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The Harpin-Induced Hypersensitive Reaction in *Nicotiana tabacum* Requires *Wall-Associated Kinase 2*

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ABSTRACT

Harpins are proteins secreted by many gram-negative, plant-pathogenic bacteria that stimulate the hypersensitive reaction (HR), a host cell death defence response, when infiltrated into plant leaves as purified proteins. This activity of harpins was first discovered in *Nicotiana tabacum* (tobacco), which manifests an especially strong and rapid harpin-activated HR that becomes evident within 12–24 h after infiltration. HrpN is the major harpin of the fire blight pathogen *Erwinia amylovora*. We discovered natural variation in the HrpN-induced HR among tobacco accessions and identified candidate genes using genetic mapping and bulked-segregant analysis with whole genome sequencing. Virus-induced gene silencing of candidate gene *Wall-Associated Kinase 2* (*WAK2*) abrogated the HR in response to HrpN and HpaG, a harpin from the soybean bacterial pustule pathogen *Xanthomonas citri* pv. *glycines*. *WAK2* silencing also compromised the avirulence activity of harpin HrpZ in the tobacco wildfire pathogen *Pseudomonas syringae* pv. *tabaci*. A natural, disruptive mutation in *WAK2* correlated with the inability of tobacco accessions to mount the harpin-mediated HR. We conclude that the predicted receptor-like kinase WAK2 is required for the strong HR induced in tobacco leaves by harpin protein infiltration and can potentially mediate resistance to bacterial pathogens based on harpin recognition.

Sixty years ago, a dramatic defence response known as the hypersensitive reaction (HR) was documented in tobacco (*Nicotiana tabacum*) leaves infiltrated with concentrated suspensions of plant-pathogenic bacteria (Klement 1963; Klement et al. 1964; Klement and Goodman 1967). Leaf tissue in the infiltrated areas turned necrotic in as little as 8 h and became dry and white or brown in 3–4 days. In the early 1990s, an unusual protein, termed HrpN, from the fire blight pathogen *Erwinia amylovora*, was discovered that triggered an HR when infiltrated as purified protein into leaves of tobacco, a fire blight nonhost (Wei et al. 1992). *E. amylovora* HrpN is required for fire blight disease development (Wei et al. 1992), is secreted through the type III protein secretion system (T3SS) (Kim et al. 1997) and assists in the translocation of T3SS-secreted effectors into the host cytoplasm (Bocsanczy et al. 2008). Following identification of *E. amylovora* HrpN as the first bacterial cell-free HR elicitor (Wei et al. 1992), T3SS-secreted proteins with cell-free HR-eliciting activities were identified in nearly all major phytopathogenic bacterial genera, including *Pseudomonas* (He et al. 1993), *Ralstonia* (Arlat et al. 1994) and *Xanthomonas* (Zhu et al. 2000; Kim et al. 2003). These proteins are collectively referred to as harpins. While harpin

sequences vary extensively between pathogens, all share the characteristic property of HR induction in tobacco leaves (Choi et al. 2013).

The question of how harpins are physically interacting with tobacco cells received some early investigation through biochemical approaches. Tobacco cells treated with *Pseudomonas syringae* pv. *syringae* harpin followed by antibody probing revealed cell-wall localised fluorescence, while harpin-treated control protoplasts showed no fluorescence (Hoyos et al. 1996). A subsequent study found that radiolabelled *P. syringae* pv. *phaseolicola* harpin bound both tobacco microsomes and protoplasts, and that the binding site was insensitive to protease treatment (Lee et al. 2001). While hinting at potential sites of action, neither study directly linked potential harpin binding sites with the HR. Genetic studies of the harpin-induced HR in tobacco have identified some downstream components and/or regulators of the cell death response such as salicylic acid (Peng et al. 2003) and mitogen-activated protein kinases (Samuel et al. 2005). However, no tobacco genes specifying the harpin-activated HR have heretofore been reported.

With the advent of whole genome sequencing and improved genetic methods, we decided to revisit the 30-year-old mystery of HR elicitation by harpins in tobacco leaves through genetic mapping. We focused our study on *E. amylovora* HrpN because it was the first harpin to be discovered (Wei et al. 1992). We began by documenting the HrpN-induced HR phenotype in 127 tobacco accessions obtained from among the c. 1900 accessions in the US *Nicotiana* Germplasm Collection (Lewis and Nicholson 2007; Dataset S1). For expediency, we conducted initial phenotyping with the synthetic Hrp37 peptide, which contains the major HR-eliciting domain of HrpN (Held et al. 2024; Shrestha et al. 2008).

