CHAPTER 5

EFFECTS OF KNOCKOUT AND OVEREXPRESSION OF ATWRKY50 AND ATWRKY28 IN TRANSGENIC PLANTS

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ABSTRACT

Transcription factors AtWRKY50 and AtWRKY28 specifically bind to the promoter of the *PR-1* gene and activate *PR-1::GUS* reporter genes in protoplast transactivation assays. Here we have studied the effects of overexpression or T-DNA knockouts of the WRKY genes in transgenic Arabidopsis plants. Overexpression of the genes did not result in enhanced expression of *PR-1* in non-induced plants, but salicylic acid (SA) treatment resulted in higher levels of *PR-1* mRNA accumulation in plants overexpressing *AtWRKY50* than wild type plants. For the plants overexpressing *AtWRKY28,* SA treatment had the opposite effect. No conclusive results were obtained for the effect of overexpression or knockout of the *WRKY* genes on resistance against *Botrytis cinerea* and *Pseudomonas syringae*.

INTRODUCTION

Upon pathogen attack plants mobilize inducible defense systems. A classic example is the systemic acquired resistance (SAR) effective against a broad range of pathogens. The signal transduction route leading to SAR involves the induced synthesis of the endogenous signal molecule salicylic acid (SA). SAR is accompanied by the de novo synthesis of pathogenesis-related (PR) proteins of which many directly affect pathogen growth and disease proliferation. Although their exact function is still not characterized, the plant-wide conserved PR-1 proteins are generally considered as marker proteins for SAR. In most plant species expression of the *PR-1* genes is under transcriptional control (Van Loon and Van Strien, 1999).

The promoters of several *PR* genes, such as Arabidopsis thaliana *PR-1*

and tobacco *PR-1a* contain as-1-(like) elements in promoter regions important for SA-induced expression. A linker scanning analysis of the region of the Arabidopsis *PR-1* promoter responsible for induced expression by the SA analog INA revealed the presence of an as-1 element with two TGACG direct repeats of which one is a positive regulatory element (LS7), while the other (LS5) mediates negative regulation of *PR-1* expression (Lebel *et al*., 1998). Through knockout analyses it was shown that the Arabidopsis bZIP transcription factors TGA2, TGA3, TGA5 and TGA6 act as redundant but essential activators of *PR-1* expression and SAR (Zhang *et al*., 2003; Kesarwani *et al*., 2007). In addition to TGAs, WRKY transcription factors are important for transcriptional programs induced in response to environmental signals (Eulgem and Somssich, 2007; Pandey and Somssich, 2009). Unlike the TGA transcription factors that are present at steady state levels (Johnson *et al*., 2003), many of the *WRKY* genes are transcriptionally activated upon biotic and abiotic stress. Of the 74 *WRKY* genes in Arabidopsis, 49 were differentially expressed upon *Pseudomonas syringae* infection or treatment with SA (Dong *et al*., 2003). Many WRKY proteins bind to the W-box, a DNA motif with the core sequence TTGAC(T/C) and the overrepresentation of this motif in several *WRKY* genes suggests their expression is regulated by WRKY transcription factors. However, for several *WRKY* genes, SA-induced expression is dependent on NPR1 and TGAs, suggesting a similar activation strategy as was originally proposed for *PR-1* (Dong *et al*., 2003; Wang *et al*., 2006).

In the same linker scanning study that identified the two as-1-like regulatory elements in the Arabidopsis *PR-1* promoter, a consensus W-box motif with a strong negative effect was identified, suggesting WRKY factors to be important for SA-mediated *PR-1* gene expression (Lebel *et al*., 1998). In the previous chapters AtWRKY50 and AtWRKY28 were identified as WRKY transcription factors that specifically bound and activated the *PR-1* promoter.

Here we generated transgenic plants overexpressing AtWRKY50 and AtWRKY28, and *AtWRKY50* T-DNA knockout plants to study the effects of the WRKYs on *PR-1* gene expression and on infection by necrotrophic and biotrophic pathogens.

