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# Common and unique Arabidopsis proteins involved in stomatal susceptibility to *Salmonella enterica* and *Pseudomonas syringae*

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One sentence summary: *S. enterica* and *P. syringae* activate alternative pathways in the guard cells to induce stomatal movement, although common players are also involved.

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## ABSTRACT

*Salmonella enterica* is one of the most common pathogens associated with produce outbreaks worldwide; nonetheless, the mechanisms uncovering their interaction with plants are elusive. Previous reports demonstrate that *S. enterica* ser. Typhimurium (STm), similar to the phytopathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000, triggers a transient stomatal closure suggesting its ability to overcome this plant defense and colonize the leaf apoplast. In order to discover new molecular players that function in the stomatal reopening by STm and *Pst* DC3000, we performed an Arabidopsis mutant screening using thermal imaging. Further stomatal bioassay confirmed that the mutant plants *exo70h4-3*, *sce1-3*, *bbe8*, *stp1,* and *lsu2* have smaller stomatal aperture widths than the wild type Col-0 in response to STm 14028s. The mutants *bbe8*, *stp1* and *lsu2* have impaired stomatal movement in response to *Pst* DC3000. These findings indicate that EXO70H4 and SCE1 are involved in bacterial-specific responses, while BBE8, STP1, and LSU2 may be required for stomatal response to a broad range of bacteria. The identification of new molecular components of the guard cell movement induced by bacteria will enable a better understanding of the initial stages of plant colonization and facilitate targeted prevention of leaf contamination with harmful pathogens.

Keywords: phytopathogen; enterobacteria; foodborne illness; stomatal movement; Arabidopsis mutant screening

## INTRODUCTION

Stomata are natural pores on the plant leaf surface surrounded by a pair of guard cells. These pores are important to promote gas exchange between the aerial parts of the plant and the atmosphere, controlling water losses during transpiration. Plants regulate stomatal movement in order to maximize fitness and survive under diverse environmental challenges, including pathogen attack (Martí'nez-Sá'nchez *et al.* 2011; Panchal and Melotto 2017). Microbe-associated molecular patterns (MAMPs),

such as the bacterial motor protein flagellin (Gómez-Gómez and Boller 2000), can induce plant basal defenses known as MAMP-triggered immunity (MTI) (Zipfel *et al.* 2004). As part of the MTI, guard cells surrounding the stomatal pores can sense bacterial flagellin and trigger stomatal closure, a process known as stomatal defense (Melotto *et al.* 2006; Zeng and He 2010). Interestingly, several plant bacterial pathogens are able to overcome the stomatal defense through the secretion of different virulence factors. For instance, *Pseudomonas syringae* pv. *tomato* (*Pst*) strain

DC3000 produces the phytotoxin coronatine that represses MTI-triggered stomatal closure, resulting in stomatal reopening. Suppression of stomatal immunity facilitates the entrance of bacterium in the leaf apoplast and subsequent colonization of internal leaf tissue, leading to the development of disease (reviewed by Melotto *et al.* 2017).

The human pathogen *Salmonella enterica* serovar Typhimurium (STm) also induces strong stomatal closure in plants, including *Arabidopsis thaliana*, lettuce (*Lactuca sativa* L.), spinach (*Spinacia oleracea*), basil (*Ocimum basilicum*) and cilantro (*Coriandrum sativum*) (Kroupitski *et al.* 2009; Roy *et al.* 2013; Roy and Melotto 2019). STm shows chemotaxis and tropism toward the leaf stomata (Kroupitski *et al.* 2009; 2011), suggesting that the stomatal pore is an important entry site for this bacterium. Interestingly, similar to the phytopathogen *Pst* DC3000, STm is capable of inducing stomatal reopening (Kroupitski *et al.* 2009; Roy *et al.* 2013). Indeed, STm colonization and survival in the plant leaf apoplast has been observed for several plant species (Dong *et al.* 2003; Iniguez *et al.* 2005; Schikora *et al.* 2008; Kroupitski *et al.* 2009; Gu *et al.* 2011; Park *et al.* 2013; Roy and Melotto 2019). Inside the leaf tissue, STm remains protected against environmental stressors such as drought and the sun light, as well as against commonly applied sanitation treatments of edible leaves (Golberg *et al.* 2011; Ziuzina *et al.* 2014).

