Tomato bHLH132 Transcription Factor Controls Growth and Defense and Is Activated by *Xanthomonas euvesicatoria* Effector XopD During Pathogenesis

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Effector-dependent manipulation of host transcription is a key virulence mechanism used by *Xanthomonas* species causing bacterial spot disease in tomato and pepper. Transcription activator-like (TAL) effectors employ novel DNA-binding domains to directly activate host transcription, whereas the non-TAL effector XopD uses a small ubiquitin-like modifier (SUMO) protease activity to represses host transcription. The targets of TAL and non-TAL effectors provide insight to the genes governing susceptibility and resistance during *Xanthomonas* infection. In this study, we investigated the extent to which the *X. euvesicatoria* non-TAL effector strain Xe85-10 activates tomato transcription to gain new insight to the transcriptional circuits and virulence mechanisms associated with *Xanthomonas euvesicatoria* pathogenesis. Using transcriptional profiling, we identified a putative basic helix-loop-helix (bHLH) transcription factor, bHLH132, as a pathogen-responsive gene that is moderately induced by microbe-associated molecular patterns and defense hormones and is highly induced by XopD during *X. euvesicatoria* infection. We also found that activation of bHLH132 transcription requires the XopD SUMO protease activity. Silencing bHLH132 mRNA expression results in stunted tomato plants with enhanced susceptibility to *X. euvesicatoria* infection. Our work suggests that bHLH132 is required for normal vegetative growth and development as well as resistance to *X. euvesicatoria*. It also suggests new transcription-based models describing XopD virulence and recognition in tomato.

Keywords: bacterial pathogenesis, bHLH transcription factor, tomato immunity, type III effectors, *Xanthomonas*

Xanthomonas species cause bacterial spot disease on cultivated tomato (*Solanum lycopersicum*) and pepper (*Capsicum annuum*), impacting vegetable production worldwide (Jones et al. 1998). Crops are more susceptible in warm and humid

climates that promote bacterial invasion, multiplication, and transmission. Bacterial spot disease is caused by four distinct *Xanthomonas* species, namely, *X. euvesicatoria*, *X. perforans*, *X. garnderi*, and *X. vesicatoria* (Jones et al. 2005). Comparative genomics of reference strains Xe85-10, Xp91-118, Xg101, and Xv1111 (Potnis et al. 2011; Thieme et al. 2005) and genome sequencing of new isolates (Schwartz et al. 2015) provide key insights to the shared and unique virulence factors.

The major determinants that control virulence and host range are the effectors translocated into host cells by the *Xanthomonas* type III secretion (T3S) system (Bonas et al. 1991). A few T3S effectors are conserved in multiple species and are referred to as ‘core’ effectors (Potnis et al. 2011; Schwartz et al. 2015). By contrast, most effectors are variable among species and are hypothesized to impose specialization to individual hosts and, thus, may be determinants of host range. Insight to the mechanisms governing bacterial spot disease in tomato and pepper has emerged from studying the biochemical structure and activity of individual *Xanthomonas* effectors from various strains. Diverse mechanisms have been uncovered, with the majority acting by suppressing host immune responses and development of disease symptoms in infected tissues (Boch and Bonas 2010; Kay and Bonas 2009; Üstün and Börnke 2014; White et al. 2009).

Notably, one of the key mechanisms underpinning *Xanthomonas* pathogenesis in tomato involves direct manipulation of host transcription (Boch and Bonas 2010). For example, the transcription activator-like (TAL) effector AvrBs3 from *X. euvesicatoria* activates the transcription of a basic helix-loop-helix (bHLH) transcription factor (UPA20) in pepper leaves to regulate cell size (Kay et al. 2007). Also, the TAL effector AvrHah1 from *X. gardneri* activates the transcription of two bHLHs (bHLH3 and bHLH6) in tomato leaves to promote water soaking (Schwartz et al. 2017). Water soaking of infected leaf tissue is associated with pathogen egress to the leaf surface and spread. AvrHah1-induced susceptibility designates bHLH3 and bHLH6 as important host susceptibility genes. By contrast, the non-TAL effector XopD from *X. euvesicatoria* represses ethylene (ET)-stimulated defense transcription in tomato by desumoylating the transcription factor ERF4 (Kim et al. 2008, 2013). Removal of SUMO from ERF4 results in its destabilization by the proteasome. The suppression of ERF4 function by XopD revealed ET-induced transcription of basal resistance genes as key factors in inhibiting *X. euvesicatoria* growth (Kim et al. 2013).

For this study, we sought to identify additional tomato loci associated with disease susceptibility or resistance, or both, given the importance of effector-dependent rewiring of host

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transcription during *Xanthomonas* infection. This work was motivated by the fact that many tomato varieties are susceptible to xanthomonads and stacking of resistance and susceptibility genes by either breeding, engineering, or both holds promise to control bacterial spot in the field (Dangl et al. 2013; Horvath et al. 2012). We used transcriptional profiling of tomato leaves elicited with Xe85-10, a strain that does not contain TAL effectors. We hypothesized that effector-dependent induction of tomato transcription may identify novel host factors governing *X. euvesicatoria* pathogenesis and uncover new virulence mechanisms for non-TAL effectors.

