the plant journal

SEB Society for Experimental Biology

The Plant Journal (2013) **73,** 483–495 doi: 10.1111/tpj.12051

AtMYB44 regulates *WRKY70* expression and modulates antagonistic interaction between salicylic acid and jasmonic acid signaling

Jae Sung Shim[1], Choonkyun Jung[1], Sangjoon Lee[1], Kyunghun Min[1], Yin-Won Lee[1], Yeonhee Choi[2], Jong Seob Lee[2], Jong Tae Song[3], Ju-Kon Kim[4] and Yang Do Choi[1,*]

[1]*Department of Agricultural Biotechnology, Seoul National University, Seoul 151-921, Korea,*
[2]*School of Biological Sciences, Seoul National University, Seoul 151-742, Korea,*
[3]*School of Applied Biosciences, Kyungpook National University, Daegu 702-701, Korea, and*
[4]*Division of Bioscience and Bioinformatics, Myongi University, Yongin 449-728, Korea*

Received 26 July 2012; revised 18 September 2012; accepted 8 October 2012; published online 10 December 2012.
*For correspondence (e-mail choiyngd@snu.ac.kr).

SUMMARY

The role of *AtMYB44*, an R2R3 MYB transcription factor, in signaling mediated by jasmonic acid (JA) and salicylic acid (SA) is examined. *AtMYB44* is induced by JA through CORONATINE INSENSITIVE 1 (COI1). *AtMYB44* over-expression down-regulated defense responses against the necrotrophic pathogen *Alternaria brassicicola*, but up-regulated *WRKY70* and *PR* genes, leading to enhanced resistance to the biotrophic pathogen *Pseudomonas syringae* pv. *tomato* DC3000. The knockout mutant *atmyb44* shows opposite effects. Induction of *WRKY70* by SA is reduced in *atmyb44* and *npr1-1* mutants, and is totally abolished in *atmyb44 npr1-1* double mutants, showing that *WRKY70* is regulated independently through both *NPR1* and *AtMYB44*. *AtMYB44* over-expression does not change SA content, but *AtMYB44* over-expression phenotypes, such as retarded growth, up-regulated *PR1* and down-regulated *PDF1.2* are reversed by SA depletion. The *wrky70* mutation suppressed *AtMYB44* over-expression phenotypes, including up-regulation of *PR1* expression and down-regulation of *PDF1.2* expression. β-estradiol-induced expression of *AtMYB44* led to *WRKY70* activation and thus *PR1* activation. AtMYB44 binds to the *WRKY70* promoter region, indicating that *AtMYB44* acts as a transcriptional activator of *WRKY70* by directly binding to a conserved sequence element in the *WRKY70* promoter. These results demonstrate that *AtMYB44* modulates antagonistic interaction by activating SA-mediated defenses and repressing JA-mediated defenses through direct control of *WRKY70*.

Keywords: R2R3 MYB transcription factor, jasmonate signaling, salicylate signaling, *WRKY70*, defense response, *Arabidopsis thaliana*.

INTRODUCTION

To cope with pathogen challenge, plants rapidly activate defense responses, which are regulated by the major signaling molecules salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). Accumulation of SA, JA or ET in response to pathogen invasion or herbivore attack activates distinct but overlapping sets of defense genes; complex networking among these signaling pathways also modulates defense responses to maximize effective defenses while minimizing cost to the plant (Reymond and Farmer, 1998; Kunkel and Brooks, 2002; Spoel *et al.*, 2003; Robert-Seilaniantz *et al.*, 2011).

JA plays a role in defense signaling against necrotrophic pathogens and herbivore attack (Thomma *et al.*, 1998; Turner *et al.*, 2002; Browse and Howe, 2008). Arabidopsis JA-mediated defense responses require the F-box protein CORONATINE INSENSITIVE 1 (COI1), which is a jasmonyl-isoleucine receptor (Xie *et al.*, 1998; Devoto *et al.*, 2002; Yan *et al.*, 2009; Sheard *et al.*, 2010). COI1 acts as part of the SCF^{COI1} complex to activate JA signaling by 26S proteasome-mediated degradation of jasmonate ZIM-domain (JAZ) proteins. JA induces degradation of JAZ proteins, and this degradation results in activation of JA-responsive gene expression (Chini *et al.*, 2007; Thines *et al.*, 2007; Chung and Howe, 2009). These JA-activated genes include that encoding the anti-microbial defensin PDF1.2, which acts against necrotrophic pathogens (Penninckx *et al.*, 1996). Ethylene, often together with JA, activates plant defenses to necrotrophic pathogens such as *Alternaria brassicicola* (Shan *et al.*, 2012).

SA plays a role in defense signaling distinct from that mediated by JA (Feys and Parker, 2000; Durrant and Dong, 2004). Accumulation of SA leads to up-regulation of defense-related genes including the pathogenesis-related (*PR*) genes *PR1*, *PR2* and *PR5*, and results in enhanced disease resistance against biotrophic pathogens (Gaffney *et al.*, 1993; Delaney *et al.*, 1994). SA-induced defense responses are mediated by an ankyrin repeat protein, NONEXPRESSOR OF PR1 (NPR1) (Cao *et al.*, 1997; Spoel *et al.*, 2003). However, *NPR1*-independent pathways have also been reported (Bowling *et al.*, 1997; Li *et al.*, 2004). For example, constitutive expression of *PR* genes in *cpr6* and *ssi2* was not compromised by the *npr1-1* mutation (Clarke *et al.*, 1998; Shah *et al.*, 2001).

In some cases, various defense signaling pathways act synergistically to enhance resistance against pathogen attack (van Wees *et al.*, 2000). In other cases, antagonistic interactions between defense signaling pathways provide focused resistance against pathogens (Kunkel and Brooks, 2002). One well-documented antagonistic interaction involves cross-talk between JA and SA. Early studies of the role of SA in tomato wounding responses revealed that exogenous SA suppressed JA-induced wound responses (Doherty *et al.*, 1988). In Arabidopsis, exogenous SA suppresses JA-dependent gene expression and defense responses against *A. brassicicola* infection (Spoel *et al.*, 2007). Transgenic plants harboring the *NahG* transgene encoding SA hydroxylase, which converts SA to catechol, showed enhanced expression of JA biosynthesis genes and defense genes during infection with *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) (Delaney *et al.*, 1994; Spoel *et al.*, 2003).

JA also suppresses SA signaling (Kunkel and Brooks, 2002). Treatment with exogenous JA inhibits the expression of SA-dependent genes (Niki *et al.*, 1998). Arabidopsis *mpk4* and *ssi2* mutants, which are impaired in JA-responsive gene expression, constitutively express SA-dependent genes and show enhanced disease resistance against *Pst* DC3000 and *Peronospora parasitica* (Petersen *et al.*, 2000; Kachroo *et al.*, 2001). The JA-insensitive mutant *coi1* also shows similar gene expression and disease resistance against *Pst* DC3000 (Kloek *et al.*, 2001).

A large set of transcription factors is involved in the regulation of plant defense (Riechmann *et al.*, 2000; Eulgem, 2005), and antagonistic interaction between SA and JA involves transcriptional reprogramming by a subset of these transcription factors. For example, WRKY transcription factors are defined by the highly conserved amino acid sequence WRKYGQK and are involved in plant defense responses. Several *WRKY* transcription factors, including *WRKY11*, *WRKY17* and *WRKY70*, play roles in antagonistic interaction between SA and JA (Li *et al.*, 2004; Journot-Catalino *et al.*, 2006). *WRKY70* plays a pivotal role in JA and SA responses (Li *et al.*, 2004). Expression of *WRKY70* is activated by SA but suppressed by JA. Over-expression of *WRKY70* leads to up-regulation of *PR* genes and down-regulation of *PDF1.2*, leading to enhanced resistance against biotrophic pathogens and enhanced susceptibility to necrotrophic pathogens.

MYB transcription factors contain a MYB domain consisting of up to four imperfect repeats of a 52 amino acid motif. Most plant MYB transcription factors belong to the R2R3-MYB family (Dubos *et al.*, 2010). Arabidopsis R2R3-MYB transcription factors have been implicated in abiotic stress responses and development (Dubos *et al.*, 2010). They also act in biotic stress responses; for example, *AtMYB30* acts as positive regulator of hypersensitive cell death (Vailleau *et al.*, 2002) and *AtMYB96* positively regulates accumulation of SA by activating *SID2* expression (Seo and Park, 2010). However, the contribution of R2R3-MYB transcription factors to regulation of the antagonistic interaction between JA and SA remains unclear.

AtMYB44 belongs to sub-group 22 of the R2R3 MYB transcription factor family. *AtMYB44* was rapidly induced by methyl jasmonate (MeJA) in Arabidopsis (Jung *et al.*, 2008). In this study, we characterized the role of *AtMYB44* in the defense responses mediated by SA and JA. It is shown that *AtMYB44* directly regulates *WRKY70*, and thus regulates *PR* genes. *AtMYB44* mediated modulation of the antagonistic interaction between SA and JA is also demonstrated by over-expression and mutation analysis.

RESULTS

AtMYB44 down-regulates defense responses against *A. brassicola*

To understand the function of *AtMYB44* in JA-mediated defense responses, we examined two previously characterized *AtMYB44* over-expressing lines: OX18 and OX21 (Jung *et al.*, 2008). To examine the JA-mediated defense responses of these plants, we challenged wild-type, *atmyb44* mutants and OX18 and OX21 over-expressing lines with the necrotrophic pathogen *A. brassicicola*. Wild-type plants showed limited necrosis at inoculation sites (Figure 1a). However, OX18 and OX21 over-expressing lines showed more severe disease symptoms with extended necrosis. The mean diameter of lesions in OX18 and OX21 plants caused by *A. brassicicola* infection was approximately six times larger than that of wild-type plants. By contrast, *atmyb44* mutant plants showed reduced lesion size.

