Journal of General Plant Pathology (2018) 84:189–201
https://doi.org/10.1007/s10327-018-0775-6

BACTERIAL AND PHYTOPLASMA DISEASES

AlgU contributes to the virulence of *Pseudomonas syringae* pv. *tomato* DC3000 by regulating production of the phytotoxin coronatine

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Received: 25 July 2017 / Accepted: 7 December 2017 / Published online: 29 March 2018

Abstract

Pseudomonas syringae pv. *tomato* DC3000 (*Pst* DC3000), which causes bacterial speck disease of tomato, has been used as a model pathogen to investigate the molecular basis of plant–pathogen interactions. The function of many potential virulence factors encoded in the *Pst* DC3000 genome and their modes of action are not fully understood. *P. syringae* is known to produce the exopolysaccharide alginate. Although AlgU, a sigma factor, is known to regulate the expression of genes such as *algD* related to alginate biosynthesis, the molecular mechanisms of AlgU in the virulence of *Pst* DC3000 is still unclear. To investigate the function of AlgU and alginate in plant–bacterial pathogen interactions, we generated Δ*algU* and Δ*algD* mutants. After inoculation with Δ*algU* but not Δ*algD*, host plants of *Pst* DC3000 including tomato and *Arabidopsis* had milder disease symptoms and reduced bacterial populations. Expression profiles of *Pst* DC3000 genes revealed that AlgU can regulate not only the expression of genes encoding alginate biosynthesis, but also the expression of genes related to type III effectors and the phytotoxin coronatine (COR). We also demonstrated that the Δ*algU* mutant showed full virulence in the *Arabidopsis fls2 efr1* double mutant, which is compromised in the recognition of PAMPs. Further, the application of COR was able to restore the phenotype of the Δ*algU* mutant in the stomatal response. These results suggest that AlgU has an important role in the virulence of *Pst* DC3000 by regulating COR production.

Keywords *Pseudomonas syringae* pv. *tomato* · Tomato · *Arabidopsis thaliana* · AlgU · Coronatine · PAMP-triggered immunity · Stomatal-based defense

Introduction

In all natural environments, plants are constantly exposed to a large number of microorganisms, including potential pathogens, and beneficial and saprophytic microorganisms. Plants have not acquired an immune system like that of animals. Therefore, they have developed monitoring systems that recognize potential invading pathogens and a wide range of immune responses to defend themselves (Hacquard et al. 2017; Jones and Dangl 2006). The first line of plant defense against invading pathogens is pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI), which recognizes conserved molecules such as flagellin and elongation factor Tu (EF-Tu) of the invading pathogens using plant pattern-recognition receptors (PRRs), such as FLS2 and EFR, respectively (Zipfel 2008; Zipfel and Felix 2005; Zipfel et al. 2006). After recognition of invading pathogens with PRRs, plants activate a number of defense mechanisms including stomatal-based defense, rapid oxidative burst, restriction of nutrient transfer from the cytosol to the apoplastic space, the accumulation of antimicrobial compounds including phytoalexins, and the activation of hormone-mediated signaling pathways leading to defense responses (Ahuja et al. 2012; Bednarek 2012; Berens et al. 2017; Chen et al. 2010; Cowan 1999; Melotto et al. 2006, 2008; O'Brien et al. 2012; Sawinski et al. 2013; Wang et al. 2012). Stomatal-based defense to restrict the entry of bacterial pathogens by stomatal closure is one of the earliest responses in PTI (Melotto et al. 2008; Underwood et al. 2007). However, successful bacterial pathogens have evolved to acquire multiple virulence factors such as phytotoxins and type III secretion system (TTSS)

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10327-018-0775-6) contains supplementary material, which is available to authorized users.

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effectors to overcome PTI and stomatal-based defense (Lozano-Durán et al. 2014; Melotto et al. 2017).

Pseudomonas syringae is a bacterial pathogen that causes economically important plant diseases (Mansfield et al. 2012; Xin and He 2013). It can induce a wide range of symptoms such as leaf blight, spots, galls, and wilt on different plants and can be classified into > 50 pathovars (pv.) based on their host plant specificities and the type of disease symptoms. *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst* DC3000), which causes bacterial speck disease of tomato, has been used as a model pathogen to investigate the molecular basis of plant–pathogen interactions because of its genetic tractability and its pathogenicity on *Arabidopsis* (Ishiga et al. 2011; Katagiri et al. 2002; Xin and He 2013). It is predicted that *Pst* DC3000 has over 200 virulence-related genes based on the whole genome sequence, including genes related to TTSS effectors, the phytotoxin coronatine (COR), extracellular polysaccharides (EPS), siderophores, and tolerance to reactive oxygen species (ROS) (Buell et al. 2003; Xin and He 2013). A comprehensive search for TTSS effectors has revealed > 30 effector molecules in the *Pst* DC3000 genome (Cunnac et al. 2009). Recent studies have revealed that several effectors including HopM1, HopF2, HopX1, AvrB, HopZ1, and XopR play an important role in overcoming PTI and stomatal-based defense in *Arabidopsis* (Gimenez-Ibanez et al. 2014; Hurley et al. 2014; Jiang et al. 2013; Lozano-Durán et al. 2014; Wang et al. 2016; Zhou et al. 2015). In addition to these effectors, COR has also been reported to suppress PTI resulting in stomatal reopening during *Pst* DC3000 infection (Lee et al. 2013; Melotto et al. 2006, 2008; Wasternack 2017; Zheng et al. 2012). The *Arabidopsis fls2 efr1* double mutant showed enhanced disease susceptibility to a COR-defective mutant because of the lack of stomatal-based defense (Zeng and He 2010). Further, when a COR-defective mutant was used to infiltrate the apoplastic space, thus bypassing stomatal-based defense, this mutant induced typical disease symptoms in *Arabidopsis*, indicating an important role for COR in overcoming stomatal-based defense (Melotto et al. 2006). Although there is a good understanding of the importance of TTSS effectors and COR in the virulence of *Pst* DC3000, the function of a large number of potential virulence factors encoded in the *Pst* DC3000 genome and their modes of action are not fully understood.

Pseudomonas syringae is considered to have two lifestyles, including an epiphytic phase on the surface of plant leaves and an endophytic phase in the apoplastic space (Melotto et al. 2008; Xin and He 2013). The epiphytic phase of *P. syringae* is one of the first steps in the infection process. Epiphytic fitness has been characterized as a virulence factor based on studies of *P. syringae* pv. *syringae* B728a (*Pss* B728a) (Yu et al. 2013, 2014). The exopolysaccharide alginate, a copolymer of mannuronic and guluronic acid and the major EPS of *P. syringae* has been reported to play a role during the epiphytic phase by protecting bacterial cells against external stresses such as oxidative and UV stresses (Keith et al. 2003; Laue et al. 2006; Schenk et al. 2006; Yu et al. 1999). Kidney bean inoculated with a *Pss* B728a *algT* mutant, deficient in the production of alginate, had reduced disease symptoms together with bacterial growth compared to the wild type (Yu et al. 1999). In addition, *Pss* B728a has a very pronounced epiphytic phase on plants, whereas *Pst* DC3000 does not colonize the leaf surface (Xin and He 2013). *P. syringae* is thought to be able to shift from the epiphytic phase to the endophytic phase during a successful infection cycle. One of the major virulence factors in *P. syringae* needed to make this shift includes effector proteins, which are delivered into the host through the TTSS to suppress PTI and to facilitate disease development (Brooks et al. 2005; Lindeberg et al. 2012; Nomura et al. 2005; Uppalapati et al. 2007). Not only type III effectors, but also phytotoxins function overcome PTI and stomatal-based defense during the transition from the epiphytic phase to the endophytic phase (Melotto et al. 2006; Uppalapati et al. 2007). However, we still have much to discover about the mechanisms of this bacterial pathogen transition because of the lack of information about the virulence factors related to infection.

