Regulation of Stomatal Defense by Air Relative Humidity

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One sentence summary: High relative humidity suppresses *Pseudomonas syringae*-triggered stomatal closure by regulating hormone signaling in guard cells.
Footnotes:

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It has long been observed that environmental conditions play crucial roles in modulating immunity and disease in plants and animals. For instance, many bacterial plant disease outbreaks occur after periods of high humidity and rain. A critical step in bacterial infection is entry into the plant interior through wounds and natural openings, such as stomata, which are adjustable microscopic pores in the epidermal tissue. Several studies have shown that stomatal closure is an integral part of the plant immune response to reduce pathogen invasion. In this study, we found that high humidity can effectively compromise *Pseudomonas syringae*-triggered stomatal closure in both *Phaseolus vulgaris* and *Arabidopsis thaliana* against, which is accompanied by early up-regulation of the jasmonic acid pathway and simultaneous down-regulation of salicylic acid pathway in guard cells. Furthermore, SA-dependent response, but not JA-dependent response, is faster in guard cells than in whole leaves suggesting that the SA signaling in guard cells may be independent from other cell types. Thus, we conclude that high humidity, a well-known disease-promoting environmental condition, acts in part by suppressing stomatal defense and is linked to hormone signaling in guard cells.
The phyllosphere is one of the most diverse niches for microbe inhabitation. Numerous bacteria can survive and proliferate on the surface of the plant without causing any harm (Lindow and Brandl, 2003). However, for a bacterial pathogen to cause disease, it must penetrate through the plant epidermis and be able to survive and proliferate inside the plant. The mode and mechanism of penetration into the plant tissue is a critical step for infection, especially for bacterial pathogens that rely on natural openings and accidental wounds on the plant surface to colonize internal tissues (Misas-Villamil et al., 2013). Stomata are an example of such openings, providing one of the main routes through which the foliar pathogen *Pseudomonas syringae* transitions from avirulent epiphytic to virulent endophytic lifestyles (Melotto et al., 2008). This abundant opening in the epidermal tissue is not a passive port that allows unrestricted entry of microbes. It has been shown that plants are able to respond to human and plant bacterial pathogens by actively closing the stomatal pore (McDonald and Cahill, 1999; Melotto et al., 2006; Gudesblat et al., 2009; Zhang et al., 2010; Roy et al., 2013; Arnaud and Hwang, 2015); a phenomenon described as stomatal immunity (Sawinski et al., 2013). Several lines of evidence point to the complexity of this response and show that stomatal closure is an integral basal plant defense mechanism to restrict the invasion of pathogenic bacteria into plant tissues (Ali et al., 2007 Melotto et al., 2008; Zhang et al., 2008; Gudesblat et al., 2009). However, certain bacterial pathogens such as *Xanthomonas campestris* pv. *campestris* (Gudesblat et al., 2009), *Pseudomonas syringae* pv. *syringae* (Pss) B728a (Schellenberg et al., 2010), *P. syringae* pvs. *tabaci*, *tomato*, and *maculicola* (Melotto et al., 2006) can successfully cause disease by producing toxins that overcome stomatal immunity. Specifically, *P. syringae* pv. *tomato* (Pst) DC3000 uses coronatine (COR) as such a toxin.

In this study, we focused on elucidating environmental regulation of stomatal-based defense against bacterial invasion. Changes in environmental conditions, such as air relative humidity (RH), light, and carbon dioxide concentration regulate guard cell turgidity that consequently alters stomatal aperture size and the basic functions of stomata in plants, *i.e.* exchange of photosynthetic gases and regulation of water loss by transpiration (Zelitch, 1969; Schroeder et al., 2001; Fan et al., 2004). In natural conditions, plants are exposed to both biotic and abiotic stresses, and guard cells need to prioritize their response to the simultaneous occurrence of these stresses. For instance, it is a common observation that severe outbreaks of bacterial disease in the field are often associated with periods of heavy rain or high air humidity (Goode and Sasser, 1980). Mechanical wounding of plant tissues by rain might be one way that allows pathogens to bypass the stomatal route and gain unprecedented access to the plant interior. Additionally, the formation of large bacterial aggregates under high humidity on the leaf surface...
(Monier and Lindow, 2004) and splashing of bacteria during rain may also contribute to the spreading of disease at a higher rate. Interestingly, to ensure infection in the laboratory, researchers commonly expose plants to very high humidity for an extended period after surface inoculation. Here, we demonstrate that high RH compromises stomatal defense in Arabidopsis and common bean against *P. syringae* allowing more bacteria to enter the leaf tissue and contributing to severe infections. Compromised bacterial-triggered stomatal closure due to high RH is accompanied by changes in plant hormone signaling in Arabidopsis. Specifically, high RH leads to activation of the jasmonic acid (JA) signaling pathway and down-regulation of the salicylic acid (SA) signaling in guard cells. These results connect plant physiology with epidemiology and advance the current understanding of foliar bacterial infection in plants.
RESULTS

Bacterium-triggered stomatal closure is compromised under high relative humidity

To assess the effect of RH on bacterium-induced stomatal closure, wild-type Arabidopsis Col-0 plants were surface-inoculated with Pst DC3000 and its COR-defective mutant Pst DC3118 and incubated at different RH conditions. Bacterium-treated leaves incubated at 60% RH showed a significant (p < 0.001) decrease in stomatal aperture width when compared with the control, mock-treated leaves at 1 h post-inoculation (Fig. 1A). As previously reported (Melotto et al. 2006), stomatal aperture re-opened in response to Pst DC3000, but not to Pst DC3118, at 4 h post-inoculation (Fig. 1A). With the increase of RH to 95%, bacterium-triggered stomatal closure in intact leaves was abolished in response to both bacteria as early as 1 h post inoculation (Fig. 1A). Although it seems that stomatal opening is more pronounced in leaves inoculated with Pst DC3000 at 1 h under high humidity as compared to the mock-inoculated leaves, no statistical significance between these means was observed (ANOVA, p<0.05). These results indicate that stomatal immunity against P. syringae is not effective under high RH condition.

