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The Arabidopsis transcription factor WRKY27 influences wilt disease symptom development caused by *Ralstonia solanacearum*

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Summary

WRKY transcription factors play a key role in modulating the plant defense transcriptome. Here we show that the Arabidopsis mutant *wrky27-1*, which lacks a functional WRKY27 transcription factor, showed delayed symptom development in response to the bacterial wilt pathogen *Ralstonia solanacearum*. Additionally, *wrky27-1* plants did not express PR marker genes upon infection, as also observed in resistant Nd-1 plants. Spatial expression of *WRKY27* correlated well with the route of bacterial infection and propagation *in planta*. Complementation experiments restored both the early wilting phenotype of wild-type Col-1 plants and activation of PR genes, not only when the WRKY27 cDNA is expressed under the control of the native promoter, but also when the *SUC2* promoter was used, suggesting that WRKY27 exerts its function in phloem companion cells. Expression studies identified genes involved in nitrogen metabolism and nitric oxide (NO) generation as potential targets of negative regulation by WRKY27. Our results show that WRKY27 negatively influences symptom development of a vascular pathogen, possibly by affecting signaling or trafficking between the phloem and the xylem.

Keywords: bacterial wilt disease, negative regulator, phloem companion cells, plant tolerance, SUC2 promoter, xylem.

Introduction

Plants have evolved specific recognition mechanisms that trigger rapid defenses at pathogen infection sites (Jones and Dangl, 2006). Basal defenses are initiated upon perception of pathogen-associated molecular patterns (PAMPs) of a micro-organism at the cell surface, and usually halt the infection process before the microbe gains access to the plant. Pathogens that can circumvent or suppress these primary defenses are often restricted by a highly specialized mechanism termed *R* gene-mediated resistance. This plant reaction triggers a more robust and prolonged defense response, resulting in rapid local cell death (the hypersensitive response, HR; Jones and Dangl, 2006). Stimulation of defense responses is not locally restricted but also occurs in distal areas of the plant as a consequence of elevated levels of the signaling hormone salicylic acid (SA) and increased expression of pathogenesis-related (*PR*) genes, a reaction termed systemic acquired resistance (SAR; Durrant and Dong, 2004). The SA-dependent pathway is part of a complex signaling network that can be influenced by other endogenous signal molecules such as jasmonic acid (JA), ethylene (ET), reactive oxygen intermediates (ROI) and nitric oxide (NO) through positive and negative regulatory interactions (Glazebrook, 2005). Activation of defense responses towards pathogen infection is associated with fine-tuned transcriptional regulation, globally altering gene expression patterns (Eulgem and Somssich, 2007).

WRKY transcription factors are encoded by large gene families in all higher plants, with 74 members in Arabidopsis (Eulgem and Somssich, 2007). Although present in early eukaryotes, their massive expansion in plants during

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evolution suggests that WRKY proteins play pivotal roles in plant-specific processes (Ülker and Somssich, 2004). WRKY factors regulate seed size, trichome development and leaf senescence (Johnson *et al.*, 2002; Luo *et al.*, 2005; Miao and Zentgraf, 2007; Ülker *et al.*, 2007). The expression of over 70% of all *WRKY* genes is influenced by diverse pathogens or by pathogen-mimicking stimuli, and recent genetic analyses have unambiguously demonstrated that WRKY factors are required for pathogen resistance (Eulgem and Somssich, 2007).

In Arabidopsis, *wrky7* mutants display heightened resistance towards a virulent *Pseudomonas syringae* strain, whereas *WRKY11* and *WRKY17* act synergistically in plant responses to both avirulent and virulent strains of this pathogen (Journot-Catalino *et al.*, 2006; Kim *et al.*, 2006). The *wrky33* mutant exhibits increased susceptibility to two necrotrophic fungi (*Botrytis cinerea* and *Alternaria brassicicola*), and reduced expression of jasmonate-regulated genes (Lippok *et al.*, 2007; Zheng *et al.*, 2006), whereas *wrky53* mutants allowed enhanced growth of the virulent bacterium *P. syringae* DC3000 (Murray *et al.*, 2007). Moreover, loss of WRKY70 function resulted in enhanced susceptibility to biotrophic (*Erysiphe cichoracearum*) and nectrotrophic (*A. brassicicola* and *B. cinerea*) fungal pathogens (AbuQamar *et al.*, 2006; Li *et al.*, 2006), and to the biotrophic oomycete *Hyaloperonospora parasitica* (Knoth *et al.*, 2007). Free SA over-accumulates in *wrky70* mutants and further increases in plants carrying mutations in the gene of its closest structural homolog WRKY54, suggesting a partial overlap of these two genes in repressing SA biosynthesis and SA signal transduction (Wang *et al.*, 2006). WRKY70 also shares partial overlapping functions with WRKY53, as *wrky53 wrky70* double mutants showed enhanced disease susceptibility to *P. syringae* pv. *maculicola* compared to single mutants (Wang *et al.*, 2006). Likewise, three closely related members, WRKY18, WRKY40 and WRKY60, have partially redundant functions as negative regulators of *P. syringae*-triggered basal defense (Xu *et al.*, 2006). Interestingly, WRKY18 acts as a positive regulator required for SAR establishment (Wang *et al.*, 2006). *wrky18 wrky40* double mutants were highly resistant towards the virulent powdery mildew *Golovinomyces orontii*, and their barley orthologs, HvWRKY1 and HvWRKY2, have been shown to act as repressors of PAMP-triggered basal defense (Shen *et al.*, 2007). Intriguingly, these data demonstrated a link between PAMP- and *R* gene-mediated resistance mechanisms by demonstrating that de-repression of PAMP-triggered basal defense in barley is achieved by nuclear interaction of an R protein (MLA) with *Hv*WRKY1 and *Hv*WRKY2 upon recognition of the fungal effector A10.

WRKY transcription factors exert their regulatory functions by binding to the W box (5′-C/TTGACC/T-3′) that is often present within short promoter stretches of many defense genes, including several *WRKY* genes (Eulgem and Somssich, 2007). In parsley, chromatin immunoprecipitation revealed the *in vivo* presence of WRKY proteins at W box promoter sites of the PAMP-induced genes *PcWRKY1* and *PcPR1-1* (Turck *et al.*, 2004). In Arabidopsis, WRKY22 and WRKY29 act positively on their own gene promoters and also regulate *FRK1*/*SIRK* expression downstream of a mitogen-activated protein kinase cascade that is triggered upon interaction of the bacterial PAMP flg22 with the membrane-associated FLS2 receptor (Asai *et al.*, 2002; Navarro *et al.*, 2004; Robatzek and Somssich, 2002). Two additional WRKY genes, *WRKY33* and *WRKY53*, were activated downstream of a mitogen-activated protein kinase signaling cascade in response to chitin, a fungal PAMP (Wan *et al.*, 2004). PAMP-dependent and W box-mediated activation was recently demonstrated for *WRKY33* (Lippok *et al.*, 2007).

Ralstonia solanacearum is a devastating soil-borne bacterium causing wilting disease in over 200 economically important plant species. *R. solanacearum* invades the intercellular spaces of the root cortex through natural wounding sites of lateral roots. From there, it crosses the endoderm barrier and enters into the vascular system, proliferating mainly in xylem vessels and traveling to the upper parts of the plant. Wilting symptoms are most likely due to reduced sap flow as a consequence of extensive bacterial exopolysaccharide slime production within xylem vessels. As with other phytopathogens, the type III secretion system is required for pathogenicity, as mutants defective in genes encoding this machinery (*hrp* genes) are unable to cause disease (Valls *et al.*, 2006). In Arabidopsis, a major *R* gene, *RRS1-R* (*WRKY52*) confers resistance towards several strains of *R. solanacearum* via its interaction with the bacterial type III effector PopP2 (Deslandes *et al.*, 2003). *RRS1-R*-mediated resistance requires SA and nonrace-specific disease resistance (NDR1) signaling, and also abscisic acid (ABA) (Deslandes *et al.*, 2002; Hernandez-Blanco *et al.*, 2007). Mutant analysis has revealed the need for another Arabidopsis gene, *NWS1*, for full virulence of the bacteria (Feng *et al.*, 2004).