In the present study, we demonstrated that the *Pst* DC3000 sigma factor AlgU regulates not only the expression of genes related to alginate biosynthesis, including *algD*, but also the expression of genes related to virulence, including TTSS effectors and the phytotoxin COR. Furthermore, the *Pst* DC3000 Δ*algU*, but not the Δ*algD* mutant showed reduced virulence during host plant infection, indicating that alginate may not have a role in virulence in *Pst* DC3000. Thus, our results suggest the importance of the downstream components of AlgU, such as COR, to overcome stomatal-based defense in the virulence of *Pst* DC3000.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana seedlings and *Solanum lycopersicum* cv. Glamour plants were used for the pathogenicity assay. *A. thaliana* ecotype Colombia (Col-0) was used as the wild-type plant in this study. The *fls2 efr1* line was obtained from Dr. Saijo (Tintor et al. 2013). Briefly, *A. thaliana* seeds were germinated and grown on 1/2 strength Murashige and Skoog (MS) medium (0.3% phytagel) with Gamborg vitamins (Sigma-Aldrich, St. Louis, MO, USA). *A. thaliana* plants were incubated in a growth chamber at 24 °C with a light intensity of 200 μE m^{-2} s^{-1} and a 12 h light/12 h dark photoperiod and used 2 weeks after germination for the pathogen assays. Tomato plants were used for pathogen

assays 4 weeks after germination and subsequent growth at 24 °C with a light intensity of 200 µE m^{-2} s^{-1} and a 12 h light/12 h dark photoperiod.

Bacterial strains and growth conditions

Pseudomonas syringae pv. *tomato* strain DC3000 (*Pst* DC3000) was used as the pathogenic strain to inoculate tomato and *Arabidopsis*. Wild-type (WT) *Pst* DC3000; mutants Δ*algU*, Δ*algD*, and COR-deficient DB29; and an *algU*-complemented line (Δ*algU/algU*) were grown at 28 °C on mannitol-glutamate (MG; Keane et al. 1970) or King's B (KB; King et al. 1954) media containing 25 µg/ml of rifampicin. Before inoculation, bacteria were suspended in sterile distilled H_2O, and the bacterial cell densities at 600 nm (OD_{600}) were measured using a JASCO V-730 spectrophotometer (JASCO, Tokyo, Japan).

Generation of Δ*algU* and Δ*algD* mutants

The genetic regions containing *algU* and *algD* and the surrounding regions were amplified using sets of PCR primers (4224_1: 5′-CGCGCTGTATGTATCCACTT-3′ and 4224_2: 5′-GCCATATCGATGCTGGTCTT-3′ for *algU*, and 1243_1: 5′-CTGCTCAACCTTGCCTCTAC-3′ and 1243_2: 5′-AACCAGCTGGACTTGTCATC-3′ for *algD*) that were designed based on the registered sequence of *Pst* DC3000 (NC_004578) with PrimeStar HS DNA polymerase (TaKaRa, Otsu, Japan), then dA was added to the 3′ end of the PCR product with 10× A-attachment mix (TOYOBO, Osaka, Japan). The resultant DNA was inserted into the pGEM-T Easy vector (Promega, Madison, WI, USA). The recombinant plasmid DNA was then used to obtain pGEM-*algU* and pGEM-*algD* as templates, and inverse PCR was carried out using a set of primers (4224_3: 5′-cgggatccTCC TCTTCCTGGGTTAGCAT-3′; 4224_4: 5′-cgggatccGTT GTTGCAGGAATCCTGA-3′ for *algU*, and 1243_3: 5′- cgggatccCCAAATATGCTGATACGCAT-3′; 1243_4: 5′-cgggatccGAAGGTATCTGCTGGTAA-3′for *algD*; lowercase letters indicate the artificial sequence for the digestion by BamHI) to delete an open reading frame for *algU* and *algD*. Then, the PCR product and template DNA were digested with BamHI and DpnI. The resultant DNA was self-ligated with T4 DNA ligase (Ligation-Convenience kit, Nippon Gene, Tokyo, Japan). The *algU*- and *algD*-deleted DNA constructs were introduced into the EcoRI site of the mobilizable cloning vector pK18*mobsacB* (Schäfer et al. 1994). The resulting plasmids containing the DNA fragment lacking *algU* and *algD* were then used to transform *E. coli* S17-1. The deletion mutant was obtained by conjugation and homologous recombination according to the method previously reported (Shimizu et al. 2003). Transconjugants were selected on KB agar containing 30 µg/ml of kanamycin (Km) and 30 µg/ml of rifampicin (Rif). We generated mutants by incubating the transconjugants on a KB agar plate containing 25 µg/ml rifampicin and 10% sucrose. The specific deletions in the Δ*algU* and Δ*algD* mutants were confirmed by PCR using the primers 4224_1 and 4224_2 for Δ*algU* and 1443_1 and 1443_2 for Δ*algD*.

To confirm whether the altered phenotype of the Δ*algU* mutant originates from a corresponding mutation, an *algU*-complemented strain was generated. The *algU*-containing DNA fragment from pGEM-*algU* was transferred into the vector pDSK519 (Keen et al. 1988) at the SacI and SphI sites to generate pDSK-*algU*. The pDSK-*algU* construct was introduced into the Δ*algU* mutant by electrophoresis to generate the complemented strain Δ*algU/algU*.

Growth curve assay

Pst DC3000 strains including the wild type, Δ*algU* mutant, and complemented line Δ*algU/algU* were grown at 28 °C for 24 h in KB broth. The strain suspensions were adjusted to an OD_{600} of 0.1 with fresh KB broth, and the bacterial growth dynamics were measured at OD_{600} for 24 h.

Spray- and flood-inoculation methods

Spray inoculations were used to observe disease symptoms on tomato leaves as described previously (Uppalapati et al. 2007). Briefly, plants were sprayed with a bacterial suspension (OD_{600} = 0.1) in sterile distilled water containing 0.025% Silwet L-77 (OSI Specialties, Danbury, CT, USA) until runoff. The plants were then incubated in growth chambers at ~ 100% RH for the first 24 h, then at ~ 70% RH for the rest of the experimental period. The inoculated plants were observed for 7 dpi for symptom development.

A flood-inoculation method was used to observe disease symptoms in *Arabidopsis* as described previously (Ishiga et al. 2011). Briefly, 40 ml of bacterial suspension made in sterile distilled H_2O containing 0.025% Silwet L-77 was dispensed onto a plate containing 2-week-old *Arabidopsis* seedlings for uniform inoculation, then the plates were incubated for 2–3 min at room temperature. After the bacterial suspension was decanted from the plates, the plates were sealed with 3M Micropore 2.5 cm surgical tape (3M, St. Paul, MN, USA) and incubated at 24 °C with a light intensity of 200 µE m^{-2} s^{-1} and 12 h light/12 h dark.

To assess bacterial growth in tomato leaves and *Arabidopsis* seedlings, we measured the internal bacterial population at several times. For determining internal bacterial growth, the inoculated leaves (tomato) and seedlings (*Arabidopsis*) were collected, and the total mass of each was measured, then the leaves and seedlings were surface-sterilized with 5% H_2O_2 for 3 min, then washed three times with sterile distilled water. The plants were then homogenized in sterile

distilled water, and the diluted samples were plated onto solid MG agar medium. The bacterial colony forming units (CFU) were normalized as CFU/mg using the total mass of the inoculated leaf and seedling samples. The bacterial population at 0 dpi was estimated using leaves harvested 1 hpi. The bacterial populations were evaluated in three independent experiments.

