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BACTERIAL AND PHYTOPLASMA DISEASES

Positive chemotaxis to plant apoplastic fluids of *Pseudomonas syringae* pv. *tabaci* 6605 and metabolome analysis

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Abstract

Pseudomonas syringae pv. *tabaci* 6605 (*Pta*6605) is a causal agent of wildfire disease in host tobacco plants. Although chemotaxis has been shown to be necessary for *Pta*6605 in tobacco infection, the chemoattractants at the site of infection are unclear. *Pta*6605 was attracted to the apoplastic fluid from not only host tobacco leaves but also non-host plant leaves, indicating that *Pta*6605 is attracted to common plant metabolites. Metabolome analysis of apoplastic fluid from tobacco leaves revealed that amino acids including γ-aminobutyric acid and organic acids are abundant, suggesting that these compounds are potential chemoattractants.

Keywords Apoplastic fluid · Chemotaxis · Chemoattractants · Metabolome · *Pseudomonas syringae*

Pseudomonas syringae pv. *tabaci* (*Pta*) 6605 is a highly motile and virulent strain of the causative agent of wildfire disease in tobacco. We revealed that flagellar motility is required for virulence in this pathogen by using flagellar motility-defective mutants (Ichinose et al. 2003, 2016). Furthermore, it was clarified that chemotaxis is required in bacterial infection (Tumewu et al. 2021b). Chemotaxis is a directional movement of an organism in response to a chemical stimulus. Bacteria recognize a variety of chemoeffectors, attractants, and repellants. Bacteria recognize these compounds by using chemoreceptors, also called methyl-accepting chemotaxis proteins (MCPs). When a chemoreceptor recognizes a chemical effector, the histidine kinase CheA2 is autophosphorylated to form CheA2-P. A phosphoryl group of CheA2-P will be transferred to CheY2, a response regulator to form active CheY2-P. CheY2-P directly interacts with flagellar motor switch protein to control the direction of the flagellar rotation (Matilla and Krell 2018; Sampedro et al. 2015). Some of the genes for proteins involved in chemotactic signaling are clustered. There are two major chemotaxis (*che*) clusters and four uninvestigated putative *che* clusters in the genome of *Pta*6605 (Tumewu et al. 2021b; Ichinose et al. 2023). Mutant analysis of *cheA1*, *cheY1*, *cheA2,* and *cheY2* revealed that CheA2 and CheY2 are indispensable for chemotaxis and virulence (Tumewu et al. 2021b). Most foliar plant pathogenic bacteria enter the apoplastic space of plants via wounds or open stomata. Melotto et al. (2006) reported that *P. syringae* pv. *tomato* (*Pto*) DC3000 gathered around open stomata but not to closed stomata. This evidence suggests that compounds abundant in the apoplastic space may attract bacteria from wounds or open stomata. Generally plant pathogenic bacteria possess more MCP genes than animal pathogens, especially bacteria living in stable and nutrient-rich environmental conditions (Lacal et al. 2010). For example, *Escherichia coli* has only 5 MCP genes (Matilla and Krell 2018), whereas plant pathogenic bacteria usually have 20–50 MCP genes (Matilla and Krell 2018). In the case of *Pseudomonas syringae*, there are about 50 MCP genes. A model plant pathogen *Pto*DC3000 is known to possess 49 MCP genes (Cerna-Vargas et al. 2019), and *Pta*6605 has 54 MCP genes (Ichinose et al. 2023). It is thought that plant pathogenic bacteria need to respond to a greater variety of environmental stimuli including preferable

