



Comparative analysis of interactions between five strains of *Pseudomonas syringae* pv. *tabaci* and *Nicotiana benthamiana*

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Abstract

Pseudomonas syringae pv. *tabaci* 6605 (*Pta* 6605), the agent of wildfire disease in tobacco, has been used as a model strain for elucidating the virulence mechanisms of *Pta*. However, the host genes involved in resistance or susceptibility to *Pta* remain largely unknown. *Nicotiana benthamiana* is a model plant species in the Solanaceae family and is useful in functional analyses of genes. We herein compared five *Pta* strains (6605, 6823, 7372, 7375, and 7380) in terms of their phenotypes on medium and interactions with *N. benthamiana*. *Pta* 6605 and *Pta* 6823 showed more active proliferation than the other strains in a high cell density culture. Moreover, *Pta* 6605 exhibited markedly higher swarming motility than the other strains. In inoculated leaves of *N. benthamiana*, *Pta* 6605 and *Pta* 6823 caused more severe disease symptoms and proliferated to a higher cell density than the other strains. However, *Pta* 6823 as well as *Pta* 7372 and *Pta* 7380 induced the high accumulation of salicylic acid (SA). Moreover, the inoculations of *Pta* 6823 and *Pta* 7372 resulted in the upregulation of ethylene biosynthesis genes. On the other hand, *Pta* 6605 induced neither SA accumulation nor the expression of ethylene biosynthesis genes, and suppressed the expression of jasmonate biosynthesis genes. Moreover, chlorosis was clearly induced in the upper uninoculated leaves of *Pta* 6605-infected plants. These results suggest that *Pta* 6605 escapes from or suppresses plant immune systems and, thus, is the most virulent on *N. benthamiana* among the five strains tested.

Keywords Chlorosis · *Nicotiana benthamiana* · Phytohormones · *Pseudomonas syringae* pv. *tabaci*

Introduction

Plant immune systems may be classified into two categories, pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones and Dangl 2006). PTI is induced by the recognition of pathogen-associated molecular patterns, such as the flagellin of bacterial pathogens. Pathogens secrete toxins and effectors to overcome PTI and establish infection. Plants recognize pathogen effectors by resistance

proteins and induce ETI, which is generally accompanied by a hypersensitive response (HR). PTI is induced by a broad spectrum of pathogens as well as non-pathogenic microbes, whereas ETI is typically induced in a strain- or race-specific manner. However, PTI and ETI activate overlapping defense responses, such as callose deposition and the generation of reactive oxygen species (Dodds et al. 2024). The induction of defense responses is mediated by phytohormones such as salicylic acid (SA), jasmonic acid (JA), and ethylene. These phytohormones induce distinct defense responses and useful ones differ depending on the lifestyle of pathogens. SA-mediated defense responses are generally effective against biotrophic and hemi-biotrophic pathogens (Peng et al. 2021), while JA- and ethylene-mediated defense responses are effective against necrotrophic pathogens (Ding et al. 2022).

Pseudomonas syringae pv. *tabaci* (*Pta*) is a Gram-negative and hemi-biotrophic pathogen. It causes wildfire disease in host tobacco (*Nicotiana tabacum*) plants. Virulence factors for *Pta* are tabtoxin, a β -lactam-type toxin

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causing chlorosis (Turner and Taha 1984), and effectors that are secreted via a type III secretion system (T3SS) (Büttner 2016). We previously used *Pta* 6605 as a model strain for elucidating the virulence mechanisms of *Pta* and revealed that flagellar motility and chemotaxis were required for invasion (Ichinose et al. 2003; Watanabe et al. 2023). The siderophore pyoverdine is also required for virulence through the production of tabtoxin (Taguchi et al. 2010) and the tabtoxin biosynthetic gene cluster is located on the genomic island designated as *Pta*GI-1 (Watanabe et al. 2025). *Pta* 6605 contains at least 22 distinct effectors (Matsui et al. 2021) and the T3SS-deficient mutant of *Pta* 6605 is not pathogenic (Kashihara et al. 2022). On the other hand, the host factors involved in resistance or susceptibility to *Pta* remain largely unknown. The tobacco cultivar N509 is resistant and induces HR against *Pta* 6605 by recognizing the effector HopAZ1 as an avirulence factor; however, host factors involved in resistance remain largely unknown (Kashihara et al. 2022).