Leaves of all 127 tobacco accessions were syringe-infiltrated with Hrp37 peptide, and their cell death responsiveness was quantified according to a custom scoring system (Figure S1a,b). This scoring system provided HR responsiveness ratings for each accession ranging from 4 to 12, with 4 indicating no HR and 12 indicating maximal observed HR. Almost two-thirds (64.6%) of the screened accessions had a responsiveness rating of ≥ 10, while nine accessions (7.1%) had a responsiveness rating of < 6 (Figure 1a).

We selected representative accessions from these 'responsive' and 'unresponsive' groups for further analysis (Figure 1b). Responsive accessions displayed HRs to Hrp37, full-length HrpN protein and the harpin peptide HaX23 (Figure 1c, Figure S2), which represents the HR-eliciting portion of the harpin HpaG from the soybean bacterial pustule disease pathogen *Xanthomonas citri* pv. *glycines* (Held et al. 2024; Kim et al. 2004). Unresponsive accessions did not respond to any of the three harpin elicitors (Figure 1c, Figure S3). All accessions produced an HR when infiltrated with the tobacco nonhost pathogen *P. syringae* pv. *tomato* (Pst) while no HR was initiated by Hrp37-S, a peptide with the same amino acid composition as Hrp37, but in a random order (Methods S1; Held et al. 2024), or buffer controls (Figure 1c; Figures S2 and S3). To evaluate the genetic uniformity of the accessions, we allowed plants grown from select original seed stocks to self-pollinate and tested the harpin responsiveness of the progeny. The progeny of self-pollinated TC 319 and TI 126 individuals showed similar Hrp37 responsiveness to the parent population (Table S1), suggesting their suitability for genetic mapping.

To investigate the genetics of the harpin response trait, we pollinated unresponsive accession TI 126 with pollen from responsive cultivar TC 319 and obtained F_1 progeny. All 20 F_1 progeny infiltrated with Hrp37 were responsive (Figure 1d), suggesting Hrp37-responsiveness was a dominant trait. An individual was deemed responsive if at least one out of four infiltrations resulted in a full HR (Figure S1a), while unresponsive individuals displayed no full HRs in response to any of the infiltrations (Figure S1a). Next, an individual from the TI 126 × TC 319 F_1 population was allowed to self-pollinate to generate an F_2 population. Hrp37 responsiveness in the F_2 progeny exhibited Mendelian segregation with a 3:1 ratio of responsive to unresponsive individuals (Figure 1d), suggesting a single dominant locus underlying the HrpN responsiveness trait.

To begin to map the genetic locus, we performed a bulked-segregant analysis (BSA; Majeed et al. 2022). Genomic DNA was extracted from the leaves of 30 responsive F_2 plants and pooled, with equal representation of each F_2 individual. A similar DNA pool was obtained for 30 unresponsive F_2 progeny. The two DNA pools were sequenced separately on a NextSeq 2000 (Illumina Inc.) using 150 bp paired-end reads at the Penn State Genomics Core Facility. Approximately 600 million reads were generated for each sample, equating to c. 40× coverage across the c. 4.5 Gb tobacco genome (Sierro et al. 2014). Reads were mapped to the chromosome-level assembly of the TC 319 tobacco genome (Sierro et al. 2024), and variant calling was performed with BCFtools (Methods S1; Li 2011; Danecek et al. 2021).

After filtering, 3.6 million variants were identified in the two samples (Dataset S2). Variant allele frequency (VAF; Strom 2016) was calculated for each variant as the proportion of sequencing reads harbouring the variant at a given locus. Responsive VAFs were subtracted from the unresponsive VAFs to generate a single VAF score for each variant, where positive values indicated variants associated with unresponsiveness. An example VAF and VAF score calculation can be found in Figure S4. When the average VAF score for all variants on each of the 24 tobacco chromosomes was determined and compared, Chromosome 2 had the highest average VAF score (Figure 1e), suggesting that the trait of interest probably resides on Chromosome 2. Interestingly, Chromosome 2 is believed to originate from *Nicotiana tomentosiformis*, the male progenitor species of allopolyploid tobacco (Sierro et al. 2024). *N. tomentosiformis* responds with an HR to Hrp37 infiltration, while *Nicotiana sylvestris*, the female progenitor species, is unresponsive (Figure S5), suggesting that harpin responsiveness might be derived from the *N. tomentosiformis* progenitor species of *Nicotiana tabacum*.