To determine whether the altered lesion size and necrosis resulted from changes in the growth of fungi in plants, the amount of fungal DNA in infected leaves was measured by quantitative PCR using *A. brassicicola* and Arabidopsis gene-specific primers. The level of *A. brassicicola*-specific DNA in OX18 and OX21 plants was approximately 30 times higher than in wild-type plants (Figure 1b). Consistent with their reduced lesion formation phenotype, the amount of

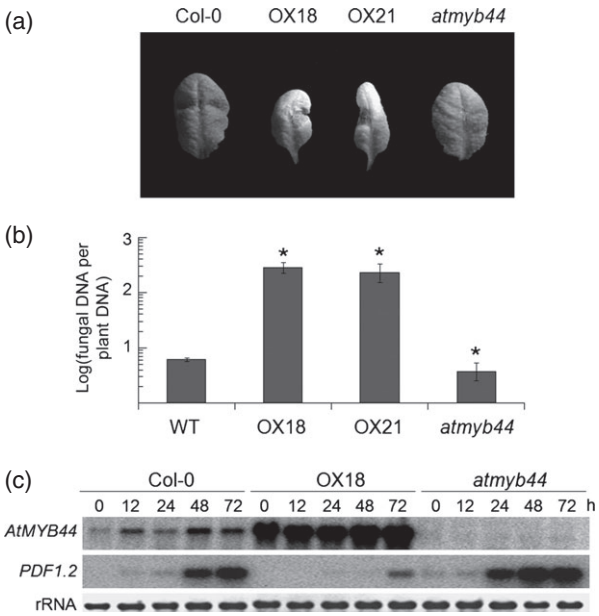

Figure 1. Suceptibility of *AtMYB44* over-expressing and *atmyb44* knockout mutant plants to the necrotrophic pathogen *A. brassicicola.*
(a) Four-week-old Arabidopsis plants were inoculated with *A. brassicicola*. OX18 and OX21 are *AtMYB44* over-expressing lines (Jung *et al.*, 2008). The photograph was taken 10 days after inoculation.
(b) Quantification of fungal growth by determination of the relative amount of fungal DNA compared with plant DNA using quantitative PCR. Values are means ± standard deviation. The experiments were repeated twice with similar results. The statistical significance of the measurements was determined using Student's *t* test (*$P < 0.01$) for comparison with the wild-type value.
(c) Four-week-old Arabidopsis plants were inoculated with *A. brassicicola*. Inoculated plants were collected for RNA extraction and analyzed by Northern blot.

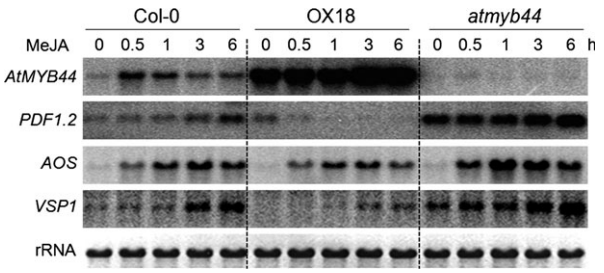

Figure 2. *AtMYB44* negatively regulates expression of JA-mediated genes. Two-week-old plants were treated with MeJA and analyzed by Northern blot with the indicated probes. rRNA was visualized by ethidium bromide staining as a loading control.

fungal DNA in *atmyb44* knockout plants was lower than in wild-type plants by a small but significant amount ($P < 0.01$).

The enhanced susceptibility to *A. brassicicola* in OX18 and OX21 plants and the increased resistance in *atmyb44* knockout mutants suggest that *AtMYB44* negatively regulates JA-mediated defenses responses to necrotrophic *A. brassicicola*. To test whether these altered disease responses depend on JA signaling, expression of the defense marker gene *PDF1.2* was examined (Figure 1c). The *AtMYB44* transcript was detected 12 h after infection in wild-type plants (Figure 1c), and expression of *PDF1.2* was induced later, 48 h after infection (Figure 1c). *PDF1.2* induction was clearly reduced in OX18 plants. By contrast, *PDF1.2* expression was induced earlier in *atmyb44* knockout plants than in wild-type plants. These results show that resistance against *A. brassicicola* and *PDF1.2* induction are inversely correlated with *AtMYB44* expression.

AtMYB44 is a negative regulator in JA signaling pathways

We next tested whether gene expression patterns induced by MeJA treatment are consistent with those produced by *A. brassicicola* infection in *AtMYB44* over-expression lines and *atmyb44* knockout mutants. Over-expression of *AtMYB44* led to delayed and reduced expression of the JA-responsive genes *ALLENE OXIDE SYNTHASE (AOS), VEGETATIVE STORAGE PROTEIN 1 (VSP1)* and *PDF1.2* after MeJA treatment (Figure 2). In contrast to the over-expression phenotype, knockout mutation of *AtMYB44* enhanced MeJA-mediated expression of these genes. Basal expression levels of *VSP1* and *PDF1.2* were constitutively up-regulated in *atmyb44* knockout plants.

AtMYB44 also affected JA-mediated growth responses. Compared to wild-type, OX18 plants are less sensitive and *atmyb44* knockout plants are more sensitive to JA-mediated root growth inhibition and root hair formation at submicromolar concentrations (Figure S1). Taken together, these results demonstrate that *AtMYB44* negatively regulates various JA-mediated responses.

AtMYB44 enhances disease resistance against *Pst* DC3000

JA and SA signaling pathways mutually antagonize each other (Kunkel and Brooks, 2002), and *AtMYB44* acts as a negative regulator of JA responses. Therefore, we next tested whether *AtMYB44* is involved in SA-mediated bacterial defense responses by testing *AtMYB44* mutant and over-expressing lines for resistance to the biotrophic bacterial pathogen *Pst* DC3000. We found that over-expression of *AtMYB44* led to enhanced resistance to *Pst* DC3000 (Figure 3a), and *atmyb44* knockout mutation led to slightly increased susceptibility. To test whether resistance resulted from inhibited pathogen growth, bacterial growth in infected leaves was measured by a colony-counting assay. We found that the titer of *Pst* DC3000 in OX18 and OX21 plants was approximately ten times lower than that in wild-type plants (Figure 3b). In *atmyb44* knockout plants, the bacterial titer was slightly increased compared to wild-type plants. This result indicates that *AtMYB44* increases resistance to *Pst* DC3000.

PR genes are up-regulated in *AtMYB44* over-expressing plants

To investigate the enhanced resistance of OX18 and OX21 plants to *Pst* DC3000, we examined the expression levels

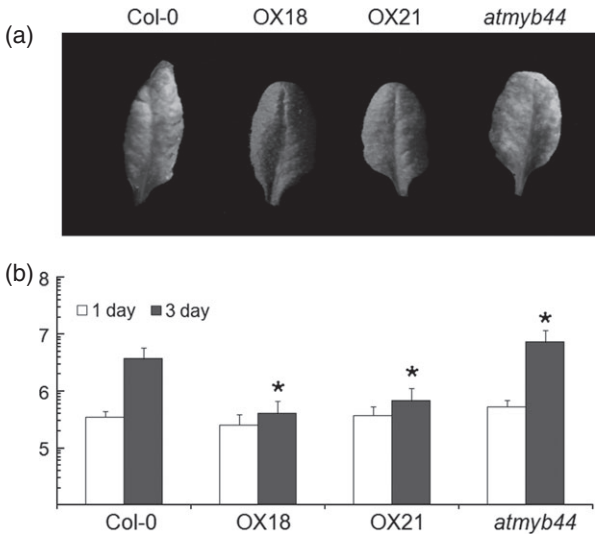

Figure 3. Resistance of *AtMYB44* over-expressing and *atmyb44* knockout mutant plants against the biotrophic pathogen *Pst* DC3000.
(a) Four-week-old Arabidopsis Col-0, *AtMYB44* over-expressing plants (OX18 and OX21) and *atmyb44* knockout mutant plants were inoculated with *Pst* DC3000. The photograph was taken 3 days after inoculation.
(b) Bacterial growth in leaves was determined 1 and 3 days after inoculation. The statistical significance of the measurements was determined using Student's *t* test (*$P < 0.01$) for comparison with the wild-type value. Values are means ± standard deviation for eight independent plants. The experiments were repeated twice and showed similar results.

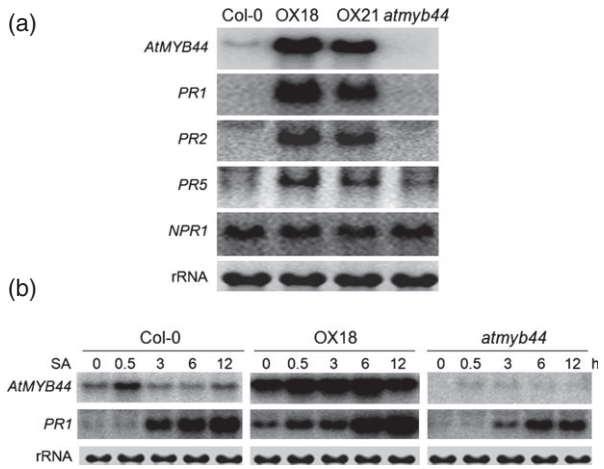

Figure 4. SA-mediated response in *AtMYB44* over-expressing and *atmyb44* knockout plants.
(a) Northern blot showing basal level expression of *PR* genes in Arabidopsis Col-0, *AtMYB44* over-expression (OX18 and OX21) and *atmyb44* knockout mutant plants.
(b) Effect of *AtMYB44* on induction of *PR1* by SA treatment. After SA treatment, total RNA was analyzed by Northern blot and rRNA was visualized by ethidium bromide staining as a loading control.

of *PR* genes, which participate in SA-mediated defense responses. Northern blot analysis demonstrated that *PR1*, *PR2* and *PR5* were constitutively over-expressed in *AtMYB44* over-expressing plants but were not expressed in untreated knockout and wild-type plants (Figure 4a).

