WRKY1 acts as a key component improving resistance against Alternaria solani in wild tomato, Solanum arcanum Peralta

Balkrishna A. Shinde1,2,3, Bhusan B. Dholakia2, Khalid Hussain1, Asaph Aharoni3, Ashok P. Giri2,* and Avinash C. Kamble1,* (API)

1Department of Botany, Savitribai Phule Pune University (Formerly University of Pune), Pune, India
2Division of Biochemical Sciences, Plant Molecular Biology Unit, CSIR-National Chemical Laboratory, Pune, India
3Department of Plant and Environmental Sciences, Weizmann Institute of Science, Rehovot, Israel

Received 17 January 2017; revised 16 January 2018; accepted 22 January 2018.
*Correspondence (Tel +91-020-25601438; fax +91-020-25690488; email ackamble@unipune.ac.in) (ACK) and (Tel +91-020-25902710; fax +91-020-25902648; email ap.giri@ncl.res.in) (APG)

Summary
Early blight (EB), caused by Alternaria solani, is a major threat to global tomato production. In comparison with cultivated tomato (Solanum lycopersicum), a wild relative, S. arcanum exhibits strong resistance against EB. However, molecular cascades operating during EB resistance in wild or cultivated tomato plants are largely obscure. Here, we provide novel insight into spatio-temporal molecular events in S. arcanum against A. solani. Transcriptome and co-expression analysis presented 33-WRKYs as promising candidates of which 12 SaWRKYs displayed differential expression patterns in resistant and susceptible accessions during EB disease progression. Among these, SaWRKY1 exhibited induced expression with significant modulation in xyloglucan endotranshydrolase 5 (XTH5) and MYB2 expressions that correlated with the disease phenotypes. Electro-mobility shift assay confirmed physical interaction of recombinant SaWRKY1 to SaXTH5 and SaMYB2 promoters. Comparative WRKY1 promoter analysis between resistant and susceptible plants revealed the presence of crucial motifs for defence mechanism exclusively in resistant accession. Additionally, many defence-related genes displayed significant expression variations in both the accessions. Further, WRKY1 overexpressing transgenic plants exhibited higher levels of EB resistance while RNAi silencing lines had increased susceptibility to A. solani with altered expression of XTH5 and MYB2. Overall, these findings demonstrate the positive influence of WRKY1 in improving EB resistance in wild tomato and this could be further utilized as a potential target through genetic engineering to augment protection against A. solani in crop plants.

Keywords: Alternaria solani, early blight, Solanum arcanum, Tomato, WRKY1, XTH5.

Introduction
Plants have evolved diverse defence mechanisms to protect themselves against pathogen attack. Rapidly activated defence responses are mediated by complex signalling pathways affecting numerous cellular and molecular processes that lead to resistance. These include generation of reactive oxygen species, cell wall lignification, accumulation of antimicrobial compounds and activation of defence-related genes (Durrant and Dong, 2004; Hammond-kosack and Jones, 1996; Jones and Dangl, 2006; Seo and Choi, 2015). These host responses to pathogen invasion are mediated by phytohormones such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Bakshi and Oelmuller, 2014; Pieterse et al., 2012). In this turn may help the plant to fine-tune the regulation of particular defence pathway and provide optimal protection (Eulgem, 2005; Pieterse et al., 2001; Rushton et al., 2010). Therefore, to understand the plant defence response, it is important to identify key regulatory factors. A number of transcription factors (TFs) orchestrating signal crosstalk have been identified in Arabidopsis and other plants. These TFs are involved in activation or inhibition of target genes alone or via interactions with other proteins (Singh et al., 2002; Tsuda and Somssich, 2015). Among these, WRKYS are one of the most important TFs playing role in both abiotic and biotic stresses (Bakshi and Oelmuller, 2014; Pandey and Somssich, 2009; Ulker and Somssich, 2004). WRKY proteins interact with W-boxes located in the promoter regions of several plant defence genes (Bakshi and Oelmuller, 2014; Ulker and Somssich, 2004). These boxes are found in clusters within the short stretches in promoters indicating potential synergistic interactions of different WRKYS (Dong et al., 2003; Eulgem et al., 1999; Eulgem and Somssich, 2007).

Tomato (Solanum lycopersicum L.) is the most important global vegetable crop; however, its production is severely affected due to biotic stresses including fungi, bacteria, viruses, insects and nematodes. Therefore, breeding tomato for disease resistance is one of the current critical necessities. Early blight (EB) is one of the most economically important tomato disease caused by Alternaria solani Jones and Grout, a foliar necrotrophic pathogen. Although EB resistance is not known to exist in cultivated tomato, robust resistance against A. solani has been identified in some of the wild relatives (Chaerani et al., 2007; Foolad et al., 2000). Significant modulations in secondary metabolites have been recently identified during wild tomato–A. solani interactions (Shinde et al., 2017). However, molecular cascades operating during EB resistance in such wild tomato against A. solani are not fully understood. Here, we developed co-expression networks of potential WRKY targets and identified differential expression of
WRKY genes in susceptible and resistant S. arcanum Peralita accessions upon A. solani inoculation at different stages of disease progression. Further, functional characterization with transgenic plants indicated that WRKY1 could influence the EB defence in tomato.

**Results**

**Co-expression analysis created networks of WRKY-targeted genes**

Considering S. arcanum closely related to cultivated tomato, co-expression analysis was performed using available S. lycopersicum transcriptomic data (Itkin et al., 2011) to find potential WRKYs and their downstream targets. Based on the literature and experimental evidences for the WRKYs which are playing role in plant defence, 33 WRKYs were selected out of 81 in preliminary bioinformatic analysis. Of these, 10 SiWRKYs displayed 1389 co-expressed genes (CEG), while SlWRKY19 and SlWRKY22 did not show any CEG with r ≤ 0.8 (Figure 1; Table S1–S3). To understand biological role of these CEG, gene ontology (GO) annotations were performed that yielded 4984 GO annotations, representing diverse functions, processes or components. From 886 CEG with significant GO terms, 306 genes indicated GO terms related to defence [i.e. response to stimulus (156 genes), immune system process (35 genes) and biological regulation (114 genes)] (Figure S1; Table S4). After removal of duplication, unique CEG were further analysed to find S’ cis-regulatory elements in their promoter regions.