N. benthamiana is a model plant species in the Solanaceae family and is useful for a functional analysis of genes by virus-induced gene silencing (VIGS) and *Agrobacterium*-mediated transient gene expression (Goodin et al. 2008). *Pta* 6605 infects and causes severe disease symptoms in *N. benthamiana* (Kashihara et al. 2022) and many effectors collectively contribute to its virulence (Kuroe et al. 2025). *Pta* 6605 may be used to investigate the host mechanisms involved in susceptibility to it. On the other hand, to investigate the host mechanisms involved in resistance to *Pta*, incompatible or low-virulence strains that are recognized by *N. benthamiana* and activate host defense responses are required. In the present study, we inoculated five strains (6605, 6823, 7272, 7375 and 7380) of *Pta* isolated in Japan into *N. benthamiana*, and compared their virulence and the host responses induced by their inoculation.

Materials and methods

Plant and bacterial materials

N. benthamiana were grown as described previously (Kotera et al. 2024). *Pta* strains (*Pta* 6605 and *Pta* 7375, isolated in Nagasaki Prefecture; *Pta* 6823, isolated in Niigata Prefecture; *Pta* 7372 and *Pta* 7380, isolated in Okayama Prefecture) were cultured in King's B (KB) medium at 30 °C.

Bacterial growth in vitro

Pta strains were cultured in KB medium at 30 °C to mid-log phase. After diluting to an optical density at 600 nm (OD₆₀₀)

of 0.1 with KB medium, they were further cultured at 30 °C and their OD₆₀₀ was measured over time.

Motility assay

Pta strains were cultured in KB medium at 30 °C to mid-log phase and collected by brief centrifugation. After washing with 10 mM MgSO₄, their concentration was adjusted to OD₆₀₀ of 0.1 with the same solution. Two-microliter aliquots were inoculated into the centers of SWM plates (0.5% peptone, 0.3% yeast extract, and 0.45% agar) for the swarming assay and into the centers of MMMF plates [50 mM potassium phosphate buffer, pH 5.7, 7.6 mM (NH₄)₂SO₄, 1.7 mM MgCl₂, 1.7 mM NaCl, and 10 mM mannitol, 10 mM fructose, and 0.25% agar] for the swimming assay. Motility was observed after 24 h (swarming assay) or 48 h (swimming assay) of incubation at 27 °C.

Pta inoculation and measurement of bacterial population

Pta strains were cultured in KB medium at 30 °C to mid-log phase and collected by brief centrifugation. After washing with 10 mM MgSO₄, they were suspended with and diluted with the same solution to be desired concentration. Bacterial solutions were infiltrated from abaxial side into fully expanded leaves of *N. benthamiana* at 4-week-old stage using a syringe without a needle. For the measurement of bacterial population, one leaf disk (10 mm diameter) was collected and ground in 1 ml of 10 mM MgSO₄. After serial dilutions with 10 mM MgSO₄, the suspension was spotted on KB agar plates. Recovered bacterial colonies were counted after a 2-day incubation at 30 °C.

SA measurement

The extraction and quantification of SA were performed as described previously (Malamy et al. 1992; Takagi et al. 2022).

RNA extraction and RT-qPCR analysis

Total RNA was extracted using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) according to the manufacturer's instructions. RT-qPCR analysis was performed as described previously (Kojima et al. 2019) with primer pairs as follows: *HSR203J*, AGGATCATCCAATAA CATGCCCGA and GCTATACAGTACAAATAAGGCGG CA; *ICS*, CACGGGCTTTTATTTTCAGAAACCGA and CA AAGCTGACCTTATTCCAACAGCA; *AOC*, CCAGAGC ATCTCAACTGACTCCAC and TAAGCAGGGCTACCA CGGTC; *AOS*, CGTTTTGCTCGACGGCAAGAG and CA

GTCGACGGCATGAAGGTCC; *ACS*, GTTATGGCTGGTGGTCAAC and TCTCTGTCCAAAGTGGTGCC; *ACO*, CTCCATCGTCGTTAACCTTGGTGA and ACGAAGCC AATGACATTCGAGTCC.

Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey's HSD was used with KaleidaGraph 5.0 software to determine significant differences among the groups. When the distribution of the data was highly skewed, log transformation of the data was applied to make the data conform more closely to the normal distribution. A P -value < 0.05 was considered statistically significant.

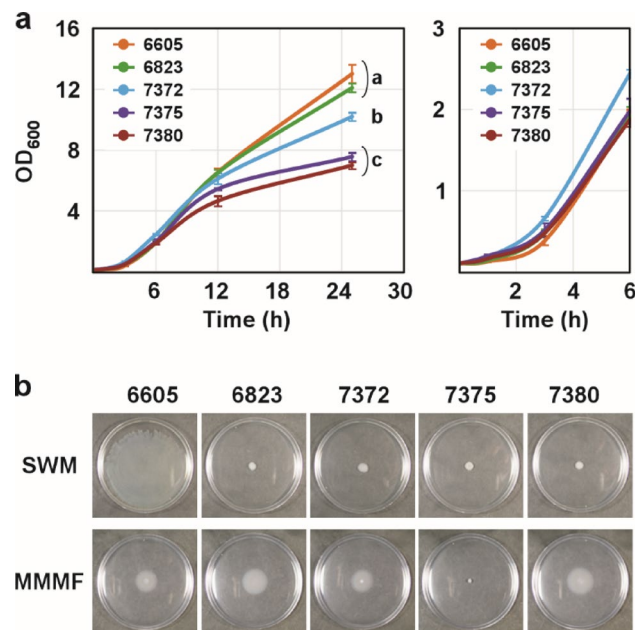


Fig. 1 Phenotypes of *Pseudomonas syringe* pv. *tabaci* strains. **a** *Pta* strains were cultured in liquid King's B medium at 30°C to the mid-log phase. After diluting to OD₆₀₀ of 0.1 with the same medium, they were further cultured at 30°C and their OD₆₀₀ was measured over time. Values are the means and standard errors of three biological replicates. The significance of differences among the groups was assessed with a one-way ANOVA followed by Tukey's HSD using KaleidaGraph 5.0 software. Lowercase letters indicate significant differences ($P < 0.05$). **b** *Pta* strains were cultured in KB medium at 30°C to the mid-log phase and collected by brief centrifugation. After washing with 10 mM MgSO₄, they were adjusted to OD₆₀₀ of 0.1 with the same solution. Two-microliter aliquots were inoculated into the center of an SWM plate containing 0.45% agar and an MMMF plate containing 0.25% agar. Photographs were taken 1 (SWM plate) or 2 (MMMF plate) days post-inoculation. Experiments were repeated at least three times with similar results

