PCR are listed in the Supplemental Table S1.

SA measurement

For SA quantification, 100-250 mg of *Pst*-infiltrated (1×10^8 cfu/ml) or mock-treated leaves of Col-0 and *ca1ca4* were harvested 24 h after infiltration and ground to a fine powder using liquid nitrogen. The hormone was extracted as described (Scala et al., 2013). Briefly, the samples were homogenized in 0.5 ml 70% methanol, spiked with 200 ng of D6-SA by shaking at 6,000 rpm for 40 sec and centrifuged at 10,000 *g* for 20 min at 4˚C. The supernatants of two extraction steps were pooled together. Hormones were quantified by liquid chromatography-mass spectrometry (LC-MS) analysis on a Varian 320 Triple Quad LC/MS/MS. Endogenous SA level was quantified by comparing the integrated surface area from each sample with its corresponding internal standard.

Stomatal aperture measurement

Stomatal aperture was measured by a modified protocol of dental resin impressions (Geisler et al., 2000). Two components of Present Light Body (Coltène, Altstatten, Switzerland) were mixed thoroughly (V:V, 1:1) and the abaxial side of plant leaves was softly pressed onto the dental resin immediately after harvesting. Leaves were removed 10 min later when the mixture had hardened. Transparent nail polish was then applied to the dental resin mold to create casts, which were fixed on microscope slides with Anutex modelling wax (Kemdent, Purton, Swindon, Wiltshire, UK) for further observation.

Stomata were examined using an Olympus microscope and Analysis D Olympus Software on the pictures taken. Stomata aperture was determined by measuring the width and length of the stomata. At least six leaves were harvested for each treatment and 20-30 observations were recorded from each leaf.

ROS measurement

ROS production was measured as previously described (Gimenez-Ibanez et al., 2009). Twelve leaf discs from 5-week-old plants were used for each condition. Luminescence of each sample was measured over 60 min by using a high-resolution photon counting system (HRPCS218, Photek) coupled to an aspherical wide lens (Sigma).

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SUPPLEMENTAL INFORMATION

Supplemental Table S1: List of qRT-PCR primers used in this study

At1g13320 _F, 5'-TAA CGT GGC CAA AAT GAT GC-3'
At1g13320 _R, 5'-GTT CTC CAC AAC CGC TTG GT-3'
CA1-F, 5'-ATG ACT TCG TCA AGG GTG CT-3'
CA1-R, 5'-CTA GTT TCG GAG AGG CCA AA-3'
CA4-F, 5'-CAT TCG TGA GAG CTG AGG TG-3'
CA4-R, 5'-TCC CAG AGA TCA AAC GTT CC-3'
*PR1*_F, 5'-CTC GGA GCT ACG CAG AAC AAC T-3'
*PR1*_R, 5'-TTC TCG CTA ACC CAC ATG TTC A-3'
*PR2*_F, 5'-CAG ATT CCG GTA CAT CAA CG-3'
*PR2*_R, 5'-AGT GGT GGT GTC AGT GGC TA-3'
*FRK1*_F, 5'-TTT CAA CAG TTG TCG CTG GA-3'
*FRK1*_R, 5'-AGC TTG CAA TAG CAG GTT GG-3'
*ICS1*_F, 5'-GGC AGG GAG ACT TAC G-3'
*ICS1*_R, 5'-AGG TCC CGC ATA CAT T-3'
*WRKY22*_F, 5'-AAA GGT TCA CCA TAT CCA AGA GGA-3'
*WRKY22*_R, 5'-TCT ATT TCG CTC CAC TTG TTT ACG-3'
*WRKY29*_F, 5'-AAG GAT CTC CAT ACC CAA GGA GT-3'
*WRKY29*_R, 5'-TCG ACT TGT TTT CTT GCC AAA CAC-3'

CHAPTER 4

Atmospheric CO_2 differentially modulates salicylic acid- and jasmonic acid-dependent defense signaling in Arabidopsis

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ABSTRACT

Atmospheric CO_2 is an important environmental stimulus that affects different hormone signaling pathways and plant immunity. Here we found that *Arabidopsis thaliana* (Arabidopsis) plants grown under high atmospheric CO_2 conditions (800 ppm) showed enhanced resistance against the necrotrophic pathogen *Botrytis cinerea*, whereas plants were more susceptible to this pathogen when grown under low atmospheric CO_2 conditions (150 ppm). Accordingly, the basal expression level of the jasmonic acid (JA)-responsive marker gene *PDF1.2* was significantly enhanced in plants grown under high atmospheric CO_2 conditions. On the contrary, susceptibility to the hemi-biotrophic pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) was enhanced under high and reduced under low atmospheric CO_2 conditions, which was associated with reduced or enhanced expression of different salicylic acid (SA)-responsive genes, respectively. The SA signaling mutant *npr1* was equally susceptible to *Pst* under high, ambient (450 ppm) and low CO_2 conditions, supporting a role for NPR1 in atmospheric CO_2-altered SA signaling, affecting resistance to *Pst*. In addition, we showed that *Pst*-induced down-regulation of the *CA1* and *CA4* genes, which encode CO_2-binding proteins that attenuate SA signaling, was enhanced by low CO_2 and reduced by high CO_2. Resistance of the *ca1ca4* mutant to *Pst* was high under all three CO_2 conditions, while *B. cinerea* resistance was unaffected by the CA mutations. This suggests the involvement of CAs in atmospheric CO_2-regulated defenses that are SA-dependent, but not in defenses that are JA-dependent. Together, our study support the notion that changed atmospheric CO_2 levels can differentially affect SA and JA defense pathways, thereby affecting disease resistance.

INTRODUCTION

Plants in nature are exposed to a wide array of pathogenic microbes and insect herbivores. Plant pathogens are typically distinguished by their biotrophic lifestyle, like the oomycete pathogen *Hyaloperonospora arabidopsidis*, or their necrotrophic lifestyle, like the fungal pathogen *Botrytis cinerea* (Glazebrook, 2005). Biotrophs commonly feed on nutrients from living host cells, whereas necrotrophs derive nutrients from killed plant tissues. Many pathogens, for example the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*), has a biotrophic and a necrotrophic infection stage and are thus referred to as hemi-biotrophs.

To defend themselves against pathogenic invaders, plants have developed a sophisticated defense system that recognizes pathogen-associated molecules and subsequently activate downstream defense cascades. The phytohormones salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA) play a pivotal role in the regulation of the plant immune signaling network (Pieterse et al., 2009). The SA signaling pathway is often associated with defense against biotrophic pathogens, whereas defenses effective against necrotrophic pathogens are primarily regulated by the JA and ET pathways (Glazebrook, 2005). Moreover, defenses against herbivorous insects are typically regulated by the JA- and ABA-dependent pathways (Howe and Jander, 2008). SA signaling is controlled by the regulatory protein NONEXPRESSOR OF PR GENES1 (NPR1), eventually leading to expression of SA-responsive genes such as the marker gene *PATHOGENESIS-RELATED1* (*PR1*) (Dong, 2004; Spoel et al., 2009; Pieterse et al., 2012). Mutations in the *NPR1* gene in *Arabidopsis thaliana* (Arabidopsis) disrupt the SA-induced expression of *PR1* and confer increased susceptibility to various biotrophic and hemi-biotrophic pathogens (Beckers and Spoel, 2006). JA/ET-dependent signaling activates the JA/ET marker gene *PLANT DEFENSIN1.2* (*PDF1.2*) (Lorenzo et al., 2003). The APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) transcription factor ORA59 is an important regulator of the JA/ET pathway, as Arabidopsis genotypes with reduced *ORA59* gene expression show enhanced susceptibility to *B. cinerea* (Anderson et al., 2004; Pré et al., 2008). JA/ABA-dependent signaling activates the expression of the marker gene *VEGETATIVE STORAGE PROTEIN2* (*VSP2*) (Lorenzo et al., 2003; Lorenzo et al., 2004). The basic-helix-loop-helix (bHLH) transcription factors MYC2, 3 and 4 are important regulators of the JA/ABA pathway, and mutants impaired in these transcription factors show enhanced susceptibility to insect herbivory (Anderson et al., 2004).

Profound crosstalk between different hormone signaling pathways indicates that the hormone-regulated defense signaling network is highly interconnected (Katagiri, 2004; De Vos et al., 2005; Kim et al., 2014). One of the best-studied examples of hormonal crosstalk is the SA-mediated suppression of JA-dependent defense signaling in which NPR1 plays an essential role (Spoel et al., 2003; Mur et al., 2006; Ndamukong et al., 2007; Spoel et al., 2007; Koornneef and Pieterse, 2008; Spoel and Dong, 2008). Exogenous application of SA strongly suppresses the expression of

JA-responsive genes, such as the marker genes *PDF1.2* and *VSP2* (Spoel et al., 2003). Antagonistic effects between JA/ET- and JA/ABA-regulated defenses have also been shown (Anderson et al., 2004; Adie et al., 2007; Mohr and Cahill, 2007; Flors et al., 2008; Verhage et al., 2011). Besides SA, JA, ET and ABA, other hormones, like brassinosteroids, gibberellins, cytokinin and auxin have been shown to be able to influence defense signaling (Denancé et al., 2013; Lozano-Durán and Zipfel, 2015), indicating that intensive interplay between the different hormone signaling pathways shape the final outcome of plant-pathogen interactions.

Various global climate change models predict a rise in the atmospheric CO_2 concentration in the coming years (IPCC, 2007). As an important environmental cue, changes in atmospheric CO_2 levels can cause profound effects on plant growth and many plant physiological processes (Bowes, 1991; Teng et al., 2006). For example, it has been reported that an increase in atmospheric CO_2 concentration can induce changes in hormone levels in many plant species (Arteca et al., 1980; Teng et al., 2006; Zavala et al., 2008; Li et al., 2011b; Li et al., 2011a). In general, SA, auxin, and gibberellin levels seem to increase under elevated CO_2 conditions, whereas ABA and JA signaling seem to decrease. Suppression of JA-related signaling by high CO_2 levels was associated with increased susceptibility of maize to *Fusarium verticillioides* (Vaughan et al., 2014) and of soybean to herbivores (Zavala et al., 2008; Zavala et al., 2013). In tomato plants, elevated CO_2 levels induced an increase in SA levels and concomitantly a decrease in JA signaling, leading to enhanced resistance against yellow leaf curl virus, tobacco mosaic virus and *Pst*, and increased susceptibility to *B. cinerea* (Huang et al., 2012; Zhang et al., 2015).

In Chapter 3, we demonstrated that two β-carbonic anhydrases, CA1 and CA4, play an important role in plant immune responses, likely through an antagonistic effect on SA signaling. Recognition of flg22, the functional epitope of the bacterial pathogen-associated molecular pattern (PAMP) flagellin, resulted in down-regulation of *CA1* and *CA4*. CAs are CO_2-binding proteins of which the activity is likely affected by changes in atmospheric CO_2 levels. Both transcript abundance and enzymatic activity of CAs were demonstrated to decrease under elevated CO_2 conditions in various C_3 plants (Porter and Grodzinski, 1984; Webber et al., 1994; Majeau and Coleman, 1996). In addition, the double *ca1ca4* mutant of Arabidopsis showed impairment in the control of stomatal aperture by atmospheric CO_2 (Hu et al., 2010).

In Chapter 2, we showed that low atmospheric CO_2 levels resulted in enhanced resistance of Arabidopsis plants to *Pst* and in reduced ABA levels. In this study, we examined the disease resistance of Arabidopsis to the necrotrophic pathogen *B. cinerea* under three different CO_2 conditions. Moreover, we investigated the role of SA- and JA-dependent signaling in the altered resistance to *Pst* and *B. cinerea* under different CO_2 conditions. We observed that high CO_2 levels enhanced the resistance of Arabidopsis plants to *B. cinerea* and increased the expression of the JA/ET-responsive marker gene *PDF1.2*. Moreover, expression of SA-responsive genes was reduced by high CO_2 and enhaced by low CO_2. Interestingly, we found no significant differences in the basal levels of SA and JAs between the plants grown

under high and ambient CO_2 conditions. Taken together, these results indicate that increased atmospheric CO_2 levels can induce resistance of Arabidopsis plants to necrotrophic pathogens and reduce resistance to hemi-biotrophic pathogens, possibly by modulating the interplay between SA, JA and ABA signaling. In addition, we found that CA1 and CA4 might play a role in the modulation of SA-dependent defenses under different atmospheric CO_2 conditions.

