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Bacteriology

e-Xtra*

Characterizing Colonization Patterns of *Clavibacter michiganensis* During Infection of Tolerant Wild *Solanum* Species

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ABSTRACT

Clavibacter michiganensis is the Gram-positive causal agent of bacterial canker of tomato, an economically devastating disease with a worldwide distribution. *C. michiganensis* colonizes the xylem, leading to unilateral wilt, stem canker, and plant death. *C. michiganensis* can also infect developing tomato fruit through splash dispersal, forming exterior bird's eye lesions. There are no documented sources of qualitative resistance in *Solanum* spp.; however, quantitative trait loci conferring tolerance in *Solanum arcanum* and *Solanum habrochaites* have been identified. Mechanisms of tolerance and *C. michiganensis* colonization patterns in wild tomato species remain poorly understood. This study describes differences in symptom development and colonization patterns of the wild type (WT) and a hypervirulent bacterial expansin knockout (ΔCmEXLX2) in wild and cultivated tomato genotypes. Overall, WT and ΔCmEXLX2 cause less severe symptoms in wild tomato species and are impeded in spread and colonization of the vascular system. Laser scanning confocal microscopy and scanning electron microscopy were used to observe preferential colonization of protoxylem vessels and reduced intravascular spread in wild tomatoes. Differences in *C. michiganensis* in vitro growth and aggregation were determined in xylem sap, which may suggest that responses to pathogen colonization are occurring, leading to reduced colonization density in wild tomato species. Finally, wild tomato fruit was determined to be susceptible to *C. michiganensis* through in vivo inoculations and assessing lesion numbers and size. Fruit symptom severity was in some cases unrelated to severity of symptoms during vascular infection, suggesting different mechanisms for colonization of different tissues.

Keywords: bacteriology, bacterial canker, *Clavibacter michiganensis*, Gram-positive, plant tolerance, tomato, wild tomatoes, xylem

Bacterial canker is one of the most economically damaging diseases affecting tomato production worldwide (de León et al. 2011; Nandi et al. 2018; Sen et al. 2015). *Clavibacter michiganensis*, formerly *Clavibacter michiganensis* subsp. *michiganensis*, is the Gram-positive causal agent of bacterial canker within the genus *Clavibacter*, which has undergone reclassification from subspecies to species rank (Li et al. 2018; Thapa et al. 2019). *C. michiganensis* systemically colonizes the tomato vascular system, leading to unilateral wilt, marginal leaf necrosis, stem cankers, and plant death (Chalupowicz et al. 2012; Sen et al. 2015). Splash dispersal of *C. michiganensis* can infect the exterior of developing tomato fruit, producing bird's eye lesions that affect fruit quality and can lead to seed contamination (Medina-Mora et al. 2001; Tancos et al. 2013). Systemic spread of *C. michiganensis* in the xylem makes it difficult to control with conventional products, and there is no commercially available resistance in tomato (de León et al. 2011; Sen et al. 2015). Quantitative trait loci (QTL) from wild tomato species that confer tolerance have been identified; however, the mechanisms of tolerance to *C. michiganensis* are poorly understood (Sen et al. 2015). Studying *C. michiganensis* colonization of tomatoes with varying susceptibility is a step toward deciphering how wild tomato species tolerate *C. michiganensis* infection.

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Wild *Solanum* spp. harbor genetic diversity that can be exploited as sources for disease resistance. Several species, including *S. arcanum*, *S. chilense*, *S. glandulosum*, *S. habrochaites*, *S. minutum*, *S. parviflorum*, *S. peruvianum*, and *S. pimpenellifolium*, have demonstrated moderate to high tolerance to *C. michiganensis* (Francis et al. 2001; Kabelka et al. 2002; Lara-Ávila et al. 2012; Sen et al. 2012, 2015; Sotirova et al. 1999; van Heusden et al. 1999; Vulkova and Sotirova 1993). Genetic studies of *S. arcanum* LA2157 (formerly *Lycopersicon peruvianum*) identified additive and codominant QTL on different chromosomes of interspecific crosses and an F_2 population (Sotirova et al. 1999; van Heusden et al. 1999). Fine mapping of *S. habrochaites* LA407 (formerly *Lycopersicon hirsutum*) identified two QTL with additive tolerance on chromosomes 2 and 5 that contribute to vascular development, morphology, and H_2O_2 defense responses in the xylem (Coaker and Francis 2004; Coaker et al. 2002, 2004; Francis et al. 2001). Despite low symptom development in wild *Solanum* spp., *C. michiganensis* is able to systemically colonize the vascular system to high densities (10^4 to 10^8 CFU per 1 mg of tissue) (Francis et al. 2001; Sen et al. 2012). To the best of our knowledge, there have been no published studies investigating wild tomato fruit susceptibility to *C. michiganensis*. The role of *C. michiganensis* colonization, spread, and plant response in the development of bacterial canker symptoms remains uncharacterized in these tolerant plant species.

C. michiganensis interacts with plant host tissues in a complex manner that is poorly understood (Chalupowicz et al. 2017; Nandi et al. 2018). Genomic analyses have revealed a pathogenicity island and two plasmids necessary for full virulence in the reference strain, NCPPB382 (Dreier et al. 1997; Gartemann et al. 2008; Jahr et al. 2000; Meletzus et al. 1993). These mobile elements contain multiple copies of genes encoding secreted serine proteases, carbohydrate active enzymes, expansins, and tomatinase (Gartemann et al. 2008). Genetic studies have demonstrated that *C. michiganensis* strains have variable gene and plasmid contents that contribute to virulence (Tancos et al. 2015; Thapa et al. 2017). In a previous study, a

hypervirulent *C. michiganensis* isolate was generated by knocking out a single-copy expansin gene encoding a secreted expansin-like protein (CmEXLX2) (Tancos et al. 2018). The role of secreted *C. michiganensis* expansin proteins during virulence and the mechanisms underlying hypervirulence remain unclear, but the hypervirulent strain ΔCmEXLX2 can be used as a tool to further our understanding of host–*C. michiganensis* interactions.

C. michiganensis poses a major threat to tomato producers, and epidemics often begin through seedling transplants harboring latent infections (de León et al. 2011). The goals of this study were to understand differences in *C. michiganensis* colonization and host symptom development between susceptible domesticated tomato and tolerant wild tomato species. Results from this study can begin to delineate the complex relationship between *C. michiganensis* and the host tissues that it colonizes.