The mechanisms by which STm induces stomatal reopening are largely unknown. To gain knowledge on this process, we have performed a thermal image-based screening of Arabidopsis mutants. The screening is a non-destructive method useful for rapid assays in plants (reviewed by Ishimwe *et al.* 2014), which enabled us to find novel components of the guard cell signaling involved in both *Pst* DC3000- and STm 14028s-mediated stomatal movement. Importantly, we discovered common and unique proteins involved in this process, suggesting that STm 14028s and *Pst* DC3000 may activate distinct pathways in the guard cells to induce stomatal movement. This study may help elucidate mechanisms targeting the protection of plants from bacterial contamination and disease development.

## MATERIALS AND METHODS

### Plant material and growth conditions

*Arabidopsis thaliana* (L. Heyhn.) mutant lines and wild type plants were used in this study. The seeds of wild type ecotype Columbia (Col-0, ABRC stock CS60000) and derived mutants were obtained through The Arabidopsis Information Resource (TAIR; https://www.arabidopsis.org/). For the genetic screening, we obtained a mutant collection (ABRC stock CS27942) that contains 3,980 individual mutant lines created by T-DNA insertion (TAIR). Additionally, we have tested mutant lines for specific genes as follows: *exo70h4-3* (SALK_003200C), *sce1-3* (SALK_05 8401), *bbe8* (SALK_117810C), *stp1* (SALK_048848C), and *lsu2* (SALK_031648C). Seeds were sown in a 1:1:1 v:v:v mixture of growing medium (Redi-earth plug and seedling mix, Sun Gro, Sacramento, CA, USA), fine vermiculite and perlite, and grown in controlled environmental chambers at $22 \pm 2°C$, $60 \pm 10\%$ relative humidity, and a 12-hour photoperiod under light intensity of $100 \ \mu mol.m^{-2}.s^{-1}$. Plants were maintained under these conditions until the completion of all experiments.

### Bacterial strains and culturing conditions

Bacterial cells of *Pst* strain DC3000 and *Salmonella enterica* subsp. *enterica* serovar Typhimurium (STm) strain 14028s were streaked on solid low-salt Luria-Bertani (LSLB; 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH = 7.0) medium from frozen glycerol stocks. Plates were kept at 28°C until the appearance of single colonies. For inoculum preparation, a single colony was transferred to liquid LSLB medium and kept at 28°C in an orbital shaker-incubator overnight. Medium was supplemented with rifampin (100 μg/mL) to grow *Pst* DC3000.

### Thermal imaging screening

A thermal imaging-based approach (Merlot *et al.* 2002) was used to access whether bacterial inoculation differentially modulates stomatal responses between each Arabidopsis mutant and the wild type Col-0. In this study, we screened ~1,000 single mutant lines that were picked randomly from the collection CS27942. Col-0 plants were included in every batch of plants for direct comparison with the mutant plants. Three- to four-week-old plants were dip-inoculated with $2 \times 10^8$ CFU.mL$^{-1}$ STm supplemented with 0.03% Silwet L-77 (Lehle Seeds Co., Round Rock, TX, USA) at 3 hours after dawn following the procedure described by Katagiri *et al.* (2002). To avoid changes in leaf temperature due to environmental conditions, experiments were conducted inside a walk-in growth chamber set at 25°C, $60 \pm 10\%$ relative humidity, and light intensity of $100 \ \mu mol.m^{-2}.s^{-1}$. At 4 hours post inoculation (hpi), pictures were taken from the rosettes using an FLIR® infrared camera (FLIR-T600). Pictures were analyzed using the FLIR Tools software, in which all pictures were set at constant range of temperature based on pictures of Col-0. Differential temperature of the center of the rosette was used to identify mutants with potential role in stomatal susceptibility to STm.