RESULTS

Changes in atmospheric CO_2 levels alter Arabidopsis resistance to the necrotrophic pathogen *B. cinerea*

As shown in Chapter 2, high atmospheric CO_2 levels reduced the resistance of Arabidopsis plants to the hemi-biotrophic pathogen *Pst*, while low CO_2 levels enhanced the resistance to *Pst*. In order to investigate if differences in atmospheric CO_2 levels also affect resistance to necrotrophic pathogens, we tested the resistance of Arabidopsis plants to *B. cinerea* at three different atmospheric CO_2 levels. Four days after inoculation, plants grown under high CO_2 conditions (800 pm) developed less disease symptoms compared with plants grown under ambient (450 ppm) and low CO_2 (150 ppm) conditions (Fig. 1a and 1b). Moreover, under low CO_2 conditions disease symptoms had developed to a much greater extent. This indicates that at increasing atmospheric CO_2 levels the susceptibility of Arabidopsis plants to *B. cinerea* declined.

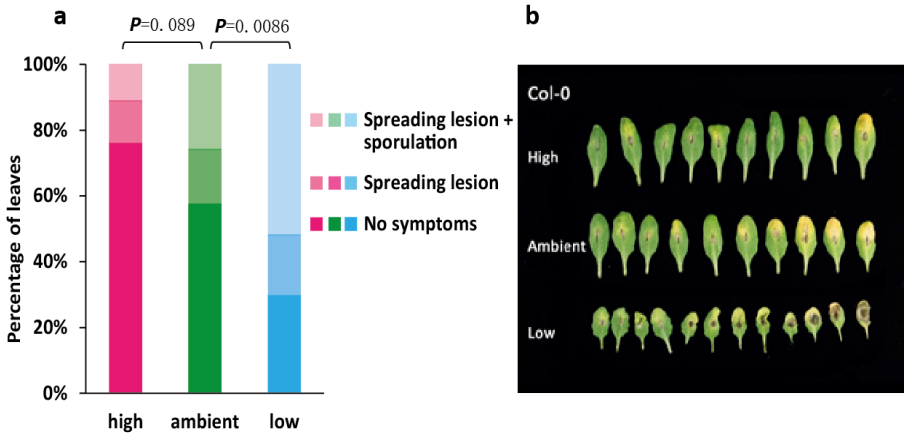

Figure 1: Effect of different atmospheric CO_2 levels on disease resistance of Arabidopsis to *B. cinerea*.
(a) Quantification of *B. cinerea* disease symptoms of Arabidopsis Col-0 plants grown under three different atmospheric CO_2 conditions. Disease severity of the inoculated leaves was scored in three classes four days after droplet inoculation. Percentage of leaves in each class was calculated per plant. Indications above the brackets specify whether there is a significant difference between different atmospheric CO_2 conditions (X^2-test; n=9 plants). **(b)** Disease symptoms on leaves of Col-0 plants grown under three different CO_2 conditions four days after inoculation with *B. cinerea*. High, 800 ppm CO_2; Ambient, 450 ppm CO_2; Low, 150 ppm CO_2.

Atmospheric CO_2 affects basal hormone levels and hormone-responsive gene expression in Arabidopsis

To investigate the mechanisms underlying atmospheric CO_2-altered defense responses in Arabidopsis plants, we analyzed the basal hormone levels and the expression of hormone-responsive genes in plants grown at three different atmospheric CO_2 concentrations. We observed no differences in basal SA levels between high and ambient atmospheric CO_2-grown plants, but basal SA levels were significantly higher in plants grown under low atmospheric CO_2 conditions (Fig. 2a). This is in accordance with the previously observed enhanced resistance to *Pst* under low atmospheric CO_2 conditions (Chapter 2). The levels of JA, its precursor 12-oxo-phytodienoic acid (OPDA), and the biologically highly active conjugate JA-isoleucine (JA-Ile) were also monitored. Accumulation of both JA and JA-Ile did not differ significantly between the three atmospheric CO_2 conditions (Fig. 2b and 2d). OPDA levels did not differ between plants grown at high and ambient CO_2 levels, but were significantly higher in plants grown under low CO_2 conditions (Fig. 2c). This suggests a complex regulation of JA biosynthesis and JA-mediated signaling by atmospheric CO_2 levels, in which there seems to be no correlation between the level of the measured JAs and the level of resistance against *B. cinerea* under the different CO_2 conditions.

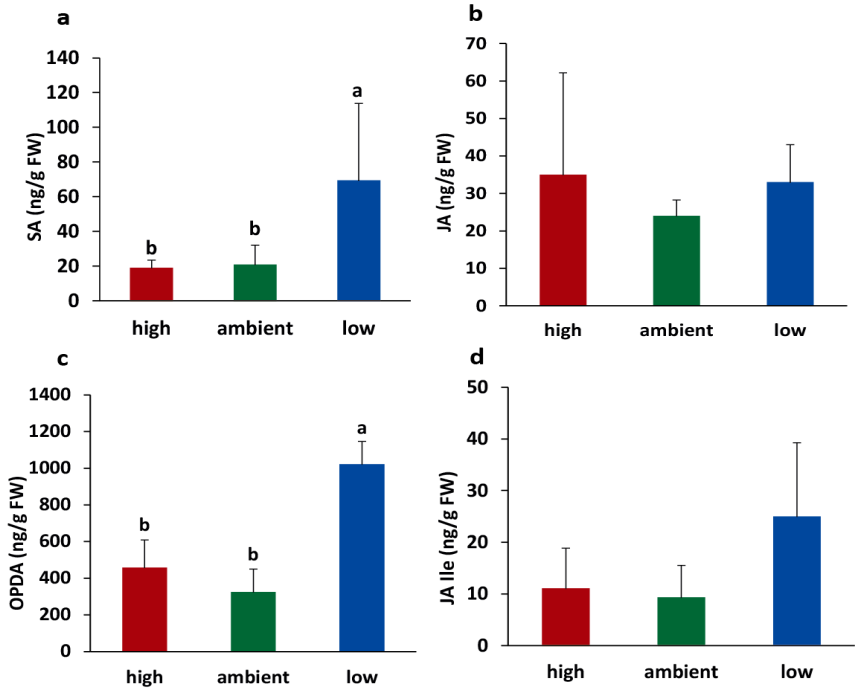

Figure 2: Effect of different atmospheric CO_2 levels on the basal production of SA, JA, OPDA and JA-Ile. Absolute levels (ng/g FW) of SA **(a)**, JA **(b)**, OPDA **(c)** and JA-Ile **(d)** in leaves of 4-week-old Arabidopsis Col-0 plants grown under high (800 ppm), ambient (450 ppm) and low (150 ppm) levels of atmospheric CO_2. Compound levels were measured by Triple Quad LC/MS/MS. Different letters indicate a statistically significant difference between the different CO_2 treatments (one-way ANOVA, Duncan's multiple range test, $P<0.05$). No statistically significant difference was found between treatments in (b) and (d). Error bars represent SD, $n=3$ plants.

Previous studies have identified *PDF1.2* as a marker gene for JA/ET-dependent defenses (Verhage et al., 2011), and *PR1*, *FLG22-INDUCED RECEPTOR-LIKE KINASE1* (*FRK1*), and *ISOCHORISMATE SYNTHASE1* (*ICS1*) as marker genes for SA-dependent defenses (Zhang et al., 1999; Métraux, 2002; Ndamukong et al., 2007; Yi et al., 2014). Analysis of the expression of these JA- and SA-responsive marker genes revealed that different atmospheric CO_2 concentrations differentially affect their basal expression (Fig. 3). More specifically, basal expression of *PDF1.2* was significantly higher in plants grown under high atmospheric CO_2 conditions than in plants grown under ambient and low atmospheric CO_2 conditions (Fig. 3a). This correlates with the increased resistance of Arabidopsis plants to *B. cinerea* under high atmospheric CO_2 conditions (Fig. 1). The basal expression level of the SA-responsive gene *FRK1* was higher at low atmospheric CO_2 levels (Fig. 3c), which correlates with the increased SA levels (Fig. 2a) and enhanced resistance to *Pst* (Chapter 2) under low CO_2 conditions. Expression of *ICS1* was reduced under the high CO_2 condition, which is in accordance with the enhanced susceptibility to *Pst* (Fig. 3d). Together, our results show that low CO_2 enhanced SA accumulation and SA-regulated defense gene expression, whereas high CO_2 enhanced JA-dependent gene expression, but suppressed SA-dependent expression.

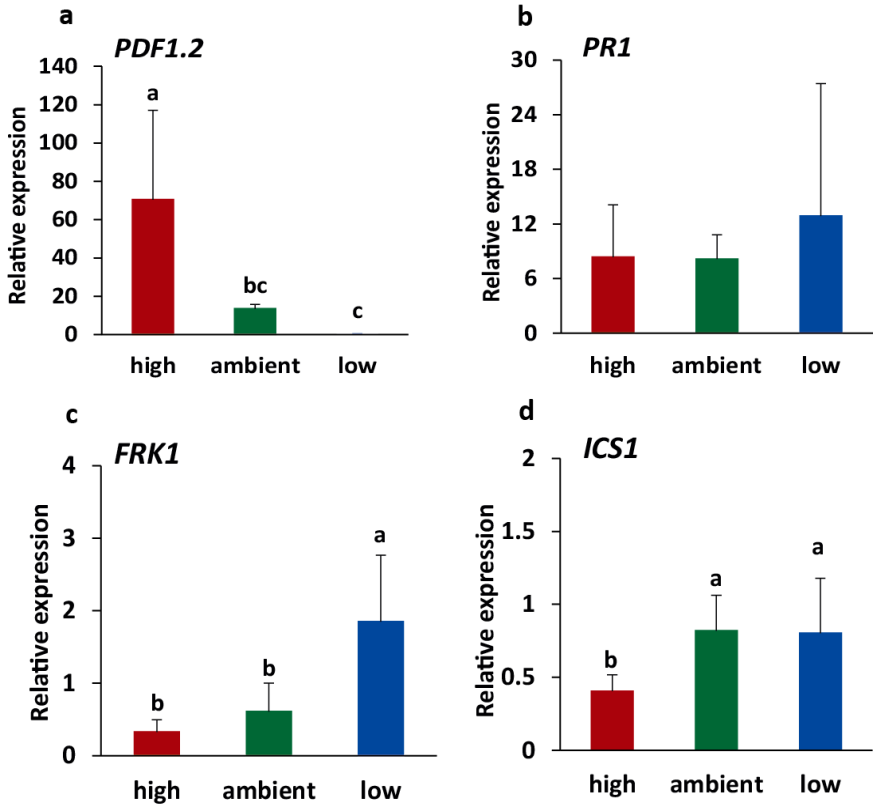

Figure 3: Effect of different atmospheric CO_2 levels on basal expression levels of SA- and JA-related defense genes.
RT-qPCR analysis of *PDF1.2* **(a)**, *PR1* **(b)**, *FRK1* **(c)**, and *ICS1* **(d)** gene expression in 4-week-old Arabidopsis Col-0 plants grown under high (800 ppm), ambient (450 ppm) and low (150 ppm) levels of atmospheric CO_2. Indicated are the expression levels relative to the reference gene *At1g13320*. Different letters indicate statistically significant differences between CO_2 treatments (one-way ANOVA, Duncan's multiple range test, $P<0.05$). Error bars represent SD, $n=3$ plants.

The role of the SA and JA pathways in Arabidopsis disease resistance under different atmospheric CO_2 conditions

To further investigate the role of the SA and JA signaling pathways in the resistance of Arabidopsis plants to *Pst* and *B. cinerea* under different atmospheric CO_2 conditions, we tested the SA signaling mutant *npr1* and the JA/ET response mutant *ora59* for their level of resistance to *Pst* and *B. cinerea*, under high, ambient and low CO_2 conditions. Consistent with previous studies, mutant *npr1* plants were significantly more susceptible to *Pst* in comparison to wild-type Col-0 plants (Fig. 4a). Furthermore, whilst the susceptibility of Col-0 to *Pst* increased with increasing atmospheric CO_2 levels, the susceptibility to *Pst* was high in *npr1* under all three atmospheric CO_2 conditions, to a level comparable to that of wild-type Col-0 grown at high CO_2 (Fig. 4a). This confirms the importance of the SA pathway in resistance of

Arabidopsis to *Pst* and suggests the involvement of NPR1 in CO_2-mediated modulation of SA signaling leading to altered resistance to *Pst*.