RESULTS

The *AtWRKY50* and *AtWRKY28* coding regions were amplified by RT-PCR on total RNA isolated from SA-treated Arabidopsis and cloned behind the *35S* promoter. After flower-dip transformation of Arabidopsis Col-0, 20 primary, hygromycin-resistant seedlings were selected for further analysis. The seedlings were transferred to soil and grown through flowering and seed set. T2 generation plants were grown for 18 of the AtWRKY50 lines and 15 AtWRKY28 lines. None of the lines produced plants that were phenotypically different from wild type Arabidopsis. The Northern blots of Fig. 1 show the expression levels of the transgenes in the plants of the T2 generation. The absence of bands in wild type Arabidopsis indicates that the expression levels of the *AtWRKY50* (Fig. 1, Panel A) and *AtWRKY28* (Fig. 1, Panel B) genes are below the level of detection, whereas a band corresponding to *AtWRKY50* mRNA is visible in all AtWRKY50 overexpression lines and similarly is *AtWRKY28* mRNA present in most of the AtWRKY28 overexpression lines. This demonstrates that the transgenes are expressed in most lines, although the expression levels vary considerably. For further analyses *AtWRKY50* overexpression lines W50#2, #8, #12 and #13, and *AtWRKY28* overexpression lines W28#2, #4 and #12 were selected for further analyses.

In addition to plants overexpressing *AtWRKY50*, homozygous plants were generated in which the gene was knocked-out through a T-DNA insertion; plants of knockout line w50#2 were used in this study. Because of the high

similarity between AtWRKY50 and AtWRKY51, we also crossed homozygous T-DNA insertion lines of both genes to obtain double homozygous plants; plants of lines w50w51#2 and #12 were used here. For all these lines the presence of the T-DNA insert and the absence of alleles containing intact genes was confirmed by PCR (data not shown).

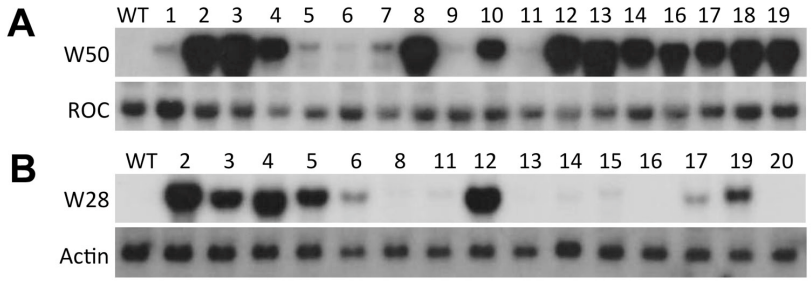

Figure 1. Transgene expression levels in transgenic plants. Northern blots containing total RNA extracted from hygromycin resistant seedlings generated from flower-dip transformed Arabidopsis were hybridized to a cDNA probe corresponding to *AtWRKY50* (A) and *AtWRKY28* (B). To check equal loading, identical blots were hybridized with probes corresponding to constitutive house-keeping genes *At4G38740* encoding rotamase cyclophilin (ROC) and *At3G18780* encoding actin 2 (Actin), respectively. Numbers above the lanes indicate the transgenic line. Samples from non-transformed Arabidopsis were electrophoresed in lanes WT.

In the previous chapters it was shown that AtWRKY50 and AtWRKY28 were each able to specifically interact with the *PR-1* promoter and activate gene expression in protoplasts. Fig. 2 shows the results of *PR-1* gene expression analyses in plants of lines W50#2 and W28#2. As was shown before, expression of *AtWRKY50* and *AtWRKY28* was below the detection level in non-induced wild type plants, but expression was induced by treatment with SA and accumulation of the corresponding mRNAs reached high levels at 6h (*AtWRKY28*) and 24h (*AtWRKY50*) after application of SA, preceding and concomitantly with *PR-1* gene expression, respectively (Chapters 2 and 4). The Northern blot in Fig. 2A shows that constitutive expression of AtWRKY50 in

the transgenic W50#2 plants, did not result in a measurable increase in *PR-1* expression, but that accumulation of *PR-1* mRNA upon SA treatment reached higher levels in the transgenic plants. Similarly, constitutive expression of AtWRKY28 did not lead to *PR-1* expression in non-treated W28#2 plants. However, in these plants SA treatment led to reduced accumulation of *PR-1* mRNA at 16h post treatment. For a more quantitative result, *PR-1* mRNA accumulation upon SA treatment was measured by qRT-PCR. The results are shown in Fig. 2B and 2C. For each of the samples the accumulation of *PR-1* transcript was calculated based on the cycle threshold (Ct) values at the indicated time points relative to that of the transcript of housekeeping gene *At1G13320* (Czechowski *et al.*, 2005).