Results

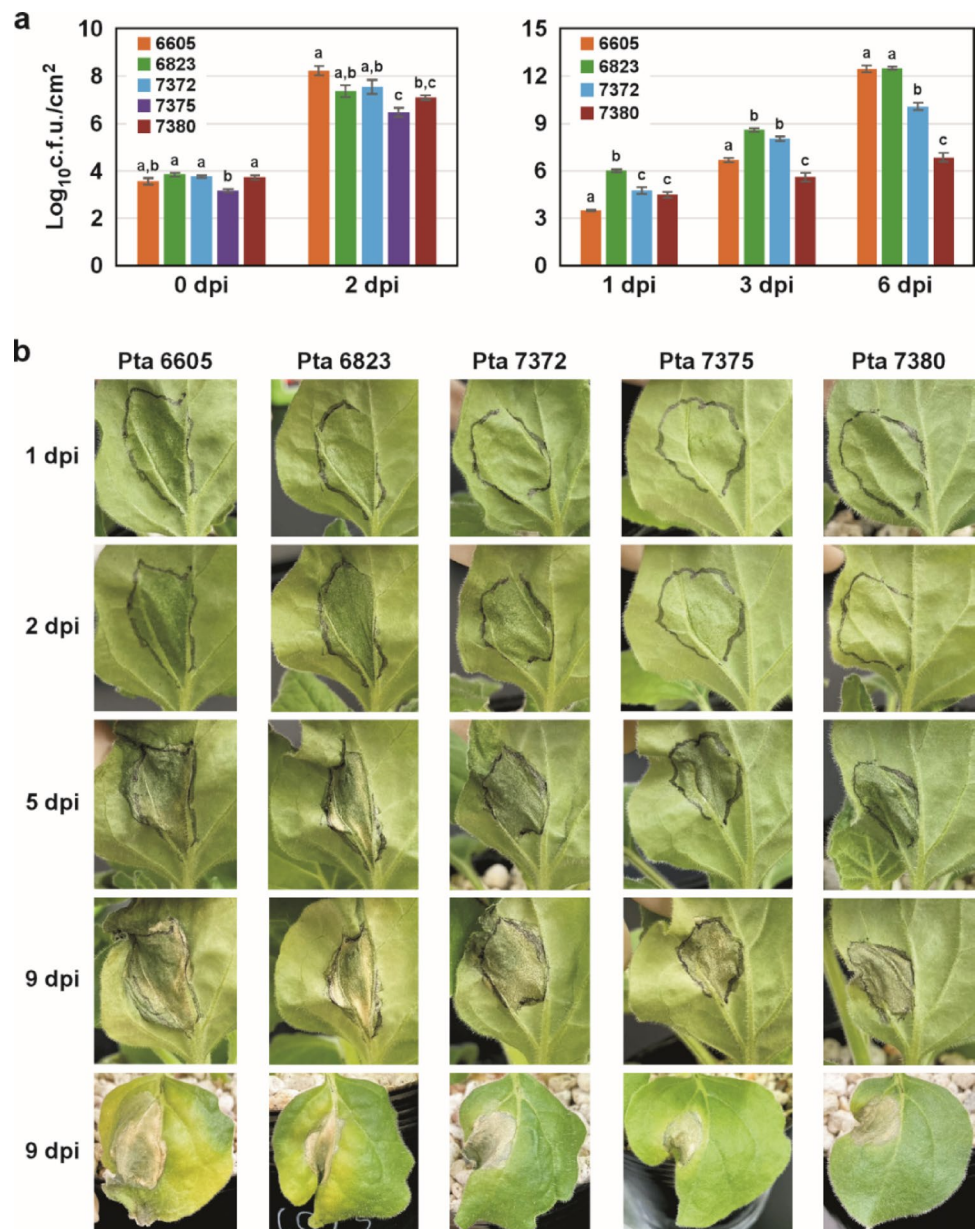
Characterization of five *Pta* strains in vitro

We compared the growth and motility of the five *Pta* strains cultured in KB medium, and growth was monitored by measuring OD₆₀₀. As shown in Fig. 1a, all strains grew similarly in the initial stage of growth; however, the cell density of *Pta* 7372 was slightly higher. In the late stage of growth, the strains were separated into three groups based on their cell density. The cell densities of *Pta* 6605 and *Pta* 6823 were approximately 1.5-fold higher than those with the lowest growth, including *Pta* 7375 and *Pta* 7380. *Pta* 7372 grew to an intermediate level between these two groups. The motilities of the *Pta* strains were compared on SWM plates for swarming and on MMMF plates for swimming. As shown in Fig. 1b, *Pta* 6605 showed more active swarming motility than the four other strains (SWM). On the other hand, all strains, except for *Pta* 7375, showed active swimming (MMMF).