**High frequency of W-box in CEG indicated promising WRKY targets**

Promoter region of 186 unique CEG and eight known tomato defence genes depicted several S’ cis-acting regulatory elements involved in phytohormones and biotic stress regulation. Core elements such as TATA, CAAT and CCAAT were located closest to the translational start site (TSS) (ATG). We selected the S’ cis-regulatory elements related to biotic stress including ERE, GCC, GT1 and W-boxes (Table S5) to better understand host response during EB disease. Interestingly, promoters of all the unique defence genes revealed high frequency of GT1 box, which is crucial against pathogen response. In few defence genes, W-boxes were distributed in proximal region while others were in distal region. Around 60% of them revealed high frequency of W-boxes (Table S6) and were further utilized for expression variations in mock- and pathogen-treated plants using qRT-PCR.

**Contrasting expression patterns of SaWRKYs upon A. solani inoculation**

EB disease severity on leaves of LA2157 (resistant) and LA1395 (susceptible) S. arcanum accessions was assessed as percentage disease index (PDI) after 1, 3 and 5 days post inoculation (dpi) of A. solani along with mock (water-treated) plants (Shinde et al., 2017). EB lesions on leaf area progressed rapidly in susceptible compared to resistant plants at all the time points, suggesting LA1395 was susceptible (henceforth, S) while LA2157 had effective resistance (henceforth, R). Experimental validation of above-mentioned 12 WRKYs showed significant differential expression between pathogen-inoculated R and S accessions during EB disease progression. Expression levels of SaWRKY1, SaWRKY3, SaWRKY8, SaWRKY19, SaWRKY23 and SaWRKY39 were significantly increased (up to 3.5-, 3.5-, 2.9-, 4.2-, 8.1- and 4.7-fold, respectively) in R as compared to S plants at 1 dpi (Figure 2). While expression of SaWRKY11 (up to 13.7-fold), SaWRKY33 (up to 29-fold) and SaWRKY40 (up to 3.9-fold) displayed significant up-regulation at 5 dpi, they remained unchanged in S accession at 1 and 3 dpi. Additionally, SaWRKY22 revealed higher expression (up to 2.4-fold) in R plants at all the time points. On the other hand, SaWRKY4 and SaWRKY54 exhibited significantly elevated (up to 5-fold and 4.7-fold, respectively) levels in S accession at 1 dpi and remained unchanged in both the accessions at 3 and 5 dpi after A. solani inoculation (Figure 2).

**High expression of co-expressed defence genes and effect of SA during EB defence**

Based on the unique CEG from WRKY co-expression network and promoter analyses, W-box containing 18 defence genes generated clear differential expression profiles in R and S accessions. Time-course expression analysis revealed significant increase in transcript levels of SaMYB2 (up to 3.5-fold), SaXTH5 (up to 13.6-fold), SaPR1 (up to 5712-fold), SaPR1 (up to 3.1-fold), SaPR6 (up to 313-fold) and SaPR12 (up to 3292-fold) in R compared to S plants upon A. solani inoculation (Figure 3). Similarly, most of other defence genes [SaJAZ1 (up to 18-fold), SaCIPK4 (up to 16.5-fold), SaMAPK3 (up to 3.8-fold), SaGEBG6 (up to 831.7-fold), SaG (up to 1213.5-fold), SaPR3 acidic (up to 922.9-fold), SaPR7 (up to 36.8-fold), SaSTPK (up to 26.4-fold), SaPR2 (up to 10.5-fold), SaPR3 basic (up to 5.6-fold) and SaMYB7 (up to 4.4-fold)] were significantly up-regulated in R accession (Figure S2). Further, key biosynthetic genes of SA (PAL and ICS1) and JA (AOS and OPR3) depicted contrasting patterns which coincided with stages of EB disease progression (Figure S3). SaCS1 and SaPAL levels were elevated (up to 3-fold and 6.7-fold, respectively) in A. solani-treated R compared to S accessions at early stages. However, there was no significant induction in these two genes in both accessions at 5 dpi. Additionally, total SA levels (including free and conjugated forms of SA) remained similar in mock samples of both accessions at different stages. However, these SA contents were significantly high in pathogen-challenged R plants compared to S (Figure 4) indicating potential involvement of SA in early EB defence. On the other hand, JA biosynthetic genes (SaAOS and SaOPR3) did not show any elevation in R accession at 1 and 3 dpi but were significantly raised (up to 11.3-fold and up to 8.9-fold, respectively) at 5 dpi. Also, SaJAZ1 (vital for JA perception) was increased (up to 6.5-fold) in R plants at 5 dpi (Figure S3). Interestingly after exogenous SA application, the EB disease progression was significantly delayed in both S. arcanum accessions which were reflected by lower PDI (>20%–50% decrease in S while >10% in R accession) (Figure S4). These plants also had significant increase in WRKY1 expression (up to 2.5-fold) levels upon SA treatment without pathogen infection in both the accessions. This increase was also accompanied by higher levels of XTH5 and MYB2 (Figure S5). Expression of these genes, further, rose (up to 14-fold in XTH5 and up to 4-fold in MYB2) along with induction in WRKY1 (up to 4.2-fold) after pathogen inoculation. Together these findings revealed possible significance of WRKY1 CEG and SA during early EB defence in wild tomato.