Real-time quantitative RT-PCR

For expression profiles of *Pst* DC3000 genes, bacteria were grown in KB broth for 24 h, then adjusted to OD_{600} of 0.2 with fresh MG broth and grown for 2 h. To analyze the expression profiles of *Pst* DC3000 genes during infection, we flood-inoculated *Arabidopsis* wild-type Col-0 plants with WT *Pst* DC3000, Δ*algU*, or Δ*algU/algU* at 5×10^6 CFU/ml for 3, 6, 12, 24, and 48 h, then the total RNAs including plant and bacterial RNAs were extracted from infected leaves and purified. Mixed plant–bacterial RNA populations were utilized for the expression profiles without bacterial RNA enrichment. Total RNA extraction and real-time quantitative RT-PCR (RT-qPCR) were done as described previously (Ishiga and Ichinose 2016). Total RNA was extracted using RNAiso Plus (TaKaRa) according to the manufacturer's protocol. Two micrograms of total RNA was treated with gDNA Eraser (TaKaRa) to eliminate genomic DNA, and the DNase-treated RNA was reverse transcribed using the PrimeScript RT reagent Kit (TaKaRa). The cDNA (1:20) was then used for RT-qPCR using the primers shown in Supplementary Table S1 with SYBR Premix Ex Taq II (TaKaRa) on a Thermal Cycler Dice Real Time System (TaKaRa). *Pst* DC3000 *oprI* (outer membrane lipoprotein precursor), *proC* (pyrroline-5-carboxylate reductase), and *rpoD* (RNA polymerase sigma factor) were used as internal controls to normalize gene expression. The *Arabidopsis UBIQUITIN EXTENSION PROTEIN 1* (*UBQ1*) was used as an internal control to normalize gene expression in *Arabidopsis*. The average CT values were calculated using the 2nd derivative maximum method, and triplicate samples were used to determine fold expression relative to the controls.

Stomatal assay

A modified method was used to assess stomatal response as described previously (Chitrakar and Melotto 2010). Briefly, *Arabidopsis* and tomato plants were grown for 4 weeks after germination as described before. *Pst* DC3000 strains including the wild type, the Δ*algD* mutant, the Δ*algU* mutant, DB29, and Δ*algU/algU* were grown at 28 °C for 48 h on MG agar. The strains were adjusted to OD_{600} of 0.2 in sterile distilled water. Dip-inoculated leaves with or without COR (100 ng/ml; Sigma-Aldrich) were directly imaged using a Leica TCS SP8 confocal microscope equipped with a white light laser (Leica, Wetzlar, Germany). A reflected image of the leaf surface was obtained by illuminating the sample with 561 nm wavelength, and reflected light was detected through a 558–566 nm filter. The stomatal aperture (width) of at least 60 stomata was measured. The average and standard error for the stomatal aperture width were calculated. The stomatal apertures were evaluated in three independent experiments.

Results

Pst DC3000 AlgU is required for full virulence in tomato and *Arabidopsis*

To assess the importance of AlgU and AlgD in the virulence of *Pst* DC3000, we constructed an *algU* mutant (Δ*algU*) and *algD* mutant (Δ*algD*). In the growth curve assay for *Pst* DC3000 wild-type (WT), Δ*algU*, and Δ*algD* in KB and MG broth, the populations of the strains did not differ significantly (Supplementary Fig. 1a, b). Next, we challenged tomato and *A. thaliana* with *Pst* DC3000 WT, Δ*algU*, and Δ*algD*, and observed disease progression. Both WT *Pst* DC3000 and Δ*algD* caused typical necrotic cell death lesions with severe chlorosis on tomato leaves at 7 dpi; however, Δ*algD*-inoculated leaves developed milder chlorosis (Fig. 1a). On the other hand, tomato leaves inoculated with Δ*algU* appeared healthy with reduced necrotic cell death and chlorosis compared with WT *Pst* DC3000 (Fig. 1a). In tomato leaves inoculated with Δ*algU*, bacterial populations were significantly lower than for WT *Pst* DC3000 and Δ*algD* (Fig. 1b).

Pst DC3000 is pathogenic not only to tomato, but also to *A. thaliana* (Ishiga et al. 2011), so did the same inoculation tests using *A. thaliana*. Consistent with the results for tomato, WT *Pst* DC3000 caused typical water soaking lesions with severe chlorosis, whereas Δ*algU* caused reduced disease symptoms with less chlorosis at 3 dpi (Fig. 2a). Interestingly, *Arabidopsis* plants inoculated with Δ*algD* developed less severe symptoms (Fig. 2a), and the Δ*algU* populations were significantly lower compared to those of WT *Pst* DC3000 and Δ*algD* (Fig. 2b). Taken together, these results clearly indicate that AlgU has a critical role in the virulence of *Pst* DC3000, whereas AlgD may partially contribute to virulence in tomato and *Arabidopsis*.

AlgU regulates the expression of virulence genes in *Pst* DC3000 in broth

It has been reported that AlgU activates the transcription of target genes related to alginate synthesis, virulence, motility, and oxidative stress (Markel et al. 2016). We thus investigated the expression profiles of these genes in