Interactions between *Pta* strains and *N. benthamiana*

We compared the growth of *Pta* strains in *N. benthamiana* leaves. *Pta* strains were inoculated into *N. benthamiana* leaves at two different concentrations. With higher concentration of inoculum, host responses are induced strongly and quickly, and the relationship between bacterial growth and induction of host responses can be investigated. On the other hand, with lower concentration of inoculum, host tissues are not damaged severely for a long time, and bacterial growth is less affected by tissue collapse. When *Pta* strains were inoculated into *N. benthamiana* leaves at OD₆₀₀ = 0.002, all strains grew well, with *Pta* 6605 and *Pta* 7375 proliferating the most and least, respectively (Fig. 2a). On the other hand, when the strains were inoculated at OD₆₀₀ = 0.00002, *Pta* 6605 and *Pta* 6823 proliferated the most and their populations were approximately five orders of magnitude larger than that of *Pta* 7380 at 6 days post-inoculation (dpi). *Pta* 7372 grew to the intermediate level between *Pta* 6605/*Pta* 6823 and *Pta* 7380. The growth of *Pta* 7375 varied under this condition and we were unable to obtain reproducible results; therefore, the data were not shown. Leaves inoculated with the *Pta* strains were observed at 1, 2, 5, and 9 dpi. As shown in Fig. 2b, water-soaked lesions initially appeared in the inoculated regions, they turned to necrotic lesions, and chlorosis was then induced around the lesions. Overall, the speed of the appearance of water-soaked lesions seemed to correlate with the proliferation of *Pta* strains, with *Pta* 6605 and *Pta* 6823 being the fastest. Chlorosis induced by

Fig. 2 Interactions between *Pseudomonas syringae* pv. *tabaci* strains and *Nicotiana benthamiana*. **a** *N. benthamiana* leaves were inoculated with any one of the *Pta* strains at $OD_{600}=0.002$ (left graph) and 0.00002 (right graph). Bacterial populations were measured at the times indicated. Values are the means and standard errors of three to nine biological replicates. The significance of differences among the groups was assessed with a one-way ANOVA followed by Tukey's HSD using KaleidaGraph 5.0 software. Lowercase letters above the bars indicate significant differences ($P<0.05$). **b** *N. benthamiana* leaves were inoculated with any one of the *Pta* strains at $OD_{600}=0.01$. Photographs were taken from the abaxial side at 1, 2, 5, and 9 days post-inoculation and from the adaxial side at 9 dpi (bottom). The regions inoculated with bacteria are marked with a black line. Experiments were repeated twice with similar results



Pta 6605 and *Pta* 6823 was more severe than that induced by the other strains.

Induction of phytohormone biosynthesis genes in response to *Pta* inoculations

We investigated the host responses induced by inoculations of *Pta* strains. The phytohormone SA plays a central role in resistance against several pathovars of *P. syringae* in *Arabidopsis thaliana* (Peng et al. 2021). Therefore, we investigated whether infection by *Pta* strains induced SA biosynthesis in *N. benthamiana*. As shown in Fig. 3a, SA accumulation was clearly induced by the *Pta* 6823 and *Pta* 7372 inoculations, but not by the inoculations of the other

strains at 2 dpi. The inoculation of *Pta* 7380 induced SA accumulation as early as 3 dpi, whereas that of *Pta* 7375 did not, even at 4.5 dpi. Leaves inoculated with *Pta* 6605 had already collapsed by 3 dpi, and we were unable to measure SA levels in the leaves. Plants have at least two distinct SA biosynthesis pathways, the so-called isochorismate synthase (ICS) and phenylalanine ammonia lyase (PAL) pathways (Peng et al. 2021). We previously reported that the PAL pathway is the primary pathway for stress-induced SA production in *N. benthamiana* (Kotera et al. 2023, 2024; Takagi et al. 2022; Yokoo et al. 2018). Therefore, we investigated whether the expression of genes in these two pathways was induced by *Pta* inoculations. *HSR203J* has long been used as a marker of HR; however, we recently found that

