The Plant Journal (2006) **48**, 592–605 doi: 10.1111/j.1365-313X.2006.02901.x

Arabidopsis WRKY33 transcription factor is required for resistance to necrotrophic fungal pathogens

Zuyu Zheng†, Synan Abu Qamar†, Zhixiang Chen* and Tesfaye Mengiste*
Department of Botany and Plant Pathology, Purdue University, 915 W. State Street, West Lafayette, IN 47907-2054, USA

Received 20 June 2006; revised 27 July 2006; accepted 31 July 2006.
*For correspondence (fax +1 765 494 0363; e-mail mengiste@purdue.edu or fax +1 765 494 5896; e-mail zhixiang@purdue.edu).
†These authors contributed equally to this study.

Summary

Plant WRKY transcription factors are key regulatory components of plant responses to microbial infection. In addition to regulating the expression of defense-related genes, WRKY transcription factors have also been shown to regulate cross-talk between jasmonate- and salicylate-regulated disease response pathways. The two pathways mediate resistance against different types of microbial pathogens, and there are numerous reports of antagonistic interactions between them. Here we show that mutations of the Arabidopsis *WRKY33* gene encoding a WRKY transcription factor cause enhanced susceptibility to the necrotrophic fungal pathogens *Botrytis cinerea* and *Alternaria brassicicola* concomitant with reduced expression of the jasmonate-regulated plant defensin *PDF1.2* gene. Ectopic over-expression of *WRKY33*, on the other hand, increases resistance to the two necrotrophic fungal pathogens. The *wrky33* mutants do not show altered responses to a virulent strain of the bacterial pathogen *Pseudomonas syringae*, although the ectopic expression of *WRKY33* results in enhanced susceptibility to this pathogen. The susceptibility of *WRKY33*-over-expressing plants to *P. syringae* is associated with reduced expression of the salicylate-regulated *PR-1* gene. The *WRKY33* transcript is induced in response to pathogen infection, or treatment with salicylate or the paraquat herbicide that generates activated oxygen species in exposed cells. WRKY33 is localized to the nucleus of plant cells and recognizes DNA molecules containing the TTGACC W-box sequence. Together, these results indicate that pathogen-induced WRKY33 is an important transcription factor that regulates the antagonistic relationship between defense pathways mediating responses to *P. syringae* and necrotrophic pathogens.

Keywords: disease resistance, *Botrytis cinerea*, *Alternaria brassicicola*, necrotroph, *Pseudomonas syringae*, WRKY33 transcription factor.

Introduction

A wide array of plant defense mechanisms involving complex networks of molecular and cellular factors are activated in plant cells upon infection by microbial pathogens. Activation of plant defense responses may occur upon recognition of pathogen-associated molecular patterns (PAMPs). For example, recognition of flagellin, a conserved component of the bacterial flagellum, activates immunity to bacterial and fungal pathogens (Asai *et al.*, 2002). In *R* gene-mediated resistance, direct or indirect recognition of race-specific elicitors (avirulence proteins) by the plant *R* gene products often leads to rapid activation of a hypersensitive response (HR) that confers a specific and effective resistance against all races of the pathogen expressing the recognized elicitor gene (Flor, 1971). Pathogen-induced HR is often associated with activation of salicylic acid (SA)-regulated defense mechanisms in both local and distal parts of the plants, leading to systemic acquired resistance (SAR). Arabidopsis plants resist infection by pathogens including the bacterial pathogen *Pseudomonas syringae*, the eukaryotic oomycete *Peronospora parasistica* and the obligate biotrophic fungal pathogen *Erysiphe orontii* via a combination of SAR, race-specific and basal resistance (Ausubel *et al.*, 1995; Cao *et al.*, 1997; Dewdney *et al.*, 2000; Glazebrook, 2005; Reuber *et al.*, 1998).

Ethylene (ET)- and jasmonate (JA)-mediated signaling pathways have been shown to play important roles in plant defense responses, particularly to necrotrophic pathogens (Penninckx *et al.*, 1996, 1998; Thomma *et al.*, 1998).

Examples of this group of pathogens include *Botrytis cinerea*, *Alternaria brassicicola*, *Plectosphaerella cucumerina*, and *Sclerotina sclerotiorum*. These pathogens produce no known host-specific toxins or race-specific Avr proteins that act as determinants of host susceptibility or resistance. The Arabidopsis *COI1* gene, which encodes an F-box protein required for JA signaling, functions in the resistance to necrotrophic pathogens, *Botrytis* and *A. brassicicola* (Li *et al.*, 2004b; Thomma *et al.*, 1999; van Wees *et al.*, 2003; Xie *et al.*, 1998). The Arabidopsis *EIN2* gene, which functions in ethylene signaling, is also important for resistance to *Botrytis* (Guzman and Ecker, 1990; Thomma *et al.*, 1999). Expression of the Arabidopsis *PDF1.2* gene, which encodes a protein with antimicrobial activity, is dependent on *COI1* and *EIN2*, and correlates with resistance to necrotrophic pathogens (Penninckx *et al.*, 1996, 1998; Thomma *et al.*, 1998). Phytoalexins are also implicated in resistance to necrotrophic pathogens (Ferrari *et al.*, 2003; Hain *et al.*, 1993). However, susceptibility to these pathogens in plants with wild-type levels of *PDF1.2* and phytoalexins has also been documented (Ferrari *et al.*, 2003; Mengiste *et al.*, 2003; Veronese *et al.*, 2004).

There are extensive interactions among the various defense response pathways that are activated in pathogen-infected plants. Antagonism between the JA/ET-dependent and the SA-regulated pathways has been clearly established, and factors that regulate these relationships are being identified (Andreasson *et al.*, 2005; Kunkel and Brooks, 2002; Petersen *et al.*, 2000; Veronese *et al.*, 2006). In Arabidopsis, pathogen-induced SA accumulation is associated with the suppression of JA signaling (Spoel *et al.*, 2003). Plants that are unable to accumulate SA produce increased levels of JA and show enhanced expression of the JA-responsive genes *LOX2*, *PDF1.2*, and *VSP* in response to infection by *P. syringae* pv. *tomato* DC3000 (PstDC3000). NPR1, whose nuclear localization is required for SA signaling, also modulates the antagonistic effect of SA on JA signaling through a novel cytosolic function (Spoel *et al.*, 2003). The Arabidopsis *MPK4* gene may also be involved in the antagonistic interactions of these pathways by contributing to repression of SA-dependent resistance and activation of JA-dependent defense gene expression (Petersen *et al.*, 2000). The *Botrytis-induced protein kinase 1* (*BIK1*) gene is a positive regulator of necrotrophic pathogen resistance but is a negative regulator of resistance to PstDC3000 in Arabidopsis (Veronese *et al.*, 2006).

Proteins containing WRKY zinc-finger motifs constitute a novel class of transcription factors encoded by a large superfamily with more than 70 members in Arabidopsis (Eulgem *et al.*, 2000). A large body of evidence indicates that many members of this protein family are involved in plant defense responses. First, a large number of WRKY genes are induced by pathogen infection or treatment with plant defense signal molecules. In Arabidopsis, 49 of 72 WRKY genes tested were differentially regulated in plants after infection with an avirulent strain of *P. syringae* or treatment with SA (Dong *et al.*, 2003). Secondly, the promoters of a large number of plant defense or defense-related genes including *PR* genes and *NPR1* contain W-box sequences that are recognized by WRKY proteins and are necessary for the inducible expression of these defense genes (Yu *et al.*, 2001). Thirdly, some WRKY proteins have been shown to be directly involved in plant defense responses. For example, two Arabidopsis *WRKY* genes (*WRKY22* and *WRKY29*) are induced by MAPK pathways that are involved in resistance responses to both bacterial and fungal pathogens, and transient expression of *WRKY29* in leaves leads to reduced disease symptoms (Asai *et al.*, 2002). Constitutive expression of Arabidopsis *WRKY18* and *WRKY70* results in constitutive or enhanced expression of defense-related genes, including *PR-1*, and increased resistance to virulent pathogens (Chen and Chen, 2002; Li *et al.*, 2004a). A more recent study has shown that Arabidopsis WRKY18 physically interacts with structurally related Arabidopsis WRKY40 and WRKY60, resulting in altered DNA-binding activities (Xu *et al.*, 2006). These three WRKY genes also interact functionally in a complex pattern of overlapping, antagonistic and distinct roles in plant responses to different types of microbial pathogens (Xu *et al.*, 2006). In tobacco, virus-induced silencing of three *WRKY* genes compromises *N* gene-mediated resistance to tobacco mosaic virus (Liu *et al.*, 2004). In addition, the resistance gene *RRS1* that confers resistance to the bacterial pathogen *Ralstonia solanacearum* encodes a novel WRKY protein, WRKY52, that combines typical TIR-NBS-LRR R protein motifs with a WRKY domain (Deslandes *et al.*, 2002; Lahaye, 2002).

Arabidopsis MAP kinase 4 (MPK4), a repressor of SA-dependent resistance, was recently found to interact with an MPK4 substrate (MKS1) that in turn interacts with Arabidopsis WRKY25 and WRKY33 (Andreasson *et al.*, 2005). In addition, WRKY25 and WRKY33 were shown to be *in vitro* substrates of MPK4. These results suggest that WRKY25 and WRKY33 may function as downstream components of the MPK4-mediated signaling pathway and contribute to repression of SA-dependent disease resistance. In the present study, we have analyzed the role of the Arabidopsis WRKY33 transcription factor in plant responses to necrotrophic and biotrophic pathogens. Two T-DNA insertion alleles of *WRKY33* exhibited increased susceptibility to the necrotrophic pathogens *A. brassicicola* and *B. cinerea*, with enhanced disease symptom and pathogen growth in inoculated plants. Ectopic expression of the gene, on the other hand, conferred increased resistance to the necrotrophic pathogens but caused enhanced susceptibility to the bacterial pathogen *P. syringae*. Altered responses to these pathogens in the mutant and over-expression plants were associated with altered expression of the JA-regulated *PFD1.2* and SA-regulated *PR-1* genes. Our findings suggest

that *WRKY33* is an important component of the regulatory cascade, mediating cross-talk between defense responses to pathogens with different mechanisms of pathogenesis.

