Xanthomonas translucens commandeers the host rate-limiting step in ABA biosynthesis for disease susceptibility

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Edited by Sean R. Cutler, University of California, Riverside, CA, and approved September 13, 2019 (received for review July 8, 2019)

Plants are vulnerable to disease through pathogen manipulation of phytohormone levels, which otherwise regulate development, abiotic, and biotic responses. Here, we show that the wheat pathogen Xanthomonas translucens pv. undulosa elevates expression of the host gene encoding 9-cis-epoxycarotenoid dioxygenase (TaNCED-SBS), which catalyzes the rate-limiting step in the biosynthesis of the phytohormone abscisic acid and a component of a major abiotic stress-response pathway, to promote disease susceptibility. Gene induction is mediated by a type III transcription activator-like effector. The induction of TaNCED-SBS results in elevated abscisic acid levels, reduced host transpiration and water loss, enhanced spread of bacteria in infected leaves, and decreased expression of the central defense gene TaNPR1. The results represent an appropriation of host physiology by a bacterial virulence effector.

Xanthomonas | ABA | TAL effector | disease susceptibility | wheat

Pathogenic bacteria acquire new virulence strategies for exploiting their hosts. This work reveals that the bacterial wheat pathogen Xanthomonas translucens uses a transcription activation-like (TAL) effector to promote virulence by directly activating the host gene 9-cis-epoxycarotenoid dioxygenase, the rate-limiting enzyme in biosynthesis of abscisic acid that is normally involved in water management within the host plant. Evolutionarily, TAL effectors are a relatively new class of virulence factors limited to a few species of pathogenic bacteria, and this work adds to the diversity of host susceptibility genes that can be exploited by pathogens through TAL effector gene function.

Significance

Pathogenic bacteria acquire new virulence strategies for exploiting their hosts. This work reveals that the bacterial wheat pathogen X. translucens pv. undulosa (Xtu), which is a pathogen of wheat. Xtu strains harbor 7 to 8 TAL effector genes (22, 23). Here, we examined the role of TAL effectors in Xtu and attempted to identify TAL effector associated susceptibility genes in wheat infections.

Results

Tal8 Is Associated with Enhanced Virulence in XT4699. Xtu causes the disease known as bacterial leaf streak of wheat (Fig. 1A) and virulence assays were conducted by injection of bacteria into young leaves with a needle-less syringe, which resulted in water-soaked streaked lesions from the inoculation site (Fig. 1B). Assays were conducted on mutants for each of 8 TAL effector genes in Xtu

Author contributions: Z.P., Y.H., A.K.B., S.P., Z.L., S.L., and F.F.W. designed research; Z.P., Y.H., J.Z., J.C.H.-T., A.K.B., S.P., S.L., and F.F.W. performed research; Z.P., Y.H., J.Z., J.C.H.-T., A.K.B., S.P., S.L., and F.F.W. analyzed data; and Z.P., Z.L., S.L., and F.F.W. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

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Data deposition: RNA sequencing data were deposited in the National Center for Biotechnology Information Sequence Read Archive (SRA) database (accession no. PRJNA485724). Xtu LW16 genome assembly was deposited under GenBank accession no. CP043540.1, https://www.ncbi.nlm.nih.gov/nuccore/CP043540.1; and P3 genome assembly was deposited under accession no. CP043500.1, https://www.ncbi.nlm.nih.gov/nuccore/CP043500.1.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1911660116/-/DCSupplemental.

First published October 1, 2019.
strain XT4699 (strains used in this study are listed in SI Appendix, Supplementary Information Text and Table S1). Mutant M6, lacking the gene tal8XT4699 (hereafter tal8) and containing the empty cloning vector (ev), incited shorter lesions in comparison to the WT strain XT4699 in 2 cultivars, the winter wheat cultivar (cv) Jagger (Fig. 1 B and C and SI Appendix, Fig. S1A) and the spring wheat cv Chinese Spring (SI Appendix, Fig. S1 B and C). The reintroduction of tal8 into M6 restored virulence in comparison to M6 (ev) on both cultivars (Fig. 1 B and C and SI Appendix, Fig. S1 A–C). No differences in total leaf and surface bacterial populations were detected between WT, M6 (ev), and M6 (tal8) in either cultivar (SI Appendix, Fig. S1 D and E). However, populations of M6 (ev) were reduced in the section of leaves distal from the inoculation site in comparison to WT and M6 (tal8), indicating that more bacteria were distributed farther up and down the leaf in the presence of tal8 (Fig. 1 D and E).