Here, we report the identification of tomato bHLH transcription factor bHLH132, a gene that plays an important role in tomato growth and development. We show that bHLH132 transcription is induced by microbe-associated molecular patterns (MAMPs) and defense hormones and specifically by the *X. euvesicatoria* effector XopD. We provide evidence that bHLH132 is required and is sufficient to protect tomato plants from *X. euvesicatoria* infection. Our work indicates that bHLH132 plays important roles in both tomato growth and immunity.

RESULTS

Tomato bHLH132 is a pathogen-responsive gene.

We hypothesized that tomato genes highly induced by *X. euvesicatoria* during the early stages of infection will define susceptibility or resistance genes playing essential roles during pathogenesis. To examine this, we analyzed a RNA sequencing dataset defining transcriptional changes that occur in tomato leaves (cultivar VF36) challenged with a diverse panel of microbial elicitors for 12, 24, and 48 h (Jeon et al. 2018). Specifically, we examined changes in gene expression induced by two MAMPs (bacterial flagellin, i.e., Flg22, and fungal chitin), two tomato-associated pathogens (Xe85-10, a virulent strain, and *Cladosporium fulvum* race 5, an avirulent strain), and three non-plant associated microbes (*Malassezia restricta*, *Staphylococcus epidermidis*, and *Propionibacterium acnes*). Our analysis revealed that one gene in particular, bHLH132, is highly induced by *X. euvesicatoria* at 24 and 48 h postinoculation (hpi) and only moderately induced by the other treatments (Supplementary Fig. S1). These data suggested that bHLH132 transcription is induced during microbial perturbation (bacterial and fungal elicitation) and specifically in response to pathogenic *X. euvesicatoria*.

Tomato bHLH132 is induced by *X. euvesicatoria* in a T3S-dependent manner.

To verify these RNAseq data, we quantified the relative abundance of bHLH132 mRNA in tomato leaves syringe-infiltrated with 10 mM MgCl₂ (mock), wild-type *X. euvesicatoria*, or the *X. euvesicatoria* T3S-deficient mutant (*X. euvesicatoria* Δ hrcV). A low level of bHLH132 mRNA was detected in response to mock and *X. euvesicatoria* Δ hrcV treatment (Fig. 1A), indicating that bHLH132 expression does not change significantly in response to nonvirulent bacteria. By contrast, bHLH132 mRNA levels dramatically increased (approximately 900-fold relative to mock) in response to wild-type *X. euvesicatoria* at 12 and 24 hpi (Fig. 1A), consistent with the RNAseq dataset (Jeon et al. 2018). The T3S-dependent induction of bHLH132 mRNA expression implicates one or more of the effectors secreted by *X. euvesicatoria* in inducing this transcriptional response.

Interestingly, bHLH132 mRNA levels also increased in tomato leaves infiltrated with another tomato pathogen, *Pseudomonas syringae* pv. *tomato* DC3000 (Fig. 1B). This increase was not effector-dependent, as bHLH132 mRNA levels were similar in leaves treated with wild-type *P. syringae* and the T3S-deficient mutant (*P. syringae* pv. *tomato* Δ hrcU) (Fig. 1B).

Next, we examined if treatment with a MAMP or defense hormones is sufficient to increase bHLH132 mRNA levels in tomato leaves, by monitoring early timepoints after treatment. bHLH132 mRNA levels increased approximately five to sevenfold in Flg22-elicited tomato leaves at 3 and 6 hpi, respectively (Fig. 1C), establishing that it is a MAMP-induced gene. High levels of bHLH132 mRNA was detected in tissue exposed to 2 mM salicylic acid (SA), 5 mM ethephon (to generate ET), and 100 μ M methyl jasmonate (Fig. 1D). Taken together, our analysis indicates that bHLH132 transcription in tomato leaves is responsive to bacteria, Flg22, defense hormones, and one or more *X. euvesicatoria* T3S effectors.

X. euvesicatoria effector XopD activates bHLH132 transcription.

To begin to elucidate the factors controlling bHLH132 transcription, we generated a transgenic tomato reporter line containing a 2-kb fragment of the bHLH132 promoter fused to the β -glucuronidase (GUS) gene (i.e., ^{Pro}bHLH132::GUS). This line was weakly induced in response to the T3S secretion mutant (*X. euvesicatoria* Δ hrcV) and was strongly induced by wild-type *X. euvesicatoria* at 24 hpi (Fig. 1E). This induction pattern mirrors that detected in wild-type tomato leaves (Fig. 1A), demonstrating that the 2-kb promoter contains the cis-regulatory elements sufficient to activate transcription of bHLH132.

We also examined the potential roles of XopD and two core effectors, XopN and XopX, which are known to play essential roles in *X. euvesicatoria* pathogenesis (Kim et al. 2008; Metz et al. 2005; Roden et al. 2004). Suspensions of wild-type *X. euvesicatoria* and effector mutant strains were inoculated into ^{Pro}bHLH132::GUS tomato leaves and GUS activity was then quantified at 24 hpi. Only the *X. euvesicatoria* Δ xopD mutant failed to induce GUS activity in the ^{Pro}bHLH132::GUS tomato leaves (Fig. 1E). Furthermore, GUS activity for Δ xopD-inoculated tissue was similar to that of Δ hrcV-inoculated tissue (Fig. 1E), indicating that XopD is major effector determinant that leads to high activation of bHLH132 transcription.