Results

Expression of WRKY33 *during activation of plant defense responses*

The *WRKY33* gene (At2g38470) encodes a transcription factor with two WRKY domains. It has been studied for its role in plant defense because its expression is significantly increased in pathogen-infected Arabidopsis plants. Based on microarray experiments, the *WRKY33* gene is expressed at low levels in healthy, uninfected wild-type plants. However, within 24-h post-inoculation (hpi) with *B. cinerea*, the transcript levels of *WRKY33* increased by an average of 5.5-fold over non-inoculated controls (AbuQamar *et al.*, 2006). To confirm the microarray results, we examined the transcript levels of *WRKY33* in *Botrytis*-infected plants using RNA blots. The transcripts of *WRKY33* were substantially enhanced within 24 h after inoculation with the fungal pathogen (Figure 1a). We previously reported that *WRKY33* was induced by an avirulent strain of the bacterial pathogen PstDC3000 (Dong *et al.*, 2003). In the present study, we analyzed its expression in response to a virulent strain of the bacterial pathogen PstDC3000. *WRKY33* transcripts rose substantially within 4 h after infiltration with the bacterial pathogen (Figure 1b). In contrast, infiltration with buffer alone induced only a low level of *WRKY33* transcripts. Thus, *WRKY33* is responsive to both virulent and avirulent strains of PstDC3000. The *WRKY33* gene was also induced rapidly and strongly by SA (Figure 1c). The transcript level of *WRKY33* increased rapidly but modestly within 1–4 h after treatment with the natural precursor of ethylene biosynthesis, 1-aminocyclopropane-1-carboxylic acid (ACC), and then returned to basal levels within 8 h. H_2O and JA treatments did not induce *WRKY33* expression (Figure 1c). Paraquat, an herbicide that generates reactive oxygen species (ROS) in exposed plant cells, also induced *WRKY33* gene expression (Figure 1d).

To determine which signaling pathways are involved in the pathogen-induced expression of *WRKY33*, its expression was studied in a set of mutants that are defective in various defense response pathways including ethylene response (*ein2*), JA signaling (*coi1*), camalexin accumulation (*pad2*), and SA accumulation (nahG; Figure 2). These mutants show increased susceptibility to *Botrytis* and/or altered responses to other pathogens (Ferrari *et al.*, 2003; Thomma *et al.*, 1999; van Wees *et al.*, 2003). As in the wild-type plants, the basal levels of *WRKY33* transcripts in *nahG*, *pad2* and *ein2* mutants were very low. However, an increased level of *WRKY33* transcript accumulated in uninfected *coi1* mutant plants (Figure 2), indicating that *COI1*, which is required for JA signaling and resistance to *Botrytis*, represses basal expression of the *WRKY33* gene. After inoculation with *Botrytis*, *WRKY33* transcripts accumulated in these mutants similarly to wild-type plants. These results indicate that expression of *WRKY33* is highly responsive to *Botrytis* through a novel signaling pathway. Our findings are similar to what was reported recently for the *WRKY70* gene; basal expression levels of *WRKY70* in the *coi1* mutant were higher than in the wild-type plants (Li *et al.*, 2004a; AbuQamar *et al.*, 2006). Unlike *WRKY70*, however, induced expression of *WRKY33* was unchanged in the *nahG* plants, suggesting that endogenous SA is not required for its basal and induced expression.

The wrky33 mutants and ectopic expression of WRKY33 result in altered responses to pathogens

In order to determine the function of *WRKY33* in disease resistance, we characterized two T-DNA insertion alleles of the *WRKY33* gene as well as transgenic lines constitutively expressing *WRKY33.* The T-DNA insertions in the mutants, designated as *wrky33-1* (SALK_006603) and *wrky33-2*

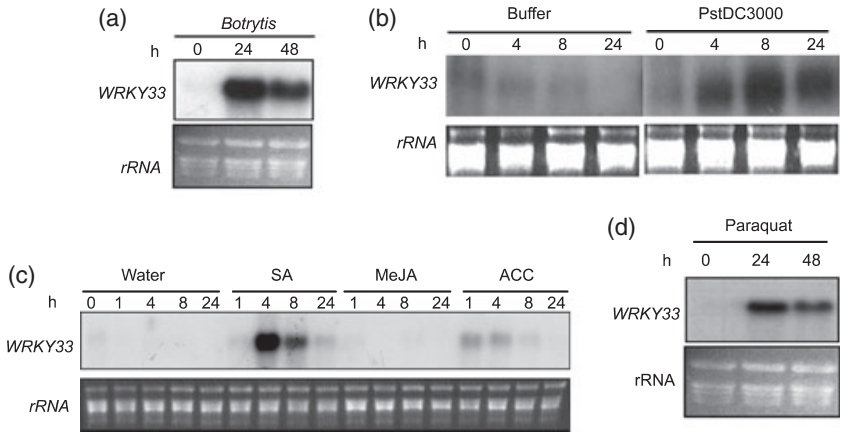

Figure 1. Induced expression of the *WRKY33* gene.
Expression of *WRKY33* in response to (a) *Botrytis*, (b) PstDC3000 inoculation, (c) treatment with plant hormones, and (d) paraquat treatment (methyl viologen). Plants were inoculated or treated with the various chemicals as described in Experimental procedures. A 5 μg aliquot of total RNA was loaded per lane. The experiments were repeated at least three times with similar results. h, hours post-inoculation; SA, salicylic acid; MeJA, methyl jasmonate; ACC, 1-aminocyclopropane-1-carboxylic acid.

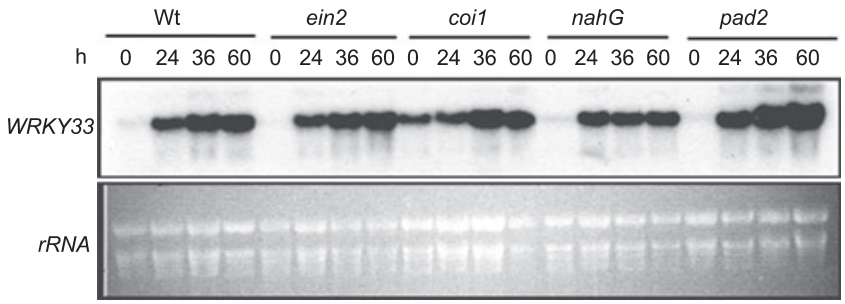

Figure 2. Expression of *WRKY33* in wild-type (Wt), *ein2*, *coi1*, *nahG*, and *pad2* plants infected with *Botrytis*.
A 5 μg aliquot of total RNA was loaded per lane. The RNA blot was repeated three times on RNA extracted from three independent experiments and similar results were obtained. h, hours after inoculation.

(GABI_324B11), were confirmed by PCR using primers specific to the *WRKY33* gene and the T-DNA insertions in the two alleles as described previously (Sessions *et al.*, 2002). In addition, the genomic region around the T-DNA insertion site was sequenced in order to verify the insertion site and determine possible genomic rearrangements. The *wrky33-1* mutant allele carries a T-DNA insertion in the second intron based on PCR results using a pair of primers corresponding to the left border of T-DNA and the *WRKY33* sequence 5′-end to the insertion site. Interestingly, PCR using primers corresponding to the *WRKY33* sequences flanking the T-DNA insertion detected another linked *WRKY33* copy that contains no T-DNA insertion but has a 33 bp deletion followed by a 23 bp duplication at the 3′-end of the second intron that would abolish proper splicing after transcription (Figure 3a, left). The second copy of *WRKY33*, derived apparently from rearrangement caused by T-DNA insertion, would therefore result in the synthesis of a larger transcript with an unspliced second intron that would be translated into a truncated protein of 209 amino acids containing no WRKY zinc-finger motif. The *wrky33-2* mutant allele contains a T-DNA insertion in the first intron of the *WRKY33* gene with no abnormal features of DNA rearrangements (Figure 3a, right). Uninoculated *wrky33* plants grow and develop normally with no obvious morphologic or developmental defects (data not shown).

To determine the status of the *WRKY33* transcript in the mutant plants, we performed an RNA blot using RNA samples from *Botrytis*-infected plants. *WRKY33* transcripts of the expected size were observed in wild-type plants after pathogen infection but were absent in plants carrying the *wrky33* mutant alleles (Figure 3b). The *wrky33-1* allele produced a transcript larger than the wild-type *WRKY33* transcript (Figure 3b). The increased transcript size produced by the *wrky33-1* allele is apparently due to the deletion and duplication at the 3′-end of its second intron

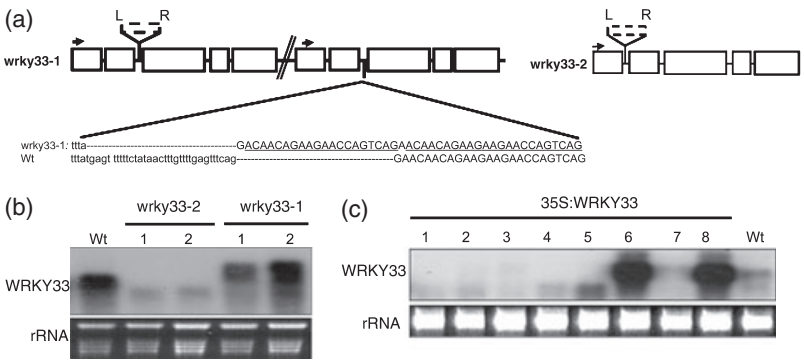

Figure 3. Characterization of the *wrky33* mutations and the *WRKY33* over-expression plants.
(a) Genomic organization of the *wrky33-1* (left) and *wrky33-2* (right) T-DNA insertion alleles. The position of the T-DNA insertion and genomic rearrangements deduced from the sequencing of the mutant alleles are shown. Sequences are comparisons of the *wrky33-1* mutant and wild-type (Wt) alleles. In the *wrky33-1* mutant, there is a second, genetically linked copy of *WRKY33* that has a 33 bp deletion and a 23 bp duplication at the 3′-end of its second intron. Duplicated sequences are underlined. The arrows show the direction of transcription. The T-DNA insertions are shown with the left (L) and right (R) borders marked.
(b) RNA blots from wild-type (Wt), *wrky33-1* and *wrky33-2* plants showing the altered transcripts of the *WRKY33* gene in the *wrky33* mutants. The numbers at the top of the gel show sibling plants used in the assay.
(c) RNA blot of 35S:*WRKY33* transgenic plants to identify lines with constitutively increased expression of *WRKY33*. The numbers at the top of the gels show independent transgenic plants.
In (b) and (c), 10 μg of total RNA was loaded per lane. The experiments were repeated at least three times with similar results. All RNA samples in (b) are from *Botrytis*-infected tissue.