Previous studies have shown that overexpression of *ORA59* conferred resistance to *B. cinerea*, whereas *ORA59* silencing caused susceptibility to this pathogen (Pré et al., 2008). In contrast, our results show that the *ora59* mutant was overall as susceptible to *B. cinerea* as wild-type Col-0 (Fig. 4b). Moreover, similar to Col-0, *ora59* became more resistant to *B. cinerea* at increasing atmospheric CO_2 levels (Fig. 4b). This indicates that other factors than ORA59 integrate CO_2 signaling with JA/ET-dependent signaling leading to differential resistance levels against *B. cinerea*.

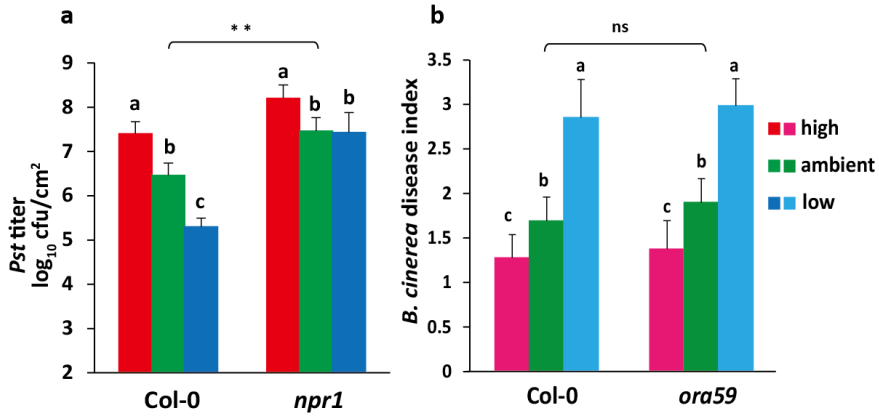

Figure 4: The role of the SA and JA pathways in *Pst* and *B. cinerea* resistance of Arabidopsis under different atmospheric CO_2 conditions.
(a) Role of NPR1 in atmospheric CO_2 level-dependent resistance against *Pst*. Arabidopsis wild-type Col-0 and the SA signaling mutant *npr1* were grown under high (800 ppm), ambient (450 ppm) and low (150 ppm) levels of atmospheric CO_2 and dip inoculated with *Pst*. Bacterial growth was determined at 5 days after inoculation. Indicated is the average of the log_{10}-transformed bacterial titer per leaf area. Different letters indicate significant differences between CO_2 treatments within the indicated genotype. Indications above the brackets specify the interaction (Arabidopsis genotype × CO_2 conditions) between the Arabidopsis genotypes Col-0 and *npr1* and the three CO_2 conditions (two-way ANOVA, Fisher's LSD test, **, P<0.05). Error bars represent SD, n=8 plants. **(b)** Role of ORA59 in atmospheric CO_2 level-dependent resistance against *B. cinerea*. Arabidopsis wild-type Col-0 and the JA/ET response mutant *ora59* were grown under three different atmospheric CO_2 conditions and inoculated with *B. cinerea*. Disease symptoms were scored 4 days after inoculation. Indicated is the average of the disease index calculated from the percentage of leaves in 4 different disease severity classes. Different letters indicate significant differences between CO_2 treatments within the same genotype. Indications above the brackets specify the interaction (Arabidopsis genotype × CO_2 conditions) between the Arabidopsis genotypes Col-0 and *ora59* and the three CO_2 conditions (two-way ANOVA, Fisher's LSD test, ns, not significant). Error bars represent SD, n=12 plants.

The potential role of CAs in SA-dependent defense signaling under different atmospheric CO_2 conditions

As we showed in Chapter 3, CA1 and CA4 inhibit Arabidopsis defense against *Pst* by antagonizing the SA pathway. Since CA1 and CA4 function in atmospheric CO_2-mediated signaling (Hu et al., 2010), we tested whether CAs are involved in the differential effects of low and high CO_2 levels in SA-dependent defenses. We examined the expression of *CA1* and *CA4* in response to *Pst* treatment under three different atmospheric CO_2 conditions. The basal expression level of *CA1* and *CA4* changed only slightly between the different atmospheric CO_2 levels, and no consistent expression pattern was found in different experiments (Fig. 5 and data for *CA4* not

shown). This variability in *CA* gene expression under different atmospheric CO_2 conditions is in line with a number of other studies (Raines et al., 1992; Fabre et al., 2007; Wang et al., 2014). Previously, we showed that *Pst* infection and treatment with the elicitor flg22 significantly reduced the expression of *CA1* and *CA4* (Chapter 3). Interestingly, the *Pst*-induced reduction of *CA1* expression was significantly stronger at the low CO_2 concentration and significant weaker at the high CO_2 concentration (Fig. 5), which parallels with the respective increase and reduction in the level of resistance to *Pst* under these conditions (Fig. 4 and Fig. 6).

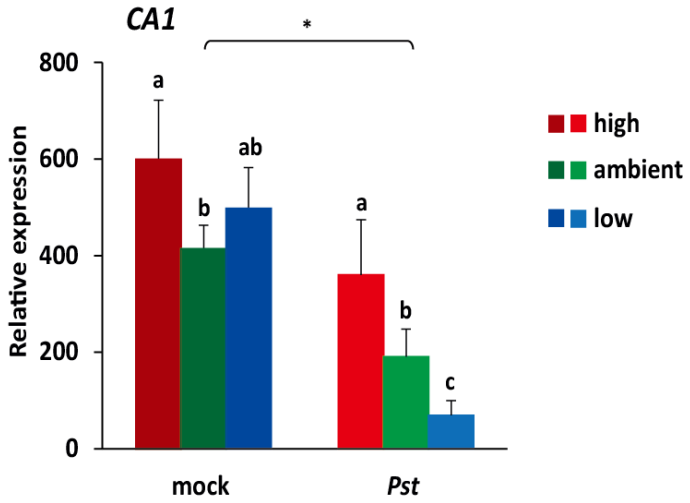

Figure 5: Effect of different atmospheric CO_2 levels on the suppression of *CA1* by *Pst* infection.
RT-qPCR analysis of *CA1* gene expression in Arabidopsis Col-0 grown under high (800 ppm), ambient (450 ppm) and low (150 ppm) levels of atmospheric CO_2. Leaf tissue was harvested 24 h after dip inoculation with *Pst* (1×10^8 cfu/ml) or a mock solution (10 mM $MgSO_4$). Indicated are the expression levels relative to the reference gene *At1g13320*. Different letters indicate statistically significant differences between different CO_2 levels within the same treatment. Indications above the brackets specify the interaction (CO_2 conditions × bacterium treatment) between the three CO_2 conditions and bacterium treatments (two-way ANOVA; Fisher's LSD test; *, $P<0.05$). Error bars represent SD, $n=3$ plants.

Subsequently, we tested the resistance of the *ca1ca4* double mutant to *Pst* at three atmospheric CO_2 levels. Disease symptoms were significantly increased in high CO_2-grown Col-0 plants at 4 days after dip inoculation with *Pst*, whereas much less disease symptoms were found in low CO_2-grown Col-0 plants (Fig. 6). Interestingly, the *ca1ca4* mutant plants were as resistant to *Pst* as low CO_2-grown Col-0 plants, under all three CO_2 conditions tested (Fig. 6), suggesting an important role for CA1 and CA4 in atmospheric CO_2-modulated resistance to *Pst* .

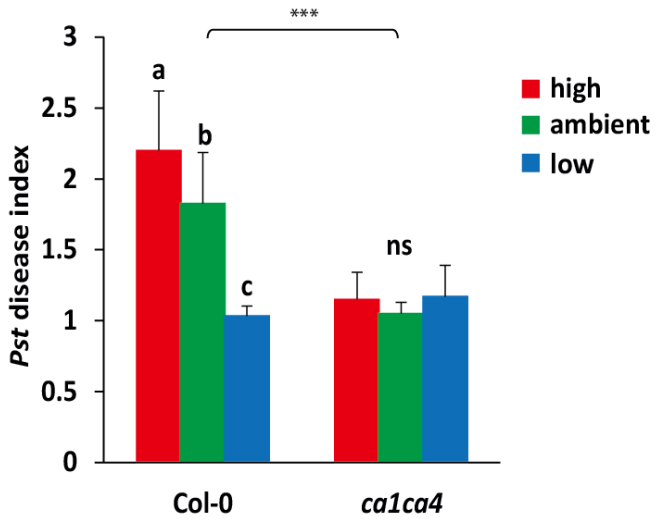

Figure 6: The role of CA1 and CA4 in atmospheric CO_2-modulated resistance to *Pst* .
Arabidopsis wild-type Col-0 and the mutant *ca1ca4* were grown under high (800 ppm), ambient (450 ppm) and low (150 ppm) levels of atmospheric CO_2 and dip inoculated with *Pst* (4×10^7 cfu/ml) or a mock solution (10 mM $MgSO_4$). Disease symptoms were scored 4 days after inoculation. Indicated is the average of the disease index calculated from the percentage of leaves in three different disease severity classes (Class 1, 0-10% diseased leaf area; Class 2, 10%-50% diseased leaf area; Class 3, >50% diseased leaf area). Different letters show significant differences between CO_2 treatments within the same genotype (ns, not significant). Indications above the brackets specify the interaction (Arabidopsis genotype × CO_2 conditions) between the Arabidopsis genotypes Col-0 and *ca1ca4* and the three CO_2 conditions (two-way ANOVA; Fisher's LSD test; ***, P<0.001, ns, not significant). Error bars represent SD, n= 12 plants.

Atmospheric CO_2 modulates JA-mediated defense signaling independently of CA1 and CA4

Next, we tested whether CA1 and CA4 are also involved in atmospheric CO_2-modulated resistance to *B. cinerea* using wild-type Col-0 plants and *ca1ca4* mutant plants grown under high, ambient and low atmospheric CO_2 conditions. Comparable with the results shown in Fig. 1, low CO_2 resulted in significantly more disease symptoms in Col-0, whereas high CO_2 led to enhanced resistance to *B. cinerea* (Fig. 7a). Interestingly, the level of disease severity in *ca1ca4* was more or less similar to that in Col-0 plants (Fig. 7a and 7b), suggesting that CA1 and CA4 do not play a major role in the modulation of JA/ET-dependent resistance to *B. cinerea* under different atmospheric CO_2 conditions.

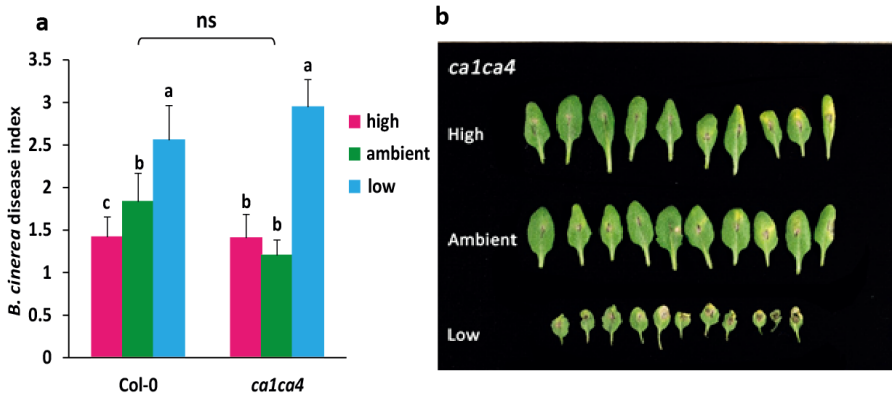

Figure 7: The role of CA1 and CA4 in atmospheric CO_2-altered disease resistance to *B. cinerea*.
(a) Quantification of *B. cinerea* disease severity. Wild-type Col-0 and *ca1ca4* mutant plants were grown under three different atmospheric CO_2 conditions and infected with *B. cinerea*. Disease symptoms were scored four day after infection. Indicated is the average of the disease index calculated from the percentage of leaves in four different disease severity classes. Different letters indicate significant differences between CO_2 treatments within one line. Indications above the brackets specify the interaction (Arabidopsis genotype × CO_2 conditions) between the Arabidopsis genotypes Col-0 and *ca1ca4* and the three CO_2 conditions (two-way ANOVA; Fisher's LSD test; ns, not significant). **(b)** Disease symptoms of *ca1ca4* grown under three different atmospheric CO_2 conditions four days after inoculation with *B. cinerea*.