Gene expression patterns induced by SA treatment were consistent with the observed changes in resistance against *Pst DC3000* infection in *AtMYB44* over-expression and *atmyb44* knockout mutant plants. In wild-type plants, *AtMYB44* was induced rapidly, within 30 min after SA treatment, but *PR1* was induced after approximately 3 h (Figure 4b). Even though the level of *PR1* was already higher in OX18 plants than that in wild-type plants, *PR1* was induced to a much higher level after SA treatment. In contrast, activation of *PR1* by SA treatment was reduced in *atmyb44* knockout plants. These results demonstrate that *AtMYB44* positively regulates SA-mediated defense responses, including activation of *PR* genes.

AtMYB44 regulates SA-mediated defense responses

AtMYB44 activates SA-mediated *PR* genes; to determine whether the effect of *AtMYB44* on *PR* gene expression is through SA signaling, we tested *AtMYB44* expression in *npr1-1* mutant and *NahG* plants. *AtMYB44* was rapidly induced by SA treatment in wild-type plants. Its induction was not affected in *npr1-1* plants, but was significantly reduced in *NahG* plants (Figure 5a). Therefore, *AtMYB44* is induced by SA but does not depend on *NPR1*. *WRKY70*, a key regulator in the SA signaling pathway, was also highly induced in wild-type plants, but was reduced in *npr1-1* and totally abolished in *NahG* plants.

To dissect the molecular components upstream and downstream of *AtMYB44* in the SA signaling pathway leading to *PR* gene expression, we determined the epistatic relationships between various components in the SA signaling pathway. OX18 plants were crossed with *npr1-1*, *NahG or sid2-2* mutant plants. *SID2* encodes an isochorismate synthase acting in SA biosynthesis. Activation of *PR1* expression by *AtMYB44* over-expression was slightly reduced in OX18 *npr1-1* plants but totally abolished in OX18 *NahG* and OX18 *sid2-2* plants (Figure 5b). These results indicate that *AtMYB44* depends on a basal level of SA to activate *PR1* expression.

To understand the role of *NPR1* and SA in *AtMYB44*-mediated disease resistance, we next challenged the OX18 *npr1-1* and OX18 *NahG* plants with *Pst* DC3000. Consistent with the expression patterns of *PR1* in Figure 5(b), bacterial resistance was enhanced in OX18 plants but completely compromised in the OX18 *NahG* plants. In OX18 *npr1-1* plants, resistance to *Pst* DC3000 was less than in OX18 but not as low as in OX18 *NahG* and wild-type (Figure 5c). Resistance of *npr1-1* and *NahG* to *Pst* DC3000 was decreased compared with Col-0 plants (Figure S2). The decreased resistance of OX18 *npr1-1* is probably due to disruption of *NPR1*-dependent pathways. *NPR1* is required for full-scale activation of *PR1* transcription mediated by *AtMYB44*. We measured the degree of resistance by quantification of bacterial growth in infected leaves (Figure 5c),

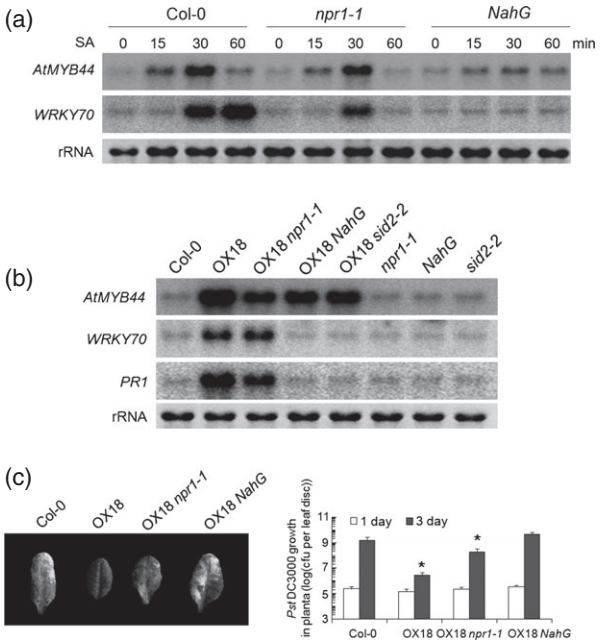

Figure 5. Role of *AtMYB44* in SA signaling.
(a) Expression of *AtMYB44* in SA signaling mutants. Plants were treated with SA for the indicated times and analyzed by Northern blot.
(b) *WRKY70* and *PR1* gene expression in SA signaling mutants in the background of *AtMYB44* over-expression. The OX18 *AtMYB44* over-expression line was crossed with SA signaling-deficient mutants or lines (*npr1-1*, *NahG* and *sid2-2*).
(c) Disease resistance against biotrophic pathogen *Pst* DC3000 in SA signaling mutants in the background of *AtMYB44* over-expression. Four-week-old Arabidopsis Col-0, *AtMYB44* over-expression plants (OX18), OX18 *npr1-1* and OX18 *NahG* plants were inoculated with *Pst* DC3000. The photograph was taken 3 days after inoculation. Bacterial growth in leaves was determined 1 and 3 days after infection. The statistical significance of the measurements was determined using Student's *t* test ($*P < 0.01$) for comparison with the wild-type value. Values are means ± standard deviation for eight independent plants. The experiments were repeated twice and showed similar results.

and found that resistance varies with the level of *PR1* gene expression (Figure 5b).

Over-expression phenotypes of *AtMYB44* are reversed by SA depletion

Because *PR1* activation in OX18 plants was abolished in the *NahG* background, we studied the growth phenotypes of OX18 *NahG* plants. Growth of OX18 and OX18 *npr1-1* plants was severely retarded, similar to plants over-expressing *PR* genes (Bowling *et al.*, 1997; Li *et al.*, 2004). However, the OX18 growth retardation was abolished in OX18 *NahG* plants (Figure 6a).

We also examined the JA response of OX18 *NahG* plants. In OX18 plants, expression of JA-responsive genes such as *VSP1* and *PDF1.2* was not substantially induced by treatment with MeJA; however, in OX18 *NahG* plants, *VSP1* and *PDF1.2* were strongly induced by MeJA treatment (Figure 6b). By contrast, OX18 *npr1-1* plants showed similar growth retardation and repression of JA-responsive genes to OX18 plants, because the *NPR1*-independent *AtMYB44* pathway leading to *PR1* is still functioning, as shown in Figure 5(b).

Because the effects of *AtMYB44* over-expression were reversed in the *NahG* background, we next measured the levels of SA in wild-type, OX18 and *atmyb44* mutant plants (Figure 6c). One-way ANOVA revealed that the endogenous levels of free SA and glucosylated SA were not significantly different among all genotypes tested at a confidence level of $P < 0.05$. Surprisingly, enhanced expression of *PR* genes in OX18 plants is not a result of enhanced SA biosynthesis.

AtMYB44 drives *WRKY70* and *PR1* expression

Because activation of *PR* genes by *AtMYB44* over-expression required SA but did not result from SA accumulation, we investigated the expression of various genes related to expression of *PR* genes in *AtMYB44* over-expression plants. We used RT-PCR, with a limited number of cycles, to screen the expression of 18 regulatory factors and SA-biosynthesis-related genes. In this assay, *WRKY70* was the only gene affected by *AtMYB44* over-expression or knockout mutation (Figure S3). SA biosynthesis and signaling genes, other WRKY transcription factors, and *TGACC MOTIF-BINDING PROTEIN (TGA)* genes, which are induced by SA and biotrophic pathogens, were not affected by *AtMYB44* over-expression or knockout mutation. We also used Northern blot analysis to confirm that *WRKY70* was constitutively over-expressed in OX18 and OX21 plants but was not expressed in *atmyb44* mutants (Figure 7a).

To demonstrate activation of *WRKY70* by *AtMYB44* over-expression, we produced transgenic Arabidopsis plants expressing *AtMYB44* under the control of the β-estradiol-inducible promoter. *AtMYB44* was induced within 6 h after β-estradiol treatment (Figure 7b). *WRKY70* was induced approximately 6 h after induction of *AtMYB44*, and *PR1* was induced after another 12 h, suggesting a hierarchical relationship among these genes. This observation supports the hypothesis that induction of *AtMYB44* mediates increased expression of *WRKY70*, which in turn mediates *PR1* gene expression.

To test whether the activation of *PR1* and suppression of *PDF1.2* (Figures 2 and 4) in *AtMYB44* over-expression plants required *WRKY70*, we generated OX18 *wrky70* double mutants. Constitutive expression of *PR1* in OX18 plants was completely abolished in OX18 *wrky70* double mutants. Also, in contrast to the OX18 plants, *PDF1.2* was induced by MeJA in the OX18 *wrky70* double mutant as in the wild-type. These results demonstrate that expression of *PR1* and suppression of *PDF1.2* in OX18 plants are mediated by *WRKY70* (Figure 7c).

