Plant breeding has successfully introduced disease resistance to pathogenic microorganisms into a wide range of economically important plant species. Plant breeders have found disease resistance in wild or cultivated relatives and, where available, have introduced these genes into crops by backcrossing. *Nicotiana tabacum* (tobacco) has a long production history as a nonfood crop worldwide and is frequently used as a molecular model in studies of plant–microbe interactions. From such studies, tobacco cultivars showing bacterial wildfire resistance (Knoche et al., 1987) have been produced; however, the molecular basis by which these disease-resistant cultivars recognize the pathogen remains unclear.

The molecular processes involved in plant disease resistance are well understood as providing two layers of defence: pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI; Jones & Dangl, 2006). Recent papers have shown that the molecular mechanisms of PTI and ETI are interlinked in a complex manner (Yuan et al., 2021). PTI functions to control opportunistic infections through the recognition of PAMPs.
by plant pattern recognition receptors. ETI suppresses pathogen infections by inducing a hypersensitive response (HR) to effectors secreted by the pathogen through direct or indirect recognition of the effectors by the plant resistance (R) gene product. Because ETI can induce strong resistance, including an HR, it has been used for molecular breeding focusing on R genes (Ayliffe, 2004); however, the pairs of R genes and their effectors required to induce ETI in many crop species remain to be identified (Kourelis & van der Hoorn, 2018).

Pseudomonas amygdali pv. tabaci (formerly Pseudomonas syringae pv. tabaci; Pta) causes bacterial wildfire disease on the foliage of host tobacco plants. The Pta virulence factors include tabtoxin, a phytotoxin involved in causing chlorosis, and type III effectors (T3Es) that are secreted via the type III secretion system (T3SS). The T3Es function as virulence factors, contributing to the establishment of infection by inhibiting the immune response of the host plant and by altering the host tissue environment to conditions suitable for pathogen growth (Block & Alfano, 2011; Xin et al., 2018). The number of T3Es in Pta strains is estimated to be about 20, based on genomic analysis of Pta strains 6605 and 11528 (Baltrus et al., 2011; Matsui et al., 2021; Studholme, 2011). The T3SS-deficient mutant of Pta 6605 is nonpathogenic. Some T3Es of Pta 11528 suppress reactive oxygen species (ROS) production and defence gene expression after flagellin peptide flg22 treatment, and HopX1Pta11528 plays a role in reopening the stomatal pores by affecting jasmonic acid signalling (Gimenez-Ibanez et al., 2014, 2018; Marutani et al., 2005). Thus, Pta is proposed to establish infections by secreting T3Es into the host plant and perturbing the host’s immune response.

In most of the studies to date, compatible interactions between Pta and tobacco plants have been investigated. In contrast, there are few studies on the incompatible interaction between Pta and tobacco plants. This study investigated tobacco cultivars showing resistance to the highly virulent Pta strain 6605 and performed experiments to elucidate how Pta-resistant tobacco cultivars recognize Pta 6605.

To confirm resistance to Pta 6605, we conducted a flood inoculation test using nine tobacco lines with different levels of Pta resistance, comprising seven N. tabacum cultivars and two other Nicotiana species (File S1). Water-soaked lesions were observed 3 days postinoculation (dpi) on N. tabacum ‘Xanthi’, ‘BY4’, ‘Matsukawa’, ‘Shiroensyu’, ‘Tsukuba-ichigou’ (hereafter Tsukuba), and Nicotiana benthamiana, but not N. tabacum ‘Burley 21’, ‘N509’, or Nicotiana longiflora (Figures 1a and S1a,b). N. benthamiana is highly susceptible to Pta 6605, and the leaf area was dramatically reduced. For this reason, the water-soaking area could not be measured in N. benthamiana. In Burley 21, N509, and N. longiflora, no reduction in leaf area was observed after infection with Pta (Figure S1c). We measured the bacterial population of wild-type (WT) Pta 6605 inoculated on these tobacco lines. Consistent with the suppression of disease symptom development, Burley 21, N509, and N. longiflora had low bacterial populations of Pta 6605 at 3 dpi. These data suggest that Burley 21, N509, and N. longiflora are resistant to Pta 6605 (Figure 1b).