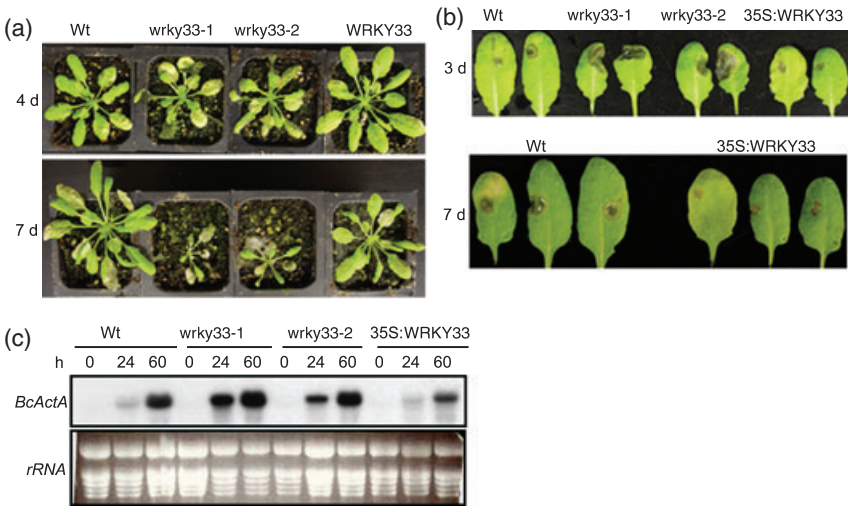

Figure 4. Altered responses of *wrky33-1*, *wrky33-2*, and 35S:*WRKY33* plants to *Botrytis*.
(a) Disease responses of spray-inoculated plants at 4 and 7 dpi.
(b) Disease responses of drop-inoculated plants at 3 and 7 dpi.
(c) Accumulation of the *Botrytis ActinA* mRNA in spray-inoculated plants.
Total RNA (12 μg) was loaded per lane. In (b), no suitable picture of the mutant leaves could be taken at 7 dpi as the leaves were completely macerated. The experiments were repeated at least three times with similar results.

that prevent proper splicing (Figure 3a). The *wrky33-2* allele produced a smaller *WRKY33* transcript at a very low level (Figure 3b).

To further characterize the role of WRKY33 in disease resistance, we constructed and analyzed transgenic Arabidopsis plants that constitutively over-express *WRKY33*. The full-length *WRKY33* cDNA was cloned behind the CaMV 35S promoter and transformed into Arabidopsis plants. RNA blotting showed that two transgenic plants contained elevated levels of the *WRKY33* transcript in the absence of pathogen infection (Figure 3c). The transgenic 35S:*WRKY33* plants flower slightly earlier than wild-type plants and have leaves with serrated margins. In all other aspects, the plants are phenotypically similar to untransformed wild-type plants (data not shown).

The *wrky33-1* and *wrky33-2* mutant alleles and the transgenic 35S:*WRKY33* plants were first tested for responses to *Botrytis* as described previously (Mengiste *et al.*, 2003). In spray-inoculated wild-type plants, *Botrytis* infection caused disease symptoms composed of necrotic spots and chlorosis with no significant spread of tissue damage (Figure 4a). In the mutant plants, most leaves were completely chlorotic or macerated at 4 dpi. At 7 dpi, the infection in the *wrky33-1* and *wrky33-2* mutants expanded, leading to severely damaged plants, whereas the majority of leaves from the wild-type plants remained green (Figure 4a). The *WRKY33* over-expression plants developed less severe disease symptoms and sustained less tissue damage than the wild-type plants, suggesting that the over-expression of *WRKY33* enhanced resistance to *Botrytis* (Figure 4a). When leaves of soil-grown plants were drop-inoculated with *Botrytis* spores, the disease lesions expanded faster in the *wrky33-1* and *wrky33-2* mutants than those in wild-type plants (Figure 4b). The 35S:*WRKY33* plants showed increased resistance to *Botrytis* compared to wild-type plants, with disease lesions that did not expand significantly after 3 dpi (Figure 4a,b). The disease lesions in the drop-inoculated mutant leaves expanded, resulting in the complete decay of mutant leaves at 7 dpi. In 35S:*WRKY33* plants, the disease lesions remained restricted with no significant growth between 3 and 7 dpi, whereas in the wild-type plants the lesions expanded until about 7 dpi when they begin to be restricted. The enhanced susceptibility to *Botrytis* was observed in *wrky33-1/wrky33-1* homozygous mutant plants but not in *wrky33-1/WRKY33* heterozygous plants (data not shown), indicating that the *wrky33-1* mutant is a recessive loss-of-function allele.

To determine whether WRKY33 contributes to limiting pathogen growth, we studied the accumulation of the *B. cinerea ActinA* gene transcript as a measure of fungal growth in inoculated plants. The transcript levels of the constitutively expressed *B. cinerea ActinA* gene correlated with fungal biomass (Benito *et al.*, 1998). Total RNA isolated from infected plants was blotted and probed with the *B. cinerea ActinA* gene (Benito *et al.*, 1998). As shown in Figure 4(c), substantially higher signals for the fungal *ActinA* gene transcript were detected in the *wrky33* mutant plants than in the wild-type plants after infection with *Botrytis*. The transgenic 35S:*WRKY33* plants had a clearly reduced *ActinA* gene transcript level relative to wild-type plants, indicating reduced fungal growth (Figure 4c). Thus, both symptom development and growth of the fungus confirmed that the *wrky33* mutant plants were more susceptible to *Botrytis*, and that the *WRKY33* gene is part of a defense response that limits *Botrytis* growth.

The *wrky33* mutants and the 35S:*WRKY33* plants were also tested for response to *A. brassicicola*, a necrotrophic fungal pathogen that causes black spot disease on cruciferous plants. Inoculation of wild-type plants with fungal spores (5×10^5 spores ml^{-1}) led to the formation of disease lesions that were largely restricted to the inoculation site, indicating that the wild-type plants are quite resistant to the

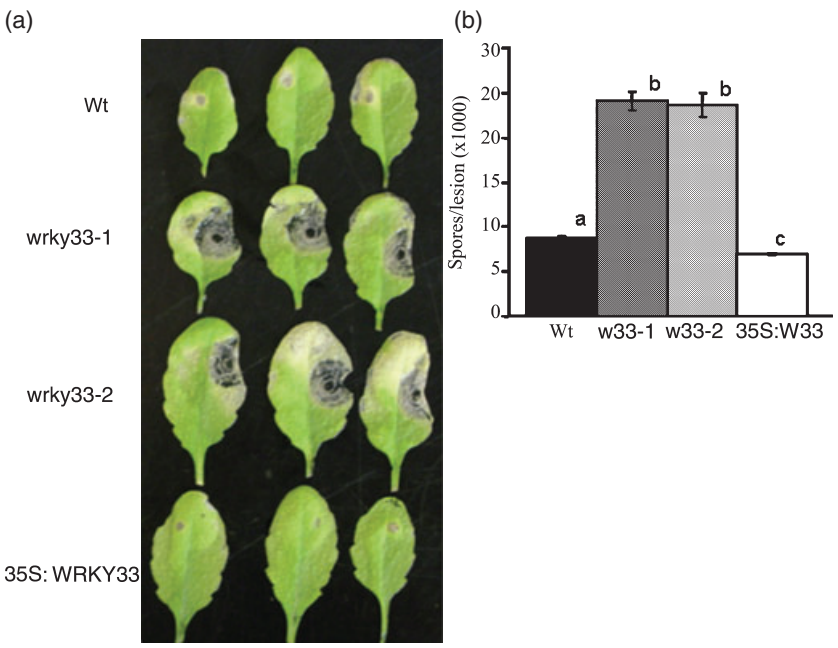

Figure 5. The *WRKY33* gene is required for resistance to *Alternaria brassicicola*.
(a) Disease symptoms in wild-type, *wrky33-1*, *wrky33-2*, and 35S:*WRKY33* plants at 5 days after inoculation with *A. brassicicola*.
(b) Mean number of spores in *A. brassicicola*-inoculated plants at 5 days after inoculation.
In (b), the data represent the mean ± SE from a minimum of 30 lesions. Analysis of variance and Duncan's multiple range test were performed to determine the statistical significance of the mean spore counts using SAS software (SAS Institute, 1999). Bars with different letters are significantly different from each other at $P = 0.05$. These experiments were repeated at least three times with similar results. Data for the 35S:*WRKY33* plants are representative of two independent transgenic lines. W33, WRKY33.

fungal pathogen (Figure 5a). In the *wrky33* mutant plants, inoculation of the fungal pathogen resulted in increased disease symptoms characterized by large lesions surrounded by extensive chlorosis (Figure 5a). In addition, the fungus produced approximately threefold more fungal spores in the mutant plants than in the wild-type plants (Figure 5b). Thus, there was an enhanced pathogen growth and reproduction after inoculation with *A. brassicicola* spores in the *wrky33* mutant plants relative to the wild-type plants. The transgenic 35S:*WRKY33* plants were more resistant to *A. brassicicola* based on disease symptoms and spore counts (Figure 5a,b). Thus, constitutive over-expression of *WRKY33* enhanced resistance to both *Botrytis* and *A. brassicicola*, while the loss-of-function alleles caused enhanced susceptibility. These results confirm that *WRKY33* plays an important role in plant defense against necrotrophic pathogens.