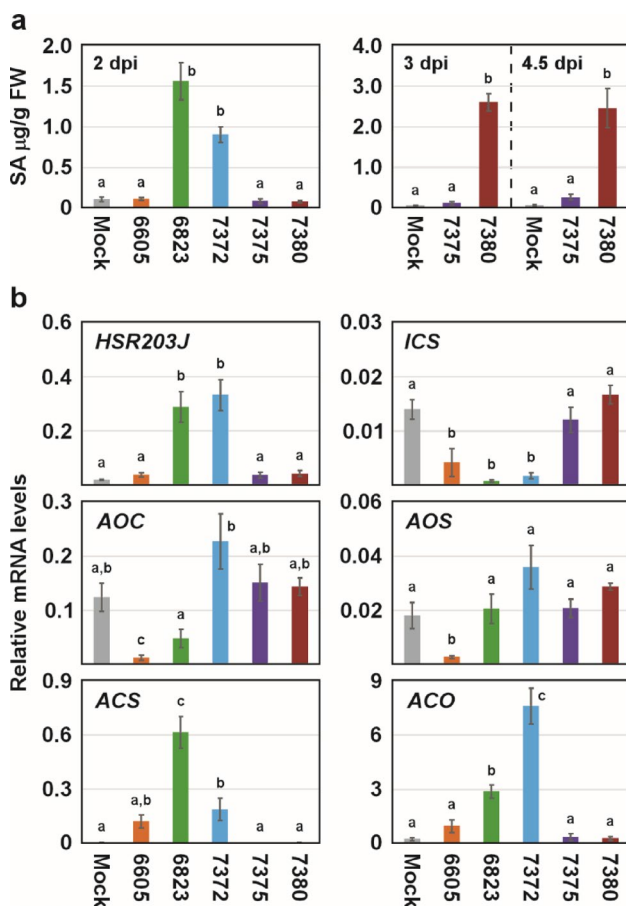


Fig. 3 Induction of salicylic acid and phytohormone biosynthesis genes in response to *Pseudomonas syringae* pv. *tabaci* inoculations. *Nicotiana benthamiana* leaves were inoculated with any one of the *Pta* strains ($OD_{600}=0.002$) or mock treated. **a** SA levels were assessed at 2, 3, and 4.5 days post-inoculation. Values are the means and standard errors of four to thirteen biological replicates. **b** The transcript levels of the indicated genes were analyzed at 2 dpi using RT-qPCR with *NbEF1a* as an internal standard. Values are the means and standard errors of five to twenty biological replicates. **a**, **b** The significance of differences among the groups was assessed with a one-way ANOVA followed by Tukey's HSD using KaleidaGraph 5.0 software. Lowercase letters above the bars indicate significant differences ($P<0.05$). ACO, 1-aminocyclopropane-1-carboxylic acid oxidase; ACS, 1-aminocyclopropane-1-carboxylic acid synthase; AOC, allene oxide cyclase; AOS, allene oxide synthase

it was required for SA biosynthesis through the PAL pathway (Takagi et al. 2022). Therefore, *HSR203J* is a marker of both SA biosynthesis and HR. As expected, the expression of *HSR203J* was clearly induced by *Pta* 6823 and *Pta* 7372 (Fig. 3b). In contrast, the expression of *ICS* was suppressed by *Pta* 6823 and *Pta* 7372 as well as *Pta* 6605. JA and ethylene, two other phytohormones, also play important roles in plant resistance against pathogens (Ding et al. 2022). The expression of the JA biosynthesis genes *allene oxide synthase* and *allene oxide cyclase* was not induced by any strains, but was suppressed by *Pta* 6605 (Fig. 3b). On the other hand, the expression of the ethylene biosynthesis

genes *1-aminocyclopropane-1-carboxylic acid synthase* and *1-aminocyclopropane-1-carboxylic acid oxidase* was induced by *Pta* 6823 and *Pta* 7372.

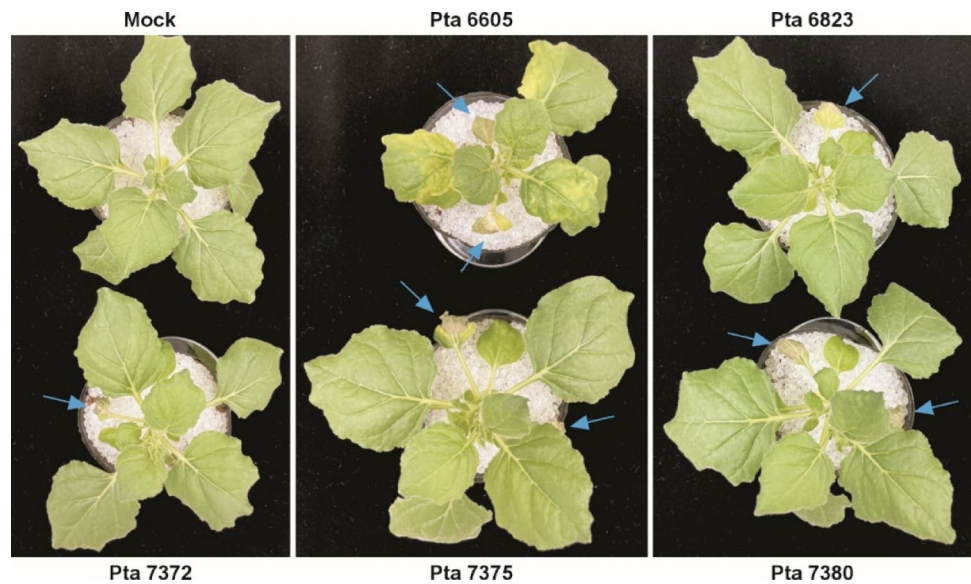
Pta 6605 induces chlorosis in upper uninoculated leaves of infected plants