To identify the gene responsible for Hrp37 responsiveness, we focused our search on protein-coding altering variants on *N. tabacum* Chromosome 2. Plotting of the positions of 821 protein altering variants on Chromosome 2 showed variants with higher VAF scores in the middle of the chromosome, in the region from approximately 20–100 Mb (Figure 1f). We selected

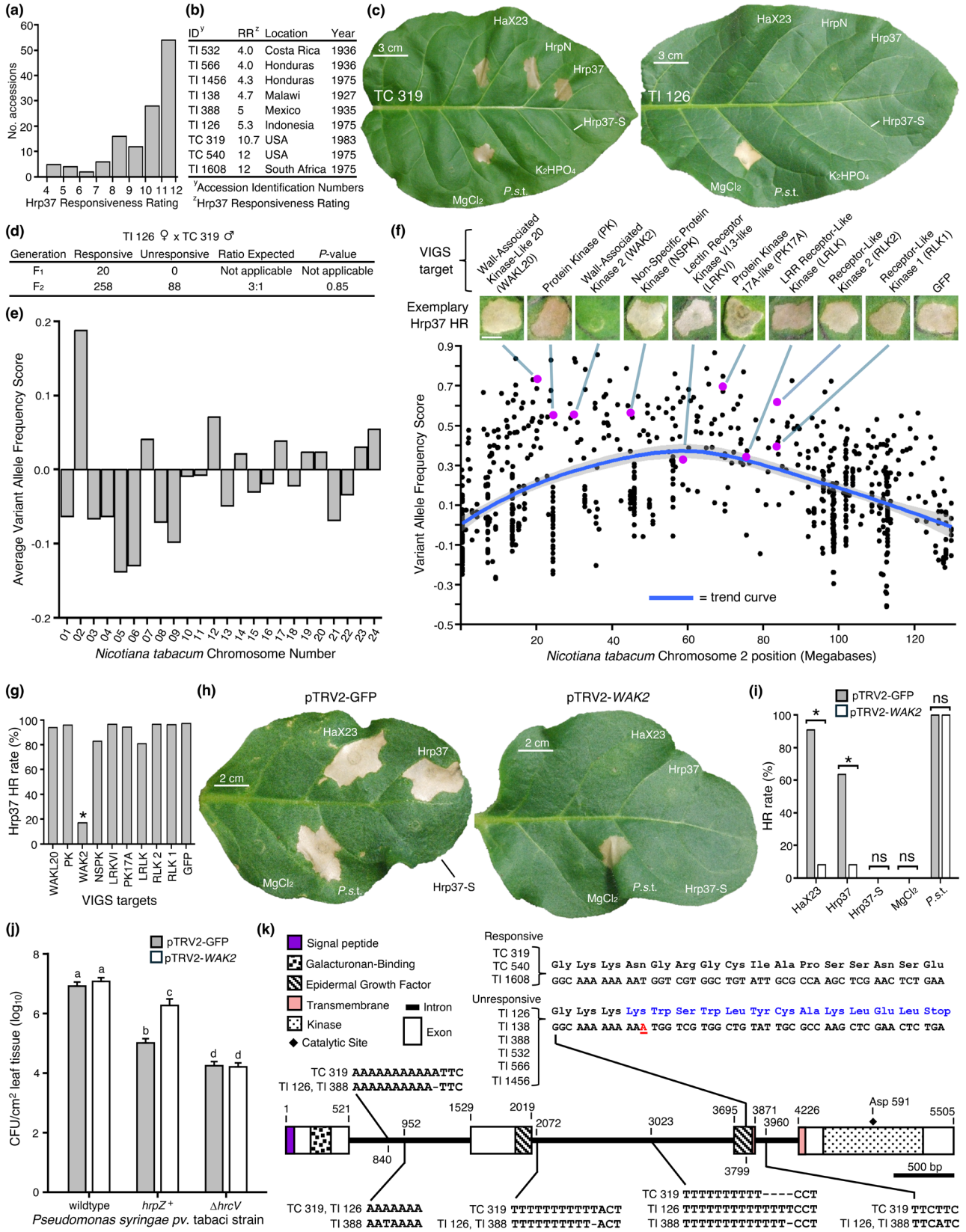

FIGURE 1 | Legend on next page.

nine variant-containing genes in this region to target with virus-induced gene silencing (VIGS; Bachan and Dinesh-Kumar 2012; Figure 1f). Six of the candidate genes encoded predicted plasma membrane receptor-like kinases, while three encoded predicted intracellular signalling kinases (Table S2). The regions targeted for silencing in the candidate genes (Dataset S3) were designed to minimise off-target silencing using the Sol Genomics Network VIGS tool (Fernandez-Pozo et al. 2015).