MATERIALS AND METHODS

Disease severity assays. Wild tomato seeds (*S. habrochaites* LA2128, *S. arcanum* LA2157, and *S. arcanum* LA2172) were washed in a 50% bleach solution for 15 min, rinsed with sterile deionized water, and then, germinated on wet sterile Whatman filter paper (Fisher Scientific) in petri dishes for 8 to 10 days. Seedlings were then transferred to a Farfard professional formula growing mix in 72-cell flats (Sun Gro Horticulture), and *Solanum lycopersicum* 'Mt. Fresh Plus' seeds were started in the mix. When seedlings had two true leaves, they were transferred to 4-inch pots and supplemented with Osmocote slow release fertilizer (Scotts Miracle-Gro Co.). Plants were grown with a 16-h light/8-h dark photoperiod in an environmentally controlled greenhouse.

To characterize plant susceptibility to *C. michiganensis*, seedlings of *S. lycopersicum* 'Mt. Fresh', *S. habrochaites* LA2128, *S. arcanum* LA2157, and *S. arcanum* LA2172 between the two- and three-true leaf stage were inoculated with wild-type (WT) *C. michiganensis* 0317 (hereafter WT) and *C. michiganensis* 0317ΔCmEXLX2 (hereafter ΔCmEXLX2) cultured in Luria–Bertani (LB) medium and LB supplemented with gentamicin (40 µg/ml), respectively, for 18 h with shaking at 160 rpm at 28°C (Tancos et al. 2018). Bacterial suspensions were adjusted to optical density 600 (OD_{600}) = 0.8 through centrifugation for 12 min at 4,000 rpm, washing the pellet with sterile water, and resuspending in sterile water (Tancos et al. 2018). Tomato seedlings (n = 15 per genotype) were arranged in three randomized blocks with two replicates of each plant genotype/*C. michiganensis* strain combination per block in the greenhouse. One mock-inoculated plant per genotype was included in each block as a control.

Plants were inoculated by dipping sterilized scissors into bacterial suspension and clipping the cotyledons (Tancos et al. 2018). Plants were assessed daily for leaflet wilting and stem canker formation. Observations continued until 21 days postinoculation (dpi). Disease severity was quantified by dividing the number of leaflets wilting each day by the total number of leaflets present on the oldest seven leaves at 21 dpi (Balaji et al. 2008; Chalupowicz et al. 2012; Tancos et al. 2018). The mean area under the disease progress curve (AUDPC) was calculated from disease severity measurements (Tancos et al. 2018). Differences in AUDPC were analyzed using a mixed effects model with strain, genotype, block, and the interaction between strain and genotype as explanatory variables and a random effect to account for block effects using packages lme4, pbkrtest, emmeans, and lsmeans in R v. 3.3.2 (Bates et al. 2015; Halekoh and Højsgaard 2014; Lenth 2016). Significant differences between treatment groups were tested using Tukey's honestly significant difference test (HSD) test ($P < 0.05$). The experiment was performed three times.

Bacterial movement and in planta growth. To characterize spread and colonization density of *C. michiganensis* in the plant vascular system, 0.5-cm sections of tomato stem tissue at the inoculation site (0 cm), 5 cm above, and 10 cm above were harvested at 21 dpi (n = 3 per genotype inoculated with WT or ΔCmEXLX2). Tissues were weighed and homogenized in 1 ml of sterile water using a sterile 5-mm stainless steel grinding bead (Qiagen) in a 2-ml Eppendorf tube using a TissueLyser (Retsch) at 30 Hz for 4 min. One hundred microliters of the homogenate was transferred to a 96-well Falcon tissue culture plate (Corning Inc), and 10 µl were serial diluted into 90 µl of sterile water for seven dilutions. Ten microliters of each dilution was spot inoculated onto D2ANX or LB plates amended with gentamycin (40 µg/ml) four times and incubated for 2 to 3 days at 28°C. Numbers of CFU were quantified at the dilution where there was between 20 and 200 CFU, and the average of four technical replicates per biological replicate was used for the final analysis. CFU counts were $\log_{10}$ transformed, and data from three individual experiments were analyzed separately and then pooled for the final analysis (n = 9 biological replicates of a host genotype inoculated with WT or ΔCmEXLX2 at 0, 5, and 10 cm from the inoculation site). A mixed effects model was generated with $\log_{10}$ CFU milligram^{-1} of tissue as the response and distance by genotype by strain as the response with random effect terms accounting for variation between experiments, blocks within experiments, and variability within a single plant. The analysis was performed using packages lme4, pbkrtest, emmeans, and lmerTest in R v. 3.3.2 (Bates et al. 2015; Halekoh and Højsgaard 2014; Lenth 2016). Significant differences between groups were tested using Tukey's HSD test ($P < 0.05$).

In vitro growth and attachment of *C. michiganensis* in xylem sap. Xylem sap for in vitro analyses was extracted from plants between the six- and eight-leaf stage by heavy watering the night before and at dawn, cutting the stem at an angle approximately 1 cm above the cotyledons; then, we tilted the pots to allow sap to drain into 15-ml conical tubes for 1 h. Sap was transferred into tubes on ice every 15 min. Sap from at least six plants was pooled and filtered through 0.2-µm polyethersulfone syringe filters (Corning Inc.) and stored at –20°C.

To measure bacterial growth in xylem sap, WT and ΔCmEXLX2 were grown as described above and resuspended to OD_{600} = 0.1 in sterile water. Five microliters of three individual WT and ΔCmEXLX2 cultures were inoculated into 12 wells (4 wells per bacterial culture) of a 96-well Falcon tissue culture plate containing 195 µl of sterile tomato sap. The plate was incubated at 28°C with shaking in a BioTek microplate reader. OD was measured at 590 nm every 2 h for 48 h (Tancos et al. 2018). OD for bacterial growth samples was normalized to mock (sterile water)-inoculated sap from each genotype. Area under the growth curve was calculated for each sample, and statistical analyses were performed using a mixed effects model including bacterial cultures as random effects using packages lme4, pbkrtest, and emmeans in R v. 3.3.2 (Bates et al. 2015; Halekoh and Højsgaard 2014; Lenth 2016). Significant differences between treatment groups were tested using Tukey's HSD test ($P < 0.05$). The experiment was performed twice.