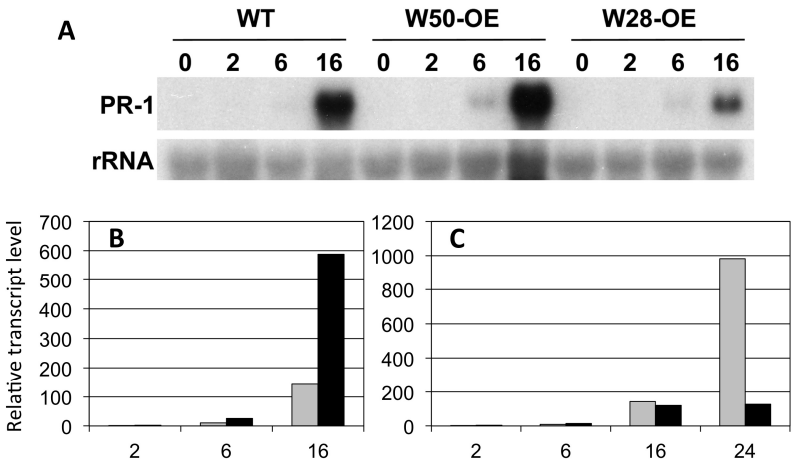

Figure 2. *PR-1* expression in WRKY overexpressing plants. (A) Time course (hours) of salicylic acid-induced *PR-1* mRNA accumulation in wild type Arabidopsis and in transgenic plants overexpressing *AtWRKY50* (W50-OE) or *AtWRKY28* (W28-OE). The band corresponding to ribosomal 25S RNA is shown as a loading control. (B, C) *PR-1* mRNA accumulation in wild type (grey bars) and transgenic (black bars) plants overexpressing *AtWRKY50* (B) or *AtWRKY28* (C) after incubation for the indicated times in 1mM salicylic acid. Transcript levels are given as 2^-ΔΔCt values relative to that of the *At1G13320* reference gene.

The results show that *PR-1* mRNA accumulation was higher in the W50#2 plants at all-time points and increased to 4-fold the level in wild type plants at 16h post treatment. Interestingly, in the W28#2 plants *PR-1* expression was higher than in wild type plants at early time points (2 and 6h post treatment), but at later time points *PR-1* mRNA accumulation leveled off. *PR-1* expression is induced upon infection by biotrophic pathogens and correlated with SAR. While the role of the PR-1 protein in enhanced defense is not clear, other PR proteins that are co-regulated with PR-1 possess antifungal activities that have been suggested to contribute to SAR (Ferreira *et al.*, 2007).

To investigate the possible role of AtWRKY50 in defense against pathogen attack, we investigated the effect of infection with the necrotrophic fungal pathogen *Botrytis cinerea* and the biotrophic bacterial pathogen *Pseudomonas syringae* of plants that constitutively express *AtWRKY50* or *AtWRKY28*, or that contain knockout *AtWRKY50* genes. The results of the *B. cinerea* assay are shown in Fig. 3. In Panel B the disease severity was scored three days after inoculation of the plants on the basis of the disease symptom index shown in Panel A. While W50#2 and W28#2 plants did not show disease scores that were statistically different from wild type Arabidopsis, the number of leaves of W50#8 and W28#4 plants showing symptoms was significantly less than that of wild type plants. However, the fact that for both types of overexpressors one line appeared less sensitive to infection with *Botrytis*, while the other did not, does not permit drawing conclusions on the role of the WRKYs on defense against *Botrytis*. Results of the infection assays with the biotrophic bacterial pathogen *P. syringae* pv. tomato DC3000 are shown in Fig. 4. In this experiment the scoring index was limited to either chlorotic symptoms or absence of symptoms (Fig. 4A). The disease scoring between the lines ranged between 30% and 70%, with the double knock out w50w51 lines and the overexpressing W28 lines showing somewhat less symptoms than wild type

plants and lines overexpressing *AtWRKY50* (Fig. 4B). The level of infection was also scored by determining the bacterial multiplication in leaf extracts of the infected plants at 3 days after inoculation. The results are shown in Fig. 5. Over all, no big differences were apparent between the different lines; although knock-out line w50w51#7 and overexpressor line W28#12 had slightly lower colony counts than the wild type, differing significantly at a P of ≤0.05 by one-way ANOVA.