To determine if expression of XopD alone or another effector is sufficient to activate bHLH132 transcription, we screened a library of *X. euvesicatoria* T3S effectors (Dubrow et al. 2018) using the transgenic ^{Pro}bHLH132::GUS tomato line. Individual His-tagged effectors (6xHis-effector) or green fluorescent protein (GFP) (negative control) were transiently expressed in leaves, using *Agrobacterium tumefaciens* 1D1249 (Wroblewski et al. 2005). At 3 days postinoculation (dpi), leaf tissue was assayed for GUS activity, using a fluorometric enzyme assay. Only transient expression of XopD resulted in a significant increase in GUS activity relative to the GFP control (Fig. 1F).

We previously reported that XopD is a SUMO protease known to repress host transcription during *X. euvesicatoria* infection by targeting the tomato transcription factor ERF4 (Kim et al. 2013). Therefore, we investigated if XopD SUMO protease activity is required to activate bHLH132 transcription. To do so, we transiently expressed His-tagged wild type (XopD-6xHis) and a catalytically inactive mutant [XopD(C685A)-6xHis] in ^{Pro}bHLH132::GUS tomato leaves and, then, GUS activity was quantified at 24 hpi. Expression of XopD(C685A)-6xHis mutant produced less GUS activity relative to wild-type XopD-6xHis (Fig. 1G). These data suggest that XopD-dependent desumoylation of a factor is required to induce bHLH132 expression.

bHLH132 is a DNA-binding protein.

The biochemical function of bHLH132 is not known. Protein structure prediction using Phyre2 (Kelley et al. 2015) shows that bHLH132 has an N-terminal bHLH domain and a

C-terminal ACT domain (Fig. 2A). ACT domains are widely present in biosynthetic enzymes (e.g., aspartate kinase, chorismate mutase, and tyrA), in which they function in allosteric regulation, often involving dimerization (Grant 2006). ACT domains are present in a few plant proteins from different bHLH subfamilies, with evidence linking the domain to transcription factor homodimerization (Feller et al. 2006).

Comparative analysis of the bHLH protein family in tomato indicates that bHLH132 is in subfamily 17 and predicts that it is a non-DNA binding protein, based on two features (Sun et al. 2015). First, bHLH132 does not contain all three of the amino residues in the HER motif (His-Glu-Arg) in the basic domain important for binding DNA (Fig. 2A). The histidine residue is not conserved. Second, bHLH132 contains four basic residues

Fig. 1. Tomato *bHLH132* is a pathogen-responsive gene and highly induced by XopD. **A**, Relative expression of *bHLH132* mRNA in tomato leaves in response to *Xanthomonas euvesicatoria* infection at 6, 12, and 24 h postinoculation (hpi). Leaves ($n = 3$) were syringe-infiltrated with a 10 mM $MgCl_2$ solution (white bars, mock) or with a bacterial suspension (2×10^8 CFU/ml) of wild-type *X. euvesicatoria* 85-10 (Xe; gray bars) or the type III secretion (T3S)-deficient mutant *X. euvesicatoria* $\Delta hrcV$ (Xe $\Delta hrcV$; black bars). **B**, Relative expression of *bHLH132* mRNA in tomato leaves in response to *P. syringae* pv. *tomato* DC3000 (Pst) infection at 3, 6, and 10 hpi. Leaves ($n = 4$) were syringe-infiltrated with a 10 mM $MgCl_2$ solution (white bars, mock) or with a bacterial suspension (2×10^8 CFU/ml) of wild-type *P. syringae* pv. *tomato* DC3000 (Pst; gray bars) or the T3S-deficient mutant *P. syringae* pv. *tomato* $\Delta hrcU$ (DC3000 $\Delta hrcU$; black bars). **C**, Relative expression of *bHLH132* mRNA in tomato leaves ($n = 4$) treated with Flg22 (100 nM) for 0 to 24 hpi. **D**, Relative expression of *bHLH132* mRNA in tomato leaves ($n = 3$) treated with salicylic acid (SA) (2 mM), ethephon (5 mM), and methyl jasmonate (MeJA) (100 μ M) for 0 to 24 hpi. *bHLH132* mRNA levels were quantified by quantitative PCR. Relative expression was determined against mock at each timepoint (A and B) and at 0 hpi (C and D). **E**, *X. euvesicatoria* activation of *bHLH132* transcription. Wild type (wt) and mutant *X. euvesicatoria* strains (1×10^8 CFU/ml) were syringe-infiltrated into tomato *Pro**bHLH132*::GUS leaves ($n = 4$) and β -glucuronidase (GUS) activity was assayed 24 hpi. **F**, Effector activation of *bHLH132* transcription. Green fluorescent protein (GFP) or 32 His-tagged Xe85-10 effectors were transiently expressed in transgenic tomato *Pro**bHLH132*::GUS leaves ($n = 3$) and GUS activity was assayed 3 days postinoculation. **G**, XopD activation of *bHLH132* transcription. Epitope-tagged wild type (XopD-6xHis, XopD wt) or protease inactive XopD [XopD(C685A)-6xHis, XopD C685A] or vector (empty) was transiently expressed in tomato *Pro**bHLH132*::GUS leaves ($n = 3$) and GUS activity was assayed 24 hpi. Relative GUS activities (mean $\pm$ standard error) were calculated against the mean of GFP normalized to 1. Different letters indicate statistically significant differences (one-way analysis of variance and Tukey's highly significant difference, $P < 0.05$).