FIGURE 1 | *Nicotiana tabacum* (tobacco) *Wall-Associated Kinase 2* (*WAK2*) is required for the hypersensitive reaction (HR) to harpins. (a) Responsiveness ratings of 127 tobacco accessions at 2 days after infiltration with 100 μM Hrp37 peptide, with higher numbers indicating more robust responses. $N = 3$ plants per accession. (b) Names, Hrp37 response ratings, locations where collected or developed and years when added to the germplasm collection, for tobacco accessions used for additional experimentation. (c) Representative HRs in leaves of accessions TC 319 and TI 126 at 3 days after infiltration with the elicitors and controls indicated on each leaf sector. Tan areas represent the HR (necrosis); HRs are evident at 24 h after infiltration and are photographed at 72 h after infiltration for clear visibility. Peptides Hrp37 and Hrp37-S were infiltrated at 100 μM, while *Erwinia amylovora* HrpN protein and HaX23 peptide were infiltrated at 2 μM. *Pseudomonas syringae* pv. *tomato* (Pst) was infiltrated at 10^8 colony-forming units (CFU)/mL. Buffer controls are 5 mM K_2HPO_4 and 10 mM $MgCl_2$ for HrpN and Pst, respectively. Hrp37-S is a scrambled-sequence version of peptide Hrp37. Concentrations used for each elicitor are consistent throughout the figure. (d) Segregation of the Hrp37 peptide responsiveness trait in F_1 and F_2 populations derived from a TI 126 × TC 319 interaccession cross. The *p* value was generated through a chi-square test. (e) Average variant allele frequency (VAF) scores for all 24 tobacco chromosomes as revealed by bulked-segregant analysis (BSA) of a TI 126 × TC 319 F_2 population. VAF was calculated for each variant as the proportion of sequencing reads harbouring a variant at a given locus. A single VAF score for each variant was calculated by subtracting its VAF in the Hrp37-responsive bulk from its VAF in the Hrp37-unresponsive bulk. The VAF scores for all variants on each chromosome were averaged. (f) Virus-induced gene silencing (VIGS) of candidate genes of interest identified by BSA. The expression of genes representing nine coding-sequence variants of interest (indicated by magenta dots) were targeted by VIGS in harpin-responsive accession TC 540 (cv. Samsun NN; Zhang et al. 2013; Zhang and Thomma 2014). Photographs of representative HRs to Hrp37 infiltration in leaves of VIGS-inoculated tobacco plants are shown. Green fluorescent protein (GFP) indicates plants that were inoculated with a GFP-targeting VIGS construct to serve as a negative control. The blue trend curve was generated through locally estimated scatterplot smoothing. Scale bar, 0.5 cm. (g) Percent of Hrp37 infiltrations resulting in HR in VIGS-inoculated TC 540 tobacco plants. Targeted genes are the same as in (f). Asterisk indicates a statistically significant ($p < 0.05$) difference relative to GFP negative control. $N = 37$–99 infiltrations per VIGS construct, with about 10 infiltrations being performed across multiple leaves on a single plant. A minimum of four plants per VIGS target were tested. (h) Representative HRs of GFP- and *WAK2*-targeted VIGS plants of tobacco accession TC 540 at 3 days after infiltration with the indicated elicitors and controls. (i) Percent of infiltrations resulting in HR in GFP- and *WAK2*-targeted VIGS plants infiltrated with the same elicitors as used in (h). Asterisks indicate statistically significant ($p < 0.05$) differences relative to GFP. Ns, not significant. $N = 11$–12 infiltrations per elicitor, with each infiltration being performed on a separate leaf across five VIGS-inoculated plants of tobacco accession TC 540. (j) Multiplication of wildtype, *hrpZ*-carrying (*hrpZ*$^+$) and type III secretion system mutant (Δ*hrcV*) *Pseudomonas syringae* pv. *tabaci* strains in GFP- and *WAK2*-targeted VIGS plants of tobacco cultivar Xanthi at 10 days after infiltration with 1.5×10^5 bacterial CFU/mL. Bars sharing a letter do not have statistically significant ($p < 0.05$) differences. $N = 20$–24 infiltrations per strain, with four infiltrations being performed across two leaves on each of five or six plants per VIGS target. Bars indicate standard error. (k) *WAK2* gene organisation, with the positions of sequence variants within analysed accessions indicated. The frameshift effect of the adenine insertion variant (indicated by the underscored red letter 'A') used to identify *WAK2* in the BSA is shown using three-letter amino acid code. Base positions within the 5505 bp *WAK2* gene sequence are indicated. For statistical tests, Fisher's exact test with Bonferroni correction (g), Fisher's exact test (i) and Tukey's multiple comparisons test (j) were used. Experiments shown in (c), (g) and (i), were performed at least three times and the experiment shown in (j) was performed twice. Experimental runs yielded similar results; representative experimental results are shown.