Here we demonstrate that Arabidopsis plants lacking a functional *WRKY27* gene exhibit delayed wilting symptoms in response to *R. solanacearum*. Complementation analyses using tissue-specific promoters revealed that *WRKY27* is required within the phloem for normal wilt disease development, suggesting that signaling between the phloem and xylem influences the infection process. Microarray analyses suggest that *WRKY27* regulates the expression of genes involved in NO biosynthesis and nitrogen metabolism. Consistent with this, the *wrky27* mutant is hypersensitive to treatment with the NO donor sodium nitroprusside (SNP). Thus, WRKY27 appears to be involved in regulating a component that is required for proper temporal development of disease.

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Results

Loss of WRKY27 function enhances tolerance to R. solanacearum *GMI1000*

In a pilot experiment aimed at identifying *WRKY* loss-of-function mutants showing altered phenotypic responses to *R. solanacearum* strain GMI1000, a set of individual homozygote DNA insertion *wrky* mutant lines (*wrky2*, *6*, *7*, *9*, *11*, *17*, *18*, *19*, *24*, *27*, *29*, *32*, *33*, *36* and *46*) were tested. *R. solanacearum* strain GMI1000 is highly virulent on various *A. thaliana* Columbia ecotypes (Col-0, -1, -5), causing severe wilting upon root inoculation, whereas the ecotype Niederzenz-1 (Nd-1) is resistant due to the presence of the major *R* gene, *RRS1-R* (Deslandes *et al.*, 2002). Of all tested mutants, only one, originally designated *wrky27ETL* but renamed *wrky27-1*, showed a clear difference compared to control Col-1 plants (Figure 1a). Wilting in the aerial parts of wild-type Columbia and all other *wrky* insertional mutant plants was already clearly visible at 8 days after root inoculation, and progressed over time until complete wilting of the leaves was observed at around 10 days after root inoculation. In contrast, Nd-1 plants showed no detectable symptoms over the entire experimental period (12 days). *wrky27-1* showed very limited symptom development at 6 days after root inoculation, and already clearly differed from the control plants at this stage. This delayed symptom development persisted to the end of the experiment, with *wrky27-1* plants eventually wilting more than 2 days after all susceptible plants had died. To substantiate these observations, we performed extensive infection experiments with a larger set of plants and three independent repetitions. Figure 1(b) confirms that there is a significant temporal difference in symptom progression between *wrky27-1* and control Col-1 plants.

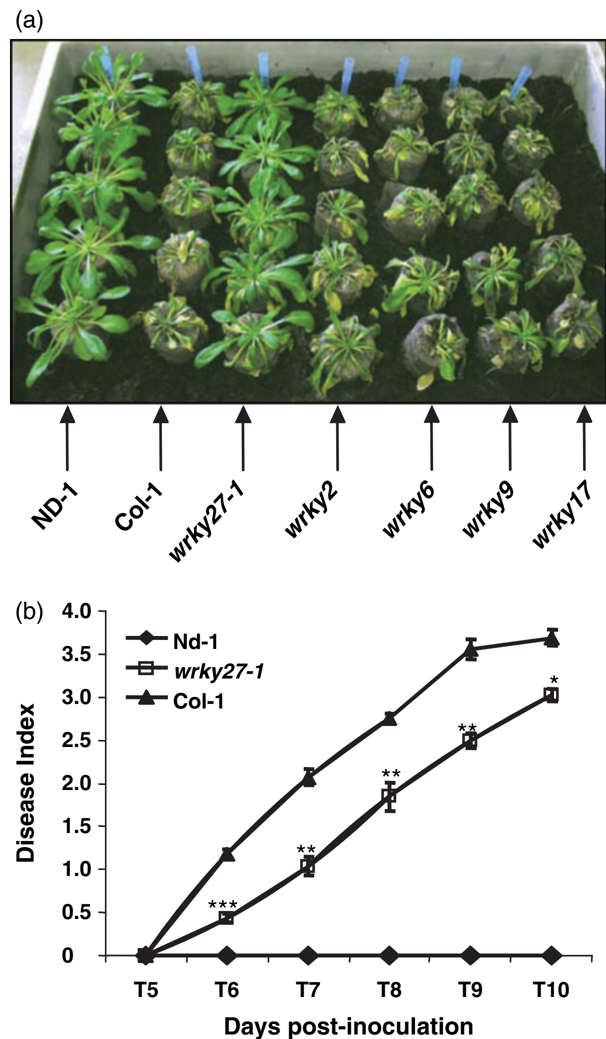

Figure 1. The *wrky27-1* mutant exhibits delayed symptom development in response to *R. solanacearum* strain GMI1000.
(a) Phenotypic wilt assay. Four-week-old Arabidopsis *wrky* mutant plants were root-inoculated with *R. solanacearum* strain GMI1000, and rows of representative plants at 8 dpi are shown. *wrky27-1* lines displayed limited wilt symptoms restricted to the oldest rosette leaves compared to ecotype Col-1 (susceptible) and the other *wrky* mutants that already showed extended wilting at this stage. The resistant ecotype Nd-1 did not develop wilt symptoms. These experiments were repeated at least three times with consistent results.
(b) The disease index score was recorded from 5 dpi (first signs of disease appearance on Col-1 leaves) to 10 dpi (complete wilting stage Col-1 plants). The disease index score (±SD) was determined according to the percentage of wilted leaves on 64 inoculated plants per genotype and repetition, with values defined as: 0 (no symptoms), 1 (>0–25% wilted leaves), 2 (25–50%), 3 (50–75%) and 4 (75–100%). The disease index score of *wrky27-1* (open squares) was lower than that of Col-1 (triangles) throughout the entire experimental period. Ecotype Nd-1 (diamonds) remained fully resistant (disease index score = 0). These experiments were repeated three times with consistent results. Values significantly different from those of wild-type Col-1 plants are indicated by asterisks: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (Student's *t*-test).

Endogenous expression of WRKY27

The level of expression of *WRKY27* (At5g52830) *in planta* is very low, and, based on publicly available microarray data, does not appear to differ significantly between tissues or be affected by tested biotic or abiotic stimuli. Using semi-quantitative RT_PCR, we detected invariable amounts of *WRKY27* transcripts in roots, shoots, leaves and flowers (data not shown), but more precise *in situ* characterization was not feasible due to very low transcript levels.

wrky27-1 is a T-DNA insertion mutant that was originally generated using a novel gene-trap approach (Babiychuk *et al.*, 1997; kindly provided by Dr S. Kushnir, University of Ghent, Belgium). The T-DNA vector is integrated into intron 2 of *WRKY27*, resulting in *in vivo* generation of a translational fusion between the coding regions of exon 1 and exon 2 of *WRKY27* and the *uidA* gene contained in the T-DNA vector (Figure 2a). The chimeric protein still contains the NLS motif of WRKY27 and functional β-glucuronidase, thereby allowing detection *in planta*, but lacks the DNA-binding domain (WRKY domain) that is essential for W box