Cultivar N509 was produced by introducing the Pta resistance of cv. Burley 21 into cv. Shiroensyu. Because the Pta resistance in cv. Burley 21 was originally introduced by crossing N. tabacum with N. longiflora (Heggestad et al., 1960), N509 is hypothesized to have inherited Pta resistance from N. longiflora. In our experiments, we used Shiroensyu as the Pta-susceptible cultivar and N509 as the Pta-resistant cultivar. To examine whether Pta resistance in N509 was due to the recognition of a T3E, we checked the HR induction using an infiltration test of the Pta 6605 ΔhrcC mutant that lacks a T3SS. As expected, the Pta 6605ΔhrcC mutant did not induce an HR (Figure S1).

We hypothesized that N509 recognizes a Pta T3E to induce disease resistance. Accordingly, we generated a series of 18 effector-deficient mutants in the genome of Pta 6605 (Table 1 and Figure S3). To search for Pta 6605 T3Es, we used the genomic data of Pta 6605 from the Pseudomonas genome database (Winsor et al., 2016) and the complete genome sequence of Pta 6605 (Matsui et al., 2021). Vector construction and the primers used in our study are described in Figure S3 and Table S1. A homologous recombination approach using pK18mobsacB (Schäfer et al., 1994) generated the Pta 6605 T3E deletion mutants. To facilitate the cloning process, we used the multicloning site of pK18mobsacB with the addition of the NotI site (pK18mobsacBN). The hopM1-avrF, hopAG1-hopAH1-hopAI1, and hopT1-hopO1 genes are located close to each other on the Pta 6605 chromosome; thus, these mutants were produced as multiple effector-deficient mutants. PCR confirmed each Pta T3E deletion mutant as lacking the target T3E gene(s).

To determine the effect of each Pta 6605 T3E deletion mutant on the disease resistance of N509, we conducted a flood inoculation test. Typically, the leaves of resistant N509 are subject to yellowing following flood inoculation with Pta 6605, but no water-soaked lesions are observed. Interestingly, when the Pta 6605 ΔhopAZ1 mutant was inoculated on N509, severe disease symptoms similar to those of the susceptible Shiroensyu were observed (Figure 2). This result suggests that the Pta resistance of N509 is possibly associated with the recognition of Pta T3E hopAZ1.

To investigate whether HopAZ1 functions as an avirulence factor against resistance in N509, we produced a complementary strain, Pta 6605 ΔhopAZ1 carrying hopAZ1 (Table 1 and File S1). Inoculation with Pta 6605 and the hopAZ1 complementation strains resulted in a resistant phenotype for the N509 cultivar (Figure 3a). No difference in the size of bacterial populations for the Shiroensyu cultivar was found between the Pta 6605 ΔhopAZ1 mutant and complementary strains compared with Pta 6605 (Figure 3b). The Pta 6605 ΔhopAZ1 mutant had an increased bacterial population compared to the Pta 6605 in N509 (Figure 3c). These results suggest that HopAZ1 is recognized by N509 as an avirulence factor and induces disease resistance against Pta 6605. Next, we examined the ability of HopAZ1 to induce an HR in N509 using a syringe-infiltration method. The Pta 6605 ΔhopAZ1 strain did not induce an HR in N509, and its ability to induce an HR on N509 was recovered on complementation with hopAZ1 (Figure 3d). Accordingly, we also confirmed an HR induction in the resistant Burley 21 and in N. longiflora. We found...
that the HopAZ1-dependent HR at 24 h postinoculation (hpi) was prominent in Burley 21 but was weak or barely detectable in N. longiflora (Figure S4). We also generated a complementary strain with 3× HA tag added to the C-terminus, but HopAZ1-3×HA complementary strains did not induce an HR in N509 and Burley 21 (Figure S5). To quantify the degree of cell death caused by HopAZ1, we measured electrolyte leakage, an indicator of cell death. Consistent with the results of HR induction, electrolyte leakage at 24 hpi was significantly increased in Pta 6605 and Pta 6605 ΔhopAZ1 (phpopAZ1) strains inoculated on N509 (Figure 3e).