Three-week-old plants were inoculated in the first true leaves with *Agrobacterium tumefaciens* strains containing the pTRV1 and pTRV2 vectors (Bachan and Dinesh-Kumar 2012). Approximately 3 weeks after VIGS inoculation, when photobleaching was evident in the leaves of *phytoene desaturase*-silenced controls, the plants were challenged with Hrp37, and the proportion of HRs was noted. Of the nine candidate genes targeted using VIGS, only the gene encoding predicted Wall-Associated Receptor Kinase 2-Like (herein referred to as Wall-Associated Kinase 2, or WAK2) resulted in significantly reduced HR rates in response to Hrp37 compared to GFP-silenced controls (Figure 1f,g). *WAK2*-targeted VIGS plants additionally showed significantly reduced HR rates when infiltrated with HaX23 relative to GFP-silenced controls, while showing no such reduced HR rate when infiltrated with Pst (Figure 1h,i). *WAK2*-targeted VIGS plants also retained the HR triggered by the oomycete elicitor INF1 (Figure S6), which is dependent on the co-receptor BAK1 (Heese et al. 2007). *BAK1*-targeted VIGS plants exhibited a reduced INF1-mediated HR and a normal HaX23-mediated HR (Figure S6), indicating that WAK2-mediated harpin responses do not depend on BAK1. *WAK2*-targeted VIGS plants showed no obvious developmental defects relative to GFP-targeted plants (Figure S7).

To determine whether *WAK2* is required for responsiveness to harpins delivered by bacteria, *WAK2*-targeted and GFP-targeted VIGS plants were infiltrated with wildtype (WT) tobacco wildfire pathogen *P. syringae* pv. *tabaci* and an engineered *P. syringae* pv. *tabaci* strain carrying an intact chromosomal copy of the *hrpZ* harpin gene (*P. syringae* pv. *tabaci hrpZ*$^+$; Figure S8). Introduction of intact *hrpZ* into *P. syringae* pv. *tabaci* was previously demonstrated to reduce *P. syringae* pv. *tabaci* multiplication and virulence in tobacco (Tsunemi et al. 2011). *P. syringae* pv. *tabaci hrpZ*$^+$ multiplication was significantly higher in *WAK2*-targeted compared to GFP-targeted VIGS plants (Figure 1j), indicating that *WAK2*-targeted VIGS plants are compromised in responsiveness to HrpZ delivered by *P. syringae* pv. *tabaci*.

Upon computer analysis of the *WAK2* gene in the TC 319 annotated tobacco genome (Sierro et al. 2024), we discovered an in-frame open reading frame encoding 141 amino acids upstream of the start codon predicted in the TC 319 genome annotation. Therefore, we designed primers to amplify the putative full c. 2500 bp *WAK2* cDNA in TC 319 and sequenced the resulting product. The updated structure of the *WAK2* gene is shown in Figure 1k. The *WAK2* variant identified in the BSA (Figure 1f) was a single-base adenine insertion resulting in a frameshift after the lysine at position 375, resulting in 12 erroneous amino

acids followed by a premature opal stop codon. This is predicted to encode a truncated protein with no transmembrane or intracellular kinase domain (Figure 1k).