The WRKY33 *cDNA rescues the disease responses of the mutant to wild-type levels*

The increased expression of *WRKY33* during infection and the altered disease response phenotypes of the two *wrky33* mutant alleles and transgenic plants support the key role of *WRKY33* in disease resistance. To further demonstrate that these responses are due specifically to the function of the *WRKY33* gene, we transformed *wrky33-1* plants with the 35S:*WRKY33* construct. Plants carrying the *wrky33-1* mutant allele and expressing the *WRKY33* cDNA were identified by PCR and RNA blots (Figure 6a,b). These plants exhibited enhanced resistance to *Botrytis* and *A. brassicicola* that is comparable to that of wild-type plants (Figure 6c,d). These results indicate that the susceptible phenotypes of the *wrky33-1* mutant are caused by loss of the functional *WRKY33* gene.

Altered responses of the 35S:WRKY33 transgenic plants to the bacterial pathogen P. syringae

We evaluated the wild-type, *wrky33-1* and *wrky33-2*, and transgenic 35S:*WRKY33* plants for responses to a virulent strain of PstDC3000. Four-week-old plants were inoculated with the bacterial pathogen and bacterial growth was determined. Bacterial growth and disease symptoms in the *wrky33-1* and *wrky33-2* mutant plants did not differ significantly from wild-type plants (Figure 7a,b). In contrast, 2 and 4 days after infiltration with PstDC3000, the 35S:*WRKY33* over-expression lines supported levels of bacterial growth that was significantly higher than either the wild-type, *wrky33-1* or *wrky33-2* mutant plants (Duncan's multiple range test, $P = 0.05$; Figure 7b). In addition, the 35S:*WRKY33* plants developed more severe disease symptoms after infection with the bacterial pathogen, including more extensive necrosis and chlorosis than wild-type plants (Figure 7a). Thus, whereas the loss-of-function alleles did not show any altered responses to PstDC3000, constitutive over-expression of *WRKY33* compromised the defense response to the bacterial pathogen. The opposing effects of the over-expression of *WRKY33* support the emerging model that different defense responses are required to combat different types of microbial pathogens (Glazebrook, 2005).

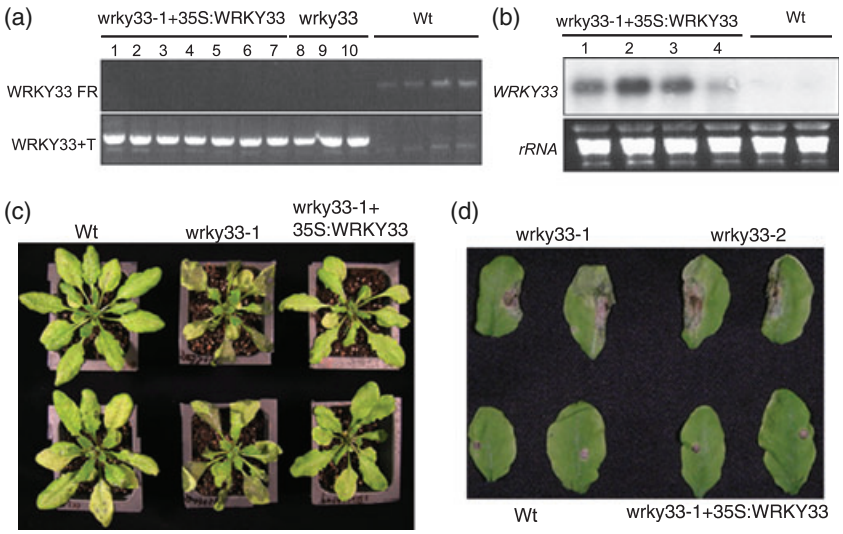

Figure 6. The disease response phenotypes of the *wrky33-1* mutant alleles are rescued by expressing *WRKY33* cDNA under the control of the CaMV 35S promoter.
(a) PCR results for the *wrky33-1* plants harboring the 35S:*WRKY33* gene.
(b) RNA blot showing the constitutive expression of 35S:*WRKY33* in the *wrky33-1* mutant.
(c and d) *WRKY33* restores the *Botrytis* (c) and *A. brassicicola* (d) susceptibility of the *wrky33-1* plants to wild-type levels. The data are representative of two independent lines. The disease assay was repeated twice with similar results. W33FR, WRKY33 gene-specific forward and reverse primers; W33 + T, *WRKY33*- and T-DNA-specific primers.

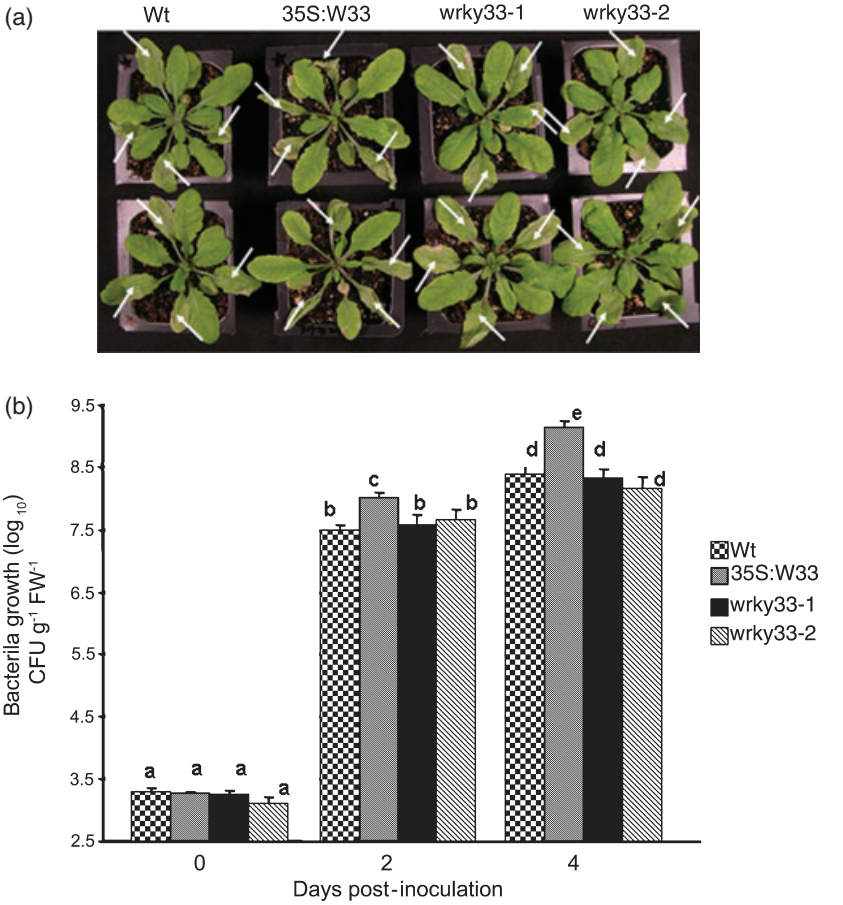

Figure 7. Responses of the *wrky33* mutant and 35S:*WRKY33* lines to *Pseudomonas syringae*.
(a) Disease symptoms on plants inoculated with a virulent strain of *P. syringae* 3 days after inoculation.
(b) Bacterial growth on plants showing the increased susceptibility of the *WRKY33* over-expression plants. In (a), arrows indicate disease symptoms. The mean bacterial count data were analyzed to determine whether mean values are statistically different from each other as described in Figure 5. Each bar represents the mean bacterial count from three independent experiments ± SE. Bars with different letters are significantly different from each other ($P = 0.05$). The bacterial disease assays were performed at least three times with similar results.

Altered defense gene expression in wrky33 mutant and WRKY33-over-expressing plants

To explore the molecular basis of the altered responses of the *wrky33* mutants and the transgenic *WRKY33* over-expression plants to necrotrophic pathogens, we characterized the expression of a number of defense-related genes in these plants after infection by *Botrytis*. Arabidopsis *PAD4* is a pathogen- and SA-induced gene encoding a lipase-like protein that may play an important role in pathogen-induced

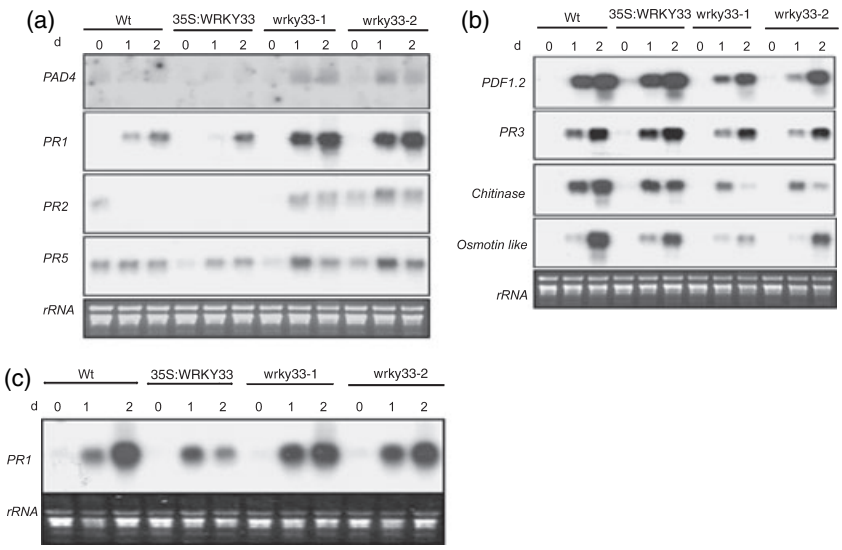

Figure 8. Expression of defense-related genes in *wrky33* mutant and 35S:*WRKY33* over-expression plants during pathogen infection.
(a) Expression of SA-regulated defense genes during *Botrytis* infection.
(b) Expression of JA-regulated defense genes during *Botrytis* infection.
(c) Expression of *PR-1* during PstDC3000 infection.
d, days after inoculation.