**TaNCED and TaERF Are Candidate Host Susceptibility Genes for Tal8.**

RNA-sequencing (RNA-seq) expression profiles of infected leaf samples were compared for both Jagger and Chinese Spring wheat cultivars with either WT or mutant bacteria (SI Appendix) (24). Candidate disease susceptibility genes corresponding to the effector Tal8XT4699 (hereafter Tal8) were selected according to relative fold-change in expression between WT and mutant treatments from the Jagger cultivar (SI Appendix). The candidate genes were then ranked by the effector binding element (EBE) prediction score (SI Appendix), and the proximity of the EBE to the ATG and the fold-change in Chinese Spring treatment were noted (Table 1). The specific EBEs were predicted from the repeat variable di-amino acid residue (RVD) region of the Tal8 protein, treating the unusual RVDs, KG, Y*, and QD as functionally equivalent to NG, N*, and HD of the consensus RVDs (SI Appendix, Fig. S2). Among the top ranked genes, 2 homeologous sets of genes were noted. Traes_5BS_B626CS22B represents a gene for 9-cis-epoxy-carotenoid dioxygenase 3 (NCED) located on the short arm of chromosome 5 of the B genome of hexaploid wheat, and was designated TaNCED_5BS (Table 1). The promoter region of TaNCED_5BS is conserved among the B, A, and D genome copies of the gene, although polymorphisms are present at the EBE region (SI Appendix, Fig. S3A). Traes_1BL_F15C52CSDF on the long arm of chromosome 1B represents a member of the apetala-2/ethylene response factor (AP-2/ERF) family and was designated TaERF_1BL (Table 1). TaERF_1BL has homeologs on the A and D genomes, and the predicted EBEs are identical (SI Appendix, Fig. S3B). The D genome copy of TaNCED was also induced as well as TaERF homeologs on the A and D genomes (Table 1 and SI Appendix, Fig. S3). No evidence was obtained for enhanced expression of TaNCED_5AS, which may be due to the TT > GC transversions in the corresponding EBE regions (SI Appendix, Fig. S3A). The proposed EBE for Tal8 lies 26 bp upstream of the putative TATA box of TaNCED_5BS (Fig. 2A). No TATA box was identifiable in TaERF_1 BL, and the EBE lies 171 bp upstream the ATG (Fig. 2B and SI Appendix, Fig. S3B).

Xtu strains occur as 2 types: A short lesion or low virulence cohort, represented here by Xtu strains LW16, XT130, and XT5791, and a high virulence cohort, represented by XT4699.

![Fig. 1](https://example.com/fig1.png)

**Fig. 1.** Tal8 is associated with enhanced virulence. (A, Left) Field bacterial leaf streak infection of wheat. (Right) Individual leaf symptoms of infected leaf. (B) Photograph of representative leaves inoculated with bacterial strains with and without tal8 at 7 d postinfection (DPI). XT4699, WT Xtu; M6(ev), tal8-deficient M6 strain harboring the empty vector pHM1; M6(tal8). M6 strain carrying tal8 in pHM1. (C) Average lesion length measurements of 10 leaves at 5 DPI. Strains are as indicated in B. Each bar signifies the range of mean ± SD. The lowercase letters show significantly different groups (P < 0.05) using Tukey’s honestly significant difference test (Tukey’s HSD). (D) Schematic of bacterial leaf population measurements in sections at 7 DPI. Section 1 included the initial inoculation site and water-soaked tissue caused by M6(ev). Section 2 had an equal length to section 1. (E) Populations of bacteria in sections 1 and 2 as described in D. The lowercase letters indicate significantly different groups (P < 0.05) with Tukey’s HSD.
Table 1. Candidate genes targeted by Tal8