To measure *C. michiganensis* attachment in vitro, WT and ΔCmEXLX2 were grown as described above and adjusted to OD_{600nm} = 0.8. Five microliters of three individual cultures of WT and ΔCmEXLX2 were inoculated to 12 wells (4 wells per bacterial culture) of a 96-well Falcon tissue culture plate containing 95 µl of filter-sterilized tomato sap. Plates were briefly shaken and statically incubated at 28°C for 5 days (Chalupowicz et al. 2012; Tancos et al. 2018; Tran et al. 2016). After incubation, the supernatant was gently removed through pipetting, and wells were washed twice with 150 µl of sterile water. The samples were fixed at 60°C for 1 h, and surface-attached bacteria were stained with 25 µl of 0.1% crystal violet for 25 min at room temperature. The plate was washed twice with 200 µl of sterile water and gently blotted on paper towels (Davey and O'toole 2000; Tancos et al. 2018; Tran et al. 2016). Crystal violet was solubilized by adding 100 µl of 30% acetic acid followed by brief agitation and quantifying the absorbance at 590 nm using the BioTek microplate reader (Davey and O'toole 2000; Tancos et al. 2018; Tran et al. 2016). Statistical analyses were performed using a mixed effects model including bacterial cultures

as random effects using packages lme4, pbkrtest, and emmeans in R v. 3.3.2 (Bates et al. 2015; Halekoh and Højsgaard 2014; Lenth 2016). Significant differences between treatment groups were tested using Tukey's HSD test ($P < 0.05$). The experiment was performed twice.

Lateral *C. michiganensis* spread in primary vascular bundles. Three seedlings of each of the four host genotypes were grown as described above. Strains of WT and ΔCmEXLX2 with stable plasmids expressing eGFP were cultured as described in LB amended with kanamycin (100 µg/ml; Fisher Scientific) or kanamycin and gentamicin (40 µg/ml), respectively (Tancos et al. 2018). Cultures were adjusted to $OD_{600} = 0.8$ and cotyledon clip inoculated into plants at the two- to three-true leaf stage ($n = 3$ per genotype per strain). Mock-inoculated plants were included as controls.

To determine the number of xylem vessels colonized by *C. michiganensis*, transverse stem sections were excised 1 cm above the inoculation site at 21 dpi. Primary vascular bundles were imaged using scanning laser confocal microscopy (Olympus BX61; Fluoview FV-300; Olympus Corp.) using argon (488-nm) and green helium neon (543-nm) lasers to excite eGFP-expressing bacteria and induce plant autofluorescence, respectively (Chalupowicz et al. 2012; Tancos et al. 2013, 2018). The number of infected and noninfected protoxylem and metaxylem vessels in primary vascular bundles was quantified for each of three plants for each genotype/strain combination. Mock-inoculated plants were included as controls for plant autofluorescence. In total, 332 fields of view were imaged for quantification. Comparisons of percentage of infected xylem vessels were calculated with analysis of variance (ANOVA) and Tukey's HSD test ($P < 0.05$) using packages agricolae and emmeans in R v 3.3.2 (Lenth 2016; Mendiburu 2015).

To visualize bacterial colonization of individual proto- and metaxylem vessels using scanning electron microscopy (SEM), transverse and longitudinal stem sections 1 cm above the inoculation site were excised 12 dpi from mock- and WT-inoculated plants ($n = 2$ per genotype). Tissue sections were fixed in 3% glutaraldehyde in sterile phosphate-buffered saline followed by application of vacuum pressure to remove air from samples. Tissues were washed four times with Sørensen's phosphate buffer (pH 7.2) and dehydrated using an ethanol series. Solvents were removed through critical point drying (030 Critical point dryer; BAL-TEC), and samples were mounted on aluminum stubs and sputter coated twice with gold (EMS500x; Electron Microscopy Sciences). Images were captured using a scanning electron microscope (S-530 SEM; Hitachi) equipped with a digital camera. Two hundred eight fields of view were imaged in total.

Fruit disease severity assays. To assess susceptibility of wild tomato fruit to *C. michiganensis*, 6-week-old plants were transferred to 3-gallon pots and fertilized with Osmocote slow release fertilizer. Eight-week-old *S. lycopersicum* 'Mt. Fresh' plants were self-pollenated using a vibrating pollinator wand (VegiBee). *S. habrochaites* LA2128 and both *S. arcanum* genotypes were manually pollinated. *C. michiganensis* strains were cultured and adjusted to $OD_{600} = 0.8$ as described above. Water, WT, and ΔCmEXLX2 were brush inoculated onto fruit between 7 and 9 days postanthesis (dpa; $n = 10$ per treatment) of four plants of each genotype. To prevent cross-contamination of treatments, fruit clusters on the same plant were required to be a minimum of 25 cm apart. Fruits were harvested 15 dpi, and the diameter and number of lesions per fruit were measured. To measure fruit lesion size, images of each fruit were taken using an Olympus SZX18 dissecting microscope (Olympus Corp.). Lesion diameter and diameter of the lesion's necrotic center were measured using Olympus cellSens software (Olympus Corp.; $n = 100$ per treatment). Comparisons of number of lesions per fruit and differences in lesion size were calculated using ANOVA and using Tukey's HSD test ($P < 0.05$) with packages agricolae, emmeans, and lsmeans in R v 3.3.2 (Lenth 2016; Mendiburu 2015). The fruit susceptibility experiment was performed twice for a total of 20 fruit per host genotype/*C. michiganensis* strain combination.

RESULTS

Wild tomato species express fewer bacterial canker symptoms. Host susceptibility and movement of *C. michiganensis* in the vascular system were assessed by inoculating seedlings of cultivated and wild tomatoes with *C. michiganensis*. Wild tomato seedlings inoculated with WT and ΔCmEXLX2 developed little to no leaflet wilt in comparison with *S. lycopersicum* 'Mt. Fresh' ($P < 0.01$) (Supplementary Fig. S1). Leaflet wilt appeared as early as 7 and 10 dpi in *S. lycopersicum* 'Mt. Fresh' plants inoculated with ΔCmEXLX2 and WT, respectively. On average, wilt appeared on *S. arcanum* LA2172 plants inoculated with ΔCmEXLX2 around 12 dpi. Wilt symptoms of *S. habrochaites* LA2128 inoculated with both strains were delayed until 17 dpi. ΔCmEXLX2 was hypervirulent in seedlings of *S. lycopersicum* 'Mt. Fresh' as well as *S. arcanum* LA2172 but not in S. *habrochaites* LA2128 and *S. arcanum* LA2157 (Fig. 1A and C).