SA accumulation (Jirage *et al.*, 1999). Arabidopsis *PR1*, *PR2*, and *PR5* are defense-regulated genes and are associated with SA-regulated defense responses (Ward *et al.*, 1991; Yang *et al.*, 1997). Uninfected wild-type plants showed low levels of *PAD4* expression (Figure 8a). In the *wrky33* mutants, the basal expression level of *PAD4* was also low, but expression of the gene was induced in *Botrytis*-inoculated mutant plants (Figure 8a). This result indicates that WRKY33 is a repressor of pathogen-induced *PAD4* expression. Likewise, we observed enhanced expression of *PR1*, *PR2*, and *PR5* in both *wrky33* mutants after infection by *Botrytis* when compared to that in wild-type plants (Figure 8a). In the transgenic 35S:*WRKY33* plants, the expression of *PAD4*, as in wild-type plants, was not induced at the time points tested. The expression of *PR1* and *PR5* was either delayed or reduced when compared to the wild-type plants (Figure 8a). These results suggest that expression of these *PR* genes is negatively regulated by *WRKY33.* However, it is also possible that reduced expression of these genes in the over-expression plants was due to reduced fungal growth.

JA-mediated defense plays an important role in regulating the expression of plant defense genes and resistance to necrotrophic pathogens. To determine whether the altered responses of the *wrky33* mutant and the transgenic 35S:*WRKY33* plants to the necrotrophic pathogens is related to altered JA-mediated defense mechanisms, we examined the expression of a number of JA-regulated defense genes after *Botrytis*infection. *PDF1.2* expression is strongly induced in wild-type plants in response to *Botrytis* infection but its induction is significantly reduced in *wrky33* mutants (Figure 8b). In transgenic 35S:*WRKY33* plants, we consistently observed an enhancement of the basal expression of *PDF1.2* relative to those in the wild-type and *wrky33* mutant plants (Figure 8b). The pathogen-induced expression of the JA-regulated *PR3* (At3g12500; Lorenzo *et al.*, 2003), chitinase- and osmotin-like genes was reduced or delayed in the *wrky33* mutant (Figure 8b). These results indicated that *WRKY33* is a positive regulator of these JA-regulated defense genes. The expression of these four defense-related genes; however, was not significantly different in the transgenic WRKY33 over-expression lines compared to wild-type plants, suggesting that WRKY33 alone may not be sufficient to alter the expression of these genes (Figure 8b).

SA-mediated defense plays a critical role in plant defense against the bacterial pathogen *P. syringae*. SA-mediated defense mechanisms are associated with the expression of pathogenesis-related (*PR*) genes, including the *PR-1* gene, which is often used as a reliable molecular marker for SA-dependent SAR. As the transgenic *WRKY33* over-expression plants are more susceptible to *P. syringae*, we examined the expression of the *PR1* gene in the transgenic plants after infection by the bacterial pathogen. As shown in Figure 8(c), *PR1* was induced at high levels in the wild-type plants after bacterial infection. The induction in the transgenic 35S:*WRKY33* plants was significantly reduced. Thus, enhanced susceptibility of 35S:*WRKY33* plants to the bacterial pathogen was associated with reduced expression of SA-regulated *PR1* (Figure 8c).

WRKY33 protein shows DNA-binding activities and localizes to the nucleus

WRKY33 encodes a protein of 512 amino acids with a molecular weight of 56 456.9 Da and an isoelectric point of 7.66. Sequence analysis shows that the WRKY33 protein contains two DNA-binding domains (WRKY domains) located at amino acid positions 177–234 and 354–412. BLAST p search (Altschul *et al.*, 1990) and sequence alignment reveals significant sequence homology to a large

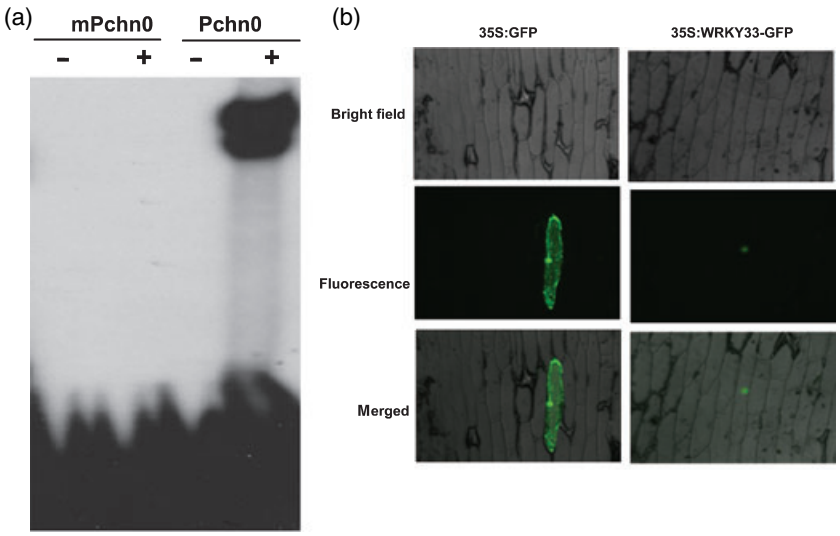

Figure 9. Sequence-specific DNA-binding activity and subcellular localization of WRKY33.
(a) Electrophoresis mobility shift assay (EMSA) showing the binding of the WRKY33 recombinant protein to the W-box (TTGACT) sequence but not to a mutated version of the W-box.
(b) The subcellular localization of the WRKY33–GFP fusion protein in onion epidermal cells. Images in the left column show the control plasmid expressing only the GFP and those in the right column show the WRKY33–GFP fusion protein expressed in onion epidermal cells. The cells were examined under the bright field (top), fluorescence (middle), and as a merged image (bottom) showing either the diffused (control plasmid) or the nuclear localization of the proteins.
In (a), the sequences shown are the wild-type (upper line) and mutant (lower line) sequences of oligonucleotides used as a probe in the EMSA. The mutations introduced are shown in lowercase letters.

number of WRKY proteins from Arabidopsis and other plant species. The Arabidopsis genome encodes 74 WRKY proteins classified into eight subfamilies. WRKY33 shows features of the group I WRKY proteins, with two WRKY DNA-binding domains, and is closely related to WRKY25 (Eulgem *et al.*, 2000).

WRKY transcription factors most likely function by binding to cognate W-box *cis*-elements in the promoter region of target genes to activate or repress their expression (Chen and Chen, 2000; Eulgem *et al.*, 2000; Rushton *et al.*, 1996; Yu *et al.*, 2001). To determine whether the WRKY33 recombinant protein binds specifically to the W-boxes, the WRKY33 protein was produced as a His-tagged protein in *Escherichia coli*, and its ability to bind to the W-box was assayed by electrophoresis mobility shift assays (EMSA) as described previously (Chen and Chen, 2000). In the presence of an oligonucleotide probe containing two W-box sequences and WRKY33 recombinant protein, specific protein–DNA complexes with reduced migration were clearly present in the EMSA assays. When the W-box sequences in the oligo probes were mutated from TTGACC to TTGAAC, the binding complexes were not detected. These results suggest that the recombinant WRKY33 protein specifically binds to the W-box in the synthesized probe (Figure 9a).

WRKY33, as a putative transcription factor, is likely to be localized in the nucleus. To verify the subcellular localization of the WRKY33 protein, we generated a C-terminal translational fusion of the *WRKY33* cDNA with the sequence encoding the green fluorescent protein (GFP). The plasmid containing 35S:*WRKY33*–GFP was bombarded into onion (*Allium cepa*) epidermal cells. The recombinant protein accumulated exclusively in the nuclei of onion cells, whereas the GFP protein alone accumulated in both the cytoplasm and the nucleus because of its small size (Figure 9b). These results suggest that WRKY33 protein is localized to the nucleus, supporting its predicted role as a transcriptional regulator.

Discussion

Although there is a large body of indirect evidence implicating plant WRKY proteins in plant defense responses, information about the biological roles of specific WRKY proteins in plant disease resistance is still very limited. The functions of a large number of WRKY proteins remain to be determined. In the present study, we analyzed the roles of the Arabidopsis *WRKY33* gene in plant defense responses and disease resistance. *wrky33* T-DNA insertion alleles were isolated and the mutants were found to exhibit enhanced susceptibility to the necrotrophic fungal pathogens *Botrytis* and *A. brasscicolia* as measured by enhanced disease symptoms and increased pathogen growth in inoculated plants. Transgenic plants constitutively expressing the *WRKY33* gene, on the other hand, were more resistant to the necrotrophic pathogens than the wild-type plants. These results indicate that Arabidopsis *WRKY33* plays an important role in resistance to necrotrophic pathogens. Although loss of *WRKY33* function appears to have little effect on plant resistance to virulent strains of PstDC3000, expression of the 35S:*WRKY33* transgene suppressed basal resistance to the bacterial pathogen. Thus, mutations and/or constitutive over-expression of *WRKY33* had contrasting effects on plant responses to different types of microbial pathogens.

Arabidopsis resistance to *Botrytis* depends on JA- and ET-signaling pathways, as mutations that block JA and ET signaling result in enhanced susceptibility (Ferrari *et al.*, 2003; Thomma *et al.*, 1999). We observed that, after *Botrytis* infection, the *wrky33* mutants showed reduced expression

of *PDF1.2*, a molecular marker of the JA- and ET-mediated defense response signaling pathways. In addition, expression of the JA-regulated chitinase gene in the *wrky33* mutants is consistent with the role of WRKY33 as a positive regulator of JA-controlled processes. This observation suggests that the JA/ET-mediated responses that are important for defense against necrotrophic pathogens might be compromised in the *wrky33* mutants. Thus, the important role for WRKY33 in plant defense against necrotrophic pathogens may be mediated by its action as a positive regulator of JA- and ET-mediated defense response signaling.

