TALE-induced bHLH transcription factors that activate a pectate lyase contribute to water soaking in bacterial spot of tomato

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AvrHah1 [avirulence (avr) gene homologous to avrBs3 and hax2, no. 1] is a transcription activator-like (TAL) effector (TALE) in *Xanthomonas gardneri* that induces water-soaked disease lesions on fruits and leaves during bacterial spot of tomato. We observe that water from outside the leaf is drawn into the apoplast in *X. gardneri*-infected, but not *X. gardneriΔavrHah1* (*XgΔavrHah1*)-infected, plants, conferring a dark, water-soaked appearance. The pull of water can facilitate entry of additional bacterial cells into the apoplast. Comparing the transcriptomes of tomato infected with *X. gardneri* vs. *XgΔavrHah1* revealed the differential up-regulation of two basic helix–loop–helix (bHLH) transcription factors with predicted effector binding elements (EBEs) for AvrHah1. We mined our RNA-sequencing data for differentially up-regulated genes that could be direct targets of the bHLH transcription factors and therefore indirect targets of AvrHah1. We show that two pectin modification genes, a pectate lyase and pectinesterase, are targets of both bHLH transcription factors. Designer TALEs (dTALEs) for the bHLH transcription factors and the pectate lyase, but not for the pectinesterase, complement water soaking when delivered by *XgΔavrHah1*. By perturbing transcriptional networks and/or modifying the plant cell wall, AvrHah1 may promote water uptake to enhance tissue damage and eventual bacterial egression from the apoplast to the leaf surface. Understanding how disease symptoms develop may be a useful tool for improving the tolerance of crops from damaging disease lesions.

TAL effector | *Xanthomonas* bacterial spot | water soaking

Bacterial spot disease caused by *Xanthomonas* sp. is a major limiting factor of agricultural yield (1). *Xanthomonas gardneri* has emerged recently as the dominant tomato pathogen in the midwestern United States and causes significant spotting on fruits (2). *X. gardneri* is also responsible for severe crop losses in Brazil (3) and appears to be spreading globally (4).

Among its repertoire of type III effectors, *X. gardneri* possesses a single transcription activator-like (TAL) effector (TALE) protein, AvrHah1 [avirulence (avr) gene homologous to avrBs3 and hax2, no. 1], which has been shown to confer enhanced water-soaked lesions in pepper (5). TALEs are secreted into host plant cells via the bacterial type III secretion apparatus and are delivered into the nucleus, where they activate the expression of target host genes (6). TALE binding specificity for host target DNA depends on the amino acid sequence of the central DNA-binding domain (DBD), which is composed of several repeats of 34–35 aa, nearly identical except for the 12th and 13th amino acids of each repeat, termed the repeat-variable diresidue (RVD) (7). Each RVD confers binding specificity to a particular nucleotide, and in combination the targeted host sequence, termed the "effector binding element" (EBE), can be predicted (7, 8). Once bound to a DNA target, the acidic activation domain (AD) of the TALE recruits the host's transcriptional machinery to activate gene expression (6).

Plants have evolved diverse resistance mechanisms in response to TALEs (9). Some plants have strategically placed "EBE traps" in the promoters of resistance genes, as in the case of the pepper *Bs3* resistance gene, which is transcriptionally activated at partially overlapping EBEs by the TALEs AvrBs3 and AvrHah1 (5, 10). Plants have developed mutations in promoter EBE regions that prevent successful activation of gene targets by TALEs, as in the case for rice *Os8N3* (11). Tomato plants use Bs4, a nucleotide-binding leucine-rich repeat (NB-LRR) resistance (R) protein, to induce a cell death, or hypersensitive response (HR), in the presence of certain TALEs (12, 13).

If a plant gene targeted by the TALE promotes pathogen growth or spread, the gene is designated as a susceptibility (*S*) gene. Identifying and characterizing *S* genes reveals pathogen strategies and is useful in the design of disease resistant plants, for example through the removal of relevant EBEs via DNA editing technologies (9). For TALEs that activate multiple host gene targets, such as those with EBEs that partially span a TATA box, it becomes increasingly challenging to identify the bona fide *S* gene(s) (14, 15). To probe single genes for pathogenicity functions, designer TALEs (dTALEs) can be constructed and tested *in planta* for virulence contributions (16).

Several examples connecting lesion development and TALE *S* gene targets have been reported in diverse plant–xanthomonad pairs. In rice, Tal2g from *Xanthomonas oryzae* pv. *oryzicoa* activates expression of *OsSULTR3;6*, which encodes a sulfate transporter (17). Mutations in *Tal2g* reduced lesion expansion and *X. oryzae* pv. *oryzicoa* surface population, but not *in planta* growth, and dTALE activation of *OsSULTR3;6* expression restored lesion expansion and surface growth to wild type levels (17). *CsLOB1*, a member of the lateral organ boundaries family from citrus, is activated by the PthA family of TALEs from

Significance

AvrHah1 [avirulence (avr) gene homologous to avrBs3 and hax2, no. 1] is a transcription activator-like (TAL) effector (TALE) in *Xanthomonas gardneri* that enhances water soaking in its known hosts tomato, pepper, and *Nicotiana benthamiana*. We observe that the water soaking conferred by AvrHah1 is due to the movement of water into the infected apoplast from a wet environment. RNA sequencing identified two basic helix–loop–helix (bHLH) transcription factors that we confirmed as targets of AvrHah1. We discovered that a pectate lyase was upregulated by both of the bHLH transcription factors. Designer TALEs (dTALEs) for both bHLH transcription factors and the pectate lyase complemented the water-soaking phenotype of *X. gardneriΔavrHah1*. This report demonstrates virulence activity from an indirect TALE target.