**Nuclear localized SaWRKY1—structurally conserved and closely related to other WRKY1**

Of these 12 WRKYs, not much is known about the role of WRKY1 in defence response in wild plants. Interestingly, WRKY1 expression was not significantly induced after A. solani...
inoculation in cultivated tomato (S. lycopersicum, which lacks EB resistance) and S accession (Figure S6). Further, SA as well as NPR1 expression levels was increased that coincided with WRKY1 in R accession at early stages of EB disease. Therefore, we selected WRKY1 as candidate to obtain its molecular insights during EB defence. On the basis of Arabidopsis WRKY classification and protein sequence analysis, SaWRKY1 and SlWRKY1 proteins had similar N- and C-terminal WRKY domains (NTD and CTD, respectively) with conserved Cys residues which belonged to Group I with zinc-finger structure of C2H2 type organized in WRKYGQK-X13-C-X4-C-X22-23-H-X1-H pattern. SaWRKY1 contained nuclear localizing sequence ‘KRRK’ as predicted by WoLF PSORT tool (Figure S7a). Further, secondary protein structure displayed stronger resemblance between SaWRKY1, SlWRKY1 and available AtWRKY1-C crystal structure (PDB ID: 2ayd). SaWRKY1 CTD and NTD shared five β-sheets (β1-β5) and β-turns with other two WRKY1 (Figure S7b). To understand the evolutionary relationship of SaWRKY1 with other known 21 plant homologs, NJ-tree was constructed with their protein sequences. This formed broadly three clusters where SaWRKY1 was closely placed with SlWRKY1 and SlWRKY23, while AtWRKY1 was separated in other clusters (Figure S7c). To ascertain cellular localization of SaWRKY1, fusion protein SaWRKY:GFP and only GFP were transiently expressed in tobacco epidermal cells. Fluorescence of GFP was visible in the nuclei from SaWRKY1 fusion protein, confirming its presence in nucleus (Figure S8).

Figure 1 WRKY Co-expression network analysis using Cytoscape 3.1.1. Co-expressed genes for 'baits' from tomato, group A–WRKY genes (SIWRKY1, SIWRKY3, SIWRKY8, SIWRKY23 and SIWRKY39) (a), group B–WRKY genes (SIWRKY11, SIWRKY33 and SIWRKY40) (b) and group C–WRKY genes (SIWRKY4 and SIWRKY54) (c). Continuous (r-value > 0.8) lines connect co-expressed genes.

SaWRKY1 interacted with SaXTH5 and SaMYB2 promoters

As SIMYB2 and SIXTH5 were co-expressed with SIWRKY1, we hypothesized that SaWRKY1 might physically interact with their promoters and regulate expression of SaMYB2 and SaXTH5. In case of SaXTH5 promoter, W-boxes were located at distal promoter region, which included five TTGAC, two TTGACT-type W-like boxes and three TGACC-type W-like boxes. Similarly in SaMYB2 promoter, W-boxes were located at proximal promoter region with two TTGAC, three TTGACT-type W-like boxes and two TGACC-type W-like boxes (Figure 5a). Further, competitive electro-mobility shift assay (EMSA) was performed using SaMYB2 and SaXTH5 native promoters, 4xW-box (TTGACC), mutated 4xW-box (TTtACC) and non-4xW-box probes (CAATTT) with rSaWRKY1 protein (Figure S9). Gel shift was clearly evident as rSaWRKY1 was bound to both native promoters and also with W-box probe (Figure 5b); however, mutated and non-W-box probes did not show any gel shift (Figure 5c). This suggested that SaWRKY1 physically bind to SaXTH5 and SaMYB2 promoters in sequence-specific manner.

Comparative promoter analysis of WRKY1 portrayed variation in TF-binding motifs

Further to understand, if nucleotide variations in WRKY1 promoter have any role in EB phenotypic variation in tomato plants, WRKY1 promoter regions were cloned from R, S and
cultivated tomato plants. After sequencing, these promoter regions were analysed for the variation in TF-binding sites. Clear differences were observed in the proximal as well as distal WRKY1 promoter regions in R compared to S and other tomato plants (Figure 6). Unique proximal region of WRKY1 promoter from R accession harboured Dof-binding motifs which are essential in plant defence while the distal region contained MYB- and AP2-binding regions (Figure 6). On the other hand, these proximal and distal regions were absent in the WRKY1 promoters from S and cultivated tomato plants. Consequently, other three different distal regions (690–699 bp, 870–885 bp and 1135–1150 bp upstream of TSS) revealed the presence of other TF-binding sites in S and cultivated tomato (Figure 6). Thus, such variations in TF-binding motifs in WRKY1 promoters might have influenced the final outcome of EB phenotype.

Overexpression and RNAi silencing plants demonstrated positive influence of WRKY1 in EB defence

Potential contribution of WRKY1 in EB defence response was further validated using SlWRKY1 overexpressing (W1OE) in T1 generation and silencing (W1RNAi) T0 generation tomato plants. As T0 W1RNAi silencing transgenic plants had poor seed setting, we were unable to get viable T1 generation. As previously shown
in cotton, this might be due to dual role of WRKY1 in plant growth and development as well as in defence (Li et al., 2014), and thus, WRKY1 silencing might have affected seed setting resulting into loss of viable T1 generation. Two independent W1OE lines (W1OE-1 and W1OE-8) exhibited significant elevation of SlWRKY1 transcripts (15- and 21-fold, respectively) compared to wild-type (WT) plants in the absence of pathogen (Figure S10a). SIMYB2 expression levels also increased significantly in W1OE-1 and W1OE-8 (1.7- and 1.6-fold, respectively) compared to WT (Figure S10b). Similarly, these two W1OE lines depicted significant increase in SlXTH5 expression (>2-fold) (Figure S10c). On the contrary, five independent W1RNAi (T0) lines (W1RNAi-15, W1RNAi-21, W1RNAi-28, W1RNAi-29 and W1RNAi-30) had reduced SlWRKY1 (up to 4-fold) levels in comparison with WT without pathogen inoculation (Figure S10a). SIMYB2 and SlXTH5 indicated significant decrease (up to 5-fold) in W1RNAi lines as compared to WT (Figure S10b,c).