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and harmful compounds, atmosphere, and redox changes than most animal pathogenic bacteria. However, little is known about the roles of chemotaxis in plant infection. We recently found that *Pta*6605 is attracted to γ-aminobutyric acid (GABA) and 19 proteinogenic amino acids except for tyrosine, and we identified McpG as a GABA chemoreceptor (Tumewu et al. 2020) and PscA, PscB, and PscC2 as chemoreceptors for proteinogenic amino acids (Tumewu et al. 2021a). These four MCPs possess the dCache_1 type ligand binding domain (LBD). PscC1 is another dCache_1 type chemoreceptor, although its attractants are not identified (Tumewu et al. 2021a). Surface swimming motility of the Δ*pscC1* and Δ*pscC2* was largely reduced, and the virulence to host tobacco plant was drastically reduced in Δ*mcpG*, Δ*pscB*, Δ*pscC1*, and Δ*pscC2* (Tumewu et al. 2020, 2021a). In *Pto*DC3000, chemoreceptor PscA specifically recognizes L-Asp, L-Glu, and D-Asp (Cerna-Vargas et al. 2019), whereas PscC specifically recognizes GABA and L-Pro (Santamaría-Hernando et al. 2022). Both mutant strains, *pscA* and *pscC*, also reduced virulence in the host tomato plant (Cerna-Vargas et al. 2019; Santamaría-Hernando et al. 2022). Therefore, recognition of amino acids is critical for plant infection of *Pta*6605 and *Pto*DC3000. In this study, we searched for potential chemoattractants in plant apoplastic fluid.

The apoplastic fluid contains compounds that contribute as nutrients and serve as chemoattractants for pathogenic bacteria. Apoplastic fluids were prepared from the leaves of 6-week-old host plants (*Nicotiana tabacum* cv. Xanthi and *Nicotiana benthamiana*), and non-host plants (8-week-old *Solanum lycopersicum*, and 6-week-old *Solanum melongena*) by the previously described method (Gentzel et al. 2019). In brief, whole leaves were entirety infiltrated with deionized and sterilized water, and apoplastic fluid was obtained by centrifuging at 1000 × *g* for 10 min at 4 °C. To assess the attraction of *Pta*6605, a microtiter plate multi-capillaries assay was carried out as described previously (Tumewu et al. 2020, 2021a, 2021b and 2022). *Pta*6605 was attracted to all plant species-derived apoplastic fluids regardless of host or non-host plant (Fig. 1), indicating that *Pta*6605 is attracted to common compounds among many plants.

The existence of a large number of MCPs suggests that *Pta*6605 can respond to a large number of compounds, and many compounds may localize in apoplastic spaces. To assess the potential attractants, metabolomes of tobacco apoplastic fluid were analyzed. Metabolites of tobacco apoplastic fluid were analyzed by Agilent CE-TOF/MS at Human Metabolome Technologies, Inc. (HMT, Tsuruoka, Yamagata, Japan). Among the 110 HMT metabolites library, 88 metabolites were detected in the apoplastic fluid (Supplementary file4). As shown in Fig. 2, amino acids including GABA, and organic acids such as malate were major metabolites. These results suggested that *Pta*6605 also might be