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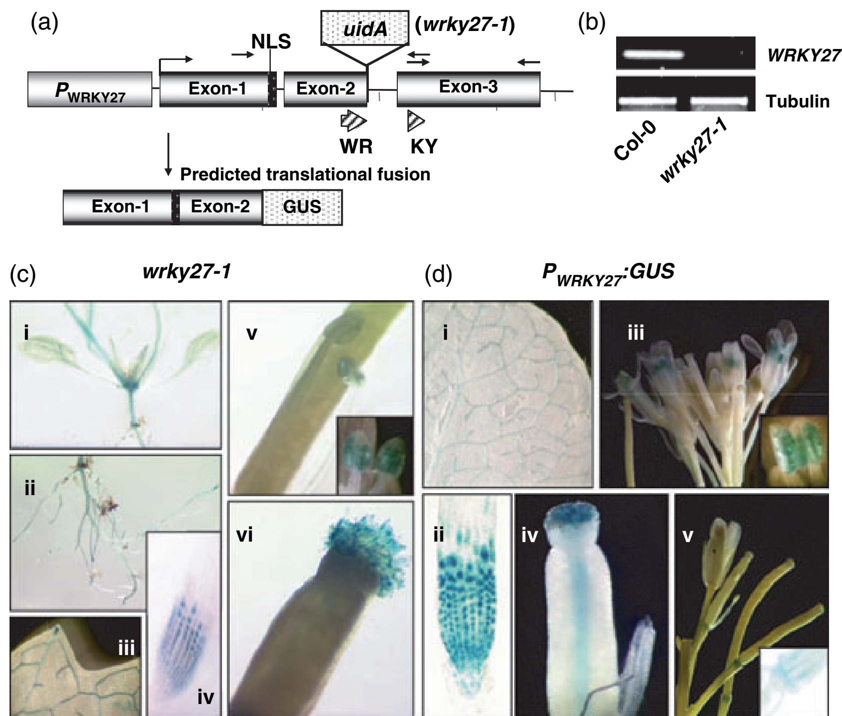

Figure 2. Characterization of the *wrky27-1* mutant.
(a) Schematic representation of the T-DNA insertion site in the *wrky27-1* mutant. The upstream region and genomic organization of *WRKY27* showing the promoter, exon 1, exon 2, exon 3 (gray boxes) and putative nuclear localization signal (NLS) motif (black dotted box) are indicated. The bent arrow illustrates the predicted translation initiation site of WRKY27. Arrows indicate the primer sites used for RT-PCR. The position of the *uidA* gene within *WRKY27* is shown. The region encoding the WRKY domain is marked by dashed arrows below exons 2 and 3. Disruption of the *WRKY27* gene leads to the generation of a translational fusion encoded by exon 1, exon 2 and the GUS reporter gene.
(b) *wrky27-1* is a loss-of-function mutant. The gel indicates the presence or absence of a *WRKY27*-specific RT-PCR amplification fragment encompassing exon 3 from RNA obtained from Col-0 and homozygous *wrky27-1* plants, respectively. The tubulin β4 subunit was used as a loading control.
(c,d) Comparative GUS expression analysis between *wrky27-1* and a representative P_{WRKY27}:GUS transgenic plant. Comparable GUS activity was detected in 7- and 14-day-old plants of both the *wrky27-1* and P_{WRKY27}:GUS lines. GUS activity was observed specifically in the vasculature of the stem (c-i) and leaves (c-i, c-iii, d-i) and in the elongation zones of primary and secondary roots (c-ii, c-iv, d-ii). In floral tissue (5-week-old plants), GUS activity was found in anthers (c-v and inset in c-v, and inset in d-iii), stigmatic papillae (c-vi, d-iii, d-iv), the transmission tract of the septum (d-iv) and flower abscission zones (d-v and inset in d-v).

binding activity. We did not detect *WRKY27* transcripts originating from exon 3 in this line (Figure 2b). Expression is under the control of the native *WRKY27* promoter, enabling us to monitor more precisely its expression pattern within specific tissues and during plant development (Figure 2c).

The WRKY27–GUS fusion protein was detected mainly in the vasculature of root, stem and leaf tissue, but its expression was particularly pronounced in the root elongation zone (Figure 2c-iv). Here, nuclear localization of the chimeric protein was clearly visible. Cross- and longitudinal sections of *wrky27-1* stems suggested that the protein is associated with phloem bundles (Figure S1). During early floral development (bud stage), GUS activity staining was transiently observed in stigmatic papillae, anthers, pollen grains and floral abscission zones. At later floral stages, GUS staining was no longer visible in mature flowers and siliques but persisted in the abscission zones. Various treatments, including wounding, 2 h cold or heat stress, UV irradiation (50 mJ cm^{-2}) or application of salicylic acid (2 mM), methyl jasmonate (100 μM), 1-aminocyclopropane-1-carboxylic acid (100 μM) or flg22 (1 μM), did not result in increased GUS activity staining in *wrky27-1* plants (data not shown).

Subcellular localization of the WRKY27 protein was demonstrated by co-expressing double 35S CaMV promoter-driven WRKY27–GFP and DsRed (red fluorescent protein) constructs in leek (*Allium porrum* L.) epidermal cells via particle bombardment. Confocal laser scanning microscopy revealed that WRKY27–GFP was confined exclusively to the nucleus, whereas DsRed was detected throughout the entire cell 24 h after transfection (Figure S2).

Complementation of wrky27-1 *with* WRKY27 *using tissue-specific promoters*

To conclusively demonstrate that loss of WRKY27 function is the cause of the delay in disease symptom development, we performed genetic complementation experiments. As the 5′ regulatory region of *WRKY27* spans over 6.7 kb, we tested

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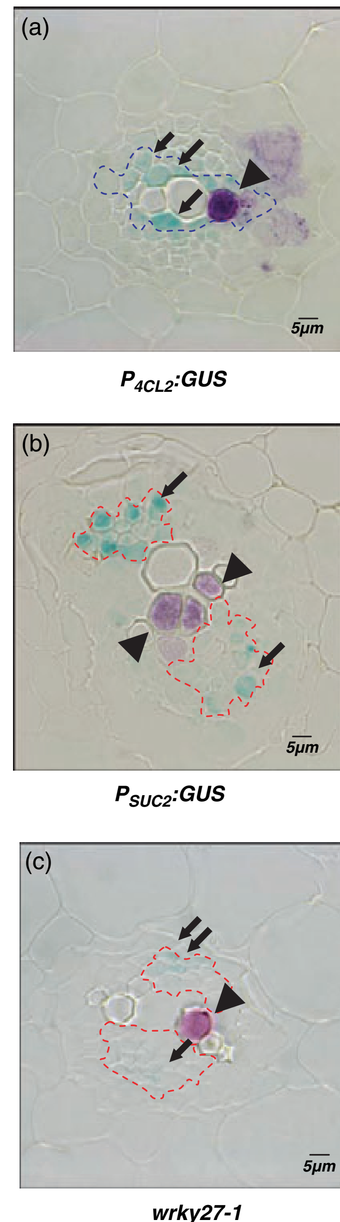
whether a shorter promoter sequence would suffice. We generated Columbia transgenic plants expressing a GUS reporter construct driven by a 2-kb native *WRKY27* promoter fragment (P_{WRKY27}:GUS). These plants were systematically analyzed for GUS activity staining during development and compared to *wrky27-1*. GUS staining in vegetative tissue was restricted to the leaf vasculature and the root elongation zone in both P_{WRKY27}:GUS plants and *wrky27-1* (Figure 2d). In floral tissue, the staining in P_{WRKY27}:GUS plants was also nearly identical to that found in *wrky27-1* plants. This comparison not only revealed a highly similar tissue-specific expression pattern between P_{WRKY27}:GUS and *wrky27-1*, but also similar GUS staining patterns during plant development (analyzed at days 7, 14, 21, 28 and 35 days after germination). Thus, we concluded that the 2 kb promoter should suffice for complementation, and therefore generated constructs expressing a full-length WRKY27 cDNA under its control. As *WRKY27* expression appeared to be restricted to the phloem, we generated additional transgenic *wrky27-1* lines expressing WRKY27 under the control of the xylem- and phloem-specific promoters *4CL2* (4-coumarate:CoA ligase 2; Soltani *et al.*, 2006) and *SUC2* (*Arabidopsis sucrose transporter 2*; Truernit and Sauer, 1995), respectively (Figure S3a and Appendix S1). As shown in Figure S3(b,c), wild-type Columbia plants expressing the GUS reporter gene under the control of these two promoters showed clear GUS activity staining in the vasculature of healthy uninfected leaf tissue. To confirm appropriate tissue-specific expression of the promoters under infected conditions, Col-0 transgenic lines expressing the GUS reporter gene under the control of either the *4CL2* or *SUC2* promoters were inoculated with a GMI1000 strain expressing a *lacZ* fusion (Vasse *et al.*, 2000). Correct xylem- and phloem-specific GUS activities were observed in transverse root sections of plants of the respective lines showing 75% wilted leaves (Figure 3a,b). The tissue specificity of both