Upon A. solani inoculation, phenotypic assessment of EB symptoms displayed improved resistance with decreased necrotic lesions on leaflets of W1OE-1 and W1OE-8 lines (T1) in comparison with WT (Figure 7a). On the other hand, all five W1RNAi lines showed increased susceptibility to EB (Figure 7a). Consequently, disease severity (PDI) was also significantly decreased (>50%) in both W1OE lines compared to WT at 3 and 5 dpi. In the case of W1RNAi lines, PDI was elevated significantly (20%–40%) than WT plants (Figure 7b). W1OE lines exhibited significant up-regulation of WRKY1, MYB2 and XTH5 (up to 20-, 2.5- and 45.9-fold, respectively) whereas in case of W1RNAi lines, these were down-regulated (up to 10-fold) upon pathogen challenge (Figure S11). Moreover, expression of SlPAL, SlICS1 and SlPR1 was also significantly elevated (up to 7.2, 118.5 and >150-fold, respectively) in W1OE lines upon A. solani inoculation while reduction (up to 3-fold) was observed in W1RNAi lines (Figure S12). Similarly, SlAOS, SlOPR3, SlPR12 and SlJAZ1 levels were significantly high (up to 130.6-, 5.7-, 8.8- and >150-fold, respectively) in these W1OE lines compared to WT (Figure S13). Contrary, expression of SlOPR3, SlPR12 and SlJAZ1 was reduced (up to 2-fold) at 3 dpi but showed elevation (up to 1.86-fold) at 5 dpi upon A. solani inoculation (Figure S13). Taken together, transgenic plants demonstrated critical involvement of WRKY1 during EB defence.

Discussion

Wild relatives of crops represent potential gene pool and thus, are primary source of important genes (Foolad, 2007). Phenotypic
Figure 4  Salicylic acid (SA) content (measured as free, conjugated and total SA \( \mu g/g \) Fw) in mock (M) and Alternaria solani-inoculated (T) leaf tissue of LA2157 (R) and LA1395 (S) Solanum arcanum accessions. The values represent means ± SE of three biological replicates each with three technical replicates. Different letters (a, b, c and d) indicate significant differences according to Duncan’s test \( (P < 0.05) \).

Comparison of LA2157 and LA1395 accessions of Solanum arcanum in EB disease severity indicated that LA2157 has robust resistance against A. solani. QTL analysis of S. lycopersicum cv. ‘Solentos’ (LA2157) population has identified six QTLs governing resistance to EB (Chaerani et al., 2007). Recently, high-throughput metabolomic study revealed positive effect of secondary metabolites from steroidal-glyco alkaloid and phenylpropanoid pathways in protecting wild tomato against EB (Shinde et al., 2017). However, there are no reports available until now describing molecular mechanism(s) of EB resistance in wild relatives or cultivated plants. Current investigation suggested significant modulation in 12 WRKY transcripts at early and late stages of EB disease progression and majority of these were significantly up-regulated in R accession indicating their potential involvement in resistance against A. solani. In Arabidopsis, AtWRKY8 expression was increased with pathogen infection, aphid and maggot feeding while loss of function mutants showed reduction in resistance to Botrytis cinerea (Chen et al., 2010). Similarly, AtWRKY19 displayed recessive resistance to several strains of Ralstonia solanacearum (Deslandes et al., 2003). Also PtWRKY23 transcripts were elevated in Populus after Melampsora medusa infection and elicitor treatments (Levée et al., 2009) and AtWRKY23 provided resistance against nematode infection (Grunenwald et al., 2008). AtWRKY22 was up-regulated against bacterial and fungal pathogens (Dong et al., 2003); however, it induced susceptibility to aphid (Kloth et al., 2016).

Earlier, WRKY1 and WRKY33 have been shown as SA responsive markers for early plant defence while WRKY33 and WRKY40 as late stage-specific response (Bakshi and Oelmuller, 2014). Nonetheless, present findings indicated that all these three WRKys together with WRKY11 were potentially involved in EB resistance. SA treatment has resulted into AtWRKY1 induced expression which is dependent on NPR1 (Duan et al., 2007) while SA-independent expression of WRKY33 is known to be essential in necrotrophic defence (Bakshi and Oelmuller, 2014). Elevated AtWRKY33 expression was reported in response to B. cinerea (Zheng et al., 2006). During BABA-induced resistance, necrotrophic inoculation resulted in higher levels of BjWRKY11 and SiWRKY11 in brassica and tomato, respectively (Chavan and Kamble, 2013; Roylawar et al., 2015). Consistency with above reports, SaWRKY11 and SaWRKY40 levels were increased in R accession. Additionally, key genes of SA and JA biosynthesis (PAL, ICS1, AOS and OPR3) as well as their defence markers (PR1, PR2, PR6 and PR12) were also up-regulated in R accession. ICS1 regulates important step in SA biosynthesis and shown to be regulated by WRKys (VanVerk et al., 2011). Similarly, PAL isoforms play critical role in SA biosynthesis and lignification (Gayoso et al., 2010) and increased lignin accumulation was observed in resistant compared to susceptible plants upon A. solani inoculation (Shinde et al., 2017). Overexpression of NtPAL resulted in the increased resistance to TMV and Cecropia nicotianae (Shadle et al., 2003) while silencing of WRKY53 has resulted in lower PAL expression with increased susceptibility to aphids in wheat (Van Eck et al., 2010). Therefore, SA could be involved at early stage of EB resistance in R based on the significant increase in SaICS1 and SaPAL transcripts. Pathogen-treated R accessions clearly displayed significantly high SA levels compared to S plants. Exogenous SA application significantly delayed the EB disease progression as well as increased WRKY1 expression in S and R accessions at early stage upon A. solani challenge. WRKY1 governs SA signalling pathway through cytokotic form of NPR1 that acts as a critical master regulator of plant defence response and is tightly governed through post-translation modifications (Saleh et al., 2015). In Arabidopsis, mutation in W-box of NPR1 promoter completely disrupted binding of WRKys and abolished NPR1 expression, resulting in susceptibility against Pseudomonas syringae (Yu et al., 2001). Similarly, Arabidopsis npr1 mutants upon P. syringae infection failed to trigger PR genes and were susceptible to a wide range of pathogens (Conrath et al., 2006). Heterologous overexpression of AtNPR1 showed resistance to Xanthomonas oryzae pv. oryzae in rice (Chern et al., 2001). Thus, significant SaNPR1 elevation in R might suggest its role in early resistance against A. solani.