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xanthomonad pathogens of citrus. Loss of PthA reduces *in planta* bacterial growth and pustule formation (18, 19). TAL20 from the vascular cassava pathogen *Xanthomonas axonopodis* pv. *manihotis* activates expression of *MeSWEET10a*, which encodes a sugar transporter (20). dTALEs activating *MeSWEET10a* complemented the reduction in water soaking and midvein bacterial growth displayed by an *X. axonopodis* pv. *manihotis TAL20* deletion strain in cassava (20). Avrb6 in *Xanthomonas campestris* pv. *malvacearum* correlates with increased water soaking and bacterial surface population in cotton (21). AvrBs3 activates the expression of pepper *UPA20*, which encodes a basic helix–loop–helix (bHLH) transcription factor that induces cell hypertrophy (22). Loss of AvrBs3 has been demonstrated to incur a bacterial fitness cost in the field (23).

AvrHah1 was found in a forward screen in search for a factor from *X. gardneri* that promotes the development of water-soaked lesions in pepper (5). We observed that AvrHah1 enables the absorption of water into the apoplast of *X. gardneri*-infected leaves, conferring a dark, water-soaked appearance. The AvrHah1-mediated intake of water can be observed in real time and can also be measured quantitatively by collection and weighting of apoplastic fluid. Furthermore, bacterial cells can be introduced into the apoplast during water soaking. We performed comparative RNA sequencing analysis of tomato leaves infected with *X. gardneri* or *X. gardneriΔavrHah1* (*XgΔavrHah1*) and identified two bHLH transcription factors that were highly up-regulated in the presence of AvrHah1. We show that two pectin-modification genes—a pectate lyase (*PL*) and a pectinesterase (*PE*)—are downstream targets of both bHLH transcription factors and are therefore indirect targets of AvrHah1. We constructed dTALEs targeting the bHLH transcription factors and the pectin modification genes for gene activation and show that dTALEs activating the bHLH transcription factor or the PL encoding genes complemented the water-soaking defect of *XgΔavrHah1*.

Materials and Methods

***X. gardneri* Mutant Construction.** *Xg153* (SM194-10) is from a collection of *X. gardneri* strains isolated between 2010 and 2012 in diseased tomato fields in Ohio and Michigan. Activation of *Bs3* in pepper 30R, Southern blot analysis, and Sanger sequencing confirmed the identity of the single TALE in *Xg153* as *avrHah1* (24). All *X. gardneri* mutants were constructed in the *Xg153* background. *XgΔavrHah1* was created by double homologous recombination using the suicide vector pLVC18 (25), such that the 13.5 repeats of the DBD were deleted in-frame (AvrHah1$_{\Delta DBD}$) (Fig. S1*A*). Deletion of the DBD was confirmed by Southern blot analysis, using 5 μg of bacterial genomic DNA restriction digested for 2 h with BamHI and run on a 0.7% agarose gel for 2 h at 100 V. DNA was transferred overnight to a Hybond-N$^+$ membrane, hybridized with a digoxigenin (DIG)-labeled probe for the first 705 bp of AvrHah1 [which includes the type 3 secretion signal (T3SS)], and imaged with chemiluminescence. The blot was stripped and rehybridized with a DIG-labeled probe for the central DBD and reimaged (Fig. S1*B*). *XgΔavrHah1* was tested on pepper 30R for loss of *Bs3* activation (Fig. S1*C*).

Complementation of *XgΔavrHah1*. All complementation constructs are driven by the *X. axonopodis* pv. *manihotis* strain 668 TAL20 promoter (^{pro}*TAL20*) (20). Gibson assembly (New England Biosciences) was used to clone ^{pro}*TAL20* upstream of *avrHah1*, *avrBs3*, or dTALEs into a gentamycin^R entry vector using SalI and XbaI sites (26). Entry clones were then Gateway cloned (Invitrogen) into the broad host-range vector pVSP61 using LR Clonase (Invitrogen). dTALEs targeting the promoters of *Solyc03g097820* (*dT bHLH3*), *Solyc06g072520* (*dT bHLH6*), *Solyc05g014000* (*dT PL*), and *Solyc11g019910* (*dT PE*) were constructed as previously described (16). Information on the dTALEs including RVD sequences and binding scores as predicted by TALE-NT (27) can be found in Table S1. Triparental matings of pVSP61 complementation plasmids into *XgΔavrHah1* or *Xanthomonas euvesicatoria 85-10* (*Xe85-10*) (28) were performed with the *Escherichia coli* helper strain pRK600, selected on rifampicin (Rif) and kanamycin, and confirmed with PCR.

Bacterial-Growth Conditions. *Xanthomonas* strains were grown on nutrient yeast glycerol agar (NYGA) supplemented, as appropriate, with 100 μg/mL Rif (all strains), 25 μg/mL kanamycin (*XgΔavrHah1* or *Xe85-10* complemented with pVSP61), and 10 μg/mL tetracycline (Tet) (*X. gardneri* Tet^R used for water-soaking inoculum). Strains were incubated at 28 °C for 48 h. Cells were adjusted to appropriate concentrations with 10 mM $MgCl_2$.

Assays for Water Soaking and *in Planta* Bacterial-Growth Assays. For water-soaking assays in *Nicotiana benthamiana*, leaves were syringe-infiltrated with a bacterial suspension adjusted to OD_{600} = 0.1 (~10^8 CFU/mL). At 48 hours postinfection (hpi), water soaking was induced in *N. benthamiana* by creating a small epidermal wound in the infected area and pipetting a 30 μL drop of water or 10^5 CFU/mL *X. gardneri* Tet^R on top of the wound. Total *in planta* bacteria were selected for on NYGA with Rif. *X. gardneri* Tet^R internalized during water soaking were selected by plating on Rif and Tet (Rif + Tet). All tomato experiments were performed in Heinz 1706 (Tomato Genetics Resource Center). For tomato water-soaking assays, leaves were infiltrated at OD_{600} = 0.1. At 48 hpi, infected leaves were submerged in water for 20 min without the introduction of surface wounds. For tomato *in planta* growth assays, six 0.5 cm^2 leaf discs were collected at time 0 (t = 0) and at 6 d postinfection (dpi). Tomato leaves were infiltrated with bacterial suspensions at 10^4 CFU/mL for the development of discrete lesions and photographed 6 dpi.

