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Research Report

Water immunity overrides stomatal immunity in plant resistance to *Pseudomonas syringae*

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Abstract

Stomata play crucial roles in the multilayered defense system against pathogens. Upon pathogen perception, stomata close promptly, establishing the first line of defense known as stomatal immunity. The bacterial pathogen *Pseudomonas syringae* (*Pst*) exploits open stomata for entry into its host. However, it can also induce stomatal closure at post-invasive stages to enhance apoplastic hydration, creating a favorable environment for *Pst* proliferation, evident as water-soaked lesions on leaves. During the post-invasive stages of *Pst* infection, plants reopen their stomata to promote apoplastic dehydration, establishing the second layer of stomatal defense termed water immunity. To evaluate the relative importance of stomatal versus water immunity, we utilized a diverse set of Arabidopsis (*Arabidopsis thaliana*) mutants with impaired stomatal function and monitored bacterial growth, stomatal behavior, and water-soaking capacity after *Pst* pv. *tomato* DC3000 infection. Most mutants with constitutively open stomata and disrupted stomatal closure were more resistant to *Pst* than wild-type plants. Also, while some mutants displayed similar stomatal behavior at the initial stages of defense, their disease outcomes were opposite, suggesting that stomatal immunity does not determine disease resistance. Instead, the water-soaking capacity, which is associated with stomatal status at later stages of infection (i.e. water immunity), dictates disease outcome. Our results show that enhanced water immunity can override the lack of stomatal immunity in plant resistance to *Pst*. We also address previous discrepancies in the literature showing contradicting results for pathogen growth on stomatal mutants, highlighting the challenges in dissecting stomatal effects on plant disease resistance.

Introduction

Stomata are pores that cover aerial plant parts and function as integral sites for gas exchange. Flanking guard cells open or close the pores in response to environmental cues, balancing CO_2 intake and water loss. Open stomata also serve as entry points for harmful organisms. Plants can restrict pathogen entry by rapidly closing stomata upon detecting pathogen-associated molecular patterns (PAMPs), a response known as stomatal immunity (Melotto et al. 2006). PAMP perception by receptors trigger signaling cascades involving intracellular kinases, calcium fluxes and reactive oxygen species burst to activate anion channels, leading to depolarization of guard cell membranes, and stomatal closure (Melotto et al. 2024). One of the most studied model pathogens that use stomata as entry point is *Pseudomonas syringae* pv. tomato DC3000 (*Pst*). Guard cells recognize *Pst* by its conserved flagellin peptide flg22, which is bound by the cell-membrane receptor complex formed by FLAGELLIN-SENSITIVE2 (FLS2), BRI1-ASSOCIATED KINASE1 (BAK1), and BOTRYTIS-INDUCED KINASE1 (BIK1). Upon activation, the signaling module initiates stomatal closure, resulting in stomatal immunity (Fig. 1A; Gómez-Gómez and Boller 2000; Chinchilla et al. 2007; Lu et al. 2010). *Pst* can further alter guard cell responses by secreting coronatine, a jasmonic acid (JA) mimic, to reopen stomata after the initial recognition to promote entry into the leaf tissues, thus bypassing stomatal defenses (Melotto et al. 2006).

Pseudomonas syringae can also induce stomatal closure at later stages of infection to form an aqueous phase inside the apoplastic spaces of the mesophyll (Xin et al. 2016). *Pst* uses type III effectors, HopM1 and AvrE1, to promote abscisic acid (ABA) signaling and stomatal closure (Hu et al. 2022; Roussin-Léveillée et al. 2022). *Pst* AvrE-family effectors can form water-permeable pores on plant cell membranes, increasing the hydration in mesophyll by releasing cell contents (Nomura et al. 2023). These factors contribute to hydration of the apoplast, observed as water-soaked lesions. To counter this post-invasive virulence mechanism, plants employ a secondary, temporally regulated defense strategy known as water immunity, also referred to as apoplastic immunity, to decrease apoplastic hydration (Jian et al. 2024; Hou et al. 2024). In water immunity, stomatal reopening is facilitated by plant-secreted peptides SMALL PHYTOCYTOKINES REGULATING DEFENSE AND WATER LOSS, as well as light-mediated salicylic acid (SA) signaling, both acting antagonistically to ABA signaling (Liu et al. 2022; Lajeunesse et al. 2023).

Early defense through stomatal immunity is considered crucial in plant defense against *Pst* and is thought to be an important determinant for disease outcomes. Studies on stomatal mutants show increased susceptibility to *Pst* due to disrupted stomatal immunity, leading to higher pathogen entry (Supplementary Table S1). However, recent research focusing on water immunity has shown that *Pst*-induced aqueous phase is prevented when stomata are open (Lajeunesse et al. 2023). Depending on the research angle, many articles, although studying the same Arabidopsis (*Arabidopsis thaliana*) genes required for stomatal regulation, show contradictory results regarding bacterial growth

