SlWRKY30 and SlWRKY81 synergistically modulate tomato immunity to *Ralstonia solanacearum* by directly regulating *PR-STH2*

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ABSTRACT

Bacterial wilt is a devastating disease caused by *Ralstonia solanacearum* that severely threatens tomato (*Solanum lycopersicum*) production. Group III WRKY transcription factors are implicated in the plant response to pathogen infection; however, their roles in the response of tomato to *R. solanacearum* infection (RSI) remain largely unexplored. Here, we report the crucial role of SlWRKY30, a group III WRKY transcription factor, in the regulation of tomato response to RSI. *SlWRKY30* was strongly induced by RSI. *SlWRKY30* overexpression reduced tomato susceptibility to RSI, and also increased H_2O_2 accumulation and cell necrosis, suggesting that SlWRKY30 positively regulates tomato resistance to RSI. RNA sequencing and reverse transcription quantitative PCR revealed that *SlWRKY30* overexpression significantly upregulated *PR-STH2* genes, *PR-STH2a*, *STH2b*, *STH2c*, and *STH2d* (hereafter *PR-STH2a/b/c/d*), in tomato, and these *PR-STH2* genes were directly targeted by SlWRKY30. Moreover, four group III WRKY proteins (SlWRKY52, SlWRKY59, SlWRKY80, and SlWRKY81) interacted with SlWRKY30, and *SlWRKY81* silencing increased tomato susceptibility to RSI. Both SlWRKY30 and SlWRKY81 activated *PR-STH2a/b/c/d* expression by directly binding to their promoters. Taken together, SlWRKY30 and SlWRKY81 synergistically regulate resistance to RSI by activating *PR-STH2a/b/c/d* expression in tomato. Our results also highlight the potential of *SlWRKY30* to improve tomato resistance to RSI via genetic manipulations.

RESULTS

Expression analysis of group III *SlWRKY* genes during RSI and exogenous application of SA

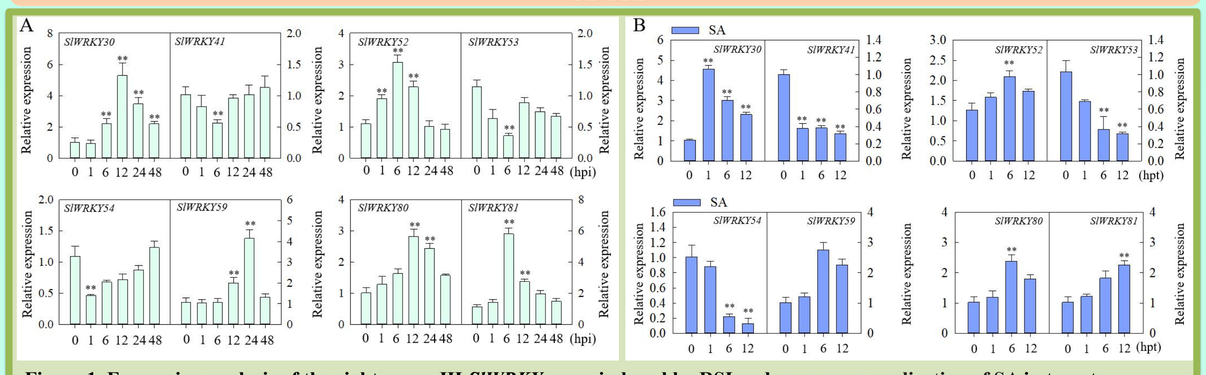


Figure 1. Expression analysis of the eight group III *SlWRKY* genes induced by RSI and exogenous application of SA in tomato.
A, Expression levels of the eight group III *SlWRKY* genes in tomato leaves analyzed by RT-qPCR from 0 to 48 hours post inoculation (hpi) with *R. solanacearum*.
B, Expression levels of the eight group III *SlWRKY* genes in tomato leaves analyzed by RT-qPCR at 0, 1, 6, and 12 hours after treatment with 200 μM SA.
Data represent the mean ± SE of three biological replicates, and asterisks indicate a significant difference compared with control plants (Student's *t* test, ***P*-value < 0.01).