in the basic domain (Fig. 2A), as opposed to the five or more typically found in basic domains with DNA-binding activity (Atchley et al. 1999; Sun et al. 2015). The sequence of the most homologous bHLH from *Arabidopsis thaliana*, AtbHLH035, and one representative bHLH containing a typical HER motif, AtbHLH067, are shown for comparison. Similar to bHLH132, AtbHLH035 lacks the conserved His residue in the HER motif. AtbHLH035 is predicted to have DNA-binding activity and target a *cis*-regulatory sequence known as the E-box (5'-CANNTG-3') (Toledo-Ortiz et al. 2003). AtbHLH067 contains the conserved residues (His-Glu-Arg) and is predicted to bind a subclass of E-boxes, known as the G-box (5'-CACGTG-3') (Toledo-Ortiz et al. 2003).

To ascertain whether or not bHLH132 binds DNA, we performed electromobility shift assays (EMSA) in vitro. Recombinant glutathione-S-transferase (GST)-tagged protein (GST-bHLH132) was purified from *Escherichia coli* and was incubated with G-box (CACGTG) DNA. We chose the G-box motif because it is more specific than the E-box sequence. Purified GST-bHLH132 but not GST altered the migration of G-box DNA in polyacrylamide gels (Fig. 2B). This binding was specific, as addition of poly(dI-dC), a nonspecific DNA fragment, did not alter bHLH132 binding to G-box DNA. These data in conjunction with our observation that bHLH132 localizes to plant nuclei upon transient expression (Supplementary Fig. S2) predict that bHLH132 operates in the nucleus as a DNA-binding protein with recognition for the G-box *cis*-regulatory element.

bHLH132 is required for tomato growth and development.

To elucidate the function of bHLH132, we engineered transgenic tomato lines that are silenced for *bHLH132* expression. *bHLH132* was silenced using a hairpin RNA construct containing a 238-base pair internal region of the *bHLH132* gene, designated hp-bHLH132. This region was used to prevent the silencing of related bHLH family members in tomato. Two independent T2 transgenic lines were characterized, to measure *bHLH132* mRNA levels and assess potential developmental phenotypes.

The phenotypes for two T2 tomato lines (i.e., lines 1 and 2) with reduced *bHLH132* mRNA levels are shown in Figure 3A. The phenotypes of the *bHLH132*-silenced lines were compared with an unsilenced transgenic control plant. The hp-bHLH132 construct appeared to be specific for the *bHLH132* gene, as it reduced *bHLH132* mRNA levels in lines 1 and 2 without affecting the mRNA levels of the most homologous tomato gene, *bHLH139* (Sun et al. 2015) (Fig. 3A).

Reducing *bHLH132* expression resulted in tomato plants with stunted growth, shorter internodes, reduced leaf expansion, and smaller leaves (Fig. 3B). These phenotypes revealed that *bHLH132* is required for tomato vegetative growth. Subsequent analysis of tissue-specific expression showed that *bHLH132* mRNA levels are the highest in roots, with much lower levels in leaves and stems (Supplementary Fig. S3). These data suggest that reduced vegetative growth for the transgenic lines silenced for *bHLH132* might be caused by one or both abnormal development or growth of roots.

bHLH132 is required for resistance to *X. euvesicatoria* infection in tomato leaves.

To determine if bHLH132 plays a role in tomato susceptibility or resistance to *X. euvesicatoria* infection, we infected the *bHLH132* RNA interference (RNAi) and control lines with wild-type *X. euvesicatoria* and the $\Delta xopD$ mutant. Both *bHLH132* RNAi lines were more susceptible to *X. euvesicatoria* and $\Delta xopD$ (Fig. 3C). Symptoms occurred much faster in the *X. euvesicatoria*-infected *bHLH132*-silenced leaves compared with control leaves (Fig. 3D). For example, yellowing or chlorosis of leaves was observed 1 to 2 days earlier in $\Delta xopD$ -infected *bHLH132* RNAi leaves compared with $\Delta xopD$ -infected control leaves (Fig. 3D). XopD is known to suppress symptom development (i.e., leaf yellowing or chlorosis) at late stages of *X. euvesicatoria* infection (Kim et al. 2008). By 12 dpi, severe disease symptoms (chlorosis and necrosis) were observed for control and RNAi lines.