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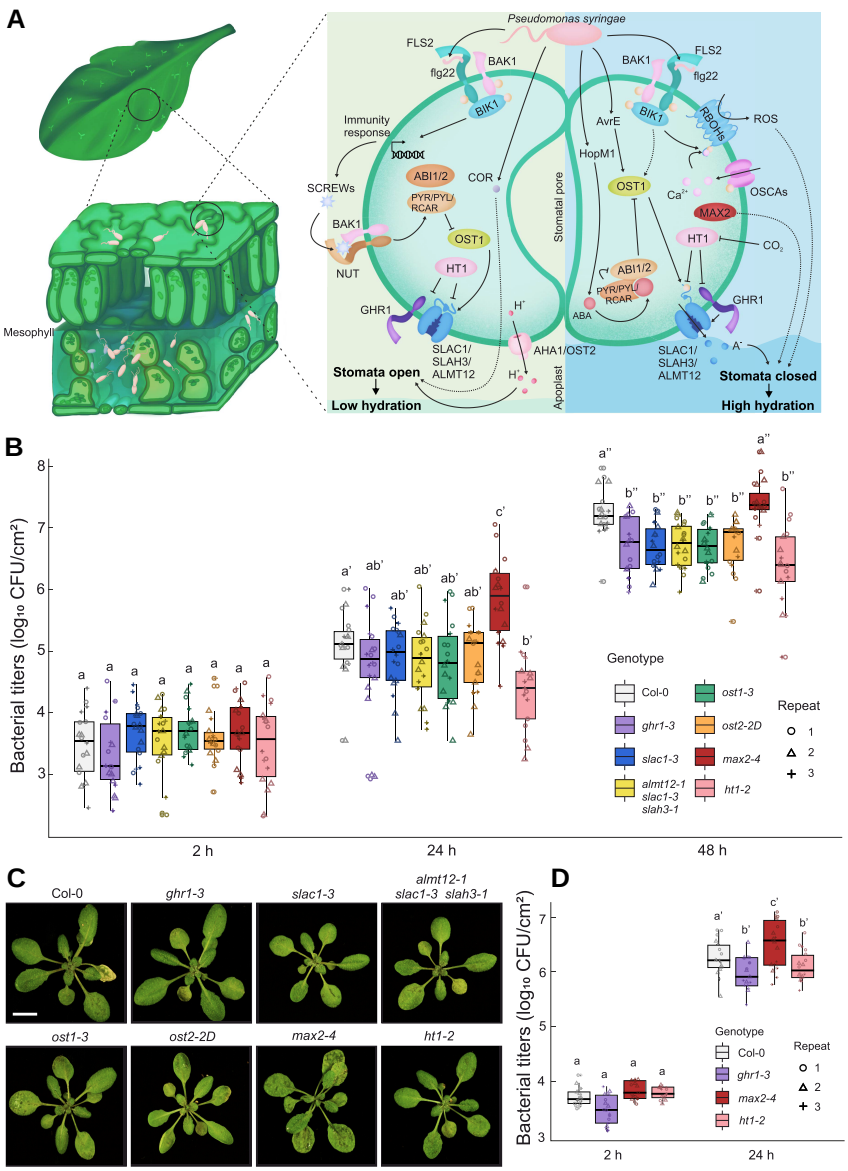

Figure 1. Signaling pathways that regulate stomatal movements during *Pst* infection and *Pst* growth in Arabidopsis mutants with stomatal dysfunction phenotypes. **A)** Apoplastic hydration in the mesophyll is crucial for *Pst* proliferation and is promoted by closed stomata. *Pst* enhances its virulence by manipulating guard cell signaling, initially promoting stomatal opening to facilitate entry during early infection (via COR and JA signaling) and later driving stomatal closure to induce water-soaking (via enhanced ABA signaling). Plants defend themselves by regulating stomatal aperture at appropriate times during infection; initial stomatal closure restricts *Pst* entry and reopening at post-invasive stages reduces apoplastic hydration. Stomatal opening involves suppression of closure pathways, leading to membrane hyperpolarization through proton pumping (AHA1/OST2) and activation of inward-rectifying potassium channels, driving osmotic flow into guard cells. Conversely, stomatal closure requires activation of SLAC1, SLAH3, and ALMT12 anion channels, leading to membrane depolarization and activation of outward-rectifying potassium channels, followed by water efflux from guard cells and stomatal closure. Numerous other components are required to process environmental and hormonal signals, and include ABA perception, kinases such as OST1, HT1, BIK1, and secondary messengers, including ROS and calcium fluxes. Other guard cell signaling components have less defined function and include the pseudokinase GHR1 and components of E3 ubiquitin ligase complexes, such as MAX2. For detailed description of the main components and pathways, see the main text. Solid lines indicate direct or experimentally validated interactions, whereas dashed lines represent indirect or hypothetical interactions. ABA, abscisic acid; ABI1/2, ABA-INSENSITIVE1/2; AHA1/OST2, AUTOINHIBITED H^+-ATPASE/OPEN STOMATA2; ALMT12, ALUMINUM-ACTIVATED MALATE TRANSPORTER12; BAK1, BRI1-ASSOCIATED KINASE1; BIK1, BOTRYTIS-INDUCED KINASE1; COR, coronatine; FLS2, FLAGELLIN-SENSITIVE2; GHR1, GUARD CELL HYDROGEN PEROXIDE-RESISTANT1; HT1, HIGH LEAF TEMPERATURE1; MAX2, MORE AXILLARY GROWTH2; NUT, PLANT SCREW UNRESPONSIVE RECEPTOR; OSCA, REDUCED HYPEROSMOLALITY-INDUCED Ca^{2+} INCREASE; OST1, OPEN STOMATA 1; PYR/PYL/RCAR, PYRABACTIN RESISTANCE1/PYR1-LIKE/REGULATORY COMPONENTS OF ABA RECEPTORS; RBOH, RESPIRATORY BURST OXIDASE HOMOLOG; ROS, reactive oxygen species; SCREW, SMALL PHYTOCYTOKINES REGULATING DEFENSE AND WATER LOSS; SLAC1, SLOW ANION CHANNEL1; SLAH3, SLAC1 HOMOLOGUE3. **B)** Bacterial titers in Arabidopsis mutants with stomatal dysfunction phenotypes. Plants were spray-infected with *Pst* ($OD_{600} = 0.2$ or 1×10^8 CFU/mL) and bacterial titers were quantified 2, 24, and 48 h post-infection. **C)** Visual symptoms of *Pst* spray-inoculated Arabidopsis rosettes 48 h post-infection. Scale bar = 10 mm for all images. **D)** *Pst* growth in Col-0, *ghr1-3*, *max2-4* and *ht1-2* after syringe infiltration. Three leaves from each plant were infiltrated with *Pst* ($OD_{600} = 0.02$ or 1×10^7 CFU/mL), and bacterial titers were quantified 2 and 24 h after infiltration. In **B)** and **D)**, colony-forming units (CFU/cm^2) were calculated from bacterial titers extracted from 3 leaves per plant. Box plots represent 3 independent repeats ($n = 18$ per genotype), shown with medians and interquartile ranges. Whiskers represent the range of data within 1.5 times the interquartile range from the first and third quartiles. Log-transformed CFU counts are displayed with different shapes indicating each repeat. A linear mixed-effects model followed by Tukey's post hoc test was applied to assess statistical differences within each time point (indicated by an apostrophe). Different letters indicate significant differences ($P < 0.05$).