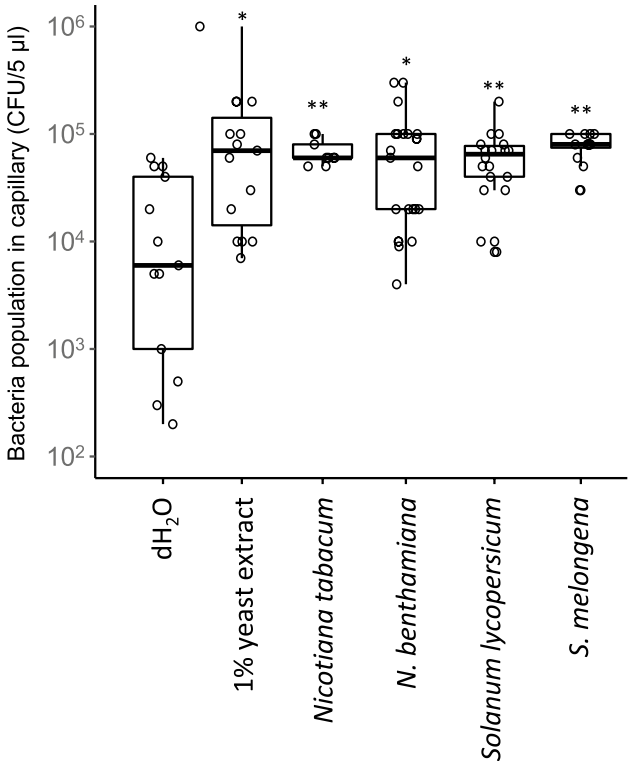

Fig. 1 Quantitative microtiter plate multi-capillaries chemotaxis assay of *Pseudomonas syringae* pv. *tabaci* 6605 (*Pta*6605) to apoplastic fluids prepared from different plants. Asterisks indicate a significant difference from water control treatment (*$P < 0.05$ and **$P < 0.01$ by Steel's multiple comparison test). As a positive control, 1% (w/v) yeast extract was used. Each circle represents a raw data point. The results of three independent experiments with three individual capillaries were combined and are illustrated in the boxplot. *Pta*6605 wild-type strain was routinely cultured on King's B medium supplemented with 50 µg/ml nalidixic acid (Nal) at 27 °C (Tumewu et al. 2020). For the chemotaxis assay, *Pta*6605 cells were prepared as described (Tumewu et al. 2021b)

attracted to GABA, proteinogenic amino acids, and organic acids in the infection stage. As mentioned above, we have identified chemoreceptor genes for GABA and proteinogenic amino acids (Tumewu et al. 2020, 2021a). Therefore, we analyzed the chemotactic response of the above mutants to the apoplastic fluid by the microtiter plate multi-capillaries method (Tumewu et al. 2020, 2021a, 2021b). In addition to the above mutants, we added another dCache_1-type receptor mutant, Δ*pscC1*, and analyzed the chemotactic responses to the yeast extracts and apoplastic fluid. As shown in Fig. 3, wild-type and all mutant strains retain the chemotactic ability to the yeast extracts and the apoplastic fluid. These results indicate a single MCP gene-deleted mutant does not lose chemotaxis to the apoplastic fluid because the apoplastic fluid contains a wide variety of attractants.

Since organic acids such as malate were also the major compounds of the apoplastic fluid, we investigated the chemotaxis of *Pta*6605 to these organic acids. However,

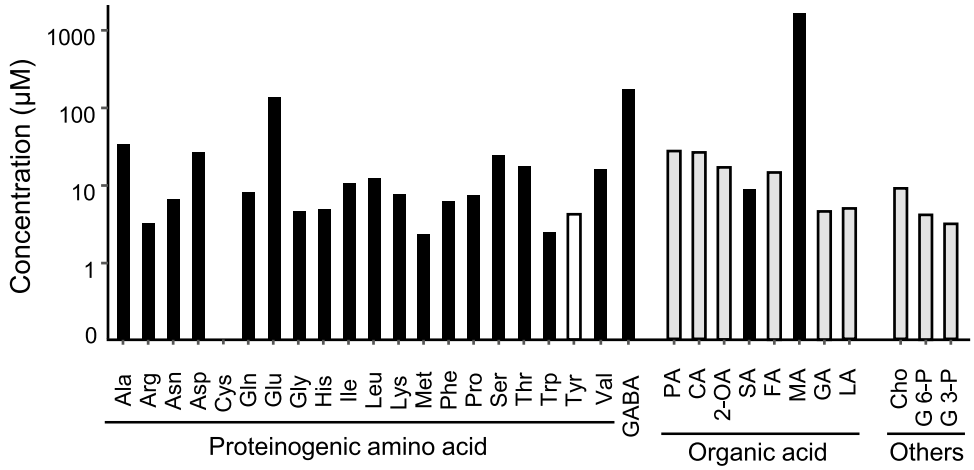

Fig. 2 Metabolome analysis of tobacco apoplastic fluid. Apoplastic fluid was harvested from 6-week-old healthy leaves of tobacco. Compounds with black bars indicate that *Pseudomonas syringae* pv. *tabaci* 6605 was attracted, and a white bar means not attracted. Compounds with grey bars indicate that chemotaxis has not been investigated yet. *PA* pyruvic acid, *CA* citric acid, *2-OA* 2-oxoglutaric acid, *SA* succinic acid, *FA* fumaric acid, *MA* malic acid, *GA* gluconic acid, *LA* lactic acid, *Cho* choline, *G6-P* glucose 6-phosphate, *G-3P* glucose 3-phosphate