We also analyzed the phenotype of bHLH132 overexpression in tomato by engineering constitutively expressed GFP-tagged bHLH132 lines using the minimal 35S cauliflower mosaic virus promoter (^{Pro}35S::GFP-bHLH132). Two T2 stable tomato lines overexpressing GFP-bHLH132 protein were characterized (Fig. 4A). Unlike the RNAi lines, the height of the bHLH132 overexpression (OE) lines was similar to that of control lines. The only visible phenotype was that the leaves of the OE lines are narrower, with sharp serrated edges (Fig. 4B). The OE lines were infected with wild-type *X. euvesicatoria* and the $\Delta xopD$ mutant. At 12 dpi, the bHLH132 OE lines harbored less wild-type and $\Delta xopD$ bacteria (Fig. 4C) compared with control wild-type lines. Notably, reduced bacterial titers in the bHLH132 OE lines resulted in less severe leaf symptoms at 12 dpi (Fig. 4D). By 15 dpi, wild-type leaves were fully collapsed (necrotic), while the bHLH132 OE leaves were either chlorotic (*X. euvesicatoria* infection) or mostly green (*X. euvesicatoria* $\Delta xopD$ infection) (Fig. 4D).

Fig. 2. Tomato bHLH132 is a DNA-binding protein. **A**, Schematic of predicted 27-kDa protein containing a bHLH and ACT domain. Amino acid sequence for basic helix loop helix domain is shown for bHLH132 and two homologs from *Arabidopsis thaliana* AtbHLH035 and AtbHLH067. Conserved amino acids (His-Glu-Arg) in HER motif required for DNA binding are denoted with bold shaded letters. **B**, bHLH132 binds G-box DNA in vitro. Biotin labeled G-box DNA (free DNA) was incubated with (+) or without (-) purified GST-bHLH132 and with increasing concentrations (0, 12.5, and 25 ng/μl) of poly(dI-dC) as a nonspecific competitor, and electromobility shift assays were performed. The arrowhead on the right denotes bHLH132-DNA complex.

Finally, we quantified mRNA levels for *bHLH132* and two defense marker genes, *PR1* and *Pti5*, in the RNAi and OE lines in response to mock (10 mM MgCl₂) or wild-type *X. euvesicatoria* infection. Compared with leaves of wild-type plants, *bHLH132* mRNA levels were decreased in the RNAi lines and were increased in the OE lines in response to *X. euvesicatoria* elicitation (Fig. 5A). *PR1* and *Pti5* mRNA levels were higher in mock-treated RNAi leaves compared with mock-treated wild-type tomato leaves (Fig. 5B and C). This suggests that these mRNAs are elevated in the *bHLH132*-silenced lines prior to pathogen elicitation. *PR1* and *Pti5*

Fig. 3. Tomato *bHLH132* silenced lines are susceptible to *Xanthomonas euvesicatoria*. **A**, Relative expression of *bHLH132* and *bHLH139* (closest homolog) mRNA in the leaves of the wild-type (wt, white bars) control (set to 1) and two *bHLH132* RNA interference (RNAi) lines (gray bars; $n = 2$; mean $\pm$ standard deviation [SD]). **B**, Growth phenotypes of 8-week-old transgenic *bHLH132* RNAi lines (lines 1 and 2) compared with wild type (wt). **C**, Growth of *X. euvesicatoria* in *bHLH132* RNAi lines. Number [log(CFU/cm²)] of wild type (Xe; gray bars) and *xopD* mutant (Xe $\Delta xopD$; white bars) bacteria in wild type and *bHLH132* RNAi lines at 10 days postinoculation (dpi) ($n = 4$; mean $\pm$ SD). Leaves were syringe-infiltrated with a 1×10^5 CFU/ml suspension of bacteria. Different letters indicate statistically significant differences (one-way analysis of variance and Tukey's highly significant difference, $P < 0.05$). **D**, Symptom development of leaves from C at 9 dpi.

mRNA levels were the highest in mock-treated *bHLH132* OE lines. Infection with wild-type *X. euvesicatoria* increased *PR1* and *Pti5* mRNA levels in both the RNAi and OE lines, relative to the mock-treated controls (Fig. 5B and C). These data suggest that *bHLH132* is not playing a direct role in the transcriptional regulation of these defense mRNAs.

Fig. 4. Tomato *bHLH132* overexpression (OE) lines are more resistant to *Xanthomonas euvesicatoria*. **A**, *bHLH132*-GFP-6xHis protein abundance in the leaves of wild type (wt) and two OE lines. Protein was detected by immunoblot analysis using anti-green fluorescent protein (GFP) sera. **B**, Leaf shapes of 6-week-old wild type and transgenic *bHLH132* OE lines (lines 1 and 2). **C**, Growth of *X. euvesicatoria* in wild type and transgenic *bHLH132*-GFP-6xHis OE (lines 1 and 2). Number [log(CFU/cm²)] of wild type (Xe; gray bars) and *xopD* mutant (Xe $\Delta xopD$; white bars) bacteria at 10 days postinoculation (dpi) ($n = 4$; mean $\pm$ standard deviation). Leaves were syringe-infiltrated with a 1×10^5 CFU/ml suspension of bacteria. Different letters indicate statistically significant differences (one-way analysis of variance and Tukey's highly significant difference, $P < 0.05$). **D**, Symptom development of leaves from C at 12 and 15 dpi.

Collectively, our work demonstrates that bHLH132 is required and is sufficient to mediate tomato disease resistance in during *X. euvesicatoria* infection.

DISCUSSION

Plant bHLHs—expanding roles in growth and immunity.