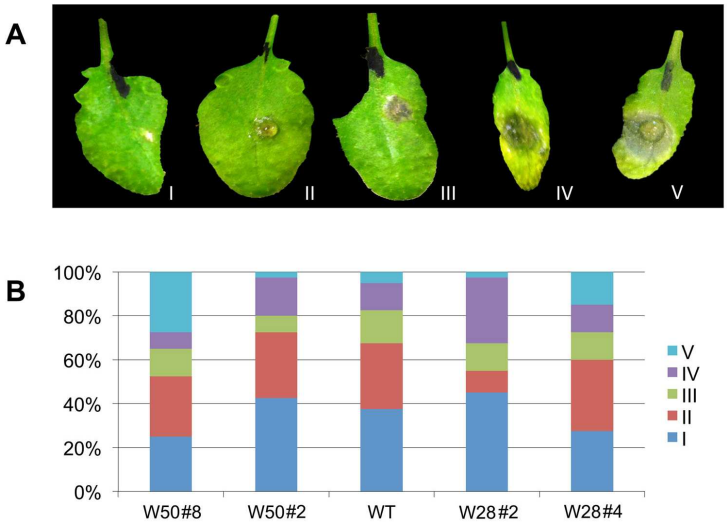

Figure 3. *Botrytis cinerea* infection assays. Disease ratings were assigned to the inoculated leaves of each plant, as described by Ton *et al.* (2002). (A) Intensity of disease symptom and lesion size was classified: 1, no visible disease symptom; 2, non-spreading lesion; 3, spreading lesion; 4, spreading lesion surrounded by a chlorotic halo; and 5, spreading lesion with extensive tissue maceration. (B) Symptoms of infection were scored 3 days after inoculation in wild type (WT) and transgenic plants of two lines each overexpressing *AtWRKY50* or *AtWRKY28*. Ratings are graphically displayed as 100% stacked columns. The differences between the genotypes were analyzed by Pearson Chi-square test.

DISCUSSION

As markers for SAR, the PR-1 proteins have since long been considered to be

involved in induced plant defense against attack by biotrophic pathogens, like many other PR proteins that are induced during the defense response. However, although other members of the group of PR proteins have been characterized as chitinases, β-1,3-glucanases or membrane leakiness provoking proteins with antifungal activities, a function for PR-1 proteins has yet to be determined (Linthorst *et al.*, 1989; Cutt *et al.*, 1989; Alexander *et al.*, 1993; Van Loon and Van Strien, 1999).