We examined the effects of local *Pta* inoculations on the subsequent growth of infected plants. Two fully expanded leaves of plants were inoculated with *Pta* or were mock treated, and the subsequent growth of infected plants was assessed. In plants inoculated with *Pta* 6605, chlorosis was clearly observed in the upper uninoculated leaves (Fig. 4). Chlorosis appeared at approximately 5 dpi, at which time the inoculated leaves had already collapsed, and the degree of chlorosis peaked at approximately 8 dpi. Since *Pta* 6605 showed very active swarming motility (Fig. 1b), we speculated that it moved from the inoculated leaves to the upper uninoculated leaves and induced chlorosis; however, *Pta* 6605 was not recovered from upper uninoculated leaves showing chlorosis.

Discussion

We herein demonstrated that among the five strains of *Pta* tested, *Pta* 6605 was the most virulent on *N. benthamiana*. *Pta* 6605 caused severe disease symptoms and proliferated to a high cell density in inoculated leaves (Fig. 2), and also induced chlorosis in not only inoculated leaves but also upper uninoculated leaves (Fig. 4). Similar to *Pta* 6605, *Pta* 6823 caused disease symptoms associated with severe chlorosis and proliferated to a high cell density in inoculated leaves (Fig. 2); however, it did not induce clear systemic chlorosis (Fig. 4). *Pta* 7375 and *Pta* 7380 were the least virulent on *N. benthamiana*. The lesions induced by *Pta* 7375 and *Pta* 7380 were associated with very mild chlorosis and their proliferation in the lesions was lower than that of *Pta* 6605 or *Pta* 6823 (Fig. 2). *Pta* 7372 was intermediate between *Pta* 6605/*Pta* 6823 and *Pta* 7375/*Pta* 7380. The molecular mechanisms underlying the different virulence of the *Pta* strains on *N. benthamiana* are currently unknown; however, there are several possibilities based on the present results. The proliferation of *Pta* 6605 and *Pta* 6823, high virulence strains, under a high cell density was more active than that of *Pta* 7375 or *Pta* 7380, low virulence strains (Fig. 1a). Furthermore, *Pta* 6605 exhibited markedly higher swarming motility than the other strains, whereas *Pta* 7375 showed low swarming and swimming motilities (Fig. 1b). We previously reported that the high motility of *Pta* 6605 contributed to its virulence on host tobacco (Tumewu et al. 2021). These results suggest that the high and low virulence

Fig. 4 *Pseudomonas syringae* pv. *tabaci* 6605 induces chlorosis in upper uninoculated leaves of infected plants. Two fully expanded leaves of *Nicotiana benthamiana* were inoculated with any one of the *Pta* strains ($OD_{600}=0.01$) or mock treated. Photographs were taken 10 days post-inoculation. Arrows indicate leaves inoculated with *Pta*. Note some inoculated leaves are covered by upper leaves and cannot be seen. Experiments were repeated three times with similar results