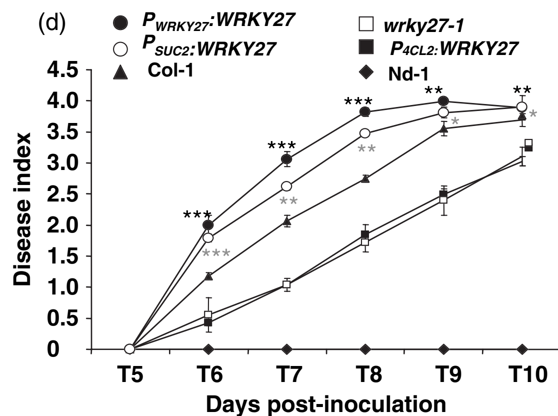


Figure 3. Functional complementation of the *wrky27-1* mutant.

Cross-sections through roots of 3-week-old P_{4CL2}:GUS–*StrepII* Col-0 (a), P_{SUC2}:GUS–*StrepII* Col-0 (b) and *wrky27-1* (c) transgenic lines inoculated with a GMI1000 strain expressing a *lacZ* reporter construct. Xylem (blue) and phloem (red) tissues are outlined. Arrows indicate cells showing GUS activity staining observed within the indicated lines. Arrowheads indicate xylem vessels containing *R. solanacearum* GMI1000-LacZ bacteria (visualized by Magenta Gal staining).

(d) Complementation assays following infection of the respective lines with *R. solanacearum* strain GMI1000. Disease index scoring was as described in Figure 1 on 64 inoculated plants per genotype per repetition at 5–10 dpi. Symptom development was slightly faster in the complementation lines *wrky27-1*/P_{WRKY27}:*WRKY27* (closed circles) and P_{SUC2}:*WRKY27* (open circles) compared to Col-1 (triangles). The disease index score for the P_{4CL2}:*WRKY27* line (closed squares) was similar to that of *wrky27-1* (open squares). Ecotype Nd-1 (diamonds) showed no wilt symptoms (disease index score = 0). These experiments were repeated three times with consistent results. Values significantly different from those of *wrky27-1* plants are indicated by black asterisks for the comparison with *wrky27-1*/P_{WRKY27}:*WRKY27* and by gray asterisks for the comparison with P_{SUC2}:*WRKY27*: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (Student's *t*-test).

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promoters therefore appears to be maintained even after *R. solanacearum* infection. Similarly, in the *wrky27-1* mutant, low levels of GUS activity staining were only detected in the phloem, thereby demonstrating maintenance of the tissue-specific expression pattern of *WRKY27* within inoculated roots (Figure 3c). Magenta Gal staining allowed visualization of the bacteria within the xylem vessels of these transgenic lines (Figure 3a–c; Deslandes *et al.*, 1998).

As shown in Figure 3(d), *wrky27-1* plants containing the P_{WRKY27}:WRKY27 construct (*wrky27-1*/P_{WRKY27}:WRKY27) showed the early wilting phenotype of Col-1 plants upon infection with *R. solanacearum* GMI1000. Thus, expression of WRKY27 cDNA in the mutant fully restored the more rapid wilting symptoms observed in wild-type plants. Moreover, a similar restoration of the early wilting phenotype was achieved by expressing WRKY27 under the control of the *SUC2* promoter. A positive correlation between the level of detectable WRKY27 transcript and wilt disease progression was observed in transgenic lines for both *wrky27-1*/P_{WRKY27}:WRKY27 and *wrky27-1*/P_{SUC2}:WRKY27 (data not shown). In contrast, the disease index score of plants expressing the cDNA under the control of the *4CL2* promoter fully overlapped with that of *wrky27-1*, demonstrating a failure of functional complementation. These results provide compelling evidence that loss of WRKY27 function is the cause of the delayed wilting phenotype associated with *R. solanacearum* GMI1000 infection. Moreover, they illustrate that this WRKY27 function appears to be restricted to companion cells of the phloem. WRKY27 cDNA expression constructs with a StrepII tag (*P_{WRKY27}:WRKY27–StrepII*) or without a tag (*P_{WRKY27}:WRKY27–terminator*) efficiently complemented *wrky27-1* (data not shown). We next tested whether the delay in symptom development of *wrky27-1* mutants is a consequence of reduced bacterial growth within aerial parts of the plant. We measured bacterial growth by two independent assays: (i) time-course sampling at 0, 3, 6 and 9 days post-inoculation (dpi), and (ii) assessment of wilting symptoms at T0 (0 dpi), T3 (3 dpi), D1 (>0–25% wilted leaves; disease index score 1) and D3 (50–75% wilted leaves; disease index score 3).

Pathogen multiplication in *wrky27-1* was monitored following inoculation of the roots with the pathogen, and compared with that of fully resistant Nd-1 or susceptible Col-1 plants, and to that of the *wrky27-1*/P_{WRKY27}:WRKY27 complementation line. Unexpectedly, we found no difference in bacterial growth between Col-1, *wrky27-1* and *wrky27-1*/P_{WRKY27}:WRKY27 plants at any time point over the entire experimental period by either assay (Figure 4 and Figure S4). The bacterial density increased at a similar rate, reaching high levels (10^{10} colony-forming units per gram of fresh weight) in these plants. Only in the resistant ecotype Nd-1 was bacterial multiplication significantly restricted, as previously reported (Deslandes *et al.*, 1998).