Syringolin A produced by Pss B728a has been described as a virulence factor that facilitates bacterial penetration into its host, the common bean (Phaseolus vulgaris). Furthermore, the syringolin-producing wild type bacterium does not induce stomatal closure (Schellenberg et al., 2010). We, therefore, assessed the effect of RH on stomatal defense in this pathosystem. Similar to what we have observed with Arabidopsis and Pst DC3118, bean seedlings (genotype G2333) infected with the syringolin A-deficient mutant Pss syl failed to close stomata only under high RH (Fig. 1B). As expected, the wild type Pss B728a did not close bean stomata regardless of the air humidity level (Fig. 1B).

Next, we tested whether the lack of stomatal closure correlated with higher levels of Pst DC3118 bacterial population in the Arabidopsis apoplast. Pst DC3118 population in the apoplast of surface-infected leaves was significantly higher (~20 fold, p = 0.02) in plants under >95% RH than under 60% RH on day 1 (Fig. 1C). High humidity seems to make COR production unnecessary for bacterial penetration into leaves as only under >95% RH does the COR-deficient mutant reaches an apoplastic population similar (no statistical significance observed) to that of wild type Pst DC3000 within 24 h after surface-inoculation (Fig. 1C). Plants infected with Pst DC3118 at 60% RH were virtually symptomless throughout the duration of the experiment (three days), similar to mock-inoculated control plants. However, plants infected under >95% RH showed disease symptoms (necrosis and mild chlorosis) in their leaves in response to the two bacteria at three days post-inoculation (Fig. 1D), which correlated with high RH.
bacterial titers in the apoplast (Fig. 1C, day 3). When \textit{Pst} DC3118 was directly infiltrated into the plant apoplast, bypassing the penetration step of the infection, RH had no effect on bacterial population counts at one day post-inoculation (Fig. 1E). Furthermore, apoplastic populations of \textit{Pst} DC3118 and \textit{Pst} DC3000 were very similar under both RH levels, as no statistical
significance was observed among all samples (Fig. 1E). It is important to note that bacterium-infiltrated leaves under continuous high RH show extensive water soaking at day 2 and leaves virtually melt after this point; thus bacterial population counts could not be taken at later time points (data not shown). These results suggest that the difference in the *Pst* DC3118 population reported in Fig. 1C is mainly due to differential ability of the bacteria to penetrate the leaf under varying RH and the penetration defect of *Pst* DC3118 can be compensated by high RH. Altogether, these findings suggest that high RH promotes disease at least in part by interfering with stomatal-based defense, which may be a common phenomenon in plant associations with bacterial phytopathogens.

High humidity activates JA signaling pathway in guard cells

Previously, we have found that stomatal closure can be interfered by the phytotoxin coronatine, a potent molecular mimic of the plant hormone JA-Ile, which induces JA signaling in plant cells (Weiler et al., 1994; Zhao et al., 2003; Melotto et al., 2006; Sheard et al., 2010). We reasoned that stomatal opening caused by high humidity could also involve components of the JA signaling pathway. Because plant response to wounding and touch is correlated with increased JA level (Chung et al., 2008; Chehab et al., 2012), plants were not moved or touched during the experimentation time and high humidity was achieved by covering plants with humidity domes pre-sprayed with sterile water. First, we assessed the expression of early JA-response genes (*JAZ1*, *JAZ8*, and *JAZ10*; Chung et al., 2008) in guard cells after exposing plants to high RH (>95%) for up to 1 h. Later time points were not included because the natural effects of the circadian rhythm on guard cells added an undesirable variable to the experimental set up. These *JAZ* genes were previously reported to be expressed in guard cells by direct RNA sequencing (Obulareddy et al., 2013) and microarray analysis (Wang et al., 2011). We observed that all three *JAZ* genes were rapidly induced (15 min to 1 h) by high RH when compared to plants under 60% RH (Fig. 2). The expression of the *LOX3* gene (Caldelari et al., 2011), which is associated with JA biosynthesis, is significantly induced 15 min after plants were exposed to high RH followed by significant repression at 1 h under high RH (Fig. 2). The *OPR3* gene (Stintzi and Browse, 2000), also involved in JA biosynthesis, was repressed in guard cells exposed to high RH (Fig. 2). The repression of *LOX3* and *OPR3* may be due to a fast negative feedback loop at the level of JA biosynthesis. These results indicate that JA signaling and JA biosynthesis are modulated by high RH, thus contributing to opening of stomata. This early and fast response correlates well with the rapid change in guard cell turgidity in response to external stimuli.
Figure 2. Regulation of JA signaling, biosynthesis by relative humidity (RH) in guard cells. The graphs show relative expression in logarithmic scale of the indicated genes 15 and 60 min after placing plants under >95% RH as compared to plants under 60% RH (time 0 min set as value 1). Data points are average of two biological replicates and 3 technical replicates (n=6) ± SE. The asterisks above the bars indicate statistical significance in comparison to the 0 h time point as calculated with two-tailed Student’s t-test (* = p <0.05, ** = p <0.01, *** = p <0.001). Significant means above 1 are considered up-regulation and significant means below 1 are considered down-regulation.