of *Pta* 6605 and *Pta* 7375, respectively, were in part due to their innate characteristics. *Pta* 6605 appeared to be able to escape from or suppress the *N. benthamiana* immune system because its inoculation did not induce SA biosynthesis or the expression of ethylene biosynthesis genes, and it inhibited the expression of JA biosynthesis genes (Fig. 3). *Pta* 6823 induced severe disease symptoms and highly proliferated in the inoculated regions, similar to *Pta* 6605 (Fig. 2), but did not induce clear systemic chlorosis (Fig. 4). Since *Pta* 6823 induced SA biosynthesis and ethylene biosynthesis gene expression and did not inhibit JA biosynthesis gene expression (Fig. 3), phytohormones may play important roles in suppressing systemic chlorosis. The population of *Pta* 7380 in the inoculated regions was smaller than that of *Pta* 6605 (Fig. 2a), and *Pta* 7380-induced lesions were surrounded by very mild chlorosis, but were associated with SA biosynthesis (Fig. 2b), suggesting that *Pta* 7380-induced lesions are HR-like lesions. However, unlike the interactions between several *P. syringae* pathovars and *A. thaliana*, the induction of SA did not necessarily result in the suppression of bacterial growth in the inoculated leaves (Figs. 2a and 3a). It will be an important subject of the future analyses to clarify the roles of SA as well as JA and ethylene in the interactions between the *Pta* strains and *N. benthamiana*.

The present results indicate that *Pta* 6605 escaped from or suppressed the *N. benthamiana* immune system (Fig. 3). Plants recognize pathogen-associated molecular patterns with the recognition of flagellin derived from flagellar filaments, and the receptor FLS2 is the most studied example (Ryu et al. 2025). Buscaill et al. (2019) recently showed that glycosylation of the flagellin polymer of *P. syringae* species including *Pta* 6605 contributed to its escape from recognition by *N. benthamiana*; however, plants secrete the glycosidase BGAL1 to hydrolyze glycans. The knockout of

BGAL1 enhanced the growth of several *P. syringae* species including *Pta* 6605 in *N. benthamiana*, suggesting that *Pta* 6605 is recognized by *N. benthamiana*, but suppresses the induction of defense responses. Effectors secreted via T3SS play central roles in suppressing host immune systems (Büttner 2016) and *Pta* 6605 has many effectors that collectively contribute to its virulence on *N. benthamiana* (Kuroe et al. 2025). We will compare the effector repertoire of *Pta* 6605 with those of other strains.

Pta 6605 induced chlorosis in upper uninoculated leaves, from which *Pta* 6605 was not recovered (Fig. 4), suggesting that *Pta* 6605 does not move to, but sends a signal to the upper leaves in order to induce chlorosis. Tabtoxin was previously found to cause chlorosis on tobacco leaves (Turner and Taha 1984); however, to the best of our knowledge, there is currently no evidence to show that tabtoxin causes systemic chlorosis. On the other hand, phaseolotoxin, a sulfodiaminophosphinyl peptide produced by *P. syringae* pv. *phaseolicola* and *actinidiae*, induced systemic chlorosis (Arvizu-Gómez et al. 2024; Mitchell 1979). Phaseolotoxin inhibits arginine biosynthesis by competitively suppressing ornithine carbamoyltransferase, which converts ornithine and carbamoylphosphate to citrulline (Arvizu-Gómez et al. 2024). Tagetitoxin secreted by *P. syringae* pv. *tagetis* is targeted to the plant apex, where it inhibits chloroplast RNA polymerase and induces chlorosis on various species of the Asteraceae family (Rhodehamel and Durbin 1989). Further studies are needed to clarify whether *Pta* 6605 produces phaseolotoxin, tagetitoxin, or similar toxins, and also if arginine levels and the homeostasis of chloroplasts are reduced in the upper leaves showing chlorosis in *Pta* 6605-infected plants.

We herein investigated the interactions between five strains of *Pta* and *N. benthamiana*. The results obtained

revealed that *Pta* 6605 was the most virulent among the strains tested and induced systemic chlorosis clearly. In the future, we intend to examine the mechanisms by which *Pta* 6605 induces systemic chlorosis and whether the suppression of host defense systems, such as SA, JA, and ethylene signaling, by VIGS enables *Pta* strains other than *Pta* 6605 to induce systemic chlorosis. In the future, we will search for *N. benthamiana* genes involved in resistance or susceptibility to *Pta* by comparing its interactions with high virulence strains, such as *Pta* 6605, and low virulence strains, including *Pta* 7380.

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Author contributions YN, YI and SK designed the research project. YN, SA and HM did the experiments. YN and SK wrote the manuscript. All authors reviewed and approved the manuscript.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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