AtMYB44 activates *WRKY70* independently of *NPR1*

WRKY70 is known to be regulated by *NPR1* (Li *et al.*, 2004). To define the contribution of *AtMYB44* to *WRKY70*

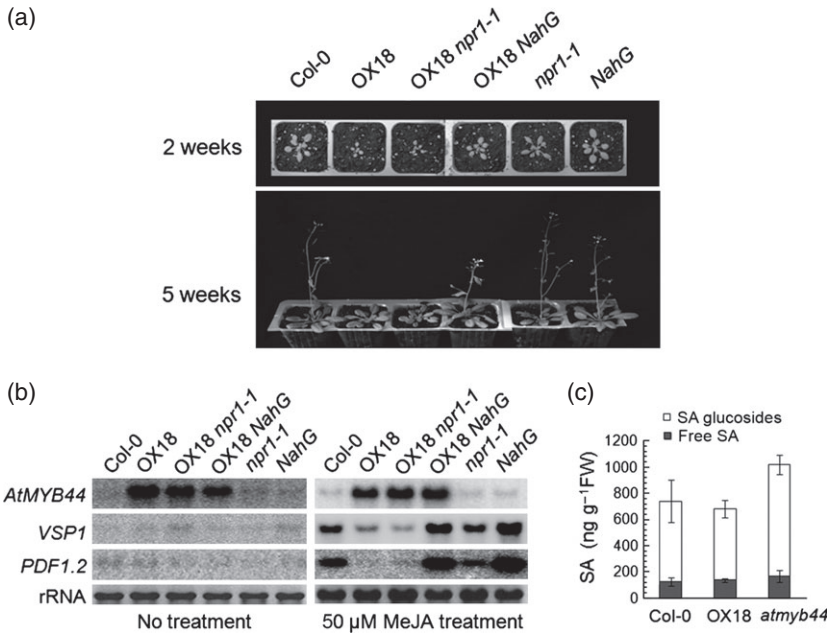

Figure 6. Effect of *npr1* mutation or *NahG* expression on *AtMYB44*-mediated responses.
(a) Growth of plants over-expressing *AtMYB44* in various mutant backgrounds. Photographs were taken 2 or 5 weeks after germination.
(b) JA response of plants over-expressing *AtMYB44* in various mutant backgrounds. Two-week-old plants were treated with MeJA for 6 h, and total RNA was analyzed by Northern blot. rRNA was visualized by ethidium bromide staining as a loading control.
(c) Quantification of free SA and SA glucosides in *AtMYB44* over-expressing plants and *atmyb44* mutant plants. Values are means ± standard deviation for three replicates. One-way ANOVA revealed no difference in SA content among control and mutant plants at a confidence level of $P < 0.05$.

expression, we generated an *atmyb44 npr1-1* double mutant. In wild-type plants, *WRKY70* was activated 15 min after SA treatment and increased continuously (Figure 8). By contrast, the increase in *WRKY70* expression was slightly delayed in *atmyb44* mutants and did not occur at all in *atmyb44 npr1-1* double mutants. In *npr1-1* mutants, *WRKY70* still appeared at an early time point but did not accumulate. These data demonstrate that *WRKY70* is regulated through both *AtMYB44* and *NPR1*, but these two factors act independently of each other.

AtMYB44 binds to the promoter region of *WRKY70*

AtMYB44 regulates transcriptional activation of *WRKY70* (Figures 7 and 8). To test the possibility that AtMYB44 acts as a direct transcriptional activator for *WRKY70*, we used a GAL4/β-galactosidase assay to determine whether AtMYB44 contains a transcriptional activation domain. Various truncated forms of AtMYB44 were fused to a GAL4 DNA binding domain and tested to determine whether they could activate transcription from a GAL4/β-galactosidase reporter plasmid (Figure S4a). The AtMYB44 C-terminal domain without the DNA binding R2R3 domain showed the highest transcriptional activation activity (Figure S4b). Therefore, AtMYB44 acts as a transcriptional activator of target gene expression. However, transcriptional activation was not observed with full-length AtMYB44 fused to the GAL4 DNA binding domain. This suggests that the structure of the DNA binding domains from two proteins may be affected by juxtaposition, and thus may have lost their DNA binding activities (Gourrierec *et al.*, 1999; : Yu *et al.*, 2011).

To determine the consensus binding sequence of AtMYB44, we performed systematic evolution of ligands by exponential enrichment (SELEX). The core binding sequence of AtMYB44, 5′-CNGTTA-3′, was deduced by alignment of the sequences identified by SELEX (Figure 9a). This consensus sequence is similar to the previously reported MYB binding consensus sequence (CNGTTA/G) (Romero *et al.*, 1998).

To test binding of AtMYB44 to the *WRKY70* promoter region *in vitro*, the DNA fragment from −381 to −284, which contains the core binding sequence, was selected and tested by electrophoretic mobility shift assay (EMSA). As the full-length protein was not as stable, the AtMYB44 R2R3 DNA binding domain (AtMYB44R2R3) was fused to glutathione S-transferase (GST) and expressed in *Escherichia coli* for the EMSA. The EMSA showed that AtMYB44R2R3 bound specifically to the probe from the *WRKY70* promoter. The GST protein did not bind to the probe containing the AtMYB44 core binding sequence. Binding of AtMYB44 R2R3 to the labeled probe was competed off in the presence of excess unlabeled probe. Probes containing a mutated binding motif did not bind to AtMYB44R2R3 (Figure 9b).

Binding of full-length AtMYB44 to the promoter region of *WRKY70* was confirmed using a yeast one-hybrid (Y1H)

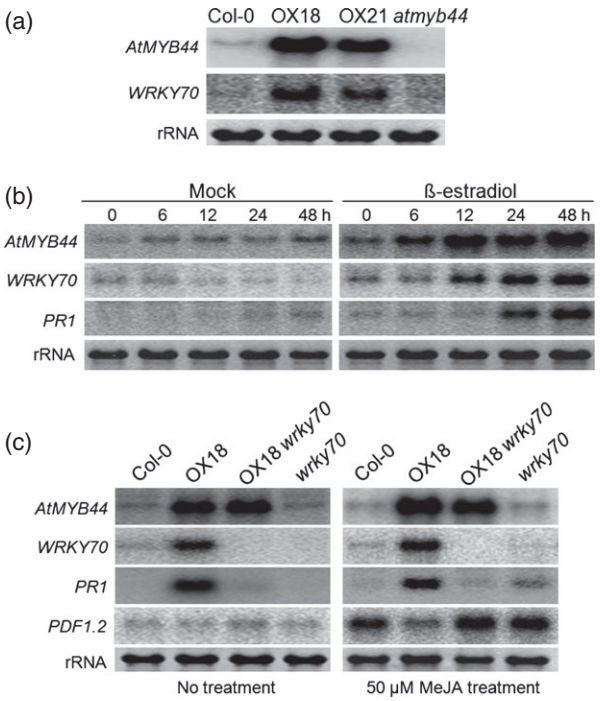

Figure 7. Role of *AtMYB44* in expression of *WRKY70* and *PR1*.
(a) Northern blot showing basal expression of *WRKY70* in Arabidopsis Col-0, *AtMYB44* over-expression (OX18 and OX21) and *atmyb44* knockout plants.
(b) Induced expression of *WRKY70* and *PR1* by *AtMYB44*. Twelve-day-old seedlings of transgenic Arabidopsis harboring *AtMYB44* under the control of a β-estradiol-inducible promoter (*XVE:AtMYB44*) were induced by transferring to MS plates containing β-estradiol. Total RNA was analyzed by Northern blot.
(c) Effect of *WRKY70* mutation on *PR1* and *PDF1.2* induction in *AtMYB44* over-expressing plants. Two-week-old plants were treated with MeJA for 6 h and total RNA was analyzed by Northern blot. rRNA was visualized by ethidium bromide staining as a loading control.

assay (Figure S5a). Thirty nucleotides from the *WRKY70* promoter region (−328 to −299) containing the AtMYB44 core binding sequence were repeated four times and placed upstream of the *HIS3* selectable marker gene. Transformation with the core binding site–*HIS3* construct and full-length AtMYB44 fused to the GAL4 activation domain made auxotrophic yeast viable on histidine selective medium. However, a mutant version of the promoter fragment did not activate HIS3 in the Y1H assay.

Chromatin immunoprecipitation (ChIP) experiments were used to test whether AtMYB44 binds directly to *WRKY70 in vivo*. Extracts from plants over-expressing AtMYB44–GFP were subjected to ChIP analysis and compared with wild-type plants. ChIP from AtMYB44–GFP over-expressing plants with anti-GFP antibody showed enrichment of the *WRKY70* promoter region containing the AtMYB44 core binding sequence (Figure 9c). A control ChIP product from wild-type plants did not show enrichment of the *WRKY70* promoter region. AtMYB44 also

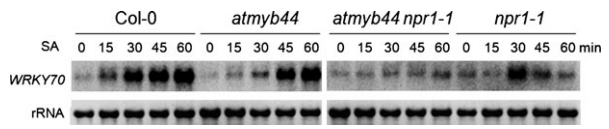

Figure 8. Effect of *atmyb44* or *npr1* mutation on expression of *WRKY70*. Two-week-old Arabidopsis plants were treated with SA, and *WRKY70* expression was analyzed by Northern blot. rRNA was visualized by ethidium bromide staining as a loading control.

bound to an upstream region of the *WRKY70* promoter containing the AtMYB44 core binding sequences (sites 1 and 2, Figure S5b). However, three negative control regions that did not contain the core binding sequence also were not enriched by ChIP; these regions include another promoter region (site 3), a coding region (site 5) and part of the 3′ UTR of *WRKY70* (site 6).