### Genotyping of arabidopsis mutants

To confirm the presence of the T-DNA insertion in selected Arabidopsis mutants (Table 1), fresh leaf samples (5 mg) were grounded in 200 μL of Edwards Solution (Edwards *et al.* 1991) and used as gDNA template in PCR reactions. Primers were designed using the T-DNA Primer Design tool of the T-DNA Express website from the Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu/cgi-bin/tdnaexpress). PCR reaction (25 μL) was performed with 1 U DNA polymerase Gotaq® (Promega, WI, USA), 1× enzyme buffer, 1.5 mM MgCl₂, 200 μM dNTP, 1 μL of gDNA template, and 400 nM of reverse and forward specific primers (Table 1). Reaction cycling parameters were: 1 cycle of 95°C for 5 min and 40 cycles of 95°C for 30 sec, 52°C for 30 sec, and 72°C for 1 min, with final extension of 72°C for 10 min. Amplicons were visualized after gel electrophoresis using the UV light on a C300 imaging system (Azure Biosystems, CA, USA).

### Stomatal bioassay

To examine the stomatal reopening of mutant plants in response to *Pst* DC3000 and STm 14028s, stomatal bioassays were conducted as previously described (Montano and Melotto 2017). Briefly, three detached whole leaves of four- to five-week old plants were floated on water or $1 \times 10^8$ CFU.mL$^{-1}$ of either *Pst* DC3000 or STm 14028s. Floating leaves were kept at 25°C and light intensity of $100 \ \mu mol.m^{-2}.s^{-1}$ for the duration of the experiment (4 hours). Stomatal images and aperture width measurements were obtained with a Nikon Eclipse 80i fluorescent microscope (Nikon Corporations, Shinagawa-ku, Tokyo, Japan), equipped with long-distance objectives. Data points represent the mean of two independent biological replicates ($n = 120$) $\pm$ standard error (SE). Student's *t*-test statistical analysis was performed by comparing the stomatal aperture

**Table 1.** List of Arabidopsis mutants and primer sequences used to validate the mutation.

| Locus number | Gene | ABRC mutant seed stock | Primer sequences |
|---|---|---|---|
| AT3G09520 | *EXO70H4* | SALK_003200C (*exo70h4-3*) | LP—AACAAACCTGAAGCCATGATG<br>RP—GTTGCTGTAGTCAGCGAGGAG |
| AT3G57870 | *SCE1* | SALK_05 8401 (*sce1-3*) | LP—TGGTGATGATGTATCCGTCTG<br>RP—GTGCAATGCCACACCATTAG |
| AT1G30700 | *BBE8* | SALK_117810C (*bbe8*) | LP—ATATCCTCCCCATCTCACCAC<br>RP—GAAACCCTTCCTCATAATCGC |
| AT1G11260 | *STP1* | SALK_048848C (*stp1*) | LP—ACACTCCCAATTCAATGATCG<br>RP—TCATATGCATAATTTCTTCATGTGG |
| AT5G24660 | *LSU2* | SALK_031648C (*lsu2*) | LP—CAGCTCATTGGGGCTATAATG<br>RP—TCGCAAAAATCAATCGGTAAC<br>T-DNA-LBb1.3–ATTTTGCCGATTTCGGAAC |

width of each mutant to that of the wild type Col-0 for each treatment.

## RESULTS

### Arabidopsis mutants screening reveals proteins involved in bacterium-induced stomatal reopening

Thermal-image screening was established >17 years ago (Merlot *et al.* 2002) and has been previously used in a number of screening assays (reviewed by Fiorani and Schurr 2013; Osakabe *et al.* 2016). Taking advantage of this technology, we first determined whether it would be suitable for our experimental setup. Indeed, Col-0 plants show an overall hotter leaf surface temperature at 2 hpi with STm when compared to the mock-inoculated plants (Fig. 1A), indicating an overall decrease of stomatal pore size . Furthermore, Col-0 rosettes became cold at 4 hpi with STm, indicating that stomatal pores reopened (Fig. 1A). This observation provided an opportunity to screen Arabidopsis mutants for compromised stomatal reopening at 4 hpi.

