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
evolution suggests that WRKY proteins play pivotal roles in plant-specific processes (Ulker 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; Ulker 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 necrotrophic (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, HvWRKMY1 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 HvWRKY1 and HvWRKY2 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 (Vaill 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.
Results

Loss of WRKY27 function enhances tolerance to R. solanacearum GM11000

In a pilot experiment aimed at identifying WRKY loss-of-function mutants showing altered phenotypic responses to R. solanacearum strain GM11000, 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 GM11000 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.

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
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).

**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 PWRKY27:GUS transgenic plant. Comparable GUS activity was detected in 7- and 14-day-old plants of both the wrky27-1 and PWRKY27: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).

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...
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 (PWRKY27::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 PWRKY27::GUS plants and wrky27-1 (Figure 2d). In floral tissue, the staining in PWRKY27::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 PWRKY27::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 P4CL2::GUS–StrepII Col-0 (a), PSUC2::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/PWRKY27::WRKY27 (closed circles) and PSUC2::WRKY27 (open circles) compared to Col-1 (triangles). The disease index score for the PWRKY27::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 PWRKY27::WRKY27 and by gray asterisks for the comparison with PSUC2::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<sub>WRKY27</sub>:WRKY27_ construct (_wrky27-1/P<sub>WRKY27</sub>: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<sub>WRKY27</sub>:WRKY27_ and _wrky27-1/P<sub>SUC2</sub>: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 Strepl tag (_P<sub>WRKY27</sub>:WRKY27-Strepl_) or without a tag (_P<sub>WRKY27</sub>: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<sub>WRKY27</sub>:WRKY27_ complementation line. Unexpectedly, we found no difference in bacterial growth between Col-1, wrky27-1 and _wrky27-1/P<sub>WRKY27</sub>: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<sup>10</sup> 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).

**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_-dependent, we challenged _wrky27-1_ plants with the *R. solanacearum* strain Rd15, 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<sub>WRKY27</sub>: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 was recorded for 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/PWRKY27 (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 fts2, ndr1, pbs1, pad2, pad4, npr1, cpr5, dnd1, edr1, mpk4, aos, jar1, etr1, ein2, eds4 and acc1). 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 strain 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/PWRKY27, 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/PWRKY27 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 Asparaginase 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/PWRKY27 and Nd-1 plants after inoculation with R. solanacearum strain 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 (Yamazaki, 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/PWRKY27 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
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

![Figure 6](image_url)

**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 *PWRKY27: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 [-estradiol. The expression values were normalized using the expression level of the tubulin β4 subunit as internal standard.

![Figure 7](image_url)

**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 *PWRKY27: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 *PWRKY27:WRKY27* taken after 3 days of 300 μM SNP treatment.
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; Badej 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 8a) (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 (Abu-Qamar 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, similarly 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 GM10000 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*<sub>SUC2</sub>·*GUS* and *P*<sub>WRKY27</sub>·*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<sub>2</sub>O<sub>2</sub> 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
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 GM1000 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 (Üker 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 s−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 [4 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 (MPQ90R; 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 GM1000 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 KPO4 pH 7.4, 0.01% Triton X-100), transferred to GUS staining buffer (0.1 M KPO4 pH 7.4,
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 SI.

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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