We performed transient GUS assays in *Nicotiana benthamiana* to confirm transactivation of *WRKY70* through the core binding sequence. The *WRKY70* promoter sequences from −328 to −299 was repeated four times (4xRE44) and fused to the GUS reporter gene. The reporter plasmid and effector plasmid, *35S:AtMYB44*, were co-infiltrated into *N. benthamiana.* The GUS reporter gene was expressed by co-infiltration of *35S:AtMYB44* with 4xRE44. However, the mutant reporter containing a mutated version of the *WRKY70* promoter (4xmRE44) was not expressed (Figure 9d).

DISCUSSION

WRKY70 is regarded as a pivotal regulator in the antagonistic interaction between SA and JA. Activation or suppression of *WRKY70* is critical step in developing an effective defense response against pathogen attack. Here we report that *AtMYB44* contributes to establishing appropriate plant defense responses by direct regulation of *WRKY70* expression in cross-talk between SA and JA.

Role of *AtMYB44* in SA- and JA-mediated defense responses

Here we show that *AtMYB44* is induced by MeJA (Figure 2). JA signaling is required for disease resistance against necrotrophic pathogens such as *A. brassicicola* (Thomma *et al.*, 1998; Seo *et al.*, 2001); expression of *AtMYB44* was also induced by *A. brassicicola* (Figure 1c). However, over-expression of *AtMYB44* led to increased susceptibility to *A. brassicicola* by suppression of JA-mediated defense gene expression. By contrast, a knockout mutation of *AtMYB44* increased resistance to *A. brassicicola* by activation of JA-mediated defense gene expression (Figures 1 and 2). In *atmyb44* plants, expression of *VSP1* and *PDF1.2* were also up-regulated without JA treatment (Figure 2). These data indicate that *AtMYB44* acts as a negative regulator of JA-mediated defense responses. The negative effects of *AtMYB44* over-expression on JA signaling were not limited to the defense response, but also

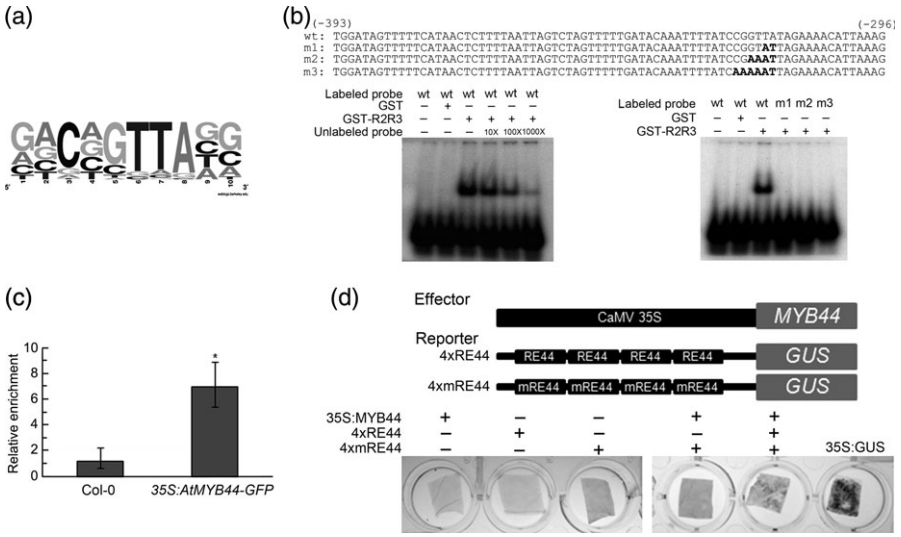

Figure 9. AtMYB44 binds to the *WRKY70* promoter region.
(a) Nucleotide frequency distribution of the AtMYB44 core binding consensus sequence as determined by SELEX. The sizes of the letters represent the relative frequency of occurrence.
(b) Electrophoretic mobility shift assay shows binding of the R2R3 domain of AtMYB44 to the *WRKY70* promoter region (−381 to −284) *in vitro*. Probe sequences of the wild-type *WRKY70* promoter region (wt) and mutant versions of the promoter (m1, m2 and m3) are shown at the top. Radiolabeled probe was competed for using an excess of unlabeled probe (left panel). Mutant probes were compared with wild-type probe (right panel).
(c) Fragmented chromatin DNA of Arabidopsis Col-0 and *AtMYB44*–GFP over-expressing plants were immunoprecipitated using anti-GFP antibody. For quantitative analysis, PCR products amplified by a primer set containing the AtMYB44 core binding sequence were analyzed by quantitative PCR. The statistical significance of the measurements was determined using Student's *t* test ($*P < 0.01$) for comparison with the wild-type value. Values are means ± standard error of the mean of three measurements for each sample.
(d) The *WRKY70* promoter sequence from −328 to −299 containing the core binding sequence (RE44) or its mutant version (mRE44) was repeated four times and fused to the GUS reporter gene. A cDNA encoding the whole AtMYB44 protein was fused to the CaMV 35S promoter as an effector. The reporter and effector constructs were infiltrated into *Nicotiana benthamiana*. Transactivation activity was detected by GUS staining assay.

affected JA-mediated root growth inhibition, root hair development (Figure S1) and anthocyanin accumulation (Jung *et al.*, 2010).

AtMYB44 over-expressing plants showed enhanced resistance against a biotrophic pathogen, but *atmyb44* knockout plants showed decreased resistance compared to wild-type (Figure 3). Suppression of the JA-mediated defense response was balanced with activation of the SA-dependent defense response (Gupta *et al.*, 2000; Kunkel and Brooks, 2002; Spoel *et al.*, 2003). The enhanced disease resistance established in over-expressing plants was accompanied by activation of SA-dependent *PR* genes (Figure 4) (Li *et al.*, 2004). Moreover, *PR1* was rapidly and strongly activated in *AtMYB44* over-expressing plants by exogenous SA treatment (Figure 4). This demonstrates that *AtMYB44* acts as a positive regulator of SA-mediated defense responses. Moreover, the antagonistic effect of SA on the JA pathway was reduced in the *atmyb44* mutant (Figure S6). Mutual antagonism between JA- and SA-mediated responses is thus observed in over-expression lines and knockout mutants of *AtMYB44*.

AtMYB44 directly regulates expression of *WRKY70*

NPR1 and TGA factors directly regulate *PR1* expression in SA signaling (Zhang *et al.*, 1999; Spoel *et al.*, 2003). However, data presented here shows that the expression levels of these direct regulators (NPR1 and TGA factors) were not affected by *AtMYB44* over-expression (Figures 4a and S3). Our data show that *WRKY70* was up-regulated, thus up-regulating *PR1* in *AtMYB44* over-expressing plants (Figure 5b). *PR* genes are activated by *WRKY70* in SA signaling, and *WRKY70* was identified as an important regulatory component in the antagonistic interaction between SA and JA (Li *et al.*, 2004, 2006). Activation of *PR* genes and suppression of JA-dependent defense genes were reported in *WRKY70* over-expressing plants (Li *et al.*, 2004, 2006). *AtMYB44* over-expressing plants showed a similar pattern of disease resistance to *WRKY70* over-expressing plants; both were resistant to a biotrophic pathogen (*Pst* DC3000) and susceptible to a necrotrophic pathogen (*A. brassicicola*). These data show that *AtMYB44* modulates SA- and JA-mediated defense responses through *WRKY70*. This conclusion is also supported by the OX18 *wrky70* double mutant phenotype comprising induction of *PDF1.2* expression and elimination of *PR1* expression (Figure 7c).

Transcription factors regulate target gene expression by binding to promoter regions and interacting with the transcription complex to effect transcriptional activation or repression. By *trans*-activation analysis, we showed that *AtMYB44* acts as a transcriptional activator (Figure S4). AtMYB44 binds to the promoter of *WRKY70*, which contains the AtMYB44 core binding sequence CNGTTA

(Figure 9). ChIP, Y1H and EMSA analyses demonstrate that AtMYB44 binds to the core binding sequence in the *WRKY70* promoter. These results show that *AtMYB44* directly regulates *WRKY70* expression. These results were consistent with elevated *WRKY70* expression in *AtMYB44* over-expressing plants and β-estradiol-induced *trans*-activation by AtMYB44 (Figure 7). Transient expression of the GUS reporter driven by the core binding sequence of the *WRKY70* promoter provides more evidence that *AtMYB44* regulates expression of *WRKY70* (Figure 9d).

The enhanced expression of *PR* genes in OX18 is not a result of increased SA content (Figure 6c). The phenotype is reminiscent of *WRKY70* over-expression plants, in which *PR1* was constitutively over-expressed without a change in SA content (Li *et al.*, 2004). Even though *AtMYB44* directly regulates expression of *WRKY70* and *PR* genes, activation of *WRKY70* by *AtMYB44* was abolished in the *NahG* or *sid2-2* background (Figure 5b). These results suggest that a basal level of SA may be essential to activate *AtMYB44* and *WRKY70*. There have been reports that expression of *WRKY70* was totally abolished in *NahG* transgenic plants even after SA treatment and biotrophic pathogen infection (Li *et al.*, 2004; Knoth *et al.*, 2007).