Moving toward a mechanistic understanding of plant molecular responses to human pathogens, we set up a medium throughput assay and phenotyped ~1,000 mutants for hotter leaf surface temperature at 4 hpi with STm by comparing with that of the wild type control. Representative images used to select mutant plants are shown in Fig. 1B. From this screening, we selected five plant mutants for further analysis. Each of these plant lines has a mutation in the following genes: *EXO70H4* (*EXOCYST SUBUNIT EXO70 FAMILY PROTEIN H4*) and *SCE1* (*SUMO CONJUGATING ENZYME 1*) involved in callose deposition (Kulich *et al.* 2015; 2018) and protein SUMOylation (Saracco *et al.* 2007), respectively; the *BBE8* (*BERBERINE BRIDGE ENZYME 8*) that is a novel protein; the hexose symporter *STP1* (*SUGAR TRANSPORTER 1*) (Shearson *et al.* 2000); and the stress mediator *LSU2* (*RESPONSE TO LOW SULFUR 2*) (Sirko *et al.* 2015). These mutants had no obvious growth and developmental defects under the conditions used for the experiment (representative images shown in Fig. 1C). Homozygous mutation in the expected gene for each of the five Arabidopsis mutants was confirmed by PCR (Fig. 1D), and mutant plants were subjected to stomatal bioassays, which comprised of direct measurements of stomatal aperture width after treatment.

### STm 14028s and *Pst* DC3000 may activate distinct pathways to induce stomatal reopening

It is well known that *Pst* DC3000 can reopen MTI-closed stomata due to the action of its phytotoxin coronatine (Melotto *et al.* 2006). STm is also capable of inducing stomatal reopening on both Arabidopsis and lettuce leaves by an unknown mechanism (Kroupitski *et al.* 2009; Roy *et al.* 2013). In order to shed light on the molecular mechanism involved in the stomatal movement evoked by these bacteria, we evaluated the stomatal movement in leaves of the five pre-selected Arabidopsis mutants (*exo70h4–3*, *sce1–3*, *bbe8*, *stp1* and *lsu2*) identified in the screen (Fig. 1) after 4 hours of exposure to either *Pst* DC3000 or STm 14028s.

All five mutants showed no defect in stomatal movement when their leaves were incubated with the water as a control for 4 hours, as the stomatal aperture width was similar between mutants and Col-0 grown and analyzed side-by-side (Fig. 2A). When interacting with the plant pathogen *Pst* DC3000, the mutants *exo70h4-3* with T-DNA insertion in the coding sequence of the *EXO70H4* gene (Kulich *et al.* 2015), and the *sce1-3* that has a T-DNA insertion upstream of the 5′ UTR of the *SCE1* (Saracco *et al.* 2007), showed no differential stomatal response in comparison to Col-0 inoculated leaves (Fig. 2B). However, the plant mutants *bbe8* with a T-DNA insertion in the first intron of the *BBE8* gene (Peng *et al.* 2014), *stp1* containing T-DNA insertion at the fourth exon of the *STP1* gene (Yamada *et al.* 2016), and *lsu2* that has a T-DNA insertion at the 5′ UTR region of the *LSU2* gene (Sirko *et al.* 2015) showed narrower stomatal aperture widths than Col-0 after incubation with the plant pathogen *Pst* DC3000 (Fig. 2B). Confirming the genetic screening data, we observed that the mean stomata aperture width in all mutants were smaller than the wild type Col-0 when leaves were incubated with STm 14028s (Fig. 2C). These results suggest that although both *Pst* DC3000 and STm 14028s induce a transient stomatal closure in Arabidopsis, these bacteria may do so by activating distinct plant signaling processes.