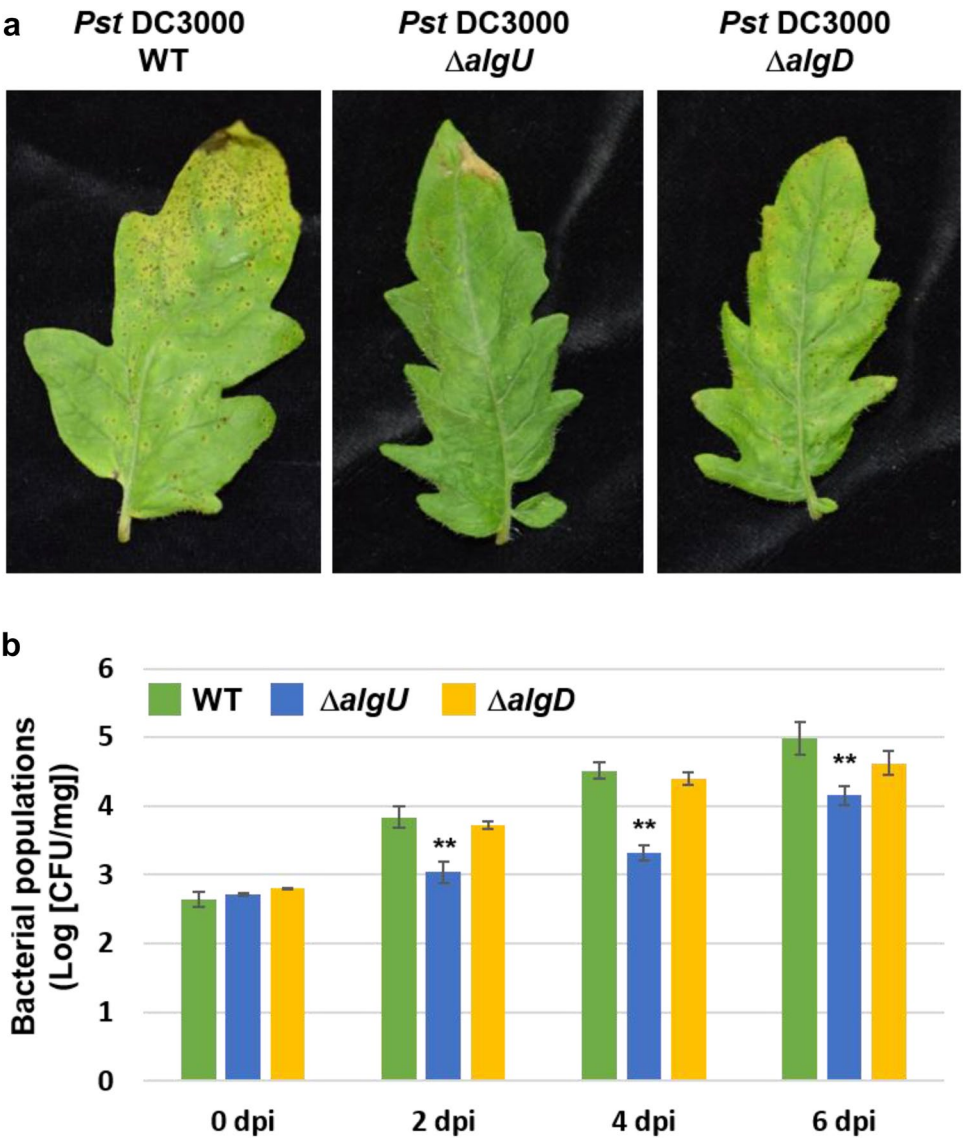


Fig. 1 Disease symptoms on tomato leaves and bacterial population dynamics in leaves after 4-week-old plants were sprayed with 1×10^8 colony forming units (CFU)/ml of the wild-type (WT) *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000), the Δ*algU* mutant (Δ*algU*), or the Δ*algD* mutant (Δ*algD*). Bacterial populations in leaves were estimated by dilution plating on selective medium as described in the methods. **a** Chlorosis and necrotic lesions on tomato leaves at 7 days post inoculation (dpi). **b** Population levels at 0, 2, 4, and 6 dpi. Vertical bars indicate standard error for three independent experiments. Asterisks indicate a significant difference from WT in a *t* test (**$P < 0.01$). (Color figure online)

WT *Pst* DC3000, Δ*algU*, and *algU*-complemented line (Δ*algU/algU*) using RT-qPCR and gene-specific primer sets (Supplementary Table S1) Our results demonstrated that the expression of *algU* was higher in Δ*algU/algU* than in WT *Pst* DC3000 (Fig. 3a), perhaps because of the higher copy number of the *algU* gene after complementation with pDSK519 plasmid. The expression of *algD* was induced in the WT *Pst* DC3000 and the Δ*algU/algU* culture, but repressed in the Δ*algU* culture (Fig. 3b), indicating that AlgU can positively regulate genes related to alginate biosynthesis.

TTSS effectors and the phytotoxin COR play central roles in the virulence of *Pst* DC3000 by suppressing plant immunity and also facilitating disease development in host plants (Buell et al. 2003; Xin and He 2013). To investigate the importance of AlgU in the regulation of genes related to virulence, we analyzed the expression profiles of genes encoding transcriptional regulators of the TTSS effectors and the COR biosynthesis genes, including *hrpL, cfl*, and *cmaB*. The expression of *hrpL, cfl*, and *cmaB* was clearly suppressed in Δ*algU* compared to their levels in WT *Pst* DC3000 and the Δ*algU/algU* (Fig. 3c–e). These results suggest that AlgU may function in modulating the expression of the genes related to the TTSS and COR biosynthesis.

Virulence of the Δ*algU* mutant was impaired in *Arabidopsis* (Fig. 2). Because PRRs such as FLS2 and EFR can recognize flagellin and EF-Tu, respectively (Zipfel 2008; Zipfel and Felix 2005; Zipfel et al. 2006), then the reduced virulence of the Δ*algU* mutant might result from increased production of PAMPs, including flagellin and EF-Tu, rather than from the reduced expression of virulence genes. To rule out this possibility, we confirmed the expression profiles of *fliC* and *tuf*, encoding flagellin and EF-Tu, respectively. As

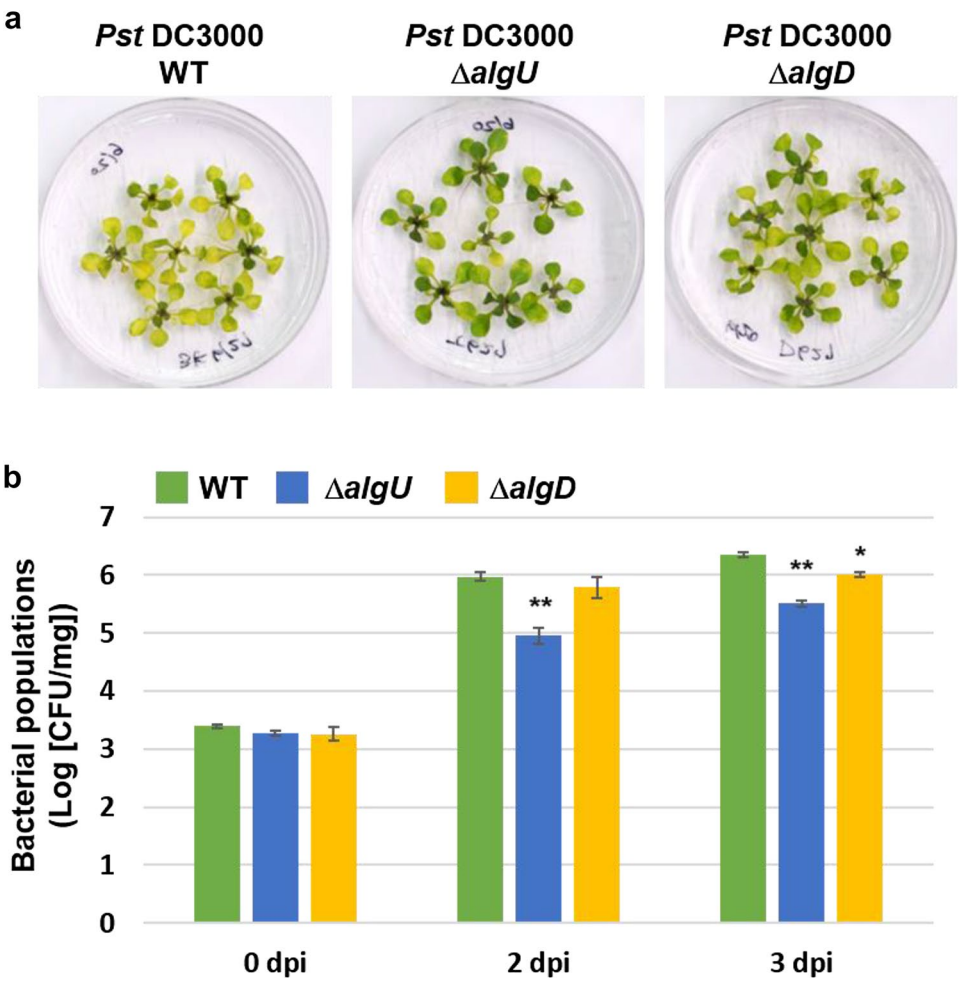


Fig. 2 Disease symptoms and bacterial population dynamics in *Arabidopsis* plants flood-inoculated with a suspension of 5×10^6 CFU/ml of the wild-type (WT) *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000), the Δ*algU* mutant (Δ*algU*), or the Δ*algD* mutant (Δ*algD*). 2-week-old *Arabidopsis* plants were flood-inoculated, and bacterial populations were estimated by dilution plating on selective medium as described in the methods. **a** Water-soaked lesions with chlorosis at 3 days post-inoculation (dpi). **b** Population levels at 0, 2, and 3 dpi. Vertical bars indicate the standard error for three independent experiments. Asterisks indicate a significant difference from WT in a *t* test (*$P < 0.05$; **$P < 0.01$). (Color figure online)

shown in Fig. 3f, g, the expression of *fliC* and *tuf* did not differ significantly between WT *Pst* DC3000 and Δ*algU*.