Moreover, SA-defective mutants (pad4 and sid2) revealed more resistance at early stages against A. brassicicola in Arabidopsis.
Figure 5 Graphical representation of native SaMYB2 and SaXTH5 promoters (a) and interaction of rSaWRKY1 protein with native SaMYB2 and SaXTH5 promoters (b) and W-box probes (c) using EMSA. 10xBB–binding buffer, pMYB2–700-bp native proximal promoter region, pXTH5–750-bp native distal promoter region, rSaWRKY1–recombinant SaWRKY1 protein (62.6 Kda), normal 4xW-box-TTGACC, mutated 4xW-box-TTtACC, and 4x-non-W-box-CAATTT as a negative control.

Figure 6 Graphical representation of WRKY1 (1–1200 bp from TSS) promoter showing binding site of different TFs (as indicated by various shapes and colour marking in the box) from different tomato genotypes. R–LA2157 (resistant) and S–LA1395 (susceptible) Solanum arcanum accessions; C–cultivated S. lycopersicum; Sol–sequence reported in Sol genomic network webpage, 1–600 bp is considered as proximal, while remaining part as distal region in WRKY1 promoter.
Taken together, a minimum threshold level of SA along with WRKY1 and other defence genes at initial stage might be critical for EB defence. Comparison between CTD and NTD of SaWRKY1 with secondary structure of AtWRKY1-C revealed similar functional domains with important conserved binding sites. Structural and phylogenetic studies revealed that SaWRKY1 was closely related to its homologs in tomato and potato (Agarwal et al., 2011). Comparative WRKY1 promoter analysis clearly depicted that variation in the TF-binding regions (Wen et al., 2016) could be responsible for the differential WRKY1 expression in R and other susceptible tomato cultivars and thus, might improve defence potential of resistance accession against EB. Furthermore, promoter analysis and EMSA clearly provided critical evidences for SaXTH5 and SaMYB2 as the targets of SaWRKY1. XTH plays important role in cell wall modulation (Hayashi and Kaida, 2011; Nishikubo et al., 2011), and LeXTH was up-regulated during incompatible tomato-Cuscuta interaction indicating its importance in plant defence (Albert et al., 2004). Similarly, MYB2 has been implicated in hormonal crosstalk during plant defence response (VanVerk et al., 2011). Transcript levels of SaXTH5 and SaMYB2 were significantly up-regulated in R accession upon A. solani inoculation and might unveil their contribution in restricting this necrotrophic pathogen. Moreover, functional relationships between WRKY1 and its targets (SIXTH5 and SIMYB2) were confirmed in SaWRKY1 transgenic tomato lines. Both W1OE T1 lines displayed improved EB resistance while five independent W1RNAi T0 lines exhibited higher EB susceptibility. However, T1 W1RNAi lines in this study failed due to poor seed setting and experimental validation of this observation (whether it is due to lethality or male sterility) may require a separate study. Interestingly, overexpression of GbWRKY1 has indicated multiple roles including organ development and fungal resistance in cotton (Li et al., 2014). Heterologous overexpression of VvWRKY1 in tobacco exhibited reduced susceptibility to several fungi (Marchive et al., 2007). Transgenic disease-resistant rice has been developed against Magnaporthe oryzae and X. oryzae pv. oryzae by expressing WRKY45 under PR promoter (Goto et al., 2011).

Figure 7 Early blight disease symptoms on leaflets of SaWRKY1 transgenic lines; a representative leaflet was photographed from each treatment at 3 and 5 dpi (a). Early blight disease scoring in terms of PDI (%) on SaWRKY1 transgenic tomato lines at 1, 3 and 5 dpi (b). (SaWRKY1 OE: W1OE-1, W1OE-8; SaWRKY1 RNAi: W1RNAi-15, W1RNAi-21, W1RNAi-28, W1RNAi-29, W1RNAi-30, WT: wild-type nontransformed). The values represent means ± SE. Bars represent the standard errors of the means. In statistical analysis, two-way ANOVA was performed followed by Bonferroni post-tests. Statistical data are significant at P-value ***p < 0.001.
Similarly, SlDRW1 has been found to regulate defence response against B. cinerea in tomato (Liu et al., 2014). Overall, significant variation in TF-binding motifs in WRKY1 promoter regions and differential XTH5 and MYB2 expression could be crucial during EB defence. Transgenic tomato lines indicated that WRKY1 might have a vital role in resistance against A. solani. Current findings offer critical evidences for the role of WRKY1 in modulation of EB resistance in tomato and could be potentially utilized to improve plant defence against A. solani in other crops.