COI1 is required for full opening of stomata, but not for stomatal response to relative humidity
As high RH induced stomatal opening (Fig. 1A white bars) and JA signaling in guard cells (Fig. 2), we sought to determine whether JA-Ile perception through COI1 was required for humidity-dependent stomatal opening. After plants were exposed to treatments, stomatal aperture width measurements were obtained directly from intact leaves without further processing of leaf samples to avoid unanticipated responses of mutant plants towards common buffers used to maintain healthy epidermal peels. Similar to the wild type plant, coi1-1 mutant showed significantly wider stomatal aperture width under >95% RH than 60% RH (Fig. 3A). However, coi1-1 stomata do not fully open as compared to the stomatal apertures of Col-0 plants under either RH levels (Fig. 3A, white bars). We further confirmed that coi1-1 plants have constitutively smaller stomatal pores by measuring stomatal aperture width in leaves of mutant and wild type plants without any treatment (Fig. 3B). As this difference could be attributed to some defect in guard cell morphology, the width of the stomatal complex, guard cell pair size, length of the stomatal complex, size of the stomatal complex, and stomatal density, were determined in both Col-0 and coi1-1 plants under normal conditions (Fig. S1). All measurements were similar between coi1-1 and Col-0, except stomatal aperture which was significantly smaller in coi1-1 plants (Fig. 3B) and is reflected by the narrower stomatal width and smaller stomatal complex size (Fig. S1).

Previously, we have determined that Col-0 and coi1 stomata close to the same extent in response to bacteria (Melotto et al. 2006). Thus, we tested whether compromised bacterial-induced stomatal closure under high RH requires COI1. Similar to the wild type, coi1-1 plants do not close stomata in response to Pst DC3118 at >95% RH (Fig. 3A). Taken altogether, these results suggest that JA-Ile perception is not required for high RH induced stomatal opening, although COI1 is required for full opening of the stomatal pore. Furthermore, stomatal closure and high humidity-dependent repression of stomatal defense are not dependent on COI1.

**JA biosynthesis and signaling genes are up-regulated by high RH in whole leaves**

To determine whether high RH also regulates the JA pathway in other cell types in the leaf, we measured the expression of JA responsive genes in whole leaf tissue of plants exposed to moderate or high RH. We observed that high RH induces the two genes involved in JA biosynthesis, LOX3 and OPR3, as early as 15 min after exposure to >95% RH and reaches the highest level (approximately 10 fold) at 1 h (Fig. 4). The expression levels of these two genes returned to basal level at 4 h and were significantly below the basal level at 8 h under high RH (Fig. 4). The rapid induction of these genes were completely dependent on the presence of COI1, however repression of LOX3 was still detected in coi1-1 mutant plants (Fig. 4) confirming
Figure 3. COI1 is required for full opening of stomata, but not for stomatal response to relative humidity (RH). A, Stomatal aperture width was measured in intact leaves of Col-0 and coi1-1 plants 4 h after dip-inoculation into bacterial suspensions (10^6 CFU.ml⁻¹) of Pst DC3118 (cor mutant) or water control (mock inoculation). Results are shown as the mean (n=60) ± SE. Different letters above the bars indicate statistical significance (p < 0.05) calculated with ANOVA. B, The graph shows stomatal aperture width in untreated intact leaves of Col-0 and coi1-1. The asterisks above the coi1-1 bars indicate statistical significance (p < 0.001) in comparison to Col-0 as calculated with two-tailed Student’s t-test.

We also observed that the expression levels of JAZ1, JAZ8, and JAZ10 were significantly induced by exposing plants to high RH and the induction was partially compromised in coi1-1 mutant plants (Fig. 4). These findings indicate the existence of COI1-dependent and independent pathways for up-regulation of JAZ genes under high RH. Similar to LOX3, JAZ1 and JAZ10 were repressed 8 h after exposure to high RH in both Col-0 and coi1-1 plants. Initially, there were 12 JAZ genes annotated in the Arabidopsis genome that were identified by homology of their encoded protein sequence (Chini et al., 2007; Thines et al., 2007). All JAZ genes seem to be regulated by relative humidity. However, the kinetics of expression of each JAZ gene differed in response to high air humidity (Fig. S2). All JAZ genes, except JAZ8 and JAZ9, were repressed after 8 h of exposure to >95% RH regardless whether they were induced
High humidity suppresses salicylic acid responsive genes in guard cells

As salicylic acid (SA) signaling is required for stomatal and apoplastic defenses against *P. syringae* *pv. tomato* (Kloek et al., 2001; Melotto et al., 2006; Zeng et al., 2011), we reasoned
that one mechanism by which high relative humidity compromises stomatal closure in response to bacteria is by suppressing SA-associated responses. We first determined whether high RH represses SA production to open stomata. In a dose-response experiment, we observed that exogenous application of SA failed to close (up to 10 µM) or partially closed (100 µM) the stomatal pore under high RH (Fig. 5A). This result suggests that repression of SA production may be partially required for high humidity to open the stomatal pore and/or high RH blocks SA action at a step downstream of biosynthesis. We then compared the expression of hallmark SA response genes, PR1 (Laird et al., 2004) and PR2 (Uknes et al., 1992), in guard cells of plants exposed to 60% and >95% RH. PR1, but not PR2, was strongly repressed in guard cells within 15 min after increasing the air RH to >95% (Fig. 5B).

Because SA-responsive genes can also be up-regulated by biotic stress such as bacterial infection (Uknes et al., 1992; Song et al., 2011), we assessed whether P. syringae could...
Similarly induce these genes in guard cells and the effect of RH on their expression. We used the coronatine-defective mutant *Pst* DC3118 for this experiment as: 1) coronatine produced by the wild type *Pst* DC3000 can repress SA-associated plant defenses (Zhao et al., 2003; Zheng et al., 2012) including SA-triggered stomatal closure (Fig. 6A), 2) JA and SA signaling pathways similarly induce these genes in guard cells and the effect of RH on their expression. We used the coronatine-defective mutant *Pst* DC3118 for this experiment as: 1) coronatine produced by the wild type *Pst* DC3000 can repress SA-associated plant defenses (Zhao et al., 2003; Zheng et al., 2012) including SA-triggered stomatal closure (Fig. 6A), 2) JA and SA signaling pathways...
may antagonize each other (Kloek et al., 2001; Van der Does et al., 2013), and 3) *Pst* DC3118
induces expression of *PR* genes 5-25 fold higher than that of *Pst* DC3000 in guard cells at 1h
after inoculation (Fig. 6B). We observed that expression of *PR1* and *PR2* genes in guard cells of
*Pst* DC3118-inoculated plants under >95% RH for 1 h is significantly reduced to approximately
10% of the expression level observed in inoculated plants kept under 60% RH (Fig. 6C). Taken
together, these results suggest that SA-related responses are also active in guard cells and high
RH represses this response even in the presence of a strong stimulus such as inoculation with a
coronatine-deficient strain of *P. syringae*. Furthermore, the fast negative regulation of SA
signaling in guard cells (< 1 h) coincides with the lack of bacterium-triggered stomatal closure
under high RH (also less than 1 h; Fig. 1).