Subcellular localization and transcriptional activity of the eight group III SlWRKY TFs

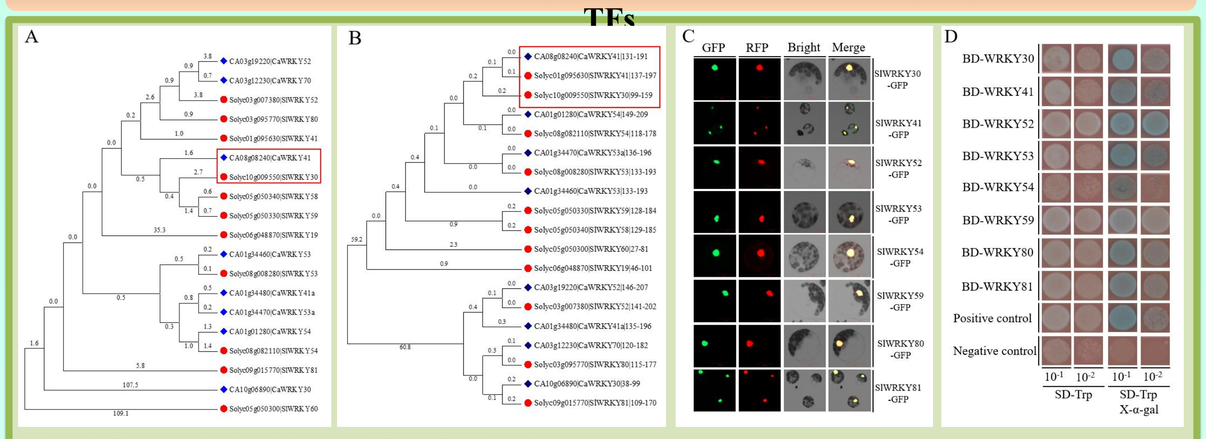


Figure 2. Characterization of the group III SlWRKY members.
A and B, Phylogenetic tree of group III SlWRKY TFs from protein (A) and domain (B) in tomato and pepper. Amino acid sequences labeled with red circles and dark blue diamonds represent the group III SlWRKY TFs from tomato and pepper, respectively. SlWRKY30 and its homolog CaWRKY41 labeled in the red box. The tree was constructed using MEGA 6.06.
C, Subcellular localization of SlWRKY-GFP fusion proteins; we transiently expressed the different *SlWRKY* genes under the control of the *35S* promoter in *Arabidopsis* mesophyll protoplasts. Protoplasts were incubated in WI buffer for 10 h after transformation and imaged using a fluorescence microscope.
D, Transcriptional activation assay of the group III SlWRKY members in yeast cells. *LacZ* reporter gene expression is indicated by blue color.

SlWRKY30 positively regulates tomato resistance to RSI

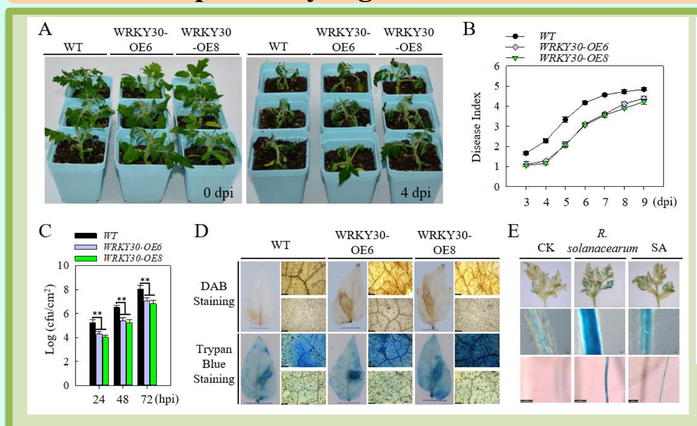


Figure 3. Figure 3. *SlWRKY30* overexpression enhances tomato resistance to RSI.
A, Resistance levels in WT, *SlWRKY30-OE6*, and *SlWRKY30-OE8* tomato plants at 0 and 4 days post inoculation (dpi) with *R. solanacearum*.
B, *R. solanacearum*-infected WT, *SlWRKY30-OE6*, and *SlWRKY30-OE8* plants were scored daily using a disease index
C, Bacterial growth in WT, *SlWRKY30-OE6*, and *SlWRKY30-OE8* leaves following RSI.
D, Increased H_2O_2 levels and cell death in *SlWRKY30-OE6* and *SlWRKY30-OE8* leaves compared with the WT at 24 hours post inoculation (hpi) with *R. solanacearum*.
E, GUS expression in transgenic tomato plants carrying the *pSlWRKY30:GUS* construct. Three-week-old *pSlWRKY30:GUS* tomato shoots and roots were treated with *R.solanacearum* or exogenous application of SA for 24 h, and then stained.