The amino acid sequence of HopAZ1 is conserved in several Pseudomonas species, but the structure and enzymatic activity of HopAZ1 as an effector has not been determined (Dillon et al., 2019; O’Brien et al., 2012). HopAZ1 of Pseudomonas savastanoi pv. savastanoi...
| Bacterial strain/plasmid | Relevant characteristics | Reference or source |
|-------------------------|--------------------------|---------------------|
| Escherichia coli        |                          |                     |
| DH5α                    | F<sup>−</sup> λ<sup>+</sup> 80dLacZ ΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(rK<sup>R</sup> m<sup>K</sup>+) supE44 thi-1 gyrA relA1 | Takara              |
| S17-1                   | thi pro hsdR hsdM<sup>+</sup>recA(chr::RP4-2-Tc::Mu-Km::Tn7) | Schäfer et al. (1994) |
| Agrobacterium tumefaciens C58C1 |                          | Holsters et al. (1980) |
| Isolate 6605            | Wild type isolated from tobacco, Nal<sup>r</sup> | Shimizu et al. (2003) |
| Pta ∆hrc                | Isolate 6605 ∆RS0106115, Nal<sup>r</sup> | Marutani et al. (2005) |
| Pta ΔavrE1              | Isolate 6605 ∆RS0100760, Nal<sup>r</sup> | This study           |
| Pta ΔavrPto4            | Isolate 6605, ΔavrPto4, Nal<sup>r</sup> | This study           |
| Pta ∆hopAB3             | Isolate 6605 ∆RS0105230, Nal<sup>r</sup> | This study           |
| Pta ∆hopAE1             | Isolate 6605 ∆RS0125645, Nal<sup>r</sup> | This study           |
| Pta ∆hopAH2             | Isolate 6605 ∆RS0116920, Nal<sup>r</sup> | This study           |
| Pta ∆hopAR1             | Isolate 6605, ∆hopAR1, Nal<sup>r</sup> | This study           |
| Pta ∆hopAS1             | Isolate 6605, ∆RS0114820, Nal<sup>r</sup> | This study           |
| Pta ∆hopAZ1             | Isolate 6605, ∆RS0124775, Nal<sup>r</sup> | This study           |
| Pta ∆hopBD1             | Isolate 6605, ∆hopBD1, Nal<sup>r</sup> | This study           |
| Pta ∆hopE1              | Isolate 6605, ∆RS0112920, Nal<sup>r</sup> | This study           |
| Pta ∆hopI               | Isolate 6605, ∆RS0109130, Nal<sup>r</sup> | This study           |
| Pta ∆hopM1ΔavrF         | Isolate 6605, ∆RS0100770, Nal<sup>r</sup> | This study           |
| Pta ∆hopO1-1ΔhopT1-1    | Isolate 6605, ∆RS01135, ∆RS01130, Nal<sup>r</sup> | This study           |
| Pta ∆hopR1              | Isolate 6605, ∆hopR1, Nal<sup>r</sup> | This study           |
| Pta ∆hopV1              | Isolate 6605, ∆hopV1, Nal<sup>r</sup> | This study           |
| Pta ∆hopW1              | Isolate 6605, ∆RS0116415, Nal<sup>r</sup> | This study           |
| Pta ∆hopX1              | Isolate 6605, ∆RS0121235, Nal<sup>r</sup> | This study           |
| Pta ∆hopAZ1(phenAZ1)    | Isolate 6605 ∆RS0124775 carrying pDSK519-hopAZ1 promoter::hopAZ1, Nal<sup>r</sup>, Km<sup>r</sup> | This study           |
| Pta ∆hopAZ1(phenAZ1<sup>1-100</sup>) | Isolate 6605 ∆RS0124775 carrying pDSK519-hopAZ1 promoter::hopAZ1<sup>1-100</sup>, Nal<sup>r</sup>, Km<sup>r</sup> | This study           |
| Pta ∆hopAZ1(phenAZ1 1-200) | Isolate 6605 ∆RS0124775 carrying pDSK519-hopAZ1 promoter::hopAZ1<sup>1-200</sup>, Nal<sup>r</sup>, Km<sup>r</sup> | This study           |
| Pta ∆hopAZ1(phenAZ1-3xHA) | Isolate 6605 ∆RS0124775 carrying pDSK519-hopAZ1 promoter::hopAZ1-3xHA, Nal<sup>r</sup>, Km<sup>r</sup> | This study           |
| Plasmids                |                          |                     |
| pHSG396                 | a pUC type of cloning vector, Cm<sup>r</sup> | Takara              |
| pK18mobsacB             | Small mobilizable vector, sucrose sensitive (sacB); Km<sup>r</sup> | Schäfer et al. (1994) |
| pK18mobsacBN            | NotI site inserted between PstI and HindIII site of MCS in pK18; Km<sup>r</sup> | This study           |
| Pta Δhrc                | ∆RS0106115 fragment-containing pK18, Km<sup>r</sup> | This study           |
| Pta ΔavrE1              | Isolate 6605 ∆RS0100760, Nal<sup>r</sup> | This study           |
| Pta ΔavrPto4            | Isolate 6605, ΔavrPto4, Nal<sup>r</sup> | This study           |
| Pta ∆hopAB3             | Isolate 6605 ∆RS0105230, Nal<sup>r</sup> | This study           |
| Pta ∆hopAE1             | Isolate 6605 ∆RS0125645, Nal<sup>r</sup> | This study           |
| Pta ∆hopAG1ΔhopAH1ΔhopAI | Isolate 6605 ∆RS0109770, ∆RS0109780, ∆RS0109785, Nal<sup>r</sup> | This study           |
Next, we analysed the subcellular localization of functional HopAZ1-GFP by a transient expression system using *N. benthamiana*. Confocal microscopy revealed that HopAZ1-GFP localized to the nucleus, cytoplasm, and plasma membrane (Figure 4d). We confirmed the subcellular localization of HopAZ1-GFP by subcellular fractionation. HopAZ1-GFP was detected in the microsomal fraction, suggesting that HopAZ1 may target proteins associated with microsome-related proteins (Figure S7).