Apoplastic Fluid Measurements. For quantitative water-soaking assays, tomato leaves were syringe-infiltrated with a bacterial inoculum adjusted to OD_{600} = 0.1. At 48 hpi, leaves were submerged in water for 20 min and blotted with a Kimwipe to remove water from the leaf surface. Two 0.5 cm^2 leaf discs were collected and placed in a 0.5 mL tube with a small hole cut in the bottom. This tube was placed in a preweighed 1.5-mL tube. The tubes were centrifuged at 3,000 × *g* for 5 min to collect the apoplastic fluid. Weights of the 1.5-mL tubes postspin were subtracted from the prespin weights to obtain a quantitative measurement of apoplastic fluid.

RNA Sequencing and TALE Prediction. *X. gardneri* and *XgΔavrHah1* were syringe-infiltrated into tomato Heinz 1706 leaves at OD_{600} = 0.25 and tissue was collected and frozen in liquid nitrogen at 24 hpi and 48 hpi. RNA from three biological replicates per time point were prepared using the Spectrum Total Plant RNA Kit (Sigma-Aldrich) and RNA sequencing libraries were prepared using the Illumina TruSeq RNA Library Prep Kit v2. Sequencing of 100-bp read length with Paired Ends was performed on a single lane of an Illumina HiSeq2000. Data analysis was performed using the CLC Genomics Workbench software to identify differentially expressed genes (at least twofold different with $P < 0.05$). Computational predictions for AvrHah1 EBEs were performed using the TALE-NT 2.0 algorithm (27) with RVDs for AvrHah1 (NN_IG_NI_NI_NI_HD_HD_NG_NN_NI_HD_HD_HD_NG) and a cutoff of 4 times the best possible score of 3.76. Up-regulation of genes of interest were confirmed with semiquantitative RT-PCR. *X. gardneri* strains were infiltrated into tomato leaves at OD_{600} = 0.25 and tissue was collected at 24 hpi. The Spectrum Plant RNA kit (Sigma-Aldrich) (with on-column DNaseI treatment) and the SuperScript III First-Strand Synthesis System were used to make cDNA from 1.5 μg of RNA. Five microliters of 1:10 diluted cDNA were used for 24 cycles of amplification using Phusion HF polymerase (New England Biolabs).

Transient Promoter::Luciferase Assays in *N. benthamiana*. Promoter sequences of 1 kb upstream of the start codon for *bHLH3* (^{pro}*bHLH3*), *bHLH6* (^{pro}*bHLH6*), *PL* (^{pro}*PL*), and *PE* (^{pro}*PE*) were Gateway (Invitrogen) cloned into the binary luciferase reporter construct pGWB35 (29). *avrHah1* and the coding regions of *bHLH3* and *bHLH6* were cloned into the binary expression vector p1776. All constructs were conjugated into *Agrobacterium* GV3101 via triparental matings. *N. benthamiana* leaves were coinfiltrated with *Agrobacterium* (OD_{600} = 0.4 for each strain) for the combinations of promoter and transcriptional activator indicated. An empty *Agrobacterium* strain was coinfiltrated with each promoter::luciferase reporter for background promoter activation measurements. At 24 hpi, the leaves were syringe-infiltrated with 1 mM luciferin. Six 0.28 cm^2 leaf punches per condition were taken and placed in separate wells of a black microtiter plate, suspended on 100 μL of water. Luciferase activity was read using a Wallace Envision plate reader.

Results

AvrHah1 Enables the Absorption of Water into the Apoplast of *X. gardneri*-Infected Leaves. We observed a dark, apoplastic water soaking when *X. gardneri*, but not *XgΔavrHah1*, was infiltrated into tomato, pepper, and *Nicotiana benthamiana*, the known hosts of *X. gardneri* (24). This symptom was enhanced when infected plants were placed in a mist chamber. We could also obtain enhanced water soaking by growing infected plants at ambient humidity and then submerging leaves in water immediately before observation.

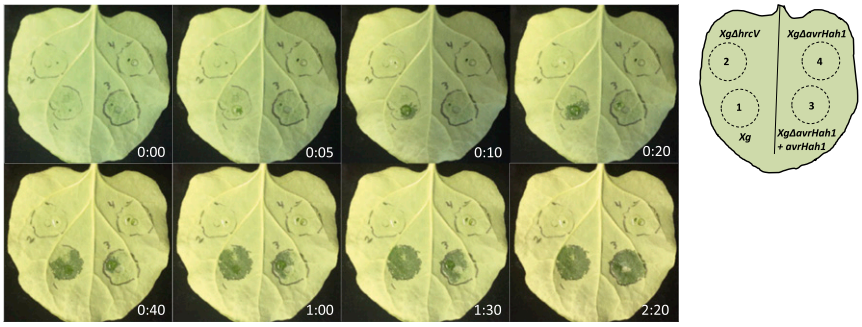

Fig. 1. AvrHah1 promotes the intake of water into the apoplast of *X. gardneri* (*Xg*)-infected plants. *N. benthamiana* was syringe-infiltrated with *X. gardneri* (1), *XgΔhrcV* (2), *XgΔavrHah1* + *avrHah1* (3), and *XgΔavrHah1* (4) at OD_{600} = 0.1 [depicted in the diagram as the bottom left area of the leaf, top left area of the leaf (2), bottom right area of the leaf (3), and top right area of the leaf (4), respectively]. At 48 hpi, a 30-μL drop of water was pipetted on top of a small epidermal wound in the infiltrated areas. Pictures were taken at the times indicated in each frame until 2 min and 20 s after application of the first water drop.