AtMYB44 is an *NPR1*-independent component of SA signaling

WRKY70 is associated with both *NPR1*-dependent and *NPR1*-independent pathways in SA signaling (Li *et al.*, 2004). For example, *WRKY70* over-expression resulted in activation of *PR* genes in the *npr1-1* mutant background. It has also been reported that *NPR1*-independent expression of *PR* genes in *snc2-1D npr1-1* was activated through *WRKY70* (Zhang *et al.*, 2010). Therefore, *WRKY70* can trigger SA-mediated activation of *PR* genes through an *NPR1*-independent pathway. Here we show that SA-induced expression of *AtMYB44* does not require *NPR1* (Figure 5a). By double mutant analysis, we show that activation of *WRKY70* and *PR1* by *AtMYB44* over-expression also did not require *NPR1* (Figure 5b). Moreover, *NPR1*-independent expression of *WRKY70* is abolished in the *atmyb44 npr1* double mutant (Figure 8). Our data demonstrate that *AtMYB44* is an *NPR1*-independent regulatory component that directly regulates expression of *WRKY70*. It has been reported that *WHIRLY TRANSCRIPTION FACTOR 1* is also induced by SA through an *NPR1*-independent pathway, which also activates *PR1* (Desveaux *et al.*, 2004). Moreover, constitutive expression of *PR1* in *cpr6* and *ssi2* mutants was not diminished in the *npr1-1* mutant background (Clarke *et al.*, 1998; Shah *et al.*, 2001). Therefore, an *NPR1*-independent branch of the SA signaling pathway does exist.

Regulation of defense responses by *AtMYB44*

The network of SA- and JA-responsive gene expression mediated by *AtMYB44* is summarized in Figure 10. Plant resistance is triggered by recognition of the invading pathogen. Plants have developed an effective defense response against pathogen attack by changing the levels of endogenous defense hormones such as SA and JA (De Vos *et al.*, 2005; Koo *et al.*, 2007; Tsuda *et al.*, 2008). *AtMYB44* transcripts are detected 12 h after *A. brassicicola* infection and 6 h after *Pst* DC3000 infection (Figures 1c and S7). *AtMYB44* is induced by SA and directly activates *WRKY70*, which activates *PR* genes; SA also independently activates *PR* genes through *NPR1*. These SA-dependent signals confer resistance against biotrophic pathogens such as *Pst* DC3000 (Cao *et al.*, 1997; Li *et al.*, 2004). *AtMYB44* is also induced by JA through COI1 (Figure S8). JA also induces expression of JA-responsive genes such as *PDF1.2* through COI1, conferring resistance against necrotrophic pathogens (Thomma *et al.*, 1998; Seo *et al.*, 2001). COI1 represses *WRKY70*, a negative regulator of the JA response, to maintain the transcription of JA-responsive downstream genes (Li *et al.*, 2004). At the same time, JA-induced expression of *AtMYB44* activates *WRKY70* (Figure S8). The expression of *AtMYB44* in response to JA is reminiscent of the *JAZ* repressor genes, which are induced in response to JA (Chini *et al.*, 2007; Thines *et al.*, 2007).

Plants pay significant costs to activate and maintain defense responses. For example, SA mutants in which defense genes are constitutively activated show growth retardation (Bowling *et al.*, 1997; Clarke *et al.*, 1998; Li *et al.*, 2004). Therefore, the plant defense response must be under tight and finely tuned regulation (Spoel *et al.*,

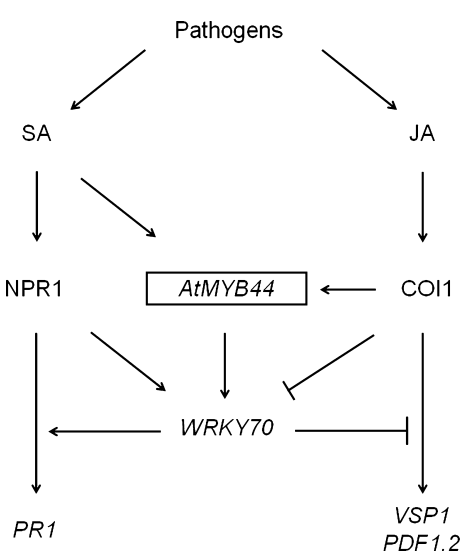

Figure 10. Role of *AtMYB44* in antagonistic interaction between SA- and JA-mediated defense signaling.
In the SA-mediated defense response, *AtMYB44* is induced by *NPR1*-independent SA signaling. Expressed *AtMYB44* regulates activation of *PR1* and suppression of JA-mediated defense genes (*VSP1* and *PDF1.2*) by direct transcriptional activation of *WRKY70*. JA-mediated expression of *AtMYB44* occurs through a COI1-dependent pathway. *AtMYB44* activated by JA acts as a negative regulator to fine-tune the JA signal. COI1 also negatively regulates *WRKY70* (Li *et al.*, 2004).

2003; Moore *et al.*, 2011). Induction of a negative regulator in response to signal molecules contributes to fine-tuning of defense responses (Journot-Catalino *et al.*, 2006; Chini *et al.*, 2007). Thus, the biological role of *AtMYB44* may be to fine-tune JA-mediated defense signals for balanced allocation of resources in plant defense responses (Spoel *et al.*, 2003; Journot-Catalino *et al.*, 2006). This possibility is supported by constitutive expression of JA-responsive genes in *atmyb44* knockout mutants (Figure 2). *AtMYB44* acts as a point of intersection for coordination of signals from JA and SA to allow cross-talk.

The function of *AtMYB44* in promoting the SA signal may be counter-balanced by the function of the other WRKYs promoting the JA signal. For example, *WRKY7*, *WRKY8*, *WRKY11* and *WRKY17* are induced by the biotrophic pathogen *Pst* DC3000, but these WRKY transcription factors suppress the SA-mediated defense response (Kim *et al.*, 2006; Chen *et al.*, 2010). Furthermore, *WRKY11* and *WRKY17* up-regulate expression of JA biosynthesis genes such as *LOXII* and *AOS* (Journot-Catalino *et al.*, 2006). These findings also support the occurrence of fine-tuned regulation of the defense response.

Another potential role of *AtMYB44* in the plant defense response was also described previously. Upon activation by *Agrobacterium* or pathogen-associated molecular patterns, mitogen-activated protein kinase 3 (MPK3) phosphorylates VirE2 interacting protein 1 (VIP1). Activated VIP1 regulates the expression of *PR1* (Djamei *et al.*, 2007). AtMYB44 was shown to be a direct target of VIP1 (Pitzschke *et al.*, 2009). It has also been reported that AtMYB44 is phosphorylated by MPK3 (Nguyen *et al.*, 2012). These results suggest that *AtMYB44* and *WRKY70* may mediate activation of *PR1* by *VIP1*.

In summary, we examine here the function of *AtMYB44* in defense responses. The differential modulation of SA- and JA-mediated defense responses by *AtMYB44* provides evidence that *AtMYB44* is a regulatory component in the antagonistic interaction between the SA and JA signaling pathways.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia (Col-0) was used throughout this study. The 35S:*AtMYB44*, 35S:*AtMYB44-GFP* and T-DNA insertion *atmyb44* mutants (SALK_039074) have been described previously (Jung *et al.*, 2008). The Arabidopsis *sid2-2* mutant, the transgenic line expressing *NahG*, and the *coi1-1* mutant were kindly provided by Frederic M. Ausubel (Harvard Medical School, Boston, MA), J. Ryals (Ciba-Geigy Agricultural Biotechnology, Research Triangle Park, NC) and J. Turner (University of East Anglia, Norwich, UK), respectively. The Arabidopsis *jar1-1*, *atmyc2*, *npr1-1* and *wrky70* mutants were obtained from the Arabidopsis Biological Resource Center (stock numbers CS8072, SALK_061267, CS3726 and SALK_025198, respectively). Strains of *Pst* DC3000 and *A. brassicicola* were kindly provided by Ingyu Hwang and Yong Hwan Lee, respectively (Seoul National University, Seoul, Korea).

Plants were grown on soil or half-strength Murashige and Skoog (MS)/agar plates in a growth chamber maintained at 22°C and 60% relative humidity under long-day conditions (16 h light/8 h dark cycle).

To examine the effect of plant hormones on gene expression, a solution of 50 μM MeJA (Sigma, http://www.sigmaaldrich.com) or 1 mM SA (Sigma) was applied to the surface of MS/agar plates in which 2-week-old seedlings were growing. For inducible expression of *AtMYB44*, an *AtMYB44* cDNA was inserted into the *Xho*I and *Spe*I sites in the pER8 vector (Zuo *et al.*, 2000). A full-length *AtMYB44* cDNA (EST 119B8) was obtained from the Arabidopsis Information Resource. Twelve-day-old transformants grown on MS/agar plates were transferred to medium containing 5 μM β-estradiol for induction of *AtMYB44*.

Plant transformation and analyses of transgenic plants

Agrobacterium tumefaciens strain C58C1 containing plasmid constructs was used to transform plants by the floral-dip method (Clough and Bent, 1998). Transgenic plants were identified by selection on half-strength MS agar medium containing 20 μg ml^{-1} hygromycin (Duchefa, http://www.duchefa-biochemie.nl/).

Analysis of transcript levels

For Northern blot analysis, total RNA was extracted from frozen sample using the phenol/SDS/LiCl method (Carpenter and Simon, 1998). Total RNA (5 μg) was separated on 1.3% formaldehyde agarose gels and transferred to Genescreen Plus hybridization transfer membranes (Perkin-Elmer, http://www.perkinelmer.com/). [α-^{32}P]-labeled cDNA probes containing gene-specific sequences were hybridized to detect signal.

For quantitative real-time PCR, a SYBR kit (Philekorea, http://www.philekorea.co.kr/) was used. Forty cycles of amplification (15 sec at 95°C, annealing for 60 sec at 68°C) after an initial step (10 min at 95°C) were performed in a Rotor-gene 2000 (Corbett, http://www.corbettlifescience.com). Primer sequences are listed in Table S1. *Actin1* was included in the assay for normalization. The quantitative real-time PCR reactions were performed using two or three biological and three technical repeats. The comparative $\Delta\Delta C_T$ method was used for relative quantification of each amplified product.