## DISCUSSION

Contamination of fresh produce with human pathogens has a strong social and economic impact due to frequent foodborne disease outbreaks (Bennett *et al.* 2018). Contrary to the widely studied Arabidopsis-*Pst* DC3000 system, useful to understand the molecular mechanisms underlying plant disease (reviewed by Xin and He 2013), very little is known about how bacterial pathogens of human are able to survive in the phyllosphere until they reach their natural host. Two bacterial species, the phytopathogen *Pst* DC3000 and the enteric STm, modulate the stomatal pore size thereby facilitating their colonization of the leaf interior (Melotto *et al.* 2006; Kroupitski *et al.* 2009; Roy *et al.* 2013). However, whether this process is controlled by different mechanisms in a bacterium-dependent manner is unknown.
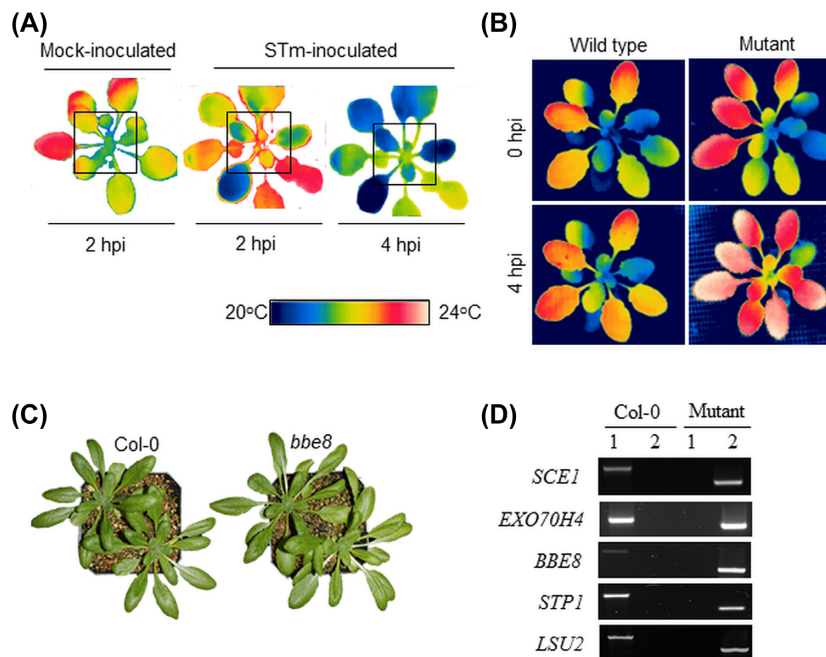
**Figure 1.** Arabidopsis mutant phenotyping and genotyping. **(A)** Representative thermal images of inoculated Col-0 plants. Three- to four-week-old plants were mock- or dip-inoculated with STm ($2 \times 10^8$ CFU.mL$^{-1}$). The thermal images were taken 2 and 4 hpi using an infrared camera (FLIR T600). Images were analyzed with the FLIR Tools Thermal Analysis and Reporting software (https://www.flir.com), in which the temperature in the center of the rosette (square) was used to assess the change in leaf temperature in bacterium-inoculated plants. Note that exposure to STm increases and decreases the temperature of the inner rosette at 2 hpi and 4 hpi, respectively. **(B)** Representative thermal images used to select plant mutants during the genetic screening. Images were taken before inoculation (0 hpi) and at 4 hpi with STm. Note that the center of the mutant rosette is hotter than the wild type rosette at 4 hpi. **(C)** Representative photographs of Arabidopsis plants used for mutant genotyping. Note that the plant mutant *bbe8* (as all other mutants reported in this study) does not have obvious defect in plant growth or development as compared to the wild type Col-0. **(D)** Genomic DNA for each T-DNA insertion mutant plant and the wild type Col-0 was used as a template in PCR amplification with gene-specific primers listed in Table 1. Reactions loaded onto lane 1 contained the LP (left primer) and RP (right primer) set to amplify the wild type allele in Col-0, whereas reactions loaded onto lane 2 contained a T-DNA specific primer and the RP primer to amplify the mutant allele.

Therefore, we sought to identify novel components of the guard cell molecular signaling that allow STm to overcome stomatal defense and establish correlations with the model system Arabidopsis-*Pst* DC3000. We first explored the guard cells movement of a collection of Arabidopsis mutants in response to STm, specifically in the context of bacterium-induced stomatal opening (*i.e.* at 4 hpi; Fig. 1). We were able to verify that five Arabidopsis mutants have impaired stomatal movement in response to STm 14028s and/or *Pst* DC3000.