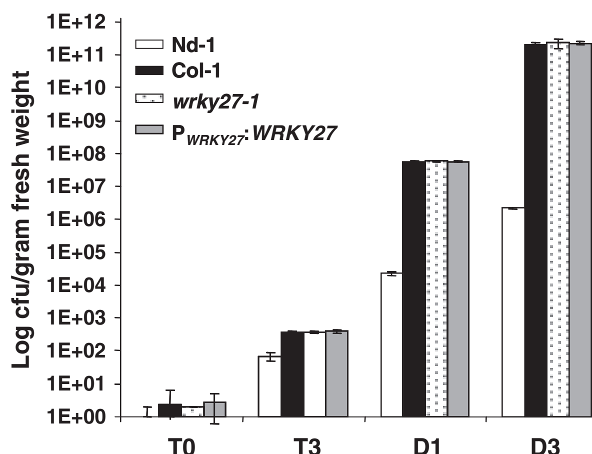

Figure 4. Bacterial growth kinetics upon infection with *R. solanacearum* strain GMI1000 based on assessment of wilt symptoms.
Bacterial multiplication in aerial parts of Nd-1, Col-1 and *wrky27-1* plants and in the *wrky27-1/P_{WRKY27}:WRKY27* complementation line were determined upon inoculation with *R. solanacearum* strain GMI1000 (10^7 bacteria per ml). Samples were collected at T0 (0 dpi), T3 (3 dpi), D1 (when disease index score in Col-1 plants = 1) and D3 (when disease index score in Col-1 plants = 3). Bacterial growth increased at the same rate in all tested plants with the exception of Nd-1. For each time point, triplicate assays were performed on nine plants per genotype.

wrky27-1 *exhibits an altered symptom response to* R. solanacearum *strain Rd15*

Resistance of Nd-1 plants to *R. solanacearum* strain GMI1000 is dependent on the genetic interaction between *RRS1-R* and the bacterial avirulence gene *PopP2* (Deslandes *et al.*, 2003). To test whether the altered disease response of *wrky27-1* is specific towards *R. solanacearum* GMI1000 and is *RRS1-R/PopP2*-dependent, we challenged *wrky27-1* plants with the *R. solanacearum* strain Rd-15, which lacks *PopP2* (Lavie *et al.*, 2004). Both Nd-1 and Col-1 plants are susceptible to this strain (Figure 5) (Yang and Ho, 1998). However, the *wrky27-1* line again showed delayed symptom development as observed using strain GMI1000 (Figure 5). Moreover, this delay in symptom development was completely negated in the *wrky27-1*/P_{WRKY27}:WRKY27 complementation line, demonstrating that loss of WRKY27 function is responsible for this response. Thus, this phenotype is not dependent on RRS1-R/PopP2 function or specific to the GMI1000 bacterial strain.

To determine how specific the altered disease response of *wrky27-1* was to *R. solanacearum*, we tested other host pathogens including *P. syringae* pv. tomato DC3000, *H. parasitica* Noco2, *G. orontii* and *Xanthomonas campestris* pv. *campestris* (*Xcc*), and the non-host pathogens *E. pisi* and *Blumeria graminis* f. sp. *hordei*. In no case could we observe a consistent altered phenotypic response compared to control plants (data not shown).

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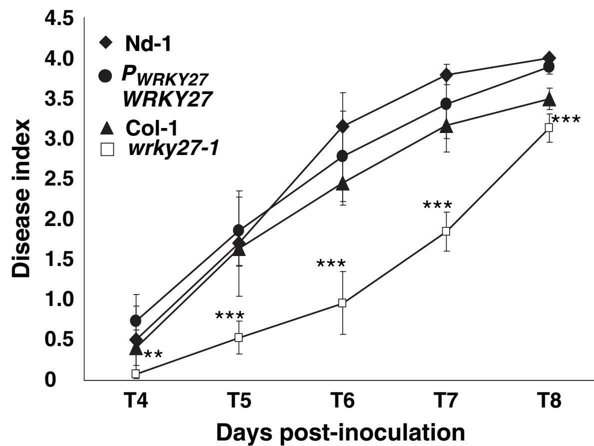

Figure 5. Response of *wrky27-1* to *R. solanacearum* strain Rd-15.
The disease index score was recorded from 4 dpi (first signs of disease appearance on Col-1 leaves) to 8 dpi (complete wilting of Col-1 plants). The disease index score for *wrky27-1* (open squares) was significantly different from those of Col-1 (triangles) and the complementation line *wrky27-1*/P_{WRKY27}:*WRKY27* (closed circles). Ecotype Nd-1 (diamonds) showed disease symptoms comparable to those of Col-1 (triangles). These experiments were repeated twice on 42 plants per genotype per repetition with consistent results. Values significantly different from those of wild-type plants are indicated by asterisks: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (Student's *t*-test).

Candidate targets for WRKY27

Using semi-quantitative PCR, we looked for differences in the basal transcript levels of *WRKY27* in wild-type Col-0 and Ler-1, and in numerous mutants affected in *R* gene, SA, JA and ethylene signaling (including *fls2*, *ndr1*, *pbs1*, *pad2*, *pad4*, *npr1*, *cpr5*, *dnd1*, *edr1*, *mpk4*, *aos*, *jar1*, *etr1*, *ein2*, *eds4* and *acd11*). Basal *WRKY27* expression was not altered in any of these plants (data not shown).

Expression of certain JA/ethylene response genes such as *PR3* and *PR4*, but not SA-responsive genes *PR1* and *PR5*, is strongly induced in Arabidopsis ecotypes that are susceptible to *R. solanacearum* GMI1000 (Hirsch *et al.*, 2002). To test whether such host genes are also affected during the delayed symptoms response of *wrky27-1* towards this bacteria, we performed quantitative real-time RT-PCR analysis. Figure 6(a) shows the results obtained for the marker genes *PDF1.2* and *PR4*. In susceptible Col-1 and the complementation line *wrky27-1*/P_{WRKY27}:WRKY27, the relative basal transcript levels of these genes were higher compared to resistant Nd-1 plants and the *wrky27-1* line. At early stages of infection (T3), the relative transcript levels in susceptible genotypes were decreased and similar to those of Nd-1 and *wrky27-1*. However, at the beginning of wilt symptom development (D1; 0–25% wilted leaves), the relative transcript levels of these genes in Col-1 and the complementation line *wrky27-1*/P_{WRKY27}:WRKY27 increased significantly and reached high levels, persisting when disease development had progressed extensively (D3; 50–75% wilted leaves). In contrast, *PDF1.2* and *PR4* expression in resistant Nd-1 plants and the more tolerant *wrky27-1* line remained suppressed throughout the entire period.

To identify additional WRKY27 target genes, we generated plants conditionally expressing *WRKY27* (Figure S5). Appropriate *wrky27-1* plants harboring MD27 (*WRKY27* cDNA) or MD (vector control) were induced for 6 h with β-estradiol, and the RNA was subsequently isolated and used for hybridization to the Affymetrix Arabidopsis Gene Chip (ATH1; NASCArrays reference number 326). Overall, we observed very few changes in gene expression within the short time period assayed. Nevertheless, independent quantitative RT-PCR analyses confirmed that *Nitrate Reductase 1* (*NR1/NIA1*), *Nitrate Reductase 2* (*NR2/NIA2*) and *Asparagine Synthase 2* (*ASN2*) transcript levels were up-regulated in the *wrky27-1* mutants complemented by MD27 (Figure 6b), whereas the transcript levels of *WRKY38* and *Acireductone Dioxygenase* (*ARD/ARD′*) family protein were slightly down-regulated (data not shown). Additional quantitative RT-PCR analyses showed that *NR1*, *NR2* and *ASN2* transcript levels were not significantly altered in Col-1, *wrky27-1*, *wrky27-1*/P_{WRKY27}:WRKY27 and Nd-1 plants after inoculation with *R. solanacearum* GMI1000 (Figure S6). The identified genes all contain W box elements within their 2 kb promoters, and are thus potential direct WRKY27 targets.