**Experimental procedures**

Co-expression network analysis of WRKYs and gene ontology prediction of CEG

Co-expression analysis was performed on available transcriptomic data (Itkin et al., 2011, 2013) using R script, and this generated a separate list of CEG for each group with \( r \leq 0.8 \) and sorted in descending order. Co-expression networks were visualized with the Cytoscape program version 3.1.0 (Shannon et al., 2003). GO annotation of CEG was performed according to Bankar et al. (2013) with plant-specific database and expression value \( \text{Se}^{-20} \) (McCarthy et al., 2006).

**S’ cis-regulatory element analysis**

For each gene with defence-related GO annotation (for categories like response to stimulus, immune system process and biological regulation), \(~1.5\) Kb of S’ regulatory region from the translational start site was retrieved from Sol genomic network (https://solgenomics.net) associated search tools (Fernandez-Pozo et al., 2015). Presence of putative S’ cis-regulatory elements was detected in these promoters using PlantPAN database 2.0 (Chow et al., 2015).

**Plant material, fungal culture, EB disease assessment and SA application**

LA2157 (R) and LA1395 (S) accessions of *S. arcanum* were procured from Tomato Genomic Research Centre, University of California, Davis, USA, while seeds of cultivated tomato (*S. lycopersicum* cv. Naina) were acquired from local market. In case of response against *A. solani* (2013) with plant-specific database and expression value prediction of CEG

Badal et al. (2016). Expression pRI101-ANvector (Takara Bio Inc., Kusatsu, Shiga, Japan). pRI101-AN:GFP clones were transformed into *Agrobacterium tumefaciens* GV3101 using a standard transformation protocol. Empty pRI101-AN vector was used as a negative control. *A. tumefaciens* cultures with respective clones were grown at 28 °C in Luria-Bertani medium containing selection markers (25 mg/ml rifampicin and 50 mg/L kanamycin) at 120 rpm for 24 h. Cells were harvested by centrifugation at 1370 \( g \) for 5 min and suspended in infiltration buffer (half strength MS medium, pH 5.6 and 200 \( \mu \)M acetosyringone). These were pelleted and re-suspended in infiltration buffer by adjusting an OD 1.0 at 600 nm. Cultures were incubated at 24 °C for 3–4 h and were infiltrated into leaves of 2-week-old *Nicotiana benthamiana* plants that were maintained at 18 to 24 °C in a growth chamber. Leaf sections were visualized for subcellular localization of GFP at 6 dpi after agro-infiltration using confocal laser scanning microscope (Zeiss LSM 710, Oberkochen, Germany).

**rSaWRKY1 expression, purification and EMSA**

CDs of SaWRKY1 and GFP was amplified (Table S7). Both SaWRKY1 and GFP were cloned in binary plant expression pRI101-ANvector (Takara Bio Inc., Kusatsu, Shiga, Japan). pRI101-AN:GFP and pRI101-AN:GFP clones were transformed into *Agrobacterium tumefaciens* strain GV3101 using a standard transformation protocol. Empty pRI101-AN vector was used as a negative control. *A. tumefaciens* cultures with respective clones were grown at 28 °C in Luria-Bertani medium containing selection markers (25 mg/ml rifampicin and 50 mg/L kanamycin) at 120 rpm for 24 h. Cells were harvested by centrifugation at 1370 \( g \) for 5 min and suspended in infiltration buffer (half strength MS medium, pH 5.6 and 200 \( \mu \)M acetosyringone). These were pelleted and re-suspended in infiltration buffer by adjusting an OD 1.0 at 600 nm. Cultures were incubated at 24 °C for 3–4 h and were infiltrated into leaves of 2-week-old *Nicotiana benthamiana* plants that were maintained at 18 to 24 °C in a growth chamber. Leaf sections were visualized for subcellular localization of GFP at 6 dpi after agro-infiltration using confocal laser scanning microscope (Zeiss LSM 710, Oberkochen, Germany).

**Cloning and promoter analysis of WRKY1**

Promoter regions of WRKY1 (1.2 Kb, upstream of TSS) from resistant (LA2157), susceptible (LA1395) *S. arcanum* accessions and cultivated *S. lycopersicum* were cloned in pcCloneJet vector (Fermentas) and were sequenced (Eurofin). The presence of putative S’ cis-regulatory elements was detected in these promoters using PlantPAN database 2.0 (Chow et al., 2015).

**Generation of WRKY1 overexpression and RNAi silencing transgenic plants**

For construction of *SiWRKY1* overexpression (*W1OE*) and silencing (*W1RNAi*) vectors, full-length CDS of *SiWRKY1* cloned in pDONR221 vector was transferred in pk2GWL7 binary vector and its 265-bp fragment cloned in pENTR/D-TOPO was transferred in
Bakshi, M. and Oelmuller, R. (2014) WRKY transcription factors: Jack of many trades in plants. Plant Signal. Behav. 9, 1–18.

Bankar, V. T., Pandesi, V. C., Kale, S. M., Qiu, S., Rollins, M., Datla, R., Gupta, V. S. et al. (2013) Genome-wide identification and characterization of microRNA genes and their targets in flax (Linum usitatissimum): characterization of flax miRNA genes. Planta, 237, 1149–1161.

Cárdenas, P.D., Sonawane, P.D., Pollier, J., Vanden Bossche, R., Devangan, V., Wetthorn, E., Tal, L. et al. (2016) GAME9 regulates the biosynthesis of steroidal alkaloids and upstream isoprenoids in the plant mevalonate pathway. Nat. Commun. 7, 1–16.

Chaerani, R., Groenwold, R., Stam, P. and Voorrips, R. E. (2007) Assessment of early blight (Alternaria solani) resistance in tomato using a droplet inoculation method. J. Gen. Plant Pathol. 73, 96–103.

Chawan, V. and Kamble, A. (2013) β-Aminobutyric acid primed expression of WRKY and defence genes in Brassica campestris against alternaria blight. J. Phytopathol. 161, 859–865.