The AvrHah1-mediated intake of water into the apoplast was observed in real time in *N. benthamiana* (Fig. 1 and Movie S1). *X. gardneri*, *XgΔhrcV* (a mutant in type III effector secretion), *XgΔavrHah1*, and *XgΔavrHah1* + *avrHah1* were infiltrated into *N. benthamiana*. At 48 hpi, a pipette tip was used to make a small epidermal wound on the infiltrated areas. Immediately after introduction of the wound, a 30 μL drop of water was pipetted on top of each wound. In the zones infiltrated with *X. gardneri* or *XgΔavrHah1* + *avrHah1*, the drop of water gradually shrunk as it was pulled into the leaf, darkening the apoplast as the advancing front proceeded away from the wound site within the boundaries of the infiltrated zone. The drop of water was completely absorbed by 2 min. In contrast, the water drop remained on top of the wound in zones infiltrated with *XgΔhrcV* or *XgΔavrHah1*.

In tomato, the water-soaking effect of *X. gardneri* was similarly dramatic and enhanced by external water, yet progressed more slowly (20 min compared with 2 min in *N. benthamiana*) (Fig. 2). Leaves infiltrated with *X. gardneri* showed dark water soaking in the infected area, whereas leaves infiltrated with *XgΔhrcV* or *XgΔavrHah1* did not develop water soaking. Complementation of full length *avrHah1* into *XgΔavrHah1* fully restored water soaking, but a mutant version of *avrHah1* with an in-frame deletion of the DBD, $AvrHah1_{\Delta DBD}$, did not enhance water soaking. Complementation of *XgΔavrHah1* with *avrBs3* induced an immune reaction, or HR, likely due to recognition of AvrBs3 by the tomato R protein Bs4.

Previous work did not detect a difference in apoplastic growth between *X. gardneri* and an *avrHah1* deletion mutant in pepper (5). We found these results to be consistent in tomato (Fig. S2). We did not detect a growth defect for *avrHah1* in the background of an *XgΔavrBs2* mutant. AvrBs2 is a type III effector protein that contributes measurably to *in planta* growth in many xanthomonads (30). Thus, it is unlikely that bacterial cell number accounts for the differential water soaking between *X. gardneri* and *XgΔavrHah1*. AvrHah1 was shown to confer enhanced water soaking to *Xe85-10* in pepper (5), and we found that result was consistent in tomato (Fig. 3). Six days after infiltration into tomato leaves, *X. euvesicatoria* + *avrHah1* developed water-soaked lesions, whereas *X. euvesicatoria* and *X. euvesicatoria* + $avrHah1_{\Delta DBD}$ developed dry, flecked lesions (AvrHah1 is able to confer enhanced water soaking to *X. euvesicatoria* at an earlier time in infection compared with the endogenous water-soaking mechanisms in *X. euvesicatoria*). Tomato infiltrated with *X. euvesicatoria* + *avrBs3* did not develop any visible lesions (likely due to activation of Bs4 resistance). Taken together, these results show that AvrHah1 enhances the intake of external water by the leaf during xanthomonad infection.

Bacteria Can Be Introduced into the Apoplast During Water Soaking. We wondered if water soaking could be a mechanism that introduces new bacteria into the apoplast. We set up a water-soaking assay in *N. benthamiana* with *X. gardneri* and *XgΔhrcV* as previously described, except we supplemented the drop of water on the wound site with Tet-resistant *X. gardneri* (*X. gardneri* Tet^R). We plated a dilution series from macerated leaf discs on Rif to measure all *X. gardneri*, or Rif + Tet to select for any newly introduced *X. gardneri* Tet^R (Fig. 4). We sampled leaf discs away from the wound site to avoid any *X. gardneri* Tet^R cells left on the leaf surface. We found that water soaking could ferry the Tet-resistant *X. gardneri* into the apoplast away from the initial wound site. No Tet-resistant *X. gardneri* were detected in the apoplasts of leaves infected with *XgΔhrcV*, which did not develop any water soaking.

AvrBs3, but Not AvrHah1, Triggers Bs4-Mediated Resistance. Bs4 is a TIR-NB-LRR type resistance protein in tomato that recognizes the TALEs AvrBs4, AvrBs3, Hax3, Hax4, but not Hax2 (12, 13, 31). Hax2 has a DBD composed of 35 amino acid repeats in contrast to its counterparts with 34 amino acid repeats (13). Although previous results showed that activation of Bs4 in response to AvrBs3 required strong expression in a transient system (31), we observed a HR in tomato in response to delivery of *avrBs3* by *XgΔavrHah1* and *Xe85-10*. Because AvrHah1 activates water soaking in tomato and not HR, we tested the possibility that water soaking was disrupting a potential Bs4-mediated cell death response. Evidence points to a direct recognition model between Bs4 and its recognized TALEs, likely involving the repeats of the DBD (12). We truncated the last 46 amino acids of *avrHah1* to delete the AD ($avrHah1_{\Delta AD}$), which removed any water-soaking ability while maintaining the structure of the DBD. Delivery of $avrHah1_{\Delta AD}$ did not induce a cell death response in tomato (Fig. 5), suggesting that Bs4 differentially recognizes AvrHah1 and AvrBs3. AvrHah1 is a structurally unique TALE

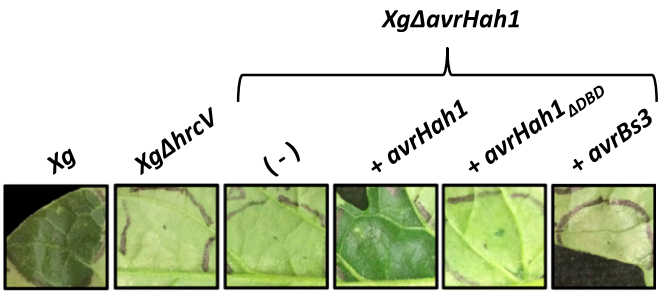

Fig. 2. AvrHah1 induces water soaking in tomato, whereas AvrBs3 activates a hypersensitive response. Tomato Heinz 1706 leaves were syringe-infiltrated with the *X. gardneri* (*Xg*) strains indicated (OD_{600} = 0.1). At 48 hpi, leaves were submerged in water for 20 min, blotted with a Kimwipe, and photographed.