Nitrate reductase is the only plant enzyme that has been conclusively shown to have NO-producing activity (Yamasaki, 2006), whereas ASN2 has been postulated to function in the recapture of nitrogen resources under stress conditions (Wong *et al.*, 2004). We used the NO donor sodium nitroprusside (SNP) to test whether sensitivity to NO is altered in the *wrky27-1* mutant. Above a certain threshold level, NO has been reported to induce hypersensitive cell death upon pathogen challenge in plants (Delledonne, 2005). We treated seedlings with various concentrations of SNP and monitored chlorotic symptoms (loss of chlorophyll) 3 days later. At a concentration of 200 μM SNP, a marked difference in the percentage of symptoms was readily observed between *wrky27-1* and Col-1 plants (Figure 7a). At 300 μM SNP, two-thirds of the *wrky27-1* seedlings were chlorotic, compared to only one-third of the Col-1 seedlings (Figure 7b). At 500 μM SNP, all mutant seedlings were chlorotic, as were 80% of Col-1 plants. This indicates that *wrky27-1* plants display lower tolerance towards exogenously supplied NO compared to wild-type plants. Again, the complementation line *wrky27-1*/P_{WRKY27}:WRKY27 behaved as wild-type (Figure 7a,b).

Discussion

Our studies have revealed that Arabidopsis plants lacking a functional WRKY27 transcription factor show enhanced tolerance towards *R. solanacearum* strains GMI1000 and Rd-15. Although such plants will ultimately succumb to this pathogen, wilting occurs significantly later than in wild-type

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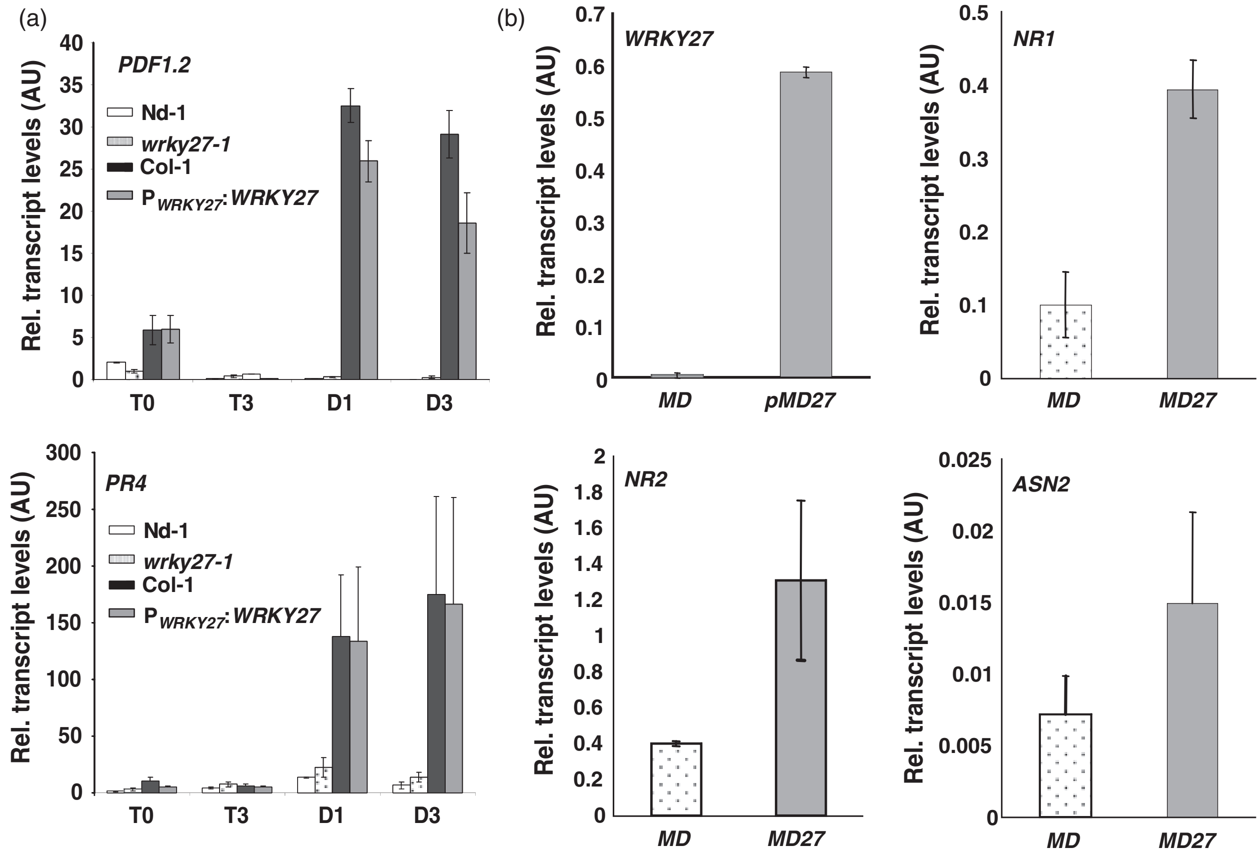

Figure 6. Expression of candidate WRKY27 target genes.
(a) Relative *PDF1.2* and *PR4* transcript levels determined by quantitative RT-PCR using cDNA generated from aerial parts of 4-week-old Nd-1 (white bars), *wrky27-1* (white dotted bars), Col-1 (black bars) and *wrky27-1/P$_{WRKY27}$:WRKY27* (gray bars) plants inoculated with *R. solanacearum* strain GMI1000 at 10^7 bacteria per ml. Samples were collected at T0 (0 dpi), T3 (3 dpi), D1 (when disease index score = 1) and D3 (when disease index score = 3). The expression values for individual genes were normalized using the expression levels for three genes (*PP2A*, At1g13320; *GAPC-2*, At1g13440; *Actin7*, At5g09810) as internal standards.
(b) Relative transcript levels of *WRKY27*, *NR1*, *NR2* and *ASN2* were determined by quantitative RT-PCR using cDNA generated from aerial parts of 18-day-old *wrky27-1* plants expressing MD (vector control; white dotted bars) or MD27 (WRKY27 cDNA; gray bars) after 6 h treatment with 10 μM β-estradiol. The expression values were normalized using the expression level of the tubulin β4 subunit as internal standard.

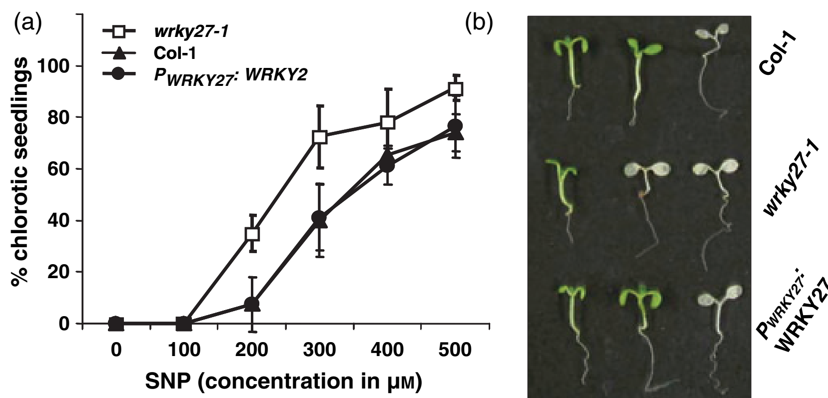

Figure 7. SNP-induced chlorosis in *wrky27-1*.
(a) Seven-day-old MS-grown seedlings were transferred into liquid MS medium containing sodium nitroprusside (SNP) concentrations as indicated. The percentage of chlorotic seedlings for *wrky27-1* (open squares), Col-1 (closed triangles) and *wrky27-1/P$_{WRKY27}$:WRKY27* (closed circles) was determined on 40 seedlings 3 days after treatment. Mean values (±SD) were calculated from the results of two independent experiments.
(b) Images of three representative seedlings of *wrky27-1*, Col-1 and *wrky27-1/P$_{WRKY27}$:WRKY27* taken after 3 days of 300 μM SNP treatment.

plants despite the fact that no differences in bacterial multiplication are observed throughout the course of infection. A similar situation has been observed for the ethylene-insensitive mutant *ein2-1*, which shows delayed symptom development in response to several virulent strains of *R. solanacearum* including GMI1000 and Rd-15, but bacterial multiplication is only slightly reduced *in planta* (Hirsch *et al.*, 2002). These findings indicate that the wilting symptoms are

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not purely the consequence of bacterial obstruction of the xylem vessels. This lack of correlation between pathogen growth and disease symptoms has been reported for effector mutants of *R. solanacearum* and *P. syringae*, and for the tomato *Never-ripe* mutant (which is impaired in ethylene perception) challenged with virulent *X. campestris* pv. *vesicatoria* and *P. syringae* pv. *tomato* (Angot *et al.*, 2006; Badel *et al.*, 2003; Lund *et al.*, 1998). Similarly, transgenic rice plants overexpressing a dominant non-functional transcription factor, rTGA2.1, or silenced for endogenous *rTGA2.1* expression, showed reduced symptom development in response to the virulent vascular pathogen *X. oryzae* pv. *oryzae* without affecting the internal bacterial load (Fitzgerald *et al.*, 2005).