A smaller stomatal aperture width was observed in the *bbe8* mutant after inoculation with *Pst* DC3000 and STm 14028s as compared to the wild type Col-0 (Fig. 2). Arabidopsis has 28 members of the BBE family and functional redundancy is expected among the family members; however, this is yet to be fully determined (Locci *et al.* 2019). Members of this family are known to promote the folding of the flavoenzyme vanillyl alcohol-oxidase (Daniel *et al.* 2016). Recently, four members of the BBE-like family were shown to be oligogalacturonides (OGs) oxidases, which may reduce the activation of plant immune responses (Benedetti *et al.* 2018). Furthermore, flavoproteins act as oxidoreductases to control redox burst processes (reviewed by Macheroux *et al.* 2011). Reactive oxygen species (ROS) burst is a hallmark of plant defense activated by microbes and function as secondary messenger to promote *Pst*-triggered stomatal closure (reviewed by Melotto *et al.* 2017). Thus, BBE8 may act on the oxidation of cell wall-derived oligosaccharides in the guard cells to facilitate stomatal opening after *Pst* DC3000 or STm inoculation.

The hexose symporter SPT1 and the sulfur-responsive protein LSU2 were also found to modulate stomatal movement in response to both *Pst* DC3000 and STm 14028s (Fig. 2). Guard cells require carbon sources to generate malate, which function as an osmotically active solute to induce influx of water and promote stomatal opening (reviewed by Santelia and Lawson 2016). Although malate can be imported by the guard cell (Lee *et al.* 2008) or synthetized through the degradation of starch and/or $CO_2$ fixation by the chloroplast, the constant supply of extracellular sugar into the guard cell is required for its proper functioning (reviewed by Santelia and Lawson 2016). In fact, STP1 was proposed to have a role in the osmoregulation of the guard cells during the day (Stadler *et al.* 2003). Therefore, deficiency in the guard cell osmoregulation in the *spt1* mutant may inhibit bacterial triggered stomatal opening. Interestingly, LSU2 was previously shown to be involved in response to several stresses, including carbon starvation and sugar treatment (Usadel *et al.* 2008). Moreover, *LSU2* is induced during oxidative stress (Davletova *et al.* 2005), its expression is required for ROS production in guard cell chloroplasts and subsequent stomatal closure during sulfur stress (Garcia-Molina *et al.* 2017), and it also participates in the apoplastic defense against *Pst* DC3000 (Mukhtar *et al.* 2011). Hence, it is possible that LSU2 functions as a hub required for proper guard cell signaling in response to bacteria. These findings suggest that STP1 and LSU2 act in guard cells in response to a broad range of bacteria, as they may participate in core processes of stomatal movement.

The mutants *exo70h4-3* and *sce1-3* showed narrower stomatal aperture width in comparison to Col-0 after 4 hours of exposure to STm 14028s, but not *Pst* DC3000 (Fig. 2). The EXO70H4 protein acts on secondary cell wall formation and callose deposition (Kulich *et al.* 2015; 2018). Callose is a highly dynamic glucose polymer that is rapidly synthesized or degraded in response
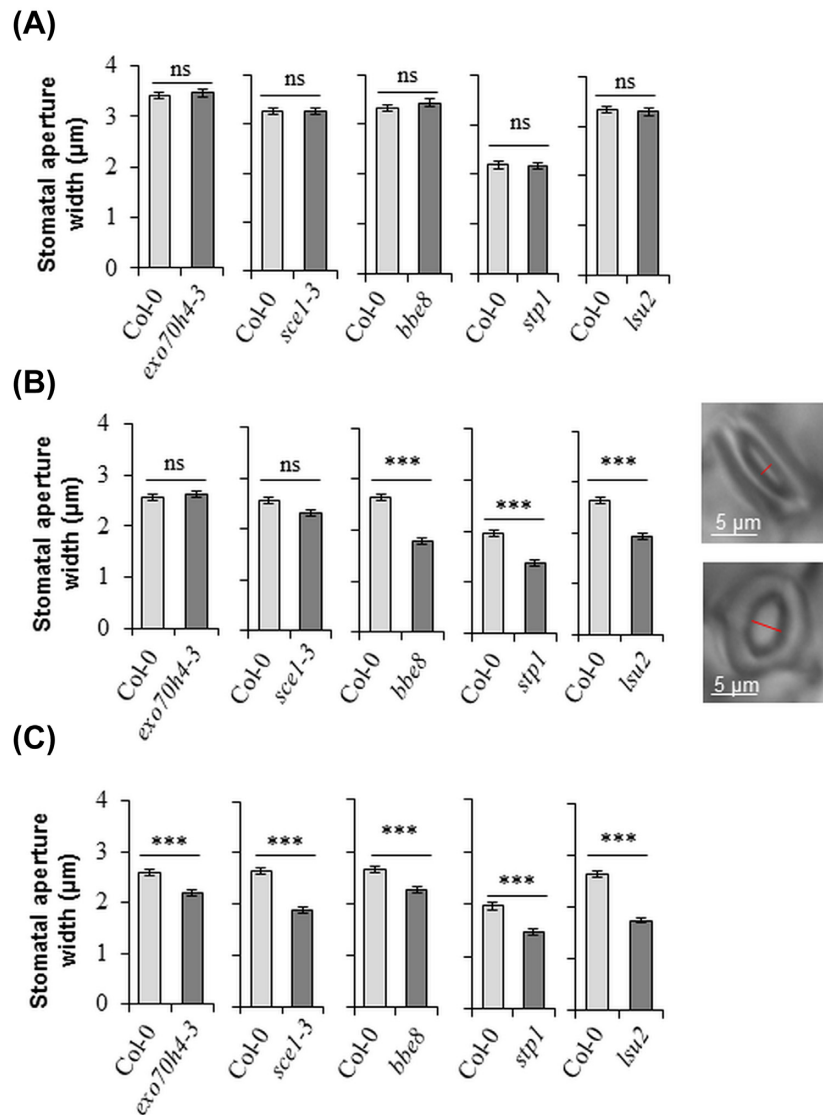
**(A)**