To assess the presence of this frameshift variant in other accessions, we cloned and sequenced the *WAK2* cDNA in an expanded set of phenotypically characterised accessions (Figure 1b). All six unresponsive accessions possessed the same frameshift variant, while the three responsive accessions had an intact *WAK2* cDNA sequence (Figure 1k). This result is consistent with an allelism cross performed between the unresponsive accessions TI 126 and TI 388, in which the resulting F_1 progeny were unresponsive to Hrp37 and HaX23 (Figure S9), indicating allelism. Crosses of TI 126 and TI 388 with the responsive accession TC 319 resulted in progeny responsive to both elicitors (Figure S9). No additional coding sequence variants within *WAK2* were observed in any of the accessions. These data strongly suggest that the *WAK2* insertion variant underlies the harpin unresponsiveness trait.

To explore the genetic independence of the unresponsive accessions, we sequenced the entire *WAK2* locus in accessions TC 319, TI 126 and TI 388. Six variants located in introns were identified in unresponsive accessions TI 126 and TI 388 relative to TC 319, with two being unique to TI 388 and one being unique to TI 126 (Figure 1k). Additionally, TI 126 and TI 388 differ in flower colour and leaf venation patterns (Figure S10). These results suggest that TI 126 and TI 388, which were originally collected at different locations and times (Figure 1b), are genetically distinct accessions.

We conclude that the predicted receptor-like kinase WAK2 is required for the harpin-induced HR in tobacco. The presence of a natural, disruptive variant of *WAK2* in diverse tobacco accessions is associated with the inability to develop an HR in response to harpin infiltration. Additionally, directly targeting the expression of *WAK2* through VIGS in harpin-responsive tobacco accessions results in a significantly diminished frequency of HR in response to harpin elicitors, while not affecting HR induction by the oomycete effector INF1. Despite the enormous size (c. 4.5 Gb) and complexity (polyploidy and 70% repetitive elements) of the tobacco genome (Sierro et al. 2014), we pinpointed *WAK2* as underlying the harpin HR phenotype. To our knowledge, this is the first successful use of BSA-facilitated mapping in tobacco, which has only one cloned resistance gene, *N*, conferring resistance to tobacco mosaic virus (Whitham et al. 1994).

Using a genome-wide association study approach, we recently reported that *Arabidopsis thaliana* (Arabidopsis) *WAK3* (*AtWAK3*) is required for the necrotic response to harpins in this model plant (Held et al. 2024). The necrotic response to harpins in Arabidopsis is clearly phenotypically distinct from the harpin-induced HR in tobacco. In Arabidopsis, the response is relatively slow and weak and does not constitute a typical HR, taking up to 5 days to become evident and often manifesting as chlorosis with limited necrosis (Held et al. 2024). In contrast, the response of tobacco to harpin infiltration is consistent with a typical HR, becoming evident within 12–24 h and characterised by complete collapse of the infiltrated area, which becomes white and dry within 3–4 days, fitting the classic phytobacterial HR definition (Klement 1963; Klement and Goodman 1967). Despite the stark differences in the necrotic responses triggered by harpins in Arabidopsis and tobacco, our previous work (Held et al. 2024) and the independent approach presented here indicate a role for WAKs in harpin responses in both species. The different harpin responses in Arabidopsis and tobacco might be due to functional differences between the WAKs or other, yet undiscovered components of the harpin recognition pathway.

Although WAKs were initially characterised as receptors of pectin and pectin fragments (Brutus et al. 2010; Decreux and Messiaen 2005; Wagner and Kohorn 2001), subsequent genetic studies have identified WAKs as underlying resistance responses to various fungal pathogens (Larkan et al. 2020; Saintenac et al. 2018; Zhong et al. 2024). Additionally, the WAK Snn1 from wheat (*Triticum aestivum*) has been shown to directly interact with a cell death-triggering fungal effector (Shi et al. 2016). *At*WAK3 and WAK2 might also sense pathogen-delivered harpins and initiate cell death, contributing to defence. Our finding that *WAK2* silencing in tobacco increases the virulence of HrpZ-bearing *P. syringae* pv. *tabaci* suggests that WAK2 participates in immune sensing of pathogen-delivered harpins and has the potential to act as a disease resistance gene. These findings are pointing to a role for WAKs in plant sensing of pathogens and potential pathogens. It is tempting to speculate that transgenic expression of tobacco *WAK2* in fire blight host plants such as apple (*Malus domestica*) could lead to increased fire blight resistance, especially considering that apple fails to activate immune responses when challenged with HrpN (Reboutier et al. 2007).

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All study data are included in the article and/or Supporting Information. DNA sequence data will be uploaded to GenBank upon acceptance of the manuscript for publication.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.