Another shared feature of *wrky27* and *ein2-1* mutants is their failure to activate the expression of ethylene response genes such as *PR3* and *PR4*, and also *PDF1.2*, upon infection, as is also observed in resistant Nd-1 plants (Figure 6a) (Hirsch *et al.*, 2002). Complementation of *wrky27-1* with the WRKY27 cDNA restored both early symptom development and *PR4* and *PDF1.2* expression levels comparable to those seen in susceptible Col-0 plants. This suggests that WRKY27 regulates, directly or indirectly, genes linked to some aspects of ET/JA signaling, which is consistent with the involvement of ethylene in wilt symptom production in the case of *ein2-1*, although other ET response mutants tested did not show the delayed wilt phenotype (Hirsch *et al.*, 2002). Thus, ethylene may actually affect alternative signaling pathways downstream of EIN2 that are in part also negatively controlled by WRKY27. In particular, components of ABA signaling may be affected. EIN2 has been shown to negatively regulate ABA synthesis (Ghassemian *et al.*, 2000), and ABA induces NO accumulation (Desikan *et al.*, 2002). Both ABA and NO are required for the defense responses towards certain pathogens (AbuQamar *et al.*, 2006; Anderson *et al.*, 2004). Moreover, three ABA mutants displayed enhanced susceptibility towards *R. solanacearum* and a fungal pathogen *Plectosphaerella cucumerina* (Hernandez-Blanco *et al.*, 2007). No *PDF1.2* expression was detected in these mutants upon pathogen challenge, similar to our observations with *wrky27*. Interestingly, similar to *wrky27-1*, Arabidopsis seedlings ectopically over-producing a pathogen-inducible cotton hemoglobin (GhHb1) involved in the regulation of cellular NO levels displayed enhanced tolerance to the fungal wilt pathogen *Verticillium dahliae*, increased tolerance to exogenous NO and constitutive expression of *PR1* and *PDF1.2* (Qu *et al.*, 2006).

Loss of WRKY27 function appears to confer tolerance rather than resistance towards the virulent bacterial strains GMI1000 and Rd-15. Tolerance has been defined as limiting or extending plant damage without actually restricting pathogen growth or viability. This has been shown for *ein2* mutants inoculated with a virulent *P. syringae* strain (Bent *et al.*, 1992), and has often been observed in various plant species challenged by vascular root-infecting wilt pathogens. The molecular basis for tolerance remains poorly studied, but, for the interaction of Craigella tomato with the *V. dahliae* isolate Dvd E6, appears to be due to the ability of the plant to actively coat xylem vessels, thereby limiting pathogen distribution (Chen *et al.*, 2004). However, in this case, subsequent mechanisms involving fungal elimination are not as effective as in resistant cultivars. Microarray studies have revealed that in the tolerant tomato–*V. dahliae* interaction, strong suppression of numerous genes is observed that in an otherwise susceptible interaction are strongly upregulated (Robb *et al.*, 2007). This suggests the importance of dedicated host transcription factors in regulating the expression of specific genes in this process. Similar to the results with WRKY27, tolerance towards the vascular pathogen *X. oryzae* pv. *oryzae* also appears to be under negative control of a transcription factor (b-ZIP-type) in rice (Fitzgerald *et al.*, 2005).

Spatial *WRKY27* expression in vegetative tissue correlates well with the vascular route of bacterial infection and propagation *in planta*. However, both histological observations and our ability to complement the mutant phenotype by expressing *WRKY27* under the control of the *SUC2* promoter are consistent with this gene being expressed mainly in phloem companion cells. In contrast, *R. solanacearum* invades, propagates and primarily resides in xylem vessels. As expression of both the P_{SUC2}:GUS and P_{WRKY27}:GUS reporter transgenes remained restricted to the phloem even in heavily infected root tissue and was not locally induced by the bacteria, it may be concluded that *WRKY27* function requires some form of communication between the phloem and xylem. Possibly, some kind of signaling occurs between these two distinct sites or trafficking of some specific component(s) is disturbed/altered in the *wrky27-1* mutant. Consistent with this, microarray analyses performed with RNA from aerial tissues at various infection stages indicate that *R. solanacearum* does not significantly alter expression of the endogenous *WRKY27* or *SUC2*, whereas expression of *4CL2* is induced (Hu *et al.*, 2008).

One potential signal candidate may be the volatile gas NO, as, of the limited number of genes up-regulated in *wrky27-1*, three (*NR1/NIA1*, *NR2/NIA2* and *ASN2*) are involved in nitrogen metabolism and NO generation (Figure 6b). Lipopolysaccharides derived from *Ralstonia* have been shown to induce nitric oxide synthase, an NO burst, and *PR* defense genes in Arabidopsis cells (Zeidler *et al.*, 2004), and the involvement of NO in *R. solanacearum*-induced hypersensitive cell death in tobacco has been reported (Huang and Knoop, 1997). Delledonne (2005) has noted that the balance between NO and H_2O_2 is critical for plant defense responses. Our pharmacological studies using the NO donor SNP indicate an enhanced sensitivity of *wrky27-1* to NO (Figure 7). However, we did not detect

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altered NO levels in healthy roots of *wrky27-1* compared to wild-type plants using the spectrofluorometric assay described by Zottini *et al.* (2007) (data not shown). Thus, loss of WRKY27 function may directly or indirectly lead to a state of nitrosative stress in this mutant rather than resulting in elevated NO levels *per se*. Induced expression of asparagine synthetase (AS/ASN) was observed in *P. syringae* pv. *tomato*-infected tomato, with immunohistochemical analyses revealing strong localization of the protein in phloem cells of the main vascular bundles (Olea *et al.*, 2004). Similarly, immunolocalization studies in rice showed that AS/ASN is localized to companion cells within vascular bundles of mature leaf sheaths (Nakano *et al.*, 2000). Additionally, transgenic tobacco seedlings containing an Arabidopsis *NR2*/*NIA2* gene promoter–GUS reporter construct and inoculated with an endophytic fungus revealed high staining activity in living cells of the vasculature (Sherameti *et al.*, 2005). Mutations within any of the cellulose synthase *CesA* genes *IRX1*, *IRX3* or *IRX5* conferred resistance to *R. solanacearum* (Hernandez-Blanco *et al.*, 2007). IRX1, IRX3 and IRX5 and their respective transcripts are highly enriched in xylem tracheary elements. All three proteins are simultaneously required for assembly of a functional complex at secondary cell-wall deposition sites (Gardiner *et al.*, 2003). Intriguingly, the majority of the genes that are constitutively up-regulated in the *irx* mutants are ABA-responsive (Hernandez-Blanco *et al.*, 2007). Indeed, recent transcriptomic studies have revealed that 40% of all up-regulated Arabidopsis genes associated with *R. solanacearum* disease development are involved in ABA biosynthesis or signaling (Hu *et al.*, 2008). As genetic evidence indicates that NR-mediated NO synthesis is also required for ABA signaling in *A. thaliana* (Desikan *et al.*, 2002), it is conceivable that loss of WRKY27 function affects a subset of ABA response genes. A more detailed analysis of the mutant in response to ABA and drought stress may clarify this point. Alternatively, or in addition, the delayed symptom phenotype of *wrky27-1* could be due to altered transcriptional responses of the host to signals generated by *R. solanacearum* originating from within the xylem.