In contrast, *P. syringae* is a biotrophic bacterial pathogen in the early stages of compatible interactions. Arabidopsis mutants defective in SA biosynthesis or signaling, including *eds1*, *pad4*, *eds5*, *sid2*, and *npr1*, allow increased growth of *P. syringae*, indicating that SA-mediated signaling mechanisms play a vital role in limiting *P. syringae* growth (Glazebrook, 2005). Although the *wrky33* mutant appeared to respond normally to *P. syringae*, transgenic plants constitutively over-expressing *WRKY33* were more susceptible to the virulent strain of *P. syringae*, with enhanced bacterial growth and disease symptoms in inoculated plants. In addition, we observed that the enhanced susceptibility of the transgenic plants to *P. syringae* was associated with reduced expression of *PR1*, a defense-related gene often associated with SA-mediated defense responses. Thus, *WRKY33* might play a negative role in SA-mediated signaling pathways, and over-expression of the gene could have a negative impact on pathogen-induced, SA-mediated defense mechanisms and thus compromise plant resistance to *P. syringae*. It should be noted that, although the *wrky33* mutant responded normally to *P. syringae*, a recently published study reported that a *wrky33* mutant had higher basal levels of *PR1* transcripts than the wild-type plants when they were grown under short day length (Andreasson *et al.*, 2005). This observation suggests that WRKY33-mediated responses to *Pst* may be conditional, which raises the possibility that certain WRKY genes may contribute to resistance under certain conditions. We did not observe constitutive expression of *PR1* in the two *wrky33* alleles, which may be attributed to the different growth conditions used in our assays. As a member of a large gene family, *WRKY33* may have functions that overlap with other *WRKY* genes, and the failure to observe stronger phenotypes, including susceptibility to the bacterial pathogen, in the loss-of-function mutants could be attributed to other functionally redundant *WRKY* genes. Indeed, phylogenetic analysis based on WRKY protein structures has revealed that WRKY33 and several other WRKY proteins including WRKY25 and WRKY26 share very similar protein structures and pathogen-induced expression (Dong *et al.*, 2003; Eulgem *et al.*, 2000). Isolation of the loss-of-function mutants for these similar WRKY proteins and construction of composite mutants for multiple *WRKY* genes should reveal whether these structurally related WRKY proteins perform overlapping functions in plant defense and disease resistance.

The role of *WRKY33* in the SA-regulated defense pathway can also be deduced from patterns of defense marker gene expression. Several SA-related marker genes, including *PR1*, *PR2*, *PR5*, and *PAD4*, are expressed at higher levels in the *wrky33* mutant than in the wild-type plants in response to *Botrytis* infection. The enhanced expression of *PR1* in the *wrky33* mutant in response to necrotrophic pathogens is similar to other *Botrytis*-susceptible Arabidopsis mutants including *bos1*, *bos3*, and *bik1* (Mengiste *et al.*, 2003; Veronese *et al.*, 2004, 2006). The *wrky33* mutants are impaired in JA signaling and show enhanced susceptibility to necrotrophic pathogens, which may in turn result in the release of the negative regulation of SA by JA.

The proposed role of WRKY33 as a negative regulator of SA-dependent defense responses is also consistent with possible regulation of WRKY33 by Arabidopsis MPK4, a repressor of SA-dependent resistance. The MKS1 interacts with the Arabidopsis WRKY25 and WRKY33 proteins (Andreasson *et al.*, 2005). In addition, WRKY25 and WRKY33 were shown to be *in vitro* substrates of MPK4, and a *wrky33* knockout mutant was found to have enhanced levels of *PR1* gene expression under short-day growth conditions (Andreasson *et al.*, 2005). These results suggest that WRKY25 and WRKY33 may function as downstream components of the MPK4-mediated signaling pathway and contribute to the repression of SA-dependent disease resistance by MPK4.

Some aspects of our observations on *WRKY33* are similar to those reported for the WRKY70 putative transcription factor. Similar to *WRKY33*, *WRKY70* is an important regulator of pathogen responses and is induced by SA and pathogens (Li *et al.*, 2004a). In addition, basal expression of both *WRKY70* and *WRKY33* was higher in the *coi1* mutant than the wild-type plants. In contrast to *WRKY70*, induced expression of *WRKY33* was unchanged in the *nahG* plants, suggesting that endogenous SA is not required for its basal and induced expression. Ectopic over-expression of *WRKY70* increased resistance to PstDC3000 and the necrotrophic bacterial pathogen *Erwinia carotovora* ssp. *carotovora* and resulted in constitutive expression of SA-induced *PR* genes. Recently, over-expression of *WRKY70* was shown to result in increased resistance to *Erysiphe cichoracearum* but increased susceptibility to *A. brassicicola* (Li *et al.*, 2006). By contrast, the constitutive expression of *WRKY33* caused resistance to necrotrophic fungi but increased susceptibility to PstDC3000. Our data suggest that WRKY33 is a negative regulator of SA responses whereas WRKY70 functions as a positive factor in SA-mediated responses.

Rapid and strong induction of *WRKY33* by pathogens and SA indicates that this gene is subject to transcriptional regulation, in addition to the possible post-translational modification by MPK4. When comparisons were made

among the three established defense signal molecules, only SA, but not JA or ET, induced *WRKY33* at high levels. However, SA is not required for pathogen-induced *WRKY33* expression as deduced from the normal induction of *WRKY33* in the transgenic *nahG* plants. These results suggest that pathogen-induced *WRKY33* expression is mediated by another signal molecule(s). As *WRKY33* is also induced by paraquat, ROS may be possible candidates for the signal molecules that mediate pathogen- and SA-induced expression of the *WRKY33* gene. Both pathogen infection and SA treatment are known to generate ROS and cause oxidative stress in plants (Lamb and Dixon, 1997). The induction of *WRKY33* through ROS-mediated signaling mechanisms is also consistent with its demonstrated role in defense against necrotrophic pathogens, a group of pathogens that are known to promote ROS generation that may contribute to induction of cell death.

Necrotrophic pathogens, including *Botrytis*, promote host cell death that is proposed to be a form of HR (Govrin and Levine, 2000). Consistent with this, cell death induced by *Botrytis* is preceded by accumulation of ROS and is associated with induction of the HR-associated gene HSR2031 in tobacco (Govrin and Levine, 2000). The accumulation of ROS and increased cell death during infection of plants by necrotrophic pathogens such as *Botrytis* enhance the susceptibility to these pathogens (Govrin and Levine, 2000). In some mutants of Arabidopsis, susceptibility to *Botrytis* correlated with sensitivity to oxidative stress (Mengiste *et al.*, 2003; Tierens *et al.*, 2002; Veronese *et al.*, 2004). In addition, induction of cell death by pre-infection with a virulent pathogen leads to increased growth of *Botrytis*, and inhibition of cell death by expressing animal antiapoptosis genes leads to enhanced resistance to the same pathogen (Dickman *et al.*, 2001). Although the *WRKY33* transcript accumulates in response to paraquat, the *wrky33* mutant plants were comparable to wild-type plants in their sensitivity to two ROS-generating compounds. This observation suggests that the susceptibility of *wrky33* plants to necrotrophic pathogens functions independently of plant sensitivity to ROS.

This study provides data on the role of *WRKY33* in defense responses. Over-expression of the gene mediates distinct responses to infection by necrotrophic and biotrophic pathogens. Antagonistic interactions between the SA-dependent and the JA-dependent defense response pathways appear to occur frequently based on the disease responses of many Arabidopsis mutants including *ssi2* (Kachroo *et al.*, 2001), *bik1* (Veronese *et al.*, 2006), *bos3* (Veronese *et al.*, 2004), and *mpk4* (Petersen *et al.*, 2000) to infection by necrotrophic and biotrophic pathogens. The molecular mechanisms of these antagonistic interactions are still poorly understood. Some of the regulatory proteins such as WRKY33 will provide tools to further elucidate the mechanisms of the antagonistic actions between defense response pathways.

Experimental procedures

Arabidopsis genotypes and growth conditions

The Arabidopsis mutants and wild-type plants used in this study are in the Columbia genetic background. The mutants are *ein2* (Guzman and Ecker, 1990), *coi1* (Xie *et al.*, 1998), and *pad2* (Glazebrook and Ausubel, 1994). The *nahG* plants express the bacterial *nahG* gene encoding salicylate hydroxylase, an enzyme that can metabolize SA (Delaney *et al.*, 1994). The homozygous *coi1* mutant plants were identified using the CAPS marker as described previously (Xie *et al.*, 1998). Procedures for growing plants for the various assays and growth conditions were as previously described (Mengiste *et al.*, 2003).

Fungal and bacterial culture and disease assays

Botrytis cinerea strain *BO5-10* and *A. brassicicola* strain MUCL20297 (Zheng *et al.*, 2000; van Wees *et al.*, 2003) were cultured on 2X V8 agar (36% V8 juice, 0.2% $CaCO_3$, 2% Bacto-agar (Becton, Dickinson and Company, Sparks, MD, USA)) and incubated at 20–25°C. Collection of *B. cinerea* and *A. brassicicola* spores and plant inoculation was performed as described previously (Mengiste *et al.*, 2003). All *Botrytis* disease assays were performed on soil-grown plants either by spraying or single-leaf drop inoculations. A single 3 μl drop of a suspension of 2.5×10^5 spores ml^{-1} in Sabouraud maltose broth (SMB) buffer was placed on each leaf. The disease assay for *A. brassicicola* was performed on detached leaves as described by Veronese *et al.* (2006).

Bacterial culture and disease assays were performed as described by Xu *et al.* (2006). For each genotype, four or five plants were infiltrated at each time point and the number of colony-forming units per gram of infected leaf tissue was determined.

Sensitivity to oxidative stress

Sensitivity of plants to photo-oxidative damage induced by paraquat (methyl viologen; Sigma, St Louis, MO, USA) was determined as described previously (Mengiste *et al.*, 2003). Sensitivity to rose Bengal (4,5,6,7-tetrachloro-2,4,5,7-tetraiodofluorescein; Sigma) was performed essentially as described previously (Rustérucci *et al.*, 2001).

Induction treatments

SA was dissolved in water as 100 mM stock solution and adjusted to pH 6.5 with KOH. Plants were sprayed with 2 mM SA solution diluted from the stock. MeJA was dissolved in 50% ethanol as 10 mM stock solution. The MeJA stock solution was diluted to 100 μM with water and sprayed onto plants. ACC was dissolved in water, and a 2 mM solution was used. In all cases, 4-week-old plants grown on soil were used. Induction by paraquat and *Botrytis* infection were performed as described by Veronese *et al.* (2006).