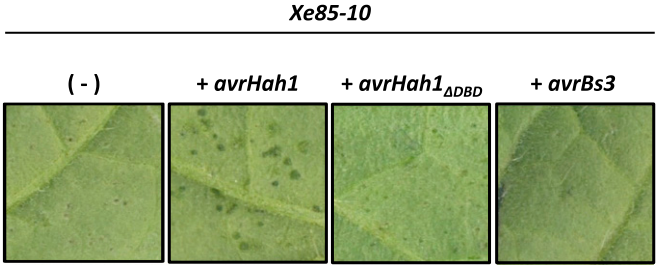

Fig. 3. AvrHah1 enhances water soaked lesions in *Xe85-10*. Tomato leaves were infiltrated at 10^4 CFU/mL with *Xe85-10* alone (–) or *Xe85-10* complemented as indicated and observed at 6 dpi. Plants were grown at ambient humidity and were not submerged in water before observation.

as its DBD is composed of both 34 and 35 amino acid repeats (5). An intriguing hypothesis is that selection pressure caused AvrHah1 to adopt its 35 amino acid repeats to evade recognition by tomato Bs4.

RNA Sequencing Reveals Direct and Indirect Targets of AvrHah1. To study the tomato genes responsible for AvrHah1-mediated water soaking, we used RNA sequencing (RNA-seq) to identify the differentially expressed genes between tomato infected with *X. gardneri* or *XgΔavrHah1*. We found that 6,292 genes were differentially up-regulated in *X. gardneri*-infected tomato leaves at 48 hpi (greater than twofold change; $P \leq 0.05$).

Of particular interest were genes that were highly differentially expressed and contained a predicted promoter EBE for AvrHah1. We used the TALE-NT 2.0 algorithm (27) to computationally predict the AvrHah1 EBEs in the tomato promoterome, defined here as the set of sequences 300 bp upstream of the start codon for annotated genes in the Heinz 1706 genome (32). This prediction resulted in 4,106 possible binding sites (on both the plus and minus strands). Among the most highly up-regulated genes in *X. gardneri*-, but not *XgΔavrHah1*-infected, tomato leaves, we identified two bHLH transcription factors, *Solyc03g097820* (*bHLH3*) and *Solyc06g072520* (*bHLH6*), that possessed EBEs for AvrHah1 (Table 1). Given that AvrBs3 targets *UPA20*, a pepper bHLH transcription factor (22), and that AvrBs3 and AvrHah1 share some binding specificity (both are capable of activating expression of the *Bs3* resistance gene at overlapping EBEs) (Fig. S3) (5), we selected the two tomato bHLH transcription factors for further study.

AvrHah1 Activates Expression from the Promoters of the bHLH Transcription Factors. We confirmed by semiquantitative RT-PCR that gene activation of *bHLH3* and *bHLH6* occurred in *X. gardneri*-infected, but not *XgΔavrHah1*-infected, tomato leaves. Additionally, expression of *bHLH3 and bHLH6* was rescued when *XgΔavrHah1* was complemented with *avrHah1* (Fig. 6*A*). To confirm that AvrHah1 was able to activate gene expression from the promoters of *bHLH3* and *bHLH6*, we used an *Agrobacterium tumefaciens* luciferase reporter assay in *N. benthamiana*. Luciferase activity driven by ^{pro}*bHLH3* or ^{pro}*bHLH6* was significantly higher when codelivered with AvrHah1, indicating that AvrHah1 is capable of activating expression of *bHLH3* and *bHLH6* (Fig. 6*B*).

The bHLH Transcription Factors Activate Expression of Two Pectin-Modification Genes. We hypothesized that genes up-regulated by bHLH3 and bHLH6 would be among the highly up-regulated genes we identified in our RNA-seq experiment but without predicted EBEs for AvrHah1 (Table 1). Using semiquantitative RT-PCR, we observed AvrHah1-specific up-regulation of two genes involved in pectin modification: *Solyc05g014000*, a *PL*, and *Solyc11g019910*, a *PE* (Fig. 6*A*). We hypothesized that *PL* and *PE* were direct gene activation targets of bHLH3 and bHLH6, and therefore indirect targets of AvrHah1. We tested the promoters of *PL* and *PE* in the transient luciferase reporter assay for activation by bHLH3 and bHLH6. Luciferase activity driven by ^{pro}*PL* and ^{pro}*PE* was significantly higher when codelivered with *avrHah1*, *bHLH3*, or *bHLH6* compared with an empty *Agrobacterium* strain (Fig. 6*B*), indicating that the bHLH transcription factors can activate expression of the pectin modification genes.

Because we observed activation of ^{pro}*PL* and ^{pro}*PE* in response to AvrHah1 in *N. benthamiana*, it is possible that AvrHah1 is activating endogenous bHLH transcription factors that are then activating the pectin modification promoters. Consistent with this prediction, two bHLH transcription factors in *N. benthamiana* (Niben101Scf00376g01004.1 and Niben101Scf01182g03011.1) have predicted AvrHah1 EBEs within 300 bp upstream of the start codon.