| Gene ID       | Gene name       | EBE score | Base pairs from ATG | Log_{2}FC in JA | Log_{2}FC in CS | Gene description                       |
|--------------|-----------------|-----------|---------------------|-----------------|----------------|----------------------------------------|
| Traes_5BS_B626C522B | TaNCED_5BS | 8        | 217                 | 7.31            | 6.73           | 9-cis-epoxycarotenoid dioxygenase 3    |
| Traes_7AS_25F9FEF52  | TaERF_1BL   | 8.91     | 467                 | 4.45            | 1.91           | Pyruvate dehydrogenase E1 component subunit a-2 |
| Traes_1BL_F0CS25DF   | TaERF_1AL   | 9.02     | 171                 | 6.50            | 7.01           | AP2-like ethylene-responsive transcription factor |
| Traes_1AL_DEF1473D   | TaNCED_5BS  | 9.02     | NA                  | 4.12            | 4.58           | AP2-like ethylene-responsive transcription factor |
| Traes_1DL_E2687E281  | TaERF_1DL   | 9.02     | 181                 | 3.82            | 3.78           | AP2-like ethylene-responsive transcription factor |
| Traes_1AL_1B76D1299  | TaERF_1BL   | 9.11     | 388                 | 4.25            | 2.42           | Unknown protein                        |
| Traes_5DL_3A49B7207  | TaERF_1DL   | 9.4      | 118                 | 3.09            | 0.20           | Heat shock protein 21                  |
| Traes_4DS_033958E87  | TaERF_1BL   | 10.04    | 220                 | 3.50            | 2.82           | Early nodulin-like protein 22          |
| Traes_2BL_172F729A7   | TaNCED_5BS  | 10.57    | 356                 | 3.44            | 2.07           | Aluminum-induced protein with YGL and LRD motifs |
| Traes_5DS_E58EBABFD  | TaNCED_5DS  | 11       | 219                 | 4.91            | 5.14           | 9-cis-epoxycarotenoid dioxygenase 3    |
| Traes_1BL_6B21370DA  | TaNCED_5DS  | 11.21    | 225                 | 3.34            | 2.47           | Eukaryotic peptide chain release factor |
| Traes_2AL_B7FC2C90   | TaNCED_5DS  | 11.47    | 261                 | 3.39            | 2.86           | Aluminum-induced protein with YGL and LRD motifs |
| Traes_1DL_E3316071   | TaNCED_5DS  | 11.92    | 147                 | 4.38            | 2.41           | Protein kinase superfamily protein     |
| Traes_1AL_61464BAA6  | TaNCED_5DS  | 11.98    | 285                 | 3.35            | 2.64           | Unknown protein                        |

CS, wheat cv. Chinese Spring; Log_{2}FC, the log_{2} value of fold-change of WT vs. M6; JA, wheat cv. Jagger; NA, missed information of translation start codon in annotation.

*EBE score value is calculated by the target finder with best possible score of 4.9 for Tal8.

LG48, XTS523, XTS5770, LB10, and P3 (Fig. 2C). Low and high expression levels of TaNCED and TaERF were associated with low and high virulence strains, respectively (Fig. 2D; primers used in this study are listed in SI Appendix, Table S2). Transfer of tal8 into the low virulence strain LW16 resulted in a strain with greater virulence (Fig. 2E and F) and enhanced expression of TaERF and TaNCED (Fig. 2G). Sequence analysis of representative low virulence strain LW16 and the high virulence strain P3 revealed that the chromosomes are colinear and contain 8 TAL effector genes in the same locations as XT4699 (SI Appendix, Fig. S4) (25, 26). The TAL effector genes of the 3 strains encode near-identical RVD repeats, with the exception that Tal8 of LW16, where the RVDs are highly dissimilar from XT4699 and P3 (SI Appendix, Fig. S5 and Table S3).