Differences in bacterial canker symptoms were observed between tomato genotypes. Severely affected leaflets of *S. arcanum* LA2172 wilted without marginal leaf necrosis, differing from typical wilt symptoms of bacterial canker in *S. lycopersicum* 'Mt. Fresh' (Fig. 1C and Supplementary Fig. S2). *S. habrochaites* LA2128 leaflets would partially wilt before the development of chlorosis followed by marginal leaf necrosis (Supplementary Fig. S2). Both *C. michiganensis* strains produced typical stem cankers at the inoculation site between 9 and 12 dpi in *S. lycopersicum* 'Mt. Fresh' (Supplementary Fig. S2). Only ΔCmEXLX2 was able to form stem cankers at the inoculation site of *S. arcanum* LA2172. No cankers were present in *S. habrochaites* LA2128 and *S. arcanum* LA2157 plants inoculated with either *C. michiganensis* strain (Supplementary Fig. S2).

***C. michiganensis* spread and colonization are reduced in wild tomatoes.** The ability for *C. michiganensis* to spread and densely colonize the vascular system is critical for systemic infection and symptom development. We quantified WT and ΔCmEXLX2 colonization density of plant stem sections 21 dpi at the inoculation site (0), 5, and 10 cm above. WT *C. michiganensis* was able to colonize to similarly high densities (10^9 CFU per 1 g of tissue) at the inoculation sites of *S. lycopersicum* 'Mt. Fresh' and *S. arcanum* LA2172 but not of *S. habrochaites* LA2128 and *S. arcanum* LA2157 (Fig. 1B and Supplementary Fig. S3). WT *C. michiganensis* populations were reduced at 5 and 10 cm above the inoculation site of all wild tomatoes (Fig. 1B and Supplementary Fig. S3). ΔCmEXLX2 only colonized at significantly higher densities relative to WT ($P < 0.05$) at varying distances in *S. arcanum* accessions (Fig. 1B and Supplementary Fig. S3). Populations of ΔCmEXLX2 in wild tomatoes were also reduced as the distance from the inoculation site increased. Both WT and ΔCmEXLX2 populations were lowest in the asymptomatic accession *S. arcanum* LA2157, but ΔCmEXLX2 colonization of *S. arcanum* LA2172 was almost equal to ΔCmEXLX2 titers in *S. lycopersicum* 'Mt. Fresh', suggesting that ΔCmEXLX2 hypervirulence is specific to certain plant genotypes within a species (Fig. 1A).

Composition of tomato xylem sap affects growth and biofilm formation. The xylem sap composition of wild tomatoes could be suboptimal for *C. michiganensis* growth, therefore reducing in planta growth and symptom development. We extracted sap from the four tomato genotypes and measured in vitro growth rates of WT and ΔCmEXLX2 for 48 h. There were no differences in growth rates between WT and ΔCmEXLX2 when cultured in sap from each of the four genotypes (Fig. 2). *C. michiganensis* grew to the highest density in sap from the most susceptible genotype, *S. lycopersicum* 'Mt. Fresh', and to the second highest density in sap from the asymptomatic genotype *S. arcanum* LA2157 (Fig. 2). *C. michiganensis* grew to lowest densities in sap from *S. habrochaites* LA2128.

Using the crystal violet attachment assay, we determined that WT *C. michiganensis* was able to aggregate to similar levels in sap from *S. lycopersicum* 'Mt. Fresh' and *S. arcanum* genotypes (Supplementary Fig. S4). Aggregation was severely reduced in sap from *S. habrochaites* LA2128, perhaps because of lower in vitro growth (Fig. 2 and Supplementary Fig. S4). ΔCmEXLX2 formed fewer aggregates in sap from both *S. arcanum* genotypes in comparison with WT but was only significantly lower in sap from *S. arcanum* LA2157 ($P = 0.0058$).

***C. michiganensis* is impeded in lateral spread in wild tomato vascular bundles.** The spread of *C. michiganensis* between xylem vessels of wild tomato species was studied by inoculating plants with eGFP-expressing WT and ΔCmEXLX2 strains and quantifying colonized proto- and metaxylem vessels in primary vascular bundles 21 dpi using laser scanning confocal microscopy (Supplementary Fig. S5). WT and ΔCmEXLX2 colonized similar proportions of protoxylem vessels in all genotypes tested (Table 1). *C. michiganensis* was present in >50% of protoxylem vessels in primary vascular bundles of all four plant genotypes (Table 1). Spread to metaxylem vessels by *C. michiganensis* was highest in the most susceptible genotypes (*S. lycopersicum* 'Mt. Fresh' and *S. arcanum* LA2172) and lowest in the most tolerant genotypes (*S. habrochaites* LA2128 and *S. arcanum* LA2157) (Table 1). Spread of WT *C. michiganensis* to metaxylem vessels was significantly higher than ΔCmEXLX2 in *S. lycopersicum* 'Mt. Fresh' ($P = 0.0012$) and *S. arcanum* LA2172 ($P = 0.0004$), the two genotypes that had more severe symptoms with ΔCmEXLX2.

To achieve a closer view of biofilm-like structures in xylem vessels, we harvested transverse and longitudinal stem sections 1 cm above the inoculation site from plants (12 dpi) inoculated with WT *C. michiganensis* for SEM. Bacterial aggregates were present in protoxylem vessels of all genotypes, and smaller aggregates were found less frequently in metaxylem vessels of *S. lycopersicum* 'Mt. Fresh', *S. habrochaites* LA2128, and *S. arcanum* LA2172 (Fig. 3) but not *S. arcanum* LA2157. *C. michiganensis* aggregates were present in xylem parenchyma and pith cells of *S. lycopersicum* 'Mt. Fresh' and *S. habrochaites* LA2128 (data not shown), but we cannot