Pathogen infection assay

A. brassicicola was cultivated on potato dextrose agar plates. Preparation and inoculation of fungi were performed as described previously (Li *et al.*, 2006). Relative fungal DNA levels of *A. brassicicola* were determined by quantitative real-time PCR using primers specific for genomic *5.8S ribosomal RNA* (GenBank accession number U05198) and primers for Arabidopsis genomic *ACT2*.

Pst DC3000 was grown in King's B medium (King *et al.*, 1954) and adjusted to 5×10^5 cfu ml^{-1} in 10 mM $MgCl_2$ solution. Inoculation of *Pst* DC3000 and determination of bacterial growth were performed as described previously (Li *et al.*, 2004).

Quantification of SA and glucosylated SA

SA and glucosylated SA were extracted from the leaves and quantified as described previously (Koo *et al.*, 2007). Extracts were separated using a Symmetry C18 HPLC column (4.6 mm internal diameter, 15 cm long, particle size 5 μm; Waters, http://www.waters.com) in a LC-6A HPLC (Shimadzu, http://www.shimadzu.

com/), and quantified using an RF-10A XL fluorescence detector (excitation 301 nm, emission 412 nm; Shimadzu). The amount of glucosylated SA was quantified by the difference between SA quantities with and without glucosidase treatment (160 units for 60 min at 37°C, Sigma). To monitor sensitivity and recovery, a known amount of SA was added to the sample and analyzed in parallel. The measurements for three biological replicates were averaged.

Systematic evolution of ligands by exponential enrichment (SELEX)

SELEX was performed as previously described (Grotewold *et al.*, 1994). After five rounds of selection, the amplified DNA was inserted into a pGEM-TEasy vector (Promega, http://www.promega.com/), and nucleotide sequences of 36 DNA fragments were determined using an ABI PRISM® 377 DNA sequencer (Applied Biosystems, http://www.appliedbiosystems.com). The obtained sequences were aligned and visualized using the Weblogo package (Crooks *et al.*, 2004; http://weblogo.berkeley.edu/).

Electrophoretic mobility shift assay (EMSA)

The R2R3 domain of *AtMYB44* (amino acid residues 1–111) was fused with the GST coding sequence through the *Bam*HI and *Eco*RI sites of the pGEX-5x-1 expression vector (GE healthcare, http://www.gelifesciences.com). The GST–AtMYB44 fusion protein was purified according to the manufacturer's instructions. DNA fragments labeled with [α-^{32}P]dCTP were incubated with 0.5 μg of purified GST–AtMYB44 protein for 20 min at 23°C in 25 μl binding buffer (20 mM HEPES pH 7.8, 50 mM KCl, 1 mM EDTA, 0.5 mM dithiothreitol, 5 μg BSA, 200 μg poly[dI-dC] and 10% glycerol). The reaction mixture was separated on 6% gels by native PAGE.

Chromatin immunoprecipitation

Two-week-old 35S:*AtMYB44-GFP* transgenic plants grown on MS/agar plates were used for chromatin immunoprecipitation (ChIP). ChIP assays were performed as described previously (Saleh *et al.*, 2008). Fragmented chromatin was immunoprecipitated using anti-GFP antibody (Clontech, http://www.clontech.com/). DNA extracts separated from the DNA–protein complex were used for quantitative real-time PCR analysis. The primer sets used in this analysis amplify various regions of the *WRKY70* locus (Figure S5b and Table S1). The ChIP experiments were performed three times.

Transient GUS assay by agroinfiltration of *Nicotiana benthamiana*

Agrobacteria were infiltrated into intact leaves of *Nicotiana benthamiana* as previously described (Kane *et al.*, 2007). After infiltration, plants were kept at 24°C for 3 days. Histochemical GUS assays were performed as previously described (Jung *et al.*, 2008).

ACKNOWLEDGEMENTS

This work was supported by a grant from the Next-Generation BioGreen 21 Program (project numbers PJ008053 to Y.D.C. and PJ007971 to J. -K.K.), Rural Development Administration, Republic of Korea through the National Center for GM Crops. A graduate research assistantship to J.S.S. and S.J.L. from the Brain Korea 21 project of the Ministry of Education, Science and Technology is also acknowledged.

SUPPORTING INFORMATION

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

Figure S1. MeJA response of *AtMYB44* over-expressing and *atmyb44* knockout plants.

Figure S2. Resistance of *npr1-1* and *NahG* plants against the biotrophic pathogen *Pst* DC3000.

Figure S3. Expression of SA signaling genes in *AtMYB44* over-expressing and *atmyb44* knockout plants.

Figure S4. Transcriptional activation domain assay of AtMYB44.

Figure S5. Direct binding of AtMYB44 to the *WRKY70* promoter *in vivo.*

Figure S6. SA-mediated suppression of the JA response in *atmyb44* knockout mutant plants.

Figure S7. Expression of *AtMYB44* in response to *Pst* DC3000.

Figure S8. Induction of *WRKY70* and *PR1* by MeJA.

Table S1. Primers used in Northern blot analysis, ChIP PCR and sub-cloning.

Data S1. Supplemental experimental procedures.

REFERENCES

Bowling, S.A., Clarke, J.D., Liu, Y., Klessig, D.F. and Dong, X. (1997) The *cpr5* mutant of Arabidopsis expresses both NPR1-dependent and NPR1-independent resistance. *Plant Cell*, **9**, 1573–1584.

Browse, J. and Howe, G.A. (2008) New weapons and a rapid response against insect attack. *Plant Physiol.* **146**, 832–838.

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.

Carpenter, C.D. and Simon, A.E. (1998) Preparation of RNA. *Methods Mol. Biol.* **82**, 85–89.

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

Chini, A., Fonseca, S., Fernandez, G. *et al.* (2007) The JAZ family of repressors is the missing link in jasmonate signalling. *Nature*, **448**, 666–671.

Chung, H.S. and Howe, G.A. (2009) A critical role of the TIFY motif in repression of jasmonate signaling by a stabilized splice variant of the jasmonate ZIM-domain protein JAZ10 in Arabidopsis. *Plant Cell*, **21**, 131–145.

Clarke, J.D., Liu, Y., Klessig, D.F. and Dong, X. (1998) Uncoupling PR gene expression from NPR1 and bacterial resistance: chracterization of the dominant Arabidopsis *cpr6-1* mutant. *Plant Cell*, **10**, 557–569.

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.

Crooks, G.E., Hon, G., Chandonia, J.M. and Brenner, S.E. (2004) WebLogo: a sequence logo generator. *Genome Res.* **14**, 1188–1190.

De Vos, M., Van Oosten, V.R., Van Poecke, R.M.P. *et al.* (2005) Signal signature and transcriptome changes of Arabidopsis during pathogen and insect attack. *Mol. Plant–Microbe Interact.* **18**, 923–937.

Delaney, T.P., Uknes, S., Vernooij, B. *et al.* (1994) A central role of salicylic acid in plant disease resistance. *Science*, **266**, 1247–1250.

Desveaux, D., Subramaniam, R., Després, C., Mess, J.N., Lévesque, C., Fobert, P.R., Dangl, J.L. and Brisson, N. (2004) A 'Whirly' transcription factor is required for salicylic acid-dependent disease resistance in *Arabidopsis*. *Dev. Cell*, **6**, 229–240.

Devoto, A., Nieto-Rostro, M., Xie, D., Ellis, C., Harmston, R., Patrick, E., Davis, J., Sherratt, L., Coleman, M. and Turner, J.G. (2002) COI1 links jasmonate signalling and fertility to the SCF ubiquitin–ligase complex in Arabidopsis. *Plant J.* **32**, 457–466.

Djamei, A., Pitzschke, A., Nakagami, H., Rajh, I. and Hirt, H. (2007) Trojan horse strategy in *Agrobacterium* transformation: abusing MAPK defense signaling. *Science*, **318**, 453–456.

Doherty, H.M., Selvendran, R.R. and Bowles, D.J. (1988) The wound response of tomato plants can be inhibited by aspirin and related hydroxy-benzoic acids. *Physiol. Mol. Plant Pathol.* **33**, 377–384.

Dubos, C., Stracke, R., Grotewold, E., Weisshaar, B., Martin, C. and Lepiniec, L. (2010) MYB transcription factors in Arabidopsis. *Trends Plant Sci.* **15**, 573–581.

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

Eulgem, T. (2005) Regulation of the Arabidopsis defense transcriptome. *Trends Plant Sci.* **10**, 71–78.

Feys, B.J. and Parker, J.E. (2000) Interplay of signaling pathways in plant disease resistance. *Trends Genet.* **16**, 449–455.

Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H. and Ryals, J. (1993) Requirement of salicylic acid for the induction of systemic acquired resistance. *Science*, **261**, 754–756.

Gourrierec, L.J., Li, Y.-F. and Zhou, D.-X. (1999) Transcriptional activation by Arabidopsis GT-1 may be through interaction with TFIIA–TBP–TATA complex. *Plant J.* **18**, 663–668.

Grotewold, E., Drummond, B.J., Bowen, B. and Peterson, T. (1994) The *myb*-homologous *P* gene controls phlobaphene pigmentation in maize floral organs by directly activating a flavonoid biosynthetic gene subset. *Cell*, **76**, 543–553.