DISCUSSION

Various climate change models predict elevation of atmospheric CO_2 levels in the future and this has boosted research on plant-pathogen interactions under different atmospheric CO_2 conditions (Chakraborty and Datta, 2003; Lake and Wade, 2009; Pangga et al., 2011; Huang et al., 2012; Zhang et al., 2015). It has been shown that altered atmospheric CO_2 levels have only a limited direct influence on the growth of plant microbes (Wells, 1974; Drigo et al., 2008). Nevertheless, altered levels of atmospheric CO_2 are likely to affect infection by plant pathogens through interference with host plant defense responses. Metabolic and transcriptional analyses of ambient and high CO_2-grown plants revealed distinct alterations in different hormone signaling pathways in different plant species (Matros et al., 2006; Teng et al., 2006; Casteel et al., 2008). It has been demonstrated that high CO_2 can affect disease resistance to pathogens and that altered activation of hormonal signaling pathways, specifically those mediated by SA and JA, plays a role in it (Zhang et al., 2015). High CO_2 levels have also been found to reduce both resistance and tolerance of plants to various herbivorous insects, likely by suppression of JA and ET signaling pathways (Casteel et al., 2008; Sun et al., 2011b; Guo et al., 2012; Zavala et al., 2013).

Hormonal signaling networks in plants play a profound role in plant immunity (Howe and Jander, 2008; Robert-Seilaniantz et al., 2011; Pieterse et al., 2012). In Arabidopsis, we found increased SA levels (Fig. 2a) as well as a generally enhanced expression of SA-responsive genes in plants grown under low atmospheric CO_2 conditions (Fig. 3b-3d), which is in accordance with the enhanced resistance against

Pst (Fig. 1 and Fig. 4a). The regulatory protein NPR1 is a master regulator in the SA-dependent defense signaling pathway (Dong, 2004). We found that mutant *npr1* plants were significantly more susceptible to *Pst* compared to wild-type Col-0 plants under all three atmospheric CO_2 conditions tested (Fig. 4a). This indicates that the enhanced resistance of low atmospheric CO_2-grown plants to *Pst* is dependent on NPR1. Moreover, in high CO_2-grown plants expression of the SA synthesis gene *ICS1* was reduced, which is in line with a reduction in resistance to *Pst* (Fig. 3d and 4a). In Chapter 2 (Fig. 5c), we showed that the observed reduction in ABA signaling in low CO_2-grown plants is likely to play a role in the enhanced resistance to *Pst* at low CO_2, at least partly by preventing stomatal reopening so that bacterial entrance to the leaves is restricted. Here, we show that SA signaling is enhanced under low CO_2 conditions, which may also be a consequence of reduced ABA signaling, decreasing the antagonistic activity on SA signaling (De Torres-Zabala et al., 2007; De Torres-Zabala et al., 2009; Cao et al., 2011).

While basal JA levels were only marginally affected by changes in atmospheric CO_2 levels (Fig. 2), the expression of the JA-responsive marker gene *PDF1.2* was significantly enhanced under high atmospheric CO_2 conditions (Fig. 3a), suggesting that increasing atmospheric CO_2 levels stimulated the JA/ET response pathway in Arabidopsis plants. This is in line with the enhanced resistance to *B. cinerea* in high CO_2-grown plants (Fig. 4b and Fig. 7). In contrast, recent findings in tomato and soybean plants showed that elevated CO_2 levels induced the SA signaling pathway and repressed the JA signaling pathway, leading to enhanced resistance to *Pst* and increased susceptibility to necrotrophic pathogens and herbivores (Zavala et al., 2008; Huang et al., 2012; Zhang et al., 2015). The different reported effects of altered atmospheric CO_2 levels on hormonal signaling might be attributed to the different plant species examined, differences in CO_2 treatments (CO_2 concentrations and duration of CO_2 treatments) or other experimental conditions applied in the different studies. Including different plant species and different experimental conditions in one study can give a better insight in how altered atmospheric CO_2 levels modulate hormone-related defense signaling pathways.

CAs act as early regulators in CO_2-mediated cellular responses, such as stomatal movements (Hu et al., 2010). In Chapter 3 we showed that CA1 and CA4 suppress resistance to *Pst*, likely through an antagonistic effect on PAMP-triggered SA signaling. In this study, we found that *ca1ca4* plants displayed a similar resistance level to *B. cinerea* as Col-0 plants grown under different atmospheric CO_2 conditions (Fig. 7), indicating that the CAs play no significant role in JA/ET-dependent defense responses. On the other hand, the *ca1ca4* plants were significantly more resistant to *Pst* and this was apparent under all three atmospheric CO_2 conditions, reaching the same level of resistance as Col-0 plants grown at low CO_2 (Fig. 6). This suggests that CA1 and CA4 do play an important role in controlling CO_2-regulated SA-dependent defense responses. In line with this, the *Pst*-induced suppression of the *CA1* gene was stronger at decreasing levels of atmospheric CO_2 (Fig. 5). This makes it likely that atmospheric CO_2 levels differentially affect the pathogen-repressed

expression level of *CA1*, thereby affecting SA-dependent defense responses. Moreover, CA activity has been demonstrated to be affected at the protein level by changes in atmospheric CO_2 (Majeau and Coleman, 1996).

In summary, our study suggests that alterations in atmospheric CO_2 levels differentially regulate the multiple defense-related hormone signaling pathways, resulting in enhanced resistance against *B. cinerea* and increased susceptibility to *Pst* as atmospheric CO_2 levels increase (Fig. 8). Moreover, we provide evidence that CAs play a role in atmospheric CO_2-regulated SA-dependent defense responses. These findings give insight into the effects of the predicted increase in atmospheric CO_2 on plant disease resistance and reveal some potential regulators of atmospheric CO_2-associated signaling. Together, this could give some useful leads for future plant breeding strategies and disease protection in the light of the envisaged steady increase in atmospheric CO_2 on our planet.

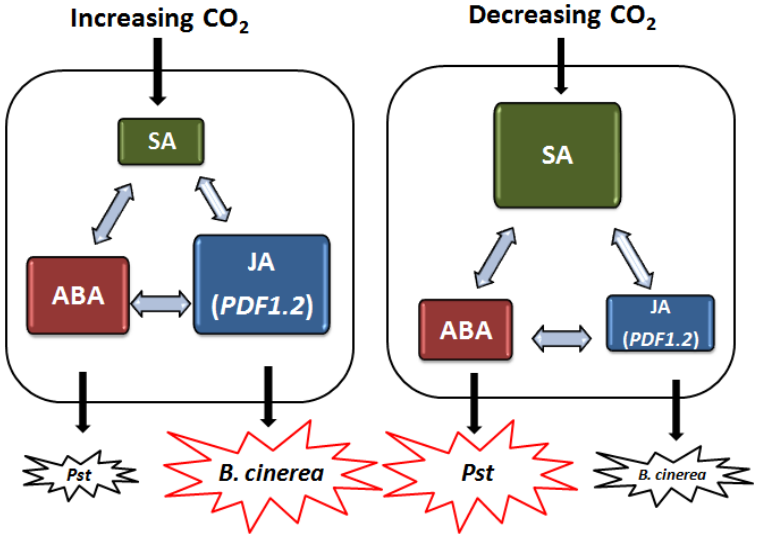

Figure 8: Schematic representation of effects of differentially modulated hormone signaling network under high and low atmospheric CO_2 conditions on disease resistance against *Pst* and *B. cinerea*.
Under increasing atmospheric CO_2 conditions, JA/ET- and the ABA-dependent signaling is induced and SA-dependent signaling is repressed in Arabidopsis plants. This results in an enhanced resistance to the necrotrophic pathogen *B. cinerea*, while defenses against the (hemi-) biotrophic pathogen *Pst* are repressed.
Under decreasing atmospheric CO_2 conditions, SA biosynthesis and SA-dependent signaling is induced and JA/ET- and ABA-dependent signaling is repressed in Arabidopsis plants. This leads to an enhanced resistance to *Pst* and enhanced susceptibility to *B. cinerea*.

MATERIALS AND METHODS

Plant material and cultivation

Seeds of *Arabidopsis thaliana* accession Col-0 and mutants *ca1ca4* (Hu et al., 2010), *npr1* (Cao et al., 1994) and *ora59* (GABI-Kat line GK-061A12 from Nottingham Arabidopsis Stock Centre) were sown on sand under ambient CO_2 conditions (450

ppm). Two weeks later, seedlings were transferred to 60-ml pots containing a sand/potting soil mixture (v/v, 5:12) that was autoclaved twice for 20 min, after which they were transferred to a growth chamber with continuous high (800 ppm), ambient (450 ppm) or low (150 ppm) atmospheric CO_2 conditions. Plants were cultivated with a 10-h day (350 µmol/m^2/s) and 14-h night cycle at 20°C with 70% relative humidity for four weeks.

B. cinerea *disease resistance assay*

Botrytis cinerea strain B05.10 (Van Kan et al., 1997) was cultivated on half-strength potato dextrose agar plates for 10 days at 22°C. *B. cinerea* spores were collected and resuspended in half-strength potato dextrose broth to a final density of 5×10^5 spores/ml. Four-week-old plants were infected by applying 10-µl drops to six leaves per plant. Symptoms were scored at four days after infection. Disease rating was expressed as the percentage of leaves showing spreading lesions and disease index was calculated as described (Van der Ent et al., 2008; Van Wees et al., 2013).

Pst *disease resistance assay*

Pseudomonas syringae pv. *tomato* DC3000 (*Pst*) (Whalen et al., 1991) was cultured on King's medium B (King et al., 1954) plates supplemented with 50 µg/ml rifampicine at 28°C. Bacteria were transferred and cultivated overnight in liquid KB medium at 28°C in an orbital shaker at 220 rpm. Subsequently, bacteria were collected by centrifugation for 10 min at 4,000 rpm and resuspended in 10 mM $MgSO_4$. The suspension was adjusted to OD_{600}=1. For dip inoculation, the bacterial inoculum was diluted to the final density of 5×10^7 cfu/ml of 10 mM $MgSO_4$ containing 0.015% (v/v) Silwet L-77 (Van Meeuwen Chemicals, Weesp, Netherlands). A 10 mM $MgSO_4$ solution was used as a mock treatment.

Leaf discs from treated plants were harvested, surface sterilized in 70% ethanol for 8 seconds, and washed immediately with water. Eight biological replicates were included for each data point. Subsequently, 200 µl of 10 mM $MgSO_4$ was added to the samples, the mixtures were ground thoroughly and 10 µl aliquots of different dilutions were plated onto KB plates containing 25 µg/ml rifampicine. After 48 h incubation at room temperature, bacterial colonies were counted and the growth of *Pst* was calculated and data was log-transformed.

RT-qPCR analysis

For gene expression analysis, total RNA was isolated as described (Oñate-Sánchez and Vicente-Carbajosa, 2008). Fermentas RevertAid H minus Reverse Transcriptase (Fermentas, St.Leon-Rot, Germany) was used to convert DNA-free RNA into cDNA. PCR reactions were performed in optical 384-well plates (Applied Biosystem) with an ABI PRISM® 7900 HT sequence detection system using SYBR® Green to monitor the synthesis of double-stranded DNA. Transcript levels were calculated relative to the reference gene *At1g13320* as described previously (Schmittgen and Livak, 2008). Primers used for RT-qPCR are listed in the Supplemental information.

Hormone analysis

For SA, JA, JA-Ile, and OPDA concentration analysis, 50-250 mg of Col-0 leaves was harvested and ground thoroughly into powder under liquid powder. The extraction was performed as described previously (Scala et al., 2013). The samples were homogenized in 0.5 ml 70% methanol, spiked with 200 ng of D6-SA, by shaking at 6,000 rpm for 40 seconds and centrifuged at 10,000 *g* for 20 min at 4˚C. The supernatants of two extraction steps were pooled together. Hormones were quantified by liquid chromatography-mass spectrometry (LC-MS) analysis on Varian 320 Triple Quad LC/MS/MS. The surface area for each daughter-ion peak was recorded for the detected analytes. Analytes were quantified using standard curves made for each individual compound.