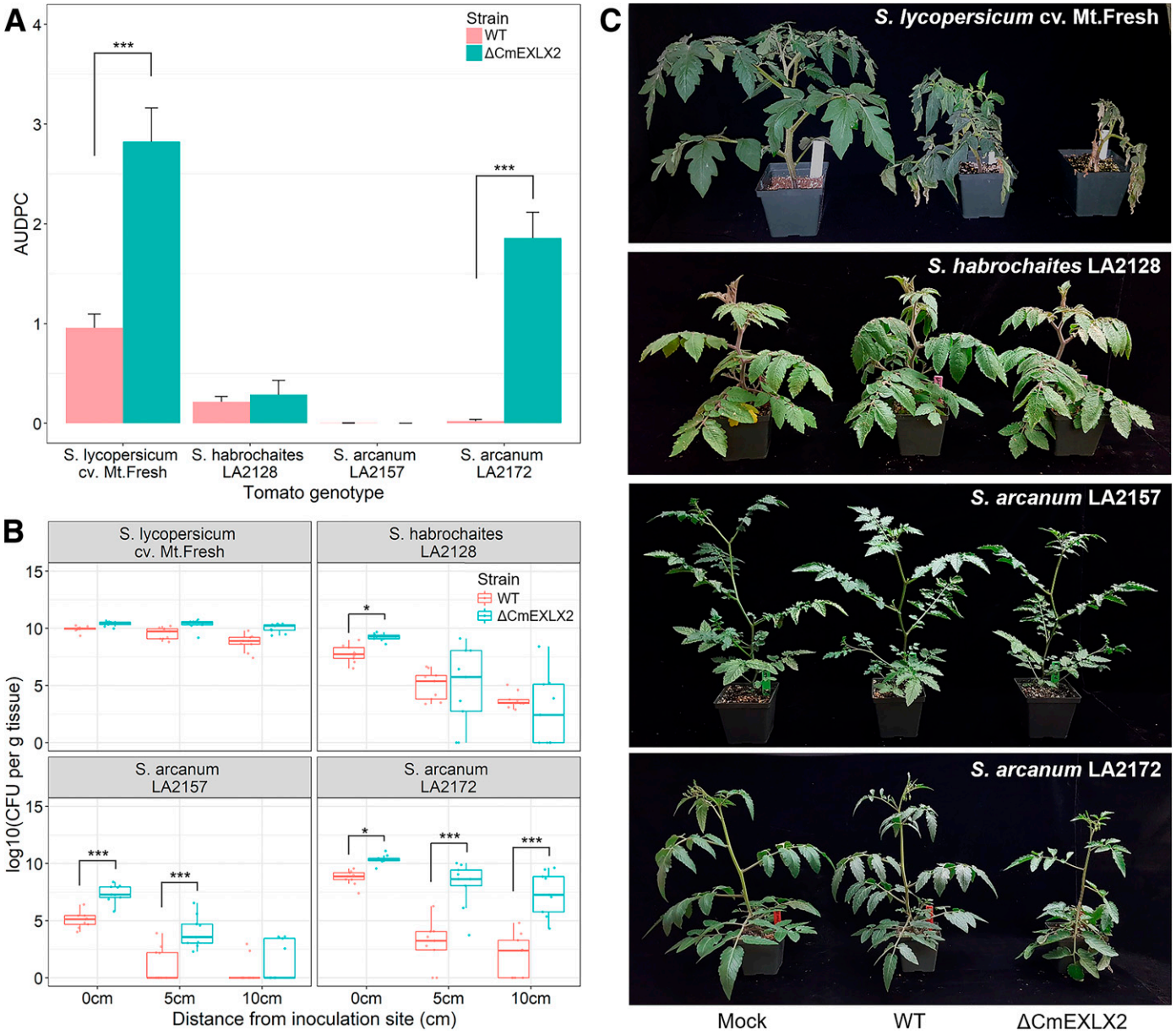

Fig. 1. A, Disease severity as area under the disease progress curve (AUDPC) of all four plant genotypes when inoculated with the wild type (WT) and ΔCmEXLX2. Bars represent standard error, and asterisks indicate significant differences between strains on the host genotype. **B,** Colonization density at the inoculation site and distally as $\log_{10}$ CFU per gram tissue. Asterisks represent significant differences in colonization density between strains at that site. $*P < 0.05$; $***P < 0.001$. **C,** Representative mock (left), WT (center), and ΔCmEXLX2-inoculated (right) plants at 21 days postinoculation.

determine whether this was an artifact of sectioning or part of the natural infection process.

Fruit susceptibility assays demonstrate that susceptibility to *C. michiganensis* is tissue specific. The ability for *C. michiganensis* strains to produce bird's eye lesions on wild tomato fruit was tested between 7 and 9 dpa. Both WT and ΔCmEXLX2 caused characteristic symptoms, which appear as a necrotic center surrounded by a white halo, although the phenotype varied by plant genotype (Supplementary Fig. S6). *C. michiganensis* formed two- to threefold more lesions in *S. lycopersicum* 'Mt. Fresh' fruit in comparison with wild genotypes (Fig. 4C). Lesion severity was determined by measuring the diameter of whole lesions and their necrotic centers. Both WT and ΔCmEXLX2 formed the largest lesions (approximately 1.3-mm diameter) on *S. lycopersicum* 'Mt. Fresh' and *S. arcanum* LA2157 fruit (Fig. 4A and Supplementary Figs. S6 and S7A). Lesion diameter of WT *C. michiganensis* was significantly larger ($P < 0.05$) in comparison with ΔCmEXLX2 lesions in *S. habrochaites* LA2128 and *S. arcanum* LA2172 fruit (Fig. 4A). ΔCmEXLX2 produced significantly larger necrotic lesions in *S. lycopersicum* 'Mt. Fresh' as has been previously reported (Tancos et al. 2018), but the size of the necrotic lesion was reduced relative to WT in *S. habrochaites* LA2128 and *S. arcanum* LA2172 (Fig. 4B).

Fruit from all four genotypes formed bird's eye lesions only when inoculated at early stages of development (7 to 15 dpa). Lesion phenotypes differed between genotypes, with the most striking difference being raised lesions in *S. habrochaites* LA2128 identified in 96% of lesions measured (Fig. 4D and Supplementary Fig. S6).

DISCUSSION

There are no commercially available sources of resistance to bacterial canker of tomato, and we are just beginning to understand the complex interactions between *C. michiganensis* and its hosts. Here, we examined colonization patterns of WT and a hypervirulent mutant *C. michiganensis* strain in xylem and fruit of tolerant wild tomato species. Previous studies characterizing wild tomato tolerance to *C. michiganensis* have inoculated older plants between 4- and 10-true leaf stages, which may prevent more severe symptoms from developing (Sen et al. 2012; Sharabani et al. 2013). To reduce the effect of plant age on assessing tolerance, we inoculated wild tomatoes seedlings during early development (two- to three-true leaf stage). Disease severity assays demonstrated that ΔCmEXLX2 was hypervirulent in *S. lycopersicum* 'Mt. Fresh' and *S. arcanum* LA2172 but not in *S. habrochaites* LA2128 and *S. arcanum* LA2157. ΔCmEXLX2 colonized both *S. arcanum* accessions to higher densities than WT at several distances from the inoculation site, but stem cankers were only present in *S. arcanum* LA2172 inoculated with ΔCmEXLX2. The lack of necrotic cankers