High RH represses bacterium induction of SA responses in whole leaves

We further analyzed *PR* gene expression in whole leaves and determined the effect of
humidity on SA-response to biotic stress. Unlike in guard cells (Fig. 6B), induction of *PR* genes
by *Pst* DC3118 was not observed at 1 h after inoculation in whole leaves of plants kept at 60%
RH (data not shown). Thus, we further incubated the inoculated plants at 60% RH for 8 h and
confirmed that *Pst* DC3118 significantly (p <0.001) induced both *PR-1* and *PR-2* expression as
compared to the mock-inoculated Col-0 and *coi1-1* plants (Fig. 7). Under high RH (>95%)
however, *PR1* and *PR2* induction by *Pst* DC3118 was significantly reduced as compared to their
expression levels in both Col-0 and *coi1-1* plants inoculated at 60% RH (Fig. 7). Low expression
level of these genes continued until 24 h post-inoculation. These results support the notion that
high RH also suppresses the defense-associated SA signaling in whole leaves allowing for
increased plant susceptibility to a weak pathogen such as *Pst* DC3118 (Fig. 1D). Furthermore,
our results also suggest that activation of SA signaling by bacteria and its repression by RH
does not require activation of JA pathway through COI1.
DISCUSSION

Environmental factors greatly influence the outcome of plant-pathogen interactions and can favor either partner in the interaction when they come into contact. In this study, we addressed the consequence of high relative humidity on the effectiveness of stomatal-based defense and

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**Figure 7.** High RH represses bacterium induction of SA responses in whole leaves. Graphs show relative expression of the PR-1 and PR-2 genes in Col-0 and coi1-1 plants 8 and 24 h after dip-inoculation with Pst DC3000 or mock control under 60% or 95% RH. Relative gene expression was calculated based on the expression levels of the ACT8 gene. Data points are average (n=3) ± SE and the letters above the bars indicate statistical significance (ANOVA, p<0.05) among the means within each time point (lower case and capital letters indicate differences within 8h and 24h, respectively).
the involvement of hormone signaling pathways in the process. We showed that bacterium-triggered stomatal closure is completely abolished under >95% RH, favoring leaf internalization and high apoplastic colonization by bacterial strains that cannot actively subvert stomatal immunity, such as the COR-deficient Pst DC3118 and the syringolin A-deficient Pss B728a. Thus, severe outbreaks of bacterial disease observed in periods of high RH in the rainy season may also be due to compromised stomatal closure.

Previously, we have determined that COR is responsible for opening PAMP-closed stomata (Melotto et al., 2006). COR shares the COI1 receptor with JA-Ile (Sheard et al., 2010), activates JA signaling pathway (Zhao et al., 2003; Brooks et al., 2005), and contributes to disease development (Kloek et al., 2001). Therefore, we reasoned that high RH might also induce JA signaling and promote plant susceptibility to COR-deficient bacteria. Indeed, a major observation in our study was that primary JA response genes (Chung et al., 2008) are up-regulated in both guard cells and whole leaves as early as 15 min after exposing plants to high RH (>95%). Both JA biosynthesis genes, LOX3 and OPR3, are induced in whole leaves, but only LOX3 is transiently induced while OPR3 is repressed in guard cells exposed to high relative humidity. Induction of JA biosynthesis genes occurs as a feedback loop to replenish JA-Ile binding to COI1. A COI1-independent mechanism for guard cell response to high RH was observed, indicating that replenishment of JA-Ile may not be required. Nonetheless, the fast repression of JA biosynthesis genes in guard cells (<1h) indicates the existence of a fine tuning of the response. These results also suggest that regulation of the JA pathway by RH is different between whole leaves and guard cells.

Only a subset of JAZ genes was induced by high humidity, similar to previous observations that not all JAZ genes are responsive to a particular stimulus, including Pst DC3000 inoculation (Demianski et al., 2012) and JA/wounding (Chung et al., 2008). Induction of the jasmonic acid pathway by high RH correlated well with increased stomatal aperture size and the high degree of susceptibility in plants inoculated with the low-virulence, COR-deficient pathogen Pst DC3118. These results suggest that high RH might compromise plant defenses to bacteria, at least in part, by the activation of the JA signaling pathway independent of COR. This conclusion is further supported by the fact that the receptor COI1 is not required for humidity-dependent suppression of stomatal closure (Fig. 3A). Furthermore, the smaller stomatal aperture width observed in coi1-1 plant compared to Col-0 plants (Fig. 3) may be due to impaired activation of JA signaling in this mutant (Fig. 4) and/or high accumulation of salicylic acid (Kloek et al., 2001; Miura et al., 2013).
Some studies have reported that methyl-JA induces stomatal closure (Suhita et al., 2004; Hossain et al., 2011). However, methyl-JA-induced stomatal closure could not be verified in other laboratories (Montillet et al., 2013) and the stomatal closure was dependent on the methyl-JA concentration used (Speth et al., 2009). Furthermore, the biologically active form of JA, that is JA-Ile (Wasternack and Xie, 2010), but not jasmonic acid, was shown to induce stomatal opening to the same extent to that of COR (Okada et al., 2009). In this study, we have demonstrated that JA-Ile perception is required for full opening of stomata, but not required for stomatal closure as the JA-Ile-insensitive coi1-1 mutant plants have constitutively narrow stomatal aperture width during the day time (Fig. 3B) and the stomatal pores in the coi1-1 mutant still close in response to bacterial infection to the extent of wild type plants (Fig. 3A). It is noteworthy to mention that stomatal closure and opening are not entirely overlapping processes (Yin et al., 2013). Furthermore, these results are consistent with previous studies showing that COR, the functional and structural mimic of JA-Ile, is responsible for opening of stomata (Melotto et al., 2006, Zhang et al., 2008). The extent to which stomatal behavior is regulated by other endogenous jasmonates such as methyl-JA and their receptors is still unclear.