ACKNOWLEDGEMENTS

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SUPPLEMENTAL INFORMATION

RT-qPCR primer list

At1g13320 _F, 5'-TAA CGT GGC CAA AAT GAT GC-3'
At1g13320 _R, 5'-GTT CTC CAC AAC CGC TTG GT-3'
CA1-F, 5'-ATG ACT TCG TCA AGG GTG CT-3'
CA1-R, 5'-CTA GTT TCG GAG AGG CCA AA-3'
CA2-F, 5'-AGC TTT GGG AGC TCC AGT TT-3'
CA2-R, 5'-CGA TGG TGA TGG TGA TGT GT-3'
CA4-F, 5'-CAT TCG TGA GAG CTG AGG TG-3'
CA4-R, 5'-TCC CAG AGA TCA AAC GTT CC-3'
PR1_F, 5'-CTC GGA GCT ACG CAG AAC AAC T-3'
PR1_R, 5'-TTC TCG CTA ACC CAC ATG TTC A-3'
PDF1.2_F, 5'-CAC CCT TAT CTT CGC TGC TCT T-3'
PDF1.2_R, 5'-GCC GGT GCG TCG AAA G-3'
FRK1_F, 5'- TTT CAA CAG TTG TCG CTG GA-3'
FRK1_R, 5'-AGC TTG CAA TAG CAG GTT GG-3'
ICS1_F , 5'- GGC AGG GAG ACT TAC G-3'
ICS1_R, 5'-AGG TCC CGC ATA CAT T-3'

CHAPTER 5

Atmospheric CO_2 levels do not affect diseases caused by soil borne fungal pathogens

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ABSTRACT

Most studies on the potential impact of elevated CO_2 levels on plant disease focused on pathogens that infect the leaves. In this study, we investigated interactions between *Arabidopsis thaliana* and two soil borne pathogens, the necrotroph *Rhizoctonia solani* and the hemibiotroph *Fusarium oxysporum* f. sp. *raphani*, at high (800 ppm), ambient (450 ppm), and low CO_2 (150 ppm) conditions. For both *R. solani* and *F. oxysporum* the disease incidence was not affected by changes in atmospheric CO_2 concentration. Infection with these pathogens caused severe plant growth reduction. Also for the growth parameter no consistent differences between high, ambient and low CO_2 conditions were observed. In the ethylene signaling mutant *ein2-1* disease severity caused by *F. oxysporum* was significantly higher than in wild-type Col-0 plants, confirming a role for ethylene signaling in resistance of Arabidopsis to *F. oxysporum*. But also for this mutant no consistent effects of the atmospheric CO_2 levels on disease severity were detected. Overall, our results revealed that atmospheric CO_2 has no effect on development of the tested soil borne fungal diseases in Arabidopsis.

INTRODUCTION

Climate change due to increasing CO_2 levels in the Earth's atmosphere may affect interactions between plants and their attackers resulting in significant effects on crop yields in agriculture. In order to secure food production for the increasing human population it is essential to anticipate on this development. Most studies that consider effects of CO_2 levels on plant disease have focused on foliar pathogens (Jwa and Walling, 2001; Braga et al., 2006; Guo et al., 2014; Li et al., 2014). Also in our previous study, we investigated the effect of atmospheric CO_2 on interactions between *Arabidopsis thaliana* (Arabidopsis) and the foliar pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) and found that increased atmospheric CO_2 levels increased disease susceptibility of Arabidopsis to *Pst* (Chapter 2).

The few studies on the effects of atmospheric CO_2 levels on soil borne diseases show ambiguous results. For example, the incidence of sheath blight in rice caused by *Rhizoctonia solani* increased under elevated CO_2 (Kobayashi et al., 2006). Similarly, increased disease incidence of *Fusarium pseudograminearum* was observed in wheat plants grown under elevated CO_2 (Melloy et al., 2014). However, elevated CO_2 increased tolerance of tomato plants to infection by *Phytophthora parasitica* (Jwa and Walling, 2001). In other studies, elevated CO_2 levels did not significantly affect disease incidence. For *Fusarium oxysporum* f.sp. *lactucae* on lettuce disease severity was not affected by elevating the atmospheric CO_2 level from 400 to 800 ppm (Ferrocino et al., 2013). In a multi-year field study elevated CO_2 did not affect sudden death syndrome in soybean caused by *Fusarium virguliforme* (Eastburn et al., 2010).

CO_2 concentrations in active soils are 10-50 times higher than the atmospheric CO_2 levels (Drigo et al., 2008). Thus it is unlikely that the increase in atmospheric CO_2 levels foreseen for the future have a direct effect on microorganisms in soil. Nonetheless, increases in atmospheric CO_2 concentration can alter plant photosynthetic rate, stimulate plant growth and lead to increased C allocation to the belowground plant tissue, resulting in changes in rhizodeposition (Drigo et al., 2008; Drigo et al., 2010). It has been postulated that the composition of rhizodeposits plays an essential role in shaping the rhizosphere microbiome (Berendsen et al., 2012). Thus, changes in atmospheric CO_2 levels may affect the richness, composition and structure of soil microbial communities through changes in carbon allocation and root exudation (Drigo et al., 2009). Such effects may also influence growth, activities and infectiousness of soil borne plant pathogens.

In this study, we investigated interactions between Arabidopsis and the soil borne plant pathogens *R. solani* and *Fusarium oxysporum* f.sp. *raphani* under high, ambient, and low atmospheric CO_2 conditions. *F. oxysporum* and *R. solani* are amongst the most detrimental soil borne plant pathogenic fungi. *R. solani* is an economically important necrotrophic fungal pathogen (Foley et al., 2013) that can affect many crops including sugar beet, potato and rice, causing collar rot, root rot, damping off and wire stem. Infection of *F. oxysporum* causes severe plant stunting and wilting, followed by

extensive chlorosis and necrosis in the leaves and ultimately plant death (Kidd et al., 2011). Plant pathogenic Fusarium species can be classified as hemibiotrophs, relying on a living host at early infection stages but finally killing the plant cells (Ma et al., 2013). Thus the two soil borne pathogens investigated in this study have contrasting strategies to infect plants.

In general, we found no significant impact of atmospheric CO_2 on these two soil borne diseases.

RESULTS

Effect of atmospheric CO_2 levels on the Arabidopsis-*R. solani* interaction

To study effects of different atmospheric CO_2 levels on the interaction between Arabidopsis and *R. solani*, four week-old seedlings, grown at ambient CO_2, were transplanted into *R. solani* inoculated soil and subsequently grown at three different atmospheric CO_2 conditions. Under ambient CO_2 condition the percentage of diseased plants reached up to nearly 50% three weeks after transfer to *R. solani* infested soil, and both high and low CO_2 conditions did not significantly affect disease incidence (Fig. 1A). *R. solani* infection significantly reduced the growth of Arabidopsis plants under the three CO_2 conditions tested, but also for this parameter no significant differences between high, ambient and low CO_2 levels were observed (Fig. 1B). These results show that varying atmospheric CO_2 conditions from 150 ppm to 800 ppm has no significant on the disease severity caused by infection of *R. solani* in Arabidopsis.

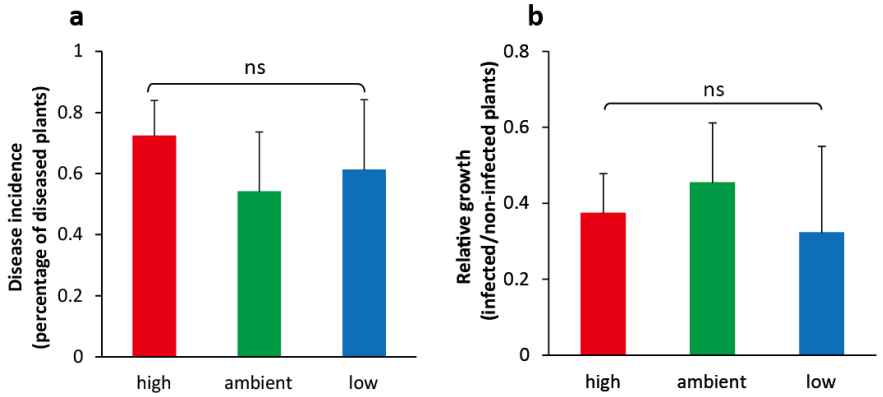

Figure 1: Effects of atmospheric CO_2 levels on disease incidence and growth reduction of Arabidopsis after infection with *R. solani*.

(a) Arabidopsis wild-type Col-0 plants were grown under ambient CO_2 conditions until two weeks old. The two-week old seedlings were then transplanted into soil mixed with *R. solani* inoculum and subsequently transferred to high, ambient, or low CO_2 conditions. Disease incidence was scored 24 days after infection and is expressed as the percentage of diseased plants. **(b)** Inhibition of plant growth by *R. solani* under three different atmospheric CO_2 conditions. Three weeks after infection the aboveground plant parts were harvested and fresh weight was recorded. The growth of *R. solani*-infected plants relative to control plants 24 days after inoculation is depicted. Error bars represent standard deviation of six replicated blocks, which contained 9 plants in each block. Statistical analysis was performed using one-way ANOVA (Duncan's multiple range test; ns, not significant).

Effect of atmospheric CO_2 levels on the Arabidopsis-*F. oxysporum* interaction

We also investigated effects of different atmospheric CO_2 levels on the performance of Arabidopsis plants inoculated with the hemibiotrophic pathogen *F. oxysporum*. Arabidopsis plants were grown under ambient CO_2 conditions for two weeks, subsequently infected with *F. oxysporum* f.sp. *raphani*, and transferred to high, ambient, or low CO_2 conditions. Disease symptoms were scored three weeks after infection. Throughout the course of the study, the disease caused by *F. oxysporum* infection in Arabidopsis plants varied significantly among experiments, with disease index ranging from 1.25 to 2.15 (Table 1). Effects of different atmospheric CO_2 levels on disease caused by *F. oxysporum* was inconsistent among the five independent experiments that were performed. In experiment 2, high and low CO_2-grown plants showed significantly less disease compared with ambient CO_2-grown plants, whereas in experiment 3, disease seemed significantly lower under high CO_2 and higher under low CO_2 condition (Table 1). Three out of these five experiments showed that atmospheric CO_2 did not significantly affect disease symptoms caused by *F. oxysporum* in Arabidopsis plants (Table 1, experiment 1, 4, and 5).

Since the disease development in these experiments was relatively low, we investigated if atmospheric CO_2 might affect disease in a more susceptible genotype of Arabidopsis. Mutations that disrupt the ethylene signaling pathway increased plant susceptibility to *F. oxysporum* (Berrocal-Lobo and Molina, 2004; Pantelides et al., 2013). Thus we included a mutant impaired in ethylene signaling (*ein2-1*) in the experiments. Indeed, four out of the five experiments, the *ein2-1* mutant exhibited a significantly higher disease index (Table 1, experiment 1, 2, 3, and 5). However, also for the more susceptible mutant the different atmospheric CO_2 levels did not influence the disease severity consistently (Table 1).

Table 1: Effect of atmospheric CO_2 levels on the *F. oxysporum* disease index in Arabidopsis.
Disease index (DI) of Arabidopsis three week after inoculation with *F. oxysporum* under different atmospheric CO_2 conditions in five independent experiments. Arabidopsis wild-type plants and a mutant defective in ET signaling (*ein2-1*) were grown in sand under ambient CO_2 until two weeks old. The two-week old seedlings were infected with *F. oxysporum* by root dipping and transferred to high, ambient, or low CO_2 conditions. Disease symptoms were scored at multiple time points starting from ten days after infection. Indicated are averages of the disease index calculated from the percentage of leaves in four different disease severity classes. For each experiment different letters indicate statistically significant differences between treatments (two-way ANOVA, Duncan's multiple-range test; $P<0.05$).

		Disease Index (DI)				
Genotype	**CO_2 level**	**exp. 1**	**exp. 2**	**exp. 3**	**exp. 4**	**exp. 5**
Col-0	**high**	1.33 a	1.67 b	1.07 c	1.72 a	1.24 a
	ambient	1.25 ab	2.15 a	1.47 b	1.64 a	1.10 b
	low	1.14 b	1.85 b	2.11 a	1.61 a	1.29 a
ein2-1	**high**	1.58 b	2.85 a	1.65 a	1.24 b	2.93 a
	ambient	1.79 a	2.43 b	1.82 a	1.24 b	2.90 a
	low	1.45 b	3.07 a	1.33 b	1.71 a	2.70 a

In addition to disease symptoms, the effects of *F. oxysporum* infection on the growth of Arabidopsis were determined. The fresh weight of infected plants relative to non-infected control plants was calculated and is presented in Table 2. The effects of CO_2 levels were again not consistent when comparing the five independent experiments that were performed. Overall, the results on both parameters suggest that there is no significant impact of atmospheric CO_2 levels on the interaction between Arabidopsis and *F. oxysporum*.

Table 2: Effect of atmospheric CO_2 levels on growth inhibition of Arabidopsis plants caused by *F. oxysporum* infection.
Growth of *F. oxy*sporum infected Arabidopsis plants relative to control plants under high, ambient and low atmospheric CO_2 conditions. Arabidopsis wild-type plants and a mutant defective in ethylene signaling (*ein2-1*) were grown in sand under ambient CO_2 until two weeks old. The two-week old seedlings were infected with *F. oxysporum* by root dipping and transferred to high, ambient, or low CO_2 conditions. The aboveground plant tissue was weighed. Indicated are averages of the growth of *F. oxysporum* infected plants relative to control treated plants. Different letters indicate statistically significant differences between CO_2 treatments of one line (two-way ANOVA, Duncan's multiple-range test; $P<0.05$). The *P* value at the bottom of the table indicates whether there is an overall statistically significant difference between wild-type Col-0 and the mutant *ein2-1*.