**(B)**



**(C)**



**Figure 2.** Arabidopsis genes involved in bacterium-mediated stomatal susceptibility. Stomatal aperture width at 4 hpi with **(A)** water, **(B)** *Pst* DC3000, **(C)** STm 14028s. Leaves of Arabidopsis wild type Col-0 and mutants were floated on water (control) or bacterial inoculum ($1 \times 10^8$ CFU.mL$^{-1}$ *Pst* DC3000 or STm 14028s). Results are shown as mean of two independent experiments ($n = 120 \pm$ SE). Statistical significance between means of each mutant and Col-0 inoculated with the same bacterium was detected using two-tailed Student's *t*-test (*** = $P < 0.001$; ns = non-significant). The pictures are representative of closed and open stomata. Red bars in the stomatal pore indicate the stomatal aperture width that was measured to obtain the graphs.

to diverse environmental stimuli or during plant development (Stone and Clarke 1992). Changes in guard cell turgor, which allows stomatal movement, require a dynamic cell wall. In the fern *Asplenium nidus* for instance, inhibition of callose synthesis reduces the stomatal ability to open (Apostolakos *et al.* 2010). SCE1 is a protein that catalyzes the attachment of the SUMO peptide to target proteins. SUMOylation is a post-translational modification that modulates protein sub-cellular localization, activity, and protein–protein interactions. Protein SUMOylation plays a major role in plant development, as well as in responses to abiotic and biotic stresses (reviewed by Verma *et al.* 2018). In fact, SCE1 interacts with FLS2 (FLAGELLIN-SENSING 2) and TPR1 (TOPLESS-RELATED 1), two proteins that activate plant immune responses, suggesting that SUMOylation can directly participate on the control of plant responses to microbes (Orosa *et al.* 2018; Niu *et al.* 2019). Therefore, our results suggest that callose deposition and protein SUMOylation in the guard cells are required

for STm 14028s to overcome stomatal immunity and induce stomatal opening.

In this study, we have found novel components of guard cell signaling pathway that function in the response to two bacteria. The results indicate that EXO70H4 and SCE1 may have a greater importance in the guard cell response to human pathogens than to plant pathogens. As callose deposition and ROS burst are common MTI responses, the distinctions observed in this study are dependent on specific interaction with each pathogen. Interestingly, the novel protein BBE8, the sugar import by STP1, and LSU2-mediated stress responses in the guard cell were common players in response to both human and plant pathogens. Altogether, the results indicate that human and plant pathogens activate alternative pathways in the guard cells to induce stomatal movement, although common players are also involved. This study provides new perspectives to assist the control of human and plant pathogen colonization of plants and

improve our knowledge on stomatal movement controlled by bacteria.

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