TaNCED Induction Contributes to High Virulence. Designer TAL effectors (dTALs) were constructed to target unique sequences in the TaNCED_5BS and TaERF_1BL promoters, respectively, in order to induce each gene independently (SI Appendix). The dTALs dNCED72 targeted the EBE for Tal8 plus 3 additional nucleotides, while 3 other dTALs (dNCED41, dNCED53, and dNCED63) targeted the TATA box region downstream of the tal8 EBE (Fig. 3A). Three dTALs—dERF3, dERF4, and dERF6—targeted the promoter region of TaNCED_5BS upstream of the original tal8 EBE (Fig. 3B). The dTALs were introduced individually into the Tal8-deficient low virulence strain LW16, which was used due to the higher natural competency for DNA transfer, and the strains were tested for the ability to promote expression of TaNCED_5BS and TaERF_1BL, respectively. The dTAL dNCED72 triggered the highest expression of TaNCED_5BS (Fig. 3C). However, dNCED72 also resulted in TaERF_1BL expression and, therefore, did not discriminate between the 2 genes (Fig. 3C). The dTALs dNCED41, dNCED53, and dNCED63 directed expression of TaNCED_5BS without concomitant expression of TaERF_1BL (Fig. 3C), and the dTALs dERF3, dERF4, and dERF6 each induced high levels of TaERF_1BL without TaNCED_5BS induction (Fig. 3D). Expression levels were so high for the ERF dTALs that we were concerned the high levels might have pleiotropic effects on the host physiology and, as a consequence, disease symptom expression. As an added caution, dERF101 was designed by the introduction of 4 RVDs with suboptimal matches with the corresponding nucleotides in the EBE for dERF3. LW16 carrying the dERF101 had lower induction on TaERF_1BL, although induction was higher in comparison to Tal8 (Fig. 3D). Upon inoculation of the strains with the dTALs on wheat, transformants carrying dTALs dNCED41, dNCED53, dNCED63, or dNCED72 enhanced virulence (Fig. 3E). Transformants harboring dERF3, dERF4, dERF6, or dERF101, targeting TaERF_1BL alone, did not promote virulence (Fig. 3E). The dTALs were also tested in the original tal8 mutant strain M6 with similar results. The dTALs for TaERF_1BL and TaNCED_5BS induced the respective target genes, while only dNCED63 enhanced virulence based on lesion length (SI Appendix, Fig. S6). Therefore, pathogen virulence, in both LW16 and XT4699 and, conversely, host susceptibility are associated with NCED expression but not ERF expression.

Tal8 Directs Expression in Tobacco. Agrobacterium-mediated transient expression assays on Nicotiana benthamiana leaves were used to corroborate Tal8-directed expression specifically from the TaNCED_5BS and TaNCED_5DS promoters (SI Appendix). When fused with the reporter gene uidA for β-glucuronidase (GUS), the native promoters of TaNCED_5BS and TaNCED_5DS resulted in low GUS activity when coexpressed with Tal8 under the control of the CaMV 35S promoter, although induction was higher in comparison to Tal8 (Fig. 3D). Upon inoculation of the strains with the dTALs on wheat, transformants carrying dTALs dNCED41, dNCED53, dNCED63, or dNCED72 enhanced virulence (Fig. 3E). Transformants harboring dERF3, dERF4, dERF6, or dERF101, targeting TaERF_1BL alone, did not promote virulence (Fig. 3E). The dTALs were also tested in the original tal8 mutant strain M6 with similar results. The dTALs for TaERF_1BL and TaNCED_5BS induced the respective target genes, while only dNCED63 enhanced virulence based on lesion length (SI Appendix, Fig. S6). Therefore, pathogen virulence, in both LW16 and XT4699 and, conversely, host susceptibility are associated with NCED expression but not ERF expression.
efficiently expressed in tobacco (27). *CsLob1* and the derivative *CsLobT* contain the EBE for the TAL effector PthA4. *CsLobT* directed expression only when containing the Tal8 EBE from TaNCED_5BS or TaNCED_5DS and cotransformed with CaMV 35S regulated Tal8 or, as a control, CaMV 35S regulated PthA4 (Fig. 3 F, Tal8+*CsLobT*, Tal8+EBeB_T, Tal8+EBeD_T, *CsLobT*, and PthA4+*CsLobT*, respectively). Again, the EBE from TaNCED_5BS (EBeB) functioned better than the EBE from TaNCED_5DS (EBeD) in the context of *CsLobT*, which is consistent with the RNA-seq data. GUS activity was undetectable with the promoter constructs EBeB_T and EBeD_T in the absence of Tal8 (Fig. 3 F, ev+EBeB_T-*CsLobT* and ev+EBeD_T-*CsLobT*, respectively). The results corroborate the hypothesis that EBeB and EBeD mediate Tal8-associated expression of TaNCED_5BS (and TaNCED_5DS).