		Relative Growth (RG)				
Genotype	**CO_2 level**	**exp. 1**	**exp. 2**	**exp. 3**	**exp. 4**	**exp. 5**
Col-0	**high**	0.76 c	0.87 ab	0.98 a	0.97 abc	0.63 b
	ambient	1.06 a	0.71 b	0.80 b	0.71 c	1.00 a
	low	0.89 b	0.91 a	0.69 b	0.82 bc	0.88 a
ein2-1	**high**	0.66 b	0.59 a	0.77 b	1.17 a	…
	ambient	0.80 b	0.81 a	0.82 ab	0.80 bc	…
	low	1.19 a	0.50 a	0.94 a	0.79 bc	…
***P* value**		**ns**	**<0.05**	**ns**	**ns**	

Finally the colonization of Arabidopsis by *F. oxysporum* was determined under low, ambient and high CO_2 conditions. Three weeks after infection Arabidopsis leaves were harvested, ground in a buffer and dilution plated on Komada agar medium that is selective for Fusarium (Komada, 1975). As shown in two independent experiments (Fig. 2A and 2B), the colonization of aboveground tissues of Arabidopsis was not affected by the atmospheric CO_2 conditions under which the plants were grown. Population densities of Fusarium ranged between 10^2 to 10^4 colony forming units per gram fresh weight irrespective of the experimental CO_2 level.

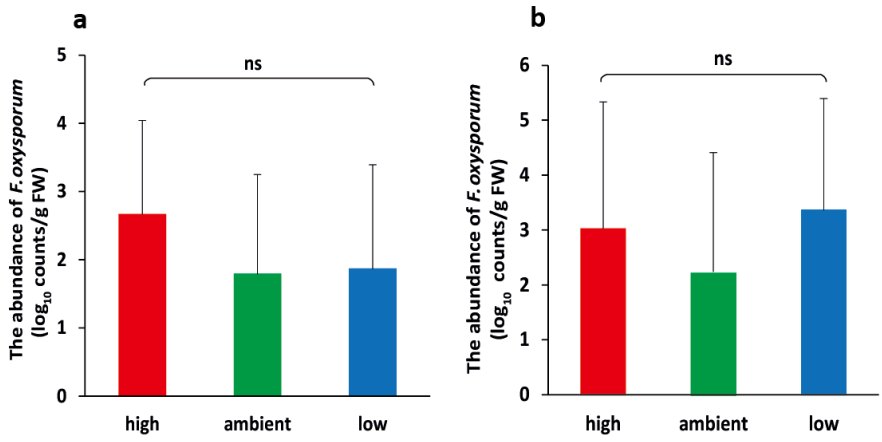

Figure 2: Effect of atmospheric CO_2 levels on colonization of Arabidopsis leaves by *F. oxysporum*.
(a and b) The density of *F. oxysporum* in Arabidopsis plants grown under three different atmospheric CO_2 conditions. Arabidopsis wild-type Col-0 plants were grown in sand under ambient CO_2 until two weeks old. The two-week old seedlings were infected with *F. oxysporum* by root dipping and transferred to high, ambient, or low CO_2 conditions. Arabidopsis leaves were harvested at 21 d after infection. The density of *F. oxysporum* was determined by dilution plating on Komada agar medium. Depicted are averages of log-transformed counts (per gram of leaf tissue) of *F. oxysporum*. Statistical analysis was performed using one-way ANOVA (Duncan's multiple range test; ns, not significant).

DISCUSSION

We studied possible effects of high, ambient, and low atmospheric CO_2 levels on interactions between Arabidopsis and two soil borne pathogens that have different strategies to infect plants, the necrotrophic *R. solani* and the hemibiotrophic *F. oxysporum* f.sp. *raphani*. Direct effects of elevated atmospheric CO_2 levels on the growth or activity of these plant pathogenic fungi seem unlikely for two reasons. First of all, the CO_2 concentrations in the active soil is 10 to 50 times higher than the concentration in the atmosphere (Drigo et al., 2008). Moreover, both Rhizoctonia and Fusarium are relatively insensitive to elevated CO_2 levels and even at 20% their growth is only marginally reduced (Durbin, 1959). However, predicted changes in atmospheric CO_2 do affect soil microorganisms in the rhizosphere due to changes in rhizodeposition (Drigo et al., 2008). It is known that atmospheric CO_2 levels affect soil borne microbes indirectly through impacts on plant-associated activities, such as plant metabolism and root secretion (Drigo et al., 2008). However such effects of elevated atmospheric CO_2 on soil borne disease can also vary in different plant species (Chakraborty et al., 2012). For example, elevated CO_2 concentrations reduced the inhibition of emergence of radish and sugar beet seedlings by *R. solani* (Papavizas and Davey, 1962), whereas in rice high CO_2 levels increased the incidence of *R. solani* (Kobayashi et al., 2006). In the latter case it was suggested that increased disease incidence was due to the higher number of tillers observed under elevated CO_2, which increased the chance for fungal sclerotia to adhere to the leaf sheath at

the water surface (Kobayashi et al., 2006). For Arabidopsis, we observed no differences in disease caused by *R. solani*, nor in the inhibition of plant growth under the three experimental atmospheric CO_2 conditions (Fig. 1A and 1B). In our case, we used two-week old Arabidopsis seedlings grown under ambient CO_2 condition for *R. solani* infection that were subsequently transferred to the three different atmospheric CO_2 conditions. Thus there were no developmental differences between the plants at the moment of inoculation with the pathogen. Also, we observed that Arabidopsis plants under the three atmospheric CO_2 conditions grew similarly during first two weeks after infection. The homogenized Arabidopsis plants before and during early stages of infection might partly account for the limited effect of atmospheric CO_2 on disease of *R. solani*.

Also for the hemibiotrophic pathogen *F. oxysporum* f.sp. *raphani*, atmospheric CO_2 levels had no consistent effects on development of disease in Arabidopsis (Table 1 and Table 2). This was not only observed for the moderately susceptible accession Col-0, but also for the more susceptible *ein2-1* mutant. Our results are consistent with a previous study in which it was found that elevated CO_2 did not influence disease severity caused by *F. oxysporum* in lettuce (Ferrocino et al., 2013). In addition, in both our study and Ferrocino's study, population densities of *F. oxysporum* remained unchanged by alterations in atmospheric CO_2 (Fig. 2A and 2B). However, several studies revealed increased susceptibility to *Fusarium* under elevated CO_2 in rice, wheat, and maize (Kobayashi et al., 2006; Melloy et al., 2014; Vaughan et al., 2014). Thus, it appears that atmospheric CO_2 can affect soil borne diseases but the effects might be different in specific host-pathogen system.

The limited effect of atmospheric CO_2 alone on Arabidopsis disease resistance to *R. solani* and *F. oxysporum* in this study does not rule out the possibility that atmospheric CO_2 affects soil borne diseases when combined with other global climate changes, such as elevated O_3, drought, and increased temperature. In some cases, the benefits of elevated CO_2 are offset by the negative effects of elevated O_3, whereas in other cases, plant disease responses to one environmental condition alter significantly when another condition is introduced (Eastburn et al., 2011). Warm and humid conditions are favorable for production of conidia, growth and virulence of many *Fusarium* species (Doohan et al., 2003). It has been projected that climate change induced increases in temperature or rainfall would directly increase risk of wheat Fusarium ear blight epidemics (Madgwick et al., 2011). Thus, atmospheric CO_2 may affect the disease severity of *F. oxysporum* in Arabidopsis at elevated temperatures or high moisture conditions.

In conclusion, our study suggests that increased atmospheric CO_2 levels will not significantly affect disease caused by the soil borne pathogens *R. solani* and *F. oxysporum*. However, further studies combined with other climate change factors, including increased temperature and humidity, are required for an accurate prediction about how soil borne diseases will respond to climate change.

MATERIALS AND METHODS

Plant material and cultivation

Seeds of *A. thaliana* accession Col-0 and mutants *ein2-1* (Alonso et al., 1999) were sown in quartz sand under ambient CO_2 condition. Two weeks later, seedlings were transferred to 60-ml pots containing a sand/potting soil mixture (v/v, 5:12) that was autoclaved twice for 20 min. Plants grew at 10-h day (350 µmol/m^2/s) and 14-h night at 20°C with 70% relative humidity. Until transplantation at 2 weeks, seedlings grew at ambient CO_2 (450 ppm), after which they were transferred to three growth chambers with different CO_2 concentrations (800 ppm, 450 ppm, 150 ppm).

Rhizoctonia solani *infection*

R. solani (anastomosis group AG2-2IIIB, (Mendes et al., 2011; Chapelle et al., 2015) was grown on half strength potato dextrose agar (PDA, Difco™) at room temperature for one week. Two-week old Arabidopsis seedlings were transferred to soil/sand mixture containing *R. solani* inoculum (0.5 petri dish/kg soil). The seedlings infected with *R. solani* were then transferred to the three different CO_2 conditions.

Fusarium oxysporum *infection*

F. oxysporum f. sp. *raphani* strain WCS600 can cause disease in *A. thaliana* (Pieterse et al., 1996) and was thus used in this study. The fungal isolate was cryopreserved by freezing a suspension of conidia in 12.5% aqueous glycerol at –80°C. Before use, the fungus was transferred to half strength PDA at 28°C for 3 days. The inoculum was prepared by transferring two PDA plugs to sucrose sodium nitrate liquid medium (Sinha and Wood, 1968) and culturing while shaking for 3 days at 28°C. For the infection, a final suspension of 10^7spores/ml was prepared in sterile distilled water (SDW).

Roots of two-week old seedlings were dipped in Fusarium inoculum. The seedlings were then transferred to soil mixed with sand (12:5) and grown under the three different CO_2 conditions.

Disease assessment

For the *R. solani* disease assessment, at least thirty plants were tested under each CO_2 condition and the percentage of plants with chlorosis and growth reduction was recorded three weeks after infection. For the *F. oxysporum* f. sp. *raphani* disease assessment, twenty plants per CO_2 treatment were analyzed and three weeks after infection disease symptoms were scored by determining the percentage of plants with Fusarium wilt. The disease index (DI) was calculated as described (Van Wees et al., 1997).

The aboveground plant parts were harvested and fresh weight was recorded. Relative growth (RG) =the weight of infected plants/the weight of control plants.

***Population densities of* F. oxysporum**

Three week after *F. oxysporum* infection, the aboveground plant parts were harvested and weighed. Around 100-200 mg of leaf material was grinded in 1 ml of 10 mM $MgSO_4$. A dilution series of this suspension was made and 10 µl of each dilution (0, 10 and 100 times diluted) was plated in duplicate on the Fusarium selective Komada medium (Komada, 1975). Komada plates were incubated at 28°C for 3-4 days. The numbers of colonies were counted to assess the population density of *F. oxysporum* in each plant. This experiment was repeated twice, with at least 12 biological replicates for each CO_2 treatment.

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CHAPTER 6

Summarizing discussion

A NEW CHALLENGE: PLANT DEFENSE UNDER INCREASING ATMOSPHERIC CO_2

With extensive attention from all over the world, climate change has been recognized as one of the greatest challenges for the 21st century. Among various global climatic changes, the increasing CO_2 level in the atmosphere is considered to be core due to its capacity of driving other climate changes, such as increasing temperatures and drought extremes (Hofmann et al., 2009; Conway and Tans, 2012; Change, 2014). The increasing atmospheric CO_2 is projected to continue and affect crop production. Previous studies have demonstrated that exposure of plants to an enriched CO_2 atmosphere results in stimulated plant photosynthesis, decreased stomatal conductance, and induced changes in secondary metabolites (Li et al., 2011c; Zavala et al., 2013). On the other hand, many crop plants suffer from a number of diseases caused by pathogens and herbivorous insects. To fight their attackers, plants have developed a complex immune system in which multiple hormonal signaling pathways play a regulatory role (Pieterse et al., 2009).