(Supplementary Table S1). For example, OPEN STOMATA1 (OST1) mutant *ost1-2* shows heightened susceptibility after vacuum-infiltration (Melotto et al. 2006), whereas *ost1-3* exhibits wild type-like susceptibility after dip-inoculation (Jalakas et al. 2017; Ou et al. 2022). Furthermore, OPEN STOMATA2 (OST2) mutant *ost2-2d* was found to be more susceptible against *Pst* after spray-infection while exhibiting similar resistance as Col-0 after syringe-infiltration, which was attributed to the impaired stomatal function of *ost2-2d* (Zhou et al. 2015).

In the light of recent insights into water immunity and its effect on later stages of *Pst* colonization, we set out to examine which layer of immunity, stomatal immunity, or water immunity, is more important for plant defense against *Pst* infection. We used mutants with constitutively open stomata or with disruptions in stomatal closure, including mutants of anion channels SLOW ANION CHANNEL1 (SLAC1), SLAC1 HOMOLOG3 (SLAH3), and ALUMINUM-ACTIVATED MALATE TRANSPORTER12 (ALMT12) (*slac1-3* and the *almt12-1 slah3-1 slac1-3* triple mutant; Vahisalu et al. 2008; Jalakas et al. 2021), ABA signaling component OST1 (*ost1-3/srk2e*; Yoshida et al. 2002), receptor-like pseudokinase GUARD CELL HYDROGEN PEROXIDE-RESISTANT1 (GHR1; *ghr1-3*; Sierla et al. 2018), F-box protein MORE AXILLARY GROWTH2 (MAX2; *max2-4*; Piisilä et al. 2015) and a mutant with overly active H^+-ATPase OST2 and stomatal opening (*ost2-2D*; Merlot et al. 2007) (Fig. 1A; Supplementary Table S2). We also included a mutant of HIGH LEAF TEMPERATURE1 (HT1) with constitutively closed stomata (*ht1-2*; Hashimoto et al. 2006). We observed that despite allowing greater pathogen entry through more open stomata, most of the stomatal mutants showed enhanced resistance, suggesting that water immunity overrides impaired stomatal immunity.

Results

Most mutants with impaired stomatal function show resistance to *Pst*

As previous literature showed discrepancies in *Pst* growth on stomatal mutants (Supplementary Table S1), we selected a broad set of mutants with impaired stomatal responses (Supplementary Table S2) for *Pst* growth assays to test plant resistance. We sprayed 3.5-week-old Arabidopsis plants with *Pst* and followed bacterial growth and disease symptom development for 2 days (Fig. 1, B and C). All mutants exhibited less bacterial growth than Col-0 except for *max2-4*, which was highly susceptible to *Pst*.

The contribution of initial stomatal openness to bacterial infection can be estimated by comparing spray infection (where bacteria enter through stomata) with syringe infiltration (where bacteria are directly injected to the interior of the leaf). Syringe infiltration thus bypasses stomatal immunity and addresses the contribution of water immunity. For syringe infiltration, we used mutants representing the extreme phenotypes from spray infection, *ht1-2*, *max2-4*, and *ghr1-3* (Fig. 1D). We anticipated more bacterial growth in *ht1-2* than in the other mutants, as more closed stomata would allow higher apoplast hydration and water-soaking. However, after 24 h post infection (HPI), we observed approximately 2 times less bacteria in *ht1-2* and *ghr1-3* compared with Col-0, while *max2-4* exhibited increased bacterial growth.

Stomatal immunity is not the main determinant for the disease outcome

Successful *Pst* infection requires the bacteria to enter the plant through stomata and induce an aqueous phase inside mesophyll apoplast (Xin et al. 2016; Aung et al. 2018). Therefore, we assessed the stomatal responses after *Pst* treatment (Fig. 2, A and B). Stomatal aperture (width/height) of spray-inoculated plants was measured 1 h after infection. All mutants had increased apertures in mock treated plants compared with Col-0, except *ht1-2*, which showed decreased aperture. *Pst* induced distinctive closure of stomata in Col-0 and, interestingly, in *ost1-3*, whereas the other mutants showed only mild or no response. Stomata of *ost1-3* was previously shown to be unresponsive to flg22 treatment (Zheng et al. 2018), but have a wild type-like closure in response to SA and live *Pst* (Prodhan et al. 2018; Ou et al. 2022), suggesting that the initial pathogen-triggered stomatal closure is not dependent on OST1. These results suggest that impaired stomatal immunity cannot explain the disease outcome except for *max2-4*.