AlgU regulates the expression of virulence genes during *Pst* DC3000 infection of *Arabidopsis*

Since the virulence of Δ*algU* was reduced in tomato and in *Arabidopsis* (Figs. 1, 2), we next determined the expression profiles of the *Pst* DC3000 genes involved in virulence during infection. In this assay, *Arabidopsis* wild-type Col-0 plants were flood-inoculated with WT *Pst* DC3000, Δ*algU*, or the Δ*algU/algU*, and at 3, 6, 12, 24, and 48 hpi, total RNAs (plant and bacterial) were purified for real-time RT-qPCR. The expression of the *Pst* DC3000 genes was not detected in the noninoculated *Arabidopsis* control plants (data not shown).

The expression of *algD* was significantly suppressed in *Arabidopsis* plants inoculated with Δ*algU* in comparison with the WT *Pst* DC3000 (Fig. 4a, b). The expression of *hrpL* was induced in *Arabidopsis* inoculated with WT *Pst* DC3000 at 24 hpi; however, *hrpL* transcripts were differentially regulated during Δ*algU* infection compared with WT *Pst* DC3000 (Fig. 4c). Interestingly, the expression of *cfl* and *cmaB* were suppressed in plants inoculated with Δ*algU* at 3 and 6 hpi in comparison with the WT *Pst* DC3000 (Fig. 4d, e). These results suggest that AlgU can positively regulate the transcription of *hrpL* and COR-related genes during *Pst* DC3000 infection.

The expression of *fliC* was induced at 12 hpi in plants inoculated with the WT *Pst* DC3000 and Δ*algU* and at late stages of Δ*algU* infection (Fig. 4f). The expression of *katE*, but not *oxyR*, was induced during late infection by WT *Pst* DC3000; however the transcript level of *katE* was lower in *Arabidopsis* plants inoculated with Δ*algU* than after inoculation with WT *Pst* DC3000 (Fig. 4h), suggesting that AlgU can positively regulate the expression of oxidative stress tolerance genes.

The Δ*algU* mutant displays full virulence on the *Arabidopsis fls2 efr1* mutant

The interactions between *P. syringae* and *Arabidopsis* have been utilized to investigate the molecular basis of plant defense responses against pathogens and have revealed a

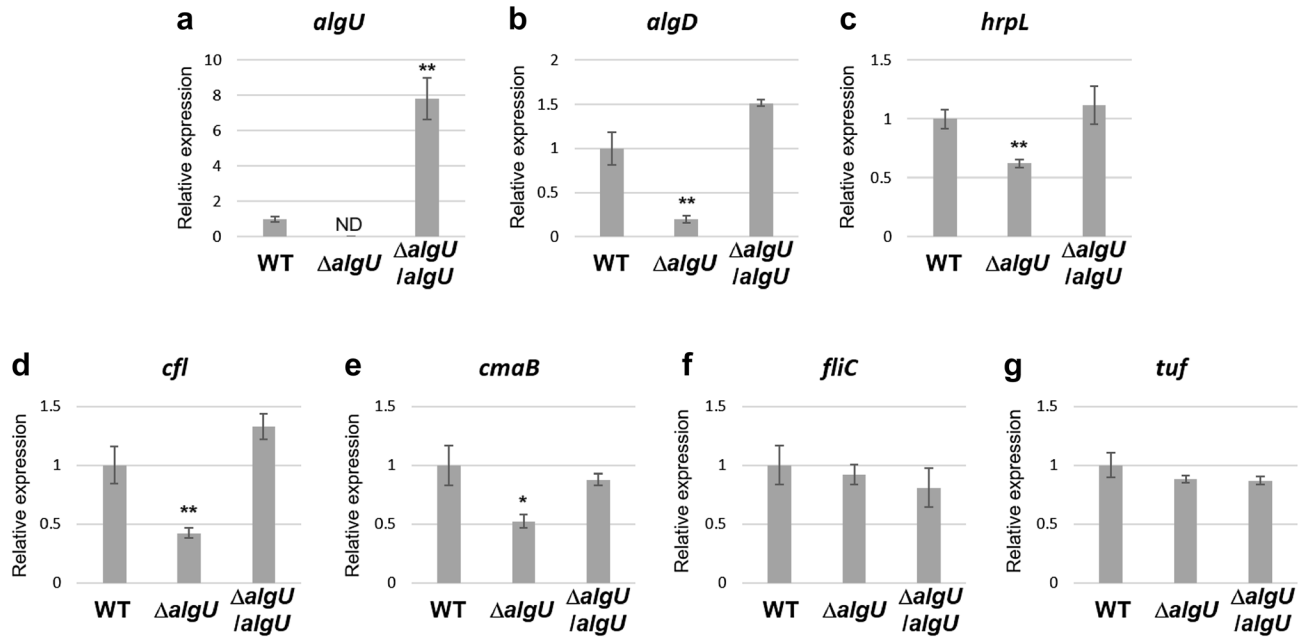
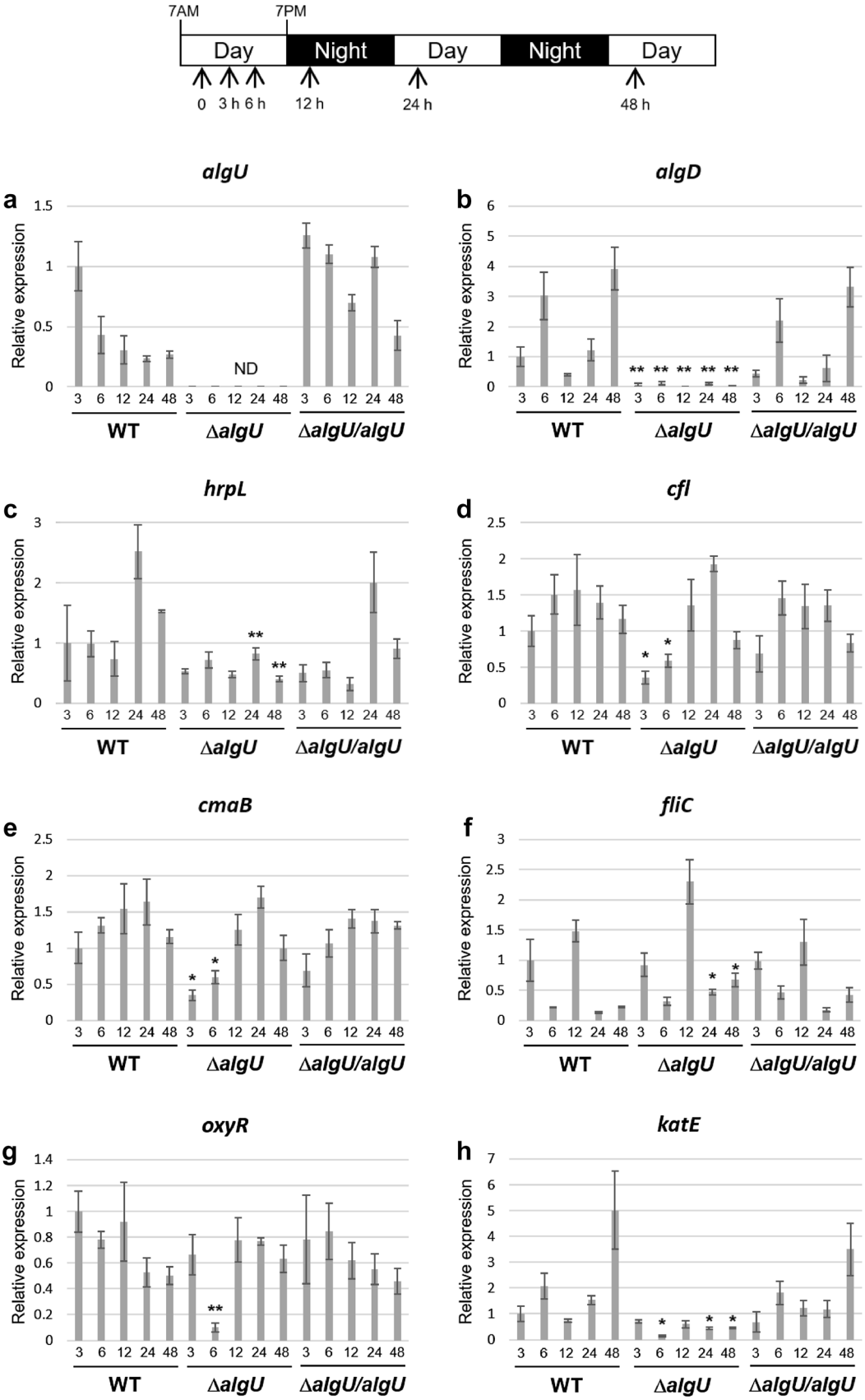