The concurrence of increased atmospheric CO_2 levels and pathogen attacks is anticipated to change interactions between plants and pathogens in the future, posing a new challenge for future strategies in plant protection. The altered photosynthetic and physiological activities in plants grown under increased atmospheric CO_2 is likely to affect plant disease resistance. Indeed, elevated CO_2 reduced both disease incidence and severity of *Tomato yellow leaf curl virus* (TYLCV) on tomato plants (Huang et al., 2012). However, doubling ambient CO_2 concentration increased the incidence of bacterial wilt and spot diseases in pepper plants (Shin and Yun, 2010). These findings suggest that elevated atmospheric CO_2 can alter disease resistance differently in different plant pathosystems (Eastburn et al., 2010; Jones and Barbetti, 2012).

It is noticed that while most studies focused on plant pathogen interactions under increased atmospheric CO_2, few studies investigated how plant disease resistance is altered under low atmospheric CO_2. The predominance of relatively low CO_2 atmosphere until the industrial revolution indicate that most plants on the Earth have evolved to be adapted to lower atmospheric CO_2 levels and this could affect plant responses to the rising atmospheric CO_2 (Sage and Coleman, 2001). In this thesis, we aimed to elucidate how disease resistance would be affected in Arabidopsis plants grown under three atmospheric CO_2 levels: 150 ppm, representing the pre-industrial CO_2 level; 450 ppm, representing the current CO_2 level; 800 ppm, representing the predicted CO_2 level 50 years from now. We investigated the molecular mechanism underlying atmospheric CO_2-regulated defenses against the hemi-biotroph *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) and the necrotroph *Botrytis cinerea* (Chapter 2 and 4). We also investigated the role of two CO_2-binding proteins, beta carbonic anhydrases (CA1 and CA4) in plant immune responses, particularly in the

PATHOGEN ASSOCIATED MOLECULAR PATTERN (PAMP)-triggered immunity (PTI) (Chapter 3). In addition, we studied the effect of atmospheric CO_2 on plant disease resistance against two soil borne pathogens, *Rhizoctonia solani* and *Fusarium oxysporum* (Chapter 5).

HORMONE SIGNALING PATHWAYS INVOLVED IN ATMOSPHERIC CO_2-REGULATED PLANT DEFENSE AGAINST FOLIAR PATHOGENS

Upon recognition of pathogens, a hormonal blend is produced depending on the type of attacker (De Vos et al., 2005). Generally, salicylic acid (SA) and its mediated signaling pathway play a key role in plant defense against biotrophic pathogens, whilst jasmonic acid (JA) and its associated signaling pathway are essential for the activation of defense against necrotrophic pathogens and insects (Glazebrook, 2005; Pieterse et al., 2012). The abscisic acid (ABA) signaling pathway plays multifaceted roles in plant defense depending on the type of pathogen, the stage of pathogen invasion, and the degree of activation of other hormone signaling pathways (Mauch-Mani and Mauch, 2005; Ton et al., 2009). ABA has also been implicated in plant defense through modulating the SA- and JA-dependent defense signaling pathways (Anderson et al., 2004; De Torres-Zabala et al., 2007; De Torres Zabala et al., 2009). Increased atmospheric CO_2 was found to cause significant changes in plant hormonal blends (Teng et al., 2006; Zavala et al., 2013). For example, ABA signaling was significantly influenced by atmospheric CO_2 levels (Leymarie et al., 1998; Li et al., 2006). It is therefore hypothesized that atmospheric CO_2 affect plant disease resistance through modulating hormonal signaling pathways.

Previous studies have found that exposure of plants to elevated CO_2 increases the SA-related responses and consequently enhances disease resistance against *Pst*, whereas elevated CO_2 reduces the JA-related responses and the defense against *B. cinerea* (Sun et al., 2011a; Zavala et al., 2013; Zhang et al., 2015). Our hormonal analysis revealed no significant difference in basal levels of SA and JA in Arabidopsis plants grown under three different atmospheric CO_2 conditions (Chapter 4). Nonetheless, we found increased levels of ABA in high CO_2-grown plants in comparison to that in ambient and low CO_2-grown plants (Chapter 2). Inoculation with *Pst* significantly induced ABA accumulation, with the *Pst*–induced ABA levels being higher in high CO_2-grown plants. This correlates well with the increased susceptibility to *Pst* under high CO_2 (Chapter 2). The ABA deficient mutant *aba2-1* showed enhanced resistance to *Pst* compared with wild-type Col-0 and more importantly, the enhanced resistance occurred under all three CO_2 conditions (Chapter 2). These results together suggest that ABA signaling plays a role in the regulation of CO_2-dependent defense against *Pst*.

During plant defense against *B. cinerea*, the ERF branch of the JA pathway is activated, resulting in the activation of its downstream target genes, such as the JA marker gene *PDF1.2* (Berrocal-Lobo et al., 2002). We found that the basal level of *PDF1.2* expression was significantly enhanced in high CO_2-grown plants, which is in

line with the increased resistance to *B. cinerea* under high CO_2 conditions (Chapter 4). Mutations in ORA59, an important regulator in JA-mediated defense signaling, enhanced the susceptibility of Arabidopsis to *B. cinerea* (Chapter 4). Moreover, *ora59* mutants displayed similar disease resistance pattern regulated by atmospheric CO_2 compared to Col-0 plants, suggesting that other regulators than ORA59 might be responsible for the altered disease resistance to *B. cinerea* under different atmospheric CO_2. The role of ABA signaling in plant defense against *B. cinerea* has been demonstrated (Audenaert et al., 2002; AbuQamar et al., 2009). Our results showed that *aba2-1* mutants displayed increased resistance to *B. cinerea* compared with wild-type Col-0 plants (data not shown), suggesting a negative role of ABA in Arabidopsis defense against *B. cinerea* (Audenaert et al., 2002). Moreover, this increased resistance of *aba2-1* to *B. cinerea* occurred under all three atmospheric CO_2 (data not shown). Thus it is likely that under high CO_2 conditions, the enhanced basal level of JA signaling functions antagonistically with ABA signaling, leading to final increased resistance to *B. cinerea*. Together, these findings highlight the importance of ABA signaling for fine tuning atmospheric CO_2–regulated defense responses.

CAs: POTENTIAL COMPONENTS CONNECTING CO_2 SIGNALING WITH PLANT DEFENSE?

CAs in plants mostly belong to the beta group with wide distributions and high abundances. It has been demonstrated that CAs play important roles in plant cellular biology (Henry, 1996). For example, as metalloenzymes they can catalyze the interconversion of CO_2 and bicarbonate. In addition, they also function in plant defense against pathogen attack (Slaymaker et al., 2002; Restrepo et al., 2005; Sun et al., 2014). Our results with two *CA* genes *CA1* and *CA4* showed that *CA1* and *CA4* were significantly down-regulated in Arabidopsis plants in response to multiple PAMPs and pathogen attack (Chapter 3). Data from Genevestigator (Perturbation module) also showed a general down-regulation pattern of these *CA* genes upon stresses such as pathogen invasion, drought and osmotic stress. This suggests that CAs might be involved in plant defense activation in response to various environmental stimuli. We further demonstrated that CA1 and CA4 play a negative role in plant defense against *Pst*, and they function likely through antagonizing the SA-mediated defense signaling pathway (Chapter 3). We thus propose a model of the function of CAs in mediating PTI: upon recognition of PAMPs, *CA1* and *CA4* are down-regulated in plants. While in healthy plants, CAs have an antagonizing effect on the SA signaling pathway, the PAMPs-mediated suppression of *CA* genes results in enhanced ROS production and increased defense-related gene expression. This ultimately leads to enhanced SA-dependent defenses and inhibition of plant growth (Chapter 3). So far, it remains unknown how these differently located CAs inhibit the SA-mediated defense signaling pathway at cellular level. CA1 has been identified as a SA-binding protein both in tobacco and Arabidopsis plants (Slaymaker et al., 2002;

Wang et al., 2009). Therefore, it might be the case that during plant defense activation, CA1 directly binds to the SA molecule, decreased free SA levels in the plant cell, thus reducing the downstream SA-mediated signaling.

As CO_2-binding proteins, CAs play an essential role in CO_2 metabolism, such as CO_2 fixation in photosynthesis and CO_2-controlled stomatal movement (Badger and Price, 1994; Hu et al., 2010). We thus speculated a role of CAs in atmospheric CO_2-regulated defense resistance. In Chapter 4, we found that high CO_2 induced susceptibility to *Pst* in wild-type Col-0 plants, and that was compromised in *ca1ca4* mutants. This suggests CA1 and CA4 play an important role in atmospheric CO_2-regulated defense against *Pst*. On the other hand, high CO_2 induced and low CO_2 decreased disease resistance to *B. cinerea* in Col-0 plants, which still occurred in *ca1ca4* mutants, suggesting CA1 and CA4 play a limited role in atmospheric CO_2-mediated defense against *B. cinerea*.

Together, our findings shed new light on the functions of CA1 and CA4 in plant defense and the potential mechanisms underlying their functions. Our study also suggests that CAs might serve as an important node connecting CO_2 and plant defense signaling.

LIMITED EFFECTS OF ATMOSPHERIC CO_2 ON PLANT RESISTANCE TO SOIL-BORNE PATHOGENS

The direct effect of elevated atmospheric CO_2 on soil-borne pathogens is unlikely as the CO_2 concentration in most soil is 10-50 times higher than current atmospheric CO_2 levels (Drigo et al., 2008). Nonetheless, the increased plant biomass and primary productivity under elevated atmospheric CO_2 conditions increase root growth and exudates through increased carbon allocation from shoot, which can cause changes in rhizosphere microbiome (Drigo et al., 2009; Drigo et al., 2010; Berendsen et al., 2012). Indeed, the enhanced C:N ratio of rhizodeposition under elevated atmospheric CO_2 favors soil fungi over bacteria, as soil bacteria have a higher requirement for N than fungi (Drigo et al., 2009). It has also shown that different soil microbes respond to elevated CO_2 in a different manner (Drigo et al., 2008). Considering the effects of elevated atmospheric CO_2 on both plant roots and soil microbes, it is anticipated that changes in atmospheric CO_2 affect soil borne disease differently in different plant pathosystems. Indeed, increased (Kobayashi et al., 2006; Melloy et al., 2014), decreased (Jwa and Walling, 2001), and no influences on soil borne disease incidence (Ferrocino et al., 2013) were reported in different plant species grown under elevated atmospheric CO_2.

Our results revealed that changes in atmospheric CO_2 do not significantly impact soil-borne diseases caused by *R. solani* and *F. oxysporum* f.sp. *raphani* (*F. oxysporum*) in Arabidopsis plants (Chapter 5). A previous study showed that elevated CO_2 increased epidemics of sheath blight caused by *R. solani* in rice plants with the higher number of tillers observed under elevated CO_2, which increased the chance for fungal sclerotia to adhere to the leaf sheath at the water surface (Kobayashi et al.,

2006). In our case, we used the two-week-old Arabidopsis seedlings for *R. solani* inoculation and there was little differences in the growth of plant grown under three atmospheric CO_2 during first two weeks after inoculation (Chapter 5). This correlates with the similar disease symptoms of Arabidopsis plants under three atmospheric CO_2 conditions. The results with little effect of atmospheric CO_2 on Arabidopsis disease resistance to *F. oxysporum* was consistent with a previous study in which it was found that elevated CO_2 had no influence on disease severity caused by *F. oxysporum* in lettuce (Ferrocino et al., 2013). These findings together support the notion that atmospheric CO_2 affects soil-borne diseases depending on plant species and soil-borne pathogen. It is known that warm and humid conditions are favorable for production of conidia, growth and virulence of many *Fusarium* species (Doohan et al., 2003). While climate changes also involve increases in temperatures and humidity (Change, 2014), it is reasonable to envisage significant changes in soil-borne diseases under future climate conditions.

FUTURE PROSPECTS

Overall, our research provides insight into mechanisms by which plant defend themselves against multiple pathogens under changed atmospheric CO_2 conditions. This knowledge can be utilized to improve crop protection and crop breeding in the face of changing future climate. Given the fact that climate changes are more than changes in atmospheric CO_2, it remains urgent to investigate how the plant immune system is altered under concurrent environmental changes, such as changed atmospheric CO_2, ozone, temperature and water supply. Furthermore, while research on climate change and plant defense has been mostly conducted under controlled growth chamber conditions, it is necessary to test the effects of climate changes on plant disease resistance under field conditions in order to make this knowledge better applicable in future agricultural improvement.