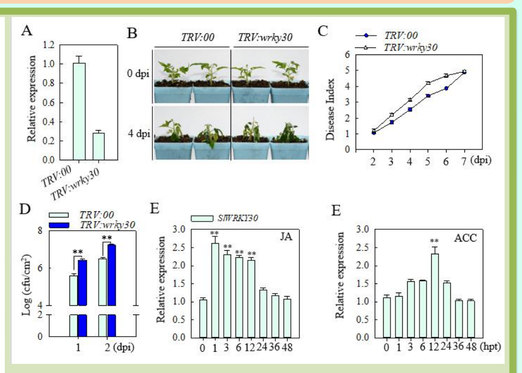


Figure 4. Silencing of *SlWRKY30* decreases tomato resistance to RSI.
A, RT-qPCR analysis of *SlWRKY30* expression in *SlWRKY30*-silenced tomato plants.
B, Resistance levels in *TRV:wrky30* and *TRV:00* (empty vector control) tomato plants at 0 and 4 days post inoculation (dpi) with *R. solanacearum*.
C and D, Disease index (C) and bacterial growth (D) in *TRV:wrky30* and *TRV:00* tomato plants following RSI.
E and F, Expression levels of *SlWRKY30* in tomato leaves analyzed by RT-qPCR at 0, 1, 3, 6, 12, 24, 36, and 48 h post treatment (hpt) with JA (jasmonic acid, 100 μM, E) and ACC (ethylene precursor, 1 μM,VF). The relative expression in phytohormone-treated plants was compared with that in control plants, which was set to 1.

SlWRKY30 functions in tomato immunity to RSI by directly regulating *PR-STH2*

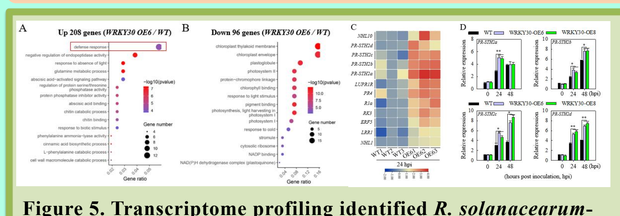


Figure 5. Transcriptome profiling identified *R. solanacearum*-induced SlWRKY30-regulated genes in tomato.
A and B, GO enrichment analysis showing that diverse terms are enriched among the DEGs regulated by SlWRKY30 in tomato at 24 hpi with *R. solanacearum*. The DEGs were identified and filtered based on the criteria of fold change ≥ 2 and false discovery rate (FDR) < 0.01.
C, Heatmap showing that SlWRKY30 upregulated defense-related genes in tomato at 24 hpi with *R. solanacearum*. The color bar indicates the $\log_2$FC (fold change).
D, Expression levels of *PR-STH2a*, *PR-STH2b*, *PR-STH2c*, and *PR-STH2d* analyzed by RT-qPCR in *WT*, *SlWRKY30-OE6*, and *SlWRKY30-OE8* tomato plants at 0, 24, and 48 hpi with *R. solanacearum*. Data represent the mean ± SE of three biological replicates, and asterisks indicate a significant difference compared with control plants (Student' *t* test, **P*-value < 0.05 or ***P*-value <0.01).

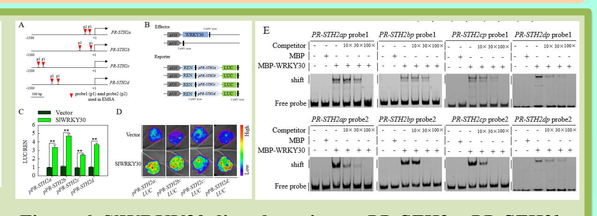


Figure 6. SlWRKY30 directly activates *PR-STH2a*, *PR-STH2b*, *PR-STH2c*, and *PR-STH2d* expression in tomato.
A, Schematic diagrams of promoter sequence selection for the EMSA. The red triangles indicate the sequence position used for the EMSA.
B, Structural schematic diagrams of the effector (*pGreenII 62-SK*) and reporter (*pGreenII-0800-LUC*) plasmids used for the dual-luciferase assay. REN: *Renilla* luciferase, LUC: Firefly luciferase.
C, Dual-luciferase assay showing that SlWRKY30 activates the expression of the *LUC* reporter gene driven by *PR-STH2a/b/c/d* promoters.
D, Transient expression assay showing that SlWRKY30 transcriptionally activates the *LUC* reporter gene (driven by the *PR-STH2a/b/c/d* promoters). At least three replicates were measured with similar results.
E, EMSA showing that SlWRKY30 directly binds to the *PR-STH2a/b/c/d* promoters.