Water-soaking capacity dictates the disease outcome

To assess water immunity, we investigated the capacity of *Pst* to induce water-soaking (Fig. 2, C and D). We sprayed plants with *Pst* and measured the water-soaked areas in leaves 24 HPI and calculated the afflicted area as a percentage of the whole leaf area (Fig. 2D). The degree of water-soaking was elevated in *max2-4*, while all other mutants showed less water-soaking than Col-0, consistent with the bacterial counts (Fig. 1B). The disease outcome could thus be explained by water-soaking capacity.

We next evaluated the water-soaking capacity of the mutants independent of *Pst* to determine whether the observed water-soaking was a consequence or a contributing factor to disease outcome. ABA combined with high humidity was used as an alternative approach to induce water-soaking. ABA and high humidity, but also high humidity alone, induced elevated water-soaking in *max2-4* (Fig. 2, E and F). A trend of ABA-induced water-soaking was also apparent in Col-0 and *ht1-2*, but not in other mutants. This suggested that the water-soaking capacity, likely associated with stomatal status, was a contributing factor to disease outcome.

Pst infection first leads to stomatal closure followed by re-opening at later stages of invasion to establish water immunity. To test if the water-soaking capacity (Fig. 2, C and D) was associated with stomatal status at later stages of infection, we sprayed mutants representing the extreme phenotypes of water-soaking, *max2-4*, and *ghr1-3*, with *Pst* and measured stomatal aperture 24 HPI (Fig. 2, G and H). The stomata of *max2-4* showed prominent closure at 24 HPI compared with mock treatment, whereas *ghr1-3* stomata remained open. Thus, the susceptibility of *max2-4* against *Pst* could be due to the combination of higher bacterial entry at the first step of infection (lack of stomatal immunity) (Fig. 2B), followed by stomatal closure at later stages (lack of water immunity) (Fig. 2H). For *ghr1-3*, the resistance is likely due to enhanced water immunity that is overriding the lack of stomatal immunity.

Expression of immunity and abiotic stress marker genes are not altered in stomatal mutants

To test if mutants had preactivated defenses before bacterial infection, we used reverse transcription quantitative PCR (RT-qPCR) to test marker genes for SA (*ICS1- ISOCHORISMATE SYNTHASE1*, a key gene in SA biosynthesis), JA (*JAZ1—JASMONATE-ZIM-DOMAIN PROTEIN1*, involved in JA signaling, and *LOX4—LIPOXYGENASE4*, a component of JA biosynthesis), ABA (*NDEC3—NINE-CIS-EPOXYCAROTENOID DIOXYGENASE3*, an ABA biosynthesis gene), and abiotic stress