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SUMMARY

The continuously increasing CO_2 levels in the atmosphere is considered to be core among climate changes and is expected to affect plant diseases in the future, posing a new challenge for future strategies in plant protection. In this thesis we explore signaling mechanisms underlying atmospheric CO_2-modulated defense responses in Arabidopsis plants. The tested CO_2 regimes are comparable to the CO_2 levels in the atmosphere before the industrial revolution (150 ppm), the current CO_2 levels (450 ppm), and the CO_2 level which is anticipated to be reached in about 50-100 years (800 ppm). We demonstrate that the disease resistance against the hemi-biotroph *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) decreases and the disease resistance against the necrotroph *Botrytis cinerea* increases as the level of atmopheric CO_2 increases. By employing genetic, physiological and biochemical analysis, we further demonstrated that ABA signaling plays a central role in CO_2-regulated defense against *Pst*. The CO_2-controlled stomatal reopening is dependent on ABA signaling in the plant, whereby the low ABA concentration under a low CO_2 regime leads to prolonged closure of the stomata after infection with *Pst*. This ABA-dependent effect on the opening of the stomata is correlated with an increased resistance against this pathogen that invades the plant through the stomata. Together, our findings highlight the importance of ABA signaling for fine tuning atmospheric CO_2-regulated defense responses.

In a search for potential components involved in CO_2-modulated defense responses, we reveal that two carbonic anhydrases (CAs), CA1 and CA4, are important regulators in pathogen associated molecular patterns (PAMPs)-triggered immunity (PTI). We demonstrate that these CAs have an antagonizing effect on the SA signaling pathway. We further propose a model for the function of CAs in mediating PTI. Upon recognition of PAMPs, *CA1* and *CA4* are down-regulated in plants, resulting in enhanced ROS production and increased defense-related gene expression. This ultimately leads to enhanced SA-dependent defenses and inhibition of pathogen growth. Moreover, we show that these two *CA* genes play a role in atmospheric CO_2-regulated defense against *Pst*. These results together suggest that CAs might serve as an important node connecting CO_2 and plant defense signaling.

Finally, our results reveal that changes in atmospheric CO_2 do not significantly influence soil-borne diseases caused by *Rhizoctonia solani* and *Fusarium oxysporum* f.sp. *raphani* in Arabidopsis. Possibly, this is caused by the fact that CO_2 levels in the soil are ready much higher than in the atmosphere.

In conclusion, our research demonstrates that hormonal signaling pathways and

CAs are important regulators in CO_2-modulated defense responses. This knowledge provides a new perspective on future investigations into the functioning of the plant immune system under changed atmospheric CO_2 conditions and ultimately can be utilized to improve crop protection and crop breeding in the face of changing climate change.

SAMENVATTING

Het voortdurend stijgende CO_2 gehalte in de atmosfeer als belangrijk onderdeel van de wereldwijde klimaatverandering heeft effect op het functioneren van het afweersysteem van planten. Om plantenziekten in de toekomst het hoofd te kunnen bieden is onderzoek naar het effect van veranderingen in atmosferische CO_2 niveaus op het afweersysteem van de plant van groot belang. In dit proefschrift is gedetailleerd onderzoek gedaan naar de veranderingen in cellulaire signaleringmechanismen onder verschillende CO_2 niveaus die het functioneren van het afweersysteem van de modelplant *Arabidopsis thaliana* (Arabidopsis) verstoren. De geteste CO_2 regimes zijn te vergelijken met de CO_2 niveaus in de atmosfeer van voor de industriële revolutie (150 ppm), het huidige CO_2 niveau (450 ppm), en het CO_2 niveau wat naar verwachting over 50-100 jaar bereikt zal zijn (800 ppm). Uit dit onderzoek blijkt dat bij hoge CO_2 niveaus Arabidopsis planten aan de ene kant vatbaarder zijn voor de hemi-biotrofe bacteriële ziekteverwekker *Pseudomonas syringae*, en aan de andere kant resistenter zijn tegen de schimmelziekte die wordt veroorzaakt door de necrotroof *Botrytis cinerea.* Genetische, fysiologische en biochemische analyses wijzen uit dat het hormoon abscisinezuur (ABA) een belangrijke rol speelt in de door CO_2-gereguleerde verandering in de afweer tegen *P. syringae*. De opening van de huidmondjes wordt beïnvloed door CO_2 op een manier die afhankelijk is van ABA signalering in de plant, waarbij de lage ABA concentratie onder een laag CO_2 regime leidt tot langdurige sluiting van de huidmondjes na infectie met *P. syringae.* Dit ABA-afhankelijke effect op de opening van de huidmondjes is gecorreleerd met een verhoogde resistentie tegen dit pathogeen dat via de huidmondjes de plant binnendringt.

Onderzoek naar de moleculaire mechanismen die ten grondslag liggen aan de door CO_2 gemoduleerde verdedigingsreacties onthult een rol voor de enzymen CO_2 anhydrase CA1 en CA4 in de door CO_2 gereguleerde veranderingen in het afweersysteem. De CAs blijken een negatief effect te hebben op de reactie van het afweersysteem van de plant op geconserveerde moleculen afkomstig van pathogenen, wat bekend staat onder de Engelse naam "*PAMP-triggered immunity (PTI)*". Omdat *P. syringae* gevoelig is voor deze reacties, zijn Arabidopsis mutanten in CA1 en CA4 resistenter tegen dit pathogeen. Tijdens de PTI-verdedigingsreacties brengen planten de expressie van de *CA* genen omlaag. Hierdoor ontwikkelt de plant een sterkere reactie op het afweerhormoon salicylzuur wat leidt tot een verhoogde afweerrespons en resistentie tegen *P. syringae.* De activiteit van de CAs is afhankelijk van het CO_2 niveau in de atmosfeer en deze enzymen blijken ook van belang te zijn

bij de door CO_2-veroorzaakte veranderingen in het afweersysteem tegen *P. syringae*. CAs lijken dus te fungeren als signaleringsknooppunt in de reactie van planten op CO_2 en pathogeeninfectie.

Verder is in dit onderzoek gekeken naar het effect van verschillende CO_2 regimes op ziekteresistentie in Arabidopsis tegen de pathogene bodemschimmels *Rhizoctonia solani* en *Fusarium oxysporum.* Veranderingen in de atmosferische CO_2 concentraties bleken geen effect te hebben op het niveau van ziekteresistentie tegen deze pathogenen, wat mogelijk verklaard kan worden door het feit dat CO_2 concentraties in de grond al veel hoger zijn.

Dit onderzoek heeft inzicht gegeven in het effect van veranderingen in de concentratie CO_2 in de atmosfeer op het functioneren van het afweersysteem van de plant, waarbij belangrijke factoren zoals ABA en CAs zijn geïdentificeerd. Met behulp van de verworven kennis is het mogelijk om de werking van het immuunsysteem van de plant onder veranderende CO_2 condities verder te onderzoeken om zo eigenschappen van de plant te identificeren die gebruikt kunnen worden in veredeling en bescherming van gewassen met het oog op de toenemende veranderingen in het klimaat.

摘要

大气中不断增长的二氧化碳(CO_2)水平被认为是全球气候变化中的一个核心特征。这个特征可能对将来植物病害产生深远影响，进而挑战未来植物保护上的策略制定。该论文探索了在拟南芥植物中大气 CO_2 调控防御反应的信号机制。文中用到了三种 CO_2 浓度：低浓度(150 ppm)，模拟工业革命以前大气中的 CO_2 浓度；中等浓度(450 ppm)，模拟当前大气中的 CO_2 浓度；以及高浓度(800 ppm)，模拟未来 50-100 年以内大气将达到的 CO_2 浓度。我们发现，植物对半活体营养型细菌 *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) 的抗病性随着 CO_2 浓度的升高而减弱。与此相反，植物对死体营养型真菌 *Botrytis cinerea* 的抗病性则随着 CO_2 浓度升高而增强。通过植物遗传，生理以及生化等方面的分析，我们进一步证明了脱落酸(ABA)信号通路在 CO_2 调节的对 *Pst* 的免疫反应中起着至关重要的作用。其中，CO_2 调控的气孔开放依赖于植物中的 ABA 信号，而低 CO_2 浓度下植物体内的 ABA 浓度较低，由此导致 *Pst* 侵染后气孔的持久闭合。这种效应与增强的对 *Pst* 的抗病性紧密相关。总体来说，我们的研究发现强调 ABA 信号通路在大气 CO_2 调节的防御反应中的关键作用。

在对 CO_2 调控的防御反应中潜在因子的探索中，我们发现两个碳酸酐酶(CAs)，CA1 和 CA4 是病原体相关分子模式(PAMP)诱导的免疫系统(PTI)中的重要调节因子。我们研究证明这两个 CAs 与水杨酸(SA)信号通路相拮抗。我们并提出了一个关于 CAs 调节 PTI 的功能模型：在识别 PAMPs 后，*CA1* 和 *CA4* 基因在植物体中的表达水平下调，导致活性氧(ROS)的产生增多以及防御相关基因的表达增强。这最终导致依赖 SA 防御机制的增强以及病原体生长的抑制，即抗病性的增强。此外，我们还发现这两个 CAs 在 CO_2 调节的对 *Pst* 的防御机制中发挥重要作用。这些研究结果表明 CAs 有可能是连接 CO_2 通路和植物防御信号通路的一个重要节点。

最后，我们还发现大气中 CO_2 浓度的变化并不显著影响由土传真菌 *Rhizoctonia solani* 和 *Fusarium oxysporum* f.sp. *raphani* 所引起的病害。这有可能是因为土壤中本来就已经很高的 CO_2 浓度。

综上所述，我们的研究表明激素信号通路和碳酸酐酶在 CO_2 调控的防御反应

中的重要调节因子。这方面的知识为将来在变化的大气 CO_2 条件下研究植物免疫系统提供了一个崭新的视角，并最终被应用到作物改良和作物育种中。

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CURRICULUM VITAE

Yeling Zhou was born the 17th of October 1986 in the county of Longhui, China. She finished her high school in Longhui in 2004. She went to the Northwest A & F University in Yangling, China for undergraduate and graduate study. She specialized in agriculture during her Bachelor studies and obtained her Bachelor Degree in 2008 with distinction. She received an exemption qualification to continue her master study. She switched her major to 'Landscape Plants and Ornamentals' and obtained her Master Diploma in 2011. In the same year, she received a fellowship from Chinese Scholarship Council (CSC) and moved to the Netherlands for continuing studies in plant pathology. In October 2011, she joined the Plant-Microbe Interactions research group at Utrecht University and started her PhD project on the effect of atmospheric CO_2 on plant immune responses under the supervision of Prof. Corné Pieterse, Dr. Saskia van Wees and Dr. Peter Bakker. The results of these studies are described in this thesis.

LIST OF PUBLICATIONS

Yeling Zhou, Irene Vos, Rob Schuurink, Corné MJ Pieterse and Saskia CM Van Wees. Atmospheric CO_2 alters resistance of Arabidopsis to *Pseudomonas syringae* by changing abscisic acid signaling and stomatal responsiveness to coronatine. *Submitted.*

Yeling Zhou, Irene Vos, Rob Schuurink, Corné MJ Pieterse, Saskia CM Van Wees and Peter AHM Bakker. Atmospheric CO_2 differentially affects Arabidopsis disease resistance against aboveground and belowground pathogens. *Submitted.*

Yeling Zhou, Irene Vos, Dieuwertje Van der Does, Rob Schuurink, Saskia CM Van Wees and Corné MJ Pieterse. Beta-carbonic anhydrases CA1 and CA4 inhibit PAMP-triggered immunity through antagonizing salicylic acid-dependent defense. *In preparation for submission.*

TALKS ON CONFERENCES

2015.7 **PhD Summer School.** '*Beta-Carbonic Anhydrases* (*CA1* and *CA4*) act as negative regulators in plant innate immunity'. **Utrecht, the Netherlands.**

2015.1 **PhD Day, Get2Gether.** '*Beta-Carbonic Anhydrases* (*CA1* and *CA4*) act as negative regulators in plant innate immunity'. **Soest, the Netherlands.**

2014.7 **XVI International Congress on Molecular Plant-Microbe Interaction (XVI IS-MPMI).** 'Atmospheric CO_2 alters pathogenicity of *Pseudomonas syringae* through altered responsiveness to coronatine and ABA signaling'. **Rhodes, Greece.**

2014.4 **NWO-ALW meeting 'Experimental Plant Sciences'.** 'Atmospheric CO_2 alters Arabidopsis resistance to *Pseudomonas syringae* in a ABA-dependent manner'. **Lunteren, the Netherlands.**

2013.11 **Course: Innate Immunity in Plants.** 'The role of ABA in CO_2-altered Arabidopsis responses to *Pst* DC3000'. **Helsinki, Finland.**

2013.8 **10th International Congress of Plant Pathology (ICPP).** 'Effect of different atmospheric CO_2 levels on plant-pathogen interactions'. **Beijing, China.**