SlWRKY30 interacts with SlWRKY52, 59, 80, and 81 during the response to RSI, SlWRKY81 positively regulates tomato immunity to RSI by regulating *PR-STH2a/b/c/d*

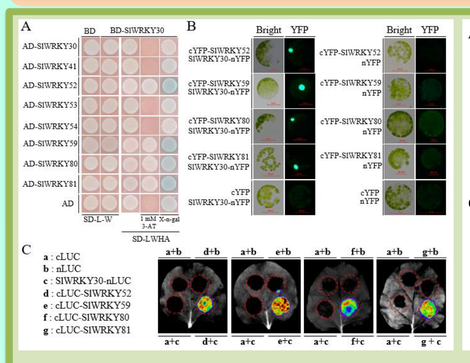


Figure 7. SlWRKY30 interacts with SlWRKY52, SlWRKY59, SlWRKY80, and SlWRKY81.
A, Y2H assay showing that SlWRKY30 interacts with SlWRKY52, 59, 80, and 81.
B, BiFC assay verifying the interactions between SlWRKY30 and SlWRKY52, 59, 80, and 81 in Arabidopsis protoplasts. Representative images are shown for protoplast cells at 10 h after incubation in WI buffer. At least three replicates were observed with similar results. Bar, 50 μm.
C, LCI assay verifying the interactions between SlWRKY30 and SlWRKY52, 59, 80, and 81 in *N. benthamiana* leaves. SlWRKY30 was fused to the N terminus of luciferase (SlWRKY30-nLUC); SlWRKY52, 59, 80, and 81 were fused to the C terminus of luciferase (cLUC-SlWRKY52, cLUC-SlWRKY59, cLUC-SlWRKY80, and cLUC-SlWRKY81).

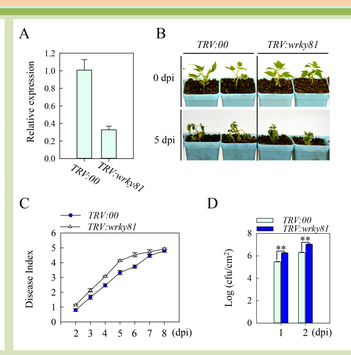


Figure 8. Silencing of *SlWRKY81* decreases tomato resistance to RSI.
A, RT-qPCR analysis of *SlWRKY81* expression in *SlWRKY81*-silenced tomato plants.
B, Resistance levels in *TRV:wrky81* and *TRV:00* (empty vector control) tomato plants at 0 and 5 days post inoculation (dpi) with *R. solanacearum*.
C and D, Disease index (C) and bacterial growth (D) in *TRV:wrky81* and *TRV:00* tomato plants following RSI.
Data represent the mean ± SE of three biological replicates, and asterisks indicate a significant difference compared with control plants (Student's *t* test, ***P*-value < 0.01).

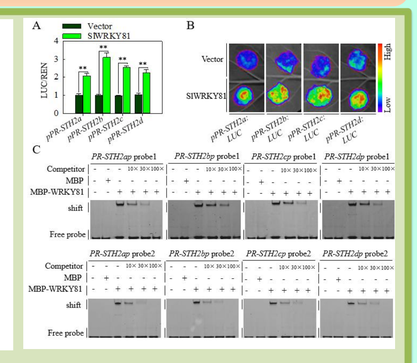


Figure 9. SlWRKY81 directly activates *PR-STH2a*, *PR-STH2b*, *PR-STH2c*, and *PR-STH2d* expression in tomato.
A, Dual-luciferase assay showing that SlWRKY81 activates the expression of the *LUC* reporter gene driven by the *PR-STH2a/b/c/d* promoters. Three independent transfection experiments were performed. Values represent mean ± SE. ***P* < 0.01 by Student's *t* test.
B, Transient expression assay showing that SlWRKY81 transcriptionally activates the *LUC* reporter gene (driven by the *PR-STH2a/b/c/d* promoters). At least three replicates were measured with similar results.
C, EMSA showing that SlWRKY81 directly binds to the *PR-STH2a/b/c/d* promoters.