Plants impaired in SA responses, such as the npr1 mutant and nahG transgenic plants, are deficient in bacterium-triggered stomatal closure and highly susceptible to P. syringae, indicating that SA plays a positive role in mediating stomatal and apoplastic defenses (Melotto et al., 2006; Zeng et al., 2011). It has also been observed that SA-dependent phenotypes are suppressed in plants grown under high RH (Yoshioka et al., 2001) and SA-dependent activation of PR genes is suppressed 24 h after shifting plants to high RH (Zhou et al., 2004). In this study, we observed suppression of PR1 gene expression also occurs in guard cells within 15 min after exposing plants to high humidity, suggesting direct regulation of this gene by high RH. This finding correlates well with the rapid change in stomatal aperture (30 min to 1 h) as well as JA signaling activation (15 min to 1 h). In addition, high RH prevented activation PR1 and PR2 genes expression by Pst DC3118 in guard cells as early as 1 h post inoculation (Fig. 6) suggesting that the SA-signaling pathway in response to biotic and abiotic stresses may overlap at the regulation of PR1, but not PR2 gene expression. In whole leaves, however, this response was observed only after 8 h post treatment and lasted at least 24 h (Fig. 7) similar to previously reported results (Zhao et al., 2003; Brooks et al., 2005). Thus, high RH seems to regulate SA signaling in both guard cells and in the entire leaf tissue; however, the kinetic of response differs (much faster response in guard cells as compared to whole leaves) indicating that SA signaling in guard cells can also occur independently of other cell types.
It is possible that the fast induction of *PR1* gene expression observed in guard cells (Fig. 6B) could be independent of SA accumulation. For instance, Moon et al. (2015) have observed the induction of the *PR1* gene by yeast extract in SA-deficient *nahG* plants. Furthermore, so far there is no evidence that EDS5, a transporter protein required for elevated SA (Chandran et al., 2014), normally accumulates in guard cells (Yamasaki et al., 2013) raising the possibility that SA may not be synthesized directly within these cells. On the other hand, the SA biosynthesis-related genes, *ICS1*, *EDS1*, and *PAD4* are induced in guard cells within 1 h of exposure to the immunity elicitor flg22 (Zheng et al., 2015) suggesting that fast SA accumulation could happen in guard cells. Due to technical impediments to quantify SA accumulation in this specialized cell type, it was not possible to distinguish between these alternatives in the present study.

We extended these findings in whole leaves and demonstrated that suppression of SA-dependent responses under high RH does not require activation of the JA pathway, as the *coi1* mutant is impaired in JA signaling (Fig. 4), but still showed wild-type patterns of SA-response to bacterium under both RH levels (Fig. 7). Collectively, these results support the idea that Arabidopsis immunity against *P. syringae* is decreased under high humidity conditions by early activation of JA signaling (<4 h) and subsequent inhibition of SA signaling (≥8 h). These two signaling pathways seem to be independent at the beginning of plant exposure to high humidity. Alternatively, if cross-talk between these pathways exists, it is downstream or independent of COI1, as observed by Van der Does et al. (2013), and/or it might occur after longer exposures to high RH. This might be another example in which the contribution of each hormone and timing of signaling determines the outcome of the plant-pathogen interaction (Gimenez-Ibanez and Solano, 2013).

It has been shown that JA signaling antagonizes SA signaling in the Arabidopsis-*P. syringae* pathosystem contributing to plant susceptibility (Kloek et al., 2001; Van der Does et al., 2013). It is possible that there is no cross-talk between JA and SA signaling in guard cells, as simultaneous induction of JA and repression of SA were observed. Hence, it can be proposed that high RH up-regulates a COI1-independent JA signaling pathway and represses SA signaling in guard cells, leading to stomatal opening. Previously, it has been shown that SA signaling functions upstream of ABA signaling to promote stomatal closure in response to *P. syringae* in Arabidopsis (Zeng and He, 2010). Thus, repression of SA signaling in the guard cell most likely will affect ABA signaling as well.

Historically, RH has been shown to regulate the abscisic acid (ABA) pathway affecting stomatal movement (Montillet and Hirt, 2013). It is possible that the lack of *P. syringae*-triggered stomatal closure under >95% RH (Fig. 1AB) is related to the fact that such high RH induces...
ABA catabolism in guard cells (Okamoto et al., 2009). By contrast, low RH (20%) induces ABA biosynthesis in guard cells leading to stomatal closure (Bauer et al., 2013a). Low RH or ABA treatment affects the expression of similar core genes indicating that ABA signaling and low RH may utilize overlapping pathways for stomatal closure (Bauer et al., 2013b). However, it is important to note that RH- and ABA-regulation of guard cells transcriptome is not entirely the same (Bauer et al., 2013b) suggesting ABA-dependent and ABA-independent mechanisms of stomatal movement controlled by RH (Okamoto et al., 2009). In addition, we have shown that high RH does not interfere with Escherichia coli O157:H7-induced stomatal closure (Roy et al., 2013) indicating that any reduction of ABA content by high RH in guard cells is not enough to abolish stomatal closure in response to all bacteria. Furthermore, our data also supports the idea that regulation of stomatal opening by high RH is not completely dependent on reducing the ABA pool in the cell (Fig. S3). Exogenous application of ABA to leaves exposed to >95% RH does not induce stomatal closure to the level of that in leaves exposed to 60% RH (Fig. S3). If high RH only works through ABA catabolism to open the stomatal pore (i.e., degrading the inducer of stomatal closure), exogenous application of ABA would cause reduction of stomatal aperture width to the extent of that in 60% RH. Although unlikely due to continuous exposure of the leaves to ABA, it could be that the high RH rapidly induces the degradation of the applied ABA diminishing the ABA effect on stomatal closure in our experiment. Even a very high concentration of ABA (100 μM) does not completely close the stomatal pore under high RH as compared to moderate RH. Thus, RH operates through additional mechanisms and pathways to control guard cell aperture size. Our results add another level of complexity to the multiple pathways that operate in guard cells exposed to variable environmental conditions. In addition, it has been suggested that various biotic and abiotic stress signals integrate with ROS and Ca\(^{2+}\) signaling, which in turn optimizes stomatal aperture sizes (Murata et al. 2015). Exactly, how all these pathways are integrated is yet-to-be elucidated.