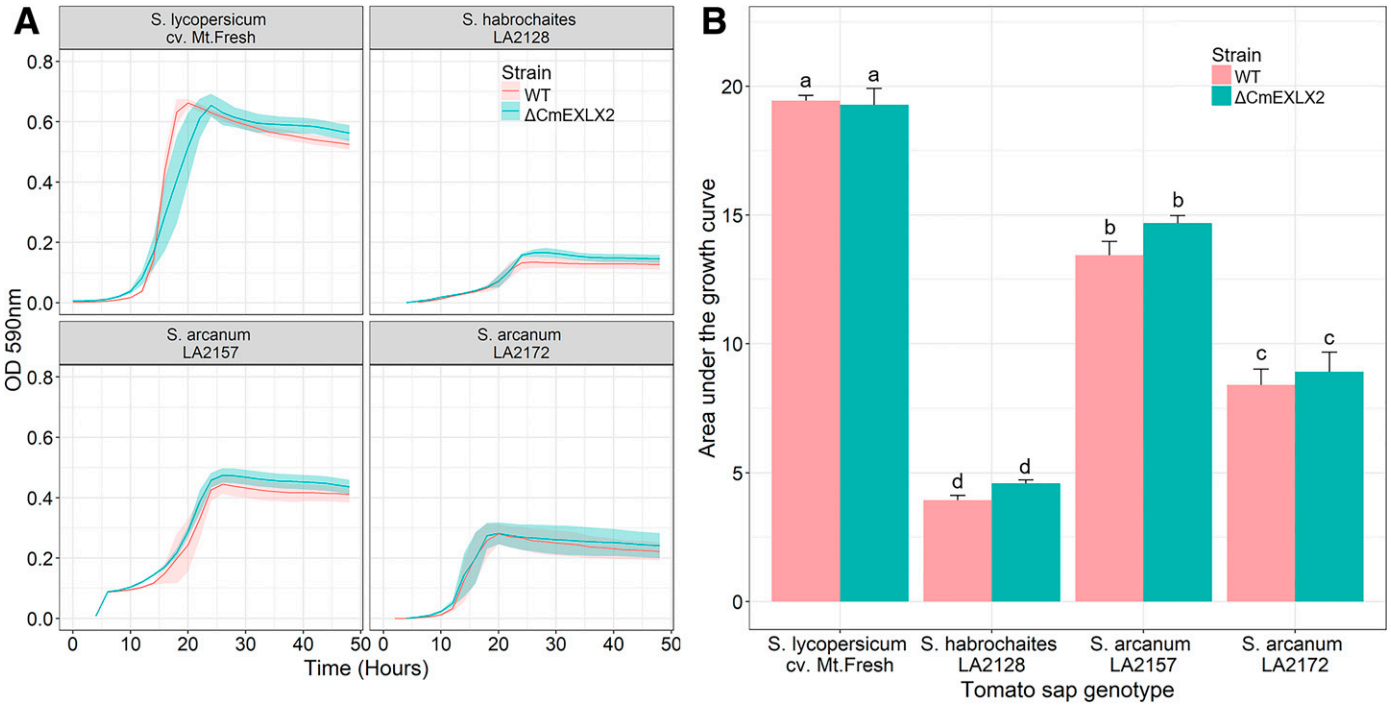

Fig. 2. A, Growth curves of the wild type (WT) and ΔCmEXLX2 cultured in sterile xylem sap collected from healthy plants measured by optical density (OD) at absorbance 590. Ribbon corresponds to 95% confidence intervals. **B,** Area under the growth curve of WT and ΔCmEXLX2 cultured in sterile xylem sap. Bars denote standard error, and letters represent significant differences ($P < 0.05$) between treatment groups using Tukey's pairwise comparisons.

TABLE 1. Percentage of protoxylem and metaxylem vessels infected by eGFP-expressing wild type (WT) and ΔCmEXLX2 *Clavibacter michiganensis* across four tomato genotypes determined using laser scanning confocal microscopy

	Percentage of infected protoxylem vessels[y]		Percentage of infected metaxylem vessels[y]	
Tomato genotype	WT	ΔCmEXLX2	WT	ΔCmEXLX2
Solanum lycopersicum 'Mt. Fresh'	76.0 ± 11.7 a	69.6 ± 3.5 A	38.2 ± 6.5 c[z]	26.6 ± 3.8 C
Solanum habrochaites LA2128	57.2 ± 8.2 a	62.1 ± 7.7 A	9.7 ± 2.7 a	13.8 ± 2.7 B
Solanum arcanum LA2157	58.4 ± 12.7 a	62.3 ± 8.3 A	7.3 ± 4.2 a	5.2 ± 1.5 A
Solanum arcanum LA2172	71.4 ± 8.6 a	64.6 ± 8.0 A	27.7 ± 3.3 b[z]	14.7 ± 1.1 B

[y] Numbers presented are the mean percentage ± standard deviation. Letters represent significant differences ($P < 0.05$) within each strain between plant genotypes. Lowercase letters represent differences or lack thereof between genotypes inoculated with WT. Uppercase letters denote differences or lack thereof between genotypes inoculated with ΔCmEXLX2.

[z] Significant differences ($P < 0.05$) between strains within a single plant genotype.

in *S. arcanum* LA2157 and *S. habrochaites* LA2128 might suggest that *C. michiganensis* is impaired in its ability to macerate stem tissue in some wild tomato genotypes. We still do not understand the molecular mechanisms underlying hypervirulence, but these results suggest that secreted CmEXLX2 (Peritore-Galve et al. 2019) interacts with specific hosts, slowing disease onset and lowering severity of symptoms (Tancos et al. 2018). Functional analysis of a secreted *Pectobacterium carotovorum* expansin protein (PcExl1) determined that cellulolytic and xylanolytic activities of commercial cellulase and xylanase were reduced in celery vascular tissue in the presence of purified PcExl1 (Tovar-Herrera et al. 2018). Taken together, these results suggest that CmEXLX2 may bind plant polysaccharides to prevent hydrolysis. This may be a conserved strategy to slow disease progress or prevent activation of damage-associated molecular pattern-triggered immunity (Choi and Klessig 2016; Gust et al. 2017).