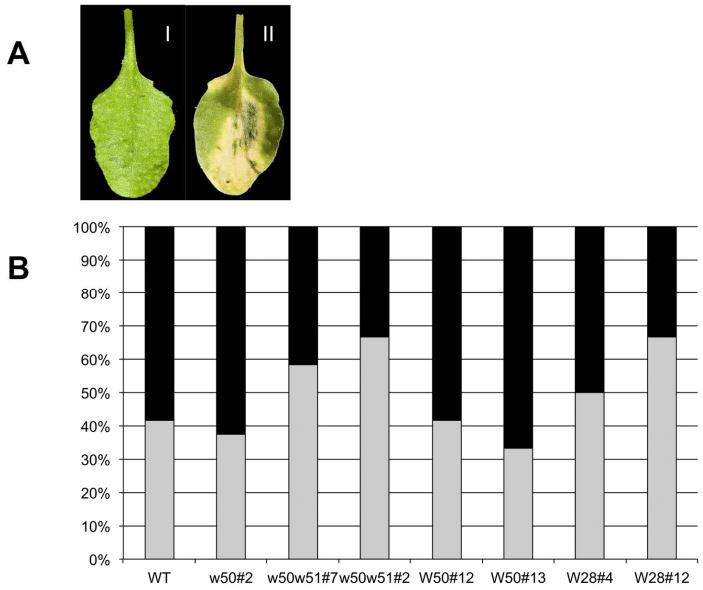

Figure 4. *Pseudomonas syringae* infection assay. Disease ratings were assessed at day 3 after infiltration. (A) Leaves were either scored as symptomless (I) or as chlorotic symptoms (II). (B) Disease ratings were assigned to each of three infiltrated leaves of 8 wild type plants (WT) and 8 plants each of lines over-expressing AtWRKY50 (W50#12 and W50#13), AtWRKY28 (W28#4 and W28#12), or knockout lines of AtWRKY50 (w50#2), or the combination of AtWRKY50 and AtWRKY51 (w50w51#2 and w50w51#7). Ratings are graphically displayed as 100% stacked columns (grey bars: no symptoms, black bars: symptoms). The differences between the genotypes were analyzed by Pearson Chi-square test

In the previous chapters we identified AtWRKY50 and AtWRKY28 as DNA-binding proteins that specifically bound to the *PR-1* promoter and activated the

expression of reporter genes under the control of this promoter in Arabidopsis protoplasts. The genes encoding these WRKY proteins were induced by treatment with SA, the signal molecule that mediates the defense response. Together these findings prompted further functional analyses of AtWRKY50 and AtWRKY28, of which we have described initial results in this chapter.

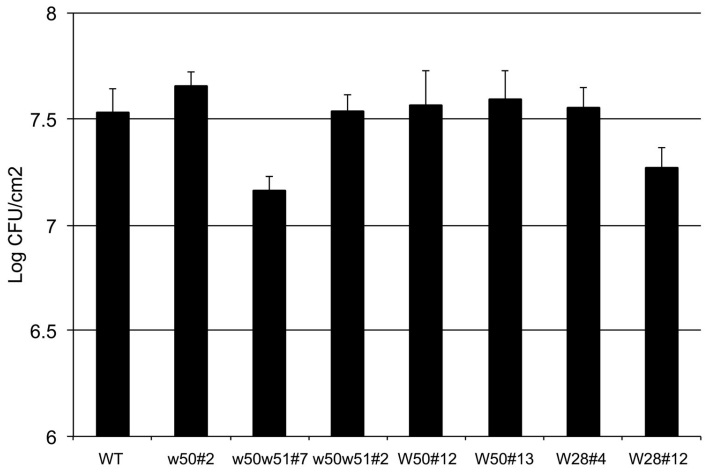

Figure 5. *Pseudomonas syringae* infection assay. Colony-forming units (CFU) of infected leave extracts from wild type plants and from transgenic plants of lines overexpressing WRKY genes or knockout lines as used in Fig. 4 were scored three days after infiltration with bacterial inoculum. Significance was assessed using One-way ANOVA.

Transgenic plants transformed with *AtWRKY50* and *AtWRKY28* genes under the control of the strong, constitutive *35S* promoter expressed high levels of the corresponding mRNAs. Under laboratory conditions, these plants appeared not phenotypically different from wild type plants. Assuming that the respective *WRKY* mRNAs were translated, this suggests that the transcription factors did not interfere with normal plant functions. In any case, the expression of the *WRKY* genes did not result in enhanced levels of *PR-1* gene expression under non-inducing conditions. However, although we haven't yet confirmed this with more transgenic lines, overexpression of *AtWRKY50* in line W50#2

resulted in a higher expression of *PR-1* at 2h, 6h and 16h of treatment with SA. This indicates that on its own, AtWRKY50 cannot trigger *PR-1* expression, but once expression is initiated, the high levels of AtWRKY50 in the transgenic plant augment *PR-1* expression. In Chapter 2 we identified two sites in the *PR-1* promoter that specifically bound the DNA-binding domain of AtWRKY50. One of these sites was located in promoter element LS10, which has a strong positive effect on *PR-1* expression (Lebel *et al.*, 1998; Pape *et al.*, 2010), suggesting that the enhanced expression of *PR-1* in the overexpression plants is mediated through AtWRKY50's binding to this element.