Delivery of dTALEs for the bHLH Transcription Factors Results in Activation of both Pectin-Modification Genes. We constructed dTALEs to activate expression of *bHLH3*, *bHLH6*, *PL*, and *PE* to study their contributions to water soaking when delivered by *XgΔavrHah1* in tomato. We first used semiquantitative PCR to confirm target gene expression. Tomato leaves infiltrated with *XgΔavrHah1* + *dT bHLH3* or *dT bHLH6* showed activation of the corresponding bHLH transcription factor gene (Fig. 7*A*). Activation of *PL* and *PE* gene expression occurred in response to *dT PL* and *dT PE*, respectively. Importantly, we also observed strong activation of *PL* and weaker activation of *PE* in response to both *dT bHLH3* and *dT bHLH6,* supporting the hypothesis that the *PL* and *PE* are downstream gene activation targets of both bHLH transcription factors. We did not observe activation of *bHLH3*, *bHLH6*, *PL*, or *PE* in response to AvrBs3, demonstrating that AvrHah1 has unique gene activation targets from AvrBs3 (AvrBs3 was reported to not cause water soaking in pepper) (5).

dTALEs for the bHLH Transcription Factors and the Pectate Lyase Complement Water Soaking in *XgΔavrHah1*. We collected and weighed the apoplastic fluid from tomato leaves infiltrated with *X. gardneri*, *XgΔavrHah1*, and *XgΔavrHah1* complemented with dTALEs to determine if the dTALE targeted genes could contribute

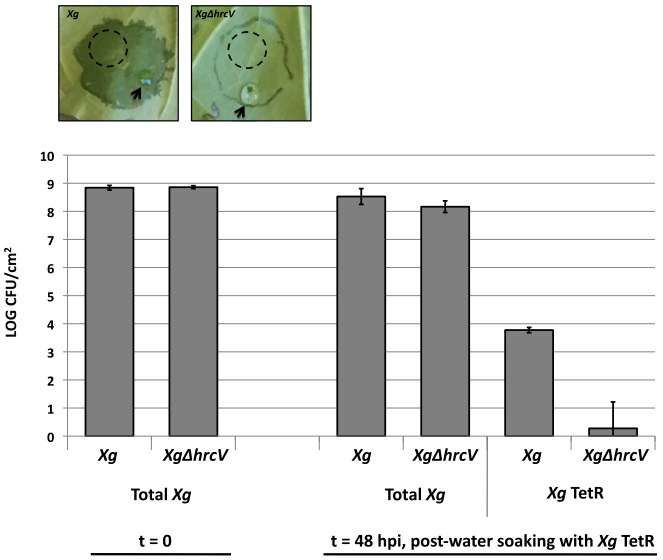

Fig. 4. Water soaking can introduce bacterial cells into the apoplast. *N. benthamiana* was syringe-infiltrated with either *X. gardneri* (*Xg*) or *XgΔhrcV* at OD_{600} = 0.1. Water soaking was induced at 48 hpi with a 30-μL drop of Tet-resistant *X. gardneri* (*X. gardneri* Tet^R, 10^5 CFU/mL in 1 0 mM $MgCl_2$) on a wound (arrow). Leaf discs were collected away from the wound site after 5 min (dashed circle), ground in 10 mM $MgCl_2$, and dilutions were plated on either Rif or Rif + Tet to select for the growth of all *X. gardneri* or the *X. gardneri* Tet^R from the water-soaking inoculum, respectively.

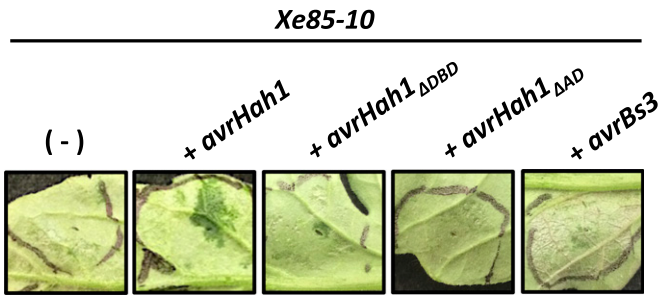

Fig. 5. AvrHah1 and AvrBs3 are differentially recognized by tomato Bs4. Tomato leaves were infiltrated with the *Xe85-10* strains indicated at OD_{600} = 0.1 and observed 48 hpi. Cell death is visible in response to delivery of *avrBs3*, whereas the beginnings of water soaking are apparent in response to *avrHah1*. Leaves were left at ambient humidity and were not submerged in water before observation.

to water soaking. Leaves were submerged in water to enhance water soaking. For comparison, we also measured the apoplastic fluid from untreated tomato leaves (also submerged in water) and leaves syringe-infiltrated with water. Little apoplastic fluid was collected from untreated leaves, whereas water-infiltrated leaves showed an approximate eightfold increase in apoplastic fluid (Fig. 7*B*). Leaves infiltrated with *X. gardneri* experienced the largest amount of water uptake, about a 12-fold increase from untreated leaves. Apoplastic fluid in *XgΔavrHah1* was significantly reduced compared with *X. gardneri* and threefold higher than untreated leaves. *XgΔavrHah1* was not fully complemented by AvrHah1 to wild type levels, however the apoplastic fluid was comparable to water-infiltrated leaves. *XgΔavrHah1* complemented with AvrBs3 showed apoplastic fluid levels similar to untreated leaves.

dT bHLH3 and *dT bHLH6* complemented water soaking back to *XgΔavrHah1* + *avrHah1* levels. *dT PL* also fully complemented water soaking back to *XgΔavrHah1* + *avrHah1* levels. We did not observe a difference in apoplastic fluid between *XgΔavrHah1* + *dT PE* and *XgΔavrHah1*. A 1:1 mixture of *dT bHLH3* and *dT bHLH6* did not further enhance water soaking above the levels of the individual dTALEs, whereas a 1:1 mixture of *dT PL and dT PE* displayed an apoplastic fluid level intermediate of the two individual dTALEs. These results indicate that the bHLH transcription factors and their proposed *PL* target contribute to AvrHah1-mediated water soaking and are therefore *S* genes of AvrHah1.