In this study, we found that *N. tabacum* 'N509' exhibited disease resistance dependent on the presence of the T3E HopAZ1. As mentioned above, N509 was derived from a cross between Shiroensyu and Burley 21, which carries Pta-resistance derived from *N. longiflora*. *N. longiflora* is a wild tobacco species from South America that was reported to be resistant to Pta strain 11528 and has a history of being used as a genetic resource for resistance to several other important diseases (Knoche et al., 1987; Schweppenhauser, 1975; Valleau et al., 1960). Because Pta strains 6605 and 11528 both possess hopAZ1 in their genomes, we assumed that Burley 21, N509, and *N. longiflora* have a common R gene that recognizes HopAZ1. Interestingly, an HR was not strongly induced in *N. longiflora* by the Pta 6605 inoculation test (Figures S4 and S5). Although the detailed molecular mechanism is unknown, *N. longiflora* seems to have a complex molecular mechanism of resistance to Pta.

Screening using a series of Pta T3E deletion mutants revealed that resistant N509 recognizes HopAZ1*Pta6605* (Figures 2 and 3). HopAZ1 is conserved in at least 12 of the 29 *Pseudomonas* strains whose genomes have been sequenced (Laflamme et al., 2020).
Comparative genome analysis has proposed the possibility that HopAZ1 is a candidate determinant of host specificity for Corylus avellana (hazelnut) in P. syringae pv. avellanae, a causative agent of decline disease in hazelnut (O’Brien et al., 2012). HopAZ1Psv3335 consists of 122 amino acids lacking the C-terminal end of the HopAZ1 encoded by other P. syringae pathovars (Figure 4; Matas et al., 2014). In addition to inhibiting the PTI response, HopAZ1Psv3335 has also been reported to inhibit ETI-like cell death (Matas et al., 2014). Because HopAZ1Psv3335 lacks the C-terminus does not induce an HR in N509 (Figures 4b and S6), the loss of HopAZ1Psv3335 may have been to avoid recognition by host defence mechanisms. The function of the HopAZ1 protein as an effector, however, is still undefined and the host factors targeted by HopAZ1 are also unknown. Future work will be required to identify the host targets of HopAZ1Psv3335.