**Increased ABA Content Is Associated with Virulence.** NCED activity is the first committed step in the biosynthetic pathway for ABA, and 3 predicted products from the wheat genes on the short arm of chromosome 5 are most closely related to OsNCED5 and OsNCED3 of rice and ZmVP14 of maize, 2 of which have been shown to be involved in ABA biosynthesis (SI Appendix, Fig. S7A) (28, 29). Indeed, the ABA content rose in infected leaf tissues upon NCED expression as measured by a competitive
ELISA (SI Appendix). Mock inoculations or inoculations with LW16 (ev), which does not induce the expression of TaNCED_5BS, were associated with low amounts of ABA in leaves (Fig. 4A). LW16 carrying dERF4 and dERF6 also did not enhance the ABA levels (Fig. 4A). Inoculations with LW16, containing dNCED41, dNCED53, dNCED63, dNCED72, or tal8, had high ABA levels (Fig. 4A). To determine if ABA was consequential to virulence, ABA was applied to leaves, which were challenged by low virulence pathogen. ABA application followed by LW16 (ev) inoculation resulted in 7-fold increase in lesion length along the leaf blade (Fig. 4B and C) and higher bacterial populations in distal sections of the leaves in comparison to the control treatment without ABA (Fig. 4D, section 2).
Tal8 and ABA Alter Water Relations in the Host. Plant pathogenic bacteria are hypothesized to engineer a favorable aqueous intracellular environment in the host (30–32). ABA, as the classic hormone in response to water-stress, might be hijacked to recruit and retain water at the site of infection. ABA induces stomatal closure and consequential reductions of gas and water transpiration. Indeed, transpiration, as well as water loss, were reduced in tissues infected with bacteria containing Tal8 (Fig. 5 A and B). Water-soaked lesions are thought to reflect release of water from mesophyll cells into the intercellular space (apoplast) upon infection. Here, we observed that ABA application by itself promoted water-soaking symptoms, indicating that the retention of water to the site of infection may also occur (Fig. 5 C). HopAM1 of P. syringae was reported to be effective in water-stressed plants (18). Here, the plants were tested under well-watered conditions. However, inoculation of plants under high humidity enhanced lesion lengths and reduced the differences between strains with and without Tal8 (Fig. 5 D), indicating the reduced water loss under higher humidity enhances disease and bacterial spread.

Tal8 Is Associated with Reduced Expression of TaNPR1. ABA-mediated disease susceptibility has been hypothesized to be due to antagonism to the salicylic acid (SA) defense response (33–36). SA levels were measured over a 3-d period after infection. SA levels, both free and total, rose over a 3-d period after infection of wheat leaves with LW16 (ev), LW16(dNCED63), and LW16 (tal8) (SI Appendix, Fig. S7 B and C). Free SA levels between the strains with and without tal8 rose to similar levels (SI Appendix, Fig. S7B). Total SA levels were also higher over the same period (SI Appendix, Fig. S7C). LW16(dNCED63) had the highest free and total SA (SI Appendix, Fig. S7 B and C), although this strain, similar to the strain with tal8, also has enhanced lesion lengths and ABA content. Therefore, SA content did not vary strictly in relation to TaNCED expression. Application of SA, both by leaf spray or soil drench, did not alter susceptibility of LW16 with or without tal8 (SI Appendix, Fig. S7 D and E). Nonetheless, changes in expression of wheat homologs of the master immune regulator and SA receptor gene TaNPR1 were observed in infected tissues by strains with or without tal8. Both A and D genome copies of the gene were lower in expression in the presence of Tal8 (Fig. 6 A). TaNPR1 suppression was correlated with NCED expression as a strain with dTALe targeting TaNCED (dNCED63) also suppressed NPR1 based on the expression of TaNPRI_3AS, while the strain with dERF101 failed to suppress (Fig. 6B). Leaf infiltration of ABA resulted in the largest suppression TaNPR1 (Fig. 6B). Reduced NPR1 expression was correlated with reduced expression of homologs of the NPR1-dependent defense gene PRI (Fig. 6 C and D). SA levels, then, may be less relevant than NPR1 levels due to suppression of NPR1 expression by ABA.
involvement of *TaERF_1BL* in virulence, independent of NCED expression. The candidate EBEs in both NCED genes directed expression in a Tal8-mediated manner in a transient expression system, and polymorphisms between the EBEs are reflected in differing levels of expression in vivo and in the transient expression system. Nonetheless, future genome editing of the EBEs, indeed, are the critical Tal8 binding sites.