Chen, L., Zhang, L. and Yu, D. (2010) Wounding-induced WRKY8 is involved in basal defense in Arabidopsis. Mol. Plant Microbe Interact. 23, 558–565.

Chern, M. S., Fitzgerald, H. A., Yadav, R. C., Canlas, P. E., Dong, X. and Ronald, P. C. (2001) Evidence for a disease-resistance pathway in rice similar to the NPR1-mediated signaling pathway in Arabidopsis. Plant J. 27, 101–113.

Chow, C.-N., Zheng, H.-Q., Wu, N.-Y., Chien, C.-H., Huang, H.-D., Lee, T.-Y., Chiang-Hsieh, Y.-F. et al. (2015) PlantPAN 2.0: an update of plant promoter analysis navigator for reconstructing transcriptional regulatory networks in plants. Nucleic Acids Res. 44, 1–7.

Conrath, U., Beckers, G. J. M., Flors, V., García-Agustín, P., Jakab, G., Mauch, F., Newman, M. et al. (2006) Priming: getting ready for battle. Mol. Plant Microbe Interact. 19, 1062–1071.

Deslandes, L., Olivier, J., Peeters, N., Feng, D. X., Khounlootham, M., Boucher, C., Somssich, I. et al. (2003) Physical interaction between RRS1-R, a protein conferring resistance to bacterial wilt, and Pop2P, a type III effector targeted to the plant nucleus. Proc. Natl Acad. Sci. USA 100, 8024–8029.

Dong, J., Chen, C. and Chen, Z. (2003) Expression profiles of Arabidopsis WRKY superfamily during plant defense response. Plant Mol. Biol. 51, 1–21.

Duan, M., Nan, J., Liang, Y., Mao, P., Lu, L., Li, L., Wei, C. et al. (2007) DNA binding mechanism revealed by high resolution crystal structure of Arabidopsis thaliana WRKY1 protein. Nucleic Acids Res. 35, 1145–1154.

Durrant, W. E. and Dong, X. (2004) Systemic acquired resistance. Annu. Rev. Phytopathol. 42, 185–209.

Eggert, K., Hoffmann, J., Hiller, B., Kruse, H. P., Rawel, H. M. and Pawelzik, E. (2010) Effects of fusarium infection on the phenolics in emmer and naked barley. J. Agric. Food Chem. 58, 3043–3049.

Eulgem, T. (2003) Regulation of the Arabidopsis defense transcriptome. Trends Plant Sci. 10, 71–78.

Eulgem, T. and Somssich, I. (2007) Networks of WRKY transcription factors in defense signaling. Curr. Opin. Plant Biol. 10, 366–371.

Eulgem, T., Rushton, P., Schmelzer, E., Hahlbrock, K. and Somssich, I. (1999) Early nuclear events in plant defence signalling: rapid gene activation by WRKY transcription factors. EMBO J. 18, 4689–4699.

Fernandez-Pozo, N., Menda, N., Edwards, J. D., Saha, S., Tecle, I. Y., Strickler, S. R., Bomarely, A. et al. (2015) The Sol Genomics Network (SGN)-from genotype to phenotype to breeding. Nucleic Acids Res. 43, D1036–D1041.

Foolad, M. R. (2007) Genome mapping and molecular breeding of tomato. J. Plant Genomics 10, 1–19.

Goto, S., Sasakura-Shimoda, F., Yamazaki, M., Hayashi, N., Suetsugu, M., Ochiai, H. and Takatsuji, H. (2016) Development of disease-resistant rice by pathogen-responsive expression of WRKY45. Plant Biotechnol. J. 14, 1127–1138.

Grundler, F., Inzé, D. et al. (2008) A role for AtWRKY23 in feeding site characterization of flax miRNA genes. Planta 228, 148–159.

Hammond-Kosack, K. E. and Jones, J. D. G. (1996) Resistance gene-dependent early blight resistance. Plant Dis. 80, 972–976.

Hayashi, N., Suetsugu, M., Ochiai, H. and Takatsuji, H. (2016) GAME9 regulates the biosynthesis of steroidal alkaloids and upstream isoprenoids in the plant mevalonate pathway. Nat. Commun. 7, 1–16.

Hebel, M., Sill, S., Worm, E., van der Maarel, S., Voorrips, R. E. and Somssich, I. (2003) Evidence for a disease-resistance pathway in rice similar to the NPR1-mediated signaling pathway in Arabidopsis. Plant J. 27, 101–113.

Igus, D., Bokhari, A., Ahmad, S. and Oelmuller, R. (2014) The WRKY superfamily during plant defense response.pdf.

Inzé, D. et al. (2008) A role for AtWRKY23 in feeding site characterization of flax miRNA genes. Planta 228, 148–159.

Jiao, M., Eulgem, T., Somssich, I., Fricke, M., Brieg, C., Gaffney, D., Newcomb, S. et al. (2008) Acoustic amplification of disease-resistance signals in Arabidopsis. EMBO J. 27, 199–209.

Kamal, A. and Barker, J. T. (2013) WRKY and defence genes in Brassica campestris against alternaria blight. J. Phytopathol. 161, 859–865.

Kapteyn, F. and Van der Velden, L. (2000) Genome-wide identification and characterization of microRNA genes and their targets in flax (Linum usitatissimum): characterization of flax miRNA genes. Planta, 237, 1149–1161.

Kasim, K., Carden, N., Pang, M., Rowlings, D., Haggar, K., Shah, S. and Inzé, D. (2008) A role for AtWRKY23 in feeding site establishment of plant-parasitic nematodes. Plant Physiol. 148, 358–368.

Kolekova, V. and Jones, J. D. G. (1996) Resistance gene-dependent plant defense responses. Plant Cell, 8, 1773–1791.