Previous studies of wild tomato tolerance have suggested that vascular morphology may play a role in tolerance to *C. michiganensis* (Coaker et al. 2002). A colonization study in cultivated tomato also demonstrated that *C. michiganensis* preferentially colonized protoxylem vessels during early infection and moved into metaxylem and parenchyma cells at later stages (Chalupowicz et al. 2012). Spread and colonization by WT and ΔCmEXLX2 varied by host genotype. WT and ΔCmEXLX2 colonized *S. lycopersicum* 'Mt. Fresh' to a high density at the inoculation site and distally, whereas colonization and spread were reduced in wild tomato genotypes. The association between *C. michiganensis* populations and symptom severity remains unclear. For example, the plasmid free derivative, Cmm100, is able to colonize and spread in *S. lycopersicum* to similar rates as the pathogenic strain Cmm382 (Meletzus et al. 1993), and other studies of wild tomato tolerance have shown that *C. michiganensis* can colonize stem tissue to high densities without causing severe symptoms (Francis et al. 2001; Sen et al. 2012). Assessment of systemic spread found that WT and ΔCmEXLX2 colonizes stem tissue to lower densities distally from the inoculation site in wild tomato species. We were unable to identify any tyloses or gels that may be obstructing the movement of *C. michiganensis* through the xylem through SEM and confocal microscopy; however, this does not rule out their presence.

Confocal microscopy revealed that both WT and ΔCmEXLX2 were present mainly in protoxylem vessels of wild tomato plants during later stages of infection (21 dpi), which has been observed in *S. lycopersicum* and hypothesized as a strategy to keep its host alive longer (Chalupowicz et al. 2012). SEM images identified larger *C. michiganensis* aggregates in protoxylem vessels relative to metaxylem at 12 dpi for all genotypes except *S. arcanum* LA2157, where *C. michiganensis* was only found in protoxylem vessels. The ability for *C. michiganensis* to colonize higher proportions of metaxylem vessels correlated with disease severity between genotypes but not for differences between strains within a single genotype. A significantly higher proportion of metaxylem vessels was colonized by WT compared with ΔCmEXLX2 in *S. lycopersicum* 'Mt. Fresh' and *S. arcanum* LA2172, which contradicts our hypothesis that metaxylem colonization is correlated to symptom severity. These data highlight the need for additional studies because the way in which ΔCmEXLX2 causes more severe symptoms than WT is unknown. Microscopy data suggest that *C. michiganensis* is impaired in its ability to spread through the vascular system of wild tomatoes, and it is possible that the restriction of *C. michiganensis* movement between xylem vessels and into other stem tissues may contribute to a reduction or elimination of symptoms in wild tomato species. However, further studies are required to delineate *C. michiganensis* colonization of xylem elements.

Biofilms play an important role in bacterial colonization and disease induction. Previous studies have shown that *C. michiganensis* is able to form biofilm-like aggregates in vitro when cultured in *S. lycopersicum* xylem sap but not in LB or minimal medium (Chalupowicz et al. 2012; Tancos et al. 2018). We hypothesized that wild tomato sap composition would hinder growth and aggregate formation in vitro. Xylem sap from wild tomato species sustained lower populations of *C. michiganensis* in vitro. In vivo bacterial populations did not correlate with growth in sap. For example, *C. michiganensis* grew to the lowest densities in *S. arcanum* LA2157 plants, but sap was able to sustain *C. michiganensis* growth at the second highest rate. The inverse was true in *S. habrochaites* LA2128, where sap was only able to sustain low levels of growth of *C. michiganensis* in vitro, but populations were only slightly reduced in planta in comparison with *S. lycopersicum* 'Mt. Fresh'. Contrasting the results of in vitro and in planta growth of *C. michiganensis* in wild tomato accessions suggests that host–*C. michiganensis* interactions are occurring in the xylem, affecting pathogen colonization. Aggregate formation was seemingly growth dependent, but attachment in vitro suggests that sap from wild tomato species provides similar conditions as sap from *S. lycopersicum* that triggers *C. michiganensis* attachment. We hypothesize that host proteins or metabolites are transported into xylem sap in response to *C. michiganensis* infection, which may lead to a reduction of bacterial growth as seen in *S. arcanum* LA2157. Metabolomics of *S. lycopersicum* xylem sap during infection by *Ralstonia solanacearum* found that the pathogen was secreting the polyamine putrescine as a virulence metabolite that altered host physiology (Lowe-Power et al. 2018). Findings in *R. solanacearum* support the idea that the xylem is an environment where pathogen and host interact to determine pathogen colonization.

Tomato fruit infection is an important disease stage for bacterial canker because it causes economic losses by blemishing fruit and can serve as a route for *C. michiganensis* infection of developing seed (Tancos et al. 2013). We tested if *C. michiganensis* would cause lesions on wild tomato fruit, and we found that wild tomatoes were susceptible to *C. michiganensis* infection and formed similar bird's eye lesions. Moreover, *C. michiganensis* was able to form bird's eye

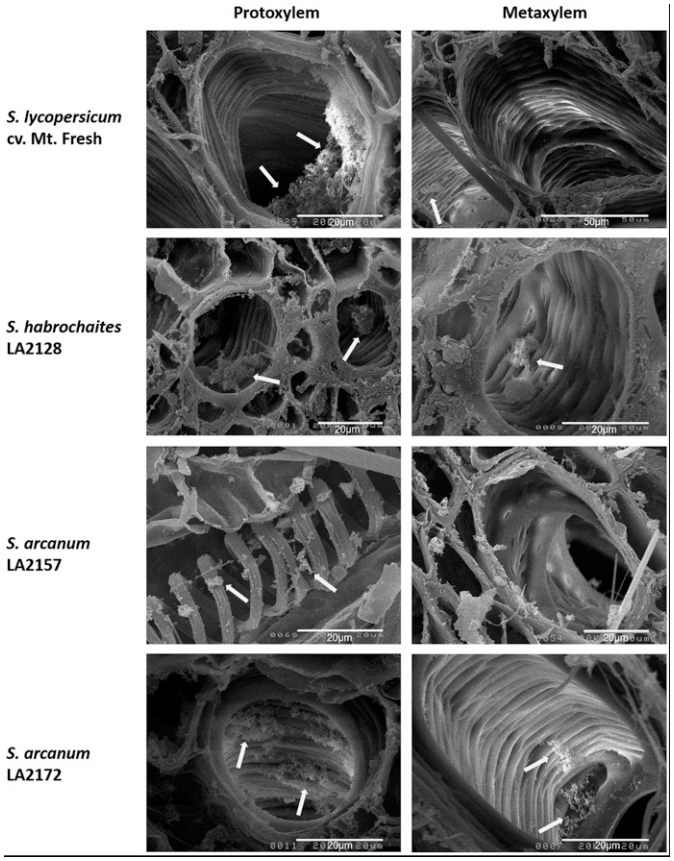

Fig. 3. Scanning electron microscope images of wild-type *Clavibacter michiganensis* aggregates in individual proto- and metaxylem vessels of all four plant genotypes. White arrows highlight bacterial aggregates.

lesions on *S. arcanum* LA2157, which did not develop any symptoms when *C. michiganensis* was inoculated into the vascular system, demonstrating that *C. michiganensis* susceptibility can vary between fruit and vascular infections. Bird's eye lesions forming on fruit from all genotypes seem to suggest that there is a conserved tomato fruit response that causes the lesion phenotype. Interestingly, bird's eye lesions on *S. habrochaites* LA2128 manifested as raised bumps, which under a microscope, seemed to be caused by host cell proliferation at the infection site. Raised lesions that we are hypothesizing to be caused by host cell proliferation might be caused by perturbations in tomato fruit by compounds produced by *C. michiganensis* or through indirect alteration of host physiology as has been observed in other Gram-positive pathogens (Thapa et al. 2019).