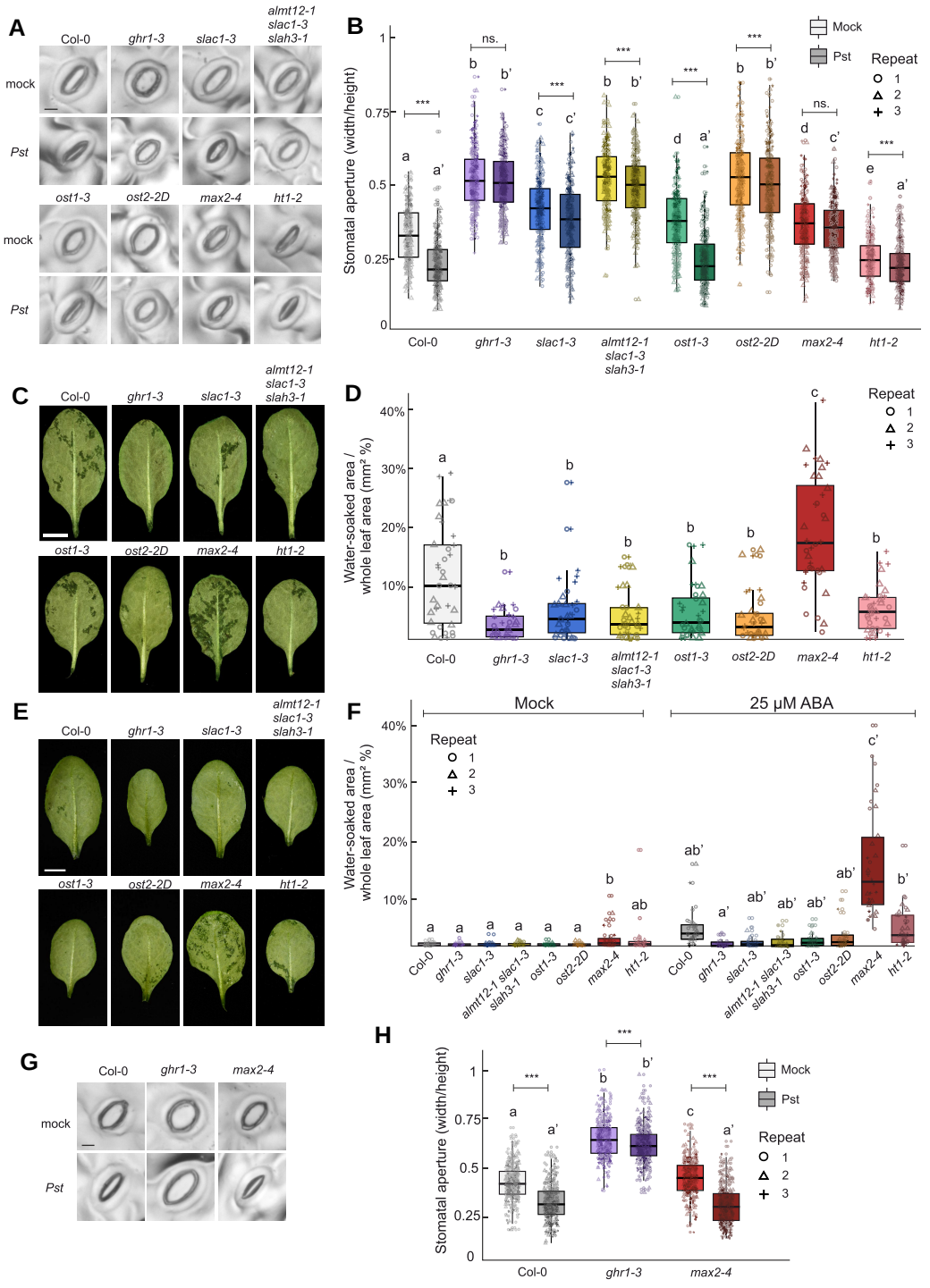

Figure 2. *Pseudomonas syringae*-induced stomatal closure and *Pst*- and ABA-induced water-soaking in stomatal mutants. **A)** and **B)** *Pst*-induced stomatal closure in experimental lines. Plants were sprayed with mock or *Pst* ($OD_{600} = 0.2$, 1×10^8 CFU/mL), and epidermal imprints were taken 1 h post-treatment. Representative images of stomata are shown **(A)**, scale bar = 10 µm for all images. Stomata from 3 plants per genotype across 3 independent experiments were analyzed ($n \approx 300$ stomata in total per genotype). **C)** and **D)** *Pst*-induced water-soaking in stomatal mutants. Plants were sprayed with *Pst*, and abaxial leaf sides were photographed 24 HPI. The water-soaked ratio (%) was determined by dividing the water-soaked area by the total leaf area. Representative images are shown **(C)**, scale bar = 5 mm for all images. Two leaves from 5 to 6 plants per genotype across 3 independent experiments were analyzed ($n = 36$ leaves in total per genotype). **E)** and **F)** ABA-induced water-soaking in stomatal mutants. Plants were treated with 25 µM ABA or mock solution, and abaxial leaf sides were photographed 24 h after treatment. Water-soaked area (%) was determined as above. Representative images of ABA-treated plants are shown **(E)**, scale bar = 5 mm for all images. **G)** and **H)** *Pst*-induced stomatal closure 24 HPI in Col-0, *ghr1*-3, and *max2*-4. Plants were sprayed with *Pst* as before, and stomatal imprints were taken 24 h post-infection. Representative images of stomata are shown **(G)**, scale bar = 10 µm for all images. Stomatal measurements ($n \approx 300$ stomata in total per genotype/treatment) were obtained from 2 leaves per plant, 4 plants per genotype across 3 independent experiments. In **B)**, **D)**, **F)**, and **H)**, box plots represent 3 independent repeats with medians, interquartile ranges, and whiskers showing 1.5× interquartile range. A linear mixed-effects model followed by Tukey's post hoc test was applied to evaluate statistical differences between genotypes. Different letters indicate significant differences ($P < 0.05$). In **B)** and **H)**, the statistical tests were conducted separately for mock and *Pst*-treatment (marked with apostrophe) groups. The effect of treatment was evaluated, with "***" indicating a significant difference ($P < 0.05$), and "ns." denoting nonsignificance.

(*P5CS1—DELTA1-PYRROLINE-5-CARBOXYLATE SYNTHASE1*, involved in catalyzing the rate limiting step in proline biosynthesis) (Fig. 3). Plants were grown as for *Pst* growth and stomatal experiments, and fully expanded leaves were used for RT-qPCR. No significant changes in gene expression were found apart from the JA marker *LOX4*, which had higher expression in *max2-4*. We conclude that the tolerance to *Pst* infection observed in *ht1-2*, *ghr1-3*, *ost2-2d*, *slac1-3*, *ost1-3* and *almt12-1 slac1-3 slah3-1* cannot be explained by these mutants having preactivated defenses.

Discussion

Susceptibility of stomatal mutants was long attributed to the disrupted stomatal immunity and enhanced pathogen entry (Supplementary Table S1). However, recent evidence indicates that enhancing transpiration and stomatal openness can improve plant resistance in both Arabidopsis and rice (Zhang et al. 2019; Liu et al. 2022; Lajeunesse et al. 2023). With the recent advances in understanding the dynamics of plant disease and stomatal regulation, we aimed to clarify the relative importance of stomatal versus water immunity responses for plant resistance. Our results suggest that despite disrupted initial stomatal immunity responses, most mutants with more open stomata displayed significantly greater resistance against *Pst* compared with Col-0, supporting recent findings (Roussin-Léveillée et al. 2022; Hu et al. 2022; Liu et al. 2022; Lajeunesse et al. 2023). This resistance was observed regardless of whether the dysfunction affects stomatal closing or opening; as mutants with disrupted closure, e.g. inactive SLAC1, as well as the mutant with constitutive opening due to hyperactive H^+-ATPase OST2, were all more resistant to *Pst* (Figs. 1 and 2). Our results suggest that stomatal opening at post-invasive stages is crucial for plant resistance in a more general manner than previously recognized and can counteract the lack of stomatal immunity (Fig. 4).