RNA blots

Total RNA was isolated from leaves using the TRIZOL reagent (BRL Life Technologies, Rockville, MD, USA) or as described by Lagrimini

et al. (1987). For RNA blots, total RNA was separated on 1.2% agarose–formaldehyde gels, blotted to nylon membranes and hybridized to ^{32}P-dATP-labeled probes according to standard procedures. The *B. cinerea ActinA* gene used as a probe was amplified from the *B. cinerea* genomic DNA (Benito *et al.*, 1998; Veronese *et al.*, 2004).

Generation of transgenic lines and identification of the wrky33-1 *and* wrky33-2 *mutant alleles*

The *WRKY33* cDNA was isolated by screening a cDNA library (Stratagene, La Jolla, CA, USA). To generate the 35S:*WRKY33* construct, the cDNA was cloned into the Arabidopsis transformation vector pOCA30 (Du and Chen, 2000). The resulting plasmid was transformed into *Agrobacterium* strain GV3101 and introduced into plants by Arabidopsis floral dip transformation (Clough and Bent, 1998). The transgenic plants over-expressing *WRKY33* were identified by RNA blotting using *WRKY33* cDNA as a probe. The seeds of the *wrky33-1* (SALK-006603) and *wrky33-2* (GABI324B11) insertion lines were obtained from the Arabidopsis Biological Resource Center (Alonso *et al.*, 2003; Columbus, OH, USA) and from Max Plack Institute (MPI) for Plant Breeding Research GABI-Kat (Cologne, Germany; Rosso *et al.*, 2003), respectively.

T-DNA insertion alleles were identified by PCR essentially as described previously (Sessions *et al.*, 2002). For the *wrky33-1* mutant, the T-DNA insertion in the *WRKY33* gene was identified by PCR using a T-DNA-specific primer (LBA1: 5′-tggttcacgtagtgggccatcg-3′) and a *WRKY33*-specific primer (pW33-1: 5′-tccactttcttcttcgttgga-3′). Homozygous *wrky33-1* mutant plants were identified by PCR using a pair of primers corresponding to sequences flanking the T-DNA insertion (pW33-2: 5′-atggacaatagcagaaccagaca-3′; pW33-3: 5′-ttcctgaaactcaaaacaaagttata-3′). The *wrky33-1* mutant contains a second *WRKY33* gene that has no T-DNA insertion but has a 33 bp deletion and a 23 bp duplication at the 3′-end of the second intron (Figure 3a). The pW33-3 primer corresponds to part of the 33 bp deleted sequence and would not amplify the second allele of *WRKY33* by PCR when used in combination with the pW33-2 primer. The T-DNA insertion in the *wrky33-2* mutant was confirmed by PCR using a T-DNA-specific primer (5′-cccatttggacgtgaatgtagacac-3′) and a WRKY33-specific primer (pW33-4: 5′-ttcagtccctctctttttctcgat-3′). Homozygous *wrky33-2* mutant plants were identified by PCR using a pair of primers corresponding to sequences flanking the T-DNA insertion (pW33-4 and pW33-5: 5′-tccactttcttcttcgttgga-3′).

For expression in the *wrky33-1* mutant background, the 35S:*WRKY33* expression cassette was cloned into the pSMB vector that carries the Basta selectable marker gene (Mylne and Botella, 1998) and transformed into the *wrky33-1* mutant. The *wrky33-1* plants expressing 35S:*WRKY33* were selected based on their resistance to spray application of 1.384% v/v Finale (Farnam Companies Inc., Phoenix, AZ, USA). Transgenic Basta-resistant plants were confirmed by PCR as harboring the *wrky33-1* mutation and by RNA blot for the constitutive expression of *WRKY33*.

Production of recombinant protein and electrophoretic mobility shift assay

To generate the WRKY33 recombinant protein, the full-length cDNA was cloned into pET32a (Novagen, San Diego, CA, USA) and transformed into *E. coli* strain BL21 (DE3). Induction of protein expression and purification of recombinant His-tagged WRKY33 protein were performed according to the protocol provided by Novagen. The purified proteins were dialyzed overnight against a nuclear extraction buffer (25 mM HEPES/KOH at pH 7.5, 40 mM KCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT and 30 mg ml^{-1} PMSF) at 4°C. Double-stranded synthetic oligonucleotides were labeled to specific activities of approximately 10^5 cpm ng^{-1} using the Klenow fragment of DNA polymerase I. Sequence-specific DNA binding was assayed with EMSA essentially as described previously (Chen and Chen, 2000). DNA and protein complexes were allowed to form at room temperature for 30 min and were resolved on a 10% polyacrylamide gel in 0.5X TBE at 4°C.

Cellular localization of WRKY33–GFP in onion epidermal cells

The *WRKY33* cDNA was cloned into a GFP vector modified from pRTL2 (Restrepo *et al.*, 1990). The plasmid was isolated using Qiagen kits (Valencia, CA, USA), concentrated to about 1 μg μl^{-1}, and used to coat the tungsten particles for bombardment experiments. A plasmid containing GFP alone was concentrated as above and bombarded in parallel as controls. Transformation of onion (*A. cepa*) epidermal cells and localization of the protein were performed essentially as described previously (Kinkema *et al.*, 2000).

Accession numbers

Sequence data for the genes described in this study can be found in the GenBank/EMBL data libraries under the following accession numbers: *WRKY33* (At2g38470), *PR1* (At2G14610), *PR2* (At3G57260), *PR3* (At3G12500), *PR5* (At1G75040), *LOX2* (At3G45140), osmotin-like (At4G11650), chitinase-like (At2G43580), *PDF1.2* (At5G44420), and *PAD4* (At3G52430).

Acknowledgements

The authors thank Dr Robert Dietrich (Syngenta Biotechnology Inc., Durham, NC, USA) and Dr Burton Bluhm (Purdue University) for critical reading of the manuscript. This research was funded by National Science Foundation (NSF) grant IOB-0422702 provided to T.M. and grant MCB-0209819 to Z.C. This is Purdue University Agricultural Program (ARP) paper number 2006-17856.

References

AbuQamar, S., Chen, X., Dahwan, R., Bluhm, B., Salmeron, J., Lam, S., Dietrich, R.A. and Mengiste, T. (2006) Expression profiling and mutant analysis reveals complex regulatory networks involved in Arabidopsis response to *Botrytis* infection. *Plant J.* **48**, 28–44.

Alonso, J.M., Stepanova, A.N., Leisse, T.J. *et al.* (2003) Genome-wide insertional mutagenesis of Arabidopsis thaliana. *Science* **301**, 653–657.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410.

Andreasson, E., Jenkins, T., Brodersen, P. *et al.* (2005) The MAP kinase substrate MKS1 is a regulator of plant defense responses. *EMBO J.* **24**, 2579–2589.

Asai, T., Tena, G., Plotnikova, J., Willmann, M.R., Chiu, W.L., Gomez-Gomez, L., Boller, T., Ausubel, F.M. and Sheen, J. (2002) MAP kinase signalling cascade in Arabidopsis innate immunity. *Nature*, **415**, 977–983.

Ausubel, F.M., Katagiri, F., Mindrinos, M. and Glazebrook, J. (1995) Use of *Arabidopsis thaliana* defense-related mutants to dissect the plant response to pathogens. *Proc. Natl Acad. Sci. USA* **92**, 4189–4196.

Benito, E.P., ten Have, A., van't Klooster, J.W. and van Kan, J.A.L. (1998) Fungal and plant gene expression during synchronized infection of tomato leaves by *Botrytis cinerea*. *Eur. J. Plant Pathol.* **104**, 207–220.

Cao, H., Glazebrook, J., Clarke, J.D., Volko, S. and Dong, X. (1997) The Arabidopsis NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell*, **88**, 57–63.

Chen, C. and Chen, Z. (2000) Isolation and characterization of two pathogen- and salicylic acid-induced genes encoding WRKY DNA-binding proteins from tobacco. *Plant Mol. Biol.* **42**, 387–396.

Chen, C. and Chen, Z. (2002) Potentiation of developmentally regulated defense response by AtWRKY18, a pathogen-induced Arabidopsis transcription factor. *Plant Physiol.* **129**, 706–716.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735–743.

Delaney, T.P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gut-Rella, M., Kessmann, H. and Ryals, J. (1994) A central role of salicylic acid in plant disease resistance. *Science*, **266**, 1247–1250.

Deslandes, L., Olivier, J., Theulieres, F., Hirsch, J., Feng, D.X., Bittner-Eddy, P., Beynon, J. and Marco, Y. (2002) Resistance to *Ralstonia solanacearum* in *Arabidopsis thaliana* is conferred by the recessive RRS1-R gene, a member of a novel family of resistance genes. *Proc. Natl Acad. Sci. USA* **99**, 2404–2409.

Dewdney, J., Reuber, T.L., Wildermuth, M.C., Devoto, A., Cui, J., Stutius, L.M., Drummond, E.P. and Ausubel, F.M. (2000) Three unique mutants of Arabidopsis identify *eds* loci required for limiting growth of a biotrophic fungal pathogen. *Plant J.* **24**, 205–218.

Dickman, M.B., Park, Y.K., Oltersdorf, T., Li, W., Clemente, T. and French, R. (2001) Abrogation of disease development in plants expressing animal antiapoptotic genes. *Proc. Natl Acad. Sci. USA* **98**, 6957–6962.

Dong, J., Chen, C. and Chen, Z. (2003) Expression profiles of the Arabidopsis WRKY gene superfamily during plant defense response. *Plant Mol. Biol.* **51**, 21–37.

Du, L. and Chen, Z. (2000) Identification of genes encoding receptor-like protein kinases as possible targets of pathogen- and salicylic acid-induced WRKY DNA-binding proteins in Arabidopsis. *Plant J.* **24**, 837–847.

Eulgem, T., Rushton, P.J., Robatzek, S. and Somssich, I.E. (2000) The WRKY superfamily of plant transcription factors. *Trends Plant Sci.* **5**, 199–206.