Discussion

External Water Plays a Major Role in Water-Soaked Lesions of Bacterial Spot. Disease outbreaks of bacterial spot are favored in periods of high humidity (33). Given our initial observation that the water soaking caused by *X. gardneri* was enhanced when the infected plants were placed in a mist chamber, we wondered how water external to the leaf was exacerbating *Xg*/AvrHah1-induced water soaking. In an agricultural setting, the external water could be from high humidity, rain, or sprinkler (overhead) watering systems. In the laboratory, we chose to submerge leaves in water before observation. We observed a relatively rapid intake of water into the apoplast, indicating that AvrHah1 may be priming the *in planta* environment such that *X. gardneri* can take advantage of sudden appearances of external water. The role of AvrHah1 and water soaking is likely to benefit the bacteria after the apoplastic growth phase of the pathogen's life cycle, such as during bacterial egression or transmission. Further tests will need to be performed to determine the role of AvrHah1-induced water soaking on pathogen fitness and the epiphytic growth phase of the pathogen.

Table 1. Differentially up-regulated genes from an RNA-seq experiment comparing *X. gardneri*- and *XgΔavrHah1*-infected tomato

		Mean RPKM		
Soly Gene ID	EBE score/bp from ATG	*X. gardneri*	*XgΔavrHah1*	Predicted protein function
Solyc02g070210	15.01/84	36.11	0.06	Phosphatidylinositol transferase
Solyc02g084010		99.00	0.14	Auxin-induced SAUR-like
Solyc02g089350		997.81	0.78	Gibberellin regulated
Solyc03g033590		55.85	0.14	Auxin-induced SAUR-like
Solyc03g097820	3.96/108	1,267.09	0.75	bHLH Transcription Factor
Solyc03g114430		133.86	0.27	Unknown Protein
Solyc03g116060		155.13	0.14	Gibberellin-regulated
Solyc04g017720		550.74	0.89	Gibberellin regulated
Solyc04g079700	13.26/173	16.26	0.07	WD-40 repeat family
Solyc04g079860		39.32	0.13	Glycosyltransferase family GT8
Solyc04g081870		442.81	1.38	Expansin
Solyc05g014000		206.02	0.25	Pectate lyase
Solyc06g067910		95.26	0.28	Unknown function DUF642
Solyc06g068360		99.04	0.4	Ethylene-resp. transcription factor 7
Solyc06g071930		461.79	2.81	Unknown Protein
Solyc06g072520	9.21/139	568.68	0.38	bHLH Transcription Factor
Solyc07g006310		129.78	0.24	Transcription factor
Solyc08g062450	8.2/105	14.70	0.14	class II heat shock
Solyc08g068720	13.74/220	196.13	0.01	Tyramine hydroxycinnamoyl transferase
Solyc08g079780		13.96	0.08	Blue copper protein
Solyc11g011210		1,436.40	8.86	Gibberellin regulated
Solyc11g019910		89.27	0.22	Pectinesterase
Solyc11g067180		94.81	0.15	Fatty acyl CoA reductase
Solyc12g009840		156.10	0.64	Pyrophosphate-energized proton pump

Mean reads per kilobase of transcript per million mapped reads (RPKM) of three biological replicates at 48 hpi are displayed. Genes are organized by Soly gene ID. Genes with predicted promoter EBEs for AvrHah1 are indicated with the score (best possible is 3.76) and distance from the start codon.

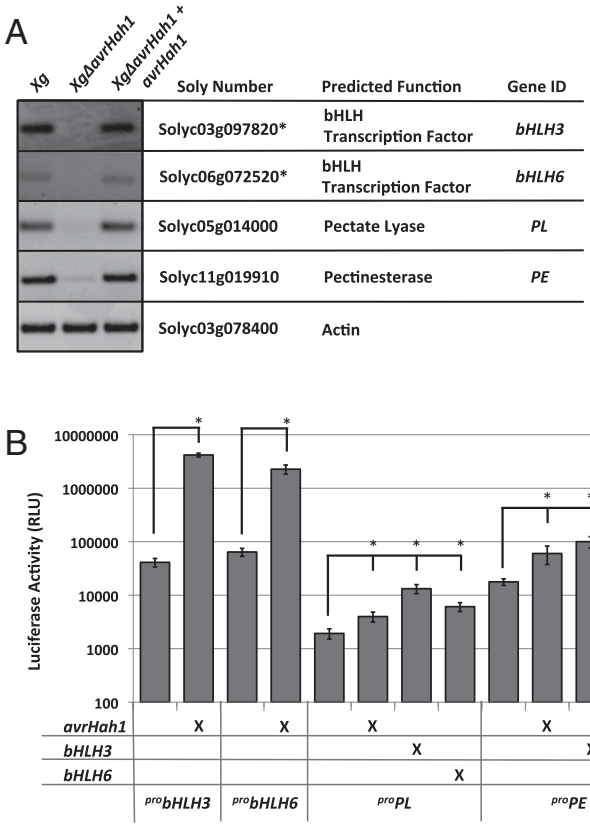

Fig. 6. Tomato gene activation in response to AvrHah1. (*A*) Semiquantitative RT-PCR in tomato inoculated with the indicated *X. gardneri* (*Xg*) strains (24 hpi; OD_{600} = 0.25); * indicates genes with predicted promoter EBEs for AvrHah1 (e.g., proposed direct targets). (*B*) Transient luciferase reporter assay in *N. benthamiana*. Promoter::luciferase constructs are displayed along the *x* axis. Expression binary vectors carrying AvrHah1 or the bHLH transcription factors are listed on the left, and an "X" signifies codelivery with a promoter::luciferase reporter; * indicates significantly different luciferase activity from promoter background (codelivered with empty *Agrobacterium*) ($P < 0.001$).

Two bHLH Transcription Factors Are *S* Gene Targets of AvrHah1. To find the downstream targets of AvrHah1 responsible for water soaking, we used RNA-seq to study the gene expression profiles of *X. gardneri*- and *XgΔavrHah1*-infected tomato leaves. We identified two highly up-regulated bHLH transcription factors, *Solyc03g097820* (*bHLH3*) and *Solyc06g072520* (*bHLH6*), which encode proteins that share 66% amino acid identity and are 86% and 64% similar, respectively, to UPA20—a bHLH transcription factor target of AvrBs3 in pepper (22). *bHLH3* and *bHLH6* are in a sister clade and part of bHLH subfamily 1 (34). *bHLH3* was found to be expressed preferentially in young fruits (annotated as bHLH022) (34). *bHLH6* was identified as a drought responsive gene in drought-tolerant tomato (annotated as bHLH048) (34, 35). We found that dTALEs activating gene expression of the identified bHLH transcription factors restored water soaking in *XgΔavrHah1*, indicating that the AvrHah1-activated *bHLH* genes encode proteins with overlapping roles in water soaking and are bona fide S gene products of AvrHah1.