Finally, the observed *WRKY27*–GUS reporter gene expression detected in floral tissue is consistent with available microarray data (NASC arrays; AtGenExpress), and suggests additional functions of WRKY27 in such tissue. The *wrky27-1* mutant shows no obvious altered floral phenotypes. In contrast, ectopic overexpression of *WRKY27* resulted in developmental defects including stunted morphology, serrated leaves, delayed anther and perianth dehiscence, and partial male sterility (data not shown). However, due to these pleiotropic effects, the response of such over-expressor lines to *R. solanacearum* strain GMI1000 could not be reliably analyzed.

As for several other WRKY transcription factors (Eulgem and Somssich, 2007), WRKY27 appears to be a negative regulator of transcription and defense responses. As WRKY27 also has intrinsic trans-activation functions when tested in transient co-bombardment assays using selected promoters containing W box elements (M.S.M. and I.E.S., unpublished results), this suggests that the WRKY27-dependent transcriptional output of a given gene *in vivo* is very likely determined by its specific association with other components of the transcription machinery at selected promoter sites. Identification of such *in vivo* binding sites and WRKY27-interacting proteins must therefore be addressed in future research studies.

Experimental procedures

Plant materials, growth conditions, insertion mutant and plant transformation

Seeds of *A. thaliana* ecotypes Col-0, Col-1, Nd-1 and the homozygote *wrky* mutant lines (*wrky2*, *6*, *7*, *9*, *11*, *17*, *18*, *19*, *24*, *27*, *29*, *32*, *33*, *36* and *46*) were grown as described previously (Ülker *et al.*, 2007). For infections, plants were grown in Jiffy pots in a growth chamber at 22°C, with a 9 h light period and a light intensity of 190 mmol photons m^{-2} sec^{-1}. The absence of *WRKY27* transcript in *wrky27-1* mutants was verified by RT-PCR analysis using primer pairs WRKY27-Exon1-FRW/WRKY27-Exon3_684Rev and W27-Exon3_FRW/WRKY27-Stop-1. Tubulin β4 subunit-specific primers (At5g44340) were used to determine equal loading of samples. Primer sequences used for RT-PCR experiments are listed in Table S1.

The correctness of all *WRKY27* transgenic constructs were confirmed by digestion with appropriate restriction endonucleases, and transformed into *A. tumefaciens* strain GV3101 (*MP90RK*; Koncz and Schell, 1986). Agro-mediated transformation into appropriate Arabidopsis genotypes (Col-0, Col-1 or *wrky27-1*) was performed using the inflorescence dipping method (Clough and Bent, 1998), and transformants were selected on 0.1% Basta. Histochemical GUS activity assays were performed as described by Jefferson *et al.* (1987).

Bacterial strains, plant inoculation and bacteria growth measurements

The *R. solanacearum* strains GMI1000 and Rd-15 are virulent on *A. thaliana* ecotype Columbia (Deslandes *et al.*, 1998; Yang and Ho, 1998). Strains were grown at 28°C in bacto-agar glucose triphenyltetrazolium chloride (BGT) medium (Boucher *et al.*, 1985), and wounded root inoculations and determination of bacterial growth curves were performed as previously described (Deslandes *et al.*, 1998). Disease symptoms after inoculation were scored daily for each individual inoculated plant according to the percentage of wilted leaves using the following scale: 0 = no wilting, 1 = 25% (considered as disease index 1; D1), 2 = 50%, 3 = 75% (considered as disease index 3; D3), 4 = 100% wilted leaves (Hirsch *et al.*, 2002).

Histochemical GUS and Magenta Gal activity assays in infected roots

Plants were incubated for 2 h in KPi buffer (0.1 M KPO_4 pH 7.4, 0.01% Triton X-100), transferred to GUS staining buffer (0.1 M KPO_4 pH 7.4,

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0.01% Triton X-100, 0.5 mM K_3 Fe(CN)$_6$, 0.5 mM K_4 Fe(CN)$_6$, 5 mM EDTA, 1 mM X-Gluc) and incubated overnight at room temperature. Samples were rinsed three times in KPi buffer. Between two fixation rounds (fixations I and II: 1 h of incubation in KPi buffer containing 1.25% and 2.5% of glutaraldehyde, respectively), Magenta Gal (5-bromo-6-chloro-3-indolyl-β-D-galactopyranoside, Biosynth Ag, http://www.biosynth.com) staining was performed by overnight incubation of the samples at room temperature in Magenta staining buffer (0.1 M KPO_4 pH = 7.4, 0.5 mM K_3 Fe(CN)$_6$, 0.5 mM K_4 Fe(CN)$_6$, 1 mM Magenta Gal). Roots were dehydrated by sequential treatments of 1 h in 70, 90 and 100% ethanol. Root samples were embedded in glycolmethacrylate (Technovit 7100; Haereus-Kulzer, http://www.heraeus-kulzer.com/hkg/web/de/laenderauswahl.html) according to the manufacturer's instructions, and sections of 7 μm thickness were analyzed. Images were acquired using an Axiocam MRc camera (Zeiss, http://www.zeiss.com/) mounted on an Axioplan 2 imaging microscope (Zeiss).

Sodium nitroprusside (SNP) treatment

Seven-day-old seedlings grown on MS plates were transferred to liquid MS medium supplemented with freshly prepared SNP solution at final concentrations ranging from 0 to 500 μM. Seedlings were scored for loss of chlorophyll 3 days later.

RNA extraction and semi-quantitative/quantitative RT-PCR analyses

RNA isolation, DNase treatment, first-strand cDNA synthesis and Northern blotting analyses were performed as described previously (Ülker *et al.*, 2007). For semi-quantitative RT-PCR analyses, 2 μl of first-strand cDNA were used as template, and reactions were standardized using primers specific to the Arabidopsis tubulin β4 subunit. Quantitative PCR was performed using a LightCycler system (Roche Diagnostics, http://www.roche.de) according to the manufacturer's recommendations, as described by Journot-Catalino *et al.* (2006). The primer sets used in the semi-quantitative and quantitative RT-PCR experiments are listed in Table S1.

Additional techniques and methods

Standard molecular biology techniques were performed as described by Sambrook *et al.* (1989). Digital photographic images were cropped and assembled using Adobe® Photoshop® 7.0 (Adobe Systems, http://www.adobe.com). Color and contrast corrections were performed on whole images only. Additional experimental procedures are described in Appendix S1.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. GUS activity staining in healthy *wrky27-1* stem and leaf tissue.

Figure S2. Subcellular localization of WRKY27–GFP fusion protein.

Figure S3. Functional complementation of the *wrky27-1* mutant.

Figure S4. Determination of bacterial growth upon infection with *R. solanacearum* strain GMI1000.

Figure S5. Inducible expression of *WRKY27* in *wrky27-1.*

Figure S6. Expression patterns of selected candidate WRKY27 target genes.

Table S1. Primers used to amplify various DNA fragments, semi-quantitative PCR and quantitative RT-PCR.

Appendix S1. Supplementary experimental procedures.

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