Ferrari, S., Plotnikova, J.M., De Lorenzo, G. and Ausubel, F.M. (2003) Arabidopsis local resistance to *Botrytis cinerea* involves salicylic acid and camalexin and requires EDS4 and PAD2, but not SID2, EDS5 or PAD4. *Plant J.* **35**, 193–205.

Flor, H.H. (1971) Current status of the gene-for-gene concept. *Annu. Rev. Phytopathol.* **9**, 275–296.

Glazebrook, J. (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* **43**, 205–227.

Glazebrook, J. and Ausubel, F.M. (1994) Isolation of phytoalexin-deficient mutants of *Arabidopsis thaliana* and characterization of their interactions with bacterial pathogens. *Proc. Natl Acad. Sci. USA* **91**, 8955–8959.

Govrin, E.M. and Levine, A. (2000) The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. *Curr. Biol.* **10**, 751–757.

Guzman, P. and Ecker, J.R. (1990) Exploiting the triple response of Arabidopsis to identify ethylene-related mutants. *Plant Cell*, **2**, 513–523.

Hain, R., Reif, H.J., Krause, E. *et al.* (1993) Disease resistance results from foreign phytoalexin expression in a novel plant. *Nature*, **361**, 153–156.

Jirage, D., Tootle, T.L., Reuber, T.L., Frost, L.N., Feys, B.J., Parker, J.E., Ausubel, F.M. and Glazebrook, J. (1999) *Arabidopsis thaliana* PAD4 encodes a lipase-like gene that is important for salicylic acid signaling. *Proc. Natl Acad. Sci. USA* **96**, 13583–13588.

Kachroo, P., Shanklin, J., Shah, J., Whittle, E.J. and Klessig, D.F. (2001) A fatty acid desaturase modulates the activation of defense signaling pathways in plants. *Proc. Natl Acad. Sci. USA* **98**, 9448–9453.

Kinkema, M., Fan, W. and Dong, X. (2000) Nuclear localization of NPR1 is required for activation of PR gene expression. *Plant Cell*, **12**, 2339–2350.

Kunkel, B.N. and Brooks, D.M. (2002) Cross talk between signaling pathways in pathogen defense. *Curr. Opin. Plant Biol.* **5**, 325–331.

Lagrimini, L.M., Burkhart, W., Moyer, M. and Rothstein, S. (1987) Molecular cloning of complementary DNA encoding the ligininforming peroxidase from tobacco: molecular analysis and tissue-specific expression. *Proc. Natl Acad. Sci. USA* **84**, 7542–7546.

Lahaye, T. (2002) The Arabidopsis RRS1-R disease resistance gene – uncovering the plant's nucleus as the new battlefield of plant defense? *Trends Plant Sci.* **7**, 425–427.

Lamb, C. and Dixon, R.A. (1997) The oxidative burst in plant disease resistance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48**, 251–275.

Li, J., Brader, G. and Palva, E.T. (2004a) The WRKY70 transcription factor: a node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense. *Plant Cell*, **16**, 319–313.

Li, L., Zhao, Y., McCaig, B.C., Wingerd, B.A., Wang, J., Whalon, M.E., Pichersky, E. and Howe, G.A. (2004b) The tomato homolog of CORONATINE-INSENSITIVE1 is required for the maternal control of seed maturation, jasmonate-signaled defense responses, and glandular trichome development. *Plant Cell*, **16**, 126–143.

Li, J., Brader, G., Kariola, T. and Tapio Palva, E. (2006) WRKY70 modulates the selection of signaling pathways in plant defense. *Plant J.* **46**, 477–491.

Liu, Y., Schiff, M. and Dinesh-Kumar, S.P. (2004) Involvement of MEK1 MAPKK, NTF6 MAPK, WRKY/MYB transcription factors, COI1 and CTR1 in N-mediated resistance to tobacco mosaic virus. *Plant J.* **38**, 800–809.

Lorenzo, O., Piqueras, R., Sanchez-Serrano, J.J. and Solano, R. (2003) ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. *Plant Cell*, **15**, 165–178.

Mengiste, T., Chen, X., Salmeron, J.M. and Dietrich, R.A. (2003) The *BOS1* gene encodes an R2R3MYB transcription factor protein that is required for biotic and abiotic stress responses in Arabidopsis. *Plant Cell*, **15**, 2551–2565.

Mylne, J. and Botella, J.R. (1998) Binary vectors for sense and antisense expression of Arabidopsis ESTs. *Plant Mol. Biol. Rep.* **16**, 257–262.

Penninckx, I.A., Eggermont, K., Terras, F.R., Thomma, B.P., De Samblanx, G.W., Buchala, A., Metraux, J.P., Manners, J.M. and Broekaert, W.F. (1996) Pathogen-induced systemic activation of a

plant defensin gene in Arabidopsis follows a salicylic acid-independent pathway. *Plant Cell*, **8**, 2309–2323.

Penninckx, I.A., Thomma, B.P., Buchala, A., Metraux, J.P. and Broekaert, W.F. (1998) Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in Arabidopsis. *Plant Cell*, **10**, 2103–2113.

Petersen, M., Brodersen, P., Naested, H. *et al.* (2000) Arabidopsis MAP kinase 4 negatively regulates systemic acquired resistance. *Cell*, **103**, 1111–1120.

Restrepo, M.A., Freed, D.D. and Carrington, J.C. (1990) Nuclear transport of plant potyviral proteins. *Plant Cell*, **2**, 987–998.

Reuber, T.L., Plotnikova, J.M., Dewdney, J., Rogers, E.E., Wood, W. and Ausubel, F.M. (1998) Correlation of defense gene induction defects with powdery mildew susceptibility in Arabidopsis enhanced disease susceptibility mutants. *Plant J.* **16**, 473–485.

Rosso, M.G., Li, Y., Strizhov, N., Reiss, B., Dekker, K. and Weisshaar, B. (2003) An *Arabidopsis thaliana* T-DNA mutagenized population (GABI-Kat) for flanking sequence tag-based reverse genetics. *Plant Mol. Biol.* **53**, 247–259.

Rushton, P.J., Torres, J.T., Parniske, M., Wernert, P., Hahlbrock, K. and Somssich, I.E. (1996) Interaction of elicitor-induced DNA-binding proteins with elicitor response elements in the promoters of parsley PR1 genes. *EMBO J.* **15**, 5690–5700.

Rustérucci, C., Aviv, D., Holt, B.F. III, Dangl, J. and Parker, J.E. (2001) The disease resistance signaling components EDS1 and PAD4 are essential regulators of the cell death pathway controlled by LSD1 in Arabidopsis. *Plant Cell*, **13**, 2211–2224.

SAS Institute (1999) *The SAS System for Windows, Release 8.0*. Cary, NC: SAS Institute.

Sessions, A., Burke, E., Presting, G. *et al.* (2002) A high-throughput Arabidopsis reverse genetics system. *Plant Cell*, **14**, 2985–2994.

Spoel, S.H., Koornneef, A., Claessens, S.M. *et al.* (2003) NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. *Plant Cell*, **15**, 760–770.

Thomma, B., Eggermont, K., Penninckx, I., Mauch-Mani, B., Vogelsang, R., Cammue, B.P.A. and Broekaert, W.F. (1998) Separate jasmonate-dependent and salicylate-dependent defense-response pathways in Arabidopsis are essential for resistance to distinct microbial pathogens. *Proc. Natl Acad. Sci. USA* **95**, 15107–15111.

Thomma, B.P., Eggermont, K., Tierens, K.F. and Broekaert, W.F. (1999) Requirement of functional ethylene-insensitive 2 gene for efficient resistance of Arabidopsis to infection by *Botrytis cinerea*. *Plant Physiol.* **121**, 1093–1102.

Tierens, K.F., Thomma, B.P., Bari, R.P., Garmier, M., Eggermont, K., Brouwer, M., Penninckx, I.A., Broekaert, W.F. and Cammue, B.P. (2002) *Esa1*, an *Arabidopsis* mutant with enhanced susceptibility to a range of necrotrophic fungal pathogens, shows a distorted induction of defense responses by reactive oxygen generating compounds. *Plant J.* **29**, 131–140.

Veronese, P., Chen, X., Bluhm, B., Salmeron, J., Dietrich, R.A. and Mengiste, T. (2004) The BOS loci of Arabidopsis are required for resistance to *Botrytis cinerea* infection. *Plant J.* **40**, 558–574.

Veronese, P., Nakagami, H., Bluhm, B., AbuQamar, S., Chen, X., Salmeron, J., Dietrich, R.A., Hirt, H. and Mengiste, T. (2006) Distinct roles of the membrane anchored *BOTRYTIS INDUCED KINASE 1* in Arabidopsis resistance to necrotrophic and biotrophic pathogens. *Plant Cell*, **18**, 257–273.

Ward, E.R., Uknes, U., Williams, S.C., Dincher, S.S., Wiederhold, D.L., Alexander, D.C., Ahl-Goy, P., Metraux, J.P. and Ryals, J.A. (1991) Coordinate gene activity in response to agents that induce systemic acquired resistance. *Plant Cell*, **3**, 1085–1094.

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

Xie, D.X., Feys, B.F., James, S., Nieto-Rostro, M. and Turner, J.G. (1998) COI1: an Arabidopsis gene required for jasmonate-regulated defense and fertility. *Science*, **280**, 1091–1094.

Xu, X., Chen, C., Fan, B. and Chen, Z. (2006) Physical and functional interactions between pathogen-induced Arabidopsis WRKY18, WRKY40, and WRKY60 transcription factors. *Plant Cell*, **18**, 1310–1326.

Yang, Y., Shah, J. and Klessig, D.F. (1997) Signal perception and transduction in plant defense responses. *Genes Dev.* **11**, 1621–1639.

Yu, D., Chen, C. and Chen, Z. (2001) Evidence for an important role of WRKY DNA binding proteins in the regulation of NPR1 gene expression. *Plant Cell*, **13**, 1527–1540.

Zheng, L., Campbell, M., Murphy, J., Lam, S. and Xu, J.R. (2000) The BMP1 gene is essential for pathogenicity in the gray mold fungus *Botrytis cinerea*. *Mol. Plant Microbe Interact*, **13**, 724–732.