A Pectate Lyase Is an Indirect *S* Gene Target of AvrHah1. To find targets of the bHLH transcription factors we selected genes in our RNA-seq dataset that were up-regulated in the presence of AvrHah1 but without predicted AvrHah1 EBEs. After confirming AvrHah1-specific activation using semiquantitative RT-PCR, we selected two pectin modification genes for further study: a *PL*, *Solyc05g014000*, and a *PE*, *Solyc11g019910*. We were interested in the pectin modification genes because several examples have implicated pectin as an important factor in plant-pathogen interactions (36, 37). We demonstrated that the promoters of *PL* and *PE* could be activated by either bHLH transcription factor using a transient luciferase reporter assay. Semiquantitative RT-PCR for *PL* and *PE* showed gene activation in response to delivery of *dT bHLH3* and *dT bHLH6* by *XgΔavrHah1*.

We constructed dTALEs to activate the transcription of the *PL* and *PE* genes. When delivered into tomato by *XgΔavrHah1*, the *PL*-specific, but not the *PE*-specific, dTALE was able to complement water soaking, suggesting that this *PL* is an indirect *S* gene target of AvrHah1. We hypothesize that *PL* activity increases the hygroscopicity of the cell wall, which—in the larger context of *X. gardneri* infection—conditions the apoplast to absorb water through breaks in the epidermis (likely caused by the lesion itself). As shown in Fig. 1, at time 0 (before the introduction of surface water) zones infiltrated with *X. gardneri* or *XgΔavrHah1 + avrHah1* appear noticeably darker and damaged compared with zones infiltrated with *XgΔhrcV* or *XgΔavrHah1*. This result is perhaps a consequence of increased tissue maceration from *PL* activity. Future experiments exploring the composition of the cell wall in response to *X. gardneri* infection may reveal a mechanism by which *PL* maceration of plant tissue promotes water soaking.

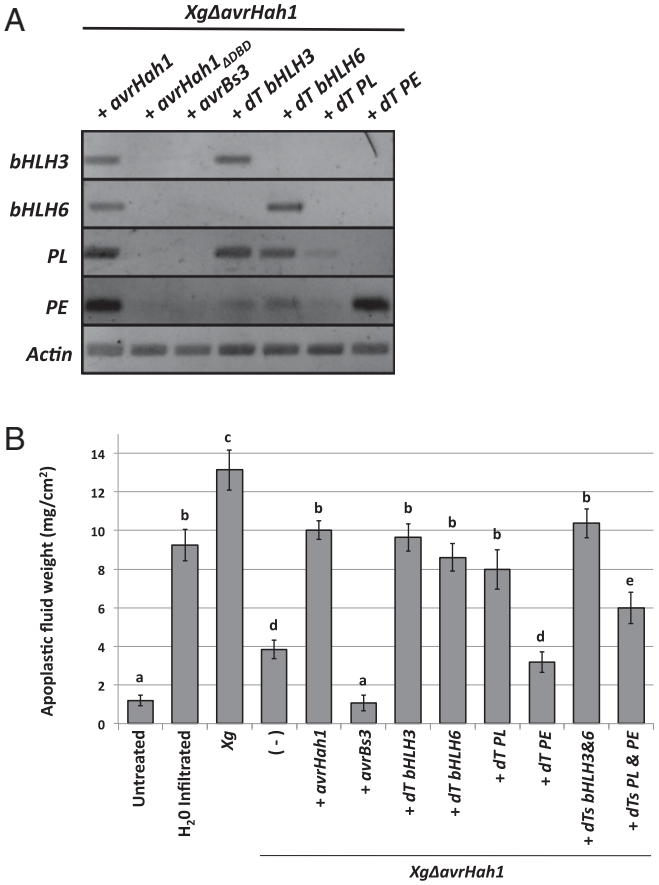

Fig. 7. dTALEs demonstrate that the bHLH transcription factors and the *PL* are *S* genes of AvrHah1. (*A*) Semiquantitative RT-PCR in tomato infected with *XgΔavrHah1* complemented with dTALEs for the bHLH transcription factors (*dT bHLH3* and *dT bHLH6*), the *PL* (*dT PL*), and the *PE* (*dT PE*) (24 hpi; OD_{600} = 0.25). (*B*) Quantitative water-soaking measurements were obtained by centrifugation and weighing of apoplastic fluid from infected tomato leaves (48 hpi; OD_{600} = 0.1; 20 min water bath). Average weights and SEs from 12 samples (each consisting of two 0.5-cm^2 leaf discs) are shown.

Diverse Strategies and Implications of Water-Soaked Lesions. The relatively fast absorption of water into the leaf apoplast due to AvrHah1 is a striking addition to the diverse mechanisms by which pathogens promote water soaking. We designed an experiment to show that bacterial cells can be introduced into the apoplast during AvrHah1-induced water soaking. Whether or not bacteria such as human pathogens can survive in the plant apoplast as a result of AvrHah1-induced water soaking will need to be determined. This finding is particularly important in the light of previous work that described how increased water soaking and foliar damage from xanthomonad pathogens facilitated the growth of the human pathogen *Salmonella enterica* on tomato leaves (38). Because bacterial manipulation of the leaf apoplast to promote an aqueous environment is required for pathogenesis (39), improving the tolerance of food crops from water-soaked lesion development as part of a multilayered disease management strategy may help reduce yield losses and even prevent the colonization of human pathogens on diseased crops.

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