We also tested the role of HopAZ1 as a virulence factor using the susceptible cv. Shiroensyu; however, there was no apparent difference in the size of bacterial populations or in disease symptoms between Pta 6605 and the Pta 6605 ΔhopAZ1 mutant (Figure 3b). It is difficult to determine the importance of HopAZ1 in the virulence of Pta 6605 using a single deletion mutation in hopAZ1. The construction of multiple mutants, such as P. syringae pv. tomato DC3000 D28E (Cunnac et al., 2011), and the reintroduction of effectors into these mutants (Wei et al., 2018), might lead to an improved understanding of their importance for virulence.

HopAZ1Pta6605-GFP mainly localized to the microsomal fraction when transiently expressed in N. benthamiana leaves (Figures 4d and S7). A previous report demonstrated that HopAZ1 of P. syringae pv. actinidiae (Pac) localizes to the cytoskeleton (Choi et al., 2017). Because the N-terminal sequence of HopAZ1Pac is shorter (219 amino acids) than that of HopAZ1Pta6605, it is possible that the N-terminal sequence of HopAZ1 is responsible for the different subcellular localization patterns.

In summary, we demonstrated that HopAZ1Pta6605 is a T3E recognized by Pta-resistant tobacco cvs Burley 21 and N509. The next
HopAZ1 induced a hypersensitive response (HR) in *Nicotiana tabacum* 'N509'. (a) Flood inoculation test of *Pseudomonas amygdali pv. tabaci* (Pta) ΔhopAZ1 and Pta ΔhopAZ1 (p hopAZ1) in the resistant cultivar N509 and the susceptible cultivar Shiroensyu. Two-week-old plants were flood-inoculated with each strain (OD$_{600}$ = 0.002) and photographed 4 days postinoculation (dpi). Inoculation tests were repeated at least twice with three independent plants with similar results. (b, c) Bacterial populations in Shiroensyu and N509 leaves at 0 and 2 dpi. The five times results were combined and illustrated in a box plot. Boxes show upper and lower quartiles of the data, and black lines represent the medians. Each dot represents raw data. The numbers of the graph indicate the total number of individuals used in the experiment. Statistical tests were performed using the Dunnett test (*p* < 0.05). (d) Photographs of HR induction in Pta-infiltrated plants. Eight-week-old plants were infiltrated with Pta wild type (WT), Pta ΔhrcC, Pta ΔhopAZ1, or Pta ΔhopAZ1 (p hopAZ1), whose concentrations were adjusted to an OD$_{600}$ = 0.02 and photographed at 24 h postinoculation (hpi). The HR assays were repeated three times with three independent plants with similar results (the total number of biological replicates; *n* = 9). (e) Measurement of ion leakage after Pta infiltration. The concentration of each Pta strain was adjusted to an OD$_{600}$ = 0.02. Leaves were inoculated by infiltration. Leaf discs were prepared from the inoculation area, floated in deionized water and measured for ion leakage 24 hpi. The two times results were combined and displayed in this boxplot. Boxes show upper and lower quartiles of the data, and black lines represent the medians. Each dot represents the raw data. Statistically significant differences are indicated by different letters (*p* < 0.01, Tukey HSD test).
challenge will be to find resistance genes that recognize HopAZ1, a challenging task given the size of the tobacco genome. Recent studies have established that a genome-wide R gene search method using a hairpin-RNAi library was suitable for isolating resistance genes in N. benthamiana (Brendolise et al., 2017). The resistance gene Rpa1 (Resistance to P. syringae pv. actinidiae 1) was also isolated using this experimental method in N. tabacum (Yoon & Rikkerink, 2020). As more genome sequence information for N. tabacum is becoming available (Sierro et al., 2014), we hope to use these technologies to decipher the HopAZ1 recognition mechanism of N. tabacum ‘N509’ in the future.

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CONFLICTS OF INTEREST
The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request. As tobacco seeds are not transferable from us, please contact the Leaf Tobacco Research Laboratory of Japan Tobacco Inc.

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SUPPORTING INFORMATION
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