Fig. 3 Expression profiles of genes involved in the virulence of wild-type (WT) *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000), the Δ*algU* mutant (Δ*algU*), and *algU*-complemented line (Δ*algU/algU*) in liquid mannitol-glutamate (MG) broth. WT *Pst* DC3000, Δ*algU*, and Δ*algU/algU* were grown in MG broth to an OD_{600} of 0.2. Gene expression of selected *Pst* DC3000 genes was normalized using housekeeping genes *oprI, proC*, and *rpoD* by real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR) with gene-specific primer sets (Supplementary Table S1). **a** *algU*, **b** *algD*, **c** *hrpL*, **d** *cfl*, **e** *cmaB*, **f** *fliC*, and **g** *tuf*. Vertical bars indicate the standard error for three biological replicates. Asterisks indicate a significant difference from WT in a *t* test (*$P < 0.05$; **$P < 0.01$). ND indicates not detected by RT-qPCR

number of key regulators for signaling pathways (Chisholm et al. 2006; Gimenez-Ibanez and Rathjen 2010; Ishiga et al. 2011; Macho and Zipfel 2014; Xin and He 2013). We next determined the expression profiles of the plant defense marker genes including *AtPR1* and *AtPR2* during infection. In this assay, *Arabidopsis* wild-type Col-0 plants were flood-inoculated with WT *Pst* DC3000 or Δ*algU* for 6, 12, 24, and 48 h, and then the total RNAs including plant and bacterial RNAs were purified for real-time RT-qPCR. The expression of *AtPR1* was induced in plants inoculated with WT *Pst* DC3000 and the Δ*algU* mutant, however the expression levels of *AtPR1* were greater in plants inoculated with the Δ*algU* mutant at 24 hpi compared to those inoculated with WT *Pst* DC3000 (Supplementary Fig. S2a). Moreover, the expression levels of *AtPR2* were greater in plants inoculated with the Δ*algU* mutant at 48 hpi compared to those inoculated with WT *Pst* DC3000 (Supplementary Fig. S2b).

Plants can recognize the bacterial invasion by PRRs leading to PTI (Zipfel 2008; Zipfel et al. 2004, 2006). To investigate the virulence of Δ*algU* on *Arabidopsis* mutants compromised in PTI, we utilized a PRRs mutant, *fls2 efr1*. Interestingly, the bacterial growth revealed enhanced susceptibility of the *fls2 efr1* mutant to the Δ*algU* mutant, whereas the populations of WT *Pst* DC3000 were comparable to those in the wild-type Col-0 (Fig. 5a, b). Moreover, the Δ*algU* mutant multiplied to almost the same levels as WT *Pst* DC3000 (Fig. 5b), indicating that the Δ*algU* mutant displays full virulence on the *Arabidopsis fls2 efr1* mutant. These results suggest that the virulence factors regulated by AlgU can affect FLS2/EFR-dependent PTI during *Pst* DC3000 infection.

The stomatal-based defense mechanism in plants is responsible for closing stomata in response to the perception of PAMPs of invading pathogens (Melotto et al. 2017). In turn, bacterial COR and TTSS effectors can prevent this PAMP-triggered stomatal closure (Melotto et al. 2017). To investigate PTI, especially stomatal-based defense, we examined the stomatal response after inoculation with WT *Pst* DC3000, the Δ*algU* mutant, and the COR-deficient mutant DB29 in *Arabidopsis*. WT *Pst* DC3000 was able to cause the stomata to reopen, whereas stomatal closure of *Arabidopsis* was triggered after inoculation with DB29 and Δ*algU* at 4 hpi; however, more stomata closed when triggered by Δ*algU* than by DB29 (Fig. 5c). Interestingly, bacterium-triggered stomatal closure was not observed on *fls2 efr1* mutant plants inoculated with WT *Pst* DC3000, Δ*algU*, or DB29 (Fig. 5c). To further clarify the role of AlgU-regulated COR, we next examined the stomatal response after inoculation with WT *Pst* DC3000, the Δ*algD* mutant, the Δ*algU* mutant, DB29, or the *algU*-complemented line (Δ*algU/algU*) with


7AM
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Day
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0 3 h 6 h
12 h
24 h
48 h
algU
algD
hrpL
cfl
cmaB
fliC
oxyR
katE
a
b
c
d
e
f
g
h
Relative expression
ND
WT
ΔalgU
ΔalgU/algU

◂**Fig. 4** Expression profiles of genes involved in the virulence of wild-type (WT) *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000), the Δ*algU* mutant (Δ*algU*), or *algU*-complemented line (Δ*algU/algU*) after inoculation of 2-week-old plants of *Arabidopsis* with a suspension of 5×10^6 colony forming units (CFU)/ml. Total RNA was extracted at 3, 6, 12, 24, and 48 h post inoculation (hpi) for use in real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR) with gene-specific primer sets. Expression of **a** *algU*, **b** *algD*, **c** *hrpL*, **d** *cfl*, **e** *cmaB*, **f** *fliC*, **g** *oxyR*, and **h** *katE* was normalized using housekeeping genes *oprI, proC*, and *rpoD* (Supplementary Table S1). Vertical bars indicate the standard error for three biological replicates. Asterisks indicate a significant difference from WT in a *t* test (*$P < 0.05$; **$P < 0.01$). ND indicates not detected by RT-qPCR

or without exogenous application of COR in *Arabidopsis* and tomato. WT *Pst* DC3000 and the Δ*algD* mutant were able to reopen stomata, whereas bacterium-triggered stomatal closure was observed in Δ*algU*- and DB29-inoculated *Arabidopsis* at 4 hpi (Fig. 5d). Interestingly, the application of COR completely restored the phenotype of Δ*algU* and DB29 (Fig. 5d). Consistent with these results in *Arabidopsis*, WT *Pst* DC3000 and the Δ*algD* mutant were able to reopen stomata, whereas bacterium-triggered stomatal closure was observed on Δ*algU*- and DB29-inoculated tomato at 4 hpi (Fig. 5e), and the application of COR was able to cause reopening of stomata in tomato inoculated with Δ*algU* and DB29 at 4 hpi. These results clearly suggest that AlgU-regulated COR production can have an important role in overcoming PTI, especially stomatal-based defense in the early virulence of *Pst* DC3000.