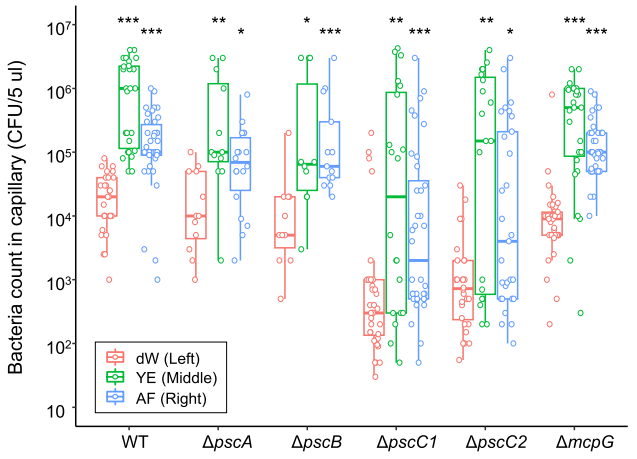

Fig. 3 Quantitative chemotaxis assay of the mutant strains for dCache_1 type chemoreceptors to yeast extract and apoplastic fluid. Chemotaxis to the distilled water (dW, left), yeast extracts (YE, middle), and apoplastic fluid (AF, right) of the mutant for GABA chemoreceptor (Δ*mcpG*), amino acids chemoreceptors (Δ*pscA*, Δ*pscB,* and Δ*pscC2*), and another mutant (Δ*pscC1*) was investigated by the microtiter plate multi-capillaries method. Bacterial numbers in 5 μl in the capillary were counted and shown with a boxplot from three independent experiments with three individual capillaries. Each circle represents a raw data point. Asterisks indicate statistically significant differences compared to the bacterial counts to distilled water (*$P<0.05$; **$P<0.01$; ***$P<0.001$ by Steel's multiple comparison test)

we couldn't observe positive chemotaxis to the malate and succinate by the microtiter plate multi-capillaries method. This was thought to be attributed to the inhibition of bacterial growth by the presence of malate since the addition of 100 mM of malate inhibited bacterial growth (data not shown). To avoid the inhibition of bacterial growth by organic acids in chemotaxis assay we applied the image analysis method for chemotaxis (Hida et al. 2015; Oku et al. 2014). This method detects bacterial cells gathering at the mouth of a microcapillary containing an attractant under microscopy. Microcapillary tubes with a diameter of about 10 μm for chemotaxis assay were prepared from glass capillary (1 mm in outer diameter, type G-1, Narishige Co., Tokyo, Japan) by using a microcapillary producing puller (PC-10, Narishige Co.). Potential attractants were prepared in 10 mM HEPES buffer (pH 7.4), mixed with an equal volume of 1% agarose (PrimeGel™ Agarose LE 1-20 K GAT, Takara Bio, Kusatsu, Japan), and embedded in the tip of the microcapillary. The microcapillary was injected into the bacterial suspension between the slide glass and the coverslip, spaced by stapler cores to observe bacterial behavior under microscopy (Olympus IX70, Tokyo, Japan). As shown in Fig. 4, bacteria migrated toward 5 mM malate and 50 mM succinate, but few bacteria were attracted to agarose as a negative control (Supplementary video). Bacteria were strongly attracted to 5 mM malate, but only weakly to 5 mM succinate, and we observed clear attraction to 50 mM succinate. A strong attraction was observed for malate than for succinate. In other *Pseudomonas* species, chemoreceptors for malate, citrate, succinate, and fumarate were characterized (Ortega et al. 2017). The *ctpM* (PA2652) in *P. aeruginosa* and *mcfS* (Pput_4520) in *P. putida* were identified to encode chemoreceptors for malate (Alvarez-Ortega and Harwood 2007; Parales et al. 2013). In *P. putida, mcfQ* (Pput_4894) was identified to encode chemoreceptors for citrate and fumarate, whereas *mcfR* (Pput_0339) was identified to encode chemoreceptors for succinate, fumarate,