As a mutant with constitutively closed stomata, we hypothesized that *ht1-2* would be more susceptible due to easier formation of an aqueous phase. However, *ht1-2* was more resistant against *Pst* after both spray- and syringe-infiltration (Fig. 1, B and D). The mechanisms of *ht1-2* resistance are unclear, but our study indicates they are not directly related to its stomatal phenotype. Interestingly, *ht1-2* exhibited similar degree of water-soaking to Col-0 after ABA treatment despite the higher resistance against *Pst* (Fig. 2F). This suggests that the resistance against *Pst* in *ht1-2* could be due to other unexplored mechanisms, for example, mesophyll properties or heightened nontranscriptional immunity responses, as *ht1-2* didn't show preactivated defenses at the gene expression level (Fig. 3). Thus, regulatory mechanisms for apoplastic hydration beyond stomatal control are worth exploring in the future.

The *max2-4* mutant exhibited a distinct susceptibility to *Pst* compared with other lines (Fig. 1, B and C). Heightened susceptibility of *max2-4* was previously attributed to the lack of stomatal immunity (Piisilä et al. 2015). However, as the other open-stomata mutants exhibited heightened resistance, disrupted stomatal immunity cannot explain the susceptibility of *max2-4* alone. We propose that *max2-4* has impaired immunity at both steps of stomatal closure and reopening, allowing more *Pst* entry early on and heightened capacity to form higher water-soaking at later stages (Fig. 4). Notably, *max2-4* exhibits a wild type-like decrease in stomatal conductance following ABA treatment (Piisilä et al. 2015). We also observed stomatal closure 24 h after *Pst* infection (Fig. 2H), possibly due to the promoted ABA signaling by *Pst*

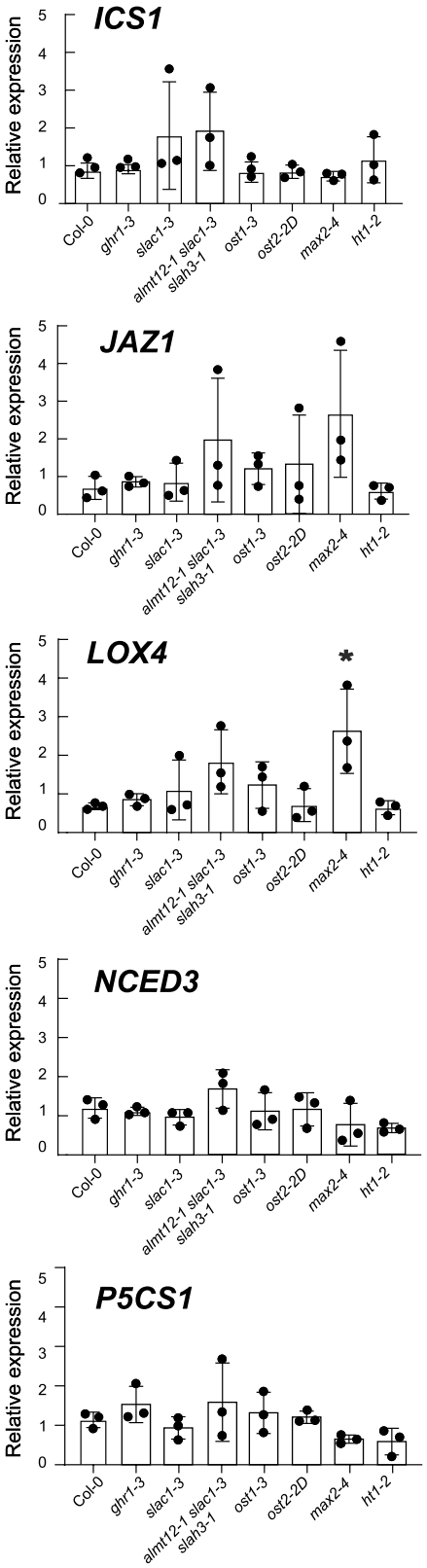

Figure 3. Expression of defense marker genes. Reverse transcription quantitative PCR (RT-qPCR) was used to assess the relative expression of *ICS1*, *JAZ1*, *LOX4*, *NCED3*, *P5CS1* in stomatal mutants and normalized against reference genes. Plants were grown under same conditions as for other experiments. Data represents 3 biological replicates, analyzed using one-way ANOVA followed by Tukey's multiple comparisons test. Error bars represent standard deviation, and an asterisk indicates a significant difference ($P < 0.05$).