In Chapter 4 we found that AtWRKY28 strongly enhanced *PR-1::GUS* expression in protoplast transactivation assays. Here we observed that in transgenic W28#2 plants overexpressing AtWRKY28, SA-induced *PR-1* mRNA accumulation was reduced in comparison to the expression in wild type plants, suggesting a role for AtWRKY28 as a transcriptional repressor of *PR-1*. An explanation for these apparently contradictory results could possibly be related to the different conditions of the two in vivo assay systems. However, other WRKYs have also been found to have opposite effects on gene expression. Examples are AtWRKY6 and AtWRKY53, which dependent on the promoter context, activate or repress gene expression (Robatzek and Somssich, 2002; Miao *et al.*, 2008). The W-box in LS4 is one of the binding sites of AtWRKY28 in the *PR-1* promoter (Chapter 4). Since the LS4 element was previously found to have a repressing effect on *PR-1* expression (Lebel *et al.*, 1998; Pape *et al.*, 2010), this suggests that the effect of AtWRKY28 could be mediated through this element. Future analyses with more overexpression and knockout lines are required to confirm these results.

Although several studies have failed to discover a clear anti-pathogen activity for PR-1 proteins of tobacco, as far as we know, a possible anti-pathogen effect of Arabidopsis PR-1 has not yet been extensively investigated.

Furthermore, AtWRKY50 and/or AtWRKY28 could possibly also be involved in regulation of other genes functioning in defense. Indeed, the sequence GACTTTTC is present in the promoter of the Arabidopsis *BGL2* gene encoding PR-2 and we determined that the region of the BGL2 promoter that contains this sequence binds AtWRKY50 in EMSA (data not shown). Nevertheless, the infection assays failed to convincingly show enhanced resistance to the necrotrophic fungal pathogen *B. cinerea* or the biotrophic bacterial pathogen *P. syringae* (Figs. 3, 4, 5). Whether the WRKYs play a role in defense against other pathogens or stresses awaits further studies.

MATERIALS AND METHODS

Construction of T-DNA mutant Plants

T-DNA knockout lines for *wrky50* (GK-650F10.01) and *wrky51* (SALK_022198) were obtained from NASC. Pollen from homozygous *wrky50* plants were used to pollinate emasculated homozygous *wrky51* flowers. F1 seedlings were grown without selection and genotyped with GABI-LB for *wrky50* and LBb1.3 for *wrky51*. The primer for genotyping was GABI-LB: 5′-GGG CTA CAC TGA ATT GGT AGC TC-3′ and for LBb1.3: 5′-ATT TTG CCG ATT TCG GAA C-3′. The gene primers used to check for homozygosity were for wrky50: 5′-GGA GGG ATG AAT AAT CCA TGG-3′ and for wrky51: 5′-TTG CTT TCA AAC CAT GCT TTG-3′. Both sets of primer were used to identify double homozygous (*wrky50wrky51*) individuals.

Construction of T-DNA plasmids and transformation of Arabidopsis

For the construction of transgenic lines constitutively overexpressing AtWRKY50 (At5g26170) The PCR was amplified by using these primer sets; 5′-ATA GCT CGA GGT ATG AAT GAT GCA GAC ACA AAC TTG-3′ and 5′-

GCC TCT AGA CGA GTC TTA GTT CAT GCT TGA GTG ATT GTG-3′ and Arabidopsis cDNA library (6h SA treated) was used a template, digested with XhoI and XbaI to clone in pRT101. The AtWRKY28 (At4g18170) open reading frame was PCR–amplified from Arabidopsis cDNA library (6h SA treated Arabidopsis) using the primer sets to cloned into pRT101. The primer sequences were WRKY28: 5′-GTC ACT CGA GAT GTC TAA TGA AAC CAG AGA TCT CTA C-3′ and 5′-CAG TGG ATC CTC AAG GCT CTT GCT TAA AGA AAA TTG-3′. The Cauliflower Mosaic Virus (*CaMV*) 35S cassette containing the *WRKY's* ORF in sense orientation was digested from pRT101 and cloned in pCAMBIA1300 (Acc. No. Af234296). The binary vector pCAMBIA1300-*WRKYs* was introduced into *Agrobacterium tumefaciens* strain containing the Vir plasmid. Arabidopsis plants were transformed using the floral dip method (Clough and Bent, 1998). Transgenic plants were selected on solid MA medium containing 100 mg/L timentin and 20 mg/L hygromycin. Transgenic plants from T2 generations were selected on MA medium containing only 20 mg/L hygromycin.