**MATERIALS AND METHODS**

**Plant material and growth conditions**

*Arabidopsis thaliana* (L. Heyhn.) ecotype Columbia (Col-0, ABRC stock CS60000) seeds were sown in a 1:1:1 v:v:v mixture of growing medium (Redi-earth plug and seedling mix, Sun Gro), fine vermiculite, and perlite (Hummert International, Earth City, MO) and grown in controlled environmental chambers at 22\(^{\circ}\)C, 60±5% relative humidity (RH), and a 12-h photoperiod under light intensity of 100 μmol.m\(^{-2}\).s\(^{-1}\). Four- to five-week old plants were used for...
all experiments. Seeds of the *Phaseolus vulgaris* (L.) genotype G2333 were surface sterilized with 50% bleach (Ultra Clorox® Germicidal Bleach, VWR, West Chester, PA) for 4 min, sown onto Jiffy peat pots (Hummert International), maintained under 16 h photoperiod at 25°C at 60±5% RH, and light intensity of 140 μmol.m⁻².s⁻¹. Eight- to ten-day old seedlings with fully expanded primary leaves were used for the experiments. *coi1-1* mutant plants (Col-0 background) were selected according to procedures described by Kloek et al. (2001). Briefly, heterozygous seed stocks were screened on 25 μM MeJA-containing Murashige & Skoog (MS) medium with MES buffer and vitamins (RPI Corporation, Prospect, IL) supplemented with 30 g.l⁻¹ sucrose for root growth sensitivity assay. Homozygous *coi1-1* plants with extended root lengths were identified 6-days after germination on culture plates and later confirmed for male sterility. Both long roots and short roots (Col-0 wild type control) seedlings from the heterozygous population were transplanted to pots and maintained as described above.

**Bacterial strains and growth conditions**

Bacterial cells were grown in low-salt Luria-Bertani medium (Katagiri et al., 2002) at 30°C for all experiments. Medium was supplemented with the appropriated antibiotic: 100 μg.ml⁻¹ rifampicin (all *P. syringae* strains), 50 μg.ml⁻¹ kanamycin (*Pst* DC3118), and 10 μg.ml⁻¹ tetracycline (*Pss* B728a syringolin A-defective mutant *syl*).

**Stomatal assay**

For experiments to assess the effect of RH on stomatal closure, plants were acclimated under varying RH of 60±5% and >95% for 12 h inside a growth chamber equipped with humidity control (Percival H2X Two Atomizer Humidifiers). Shoots of plants from each RH condition were dip-inoculated in the morning (2-3 h after lights were turned on) as described below and leaves were collected over time for stomatal aperture measurements.

To assess the effect of pure chemicals (SA, ABA, COR obtained from Sigma) on stomatal movement, plants were kept under light for at least 3 h in the morning, leaves were excised and floated (abaxial side touching the solution) on chemicals or on water as a control, and maintained under light and indicated air RH for the duration of the experiment. Stomatal assays with intact leaves were performed as previously described (Chitrakar and Melotto, 2010), except that leaves were directly imaged without propidium iodide staining. Stomatal aperture width was measured with a Nikon Eclipse 80i fluorescent microscope equipped with DIC and long distance objectives (Nikon Corporations, Shinagawa-ku, Tokyo, Japan) to avoid the use cover slip. All experiments were completed by 2 pm.
Plant inoculation

Prior to inoculation, all plants were acclimated for 12 h under 60±5% or >95% RH at 25°C; these conditions were maintained for the duration of the experiment. Highly humid conditions (>95% RH) were obtained by keeping well-watered plants covered with plastic domes in controlled environmental chambers. The level of humidity was monitored with a digital hygrometer (Traceable®, VWR).

Plants were surface-inoculated by dipping the shoots into 1 x 10^8 CFU.ml^{-1} bacterial suspension or water containing 0.02% Silwet. Alternatively, plants were vacuum-infiltrated with 1 x 10^6 CFU.ml^{-1} bacterial suspension or water containing 0.008% Silwet. Apoplastic bacterial population count was assessed by the serial dilution method as previously described (Katagiri et al., 2002; Sabaratnam and Beattie, 2003). To ensure removal of leaf surface bacteria, each leaf was surface sterilized with 70% ethanol for 2 min followed by a rinsing step with water.

Tissue sampling for gene expression analysis

Arabidopsis plants were acclimated under 60±5% RH at 25°C for 16 h starting at 6-7 pm. Each experiment started between 10-11 am to be consistent with the guard cell circadian rhythm. The photoperiod was the same as the one used for growing plants. To check the effect of high humidity on JA- and SA-responsive gene expression, plants were covered with a clear plastic humidity dome covered with a fine mist on the inside so that a 95±5% RH was immediately reached. Plants were not moved or disturbed during changes of RH. Plants from different pots were used to collect leaf tissue at different time points to avoid induction of genes by touching or movement.