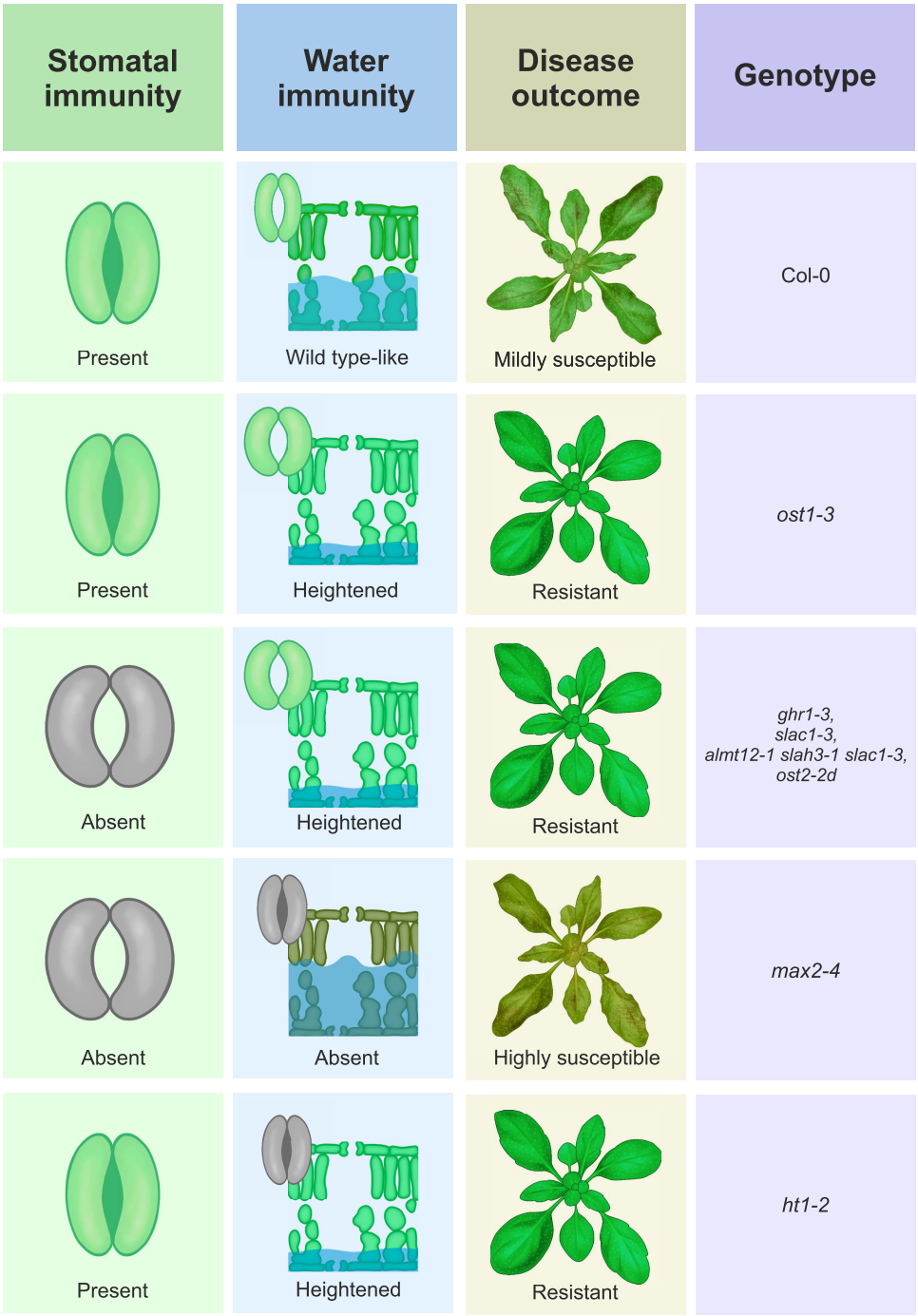

Figure 4. Model illustrating the relationship between temporal stomatal behavior, apoplastic hydration, and disease outcome. Stomatal immunity (stomatal closure in response to pathogens) and water immunity (stomatal reopening and low apoplastic hydration) are shown alongside disease outcomes for different genotypes. Impaired stomatal function is indicated with gray color, showing the absence of functional closure or reopening in response to *Pst* infection. Water level (in blue) indicates *Pst*-induced apoplastic hydration, measured as water-soaking symptoms. Water immunity is the main determinant of disease outcome and can override the absence of stomatal immunity.

(De Torres-Zabala et al. 2007; Hu et al. 2022; Roussin-Léveillée et al. 2022). Moreover, ABA alone induced a higher degree of water-soaking in *max2-4* compared with other lines (Fig. 2F). Increased water accumulation of *max2-4* could thus be due to sensitivity to ABA signaling and/or other, e.g. anatomical properties.

The mutants *ht1-2* and *max2-4* further highlight the importance of experimental design in pathogen growth assays. For example, if only spray infection was used or a single time point early in the infection, it could be possible to speculate from our results that the resistance of *ht1-2* is due to its closed-stomata phenotype, while the susceptibility of *max2-4* is related to its disrupted stomatal immunity responses. Our study underscores how the temporally varying nature of *Pst* pathogenesis can easily lead to misunderstandings, often due to the oversights in experimental design. Previously, the importance of stomatal immunity has been emphasized in literature, potentially leading to inaccurate conclusions about mutant susceptibility.

Accurate reporting of experimental design and methods is also crucial. For example, it is well known that maintaining high humidity is important for successful *Pst* pathogenesis, often

achieved by adding a sealed cover on infected plants (Katagiri et al. 2002). Indeed, elevated humidity levels are associated with increased *Pst* proliferation inside plant tissues (Xin et al. 2016). Furthermore, light also has a role in controlling plant immunity both on stomatal and whole-plant level; for example, it was shown that light can mitigate *Pst* infection possibly by enhancing SA signaling (Lajeunesse et al. 2023). However, increased light intensity can also reopen stomata, thus decreasing water-soaking (reviewed by Inoue and Kinoshita 2017).

Here we have shown that stomatal dysfunction mutants exhibit increased resistance to *Pst* despite impaired initial closure of stomata. Our findings highlight the importance of water immunity in plant resistance against *Pst* in determining the disease progression (Fig. 4). Although recent articles have suggested that water immunity (i.e. the prevention of aqueous space) is controlled by stomatal status, direct experimental evidence is scarce. Our study shows that stomatal regulation plays a significant role in managing apoplastic water status and, consequently, plant resistance.