Discussion

AlgU, one of the sigma factors that regulates genes responsive to the environment, also regulates the expression of genes related to alginate biosynthesis enzymes, such as AlgD (Okkotsu et al. 2014). The EPS alginate produced by *Pseudomonas* spp. contributes to biofilm formation and resistance to various external stresses (Keith et al. 2003). In the present mutant analysis of AlgU and AlgD, we found that the Δ*algU* mutant clearly showed reduced disease symptoms as well as reduced bacterial populations in host tomato and *Arabidopsis*, suggesting that AlgU plays a critical role in the virulence of *Pst* DC3000 (Figs. 1, 2). The host plants inoculated with Δ*algD* mutant showed milder disease symptoms, suggesting that AlgD may partially contribute to virulence. Gene expression profiles indicated that *algD* was differentially expressed in the Δ*algU* mutant background, suggesting that AlgU plays a role in regulating gene expression of alginate biosynthesis. Based on the gene expression profiles in Figs. 3c, 4c, it is tempting to speculate that AlgU can regulate the expression of *hrpL* during *Pst* DC3000 infection and that AlgU-regulated TTSS effectors may have roles in the virulence of *Pst* DC3000. Our results were consistent with a previous study describing interactions between tomato and *Pst* DC3000 (Markel et al. 2016). Markel et al. (2016) characterized the phenotype of a Δ*algU* mutant and reported that AlgU, but not AlgD, plays an important role in virulence. On the basis of their transcriptome analysis (RNA-seq) to characterize the AlgU regulon, they suggested that AlgU regulates the expression of TTSS effectors, resulting in modulation of virulence. Thus, AlgU can function as a virulence factor by regulating the expression of genes related to TTSS effectors.

In addition to genes related to TTSS effectors, we also demonstrated that the expression of genes related to the virulence of *Pst* DC3000 were differentially regulated in the Δ*algU* mutant background. Interestingly, the expression of genes related to biosynthesis of the phytotoxin COR, such as *cfl* and *cmaB* were clearly suppressed in the Δ*algU* mutant background compared to WT *Pst* DC3000 and *algU*-complemented line (Fig. 3d, e). Moreover, the expression of these genes was suppressed in the early stage of Δ*algU* mutant infection (Fig. 4d, e). The expression of genes related to COR is activated by the sigma factor, HrpL, and CorR, and COR contributes to the virulence of *Pst* DC3000 by suppressing the host defense response (Sreedharan et al. 2006; Uppalapati et al. 2007). Furthermore, COR can suppress PTI, especially stomatal-based defense in the early stage of *Pst* DC3000 infection in *Arabidopsis* and tomato (Lee et al. 2013; Melotto et al. 2006, 2008; Wasternack 2017; Zheng et al. 2012). Based on the gene expression profiles of *cfl* and *cmaB* (Fig. 4d, e), AlgU apparently can regulate the production of COR and contribute to virulence by suppressing stomatal-based defense in the early stage of infection. Consistent with the hypothesis, we demonstrated that the virulence phenotype of the Δ*algU* mutant was identical to the COR-deficient mutant, DB29, with respect to stomatal-based defense in tomato (Fig. 5e). Interestingly, the application of COR was able to restore the ability of the Δ*algU* mutant to reopen stomata in *Arabidopsis* and tomato (Fig. 5d, e). We also showed the Δ*algU* mutant displayed full virulence on the *Arabidopsis fls2 efr1* mutant, which is compromised in PTI and stomatal-based defense (Fig. 5c). *Arabidopsis* double mutant *fls2 efr1* is more susceptible to a COR-defective mutant because it lacks the stomatal-based defense (Zeng and He 2010). Together, these results suggest that AlgU contributes to the virulence of *Pst* DC3000 by regulating COR production to overcome PTI, especially early stomatal-based defense.

Our stomatal response assay demonstrated that DB29 and the Δ*algU* mutant induced stomatal closure compared to the WT *Pst* DC3000 in tomato and *Arabidopsis* (Fig. 5c–e). However, Δ*algU*-triggered stomatal closure was greater than that triggered by DB29 in *Arabidopsis* (Fig. 5c). It is important to note that the differences between tomato and *Arabidopsis* in Δ*algU*-triggered stomatal closure compared