© 2018 The Authors. Plant Biotechnology Journal published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd., 16, 1502–1513
Hayashi, T. and Kaida, R. (2011) Functions of xyloglucan in plant cells. Mol. Plant 4, 17–24.

Itkin, M., Rogachev, I., Alkan, N., Rosenberg, T., Malitsky, S., Masini, L., Meir, S. et al. (2013) Glycolaldehyde metabolism is required for steroidal alkaloid glycosylation and prevention of phytotoxicity in tomato. Plant Cell 25, 4507–4525.

Itkin, M., Heing, U., Tzfaadia, O., Bhide, A.J., Shinde, B., Cardenas, P.D., Bocobza, S.E. et al. (2013) Biosynthesis of antitranslational alkaloids in solanaceous crops is mediated by clustered genes. Science 341, 175–179.

Jones, J. and Dangl, J. (2006) The plant immune system. Nature 444, 323–329.

Kim, K., Fan, B. and Chen, Z. (2006) Pathogen-induced Arabidopsis WRKY1 is a transcriptional repressor and enhances plant susceptibility to Pseudomonas syringae. Plant Physiol. 142, 1180–1192.

Kloth, K.J., Wiegers, G.L., Lusscher-Jang, J., van Haarst, J.C., Kruijer, W., Saleh, A., Withers, J., Mohan, R., Marques, J., Witney, S. et al. (2017) Dynamic metabolic reprogramming of steroidal glycol-alkaloid and phenylpropanoid biosynthesis may impact early blight resistance in wild tomato (Solanum arcanum Peralta). Plant Mol. Biol. 95, 411–423.

Singh, K.B., Foley, R.C. and Oi-Sate-Sánchez, L. (2002) Transcription factors in plant defense and stress responses. Curr. Opin. Plant Biol. 5, 430–436.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.

Tsuda, K. and Sossmich, I.E. (2015) Transcriptional networks in plant immunity. New Phytol. 206, 932–947.

Ulker, B. and Somssich, I. (2004) WRKY transcription factors: from DNA binding towards biological function. Curr. Opin. Plant Biol. 7, 491–498.

Van Eck, L., Schultz, T., Leach, E.J., Srofol, S.R., Pearls, F.B., Botha, A.M. and Lapitan, N.L.V. (2010) Virus-induced gene silencing of WRKY53 and an inducible phenylalanine ammonia-lyase in wheat reduces aphid resistance. Plant Biotechnol. J. 8, 1023–1032.

VanVerk, M.C., Bol, J.F. and Linthorst, H.J.M. (2011) Prospecting for Genes involved in transcriptional regulation of plant defenses, a bioinformatics approach. BMC Plant Biol. 11, 1–12.

VanWees, S.C.M., Chang, H.-S., Zhu, T. and Glazebrook, J. (2003) Characterization of the early response of Arabidopsis to Alternaria brassicola infection using expression profiling. Plant Physiol 132, 606–617.

Wen, C.L., Cheng, Q., Zhao, L., Mao, A., Yang, J., Yu, S., Weng, Y. et al. (2016) Identification and characterisation of Dof transcription factors in the cucumber genome. Sci. Rep. 6, 1–11.

Yu, D., Chen, C. and Chen, Z. (2001) Evidence for an important role of WRKY DNA binding proteins in the regulation of NPR1 gene expression. Plant Cell 13, 1527–1539.

Zheng, Z., Qamar, S.A., Chen, Z. and Mengiste, T. (2006) Arabidopsis WRKY33 transcription factor is required for resistance to necrotrophic fungal pathogens. Plant J. 48, 592–605.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 Gene ontology (GO) analysis of all CEcs.

Figure S2 Expression profiles of selected defence genes.

Figure S3 Expression profiles of key SA and JA biosynthetic genes.

Figure S4 Early Blight disease scoring of R and S plants after exogenous SA application.

Figure S5 Expression analysis of SIWRKY1, SIXTHS and SIMYB2 after exogenous SA application.

Figure S6 Expression profiles of WRKY1 in Solanum lycoctonum (Sl), susceptible and resistant Solanum arcanum accessions.

Figure S7 (a) Protein sequence alignment of SaWRKY1 and SIWRKY1; (b) structure based sequence alignment of SaWRKY1 C and N terminal domains with tomato and Arabidopsis WRKY1; and (c) phylogenetic analysis of WRKY1 using amino acid sequences from 21 plant homologs.

Figure S8 Subcellular localization of GFP in absence and presence of SaWRKY1.

Figure S9 SDS-PAGE of recombinant SaWRKY1 protein.

Figure S10 Expression analysis of SIWRKY1, SIXTHS and SIMYB2 in T2 transgenic tomato lines.
Figure S11 Expression analysis of SlWRKY1, SlXTH5 and SlMYB2 using qRT-PCR in transgenic tomato W1OE and W1RNAi lines.

Figure S12 Expression analysis of key genes involved in SA mediated defence response (SlPAL, SlICS1 and SlPR1) using qRT-PCR in transgenic tomato W1OE and W1RNAi lines.

Figure S13 Expression analysis of key genes involved in JA mediated defence response (SlAOS, SlOPR3, SlJAZ and SlPR12) using qRT-PCR in transgenic tomato W1OE and W1RNAi lines.

Table S1 List of co-expressed genes with five WRKYs (WRKY1, 3, 8, 23, and 39).

Table S2 List of co-expressed genes with three WRKYs (WRKY11, 33, and 40).

Table S3 List of co-expressed genes with two WRKYs (WRKY4, and 54).

Table S4 Defence gene with GO terms—Biological regulation, Immune system process and response to stimulus.

Table S5 Potential 5’ cis-acting elements from identified defence-related genes and their predicted functions.

Table S6 Frequency of 5’ cis elements in all defence-related WRKY co-expressed genes.

Table S7 List of primers used in present study.