The benefits of Tal8 and consequential high ABA levels could be due to multiple factors. The effects of ABA on disease susceptibility has been attributed to 3 possible factors, which are not mutually exclusive: Suppression of SA pathway defense responses, inactivation of defense-related MAP kinases, and alteration of water availability at the infection site (17, 18, 33). The effects of humidity on lesion development and reduction of the differences between bacteria with and without Tal8 corroborates the importance of water relations. Rice mutants, for example, with severely reduced ABA levels, are resistant to infection by *Xanthomonas oryzae* pv. *oryzae*, the agent of bacterial blight of rice, and reductions in stomatal conductance, water transpiration, and higher ABA content accompany bacterial blight infections of normal plants (38). In the latter study, no evidence could be found to support an alteration in SA signaling in contrast to 2 other studies of ABA content in disease susceptibility (34, 36). We found variable changes in free and total SA content of infected rice leaves in association with TALe-mediated ABA increases and could not discern a firm contribution to disease susceptibility. At the same time, some contribution of SA cannot be ruled out. NPR1 plays essential roles in localized and systemic acquired resistance in many plant species and overexpression of *NPR1* in wheat enhances disease resistance (39, 40). Reduced expression of *TaNPR1*, which is involved in SA perception, was observed and, coincidently, is a unique finding for an effector function. These data indicate that ABA production induced by Tal8 may impact susceptibility to *Xtu* by suppressing the ability of the plant to perceive SA. Similar antagonistic relationships between ABA and NPR1 have been observed in other plant species, for example NPR1 protein degradation is stimulated by ABA in *Arabidopsis* (41). Suppression of NPR1 expression by ABA has also been observed in rice (35, 36), indicating that ABA-mediated regulation of NPR1 maybe a conserved function of ABA in phytohormone cross-talk. The effects of Tal8 on ABA, directly, on MPK6 and MPK3 phosphorylation were observed in other plant species, for example NPR1 has been attributed to the elevated access to sugar as a nutrient for pathogen growth (45). Three host transcription factor genes are known to be targeted in diseases of pepper, citrus, and tomato (27, 31, 46). The latter 2 cases are associated with coexpression of cell wall degradative enzymes, which may release of bacteria from the infection site or alter water content of the infected tissue (31). In the case of Xtu, increased release of bacteria from the leaf surface was not detected. Nonetheless, bacteria were found to spread farther up and down the leaf in the presence of NCED expression. The spread could be driven by water flow into the infected area due to stomatal closure, enhanced bacterial growth by a more aqueous environment, or a combination of ABA effects. A variety of additional TAL effector host targets are associated with virulence effects (47–49). TAL effectors, therefore, represent a
versatile method to manipulate host susceptibility, and the list of TALe-mediated changes in plant physiological processes potentially affecting disease susceptibility is growing (50). The mechanisms by which known host S genes mediate enhanced virulence are poorly understood. Due to the extensive knowledge of ABA function, the consequences of Tal8 seem amenable for further elucidation.

Materials and Methods

The wheat plants were grown and inoculated for virulence assays in growth chambers with control conditions as described in SI Appendix. Bacteria and plasmids used in this study are provided in SI Appendix, Table S1. Methods on RNA-seq analyses, prediction of candidate S genes for Tal8 and heatmap constructions, and sequence analysis of high and low virulence strains of Xtu are provided in detail in SI Appendix. Repeats of dTALes were assembled following methods described previously (51, 52). dTALes were cloned into broad host-range vector pHM1 and transformed into Pseudomonas syringae to functional tests. Further methods on qRT-PCR analyses, transient GUS assay in N. benthamiana, ABA quantification assay, application of ABA and effect of Tal8 in wheat leaves, and SA measurement and application are described in detail in SI Appendix.

ACKNOWLEDGMENTS. The authors thank Dr. Alina Akhunova and the staff of the Kansas Integrated Genomic Facility at Kansas State University for RNA sequencing; Dr. Matthew Dommel, and Dr. Zhonglin Mou for assistance with salicylic acid assays; and Dr. Sixue Chen and Mr. Qijie Guan for assistance with leaf transpiration measurements. This research was supported by funds from the National Science Foundation research Grants 1238189 (to F.F.W.) and 1741090 (to S.L., S.P., and F.F.W.), and the Institute of Food and Agricultural Sciences, University of Florida (F.F.W.).

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