SlWRKY30 and SlWRKY81 directly and synergistically regulate *PR-STH2a/b/c/d* expression

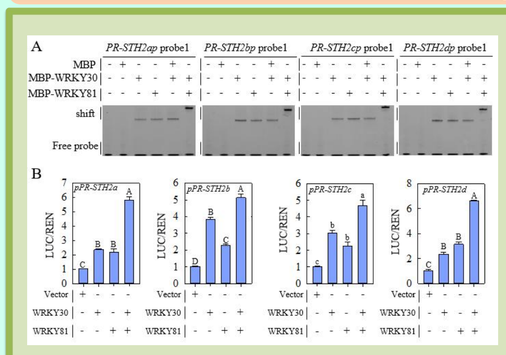


Figure 10. SlWRKY30 and SlWRKY81 synergistically activate *PR-STH2a*, *PR-STH2b*, *PR-STH2c*, and *PR-STH2d* expression.
A, EMSA showing that addition of the MBP-SlWRKY81 recombinant protein affected the mobility of MBP-SlWRKY30-bound DNA probe 1 (generated from the *PR-STH2a/b/c/d* promoters).
B, Transient expression assay in *N. benthamiana* leaves showing that SlWRKY81 significantly enhanced the transcriptional activation activity of SlWRKY30 on *pPR-STH2a:LUC*, *pPR-STH2b:LUC*, *pPR-STH2c:LUC*, and *pPR-STH2d:LUC*. Values represent mean ± SE. Significant differences are indicated by letters (lowercase letters, $P < 0.05$; uppercase letters, $P < 0.01$, Tukey's multiple comparisons test).

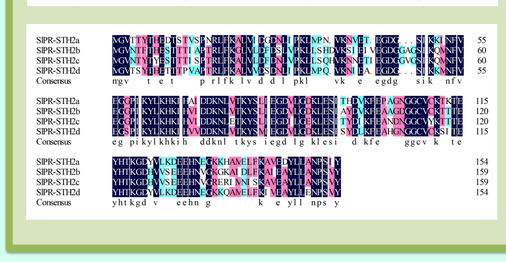
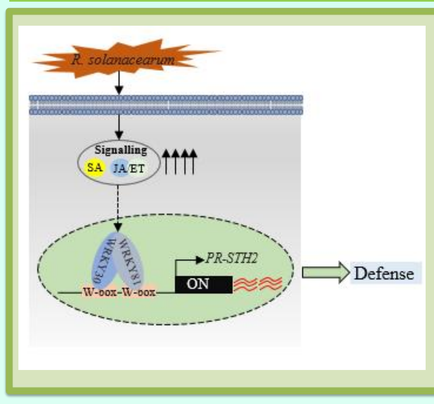


Figure 11. Alignment of deduced amino acid sequences of PR-STH2a, PR-STH2b, PR-STH2c, and PR-STH2d in tomato.
PR-STH2 genes *PR-STH2a* (Solyc09g090970), *PR-STH2b* (Solyc09g090980), *PR-STH2c* (Solyc09g090990), and *PR-STH2d* (Solyc09g091000), which are closely related and share approximately 70% identity in their deduced amino acid sequences

CONCLUSIONS



Discovery of genes that confer resistance to RSI is crucial to prevent bacterial wilt outbreaks in tomato production. We identified two group III SlWRKY TFs, SlWRKY30 and SlWRKY81, that were upregulated by RSI and positively regulated tomato immunity by directly targeting and regulating *PR-STH2a/b/c/d*. The function of SlWRKY30 might be modulated via protein-protein interactions with SlWRKY52, 59, 80, and 81. Based on these results, we proposed a model of the mechanism by which SlWRKY30 regulates immunity to RSI (Figure 12).

Figure 12. Proposed working model of the SlWRKY30-SlWRKY81 module in regulating tomato resistance to RSI.
R. solanacearum and phytohormones, such as SA and JA/ACC, induce *SlWRKY30* and *SlWRKY81*. Then, SlWRKY30 interacts with SlWRKY81 to directly and synergistically activate the expression of *PR-STH2a/b/c/d*, increasing tomato resistance to RSI.

ACKNOWLEDGEMENTS

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