For whole leaf gene expression, leaves were flash frozen in liquid nitrogen at different time points. It is estimated that only 15% of the total leaf mRNA derives from epidermal tissue (Endo et al., 2014) and the guard cells represent 21-26% of all cells in the epidermis (Casson et al., 2009). Thus, guard cells are a significantly small fraction of all cells in a leaf and assessing gene expression in this organ represents the transcriptional profile of a sample enriched by mesophyll cells (77%) (Endo et al., 2014). For guard cell gene expression, 50-75 leaves were harvested at different time points and blended in water containing the transcription inhibitors, cordycepin (0.01%) and actinomycin D (0.0033%) to avoid gene expression changes due to mechanical damage. Guard cell protoplasts were isolated and flash frozen for RNA extraction using the fast protocol according to Obulareddy et al. (2013). This procedure allows for the isolation of highly...
pure guard cell samples (>97%) within 1.5 h and preserves the transcriptome of the guard cells during preparation (Obulareddy et al., 2013).

To assess the effect of RH on bacterium-induced expression of SA-responsive genes, plants were dip-inoculated in bacterium suspension or mock inoculated and then distributed in two different humidity conditions, 60±5% and 95±5% RH. Leaf tissue was collected at different time points and flash frozen for RNA extraction or used for guard cell extraction as described above.

Gene expression analysis

Total RNA was extracted from rosette leaves or guard cell protoplasts using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA) and quantified using a NanoDrop spectrophotometer (Thermo Scientific, Rockford, IL). Total RNA (1 µg) was synthesized into cDNA using the Takara RNA PCR kit (AMV) (Clontech, Montain View, CA). Quantitative PCR (qPCR) reaction (20 µL) was performed with 10 µL of iTaq Fast SYBR Green Supermix (BioRad, Hercules, CA), 2 µL of cDNA template from the reverse transcriptase reaction described above, and 200 nM of reverse and forward gene-specific primers. Reactions were carried out in an Applied Biosystems 7300 thermocycler (Applied Biosystems, Foster City, CA) using the following cycling parameter: 1 cycle 95°C for 5 min and 40 cycles of 95°C for 10 sec and 58°C for 30 sec. Gene expression levels were normalized based on the expression of the housekeeping gene ACT8 and fold change expression relative to the control was calculated using the ΔΔCt method (Livak and Schmittgen, 2001). The 2^{-ΔΔCt} method (Livak and Schmittgen, 2001) was used when gene expression in two plant genotypes (Col-0 and coi1-1) was directly compared (Fig. 7). In this case, changes in the expression of the target gene were calculated relative to the expression of the ACT8 gene. Baseline expression of ACT8 gene was the same in both Col-0 and coi1-1 genotypes. Two biological replicates with three technical replicates were performed.

Gene-specific primer sets that span an intron region were designed using the primer quest software from IDT-SciTools (http://www.idtdna.com/Primerquest/Home/Index) for qPCR analysis. To assess reaction efficiencies, standard curves were created using a five-fold serial dilution of the cDNA pool. A linear regression between the amount of cDNA template and the cycle threshold (C_T) value was calculated to obtain a correlation coefficient (R^2) >0.97. The PCR efficiency was determined according to Schmittgen and Livak (2008). All gene-specific primers are described in the Table S1.
Statistical analysis

Statistical significance of the results was calculated using ANOVA followed by Tukey-Kramer HSD at a 95% confidence limit (InfoStat version 2012) or 2-tailed Student’s t-test (Microsoft Office Excel version 2010). All experiments reported here were repeated at least two times with similar results.

LIST OF SUPPLEMENTAL MATERIALS

Table S1. Sequence of primers used to detect transcript of hormone responsive genes.

Figure S1. Measurements of stomatal complex in Col-0 and coi1-1 intact, non-treated leaves 3 h after lights were turned on in the morning.

Figure S2. JAZ genes are regulated by air relative humidity (RH) levels.

Figure S3. Addition of exogenous abscisic acid is not enough to completely close the stomatal pore under high relative humidity (RH).

Figure S1. Measurements of stomatal complex in Col-0 and coi1-1 intact, non-treated leaves 3 h after lights were turned on in the morning. A, The diagram represents the measurements taken from stoma-forming guard cells. B, Stomatal density. C, Guard cell pair width measured based on stomatal complex width minus the stomatal pore width. D, Stomatal length. E, Width of the stomatal complex. F, Stomatal complex size calculated by multiplying the complex width by the length. Results are shown as means (n≥60) ± SE and the asterisks above the coi1-1 bars indicate statistical significance in comparison to Col-0 as calculated with two-tailed Student’s t-test (** = p <0.01). Note that all measurements taken were similar between coi1-1 and Col-0, except that stomatal aperture was significant smaller in coi1-1 plants (Fig. 3B) which reflected on narrower stomatal width (E) and smaller stomatal complex size (F).

Figure S2. JAZ genes are regulated by air relative humidity (RH) levels. Data points indicate the relative expression of indicated genes after placing Col-0 plants under >95% RH (grey-shaded
bars) as compared to plants kept at 60% RH (white bar time 0 h set as value 1). Results are shown as average (n=3) with standard error (SE) bars. The asterisks above the bars indicate statistical significance in comparison to the 0 h time point as calculated with two-tailed Student's t-test (* = p < 0.05, ** = p < 0.01, *** = p < 0.001). Significant means above 1 are considered up-regulation and significant means below 1 are considered down-regulation. Note that error bars for some data points are very small and do not appear in the graph. Two biological replicates were performed with similar results.

Figure S3. Addition of exogenous abscisic acid is not enough to completely close the stomatal pore under high relative humidity (RH). Stomatal aperture width in intact leaves of Col-0 2 h after exposure to the varying concentrations of abscisic acid. Data points are average (n>60) ± SE and different letters above the bars indicate statistical significance (p<0.05) calculated with ANOVA.