Land plants contain approximately 100 to 170 bHLHs (Feller et al. 2006; Pires and Dolan 2010). Most bHLHs have been characterized in *Arabidopsis thaliana* (Toledo-Ortiz et al. 2003), with sparse information from crop plants. bHLHs can act as activators or repressors either by binding to DNA, by forming homo- and hetero-dimers with other bHLHs or HLHs, or both. Such interactions contribute to DNA-target specificity and combinatorial gene regulation (Toledo-Ortiz et al. 2003). Most bHLHs are known to play roles in development, though a few are linked to flavonoid biosynthesis, phytochrome signaling, hormone signaling, and stress responses, including immunity against bacteria and fungi (Dombrecht et al. 2007; Fan et al. 2014; Fernández-Calvo et al. 2011; Kim et al. 2012; Kiribuchi et al. 2005; Malinovsky et al. 2014; Mertens et al. 2016; Xu et al. 2014).

Importantly, some bHLHs have been implicated in the control of growth and defense circuits, providing insight to some of the molecular mechanisms underpinning growth-defense tradeoffs. For example, antagonism between the brassinosteroid pathway and MAMP-triggered immunity in *Arabidopsis thaliana* is controlled by HBI1, a bHLH that activates growth-related genes and suppresses defense-related genes induced by MAMPs (Fan et al. 2014; Malinovsky et al. 2014). Similar antagonism was reported in potato plants infected with *Phytophthora infestans* (Turnbull et al. 2017). These studies suggest that bHLHs may integrate signals for transcription circuits well beyond those controlling classical developmental pathways.

The role of tomato bHLH132 in growth and defense.

Our genetic analyses indicate that tomato bHLH132 is required for both growth and defense programs, deviating from models implicating bHLHs in the growth-defense paradigm. In terms of defense, both the wild-type *X. euvesicatoria* and mutant $\Delta xopD$ strains were able to grow to higher titers in *bHLH132*-silenced tomato leaves compared with similarly infected wild-type leaves. These genetic data along with biochemical data showing that bHLH132 specifically binds a G-box *cis*-regulatory element (Fig. 2B) suggest that bHLH132

encodes a transcription factor that regulates the expression of genes that influence *X. euvesicatoria* multiplication.

bHLH132 transcription in tomato leaves is induced by multiple biological factors, including two MAMPs (Flg22, chitin), defense hormones (SA, ET, jasmonic acid), compatible tomato pathogens (*P. syringae*, *X. euvesicatoria*), and, specifically, by the effector XopD (Fig. 1). The magnitude of *bHLH132* transcriptional activation varied among elicitor types under the conditions tested. Of the hormones tested, the relative fold induction of *bHLH132* transcription was highest for ET and SA. While these hormone treatments are each sufficient to activate *bHLH132* transcription using this reporter assay, they are not sufficient to do so during *X. euvesicatoria* infection. During *X. euvesicatoria* infection, ET and SA levels are highest in leaves infected with *X. euvesicatoria* $\Delta xopD$ compared with those infected with wild-type *X. euvesicatoria* (Kim et al. 2008, 2013). However, activation of *bHLH132* transcription is highest in wild-type *X. euvesicatoria*-infected leaves and much lower in *X. euvesicatoria* $\Delta xopD$ -infected leaves (Fig. 1). These data suggest that ET and SA produced during *X. euvesicatoria* are not sufficient to activate *bHLH132* expression and another host factor is involved, most likely one linked to XopD action. Activation of *bHLH132* transcription by XopD in *A. tumefaciens*-infected leaves (Fig. 1) is consistent with this model.

The elicitor treatments suggest that bHLH132 may be involved in transcription networks that operate in response to diverse biotic stresses. Analysis of susceptible *X. euvesicatoria* infections in tomato leaves with altered levels of bHLH132 shows that bHLH132 plays an indirect role in defense gene induction (Fig. 5). Interestingly, the closest bHLH homolog in *Arabidopsis thaliana*, bHLH35, is induced during effector-triggered immunity in response to *P. syringae* pv. *tomato* DC3000 expressing the AvrRpm1 effector (Truman et al. 2006). Notably, we found that tomato bHLH132 was also induced during an incompatible *X. euvesicatoria* interaction governed by the AvrBs2 effector and the Bs2 disease resistance gene (Tai et al. 1999b) (Supplementary Fig. S4). Collectively, our data indicate that tomato bHLH132 is responsive to both MAMP-triggered immunity and effector-triggered immunity.

It is intriguing that a tomato gene that is expressed at high levels in roots and very low levels in leaves is induced in leaves during pathogen infection. We speculate that bHLH132 may control transcription networks regulating one or both nutrient uptake and mobilization within the plant, fueling both growth and defense signaling. This could explain why silencing *bHLH132* limits vegetative growth as well as basal disease

Fig. 5. Abundance of *bHLH132* and defense-associated mRNAs in wild type (wt) and *bHLH132* RNA interference (RNAi) and *bHLH132* overexpression (*bHLH132*-GFP-6xHis) tomato lines during *Xanthomonas euvesicatoria* infection. Leaves were syringe-infiltrated with 10 mM MgCl₂ (white bars) or a 5 × 10⁸ CFU/ml suspension of wild type (Xe; gray bars). After 12 h postinoculation, mRNA abundance for A, *bHLH132*, B, *PR1*, and C, *Pti5* was determined by quantitative PCR. The numbers above the bars indicate the relative expression values (n = 3; mean ± standard deviation).

resistance (Fig. 3). The biological significance of XopD-dependent activation of *bHLH132* transcription is currently unclear. We speculate that this might be a host compensation mechanism activated in an attempt to combat successful colonization by *X. euvesicatoria*. Notably, OE of *bHLH132* in tomato leaves significantly reduces the growth of wild-type *X. euvesicatoria* and mutant $\Delta xopD$ strains (Fig. 4). These data indicate that increased biosynthesis of *bHLH132* is protective, implicating *bHLH132* target genes in host disease resistance physiology. Elucidating the *bHLH132* transcription network during tomato development and in response to biotic stress is expected to provide insight to the factors and pathways involved.