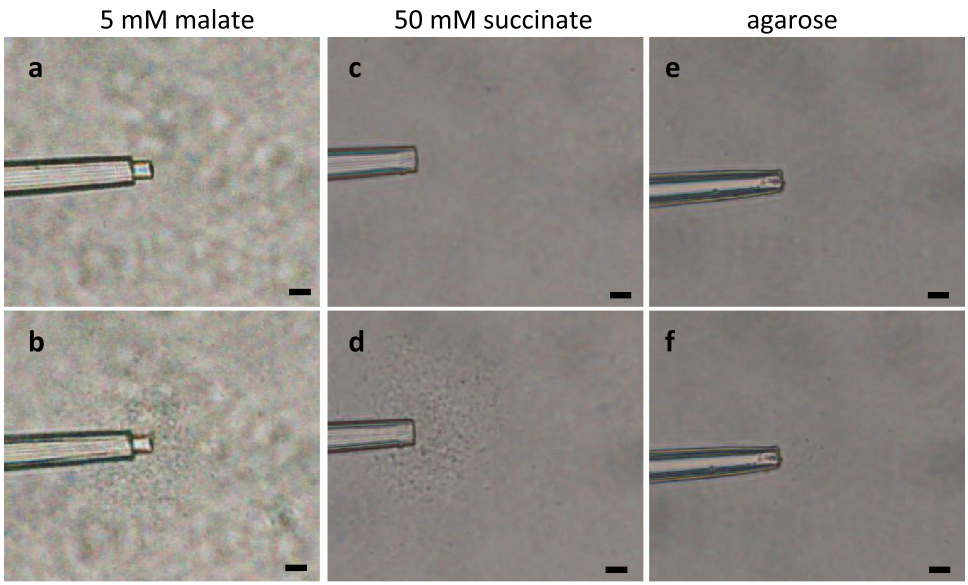

Fig. 4 Chemotaxis to malate and succinate. Photographs show bacterial behavior to 5 mM malate **a** and **b**, 50 mM succinate **c** and **d** and agarose as a negative control (**e**) and (**f**). Experiments were independently carried out two times. In one experiment, independent three microcapillaries with the same chemical were analyzed. Representative photographs were taken immediately **a**, **c** and **e** and 60 s after injection (**b**), (**d**) and (**f**). Bar = 10 µm

and malate (Parales et al. 2013). In *P. fluorescens, mcpS* (Pfl01_0728) and *mcpT* (Pfl01_3768) were identified to encode chemoreceptors for malate, succinate, and fumarate (Oku et al. 2014). However, the LBD type of CtpM and McpT is single calcium channels and chemotaxis receptors (sCache), that of McfS, McfQ, and McpS is helical bimodular (HBM), and that of McfR is 4-helix-bundle (4HB) type. In plant-pathogenic bacteria, soil pathogen *Ralstonia pseudosolanacearum mcpM*, a gene encoding a chemoreceptor for malate was identified (Hida et al. 2015). We summarized previously identified chemoreceptors for organic acids in Supplementary file5. The predicted LBD of McpM was a 4HB type. Thus, the malate chemoreceptor has a variable type of LBD. In *Pta*6605, there are two chemoreceptor genes with sCache type LBD, 7 chemoreceptor genes with HBM type LBD, 16 chemoreceptor genes with 4HB type LBD of class Ia, and 1 chemoreceptor gene with 4HB type LBD of class 1b (Ichinose et al. 2023). Therefore, there are too many candidate chemoreceptors for malate or other organic acids, and it is difficult to identify them at this stage. In *R. pseudosolanacearum*, the *mcpM* mutant strain was less infectious than the wild-type strain when tested by sand soak inoculation assay (Hida et al. 2015). Since malate was most abundant (1637 µM) in the apoplastic fluid (Fig. 2, Supplementary file4), it might navigate *Pta*6605 to the entry sites to apoplast via a chemoreceptor for malate.

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Author contributions HM, MY, YN, KT, and YI conceived the study and designed the experiments. YW, SAT, and HY performed experiments and analyzed data. YI drafted the manuscript, and SAT, YW, HM, MY, YN, and KT contributed to data interpretation and the critical revision of the manuscript. All authors contributed to the article and approved the submitted version.

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Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical approval The manuscript does not contain experiments using animals and human studies.

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