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FIGURE LEGENDS

Figure 1. Bacterium-triggered stomatal closure is compromised under high relative humidity (RH). A, Arabidopsis Col-0 plants were dipped into bacterial suspensions (1x10^8 CFU.ml^-1) of bacteria or water control (mock inoculation) and stomatal aperture width was measured 1 h and 4 h post inoculation. Results are shown as the mean (n=60) ± SE. B, Bean plants were dipped into bacterial suspensions of Pss B728a, its syl- (syringolin A) mutant, or water control (mock inoculation) and stomatal aperture width was measured 1 h post inoculation. Results are shown as the mean (n=60) ± SE. C, Bacterial population in the apoplast of surface-inoculated plants under 60% (white bars) and >95% RH (grey bars). D, Disease symptoms observed in Col-0 plants three days after dip-inoculation with Pst DC3118 or Pst DC3000 under two RH levels. Note that some necrosis and yellowing on the leaves appeared only on leaves inoculated with
Pst DC3118 kept at >95% RH and symptoms become severe with virulent pathogen *Pst* DC3000 at >95% RH. E, Bacterial population in the apoplast of vacuum-infiltrated plants under 60% (white bars) and >95% RH (grey bars). Different letter on the top of each bar of all graphs indicates significant statistical difference between the means calculated with ANOVA (p<0.05).

**Figure 2.** JA biosynthesis and signaling are regulated by relative humidity (RH) in guard cells. The graphs show relative gene expression in logarithmic scale of the indicated genes 15 and 60 min after placing plants under >95% RH as compared to plants under 60% RH (time 0 h set as value 1). Data points are average of two biological replicates and 3 technical replicates (n=6) ± SE. The asterisks above the bars indicate statistical significance in comparison to the 0 h time point as calculated with two-tailed Student’s *t*-test (* = p <0.05, ** = p <0.01, *** = p <0.001). Significant means above 1 are considered up-regulation and significant means below 1 are considered down-regulation.

**Figure 3.** COI1 is required for full opening of stomata, but not for stomatal response to relative humidity (RH). A, Stomatal aperture width was measured in intact leaves of Col-0 and *coi1-1* plants 4 h after dip-inoculation into bacterial suspensions (10⁸ CFU·ml⁻¹) of *Pst* DC3118 (*cor* mutant) or water control (mock inoculation). Results are shown as the mean (n=60) ± SE. Different letters above the bars indicate statistical significance (p < 0.05) calculated with ANOVA. B, The graph shows stomatal aperture width in untreated, intact leaves of Col-0 and *coi1-1*. The asterisks above the *coi1-1* bars indicate statistical significance (p <0.001) in comparison to Col-0 as calculated with two-tailed Student’s *t*-test.

**Figure 4.** JA biosynthesis and signaling genes are up-regulated by high RH in whole leaves. The graphs show relative expression of the indicated genes in whole leaves after placing plants under >95% RH (grey-shaded bars) as compared to plants under 60% RH (white bars set as value 1). Data points are average (n=3) ± SE. The asterisks above the bars indicate statistical significance in comparison to the 0 h time point calculated with two-tailed Student’s *t*-test (* = p <0.05, ** = p <0.01, *** = p <0.001). Significant means above 1 are considered up-regulation and significant means below 1 are considered down-regulation. Note that error bars for some data points are very small and do not appear in the graph. Two biological replicates were performed with similar results.

**Figure 5.** High RH represses SA-responsive genes in guard cells. A, Stomatal aperture width in intact leaves 2 h after exposure to varying concentrations of SA. Data points are average (n>60) ± SE and different letters above the bars indicate statistical significance among the means (p<0.05) calculated with ANOVA. B, The graph shows relative expression of *PR1* and *PR2*.
genes after placing plants under >95% RH (grey-shaded bars) as compared to plants under 60% RH (white bar), which was set as 1. Data points are average (n=3) ± SE. The asterisks above the bars indicate statistical significance between the means represented by adjacent bars calculated with the Student’s *t*-test (** = p <0.001). Significant means above 1 are considered up-regulation and significant means below 1 are considered down-regulation.

**Figure 6.** High RH represses bacterium-induced SA-responsive genes in guard cells. **A,** Stomatal aperture width in intact leaves 2 h after exposure to the indicated chemicals. Data points are average (n>60) ± SE and different letters above the bars indicate statistical significance (p<0.05) calculated with ANOVA. **B,** Relative expression of the *PR1* and *PR2* genes in guard cells of Col-0 plants at 1 h after dip-inoculation with either *Pst* DC3000 or *Pst* DC3118 under 60% RH. Relative expression was calculated based on the expression levels of *Pst* DC3000-inoculated plants (white bars), which was considered 1. **C,** Relative expression of the *PR1* and *PR2* genes in guard cells of Col-0 plants at 1 h after dip-inoculation with *Pst* DC3118 under 60% or >95% RH. Relative expression was calculated based on the expression levels of plants kept under 60% RH (white bars), which was considered 1. For graphs showing gene expression (panels B and C), data points are average (n=3) ± SE. The asterisks above the bars indicate statistical significance between the means represented by adjacent bars calculated with the Student’s *t*-test (** = p <0.001). Significant means above 1 are considered up-regulation and significant means below 1 are considered down-regulation. Note that error bars for some data points are very small and do not appear in the graph.

**Figure 7.** High RH represses bacterium induction of SA responses in whole leaves. Graphs show relative expression of the *PR-1* and *PR-2* genes in Col-0 and *coi1-1* plants 8 and 24 h after dip-inoculation with *Pst* DC3118 or mock control under 60% or 95% RH. Relative gene expression was calculated based on the expression levels of the *ACT8* gene. Data points are average (n=3) ± SE and the letters above the bars indicate statistical significance (ANOVA, p<0.05) among the means within each time point (lower case and capital letters indicate differences within 8h and 24h, respectively).


McDonald KL, Cahill DM (1999) Evidence for a transmissible factor that causes rapid stomatal closure in soybean at sites adjacent to and remote from hypersensitive cell death induced by Phytophthora sojae. Physiol Mol Plant Pathol 55: 197-203.


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