Activation of *bHLH132* transcription requires the XopD SUMO protease activity.

How and why XopD specifically activates *bHLH132* transcription in tomato leaves during a susceptible interaction is not clear. One model is that XopD activity in the nucleus interferes with a transcription complex that represses *bHLH132* transcription. XopD-dependent cleavage of SUMO- (Hotson et al. 2003; Kim et al. 2013) or potentially ubiquitin-isopeptide (Pruneda et al. 2016) linkages with lysine residues of a repressor or corepressor complex could lead to activation of *bHLH132* transcription. Alternatively, XopD could be directly binding to the *bHLH132* promoter and, in conjunction with isopeptide cleavage, activate transcription. This would likely require a conformational change of XopD or a specific protein-protein interaction to localize XopD to the *bHLH132* promoter, given that XopD has nonspecific DNA-binding activity in vitro (Kim et al. 2008). This model assumes that XopD-dependent activation of *bHLH132* transcription in *Xanthomonas*-infected leaves is associated with XopD virulence.

A contrasting, second model is that XopD protease activity in the nucleus may be guarded by a transcription complex and perturbation of the complex results in activation of a disease resistance response that involves *bHLH132* transcription. Such a model would predict that *bHLH132* functions in host defense. Silencing and OE of *bHLH132* resulted in tomato leaves with enhanced susceptibility and resistance, respectively. These data are consistent with a role for *bHLH132* in tomato growth and immune responses that limit *Xanthomonas* pathogenesis.

Conclusion.

We provide evidence that the non-TAL effector XopD strongly activates the transcription of *bHLH132* in *X. euvesicatoria*-infected tomato leaves. We provide evidence that *bHLH132* is required and is sufficient to protect tomato plants from *X. euvesicatoria* infection. In addition, our work illustrates that *bHLH132* plays a central role in tomato plant growth and protective responses to multiple biotic perturbations.

MATERIALS AND METHODS

Bacterial strains.

Strains used in this study were as follows: *Escherichia coli* DH5a and BL21 Star (DE3) (Invitrogen); *Agrobacterium tumefaciens* strains C58C1 (pCH32) (Tai et al. 1999a), 1D1249 (Wroblewski et al. 2005), and LBA4404 (obtained from B. Staskawicz); Xe85-10: wild type, $\Delta hrcV$, $\Delta xopD$, $\Delta xopN$, $\Delta xopF2$, $\Delta xopX$, and $\Delta xopZ1$ (Kim et al. 2009, 2011; Roden et al. 2004; Stork et al. 2015); $\Delta xopZ1$ (obtained from G. Sessa). *E. coli* and *A. tumefaciens* were grown on Luria-Bertani or yeast extract peptone agar medium (for *A. tumefaciens* 1D1249) at 37 and 28°C, respectively. *X. euvesicatoria* strains were grown on nutrient agar (Turner et al. 1984) at 28°C.

Plant lines.

Solanum lycopersicum cultivar VF36 (susceptible to Xe85-10) and *Nicotiana benthamiana* were used in this study.

Plasmid constructs.

Effector genes from Xe85-10 were cloned into pCR8/GW/TOPO previously (Dubrow et al. 2018) and were recombined into the pEAQ-HT-DEST2 (Sainsbury et al. 2009) to create 32 pEAQ-HT-DEST2(6xHis-effector) plasmids encoding for 6xHis-effector fusion proteins using LR clonase II (ThermoFisher). The PCR-amplified internal region (238 bp) of *bHLH132* was cloned into pCR8/GW/TOPO and was recombined into the pHellsgate12 (Helliwell and Waterhouse 2003), to create the RNAi construct pHellsgate12(hp-*bHLH132*). To construct the ^{PRO}*bHLH132*-GUS reporter, the 2-kb *bHLH132* promoter region was PCR-amplified (primers listed in Supplementary Table S1) from tomato VF36 genomic DNA and was subcloned into pEZRK-LCY(*GUSplus*) (Kim et al. 2008) to create pEZRK-LCY(^{PRO}*bHLH132-GUSplus*). For overexpression of *bHLH132* in tomato, the coding sequence of *bHLH132* was PCR-amplified from tomato VF36 cDNA and was cloned into pCR8/GW/TOPO to create pCR8/GW/TOPO(*bHLH132*). Then the plasmid was recombined into pMDC83 (Curtis and Grossniklaus 2003) to create pMDC83(*bHLH132-GFP-6xHis*). For overexpression of *bHLH132* in *E. coli*, pCR8/GW/TOPO(*bHLH132*) was recombined into pDEST15 (ThermoFisher) to create pDES15(*GST-bHLH132*).