Growth of plant materials

Arabidopsis thaliana ecotype Columbia (Col-0) seeds were surface sterilized by incubation for 1 min in 70% ethanol, 15 min in 50% bleach, and five rinses with sterile water. Alternatively, seeds were surface-sterilized in a closed container with chlorine gas for 3-4 hours. Surface-sterilized seeds were grown on plates containing MA (Masson and Paszkowski, 1992) medium supplemented with 0.6% agar. Following stratification for 3 days at 4^0C, seeds were incubated at 21^0C in a growth chamber (16h light / 8h dark, 2500 lux) for 10-12 days. Immediately after harvesting, the material was frozen in liquid nitrogen and kept at -80°C until use.

RNA extraction and Northern blot analyses

Total RNA was extracted from the 10-12 days old seedlings that were frozen in liquid nitrogen by phenol/chloroform extraction followed by overnight precipitation with 8M lithium chloride, washed with 70% ethanol, and re-suspended in water. For RNA blot analysis 10µg RNA samples were subjected to electrophoresis on 1.5% agarose, 1% formaldehyde gels and blotted to Gene Screen nylon membranes (Perkin-Elmer Life Sciences). All probes were ^{32}P-labelled by random priming. Pre-hybridization of blots, hybridization and subsequent washings were performed as described (Memelink *et al.*, 1994), with minor modifications. Blots were exposed on X-ray films (Fuji, Tokyo). DNA fragments used as probes were PCR amplified using the sets of primers used to clone the gene for overexpressing.

***B. cinerea* infection assay**

B. cinerea was grown on potato dextrose agar plates for 2 weeks at 22°C. Spores were harvested as described by Broekaert *et al.* (1990). Plant seedlings germinated on plates were transferred to individual pots containing sterile soil and randomly distributed in trays. Seedlings were cultivated for another 3 weeks in a growth chamber with an 8 h day (1400 lux at 24°C) and 16 h night (20°C) cycle at 65% humidity. For inoculation with fungal pathogens, 5 µL droplets of spore suspension were deposited on two matured leaves of each plant. Inocula consisted of 7.5 X 10^5 spores/mL. *B. cinerea* spores were incubated in half strength potato dextrose broth for 2 hours prior to inoculation. After inoculation, plants were maintained under high relative humidity with the same temperature and photoperiod conditions. In each experiment, 20 plants per genotype were inoculated. Control plants were not inoculated but kept under the same growing conditions. Disease ratings were assessed at day 2 and day 3 after inoculation with *B. cinerea*. Disease ratings were assigned to the

inoculated leaves of each plant, as described by Ton *et al.* (2002). Intensity of disease symptom and lesion size was classified: 1, no visible disease symptom; 2, non-spreading lesion; 3, spreading lesion; 4, spreading lesion surrounded by a chlorotic halo; and 5, spreading lesion with extensive tissue maceration. Disease resistance test were performed at the same time for all genotypes. The differences between the genotypes were analyzed by Pearson Chi-square test.

***P. syringae* infection assay**

Inoculations with the bacterial leaf pathogen *P. syringae* pv. tomato DC3000 were performed as described previously (Van Wees *et al.*, 1999). Briefly, *P. syringae* pv. tomato DC3000 with the plasmid pV288 carrying avirulence gene avrRpt2 (Kunkel *et al.*, 1993) was cultured overnight at 28°C in liquid King's medium B (King *et al.*, 1954), supplemented with kanamycin at 25 mg L^{-1} to select for the plasmid. Subsequently, bacterial cells were collected by centrifugation and resuspended in 10mM $MgSO_4$ to a final density of 10^7 CFU ml^{-1}. Wild-type Col-0 plants were inoculated by pressure infiltrating a suspension of *P. syringae* at 10^7 CFU ml^{-1} into fully expanded leaves of 5-week-old plants. After infiltration, plants were maintained under high relative humidity at the same temperature and photoperiod conditions. In each experiment, 3 leaves of 8 plants per genotype were infiltrated. Control plants were infiltrated with 10 mM $MgSO_4$ and kept under the same growing conditions. Disease ratings were assessed at day 2 and day 3 after infiltration. Disease ratings were assigned to the infiltrated leaves of each plant. Disease resistance tests were performed at the same time for all genotypes. The differences between the genotypes were analyzed by Pearson Chi-square test. The CFU scores from the leaf extracts at three days after infiltrations were assessed with One-way ANOVA.

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