Materials and methods

Plant material and growth conditions

Arabidopsis lines and their origins are listed in Supplementary Table S2. Plants were grown in 1:1 mixture of peat and vermiculite in chambers (Fitotron SGC120, Weiss Technik) under 12 h light (170 μmol m−2 s−1)/12 h dark cycle, 23 °C/19 °C (day/night), 60%/70% relative humidity. For all experiments, 3.5-week-old plants were used.

Bacterial growth Assays

Pseudomonas syringae pv. *tomato* DC3000 was cultured overnight at 28 °C in liquid LB, diluted 1:50 in fresh LB, and cultured for 12 h. Bacteria was collected by centrifugation at 4000 rpm for 2 min and resuspended in 10 mM $MgCl_2$, followed by 2 identical wash steps. Cell density was adjusted to OD600 nm = 0.2 (1×10^8 CFU/mL) or 0.02 (1×10^7 CFU/mL) in 10 mM $MgCl_2$ for spray and syringe inoculation, respectively, and 0.02% (v/v%) of Silwet L-77 was added.

For spray-infections, adaxial leaf surfaces were saturated with an airbrush spray. For syringe-infections 3 fully expanded leaves (leaf numbers 5 to 7) were infiltrated with a 2 mL needleless syringe until saturation. Infections were performed at mid-day. Trays were covered with plastic domes 2 h prior to infections and again after the infections to maintain high humidity.

For bacterial counting, 3 leaves per plant were excised, surface-sterilized in 70% ethanol and washed twice in sterile milliQ. One leaf disc from each leaf was collected with 0.7 cm cork borer and ground in 10 mM $MgCl_2$. Serial dilutions were plated on LB-Miller-plates and incubated at RT for ~40 h. Colonies were counted to determine colony-forming units (CFU). In each repeat, 6 plants were sampled, and the experiment was repeated 3 times.

Water-soaking Assays

Pst spray-inoculation was conducted as above. For ABA spraying, 25 μM ABA with 0.02% (v/v%) Silwet L-77, and 0.1% ethanol in sterile milliQ were used. The same solution without ABA was used as a mock treatment for ABA. Plants were sprayed at mid-day and trays covered with plastic domes to maintain high humidity. At 24 h post-spraying, 2 leaves per plant were excised, and the abaxial sides were photographed. For each genotype and treatment, 5 to 6 plants were photographed per experiment, and the experiment was repeated 3 times. The water-soaked area was measured using ImageJ and calculated as described by Wu et al. (2023). The water-soaking area was divided by total leaf area (mm^2) to obtain water-soaked ratio (%).

Stomatal Assays

Pst spray-inoculation was conducted as above. At indicated times, 2 leaves (leaf numbers 5–7) per plant were excised, and a dental resin mixture (Xantopren M Mucosa; Kulzer) was applied on the abaxial side. After solidifying, the impressions were removed, and a cast of the epidermis was created with clear nail varnish. Nail varnish was removed with clear tape and applied to a microscopy slide. Images were acquired with a Leica DMLB microscope using Leica 20× Fluotar objective, and stomatal aperture ratio (width/height) was measured using ImageJ. Sampling of leaves was done in a randomized order, and acquisition of images and measurements was performed blind.

RNA isolation and reverse transcription quantitative PCR

RNA was isolated using Spectrum Plant Total RNA Kit (Sigma-Aldrich). Four leaves (leaf numbers 5–7) were pooled together from 4 plants. Extracted RNA (2.5 μg) was treated with DNase I (ThermoFisher Scientific) and used for cDNA synthesis with Maxima Reverse Transcriptase (ThermoFisher Scientific). After cDNA synthesis, final volume was diluted to 100 μL, and 1 μL used for qPCR with 2X FastDye qPCR mix (KleverLab). Reactions were run on CFX Opus 384 System (Bio-Rad). Primers used for qRT–PCR, and their amplification efficiency are listed in Supplementary Table S4. Analysis of qPCR results was performed using the qBase3.4 program (CellCarta), (Hellemans et al. 2007). Three reference genes (*PP2AA3*, *TIP41*, *YLS8*) were used for normalization. Expression stability of reference genes (*M*-value) was evaluated with geNorm in qBase (Supplementary Table S4). Statistical analysis of qPCR data using 3 biological repeats was done with 1-way ANOVA and Tukey multiple comparisons test (GraphPad 10.2.3).

Statistical analysis

Statistical analyses were conducted with R version 4.4.1 (R Core Team 2022, https://www.R-project.org). Linear mixed-effect models with biological repeats as a random grouping factor were fitted using function lmer from package lme4 (Bates et al. 2015). Pairwise-comparisons (Tukey's method) were performed using emmeans package (Lenth 2022).

Accession numbers

Sequence data from this article can be found at The Arabidopsis Information Resource (https://www.arabidopsis.org) under the accession numbers listed in Supplementary Tables S2 and S4.

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Author contributions

J.K., M.B., and M.S. conceived and designed the research. J.K., M.P., and M.B. performed research. J.K., S.E., M.B., and M.S. analyzed data. J.K., M.B., and M.S. wrote the manuscript. All authors discussed and interpreted the data and edited and commented the manuscript.

Supplementary data

The following materials are available in the online version of this article.

Supplementary Table S1. Literature describing discrepancies in mutant resistance to *Pst* and differing growth conditions.

Supplementary Table S2. Mutant lines used in this study with their gene function and known stomatal phenotypes.

Supplementary Table S3. Primers for genotyping mutants by PCR.

Supplementary Table S4. RT-qPCR primers and expression stability of reference genes.

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Conflict of interest statement. None declared.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

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