In conclusion, our data provide evidence that *C. michiganensis* colonizes vascular and fruit tissues of wild tomato species but is reduced in systemic spread. *C. michiganensis* growth in sap and in planta was not correlated, suggesting that interactions between host and *C. michiganensis* impact pathogen growth in xylem vessels. Confocal microscopy was used to observe preferential protoxylem colonization and reduced spread to metaxylem in wild tomato genotypes, which may play a role in reducing disease severity. Finally, fruit inoculations revealed that asymptomatic responses to *C. michiganensis* can be specific to the inoculated tissue in wild

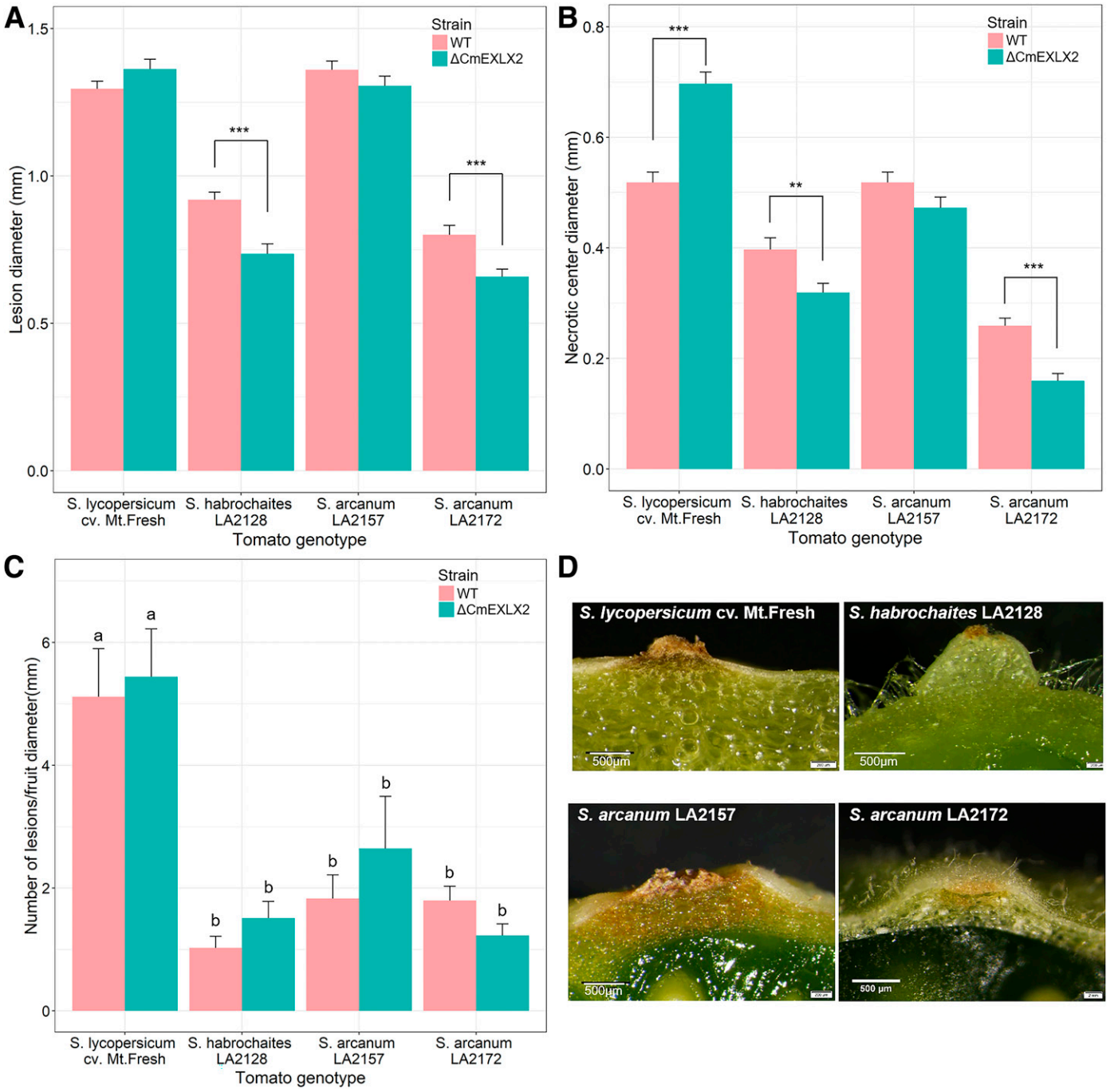

Fig. 4. A, Mean diameter (millimeters) of bird's eye lesions caused by the wild type (WT) and ΔCmEXLX2 on fruit from all four genotypes. **B,** Mean diameter (millimeters) of necrotic centers of bird's eye lesions. **C,** Disease severity on fruit represented as number of lesions per fruit divided by fruit diameter (millimeters). Bars for all graphs represent standard error. Asterisks denote significant differences between strains, and letters denote significant differences ($P < 0.05$) across all treatments as calculated through Tukey's pairwise comparisons. **D,** Transverse cross-sections of fruit lesions on all four genotypes caused by WT *Clavibacter michiganensis* demonstrating phenotypic differences of lesions. Scale bar is 500 µm. $^{**}P < 0.01$; $^{***}P < 0.001$.

tomato species. This is important to know because breeding efforts have focused largely on susceptibility of plants to *C. michiganensis* when inoculated into the vascular system but not onto fruit. Bacterial canker continues to be one of the largest threats to the tomato industry; therefore, understanding the pathology of this Gram-positive bacterium in tolerant and susceptible tomato genotypes can begin to untangle the complex interactions between host and pathogen.

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