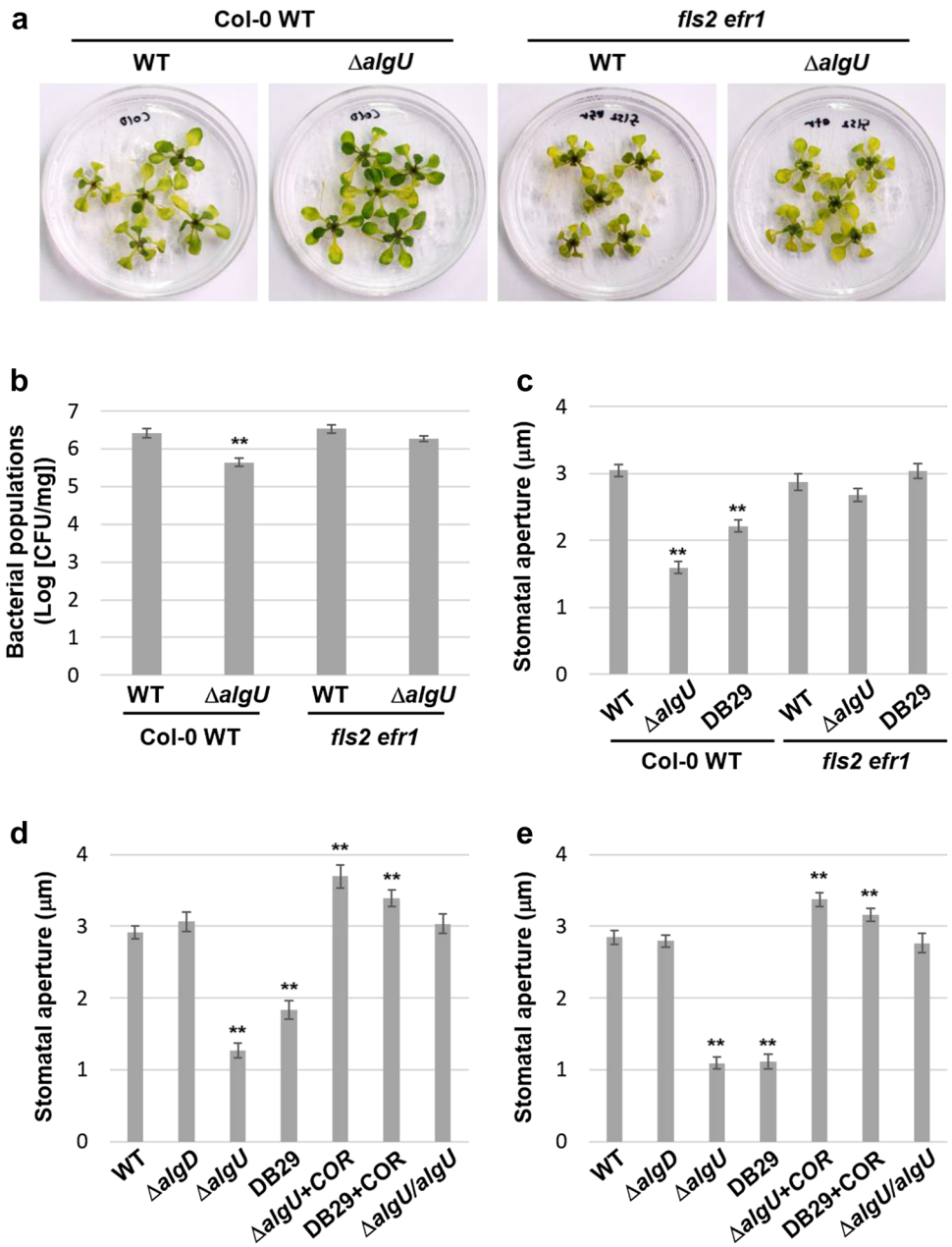

Fig. 5 Disease symptoms on wild-type *Arabidopsis* Col-0 and *fls2 efr1* mutant after flood-inoculation with 5×10^6 colony forming units (CFU)/ml of wild-type (WT) *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) or the Δ*algU* mutant (Δ*algU*) or dipping into WT *Pst* DC3000, Δ*algD*, Δ*algU*, DB29, or *algU*-complemented line (Δ*algU/algU*) with or without coronatine (COR). 2-week-old plants were flood-inoculated, and then the bacterial populations were measured as described in methods. **a** Water-soaked lesions with chlorosis at 3 dpi. **b** Bacterial populations in leaves estimated by dilution plating on selective media at 2 dpi. **c** Stomatal aperture width on intact *Arabidopsis* leaves 4 h after dip-inoculation with WT *Pst* DC3000, Δ*algU*, or DB29. **d** Stomatal aperture width on intact *Arabidopsis* leaves 4 h after dip-inoculation with WT *Pst* DC3000, Δ*algD*, Δ*algU*, DB29, or *algU*-complemented line (Δ*algU/algU*) with or without COR (100 ng/ml). **e** Stomatal aperture width in intact tomato leaves 4 h after dip-inoculation with WT *Pst* DC3000, Δ*algD*, Δ*algU*, DB29, and Δ*algU/algU* with or without COR (100 ng/ml). In all bar graphs, vertical bars indicate the standard error for three biological replicates. Asterisks indicate a significant difference from WT in a *t* test (**$P < 0.01$)

to that triggered by DB29 might be explained by the differential responses of the hosts to COR; purified COR induces chlorosis on leaves of tomato, but not on *Arabidopsis* leaves (Bent et al. 1992). We also previously demonstrated that purified COR suppresses the expression of genes related to chloroplast-localized ROS-detoxifying enzymes in tomato, but not in *Arabidopsis* (Ishiga et al. 2012). Therefore, further characterization of responses to COR in tomato and in *Arabidopsis* is needed to understand the molecular mechanisms of COR in the virulence of *Pst* DC3000.

Expression profiles in this study revealed a complex effect by AlgU on expression of virulence genes including *algD, hrpL, cfl, cmaB, oxyR*, and *katE*. Expression profiles of *algD* were clearly correlated with that of *algU* not only in vitro (Fig. 3b), but also during infection by *Pst* DC3000 (Fig. 4b), suggesting that AlgU can directly regulate the expression of *algD*. On the other hand, the expression profile of other virulence genes differed from that of *algU* (Figs. 3, 4). The different expression profiles among virulence genes in Δ*algU* mutant background might be explained by complex regulation of virulence genes by multiple transcriptional regulators. Previous studies demonstrated that CorR, a positive regulator for coronafacic acid (CFA) and coronamic acid (CMA), which are intermediates in the COR biosynthesis, bind the promoter region of *hrpL* and regulate the expression (Sreedharan et al. 2006). Therefore, further identification of

complex gene regulatory networks that function to modulate the virulence during *Pst* DC3000 infection is needed to understand the mechanisms by which AlgU and other regulators, such as HrpL and OxyR function in the virulence of *Pst* DC3000.

EPS production in planta is correlated with virulence in numerous phytopathogenic bacteria including *Ralstonia solanacearum, Erwinia stewartii*, and *Xanthomonas campestris* (Dolph et al. 1988; Kao et al. 1992; Katzen et al. 1998; Saile et al. 1997). Moreover, several studies have shown that alginate plays an important role in the virulence of *P. syringae*. In bean leaves inoculated with an alginate-defective mutant of *P. syringae* pv. *syringae*, disease symptoms and bacterial populations were significantly reduced compared to the wild-type (Yu et al. 1999). In the present study, the Δ*algD* mutant induced milder disease symptoms than the WT *Pst* DC3000 did in tomato and *Arabidopsis* (Figs. 1, 2), suggesting that alginate may partially contribute to virulence in all *P. syringae* pathovars. Gene expression analysis of *algD* in the interaction between *Pst* DC3000 and tomato showed that the promoter activity of *algD* was only detected in leaves showing necrotic cell death (Keith et al. 2003). Similarly, our previous studies also showed that the expression of *algD* was induced in the late stage of *Pst* DC3000 infection in *Arabidopsis* (Ishiga and Ichinose 2016). These results suggest that alginate may play a role in the virulence of *Pst* DC3000, especially in modulating disease-associated necrotic cell death. Further characterization of alginate and other EPS is needed to understand the interactions between the pathogenic bacteria and plants.

Light is known to function as a key factor regulating virulence, including the motility of *Pst* DC3000 (Río-Álvarez et al. 2014). Several studies demonstrated that motility is a critical virulence factor during *P. syringae* infection (Clarke et al. 2016; Nogales et al. 2015; Shimizu et al. 2003). Light inhibits the motility of *Pst* DC3000 by regulating the expression of genes related to flagellum-dependent motility, such as *fliC* (Río-Álvarez et al. 2014). Based on gene expression profiles, the expression of *fliC* was clearly induced in the dark (Fig. 4f), but transcript levels of *fliC* in the WT *Pst* DC3000 and the Δ*algU* mutant were not affected (12 hpi), suggesting that AlgU does not regulate motility in the virulence of *Pst* DC3000.

OxyR, a key transcription factor related to the virulence and oxidative stress tolerance of *Pst* DC3000, regulates the expression of genes related to H_2O_2-detoxifying enzymes, such as the *kat* genes (Ishiga and Ichinose 2016). Based on gene expression profiles, the expression of *oxyR* was suppressed in the Δ*algU* mutant at 6 hpi (Fig. 4g). The expression of *katE*, which is downstream of *oxyR*, was suppressed at 6, 24, and 48 hpi (Fig. 4h), suggesting that AlgU regulates not only the genes related to virulence factors including TTSS effectors and COR, but also the genes related to oxidative stress tolerance through the regulation of OxyR. ROS also play a key role in the defense responses of plants. A study utilizing the *Arabidopsis* NADPH oxidase *rbohD* mutant showed that the ROS burst via RbohD plays an important role in PTI (Kadota et al. 2014). Thus, it is tempting to speculate that not only type III effectors and COR, but also OxyR and oxidative stress tolerance mechanisms play an important role in avoiding PTI in the early infection stage in *Arabidopsis*. Therefore, further identification of regulators that function to modulate the virulence factors during infection and characterization of their modes of action in conjunction with AlgU are needed to understand the mechanisms of AlgU and other regulators in plant–bacterial pathogen interactions.

Acknowledgements We thank Dr. Christina Baker for editing the manuscript. This work was supported, in part, by JST ERATO NOMURA Microbial Community Control Project, JST, Japan.

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