Tomato transgenic lines.

To generate *bHLH132* silencing, OE, and reporter transgenic lines, constructs were mobilized into *A. tumefaciens* LBA4404 and were then used to transform VF36 tomato transgenic lines using standard methods (McCormick 1991). hp-*bHLH132* lines were analyzed by quantitative PCR (qPCR) to measure *bHLH132* mRNA levels, using gene-specific primer. *bHLH132*-GFP-6xHis lines were analyzed by immunoblot analysis. ^{PRO}*bHLH132*-GUS lines were analyzed using GUS report.

GST-*bHLH132* protein expression and purification.

GST-*bHLH132* and GST were expressed in *E. coli* Rosetta 2 (DE3) cells (Novagen) and were purified by GST affinity chromatography. Cells were grown in 2× yeast extract/tryptone media at an optical density at 600 nm = 0.6 to 0.8 and were then induced with 500 μM isopropylthio-β-galactoside (Roche) for 4 h at room temperature. Cells were lysed in phosphate buffered saline, pH 8, 1% Triton X-100, 0.1% β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich) with a sonicator (Branson). Protein bound to glutathione sepharose 4B beads (Amersham Biosciences) was eluted by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and was quantified using Bradford protein assay (Pierce).

EMSA.

bHLH132 DNA binding assays were done using the Light-shift Chemiluminescent EMSA kit as described (Pierce). A 14-bp DNA containing G-box (CACGTG) was labeled with biotin, using the Biotin 3' end DNA labeling kit (Pierce), and was then incubated with purified GST or GST-*bHLH132* and an increasing concentration (0, 12.5, and 25 ng/μl) of poly(dI-dC), as nonspecific competitor, in binding buffer (10 mM Tris, pH 7.5, 50 mM KCl, 5 mM MgCl₂, 5% glycerol, and 1 mM dithiothreitol) for 30 min at 28°C. The final concentration of each protein in the reaction was 1 μM. Mixtures were separated on a 6% polyacrylamide gel in 0.5× Tris-borate-EDTA buffer and

were transferred to nitrocellulose membranes (Amersham Biosciences), to detect DNA by chemiluminescence.

Bacterial growth curves.

To monitor *X. euvesicatoria* growth in tomato leaves, bacterial suspensions (1×10^5 CFU/ml in 10 mM MgCl₂) were inoculated into leaves using a needleless syringe. Leaves of the same age on the same branch were used for each test. Plants were kept under 16-h of light per day at 28°C. Under these conditions, small black bacterial lesions or spots generally appear by 6 days postinfection. Four leaf discs (0.5 cm²) per treatment per timepoint were ground in 10 mM MgCl₂ and were diluted and spotted onto nutrient agar plates in triplicate to determine bacterial titer. Three biological replicates (i.e., three plants) were used, and the experiment was repeated at least three times. Average bacterial titer $\pm$ standard deviation is reported.

Transient protein expression.

A. tumefaciens C58C1 pCH32 (for *Nicotiana benthamiana*) or 1D1249 (for tomato) were incubated in induction media (10 mM MES, pH 5.6, 10 mM MgCl₂, and 150 μ M acetosyringone) for 2 h. Leaves were inoculated with bacteria (0.2×10^9 to 0.5×10^9 CFU per milliliter) and, then, plants were incubated at ambient temperature under continuous low light for 48 to 72 h.

Immunoblot analysis.

Proteins were separated by SDS-PAGE, were transferred to nitrocellulose, and were then detected by ECL prime (GE) using anti-GFP (Covance) and horseradish peroxidase-conjugated secondary antibodies (Bio-Rad). Primary and secondary antibodies were typically diluted 1:5,000 and 1:3,000, respectively.

Tomato GUS reporter assays.

To monitor effector induction of bHLH132 transcription, *A. tumefaciens* 1D1249 cells harboring effector plasmids [pEAQ-HT-DEST2(6xHis-effector)] were inoculated into leaves as described above. After 72 h, leaf discs (0.6 cm²) were ground in 200 μ l of GUS extraction buffer (50 mM sodium phosphate, pH 7, 10 mM EDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100, and 10 mM β -mercaptoethanol) and were centrifuged at $16,000 \times g$ for 10 min at 4°C. Supernatant (10 μ l) was mixed with 90 μ l of GUS extraction buffer with 1 mM 4-methylumbelliferyl-D-glucuronide (Sigma-Aldrich). The mixture was incubated at 37°C for 1 h. GUS activity was determined using the Wallac 1420 multilabel counter (Perkin Elmer). Total protein concentration was determined using Bradford protein assay.

qPCR.

Total RNA was isolated from leaves using Trizol reagent (Invitrogen) according to manufacturer instructions, and 5 μ g RNA was used for cDNA synthesis. qPCR was performed using the cDNA and gene-specific primers. Each cDNA was amplified using HotStart-IT SYBR green qPCR master mix (USB) and the MJ Opticon 2 (Bio-Rad). Tomato *ACTIN* expression was used to normalize expression value in each sample, and relative expression values were determined against buffer or wild-type *X. euvesicatoria*, using the comparative cycle threshold method ($2^{-\Delta\Delta C_t}$) (Pfaffl 2001).

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