Gupta, V., Willits, M.G. and Glazebrook, J. (2000) *Arabidopsis thaliana EDS4* contributes to salicylic acid (SA)-dependent expression of defense responses: evidence for inhibition of jasmonic acid signaling by SA. *Mol. Plant–Microbe Interact.* **13**, 503–511.

Journot-Catalino, N., Somssich, I.E., Roby, D. and Kroj, T. (2006) The transcription factors WRKY11 and WRKY17 act as negative regulators of basal resistance in *Arabidopsis thaliana*. *Plant Cell*, **18**, 3289–3302.

Jung, C., Seo, J.S., Han, S.W., Koo, Y.J., Kim, C.H., Song, S.I., Nahm, B.H., Choi, Y.D. and Cheong, J.-J. (2008) Overexpression of *AtMYB44* enhances stomatal closure to confer abiotic stress tolerance in transgenic Arabidopsis. *Plant Physiol.* **146**, 623–635.

Jung, C., Shim, J., Seo, J., Lee, H., Kim, C., Choi, Y. and Cheong, J.-J. (2010) Non-specific phytohormonal induction of *AtMYB44* and suppression of jasmonate-responsive gene activation in *Arabidopsis thaliana*. *Mol. Cells*, **29**, 71–76.

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.

Kane, N.A., Agharbaoui, Z., Diallo, A.O., Adam, H., Tominaga, Y., Ouellet, F. and Sarhan, F. (2007) TaVRT2 represses transcription of the wheat vernalization gene *TaVRN1*. *Plant J.* **51**, 670–680.

Kim, K.-C., Fan, B. and Chen, Z. (2006) Pathogen-induced Arabidopsis WRKY7 is a transcriptional repressor and enhances plant susceptibility to *Pseudomonas syringae*. *Plant Physiol.* **142**, 1180–1192.

King, E.O., Ward, M.K. and Raney, D.E. (1954) Two simple media for the demonstration of pyocyanin and fluorescin. *J. Lab. Clin. Med.* **44**, 301–307.

Kloek, A.P., Verbsky, M.L., Sharma, S.B., Schoelz, J.E., Vogel, J., Klessig, D.F. and Kunkel, B.N. (2001) Resistance to *Pseudomonas syringae* conferred by an *Arabidopsis thaliana* coronatine-insensitive (*coi1*) mutation occurs through two distinct mechanisms. *Plant J.* **26**, 509–522.

Knoth, C., Ringler, J., Dangl, J.L. and Eulgem, T. (2007) *Arabidopsis* WRKY70 is required for full *RPP4*-mediated disease resistance and basal defense against *Hyaloperonospora parasitica*. *Mol. Plant–Microbe Interact.* **20**, 120–128.

Koo, Y., Kim, M., Kim, E. *et al.* (2007) Overexpression of salicylic acid carboxyl methyltransferase reduces salicylic acid-mediated pathogen resistance in *Arabidopsis thaliana*. *Plant Mol. Biol.* **64**, 1–15.

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

Li, J., Brader, G. and Palva, E.T. (2004) The WRKY70 transcription factor: a node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense. *Plant Cell*, **16**, 319–331.

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.

Moore, J.W., Loake, G.J. and Spoel, S.H. (2011) Transcription dynamics in plant immunity. *Plant Cell*, **23**, 2809–2820.

Nguyen, X.C., Hoang, M.H., Kim, H.S., Lee, K., Liu, X.M., Kim, S.H., Park, H.C. and Chung, W.S. (2012) Phosphorylation of the transcriptional regulator MYB44 by mitogen activated protein kinase regulates Arabidopsis seed germination. *Biochem. Biophys. Res. Commun.* **423**, 703–708.

Niki, T., Mitsuhara, I., Seo, S., Ohtsubo, N. and Ohashi, Y. (1998) Antagonistic effect of salicylic acid and jasmonic acid on the expression of pathogenesis-related (PR) protein genes in wounded mature tobacco leaves. *Plant Cell Physiol.* **39**, 500–507.

Penninckx, I.A.M.A., Eggermont, K., Terras, F.R.G., Thomma, B.P.H.J., Samblanx, G.W.D.S., Buchala, A., Métraux, J.-P., Manneqa, 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.

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

Pitzschke, A., Djamei, A., Teige, M. and Hirt, H. (2009) VIP1 response elements mediate mitogen-activated protein kinase 3-induced stress gene expression. *Proc. Natl Acad. Sci. USA*, **106**, 18414–18419.

Reymond, P. and Farmer, E.E. (1998) Jasmonate and salicylate as global signals for defense gene expression. *Curr. Opin. Plant Biol.* **1**, 404–411.

Riechmann, J.L., Heard, J., Martin, G. *et al.* (2000) Arabidopsis transcription factors: genome-wide comparative analysis among eukaryotes. *Science*, **290**, 2105–2110.

Robert-Seilaniantz, A., Grant, M. and Jones, J.D.G. (2011) Hormone crosstalk in plant disease and defense: more than just jasmonate–salicylate antagonism. *Annu. Rev. Phytopathol.* **49**, 317–343.

Romero, I., Fuertes, A., Benito, M.J., Malpica, J.M., Leyva, A. and Pazares, J. (1998) More than 80 *R2R3-MYB* regulatory genes in the genome of *Arabidopsis thaliana*. *Plant J.* **14**, 273–284.

Saleh, A., Alvarez-Venegas, R. and Avramova, Z. (2008) An efficient chromatin immunoprecipitation (ChIP) protocol for studying histone modifications in Arabidopsis plants. *Nat. Protoc.* **3**, 1018–1025.

Seo, P.J. and Park, C.-M. (2010) MYB96-mediated abscisic acid signals induce pathogen resistance response by promoting salicylic acid biosynthesis in Arabidopsis. *New Phytol.* **186**, 471–483.

Seo, H.S., Song, J.T., Cheong, J.-J., Lee, Y.-H., Lee, Y.-W., Hwang, I., Lee, J.S. and Choi, Y.D. (2001) Jasmonic acid carboxyl methyltransferase: a key enzyme for jasmonate-regulated plant responses. *Proc. Natl Acad. Sci. USA*, **98**, 4788–4793.

Shah, J., Kachroo, P., Nandi, A. and Klessig, D.F. (2001) A recessive mutation in the Arabidopsis *SSI2* gene confers SA- and NPR1-independent expression of *PR* genes and resistance against bacterial and oomycete pathogens. *Plant J.* **25**, 563–574.

Shan, X., Yan, J. and Xie, D. (2012) Comparison of phytohormone signaling mechanisms. *Curr. Opin. Plant Biol.* **15**, 84–91.

Sheard, L.B., Tan, X., Mao, H. *et al.* (2010) Jasmonate perception by inositol-phosphate-potentiated COI1–JAZ co-receptor. *Nature* **468**, 400–405.

Spoel, S.H., Koornneef, A., Claessens, S.M.C. *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.

Spoel, S.H., Johnson, J.S. and Dong, X. (2007) Regulation of tradeoffs between plant defenses against pathogens with different lifestyles. *Proc. Natl Acad. Sci. USA*, **104**, 18842–18847.

Thines, B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, G., Nomura, K., He, S.Y., Howe, G.A. and Browse, J. (2007) JAZ repressor proteins are targets of the SCF^{COI1} complex during jasmonate signaling. *Nature*, **448**, 661–665.

Thomma, B.P.H.J., Eggermont, K., Penninckx, I.A.M.A., 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.

Tsuda, K., Sata, M., Glazebrook, J., Cohen, J.D. and Katagiri, F. (2008) Interplay between MAMP-triggered and SA-mediated defense responses. *Plant J.* **53**, 763–775.

Turner, J.G., Ellis, C. and Devoto, A. (2002) The jasmonate signal pathway. *Plant Cell*, **14**, S153–S164.

Vailleau, F., Daniel, X., Tronchet, M., Montillet, J.-L., Triantaphylidès, C. and Roby, D. (2002) A R2R3-MYB gene, *AtMYB30*, acts as a positive regulator of the hypersensitive cell death program in plants in response to pathogen attack. *Proc. Natl Acad. Sci. USA*, **99**, 10179–10184.

van Wees, S.C.M., de Swart, E.A.M., van Pelt, J.A., van Loon, L.C. and Pieterse, C.M.J. (2000) Enhancement of induced disease resistance by simultaneous activation of salicylate- and jasmonate-dependent defense pathways in *Arabidopsis thaliana*. *Proc. Natl Acad. Sci. USA*, **97**, 8711–8716.

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.

Yan, J., Zhang, C., Gu, M. *et al.* (2009) The Arabidopsis CORONATINE INSENSITIVE1 protein is a jasmonate receptor. *Plant Cell*, **21**, 2220–2236.

Yu, Y., Xu, W., Wang, S., Xu, Y., Li, H.E., Wang, Y. and Li, S. (2011) *VpRFP1*, a novel C4C4-type RING finger protein gene from Chinese wild *Vitis pseudoreticulata*, functions as a transcriptional activator in defence response of grapevine. *J. Exp. Bot.* **62**, 5671–5682.

Zhang, Y., Fan, W., Kinkema, M., Li, X. and Dong, X. (1999) Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the *PR-1* gene. *Proc. Natl Acad. Sci. USA*, **96**, 6523–6528.

Zhang, Y., Yang, Y., Fang, B., Gannon, P., Ding, P., Li, X. and Zhang, Y. (2010) Arabidopsis snc2-1D activates receptor-like protein-mediated immunity transduced through WRKY70. *Plant Cell*, **22**, 3153–3163.

Zuo, J., Niu, Q.-W. and Chua, N.-H. (2000) An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. *Plant J.* **24**, 265–273.

